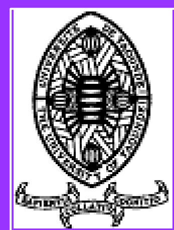
UNIVERSITY OF YAOUNDE I
****
FACULTY OF SCIENCE
****
CENTRE FOR RESEARCH AND TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENTAL SCIENCES
****
DEPARTMENT OF BIOCHEMISTRY



UNIVERSITE DE YAOUNDE 1
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FACULTE DES SCIENCES
****
CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE, SCIENCES DE LA VIE, SANTE ET ENVIRONNEMENT
****
DEPARTEMENT DE BIOCHIMIE

BIOTECHNOLOGY CENTRE NKOLBISSON

LABORATORY OF PHYTOPROTECTION AND VALORIZATION OF GENETIC RESOURCES (LPVGR)

Effects of two formulations based *on Streptomyces cameroonensis* on growth and resistance of two populations of cocoa hybrids ((♂) SNK413 × (♀) T79/467); ((♂) UPA134 × (♀) SCA12) from Cameroon

THESIS

Submitted in fulfillment of the requirement for the award of a Doctorate/Ph.D. in Biochemistry

Option: Biotechnology and Development

By:

DZELAMONYUY Aristide

Registration Number: 10R1186
Master of Science

Supervised by:

EFFA ONOMO Pierre
Associate Professor
University of Yaoundé 1

BOUDJEKO Thaddée
Professor
University of Yaoundé 1

EWANE Cécile Annie
Associate Professor
University of Yaoundé 1

Year 2025

UNIVERSITY OF YAOUNDE 1

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FACULTY OF SCIENCE

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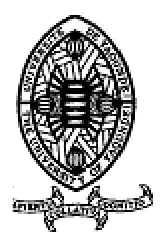
DEPARTMENT OF BIOCHEMISTRY



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FACULTÉ DES SCIENCES

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CENTRE DE RECHERCHE ET DE FORMATION DOCTORALE, SCIENCES DE LA VIE, SANTÉ ET ENVIRONNEMENT

****

DEPARTMENT DE BIOCHIMIE

THE BIOTECHNOLOGY CENTRE OF NKOLBISSON

CENTRE DE BIOTECHNOLOGIE DE NKOLBISSON

LABORATORY OF PHYTOPROTECTION AND VALORIZATION OF GENETIC RESOURCES

LABORATOIRE DE PHYTOPROTECTION ET DE VALORISATION DES RESSOURCES GÉNÉTIQUES

Effects of two formulations based on *Streptomyces cameroonensis* on growth and resistance of two populations of cocoa hybrids [(♂) SNK413 × (♀) T79/467. [(♂) UPA134 × (♀) SCA12] from Cameroon

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University of Yaoundé 1

Year 2025

<table>
<tr><td>UNIVERSITÉ DE YAOUNDÉ I
Faculté des Sciences
Division de la Programmation et du Suivi des Activités Académiques</td><td> </td><td>THE UNIVERSITY OF YAOUNDE I
Faculty of Science
Division of Programming and Follow-up of Academic Affaires</td></tr>
<tr><td>LISTE DES ENSEIGNANTS PERMANENTS</td><td colspan="2">LIST OF PERMANENT TEACHING STAFF</td></tr>
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LIST OF PERMANENT TEACHING STAFF

ACADEMIC YEAR 2024/2025

(By Department and Grade)

DATE OF UPDATE 16 Januuary 2025

<u>ADMINISTRATION</u>

1. **DEAN:** OWONO OWONO Luc Calvin, *Professor*
2. **VICE-DEAN/ DPSAA:** NDJIGUI Paul-Désiré, *Professor*
3. **VICE-DEAN / DSSE:** NYEGUE Maximilienne Ascension, *Professor*
4. **VICE-DEAN / DRC:** NOUNDJEU Pierre, *Associate Professor*
5. **Head of Administrative and Financial Division:** NDOYE FOE Florentine Marie Chantal, *Associate Professor*
6. **Head of Academic Affairs, education and Research division/ DAARS:** AJEAGAH Gideon AGHAINDUM, *Professor*

1- DEPARTMENT OF BIOCHEMISTRY (BC) (44)			
N°	**NOMS ET PRÉNOMS**	**GRADE**	**OBSERVATIONS**
1.	BIGOGA DAIGA Jude	Professor	In Office
2.	FEKAM BOYOM Fabrice	Professor	In Office
3.	KANSCI Germain	Professor	In Office
4.	MBACHAM FON Wilfred	Professor	In Office
5.	MOUNDIPA FEWOU Paul	Professor	*Head of DEPARTMENT*
6.	NGUEFACK Julienne	Professor	In Office
7.	NJAYOU Frédéric Nico	Professor	In Office
8.	OBEN Julius ENYONG	Professor	In Office
9.	ACHU Merci BIH	Associate Professor	In Office
10.	BEBEE Fadimatou	Associate Professor	In Office
11.	BEBOY EDJENGUELE Sara N.	Associate Professor	In Office
12.	FONKOUA Martin	Associate Professor	In Office
13.	AKINDEH MBUH NJI	Associate Professor	In Office
14.	ATOGHO Barbara MMA	Associate Professor	In Office
15.	AZANTSA KINGUE GABIN BORIS	Associate Professor	In Office
16.	BELINGA née NDOYE FOE F. M. C.	Associate Professor	*Head DAF / FS*
17.	DAKOLE DABOY Charles	Associate Professor	In Office
18.	DONGMO LEKAGNE Joseph Blaise	Associate Professor	In Office
19.	DJUIDJE NGOUNOUE Marceline	Associate Professor	In Office
20.	DJUIKWO NKONGA Ruth Viviane	Associate Professor	In Office
21.	EFFA ONOMO Pierre	Associate Professor	*VD/FS/Univ Ebwa*
22.	EWANE Cécile Annie	Associate Professor	In Office
23.	KENGNE NOUEMSI Anne Pascale	Associate Professor	In Office
24.	KOTUE TAPTUE Charles	Associate Professor	In Office
25.	LUNGA Paul KEILAH	Associate Professor	In Office
26.	MANANGA Marlyse Joséphine	Associate Professor	In Office
27.	MBONG ANGIE M. Mary Anne	Associate Professor	In Office
28.	MOFOR née TEUGWA Clotilde	Associate Professor	*DEAN FS / UDs*
29.	NANA Louise épouse WAKAM	Associate Professor	In Office

30.	NGONDI Judith Laure	Associate Professor	In Office
31.	Palmer MASUMBE NETONGO	Associate Professor	In Office
32.	PECHANGOU NSANGOU Sylvain	Associate Professor	In Office
33.	TCHANA KOUATCHOUA Angèle	Associate Professor	In Office

34.	BAKWO BASSOGOG Christian Bernard	Lecturer	In Office
35.	ELLA Fils Armand	Lecturer	In Office
36.	EYENGA Eliane Flore	Lecturer	In Office
37.	FOUPOUAPOUOGNIGNI Yacouba	Lecturer	In Office
38.	KOUOH ELOMBO Ferdinand	Lecturer	In Office
39.	MADIESSE KEMGNE Eugenie Aimée	Lecturer	In Office
40.	MANJIA NJIKAM Jacqueline	Lecturer	In Office
41.	MBOUCHE FANMOE Marceline J.	Lecturer	In Office
42.	OWONA AYISSI Vincent Brice	Lecturer	In Office
43.	WILFRED ANGIE ABIA	Lecturer	In Office
44.	WOGUIA Alice Louise	Lecturer	In Office

2- DEPARTMENT OF BIOLOGY AND ANIMAL PHYSIOLOGY (BPA) (49)			
1.	AJEAGAH Gideon AGHAINDUM	Professor	*DAARS/FS*
2.	DJIETO LORDON Champlain	Professor	In Office
3.	DZEUFIET DJOMENI Paul Désiré	Professor	In Office
4.	ESSOMBA née NTSAMA MBALA	Professor	*HoD and Vice DEAN/FMSB/UYI*
5.	KEKEUNOU Sévilor	Professor	*Head of DEPARTMENT*
6.	MEGNEKOU Rosette	Professor	In Office
7.	NJAMEN Dieudonné	Professor	In Office
8.	NOLA Moïse	Professor	In Office
9.	TAN Paul VERNYUY	Professor	In Office
10.	TCHUEM TCHUENTE Louis Albert	Professor	*Service Inspector / Coord.Progr./MINSANTE*
11.	ZEBAZE TOGOUET Serge Hubert	Professor	In Office

12.	ALENE Désirée Chantal	Associate Professor	*Vice DEAN/ UEb*
13.	ATSAMO Albert Donatien	Associate Professor	In Office
14.	BILANDA Danielle Claude	Associate Professor	In Office
15.	DJIOGUE Séfirin	Associate Professor	In Office
16.	GOUNOUE KAMKUMO Raceline épse FOTSING	Associate Professor	In Office
17.	JATSA BOUKENG Hermine épse MEGAPTCHE	Associate Professor	In Office
18.	KANDEDA KAVAYE Antoine	Associate Professor	In Office
19.	LEKEUFACK FOLEFACK Guy B.	Associate Professor	In Office
20.	MAHOB Raymond Joseph	Associate Professor	In Office
21.	MBENOUN MASSE Paul Serge	Associate Professor	In Office
22.	MOUNGANG Luciane Marlyse	Associate Professor	In Office
23.	NOAH EWOTI Olive Vivien	Associate Professor	In Office
24.	MONY Ruth épse NTONE	Associate Professor	In Office
25.	MVEYO NDANKEU Yves Patrick	Associate Professor	In Office
26.	NGUEGUIM TSOFACK Florence	Associate Professor	In Office
27.	NGUEMBOCK	Associate Professor	In Office
28.	TADU Zephyrin	Associate Professor	In Office
29.	TAMSA ARFAO Antoine	Associate Professor	In Office
30.	TOMBI Jeannette	Associate Professor	In Office
31.	YEDE	Associate Professor	In Office

32.	AMBADA NDZENGUE GEORGIA ELNA	Lecturer	In Office
33.	BASSOCK BAYIHA Etienne Didier	Lecturer	In Office
34.	ETEME ENAMA Serge	Lecturer	In Office
35.	FEUGANG YOUMSSI François	Lecturer	In Office
36.	FOKAM Alvine Christelle Epse KENGNE	Lecturer	In Office
37.	FOSSI TANKOUA Olivia Epse DJEUTCHOUANG SAYANG	Lecturer	In Office
38.	GONWOUO NONO Legrand	Lecturer	In Office

39.	KOGA MANG Dobara	Lecturer	In Office
40.	LEME BANOCK Lucie	Lecturer	In Office
41.	MAPON NSANGOU Indou	Lecturer	In Office
42.	METCHI DONFACK Mireille Flaure EPSE GHOUMO	Lecturer	In Office
43.	NDENGUE Jean De Matha	Lecturer	In Office
44.	NGOUATEU KENFACK Omer Bébé	Lecturer	In Office
45.	NJUA Clarisse YAFI	Lecturer	*Head of Div. U. Bamenda*
46.	NWANE Philippe Bienvenu	Lecturer	In Office
47.	YOUNOUSSA LAME	Lecturer	In Office
48.	ZEMO GAMO Franklin	Lecturer	In Office
49.	KODJOM WANCHE Jacguy Joyce	Assistante	In Office

3- DEPARTMENT OF BIOLOGY AND PLANT PHYSIOLOGY (BPV) (37)			
1.	AMBANG Zachée	Professor	*Head of DEPARTMENT*
2.	BIYE Elvire Hortense	Professor	In Office
3.	DJOCGOUE Pierre François	Professor	In Office
4.	MBOLO Marie	Professor	In Office
5.	NDONGO BEKOLO	Professor	In Office
6.	ZAPFACK Louis	Professor	In Office

7.	ANGONI Hyacinthe	Associate Professor	In Office
8.	DJEUANI Astride Carole	Associate Professor	In Office
9.	MAHBOU SOMO TOUKAM Gabriel	Associate Professor	In Office
10.	MALA Armand William	Associate Professor	In Office
12.	NGALLE Hermine BILLE	Associate Professor	In Office
13.	NGONKEU MAGAPTCHE Eddy L.	Associate Professor	*CT/MINRES I*
14.	TONFACK Libert Brice	Associate Professor	In Office
15.	TSOATA Esaïe	Associate Professor	In Office
16.	ONANA Jean Michel	Associate Professor	In Office

17.	DIDA LONTSI Sylvere Landry	Lecturer	In Office
18.	GONMADGE Christelle	Lecturer	In Office
19.	MAFFO MAFFO Nicole Liliane	Lecturer	In Office
20.	MANGA NDJAGA JUDE	Lecturer	In Office
21.	NNANGA MEBENGA Ruth Laure	Lecturer	In Office
22.	NOUKEU KOUAKAM Armelle	Lecturer	In Office
23.	NSOM ZAMBO EPSE PIAL Annie Claude	Lecturer	*On Secondment/UNESCO MALI*
24.	GODSWILL NTSOMBOH NTSEFONG	Lecturer	In Office
25.	KABELONG BANAHO Louis-Paul-Roger	Lecturer	In Office
26.	KONO Léon Dieudonné	Lecturer	In Office
27.	LIBALAH Moses BAKONCK	Lecturer	In Office
28.	LIKENG-LI-NGUE Benoit C	Lecturer	In Office
29.	TAEDOUNG Evariste Hermann	Lecturer	In Office
30.	TEMEGNE NONO Carine	Lecturer	In Office
31.	BOLIE Hubert	Assistant	In Office
33.	MACHE NKOUANDEU Pasma	Assistant	In Office
34.	MAFFO FOKOU Adèle	Assistant	In Office
35.	METSEBING Blondo-Pascal	Assistant	In Office
36.	NTONMEN YPNKEU Amandine Flore	Assistant	In Office
37.	ONANA EBODE Clotaire	Assistant	In Office

4- DEPARTMENT OF INORGANIC CHEMISTRY (CI) (28)			
1.	GHOGOMU Paul MINGO	Professor	*Minister in Charge of Mission PR*
2.	NANSEU NJIKI Charles Péguy	Professor	In Office
3.	NDIFON Peter TEKE	Professor	*CT MINRESI*
4.	NGOMO Horace MANGA	Professor	*Vice Chancelor/UB*
5.	NJIOMOU C. épse DJANGANG	Professor	In Office
6.	NJOYA Dayirou	Professor	In Office

7.	ACAYANKA Elie	Associate Professor	In Office
8.	EMADAK Alphonse	Associate Professor	In Office
9.	KAMGANG YOUBI Georges	Associate Professor	In Office
10.	KEMMEGNE MBOUGUEM Jean C.	Associate Professor	In Office
11.	KENNE DEDZO GUSTAVE	Associate Professor	In Office
12.	MBEY Jean Aimé	Associate Professor	In Office
13.	NDI Julius NSAMI	Associate Professor	*Head of DEPARTMENT*
14.	NEBAH Née NDOSIRI Bridget NDOYE	Associate Professor	*Senator/SENATE*
15.	NYAMEN Linda Dyorisse	Associate Professor	In Office
16.	PABOUDAM GBAMBIE AWAWOU	Associate Professor	In Office
17.	TCHAKOUTE KOUAMO Hervé	Associate Professor	In Office
18.	BELIBI BELIBI Placide Désiré	Associate Professor	*Head of Service/ ENS Bertoua*
19.	CHEUMANI YONA Arnaud M.	Associate Professor	In Office
20.	KOUOTOU DAOUDA	Associate Professor	In Office

21.	MAKON Thomas Beauregard	Lecturer	In Office
22.	NCHIMI NONO KATIA	Lecturer	In Office
23.	NJANKWA NJABONG N. Eric	Lecturer	In Office
24.	PATOUOSSA ISSOFA	Lecturer	In Office
25.	SIEWE Jean Mermoz	Lecturer	In Office
26.	BOYOM TATCHEMO Franck W.	Assistant	In Office
27.	DANTIO NGUELA Christian Brice	Assistant	In Office
28.	LEKENE NGOUATEU Reine	Assistant	In Office

5- DEPARTMENT OF ORGANIC CHEMISTRY (CO) (36)			
1	Alex de Théodore ATCHADE	Professor	*DEPE/Univ. Bertoua*
2	DONGO Etienne	Professor	*Vice-DEAN/FSE/UYI*
3	NGOUELA Silvère Augustin	Professor	*Head of DEPARTMENT/UDS*
4	PEGNYEMB Dieudonné Emmanuel	Professor	*Rector UBertoua/ Head of DEPARTMENT*
5	MBAZOA née DJAMA Céline	Professor	In Office

6	MKOUNGA Pierre	Professor	In Office

7	AMBASSA Pantaléon	Associate Professor	In Office
8	EYONG Kenneth OBEN	Associate Professor	*Director/HTTTC/UBda*
8	FOTSO WABO Ghislain	Associate Professor	In Office
10	KAMTO Eutrophe Le Doux	Associate Professor	In Office
11	KENMOGNE Marguerite	Associate Professor	In Office
12	MVOT AKAK CARINE	Associate Professor	In Office
13	NGOMO Orléans	Associate Professor	In Office
14	NGO MBING Joséphine	Associate Professor	*Head of Unit MINRESI*
15	NGONO BIKOBO Dominique Serge	Associate Professor	*Head of Div./MINESUP*
16	NOTE LOUGBOT Olivier Placide	Associate Professor	*Dir ENS/Uty Bertoua*
17	NOUNGOUE TCHAMO Diderot	Associate Professor	In Office
18	TABOPDA KUATE Turibio	Associate Professor	In Office
19	TAGATSING FOTSING Maurice	Associate Professor	In Office
20	OUAHOUO WACHE Blandine M.	Associate Professor	In Office
21	ZONDEGOUMBA Ernestine	Associate Professor	In Office

22	MELONG Radius	Lecturer	In Office
23	MESSI Angélique Nicolas	Lecturer	In Office
24	MUNVERA MFIFEN Aristide	Lecturer	In Office
25	NGNINTEDO Dominique	Lecturer	In Office
26	NONO NONO Éric Carly	Lecturer	In Office
27	OUETE NANTCHOUANG Judith Laure	Lecturer	In Office
28	SIELINOU TEDJON Valérie	Lecturer	In Office
29	TCHAMGOUE Joseph	Lecturer	In Office
30	TSAFFACK Maurice	Lecturer	In Office
31	TSAMO TONTSA Armelle	Lecturer	In Office
32	TSEMEUGNE Joseph	Lecturer	In Office
33	NDOGO ETEME Olivier	Assistant	In Office
34	NGUEMDJO CHIMEZE Valery Wilfried	Assistant	In Office

6- DEPARTMENT OF RENEWABLE ENERGIES (ER) (1)

1.	BODO Bertrand	Professor	Head of Department

7- DEPARTMENT OF COMPUTER SCIENCE (IN) (25)			
1.	ATSA ETOUNDI Roger	Professor	*Head of Division SI/MINESUP*
2.	FOUDA NDJODO Marcel Laurent	Professor	*Academic Inspector General / MINESUP*
3.	NDOUNDAM René	Professor	In Office
4.	ABESSOLO ALO'O Gislain	Associate Professor	*CT1/MINFOPRA*
5.	MELATAGIA YONTA Paulin	Associate Professor	In Office
6.	TSOPZE Norbert	Associate Professor	In Office

7.	AMINOU HALIDOU	Lecturer	*Head of DEPARTMENT*
8.	DJAM Xaviera YOUH - KIMBI	Lecturer	In Office
9.	DOMGA KOMGUEM Rodrigue	Lecturer	In Office
10.	EBELE Serge Alain	Lecturer	In Office
11.	EKODECK Stéphane Gaël Raymond	Lecturer	In Office
12.	HAMZA Adamou	Lecturer	In Office
13.	JIOMEKONG AZANZI Fidel	Lecturer	In Office
14.	KOUOKAM KOUOKAM E. A.	Lecturer	In Office
15.	MESSI NGUELE Thomas	Lecturer	*Head of DEPARTMENT/ Computer Eng./U Ebolowa*
16.	MONTHE DJIADEU Valery M.	Lecturer	In Office
17.	NZEKON NZEKO'O Armel Jacques	Lecturer	In Office
18.	OLLE OLLE Daniel Claude Georges Delort	Lecturer	*Deputy Director/ ENSET Ebolowa*
19.	TAPAMO Hyppolite	Lecturer	In Office
20.	BAYEM Jacques Narcisse	Assistant	In Office
21.	MAKEMBE. S. Oswald	Assistant	*Director CUTI*
22.	MAXWELL NDOGNKON MANGA	Assistant	In Office
23.	NDOM Francis Rollin	Assistant	In Office

24.	NGUIMEYA TSOFACK Baudoin	Assistant	In Office
25.	NKONDOCK. MI BAHANACK. N.	Assistant	In Office

8- DEPARTMENT OF MATHEMATICS (MA) (36)			
1.	AYISSI Raoult Domingo	Professor	*Head of DEPARTMENT/D. ENSPY*

2.	KIANPI Maurice	Associate Professor	In Office
3.	MBANG Joseph	Associate Professor	In Office
4.	MBEHOU Mohamed	Associate Professor	*Head de Division/ENSPY*
5.	MBELE BIDIMA Martin Ledoux	Associate Professor	In Office
6.	NOUNDJEU Pierre	Associate Professor	*VDRC/FS/UYI*
7.	TAKAM SOH Patrice	Associate Professor	In Office
8.	TCHAPNDA NJABO Sophonie B.	Associate Professor	*Director/AIMS Rwanda*
9.	TCHOUNDJA Edgar Landry	Associate Professor	In Office

10.	AGHOUKENG JIOFACK Jean Gérard	Lecturer	*Head Cellule MINEPAT*
11.	BOGSO ANTOINE Marie	Lecturer	In Office
12.	BITYE MVONDO Esther Claudine	Lecturer	In Office
13.	CHENDJOU Gilbert	Lecturer	In Office
14.	DJIADEU NGAHA Michel	Lecturer	In Office
15.	DOUANLA YONTA Herman	Lecturer	In Office
16.	KIKI Maxime Armand	Lecturer	In Office
17.	KOKOMO AYISSI Eric Brice	Lecturer	In Office (Transfer from the University of Douala)
18.	LOUMNGAM KAMGA Victor	Lecturer	In Office
19.	MBAKOP Guy Merlin	Lecturer	In Office
20.	MBATAKOU Salomon Joseph	Lecturer	In Office
21.	MENGUE MENGUE David Joël	Lecturer	*Head Dpt /ENS Uty Ebolowa*
22.	MBIAKOP Hilaire George	Lecturer	In Office
23.	NGUEFACK Bernard	Lecturer	In Office

24.	NIMPA PEFOUKEU Romain	Lecturer	In Office
25.	OGADOA AMASSAYOGA	Lecturer	In Office
26.	POLA DOUNDOU Emmanuel	Lecturer	*Internsihip*
27.	TENKEU JEUFACK Yannick Léa	Lecturer	In Office
28.	TCHEUTIA Daniel Duviol	Lecturer	In Office
29.	TETSADJIO TCHILEPECK M. Eric.	Lecturer	In Office

30.	EBODE ATANGANA Pie Désiré	Assistant	In Office
31.	FOKAM Jean Marcel	Assistant	In Office
32.	GUIDZAVAI KOUCHERE Albert	Assistant	In Office
33.	MAMA ASSANDJE Prosper	Assistant	In Office
34.	MANN MANYOMBE Martin Luther	Assistant	In Office
35.	MEFENZA NOUNTU Thiery	Assistant	In Office
36.	NYOUMBI DLEUNA Christelle	Assistant	In Office

9 - DEPARTMENT OF MICROBIOLOGY (MIB) (25)

1.	ESSIA NGANG Jean Justin	Professor	*Head of DEPARTMENT*
2.	KOUITCHEU MABEKU Epse KOUAM Laure Brigitte	Professore	In Office
3.	MUNE MUNE Martin Alain	Professor	In Office
4.	NYEGUE Maximilienne Ascension	Professore	*Vice-DEAN / DSSE*
5.	RIWOM Sara Honorine	Professore	In Office
6.	SADO KAMDEM Sylvain Leroy	Professor	In Office

7.	ASSAM ASSAM Jean Paul	Associate Professor	*DEAN/FASA/UDs*
8.	BOUGNOM Blaise Pascal	Associate Professor	In Office
9.	NJIKI BIKOÏ Jacky	Associate Professor	In Office
10	TCHIKOUA Roger	Associate Professor	*Head of the Academic Affairs*

11	EHETH Jean Samuel	Lecturer	In Office
12	ESSONO Damien Marie	Lecturer	In Office
13	EZO'O MENGO Fabrice Télésfor	Lecturer	In Office

14	LAMYE Glory MOH	Lecturer	In Office
15	MEYIN A EBONG Solange	Lecturer	In Office
16	MONI NDEDI Esther Del Florence	Lecturer	*Chief of service/DAAC/UYI*
17	NKOUDOU ZE Nardis	Lecturer	In Office
18	NKOUE TONG Abraham	Lecturer	In Office
19	NGOUENAM Romial Joël	Lecturer	In Office
20	NJAPNDOUNKE Bilkissou	Lecturer	In Office
21	TAMATCHO KWEYANG Blandine Pulchérie	Lecturer	In Office
22	SAKE NGANE Carole Stéphanie	Lecturer	In Office
23	TOBOLBAÏ Richard	Lecturer	In Office

24	ZO'O EZO'O Fabrice Télesfor	Assistant	In Office
25	MAYI Marie Paule Audrey	Assistante	In Office

10. DEPARTEMENT OF PHYSICS (PHY) (47)			
1.	BEN- BOLIE Germain Hubert	Professor	In Office
2.	BIYA MOTTO Frédéric	Professor	*DG/HYDRO Mekin*
3.	DJUIDJE KENMOE ép. ALOYEM	Professor	In Office
4.	EKOBENA FOUDA Henri Paul	Professor	*Vice-Rector. Uty Ngaoundéré*
5.	ESSIMBI ZOBO Bernard	Professor	In Office
6.	EYEBE FOUDA Jean sire	Professor	In Office
7.	FEWO Serge Ibraïd	Professor	In Office
8.	HONA Jacques	Professor	In Office
9.	NANA ENGO Serge Guy	Professor	In Office
10.	NANA NBENDJO Blaise	Professor	*Head of DEPARTMENT/Uty. Bertoua*
11.	NDJAKA Jean Marie Bienvenu	Professor	*Head of DEPARTMENT*
12.	NJANDJOCK NOUCK Philippe	Professor	In Office
13.	SAIDOU	Professor	*Head of centre/IRGM/MINRESI*
14.	SIEWE SIEWE Martin	Professor	In Office

15.	SIMO Elie	Professor	In Office
16.	TABOD Charles TABOD	Professor	*DEAN FS/Univ/Bda*
17.	TCHAWOUA Clément	Professor	In Office
18.	WOAFO Paul	Professor	In Office
19.	ZEKENG Serge Sylvain	Professor	In Office
20.	VONDOU Derbetini Appolinaire	Professor	In Office

21.	ENYEGUE A NYAM épse BELINGA	Associate Professor	*Head of Div./ENSPY*
22.	FOUEJIO David	Associate Professor	*Head of Unit/MINADER*
23.	MBINACK Clément	Associate Professor	In Office
24.	MBONO SAMBA Yves Christian U.	Associate Professor	In Office
25.	MELI'I Joelle Larissa	Associate Professor	In Office
26.	MVOGO ALAIN	Associate Professor	In Office
27.	NDOP Joseph	Associate Professor	In Office
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Numerical Distribution of Faculty Members of the Faculty of Sciences at the University of Yaoundé I

NOMBRE D'ENSEIGNANTS					
DEPARTMENT	**Professors**	**Associate Professors**	**Lecturers**	**Assistants**	**Total**
BCH	08 (01)	25 (15)	11 (05)	00 (00)	**44 (21)**
BPA	12 (02)	18 (06)	16 (06)	01 (01)	**47 (15)**
BPV	08 (02)	10 (03)	14 (06)	06 (03)	**39 (14)**
CI	06 (01)	14 (02)	05 (01)	03 (01)	**28 (05)**
CO	06 (01)	14 (05)	12 (03)	02 (00)	**33 (09)**
ER	01 (00)	/	/	/	**01 (0)**
IN	03 (00)	03 (00)	13 (01)	06 (00)	**24 (01)**
MAT	01 (00)	08 (00)	20 (01)	07 (01)	**36 (02)**
MIB	05 (03)	05 (01)	13 (06)	01 (01)	**24 (11)**
PHY	17 (01)	11 (04)	15 (01)	04 (02)	**45 (08)**
ST	10 (00)	14 (05)	09 (02)	00 (00)	**33 (07)**
Total	**67 (11)**	**122 (41)**	**128 (32)**	**30 (09)**	**342 (88)**

For a total of **360 (94),** including:

- Professors: **67 (11)**
- Associate Professors: **122 (41)**
- Senior Lecturers: **128 (32)**
- Assistants: **30 (09)**
- () = Number of Women **94**

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DEDICATION

This Dissertation is dedicated to

the memory of late

Professor BOUDJEKO Thaddée.

May His Soul Rest In Peace Forever …

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ABSTRACT

Cocoa (*Theobroma cacao* L.) is one of the most important cash crops in Cameroon and other producing countries, as it is a source of income for producers and a raw material for the $100 billion global chocolate industry. Black pod disease caused by *Phytophthora megakarya* is a major constraint that threatens cocoa production. Control of this disease is mostly achieved by chemical fungicides, but the latter constitute a major public health safety concern, so a new strategy is needed to limit the use of chemical fungicides and improve the productivity of cocoa. *Streptomyces cameroonensis*, an actinomycete isolated from uncropped soil in Yaounde, Cameroon, has been shown to exhibit strong abilities to promote plant growth and protect cocoa against *P. megakarya.* This study was carried out to develop a liquid and powder formulation based on *S. cameroonensis* that are convenient for agricultural systems and better suited for commercial use. The viability, efficacy and phytotoxicity of the formulations were evaluated *in vitro* for two populations of cocoa hybrids [(♂) SNK413 × (♀) T79/467 and (♂) UPA134 × (♀) SCA12]. The effects of the formulations against *P. megakarya* and its ability to impact the growth and resistance of cocoa seedlings were evaluated by biochemical analysis as well as gene expression of plant defense markers. The powder formulation showed a high shelf-life of 1.07 x 10^6 CFU/g after six months at an optimal storage of 4 °C and a 100% Antimicrobial effect against *P. megakarya* at 10% w/v. The liquid formulation developed from a crude extract of *S. cameroonensis* secondary metabolites at 0.1% v/v had a non-toxic effect against young cocoa leaves and an 85% inhibition capacity against *P. megakarya*. The two formulations, when tested on cocoa seedlings, significantly improved the growth of the plants in the nursery after 3 months averagely by 27%. A neutral leaf assay showed a significant reduction in the disease severity index of about 67% and 57% respectively for the powder and liquid formulation. Analysis of biochemical markers showed a significant increase in total polyphenol, flavonoid, and total protein contents and an increase in the activities of polyphenol oxidases, peroxidases, chitinases, and β-1,3-glucanases when cocoa plants were treated with both formulations. To better understand the molecular mechanisms by which these formulations promote growth and resistance, we used real time-Polymerase Chain Reaction (rt-PCR) to compare the effects of the formulations on the relative expression levels of TcPer-1, TcGlu1, TcChiB, and TcMYBPA genes before and after infection. We observed an up-regulation in the expression of these defense related genes upon treatment of the plants with

our formulations. Hence, our results show that *S. cameroonensis*-based formulations are stable and effective at suppressing black pod disease in cocoa. This provides a basis for the optimization of beneficial microorganisms and a viable alternative to chemical fungicides used in disease suppression.

Keywords: Cocoa seedlings, *Phytophthora megakarya*, *Streptomyces cameroonensis,* formulation, biochemical markers, resistance, genes, rt-PCR.

RESUME

Le Cacao (*Theobroma cacao* L.) est l'une des cultures de rente les plus importantes pour les pays producteurs, à l'instar du Cameroun. C'est la principale source de matière première de l'industrie chocolatière à l'échelle mondiale, dont la valeur totale dépasse les 100 milliards de dollars. C'est également une source importante de revenus pour les producteurs locaux. Sa culture fait face à la pourriture brune de cacao causée par *Phytophthora megakarya,* qui est responsable d'une baisse significative du rendement pouvant atteindre 100 % en l'absence de traitement. La lutte contre cet agent pathogène est principalement assurée par des fongicides chimiques. Cependant, ces derniers constituent une préoccupation majeure pour l'environnement et la santé des producteurs, en raison de leur exposition aux residues de pesticides. Ainsi, le recours a des strategies de gestion durable et éco-responsable de cette maladie est nécessaire pour limiter l'utilisation intensive de ces pesticides chimiques de synthèse, afin d'améliorer la productivité du cacao. À cet effet, *Streptomyces cameroonensis,* une actinomycète isolé à partir du sol non cultivé à Yaoundé, Cameroun, a prouvé son éfficacité protectrice envers les plants de cacoyers vis-à-vis de *P. Megakarya,* ainsi que sa capacité à stimuler la croissance des plants de cacaoyers en pépinière. Le but de cette étude était de développer deux formulations à base de *S. cameroonensis* pour la protection durable des plants de cacaoyers vis-à-vis de *P. megakarya*. La viabilité, l'efficacité et la phytotoxicité de ces formulations ont été évaluées *in vitro* sur deux populations d'hybrides de cacao [(♂) SNK413 × (♀) T79/467 et (♂) UPA134 × (♀) SCA12]. Les effets des formulations contre *P. megakarya* et leur capacité à stimuler la croissance ainsi que la résistance des plants de cacaoyers ont été évalués par analyse des marqueurs biochimique de résistance, ainsi que par l'expression des gènes de ces marqueurs de défense. Il ressort que la formulation en poudre, à la concentration de 10% p/v, a une durée de conservation de six mois à 4 °C, avec une charge bactérienne de l'ordre de 1,07 x 106 CFU/g. Elle présente un effet antifongique de 100 % contre *P. megakarya*. La formulation liquide à la concentration 0.1% v/v, développée à partir d'un extrait brut de métabolites secondaires de *S. Cameroonensis,* à démontré un effet non toxique contre les jeunes feuilles de cacao et une capacité d'inhibition de 85 % contre *P. megakarya.* Ces deux formulations, testées individuellement pour leur effet sur la croissance sur des plants de cacaoyers, ont amélioré significativement les paramètres de croissance des plants en pépinière après 3 mois, avec croissance avec un taux d'augmentation moyen de l'ordre de 27%. Le test d'inoculation des feuilles par *P. megakarya* a montré une réduction

significative de l'indice de sévérité de la maladie de l'ordre de 67 % et 57 % respectivement pour la formulation en poudre et la formulation liquide comparativement, aux témoins. L'analyse des marqueurs biochimiques a montré une augmentation significative de la teneur totale en polyphénols, flavonoïdes et protéines totales ainsi qu'une augmentation des activités des polyphénols oxydases, des peroxydases, des chitinases et des β-1,3-glucanases après le traitement de plants de cacaoyers avec les deux formulations. L'analyse du niveau d'expression des gènes TcPer-1, TcGlu-1, TcChiB et TcMYBPA par réaction en chaîne par polymérase en temps réel (PCR en temps réel) a montré une régulation positive de l'expression de ces gènes, caractérisé par une surexpression observée avant et après infection. Ainsi, nos résultats montrent que les formulations à base de *S. cameroonensis* sont stables sur une longue période et efficaces pour la protection des plants de cacaoyers vis à vis de *P. megakarya*. Ces résultats constituent une base de données pour l'optimisation des micro-organismes bénéfiques pour les plantes, et une alternative vitale aux fongicides chimiques utilisés dans la lutte contre les agents pathogènes.

Mots clés : Plants de cacaoyer, P*hytophthora megakarya*, *Streptomyces cameroonensis*, formulation, marqueurs biochimiques, résistance, gènes, PCR en temps réel

LIST OF ABBREVIATIONS

16S RNA:16S ribosomal ribonucleic acid

ACC: 1-aminocyclopropane-1-carboxylate

BSA: Bovin Serum Albumin

BTC: Biotechnology Centre

C: Chemical Treatment

cDNA: complementary DeoxyRibonucleic Acid

CEC : Cation Exchange Capacity

F CFA : Francs de la Communauté Financière Africaine

CFU: Colony Forming Unit

CMC: CarboxyMethylCellulose

dNTPS : Dexoxyribonucleotides triphosphate

EU : Enzymatic Units

FW: Fresh Weight

GDP: Gross Domestic product

HR: Hypersensitive Response

I.S.P: International *Streptomyces* Project

IAA: Indole Acetic Acid

ICCO: International cocoa organization

ICS: Imperial College Selection

IFOAM: International Federation of Organic Agriculture Movements

ISR: Induced Systemic Resistance

KMB: King Medium Base

LAR: Local Acquired Resistance

LL-DAP: LL-diaminopimelic acid

LPVGR: Laboratory of Phytoprotection and Valorization of Genetic Resources

MAP: Mitogen-Activated Protein

NT: Non-Treated

ONCC : Office National du Cacao et du Café

PA: Proanthocyanidin

PAMPs: pathogen-associated molecular patterns

PDA: Potato Dextrose Agar

PGPR: Plant growth promoting rhizobacteria.

PM: *Phytophthora megakarya*

POX: Peroxidase

PPO: Polyphenoloxidase

PR: Pathogenesis Related

PRRs: Plasmalemma-Spanning Pattern Recognition Receptors

qPCR: quantitative Polymerase Chain Reaction

RLKs: Receptor-Like Protein Kinases

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rt-PCR: real-time PCR

SA: Salicylic Acid

SAR: Systemic Acquired Resistance

SCA: Scavina

SNK : Selection de NKoemvone

SODECAO : Société de Développement du Cacao

T: Formulation treatment

T79/467: Taffo 79/467

UPA: Upper Amazon

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Introduction

I. INTRODUCTION

The economic and social development of several regions of Africa and Cameroon depends primarily on agriculture and agronomy. This sector is constantly subjected to many technological and scientific innovations aimed at combating the many constraints it faces, including constant parasitic pressures. Recent discoveries on physiological exchange mechanisms, gene therapy research, plant vaccine trials and biocontrol against parasitic attacks are all examples of technologies that have been used in modern agriculture to increase the yield of agro-food products while reducing world hunger and environmental degradation (Adenle *et al.,* 2019).

Cocoa (*Theobroma cacao* L.) is a perrenial tree crop cultivated worldwide, from the Americas to Asia and Africa. It is cultivated for its beans which serves as raw material for a wide range of uses, including mainly chocolate production with an international trade valued at US $103 billion per year (Rego *et al.,* 2022). In Africa, it is one of the main cash crops in tropical countries (Ivory Coast, Ghana, Cameroon, Brazil, Nigeria, etc.) accounting for over 74.5% of global cocoa production. In Cameroon, its productivity and importance to the country's economy have grown steadily over the past ten years accounting for 5.3% of global production and with over 266,725 tons produced in 2020 (ICCO 2023, ONCC 2023). Cocoa production in Cameroon contributes to the national GDP by 1.2% and generates annual revenue of over $450 million, providing more than 600 000 jobs (Kongor *et al*., 2024). However, cocoa production is being confronted by poor yields globally, particularly due to parasitic attacks leading to losses up to 38% of the annual global cocoa harvest (Marelli *et al.,* 2019).

The most economically important disease of cocoa is black-pod disease, which is caused by *Phytophthora* species that infect the fruits and is responsible for losses of 20-25% on a global scale (Adeniyi, 2019). In Cameroon, black pod disease is caused primarily by *Phytophthora megakarya* Brasier and Griffin (Ndoumbe-Nkeng *et al.,* 2004). Annual losses are estimated at 40% and can reach up to 90% if no proper control measures are taken (Boudjeko *et al*., 2007, Kongor *et al*., 2024). Another huge constraint in the cocoa production chain in Cameroon is the unavailability of healthy seedlings. Seedling production is a key step in the production chain of cocoa and the establishment of new plantations (Téné *et al*., 2019). Parasitic attacks in nurseries and farms can lead to an unavailability of healthy seedlings and consequently a disruption in the cocoa production chain. This can equally have serious repercussions on the quality of the beans produced, resulting in a drop in cocoa prices on the world market.

To counter this problem, several control methods have been set up, including chemical control. This entails the use of chemical fungicides, but the latter creates many problems, including the emergence of resistant pathogens, biodegradation of soil, and concerns about human health that constitute a separate dimension of the problem (Walters *et al*., 2005, Oyekale, 2018). It has also been shown that hybridization is a reliable method of ameliorating cocoa production. In Cameroon, research has been initiated towards genetic control by breeding cocoa cultivars less susceptible to the disease. This involves exploiting field resistance factors to find more resistant hybrids with quality seedlings. However, it has been difficult to find a balanced mechanism between disease-resistant hybrids and higher-quality seedlings (Ondobo *et al.,* 2017).

In today's world, where environmentally friendly approaches are increasingly valued, current options are shifting toward sustainable agriculture, which is based on the use of fewer chemical fertilizers and encourages the use of organic products, thus playing an important role in the fight against environmental and health problems caused using chemical fungicides (Selmi, 2016). Prospects for biological control in phytoprotection have been regularly explored in the eradication of soil-borne disease in recent decades, and its use is widespread in modern agriculture. Worldwide, intensive research on plant growth promoting rhizobacteria (PGPR) is being conducted to develop biofertilizers and biocontrol agents (Tamreihao *et al.,* 2016).

Actinobacteria, among all bacteria, have been reported to comprise several biocontrol agents that suppress plant disease. Actinomycetes produce about 45% of the antibiotics currently in use, among which the genus *Streptomyces* alone produces 73% of those known metabolites capable of suppressing plant diseases (Liu *et al*., 2012). *Streptomyces cameroonensis,* an actinomycete isolated from the *Chromolaena odorata* rhizosphere in Yaoundé (Cameroon), has been shown to exhibit extensive antimicrobial effects against a wide range of microorganisms while also possessing PGPR-like traits (Boudjeko *et al.,* 2017). During cocoa plantlet assays, this strain demonstrated strong abilities to promote plant growth and protect against *P. megakarya*. The mechanism of disease suppression by *S. cameroonensis* involves the production of antibiotics like geldanamycin, the production of cell wall-degrading enzymes, hyperparasitism, the production of volatile compounds, competition, and the induction of host resistance. This strain, like other *Streptomyces* species, is an equally effective root colonizer and can improve plant growth by enhancing iron availability through production of siderophores and production of indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate deaminase activity, nitrogen fixation and solubilization of phosphates (Alexander, 1997; Palaniyandi *et al.,* 2013; Boudjeko *et al*., 2017). These bacteria can also cause Induced

Systemic Resistance (ISR), an important resistance mechanism that prepares plants to respond quickly to pathogenic microorganism attacks (Pieterse *et al.,* 2014). These beneficial microorganisms are also able to control plant pathogens by establishing intimate contact with the host pathogen and attacking their living hyphae (Rey *et al.,* 2008).

Once the biocontrol abilities of this microbe have been proven, the next step is to put them into forms that are easier for agricultural and commercial use. To achieve this milestone, we aim to establish (1) a link between the efficacy of the formulation, (2) the ease and safety of application, (3) the limited eco-toxicological risks, and (4) the mechanism by which we expect this formulation to function. Many biocontrol agents have been formulated in various forms of powder, granules, or liquid. Powder formulations are easy to transport, suitable for easy storage, and have longer shelf lives. Furthermore, the powder formulation can be converted into liquid or water-based suspensions for use in spraying, root-dipping, or seed drenching applications (Vidhyasekaran *et al*., 1997; Tamreihao *et al*., 2016). Liquid formulations based on secondary metabolite extracts have a high potential for suppressing pathogenic attack on plant aerial parts.

Research Questions

- Can *Streptomyces cameroonensis* be formulated into a powder and a liquid formulation with a longer shelf life and effectiveness against plant pathogens?
- Are these formulations able to stimulate cocoa seedlings in a nursery to grow faster and be more resistant to *Phytophthora megakarya*?
- Can these formulations activate the same mechanisms of priming target defense genes in cocoa as freshly prepared cultures of beneficial microorganisms?

General Objective

Evaluate the effect of two formulations of *Streptomyces cameroonensis* on the stimulation of the defense systems of cocoa after infection by *Phytophthora megakarya.*

Specific Objectives

1. Develop and test two formulations based on *Streptomyces cameroonensis* that can be used to treat seedlings in nurseries.
2. Evaluate the effect of the formulations on the growth and resistance of cocoa seedlings in nursery following treatment.
3. Analyze the effect of the formulations on the expression of defense related genes when confronted with *Phytophthora megakarya.*

Literature Review

CHAPTER I : LITERATURE REVIEW

I.1. Generalities on Cocoa

I.1.1. Origin

Cocoa (*Theobroma cacao* L.) is a perennial tropical tree crop that belongs to the Malvaceae family (Whitlock *et al*., 2001). It originated from the humid tropical forests of Central and South America, the Amazon basin, and the Guyana Regions (Almeida and Valle, 2007). Over 50 countries in the world are involved in cocoa production ranging from South America to Asia and Africa being the major zone of culture. Cocoa production was first introduced in Africa in Ghana during the colonial period in the 19th Century. It was introduced in Cameroon by the Germans in 1892 (Nya-Ngatchou *et al*., 1979). Building upon its origins, the classification of cocoa reveals the rich diversity within the *Theobroma* genus.

I.1.2. Taxonomy and Biological Diversity of Cocoa Species

Cacao belongs to the family Malvaceae and the genus *Theobroma* (Whitlock *et al*., 2001). The genus has been classified into 22 different species and are also sub-divided into six sections (Cuatrecasas, 1964). Understanding the taxonomy of cocoa sets the stage for examining the distinct morphological traits that characterize the cocoa plant.

Table I: Section and Species of the genus *Theobroma* (Cuatrecasas).

Sections	Species
Rhitidocarpus	*T. bicolor*
Oeanthes	*T. sylvestre, T. speciosum, T. velutinum, T. glaucum, T. bernouilli*
Theobroma	*T. cacao*
Telmatocarpus	*T. gileri, T. microcarpum*
Glossopetalum	*T. cirmolinae, T. stipulatum, T. simiarum, T. chocoense, T. angustifolium, T. grandifrorum, T. obovatum, T. sinuosum, T. canumanense, T. subincanum, T. hylacum, T. nemorale*
Andropetalum	*T. mammosum*

Cocoa (*Theobroma cacao* L.) is a simple diploid with ten chromosomes ($2n = 2x = 20$) and a small genome, 0.4 pg/1C (Lanaud *et al*., 1992) and it is the most commercially important member of this genus.

Table II: Taxonomy of *Theobroma cacao* L. (Cuatrecasas, 1964).

Kingdom	Plantae
Branch	Spermaphytes
Sub-branch	Angiospermes
Class	Dicotyledonae
Sub-class	Dilleniidae
Order	Malvales
Family	Malvaceae
Genus	Theobroma
Species	*Theobroma cacao* L.

The Theobroma genus comprises of many species of cocoa which can be classified in 3 main morpho-geographical groups, namely: Criollo, Forastero and Trinatario (Ondobo, 2014). This global representation of the genetic diversity in *T. cacao* is still largely used despite its imprecision and all the recent changes in genetic classification (Motamayor, 2001). Despite being the first model of classification, these three groups no longer reflect the correct dimension, structural and genetic diversity of this species. The large diversity of the *T. cacao* species justifies a new analysis of the classification of its genetic diversity as described by the works of Loor Solorzano (2007).

The **Criollos** were originally cultivated in Central America, Mexico, and Venezuela. Nowadays, this variety can be found in Colombia, Madagascar, and the West Indies. The cocoa pods are elongated, with ridges and deep furrows. They are green or purple before maturity and yellow or orange after maturity. The Criollos provide fine and delicate cocoa beans which are white or purple, pale and round; highly aromatic with a slight of bitterness and are used in chocolate industry for the manufacture of luxury products. Despite their great qualities, they represent only 1 to 5% of global production due to their reduced vigor and high sensitivity to black pod disease (Loor Solorzano, 2007; Oro, 2011; Ondobo, 2014).

The **Forasteros** group together all other types of "non-Criollo" cocoa varieties. Most of this variety is cultivated in Latin America, West and Central Africa. This group is particularly diverse, contains genotypes that are very resistant to diseases of cocoa and represents 75 to 80% of global production (Oro, 2011). It is characterised by round pods with shallow furrows, green before maturity and yellow after maturity. The beans are dark violet in colour and flattened in shape (Ondobo, 2014).

The **Trinitarios** (From the Island of Trinidad) are hybrid populations of cocoa which originated from crossings between Criollos. They are cultivated mainly in Central American countries and some countries of South America, Africa, and Southeast Asia. As heterogeneous natural hybrids, they are very resistant to diseases and represent about 20 to 25% of global production (Oro, 2011).

There exist other varieties such as Nacional, Catongo and Capuaçu which are concentrated only in the Americas but rare in the rest of the world. In Cameroon, the most cultivated varieties are Forastero and Trinitario (Blaha et Lotode, 1976) with more than 80 clones. Some of the most cultivated clones and hybrids and their origins are shown in the Table III below.

Table III: Identification and origin of some cocoa clones and hybrids cultivated in Cameroon (Blaha et Lotode, 1976, Ondobo, 2014, Effa *et al.*, 2017).

<table>
<tr><th colspan="2">Genotypes</th><th>Origin</th><th>Collections</th><th>Group</th><th>Sensibility</th></tr>
<tr><td rowspan="8">Clones</td><td>SNK 13</td><td rowspan="3">Cameroon</td><td rowspan="3">Nkoemvone</td><td rowspan="3">Trinitario</td><td rowspan="4">Moderately tolerant</td></tr>
<tr><td>SNK 16</td></tr>
<tr><td>SNK 413</td></tr>
<tr><td>ICS 40</td><td>Trinidad</td><td>/////</td><td rowspan="5">UPA</td></tr>
<tr><td>UPA 134</td><td rowspan="3">Ghana</td><td>Wacri</td><td>Sensible</td></tr>
<tr><td>T 79/467</td><td>Tafo</td><td rowspan="2">Tolerant</td></tr>
<tr><td>T 79/501</td><td>Tafo</td></tr>
<tr><td>SCA 12</td><td>Ecuador</td><td>/////</td><td rowspan="7">Moderately sensible</td></tr>
<tr><td rowspan="8">Hybrids</td><td>SCA 12 × SNK 16</td><td rowspan="8">Cameroon</td><td rowspan="8">Mengang</td><td rowspan="8">UPA x T</td></tr>
<tr><td>SNK 16 x SCA 12</td></tr>
<tr><td>T 79/501 x SNK 413</td></tr>
<tr><td>SNK 413 X T 79/501</td></tr>
<tr><td>UPA 124 X ICS 40</td></tr>
<tr><td>ICS 40 x UPA 134</td></tr>
<tr><td>T 79/467 x SNK 13</td><td rowspan="2">Susceptible</td></tr>
<tr><td>SNK 13 x T 79/467</td></tr>
</table>

SNK= selection Nkoemvone; ICS= Imperial college selection; UPA= Upper amazon; T= Tafo; SCA= Scavina; Trinitario=T; Forastero=F.

I.1.3. Morphology of the Cocoa Plant

With a foundational understanding of the taxonomy of cocoa species, we can now further explore the intricate morphology of the cocoa plant, which is essential for appreciating its growth and development. The cocoa plant is a tree grown from its seeds and its morphology refers to the description of the different parts of the tree including roots, stems, leaves, flower, fruits and the grains or seeds.

The germinating seedling is made up on the one hand of the tap root that penetrates about 2 m deep into the soil and on the other hand of superficial adventitious root system that grows laterally. The tap root can reach a diameter of about 30 to 40 cm in about 4-5 months and 70 to 80 cm in about 5-6 years. At age 10, the entire root system reaches its final development stage with abundant lateral roots distributed superficially on the surface of the humus layer of the soil (Oro, 2001; Adden, 2017). Most of the root system remains confined within the first 50 cm from the soil surface and within a radius of 5 to 6 cm.

After the appearance of the first leaves on the young plant, the terminal bud and the trunk continue to grow vertically (orthotropic axis). The trunk is usually straight with a think bark of greyish brown color. Its diameter on average is 20 cm (Adden, 2017). From the axils of the leaves, one or more axillary buds follow the 3/8 phyllotaxis which can vary from 3/8 to 5/13. The growth in the height of the stem stops at 18 months. The end of the stem shows a characteristic cluster appearance of 5 axillary buds that can give rise to 5 plagiotropic branches forming a crown. During this time, the terminal bud disappears, and the axillary bud grows to give rise to a new orthotropic axis which behaves like the first stem (Oro, 2011).

An average cocoa tree is evergreen with leaves that are 20 to 30 cm long and about 7 to 12 cm wide. Young leaves are soft and tender with varying coloration from very light green to various shades of red depending on the genotype and its anthocyanin content. As they age, these leaves become rigid and brittle (Oro, 2011). The intensity of light affects the number, size, thickness, and chlorophyll content. Thus, the leaves developing under shades are larger and greener than those exposed to direct light (Loor Solorzano, 2007). Being a diploid plant (2n = 20 chromosomes and genome of size 430 Mb), cocoa flowers are small with a varying diameter of 0.5 to 1 cm supported by a peduncle of 3 cm. The flowers are hermaphrodite and pentameric. They are carried on the trunk (caulifloria) or on the branches (ramifloria). They can appear solitary or grouped which can successively small green fruits and finally ripe fruits. The cocoa pods can come in a variety of colors, shapes, texture, and sizes depending on the genotype and origin of the tree. Pods are usually green or red before maturity and yellow, red, or orange when matured. A ripe pod can be 6 to 10 inches long, 3 to 6 inches wide, and its average weight

ranges from 400 to 500 g (Adden, 2007). Each pod is protected by a hard, thick outer cortex and can contain between 20 and 50 beans distributed in 5 to 8 longitudinal furrows (Leon, 1987). Usually unlike most fruits, the pod is indehiscent and stick to the tree and matures between 4 and 6 months after flowering. With a firm grasp of its morphology, we can now explore how the cocoa plant's growth habits influence its geographical distribution across the tropics.

I.1.4. Geographical Distribution of the Cocoa Plant

Cocoa tree is a tropical tree crop that grows in humid zones particularly between Latitudes 20°N and 20°S at an altitude generally less than 1250 m. Cocoa culture is believed to have originated millions of years ago in South America, to the east of the Andes. Recent archaeological findings reveal that cocoa beans were used by the natives of Southern Ecuador 5300 years ago before domestication of the tree. Pre-Colombian civilization including the Mayas (current Mexico, Guatemala, and Honduras), the Incas (current Ecuador, Peru, Colombia, and Bolivia) and the Aztecs (Current Southern Mexico) are some of the civilizations associated with early use of cocoa for food ingredients, beverage and currency for trade or rituals. Cocoa and chocolate drinks were introduced in European civilization around the 14th Century by the Spaniards who brought it from Mexico. To meet European demand, cocoa cultivation was slowly extended during the colonization period to the three main current cocoa producing areas detailed below: Asia, Africa, Latin America, and the Caribbean. Its quick cultivation spread to Central America in the Caribbean Islands, Dominican Republic, Trinidad, and Tobago as well as to South America precisely Ecuador, Brazil, Venezuela, and Colombia. The culture gradually spread from the 16th to the 19th Century to other tropical areas with a humid tropical climate, through SouthEast Asia including Malaysia, Indonesia, India, and Sri Lanka, but also to Papua New Guinea. The 19th and 20th Century saw the expansion of cocoa to West Africa during the colonial era by Europeans. It was first introduced in Principe in 1822, Sao Tomé in 1830 and Fernando Po in 1854, then in Nigeria in 1874 and Ghana in 1879. In Cameroon, cocoa was introduced during the colonial period of 1925 to 1939. Large plantations were later established and are flourishing particularly in Cameroon, Nigeria, Ghana, and Côte d'Ivoire which currently accounts for 77% global production (Adden, 2017; Téné, 2020; ICCO, 2021). With a firm grasp of its morphology, we can now explore how the cocoa plant's growth habits influence its geographical distribution across the tropics. Recognizing the geographical spread of cocoa cultivation allows us to better understand the ecological requirements of the cocoa plant, which are crucial for its successful growth and productivity.

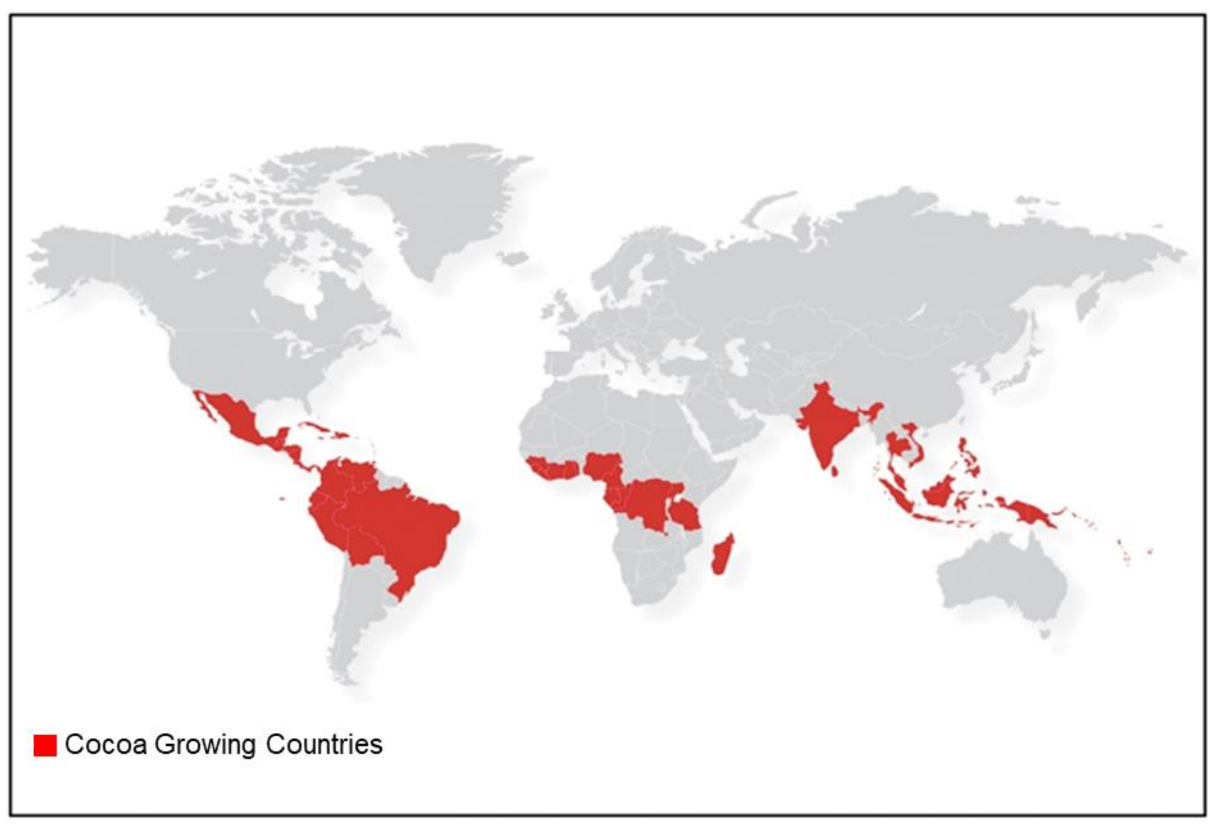


Figure 1: Main cocoa-producing countries in the world (Map from the ICCO).

I.1.5. Ecology of the Cocoa Plant

The cocoa tree grows very well within the equatorial ecological zone (between latitudes 10° north and south). Cocoa grows well in zones with temperatures ranging between 21 to 32 °C. The amount of rainfall is the most important climatic factor that affects cocoa production requiring an average annual rainfall of between 1500 mm to 2000 mm. Cocoa trees are very sensitive to a soil water deficiency and dry spells, where rainfall is less than 100 mm per month, should not exceed three months. Humidity is also a very important climatic aspect for cocoa culture. Relative humidity needs to be generally high for optimal cocoa production often as much as 100% during the day, falling to 70-80% during the night. Availability of light is essential for cocoa trees especially as the trees are naturally grown well under shades. Its natural environment is the evergreen rainforest which provides natural shade trees such as the Amazonian rainforest or the Congo Basin rainforest. Shading is indispensable in a cocoa tree's early years. Reasonable quantity of nutrients is required for soil needed for cocoa growth, with at least a 1.5 m depth of soil rich in coarse materials to allow the development of a good root system and good drainage of excess water. The cocoa tree is sensitive to a lack of water, so the soil must have both water retention properties and good drainage. Cocoa can grow in both

acidic and alkaline soils with a pH in the range of 5.0-7.5. However, excessive acidity (pH 4.0 and below) or alkalinity (pH 8.0 and above) must be avoided. Organic content of topsoil needs to be high enough (3.5% in the top 15 centimetres of soil) and must have certain anionic and cationic balances. Exchangeable bases in the soil should amount to at least 35% of the total cation exchange capacity (CEC), otherwise nutritional problems are likely. The optimum total nitrogen/total phosphorus ($_TN/_TP$) ratio should be around 1.5.

In Cameroon, favourable ecological zones for cocoa culture are in the south where the soil and climatic conditions are proper for cocoa culture. Except for just the two northern Regions (North and Far North), cocoa is cultivated in Adamawa and in all the 7 southern regions (Centre, West, North-west, South-west, South, East, Centre, and Littoral). The centre and the southwest Regions are responsible for more than 80% of the national cocoa production (ONCC, 2017). Having established the crucial ecological conditions necessary for the thriving of cocoa plants, it is imperative to explore the broader implications of cocoa culture, highlighting its economic significance and the pivotal role it plays in the livelihoods of communities, especially in Cameroon.

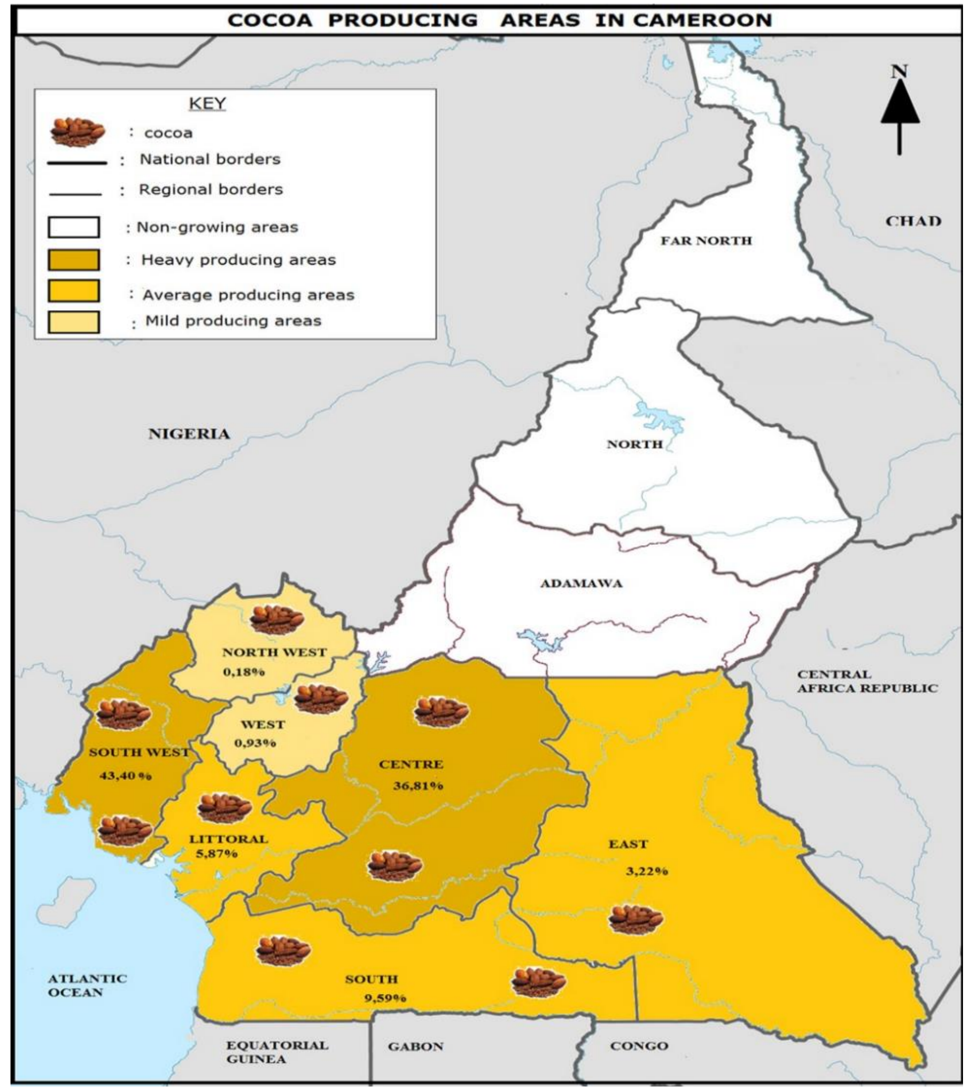


Figure 2: Cocoa Producing Zones in Cameroon (ONCC).

I.1.6. Importance of Cocoa Culture

Significantly, cocoa is one of the main cash crops in tropical countries. Africa alone accounts for 77% of global cocoa production with Cameroon ranked fifth in global cocoa production rankings (ICCO, 2023). Cocoa production in Cameroon contributes to the national GDP by 1.2%, equivalent to 8.2% of agricultural GDP. It generates an annual revenue of over $ 450 million, providing more than 600000 jobs (Lescuyer *et al*., 2019, Kongor *et al.,* 2024). According to ICCO (2021), cocoa production is predominantly carried out by smallholders, as more than 90% of world cocoa farmers have plots of land of between 2-5 hectares. This activity survives hundreds of thousands of households in Cameroon. The 2020-2021 cocoa seasons in Cameroon saw 292.472 tons, 465 kg of the crops traded at the domestic level, with 334.8 billion CFA F as claims of the sector's contribution to the country's economy in 2021. Cocoa is

cultivated for its beans which serve as raw material for food, cosmetic and pharmaceutical industries worldwide. The most economically important product of cocoa is chocolate.

The chocolate industry is valued globally at about US $100 billion (ICCO, 2021). Chocolate is consumed worldwide as a high-calorie food capable of improving a person's general mood and fighting symptoms linked to depression, thus leading to a better quality of life. Research findings also indicate that some components in cocoa can help prevent cardiovascular diseases and reduce the risk of cancer. Additional health benefits include helping with the management of one's weight and providing a source of antioxidants through cocoa fibres or reducing the risk of developing type 2 diabetes through cocoa phytochemical flavanols. There is also emerging evidence which suggests that cocoa and chocolate may contribute to reducing inflammation, better blood flow, lower blood pressure, improving cholesterol and blood sugar level. These beneficial properties originate from another type of phytochemical in cocoa, called polyphenols. Therapeutically, leaves, stems and barks of cocoa trees are used in traditional medicine to treat burns, fever, malaria, rheumatism, snake bites and injuries.

On an exponentially expanding scale is the demand for organic cocoa which is rapidly surpassing supply. Organic cocoa commands a higher price in the world cocoa market than conventional cocoa (AGENCEBIO, 2017). The aim of producing cocoa organically is to establish a production system that is socially, economically, and environmentally sustainable in the long term. This incorporates several principles comprising agroforestry systems, incorporate crop successions, no chemical pest control, no chemical fertilizer, use of bio-fertilizers and biocontrol agents. This strategy benefits the farmer by lowering input costs, increasing their income, decreasing environmental and health risks, as well as increasing the sovereignty of farmers. By 2016, world production of organic cocoa stood at about 3.5 % of global cocoa production and it is expected only to rise to meet the ever-expanding demand. Africa accounts for only 15% of global organic cocoa production. Cameroon does not appear on the list of countries that are engaged in organic cocoa production (IFOAM, 2017). While cocoa culture is vital for economic and social stability, it faces significant challenges that threaten its sustainability and productivity.

I.2. Challenges of Cocoa Cultivation

Unparalleled growth and sometimes decrease in productivity of cocoa is because of several problems that plague this industry. Despite the economic and social importance of cocoa as well as the efforts made to increase productivity, these problems persist. These challenges include declining soil fertility, insufficient or lack of healthy cocoa seedlings, outdated production systems, ageing of plantations, changing weather and environmental effects, declining prices in the world market, more importantly parasitic attacks by fungi, oomycetes, viruses, and insects.

I.2.1. Pests and Diseases of Cocoa

Cocoa is affected by a range of pests and diseases, with some estimates putting losses as high as 30% to 40% of global production (ICCO, 2021). These parasitic attacks include the following biotic factors.

- Witches broom caused by the fungus *Moniliophthora pernicosa* predominant in Central and South America. This fungus attack only actively growing tissue (shoots, flowers, and pods) causing cocoa trees to produce branches with no fruit and ineffective leaves. The pods show distortion and present green patches that give the appearance of uneven ripening.
- Frosty pod rot caused by basidiomycete *Moniliophthora roreri*. It is found in all north-western countries in South America. The fungus infects only actively growing pod tissues, especially young pods. The time from infection to the appearance of symptoms is about 1-3 months. The most outstanding symptom is the white fungal mat on the pod surface.
- Attacks from destructive insects such as mirids which are the major insects that affect cocoa worldwide. The most common species in Ghana and West African countries are *Distantiella theobroma* and *Sahlbergella singularis*. Mirid damage alone, if left unattended for three years, can reduce yields by as much as 75%. Cocoa mirids pierce the surface of cocoa stems, branches and pods, killing the penetrated host cells and producing unsightly necrotic lesions. Mirids feeding on shoots often result in the death of terminal branches and leaves, causing dieback.
- *Phytophthora* pod rot also called black pod is caused by the fungus *Phytophthora* spp. which is widespread all over the world but especially in Africa where some species of

Phytophthora are specific. Three fungal species of the same genus responsible for this disease include:

- *Phytophthora palmivora* present in tropical and sub-tropical zones hence present all over the cocoa producing regions of the world and it causes global yield loss of 20-30% as well as tree deaths of 10% annually.
- *Phytophthora capsici* widespread in Central and South America, causing significant losses in favourable environments.
- *Phytophthora megakarya* only present in Central and West Africa principally Cameroon, Nigeria, Ghana, and it is the most virulent, therefore responsible for greater losses.

Importantly, *Phytophthora megakarya* has been identified as the only species of *Phytophthora* in Cameroon responsible for black pod disease (Nyasse, 1992). It has been reported that this parasite most have been transmitted from a forest host, possibly *Irvingia* (Holmes *et al*., 2003) unto the cocoa plant. Obvious symptoms are the rotting or necrosis of pods. Pods can be attacked at any stage of development, and the initial symptoms are small, hard, dark spots on any part of the pod. Internal tissues, including the beans are colonized and shrivel to form a mummified pod. In addition to pests and diseases, the insufficiency of resistant seedlings poses a significant barrier to improving cocoa production.

I.2.2. Insufficiency of Resistant Seedlings

Unavailability of sufficient and healthy cocoa seedlings has been identified as one of the most important challenges to cocoa production in several cocoa producing countries including Cameroon (Téné, *et al.,* 2019). The seeds are at the epicenter for the establishment of a cocoa plantation. According to Téné *et al.*, (2019), the market for cocoa seedlings is highly unbalanced with demand far surpassing supply. This thus puts a big dent in the cocoa production chain affecting the general outcome. This shortage can be attributed to poor farming practices by farmers using soil with heavy loads of microbial pests and unverified beans for nurseries. Establishing a successful production chain for seedlings that aren't only healthy but resistance to pathogenic attacks and in sufficient numbers for the revitalization of already ageing plantations thus becomes a priority.

I.3. Generalities on *Phytophthora megakarya*

I.3.1. Origin, Taxonomy and Biology

I.3.1.1. Origin

The genus *Phytophthora* was first described in 1875 by Anton de Barry who explained the life cycle of *Phytophthora infestans*, the pathogen that attacks potatoes and eventually laid the foundation for the plant pathology (Matta, 2010). *Phytophthora megakarya* was first described by Brasier and Griffin (1979). This pathogen appears to be confined to West and Central Africa specifically in Nigeria and Cameroon. In Cameroon, it is the only species responsible for black pod disease of cocoa (Nyasse, 1992). Understanding the taxonomy of *P. megakarya* is critical for implementing effective management strategies against its impact on cocoa production.

I.3.1.2. Taxonomy

- Kingdom: Chromista
- Phylum: Oomycota
- Class: Oomycetes
- Order: Peronosporales
- Family: Peronosporacea
- Genus: *Phytophthora*
- Species: *Phytophthora megakarya*

Exploring the biology of *P. megakarya* further illuminates its pathogenicity and the mechanisms by which it infects cocoa plants.

I.3.1.3. Biology

Like all Oomycetes, *P. megakarya* is a diploid organism possessing two flagella, a property that mimics algae rather than fungi. However, alga can be distinguished from bacteria by the presence of green pigment chlorophyll. According to Brasier and Griffin (1979), *P. megakarya* is a member of Group II of *Phytophthora*. Sporangia are limoniform, obpyriform or ellipsoid with rounded bases, varying from 20-60 x 13-41 µm, with a length-breadth ratio of 1.2-1.6 and are formed in a sympodium. Oogonia are produced in paired cultures of A_1 and A_2 compatibility types only. The A_1 compatibility type is most frequently isolated. Oogonia range in size from 19-37 (av. 27) µm and taper to a funnel-shaped base at the oogonial stalk. Antheridia are amphigymous, spherical, averaging 13 µm long. Oospores are plerotic 23-28 µm diameter with a wall thickness ranging from 1.5-3 µm. The vegetative organ of *P. megakarya* is the filamentous thallus formed from coenocytic mycelia present on the ramified

hyphae. Oomycetes are organisms that do well in humid conditions and possess a cell wall rich in chitin.

Phytophthora can multiply sexually or asexual (Attard *et al.*, 2008). Asexual reproduction occurs frequently under natural conditions (Nyasse, 1997). It involves production of zoospores that are highly pathogenic. During unfavorable conditions, *P. megakarya* produces thick-walled chlamdospores that serve as conservation structures in soil and plant debris (Erwin and Ribeiro, 1996). When conditions become favorable, the sporangia proliferate, liberating zoospores that swim in water by chemotaxis towards the target organs for infection within a period varying between a few minutes to some hours (depending on temperature and pH of the medium) after which they encyst (Judelsen and Blanco, 2005). Thirty minutes after encystment, germination starts, and the hypha penetrates the host tissue within 48 hours under very humid conditions marking the starting point of the infection process.

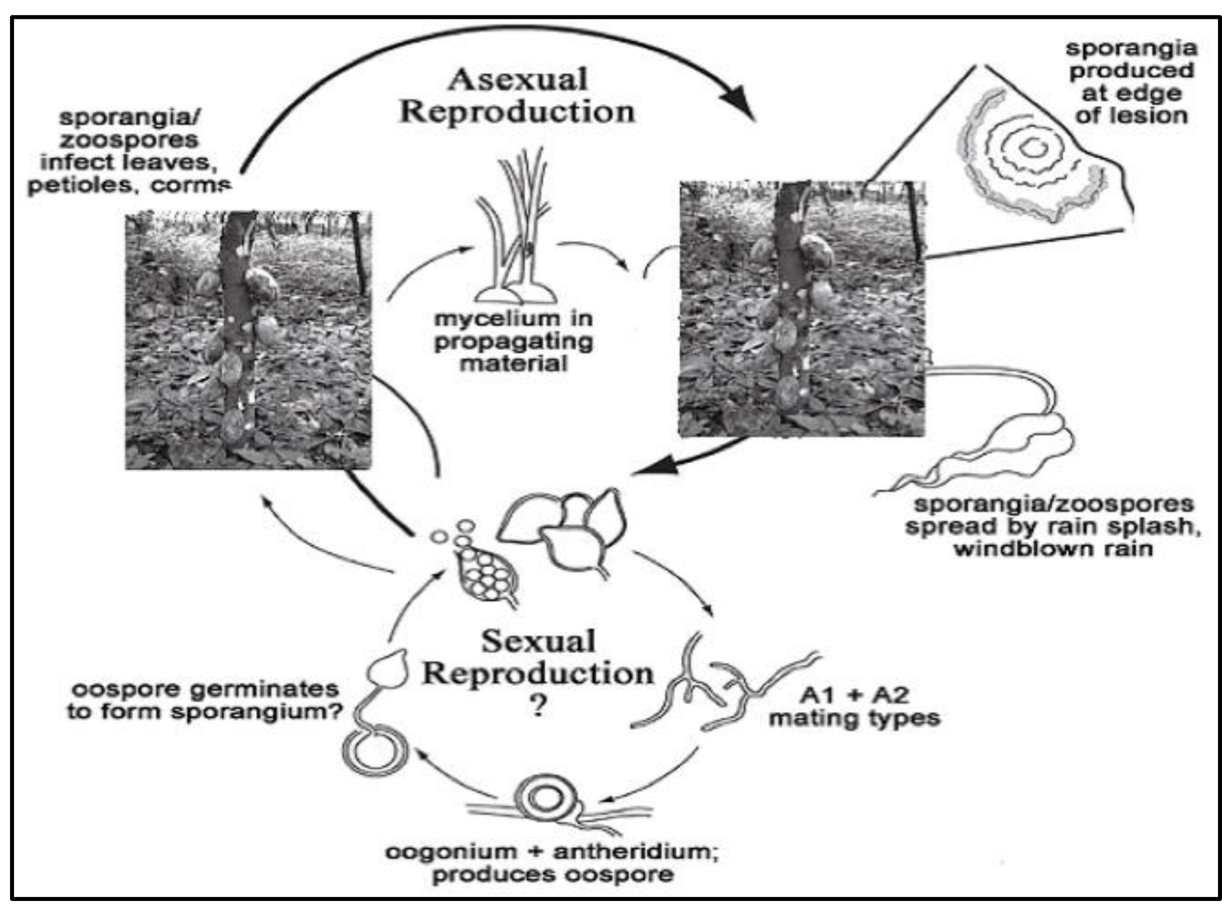


Figure 3: The life cycle of *Phytophthora* species (Adapted from Hardham, 2005).

The role of sexuality in nature in producing oospores is unclear. However, Sexual reproduction occurs only during favorable environmental conditions. Here, there is formation of spherical oocytes by fusion of antheridia with the oogonia under the control of mat hormones (Campbell and Husband, 2005). The gametes fuse in the presence of sterols leading to the formation of spherical thin-walled oospores (Figure 3). These oospores can resist adverse conditions in nature in the absence of the host. Having explored the biological characteristics of *Phytophthora megakarya*, it is crucial to understand its implications for cocoa crops, particularly through its host-parasite interactions.

I.3.2. Host-Parasite Relationship

I.3.2.1. Epidemiology, Symptoms and Development of Black Pod Disease

Black pod disease of cocoa in Cameroon is caused almost exclusively by *P. megakarya* and remains one of the most serious constraints on cocoa production. The disease is clearly polycyclic and the structures of the oomycete responsible for disseminating the parasite include hyphae, caduceus sporangia and spores (Gidoin, 2013). Sporangia form on the surface of infected pods at relative humidity in the range 60-80% and temperatures between 20-30 °C (Gregory,1983). Sporangia can germinate directly via a germ tube or indirectly to release about 30 zoospores. The primary source of inoculum is the soil, however root infection maintained a reservoir of inoculum, allowing zoospores to be released into the soil surface water (Gregory and Madison, 1981). From there, the zoospores spread up the plant by small splash droplets in convection currents into the leaf canopy. Generally, pods closest to the ground are first infected, with the disease rapidly spreading to affect fruit on the entire tree. Insects, particularly the small black ant (*Crematogaster striatula*), are also responsible for moving inoculum from the soil to the canopy (Evans, 1973). These ants also use old, infected pods to construct tents around the pod peduncle, and this can lead to infection from the peduncle region.

Several parts of the cocoa tree, namely the trunk, the young branches, the beans and the leaves can be affected; but the attack of the disease is readily recognized through the presence of a brown to black spot on the pod, which eventually spreads to encompass the entire pod. Under conditions of high humidity, a white bloom comprising fungal mycelia and sporangia may be present on the surface. In advanced stages, the fungus invades the internal tissues of the pod, including the seeds and diseased pods eventually mummify. *P. megakarya* can also cause seedling blight and trunk cankers, but its capacity to cause root rot is equivocal. Pods are susceptible at all stages of development and may be infected at any place on the surface. Most of the attacks are in the apical zone or in the peduncular zone of the fruit where rainwater

stagnates. Attacks on the lateral region can be made at the point where the pod is in contact with the trunk or with another pod. The first symptom is a brown to black spot on the pod, which spreads rapidly in all directions and eventually covers the whole pod. The beans become infected internally about 15 days after the initial infection and are soon of no commercial value. The attack on leaves is mostly limited to nurseries (Téné, 2020). There is also a possibility of infection by flower pads (Babacauch, 1980), mostly by insects. Understanding the epidemiology and symptoms of black pod disease sets the stage for examining the specific mechanisms of parasitism employed by *Phytophthora megakarya*.

Figure 4: Cocoa tree showing **(a)** symptoms of black pod diseas, **(b)** Infected leaf and **(c)** Internal rotting partially or completely damages the seeds [Photos **a** et **c** (Phillips-Mora, 2009); Photo **b** (credit Dzelamonyuy, 2015)].

I.3.2.2. Parasitism of *Phytophthora megakarya*

Phytophthora megakarya employs both sexual and asexual spores (sporangia) for dissemination and host infection. Colonization by most oomycetes begins when an asexual sporangium releases zoospore, which encyst and form a germ tube (Figure 5). Many aspects of spore behavior are influenced by plant signals. Host signals can be sensed by asexual sporangia since they are fully hydrated and metabolically active prior to germination, unlike most fungal

spores which are desiccated (Judelson and Ah-fong, 2019). As water molds, most oomycetes prefer to grow in moist environments such as the apoplast. Entry into the plant may occur when zoospores or germ tubes pass through stomata or other natural openings, transit through wound or actively grow between root epidermal cells by lysis using parasite-produced hydrolases. The appearance of necrotic spots on the surface of the epidermis characterizes the invasion of the plant by the parasite. The plant's system reacts through a sophisticated mechanism involving the binding of pathogen-associated molecular patterns (PAMPs) to plasmalemma-spanning pattern recognition receptors (PRRs) such as plant's Receptor-Like Protein Kinases (RLKs) which activates PAMP-triggered immunity (Saijo *et al*., 2018) involving the salicylic acid (SA) and jasmonic acid pathways as well as constituents of the cell wall or plasma membrane. During this phase, defense molecules are delivered to plant-oomycete interfaces, including pathogenesis-related (PR) proteins, callose for thickening cell walls and microbial toxins.

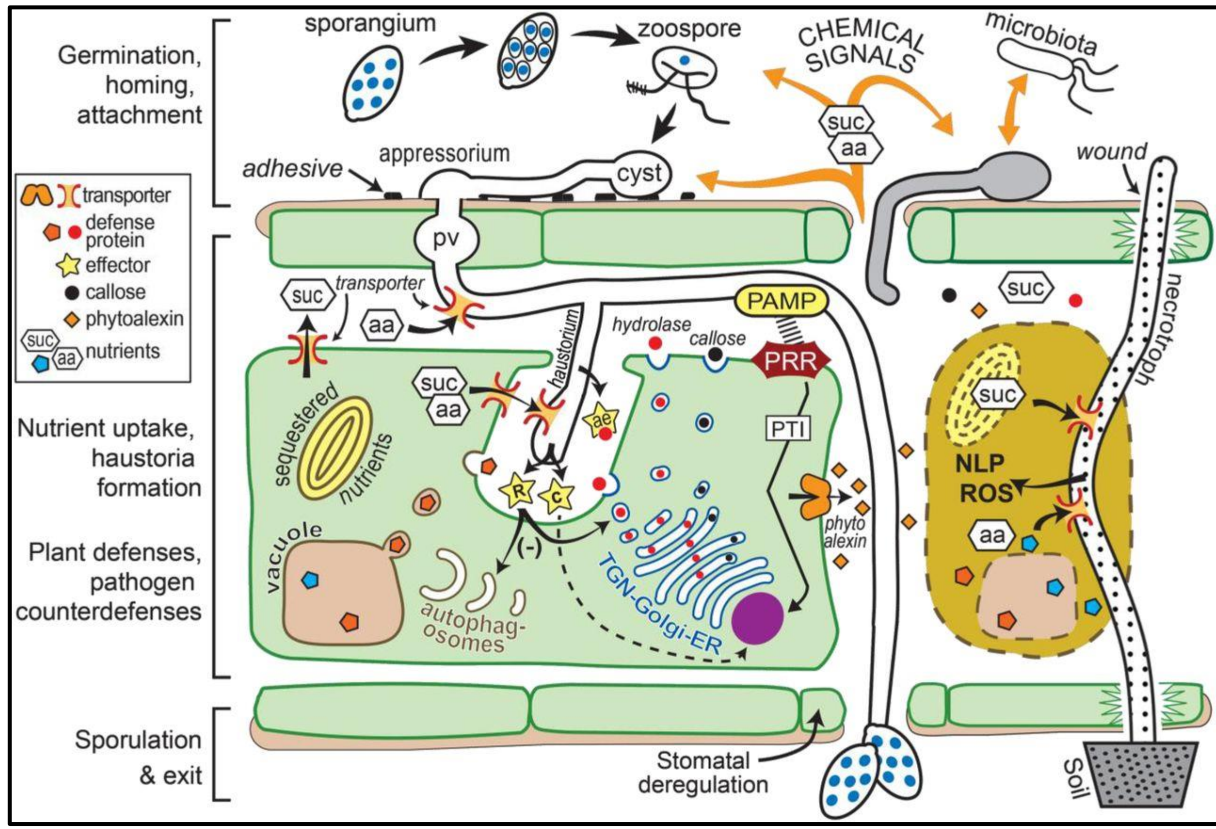


Figure 5: Interactions at plant-oomycete interfaces (Judelson and Ah-fong, 2019).

Effector triggered immunity reinforces and expands these responses and often leads to hypersensitive cell death. Reactive oxygen species (ROS) are also delivered to plant-*P. megakarya* interfaces through several pathways. These defenses may combine to produce apoplastic (or intracellular) environments that are unfavorable to *P. megakarya* (Judelson and

Ah-fong, 2019). The pathogenic strategies of *Phytophthora megakarya* highlight the urgency for effective control measures to combat black pod disease.

I.3.3. Methods of Control against *Phytophthora megakarya*

In Cameroon, black pod disease stands out as one of the most economically destructive diseases of cocoa (Ondobo *et al.,* 2017) since *P. megakarya* appears to be confined to West Africa. Annual losses are estimated at 40% and could potentially reach up to 90% if no proper control measures are taken (Boudjeko *et al.,* 2007). The control of black pod disease is a major challenge for world cocoa production. It is therefore essential to implement strategies to limit its spread, particularly in nursery material. Several methods have been adopted by stakeholders and farmers to control disease caused by *Phytophthora* species in cocoa. The most used control methods include cultural practices, biological, genetic control, and chemical control.

I.3.3.1. Cultural Practices

Inoculum levels of *P. megakarya* are rapidly reduced in the absence of the host, and cocoa is the main known host. This affords opportunities to limit the spread through ensuring that disease-free nursery material is planted when biologically controlled material is used for propagation. Frequent and complete harvesting, farm sanitation and appropriate disposal of pod mummies, infected pods and pod husks can reduce the disease (Akrofi *et al*., 2015). Improved control is also obtained by avoiding bare earth (reducing thus spore splash) within the plantation. Management of the amount of light entering the canopy is also critical, to ensure improved aeration and to promote the drying of the pod surface. Shade is critical in young trees to promote development of the most productive canopy shape. Clear felling of jungle, followed by planting of temporary and permanent shade trees, allows more effective regulation of light. These practices are cheaper but their efficiency in the field is limited. Hence, they must be associated to with other control strategies for optimal control. While cultural practices lay the groundwork for disease management, the integration of genetic control methods is vital for long-term sustainability

I.3.3.2. Breeding for Resistance to Disease (Genetic Control)

It is generally believed that the replacement of susceptible cultivars by breeding for ones showing durable resistance to the pathogen is one of the most effective and sustainable control methods of black pod disease (Iwaro *et al.,* 2000). So, for a long time, a lot of hope has been placed on the selection and multiplication of cocoa plants resistant to black pod disease

(Pokou *et al*., 2008). However, it has been difficult to find a cultivar that has been completely resistant to *P. megakarya*. In addition to breeding for resistance, chemical control methods offer another layer of defense against the pathogen.

I.3.3.3. Chemical Control

The chemical control method is generally the most used through application of chemical fungicides (mainly copper-based fungicides), very expensive for small scale farmers and only economically viable when disease levels are extremely high. It can be an effective way of controlling and delaying the development of these fungal diseases. Phenylamide type fungicides (Metalaxyl) are the most widely used. This type of molecule interferes with RNA biosynthesis; by targeting RNA polymerases, enzymes present in all living organisms (Jespers *et al*., 1994). However effective this method is, it has a lot of disadvantages such as necessity for frequent application, high cost of fungicides, pollution, toxicity, accumulation of these products and sub-products in different links of the food chain and resistance developed by pathogens against the most used fungicides. Therefore, fungicidal control should be limited to suit only situations where other control methods are futile. While chemical control remains a common approach, biological control presents a promising alternative with unique advantages.

I.3.3.4. Biological control

Biological control refers to the purposeful utilization of introduced or resident living organisms other than disease resistant host plants, to suppress the activities and populations of one or more plant pathogens (Pal and Gardener, 2006). This may involve the use of microbial inoculants to suppress a single type or class of plant diseases; or this may involve managing soils to promote the combined activities of native soil- and plant-associated organisms that contribute to general suppression (Haas and Defago, 2005). More broadly, the term biological control has also been applied to the use of natural products extracted or fermented from various sources. While such inputs may mimic the activities of living organisms, non-living inputs should more properly be referred to as bio-pesticides or bio-fertilizers, depending on the primary benefit provided to the host plant (Pal and Gardener, 2006).

In recent decades, the use of biological is extensive in modern-day agriculture. Many biocontrol agents have been used to control black pod disease such as the use of *Trichoderma asperellum* (Tondje *et al*., 2007), *Arbuscular mycorrhizal* fungi (Tchameni *et al*., 2011), actinomycetes such as *Streptomyces cameroonensis* (Boudjeko *et al*., 2017) as well as endophytic fungi isolated from cocoa leaves. Intensive research on plant growth-promoting

rhizobacteria (PGPR) is being conducted worldwide to develop bio-fertlilizers and biocontrol agents that can be used to enhance biological control and improve plant protection. Nevertheless, biological control should not be considered as the negation of chemical control, but it should be complementary to the latter. As biological control methods gain prominence in the fight against diseases like black pod disease, the exploration of *actinomycetes and Streptomyces*, potential biocontrol agents is critical to understanding their role, properties and mechanism of action.

I.3.3.5- Natural control

Natural control of black pod disease in cocoa, often referred to as integrated control, involves a synergistic combination of cultural practices, biological agents, resistant varieties, and, when necessary, chemical interventions. This multi-pronged strategy reduces pathogen inoculum, modifies the crop environment to deter *Phytophthora* spp., and strengthens plant defenses, all while minimizing reliance on synthetic fungicides. When effectively implemented, each component complements the others, enhancing overall disease suppression and aligning with sustainable cocoa farming systems (Peter and Chandramohanan, 2014; Thorold, 1959; Umaharan, n.d.). The strength of this approach lies not in any single tactic, but in its integration—creating a resilient, adaptive framework for long-term disease management in diverse agroecological settings.

I.4. Generalities on Actinomycetes and *Streptomyces*

I.4.1. Definition and Principal Characteristics of Actinomycetes

The term actinomycete was first used by Bollinger in 1877 to designate the causative agent of a livestock disease, coined from the Greek word «aktino, mycetes» signifying «radiating fungi». These microorganisms have long been rejected from being considered as bacteria and have been confused with the fungi because of the mycotic appearance of the diseases they cause (Gazenko *et al*., 1998) and due to their fungi-like morphology (branched filaments), as well as their sporulation organs (Reponen *et al*., 1998). Nowadays, this problem has been resolved, and this group of microorganisms is classified amongst bacteria (prokaryotes) because of their physiological, genetic, immunologic, and chemical properties.

Their cell wall contains neither chitin nor cellulose but a glycoprotein containing lysine or diaminopimelic acid and their cytology is that of bacteria (Mohamed, 2010). Actinomycetes do not have a nuclear membrane. They have flagella resembling those of bacteria. The diameter of their mycelia is approximately one-tenth that of most fungal hyphae (usually 0.7–0.8 µm) (Prescott *et al*., 2010) and they are mostly sensitive to lysozymes and antibacterial agents.

Actinobacteria are gram-positive bacteria characterized by a genome with a high chargraff coefficient (G+C) generally between 60-75% (Mohamed, 2010). They are ubiquitously distributed in all aquatic and terrestrial ecosystems (Barka *et al*., 2016) and consist of a wide variety of bacteria widely dispersed in the ecosystem. Among the most common genus are *Nocardia, Actinomyces, Mycobacterium, Corynobacterium, Streptomycetes and Bifidobacterium,* all having very similar characteristics (Mariat and Sebald, 1990). Their average growth time of 2 to 3 hours is slower than that of other bacteria. On solid media, actinomycetes form within a week, pigmented colonies (grey, green, red ...) coming from the accumulation of branched hyphae with a smooth or indented outline with a compact appearance.

Generally, actinomycetes are heterotrophs, but several species are capable of chemo-autotrophic growth (Ensign *et al*., 1993). Some have nutritional requirements such as vitamins and certain amino acids. They can degrade proteins, cellulose, and other organic materials such as paraffin (Hernandez-Coronado *et al.,* 1997) as well as plant residues in soil. Actinomycetes prefer a neutral or slightly alkaline pH and they are generally mesophilic while others are thermophilic tolerating temperatures ranging from 50 °C to 60 °C (Mohamed, 2010). Having established the foundational definition and principal characteristics of actinomycetes, we now turn our attention to their taxonomy, which has undergone significant evolution over the past four decades, further refining our understanding of these diverse microorganisms.

I.4.2. Taxonomy of Actinobacteria

Classification of actinobacteria has been evolving for the past 40 years. In 1964, a study called "International *Streptomyces* Project" (I.S.P) was employed to evaluate the characteristics of actinobacteria (Shirling and Gotlieb, 1969). The criteria used for the identification of actinomycetes in their respective genus and species are based on the ecological, morphological, cultural, and physiological aspects.

The main morphological criteria considered to determine the family of an actinomycetes strain includes the presence or absence of a true mycelium, the production or not of an aerial mycelium, the color of the aerial mycelium, the fragmentation or not of the vegetative mycelium and its color, production or not of special structures such as sporangia, sclerotia and zoospores. Indeed, *Streptomyces* are divided into seven groups based on the color of the aerial mycelium, that is: "white, gray, red, yellow, blue, green, or purple.

The other criteria that have been used are based on the chemo-taxonomic properties such as the composition in amino acids and carbohydrates and the composition of their cell

membranes. Some examples of cellular membrane constituents include LL-diaminopimelic acid (LL-DAP) and glycine for *Streptomyces,* meso-diaminopimelic acid and glycine for *Micromonospora*, Lysine and ornithine for *Actinomyces* etc.

Recently, digital taxonomy, which involves the combination of computer tools and physiological tests to differentiate between species of the same genus, has been used to classify actinobacteria (Smaoui, 2010). The degrees of similarity between individual species are represented in the form of a dendrogram, making it possible to bring together similar species in the same class (Prescott *et al.,* 2003). Other modern tools widely used to classify actinobacteria include the use of molecular biology techniques based on 16S RNA analysis and DNA/DNA hybridization (Maidak *et al.,* 1999).

Following the above-mentioned criteria, actinobacteria are classified in the kingdom prokaryotes, division Firmicutes, class of Thallobacteria (Gram-positive bacteria), in which we find the order Actinomycetales (Murray *et al*., 1989; Ouhdouch *et al.*, 2001). The actinomycete group currently includes more than 40 genera and a few hundred species (kitouni, 2007), principal among which we have the genus *Streptomyces.* With a clearer taxonomy in place, we can now focus on the genus *Streptomyces*, which represents the most abundant and ecologically significant group within the actinomycetes, accounting for over 90% of these bacteria found in nature.

1.4.3. Presentation of the genus *Streptomyces*

The genus *Streptomyces* represents more than 90% of actinomycetes present in nature, and it is the most widespread (Bastide *et al*., 1986; Thakur *et al.*, 2007). Actinomycetes of the genus *Streptomyces* are aerobic, Gram-positive, non-acid-alcohol resistant, catalase-positive, filamentous, and spore-producing bacteria. Most *Streptomyces* inhabit the soil as saprophytes. Their sole source of carbon is from organic compounds and nitrogen is obtained in mineral form. Filamentous, they form a highly branched mycelium which rarely fragments. When mature, the aerial mycelium produces chains of spores of varying lengths. The colonies of spores formed are round, powdery, often colored, and slightly embedded in the agar. The colorings of the spores/colonies are very varied, and this can be explained by the presence of pigments of very different natures. The pigmentation is most often different between the substrate mycelium and the spores. As a result, the coloration is rarely homogeneous at the colony level. In addition, diffusible pigments may be present, thus the coloration is pH sensitive. Seven different series of colors can be identified depending on the type of spores. These include gray (from gray to brown), white, red (bronze, pink and pale pink), yellow

(yellowish to yellow greenish), blue (bluish to blue-grayish pale), green (greenish to pale greenish grey) and violet (Mohamed, 2010). Many *Streptomyces* strains produce antibiotics and most of their species especially those with abundant and vegetative mycelia have an earthy or musty odour, sometimes fruity. They could be due to the presence of organic amines (soluble in ether) which would be more abundant in media containing glycerol.

Streptomycetes are abundant in humus soils rich in decomposing organic matter as well as the rhizosphere of cultivated plants (Mohamed, 2010). Most *Streptomyces* species flourish at an optimum pH of 6.5-8.5 and temperatures between 25 °C and 35 °C. Understanding the characteristics of the genus *Streptomyces* lays the groundwork for examining its life cycle, which is a fascinating process that underscores the complexity of these microorganisms and their ecological roles.

I.4.4. Life Cycle of *Streptomyces*

The life cycle of Streptomycetes starts when a spore settles in a nutrient rich medium. This stimulates the spore to exit its dormant state, undergo germination and form germ tubes, that grow by tip elongation and the cells don’t undergo binary fission. Through extension and branching, the germ tubes give rise to a network of filaments which grow into and across the surface of an agar plate. This network is called the substrate mycelium (Flärdh and Buttner, 2009). As the colony continues to grow, the mycelium in the center of the colony starts to differentiate. Differentiation results in the formation of a new cell type, the aerial hyphae. When growth of the spiraling, multi-genomic aerial hyphae stop, the aerial hyphae undergo synchronous cell division giving rise to monoploid compartments each of which will develop into a resistant spore (Kieser *et al*., 2000). The life cycle of a *Streptomyces* sp. has been studied by the Robinow HC1-Giemsa method of nuclear staining and is described in the subsequent manner: (1) initial nuclear division phase, (2) primary mycelium, (3) secondary mycelium (including aerial) and (4) the formation of spores. The primary mycelium develops after the initial nuclear division phase and produce side divisions, then later gives rise to single swellings in the hyphae. These swellings grow to form large round cells, each of which contains many nuclei. The secondary mycelium develops, a part of which become aerial and terminates in the form of chains of spores (Mc Gregor,1954). As we explore the life cycle of *Streptomyces*, it becomes evident that their habitat plays a critical role in their development and ecological function, providing the necessary nutrients and conditions for their growth.

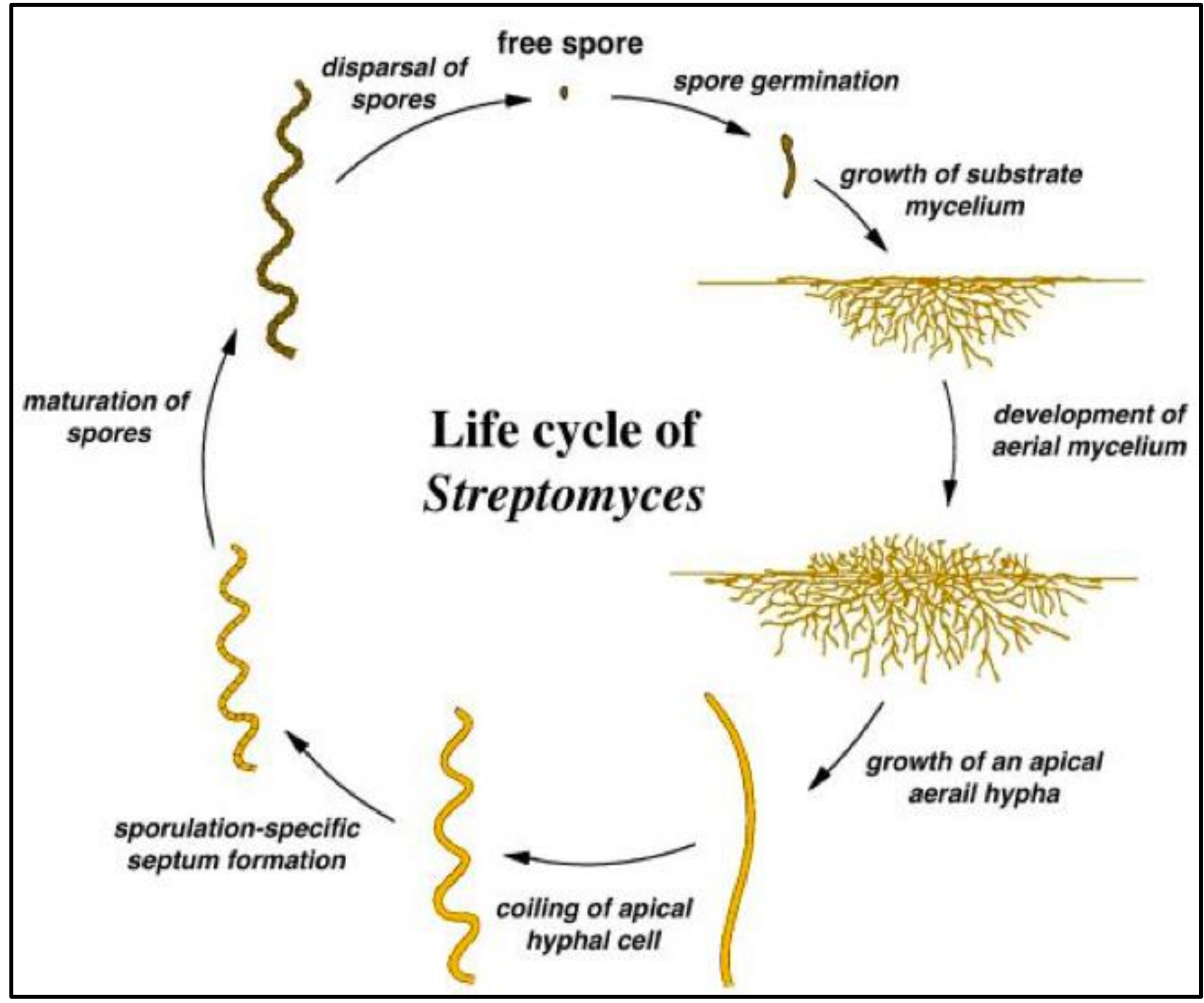


Figure 6: Schematic representation of life cycle of *Streptomyces* adapted from Barka *et al.* (2016).

I.4.5. Habitat of *Streptomyces*

Streptomyces are ubiquitous microorganisms, widely distributed in different natural habitats including different types of soil, fresh or salty water, plant tissues, antarctic sub-glacial ice, rivers' sediments, and bottoms of lakes where they play an important role in the decomposition of plant debris and give the water its earthy smell and flavor (Boudjeko *et al.,* 2017; Gos *et al.,* 2017). However, they are particularly abundant in the soil, especially in the alkaline soils and soils rich in organic matter where they constitute a significant part of the microbial population (Djuidjé, 2020).

Among actinomycetes present in the soil, Streptomycetes make 40% of soil bacteria (Boone *et al.,* 2001) and 95.30% of the isolation frequency (Mohamed, 2010). *Streptomyces* are therefore very abundant in the soil and play a crucial role in nutrient recycling (Kinkel *et*

al., 2012) and are widely studied because of their ability to produce antibiotics and many other bioactive secondary metabolites (Davelos *et al.,* 2004).

I.4.6. Importance of Streptomycetes

Understanding the ecological significance of Streptomycetes sets the stage for recognizing their broader importance

I.4.6.1 Ecological Importance of Streptomycetes

Streptomycetes like most actinomycetes are present in every ecological niche. They play an essential role in soil ecology. The main ecological function of Streptomycetes within ecosystems is the decomposition of organic substances (Prescott *et al*., 2010). This can be attributed to their ability to produce a wide range of hydrolytic enzymes, such as proteases, nucleases, lipases (Prakash *et al.,* 2012), and enzymes capable of hydrolyzing complex sugars like cellulose and hemicellulose (Maier *et al*., 2009). At the level of the rhizosphere, actinobacteria form symbiotic relationships with plant roots, while promoting their growth through direct and indirect effects (Barreto *et al*., 2008). The direct effects include phosphate solubilization, nitrogen fixation and production of phytohormones (El-Mehalawy *et al*., 2004). The indirect effects are the control of pathogens through the production of antibiotics (Barreto *et al.,* 2008), or through competition for nutrients or space (Getha *et al*., 2005). Other studies have also focused on the isolation of new and rare actinomycetes strains for use either as biological control agents or as biopesticides (Toumatia *et al.,* 2010). In addition to their ecological roles, the biotechnological applications of Streptomycetes further emphasize their significance.

I.4.6.2. Biotechnological Importance

Streptomyces like other actinobacteria is of great importance in the field of biotechnology especially in the field of antibiotics. Actinobacteria are prolific antibiotic producers, which produce about 45% of the antibiotics currently in use (Liu *et al*., 2012). They produce diverse natural products that accounts for approximately 10,000 compounds (Liu *et al.* 2012). The genus *Streptomyces* alone accounts for over 70% of the antibiotics produced by these actinomycetes (Hassan *et al*., 2011). Streptomycetes have high potential to produce secondary metabolites such as antibiotics (MCIntyre, 2002), anthelminthic enzymes, herbicides (Kariminik and Baniasadi, 2010), anti-cancer drugs (Berdy, 2005), growth factors like vitamin B12 (Bibb, 2005) and immuno-modulators (Mann, 2001). They produce over two-

thirds of the clinically useful antibiotics of natural origin (e.g., neomycin and chloramphenicol). Nowadays, 80% of the antibiotics are derived from *Streptomyces* sp. (Kharat *et al.,* 2009). Some of the examples of antibiotics produced by *streptomyces* include geldanamycine, cycloheximide, streptomycine, etc (Palaniyandi *et al.,* 2013). Streptomycetes also produce other molecules that have many biotechnological applications such as antitumors, antivirals, antiparasitics and biopesticides (Wang *et al.,* 2015).

Streptomycetes are also important sources of extracellular enzymes of agricultural and industrial interest (Saugar *et al*., 2002; Basilio *et al.*, 2003). Several enzymes can also be produced by *Streptomyces* such as chitinases (*Streptomyces viridificans, Streptomyces violaceusniger* XL-2), cellulases (*S. albidoflavus*), protease, phospholipase, etc (Djuidjé, 2020).

They also play an important role in improving the agricultural quality of soil (Demain and Sanchez, 2009). They are known to be sources of important agro-active compounds which are important in the breakdown of cellulose and chitin (Tyc *et al*., 2017). Streptomycetes like other actinomycetes are increasingly used in biotechnology to produce biofertilizers that increase the supply or availability of primary nutrients to the host plant and stimulate plant growth through the production of growth promoting substances (Doolotkeldieva *et al*., 2015). They equally contribute to the maintenance of soil particles, provide for water infiltration, and thus allow for good aeration thanks to their filamentous structure and their ability to produce polysaccharides (Kennedy, 1999). In addition to soil structure and fertility, *Streptomyces* increase the suppressive power of the soil.

I.4.7. Mechanisms of Action of the *Streptomyces* in Plant Defense

The diverse applications of *Streptomyces* as both ecological and biotechnological agents lead us to examine their specific mechanisms of action in plant defense. The mechanisms by which microorganisms (*Streptomyces*) operate as biocontrol agents can be classified into direct effects against the pathogenic agent, effects on pathogenesis and indirect effects through the plant.

Among the direct effects of the pathogen, **antibiosis** is probably the oldest known mechanism. This is the production of toxic metabolites by the biocontrol agent against the pathogen. Several *Streptomyces* strains such as *S. griseoviridis* (K61), *S. corchorusii* (UCR3-16) are some known biocontrol agents presently used based on the antibiose effects (Nicot, 2002; Tamreihao *et al*., 2016). Antibiosis by biocontrol agents allows for both preventive and curative use of their rapid action that destroys the pathogen or blocks its development.

Another direct mode of action of *Streptomyces* that is specific to the microbial agent is **hyperparasitism**. This is most often associated with the production of enzymes capable of degrading the cell walls of pathogens such as chitinases, β-1,3 glucanases, etc (Fernandes, 2006). Recently, **mycoparasitism** as a mechanism of antagonism towards fungal pathogens has been demonstrated in *S. phaeopurpureus* ExPro138 (Palaniyandi *et al.* 2013). This strain exhibited a combination of coiling and lysis as a mechanism of mycoparasitism.

Another frequently cited mechanism of action of *Streptomyces* is **competition** with the pathogen, mainly for nutrients essential for growth such as organic acids, amino acids, sugars, vitamins, enzymes, purines/nucleosides, inorganic ions, and gases, phytosiderophores, phenolics, flavonoids and root border cells exuded by plant roots. Among these substances, phenolic and flavonoid compounds influence symbiosis with beneficial *Streptomyces* whereas organic acids, sugars, amino acids, inorganic ions, purines, and vitamins serve as essential nutrients (Dakora and Phillips, 2002) for microbes present in soil.

The effectiveness linked to such a mechanism is based on the exclusion of pathogenic agents, especially those whose spores require exogenous nutrient sources to germinate (Elad, 2000). Pathogen suppression through competition for iron has been the most examined type of competition. Iron concentration in the rhizosphere is extremely low (Pal and Gardener 2006). Microbes produce high affinity iron chelators called siderophores to sequester iron in such conditions. Microorganisms producing the highest affinity siderophore efficiently colonize the rhizosphere, whereas the one producing low affinity siderophores are eliminated. Involvement of siderophores in pathogen suppression has been reported for *Streptomyces albovinaceus, S. griseus and Streptomyces virginiae* which inhibit the germination of basidiospores of *Moniliophthora perniciosa* (Macagnan *et al.*, 2008).

Apart from direct action against pathogen survival, some biological protection agents are also capable of interfering with pathogenicity. This may involve production of proteases which inhibits the activity of enzymes used by the pathogen to break down cell walls of plants (Elad, 2000). This may also involve the secretion of surfactants which may interfere with adhesion of pathogen spores to the plant.

In addition, some *Streptomyces* strains are known for their effect in inducing non-specific defense [induced systemic resistance (ISR)] in the host plant that confers resistance to a broad spectrum of pathogens (Pieterse *et al.,* 2014). The biological control agent triggers in the plant mechanisms of defense (thickening of plant walls, production of defense molecules, etc.) which oppose the development of infection by the pathogen. This examination of the mechanisms of action underscores the dual role of *Streptomyces* in both plant defense and

growth promotion, leading us to further explore the specific growth-promoting pathways attributed to these microorganisms.

I.4.8. Mechanisms of Action of the *Streptomyces* in Plant Growth Promotion

Streptomyces like other plant growth promoting rhizobacteria (PGPR) can directly influence plant growth throw several pathways. Some of these pathways include phytohormone production nitrogen fixation, phosphate solubilization and increasing iron availability.

Growth promotion by plant growth regulators is an important mechanism exhibited by *Streptomyces* strains. Phytohormones are chemical messengers that play a major role in plant growth through seed growth and germination, flowering, leaf formation and reduction of senescence of leaves and fruits. These plant growth regulators regulate many physiological processes in the plant such as cellular division and growth, vegetative and reproductive development, and stress responses. The most studied plant growth regulator produced by PGPR is indole-acetic acid (IAA), the natural auxin. IAA production and plant growth promotion has been reported for several actinobacteria (Khamna *et al*., 2010; Legault *et al*., 2011; Rungin *et al.,* 2012). The production of IAA in *Streptomyces* is L-tryptophan dependent, and the pathway is through indole-3-acetamide (Lin and Xu, 2013). Stimulation of lateral root development, root hairs and release of sugars are some of the physiological effects of IAA in plants (Davies, 2014). Other plant growth regulators include cytokinins, gibberellins, abscisic acid, ethylene, polyamines, jasmonates and salicyclic acid. *Streptomyces olivaceoviridis, S. rimosus and S. rochei* have been shown to produce auxins, gibberellins and cytokinin-like substances and enhance the growth of wheat plants (Aldesuquy *et al*., 1998).

Nitrogen fixation is a process by which atmospheric nitrogen (N2) is converted into ammonia (NH_3), which can be assimilated by plants for the synthesis of nitrogenous biomolecules (Wagner 2012). Nitrogen is important nutrient for plant growth and productivity. This nitrogen fixing rhizobacteria establish symbiosis in the roots of plants through a complex interaction between the host and symbiont resulting in the formation of the nodules (Lisa, 2019). A *Streptomyces* strain ie. *S. thermoautotrophicus*, isolated from burning charcoal pile is a well-studied nitrogen fixing actinobacteria which has been shown to utilize N_2 as a sole nitrogen source. Bacteria which can fix N_2 are economically beneficial and environmentally sound alternatives to chemical fertilizers.

Soil phosphorus (P) content is generally very low and is available in the form of insoluble metallic complexes (with iron, aluminium, silicium ...) in acidic soil or calcium carbonate in alkaline soil. Therefore, only a small fraction of 1 mg kg^{-1} or less Phosphorus is

available for plant growth (Hamdali *et al.* 2008). Phosphate deficiency is one of the limiting factors in crop production. Microbes can solubilize insoluble phosphates in metallic complexes or in hydroxyapatite and release free phosphates (Rodríguez and Fraga, 1999) leading to plant growth promotion. The major mechanism of phosphate solubilization is based on organic acid secretion by the PGPR through sugar metabolism. PGPR utilize sugars from root exudates and produce organic acids. These acids are excellent chelators of divalent Ca^{2+} cations, thereby releasing phosphates from insoluble phosphate compounds. Actinobacteria such as *Streptomyces* have been previously reported to solubilize rock phosphate and shown to suppress damping off caused by *Pythium ultimum* as well as promote the growth of wheat in a phosphate-deficient soil (Hamdali *et al.*, 2008). These results indicate that plant growth-promoting traits by *Streptomyces* strains are desirable in addition to biocontrol traits to achieve biological control in nutrient deficient soils.

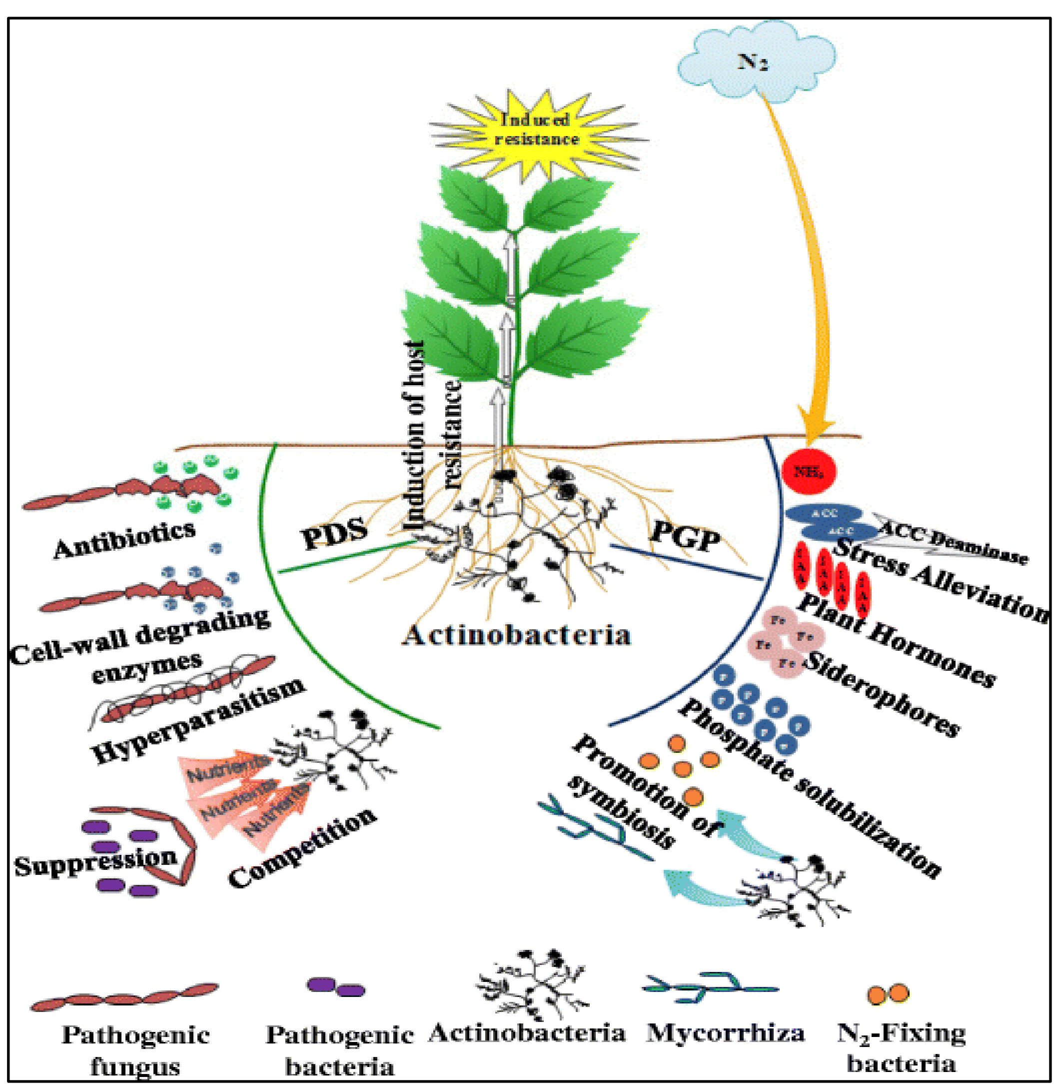


Figure 7: Mechanisms exhibited by plant-associated *Streptomyces* in plant disease suppression and plant growth promotion (Palaniyandi *et al.*, 2013).

Iron is an important mineral for plant growth and is available in soil as insoluble ferric ion (Fe^{3+}). Plants and microbes can readily uptake iron in the more soluble ferrous ion (Fe^{2+}) form (Francis *et al*., 2010).

Microbes such as *Streptomyces* that can reduce Fe^{3+} to Fe^{2+} can enhance the bioavailablity of iron in the plant rhizosphere. Siderophores produced by microorganisms in the rhizosphere can increase the availability and uptake of iron. *Streptomyces acidiscabies* E13 strains have been reported to produce hydroxamate siderophores (Palaniyandi *et al*. 2013; Dimkpa *et al*., 2008) which enhanced iron acquisition and promote growth of *Vigna unguiculata* under nickel stress. In this context, we now turn our attention to a particularly intriguing subgroup within the Streptomyces genus: *Streptomyces cameroonensis.* This species not only showcases unique properties but also plays significant roles in both biological control and plant growth promotion, as will be discussed in the next section.

I.5. Presentation of *Streptomyces cameroonensis*

Streptomyces cameroonensis is an actinomycete isolated from the rhizosphere of *Chromolaena ordorata* in a project in the Laboratory of Phytoprotection and Valorization of Genetic Resources, Biotechnology Centre, Nkolbisson, Yaounde, Cameroon, aimed at isolating rhizobacteria as candidates from biocontrol of *Theobroma cacao* (Boudjeko *et al.,* 2017). It is a saprophytic, multicellular, aerobic, filamentous sporulating and Gram-positive actinobacterium characterized by a high G+C genome. It grows well on ISP-2 medium forming white aerial hyphae that differentiates to grey, moist and tight spiral spore chains after about 4 days of growth. Growth occurs between 25 °C to 37 °C and at a pH of 5 to 9. Phylogenetic and phenotypic tests performed classified the strain in the *Streptomyces violaceusniger* clade (Boudjeko *et al.,* 2017). According to “Trends in Microbiology”, the taxonomy of this strain is as follows:

Bacteria

Phylum: Actinobacteria

Order: Actinomycetales

Suborder: Streptomicineae

Family: Streptomycetaceae

Genus: *Streptomyces*

Species: *Streptomyces cameroonensis*

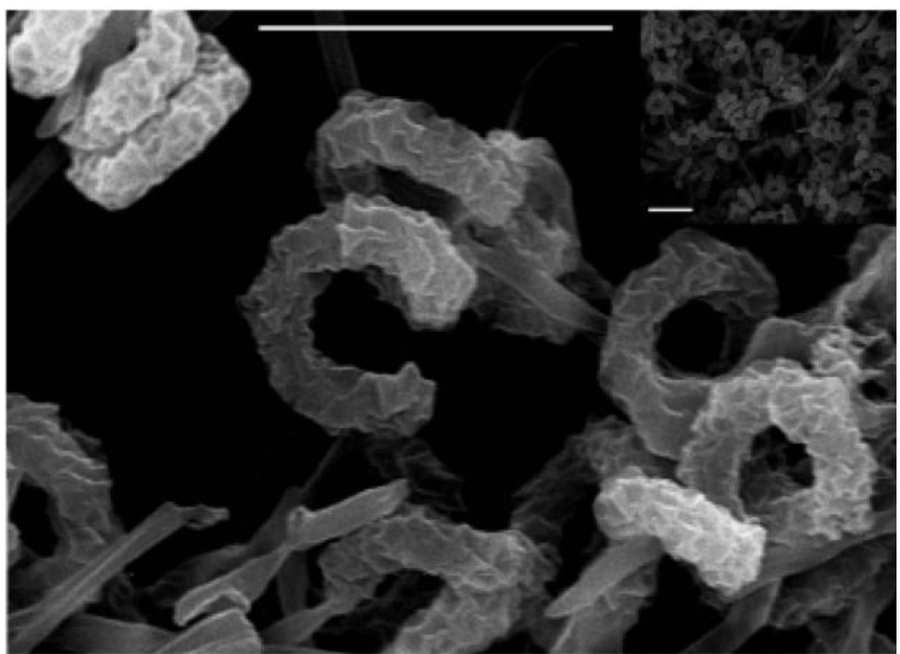


Figure 8: Scanning electron micrograph showing formation of spiral chains of spores by *S. cameroonensis* grown on ISP-medium (Boudjeko *et al*., 2017).

Streptomyces cameroonensis exhibits antimicrobial activities against a variety of fungi and oomycetes including *Fusarium oxysporum, Aspergillus niger, Phytophthora infestans, Pythium myriotylum, Phytophthora megakarya, … and* antibacterial activity against *Agrobacterium tumefaciens, Streptomyces scabei*, etc. It has the capacity to produce a plethora of natural products and can induce growth and defense in plants directly and indirectly. It has been demonstrated that *S. cameroonensis* produced siderophores and indole-acetic acid, solubilized phosphate and is capable of degrading 1-aminocyclopropane-1-carboxylate (ACC deaminase activity), an intermediary product in the biosynthetic pathway of the plant hormone ethylene (Boudjeko *et al*., 2017). These products are typical of plant growth promoting rhizobacteria (PGPR), thus suggesting strongly that *S. cameroonensis* is a PGPR. Boudjeko *et al.* (2017) effectively demonstrated the ability of a fresh culture of *S. cameroonensis* applied to the substrate promoted the growth of *Theobroma cacao* seedlings in nursery first by accelerating germination and then an increase in the root and aerial biomass of cocoa seedlings. This strain also exhibited antimicrobial activities against a broad range of microorganisms. This activity was related partly to the increase production of the secondary metabolite geldanamycin, a type-1 polyketide compound known for its antibacterial and antimicrobial activities. Boudjeko *et al*. (2017) demonstrated that the necrosis index on leaves of cocoa plants grown on substrate inoculated with a fresh culture of *S. cameroonensis* reduced considerably. This suggest that *S. cameroonensis* had a protective effect on cocoa plants by stimulating systemic induced resistance. *Streptomyces cameroonensis* like other geldanamycin-producing

actinobacteria can live as endophytes within the plant (Palaniyandi *et al*., 2013), hence will provide a direct effect against pathogens within the plant. It is therefore essential to explore the practical applications of *S. cameroonensis* in biological control strategies.

I.5.1. Possibilities of Using *Streptomyces cameroonensis* in Biological Control

In the case of plant pathology, biological control is the fight against plant diseases by means of antagonistic microorganisms. It is an attractive alternative to chemical control. The properties exhibited by *S. cameroonensis* strains makes it an ideal antagonist. These criteria suggest that *S. cameroonensis* may play a key role in the field of plant protection against their bio-aggressors. Its multiplication rate is also very high and strong sporulation allows for important dissemination. They have a high rate of the rhizosphere colonization and can survive well in unfavorable conditions. In fact, its ability to adapt to aerial and underground life puts it in direct contact with many pathogens.

On the other hand, the plant has its own resistance mechanisms (Franceschi *et al*., 2005), but this biocontrol can contribute to this defense by excreting substances limiting the growth of phytopathogenic oomycete (Vassilev *et al.,* 2006) or stimulating plant defense systems; this phenomenon of elicitation was reported by Lehr *et al*. (2008). *Streptomyces cameroonensis* is thus a good candidate as an agent of biocontrol against the pathogen *Phytophthora megakarya.* As the potential of *Streptomyces cameroonensis* in biological control becomes increasingly evident, exploring the formulation of effective bio-inoculants and commercial products emerges as a crucial next step to harness its benefits in agriculture.

I.6. Formulation of Bio-inoculants and Commercial Products

Formulation in this context refers to the process of integrating microbial agents or their bioactive metabolites such as antibiotic secondary metabolites and cell wall-degrading enzymes into inert carriers often supplemented with nutritional additives to support microbial viability. The goal is to preserve microbial viability and enhance efficacy, stability and usability of the product during storage and field applications. Researchers have attributed the inconsistency and failure of biocontrol agents under field conditions to the lack of proper formulation of the biocontrol agents (Macagnan *et al.,* 2006). The type of formulation depends on the nature of the biocontrol agent (either live microbe or microbial products), site of application (soil, seed treatment or foliar application), stability and delivery at the site of action as well as the target pathogen (Sabaratnam and Traquair 2002). Traditionally, biocontrol

formulations are prepared as liquids or powders (Schisler *et al*., 2004), while dry formulations (granules or powders) are generally preferred because of extended shelf life, easy transportation, and storage, which can be suspended in liquid (oil, water, or oil in water emulsion) at the time of application (Sabaratnam and Traquair, 2002).

Several commercial products derived from actinobacteria, and *Streptomyces* strains are available for use in crop protection and growth promotion. *Streptomyces griseoviridis* (Mycostop) was the first actinobacterial biocontrol agent made commercially available for crop protection. Mycostop was marketed as wettable powder for use against soilborne fungal pathogens such *as Alternaria, Botrytis, Fusarium, Phomopsis, Pythium, Phytophthora,* and *Rhizoctonia* (Palaniyandi *et al*., 2013). Another well-studied biocontrol agent (BCA) is *Streptomyces lydicus* WYEC108, which is marketed with the commercial name Actinovate by Natural industries, Inc, Houston, USA (Elliott *et al*., 2009). Actinovate is formulated as water-dispersible granules, which can be used as soil drench or seed treatment for the control of soilborne pathogens and as spray for foliar pathogens. *S. melanosporofaciens* strain EF-76, a geldanamycin producer and an antagonist of *S. scabies* was formulated with chitosan and resulted in enhanced protection against potato scab (Beausejour *et al*., 2003).

Streptomyces cameroonensis exhibits several mechanisms of pathogen suppression and plant growth promotion. Formulation of the *S. cameroonensis* is necessary to utilize these activities in sustainable agriculture for crop protection and production. As we delve deeper into the formulation process, the importance of selecting the right inert and nutritional supplements for biofertilizer formulations becomes paramount, ensuring the efficacy and longevity of our microbial allies in agricultural practices.

I.7. Inert and Nutritional Supplements of Biofertilizer Formulations

The survivability and efficiency of rhizobacterial inoculants in biofertilizer formulations are greatly dependent on the choice of carrier materials and storage conditions (Sohaib *et al*., 2020). The carrier provides a temporary protective surface to the microorganism and must demonstrate the ability to support the growth of the target organism and maintain desired populations over an acceptable period (Macik *et al*., 2020). Carriers must have high water holding capacity, be well-buffered, environmentally safe, easy to use and cost-effective. Some of the common carriers and nutritional supplements used in our formulations are discussed below. These supplements are stable at room temperature but can be stored at 4°C to prolong bacterila shelf-life.

I.7.1. Cassava Starch

Starch is a polysaccharide of plant origin. It is the main reserve carbohydrate substance of higher plants. It represents a significant weight fraction of agricultural raw materials. It is found in the storage organs of plants such as cereals i.e., 30-80% of dry matter (dm), tubers (60-90% dm) and legumes (20-25% dm). It is a carbohydrate with the molecular formula $C_6H_{10}O_5$ consisting of 98-99% of a mixture of two natural polymers: amylose and amylopectin which consist of molecules of α-D-glucopyranose (or α-D- glucose or anhydroglucose) and are in cyclized form. Amylose is an almost linear macromolecule, and amylopectin is a highly branched macromolecule. The other constituents (1-2%) of starch are lipids, proteins, minerals, and phosphorus located both on the surface of the starch and inside. Starch is recognized for its many properties, namely, its biodegradability, its renewability, and its importance in the food industry. Starch is the main source of energy for human and animal nutrition. It is an abundant, renewable, and inexpensive nutritional compound (Liu *et al.,* 2012). Many studies have already made use of this polysaccharide as a vehicle for biocontrol agents such as Lee *et al.* (2006) regarding the establishment of a formulation of *Bacillus Licheniformis* based on starch. Tamreihao *et al.* (2016) used corn starch as a carrier in the bioformulation of *S. corchorusii* strain UCR3-16.

I.7.2. Talc Powder

Talc is a mineral species composed of double hydroxylated magnesium silicate which may contain traces of nickel, iron, calcium, and sodium. It is an inert material that has no phytotoxic effect on the plant, and dissolves well in water giving the ability to release spores easily. Several researchers have in recent years used talc as common carrier for the development of formulations based on biocontrol agents. Anitha and Rabeet (2009) developed a bioformulation of *S. griseus* using talc powder as a carrier. Talcrum powder-based formulation of *S. corchorusii* strain UCR3-16 significantly increased the growth and grain yield production of rice plant under pot and field conditions (Tamreihao *et al.,* 2016). Moreover, talc-based formulation of *Ochrobactrum anthropi* was prepared and its survival determined every month up to a period of 12 months (Chakraborty *et al*., 2009). Talc-based carriers are thus known to show great potential to assure viability of bacterial spores for many months and retain their ability to colonize the rhizosphere and achieve plant beneficial activity.

I.7.3. Carboxymethylcellulose

Described by several researchers as an adhesive element, carboxymethylcellulose (CMC) can also act as preservatives for the long-term viability of bacteria. It had been previously reported that adhesives and stickers increase the effectiveness of biological agents by protecting them from desiccation and death (Ibrahim *et al*., 1999). Sticking agents such as CMC are often added to peat-based biofertilizers to evenly distribute and stabilize the microorganism (Novinscak and Filion, 2020). Thus, the incorporation of CMC in the formulations serves as a sticker in the uniform coating of the spores of biocontrol agents. It was highlighted that a bioformulation of *Pseudomonas fluorescens* Pf1 based on Talc and CMC showed a very high spore viability (Chakravarty and Kalita, 2011).

I.7.4. Calcium Carbonate

Calcium carbonate ($CaCO_3$) can be used as a bacteria growth stimulator. It is also used in formulations to adjust the pH of the formulation to neutrality (Charkravarty and Kalita, 2011). $CaCO_3$ was recently used to adjust the pH of peat and talc-based formulations of *Pseudomonas fluorescens* and *Pseudomonas synxantha* bioformulations for promoting plant growth (Novinscak and Filion, 2020).

I.7.5. Gum Arabic

Gum arabic is a natural exudate harvested from the trunk and branches of shrubs mainly from the acacia family. It is a complex hydrocolloid, used in the food industry (aromatic drinks, confectionery, additives, …) and as an adhesive in pharmaceutical, offset printing and fabrics, ceramic foundry, cosmetics, fertilizers, and explosives. It is the only natural gum that can be directly used as an adhesive. Two main plant species namely *Acacia senegal* and *Acacia seyal* produce gum arabic. Gum Arabic was reported to be more tenacious as it provides excellent adhesion, its presence can have enhanced and promote early nodulation as well as improved growth in roots of plant when combined with *Rhizobium* spp. and *Bradyrhizobium* spp. on inoculated seeds (Hoben *et al*., 1991). Recently, gum Arabic was used as an adhesive material in the liquid bioformulation of *Bacillus velezensis* which enhanced the seed coating efficiency of the formulation (Raj *et al*., 2019).

Having explored the intricate interactions between plant-associated microorganisms and their impact on plant defense and growth against pathogens, we now delve into the cocoa plant's remarkable defense mechanisms, illuminating how it equips itself against various biotic threats in its environment.

I.8. Defense Mechanisms in Plants

Plants are constantly being exposed to attacks by pathogens and are thus equipped with an arsenal of defense mechanisms that are put in place to delay or stop invasion by these aggressors. Metabolic factors that participate in the fight against this aggression are systematically found in three main categories of reactions: (1) the strengthening of cell walls, (2) the production of antibiotics (phytoanticipins and? phytoalexins) and (3) defense proteins (PR proteins). These changes are often preceded by the self-destruction of infected cells (hypersensitive response), which limits the progression of the pathogen.

I.8.1. Hypersensitive Response

Hypersensitive Response (HR) is genetically programmed cell death in a host plant at the site of infection by the pathogen and this sacrifice of a few cells allows others to stay alive. HR is thus the culmination of plant defense responses initiated by the recognition of specific pathogen by the plant producing signal molecules in the apoplast called elicitors that would serve as signals for the induction of defense reactions in cells neighboring or distant from the site of infection (Dangl *et al*., 1996).

I.8.2. Strengthening of the Cell Wall

Following the immediate response, plants can further reinforce their defenses through the strengthening of the cell wall, enhancing their structural barriers against potential invaders. The cell wall is a very effective natural physical barrier recognized as passive resistance, that can be strengthened to fight against aggression. Indeed, most microorganisms are generally unable to cross the external protective barriers of plants. In addition to this passive resistance, defense responses activated by the aggressors will lead to further modifications of the cell wall. These modifications include thickening and reinforcement of the cell wall by the deposition of molecules such as callose, glycoproteins rich in hydroxyprolines, phenolic compounds ie. lignin and suberin. The deposition of these highly cross-linked molecules arms the cell wall against cell wall degrading enzymes produced by certain pathogens but also allows it to limit the diffusion of these enzymes as well as toxins (Benhamou, 1996).

I.8.3. Production of Antibiotics

In addition to structural fortifications, plants also engage in chemical warfare by producing antibiotics that inhibit the growth and spread of pathogens. The stimulation of secondary metabolite pathways plays an essential role in the resistance of plants to pathogens through the synthesis of molecules which will inhibit or block the spread of the pathogen. The enzymes involved in this synthesis are induced in numerous plant/pathogen interactions (Bischoff *et al.,* 1996). These molecules may be pre-existing compounds (phytoanticipins) or newly synthesized compounds (phytoalexins). Their nature and concentration vary according to genotype, age and factors external to the host. Phytoanticipins can be: diterpernoides, saponins, isoflavonoids, defensins and lysozymes. Phytoalexins are small molecular weight, lipophilic and antimicrobial compounds that rapidly accumulate around sites of necrosis (Hammerschmidt, 2004). Complementing these biochemical defenses, plants also employ a diverse range of defense proteins that act directly or indirectly against invading pathogens.

I.8.4. Defense Proteins (PR proteins)

The defensive arsenal of plants includes many defense proteins induced after infection by pathogens. These proteins are thus referred to as pathogenesis-related (PR) proteins. These proteins can interact directly against the pathogen by degrading the pathogen cell wall or indirectly by generating elicitors capable of stimulating the host plant defense responses. They are likely to accumulate locally at the site of penetration of the pathogenic agent, but also systemically in organs distant from the site of infection (Ethan *et al*., 2018). Some are glycosidic hydrolases like glucanases (PR-2) and chitinases (PR-3) or detoxification enzymes like peroxidases (PR-9) (Oliveira *et al.,* 2014).

I.8.5. Plant Resistance

The strategic deployment of these defense proteins contributes to a plant's resistance, which can manifest in two distinct forms: Local Acquired Resistance (LAR) and Systemic Acquired Resistance (SAR).

I.8.5.1. Local Acquired Resistance (LAR)

LAR is a phenomenon which is induced by dying cells via the transmission of molecular signals (jasmonic acid, salicylic acid, and ethylene). These molecular signals surround the zone where the hypersensitive response occurs (Dorey *et al*., 1999). It is a typical zone where numerous antimicrobial substances such as phenolic composites and PR-Proteins

are produced. The resistance is very high at the level of this zone, hence restricting progression of pathogenic agents.

I.8.5.2. Induced Resistance in Plants

Induced resistance is a physiological state of enhanced defensive capacity, whereby the plant's innate defenses are potentiated against subsequent biotic challenges (Van Loon, 1998). This enhanced state of resistance is effective against a broad range of pathogens and parasites including oomycetes, fungi, bacteria, viruses, nematodes, parasitic plants and even insect herbivores (Benhamou and Nicole, 1999). It occurs when the plant is protected entirely or systemically after application of an inducing agent to a particular part of the plant. The trigger factor is an event or process leading to the production of a signal which is a trans-locatable host factor that conditions the host to respond in a resistant manner. Once the resistance mechanisms are induced, the protection of the plant is independent of the concentration of elicitor and its presence or not in the tissues of the plant (Hoffland *et al.,* 1995). This protection requires latency between treatment with the inducing agent and the pathogen (Leeman *et al.,* 1995). Moreover, the reactions of stimulation of defense mechanisms of the plant are not specific. They can be induced by different agents such microbial agents, plant extracts, organic composites etc. The most clearly defined forms of induced resistance are systemic Acquired resistance (SAR) and Induced Systemic Resistance (ISR).

Systemic Acquired Resistance occurs in response to an exposure of roots or foliar tissue to elicitors (Van Loon, 2000). The classic form of SAR can be triggered by exposing the plant to virulent, avirulent, and non-pathogenic microbes, or artificially with chemicals such as salicylic acid (Ali *et al*., 2018). Depending on the plant and elicitor, a set period is required for the establishment of SAR that corresponds to the time required for the coordinated accumulation of pathogenesis-related proteins (PR-proteins) and salicylic acid (SA) throughout the plant (Durant and Dong, 2004).

Induced Systemic Resistance is elicited by beneficial soil-borne microorganisms such as mycorrhizal fungi and plant growth promoting rhizobacteria (Van Loon, 2000). It is not mediated by the SA dependency signaling pathway. Instead, it requires functioning Jasmonic acid and ethylene (Pangesti *et al*., 2016). Finally, to better understand the plant's overall defense strategy, we must examine the various markers that signify these defensive responses and their implications in plant health. Induced systemic resistance (ISR) in plants results in the production of several defensive compounds and changes that enhance plant health and resistance to pathogens. These include increased expression of defense-related genes,

production of pathogenesis-related (PR) proteins, and the accumulation of secondary metabolites like phytoalexins.

I.8.6. Plant Markers of Defense

Plants markers of defense are molecules secreted by plants implies in many plants defense mechanism such as phenolic compounds, polyphenol oxidase, peroxidase, chitinase, glucanase etc.

I.8.6.1. Phenolic Compounds

Phenolic compounds are secondary metabolites that play multiple roles in the defense of plants against pathogens. They can act as phytoanticipines (constitutive phenols), phytoalexins (induced phenols), structural barriers (precursors of synthesis of lignin) and/or activators of the expression of defense genes such as acid salicylic acid (Hammerschmidt, 2004). These compounds are produced and accumulate at a faster rate especially after infection in highly resistant varieties of plants. The main classes of phenolic compounds are simple phenols (catechol, resorcinol, …), hydrobenzoic acids (salicylic acid, gallic acid, …), hydroxycinnamic acids (ferulic acids, flavonoids, …), hydroxycoumarins, lignans and polyflavans (Dicko *et al*., 2006). These compounds play an important role in metabolism, but also in the protection of plants against biotic and abiotic stresses (Holly *et al.,* 2013). Several authors have shown the involvement of different classes of phenolic compounds as markers of resistance in the *T.cacao/P.megakarya* interaction like isoflavones, luteolin derived compounds, caffeic acid, chlorogenic acid and proantocyanidins (Boudjeko *et al.,* 2007; Minyaka *et al.,* 2017; Manga *et al*., 2018). To this end, several genes are involved in the regulation of the expression of phenolics compounds including the TcMYBPA transcription factor (Jalali *et al.*, 2006). This gene is involved in the regulation of the biosynthesis of anthocyanidins such as flavonoids (Yi *et al.,* 2015). This transcription factor activates the increased expression of several key genes encoding the main structural enzymes of the biosynthetic pathway for flavonoids and anthocyanidin, including dihydroflavanol reductase, leucoanthocyanidin dioxygenase and anthocyanidin reductase (Yi *et al.,* 2010). Several works have demonstrated the existence of a direct correlation between the degree of resistance of the cocoa tree against blackpod and the high content of phenolic compound (Djocgoué *et a*l., 2011; Effa *et al*., 2016; Tchameni *et al*., 2017, Ewané *et al*., 2019).

I.8.6.2. Polyphenol Oxidase

Polyphenol oxidase are enzymes associated to the oxidation of the phenolic composites of plants (Tyagi *et al.*, 2000) to produce hydroxyphenols and quinones. The Oxidation of phenols is a common phenomenon in all plants/pathogen interactions. Indeed, tissue oxidation is noted at the site of infection (hypersensitivity reaction). This oxidation leads to the formation of quinones and the accumulation of free radicals which can inactivate the enzymes produced by the pathogen (Appel, 1992). In addition, these quinones have antimicrobial activity and can therefore act directly on the pathogen as well as stop its development (Sivaprakasan and Vidhyasekaran, 1993). This phytotoxic property of quinones causes certain plants to accumulate in the cells adjacent to the site of infection, pigments capable of detoxifying these toxic metabolites. Several studies have shown the role of phenolic compounds in plant protection against pathogens. The PPO are also considered to have general stress response to reactive oxygen species (ROS) that are produced in excess amounts (Ondobo *et al.*, 2017). When Polyphenoloxidase are inactivated under severe stress conditions, toxic properties of H_2O_2 are inhibited by another antioxidant enzyme such as peroxidase. The increase in polyphenoloxidase activity may contribute to the reduction of H_2O_2 content (Baysal and Tipirdamaz, 2010).

I.8.6.3. Peroxidases

Peroxidases (PR-9) are hemoproteins that represent the most abundant group in the defense category of the up-regulated proteins. They are subdivided into three classes (Welinder, 1992).

- Class I: Intracellular peroxidases of prokaryotic origin (EC 1.11.1.5/.6/.11)
- Class II: Peroxidases secreted by oomycete and fungi (EC 1.11.13/.14)
- Class III: Peroxidases secreted by plants (EC 1.11.1.7)

There exist some similarities between these classes of POX, thus the suggestion that the peroxidases of class II and Class III might have been derived from Peroxidases of prokaryotes. Class III POX are present in all plant species. Their mechanism of defense is through the activation of pre-existing compounds and hence they act as biochemical markers for resistance. They can be associated to cell walls leading to oxidation of several compounds mainly phenolic compounds into substances toxic to the pathogen. It has been reported that Class III peroxidase-mediated H_2O_2-dependent cross linking of cell wall components with extension and ferulic acid reinforces cell walls through the formation of physical barriers composed of lignification or

suberization which limit pathogen invasion. POX activity in cocoa has been correlated with the resistance of cocoa plants to be? attacks by *P. megakarya* (Nana *et al*., 2016; Téné e*t al*., 2019).

I.8.6.4. Chitinases

Chitin (PR-3), an insoluble β-1,4-linked polymer of N-acetylglucosamine, is the second most abundant polysaccharide in nature and a major constituent of the cell walls of many oomycete, fungi, insect exoskeletons and crustacean shell. Degradation of chitin is essentially catalyzed by chitinases (Essghaier *et al*., 2014). Chitinases (E.C. 3.2.1.14) are found in bacteria, oomycetes, fungi, virus and higher plants, and they belong to PR-protein class 3. In chitin-containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division, whereas plants produce chitinases as part of their defense against fungal pathogens. Chitinases enzymes therefore act on cell wall chitin of oomycetes by hydrolyzing the cell wall polysaccharide and thus disabling the microbe (Essghaier *et al*., 2014). Chitinases are located on the leaves and do not intervene in the basal metabolism of the plant (Saboki *et al.,* 2011). In response to attack by the oomycets like *Phytophthora capsici*, the cocoa plant stimulates the expression of the TcChiB gene leading to the increase synthesis of chitinases (PR-3) to degrade the wall of this fungal and inhibit its spread (Zhang *et al*., 2015).

I.8.6.5. β-1,3-Glucanases

Plant β-1,3-glucanases (PR-2) are pathogenesis-related proteins belonging to the family of PR-2 proteins and they play an important role in plant responses against pathogens. This enzyme can catalyze the cleavage of the β-1,3-glucoside bond of β-1,3- glucans, a major structural component of the cell wall of several fungal pathogens such as *P. megakarya,* causative agent black pod disease of cocoa (Oliveira *et al.,* 2014; Téné, 2020). β-1,3-glucanases are generally expressed at low concentrations in plants, but when plants are infected by a pathogenic agent, this induces an exponential increase in its synthesis, which can be localized or systemical. This enzyme can be induced by the presence of pathogen or components from degradation of the cell surface of the pathogen (β-glucan, chitin, …) released by the host enzymes (Saboki *et al.,* 2011). Leelasuphakul *et al.* (2006) demonstrated that chitin at 0.3% acting as extra carbon source supplementing the growth medium induced production of glucanases. Indeed, Téné *et al.* (2019) demonstrated that soil amendment with oyster shell induced an increased synthesis of β-1,3-glucanase in cocoa plants following infection by the pathogenic agent *P. megakarya*. *In vitro* experiments have demonstrated that the antimicrobial

effects of chitinases and β-1,3-glucanases are synergistically enhanced when both enzyme classes are present (Lawrence *et al*., 1996).

Chapter II:
Material and Methods

CHAPTER II: MATERIAL AND METHODS

II.1. MATERIAL

II.1.1. Edaphic Material

The soil used was black humus obtained from Nkolbisson, (Yaoundé, Centre Region, Cameroon, 3°52'24.4''N-11°26'7.8''E). This is the soil often used by locals operating small scale nurseries for cocoa seedlings and other plants. The sand used was purchased from a quarry of deposits obtained from the river Sanaga in Yaoundé, Centre-Region, Cameroon.

Figure 9: Photos of Edaphic Material; (**A**): Black humus soil, (**B**) River sand (Photographed by Dzelamonyuy, 2019).

II.1.2. Plant Material

Hybrid pods of *Theobroma cacao* [H1: (♀) SNK413 × (♂) T79/467) and H2: ((♀) UPA 134× (♂) SCA 12] produced by hand pollination were obtained from the SODECAO ("Société de Développement du Cacao") experimental farm in Mengang, Center Region, Cameroon. (Figure 10).

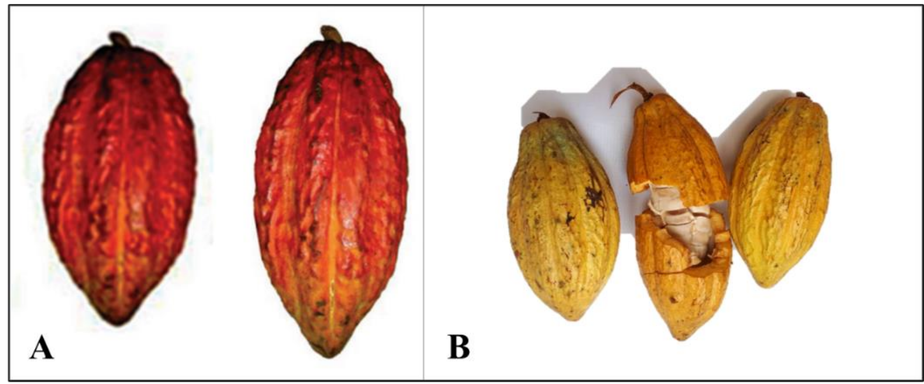


Figure 10: Photos of plant material; (A): H1 Hybrid [(♀) SNK413 × (♂) T79/467, (B): H2 Hybrid ((♀) UPA 134× (♂) SCA 12] (A) Adapted from Youbi *et al.,* (2020) and Photographed by Dzelamonyuy, 2019).

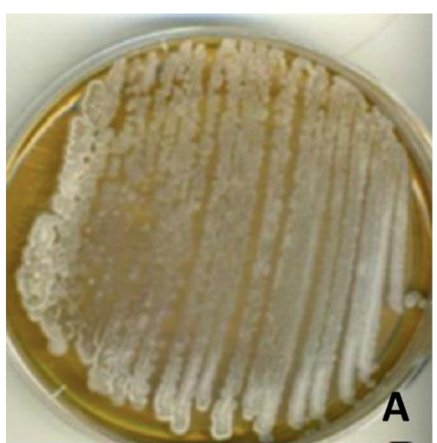
Table IV: Characteristics of the hybrids used, (Effa *et al*., 2017).

Genotype	**Origin**	**Collection**	**Group**	**Sensitivity to *P. megakarya***
SNK413	Cameroon	Nkoemvone	Trinitario	Moderately resistant
T79/467	Ghana	Tafo	UAF	Tolerant
UPA134	Ghana	Wacri	UAF	Sensitive
SCA 12	Ecuador		UAF	Moderately resistant
SNK413 × T79/467	Cameroon	Mengang	T x UAF	Tolerant
UPA134 × SCA 12	Cameroon	Mengang	UAF x UAF	Sensitive

SNK= selection Nkoemvone; UPA= Upper amazon; T= Tafo; SCA= Scavina; Trinitario=T; UA=Upper Amazon Forastero

II.1.3. Microbial Material

Streptomyces cameroonensis and *Phytophthora megakarya* strains (Figure 11) were obtained from the microorganism bank of the Laboratory of Phytoprotection and Valorization of Genetic Resources (LPVGR) of the Biotechnology Centre (BTC) of the University of Yaounde 1, Cameroon.



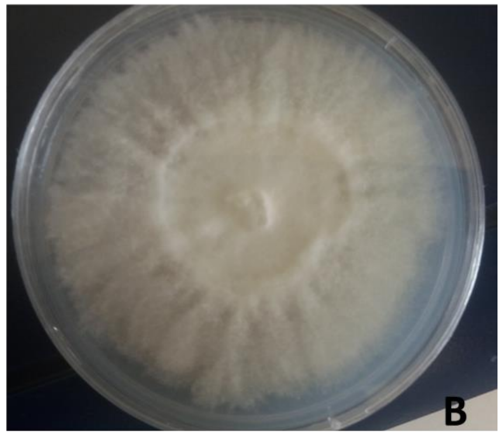


Figure 11: Strains of microbial material (A) *S. cameroonensis* (B) *P. megakarya* (Photographed by Dzelamonyuy, 2019).

II.1.4. Organic Material and the Chemical Product

The organic material used in the framework of our study were purchased from the local market of Mokolo in the City of Yaoundé. These include Talc powder and cassava starch as vehicles for the bacterial spores in the powder formulation, gum arabic as adhesive/sticker for the liquid formulation.

The chemical product used was purchased from an agricultural shop in Mokolo with the commercial name callomil Super 66WP with metalxyl-M (60g/kg) and copper oxide (600g/kg) as the active ingredients (Figure 12).

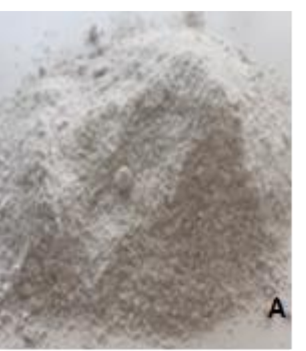


Figure 12: Photos of Organic material; (A): Cassava Starch; (B): Talc Powder (C): Gum arabic and (D): The Chemical product Callomil Super 66WP (Photographed by Dzelamonyuy, 2019).

II.2. METHODS

II.2.1. Culture of *Streptomyces cameroonensis* and Preparation of the Sporal Suspension

II.2.1.1 Culture of *S. cameroonensis*

This strain was cultivated on the International *Streptomyces* Project-2 medium (ISP-2 medium). This medium was prepared and autoclaved at 121 °C for 15 minutes, then poured on sterile Petri dishes. Spores from the sample Petri-dish were streaked on freshly prepared ISP-media on Petri dishes and incubated at 30 °C for 7 days for full sporulation.

II.2.1.2. Preparation of a Sporal Solution of *S. cameroonensis*

The spores were collected using sterile glass beads over sporulating colonies. The beads were washed in glycerol (20% v/v) at -20 °C. After freezing for 24 hours at -20 °C, the purity of the sample was checked for possible contamination. The sporal solution was then adjusted to 10^9 CFU/mL and stored in a 20% glycerol solution at -20 °C for further use.

II.2.2. Culture of *Phytophthora megakarya* and Preparation of a Sporal Solution

II.2.2.1. Culture of *Phytophthora megakarya*

Phytophthora megakarya strain was cultured on PDA medium (Potato Dextrose Agar). The medium was prepared and autoclaved at 121 °C for 15 min and then poured into sterile petri dishes. The 5 mm diameter mycelial discs taken from the surface of the sample culture of *Phytophthora megakarya* were transferred unto the Petri dishes containing PDA medium and incubated at 2 ±1 °C in the dark. This culture was carried out every three days to maintain the virulence of the strain.

II.2.2.2. Preparation of a Sporal Solution of *Phytophthora megakarya*

The spores of *P. megakarya* were obtained from necrosis formed on healthy cocoa pods because of artificial inoculation with an inoculum of *P. megakarya* (Tondje *et al*., 2007). Healthy cocoa pods were harvested, washed abundantly with tap water and then with 0.2% sodium hypochlorite, then rinsed with sterilized distilled water. The sterilized pods were then dried in a laminar flow hood; and an incision of about 5 mm in diameter made on the cortex of the pod with a sterilized surgical blade. The 5 mm mycelial discs of *P. megakarya* were extracted and aseptically deposited in the incision of each pod. The mycelial disc on the incision was then covered with a piece of cotton soaked in sterilized distilled water; then placed in sterilized plastic trays lined with paper towels moistened to maintain a relative humidity (75-100%) conducive for sporal germination (Téné, 2020). The trays were hermetically sealed and incubated in the dark to create favorable conditions for the development of *P. megakarya*. Six days afterwards, the infected pods were washed and the sporocyst suspension harvested in sterilized distilled water. The zoospores were released from the sporocysts by heat shock by subjecting the mixture at 4 °C for 5 minutes, then at room temperature in the dark for 30 minutes. The zoospores were counted using the Neubauer cell counting chamber mounted on a light microscope. The number of zoospores was calibrated at 10^6 zoospores/mL and stored at -20 °C for further use.

II.2.3. Preparation of the Powder Formulation of *Streptomyces cameroonensis*

II.2.3.1. Screening of the Best Vehicle Mixture and Formulation Composition

The preparation of the powder formulation started with the sterilization of the vehicles (cassava starch and talc powder) in different proportion mixtures (Table V). The mixtures were sterilized in an autoclave at 121 °C twice. The sterilized mixtures (100 g) upon cooling were each supplemented with 1.5 g calcium carbonate ($CaCO_3$) and 1 g carboxymethyl cellulose

(CMC). The total mixtures were then mixed under sterile conditions with a 40 mL of the filtered sporal solution of *S. cameroonensis* spores previously prepared and calibrated at 10^9 CFU/mL. The resulting mixture was shade dried in a laminar flow hood, ground to powder form using a mortar and pestle then passed through an 8 mm sieve to obtain fine homogenous powder. The resulting powder was then packaged in pre-sterilized polypropylene bags, sealed and stored for further analysis.

Table V: Table of various constituents for the formulation trials.

Formulation	Talc Powder (g)	Cassava starch (g)	CMC (g)	$CaCO_3$ (g)	10^9 CFU/ml of *S. cameroonensis* spores (ml)
T1	0	100	1	1.5	40
T2	25	75	1	1.5	40
T3	50	50	1	1.5	40
T4	75	25	1	1.5	40
T5	100	0	1	1.5	40

II.2.3.2. Determination of Stability of Formulations by Spore Viability

The viability of the spores in the different formulations in Table V above were determined using spread plate technique to determine the number of viable spores in CFU/mL. One (01) g of the powder from each of the formulations were dissolved in 10 mL of sterilized distilled water. A series of dilutions were made from the original solution (1:10. 1:100, 1:1000, 1:10 000, 1:100 000) and 10 µL each deposited on a solidified King medium base (KMB) on a Petri dish and spread uniformly across the surface with an L-shaped glass rod. The Petri dishes were then sealed and incubated for 3-4 weeks, and the number of colonies formed calculated. The formulation with the highest spore viability was selected as our best formulation that will be produced for further analysis.

II.2.3.3. Determination of Shelf-Life of theMost Stable Formulation

One (01) kg of most stable formulation (T4), that is the formulation with the highest spore viability and better texture was prepared as previously described. The formulation prepared was divided into two equal parts in pre-sterilized propylene bags and stored at 25 °C and 4 °C separately. The shell lives of the formulation at the different storage temperatures were determined using the standard dilution count method described in **II.2.3.2** above. The

shell-life was determined every 30 days over a period of 180 days and the projection for 12 months calculated.

II.2.3.4. Evaluation of the efficacy of the formulation

The Efficacy of the formulation was evaluated by the spread plate technique method. One (01) g of the powder formulation was dissolved in 1 mL distilled water. Various concentrations of the dilutions were prepared (50% w/v, 25% w/v, 10 % w/v, 1% w/v and 0.1 % w/v) PDA media were prepared and allowed to cool. 10 µL of each concentration was dropped on the plate, spread with a glass stirrer and allowed to dry. Sterilized distilled water was used as control. A 10 mm in diameter mycelial disc of *P. megakarya* isolate collected from a pre-prepared pure culture was placed in the middle of the solid PDA media. All dishes were sealed and incubated at 28±2 °C for 7 days. The growth of the pathogen in each concentration of formulation was measured and the comparison was made with that of the control dish. The percentage radial growth inhibition was calculated after 7 days according to the formula below.

$$\text{Inhibition } (\%) = \frac{\text{Radial growth of control} - \text{Radial growth of treatment}}{\text{Radial growth of control}} \times \mathbf{100}$$

II.2.4. Preparation of the Liquid Formulation.

II.2.4.1. Preparation of a Crude Extract of Secondary Metabolites of *S. cameroonensis*

A Crude extracts of *S. cameroonensis* secondary metabolites was prepared using the solid fermentation process with wheat bran as substrate according to the modified protocol of Selvameenal *et al.* (2009). A pure *S. cameroonensis* strain was inoculated in tight streaks on ISP-2 agar medium and incubated at 28° C for 7 days. The spores formed were scraped aseptically using a sterilized platinum handle and introduced into 25 mL of broth ISP-2 culture contained in a 250 mL conical flask and incubated in a rotary incubator at 28 ± 2 °C and at 150 rpm for 48 hours to obtain a pre-culture. The pure pre-cultures obtained were transferred to 250 mL of ISP-2 broth contained in 1000 mL conical flasks and shook at 150 rpm at 28 ± 2 ºC for 7 days. The cultures obtained were used at a rate of 10% of the inoculum to aseptically inoculate 50 g of sterilized wheat bran previously prepared and soaked in 50 mL of distilled water sterilized at 121 °C for 15 min at a pressure of 1 bar. The sterilized wheat bran inoculated with a pure culture of the *S. cameroonensis* was incubated at 28 ± 2°C for 30 days. After fermentation, the fermented substrate was macerated in ethyl acetate in the proportions 2/5 (m (Kg)/v (L)) every 4 hours for 72 hours (3× 24 h). After extraction, the filtrates obtained were evaporated using a rotary evaporator to remove the extract crude from the remaining ethyl

acetate. The raw extract of secondary metabolites obtained was then dried in an oven at 60 °C for three days. After drying, the crude extract was weighed on a mass balance and stored in a sterilized container protected from light for later use. The extraction yield was calculated using the formula.

$$Yield\ (\%) = \frac{Final\ mass\ of\ the\ extract}{Total\ mass\ of\ the\ Biomass} \times 100\%$$

II.2.4.2. Preparation of a 15% w/v Solution of Gum Arabic

500 g of gum Arabic were crushed then sieved to remove impurities and hard materials. 15 g of gum Arabic powder was measured on a mass balance and diluted in 100 mL of distilled water. The solution was stored at 4°C for later use.

II.2.4.3. Preparation of Different Formulation Trials

The establishment of the different formulations based on the crude extract of secondary metabolites synthesized by *S. cameroonensis*, gum Arabic as absorbent and Tween 80 as adjuvant were carried out using STATISTICA software (version 10.0. 228.8 for Windows). The different modelings of the various formulations considering the proportions of each input (µL) for a final volume of 1mL were carried out (Table VI), following a model developed by Bhattacharya and Dixit (2015).

Table VI: Different trials for the liquid formulation.

Formulation Test	Proportion of each component (%)		
	Secondary metabolites (mL)	**Tween 80 (mL)**	**Gum arabic (mL)**
A	33.3	33.3	33.3
B	90	5	5
C	5	90	5
D	5	5	90
E	61.6	33.3	5.0
F	61.6	5.0	33.3
G	33.3	61.6	5.0
H	33.3	5.0	61.6
I	5.0	61.6	33.3
J	5.0	33.30	61.6
K	61.6	19.2	19.2
L	19.2	61.6	19.2
M	19.2	19.2	61.6

II.2.4.4. Evaluation of the Stability of the Formulations

The stability of the formulations was evaluated according to the modified protocol of Bhattacharya and Dixit (2015). The various trials were stored at room temperature away from light, in conical glass tubes for the evaluation of their stability. The percentage of stability was evaluated after 0, 1, 2, 4 and 24 hours for each trial according to the homogeneity of the emulsions (phase separation) according to the scale below.

- 0% stability = oily emulsions
- 25% stability = milky and oily emulsions
- 50% stability = milky, creamy and oil emulsions
- 75% stability = milky and creamy emulsions
- 100% stability = milky media

The formulations with a clear separation of oil droplets which flocculates after 24 h were considered as unstable whereas those which were just milky or milky and having the cream which flocculates were considered as stable.

Furthermore, the colour and odour of the stable formulations were observed by visualisation. The size of the micelles formed on the various stable formulations after 24 hours which were observed under the light microscope. To this end, 20 µL of each stable test were taken and deposited on a slide and covered with a coverslip and observed at a magnification of 400X. The most stable formulation obtained from this phase was stored at both 4 °C and 25 °C for further tests.

The microbiological stability of the formulation obtained previously was evaluated according to the modified dilution protocol of Scorzoni *et al.* (2007). It was done by culturing the formulation on PDA medium. Hence, the formulation stored at 4 °C and at 25 °C in the absence of air and that kept at room temperature in contact with air were mixed with the PDA medium, then poured into Petri dishes. After solidification, the dishes were sealed with film paper and incubated for 15 days at 28±2 °C. Stability was assessed by observing the development of microbial colonies and mycelia.

II.2.4.5. Evaluation of the Antimicrobial Efficacy of the Formulation *vis-a-vis P. megakarya*

The antimicrobial effect of the most stable formulation was evaluated against the fungal pathogen of cocoa *P. megakarya* according to the modified dilution protocol of Scorzoni *et al.* (2007). The PDA medium was prepared and divided into 7 batches (the negative control, a

positive control and 5 concentrations of the formulation) of 100 mL each before being autoclaved at 121 °C under a pressure of one bar for one hour. The different batches were supplemented after cooling with the biopesticide formulation, sterilized distilled water and the chemical fungicide as follows:

- Negative control: 50 mL of PDA medium + 50 mL sterilized distilled water.
- Positive control: 50 mL of PDA medium were added to 50 mL of chemical fungicide CALLOMIL SUPER 66 WP (3.33 % w/V as recommended by the manufacturer);
- Formulation: 90 mL of PDA medium were added to 10 mL of formulation to have the mixture at 100 %. Dilutions were then made to have the formulation at 1% v/v and 0.1% v/v.

The mixtures were introduced into the 9.6 cm diameter Petri dishes and allowed to solidify for 30 minutes. After solidification, discs (0.6 cm in diameter) of the pure cultures of *P. megakarya* obtained from a six-day-old culture were placed in the center of the dishes and sealed hermetically. The latter were incubated for seven days at 28±2 °C for 7 days after which the mycelial growth diameters were measured. The experiment was carried out in triplicates. The percentage mycelial growth inhibition calculated.

$$\text{Inhibition (\%)} = \frac{\text{Mycelial growth diameter of control} - \text{Mycelial growth diameter of treatment}}{\text{Mycelial growth diameter of control}} \times \mathbf{100}$$

II.2.4.6. Evaluation of the Phytotoxic Effect of the Formulation on Cocoa Leaves

The sensitivity of young cocoa leaves to the formulation with the minimum inhibitory concentration obtained from the antimicrobial analysis above was evaluated according to the modified protocol of Zhang *et al*. (2015). The formulation was applied at different concentrations to the detached leaves of one-month-old SNK413 × T79/467 hybrid cocoa seedlings. The leaves were divided into 5 batches.

- Negative control (NT)
- Formulation at 100 % v/v
- Formulation at 10% v/v
- Formulation at 1% v/v
- Formulation at 0.1 v/v%

The detached leaves were sprayed with the 500 µL formulation at 100%, then with diluted concentrations of the formulation at 10%, 1% and 0.1% and distilled water (NT) for 4

successive days and the level of phytotoxicity recorded every 24 hours for 96 hours according to the scale below.

- 0 = No observation
- 1 = leaf deformation
- 2 = leaf discoloration
- 3 = discoloration + burning
- 4 = burn

II.2.5. Effect of the Formulations on the Quality of Cocoa Seedlings in the Nursery

II.2.5.1. Sterilization of Cocoa Seedlings

Cocoa pods of two hybrids [H1 (♀) SNK413 × (♂) T79/467 and H2 (♀) UPA 134× (♂) SCA 12] were dehusked, and the seeds washed with sand and distilled water to remove the mucilage. The washed seeds were surface sterilized with 70% ethanol for 5 minutes then later with 0.2% sodium hypochlorite solution for another 5 minutes. The sterilized seeds were then rinsed with sterilized distilled water.

II.2.5.2. Treatment and Production of Cocoa Seedlings with Powder Formulation

A water suspension of the powder formulation (0.1mg/mL) was prepared. Cocoa seeds from each hybrid were divided into three groups. The first group was soaked in the formulation suspension, the second group in distilled water and the third group in a suspension of the chemical fungicide CALLOMIL SUPER 66 WP (3.33% w/v). The soaked seeds were incubated at 150 rpm at 28 °C for 24 hours prior to planting.

The treated seeds were then seeded in black polythene bags containing a mixture of 1 kg soil and river sand mixed in the ratio 3:1. The experimental set up was divided into three major lots. Each major lot had two minor lots corresponding to the two hybrids. The experimental as follows.

- Lot 1: Seedlings treated with powder formulation (T)
  - Hybrid 1 (H1T)
  - Hybrid 2 (H2T)
- Lot 2: Seedlings treated with CALLOMIL SUPER 66 WP (Positive control C)
  - Hybrid 1 (H1C)
  - Hybrid 2 (H2C)
- Lot 3: Untreated seedlings (Negative control NT)

- Hybrid 1 (H1NT)
- Hybrid 2 (H2NT)

Each major treatment was in duplicates of 60 seedlings. The pots were kept in controlled conditions and watered every two days for a period of 12 weeks while the chemically treated were sprayed every two weeks with 3.33% w/v CALLOMIL SUPER 66 WP as recommended by the manufacturer. The treated seedlings and the negative control were each sprayed with sterilized distilled water. Stem length, leaf number and leaf surface area were measured every 4 weeks. At the end of the 12th week, the dry and fresh weight of roots and shoot were also measured. The experiment was a completely randomized design and was independently repeated twice.

II.2.5.3. Production and Treatment of Cocoa Seedlings with the Liquid Formulation

Cocoa seeds used to evaluate the effect of the liquid formulation were from the H1 hybrid [H1 (♀) SNK413 × (♂) T79/467]. After dehusking, the seeds were sterilized as described in paragraph **II.2.5.1.** The experimental set up constituted of these seedlings seeded in a 1:3 w/w of soil/sand mixture previously collected as shown below.

- Lot 1: Seedlings treated with formulation (T)
  - Seedlings treated with Formulation only (TS)
  - Seedlings treated with Formulation + *P. megakarya* inoculum (TS + PM)
- Lot 2: Seedlings treated with CALLOMIL SUPER 66 WP (Positive control C)
  - Seedlings treated with Chemical Fungicide (C)
  - Seedlings treated with Chemical Fungicide + *P. megakarya* inoculum (C + PM)
- Lot 3: Untreated seedlings (Negative control NT)
  - Untreated (NT)
  - Untreated Seedlings + *P. megakarya* inoculum (NT + PM)

Each treatment was in duplicates of 15 seedlings. The pots were kept in a greenhouse and watered every two days for a period of 12 weeks. The treated seedlings were sprayed every two weeks with a 0.1% v/v of the liquid formulation while the chemically treated seedlings were sprayed every two weeks with 3.33% w/v CALLOMIL SUPER 66 WP as recommended by the manufacturer. The negative control was sprayed with distilled water. Agro-morphological parameters (stem length, leaf number and leaf surface area) were measured every four weeks.

II.2.5.4. Pathogenicity test

The degree of resistance of plants was done following the protocol of Nyassé *et al*. (1995). This involved measuring the resistance index on leaf discs of diameter 15 mm and on whole leaves. The leaves were collected after 12 weeks of growth from each of the treatments, all in the same physiological state. The leaves were then washed with distilled water and surface sterilized with ethanol (70% v/v) for 30 s and again rinsed with distilled water. The leaves were then divided into two groups of 6 leaves each and laid up on a sterile tissue soaked in distilled water in lightproof trays and hermetically sealed in chamber at 25 ^{o}C overnight to make the lamina more receptive to inoculation. Leaf discs were also cut out with a 15 mm diameter metal disc (10 discs per sheet) and placed in randomized Petri dishes (8 discs per dish and four dishes per treatment). One group of leaves and discs per treatment were each inoculated with a 10µL of 10^6 zoospore/mL suspension of *P. megakarya* on the underside leaf surface while the other group for the control was inoculated with an equivalent amount of sterilized distilled water. The trays hermetically sealed while the Petri dishes were covered, and all were incubated under darkness at 26 °C. The rating scale as described by Nyassé *et al.* (1995) was used to calibrate level of infection in which observations were made after 6 days on a basis of 0-5 aspect, where:

- 0= No symptom development
- 1= Penetrations observed at the inoculation site
- 2= Connected points
- 3= Reticulate necrotic aspect
- 4= Marbled necrosis
- 5= True necrosis

This experiment was repeated twice, and the severity of disease was determined for each treatment by calculating the ratio of the sum of individual scores to the total number of discs used. The disease severity index used to express the resistance level was done following the scale described by Paulin *et al*. (2008).

- 0 < Index≤ 1: very resistant
- 1 < Index≤ 2: resistant
- 2 < Index ≤ 2.5: moderately resistant
- 2,5 < Index ≤ 3: susceptible

- Index >3: very susceptible

12-week-old whole leaves were used to constitute our sample for the extraction and quantification of biochemical markers. The samples were collected at about 2 cm away from the infected area on the leaves.

II.2.6. Biochemical Analysis

II.2.6.1. Extraction and Quantitative Dosage of Phenolic Composites

II.2.6.1.1. Extraction

The extraction of phenolic compounds was done following the modified protocol described by Boudjeko *et al.* (2007). 1 g of tissue extract (leaf) was ground in 5 mL methanol 80% (V/V). The sample was incubated at 4 ^{o}C and centrifuged at 10 000 xg for 5 min at room temperature using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected, and the precipitate re-suspended in 3 mL methanol, and incubated at room temperature for 15 min followed by another centrifugation. The second supernatant was collected and mixed with the first to constitute the extract (Figure 13).

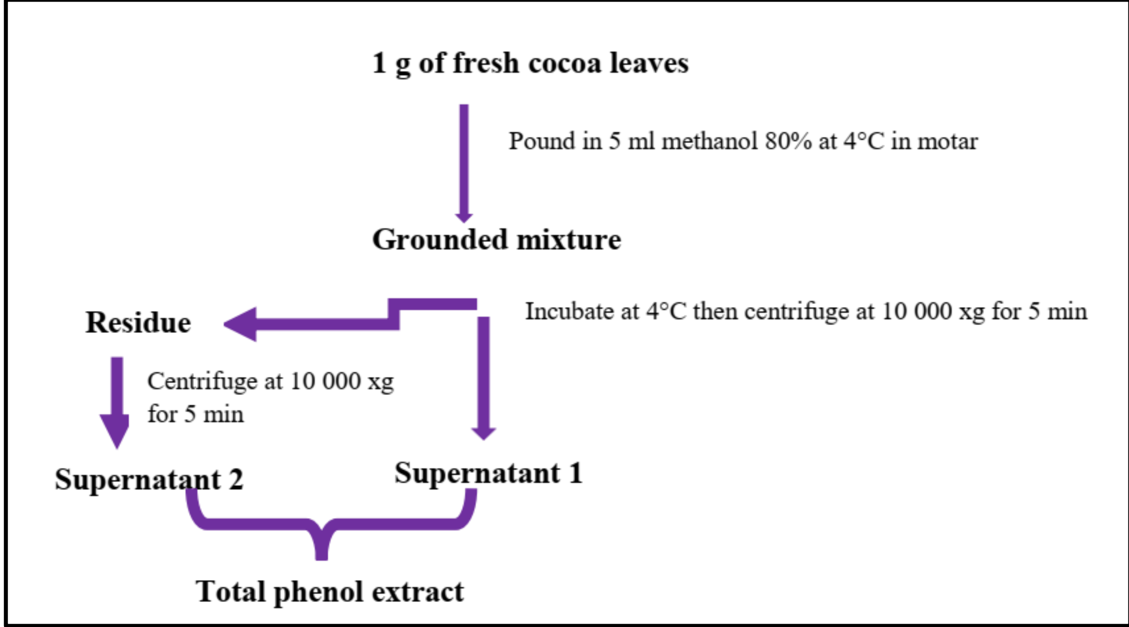


Figure 13: Protocol for extraction of phenolic compounds.

II.2.6.1.2. Quantitative Dosage

The quantification of phenolic compounds determined according to the protocol set by Marigo (1973) using the Folin-Ciocalteu reagent.

Principle

The Folin-Ciocalteu reagent being a strong acid (colored yellow) and made up of phosphotungstic acid ($H_3PW_{12}O_{40}$) and phosphomolybdic acid ($H_3PMo_{12}O_{40}$) undergoes reduction in an alkaline medium under heated conditions by oxidizing phenolic composites. The product of oxidation is blue oxidized tungsten and molybdenium. The blue coloration is therefore responsible for the maximum absorption at 760 nm which is proportional to the quantity of phenolic compounds present in the extract.

Procedure

In a test tube, the following were introduced successively:

- 10 µL extract
- 500 µL distilled water.
- 75 µL Folin-Ciocalteu
- 500 µL Na_2CO_3.

The mixture obtained was incubated for 30 min at 25 °C. In the presence of phenolic compounds, the Folin-Ciocalteu reagent appears blue. The color intensity is proportional to the quantity of phenolic compounds present in solution. Absorbance was measured using the Shimadzu U.V-1605 spectrophotometer at 760 nm against a standard where the extract is replaced by distilled water. Three repetitions were carried out. The quantity of phenolic compounds expressed in mg/g of fresh matter equivalent on a standard curve of gallic acid (0.1 g/mg).

II.2.6.2. Extraction and Dosage of Proteins

II.2.6.2.1. Extraction of Proteins

Extraction of proteins was carried out following the modified protocol of Téné *et al.* (2019). Initially, 1 g of the fresh plant leaves were ground in 5 mL of the Tris-Maleate buffer (Tris-HCl 10 mM, Triton X-100 1%, pH 7.5) using a pestle and mortar at 4 °C. After grinding, the mixture was vortexed for 10 min and was kept on ice. It was then centrifuged at 10 0000 × g for 25 min under cold conditions using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected and conserved while the remaining residue was centrifuged again at 20 000 x g for 20 min. The two supernatants obtained were added and constituted our extract and conserved at -20 °C pending quantification of proteins (Figure 14).

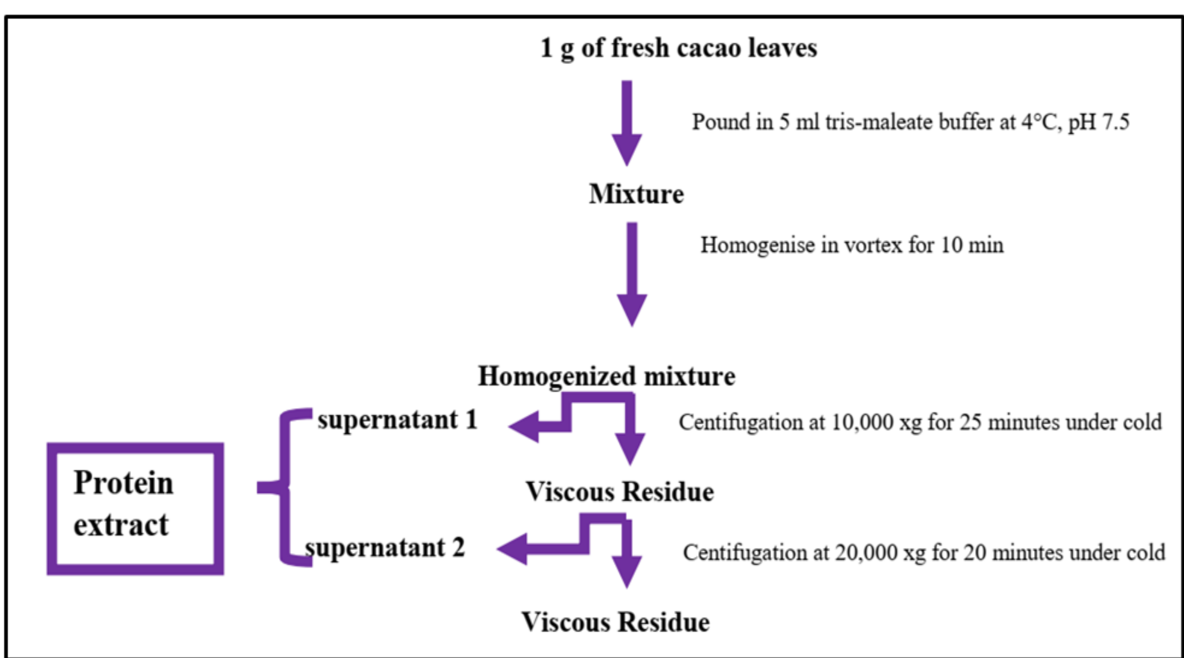


Figure 14: Protocol for extraction of proteins (Téné *et al.*, 2019 modified).

II.2.6.2.2. Quantitative dosage of proteins

The dosage of proteins was carried out following the method of Bradford (1976), using the reagent Coomasie blue G 250.

***Principle*:**

This method measures the concentration of proteins based on a Colorimetric reaction between proteins and the colorant Coomasie Blue G250 (Bradford's reagent) with a clear brown color. In an acidic medium, Coomasie blue G250 fixes on the hydrophobic residues of amino acids which make up the protein forming a blue coloration with an absorbance of 595 nm and the intensity of coloration is equivalent to the quantity of protein residues in the medium.

Procedure

In each of the test tubes, add the following:

- 5 µL extract
- 495 µL distilled water
- 500 µL Bradford's reagent

The mixture was incubated for 15 min under ambient temperature and the optical density was read at 595 nm using the Shimadzu spectrophotometer UV-1605 against a control in which

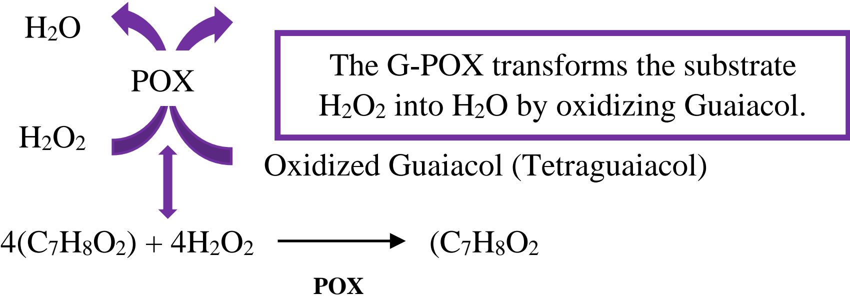
the extract was replaced by distilled water. For each extract, three repetitions were carried out. The concentration of the protein presents was expressed in mg/g of fresh matter, reference of an extrapolation on a standard curve realized under the same conditions as those with the samples using the BSA (Bovine Serum Albumin) at 0.1 mg/mL.

II.2.6.3. Measurement of Peroxidase Activity in the Protein extract

Dosage of peroxidase (POD) was carried out following the method designed by Baaziz et El Hadrami (1995) modified.

Principle

Its principle involves determining the enzymatic residue using H_2O_2 to form a colored product in the presence of Guaiacol with an absorbance of 470 nm.



Procedure

The Reaction medium consist of:

- 925 µL of Tris-Maleate buffer 0.1 M, pH 7.2 containing 1 g Guaiacol.
- 25 µL extract
- 50 µL H_2O_2 10%

After homogenization, the reaction mixture is incubated at ambient temperature for 3 min. The activity of peroxidase was determined following the formation of tetraguaiacol with an absorbance at 470 nm using the Shimadzu UV-1605 spectrophotometer. The enzymatic activity was expressed in enzymatic units per gram of fresh weight (Δ_{470}/min (EU)/g FW). The control was done using the same procedure as samples except that the plant extract was replaced with water. The enzymatic unit correspond to the quantity of enzyme which leads to a 0.1 variation in optical density.

The enxymatic activity was calculated following the the formula below.

$$Enzymatic\ activity = \frac{\Delta OD\ x\ V\ ext}{\Delta t\ x\ TS\ x\ g\ (FW)\ x\ 0.1}$$

V_{ext} = Extraction volume TS= No of test specimens FW = fresh Weight

ΔO. D = Change in optical density Δt = change in time

II.2.6.4. Measurement of Polyphenol Oxidase Activity in the Protein Extract

Polyphenol oxidase (PPO) activity was also assayed spectrophotometrically as described by Van Kammenn and Broumer (1964) using catechine as a substrate.

Procedure

The reaction mixture was as follows:

- 500 µL phosphate buffer pH 7, 0.66 M
- 150 µL catechine (10 mM)
- 35 µL extract

The mixture was incubated at 25 °C for 30 s. Changes in the absorbance at 330 nm were measured after 1 min using Shimadzu UV-1605 spectrophotometer. Enzyme activity was expressed as ΔA330 nm /min/g fresh weight.

The enxymatic activity was calculated following the the formula below.

$$Enzymatic\ activity = \frac{\Delta OD\ x\ V\ ext}{\Delta t\ x\ TS\ x\ g\ (FW)}$$

V_{ext} = Extraction volume TS= No of test specimens FW = fresh Weight

ΔOD = Change in optical density Δt = change in time

II.2.6.5. Measurement of chitinase activity in the protein extract

Chitinase activity was determined following the modified protocol of Pirovani *et al.* (2008).

Principle

The activity was determined by colorimetric assay using biopolymeric substrate colloidal Chitin-RBV. The principle involved quantifying the amount of the reaction product N-acetyl glucosamine released by the chitinase by hydrolyzing chitin.

Procedure

The reaction mixture was as follows:

- 200 µL Colloidal chitin
- 300 µL protein extract
- 300 µL Tris HCl 10 mM pH 7.5 Triton 1%

This mixture was then incubated at 37 ºC for 3 hours. The reaction was stopped with 200 µL of 2 M HCl. The samples obtained were cooled for 15 min to eliminate unhydrolyzed substrate and then centrifuged at 10,000 × g for 20 min to remove non-degraded substrate. The spectrophotometric analysis of the supernatant was done at 500 nm. Chitinase activity is described by unit/g fresh matter/h. One-unit chitinase activity corresponds to an increased absorbance of 0.1. For each sample, three independent repetitions were used.

The enxymatic activity was calculated following the the formula below.

$$Enzymatic\ activity = \frac{EU}{\Delta t\ x\ g\ (FW)}$$

FW = fresh Weight

EU= unit of chitinase activity corresponds to an increased absorbance of 0.1

Δt = change in time in hours

II.2.6.6. Measurement of β-1,3-glucanase activity in the protein extract

The assay was performed according to the method of Leelasuphakul *et al*. (2006) modified. This was done using the substrate laminarine (2.5% m/V), a polymer of β-1,3-glucan.

Principle

The activity of the enzyme liberates glucose which is quantified. The activity of β-1,3-glucanases was determined in µmoles of glucose liberated per hour (UE) for 100 mg of fresh matter.

Procedure

The following reagents were added as follows:

- 90 µL Na acetate buffer containing laminarine pH 4.5, 0.1 M.
- 10 µL extract

Incubate the mixture for 10 min at 40 °C.

- Then add 200 µL HCl 2M to stop the reaction.

Place in an ice bath to cool.

- Add 900 µL distilled water.

The quantity of glucose released was determined spectrophotometrically as changes in absorbance were measured at 540 nm using the Shimadzu UV-1605 spectrophotometer. The amount of reducing sugars released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in units (mg glucose equivalent per min). The enzymatic activity was calculated from the standard graph of glucose.

II.2.8. Effects of Powder and Liquid formulations on Expression Levels of Cocoa Defense Genes

II.2.8.1. Production of Plants

Cocoa seedlings from the H1 hybrid [H1 (♀) SNK413 × (♂) T79/467] were obtained and treated with the powder formulation as described in paragraph **II.2.5.2.** The treated seedlings were seeded in polythene bags containing a mixture of soil and sand in the proportion 3:1 w/w and kept in the greenhouse. They were sprayed every two weeks with the liquid formulation as described in paragraph **II.2.5.3.** The positive control consisted of seedlings seeded in polythene bags and were treated with the chemical fungicide CALLOMIL SUPER 66 WP while the negative control was sprayed with sterilized distilled water. The experimental set up was randomized as shown below.

- Lot 1: Seedlings treated with powder formulation (H1T)
- Lot 2: Seedlings treated with CALLOMIL SUPER 66 WP (H1C)
- Lot 3: Untreated seedlings (H1NT)

The seedlings were kept in the greenhouse for a period of one and a half months and watered consistently with sterilized distilled water. After one and half months of growth, 1 month old leaves were harvested from each treatment and conditioned in plastic sachets previously labelled and transported to the laboratory. The leaves were washed, then surfaced sterilized with 70% v/v ethanol and rinsed with sterilized distilled water. For each treatment, the leaves were divided into two equal parts per treatment and laid up on a sterile tissue paper moistened with sterilized distilled water in lightproof trays. For the first group, leaves were inoculated on the dorsal surface with 10µL of 10^6 zoospore/mL suspension of *P. megakarya* previously prepared while. The control group was inoculated with 10µL of sterilized water. The trays were hermetically sealed and kept in the dark at 28±2 °C for six days. At the end of

the 6 days, samples were collected at about 2 cm away from the infected area on the leaves and used for the molecular analysis.

II.2.8.2. Extraction of Total RNA from Plant Leaves

RNA was extracted using the *AccuPrep*® Universal RNA Extraction Kit (K-3140, BIONEER, Republic of Korea). 100 mg of fresh infected and uninfected plant leaves were ground under liquid nitrogen to a fine powder using a mortar and pestle. The tissue powder and the liquid nitrogen were then transferred to an appropriately sized tube and the liquid nitrogen allowed it to evaporate. 500 µL of lysis buffer RB was added and vortexed vigorously. The mixture was incubated 60 °C for 3 min, then centrifuged at full 14000 rpm for 2 minutes. The supernatant was recovered and transferred to a new microcentrifuge tube. RNA was precipitated by adding 0.5 volume of ethanol (96-100%) and mixed immediately by pipetting up and down. The sample was then transferred to the AccuPrep® Binding Column-III in a 2 mL collection tube, the lid closed and centrifuge at 14000 rpm for 20 sec. The flow-through was discarded from the collection tube and the column placed back in the tube.

The column was washed first by adding 700 µL of RWA1 Buffer without wetting the rim, the tubed closed, and centrifuge at 14000 rpm for 20 sec. The flow-through was discarded, and the column placed back in the tube. A second washing was done by adding 500 µL of RWA2 Buffer without wetting the rim, the tube closed, and centrifuged at 14000 rpm for 20 sec. The flow-through was discarded and the third washing was done by adding again 500 µL of RWA2 Buffer without wetting the rim and centrifuged at 14000 rpm for 2 mins. The flow-through was discarded and the column placed back in the collection tube. The empty column and collection tube were centrifuged one more time at 14000 rpm for 1 min to completely remove ethanol and checked that there were no droplet clinging to the bottom of the AccuPrep® Binding Column-III tube. The column was then transferred into a new 1.5 mL centrifuged tube, and 100 µL elution buffer ER added onto AccuPrep® Binding Column-III and allowed to stand for at least 1 min at room temperature. It was then centrifuged at 10000 rpm for 1 min to elute the RNA. The eluted RNA was stored at -80 °C for further use.

II.2.8.3. Treatment of RNA with DNase

The synthesized RNA was treated with RNase-free DNase to remove any residual genomic DNA that may be present in the RNA. DNase master mix was prepared as shown in Table VII.

Table VII: Preparation of master mix for Dnase.

Ingredient	Per reaction (µL)	For 6 reactions (µL)
RNase-free DNase I (10 U µL1^{-})	1.8	X 1.8
RNA Guard	0.3	X 0.3
25 mM $MgCl_2$	2.4	X 2.4
Total	4.5	X 4.5

X: number of treatments = 6

1.2 µg of RNA was added to 4.5µL of master mix and the total volume made up to water 30 µL nuclease free water into 200-µL PCR strip tubes. They were gently mixed by flicking and briefly spinning on a minicentrifuge. The mixture was then incubated in Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific – US) at 37 °C for 10 min and then 90 °C for 5 min to inactivate the DNase.

II.2.8.4. Complementary DNA Synthesis from RNA by Reverse Transcription (RT)

The synthesis of DNA from RNA template via reverse transcription results in complementary DNA (cDNA), which will serve as a template for the downstream application of gene expression.

Principle

The synthesis of a complementary DNA strand is catalyzed by RNA-dependent DNA polymerase (Reverse Transcriptase) and uses a primer with a free 3'-OH end. When the RNA to be amplified are 3′ polyadenylated (eukaryotic mRNA), the primer chosen can be a polyT sequence consisting of a succession of deoxythimidines. In this case, all the mRNAs are copied into cDNA. Amplification is accomplished by the replication of denaturation cycles, hybridization and elongation that ensure exponential multiplication of the hybrid cDNA molecule resulting from reverse transcription.

Protocol

The master mix was prepared as shown in Table VIII, accounting for one to two additional reactions per gene. 25 µL of the reverse transcription master mix was added to 200-µL PCR strip tubes, properly labelled with the date and sample number on the side.

25 µL of the DNase-treated RNA was added to each master mix tubes (equivalent to 1 µg of RNA) and then incubated at the following temperatures using an Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific - US: 26 °C for 10 min (to allow

the random hexamers to anneal), 42 °C for 45 min (reverse transcription) and 75 °C for 10 min (to inactivate the reverse transcriptase). The resulting cDNA was stored at -80 °C for subsequent real time PCR analysis.

Table VIII: Preparation of master mix for cDNA synthesis.

Ingredients	Per reaction (µL)	For X reactions (µL)
5x superscript II buffer	10	X 10
10mM dNTPs	5	X 5
0.1 M DTT	5	X 5
BSA (RNase free)	1.25	X 1.25
Random Primers	0.25	X 0.254
RNA guard	1.25	X 1.25
Superscripts II reversed transcriptase	1.25	X 1.25
Molecular grade water	1	X 1
Total	25	X25

X: 6 samples, BSA: bovine serum albumin

II.2.8.5. Real time PCR (rt-PCR)

Quantifying gene expression levels has become a staple of most molecular biological laboratories. By measuring the amount of cellular RNA, one can determine to what extent that gene is being expressed. Real-time PCR (rt-PCR) is a powerful tool to quantify gene expression. The quantitative endpoint for real-time is the threshold cycle (*C*T). The *C*T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. Real-time PCR fuses the chemistry of PCR with the use of fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. The relative level of expression of the TcChiB, TcGlu-1, TcPer-1 and TcMYBPA genes were assessed using the Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific-US). The rt-PCR was performed according to the manufacturer's instructions for the Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific-US).

Principle

Real time PCR technology relies on the attachment of the fluorescent dye SYBR Green on double-stranded DNA (dsDNA). The SYBR Green is inserted at the level of the double-stranded DNA chains and becomes fluorescent. During the hybridization phase (A) of the qPCR cycle, the PCR primers hybridize to the target and form small regions of dsDNA or the

SYBR green intercalates, and a weak fluorescent signal is observed. In the elongation phase (B), more of dsDNA are formed and more SYBR Green dyes can intercalate, and the fluorescent signal observed is higher. At the end of the elongation phase, all the DNA molecules become double-stranded and the maximum amount of SYBR green is inserted. SYBR green has maximum and excitation emission at 491 nm and 521 nm respectively. The fluorescence is thus measured at the end of each elongation phase. This fluorescence is used to measure the accumulation of PCR products after each of its cycles. Fluorescence measurements make it possible to extrapolate the amount of target DNA present in the sample prior to amplification.

Protocol

The *AccuPower®* 2X *GreenStar™* qPCR MasterMix (BIONEER, Republic of Korea) was used. This is a ready-to-use reagent containing all components for real-time PCR reaction, except for target-specific primers. This kit is supplied in a convenient 2X concentration Master Mix to perform real-time PCR. It contains.

- SYBR Green, I Dye
- Hotstart DNA polymerase (1 U)
- dNTP Mixture (each 250 uM)
- Optimized buffer components (contains Tris-HCl, 60 mM KCl, 1.5 mM $MgCl_2$)
- Stabilizer

The cDNA template was removed from the -80°C and thawed and for each of the 6 samples, the AccuPower® 2X GreenStar™ qPCR MasterMix, template, template DNA and primers (thawed) were added into PCR tubes as shown in Table IX below.

Table IX: Reaction Mixture for rt-PCR.

Ingredient	Per 50µL reaction (µL)	For X reactions (µL)
2X GreenStar Master Mix	25	X 25
Template DNA	100 ng	X 100 ng
Forward primer (10 pmol/µL)	2 µL	X 2 µL
Reverse Primer (10 pmol/µL)	2 µL	X 2 µL
(Optional) Rox dye	5 µL	X 5 µL
PCR grade water	Variable	Variable

X: 2 duplicates X 6 samples =12

The PCR 8 strip tubes were sealed using optically clear cap strips and completely mixed by pipetting up and down several times. The reactions mixtures were then centrifuged at 3000 rpm for two minutes to bring all the droplets into the reaction mixture. The samples were then loaded into the Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific - US) and qPCR performed according to the following conditions, 1 cycle of pre-denaturation at 95 °C for 2 minutes, followed by 45 cycles of denaturation at 95 °C each at 5 seconds and an annealing extension at 60 °C at 5 seconds each. This was followed by an extension at 72 °C for 30 seconds.

Melting curve analysis was performed immediately after final extension for validating the amplification of a single product.

The oligonucleotide primers were designed according to Accupower/BIONEER recommendation. The primers lengths were 21 bp with a 40-60% GC.

Table X: List of primers used to assess the relative level of expression of the TcChiB, TcGlu-1, TcPer-1 and TcMYBPA genes.

Reference gene (**β-**actin)	**Forward Primer** *5'…………………………….3'*	Reverse Primer *5'…………………………….3'*
β- Actin	GTGGGCCGCTCTAGGCACCAA	TCATACTCTGCCTTAGCAATCC
Target gene		
TcChiB	GTGGCTTTGCTTGTGAATCTC	CACTGCTTCTCACCCTTATGT
TcGlu-1	GCTATGATTCCCTTCCCTCTTC	CAGGCCAAGTGCTAGGATAAG
TcPer-1	TGCGCTGATATTCTCGCTATT	CTGTGAACCCATCCCTTCTT
TcMYBPA	GATGGGAAGGGCTCCTTGTTG	ATCTCGTTATCGGTTGGACCAG

β-Actin a housekeeping gene whose expression was unchanged for all exposure conditions was used as internal control.

II.2.8.6. Relative Gene Expression Analysis using Comparative CT Method ($2^{-\Delta\Delta CT}$)

Data were analyzed using Sequence Detection System software (ver. 2.2, Applied Biosystems). The cycle threshold (Ct), i.e., the cycle at which an increase in the fluorescence level above the background was statistically significant, was chosen in the exponential phase of the amplification.

The comparative gene expression analysis of target genes (***TcChiB, TcGlu-1, TcPer-1 and TcMYBPA genes***) in terms of fold change was calculated using delta $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), where:

$\Delta Ct = Ct\ (target) - Ct\ (internal\ reference\ i.e\ \beta\text{-}actin)$; while

$\Delta\Delta Ct = \Delta Ct\ (target) - \Delta Ct\ (Internal\ calibrator)$.

All the relative expression analysis was carried out in triplicates for every condition in an experiment and the average fold change value was considered for calculating the relative $2^{-\Delta\Delta CT}$ value. The abundance of targeted gene transcripts was normalized to actin (internal control) relative to control experiments (uninfected) according to the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

II.2.9. Statistical Analysis

The experimental designs were randomized complete block and each value reported is the average of three repeats. The raw data was imported into Microsoft Excel 2016 for calculations and graphic representation. Statistix software version 9.01 for windows was used for analysis of variance and IBM SPSS software version 28.0.1.1 (15) was used for analysis of molecular data. Quantitative changes of parameters were evaluated through analysis of variance (one-way ANOVA), with Turkey multiple comparison tests at $P \leq 0.05$ to find out significant differences among treatments. All results are presented as the means ± standard deviation (SD).

Chapter III:
Results and Discussion

CHAPTER III: RESULTS AND DISCUSSION

III.1. Powder and Liquid Bioformulations Based on *S. cameroonensis* against *P. megakarya* Attacking Cocoa Seedlings Nursery

III.1.1. Powder Formulation of *S. cameroonensis*

III.1.1.1. Composition of Mixtures and Texture of Formulations

The mixture of different composites of the formulation at different proportions shows the T4 and T5 formulations with the finest textures compared to the rest with a more granular texture (Table XI). Particle size ranges between]0.1-2] mm. The colour of the formulations varies from greyish to white. All the formulations present a hygroscopic power of over 80% after drying.

Table XI: Characteristics of different formulations.

Formulations	Starch/Talc (w/w%)	Coloration	Aspect	Granulometry	Photos
T1	100 /0	Whitish	Very granular	≤ 2,5mm	
T2	75/25	Greyish	Granular	≤ 2mm.	
T3	50/50	Greyish	Granular	≤ 2mm	
T4	25/75	White	Fine	≤ 0.1 mm	
T5	0/100	White	Fine	≤ 0.1 mm	

III.1.1.2. Viability of Spores in the Formulations

The evidence of spore viability was confirmed by the formation of colonies of *S. cameroonensis* on the King Medium Base (KMB) medium following the spread plate technique. The plate with countable colonies from a suitable dilution was used to determine the population of bacteria spores present in the formulation (Figure 15 B).

The T4 formulation has the highest population of viable colonies of spores (2.78 x 10^6 CFU/g) compared to the other proportions of the formulation which had less (Figure 15A). The concentration of the cassava starch influences the viability of the spores at a concentration of 25% while the talc powder concentration in the formulation supports the maximum spore viability at 75%.

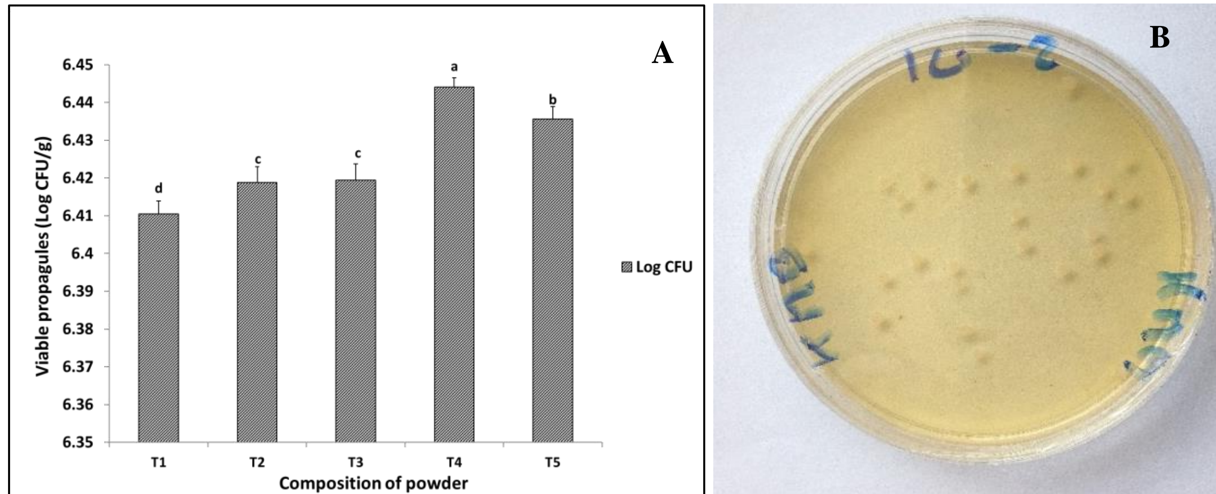


Figure 15: *Streptomyces cameroonensis:* A) spore viability in various proportions of cassava starch/talc powder after 1 month, B) countable colonies from a 10^{-2} dilution of the powder formulation on KMB medium.

*Values with the same letter within a column are not significant at $P \leq 0.05$. note: T1: 0%talc/100% w/w cassava starch; T2: 25%talc/175% w/w cassava starch; T3: 50%talc/50% w/w cassava starch; T4: 75%talc/25% w/w cassava starch; T5: 100%talc/ 0% w/w cassava starch.

III.1.1.3. Shelf-life of the T4 Formulation

Following the screening of the formulation and spore viability, T4 was selected for subsequent evaluation. After 180 days (6 months) of storage, the viable population of *S. cameroonensis* in the T4 formulation remained within acceptable thresholds at both 4 °C and 25 °C. However, the formulation stored at 4 °C exhibited greater stability and shelf-life preservation compared to that stored at 25 °C. Thus, after 180 days, cell count in powder

formulation stored at 4 ℃ is 1.07 × 10^6 CFU/g compared to 3.53 × 10^5 CFU/g for the formulation stored at room temperature.

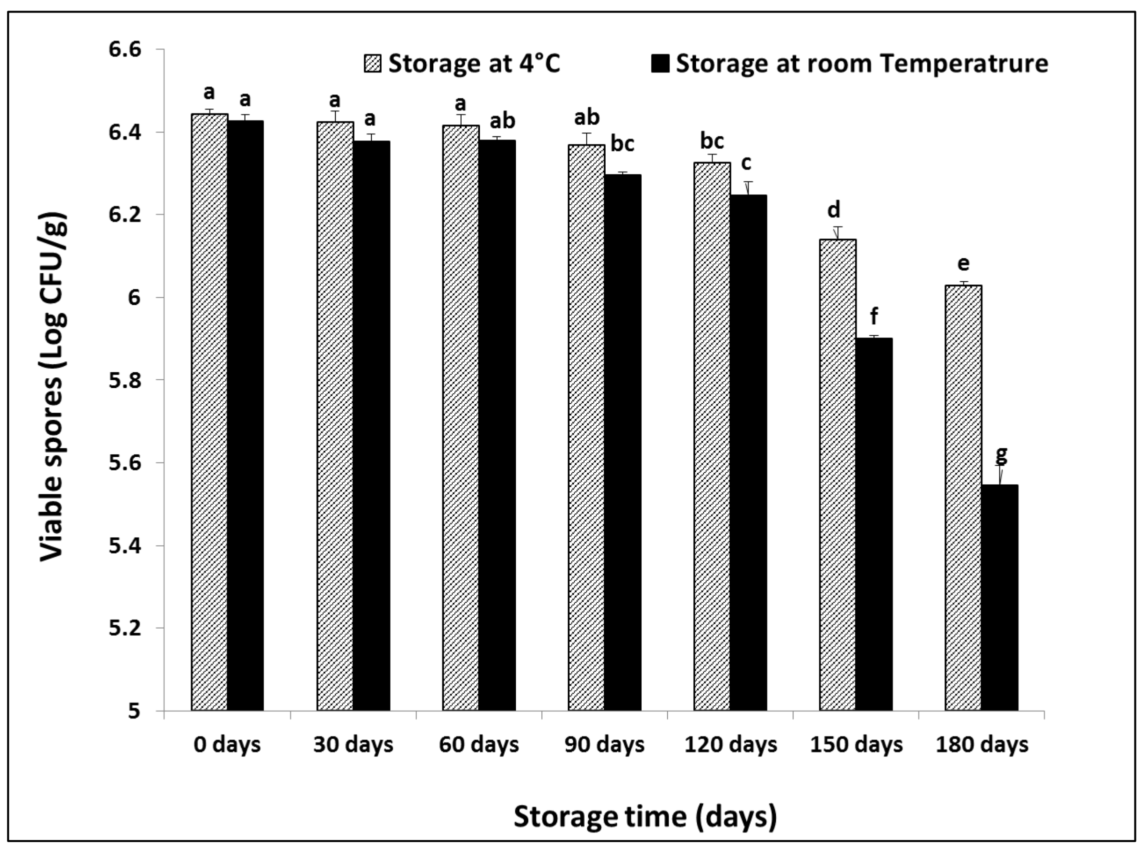


Figure 16: Shelf- life of the T4 powder formulation of *S. cameroonensis* at different temperature for over a period of 6 months.

*Values with the same letter within a column are not significant at $p \leq 0.05$.

This therefore signifies that 4 °C is a better storage temperature for the formulation. By extrapolating from the results, we derive an equation of the graphs of log CFU for viable spores against time in days (Figure 16) which gives an estimate of the spores in the formulation after a year.

- For Storage at 4°C. $\boldsymbol{y = -0.186 \ln x + 6.5427}$
- For Storage at room temperature. $\boldsymbol{y = -0.357 \ln x + 6.6012}$

y = log CFU

x = number of days

From the above equations, we calculate that even after 360 days, the shelf-life for the formulations stored at 4 °C and room temperature would be approximately 3.62 x 10^5 CFU/g and 3.16 x 10^4 CFU/g, respectively (Figure 17). Hence, still viable for use in soil amendment.

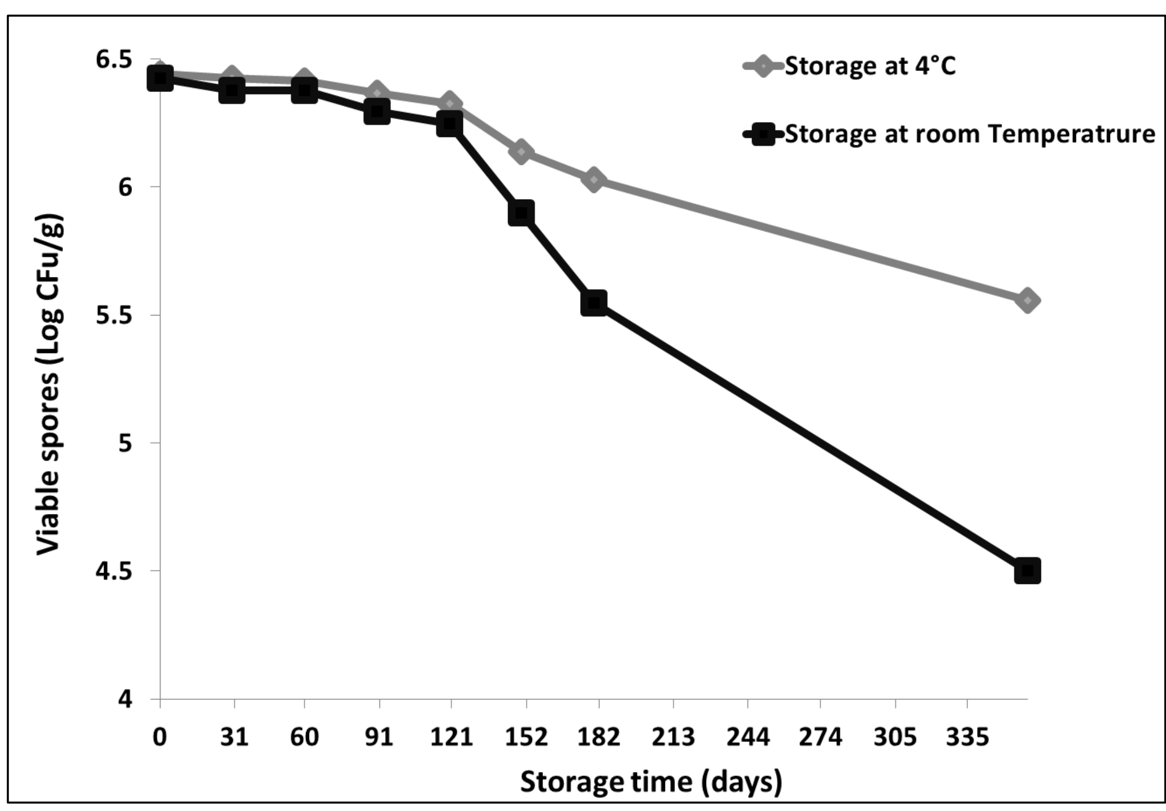
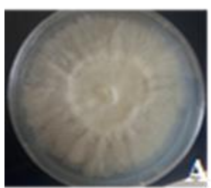
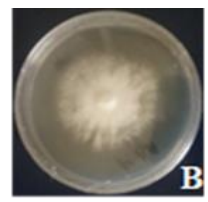


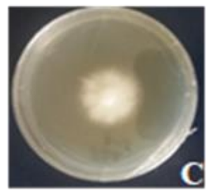
Figure 17: Shelf-life of the cassava starch/talc powder formulation of *S. cameroonensis* at different temperatures with projections for a year (360 days).

III.1.1.4. Biocontrol Efficacy of *S. cameroonensis*-Based Powder Formulation

The Powder formulation exhibits a high biocontrol efficacy against the causal agent of black pod disease of cocoa, *Phytophthora megakarya* (Figure 18). At a formulation concentration of 10% (w/v) supplemented on a PDA medium, we observe a 100% mycelial inhibition against *P. megakarya*. The mycelial growth inhibition percentage at very low concentrations of 1% (w/v) and 0.1% (w/v) was at 76.2% and 60.5 % respectively as shown in Figure 19 below.







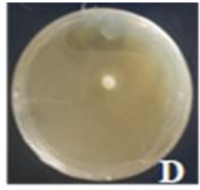


Figure 18: Photos of the inhibition diameters of various formulation dilutions against *P. megakarya* on PDA media: (**A**) Control (No inhibition), (**B**) 0.1% (60% inhibition), (**C**) 1% (76% inhibition) and (**D**) 10% (100% inhibition).

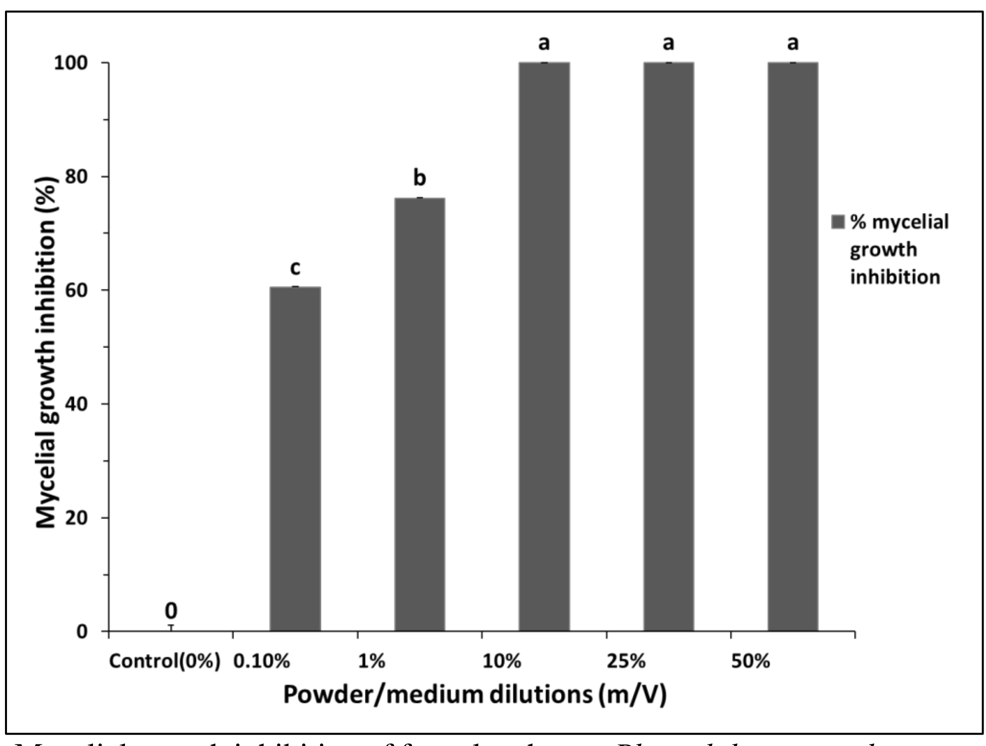


Figure 19: Mycelial growth inhibition of fungal pathogen *Phytophthora megakarya* at various concentrations of the powder formulation.

*Values with the same letter within a column are not significant at *P*≤ 0.05.

Note: **0%** (Control): 0% formulation; **0.1%:** 0.1%w/v formulation concentration; **1%:** 1% w/v formulation concentration**;** **10%:** 10% w/v formulation concentration; **50%:** 50% w/v formulation concentration, and **100%:** 100% w/v formulation concentration.

III.1.2. Liquid Formulation and its Characteristics

III.1.2.1. The Crude Extract of Secondary Metabolites of *S. cameroonensis*

The crude extract of secondary metabolites synthesized by *S. cameroonensis* is characterized by a strong unpleasant odour, dark black coloration and a viscous appearance. The yield obtained is 2.77% with respect to the mass of the biomass (Figure 20).

Figure 20: Crude extract of secondary metabolites of *S. cameroonensis.*

III.1.2.2. Stability of the Formulation

The evaluation of the stability of the various mixtures of the formulation in course of time shows that the formulations E (61.6/33.3/5.0), G (33.3/61.6/5.0), L (19.2/61.6/19.2) % v/v/v of crude extract/Tween 80/gum Arabic are the most stable after 24 hours of storage. They show no precipitation after 24 hours presenting a maximum stability score of 4 (Table XII).

Table XII: Stability scores for the various formulations after 24 hours.

Formulation	Stability score				
	0h	1h	2h	4h	24h
A	+	+	+	+	+
B	++++	++++	++++	+++	++
C	++++	++++	++++	+++	+++
D	+++	+++	+++	++	++
E	++++	++++	++++	++++	++++
F	++	++	++	+	+
G	++++	++++	++++	++++	++++
H	+++	+++	+++	+++	+
I	+++	+++	+++	+++	
J	+	+	+	+	
K	+++	+++	+++	+++	+++
L	++++	++++	++++	++++	++++
M	++	+	+	+	+

+: 25% stability ++ : 50% stability +++ : 75% stability ++++ : 100% stability - : 0% stability

III.1.2.3. Characteristics of the Stable Formulations

The evaluation of the physical properties and micelle formation of formulations E, G and L show that all are the three were viscous with an unpleasant smell and brown in colour. However, only formulation L do not show formation of micelles when viewed under the light microscope at a magnification of 400X (Table XIII). Thus, the formulation L was chosen for further analysis based on the above observations.

Table XIII: Characteristics of liquid formulations.

Formulation	State	Homogeneity	Odour	Colour		Image under 400 X magnification
E	Viscous	100%	Unpleasant	Dark brown		
G	Viscous	100%	Unpleasant	Dark Brown		
L	Viscous	100%	Unpleasant	Brown		

E (61.6/33.3/5.0), G (33.3/61.6/5.0) and L (19.2/61.6/19.2) % v/v/v of crude extract/Tween 80/gum Arabic

III.1.2.4. Biocontrol Efficacy of Liquid Formulations Against *P. megakarya*

In evaluating the efficacy of the liquid formulations, we observe a 100% inhibition rate for all formulations [E (61.6/33.3/5.0), G (33.3/61.6/5.0), L (19.2/61.6/19.2] % v/v/v of crude extract/Tween 80/gum Arabic) at concentration of 10% and above (Table XIV). However, at concentrations of 1%v/v, the formulation L with no micelle formation shows a 100% inhibition capacity and an 85% inhibition at a concentration of 0.1% w/v (Figure 21).

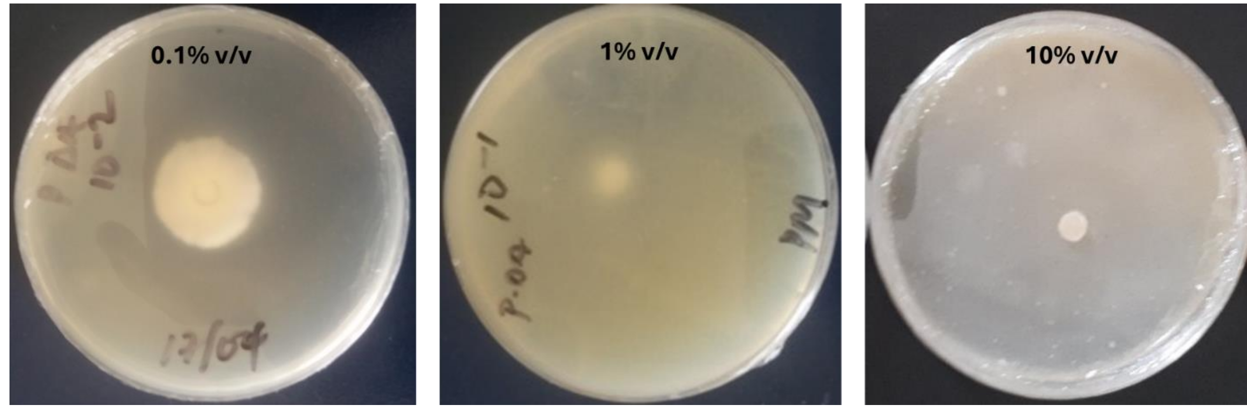


Figure 21: Inhibitory effects of different concentrations of the formulation L against *P. megakarya* on PDA medium.

Table XIV: Antimicrobial Efficacy of the liquid formulations (E, G and L) at different concentrations against *P. megakarya.*

Formulation	Concentration (% v/v)	Inhibition of *P. megakarya* (%)
E	10	100 ± 0a
	1	100 ± 2.16a
	0.1	90.00± 2.57b
G	10	100 ± 0a
	1	100. ± 1.31a
	0.1	90± 2.91c
L	10	100 ± 00a
	1	100 ± 00a
	0.1	85.00 ± 1.57d
Positive control (33 % w/v)	50	100 ± 00a

E (61.6/33.3/5.0), G (33.3/61.6/5.0), L (19.2/61.6/19.2) % v/v/v of crude extract/Tween 80/gum Arabic
*Values with the same letter within a column are not significant at $P \leq 0.05$.

III.1.2.5. Microbial Stability of the Formulations

The formulations remain stable at room temperature and free of microbial contaminants provided they are kept sealed. When left exposed to air, the formulation gets contaminated by microbes within a short period of time (12 hrs) (Figure 22).

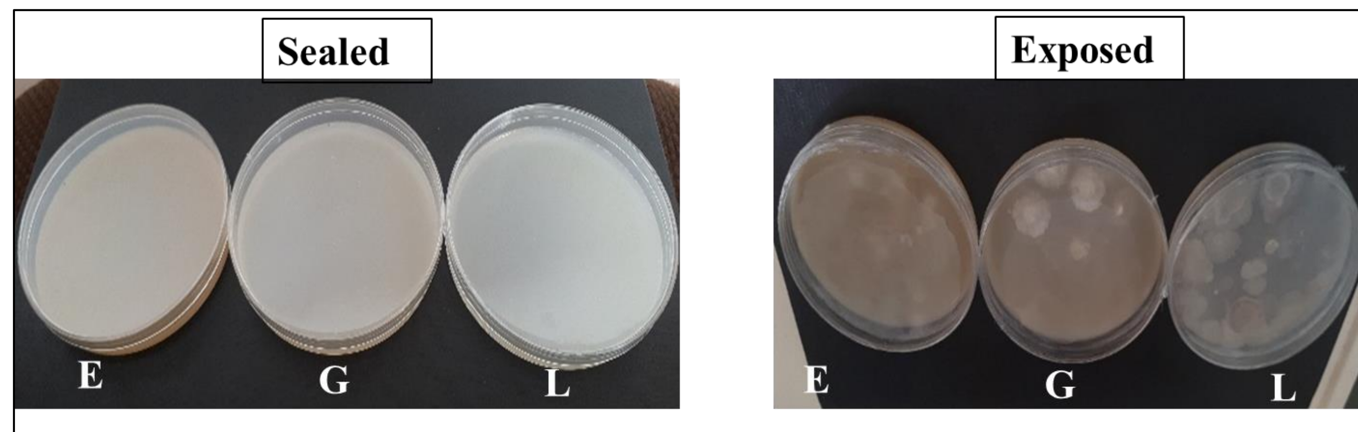


Figure 22: Storage stability of the Formulations A) sealed and stored at 25 °C and **B**) exposed. At room temperature for 12 hrs

III.1.2.6. Phytotoxic Effect of the L Formulation on Young Cocoa Leaves

The phytotoxicity of the L formulation was assessed at varying concentrations. At 100%, severe leaf burn was observed on very young cocoa leaves within 96 hours (Table XV). At 10%, moderate leaf discoloration and slight burning occurred. At 1%, only mild discoloration and deformation were noted. However, at the lowest tested concentration of 0.1%, no visible phytotoxic symptoms were recorded (Figure 23).

Table XV: Phytotoxic effect of the L formulation on young cocoa leaves.

Concentration (%)	24 H	48 H	72 H	96 H
0	–	–	–	–
0.1	–	–	–	–
1	–	–	+	++
10	–	+	++	+++
100	+	+++	++++	++++

- No observation; (+) leaf deformation; (++) leaf discoloration; (+++) discoloration + slight burning; (++++) Total burn

Figure 23: Phytotoxicity effect of different concentrations of the L formulation on young cocoa leaves after 96 hours.

III.1.3. Partial Discussion I

The aim of this first part was to develop and test the efficacy of formulations based on *Streptomyces cameroonensis* that can be used to stimulate the growth and resistance of cocoa seedlings against black pod disease in nurseries and farms. The selection of *S. cameroonensis* as our biocontrol agent is based on preliminary research that shows *S. cameroonensis* has antimicrobial activity against a wide range of pathogens including *P. megakarya*. Indeed, fresh cultures of *S. cameroonensis* promote plant growth and protect cocoa seedlings in nurseries against *P. megakarya* infection (Boudjeko *et al*. 2017). As a result, we decided to develop two formulations based on *S. cameroonensis* that are simple to apply, transport and store. Hence, we developed a powder formulation for treating cocoa beans before and after planting, as well as a liquid formulation for surface spraying.

The powder formulation of *S. cameroonensis* was developed using a combination of talc powder and cassava starch as carriers of *S. cameroonensis* spores. Calcium carbonate and carboxymethyl cellulose were used as additives. The first stage of development of the powder formulation involved testing the form and viability of *S. cameroonensis* spores in different proportions of mixtures of talc and cassava starch. The proportion of talc powder and cassava starch that demonstrated the finest powder with the highest spore viability after a period of one month was the T4 with 75%/25% w/w talc and cassava starch, and a spore viability of 2.78 x 10^6 CFU/g. The shelf-life of the spores in the powder mixture is six months when stored at 25 °C and could extend for a year based on the graphical extrapolation. The ability of S. *cameroonensis* spores to remain stable after such a long period of time is related to bacteria spores' ability to withstand high temperatures, humidity and other unfavorable environmental conditions. These findings are consistent with a previous study which report that a powdered form of *Streptomyces griseus* based on talc was stable up to the 105th day at both 4 °C and 30 °C storage conditions with 10^7 CFU/g (Anitha and Rabeeth, 2009; Mareeswaran and Radhakrishnan, 2016). *Streptomyces* species, as well as other plant growth-promoting rhizobacteria (PGPR) have been shown to survive in dry formulations containing talc, corn starch, xanthan gum and peat (Vidhyasekaran *et al.,* 1997, Liu *et al*., 2012, Tamreihao *et al*., 2013, Novinscak and Filion, 2020). The fact that talc and cassava starch are suitable for enhancing *S. cameroonensis* spore survival is probably related to the nature of their lipopolysaccharides and nutritional, as well as their coating properties. Talc has been shown in several powder formulations to favor an increased shelf-life for biocontrol agents ranging from 45 days to 12 months (Sahai and Kumar, 2019), since starch serves as an important source of

carbon and nutrients. The correct combination of these carriers with the adhesive carboxymethylcellulose (CMC)and stimulator calcium carbonate ($CaCO_3$) provides a temporary protective surface for *S. cameroonensis* spores, allowing them to maintain a high population count for an extended period. Recently, additional additives have included protective compounds such as sugars, polymers and amino acids in powders and granules to enhance the shelf-life of the microorganisms (Berninger *et al*., 2018). In evaluating the antimicrobial effect of the powder formulation (T4) against *P. megakarya in vitro*, the formulation at 10% m/V was shown to suppress the growth *of P. megakarya* by 100%. This can be explained by the fact that the presence of *S. cameroonensis* spores in the powder allows them to digest starch and powder and disseminate, while in a moist environment to exercise their antimicrobial effect. *Streptomyces cameroonensis* possesses antimicrobial properties, as demonstrated by the production of bioactive molecules such as geldanamycin, which can inhibit the growth of oomycetes (Boudjeko *et al.,* 2017). *Streptomyces* species are also known to produce major fungal degrading cell wall enzymes such as chitinase and β-1,3-glucanase, as well as volatile compounds, siderophores and other compounds that inhibit fungal pathogen mycelial growth (Fernandes, 2006, Dimkpa *et al*., 2008, Khan *et al.,* 2023). Moreover, a powder formulation of *Streptomyces corchorusii* strain UCR3-16 have been shown to inhibit rice fungal pathogens such as *Bipolaris oryzae*, *Rhizoctonia solani, Fusarium oxysporum* and others (Tamreihao *et al.*, 2016). This antimicrobial activity initiated by this *Streptomyces* strain corresponds with our results previously described above. The presence of *S. cameroonensis* spores in the powder as demonstrated by our viability study can explain the antimicrobial activities of this formulation suggesting the production of anti-fungal metabolites, volatile compounds, siderophores and cell wall degrading enzymes that inhibit pathogen growth.

The liquid formulation was prepared from the secondary metabolites of *S. cameroonensis* as the main active ingredient, with Tween 80 as an emulsifier and 15% w/v gum arabic as an adhesive. Analysis of stability showed that the trials E (61.6/33.3/5.0), G (33.3/61.6/5.0) and L (19.2/61.6/19.2) % v/v/v of crude extract/Tween 80/gum arabic were the most stable after 24 hours of preparation, showing 100% homogeneity. Observation under the light microscope showed no micelles formed only with the L formulation. Indeed, the stability of a mixture of oily emulsions is influenced by the 6-80% content of Tween 80 in the presence of other compounds such as formamide, ethylene glycol, and glycerol (Bhattacharya and Dixit, 2015, Udomrati *et al*., 2020). In the presence of the oily secondary metabolites and the adhesive gum arabic, our most stable mixtures had tween content ranging from 33 to 62%. In combination with the shown quantities of the adhesive and active ingredient, the formulation

remained stable after 24 hours of storage. Adhesives, thickeners, dispersants, and thickening agents provide good stability as well as keep the active ingredient from sedimentation (Bejarano and Puopolo, 2020). Our formulations were stored at room temperature without any contamination, provided they were sealed. The formulation kept in contact with the air would thus be colonized by certain ambient microorganisms. The formulation could well be stored at 4 °C for better preservation. The biocontrol efficacy of the formulation showed 100% inhibition of the pathogen (*P. megakarya*) for all 3 stable mixtures (E, G and L) at concentrations of 1%. This biocontrol efficacy can be directly correlated with the presence of a crude extract of secondary metabolites of *S. cameroonensis*. Secondary metabolites of *Streptomyces* are rich in antibiotics, volatile compounds, and fungal cell wall-degrading enzymes. The antibiotic geldanamycin produced by *S. cameroonensis* is known to inhibit the growth of oomycetes such as *P. megakarya (*Boudjeko *et al*., 2017*).* Furthermore, it has been shown that volatile substances from *Streptomyces* could cause several morphological abnormalities in fungi and oomycetes (Moore-Landecker and Stotzky, 1973; Jones *et al.,* 2017). As a result, volatile compounds found in the crude extract of *S. cameroonensis* secondary metabolites have the potential to act as biofumigants in the formulation. This crude extract has the potential of containing hydrolytic enzymes like chitinases and β-1,3-glucanases (Boudjeko *et al*., 2017). These enzymes capable of degrading fungal and bacterial cell walls, cell membranes, cell membrane proteins and extracellular virulence factors have been implicated in biocontrol (Pal and Gardener, 2006). The formulation L, which is homogenous with no micelles formed, showed good antimicrobial activity agains*t P. megakarya*, precisely at 0.1% (85% efficacy), had no phytotoxic effect against young, detached cocoa leaves. It is important to point out in this case that phytotoxicity is one of the most important determinants of a good formulation and can be observed on plants at emergence, growth, and harvest (Bejarano and Puopolo, 2020). Symptoms of phytotoxicity can include changes in the development cycle, thinning, color changes, necrosis, deformations, effect on quantity and quality of yield may be temporary or long-lasting affecting thus the entire plant or specific plant parts. The phytotoxicity of the liquid formulation was determined by changes in color, necrosis, and deformation of the leaves. We noticed our formulation at very low concentrations of 0.1% (85% antimicrobial efficacy) had no phytotoxic effect on the leaf. The high levels of phytotoxicity at 100%, 10% and 1% can be explained by the high concentrations of Tween 80 and its mixtures with oily substance of secondary metabolites. It has been recently shown that high concentrations of Tween 80 and oily substances at a concentration of 10 ml/L led to 20% mortality in Baikal oligochaetes *Mesenchytraeus bungee* (Zyuzina *et al*., 2021). This is consistent with our findings, which

show that higher concentrations of the formulation (Tween 80 and the oil crude secondary metabolite) were phytotoxic to our plants. However, at these concentrations, the action of individual substances is harmless to the plant (Zyuzina *et al*., 2021).

Partial Conclusion I

The present study had as its aim the development of two formulations based on *S. cameroonensis* (a powder formulation for seed treatment and a liquid formulation for spraying). A powder formulation and a liquid formulation were effectively developed and showed high levels of efficacy *in vitro* against the fungal pathogen *P. megakarya*. The powder formulation checked through all the criteria for an effective bioformulation. When stored at 4 °C and 25 °C, the formulation had a shelf life of 1.07 x 10^6 CFU/g and 3.53 x 10^5 CFU/g, respectively. At 4 °C, the spores can remain viable for over a year. The ability of *S. cameroonensis* to form spores gives it the ability to tolerate adverse environmental conditions. The efficacy of the formulation against *P. megakarya* was 100% at concentrations of 10% w/w. The formulation is also compatible with other agrochemicals and the carriers used for formulation development are inexpensive and readily available, making the formulation not only effective against *P. megakarya* and potentially other oomycetes, but also cost effective. The liquid formulation of *S. cameroonensis*-based secondary metabolites developed was 85% effective at a very low and non-phytotoxic concentration of 0.1% v/v. This formulation can be stored at room temperature in a closed container for several months. The next phase of this work involves evaluating the antimicrobial and plant growth-promoting properties of the formulations on cocoa seedlings in the nursery challenged with *P. megakarya*.

III.2. Agromorphological and Biochemical Effects of Formulations on Cocoa Seedlings' Growth and Resistance in Nursery

III.2.1. Effect of Formulations on the Growth Parameters of the Cocoa Seedlings in the Nursery

III.2.1.1. Effects of Powder Formulation on Growth of Cocoa Seedlings in the Nursery

Powder formulation-treated plants show a significant increase in plant height over the non-treated and chemically treated plants after 12 weeks of growth for both hybrids. Plant height in formulation-treated plants for H1 is significantly higher (30.4±0.74, 36.00±1.02 and 37.30±0.81cm for the non-treated (NT), chemically treated (C), and formulation-treated (T) plants respectively, compared to H2 hybrids (23.76±0.24, 24.67±0.94 and 26.43±0.74 for the NT, C, and T plants respectively). After the 12th week of growth, the H1 and H2 hybrids have a percentage increase in stem length of 22.7% and 11.2% respectively when compared to the non-treated (Figure 24A).

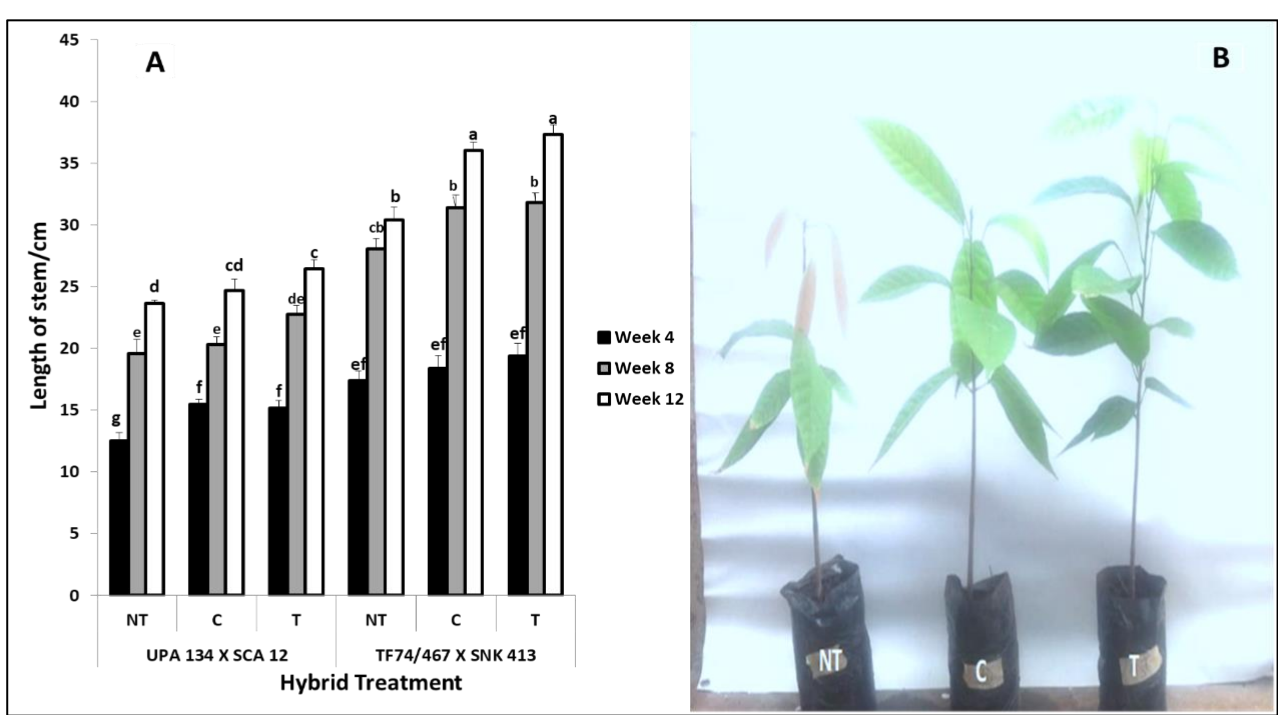


Figure 24: Effects of powder formulation treatment: (A) on stem length of cocoa hybrids in the nursery, (B) photos of 12-week-old [H1 (♀) SNK413 × (♂) T79/467] hybrid cocoa plants treated (photographed by Dzelamonyuy, 2020).

*Values with the same letter within a column are not significant at $p \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

In addition, treatment with our powder formulation significantly increases the number of leaves at all stages of plant development. This increase is remarkably large at the twelfth week for both the H1 and H2 hybrids. We have an average of 11.5±0.7, 12.5±0.92 and 12.8±0.74 leaves for the non-treated, chemically treated and formulation treated (Figure 25A).

The surface area of a plant's leaves is very different between formulation-treated and non-treated plants. Leaf surface area in formulation-treated plants for H1 is significantly higher (56.68±2.19, 58.01±1.59 and 83.78±3.71 cm^2 for the non-treated (NT), chemically treated (C), and formulation-treated (T), respectively) compared to H2 hybrids (38.23±1.54, 43.41±1.78, 47.50±1.48 cm^2 for NT, C, and T, respectively). This shows a percentage increase of 47.8% for the H1 hybrid after treatment compared to 24.25% for the H2 hybrid. However, after the 12th week of growth, the chemically treated formulations show a lower percentage increment of 2% and 13.2% for H1 and H2 hybrids respectively (Figure 25B).

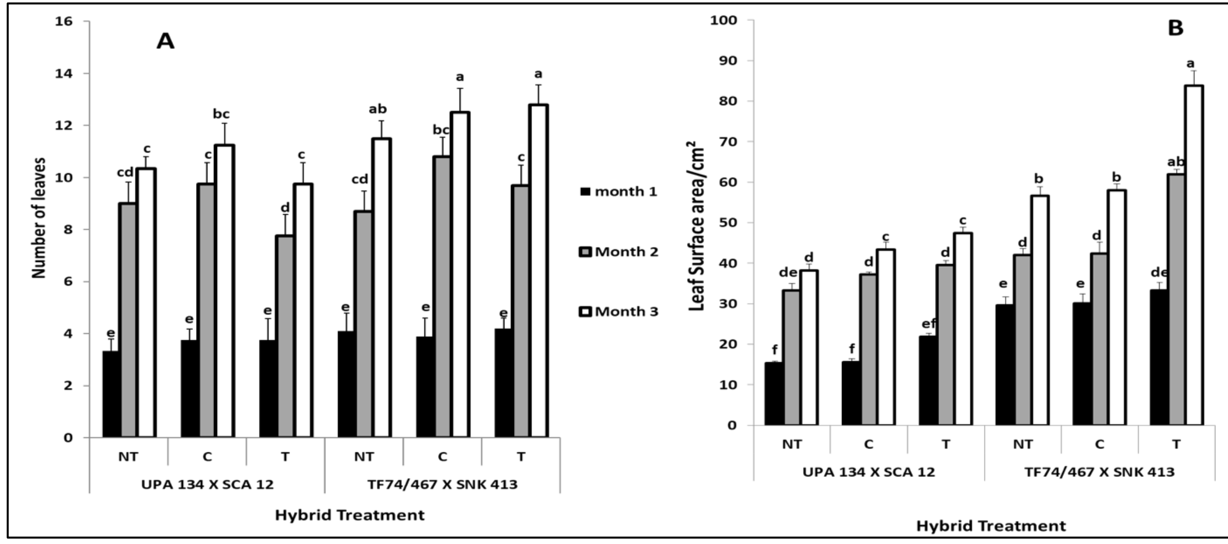


Figure 25: (A): Effects of powder formulation treatment on the number of leaves (A) and leaf surface area (A) of cocoa seedlings in nursery.

*Values with the same letter within a column are not significant at $p \leq 0.05$.
Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

In the presence of the powder formulation, the dry weights of the roots and shoots are higher in the H1 hybrid (1.80±0.18, 2.30±0.11, 3.08±0.14 g and 7.14±0.54, 7.56±0.85, 8.51±0.31 g respectively) for the non-treated (NT), chemically treated (C) and formulation treated (T) plants respectively; compared to the H2 hybrid (1.16±0.14, 1.40±0.10, 1.44±0.09 and 3.58±0.31, 4.33±0.38, 3.81±0.40) respectively (Figure 27). This implied a percentage increase of 71% and 19% for dry root and shoot weight for the H1 hybrid compared to 24% and 8% for the H2 hybrid.

Figure 26: Photos of 12-week-old dry roots of cocoa plants treated with the powder formulation and chemical product

*Note: A: Hybrid H1 and B: hybrid H2, NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (With Callomil Super 66WP).

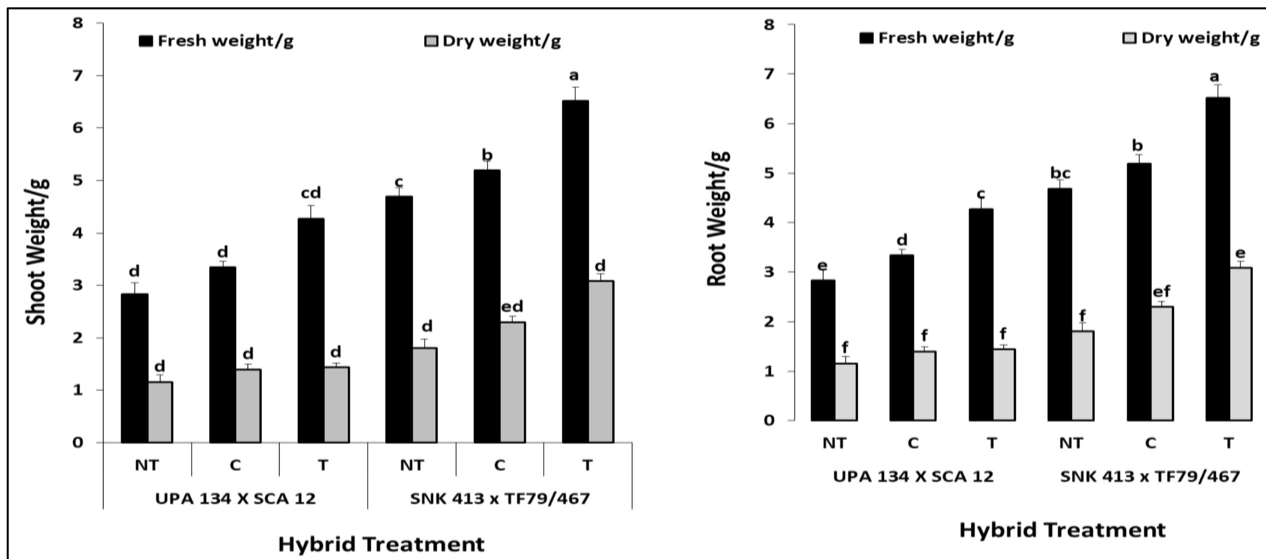


Figure 27: Variation of the fresh and dry weights of roots (A) and shoots (B) with respect to the treatments after 12 weeks of growth.

*Values with the same letter within a column are not significant at $p \leq 0.05$.
Note: NT: non-treated (negative control), T: treated (with bioformulation) and C: chemically treated (with CALLOMIL SUPER 66WP).

III.2.1.1. Effects of Liquid Formulation on Growth of Cocoa Seedlings in Nursery

Seedling treatments with the liquid formulation shows a significant increase ($P \leq 0.05$) of the agromorphological parameters (stem height, number of leaves and leaf surface area) at all stages of growth compared to the non-treated cocoa seedlings (Figure 28).

In effect, the average stem height after 12 weeks of growth stands at 29.2±0.5 cm, compared to 22.7±0.6 cm for the non-treated seedling. No significant increase is observed in seedlings treated with the chemical fungicide (Figure 28A). We equally observe a significant increase in the number of leaves in batches treated with our liquid formulation compared to the chemically treated and the non-treated, with an average 9.4±0.5 for the formulation treated compared to 8.4±0.5 and 7.6±0.5 leaves for the chemically treated as well as non-treated respectively (Figure 28B). The average leaf surface area at the 12th week is observed to be significantly higher with 77.30±2.82 cm^2 for the formulation treated compared to 71.39±2.59 cm^2 and 69.29±1.87 cm^2 for the chemically treated and non-treated batches respectively (Figure 28C).

Infection of seedlings with the pathogenic agent *P. megakarya* slows down growth. We observed an average slowdown in growth rates of 15%, 14.7% and 9.3%, respectively for the non-treated, chemically treated and formulation-treated seedlings because of *P. megakarya* infection. However, the formulation-treated seedlings maintained a higher growth rate than the rest of the treatments despite the slowing effects of *P. megakarya*. Indeed, we observe that cocoa seedlings treated with our liquid formulation and infected with *P. megakarya* show a higher plant height growth rate than the chemically treated (23%), as well as the non-treated (27%) ones (Figures 28).

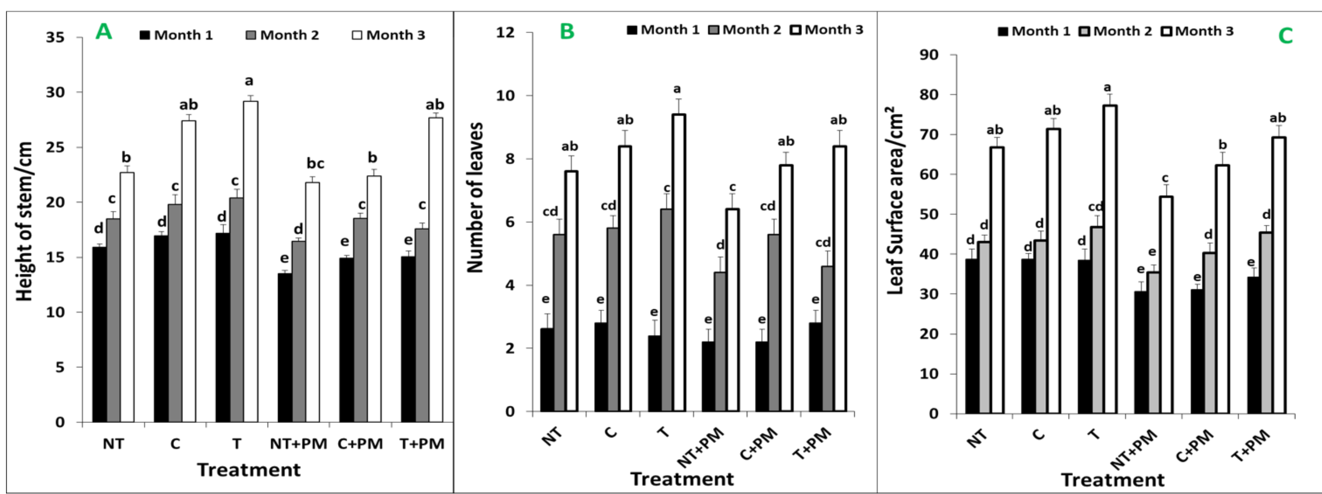


Figure 28: Effects of liquid formulation on (**A**) height of plant stem, (**B**), number of leaves, (**C**) leaf surface area over a period of three months in nursery.

*Values with the same letter within a column are not significant at *P*≤ 0.05.

Note: NT: non-treated (negative control), T: treated (with liquid formulation), C: chemically treated (with CALLOMIL SUPER 66WP), NT+PM: non-treated infected with *P. megakarya*, C+PM: chemically treated infected with *P. megakarya* and L: liquid formulation treated infected with *P. megakarya.*

III.2.2. Disease Severity

II.2.2.1. Effect of Powder Formulation on Disease Severity

Six days after the *P. megakarya* inoculum was applied to all leaves, necrotic lesions were discovered but no lesions were present on the leaves exposed to sterilized distilled water. Analysis of variance demonstrates that the disease expression is significantly different for the various treatments ($P \leq 0.05$). For both hybrids, plants treated with the *S. cameroonensis*-based powder formulation shows significantly lower disease severity indices than untreated plants, with roughly 67% for H1 and 55% for H2 (Figure 29). We may conclude that they are very resistant with a necrosis index of 0.83 for the H1 plants treated with the formulation, whereas the necrosis index for the H2 plants treated with the formulation is 1.5 indicating resistance.

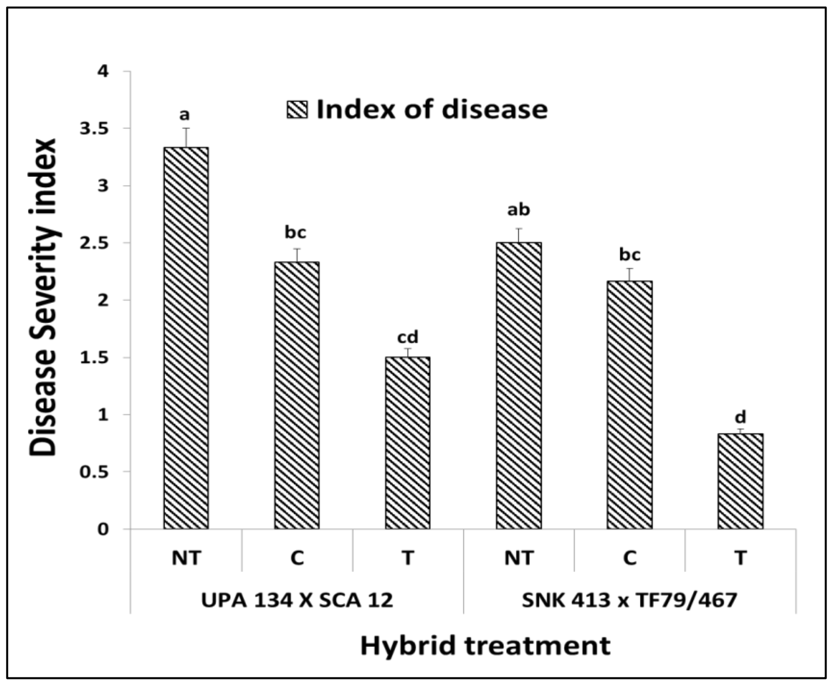


Figure 29: Disease severity index of plants under different treatments with the *S. cameroonensis*-based powder formulation six days after inoculation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.
Note: NT: non-treated (negative control), T: treated (with bioformulation) and C: chemically treated (with CALLOMIL SUPER 66WP).

The chemically treated plants show a necrosis index of 2.1 for H1 and 2.3 for H2, respectively, which classifies them as moderately resistant. Whereas the non-treated H2 plants are categorized as vulnerable with a necrosis index of 3.3, the non-treated H1 plants exhibit moderate resistance with a necrosis value of 2.5.

III.2.2.2: Effect of Liquid Formulation on Disease Severity

To assess resistance levels, leaves from each treatment group; non-treated (NT), chemically treated (C), and liquid formulation-treated (T), were inoculated with *Phytophthora megakarya* following Nyasse *et al.* (1995). This explains the presence of disease symptoms even in seedlings not previously exposed to the pathogen in soil. In a separate setup, denoted with "+PM," seedlings were grown in soil pre-infected with *P. megakarya* to evaluate treatment effects under active pathogen pressure.

Six days after inoculation, the disease severity index (DSI) was lowest in T-treated seedlings (DSI = 1.0, very resistant), followed by C (1.4, resistant) and NT (2.3, moderately resistant). A similar pattern was observed in the infected groups, with severity increasing across the board: T+PM (1.7, resistant), C+PM (2.2, moderately resistant), and NT+PM (3.3, susceptible). This confirms the protective effect of the formulation both systemically and under real infection conditions.

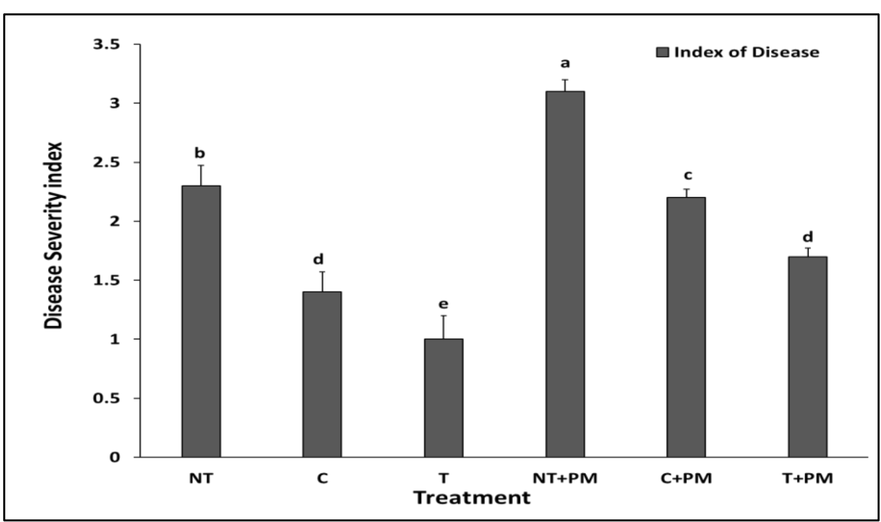


Figure 30: Variation in height of cocoa seedlings after treatment or non-treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: non-treated (negative control), T: treated (with liquid formulation), C: chemically treated (with CALLOMIL SUPER 66WP), NT+PM: non-treated infected with *P. megakarya*, C+PM: chemically treated infected with *P. megakarya* and L: liquid formulation treated infected with *P. megakarya*.

III.2.3. Biochemical Analysis

III.2.3.1. Effect of Powder Formulation on Biochemical Parameters of Resistance

III.2.3.1.1. Variation in the Content of Total Phenolic Compounds

Quantitative analysis of total phenolic compounds in the leaves of cocoa seedlings for both hybrids before and after infection shows significant differences ($P < 0.05$) between the various treatments. For both hybrids, the total phenolic content of plants treated with the powder formulation based on *S. cameroonensis* is higher than that of chemically treated and untreated seedlings. In comparison to the chemical control and the untreated plants, the *S. cameroonensis*-based formulation-treated plants exhibit a significantly higher level of total phenol content. After inoculation in the formulation-treated plants, H1 exhibits a 40% larger variation in phenol content compared to H2 (Figure 31). After inoculation of cocoa leaves with *P. megakarya*, total phenol content in formulation-treated plants increased by 16% for H1 and 85% for H2 (Figure 31).

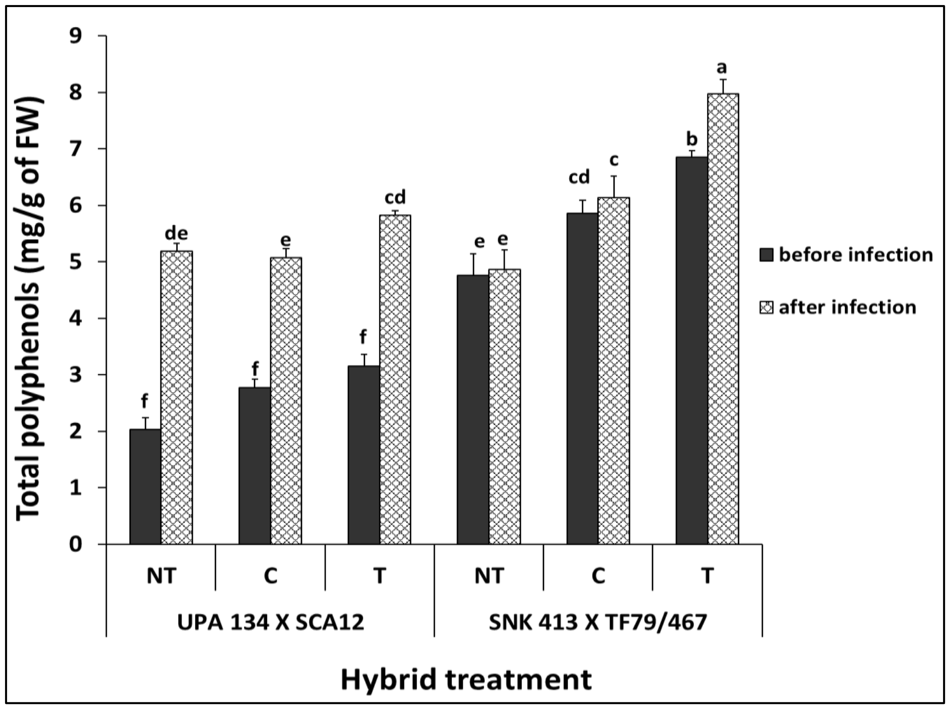
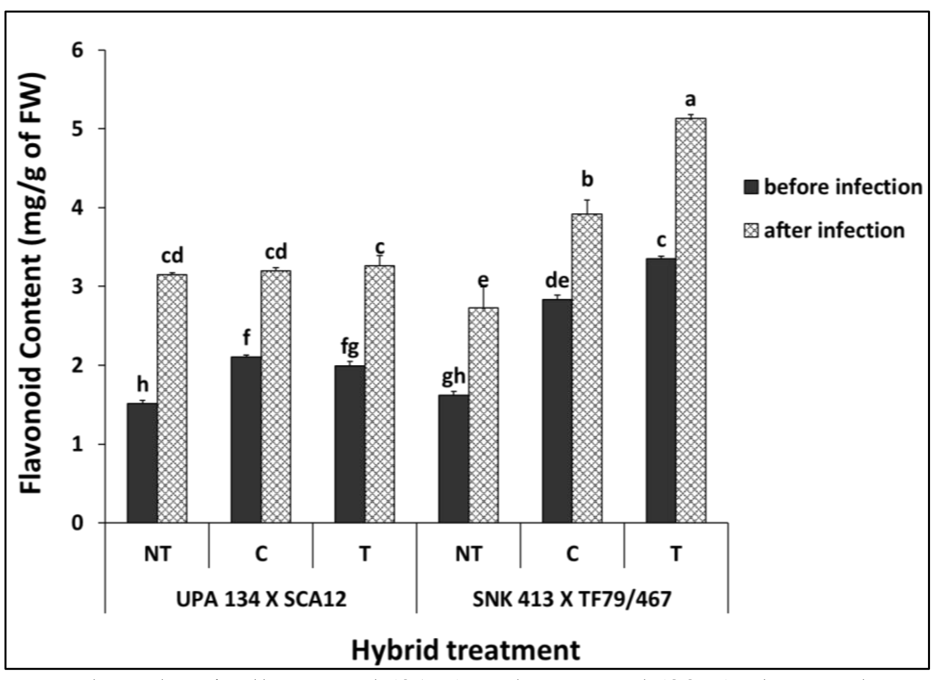


Figure 31: Total polyphenol content in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P \leq 0.05$.
Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.2. Variation in Flavonoid Content

A quantitative analysis of the flavonoid content in the leaves of the H1 hybrid cocoa seedlings before and after infection reveals significant differences ($P \leq 0.05$) between all treatments. After inoculation, formulation treated H1 plants display a considerably greater



flavonoid content than chemically treated (31%) and untreated (88%) plants, whereas changes in the flavonoid content of H2 plants are not significant (Figure 32).

Figure 32: Flavonoid content in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P \leq 0.05$.
Note: NT: Non-treated (negative control); T: Treated (with bioformulation); C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.3. Variation in total protein content

The formulation-treated plants from both the H1 and H2 hybrids have a much higher protein content before and after infection with a sporal solution of *P. megakarya* than chemically treated plants or plants that were not treated at all (Figure 33). For all treatments and hybrids, there is an increase in total protein synthesis concurrent with the infection of leaves. The protein content of the H1 hybrids treated with the formulation increased by 71% (5.30 to 9.06 mg equivalent of BSA/g of fresh weight (FW)), whereas the protein content of the H2 hybrid increased by 67% (2.94 to 4.92 mg equivalent of BSA/g of FW). In contrast, chemically treated plants showed lower protein synthesis following infection of 11% and 30%, respectively, for the H1 and H2 hybrids. Treatment of cocoa seedlings with the formulation led to an increase in total protein synthesis of 42% and 46% for the H1 and H2 hybrids respectively in comparison to the untreated plants (Figure 33).

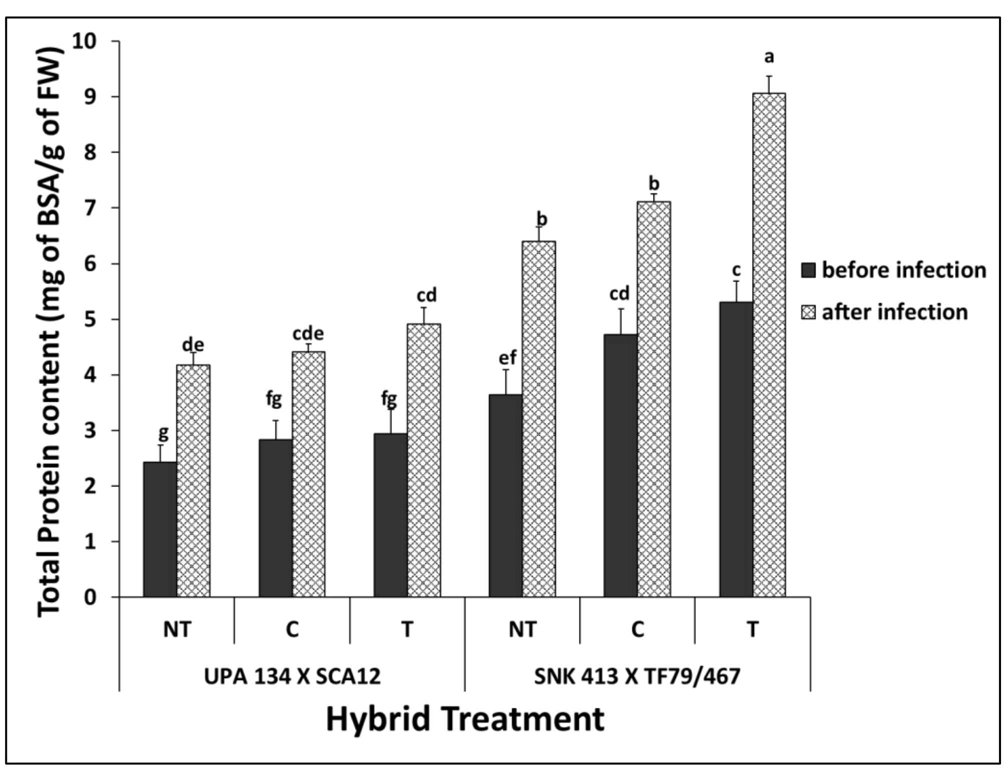


Figure 33: Total protein content in *S. cameroonensis* based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P \leq 0.05$.
Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.4. Variation in the Activity of Polyphenol Oxidases

For both hybrids H1 and H2, there is a significant difference in the enzymatic activity of polyphenoloxidases (PPO) in the leaves of cocoa seedlings between treatments before and after infection ($P \leq 0.05$). The formulation-treated seedlings exhibit a substantial increase in polyphenol oxidase activity (Figure 34). This significant rise in enzyme activity in all treatments is a feature of *P. megakarya* infection of leaves (Figure 34). In all formulation-treated seedlings, this action is more prominent, more so in the H1 hybrid than the H2 hybrid. For the formulation treated H2 and H1 hybrids, the rate of increased PPO activity after infection is in the range of 17% (4.18 to 4.91 Abs at 330 min/g of FW) and 39% (5.34 to 7.44 Abs at 330 min/g of FW) respectively (Figure 34). In comparison to the untreated group, treatment with the formulation boosted PPO activity by 25% for the H1 hybrid and by 24% for the H2 hybrid, whereas following infection PPO activity increased by 8% and 6%, respectively for the chemically treated group (Figure 34).

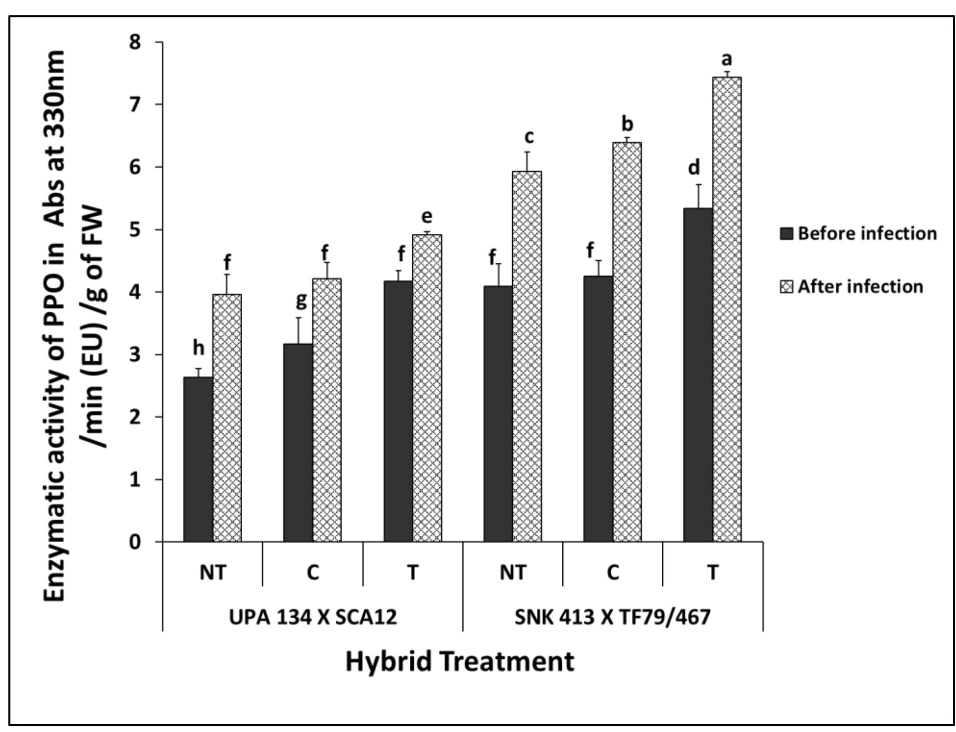


Figure 34: Enzymatic activity polyphenoloxidase in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P \leq 0.05$.
Note: NT: Non-treated (negative control); T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.5. Variation in the Activity of Peroxidases

The peroxidase (PR 9) activities in the total proteins extract observed vary according to the health status of the plant. Peroxidase activity is observed to be high in all batches treated with the *S. cameroonensis*-based powder formulation (Figure 35). A noticeable surge in peroxidase activity is a hallmark of *P. megakarya* infection in all treatments, with a more prominent activity in batches treated with our powder formulation (Figure 35). As a result, for the H1 and H2 hybrids treated with the powder formulation, increases of 21% (5.34 to 6.54 EU/min/g of FW) and 40% (3.91 to 5.50 EU/min/g of MF) respectively are seen. The activity of peroxidase is increased by 58% for the H1 hybrid and 54% for the H2 hybrid after treatment with the formulation as opposed to the untreated (Figure 35).

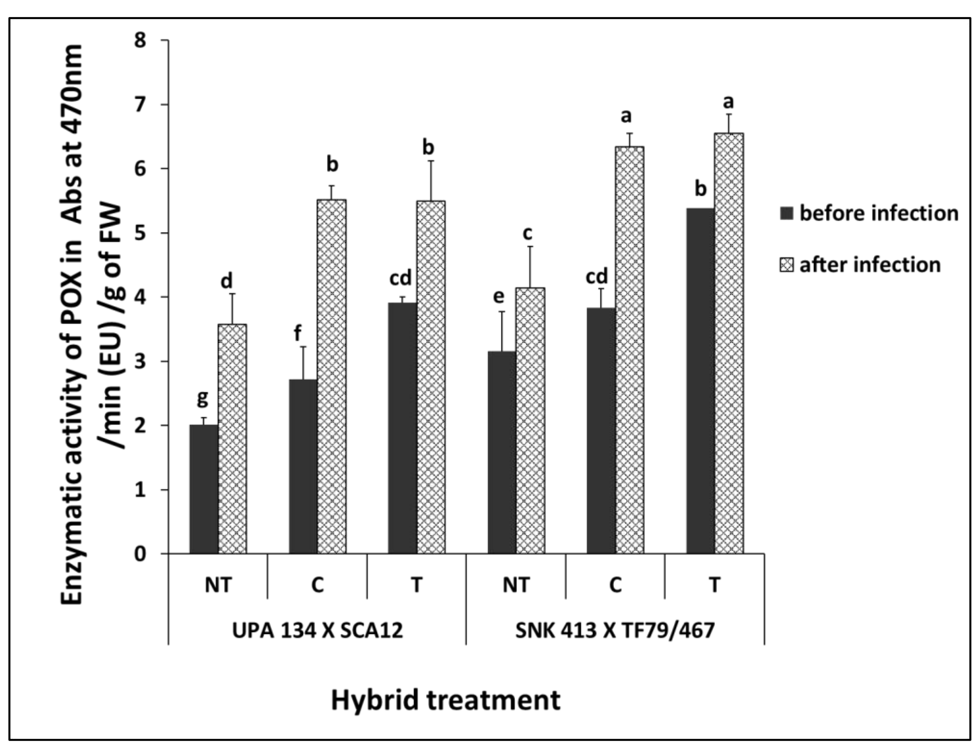


Figure 35: Enzymatic activity of peroxidase in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P\leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.6. Variation in the Activity of Chitinases

Depending on the treatments and the plant's health, the activity of chitinases (PR 2) varies considerably (Figure 36). Its activity is observed to be higher in *S. cameroonensis*-based powder formulation treatments before and after infection. Increased chitinase activity is a hallmark *of P. megakarya* infection in all treatments, with higher activity in batches treated with our powder formulation (Figure 36). Following *P. megakarya* infection, the activity of chitinases is seen to rise by 49% (1.49 to 2.22 EU/g of FW/h) and 43% (1.35 to 1.93 EU/g of FW/h) for the H1 and H2 hybrids respectively in the powder formulation treatments. After infection with *P. megakarya,* chemical treatment had no discernible impact on the production of chitinases, but treatment with the formulation enhanced the activity of chitinases by 19% for the H1 hybrid compared to 54% for the H2 hybrid (Figure 36).

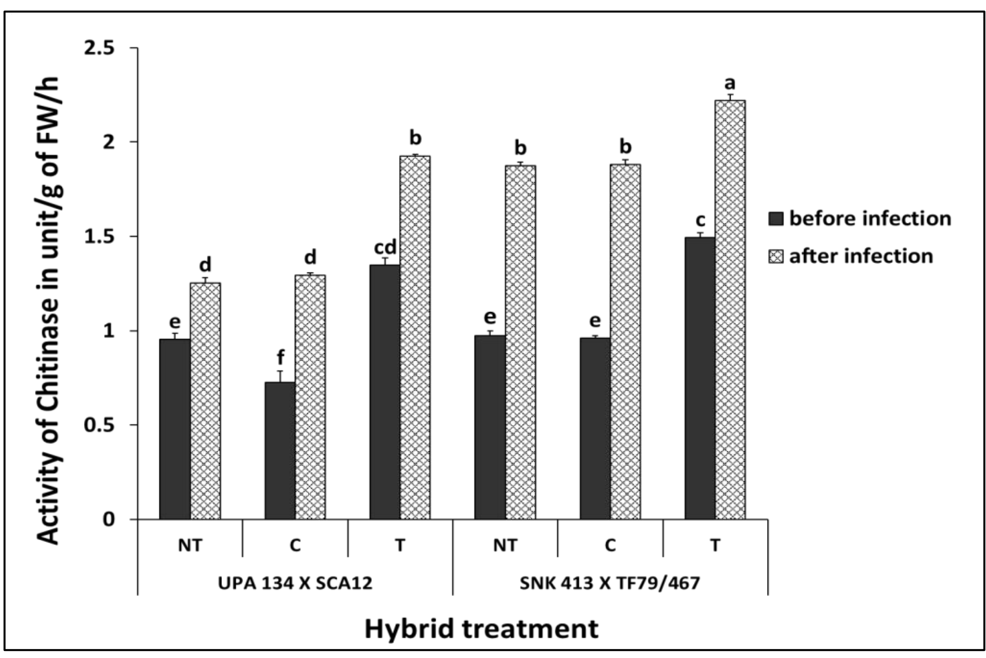


Figure 36: Enzymatic effect of Chitinase in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation with *P. megakarya.*

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.1.7. Variation in the Activity of β-1,3-Glucanases

Treatments using the *S. cameroonensis*-based powder formulation are observed to have a generally higher enzymatic activity of β-1,3-glucanase (PR 3) in comparison to other treatments (Figure 37). After infection with *P. megakarya,* all the treatments show a considerable rise in β-1,3-glucanase enzymatic activity, although the plants treated with the powder formulation show a more pronounced activity (Figure 37). For the H1 hybrid and H2 hybrid, the rate of β-1,3-glucanase activity rises throughout the powder formulation treatments by 89% (1328 to 2513 µmoles of glucose released/min/g of FW) and 205% (503 to 1535 µmoles of glucose released/min/g of FW) respectively. In contrast to chemical therapy, which has a negligible impact on the production of chitinases after infection with *P. megakarya*, treatment with the formulation raises the activity of chitinases by 93% for the H1 hybrid compared to 47% for the H2 hybrid (Figure 37).

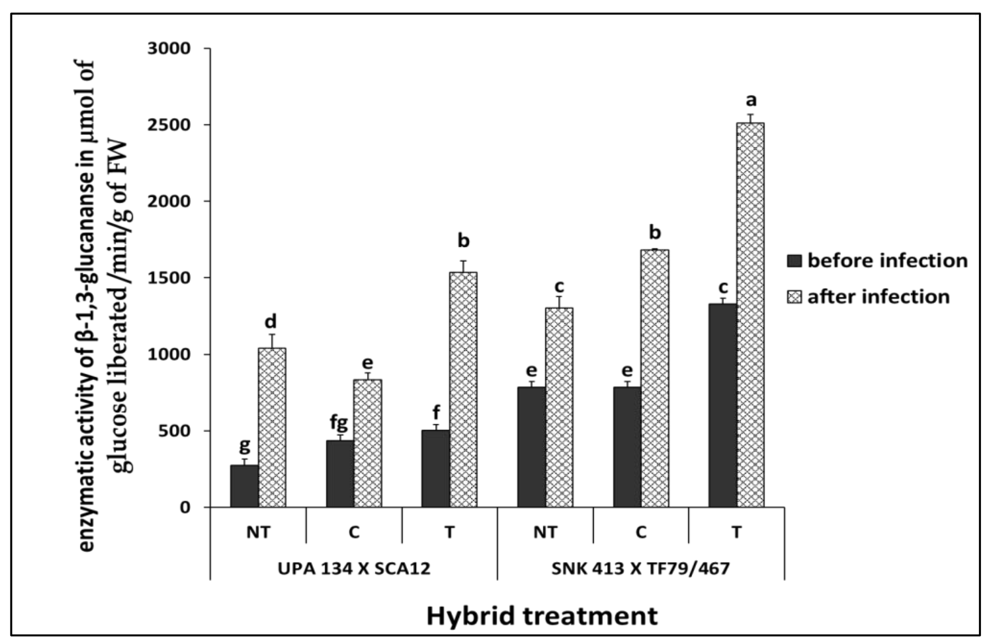


Figure 37: Enzymatic effect of β-1,3-glucanase in *S. cameroonensis*-based powder formulation treated and non-treated plants before and after inoculation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with bioformulation) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.2.3.2. Effect of Liquid Formulation on Biochemical Parameters of Resistance

III.2.3.2.1. Variation in the Content of Total Phenolic Compounds

Quantitative analysis of total phenolic compounds in cocoa leaves reveals significant differences ($P \leq 0.05$) in all treatments before and after infection (Figure 38). Infection of leaves by *P. megakarya* is characterized by a significant increase in the content of phenolic compounds in all treatments. In effect, treatment with our liquid formulation leads to an increase of 46% (from 0.91 to 1.33 mg/g of FW), 43% (0.90 to 1.28 mg/g of FW) and 37% (0.87 to 1.19 mg/g of FW) respectively for Formulaton treated (T), Chemically treated (T) and Non-treated (NT) seedlings (Figure 38). We have seen a similar trend with the introduction of *P. megakarya* into soil. However, the confirmed presence of *P. megakarya* in soil leads to a significant average decrease of 22% in the content of phenolic compounds in all treatments both before and after infection.

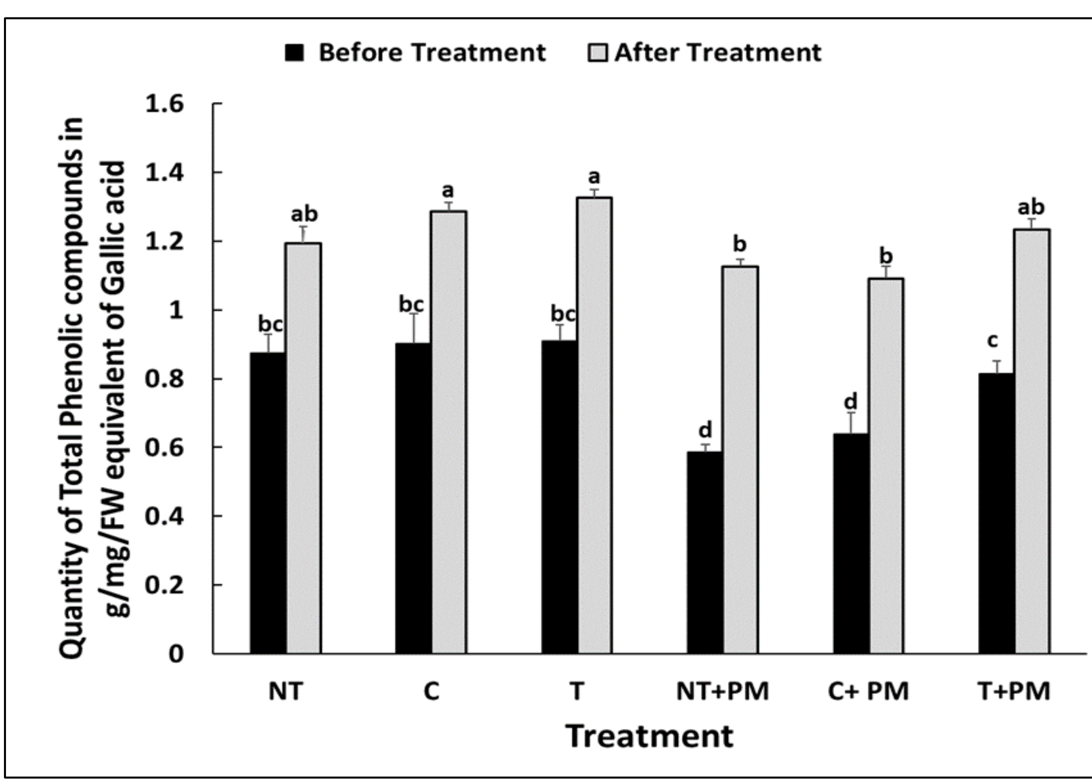


Figure 38: Variation in the content of Total Phenolic compounds after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

III.2.3.2.2. Variation in the Content of Flavonoids

In our treatments with the liquid formulation, the content of flavonoids is substantially higher than in the chemically treated and untreated batches (Figure 39). Analysis of variance shows that the flavonoids? content is significantly different in all treatments after infection. Infection of leaves with *P. megakarya* causes a significant increase in flavonoids across all treatments. However, treatment with our liquid formulation leads to a more noticeable accumulation of these flavonoids in the order of 122% (0.27 to 0.59 mg/g of FW) compared to 116% (0.26 to 0.58 mg/g of FW) for the chemical treatment and 66% (0.26 to 0.43 mg/g of FW) for the untreated cocoa seedlings. Moreover, when *P. megakarya* is introduced into the soil, the content of flavonoids in all treatments drops by an average of 19% before infection and 31% after infection.

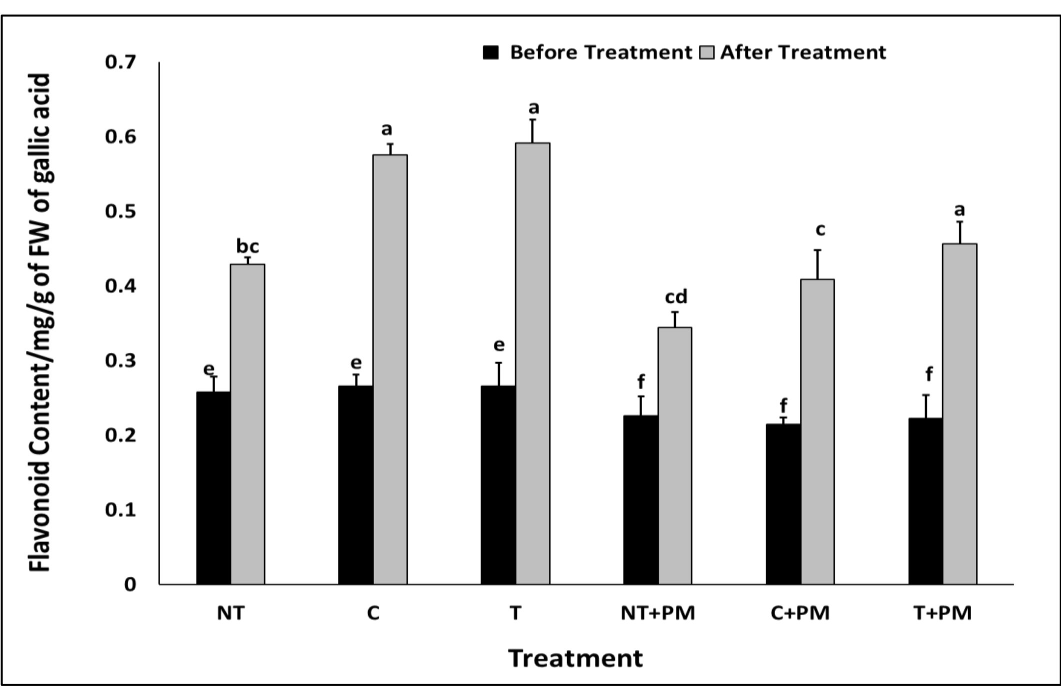


Figure 39: Variation in the content of Flavonoids after treatment with the liquid formulation. *Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

III.2.3.2.3. Variation in the Content of Total Proteins

Quantitative analysis of total protein content in cocoa leaves before and after infection shows significant differences ($P < 0.05$) between the various treatments (Figure 40). This is highly observed in seedlings treated with our liquid formulation both before and after infection (Figure 40). Infection of leaves by *P. megakarya* is characterized by a significant increase in the synthesis of total proteins. Treatment with our liquid formulation leads to an average increase of 0.6 mg/g of FW (30%) compared to the non-treated. The presence of *P. megakarya* led to an average reduction of 22% in the content of total proteins in the leaves of plants treated with our liquid formulation.

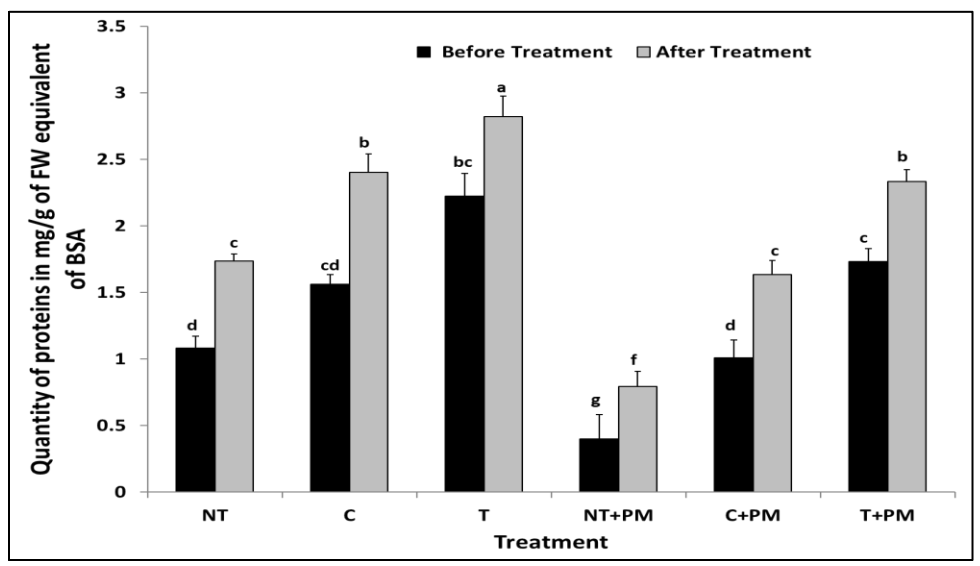


Figure 40: Variation in the content of total proteins after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

II.2.3.2.4. Variation in the Enzymatic Activity of Polyphenol Oxidase

The enzymatic activity of polyphenol oxidase (PPO) in the total protein extract varies in all treatments (Figure 41). In comparison to the non-treated and chemically treated batches, the formulation treated batch's PPO activity is significantly ($P \leq 0.05$) more pronounced. Hence, with the Formulaton treated (T), Chemically treated (T) and Non-treated (NT) seedlings respectively, we see increases of 90% (0.61 to 1.17 EU/min/g of FW), 85% (0.60 to 1.11 EU/min/g of FW) and 84% (0.53 to 0.98 EU/min/g of FW). PPO activity is significantly reduced when *P. megakarya* is present in the soil both before and after infection. *P. megakarya* infection causes an average decline in PPO activity of 40% prior to infection and 15% following infection.

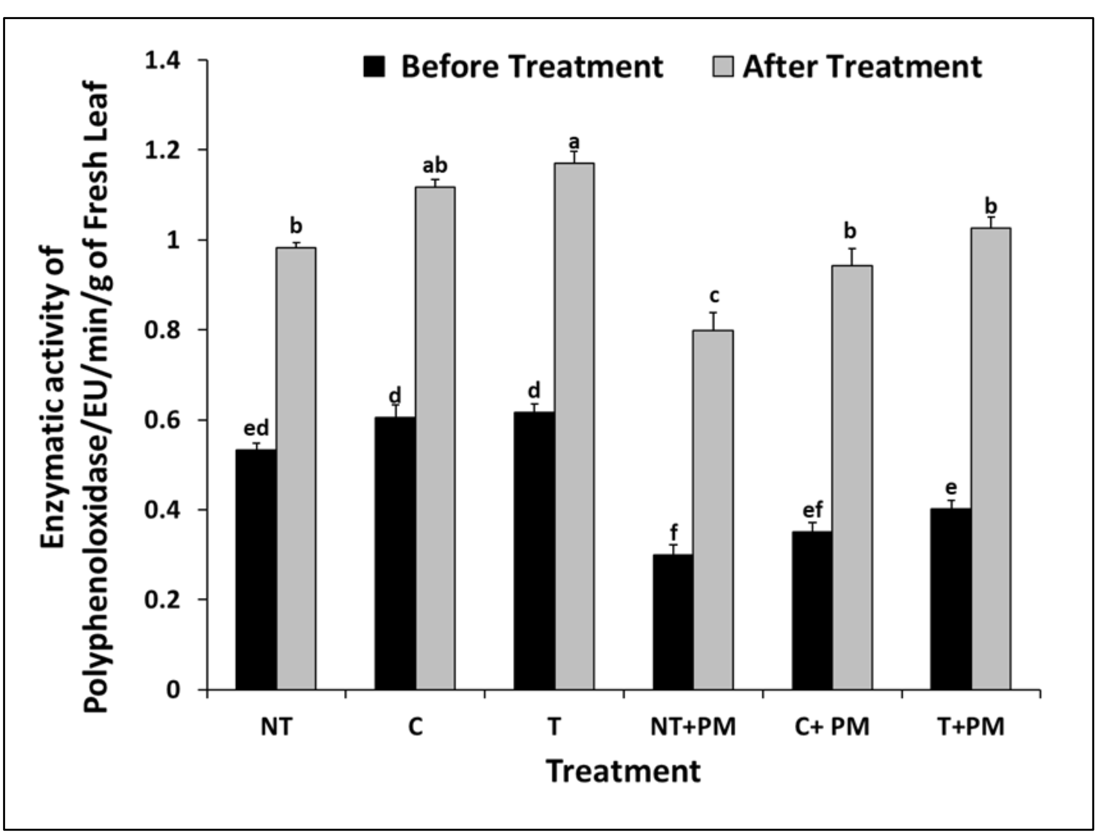


Figure 41: Variation in the enzymatic activity of polyphenoloxidase after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

III.2.3.2.5. Variation in the Enzymatic Activity of Peroxidases

The enzymatic activity of peroxidases in cocoa leaves is significantly different ($P <$ 0.05) between all treatments before and after infection (Figure 42). Compared to untreated and chemically treated batches, the activity of peroxidases is more prominent in batches with seedlings treated with our liquid formulation (Figure 42). In effect, an increase of 96% (2.00 to 3.92 EU/min/g of FW), 79% (1.95 to 3.49 EU/min/g of FW) and 70% (1.95 to 3.32 EU/min/g of FW) respectively for the Formulaton treated (T), Chemically treated (T) and Non-treated (NT) seedlings is observed. The presence of *P. megakarya* in the soil leads to a significant decrease in peroxidase activity before and after infection. For the non-treated (NT), chemically treated (C) and formulation treated groups respectively and we observed an average decline of 26%, 16% and 14%.

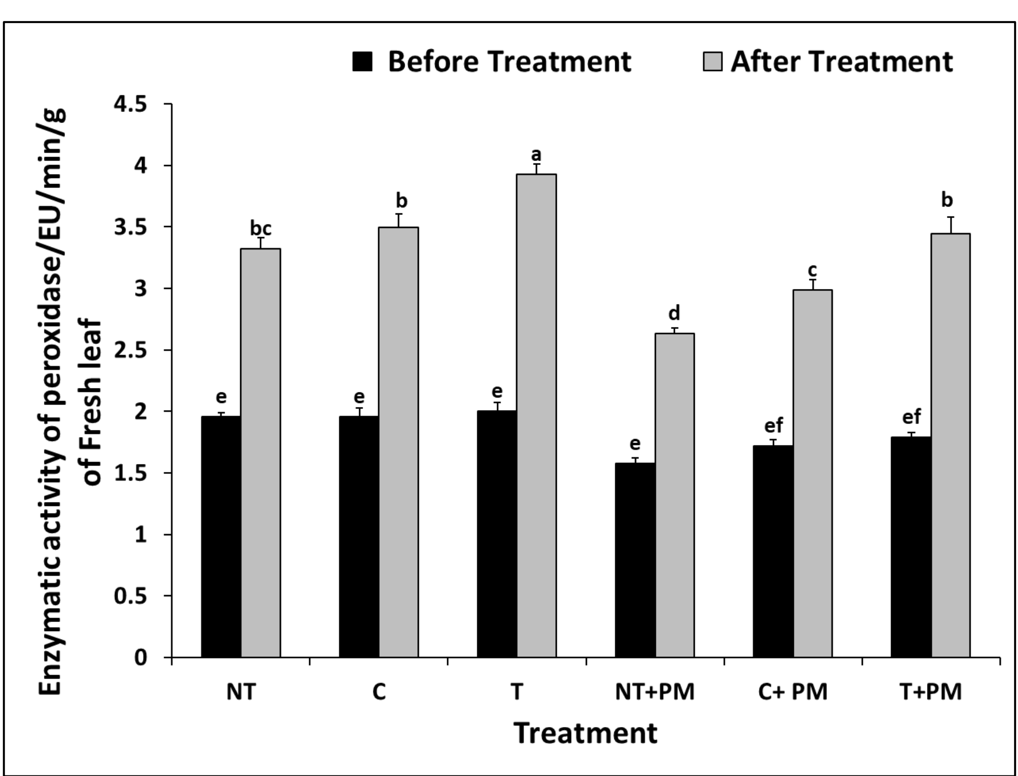


Figure 42: Variation in the enzymatic activity of peroxidase after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

II.2.3.2.6. Variation in the Enzymatic Activity of β-1,3-Glucanase

The enzymatic activity of β-1,3-glucanase increases significantly ($P < 0.05$) in cocoa leaves from batches treated with our liquid formulation compared to the other batches (Figure 43). This increase is noticed both before and after the infection of leaves with *P. megakarya*. This increase is in the order of 69% (4.79 to 8.80 mg of glucose per g of FW), 65% (4.19 to 6.9 mg of glucose per g of FW) and 62% (3.75 to 6.10 mg of glucose per g of FW) Formulaton treated (T), Chemically treated (T) and Non-treated (NT) seedlings respectively. The introduction of *P. megakarya* in the soil leads to a significant decrease in the activity of β-1,3-glucanases. We note an average reduction in the activity of β-1,3-glucananse of 24% prior to infection and 16% following infection (Figure 43).

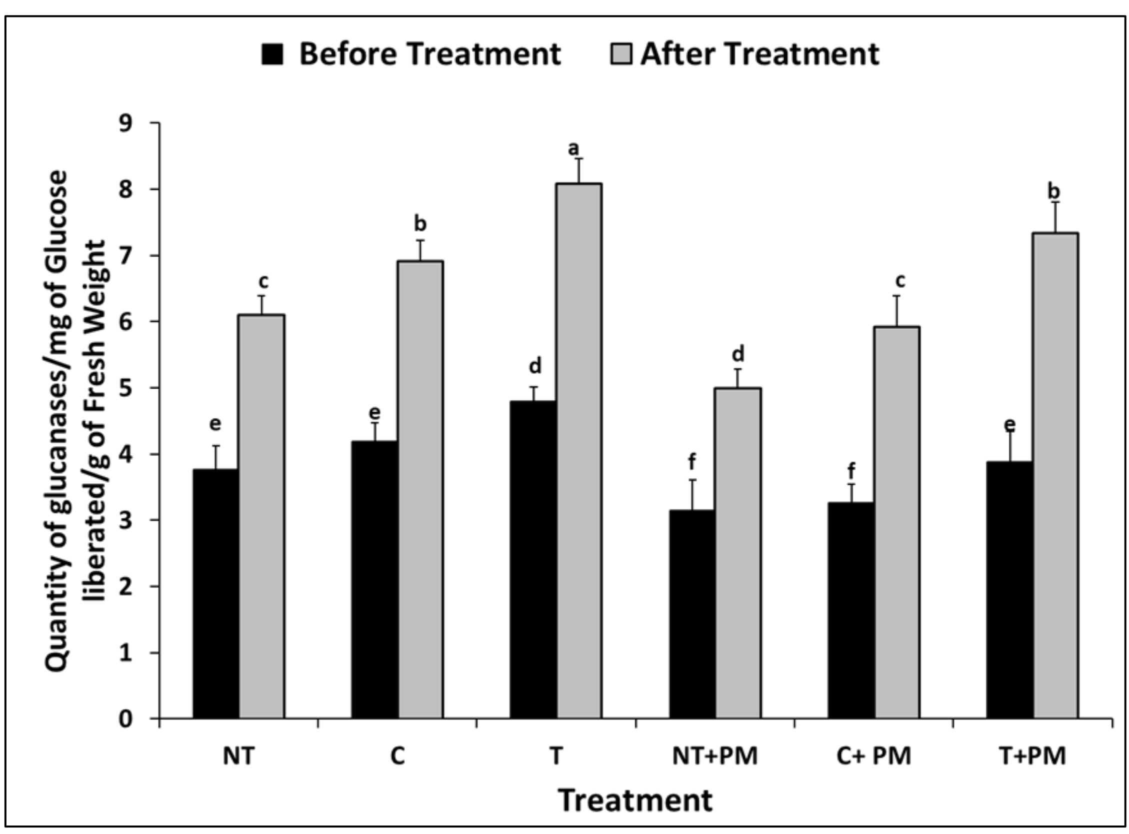


Figure 43: Variation in the enzymatic activity of β-1,3-glucanase after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

II.2.3.2.7. Variation in the Enzymatic Activity of Chitinases

The enzymatic activity of chitinases significantly varies ($P < 0.05$) among all treatments both before and after infection of cocoa leaves *with P. megakarya* (Figure 44). This increase is more pronounced in plants treated with our liquid formulation. We observe an increase of 92% (0.64 to 1.15 EU/g of FW/h), 90% (0.61 to 1.15 EU/g of FW/h) and 85% (0.54 to 0.99 EU/g of FW/h) respectively for Formulaton treated (T), Chemically treated (T) and Non-treated (NT) seedlings. The presence of *P. megakarya* in the soil leads to a significant decrease in chitinase activity before and after infection. In addition, *P. megakarya's* presence causes the activity of chitinase to decline on average by 11% (Figure 44).

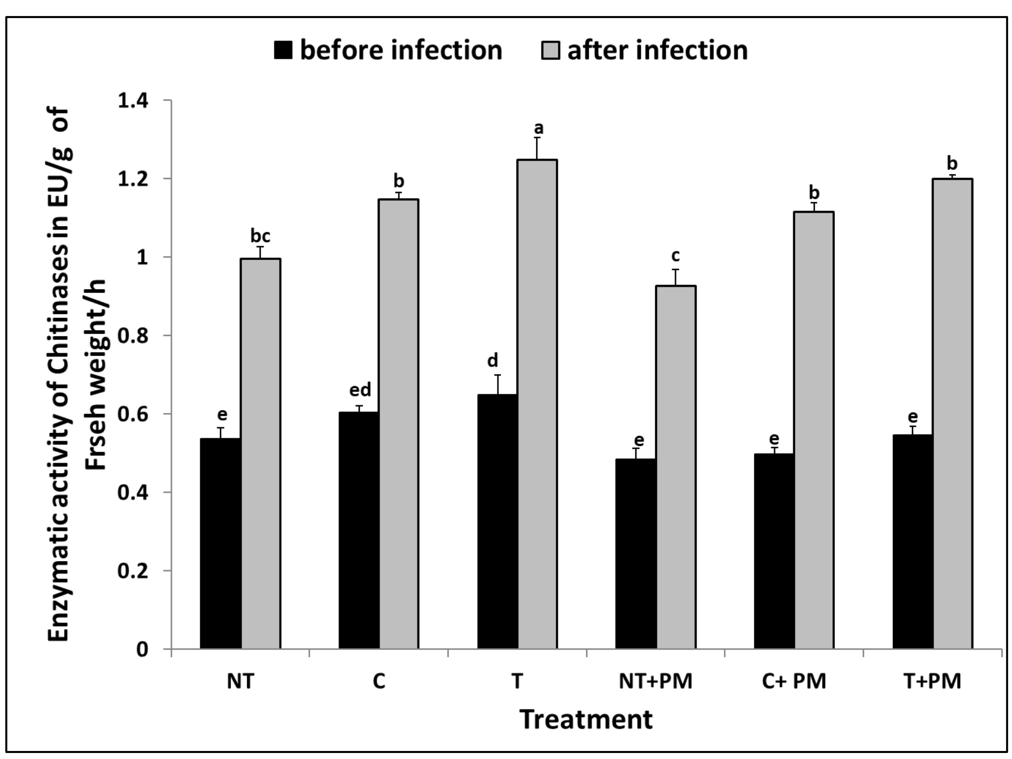


Figure 44: Variation in the enzymatic activity of chitinases after treatment with the liquid formulation.

*Values with the same letter within a column are not significant at $P \leq 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with Liquid formulation), C: Chemically treated (with CALLOMIL SUPER 66WP), NT+PM (Non-Treated infected with *P. megakarya*), C+PM (Chemically treated infected with *P. megakarya*) and (Liquid formulation treated infected with *P. megakarya*).

III.2.4. Partial Discussion II

The purpose of this part was to assess the impact of our formulations on the quality of cocoa seedlings in the nursery by evaluating their effects on growth and resistance. Firstly, the effect of the powder formulation was evaluated by enrobing cocoa grains from two cocoa hybrids (the tolerant SNK413xT79/467 and the susceptible UPA134 X SCA 12) with a 0.1 g/ml water suspension of the formulation. The effect of the liquid formulation was evaluated by surface spraying seedlings of the hybrid SNK413xT79/467 in nurseries grown in sterilized and unsterilized soil. Our research revealed that, when compared to untreated and chemically treated seedlings, cocoa seedlings grown from grains treated with the formulations had significantly higher values for the agro-morphological parameters (stem length, number of leaves, leaf surface area, root and shoot fresh and dry weight). The improved agro-morphological characteristics of the treated seedlings are an indication of the plants' robust health. The growth-promoting qualities of *S. cameroonensis* spores present in the powder formulation as well as the presence of the active ingredient, the secondary metabolites of *S. cameroonensis*, in our liquid formulation, may explain this increase in the agro-morphological parameters. Previous studies have shown that fresh cultures of *S. cameroonensis* enhances the germination and growth of cocoa seedlings in nurseries (Boudjeko *et al.,* 2017). Like other PGPRs, *S. cameroonensis* possess growth-promoting properties such as the production of siderophores and IAA, the solubilization of phosphates and the degradation of 1-aminocyclopropane-1-carboxylate, an intermediary product in the biosynthetic pathway of the plant hormone ethylene (Boudjeko *et al.,* 2017). IAA stimulates lateral root development, expansion of root hairs and the release of sugars (Davies, 2004, Barbaccia *et al.,* 2022). This strain is also capable of synthesizing phytohormones involved in growth, germination, flowering, leaf formation and the reduction of senescence in leaves and fruits. Other mechanisms associated with *Streptomyces* and growth in plants involve their ability to fix nitrogen, thereby establishing symbiosis between plant roots and the *Streptomyces* strain. The ability of *Streptomyces* strains to chelate iron with the help of siderophores enhances growth, as iron is one of the important minerals involved in plant growth. *Streptomyces acidiscabies* E13 strains have been reported to produce hydroxamate siderophores (Dimkpa *et al*., 2008), which enhanced iron acquisition and promoted growth of *Vigna unguiculata* under nickel stress. This improvement in growth parameters following application of our formulations is consistent with our earlier findings revealing the existence and viability of *S. cameroonensis* spores in our formulation, which impacts their growth-promoting capabilities on the cocoa seedlings. The growth properties varied among the hybrids, with the tolerant hybrid showing

better growth than the susceptible hybrid. This could be related to the genetic variability of the tolerant hybrid conferring more resistance against the pathogen and as a result, this hybrid undergoes less stress, providing room for enhanced growth. This sums up the integrated approach of biological control and genetic control to enhance plant growth and resistance.

Treatment of cocoa seedlings with our formulations significantly reduced the disease severity index. These outcomes support the findings of Boudjeko *et al. (*2017), who showed that cocoa seedlings planted in substrate treated with fresh cultures of *S. cameroonensis* showed a reduced necrosis index in foliar discs compared to the control. The systemic protection that our formulation provided to cocoa seedlings may have been caused by the presence of S. *cameroonensis* spores and their capacity to induce systemic plant resistance, a property shared by other *Streptomyces* strains. Given that the powder formulation was used as a seed treatment by enrobing cocoa grains, the bacteria will well establish themselves on the seed surface and colonize the roots following germination when the treated seeds are sown. Our findings support previous studies that showed the capacity of talc-based powder formulations of *Pseudomonas fluorescens* and *Streptomyces corchorusii* respectively, applied as seed treatments under pathogen-challenged conditions to suppress pathogens in leaves and roots of rice (Vidhyasekaran *et al*.,1997; Tamreihao *et al*., 2008, Pahari *et al.,* 2017). In addition, these biocontrol agents could be detected on leaf sheaths and root cortex (Vidhyasekaran *et al*., 1997). The liquid formulation equally enhances the resistance of plants, as seen through a reduction in the disease severity index. This formulation's mode of action is equally linked to the systemic induction of resistance. The main active ingredient of this formulation is the crude extract of *S. cameroonensis* secondary metabolites which contains antibiotic substances like geldanamycin known to inhibit the growth of oomycetes (Agbessi *et al.,* 2003; Boudjeko *et al.,* 2017, Djuidje *et al.,* 2022). The presence of volatile antibiotics in the crude extract can also be identified as responsible for this disease suppression effect after treatment (Palaniyandi *et al.,* 2013).

When a pathogen infects a plant that has been treated with our formulations, the plant synthesizes more biochemical defense markers. This shows that the cell is becoming more resistant. In fact, when a pathogen infects a plant, the plant's hypersensitive response kicks in quickly. This causes nearby cells to die, which stops the pathogen from spreading. Thus, PR proteins and phytoalexins (phenolic compounds) take over to restrict the threat via fungal cell wall degrading enzymes such β -1,3-glucanase and chitinase, as well as oxidative enzymes (Oliveira *et al.,* 2014). The role of phenolic compounds and flavonoids in plant defense is well documented (Djocgoué *et al.,* 2011; Ewané *et al*., 2012; Simo *et al.,* 2014; Effa *et al.,* 2016;

Tchameni *et al*., 2017), as these metabolites tend to accumulate at different levels in infected tissues in response to pathogen attack. The production of these metabolites is significantly increased when our formulations are used, which confirms the accompanying improvement in growth and resistance to pathogen attack in cocoa seedlings. In formulation-treated seedlings, defense-related enzymes such as polyphenoloxidase, peroxidase, chitinase, and β -1,3-glucanase showed significantly greater increases in activity compared to the controls. Enhancing systemic resistance and reducing plant disease are both positively correlated with the increased activity of these enzymes in plant tissues (Chandrasekaran and Chun, 2017; Téné *et al*., 2019, Ewané *et al.,* 2020). It is commonly known that POX is important in scavenging for H_2O_2 in cells, which is an important element in disease resistance to pathogens. Moreover, PPO oxidizes phenolic compounds during pathogen-plant interaction, leading to the formation of quinones and the accumulation of free radicals that can inactivate the enzymes produced by the pathogen (Appel, 1992, Taranto *et al.,* 2017). Chitinases and β-1,3-glucanases have been demonstrated by many authors to be fungal pathogen cell wall degrading enzymes (Oliveira *et al.,*2014*,* Téné *et al.,* 2019, Ewané *et al.,* 2020, Perrot *et al.,* 2022). A recent study has also reported an increase in the activities of these defense-related enzymes because of induction by microbial antagonists in hosts (Chandrasekaran and Chun, 2017). Indeed, root inoculation with *Streptomyces* GB 4-2 provided Norway spruce with systemic resistance to the needle pathogenic fungus *B. cinerea* (Lehr *et al*., 2008). Moreover, an increase in the activities of peroxidase, phenylalanine ammonia-lyase and β-1,3-glucanase in cucumber leaves after treatment with a culture filtrate from *Streptomyces bikiniensis* HD-087 was also reported (Zhao *et al*., 2012). These results corroborate our findings that treating seedlings of cocoa with our *S. cameroonensis*-based formulations induce systemic resistance in the seedlings by increasing the synthesis of defense markers. This systemic resistance is mediated by functioning jasmonate and ethylene pathways, leading to the synthesis of the above-mentioned defense molecules and thickening of the plant cell wall (Shoresh *et al.,* 2004, Pangestic *et al.,* 2016, Yuan *et al*., 2019, Wilson *et al.,* 2023). Hence, the accumulation of these biochemical markers of defense in plants treated with our formulations provides a basis for systemically induced resistance and the associated pathways, which appear as a credible mechanism for the efficacy of our formulations.

Partial conclusion II

This study was set out to determine how our formulations impacted the development and resistance of cocoa seedlings in the nursery. We can conclude that enrobing cocoa grains with a 10% w/v water suspension of our powder formulation before planting increases the plant height of the tolerant H1 [(♀) SNK413 × (♂) T79/467] hybrid (30.4 to 37.3 cm), the number of leaves (11.5 to 12.8 cm), the leaf surface area (38.23 to 47.50 cm2), the dry weight of roots (1.8 to 3.1 g) and the dry weight of shoots (7.4 to 8.51g) compared to the negative control. Similarly, spraying our seedlings with the liquid formulation led to an observed increase in plant height (28%), number of leaves (24%) and leaf surface area (11.5%). Leaf disc assay showed a low disease severity index of 0.83 (resistant) for the H1 hybrid treated with our powder formulation. The H1 plants treated with our liquid formulation were also classified as resistant (necrotic index of 1). This reduced disease severity index was correlated with an increased synthesis of biochemical markers of defense (total phenolic compounds, flavonoids, total proteins) and an observed increased activity of peroxidases, polyphenoloxidase, chitinases and β-1,3-glucanase in all plants both before and after infection, hence aligning plant resistance with the increased synthesis of these markers. According to our findings, both of our formulations exhibit an improved protective impact against *P. megakarya* as well as a growth-inducing effect on cocoa plants.

III.3. Expression of PR-Protein Genes in Cocoa Seedlings Challenged with *Phytophthora megakarya* in Response to Treatment with *S. cameroonensis*-Based Formulations

The effect of *S. cameroonensis*-based formulations on the expression of the PR-protein genes (TcChiB, TcGlu-1 and TcPer-1 for chitinases, glucanases and peroxidases respectively) and the transcription factor for some phenolic compounds (TcMYBPA) in cocoa seedlings was studied by real-time PCR both before and after infection of young cocoa leaves with *P. megakarya*.

The relative gene expression ($2^{-\Delta\Delta CT}$) of the TcChiB gene in *T. cacao* seedlings after treatment with our formulations was studied by rt-PCR showed a significant ($P< 0.05$) up-regulation (Figure 45A). In fact, seedlings treated with our formulation exhibited a relative fold expression of 1.33 as opposed to the chemically treated and untreated batches' respective values of 1.02 and 1.04. In all treatments, *P. megakarya* infection of leaves was characterized by an increase in the expression of the TcChiB gene. Compared to chemically treated and untreated seedlings, this increase was more evident in seedlings treated with our formulation. Indeed, we observed an increased fold expression of 1.02 for the formulation-treated seedlings compared to an increase of 0.72 for the chemically treated, and an increase of 0.78 for the non-treated, giving an average of 74% across all treatments (Figure 45 A).

The relative fold expression ($2^{-\Delta\Delta CT}$) of the TcPer-1 gene was significantly ($P< 0.05$) up-regulated in seedlings treated with our formulation both before and after infection of leaves with *P. megakarya* compared to chemically treated and untreated seedlings (Figure 45B). Seedlings treated with our formulation showed a relative fold expression of 2.07 as opposed to chemically treated and untreated seedlings' respective values of 1.61 and 0.79. In all treatments, *P. megakarya* leaf infection resulted in an up-regulation of the TcPer-1 gene. In effect, we observed an increase in fold expression of 0.84, 0.97 and 0.79 for the formulation-treated, chemically treated and non-treated batches respectively, giving an average increase in gene fold expression of 67% across all treatments (Figure 45B).

The relative fold expression ($2^{-\Delta\Delta CT}$) of the TcGlu-1 gene was seen to be significantly up-regulated ($P< 0.05$) in cocoa seedlings treated with our formulation both before and after infection of cocoa leaves with *P. megakarya* (Figure 45C). In fact, we saw a relative fold expression of 2.53 for seedlings treated with formulation as opposed to 1.99 and 1.05 for chemically treated and untreated seedlings respectively. Infections with the pathogenic agent *P. megakarya* triggered an up-regulation in the expression of this gene in all treatments. In

effect, we observed an increase in fold expression of 4.34, 3.86 and 2.17 for the formulation-treated, chemically treated and non-treated batches respectively. As a result of the *P. megakarya* infection, we saw an average increase in gene fold expression of 191% across all treatments (Figure 46C).

The relative fold expression (2^-ΔΔCT) of the TcMYBPA gene was observed to be significantly up-regulated ($P < 0.05$) in seedlings treated with our formulations (Figure 45D). Our formulation-treated seedlings exhibited a relative gene fold expression of 1.35, as opposed to 0.89 and 0.79 for chemically treated and untreated seedlings respectively. Infection of leaves with P. *megakarya* led to a significant up-regulation in the expression of the TcMYBPA gene across all treatments. Indeed, we observed an increased fold expression of 1.75 for the formulation-treated seedlings compared to an increase of 1.49 for the chemically treated and an increase of 1.03 for the non-treated. In effect, because of the infection of leaves with *P. megakarya,* we observed an average increase of 143% in the gene fold expression across all treatments (Figure 45D).

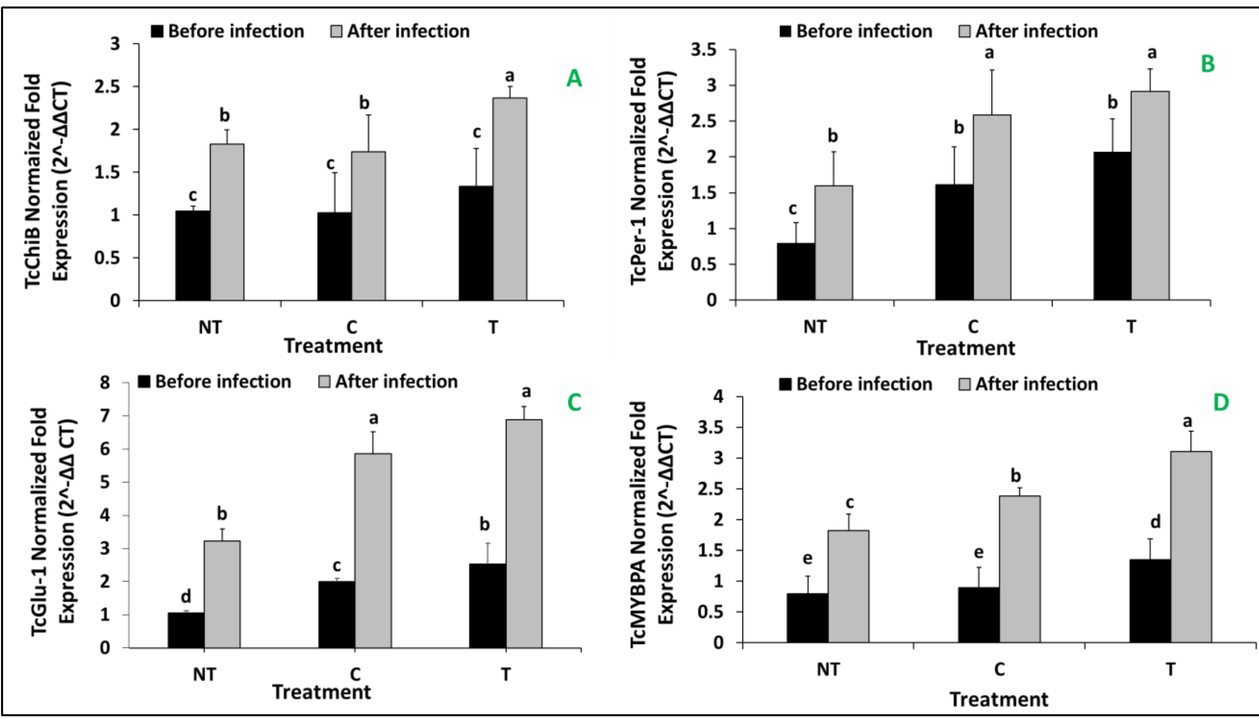


Figure 45: Quantitative reverse transcriptase-PCR analysis of (A) chitinase (B) Peroxidase (C) β-1,3-glucanase and (D) anthocyanidin transcription factor related gene expression of cocoa seedling leaves treated with our *S. cameroonensis*-based formulations and challenged with *P. megakarya*.

* Each value represents the mean ± SD (n = 2). Different letters above the columns indicate significant differences at $P < 0.05$.

Note: NT: Non-treated (negative control), T: Treated (with formulations) and C: Chemically treated (with CALLOMIL SUPER 66WP).

III.3.5. Partial Discussion III

In this study, two formulations based on *S. cameroonensis* have been shown to enhance the growth and resistance of cocoa seedlings in a nursery when challenged with *P. megakarya*. This part of the study analyzed the expression patterns of three defense-related genes: TcChiB, TcGlu-1 and TcPer-1, which code for chitinases, glucanases and peroxidases, as well as the Tc-MYBPA gene, which encodes a transcription factor involved in the regulation of phenolic compounds involved in plant defense systems like proanthocyanidin synthesis in cocoa (Liu *et al*., 2015). Through the priming effect, our study showed that the powder formulation applied by coating cocoa grains with 10% w/v of the formulation before planting and then spraying the plant from above with 0.1% v/v of the liquid formulation every two weeks led to a significant up-regulation of these target genes.

After treatment with our formulations, we observed an up-regulation of the TcPer-1 gene, which codes for the PR-9 protein that is peroxidase. Normalized fold expression of the gene went up by 82% when our formulations were used. In effect, the TcPer-1 gene is highly expressed in young leaves of cocoa upon mechanical wounding and treatment with ethylene (Bailey *et al*., 2005). These results correspond with ours, where we observed an up-regulation of the TcPer-1 gene in 1 month-old, detached cocoa leaves treated with our *S. cameroonensis*-based formulations and infected with a sporal solution of *P. megakarya*. The high expression of TcPer-1 in young leaves is related to the high expectations of expansion and lignification at this early stage of growth. The activity of peroxidases is an integral part of the lignification process of plant cell walls as a response to elicitors of cell death like pathogenic attacks (Christensen *et al.,* 2001; Bolwell *et al*., 2002; Bailey *et al.,* 2005).

The TcGlu-1 gene is expressed more in plants treated with our formulations, especially when challenged with *P. megakarya.* It shows a high degree of homology to genes encoding for β-1,4-glucanases in cocoa. In the one hand, endo-1,4-β-glucanases are believed to be involved in cell wall loosening during reorganization or degradation processes (Bailey *et al.,* 2005). The β-1,3-glucanases, on the other hand, within the glycosyl hydrolase family, are composed of complex families with expression patterns that are both developmentally regulated and/or inducible by divergent stimuli, including plant hormones and inducers of plant defense (Thomas *et al*., 2000). Polymers of 1,3-glucan are components of the cell walls of *Phytophthora* spp. and other oomycetes (Kamoun *et al.,* 2003). Thus, the high expression of the TcGlu-1 genes because of induction by our formulation will likely destabilize the invading *P. megakarya,* providing thus both a protective effect and an inducing effect.

Expression of the TcChiB gene is observed to be up-regulated in cocoa leaves treated with our formulations and challenged with *P. megakarya*. In effect, a previous study have shown thet the TcChiB gene is associated with several putative chitinases from *Gossypium hirsutum*, including a salicylic acid-activated class VII chitinase (Li and Liu, 2003). The insoluble -1,4-linked polymer of N-acetylglucosamine, the substrate for chitinases, is a major constituent of the cell walls of many oomycetes including *Phytophthora* spp. Therefore, chitinase enzymes act on the chitin of the cell wall of oomycetes by hydrolyzing the cell wall polysaccharide and thus disabling the microbe (Essghaier *et al*., 2014). It should be noted that chitinases are located on the leaves and do not intervene in the basal metabolism of the plant (Saboki *et al*., 2011). As a result, our formulation's induction of the TcChiB genes, which results in high TcChiB gene expression and synthesis of chitinases, will probably disrupt the invading *P. megakarya* while also having a protective effect on cocoa seedlings. Our results are consistent with a previous study that have demonstrated the ability of cocoa plants, in response to attack by the fungus *Phytophthora capsici* to stimulate the expression of the TcChiB gene, leading to an increase in the synthesis of chitinases that degrade the wall of this fungal pathogen and inhibit its spread (Zhang *et al.,* 2015).

Expression of the TcMYBPA gene was observed to be significantly upregulated in cocoa plants treated with our formulation. This expression was observed to even increase more after the plants were challenged with *P. megakarya*. TcMYBPA encodes an R2R3-MYB transcription factor involved in regulating the biosynthesis of cocoa proanthocyanidin (PA), a derivative of flavonoids that contributes to plant defense mechanisms against biotic and abiotic stress (Mellway *et al*., 2009; Terrier *et al*., 2009; Liu *et al*., 2015). This transcription factor highlights the initial steps of the transduction pathway leading to cacao resistance of *P. megakarya*. The development of cocoa and flavonoid (mainly anthocyanin) synthesis has been described previously by Wright *et al*. (1982). The increase in the expression of TcMYBPA genes in cocoa following infection with *P. megakarya* and treatment with our formulations is consistent with previous research, which has demonstrated that in *Arabidopsis*, several genes encoding MYB transcription factors are up-regulated after infection by *Pseudomonas syringae* and act as a positive regulator of the hypersensitive response as well as in response to bacterial pathogens (Raffaele *et al.,* 2006).

Regarding the mechanism by which this priming by our *S. cameroonensis-based* formulation and infection with *P. megakarya* occur, several studies have shown the involvement of plant defense responses regulated by signal molecules, such as salicylic acid (SA), abscissic acid (ABA), jasmonate (JA) and ethylene (Pereira Menezes *et al*., 2014); with

a direct relationship to systemic induced resistance, systemic acquired resistance and hypersensitive response. These early responses elicited in the plant may also involve mitogen-activated protein (MAP) kinase cascade activation and the reactive oxygen species (ROS) production (Verhagen *et al*., 2010). The mechanism by which priming by the S. cameroonensis b-based formulation occurs in two phases. Firstly, the pathogen encapsulation of the plant surfaces and the germination of the zoospores before penetration into the hypodermal cells can occur within two hours (Widmer *et al.,* 1998). During this phase, a pattern of elicitors from the pathogen is recognized by a pattern of plant receptors (Gomez and Gomez, 2004). This recognition stimulates the transduction of the signal into the plant cells, which consequently activates the transcription of defense genes (Zhang *et al*., 2013). Secondly, pathogen signaling is relayed by phytohormones, which operate the mechanisms of systemic acquired resistance and systemic induced resistance (Vallad and Goodman, 2004). The interaction between plants and beneficial microbes such as *S. cameroonensis* has been linked to these pathways. This suggests that our powder formulation contains. *S. cameroonensis* spores which initiate these responses linked to these pathways because of the interaction between the cocoa plant and *S. cameroonensis.* This early response can also be because the cocoa plant may initially perceive *S. cameroonensis* as a potential invader, resulting in activation of the plant immune system (Zamiuodis and Pieterse, 2012). The *Streptomyces* genus has been reported as a versatile group that impacts growth and resistance in plants by affecting their metabolism (Salla *et al.,* 2014; Ait Barka *et al.,* 2016). The *Streptomyces*-induced resistance in Arabidopsis seems to be dependent on salicylic acid but not on the jasmonate/ ethylene pathways (Conn *et al.,* 2008). Our two formulations can be seen to elicit such responses from the plant by causing an up regulation of these target genes.

GENERAL DISCUSSION

Biological control of plant diseases and promotion of growth using beneficial microorganisms have been proven in numerous studies aimed at preserving plant health and enhancing crop yield. These beneficial microorganisms can be optimized for the development of novel biofertilizers that are better suited for agricultural systems, usage, transportation, storage, and durability (Anitha and Rabeeth, 2009; Tamreihao *et al.*, 2016; Dzelamonyuy *et al.,* 2022). Our study has shown that *Streptomyces cameroonensis* can be formulated successfully in powder and liquid bioformulations that are easy to use and have longer shelf lives. These bioformulations have been shown to have similar antimicrobial and growth-promoting properties as fresh cultures of *S. cameroonensis*. Application of these formulations to cocoa seedlings in nurseries improved the agronomic characteristics of the treated seedlings. The growth-promoting effects of *S. cameroonensis*-based formulations can be directly linked to the presence of *S. cameroonensis* spores and metabolites in the powder and liquid formulations respectively. As such, these formulations can initiate the generation of siderophores and indole acetic acid (IAA), the solubilization of phosphates and the fixation of nitrogen among others (Boudjeko *et al.,* 2017). This strain is also capable of synthesizing phytohormones involved in germination and growth (Dzelamonyuy *et al.,* 2022). This improvement in growth parameters following application of *S. cameroonensis*-based formulations is consistent with previous studies that demonstrate the growth-promoting properties of formulations based on beneficial microorganisms (Vidhyasekaran *et al.,* 1997; Keswani *et al.,* 2016; Khan, 2023). When cocoa seedlings were infected with *P. megakarya*, our formulations significantly reduced the disease severity index, thus demonstrating an increase in the ability of the plant to counter parasitic attacks. This result backs up what Boudjeko *et al*. (2017) found, which was that cocoa seedlings planted in soil treated with fresh cultures of *S. cameroonensis* had a lower necrosis index in leaf discs than the control. This systemic protection that our formulations provided to cocoa seedlings may have been caused by the presence of *S. cameroonensis* spores and metabolites as well as their capacity to induce systemic plant resistance, a property shared by other *Streptomyces* strains. When a pathogen infects a plant, the plant’s hypersensitive response kicks in quickly. This causes nearby cells to die, which stops the pathogen from spreading. The plant then starts to synthesize more biochemical defense markers. This shows that the cell is becoming more resistant. Thus, PR proteins and phytoalexins (like phenolic compounds) take over to restrict the threat via oomycete cell wall degrading enzymes such as β-1,3-glucanase and chitinase, as well as

oxidative enzymes (Oliveira *et al.,* 2014). In response to pathogen assault, phenolic compounds and flavonoids tend to concentrate at various levels in diseased tissues, which has been extensively shown to play a role in cocoa plant defense (Simo *et al.,* 2014; Effa *et al.,* 2016; Ondobo *et al*., 2017). The production of these metabolites is significantly increased when our formulations are applied as a treatment, which confirms the accompanying improvement in growth and resistance to pathogen attack in cocoa seedlings. Previous research has shown that the introduction of microbial antagonists into hosts results in an increase in the activity of these defense-related enzymes (Lehr *et al.,* 2008; Zhao *et al.,* 2012; Chandrasekaran and Chun, 2017). The findings support our hypothesis that our *S. cameroonensis*-based formulation treatments of cocoa seedlings trigger systemic resistance in the seedlings by enhancing the production of defense markers. Thus, enhancing systemic resistance and reducing plant disease are both positively correlated with the increased activity of these enzymes in plant tissues. This systemic resistance is mediated by functioning jasmonate and ethylene pathways leading to the synthesis of the above-mentioned defense molecules and thickening of the plant cell wall (Wan and Xin, 2022). Plants treated with our formulations showed higher defense-related gene expression after one month of growth for the TcChiB, TcGlu-1 and TcPer-1 genes, which code for chitinases, glucanases and peroxidases, and the Tc-MYBPA gene, which encodes a transcription factor involved in the regulation of phenolic compounds involved in plant defense systems like proanthocyanidin synthesis in cocoa. This early impact of the formulations on the molecular mechanisms of defense in cocoa seedlings can be attributed to the priming or pre-immunization following the activation of microbe-associated molecular patterns (MAMPSs) or pathogen-associated molecular patterns (PAMPs) upon interaction of the plant with *S. cameroonensis*-based formulations and/or *P. megakarya.* Priming is the phenomenon whereby plant tissues are preconditioned to respond in a more rapid and robust manner to very low levels of stimulus (Jung *et al.,* 2012). Increased expression of these defense-related genes is associated with significant fitness benefits in this primed state. Our results correspond with several studies that have shown the early up-regulation of the TcChiB, TcGlu-1, TcPer-1 and Tc-MYBPA genes in cocoa and other plants when challenged by both biotic and abiotic factors (Bailey et al., 2005; Thomas *et al.,* 2000; Zhang *et al.,* 2015; Liu *et al.,* 2015; Téné *et al.,* 2023). This priming mechanism is mediated by signal molecules including salicylic acid (SA), abscissic acid (ABA), jasmonate (JA) and ethylene (Pereira Menezes *et al.,* 2014), with a direct relationship to systemic induced resistance, systemic acquired resistance, and hypersensitive response. This suggests that the observed responses in cocoa seedlings following treatment with our *S. cameroonensis*-based formulations are directly linked to these pathways.

Conclusion and Perspectives

CONCLUSION AND PERSPECTIVES

GENERAL CONCLUSION

Our work focused in contributing to the development of a biological control method against *Phytophthora megakarya* in cocoa production by developing effective formulations based on *Streptomyces cameroonensis*, with the aim to improve the quality of cocoa seedlings produced in Cameroon. This would improve the quality of cocoa seedlings grown in Cameroon. It required creating two formulations based on *S. cameroonensis* with two different modalities of treatment, analyzing the effects of those formulations on two well-known cocoa hybrids produced in Cameroon, as well as providing a mechanism for how they work and how effective they are.

At the end of the work, we established that:

- ❖ A starch/talc powder formulation of *S. cameroonensis* maintained a stable shelf life of 3.53 x10^5 CFU/mL after 6 months at room temperature and can retain stability for more than a year when stored at 4 °C. With no discernible negative effects on cocoa seedlings, this formulation demonstrated a 100% inhibitory efficacy *in vitro* against *P. megakarya* at a concentration of 10% W/W. A liquid formulation based on the secondary metabolites of *S. cameroonensis* stabilized with Tween 80 and made adhesive by adding 15% w/v gum arabic was seen to be stable. At a dosage of 0.1% v/v, this liquid formulation demonstrated no phytotoxic effects on cocoa seedlings and had an 85% inhibitory capacity against *P. megakarya.*
- ❖ The treatment of cocoa seedlings with our powder formulation (10% w/v) and the liquid formulation (0.1% w/v) significantly increased the growth of cocoa seedlings in the nursery after 3 months by an average of 33% and 21% respectively. Application of the powder and liquid formulations also significantly induced the resistance of cocoa seedlings against *P. megakarya* by reducing the disease severity index by 67% and 57% respectively. This increased resistance was correlated with increased production of biochemical markers of defense (total phenolic compounds, flavonoids, and total proteins), as well as observed increased activity of peroxidases, polyphenol oxidase, chitinases and β-1,3-glucanase. This was particularly evident in the more resistant hybrid H1[(♀) SNK413 × (♂) T79/467]. This positive correlation between an increase in growth parameters, biochemical markers of defense and a reduction in disease severity in both cases of treatment affirms both the protective and inducing effects of

our *S. cameroonensis*-based formulations and outlines a possible basis for the mechanism by which the formulation functions.

- ❖ The combined effect of our two formulations significantly reduced the disease severity index in cocoa when challenged with *P. megakarya* in the early stages of growth. This reduction in the severity of the disease was translated by the up-regulation of defense-related genes TcChiB, TcGlu-1, TcPer-1 and TcMYBPA that encode chitinases, glucanases, peroxidases and the transcription factor involved in the regulation of the flavonoid derivative proanthocyanidin respectively. These biomolecules are produced in response to biotic and abiotic stress, like the introduction of our formulation or pathogenic attacks. This knowledge lays the groundwork for understanding both the molecular markers involved in the response of cocoa to both pathogenic and beneficial microbes, as well as the process underlying the interaction between our formulations, the cocoa plant, and the pathogenic agent *P. megakarya.*

PERSPECTIVES

To add value to this work, it will be important to:

- ✓ Optimize the formulations for better activity and durability, which can serve as a benchmark for the valorization of beneficial microorganisms in disease management, as well as set up pilot studies for scaling up the formulations for mass production.
- ✓ Follow up the treatment from the nursery to the farm to evaluate the effect of the formulation on the final product of cocoa on the farm, thereby investigating the sanitary and nutritional quality of the treatment on cocoa beans and establishing a technical platform for healthy organic cocoa production.
- ✓ Certify the biopesticide as a commercial product and secure a patent.

RECOMMENDATIONS

This study provides not only a pathway for the optimization of the numerous beneficial microorganisms that are constantly being isolated and characterized but also presents a technical platform to produce healthy, resistant, and organic cocoa seedlings in Cameroon. We suggest that while these sustainable methods for making and using biocontrol formulations are being worked on, the guidelines for evaluating efficacy and testing in the field should be constantly updated. This would help provide proof of efficacy and aid in the development of

biopesticides by putting in place effective regulations for the use of biological products in the agricultural market.

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Appendices

APPENDICES

APPENDIX I: CULTURE MEDIA

ISP-2 medium

Yeast Extract	4 g
Malt Extract	10 g
Glucose	4 g
Agar	20 g
Distilled Water	1000 mL
Final pH (at 25°C)	7.3

Kings Medium base (KMB)

Proteose peptone	20 g
K_2HPO_4	1.5 g
$MgSO_4.7H_2O$	1.5 g
Agar	15 g
Distilled water	1000 mL
Final pH (at 25°C)	7.2

Milieu PDA

Potatoes, Infusion from	200 g
Dextrose	20 g
Agar	30 g
Distilled water	1000 mL
Final pH (at 25°C)	5.6

N.B: All media autoclaved at 121°C for 15 mins

APPENDIX II: SOLUTIONS AND REAGENTS

BSA (BOVIN Serum Albumin)

1 mg/ml BSA solution was prepared by dissolving 2 mg BSA powder in 2 ml distilled water.

Bradford's Reagent

Bradford's reagent was prepared by dissolving 50 mg of Coomasie Blue G 250 in 25 ml 95% ethanol. The mixture was shaken for 30 min, then 50 ml orthophosphoric acid 85% was added and homogenized for 10 min. The volume was made up to 500 ml with distilled water, filtered and conserved at 4°C.

20% Na_2CO_3 Solution

It was prepared by dissolving 4 g of Na_2CO_3 powder in 20 ml distilled water.

Preparation of 1% Triton X-100C

- Measure 2.5 ml Triton X-100
- Complete with 100 ml distilled water
- Conserve at ambient temperature

Tris-HCl, Triton X-100C, 100 mM, pH 7.5, Volume 1000 ml

- Weigh 1.58 g of Tris base.
- Dissolve in 500 ml distilled water
- Add 100 ml Triton X-100C.
- Make up the volume to 1000 ml with distilled water.
- Adjust pH to 7.5.

Catechine 10 mM solution

Weigh 1 mg catechine and dissolve completely in 10 ml distilled water.

Methanol 80% solution, Volume 100 ml

Measure 80 ml pure methanol solution and add unto it 20 ml distilled water.

Sodium acetate buffer C=0.1 M, pH 4.5 V 500 ml

- Weigh 4.1 g Sodium acetate and dissolve in 400 ml distilled water.
- Add 12.5 mg laminarine.
- Add 1.62 ml acetic acid.
- Adjust pH to 4.5.

Preparation of Phosphate buffer pH 7, 0.66 M

- Weigh 45 g KH_2PO_4 and 57.5 g K_2HPO_4.
- Dissolve in500 mL distilled water
- Adjust pH to 7.

Tris-maleate buffer pH 7.2, 0.1 M V=500 ml

- Weigh 6 g Tris-base.
- Dissolve in 400 mL distilled water
- Add 6.7 g malic acid.
- Add 277 mg $CaCl_2$.
- Add 1 g Guiacol and make up the volume to 500 ml.
- Adjust pH to 7.2.

1 mg/ml Colloidal Chitine RBV

- 1.5 g colloidal chitin
- Dissolve in 6 ml 95% acetic acid,

- Make the volume up to 100 mL with distilled water.

Laminarine Solution

- 2 g Laminarine
- 100 mL distilled water

APPENDIX III: STERILIZATION OF SOIL, SEEDLINGS AND APPLICATION OF FORMULATIONS

Pretreatment of soil

- The soil is dried at ambient temperature for 72 hrs and sieved to eliminate debris like stones and roots, then mixed with sand in the ration 1:3 w/w.

Sterilization of soil

The soil was sterilized in an oven (121°C) for 4 hr. and repeated thrice.

Sterilization of Seedlings

-Dehusked the pods

- Wash the beans with distilled water and sand to removed mucilage, rinse with distilled water

- Sterilize the washed seeds by soaking in 70% ethanol for 5 mins, then 0.2% Sodium Hypochlorite for 5 mins

- Rinse with Sterilized distilled water.

Treatment of seedlings with powder formulation

- Prepare a water solution of 0.1g/ml of the powder formulation.
- For 1 kg seedlings, 10 g of formulation is required.
- Soak the seedlings in the formulation for 24 hours to allow for adhesion of spores before planting.

Treatment of seedlings with liquid formulation.

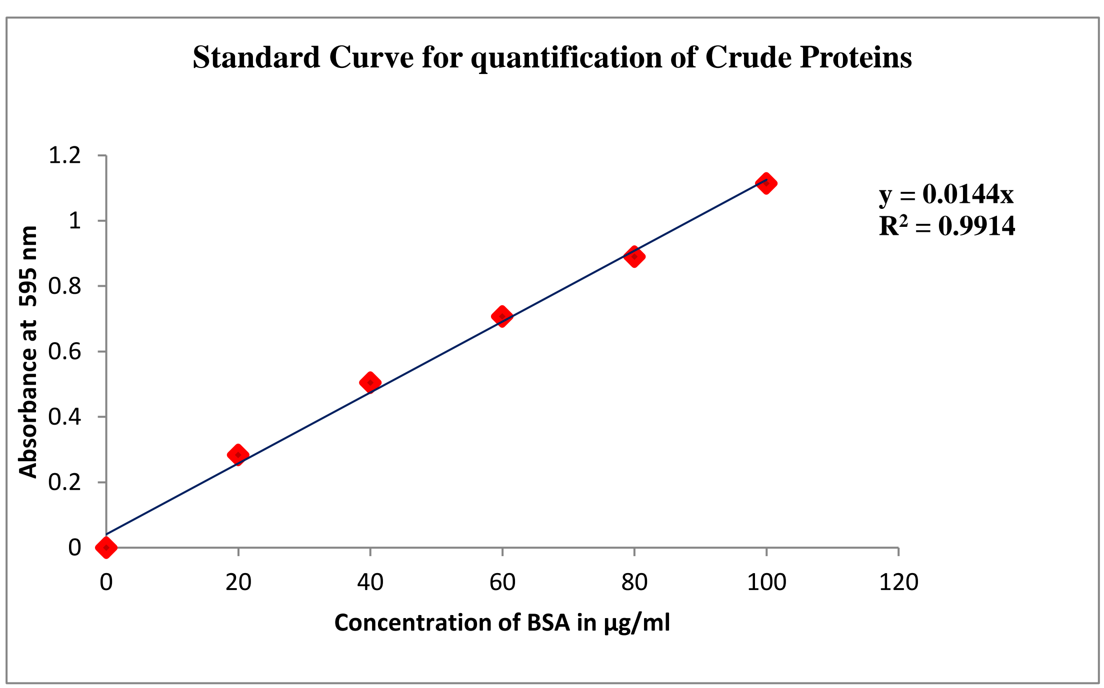
- Seedlings of at least two weeks old are sprayed with the formulation (0.1%v/v) every two weeks.

Treatment of seedlings with Chemical formulation.

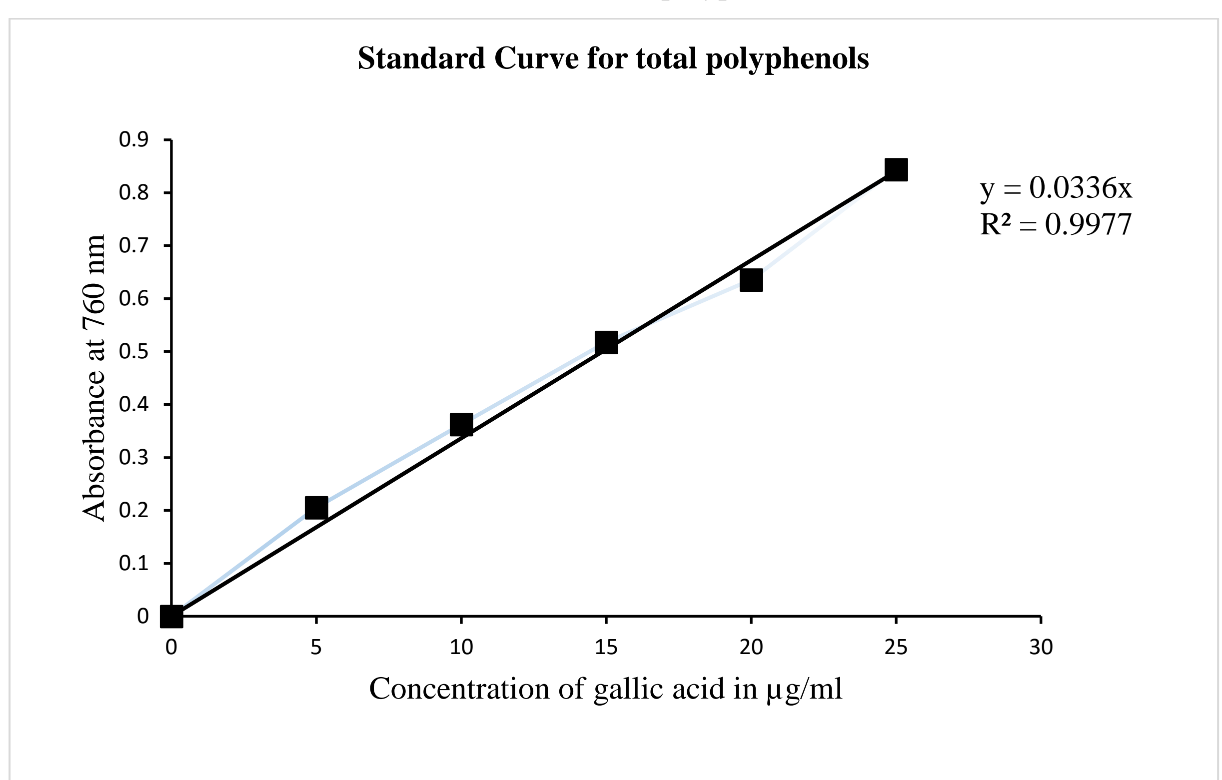
- 3.33% w/v CALLOMIL SUPER 66 WP (recommended by the manufacturer) is prepared and used to spray the seedlings every two weeks as from the second week of growth.

APPENDIX IV: STANDARD CURVES

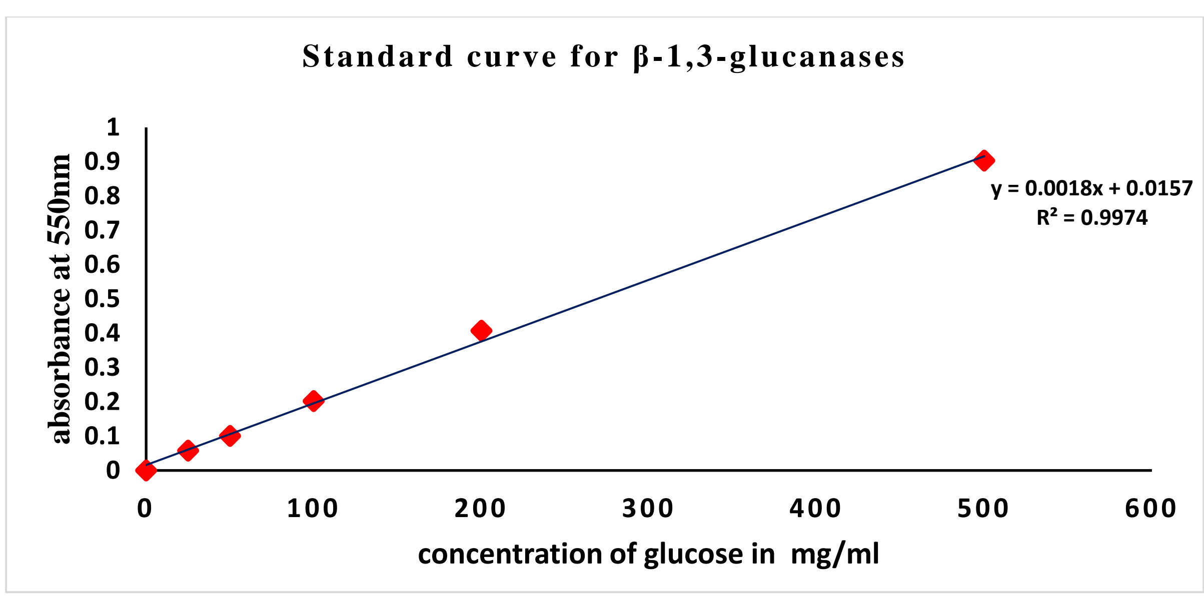
➢ Standard Curve for quantification of Proteins



➢ Standard Curve for Quantification of Total polyphenols

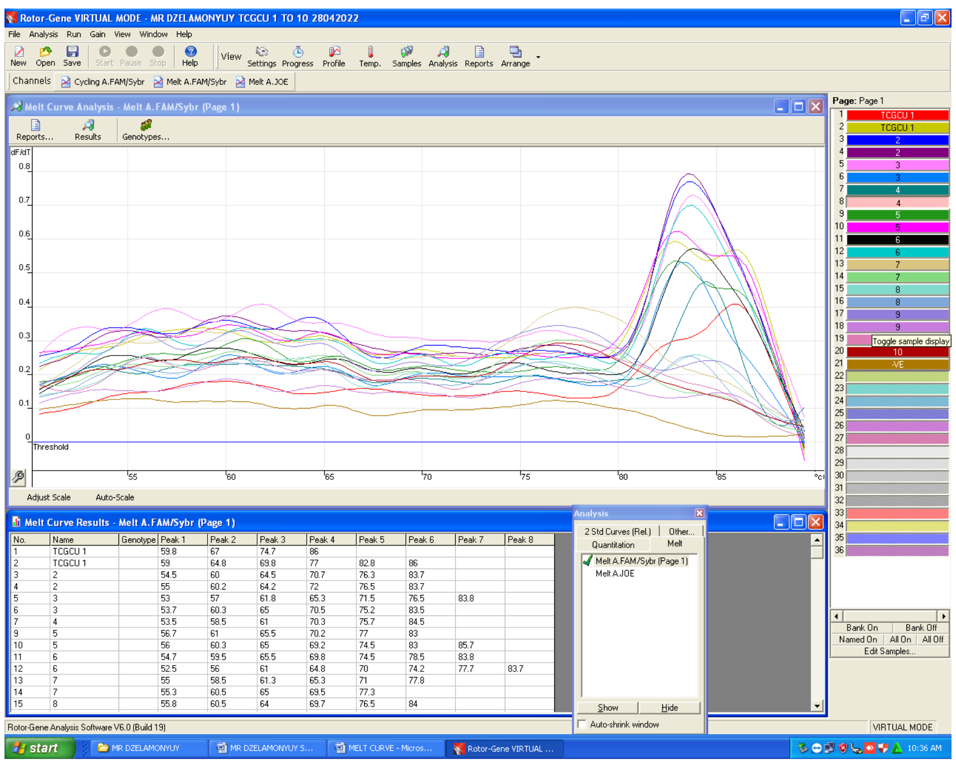


➢ Standard Curve for the quantification of β-1,3- glucanases



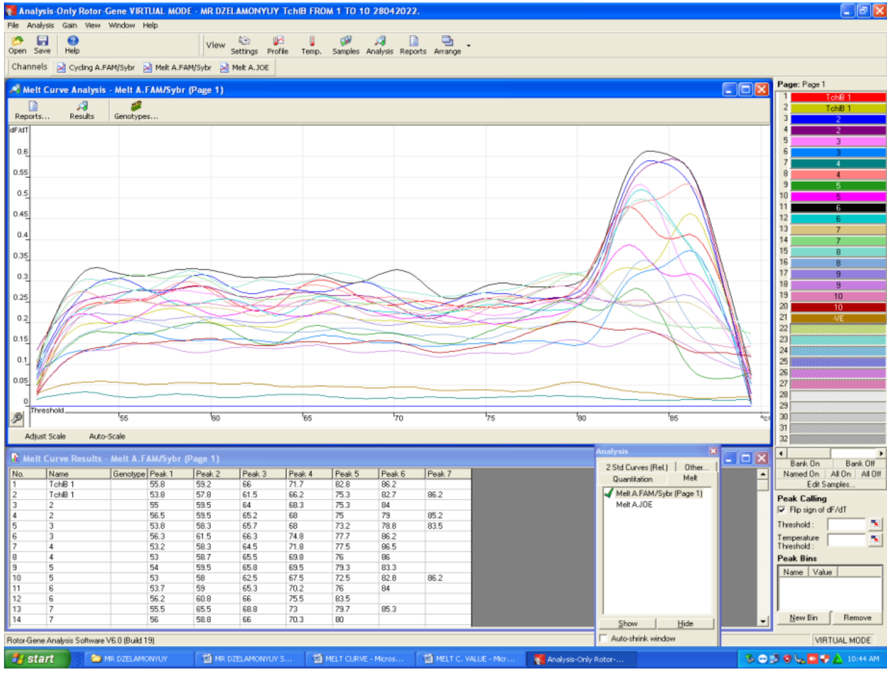
APPENDIX V: RT-PCR MELT CURVES

TcGlu-1

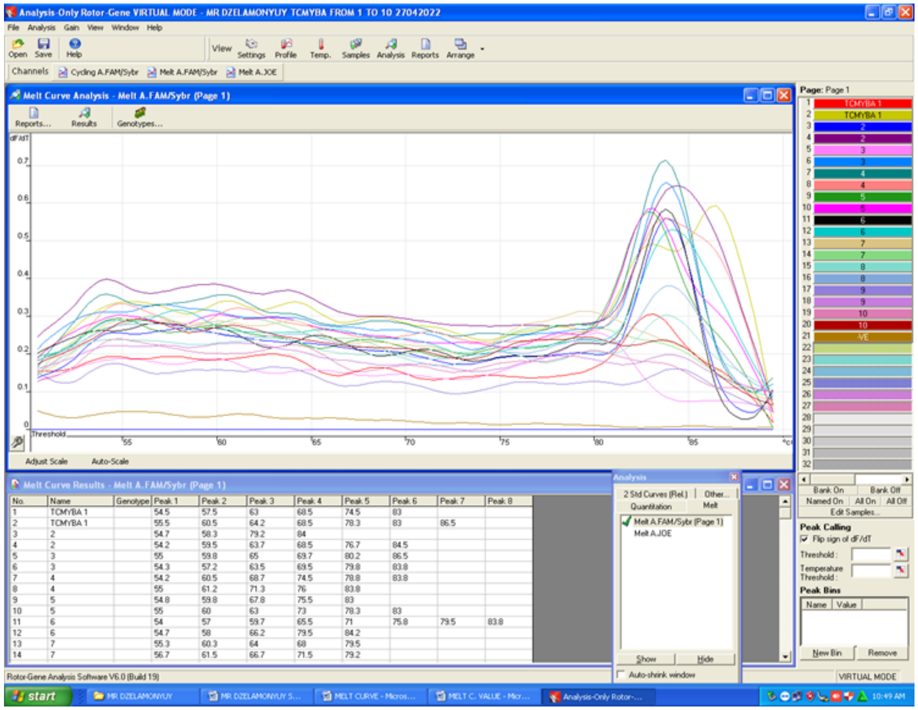


No.	Name	Genotype	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
1	TCGCU 1		59.8	67	74.7	86				
2	TCGCU 1		59	64.8	69.8	77	82.8	86		
3	2		54.5	60	64.5	70.7	76.3	83.7		
4	2		55	60.2	64.2	72	76.5	83.7		
5	3		53	57	61.8	65.3	71.5	76.5	83.8	
6	3		53.7	60.3	65	70.5	75.2	83.5		
7	4		53.5	58.5	61	70.3	75.7	84.5		
9	5		56.7	61	65.5	70.2	77	83		
10	5		56	60.3	65	69.2	74.5	83	85.7	
11	6		54.7	59.5	65.5	69.8	74.5	78.5	83.8	
12	6		52.5	56	61	64.8	70	74.2	77.7	83.7
13	7		55	58.5	61.3	65.3	71	77.8		
14	7		55.3	60.5	65	69.5	77.3			
15	8		55.8	60.5	64	69.7	76.5	84		

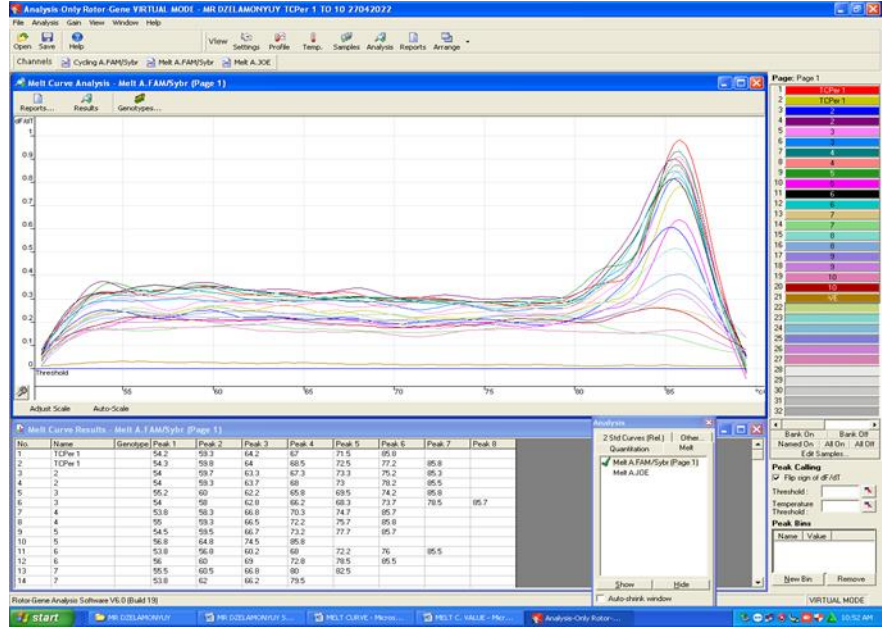
TcChiB



TcMYBA



TCPer 1-10



APPENDIX VI: LIST OF PUBLICATIONS

<u>Dzelamonyuy A</u>., Téné T. P. M., Ngassam N. E. R., Lele B. G., Foka T. E., Magni P. T. F., and Boudjeko T. (2022). Effects of a Powder Formulation of *Streptomyces cameroonensis* on Growth and Resistance of Two Cocoa Hybrids from Cameroon against *Phytophthora megakarya* (Causal Agent of Black Pod Disease). *Journal of Microbiology and Biotechnology*. 32:160-169. https://doi.org/10.4014/jmb.2110.10006

<u>Dzelamonyuy A.,</u> Tene T.P.M, Bopda W. A., Effa O.P., Ewane C.A., and Boudjeko T. (2023). Primed Expression of Defense-Related Genes by *Streptomyces cameroonensis*-Based Bioformulation (SCaB) on Cocoa Seedlings in a Nursery Challenged with *Phytophthora megakarya*. *American Journal of Plant Sciences*. **14**:1480-1497. doi:10.4236/ajps.2023.1412100

J. Microbiol. Biotechnol. 2022. 32(2): 160–169
https://doi.org/10.4014/jmb.2110.10006

Effects of a Powder Formulation of *Streptomyces cameroonensis* on Growth and Resistance of Two Cocoa Hybrids from Cameroon against *Phytophthora megakarya* (Causal Agent of Black Pod Disease)

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In the present study we evaluated the efficacy of a bioformulation of *Streptomyces cameroonensis* for control of black pod disease in cocoa and enhancement of seedling growth. The formulation developed using talc powder and cassava starch as carriers showed high shelf-life of 1.07 × 10^{6} CFU/g after six months storage at 4°C. The formulation was tested for inhibition of spore germination in *Phytophthora megakarya* and showed 100% inhibition at 10% (w/v) of formulation. To determine the efficacy of the formulation, we performed an *in planta* assay in the greenhouse on two hybrids of cocoa seedlings, the tolerant SNK413 × (♂) T79/467 and the susceptible UPA 134× (♂) SCA 12. Detached leaf assay showed a significant reduction in the disease severity index of about 67% for the tolerant hybrid and 55% for the susceptible hybrid compared to non-treated plants. A significant enhancement in stem length, leaf surface area and root weight was observed. Analysis of biochemical markers of defense showed a significant increase in total polyphenol, flavonoid, and total protein contents. There was also significant upregulation of PR-proteins such as chitinases, peroxidases and β-1, 3-glucanases following treatment of both tolerant and susceptible hybrids, though with a higher level of synthesis in the tolerant hybrids. A significant increase was also observed in polyphenol oxidase activities in plants treated with the formulation. This work demonstrated the stability and effectiveness of the *S. cameroonensis* powder formulation in suppressing black pod disease in cocoa and subsequently enhancing the growth of seedlings.

Keywords: *Streptomyces cameroonensis*, bioformulation, cocoa, *Phytophthora megakarya*

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Introduction

Cocoa (*Theobroma cacao* L.) is one of the main cash crops in tropical countries, and in Cameroon its production contributes to the national GDP by 1.2%, generating annual revenue of over €400 million and providing more than 400,000 jobs [1]. However, cocoa cultivation has been confronted by poor yields of the farmed varieties, unavailability of healthy seedings and parasitic attacks such as black pod disease caused by the soil-borne fungal pathogen *Phytophthora megakarya* [2].

In Cameroon, black pod disease stands out as one of the most economically destructive diseases of cocoa [3]. Annual losses are estimated at 40% and could potentially reach up to 90% if no proper control measures are taken [4]. Seedling production is a key step in the production chain of cocoa and in the establishment of new plantations [5]. Attacks of disease at nurseries and farms can lead to unavailability of healthy seedlings.

Countering this problem often requires the use of chemical fungicides but the latter creates many additional problems including the emergence of resistant pathogens, biodegradation of soil, and concerns about human health that constitute a separate dimension to the predicament [6]. However, hybridization has been shown to be a reliable method of ameliorating cocoa production. In Cameroon, research has been initiated towards genetic

control by breeding cocoa cultivars less susceptible to the disease and exploiting field resistance factors to find more resistant hybrids with quality seedlings. Several parental cocoa clones, with different sensibility to *P. megakarya* and available in gene banks of the Cameroon Cocoa Development Corporation (SODECAO), for example SNK413 and T79/46, have been bred and analyzed and some of the progenies have demonstrated tolerance to pathogen attack while also showing production of defense markers that complement the tolerance [7]. However, it has been difficult to find a balanced mechanism between disease-resistant hybrids with higher quality seedlings. It is therefore necessary to explore alternative methods of control that are both environmentally safe and effective.

In recent decades, the prospects of phytoprotection through biological control have been regularly explored in the eradication of soil-borne disease [8], and its use is extensive in modern- day agriculture. Intensive research on plant growth-promoting rhizobacteria (PGPR) is being conducted worldwide to develop biofertlilizers and biocontrol agents [9]. Among bacteria communities, Actinobacteria have been reported to comprise several biocontrol agents that suppress plant disease. Actinomycetes produce about 45% of the antibiotics currently in use, among which the genus *Streptomyces* alone produces 73% of the metabolites known to be capable of suppressing plant diseases [10]. *Streptomyces cameroonensis*, an actinomycete isolated from the *Chromolaena odorata* rhizosphere in Yaoundé (Cameroon), has been shown to exhibit extensive antimicrobial effects against a wide range of microorganisms while also possessing PGPR-like traits [11]. This strain demonstrated strong abilities to promote plant growth and protect against *P. megakarya,* the main causal agent of black pod disease during assays performed on cocoa plantlets. The mechanism of disease suppression by *S. cameroonensis* involves production of antibiotics like geldanamycin, production of cell wall-degrading enzymes, hyperparasitism, and production of volatile compounds, competition, and induction of host resistance. This strain is an equally effective root colonizer and can improve plant growth by enhancing iron availability through production of siderophores and production of indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate deaminase activity, nitrogen fixation, and solubilization of phosphates [1]. Having established this microbe as a biocontrol agent, the challenge lies in putting it into forms that are convenient in agricultural systems and better suited for commercial use. Many biocontrol agents have been formulated in various types of powders, granules or liquid. Powder formulations are easy to transport, suitable for easy storage and have longer shelf lives. In addition, the powder formulation can be made into liquid or water-based suspensions for various applications that can involve spraying, root-dipping or seed drenching [9].

In the present study, *S. cameroonensis* isolated from uncropped soil in Yaounde was used in a powder formulation at a nursery in Cameroon to study its shelf-life and efficacy against *P. megakarya* in two hybrids of cocoa as well as its impact on growth and defense-related markers.

Materials and Methods

Soil

The soil used was black humus often used by farmers producing cocoa seedlings and was obtained from Nkolbisson, (Yaounde, Centre Region, Cameroon, 3°52′24.4′′N-11°26′7.8′′E). It was dried and sieved to eliminate hard materials and debris, then mixed with river sand obtained from the River Sanaga (Centre Region) at the proportion of 3:1 (w/w).

Bacterial Strain and Pathogenic Fungi

S. cameroonensis was obtained from the microorganism bank of the Laboratory of Phytoprotection and Plant Valorization (LPPV) of the Biotechnology Centre of the University of Yaoundé 1, Cameroon. The strain was cultivated on the International Streptomyces Project-2 medium (ISP-2 medium) and incubated at 30°C for 7 days. The bacterial cells were then harvested using glass beads rolled over sporulating colonies. The beads were washed in glycerol (20% v/v), adjusted to 10^9 CFU/ml and stored at -20°C for further use according to [11].

Phytophthora megakarya isolate used in this study was obtained from the microorganism bank of the Laboratory of Phytoprotection and Plant Valorization (LPPV) of the Biotechnology Centre of the University of Yaoundé 1, Cameroon. Zoospore suspensions of *P. megakarya* isolate PM5 were obtained as described previously [12].

Powder-Based Formulation of *S. cameroonensis*

The powder formulation was developed as described by Anitha and Rabeeth [13] with some modifications. A mixture of 1 kg cassava starch and talc powder were mixed in different proportions (100:0% v/v, 25:75% v/v, 50:50% v/v, 75:25% v/v, 0:100% v/v, respectively) and sterilized twice at 121°C in an autoclave. Upon cooling, the various mixtures were supplemented with 15 g calcium carbonate and 10 g methyl cellulose. The powder mixture was then mixed under sterile conditions with 400 ml of sporal solution obtained previously containing 10^9 CFU/ml filtered broth of *S. cameroonensis*. The various mixtures were shade dried, ground to powder form and passed through a 0.8 mm sieve to obtain homogenous powder. The resulting powder was then packaged in pre-sterilized polypropylene bags and sealed. The different formulations were then tested for spore viability after 30 days using the spread plate technique. The formulation with the highest spore viability was selected for further testing.

Study of the Shelf Life of Powder-Based Formulation of *S. cameroonensis*

The formulation with the most viable spore count was stored at 4°C and 25°C, respectively. The shelf lives of the formulation at the two temperatures were determined using the standard dilution plate count method. Samples were taken periodically at 1 month intervals over a period of six months. Three plates were used for each

treatment. Humidity, form and texture were evaluated to determine the best conditions of storage as described previously [13].

Efficacy of *S. cameroonensis* Powder-Based Formulation

The efficacy of the formulation was evaluated in vitro against *P. megakarya.* This was done using the agar well diffusion method. One gram of the powder formulation was dissolved in 1 ml distilled water. PDA media were prepared and supplemented with the water-based suspension of the formulation at various concentrations (50% w/v, 25% w/v, 10 % w/v, 1% w/v and 0.1% w/v). *P. megakarya* was cultured in the middle of the solid PDA media and the percentage radial growth inhibition was calculated after 7 days using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Radial growth of control} - \text{Radial growth of treatment}}{\text{Radial growth of control}} \times 100.$$

Seed Treatment Using *S. cameroonensis* Powder-Based Formulation

Two hybrids of cocoa, (H1 (♀) SNK413 × (♂) T79/467) and H2 ((♀) UPA 134× (♂) SCA 12) produced by manual pollination, were obtained from the SODECAO ("Société de Développement du Cacao") experimental farm in Mengang, South Region, Cameroon. The cocoa pods were dehusked and the seeds washed with sand and distilled water to remove the mucilage. The washed seeds were surface sterilized with 70% ethanol for 5 min and then later with 0.2% sodium hypochlorite solution for another 5 min. The sterilized seeds were then rinsed with sterilized distilled water to prepare a 0.1 g/ml water suspension of the formulation. The cocoa seeds were divided into three groups. The first group was soaked in the formulation suspension, the second group in distilled water and the third group in a suspension of the chemical fungicide MANCOXYL PLUS 720WP. The soaked seeds were incubated at 150 rpm at 28°C for 24 h prior to planting.

Production of Cocoa Plant Seedlings under Greenhouse Conditions and Evaluation of Agro-Morphological Parameters

The treated seedlings were then planted in pots containing soil and river sand mixed at the ratio of 3:1. Each treatment was in duplicates of 60 seedlings. The pots were kept in a greenhouse and watered with distilled water every two days for a period of 12 weeks. Stem length, leaf number and leaf surface area were measured every 4 weeks. At the end of the 12th week, the dry and fresh weight of roots and shoots were also measured. The experiment was a completely randomized design with 3 treatments per hybrid, formulation-treated seedlings (H1T and H2T), non-treated seedlings (H1NT and H2NT) and chemically-treated seedlings (H1C and H2C).

Assessment of Induced Resistance

The formulation was assessed for its efficacy in controlling the incidence of *P. megakarya* using detached leaf inoculation test [5] with some modifications. Briefly, young leaves were detached from cocoa seedlings in the greenhouse after 12 weeks of growth. The leaves were washed with distilled water and surface sterilized with ethanol (70% v/v) for 30 s. For each treatment, leaves were divided into two, one group for the inoculation with 10 µl of 10^6 zoospore/ml suspension of *P. megakarya* on the underside leaf surface while the other group for the control was inoculated with an equivalent amount of sterilized distilled water. The inoculated leaves were incubated in a humid dark chamber at 25 ± 1°C. Each treatment consisted of three replicates of 6 leaves each. Disease expression was rated six days after and by using Nyassé's rating scale [14]. This experiment was repeated twice and the severity of disease was determined for each treatment by calculating the ratio of the sum of individual scores to the total number of leaves used. The disease severity index used to express the resistance level [15] was as follows: Highly Resistant (HR: $0 < \text{index} \leq 1$); Resistant (R: $1 < \text{index} \leq 2$); Moderately Resistant (MR: $2 < \text{index} \leq 2.5$); Susceptible (S: $2.5 < \text{index} \leq 3.5$); and Highly Susceptible (HS: $3.5 < \text{index} \leq 5$).

Biochemical Analysis

Biochemical analyses were carried out by assessing the level of infections on whole detached leaves after seven days of inoculation with *P. megakarya* sporal solution. The samples involved were cut at about 1 cm from beyond the necrosis point where no symptoms were noticed. For biochemical analyses, each treatment was repeated twice.

Determination of the Content of Total Phenolic Compounds and Flavonoids

The extraction of phenolic compounds was done following the modified protocol [4]. One gram of tissue extract (leaf) was ground in 5 ml methanol 80% (v/v). The sample was incubated at 4°C, and centrifuged at 10,000 ×*g* for 5 min at room temperature using the Beckman-Coulter Microfuge 20R centrifuge. The supernatant was collected and the precipitate re-suspended in 3 ml methanol, and incubated at room temperature for 15 min followed by further centrifugation. The second supernatant was collected and mixed with the first to constitute the extract.

Concentration of phenolic compounds was determined spectrophotometrically at 725 nm according to the method of [16], using the Folin-Ciocalteu reagent. Total content of phenolic compounds was expressed in milligrams of gallic acid equivalents per gram of fresh weight. Flavonoid content was determined in phenolic extract according to the method described by Kramling and Singleton [17] with some modifications. Briefly, 400 µl of phenolic extract, 200 µl of HCl (50%) and 200 µl of formaldehyde (8 mg/l) were incubated 15 min at 4°C and centrifuged at 3,000 ×*g* for 5 min at 4°C. The supernatant was collected and used for non-flavonoid

quantification spectrophotometrically at 725 nm [16].

Determination of Total Protein Content

For the determination of total protein content, extraction was performed as described by [13] with some modifications. One gram of plant samples (inoculated and healthy leaves) were crushed separately in a pestle and homogenized in 5 ml of the Tris-Maleate buffer (Tris-cHcl 1 HCl 10 mM, Triton X-100 1%, pH 7.5) at 4°C. The homogenate was centrifuged at 10,000 ×*g* for 25 min at 4°C using the Beckman-Coulter Microfuge 20R centrifuge. The supernatant was collected and the precipitate re-suspended in 3 ml buffer followed by further centrifugation. The second supernatant was collected, mixed with the first in 1.5 ml Eppendorf tubes and stored at -20°C. Quantification of the concentration of total proteins was done using the standard Bradford assay. The absorbance was measured at 595 nm using a UV-Vis 1605 Shimadzu spectrophotometer. For each extract, three repetitions were carried out. Bovine Serum Albumin was used as the standard. The concentration of the protein present was expressed in mg/g of fresh matter.

Evaluation of the Enzymes Activities

Peroxidase activity was assayed spectrophotometrically at 470 nm in the total protein extract [4]. The enzymatic activity was expressed in enzymatic units per gram of fresh weight (Δ470/min (UE)/g of Fresh Weight). Polyphenoloxidase activity was assayed spectrophotometrically [18], using catechine as a substrate. Enzyme activity was expressed as "A330 nm/min/g fresh weight". Chitinase activity was determined by colorimetric assay using bipolymeric substrate colloidal Chitin-RBV [19]. Chitinase activity is expressed in unit/g fresh matter/h. One unit of chitinase activity corresponds to an increased absorbance of 0.1. The β-1,3-glucanase activity was assayed using laminarin as a substrate. The quantity of glucose released was determined spectrophotometrically as changes in absorbance were measured at 540 nm. The amount of reducing sugars released was calculated from a standard curve prepared with glucose and the glucanase activity was expressed in units (μg glucose/min/mg protein).

Statistical Analysis

All experiments were conducted in triplicates and all data were expressed as means ± SD and subjected to one-way ANOVA. Tukey's test and probability values of $p \leq 0.05$ were considered significant using the Statistix software version 9.0.

Results

Dry Formulations of *S. cameroonensis*

Dry powder formulations of cassava starch and talc powder with the percentage proportions of 25/75% (w/w) yielded the maximum population of bacterial colonies (2.78×10^6 CFU/g) than the rest of the proportions (Fig. 1). The concentration of the cassava starch influenced the viability of the spores at a concentration of 25% while the talc powder concentration in the formulation supported the maximum spore viability at 75% more than at 100%. This particular formulation produced the finest powder.

Shelf Life Stability of *S. cameroonensis* in Starch/Talc-Based Powder Formulation

The shelf life of the formation remained stable after 6 months. The formulation stored at 4°C showed longer shelf life than the formulation stored at 25°C. After 180 days, cell count in powder formulation stored at 4°C was 1.07×10^6 CFU/g compared to 3.53×10^5 CFU/g for the formulation stored at 25°C (Fig. 2).

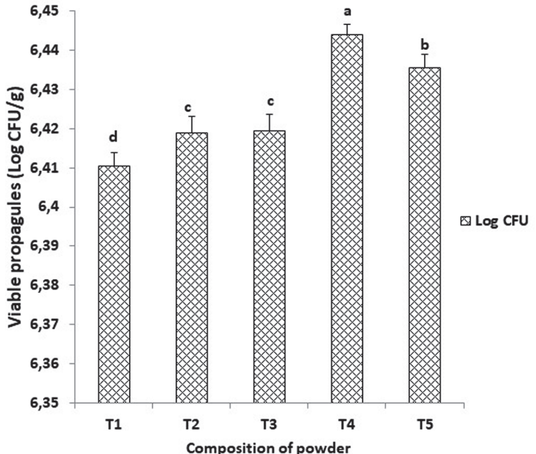

Fig. 1. *S. cameroonensis* spore viability in various proportions of cassava starch/talc powder after 1 month.
*Values with the same letter within a column are not significant at $p \leq 0.05$. Note: T1: 0%talc/100% w/w Cassava starch; T2: 25%talc/175% w/w Cassava starch; T3: 50%talc/50% w/w Cassava starch; T4: 75%talc/25% w/w Cassava starch; T1: 100%talc/0% w/w Cassava starch.

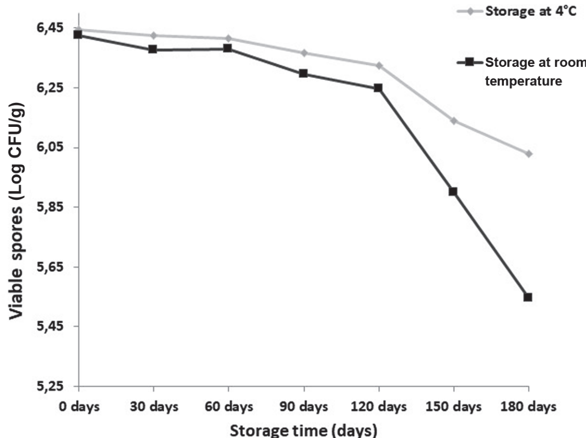

Fig. 2. Shelf-life of the cassava starch/talc powder formulation of *S. cameroonensis* at different temperatures.

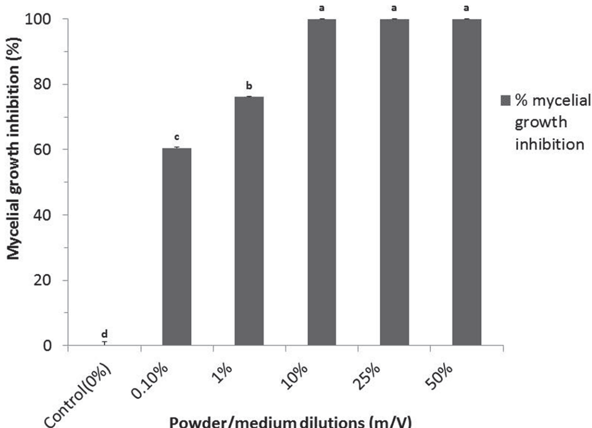

Fig. 3. Mycelial growth inhibition of fungal pathogen *Phytophthora megakarya* at various concentrations of the powder formulation. *Values with the same letter within a column are not significant at $p \leq 0.05$. Note: **0% (Control)**: 0% formulation; **0.1%:** 0.1%w/v formulation concentration; **1%**: 1% w/v formulation concentration; **10%:** 10% w/v formulation concentration; **50%**: 50% w/v formulation concentration; **100%**: 100% w/v formulation concentration.

Biocontrol Efficacy of *S. cameroonensis*-Based Powder Formulation

The formulation powder exhibited a high biocontrol efficacy (Fig. 3). At very low concentrations of 10% (w/v), the formulation exhibited a 100% mycelial inhibition against *P. megakarya*. The percentage mycelial growth inhibition at very low concentrations of 1% (w/v) and 0.1% (w/v) were at 76.2% and 60.5 %, respectively.

Growth Promotion under Greenhouse Conditions

Formulation-treated plants showed significant increase ($p \leq 0.05$) in plant height, number of leaves, leaf surface area, fresh and dry weight of shoots and roots over the non-treated and chemically treated plants after 12 weeks of

Table 1. Effect of *S. cameroonensis*-based powder formulation on different growth parameters of cocoa seedlings in nursery after 12 weeks of growth.

Hybrid	Treatment	Leave number/ plant	Stem length (plant/cm)	Leaf surface area (plant/cm^2)	Shoot fresh weight (g/plant)	Shoot dry weight (g/plant)	Root fresh weight (g/plant)	Root dry weight (g/plant)
SNK 413 x TF79/ 467(H1)	NT	12 ± 0.67^{ab}	30.4 ± 0.74^{b}	56.68 ± 2.19^{b}	13.92 ± 0.46^{c}	7.14 ± 0.54^{ab}	4.69 ± 0.17^{bc}	1.80 ± 0.18^{cd}
	C	13 ± 0.92^{a}	36.00 ± 1.02^{a}	58.01 ± 1.59^{b}	14.74 ± 0.80^{b}	7.56 ± 0.85^{ab}	5.19 ± 0.18^{b}	2.30 ± 0.11^{b}
	T	13 ± 0.74^{a}	37.30 ± 0.81^{a}	83.78 ± 3.71^{a}	16.04 ± 0.54^{a}	8.51 ± 0.31^{a}	6.52 ± 0.27^{a}	3.08 ± 0.14^{a}
UPA 134 x SCA 12 (H2)	NT	10 ± 0.47^{c}	23.76 ± 0.24^{d}	38.23 ± 1.54^{d}	10.44 ± 0.38^{d}	3.58 ± 0.31^{d}	2.83 ± 0.22^{e}	1.16 ± 0.14^{e}
	C	11 ± 0.83^{bc}	24.67 ± 0.94^{cd}	43.41 ± 1.78^{c}	9.93 ± 0.46^{d}	4.33 ± 0.38^{cd}	3.34 ± 0.13^{d}	1.40 ± 0.10^{d}
	T	10 ± 0.83^{c}	26.43 ± 0.74^{c}	47.50 ± 1.48^{c}	10.73 ± 0.57^{d}	3.81 ± 0.40^{d}	4.27 ± 0.25^{c}	1.44 ± 0.09^{d}

*Values with the same letter within a column are not significant at $p \leq 0.05$. **NT**: Non-treated (negative control); **T**: Treated (with bioformulation); **C**: Chemically treated (With MANCOXYL PUS 720WP)

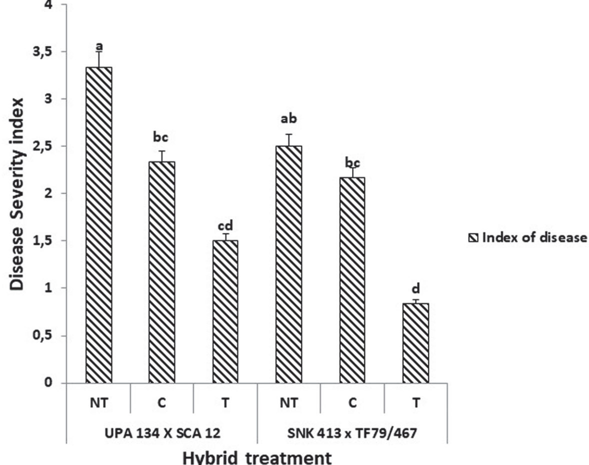

Fig. 4. Disease severity index of plants under different treatments six days after inoculation. *Values with the same letter within a column are not significant at $p \leq 0.05$. Note: **NT**: Non-treated (negative control); **T**: Treated (with bioformulation); **C**: Chemically treated (With MANCOXYL PUS 720WP).

growth (Table 1). The tolerant hybrid (H1: (♀) SNK413 × (♂) T79/467) showed a greater degree of growth than the susceptible hybrid (H2: (♀) UPA 134× (♂) SCA 12) in all cases of treatment. Plant height in formulation-treated plants for H1 was significantly higher (37.30 ± 0.81 cm) compared to non-treated plants (30.4 ± 0.74 cm). The same trend was observed for H2 in the order of 26.43 ± 0.74 cm and 23.76 ± 0.24 respectively for formulation-treated (T) and non-treated (NT). The same trend was observed for leaf surface area (Table 1). The dry and fresh weight of roots and shoot for both hybrids showed a significant increase in formulation-treated than the non-treated and chemical treatments. However, H1 showed higher levels of dry and fresh weight than H2.

Assessment of Disease Severity on Inoculated Leaves

Necrotic lesions were noticed on all leaves inoculated with the *P. megakarya* inoculum six days after inoculation while no lesions were found on leaves inoculated with sterilized distilled water. A significant lowest disease severity index was noticed on plants treated with the *S. cameroonensis*-based formulation compared to the non-treated for both hybrids with about 67% for H1 and 55% for H2 (Fig. 4). This led us to classify the H1 plants treated with the formulation as highly resistant with a necrosis index of 0.83, while the H2 plants treated with the formulation were then classified as resistant with a necrosis index of 1.5. The chemically treated plants were classified as moderately resistant with a necrosis index of 2.1 and 2.3 for H1 and H2, respectively. The non-treated H1 plants also showed moderate resistance (necrosis index of 2.5) while the non-treated H2 plants were classified as susceptible with a necrosis index of 3.3.

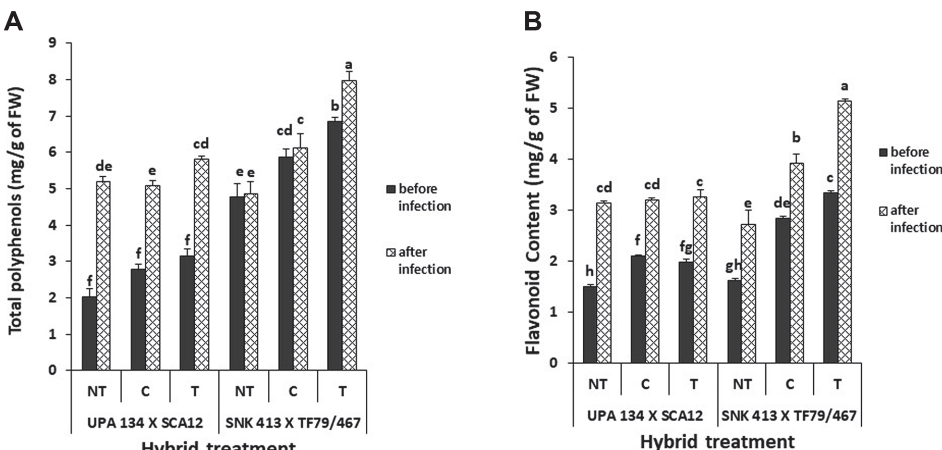

Fig. 5. Total polyphenol content (a) and flavonoid content (b) in *S. cameroonensis* based powder formulation treated and non-treated plants before and after inoculation. *Values with the same letter within a column are not significant at $p \leq 0.05$. Note: NT: Non-treated (negative control); T: Treated (with bioformulation); C: Chemically treated (With MANCOXYL PUS 720WP).

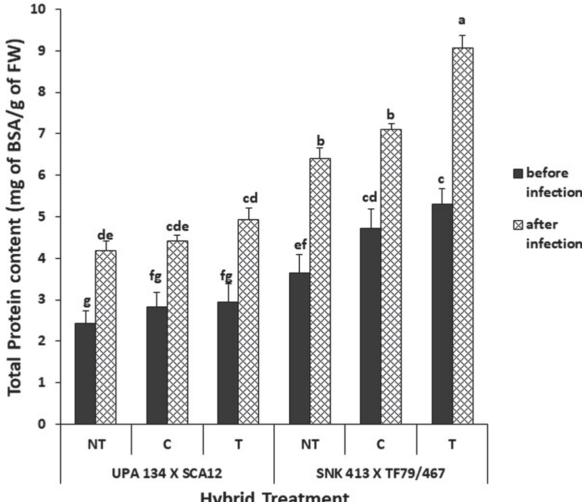

Fig. 6. Total protein content in *S. cameroonensis* based powder formulation treated and non-treated plants before and after inoculation. *Values with the same letter within a column are not significant at $p \leq 0.05$. Note: NT: Non-treated (negative control); T: Treated (with bioformulation); C: Chemically treated (With MANCOXYL PUS 720WP).

Biochemical Analysis

Total phenol and flavonoid content. The *S. cameroonensis*-based formulation-treated plants showed a significant increase in total phenol content as compared to the chemical control and the non-treated plants. H1 also showed a greater difference in phenol content than H2 by 40% after inoculation for the formulation-treated plants (Fig. 5A). Formulation-treated plants showed a percentage increase of 16% for H1 and 85% for H2 after inoculation while the chemically treated showed an increase of 5% and 83% respectively for both hybrids, H1 and H2. Flavonoid content in inoculated leaves was significantly higher than in the non-inoculated leaves for both hybrids. Formulation-treated H1 plants showed a significantly higher flavonoid content than chemically treated (31%) and non-treated plants (88%) before and after inoculation, while that for H2 was not significant. (Fig. 5B)

Total protein content. Formulation-treated plants showed a significantly higher protein content than plants chemically treated and non-treated for both hybrids H1 and H2 (Fig. 6). H1 however showed a higher protein content after inoculation equivalent to 5.30 mg equivalent of BSA/g of fresh weight (FW) and 2.94 mg equivalent of BSA/g of fresh weight for H2, which rose significantly by 71% and 67% respectively after inoculation.

Enzymatic Activities

Formulation-treated plants had a higher peroxidase activity than the chemically treated and the non-treated

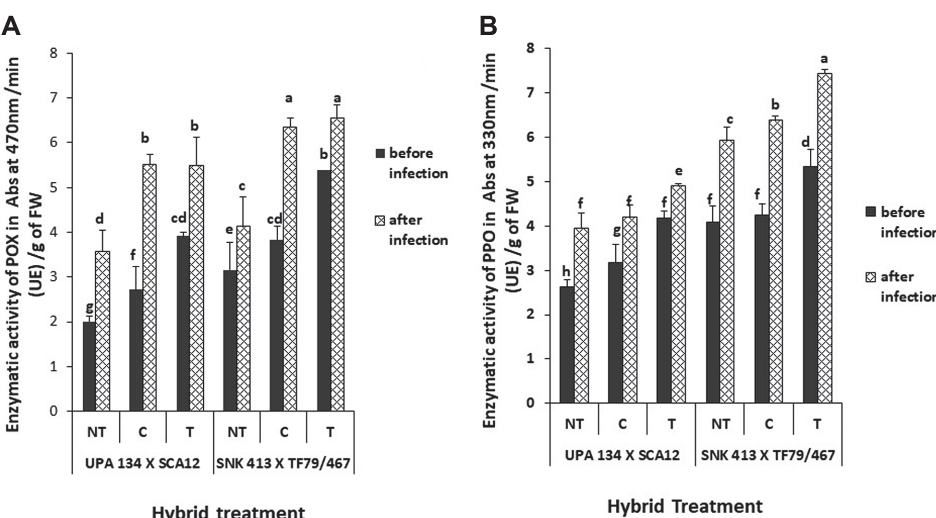

Fig. 7. Enzymatic activity of peroxidase (A) and polyphenoloxidase (B) in *S. cameroonensis* based powder formulation treated and non-treated plants before and after inoculation. *Values with the same letter within a column are not significant at $p \leq 0.05$. Note: NT: Non-treated (negative control); T: Treated (with bioformulation); C: Chemically treated (With MANCOXYL PUS 720WP).

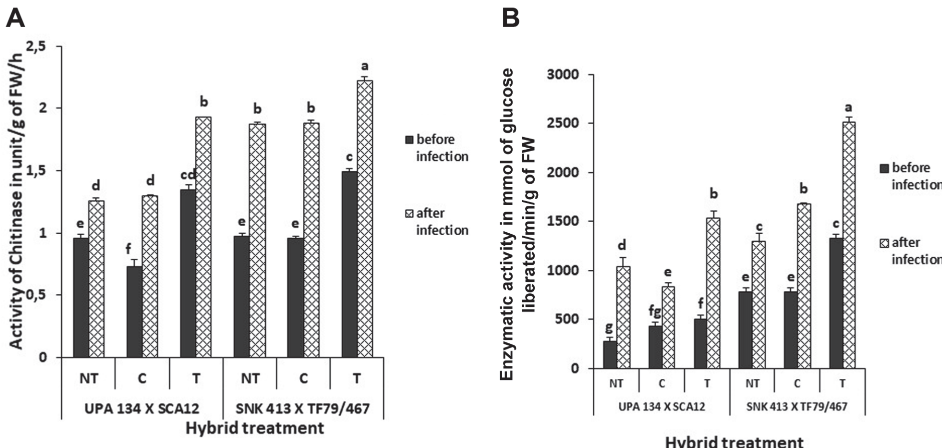

Fig. 8. Enzymatic effect of Chitinase (A) and β-1,3-glucanase (B) in *S. cameroonensis* based powder formulation treated and non-treated plants before and after inoculation. [*]Values with the same letter within a column are not significant at $p \leq 0.05$. Note: NT: Non-treated (negative control); T: Treated (with bioformulation); C: Chemically treated (With MANCOXYL PUS 720WP).

plants. In H1, the formulation-treated plants showed a significantly higher peroxidase activity (5.34 UE/g FW) than the chemically treated (4.25 UE/g FW) and the non-treated (4.08 UE/g FW). The activity of peroxidase effectively rose by 39%, 50% and 45%, respectively, after inoculation (Fig. 7A). H2 showed a similar pattern but with generally less UE/g of fresh weight before and after inoculation. Polyphenoloxidase activities were higher in the formulation-treated plants than the controls before and after inoculation. In H1, the formulation-treated plants showed a significantly higher polyphenoloxidase activity (5.34 UE/g FW) which rose by 39% after inoculation. H2 showed a lower activity (4.17 UE/g FW), which rose by 18% after inoculation (Fig. 7B). There was a significant increase in the activity of chitinase in formulation-treated plants compared to the chemically treated plants and non-treated plants. In H1, the activity of chitinase for formulation-treated plants stood at 1.49 UE/g of fresh weight and rose by 49% after inoculation. H2 followed a similar pattern with 1.35 UE/g of fresh weight that rose to 43% after inoculation (Fig. 8A). As shown in Fig. 8B, formulation-treated plants (T) had a significant increase in β-1, 3-glucanase production as compared to the non-treated (NT) and chemically treated (C). There was a general significant increase in β-1, 3-glucanase after inoculation for both hybrids and all treatments. The activity of β-1, 3-glucanase increased by 89% after inoculation for formulation-treated H1 plants while that of H2 increased more than 200% (Fig. 8B).

Discussion

In this present study, *S. cameroonensis*, an actinomycete isolated from uncropped soil in Yaoundé, has been shown to survive well in a mixture of talc/cassava starch powder-based formulations (75%/25% w/w) for more than 6 months at 4°C as well as at room temperature. *Streptomyces* species and other plant growth-promotion rhizobacteria (PGPR) have been shown to survive in dry formulations including talc, corn starch, xanthan gum, and peat [9, 13, 20]. The talc and cassava starch suitable in enhancement of Actinobacteria cell survival is probably related to the nature of their lipopolysaccharides and coating properties. Talc/cassava starch formulation of *S. cameroonensis* strongly suppressed the growth of *P. megakarya* in vitro even at very low concentrations in PDA medium. Boudjeko *et al.* [11] showed that *S. cameroonensis* possessed antifungal properties by producing bioactive molecules like geldanamycin which has the ability to inhibit growth of oomycetes. *Streptomyces* species are also known to produce major fungal degrading cell wall enzymes such as chitinase and β-1,3-glucanase which tend to inhibit mycelial growth of fungal pathogens [9, 21]. These results are in agreement with our findings.

The efficacy of this formulation was tested on two hybrids of cocoa commonly used in Cameroon, H1: SNK413xTF79/467 (tolerant) and H2:UPA134 X SCA 12(susceptible) [22]. The formulation enhanced significantly the growth of cocoa seedlings of the two hybrids under greenhouse conditions and greatly reduced the severity of *P. megakarya* infection on leaves of cocoa inoculated with *P. megakarya* in vitro. Boudjeko *et al.* [11] previously demonstrated that *S. cameroonensis* possess growth-promoting properties such as production of siderophores and indole acetic acid, solubilization of phosphates, and degradation of 1-aminocyclopropane-1-carboxylate, an intermediary product in the biosynthetic pathway of the plant hormone ethylene. The production of these substances has been reported in several cases where treatment with *Streptomyces* species has enhanced growth of *Vigna unguiculata* and *Arabidopsis* in vitro [23, 24].

Both hybrids exhibited a low disease severity index after treatment with the *S. cameroonensis*-based powder formulation. Boudjeko *et al.* [11] previously reported that the necrosis index for cocoa foliar discs inoculated with sporal solution of *P. megakarya* for plants grown in substrate inoculated with *S. cameroonensis* reduced significantly compared to the control by stimulating systemic resistance in cocoa plants. Given that the powder formulation was applied as seed treatment by enrobing cocoa grains, when the treated seeds are sown, the bacteria

will establish well into the seed surface and colonize the roots after germination. Previous studies have shown that a talc-based powder formulation of *Pseudomonas fluorescens* when applied as seed treatment controlled foliar infection of rice blast and could be detected on the root cortex of leave sheaths [20]. Talc-based formulations of *Streptomyces griseus* suppressed *Fusarium* wilt of tomato in greenhouse [13]. Similarly, *Streptomyces corchorusii* formulated on talc strongly enhanced the growth of rice plants under pathogen-challenged conditions and suppressed root disease caused by *Macrophomina phaseolin*a under greenhouse conditions [9, 25]. This antifungal activity could as well be attributed to the production of antimicrobial compounds like geldanamycin or the production of many fungal cell wall- degrading enzymes.

The increase in resistance was translated by the increased synthesis of biochemical markers of defense. The level of disease severity was lower in hybrid H1, which was the more tolerant and displayed higher levels of phenol content, flavonoid content, protein content and increased activities of polyphenoloxidase, peroxidases, chitinase and β-1,3-glucanases. Previous studies have shown that the tolerant and more productive species displayed high phenol content while less tolerant ones displayed less phenol content [26]. The role of phenolic compounds and flavonoids in plant defense is well documented [27] as these metabolites tend to accumulate in different levels in infected tissues in response to pathogen attack. The use of *S. cameroonensis* powder formulation led to a significant increase in the synthesis of these metabolites signifying a corresponding increase in growth and resistance against pathogen attack in cocoa seedlings. The activities of defense-related enzymes like PPO, POX, chitinase and β-1,3-glucanase increased higher in *S. cameroonensis* powder formulation-treated plants compared to the controls. Studies have shown that POX is important in scavenging for H_2O_2 in cells which is an important element in disease resistance to pathogens. The enhanced activities of these enzymes in plant tissues are positively associated to induce systemic resistance and plant disease suppression [5, 28]. Chitinases and glucanases have been demonstrated by many authors to be fungal pathogen cell wall-degrading enzymes [5]. Previous studies have also reported an increase in activities of these defense-related enzymes as a result of induction by microbial antagonist in hosts [28]. Studies have shown that root inoculation with *Streptomyces* GB 4–2 provided Norway spruce with systemic resistance to the needle pathogenic fungus *B. cinerea*. Treatment of cucumber leaves with a culture filtrate from *Streptomyces bikiniensis* HD-087 showed an increase in the activities of peroxidase, phenylalanine ammonia-lyase, and beta-1,3-glucanase as well as increased levels of chlorophyll and soluble sugars [30]. These results corroborate our findings that treating seedlings of cocoa with a powder formulation of *S. cameroonensis* induces systemic resistance in the seedlings by causing an increased synthesis of defense markers. Hence, the accumulation of these defense-related enzymes in this study appears as a credible mechanism for the efficacy of this *S. cameroonensis* powder formulation.

In conclusion, the present study clearly demonstrates that *S. cameroonensis* fused in a mixture of talc and cassava starch in the bioformulation performs well in the control of *Phytopthora megakarya,* the causal agent of black pod disease in cocoa. This formulation significantly increased the growth and resistance of two hybrids of cocoa commonly cultivated in Cameroon and significantly so for the more susceptible one by demonstrating an increase synthesis of biochemical markers of resistance. Hence, this formulation could be used as an effective biofungicide for the biocontrol of black pod disease of cocoa. Moreover, based on this present study and other reports, we recommend investigating the genetic basis of the interactions between the formulation and the cocoa seedlings, the concentration of the biocontrol agent in the plant and soil after treatment as well as the follow-up of the treatment to evaluate the effect of the formulation on the final product of cocoa in the farm. This will lay the basis for identifying the upregulated genes corresponding to increased resistance as well as metabolic and transcriptomic profiling and will help us better understand the mode of action of the formulation by studying the plant-bacteria interaction.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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Primed Expression of Defense-Related Genes by *Streptomyces cameroonensis*-Based Bioformulation (SCaB) on Cocoa Seedlings in a Nursery Challenged with *Phytophthora megakarya*

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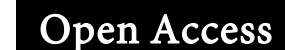

Abstract

A *Streptomyces cameroonensis* based bioformulation (SCaB) has been developed and shown to be stable and effective in controlling the early proliferation of *P. megakarya* and promoting the growth of cocoa seedlings in nursery. This study was carried out to explore the molecular mechanisms associated with the interaction of SCaB, cocoa seedlings, and the pathogen during the early stages of seedling growth in the nursery. For this purpose, seedling treatment with 10% W/W SCaB under greenhouse conditions evaluated SCaB's capacity to stimulate the defense mechanisms in cocoa. Agronomic growth parameters and the level of induction of defense-associated compounds were analyzed. Real-time (rt) PCR was used to assess the level of expression of defense genes. Here, we showed that the application of SCaB as a seedling treatment enhanced the growth of cocoa seedlings in the nursery by an average of 15.6% after 30 days of growth and led to an average reduction in disease severity of 64% when challenged with *P. megakarya*. The latter led to an increased synthesis of total phenolic compounds, flavonoids, chitinases, peroxidases, and β-1,3-glucanases and an induced up-regulation of TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes. This research provides a basis for the optimization of beneficial microorganisms as a viable alternative

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[#]The supervisor and original initiator of this project.

to chemical fungicides used in disease suppression.

Keywords

Cocoa Seedlings, *Phytophthora megakarya*, *Streptomyces cameroonensis*, Bioformulation, Priming

1. Introduction

Cocoa (*Theobroma cacao*), cultivated for its beans, is the source of the raw material for the global chocolate industry, valued at over US $103 billion [1]. In Cameroon, cocoa culture is an important economic activity that generates over 1.2% of the national gross domestic product (GDP) and provides employment to hundreds of thousands of Cameroonians [2]. Parasitic attacks are a major threat to cocoa production in Cameroon and worldwide [3] [4]. Black pod disease, caused by the soil-borne fungal pathogen *Phytophthora megakarya* (Brasier and Griffin), is the main disease of cocoa in Cameroon as well as in Central African countries and can cause losses of over 80% in farms and nurseries if unchecked [3] [5]. Healthy and available seedlings constitute the basis for plants to grow and flourish well in the future, hence the necessity to deter the effects of these parasitic attacks at an early stage. Chemical control is often used, but the latter causes a wide range of challenges, including the proliferation of diseases with heightened resistance, the degradation of soil, and concerns about human health and the environment. This therefore highlights the necessity for more environmentally friendly methods of disease control. Prospects for biological control in Phytoprotection have been regularly explored in the eradication of plant diseases in recent decades, and it is frequently employed in contemporary agriculture. Biological control involves the use of microbial inoculants or naturally fermented products to suppress the activities and populations of one or more plant pathogens. Worldwide, intensive research on plant growth-promoting rhizobacteria (PGPR) is being conducted to develop biofertilizers and biocontrol agents [2] [6]. These beneficial microbes provide cross-protection in plants against multiple stress factors in a plethora of ways. These include the production of antibiotics like geldanamycin, cell wall-degrading enzymes like chitinases and β-1,3-glucanases, stress-alleviating enzymes such as the 1-aminocyclopropane-1-carboxylate (ACC) deaminase, volatile compounds, the synthesis of siderophores and phytohormones, the solubilization of inorganic phosphorus (P), the formation of biofilm, and induced mechanisms of host resistance, among others [7].

The molecular mechanisms by which these biocontrol agents and biofertilizers initiate resistance in plants associated with pathogen interaction have been linked to priming. Priming is the phenomenon whereby plant tissues are preconditioned to respond in a more rapid and robust manner to very low levels of stimulus [8]. Priming confers important fitness benefits to the plant by setting it in a state of readiness to respond faster and stronger to attacks compared to

plants not exposed to priming agents. Studies on the genetic control of cocoa resistance to *Phytophthora* species have shown quantitative trait loci (QTLs) located in many genomic regions associated with *Phytophthora* species, thus offering the possibility of improving resistance in cocoa by a possible accumulation of many different resistance genes, such as those coding for pathogenesis-related proteins (PRs) [9]. Several studies have reported the systemic induction of these defense gene transcripts, including those that code for chitinases (PR-3, 8, and 11), glucanases (PR-2), peroxidases (PR-9), or transcription factors during *Phytophthora* species interaction with plants [10] [11] [12]. Induced systemic resistance leads to the rapid production of these PR proteins and other defense-related biomolecules when plants are attacked [13] [14]. The mechanism of priming is based on induced systemic resistance, often observed in the interaction between plants and beneficial microorganisms [15]. Several beneficial microorganisms and their products have been identified as priming agents in plants. Seedlings of *Arabidopsis* and rice exposed to bacterial cultures of *Streptomyces* species have been shown to significantly reduce disease severity [5] [7] [16]. Our previous work focused on developing and testing a bioformulation based on the biocontrol agent *Streptomyces cameroonensis* (labeled as ScaB) against *Phytophthora megakarya* and its effects on the growth and resistance of selected varieties of cocoa seedlings in a nursery [2]. *Streptomyces cameroonensis,* like other actinomycetes and other classes of plant growth-promoting rhizobacteria, has been shown to ameliorate the growth and resistance of plants using various similar mechanisms [7]. Recent studies have shown formulations of these biocontrol agents to be better suited for agricultural systems and effective in field applications in improving the growth and yield of seedlings [2] [6] [17] [18]. In this study, we investigate the ability of our SCaB treatment to induce growth and resistance in cocoa seedlings in the nursery against *P. megakarya* by quantifying the primed induction of defense metabolites and expression of stress response genes in cocoa leaves using rt-PCR.

2. Material and Methods

2.1. Material

The soil used to grow the cocoa seedlings was black humus soil, often used by cocoa seedling producers in the environs of Yaoundé, Centre Region, Cameroon. The soil collected was air dried, sieved through a 10 mm sieve to get rid of hard material and debris, and then mixed with river sand in a ratio 3:1 w/w. The *Streptomyces cameroonensis* and *Phytophthora megakarya* PM5 strains were obtained from the microorganism bank of the Laboratory of Phytoprotection and Valorization of Genetic Resources (LPVGR) of the Biotechnology Centre (BTC) of the University of Yaoundé 1, Cameroon. Hybrid pods of *Theobroma cacao* ((♀) SNK413 × (♂) T79/467) produced by hand pollination were obtained from the SODECAO ("Société de Développement du Cacao") experimental biclonal farm in Mengang, Center Region, Cameroon.

2.2. Culture of *S. cameroonensis* Spores and Storage Conditions

This strain was cultivated on the International *Streptomyces* Project-2 medium (ISP-2 medium). This medium was prepared and autoclaved at 121˚C for 15 minutes, then poured onto sterile Petri dishes. Spores from the stock were streaked on freshly prepared ISP-2 media on Petri dishes and incubated at 30˚C for 7 days for full sporulation. The spores were later collected using sterile glass beads over sporulating colonies. The beads were washed in glycerol (20% v/v) and stored at −20˚C. After freezing for 24 hours at −20˚C, the purity of the inventory was checked for possible contamination. The sporal solution was then adjusted to 10^9 CFU/ml and stored in a 20% glycerol solution at −20˚C for further use.

2.3. Culture of *S. cameroonensis* Spores and Storage Conditions

The *P. megakarya* PM5 strain was cultured on PDA medium (Potato Dextrose Agar). The medium was prepared, autoclaved at 121˚C for 15 minutes, and then poured into sterile Petri dishes. The 5 mm-diameter mycelial discs taken from the surface of the stock culture of *P. megakarya* were transferred to the Petri dishes containing PDA medium and incubated at 26˚C ± 1˚C in the dark. A sporal solution of *P. megakarya* was obtained according to the protocol described by [19]. The number of zoospores was calibrated at 10^6 zoospores/ml and stored at −20˚C for further use.

2.4. Powder-Based Bioformulation of *S. cameroonensis* (SCaB)

Streptomyces cameroonensis based bioformulation (SCaB) was prepared as described [2]. The viability of the spores in the powder bioformulation was determined using the spread plate technique, and the number of CFU/g of powder was calculated.

2.5. Evaluation of the Effect of SCaB on Growth of Cocoa Seedlings

Freshly harvested cocoa pods were dehusked, the seeds were washed with sand and distilled water to remove the mucilage. The washed seeds were surface sterilized with 70% ethanol for 5 minutes, then later with 0.2% sodium hypochlorite solution for another 5 minutes. The sterilized seeds were then rinsed with sterilized distilled water. A water suspension of SCaB (0.1 mg/mL) was prepared, while sterilized cocoa seeds were divided into three equal groups. The first group was treated with 0.1 mg/ml of the bioformulation suspension as described by [2]. The second and third groups served as positive and negative controls respectively. The positive control consisted of seedlings treated with the chemical fungicide CALLOMIL SUPER 66 WP following the manufacturer's protocol, while the negative control was made up of untreated seedlings. Each treatment consisted of 60 seedlings. The different groups of seedlings were planted in polythene bags each containing a mixture of 1 kg of soil and river sand mixed in the ratio of 3:1, kept in the greenhouse for a period of 30 days and watered consis-

tently with sterilized distilled water. At the end of this period, stem length, leaf surface area, number of leaves, root and shoot fresh weights were sampled and measured.

2.6. Evaluation of the Effect of SCaB on Disease Severity

After 30 days of growth, leaves were harvested from each treatment, conditioned in plastic sachets, and transported to the lab. The leaves were washed, then surface sterilized with 70% v/v ethanol and rinsed with sterilized distilled water. Leaf discs were cut out with a 15-mm-diameter cock borer and placed on their abaxial surface in randomized Petri dishes lined with tissue paper soaked in sterilized distilled water (8 discs per dish and two dishes per treatment). One group of leaf discs was inoculated on their abaxial surface with 10 µL of 10^6 zoospore/ml suspension of *P. megakarya,* while the other group, which serves as the control, was inoculated with an equivalent amount of sterilized distilled water. The Petri dishes were covered and incubated under darkness at 25˚C ± 2˚C. Disease expression was rated six days later using a rating scale described by [20]. This experiment was repeated twice, and the severity of the disease was determined for each treatment by calculating the ratio of the sum of individual scores to the total number of discs used. The disease severity index used to express the resistance level was done following the scale described by [21] as follows: 0 < Index ≤ 1: very resistant; 1 < Index ≤ 2: resistant; 1 < Index ≤ 2.5: moderately resistant; 2.5 < Index ≤ 3: susceptible; Index ≤ 3.5: very susceptible.

2.7. Evaluation of the Effect of SCaB on Induction of Biochemical Markers

For each treatment, two groups of 10 leaves per treatment were selected, sterilized, and arranged separately in lightproof trays as described by [2]. One group of leaves was inoculated on their abaxial surface with 10 µL of 10^6 zoospore/ml suspension of *P. megakarya,* while the other group, which serves as the control, was inoculated with an equivalent amount of sterilized distilled water. The trays were hermetically sealed and incubated under darkness at 25˚C ± 2˚C. Six days after inoculation, samples for biochemical and molecular analysis were collected about 1 cm away from the infected area on the leaves.

2.7.1. Extraction and Quantification of Total Polyphenols and Flavonoids

The extraction and quantification of phenolic compounds were done following the modified protocol described by [23]. One g of tissue extract (leaf) was ground in 5 mL of 80% methanol (V/V). The sample was incubated at 4˚C and centrifuged at 10,000 g for 5 min at room temperature using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected, and the precipitate was re-suspended in 3 mL of methanol and incubated at room temperature for 15 min, followed by another centrifugation. The second supernatant was collected and mixed with the first to form the extract.

The quantification of phenolic compounds was determined spectrophotomet-

rically at 725 nm according to the protocol set by [24] using the Folin-Ciocalteu reagent. In a test tube, 10 µL of phenolic extract was added to 500 µL of distilled water, followed by 75 µL of Folin-Ciocalteu and 500 µL of Na_2CO_3. The mixture obtained was incubated for 30 minutes at 25˚C. In the presence of phenolic compounds, the Folin-Ciocalteu reagent appears blue. The color intensity is proportional to the quantity of phenolic compounds present in solution. Absorbance was measured using the Shimadzu UV-1605 Spectrophotometer at 725 nm against a standard where the extract was replaced by distilled water. Three repetitions were carried out. The quantity of phenolic compounds is expressed in mg/g of fresh matter equivalent to a standard curve of gallic acid (0.1 g/mg).

Flavonoid content was determined in phenolic extract according to the method described by [25] with some modifications. Briefly, 400 µL of phenolic extract, 200 µL of HCl (50%) and 200 µL of formaldehyde (8 mg/L) were incubated for 15 min at 4˚C and centrifuged at 3000 ×*g* for 5 min at 4˚C. The supernatant was collected and used for non-flavonoid quantification spectrophotometrically at 725 nm [24].

2.7.2. Extraction of Total Proteins and Estimation of the Enzymatic Activities of Peroxidases, *β*-1,3-Glucanasea, and Chitinases

The extraction and quantification of proteins were carried out following the modified protocol of [22]. Initially, 1 g of the fresh plant leaves were ground in 5 ml of the Tris-Maleate buffer (Tris-HCl 10 mM, Triton X-100 1%, pH 7.5) using a pestle and mortar on ice. After grinding, the mixture was vortexed for 10 minutes and kept on ice. It was then centrifuged at 10,000 g for 25 min under cold conditions using the Beckmann-Coulter microfuge 20 R centrifuge. The supernatant was collected and conserved, while the remaining residue was centrifuged again at 20,000 g for 20 min. The two supernatants obtained were added and constituted our extract, which was conserved at −20˚C pending quantification of protein. The protein content was determined according to the standard Bradford assay. The absorbance was read at 595 nm using the Shimadzu spectrophotometer UV-1605 against control in which the extract was replaced by distilled water. For each extract, three repetitions were carried out. The concentration of the total protein present was expressed in mg/g of fresh matter, reference to an extrapolation on a standard curve realized under the same conditions as those with the samples using BSA (Bovine Serum Albumin) at 0.1 mg/mL.

The activity of peroxidase (POD) was measured following the method described by [23] with some modifications. 925 µL of Tris-Maleate buffer (0.1 M, pH 7.2, 1 g Guaiacol), was added to 25 µL protein extract, followed by 50 µL H_2O_2 (10%). After homogenization, the reaction mixture was incubated at ambient temperature for 3 minutes. The activity of peroxidase was determined following the formation of tetraguaiacol with an absorbance of 470 nm using the Shimadzu UV-1605 spectrophotometer. The enzymatic activity was expressed in enzymatic units per gram of fresh weight (Δ_{470}/min (EU)/g FW). The control was done using the same procedure as the samples, except that the plant extract

was replaced with water. The enzymatic unit corresponds to the quantity of enzyme, which leads to a 0.1 variation in optical density.

The activity of *β*-1,3-glucanases was determined according to [26] using laminarine as a substrate. 500 µL of phosphate buffer (pH 7, 0.66 M), was added to 150 µL of catechin (10 mM), followed by 35 µL of protein extract. The mixture was incubated at 25˚C for 30 s. The quantity of glucose released was determined spectrophotometrically, as changes in absorbance were measured at 540 nm using the Shimadzu UV-1605 spectrophotometer. The amount of reducing sugars released was calculated from a standard curve prepared with glucose, and the glucanase activity was expressed in units (g of glucose equivalent per g of fresh weight). The enzymatic activity was calculated from the standard graph of glucose.

Chitinase activity was determined by colorimetric assay using a biopolymeric substrate, colloidal chitin-RBV, following the modified protocol of [27]. 200 µL of colloidal chitin was added to 300 µL protein extract, followed by 300 µL Tris HCl (10 mM, Triton 1%, pH 7.5). This mixture was then incubated at 37˚C for 3 hours. The reaction was stopped with 200 µL of 2 M HCl. The samples obtained were cooled for 15 min to eliminate unhydrolyzed substrate and then centrifuged at 10,000 g for 20 min to remove non-degraded substrate. The spectrophotometric analysis of the supernatant was done at 500 nm. Chitinase activity is described by units per g of fresh matter/h. One-unit chitinase activity corresponds to an increased absorbance of 0.1. For each sample, three independent repetitions were used.

2.8. Evaluation of the Effect of SCaB on the Gene's Expression Level

This evaluation was done through a real-time PCR and analysis of PR protein gene expression in response to infection with *P. megakarya* and SCaB treatment.

2.8.1. Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 100 mg of fresh infected and uninfected cocoa plant leaves using the AccuPrep® Universal RNA Extraction Kit (K-3140, BIONEER, Republic of Korea) according to the manufacturer's protocol. The extracted RNA samples were treated with RNase-free DNase to remove any residual genomic contamination. The purity and concentration of the total RNA extract were determined spectrophotometrically (NanoDrop ONE^{C}; Thermo Fisher Scientific, USA). cDNA was synthesized from RNA using the AccuPower RT PreMix (Bioneer, Korea). The template Dnase-treated RNA (1 µg) was mixed with 1 µL (25 pmol) of oligo dT reverse primer in a sterile tube and incubated at 70˚C for 5 min, then placed on ice. The mixture was transferred into the AccuPower® RT PreMix tube, and the total reaction volume was filled up to 20 µL with DEPC-DW (BIONEER). cDNA synthesis was carried out at 42˚C for 60 min and then RTase inactivation at 94˚C for 5 min using the Applied Biosystems® 7500 fast real-time PCR system (Thermo Fisher Scientific, USA).

2.8.2. Quantitative Real-Time PCR (rt-PCR) Assays

The relative levels of expression of the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes were assessed by qPCR. The qPCR reaction was performed in a 50 μL reaction mixture containing 100 ng of cDNA sample, 25 μL of the AccuPower® 2X GreenStar™ qPCR MasterMix (Bioneer, Korea), 2 μL each of the forward and reverse primers, 5μL of ROX dye, and the volume made up to 50 μL with DEPC-DW (Bioneer). The sequences of primers used for RT-PCR analysis are presented in **Table 1**. The qPCR reactions were carried out in the Applied Biosystems® 7500 fast real-time PCR system (Thermo Fisher Scientific, US) using the following PCR cycling conditions: 1 cycle of pre-denaturation at 95˚C for 2 minutes, followed by 45 cycles of denaturation at 95˚C each at 5 seconds, and annealing extension at 60˚C at 5 seconds each. This was followed by an extension at 72˚C for 30 seconds. The Arabidopsis *β*-Actin gene was used as the housekeeping gene for normalizing the expression of the target genes. The comparative gene expression analysis of target genes (TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes) was normalized to the *β*-Actin (internal control) relative to control experiments (uninfected) according to the $2^{-\Delta\Delta CT}$ method [28]. Data was analyzed using Sequence Detection System software (version 2.2, Applied Biosystems). The cycle at which an increase in the fluorescence level above the background was statistically significant (cycle threshold (Ct), was chosen in the exponential phase of the amplification. All the relative expression analysis was carried out in triplicate for every condition in the experiment.

2.9. Statistical Analysis

The experimental designs were randomized complete blocks, and each value reported is the average of multiple repeats. The raw data was imported into Microsoft Excel 2010 for calculations and graphic representation was done using GraphPad Prism 8.0.1. IBM SPPS software version 28.0.1.1 (15) was used for the analysis of the data. Quantitative changes in parameters were evaluated through analysis of variance (one-way ANOVA) with Turkey multiple comparison tests at $P \leq 0.05$ to find significant differences among treatments. All results are presented as the means ± standard deviation (SD).

Table 1. List of primers used to assess the relative level of expression of the TcChiB, TcGlu-1, TcPer-1, and TcMYBPA genes.

Reference gene (*β*-actin)	**Forward Primer** *5′……………………………3′*	Reverse Primer *5′……………………………3′*
β-Actin	GTGGGCCGCTCTAGGCACCAA	TCATACTCTGCCTTAGCAATCC
Target gene		
TcChiB	GTGGCTTTGCTTGTGAATCTC	CACTGCTTCTCACCCTTATGT
TcGlu-1	GCTATGATTCCCTTCCCTCTTC	CAGGCCAAGTGCTAGGATAAG
TcPer-1	TGCGCTGATATTCTCGCTATT	CTGTGAACCCATCCCTTCTT
TcMYBPA	GATGGGAAGGGCTCCTTGTTG	ATCTCGTTATCGGTTGGACCAG

β-Actin a housekeeping gene whose expression was unchanged for all exposure conditions was used as internal control.

3. Results

3.1. Effect of SCaB on Growth of Cocoa Seedlings

Cocoa seedling treatment with SCaB showed significant increases ($P \leq 0.05$) in the agronomic metrics of growth compared to the untreated seedlings (Table 2). Thus, after treating the seedlings with SCaB increases were seen in the number of leaves, stem length, leaf surface area, shoot fresh weight, and root fresh weight of 8%, 12%, 4%, 15%, and 39%, respectively compared to the untreated seedlings.

3.2. Effect of SCaB on Disease Severity

Necrotic lesions appeared on leaf disc samples from all treatments six days after inoculation. Leaf discs inoculated with sterilized distilled water showed no necrotic lesions across all treatments (Figure 1). Following *P. megakarya* inoculation, plants treated with SCaB had the lowest disease severity index compared to chemically treated and untreated plants. In effect, the lowest disease severity index of 0.83 (very resistant) was observed for SCaB-treated seedlings compared to 1.83 (resistant) for the chemically treated seedlings and 2.3 (moderately resistant) for the untreated seedlings. Indeed, a 64% reduction in disease severity following treatment with SCaB was observed compared to the untreated seedlings.

3.3. Effect of SCaB on Induction of Biochemical Markers

3.3.1. Effect of SCaB on Total Phenolic Compounds and Flavonoids

Treatment of cocoa seedlings with SCaB significantly induced the synthesis of total polyphenols, and flavonoids, both before and after infection of cocoa leaves with *P. megakarya* (Figure 2a and Figure 2b). Production of these compounds increased significantly ($P \leq 0.05$) across all treatments when leaves were infected with *P. megakarya*. In fact, after treatment with our bioformulation, percentage increases of 35% and 28% were seen for total polyphenols, and flavonoids, before infection and 40% and 47% after infection compared to the untreated seedlings (Figure 2).

3.3.2. Effect of SCaB on Total Protein Content and Enzymatic Activities of Peroxidases, β-1,3-Glucanases, and Chitinases

Cocoa seedlings treated with SCaB showed an increase in the content of total proteins and the enzymatic activities of peroxidases (PR-9), β-1,3-glucanases (PR-2) and chitinases (PR-3), both before and after infection compared to the chemically treated and untreated seedlings (Figure 3). Inoculation of leaves with *P. megakarya* is characterized by an increase in total protein content and the activity of these enzymes across all treatments. When compared to untreated seedlings, total protein content by 21% for seedlings treated with SCaB before infection and by 30% after infection with *P. megakarya* (Figure 3a) The enzymatic activities of peroxidases, β-1,3-glucanases and chitinases increased for seedlings treated with SCaB by 39%, 40% and 25% respectively before infection and by 58%, 59% and 33% after infection (Figures 3b-d).

Table 2. Effect of SCaB on agronomic parameters of cocoa seedlings in nursery after 30 days of growth.

Treatment	Leave number/ plant	Stem length (Plant/cm)	Leaf Surface area (Plant/cm²)	Shoot Fresh weight (g/plant)	Root Fresh weight (g/plant)
NT	4.0 ± 0[a]	17 ± 1[b]	30.31 ± 2.16[a]	4.64 ± 0.18[b]	1.56 ± 0.07[b]
C	4.3 ± 0.58[a]	19 ± 1[a]	31.77 ± 1.44[a]	4.91 ± 0.33[b]	1.73 ± 0.07[b]
T	4.3 ± 0.58[a]	19 ± 1[a]	34.62 ± 1.64[a]	5.34 ± 0.22[a]	2.17 ± 0.11[a]

*Each value represents the mean ± SD (n = 60). Values with the same superscript letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

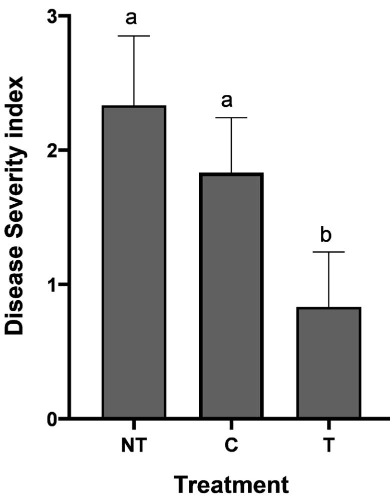

Figure 1. Disease Severity of cocoa plants treated with SCaB six days after inoculation. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66 WP); T: Treated (with SCaB).

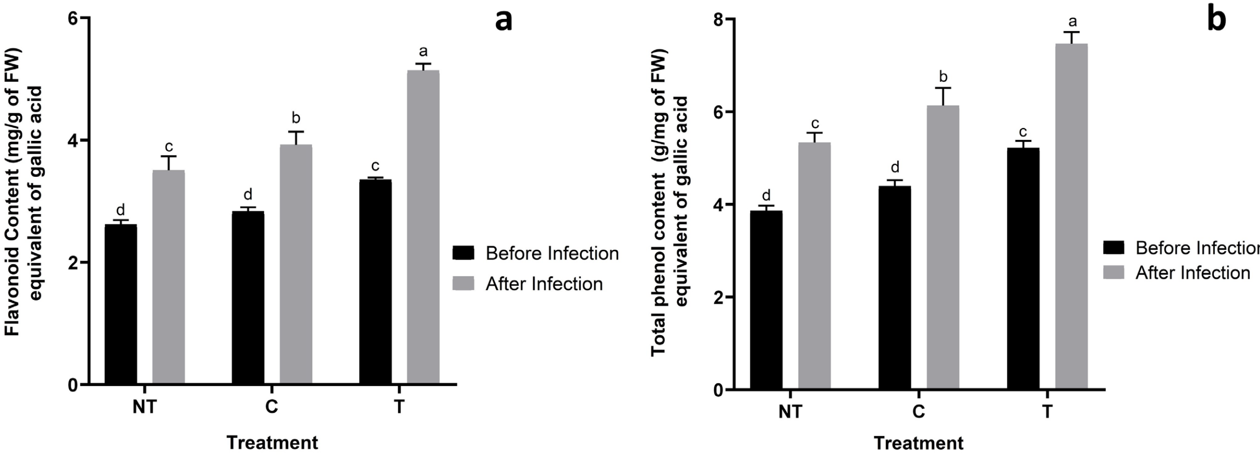

Figure 2. Effect of SCaB on (a) Total phenolic content (b) flavonoid content in 30 days cocoa leaves challenged with *P. megakarya*. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66 WP); T: Treated (with SCaB).

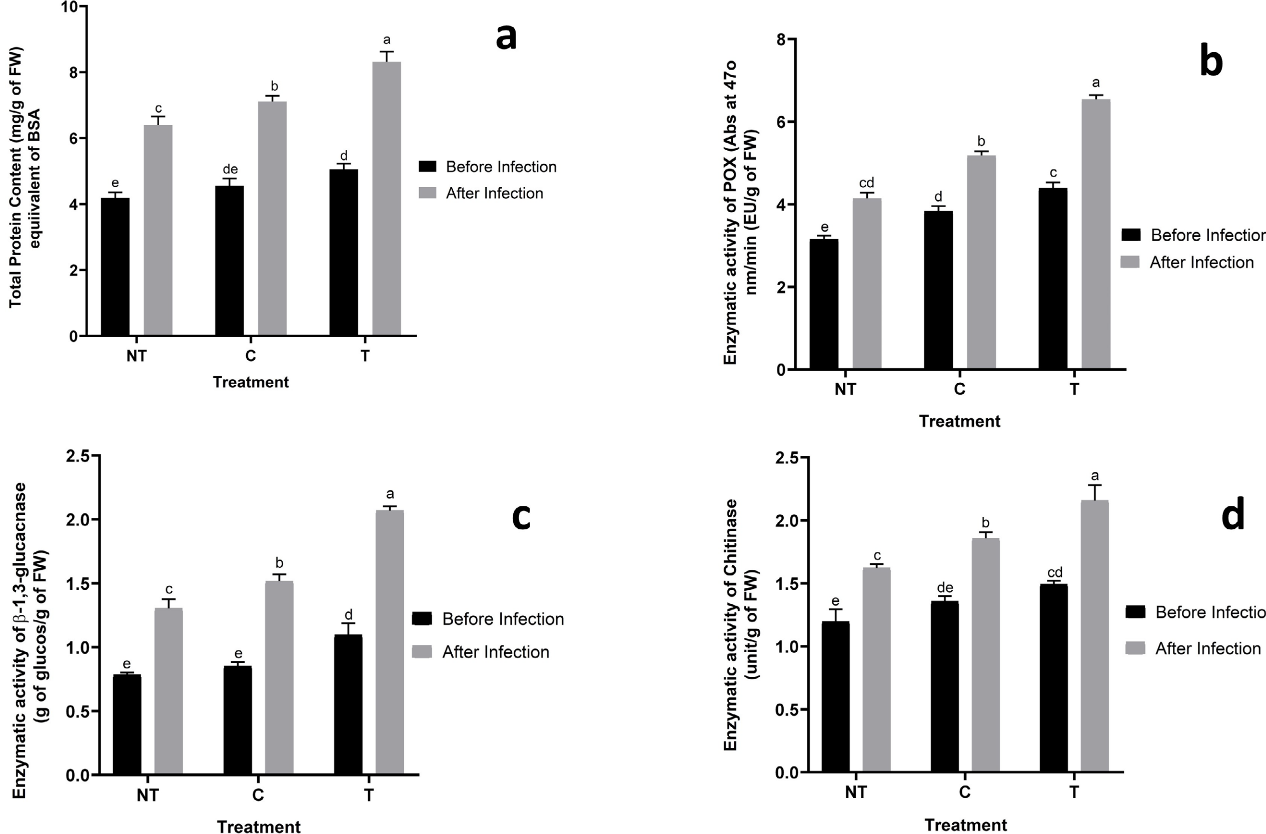

Figure 3. Total protein content (b) Total phenolic content and the enzymatic activities of (b) peroxidase, (c) β-1,3-glucanase and, (d) Chitinase in leaves of 30 days old cocoa seedlings challenged with *P. megakarya*. Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

3.4. Effect of SCaB on the Level of Gene Expression

Treatment of cocoa seedlings with SCaB significantly induced an increased expression of defense-related genes that code for peroxidase (TcPer-1), glucanase (TcGlu-1), chitinase (TcChiB), and the transcription factor involved in the regulation of phenolic compounds (TcMYBPA) in cocoa leaves both before and after infection with *P. megakarya* when compared to the chemically treated and untreated seedlings (Figure 4). Seedlings treated with SCaB showed a relative fold expression of 2.07, 2.33, 1.33, and 1.35, respectively, for TcPer-1, TcGlu-1, TcChiB and TcMYBPA genes, as opposed to a relative fold expression of 0.79, 1.04, 1.05, and 0.79 for untreated seedlings. In fact, when compared to untreated seedlings, treatment with SCaB increased the expression of the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes by 122%, 128%, 29%, and 70% respectively (Figure 4). Infections with the pathogenic agent *P. megakarya* triggered an upregulation in the expression of these genes across all treatments. As a result of *P. megakarya* infection, we see a 74%, 191%, 67%, and 143% increase in the gene fold expression for the TcPer-1, TcGlu-1, TcChiB, and TcMYBPA genes across all treatments.

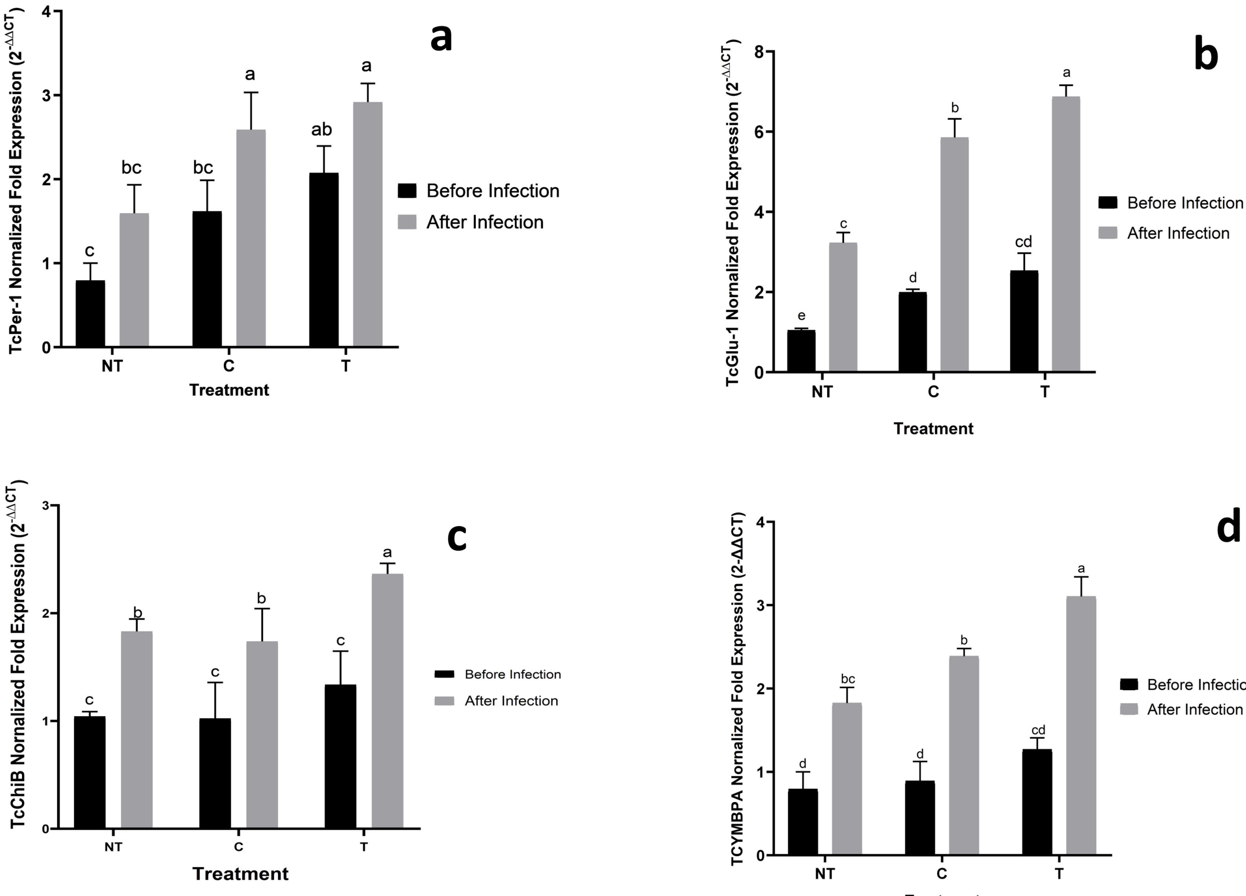

Figure 4. Quantitative reverse transcriptase-PCR analysis of the expression (a) Peroxidase (TcPer-1) (b) glucanase (TcGlu-1) (c), Chitinase (TcChiB), and (d) anthocyanidin transcription factor (TcMYBPA) defense-related genes of cocoa seedling leaves treated SCaB challenged with *P. megakarya.* Each value represents the mean ± SD (n = 3). Values with the same letter within a column are not significant at $P \leq 0.05$. Note: NT: Non-treated (negative control) C: Chemically treated (With CALLOMIL SUPER 66WP); T: Treated (with SCaB).

4. Discussion

Biological control of plant diseases and promotion of growth using beneficial microorganisms have been proven in numerous studies aimed at preserving plant health and enhancing crop yield. These beneficial microorganisms can be optimized for the development of novel biofertilizers that are better suited for agricultural systems, usage, transportation, storage, and durability [2] [6]. The mechanisms by which these novel bio formulations interact with the plant and the pathogenic agent have not been fully discussed. Our present study has shown that a bioformulation based on *Streptomyces cameroonensis* (SCaB) can improve growth, resistance and the expression of genes associated with defense in cocoa seedlings. Given that the SCaB is applied as seed treatment by enrobing cocoa grains when the treated seeds are sown, the bacteria spores will establish well into the seed surface and colonize the roots after germination [2]. The presence of the *S. cameroonensis* spores in the root system of the plant and soil is thus expected to account for the changes in the growth and disease-resistant

properties of the cocoa seedlings [7]. The SCaB treatment significantly increased the agronomic parameters by 18% and reduced the disease severity by 64% after a 30-day period when the cocoa seedlings were challenged with *P. megakarya*, thus raising the health status of the seedlings from tolerant to highly resistant. The improved agronomic characteristics and reduced disease severity at this early stage of growth can be attributed to the priming or pre-immunization of the seedlings with SCaB, following the activation of microbe-associated molecular patterns (MAMPSs) or Pathogen-associated molecular patterns (PAMPs) upon interaction of the plant with spores of *S. cameroonensis* and/or *P. megakarya*. This reduction in disease severity index is translated by an increased synthesis of primed defense biomolecules like phenolic compounds, flavonoids, oxidative enzymes, and PR-proteins that are highly implicated in the cocoa plant defense system against *P. megakarya.* A Previous study [2] has shown that the growth-promoting and disease-resistant effects of SCaB are directly linked to the presence of *S. cameroonensis* spores in the bioformulation.

Beneficial bacteria, such as *Streptomyces*, have been demonstrated to be reliant on the ethylene, salicylic acid, and jasmonic acid (JA) pathways in *Arabidopsis*. These pathways may be used to induce systemic resistance [29] [30]. which underlines this priming mechanism by SCaB in cocoa. This primed state is accompanied by important fitness benefits associated with an accumulation of defense-related molecules [8]. This priming mechanism occurs in two major phases, namely, the post-challenged primed state and the priming phase [5]. Firstly, the recognition of the microbe or pathogen by the host cells occurs when a pattern of elicitors from the microbe (MAMPs) is recognized by a pattern of plant receptors. This recognition stimulates the transduction of the signal into the plant cells, which consequently activates the transcription of defense genes [31]. In our study, the primed state is accompanied by changes in the agro-morphological, transcriptional, and metabolic states of the plant. An increase in the expression of target transcription factors and defense-related genes was observed. In this study, plants treated with SCaB showed higher defense-related gene expression for the TcChiB, TcGlu-1, and TcPer-1 genes, which code for chitinases, glucanases, and peroxidases, and the Tc-MYBPA gene, which encodes a transcription factor involved in the regulation of phenolic compounds involved in plant defense systems like proanthocyanidin synthesis in cocoa [32]. The TcPer-1 gene is highly expressed in young leaves of cocoa upon mechanical wounding and treatment with ethylene [12]. These results correspond with ours, where an up-regulation of the TcPer-1 gene in 30 days, detached cocoa leaves treated with SCaB and infected with a sporal solution of *P. megakarya* was observed. The high expression of the TcPer-1 gene in young leaves is related to the high expectations of expansion and lignification at this early stage of growth. The TcGlu-1 gene was transiently primed by treatment with SCaB. This gene shows a high degree of homology to genes encoding for β-1,4-glucanases in cocoa. Endo-1,4-glucanases are believed to be involved in cell wall loosening during reorganization or degradation processes [12]. The

β-1,3-glucanases of the glycosyl hydrolase family are made up of diverse groups with distinct patterns of expression that are modulated and induced by a variety of stimuli, such as inducers of plant defense [33]. The TcChiB gene was overexpressed in leaves treated with SCaB and infected with *P. megakarya*. The TcChiB gene is associated with several putative chitinases from *Gossypium hirsutum*, including a salicylic acid-activated class VII chitinase [34]. Our results are consistent with previous research that has demonstrated the ability of cocoa plants, in response to attack by the fungus *Phytophthora capsici,* to stimulate the expression of the TcChiB gene, leading to an increase in the synthesis of chitinases (PR3) that degrade the wall of this fungal pathogen and inhibit its spread [31]. Thus, the high expression of these genes because of induction by SCaB will likely destabilize the invading *P. megakarya*, providing both a protective and an inducing effect. Expression of the TcMYBPA gene was observed to be significantly upregulated in cocoa plants treated with SCaB and infected with *P. megakarya*. TcMYBPA encodes an R2R3-MYB transcription factor involved in regulating the biosynthesis of cocoa proanthocyanidin (PA), a derivative of flavonoids that contributes to plant defense mechanisms against biotic and abiotic stress [32] [35]. This transcription factor highlights the initial steps of the transduction pathway leading to cocoa resistance against *P. megakarya*. The development of cocoa and flavonoid (mainly anthocyanin) synthesis has been described previously [36]. The increase in the expression of TcMYBPA genes in cocoa following infection with *P. megakarya* and treatment with SCaB is consistent with previous research, which has demonstrated that in *Arabidopsis*, several genes encoding MYB transcription factors are upregulated after infection by *Pseudomonas syringae* and act as a positive regulator of the hypersensitive response and in response to bacterial pathogens [37]. The generation of reactive oxygen species and activation of the mitogen-activated protein (MAP) kinase pathways may also be some of the early responses elicited in the plant as a result of priming [29]. In the second stage of priming, pathogen signaling is relayed by phytohormones, which operate the mechanisms of systemic acquired resistance and systemic induced resistance [38]. The interaction between plants and beneficial microbes such as *Streptomyces Ssp*, *Trichoderma Ssp*, *Bacillus Ssp have* been linked to these pathways [2] [6] [17] [39]. This suggests that SCaB containing *S. cameroonensis* spores initiates these responses linked to these pathways. This primed response can also be because the cocoa plant may initially perceive *S. cameroonensis* as a potential invader, resulting in a rapid activation of the plant immune system [40]. SCaB can be seen to elicit such early and robust responses from the plant, leading to an up-regulation of these target genes.

5. Conclusion

The treatment of cocoa seedlings with SCaB led to an 18% increase in the growth parameters of our cocoa seedlings in the nursery and an average reduction of about 64% in the disease severity index when challenged with the pathogen *P. megakarya* at an early stage of growth. This reduction is characterized by in-

creased synthesis of total proteins, total polyphenols, and flavonoids, increased enzymatic activity of peroxidases, *β*-1-3-glucanases and chitinases, and an up-regulation of defense-related genes like TcPer-1, TcGlu-1, TcChiB, and the TcMYBPA gene. This knowledge gain provides a basis for understanding the priming mechanisms involved in the interaction between formulations derived from biocontrol agents, the cocoa plant, and the pathogenic agent *P. megakarya*. It also lays the framework for the optimization of beneficial microbes to induce an early alert state of defense responses in cocoa seedlings against potential pathogen invasion, reducing dependence on chemical pesticides along the way. In future studies, the changes observed in the expression of these defense-related genes and transcription factors may also serve as molecular markers for the response of cocoa to formulations of beneficial microorganisms and infection by *P. megakarya*.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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