THE UNIVERSITY OF YAOUNDE I
UNIVERSITE DE YAOUNDE I

FACULTY OF SCIENCE
FACULTE DES SCIENCES

CENTRE FOR RESEARCH AND DOCTORAL TRAINING IN GRADUATE STUDIES IN LIFE, HEALTH AND ENVIRONMENT

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

UNIT FOR RESEARCH AND DOCTORAL TRAINING IN GRADUATE STUDIES IN LIFE SCIENCE

UNITE DE RECHERCHE EN SCIENCES DE LA VIE

DEPARTMENT OF BIOCHEMISTRY
DEPARTEMENT DE BIOCHIMIE

EFFECT OF DROUGHT STRESS ON PHYSIO-METABOLOMICS ATTRIBUTES OF *Theobroma cacao* (L.)

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of « Doctorate/PhD »

Option: Biochemistry
Specialty : Biotechnology and Development

By **:**

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Msc. Science
Matricule: 05U228

Direction of

NIEMENAK Nicolas
Professor

2021

LIST OF PERMENENT TEACHING STAFF OF THE FACULTY OF SCIENCE, UNIVERSITY OF YAOUNDE I

UNIVERSITE DE YAOUNDE I **FACULTE DES SCIENCES** Division de la Programmation et du Suivi des Activités Académiques		**THE UNIVERSITY OF YAOUNDÉ I** **FACULTY OF SCIENCE** Division of Programming and follow-up of Academic Affaires
LISTE DES ENSEIGNANTS PERMANENTS		**LIST OF PERMENENT TEACHING STAFF**

ANNEE ACADEMIQUE 2020/2021

(Par Département et par Grade)

ADMINISTRATION

Doyen : TCHOUANKEU Jean-Claude, *Maitre de Conférences*

Vice-Doyen / DPSAA : DONGO Etienne, *Professeur*

Vice-Doyen / DSSE : AJEAGAH Gideon AGHAINDUM, *Professeur*

Vice-Doyen / DRC : ABOSSOLO Monique, *Maitre de Conférences*

Chef Division Affaires Académiques, Scolarité et Recherche : MBAZE MEVA'A Luc Léonard, *Professeur*

Chef Division Administrative et Financière : NDOYE FOE Marie C. F., *Maitre de Conférences.691906711*

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N°	NOMS ET PRENOMS	GRADE	OBSERVATIONS
1	BIGOGA DIAGA Jude	Professeur	En poste
2	FEKAM BOYOM Fabrice	Professeur	En poste
3	FOKOU Elie	Professeur	En poste
4	KANSCI Germain	Professeur	En poste
5	MBACHAM Wilfried	Professeur	En poste
6	MOUNDIPA FEWOU Paul	Professeur	**Chef de Département**
7	NINTCHOM PENLAP V. épse BENG	Professeur	*Coordonnateur CRFD-STG*
8	OBEN Julius ENYONG	Professeur	En poste
9	ACHU Merci BIH	Maître de Conférences	En poste
10	ATOGHO Barbara Mma	Maître de Conférences	En poste
11	AZANTSA KINGUE Gabin B.	Maître de Conférences	En poste
12	BELINGA née NDOYE FOE M.C.F.	Maître de Conférences	**Chef DAF / FS**
13	BOUDJEKO Thaddée	Maître de Conférences	En poste
14	DJUIDJE NGOUNOUE Marcelline	Maître de Conférences	En poste
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16	NANA Louise épse WAKAM	Maître de Conférences	En poste
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18	NGUEFACK Julienne	Maître de Conférences	En poste
19	NJAYOU Frédéric Nico	Maître de Conférences	En poste
20	MOFOR née TEUGWA Clautilde	Maître de Conférences	Insp. service MINESUP
21	TCHANA KOUATCHOUA Angèle	Maître de Conférences	En poste
22	EWANE Cécile Anne	Maître de Conférences	*En disponibilité*
23	KOTUE KAPTUE Charles	Chargé de Cours	En poste
24	AKINDEH MBUH NJI	Chargé de Cours	En poste
25	BEBOY EDJENGUELE Sara N.	Chargé de Cours	En poste
26	DAKOLE DABOY Charles	Chargé de Cours	En poste
27	DJUIKWO NKONGA Ruth V.	Chargé de Cours	En poste
28	DONGMO LEKAGNE Joseph B.	Chargé de Cours	En poste
29	FONKOUA Martin	Chargé de Cours	En poste
30	LUNGA Paul KEILAH	Chargé de Cours	En poste
31	MANANGA Marlyse Joséphine	Chargé de Cours	En poste
32	BEBEE FADIMATOU	Chargé de Cours	En poste

33	MBONG ANGIE M. Mary A.	Chargé de Cours	En poste
34	PECHANGOU NSANGOU S.	Chargé de Cours	En poste
35	Palmer MASUMBE NETONGO	Chargé de Cours	En poste
36	MBOUCHE FANMOE Marcelline J.	Assistante	En poste
37	OWONA AYISSI Vincent Brice	Assistant	En poste
38	WILFRIED ANGIE Abia	Assistante	En poste
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4	DJIETO Lordon Champlain	Professeur	En poste
5	ESSOMBA née NTSAMA MBALLA	Professeur	*VDRC/Chef dépt FMSB*
6	FOMENA Abraham	Professeur	En Poste
7	KAMTCHOUING Pierre	Professeur	En poste
8	NJAMEN Dieudonné	Professeur	En poste
9	NJIOKOU Flobert	Professeur	En Poste
10	NOLA Moïse	Professeur	En poste
11	TAN Paul VERNYUY	Professeur	En poste
12	TCHUEM TCHUENTE Louis	Professeur	*Coord. Progr. MINSANTE*
13	ZEBAZE TOGOUET Serge H.	Professeur	En poste
14	KEKEUNOU Sévilor	Professeur	En poste
15	DZEUFIET DJOMENI Paul D.	Professeur	En poste
16	BILANDA Danielle Claude	Maître de Conférences	En poste
17	DJIOGUE Séfirin	Maître de Conférences	En poste
18	JATSA MEGAPTCHE Hermine	Maître de Conférences	*En poste*
19	MEGNEKOU Rosette	Maître de Conférences	En poste
20	MONY Ruth épse NTONE	Maître de Conférences	En poste
21	NGUEGUIM TSOFACK Florence	Maître de Conférences	En poste
22	TOMBI Jeannette	Maître de Conférences	En poste
23	GOUNOUE KAMKUMO Raceline	Maître de Conférences	En poste
24	ALENE Désirée Chantal	Chargé de Cours	En poste
25	ATSAMO Albert Donatien	Chargé de Cours	En poste
26	BELLET EDIMO Oscar Roger	Chargé de Cours	En poste
27	DONGFACK Mireille	Chargé de Cours	En poste

28	ETEME ENAMA Serge	Chargé de Cours	En poste
29	KANDEDA KAVAYE Antoine	Chargé de Cours	En poste
30	LEKEUFACK FOLEFACK Guy B.	Chargé de Cours	En poste
31	MAHOB Raymond Joseph	Chargé de Cours	En poste
32	MBENOUN MASSE Paul S.	Chargé de Cours	En poste
33	MOUNGANG NGAMENI Luciane	Chargé de Cours	En poste
34	MVEYO NDANKEU Yves P.	Chargé de Cours	En poste
35	NGOUATEU KENFACK Omer B.	Chargé de Cours	En poste
36	NGUEMBOCK	Chargé de Cours	En poste
37	NJUA Clarisse YAFI	Chargé de Cours	Chef Div. UBA
38	NOAH EWOTI Olive Vivien	Chargé de Cours	En poste
39	TADU Zéphirin	Chargé de Cours	En poste
40	TAMSA ARFAO Antoine	Chargé de Cours	En poste
41	YEDE	Chargé de Cours	En poste
42	BASSOCK BAYIHA Etienne D.	Assistant	*CT2 MIN. ENERGIE*
43	ESSAMA MBIDA Désirée S.	Assistante	*C.E. MINRESI*
44	KOGA MANG DOBARA	Assistant	En poste
45	LEME BANOCK Lucie	Assistante	En poste
46	YOUNOUSSA LAME	Assistant	En poste
3-DEPARTEMENT DE BIOLOGIE ET PHYSIOLOGIE VEGETALES (B. P. V.) (31)			
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2	BELL Joseph Martin	Professeur	En poste
3	DJOCGOUE Pierre François	Professeur	En poste
4	MOSSEBO Dominique C.	Professeur	En poste
5	YOUMBI Emmanuel	Professeur	**Chef de Département**
6	ZAPFACK Louis	Professeur	En poste
7	MBOLO Marie.	Professeur	En poste
8	ANGONI Hyacinthe	Maitre de Conférences	En poste
9	BIYE Elvire Hortense	Maitre de Conférences	En poste
10	MALLA Armand William	Maitre de Conférences	En poste
11	MBARGA BINDZI Marie Alain	Maitre de Conférences	*CT/ MINESUP*
12	NGODO MELINGUI Jean Pierre	Maitre de Conférences	En poste
13	NDONGO BEKOLO	Maitre de Conférences	*CE/MINRESI*
14	NGONKEU MAGAPTCHE Eddy L.	Maitre de Conférences	En poste

15	TSOATA Esaïe	Maitre de Conférences	En poste
16	TONFACK Libert Brice	Maitre de Conférences	En poste
17	DJEUANI Astride Carole	Chargé de Cours	En poste
18	GOMANDJE Christelle	Chargé de Cours	En poste
19	MAFFO MAFFO Nicole L.	Chargé de Cours	En poste
20	MAHBOU SOMO TOUKAM Gabriel	Chargé de Cours	En poste
21	NGALLE Hermine BILLE	Chargé de Cours	En poste
22	NNANGA MEBENGA Ruth Laure	Chargé de Cours	En poste
23	NOUKEU KOUAKAM Armelle	Chargé de Cours	En poste
24	ONANA Jean Michel	Chargé de Cours	En poste
25	GODWILL NTSOMBAH N.	Assistant	En poste
26	KABELONG BANAHO Louis-P. R.	Assistant	En poste
27	KONO Léon Dieudonné	Assistant	*En poste*
28	LIBALAH Moses BAKONCK	Assistant	En poste
29	LIKENG-LI-NGUE Benoit C	Assistant	En poste
30	TAEDOUNG Evariste Hermann	Assistant	En poste
31	TEMEGNE NONO Carine	Assistant	En poste
4-DEPARTEMENT DE CHIMIE INORGANIQUE (C.I.) (35)			
1	AGWARA ONDOH Moïse	Professeur	*Vice Recteur Univ Bamenda*
2	ELIMBI Antoine	Professeur	En poste
3	Florence UFI CHINJE épse MELO	Professeur	Recteur Univ Ngaoundéré
4	GHOGOMU Paul MINGO	Professeur	*Ministre chargé de Miss. PR*
5	NANSEU Charles Péguy	Professeur	En poste
6	NDIFON Peter TEKE	Professeur	*CT MINRESI/Chef de Dépt*
8	NDIKONTAR Maurice KOR	Professeur	*Vice-Doyen/Ubda*
9	NENWA Justin	Professeur	En poste
10	NGAMENI Emmanuel	Professeur	*Doyen FS/ UDS*
12	DJOUFAC WOUMFO Emmanuel	Professeur	En poste
11	BABALE née DJAM DOUDOU	Maître de Conférences	*Chargée mission P.R.*
13	KAMGANG YOUBI Georges	Maître de Conférences	En poste
14	KEMMEGNE MBOUGUEM Jean C.	Maître de Conférences	En poste
15	KONG SAKEO	Maître de Conférences	En poste
16	NDI Julius NSAMI	Maître de Conférences	En poste
17	NJIOMOU Chantale épse DJANGANG	Maître de Conférences	En poste

18	NJOYA Dayirou	Maître de Conférences	En poste
19	YOUNANG Elie	Maître de Conférences	En poste
20	ACAYANKA Elie	Maître de Conférences	En poste
21	BELIBI BELIBI Placide Désiré	Chargé de Cours	En poste
22	CHEUMANI YONA Arnaud	Chargé de Cours	En poste
23	EMADACK Alphonse	Chargé de Cours	En poste
24	KENNE DEDZO Gustave	Chargé de Cours	En poste
25	KOUOTOU DAOUDA	Chargé de Cours	En poste
26	MAKON Thomas Beauregard	Chargé de Cours	En poste
27	MBEY Jean Aime	Chargé de Cours	En poste
28	NCHIMI NONO Katia	Chargé de Cours	En poste
29	NEBA née NDOSIRI Bridget NDOYE	Chargé de Cours	CT/MINFEM
30	NYAMEN Linda Dyorisse	Chargé de Cours	En poste
31	PABOUDAM GBAMBIE Awaou	Chargé de Cours	En poste
32	TCHAKOUTE KOUAMO Hervé	Chargé de Cours	En poste
33	NJANKWA NJABONG N. Eric	Assistant	En poste
34	PATOUOSSA ISSOFA	Assistant	En poste
35	SIEWE Jean Mermoz	Assistant	En poste
5-DEPARTEMENT DE CHIMIE ORGANIQUE (C.O.) (34)			
1	DONGO Etienne	Professeur	Vice-Doyen/ PSAA
2	GHOGOMU TIH ROBERT RALPH	Professeur	*Directeur I.B.A.F/UDA*
3	NGOUELA Silvère Augustin	Professeur	*Chef de dépt. Uds*
4	NKENGFACK Augustin Ephraïm	Professeur	Chef de Département
5	NYASSE Barthélemy	Professeur	*Vice- Recteur UBda*
6	PEGNYEMB Dieudonné Emmanuel	Professeur	*Directeur au MINESUP*
7	WANDJI Jean	Professeur	En poste
8	Alex de Théodore ATCHADE	Maître de Conférences	*CS Rectorat/ UYI*
9	EYONG Kenneth OBEN	Maître de Conférences	En poste
10	FOLEFOC Gabriel NGOSONG	Maître de Conférences	En poste
11	FOTSO WABO Ghislain	Maître de Conférences	En poste
12	KEUMEDJIO Félix	Maître de Conférences	En poste
13	KENMOGNE Marguerite	Maître de Conférences	En poste
14	KOUAM Jacques	Maître de Conférences	En poste
15	MKOUNGA Pierre	Maître de Conférences	En poste

16	NOTE LOUGBOT Olivier	Maître de Conférences	Chef Service MINESUP
17	NGO MBING Joséphine	Maître de Conférences	Sous Direct. MINERESI
18	NGONO BIKOBO Dominique	Maître de Conférences	En poste
29	NOUNGOUE TCHAMO Diderot	Maître de Conférences	En poste
20	TABOPDA KUATE Turibio	Maître de Conférences	En poste
21	TCHOUANKEU Jean-Claude	Maître de Conférences	*Doyen /FS/ UYI*
22	TIH née NGO BILONG E. A.	Maître de Conférences	En poste
23	YANKEP Emmanuel	Maître de Conférences	En poste
24	AMBASSA Pantaleon	Maître de Conférences	En poste
25	MVOT AKAK CARINE	Chargé de Cours	En poste
26	KAMTO Eutrophe Ledoux	Chargé de Cours	En poste
27	NGINTEDO Dominique	Chargé de Cours	En poste
28	NGOMO Orléans	Chargé de Cours	En poste
39	OUAHOUO WACHE Blandine M.	Chargé de Cours	En poste
30	SIELINOU TEDJON Valérie	Chargé de Cours	En poste
31	TAGATSING FOTSING Maurice	Chargé de Cours	En poste
32	ZONDEGOUMBA Ernestine	Chargé de Cours	En poste
33	MESSI Angélique Nicolas	assistant	En poste
34	TSEMEUGNE Joseph	Assistant	En poste
6-DEPARTEMENT D'INFORMATIQUE (IN) (26)			
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3	NDOUNDAM René	Maître de Conférences	En poste
4	AMINOU Halilou	Chargé de Cours	En poste
5	DJAM Xaviera Youth KIMBI	Chargé de Cours	En poste
6	EBELE Serge	Chargé de Cours	En poste
7	KOUOKAM KOUOKAM Etienne A.	Chargé de Cours	En poste
8	MELATAGIA YONTA Paulin	Chargé de Cours	En poste
9	MOTO MPONG Serge Alain	Chargé de Cours	En poste
10	TAPAMO KENFACK Hyppolite	Chargé de Cours	En poste
11	ABESSOLO ALO'O Gislain	Chargé de Cours	En poste
12	KAMGUEU Patrick Olivier	Chargé de Cours	En poste
13	MONTHE DJIADEU Valery M.	Chargé de Cours	En poste
14	OLLE OLLE Daniel Claude Delort	Chargé de Cours	CT Enset. Ebolowa

15	TINDO Gilbert	Chargé de Cours	En poste
16	TSOPZE Norbert	Chargé de Cours	En poste
17	WAKU KOUAMOU Jules	Chargé de Cours	En poste
18	DOMGA KOMGUEM Rodrigue	Assistant	En poste
19	BAYEM Jacques Narcisse	Assistant	En poste
20	EKODECK Stéphane Gael R.	Assistant	En poste
21	HAMZA Adamou	Assistant	En poste
22	JIOMEKONG AZANZI Fidel	Assistant	En poste
23	MAKEMBE S. Fritz Oswald	Assistant	En poste
24	MESSI NGUELLE Thomas	Assistant	En poste
25	MEYEMDOU Nadège Sylvianne	Assistante	En poste
26	NKONDOCK MI. BAHANACK. N.	Assistant	En poste
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3	NKUIMI JUGNIA Célestin	Maître de Conférences	En poste
4	NOUNDJEU Pierre	Maître de Conférences	En poste
5	MBEHOU Mohamed	Maître de Conférences	En poste
6	TCHAPNDA NJABO Sophonie Blaise	Maître de Conférences	Directeur/AIMS Rwanda
7	TCHOUNDJA Edgar Landry	Maître de Conférences	En poste
8	MBELE BEDIMA Martin	Maître de Conférences	En poste
9	TAKAM SOH Patrice	Maître de Conférences	En poste
10	AGHOUKENG JIOFACK Jean Gérard	Chargé de Cours	Chef Cellule MINPLAMAT
11	CHENDJOU Gilbert	Chargé de Cours	En poste
12	DJIADEU NGAHA Michel	Chargé de Cours	En poste
13	DOUANLA YONTA Hermann	Chargé de Cours	En poste
14	FOMEKONG Christophe	Chargé de Cours	En poste
15	KIANPI Maurice	Chargé de Cours	En poste
16	KIKI Maxime Armand	Chargé de Cours	En poste
17	MBAKOP Guy Merlin	Chargé de Cours	En poste
18	MBANG Joseph	Chargé de Cours	En poste
19	MENGUE MENGUE David Joe	Chargé de Cours	En poste
20	NGUEFACK Bertrand	Chargé de Cours	En poste
21	NIMPA PEFOUKEU Romain	Chargé de Cours	En poste

22	POLA DOUNDOU Emmanuel	Chargé de Cours	En poste
23	TCHANGANG Roger Duclos	Chargé de Cours	En poste
24	TETSADJIO TCHILEPECK M. E.	Chargé de Cours	En poste
25	TIAYA TSAGUE N. Anne- Marie	Chargé de Cours	En poste
26	MBIAKOP Hilaire George	Assistant	En poste
27	BITYE MVONDO Esther Claudine	Assistante	En poste
28	MBATAKOU Salomon Joseph	Assistant	En poste
29	MEFENZA NOUNTU Thiery	Assistant	En poste
30	TCHEUTIA Daniel Duviol	Assistant	En poste
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4	NWAGA Dieudonné M.	Maître de Conférences	En poste
5	RIWOM Sara Honorine	Maître de Conférences	En poste
6	SADO KAMDEM Sylvain Leroy	Maître de Conférences	En poste
7	ASSAM ASSAM Jean Paul	Chargé de Cours	En poste
8	BODA Maurice	Chargé de Cours	En poste
9	BOUGNOM Blaise Pascal	Chargé de Cours	En poste
10	ESSONO OBOUGOU Germain G.	Chargé de Cours	En poste
11	NJIKI BIKOÏ Jacky	Chargé de Cours	En poste
12	TCHIKOUA Roger	Chargé de Cours	En poste
13	ESSONO Damien Marie	Assistante	En poste
14	LAMYE Glory MOH	Assistant	En poste
15	MEYIN A EBONG Solange	Assistante	En poste
16	NKOUDOU ZE Nardis	Assistant	En poste
17	SAKE NGANE Carole Stéphanie	Assistante	En poste
18	TOBOLBAI Richard	Assistant	En poste
9-DEPARTEMENT DE PHYSIQUE (PH) (40)			
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2	ESSIMBI ZOBO Bernard	Professeur	En poste
3	KOFANE Timoléon Crépin	Professeur	En poste
4	NANA NBENDJO Blaise	Professeur	En poste

5	NDJAKA Jean Marie Bienvenu	Professeur	**Chef de Département**
6	NOUAYOU Robert	Professeur	En poste
7	NJANDJOCK NOUCK Philippe	Professeur	*Sous Directeur/. MINRESI*
8	PEMHA Elkana	Professeur	En poste
9	TABOD Charles TABOD	Professeur	Doyen Univ/Bda
10	TCHAWOUA Clément	Professeur	En poste
11	WOAFO Paul	Professeur	En poste
12	DJUIDJE KENMOE épse ALOYEM	Professeur	En poste
13	ZEKENG Serge Sylvain	Professeur	En poste
14	BIYA MOTTO Frédéric	Maître de Conférences	*DG/HYDRO Mekin*
15	BODO Bernard	Maître de Conférences	En poste
16	EKOBENA FOUDA Henri Paul	Maître de Conférences	*Chef Division UN*
17	EYEBE FOUDA Jean Sire	Maître de Conférences	En poste
18	FEWO Serge Ibraïd	Maître de Conférences	En poste
19	HONA Jacques	Maître de Conférences	En poste
20	MBANE BIOUELE	Maître de Conférences	En poste
21	NANA NBENDJO Blaise	Maître de Conférences	En poste
22	NDOP Joseph	Maître de Conférences	En poste
23	SAIDOU	Maître de Conférences	MINRESI
24	SIEWE SIEWE Martin	Maître de Conférences	En poste
25	SIMO Elie	Maître de Conférences	En poste
26	VONDOU DERBETINI Appolinaire	Maître de Conférences	En Poste
27	WAKATA née BEYA Annie	Maître de Conférences	*Sous Directeur/ MINESUP*
28	ENYEGUE A NYAM épse BELINGA	Maître de Conférences	En Poste
29	MBINACK Clément	Maître de Conférences	En Poste
30	EDONGUE HERVAIS	Chargé de Cours	En poste
31	FOUEDJIO David	Chargé de Cours	Chef Cell. MINADER
32	MBONO SAMBA Yves Christian U.	Chargé de Cours	En poste
33	MELI'I Joelle Larissa	Chargé de Cours	En poste
34	ABDOURAHIMI	Chargé de Cours	En poste
35	MVOGO ALAIN	Chargé de Cours	En poste
36	WOULACHE Rosalie Laure	Chargé de Cours	En poste
37	OBOUNOU Marcel	Chargé de Cours	*Dir.acad/Univ.Int.Etat Sang*
38	CHAMANI Roméo	Chargé de Cours	En poste
39	AYISSI EYEBE Guy Francois V.	Assistant	En poste

40	TEYOU NGOUPOU Ariel	Assistant	En poste
10- DEPARTEMENT DE SCIENCES DE LA TERRE (S.T.) (43)			
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2	FOUATEU Rose épouse YONGUE	Professeur	En poste
3	KAMGANG Pierre	Professeur	En poste
4	NDJIGUI Paul-Désiré	Professeur	*Chef de Département*
5	NDAM NGOUPAYOU Jules-Remy	Professeur	En poste
6	NGOS III Simon	Professeur	*D.A.A.C./Uma*
7	NKOUMBOU Charles	Professeur	En poste
8	NZENTI Jean-Paul	Professeur	En poste
9	ABOSSOLO née ANGUE Monique	Maître de Conférences	**Vice-Doyen/DRC**
10	GHOGOMU Richard TANWI	Maître de Conférences	*Chef de Dépt IMIP/Maroua*
11	MOUNDI Amidou	Maître de Conférences	*CT/MINMIDT*
12	NGUEUTCHOUA Gabriel	Maître de Conférences	CEA MINRESI
13	NJILAH Isaac KONFOR	Maître de Conférences	En poste
14	ONANA Vincent	Maître de Conférences	En poste
15	BISSO Dieudonné	Maître de Conférences	*Dir. Projet Barage Memve'ele*
16	EKOMANE Emile	Maître de Conférences	En poste
17	GANNO Sylvestre	Maître de Conférences	En poste
18	NYECK Bruno	Maître de Conférences	En poste
19	TCHOUANKOUE Jean-Pierre	Maître de Conférences	En poste
20	TEMDJIM Robert	Maître de Conférences	En poste
21	YENE ATANGANA Joseph Q.	Maître de Conférences	En poste
22	ZO'O ZAME Philémon	Maître de Conférences	En poste
38	TCHAKOUNTE J. épse NOUMBEM	Maître de Conférences	*Chef. Cell/ MINRESI*
23	ANABA ONANA Achille Basile	Chargé de cours	*En Poste*
24	BEKOA Etienne	Chargé de cours	En poste
25	ELISE SABABA	Chargé de cours	En poste
26	ESSONO Jean	Chargé de cours	*Sous Directeur MINMIDT*
27	EYONG JOHN TAKEM	Chargé de cours	En poste
28	FUH Calistus Gentry	Chargé de cours	En poste
29	LAMILEN BILLA Daniel	Chargé de cours	En poste
30	MBESSE CECILE OLIVE	Chargé de cours	En poste
31	MBIDA YEM	Chargé de cours	En poste

32	METANG Victor	Chargé de cours	En poste
33	MINYEM Dieudonné	Chargé de cours	En poste
34	NGO BELNOUN Rose Noël	Chargé de cours	En poste
35	NGO BIDJECK Louise Marie	Chargé de cours	En poste
36	NOMO NEGUE Emmanuel	Chargé de cours	En poste
37	NTSAMA ATANGANA Jacqueline	Chargé de cours	*En Poste*
39	TCHAPTCHET TCHATO De P.	Chargé de cours	En poste
40	TEHNA Nathanaël	Chargé de cours	CS/ *MINMIDT*
41	TEMGA Jean Pierre	Chargé de cours	En poste
42	FEUMBA Roger	Assistant	En poste
43	MBANGA NYOBE Jules	Assistant	En poste

Répartition chiffrée des enseignants de la Faculté des Sciences de l'Université de Yaoundé I

Nombre d'enseignants					
Département	**Professeur**	**Maître de Conférences**	**Chargé de Cours**	**Assistant**	**Total**
BC	8 (1)	13 (09)	12 (05)	3 (2)	**38 (16)**
BPA	15 (0)	07 (06)	17 (04)	05 (2)	**46 (13)**
BPV	07 (1)	9 (01)	08 (06)	07 (01)	**31 (9)**
C.I.	12 (2)	09 (01)	11 (04)	03 (0)	**35 (5)**
C.O.	7 (0)	16 (03)	7 (04)	03 (0)	**35 (7)**
IN	2 (0)	1 (0)	14 (01)	9 (01)	**26 (3)**
MA	2 (0)	7 (0)	17 (01)	5 (01)	**30 (2)**
MB	2 (1)	4 (01)	06 (01)	6 (03)	**17 (5)**
PH	14 (2)	16 (02)	9 (02)	02 (0)	**40 (4)**
ST	9 (0)	15 (01)	19 (04)	02 (0)	**43 (6)**
Total	**78 (7)**	**99 (24)**	**119 (32)**	**45 (10)**	**341 (73)**

Soit un total de : **341 (73)**dont :
- Professeurs **78 (7)**
- Maîtres de Conférences **99 (24)**
- Chargés de Cours **119 (32)**
- Assistants **45 (10)**
- ( ) = Nombre de femmes.

Le Doyen de la Faculté des Sciences

Pr TCHOUANKEU Jean-Claude

DEDICATION

To my parents, M. Pouengue Augustin and the late Njuissi Kezembou Augustine, who showed me how to love. You are the best teachers I ever have.

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II. LIST OF ABREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acide
BAP	6-Benzylaminopurine
CSSV	Cacao Swollen Shoot Virus
DKW	Driver and Kuniyuki Walnut
ED	Embryo Development medium
FI	Primary plagiotropic fan branch
FII	Secondary plagiotropic fan branch
IRAD	Institute of Agricultural Research and Development
ICCO	International Cocoa Organization
Kin	kinetin (6-Furfurylaminopurine)
MA	Maraňao
NMR	Nuclear magnetic resonance
NS	Non-stressed sample
OS	Orthotropic stem
PCG	Primary Callus Growth medium
PEG	Polyethylene Glycol 8000
PCA	Principal component analysis
PLS-DA	Partial least square – discriminant analysis
POX	Peroxydase
RAPD	Random Amplification of Polymorphic DNA
RD	Root Development medium
RFLP	Restriction Fragment Length Polymorphism
SCA	Scavina
SCG	Secondary Callus Growth medium
SE	Somatic embryos
SSE	Secondary somatic embryos
T. cacao	*Theobroma cacao* L.
TDZ	Thidiazuron [1-Phenyl-3-(1, 2, 3-thiadiazol-5-yl) urea]
v/v	Volume per volume
w/v	Weight per volume
WS	Water-stressed sample

III. LIST OF TABLES

IV. ABSTRACT

Theobroma cacao L. is a strategic crop to fight against poverty in several African countries. Its dry beans are commonly used in chocolate industries. Nowaday, the search for elite clones capable of withstanding the effects of climate change is a challenge for the future of cacao farming. The elite clone in this context will be a genotype which is capable to produce specific metabolites favoring adaptation in a new climatic condition. Therefore, using cacao calluses and seedlings, we report here a global analysis of metabolome dynamics aiming to unravel mechanisms regulating response of plant to drought stress. Callus and embryogenesis were induced using staminodes and petals from immature flower buds. The impact of the position of a flower bud in a single tree was evaluated. Results show that the position of the floral explant influences its ability to produce embryos. The embryogenesis frequencies were ca 2-fold higher in staminodes-derived callus from secondary fan branch (FII) and primary fan branch (FI) than orthotropic main stem (OS). During embryos induction stage, phenolics were more accumulated in explants from OS, while sugars and proteins were more accumulated in explants from FI and FII, respectively. When embryos are induced, explant from FII displayed the lowest peroxydase activities. This set of information suggests that flower buds from fan branches are suitable for cacao somatic embryogenesis. The impact of water stress was first studied during the embryos establishment stage. For this, 28-days-old callus of SCA12, SCA6 and MA12 genotypes were placed on DKW media supplemented with 2.5; 5 and 7.5% (w/v) of polyethylene glycol-8000 (PEG). The impact of water stress was also studied during maturation stage, through culturing of 6-months-old secondary embryos of SCA6 genotype in DKW media supplemented with 5; 7.5 and 10% (w/v) of PEG, mannitol or sorbitol. Results show that water stress influences the capacity of callus to produce embryos. SCA12 genotype exhibited the best conversion capacities under severe conditions (7.5% (w/v) of PEG) and was considered as drought-tolerant, followed by SCA6 (less sensitive) and MA12 (sensitive). The biochemical analysis carried out on these explants show that the contents of phenolics and total flavonoids were reduced under water stress conditions in SCA12 and SCA6. The maximum reduction rate was obtained with SCA12 genotype in the media supplemented with 5 and 7.5% (w/v) of PEG, for phenolics and total flavonoids contents respectively, as compared to the control. 7.5% (w/v) of PEG during maturation stage was found to be the best supplement tested for secondary embryos development with SCA6 genotype which promotes roots and shoot differentiation, as compared to mannitol and sorbitol, regardless of the concentration used.

Metabolomics changes associated with callus differentiation and seedling establishment under water stress induced with PEG was analyzed using ^{1}H-NMR. Multivariate analysis (PCA and PLS-DA) allowed the identification of discriminant metabolites. Metabolites involved in water stress tolerance in callus include trans-aconitic acid, aspartic acid, fructose and sucrose (more accumulated) and stachyose (with reduced content). In cotyledons from seeds germinated under water stress conditions, proline, lactic acid and 1-O-ethyl-β-D-glucoside were more accumulated whereas the content of β-glucose was higly reduced. This study emphasizes the utility of these datasets as an essential resources for the characterization of the drought-tolerant behaviour of *T. cacao*.

Key words: Cacao, somatic embryogenesis, seeds, drought, water deficit, germination, metabolites.

V. RÉSUMÉ

La culture cacaoyer (*Theobroma cacao* L.) est un moyen stratégique pour la lutte contre la pauvreté dans plusieurs pays d'Afrique. Il est cultivé surtout pour ses fèves qui ont une grande valeur marchande car, elles constituent la principale matière première du chocolat. De nos jours, la recherche des clones élites capables de résister aux effets des changements climatiques est un véritable challenge pour la culture du cacaoyer. Le clone élite dans ce contexte sera donc ce génotype capable de produire des métabolites spécifiques favorisant l'adaptation à une nouvelle condition climatique. Ainsi, utilisant les cals et les plantules régénérées à partir des graines, cette étude présente une analyse globale de l'évolution du métabolome dans le but de permettre une meilleure compréhension des mécanismes régulant la réponse des plantes au stress hydrique. Les explants utilisés pour la production des cals et des embryons sont les staminodes et les pétales des boutons floraux immatures. L'influence de la position des boutons floraux sur le même arbre a été évaluée. Les résultats montrent que la position de l'explant floral influence ses capacités à produire les embryons. Les fréquences d'embryogénèse somatique étaient 2 fois plus élevées dans les explants issus des cals provenant des staminodes des branches plagiotropes secondaires (BII) et primaires (BI) comparés à ceux des tiges (Ti). Au cours de la phase d'établissement des embryons, les polyphénols sont plus accumulés dans les explants provenant de Ti tandis que les sucres et les protéines sont plus accumulés dans les explants provenant de BI et BII, respectivement. Lorsque les embryons sont établis, les explants provenant de BII présentent les plus faibles activités péroxydasique. Cet ensemble de données suggère que les boutons floraux situés sur les branches plagiotropes sont plus aptes à l'embryogénèse somatique chez le cacaoyer. L'influence du stress hydrique a par la suite été étudiée premièrement au cours de la phase d'établissement des embryons. Pour cela, les cals des génotypes SCA12, SCA6 et MA12, âgés de 28 jours ont été placés sur milieu DKW contenant du polyéthylène glycol-8000 (PEG) à des concentrations de 2,5 ; 5 et 7,5 % (p/v). L'influence du stress hydrique a ensuite été étudié au cours de la phase de maturation, à travers la culture des embryons du génotype SCA6, âgés de 6 mois sur milieu DKW contenant du PEG, du mannitol ou du sorbitol à des concentrations de 5 ; 7,5 et 10% (p/v). Les résultats montrent que le stress hydrique influence les capacités des cals à produire les embryons. Le génotype SCA12 a présenté les meilleures capacités de conversion en condition de stress sévère (PEG à 7,5% (p/v)) et est de ce fait considéré comme tolérant au stress hydrique, suivi de SCA6 (moyennement tolérant) et MA12 (sensible). Les analyses biochimiques faites sur ces explants montrent que les teneurs en polyphénols et en flavonoïdes totaux sont réduites chez les

génotypes SCA12 et SCA6 au cours du stress hydrique. Le taux de réduction maximal est observé chez le génotype SCA12 en présence de PEG à 5 et 7,5 % (p/v), pour les polyphénols et les flavonoïdes totaux respectivement, comparé au contrôle. Le PEG à 7,5% (p/v) au cours de la maturation s'est révélé comme le meilleur traitement pour le développement des embryons secondaire du génotype SCA6 favorisant la différentiation des racines et des tiges, comparé au mannitol et au sorbitol, quel que soit la concentration utilisée.

Les changements métaboliques associés à la différentiation des cals et à l'établissement des plantules provenant des graines en condition de stress hydrique induit par le PEG ont été analysés en utilisant la RMN-^{1}H. Les analyses multi-variées (ACP et PLS-DA) ont permis la mise en évidence des métabolites discriminants. Les indicateurs métaboliques de la tolérance au stress hydrique dans les cals regroupent l'acide trans-aconitique, l'acide aspartique, le fructose et le saccharose (qui sont fortement accumulés) et le stachyose (dont la teneur diminue). Dans les cotylédons issus des graines germées sous stress hydrique, la proline, l'acide lactique et le 1-O-ethyl-β-D-glucoside sont fortement accumulés tandis que le β-D-glucose présente une forte réduction de sa teneur. Cette étude souligne l'importance de ces données comme une ressource essentielle pour la caractérisation du comportement tolérant de *T. cacao* vis-à-vis du stress hydrique.

Mots clés : Cacao, embryogénèse somatique, graines, sècheresse, stress hydrique, germination, métabolites.

INTRODUCTION

Cacao (*Theobroma cacao* L.) or the chocolate tree belongs to the *Malvaceae* family (Alverson et *al*., 1999). According to Cheesman (1944), the cacao species can be divided into three main groups : the Criollo originally domesticated by Maya people in Central America, 3000 years ago, the « non-criollo » or amazonian Forastero, and the Trinitario complex, resulting from the hybridization between Criollo and amazonian Forastero. Under natural conditions the tree can reach 20 to 25 m in height (Lachenaud et *al*., 1997), whereas under cultivation it varies from 3 to 5 m. Cacao is commercially exploited for its dry beans (named "cocoa") mainly destined for chocolate, food and beverage industries.

During the crop year 2018/19, the world cocoa production was estimated to 4780 thousand tons, African countries are responsible of more than 75 per cent of this production and almost 90 per cent of the total production was exported in consumers' countries (Anonymous 1, 2020). The global cocoa market is expected to grow at a compound annual growth rate (CAGR) of 7.3 per cent from 2019 to 2025 to reach USD 16.32 billion (Anonymous 2, 2019). According to euromonitor, increased demand for chocolate with perceived health benefits and more exotic flavours is expected in Western Europe and North America, which are the traditionnal consuming markets (Yu, 2017) and also in emerging economies such as China and India (Anonymous 3, 2015; Anonymous 4, 2018). Cameroun, with a production of 280 thousand tons during the crop year 2018/19, is the fourth world producer and exporter of cocoa after Ivory Coast, Ghana and Ecuador (Anonymous 1, 2021).

Cacao is a shrub grown preferentially under the shade of a selectively thinned forest (Lobao et *al*., 2007). However, shading primary forest, traditionally used, is becoming less available due to deforestation caused by population pressure, industrial exploitation, and climate change (Bidzanga et *al*., 2005). Climatic upheavals lead to irregular rainfall and a drop in hydrometry. Under these conditions, a significant reduction in crop production potential and developmental abnormalities is observed (Moser et *al*., 2010), and in extreme case lead to the death of the tree. Despite its susceptibility to drought, field observations indicated that some cacao genotypes exhibited a satisfactory level of tolerance to drought with sufficient productivity (Balasimha et *al*. 1999; Apshara et *al*., 2016). Morpho-physiological responses of young cacao hybrids to different water stress showed significant differences under controlled conditions (Alban et *al*., 2016). However, in perennial plants, field studies are difficult, requiring six to eight consecutive years of observation to integrate production programs (Paulin and Eskes, 1995).

The approach of cultural practices was aimed to stabilize environmental condition which is less favorable for the deleterous effects of climate changes. Resistant planting materials should be more effective and durable in addressing the tolerance to drought stress than other approaches but it need a long time process and need more fund for replanting existing farm. Furthermore, strategy on controlling drought effects should be directed in short term or long term approaches depending on the level of drought severity; however, studies reporting the physiological effects of water stress and the molecular markers involved in cacao are scarces. Indeed, cacao is a plant very sensitive to drought (Cuatrecasas et *al*., 1964).

Cacao somatic embryogenesis allows rapid vegetative and large-scale multiplication of homogenous elite genotypes, genetic improvement, collection and conservation of cacao germoplasm, efficient diffusion of plant material as plantlets to farmers, etc. However, the limiting factor in using somatic embryogenesis to vulgarize an elite cacao genotype is its somatic embryogenic response/recalcitrance (Minyaka et *al*., 2017). The composition of the culture medium can be altered in order to allow a better somatic embryogenic response in cacao genotypes (Tan and Furtek, 2003; Niemenak et *al*., 2008; Minyaka et *al*., 2010). The genetic changes called somaclonal variation (Evans and Sharp, 1998) during the callogenesis phase of plant cells cultured *in vitro* are now considered a new source of changes intended to enrich the genetic resource for the improvement of plant species (Larkin, 1981). Therefore, somatic embryogenesis represents an advantageous experimental method for assessing plant responses to climate changes.

Morphological and physiological responses are linked to the biochemical mechanisms which take place into the tissues. It is therefore necessary to identify key metabolites involved in tolerant genotypes during adaptation to local agro-climatic conditions which varied recently under climate changes pressure. In fact, studies carried by Le Gall et *al*. (2017) through metabolomics showed a high accumulation of proline, sucrose and maltose in *Miscanthus sinensis* in response to abiotic stress such as cold during acclimation. In cacao, works done by Noah (2016) on the germination and seedling establishment through proteomics permitted the identification of approximately 1698 proteins. A large number of antioxidant proteins were revealed including: glutaredoxin, thioredoxin, glutathione reductase, glutathione S-transferase and peroxyredoxine. In addition, the work of Niemenak et *al*. (2015) on the maturation of somatic and zygotic embryos subjected to osmotic stress revealed 72 protein spots with about twenty unidentified. Spots proteins involved in the stress response and in photosynthesis were also identified. These authors revealed the abundance of enzymes such as ascorbate peroxidase

and glutathione S-transferase. These are proteins of antioxidant systems with protective effect against drought (Reddy et *al*., 2004).

Nowadays, callus from which embryos are produced is considered as an essential stage of *in vitro* development of cacao plant. According to Niemenak et *al*. (2010), callus formation corresponds to roots emergence in natural germination. Hence, understanding molecular events involved in drought tolerance in calluses is of prime interest to ameliorate cacao production and development in breeding programme. Metabolomics constitute one of the most promising technique for studying plants undergoing abiotic stress such as drought. This technique, which is a non-targeted analytical method, is able to provide a wide range of information about the metabolite content in different organs and thus on the overall plant metabolic status in a relatively short period of time on a large number of samples (Verpoorte et *al*., 2007). In this thesis, through the ^{1}H-NMR metabolomics, we highlight the differential metabolites produced in cacao genotypes during callus and seedlings cultures in response to water stress. In conjunction with metabolomic analysis, physiological initiative was used to determine the levels of tolerance to water stress of some genotypes. The research hypotheses are:

- the microclimate (microenvironment) could influence the regenerative capacities of cacao tissues *in vitro*;
- the physiological mechanisms involved in the adaptation of cacao to water stress due to climate changes in production areas could vary depending on the genotypes and the intensity of the water stress;
- key metabolites could be involved in drought tolerance in some cacao genotypes.

The general objective of this thesis is to evaluate the physiological and metabolomical responses of cacao subjected to water stress through somatic embryogenesis and seedlings establishment**.**

The specific objectives are:

- to determine the influence of the position of flower buds in a single tree on somatic embryogenesis of *T. cacao*. Flower buds from superior stages on the branches are more exposed to ligth (and consequently to water stress) than those from the main orthotropic axis, more shaded by forest species;
- to evaluate the physiological responses of *T. cacao* to water stress due to osmotic agents;
- to characterize metabolites involved in drought tolerance in *T. cacao*.

CHAPTER I: LITTERATURE REVIEW

I.1 Taxonomy and Morphology of *Theobroma cacao*

The genus *Theobroma* has evolved in the Amazon basin and was naturally dispersed through the tropical rainforest up to Southern Mexico. Earlier publications still describe *Theobroma* as belonging to the family *Sterculiaceae* (Angiosperm phylogeny Group, 2003). This genus contains 22 species and the most representative are *Theobroma cacao* (L.), *Theobroma grandiflorum* (Willd. ex Spreng.).

T. cacao is a perennial, allogamous and diploid (2n=20) crop. Cacao is characterized by its dimorphic architecture, with both orthotropic (upright) and plagiotropic (lateral) branching (Fig. 1). Germinating cacao seeds (often referred to as 'beans') first produce a root. Then the hypocotyl elongates to raise the cotyledons above ground level. Once the cotyledons have opened out, the plumule usually produces four leaves with very short internodes. The inflorescence is a highly compressed dichasial cyme, emerging from a leaf axil (cushion). The flowering intensity and the growth rhythms of the inflorescences are affected by environmental factors such as rainfall/water availability, light intensity, existing fruit load, and genetic factors. Flowering in cacao is manifested by the production of a minimum of 50 000 flowers during the term with less than 5% of production pods (Lass, 1999).

The five fertile stamens have two anthers which are allocated in the concave part of the petals (Fig. 2A). The self-incompatibility is observed in some genotypes of cacao and is responsible for the increasing of allogamy in that species. After fertilization, the superior ovary grows to form the young cacao fruit, called cherelle (Fig. 2B). The synchronized mass manual pollination produces cherelles of uniform age with little competition between them, which circumvents the normal crop-limiting mechanism, cherelle wilt (Fig. 2C). The normally developing pods reach maturity (Fig. 2D) after approximately 130 days and can form fruits of 20 cm to 30 cm in length. The number of seeds in a pod is variable, but up to 40 seeds can be found in one pod. In the pod, white sweet mucilage is covering the seeds which are fixed on the placenta (Daymond and Hadley, 2008).

The cacao seeds are characterized by the absence of maturation drying; most of them have a high water content and active metabolism when they are shed from the mother plant. They are sensitive to drying and low temperatures, and quickly lose viability during storage (Vertucci and Farrant, 1995).

Figure 1. The architecture of the cacao tree (picture was taken in the germplasm collection at the seeds garden of Mengang, Center region of Cameroon).

Figure 2. Cacao cauliflory and fructification. A- Inflorescences (cushion) with opened flower (i) and immature flower buds; B- Trunk with a non-aborted cacao cherelle (white narrow); C- Aborted cherelle ("cherelle wilt") and D- Mature pod (14 weeks) (Boutchouang, 2019).

I.2 Seeds germination

The germination is a key process in plant metabolism, responsible for embryo growth and development into a complete plant (Bewley et *al*., 2013). The germination comprises four phases:

(i) Water imbibition;
(ii) Cell stretching;
(iii) Cell division and;
(iv) Cell differentiation into tissues (Popinigis and Popinigis, 1985). The knowledge about seed biology and the germination process of each species is fundamental to understanding the establishment of a plant community (Izquierdo et *al*., 2017).

I.2.1 Factors governing seed germination in plants

The emergence of the radicle from the seed coat marks the end of germination, which is immediately followed by seedling establishment. The abscisic acid (ABA) is reported to inhibit

germination through dormancy maintenance while promoting embryo development and, gibberellin acid (GA) is known to promote germination (Chibani et *al*., 2006; Ogawa et *al*., 2003; Lee et *al*., 2002). The downstream of this hormonal regulation, biochemical machinery ensures timely and ordered mobilization of this stored reserves to power germination until photosynthetic apparatus is fully functional and the seedling has achieved autotrophic competence (Bewley et *al*., 1997). The germination efficiency is affected by reserve accumulation during seed development or their mobilization during seed germination as well as several unknown factors (Rosental et *al*., 2014). The protein mobilization releases reduced nitrogen needed for growth and development (Calli, 1995). The sugar compounds such as starch and sucrose act as energy suppliers with lipid reserves (Elamrani et *al*., 1992).

I.2.2 Seeds germination and vegetative propagation of *T. cacao*

The early stage of development from seed germination to initiation of the leading shoot, including growth stages ranging from BBCH00 to BBCH09, during description of cacao tree development based on the BBCH (BASF, Bayer, Ciba-Geigy, Hoeschst) (Niemenak et *al*., 2010). Imbibition is completed within 3 days and radicle protrusion appears around day 5 (BBCH 02). Radicle and hypocotyl elongation proceeds, hypocotyl is visible (BBCH 03) and the formation of root hairs follows (BBCH 05). Between days 7 and 18, depending on the conditions, seedlings have emerged from the soil (emergence) and the hypocotyl forms a hook (3-5 cm long) lifting the cotyledons from the ground (BBCH 07, Fig. 1b and Fig. 1c); the cotyledons are upright but still closed (BBCH 09) (Hunter, 1959; Rohsius, 2000). The period from onset of imbibition to seedling emergence takes approximately 25 days. The development of the first leaves (eophylls) from seeds begins after the cotyledons start to unfold (BBCH 10, Fig. 1d). The subsequent leaves occur as a succession of flushes. Each flush emerging from the main shoot or from a fan branch is counted up to the 9th flush and assigned BBCH codes 11 to 19, respectively and is referenced as **principal growth stage 1**. The seedling bear leaves along the main stem and up to a certain height the growth on the terminal bud of the up-right shoot (orthotropic growth) cease, replaced by the development of lateral branches (plagiotropic growth). When leaves expand and display 20% or 50% of their final size, BBCH stage 112 (Fig 1e) or 115 is reached. The mature, flush–bearing, already hardened leaves with dark green colour and constant size are classified as BBCH stage 119 (Fig 1f) (Fig. 3). The fruit production starts when trees are 3 years old. The highest yield is reached at the age of 20-40 years old.

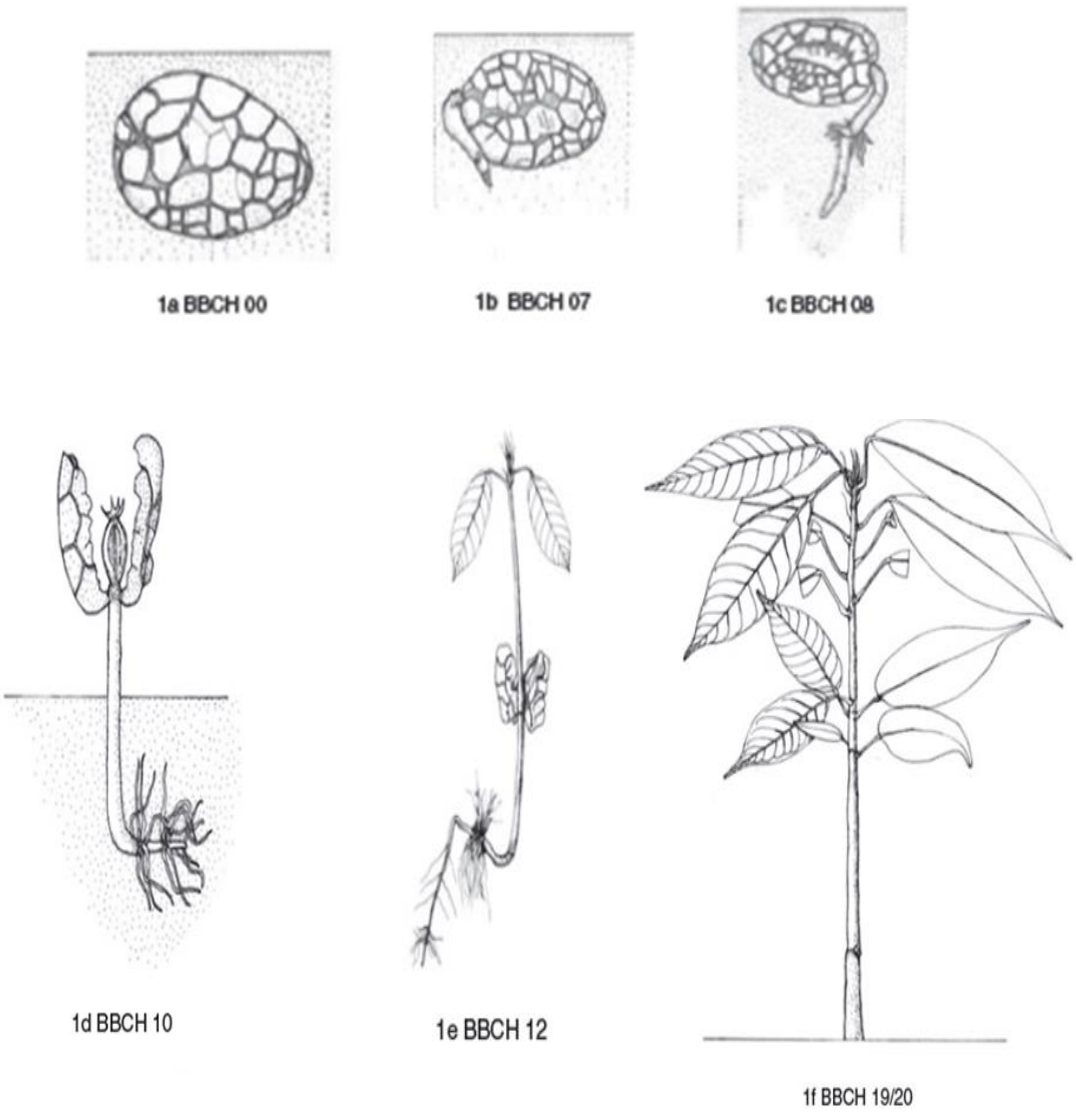


Figure 3. Principal growth stage of cacao plant 0 and 1. (Niemenak et *al*., 2010)

I.3 Ecology and Geographical distribution

I.3.1 Ecology

This species needs for its good development an average annual temperature of 25°C, a rainfall between 1500 and 2500 mm^3 of water/year with uniform repartition and a humid atmosphere. It is recommended to establish plantations by systems that offer shade conditions. The soils with good drainage are necessary to avoid roots asphyxiation, as well as water retention, and their level of acidity should be comprised between 6 and 7.

I.3.2 Geographical distribution

It is believed that the cacao center of origin is South America, more specific the Amazon basin in the border region between Peru, Ecuador, Colombia and Brazil. The high diversity of cacao found in this region supports this assumption (Thomas et *al*., 2012). The indigenous people used the seeds and the pulp extensively, mainly as alcoholic-drinks and currency. The first domestication of cacao is supposed to have been carried out by Olmec populations who were known as active traders. The distribution of large populations of cacao to African and Asian producer countries was strongly facilitated by human intervention. The objective was to increase the genetic variety in order to overcome the various diseases occurring in the respective countries.

Today, cacao growth and production is an important economic factor in all countries with suitable conditions for cacao cultivation.

I.4 Genetic variability, cultivation and propagation of cacao

I.4.1 Genetic variability

Classification of cacao tree cultivated is mitigated since it was only based on morphological characteristics of pods, flowers or seeds, which all present a very high diversity. Fig. 4 presents different aspects of cacao pods, with their corresponding name just above. Nowadays, based on morphological aspect of cacao pods and geographical distribution of the trees, *Theobroma cacao* were subdivided into three main groups, namely Forastero, Criollo and Trinitario complex.

Figure 4. Different aspects of cacao pods (Demol, 2002).

- **Forastero** group

They are originated in the Amazon region of South America and possess green pods with predominantly purple seeds. Most cultivated clones are Forastero which represent over 80 per cent of the world cacao production. Forastero were subdivided into Upper Amazon and Lower Amazon because of their geographical locations and some distinctive characteristics (e.g. precocity and disease resistance).

- **Criollo** group

There are the first domesticated cacaos and originated in Central America and in the north of South America. They possess large white or rosy beans that give a desirable chocolate. The Criollo are found today as small groups of isolated trees or in small very old plantations. In spite of the morphological variations, Criollo cacao is self-compatible and is nearly fully homozygous as revealed by SSR and SNP markers (Ji et *al*. 2013; Motilal et *al*. 2010).

- **Trinitario** complex

They are resulting from hybridization between Criollo and Amazon Forastero (Cheesman, 1944), combines the quality characteristics of the first with the hardiness of the latter. The name of the genotype is generally referring to station where it was selected e.g. ICS (selected in Trinidad at the Imperial College of Tropical Agriculture); UF (selected in Costa Rica at the United Fruit); SNK (selected in Cameroon at the "Nkoemvone" station).

The recent molecular studies of cacao populations distributed around the world revealed a more variable number of cacao groups which nowadays can be differentiated genetically and geographically (Motamayor *et al*., 2008; Susilo *et al*., 2011; Smulders *et al*., 2012). According to these studies, the cacao populations of Central and South America were subdivided into 10 genetic clusters: Amelonado, Contamana, Curaray, Criollo, Guiana, Iquitos, Maranõn, Nacional, Nanay and Purús, these occur in the Amazon basin and Bahia.

I.4.2 Cultivation

There is a growing interest in cacao these last years due to its high content of polyphenols which prevent brain and cardiovascular diseases (Rimbach et *al*., 2009; Kris-Etherton et *al*., 2002; Lee et *al*., 2003). This biological activities are thought to be attributed to a group of polyphenol compounds present in cacao, including the flavan-3-ol monomers, (-)-epicatechin and (+)-

catechin and several procyanidin oligomers built upon these monomeric units. *Theobroma cacao* L. trees are grown in the humid tropics to produce cacao beans which are a cash crop and an important raw material for the chocolate industry. During the year 2018/2019 the largest producers and exporters were Ivory Coast (2154 t. tons), Ghana (812 t. tons), Ecuador (322 t. tons) and Cameroon (280 t. tons) (ICCO Quarterly Bulletin of Cacao statistics, 2021).

Table 1. The evolution of world cocoa market and forecast for the year 2020/2021 (Anonymous 1, 2020; 2021).

Cocoa year	2018/2019	Estimates 2019/2020	Forecasts 2020/2021
World production (x1000 t)	4784	4728	4843
World grinding (x1000 t)	4784	4671	4693

I.4.3 **Propagation**

Theobroma cacao L., has a strong history of seed (seminal) propagation in Central America, where it was first domesticated (Cruz et *al*., 1995). Seeds from mature fruits are recalcitrant and rapidly lose viability after ripening (Barton, 1965). Nevertheless, seed propagation is the principal means for the establishment and latter for the expansion of production plantings among the more than 50 countries currently growing cacao.

Cacao, however, is a heterezygous plant (N'Goran et *al*., 2000) with a characteristically wide variability for agronomic traits (Figueira and Janick, 1995).

I.5 Constraints of cacao cultivation

I.5.1 Pathogens attacks

- Fungi diseases

The most common disease, known as "black pod" disease, is caused by oomycetes of the genus *Phytophthora* (*P. palmivora, P. megakarya, P. capsici and P. citrophothora*), spread all the world and causing significant losses, mainly in Africa (Fig. 1A and B). *Phytophthora* fungi can cause the loss of almost 90-100%, dependently from the localization, the cultivar, the pathogenic strain and environmental conditions (Iwaro et *al*., 1997). Other strains include Vascular Streak Dieback (VSD), a disease caused by the fungus *Oncobasidium theobromae* in

Southeast Asia and parts of the Far East. In South America and especially in Ecuador, it is the monilia (Fig. 1C) and the disease of the "witch's broom" which constitute the most devastating attacks. The fungi responsible for these diseases are *Moniliophthora roreri* (monilia), which affects fruit, and *Moniliophthora perniciosa* (witch broom disease).

- Viral disease

The virus swollen cacao stalks (cacao swollen shoot virus-CSSV) mainly affects West Africa (Fig. 5D) and more particularly Ghana and Togo (Falque, 1994). The virus-CSSV, an adenovirus is transmitted by at least 14 species of mealybugs of the *Pseudococcidae* family belonging to *Coccoidae* (Roivainien, 1976).

- Insects and animals

The cacao tree is the target of several pests including: the trunk perforators (*Xyleborus ferrugineus*), the acares (*Floracarus theobramae Keiffer*), the thrips (*Selanothrips rubrocintus*), and the fruit perforators (*Carmenta theobromae*, *Anadasmus porinodes*, *Gymnandrosoma aurantium* and *Synanthe don* sp.) (Fig. 5E). According to Falque (1994), the main insect pests of cacao in Africa are bedbugs of the genera *Sahlbergella*, *Distantiella*, *Helopeltis* and *Menalonion* (Heteropthera: Miridae), whose larvae bite branches, which can lead to the death of the tree.

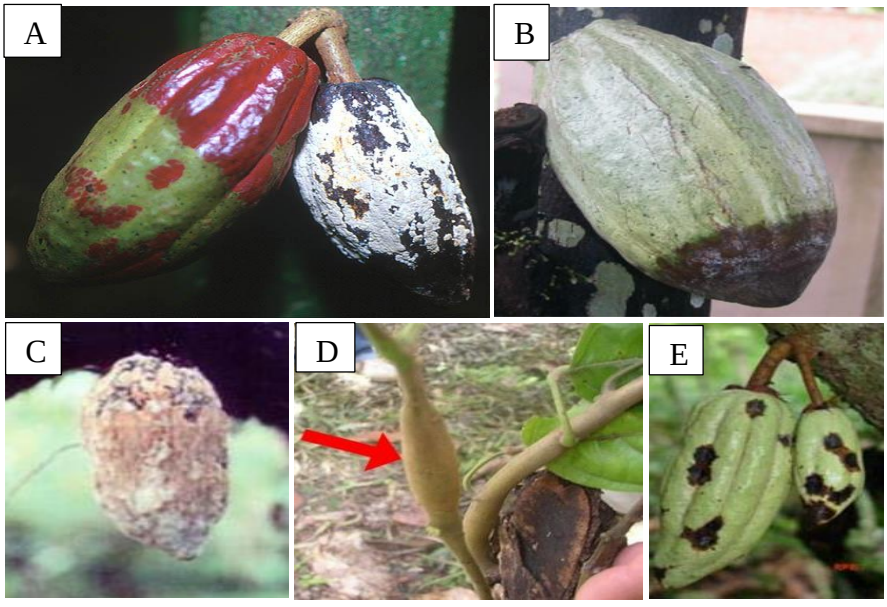


Figure 5.The cacao diseases and pests. A- pods infected with *Phytophtora Palmivora*; B- pods infected with *Phytopthtora Megakarya*; C- typical symptoms of monilia (*Moniliophtora roreri*) on pods; D- young cacao plant showing symptoms of viral swollen shoot disease (red narrow) and E- damage caused (dark spots) by mirids on young fruits.

I.5.2 Drought stress

The drought is a meteorological term and is commonly defined as a period without significant rainfall. Generally drought stress occurs when the available water in the soil is reduced and atmospheric conditions cause continuous loss of water by transpiration or by evaporation.

I.5.2.1 Impacts of drought stress on plants

I.5.2.1.1 Impacts on plant morphology and yield

The first and foremost effect of drought is impaired germination and poor stand establishment (Harris et *al*., 2002). The cell growth is one of the most drought-sensitive physiological processes due to the reduction in turgor pressure (Taiz and Zeiger, 2007). The cell elongation of higher plants can be inhibited by interruption of water flow from the xylem to the surrounding elongating cells (Nonami, 1998). The cell elongation and expansion result in reduced plant height, leaf area and crop growth under drought (Nonami, 1998; Kaya et *al*., 2006; Hussain et *al*., 2008). The drought-induced yield reduction has been reported in many crops species, which depends upon the severity and duration of the stress period. In pearl millet (*Pennisetum glaucum*), co-mapping of the harvest index and panicle harvest index with grain yield revealed that greater drought tolerance was achieved by greater partitioning of dry matter from stover to grains (Yadav et *al*., 2004).

I.5.2.1.2 Impacts on plant physiology

- *Photosynthesis*

A major effect of drought is reduction in photosynthesis, which arises by a decrease in leaf expansion, impaired photosynthetic machinery, premature leaf senescence and associated reduction in food production (Wahid and Rasul, 2005). When stomatal and non-stomatal limitations to photosynthesis are compared, the former can be quite small. This implies that other processes besides CO_2 uptake are being damaged. Another important effect that inhibits the growth and photosynthetic abilities of plants is the loss of balance between the production of reactive oxygen species and the antioxidant defense (Fu and Huang, 2001; Reddy et *al*., 2004).

- *Water and nutrients relations*

The relative water content, leaf water potential, stomatal resistance, rate of transpiration, leaf temperature and canopy temperature are important characteristics that influence plant water relations. Exposure of the plants to drought stress substantially decreased the leaf water potential, relative water content and transpiration rate, with a concomitant increase in leaf temperature (Siddique et *al*., 2001). The studies show a positive response of crops to improved soil fertility under arid and semi-arid conditions.

I.5.2.1.3 Impacts on plant metabolism

- *Amino acids and other nitrogen compounds*

These osmolytes, which are known to increase their synthesis during osmotic stress, including proline, accumulate to high concentrations without interfering with cell metabolism (Bray, 1993). As well as proline, other nitrogen compounds could be accumulating in plants in response to drought stress (Diaz et *al*., 2005). The amino acid metabolism may play an important role in plant stress tolerance, by osmotic adjustment through the accumulation of compatible osmolytes by detoxification of reactive oxygen species, xenobiotics and heavy metals, and by intracellular pH regulation (Alia et *al*., 2001).

- *Non-structural sugars*

The carbohydrates produced from photosynthesis are major energy sources and building blocks for production of biomass and its maintenance. The stressed plants accumulate carbohydrates with the reduction in osmotic potential (Afzal et *al*., 2017). De Roover et *al*. (2000) studied the impact of water stress at the chicory seedling stage and demonstrated that water shortage increased glucose, fructose, and sucrose concentrations in the roots and leaves of stressed plants, leading to increased fructan concentrations in the roots.

- *Total phenolics*

The phenolic compounds are the most widespread substantial groups of plant secondary metabolites that exhibit antioxidant properties (Quan et *al*., 2016). These compounds can scavenge ROS (Quan et *al*., 2016) and prevent lipid peroxidation, protein denaturation and DNA damage (Mittler et *al*., 2011; Król et *al*., 2014). The drought stress caused accumulation of total phenolic compounds in grapevine roots, which may indicate that these compounds play

an important role in the adaptation of roots to growth under stress conditions (Weidner et *al*., 2009).

I.5.2.2 Plants responses to drought stress

I.5.2.2.1 Morpho-physiological responses

Water limitation is one of the major threats in crop production and it is projected to get considerably worse in the coming decades (Cominelli et *al*., 2009). Plants have developed numerous strategies in response to this environmental adverse factor (Ogbaga et *al*., 2014), most of them compatible with maintaining crop productivity.

- *Escape*

The drought escape occurs when phenological development is successfully matched with periods of soil moisture availability, where the growing season is shorter and terminal drought stress predominates (Araus et *al*., 2002). In developing short-duration varieties has been an effective strategy for minimizing yield loss from terminal drought, as early maturity helps the crop to avoid the period of stress (Kumar and Abbo, 2001). However, yield is generally correlated with the length of crop duration under favorable growing conditions, and any decline in crop duration below the optimum would tax yield (Turner et *al*., 2001).

- *Avoidance*

The drought avoidance consists of mechanisms that reduce water loss from plants, due to biosynthesis of abscisic acid (ABA) to stomatal closure thereby reducing water loss through transpiration, and also maintain water uptake through an extensive and prolific root system (Turner et *al*., 2001; Kavar et *al*., 2007). The root characters such as biomass, length, density and depth are the main drought avoidance traits that contribute to final yield under terminal drought environments (Turner et *al*., 2001). A deep and thick root system is helpful for extracting water from considerable depths (Kavar et *al*., 2007).

I.5.2.2.2 Metabolic regulation

A precise metabolic regulation through the regulation of photosynthesis and accumulation of compounds such as osmolytes (Slama et *al*., 2015) is another strategy of plants to cope with drought stress. As a consequence of an increase in osmolytes the osmotic potential of the cell

is lowered, and water is drawn into the cell to maintain turgor pressure. Osmolytes include compounds such as soluble carbohydrates (e.g., glucose, sucrose, trehalose) and carbohydrate alcohols (e.g., mannitol, sorbitol); oligosaccharides (raffinose, stachyose, and verbascose); different amino acids, such as proline, betaine, valine, leucine, isoleucine, and agmatine (as precursor of polyamines); quaternary ammonium compounds (e.g., glycine betaine, b-alanine betaine, proline betaine, choline-O-sulfate); as well as polyamines (e.g., putrescine, spermidine, and spermine) (Ruan and Teixeira da Silva, 2011; Arbona et *al*., 2013). These compounds have an osmoprotective role during stress, repair damaged tissues, and support growth. In general, sugar alcohols are classified as acyclic (e.g., mannitol) and cyclic (e.g., pinitol) polyols. The accumulation of polyols may have dual functions: facilitating osmotic adjustment and supporting redox control. The up- and down-regulation of specific primary and secondary metabolites contribute to a defense mechanism to better tolerate the stress, and a number of them can be analyzed to assess the stress status of plants (Conde et *al*., 2014; Patel and Williamson, 2016).

I.6 Improvement of cacao cultivation and propagation

I.6.1 Control of pests and diseases incidence

The primary method of controlling incidence of the disease in the cacao plantation is through frequent removal of weeds and other plants capable of increasing the relative humidity, as well as removal and burning of the infected cacao pods. However, the costs of treatment and environmental damages make this practice unfeasible (Phillips-Mora *et al.*, 2007; Thevenin *et al.*, 2005). The studies concerning biological control of the diseases as well as genetic interference in plants and microorganisms physiology are in progress (Ondobo et *al*., 2014).

I.6.2 Selection

The classic breeding programs and domestication practices established during the last hundred years play and essential role in the amelioration of cacao. The aim of these programs is the vulgarization of plant materials with special agronomic traits through genetic amelioration of plants. These agronomics traits include: a) the productivity (which refer to the number of pods per tree, weight of pods and adaptation in farm) and b) resistance to diseases and insects, efficient strategy to control pathogens (Ahnert, 2000). The precocity of the production of genotypes; the physical quality (the dimension of the seeds; the rate of butter, the color of

cotyledons) and organoleptic (aromas and viscosity) of dry seeds are important criteria also considered during selection.

I.6.2.1 Clonal selection

The clonal selection consists basically to choose trees according to their specific character and multiply through vegetative method (cutting). This method is less successful because it is expensive and the susceptibility of plantlets obtained to climatic developmental factors (Mooleedhar, 1998).

I.6.2.2 Generative selection (cross breeding)

The generative selection is the most used and contributes to the creation of seeds garden. Cacao hybrids between parent clones of different geographic origin are common. Frequently the crossing strategy adopted aims at complementation of traits, with the purpose of correcting faults. Lately, species diversity were investigated through multivariate methods applied to morphological (Bekele *et al*., 1994), enzymatic (Ronning and Schnell, 1994), and DNA-based molecular markers (Laurent, 1993; N'Goran *et al*., 1994). The restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNA (RAPD) are used as markers. Advances in cacao breeding due to introduction of these DNA markers were examined (Dias, 1995). But currently, microsatellite markers (SSRs) can be considered as the most used type of markers. SSRs can be used to conserve valuable genetic material and to select promising cacao cultivars which are disease resistant, high yielding, and fine flavored (Everaert et *al*., 2017). These markers consist of short tandem repeats of a simple motif sequence (Lima et *al*., 2010), with a variable length, and can be assayed by PCR-based analysis and subsequent (gelorcapillary) electrophoresis (Livingstone et *al*., 2011; 2012). Disadvantageous is the ambiguity of the results, which obstructs the comparison between different laboratories (Fang et *al*., 2014). The second is the non matching type, where the cultivars have unique SSR profiles but do not match with the International Cocoa Germplasm Database (ICGD).

I.6.3 Vegetative and clonal propagation

Generally, the propagation of cacao is made from scions from plagiotropic or orthotropic shoots.The plagiotropic material, when propagated, results in a sprawling, bush-like architecture lacking the normal dimorphic growth habit. Plagiotropic derived plants are sensible to wind. Orthotropic materials develop the seedlings-architecture but they are limited in

availability and poor in rooting and/or establishment (Miller and Guiltinan, 2003). Farmers use seeds from superior clones for establishment of plantations, but this action results in a lack of vigor and in the susceptibility to diseases. Furthermore, only a few of the clones are self-compatible (Bennet, 2003).

The clonal propagation method offers a suite of new approaches to speed up the development and the deployment of genetically-improved genotypes because of its potentially very high multiplication rate and scalability (Traore *et al*., 2003; Miller and Guiltinan, 2003; Maximova *et al.*, 2008; Pilatti *et al*., 2011). The main advantages of cacao tissue-culture methods include the genetic gain, the production of orthotropic plants with normal dimorphic architecture, production and testing of disease-free materials and germplasm conservation *via* cryopreservation.

I.7 Somatic embryogenesis of *Theobroma cacao*

I.7.1 Totipotent cells

In nature, as an alternative to the propagation via seeds, plants developed strategies like organogenesis, shoot proliferation via axillary buds and somatic embryogenesis (Zhang and Ogas, 2009). In order to respond to environmental signals and to guarantee continuous vegetative growth (organogenesis) plants developed special groups of cells called meristem. The totipotency of these cells allows reversibility and differentiation of somatic cells which under extreme conditions can change their specificity depending on the needs (Harada *et al*., 2010; Clark, 1997). These stem cells are used as a key for the initiation of somatic embryogenesis (Fehér *et al*., 2003). The somatic embryogenesis has been used as a strategy for large-scale propagation of clones as well as a model for morphological and physiological studies concerning plant development. However, the process of somatic embryogenesis presents limitations depending on the biological system. Most of the investigations attempt for studies concern regulatory mechanisms of somatic embryogenesis as well as the comparison to zygotic embryos (Dodeman *et al*., 1997) (Fig. 6).

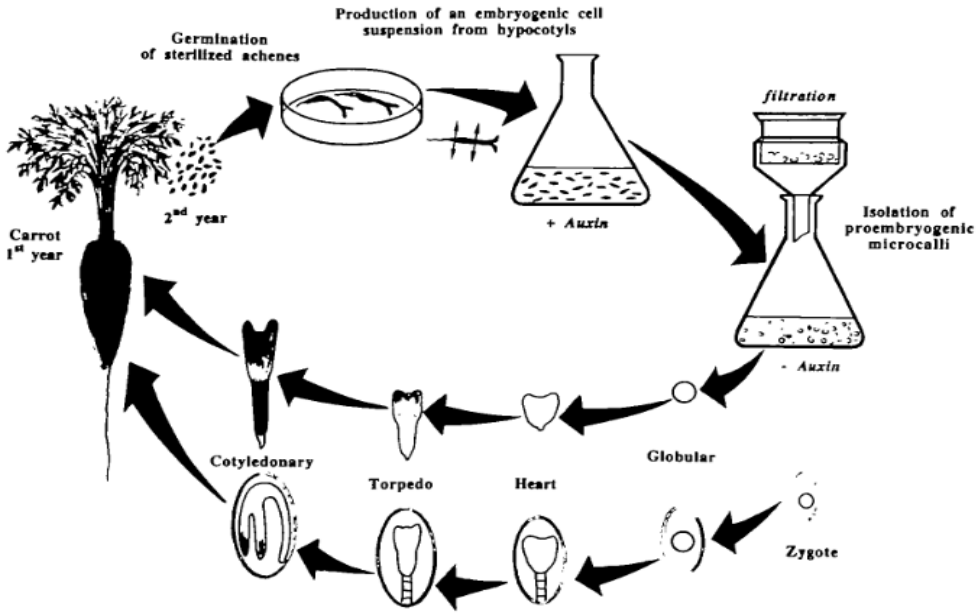


Figure 6. Cartoon of zygotic and somatic embryogenesis using *Daucus carota* L. as an example (Dodeman et *al*., 1997).

I.7.2 Somatic embryogenesis of *Theobroma cacao*

A go forward was achieved by studying cacao somatic embryogenesis in order to develop an efficient tissue culture system for a rapid and efficient vegetative multiplication of elite genotypes (Minyaka et *al*., 2010; Maximova *et al*., 2014). The current results indicate that many genotypes are recalcitrant whereas somatic embryogenesis response is affected by genotypes and culture medium composition. The culture media currently used for cacao somatic embryogenesis are made of MS or DKW, in some cases differing in plant growth regulators and minerals concentrations (Minyaka *et al*., 2008). Besides, Alemanno *et al.* (2007) identified the homologous gene *leafy cotyledon1-like* in cacao somatic embryogenesis cultures (*TcL1L*). The transcripts of this gene are mainly accumulated in young somatic and zygotic embryos and related to embryogenic cells from shoot and root meristem as well as protodermal and epidermal cells. Another important gene involved in embryogenetic processes is the gene *SERK* (*somatic embryogenesis receptor-like kinase*). It is considered to be responsible for the embryogenic capacity of tissues of species like *Daucus carota*, *Zea mays* and *Arabidopsis thaliana* (Santos and Aragão, 2009). A copy of this gene was found in cacao and seems to be as functional as in others species. The importance of this gene in the cacao somatic embryos development by restricting the amino acid substitutions was showed (Santos *et al*., 2005).

The histological studies were carried out to investigate the structural formation of cacao somatic embryos. During formation of cacao primary somatic embryos high amounts of phenolic compounds were found in explants of flower pieces and in the produced calluses which show non-embryogenic features (Alemanno *et al*., 2003). The primary somatic embryos of cacao regenerated from floral explants seem to have a multicellular origin (Maximova *et al*., 2002; Alemanno *et al*., 1996). Maximova *et al*. (2002) investigated the morphogenic formation of primary and secondary somatic embryos and showed differences between both processes. Primary cacao somatic embryos evolve from a group of stem cells while secondary somatic embryos are formed from a single cell (Maximova *et al*., 2002).

The somatic embryos of cacao developed from primary or secondary somatic embryogenesis present normal morphological development with respect to embryonic stages defined for angiosperms (Fehér *et al*., 2003). The development of the zygotic embryo can be conceptually divided into two stages: a first morphogenic stage where the basic structure of the embryo is established and a second metabolic stage characterized by biochemical activities that prepare the embryo for quiescence (Lopes and Larkins, 1993). At this point morphogenesis is considered to be completed and the second phase, the so-called late development, begins. (Harada *et al*., 2010; Zhang and Ogas, 2009; Von Arnold *et al*., 2002). There are many investigations concerning morphology and physiology of the early development of cacao somatic embryos (Niemenak et *al*., 1998; Maximova et *al*., 2014; Noah *et al*., 2013).

Somatic embryos of cacao is generated from many different genotypes and somatic embryo-plants were grown under field conditions, and demonstrated growth similar to plants propagated by seeds (Maximova *et al*., 2008; Li *et al*., 1998). Although the system is sufficiently efficient to be utilized commercially, it includes several steps that are highly genotype-dependent. One of the most inefficient steps of the procedure is somatic embryo conversion, which involves transition of mature somatic embryos into whole plants (Traore *et al*., 2003). The comparison of zygotic and somatic embryogenesis of cacao led to the identification of some factors which may limit cacao somatic embryo development. The morphological abnormalities of the somatic embryos population, lack of protein and starch accumulation in the cotyledons and a significantly higher water content in the cells than the proportional content in zygotic embryos in the maturation stage were reported (Alemanno *et al*., 1996). However, the deficiency in the rooting of the somatic plantlets has been identified as the critical point during conversion of the cacao somatic embryos into plantlets (Maximova *et al*., 2005, 2008; Niemenak *et al*., 2008).

The supplementation of the maturation media with high concentrations of sugars showed positive effects on the accumulation of storage compounds in cacao somatic embryos and improved their development, but these effects reached only in presence of abscisic acid. The effect of different carbon sources on the induction of cacao somatic embryogenesis was achieved by Traore and Guiltinan (2006). These authors showed that, during embryo maturation and conversion, has no significant difference among glucose, fructose, maltose or sucrose for embryo weight, total shoot and root development. In cacao, temporary immersion system (TIS) allows for an increase in embryo numbers and quality as showed by Niemenak *et al*. (2008).

The establishment of somatic plantlets of cacao in field conditions revealed that some of these plantlets showed growth and architecture similar to seedlings. These characteristics favor this method as an alternative propagation for cacao genetic breeding programs (Maximova *et al*., 2008).

I.8 Metabolomics

I.8.1 Overview

The term "metabolome" is usually restricted to metabolite with molecular weight under 1,500 Da (Wishart *et al*., 2007). Metabolomics is both a quantitative and qualitative method to characterise small molecules that participate in metabolic reactions in general and that are required for the maintenance, growth and normal function of a cell (Kim et *al*., 2011). Metabolomics is now a well-known technique for studying plants undergoing abiotic stress or applied in bioenergy (Abdelnur et *al*., 2014). Metabolomic studies have been developed in the last decade for numerous applications in the field of plant sciences. The objectives of these studies include the classification and characterization of different plant species, monitoring the response to stress, wounding, herbivory or infection, discrimination of wild/transgenic plants or different genotypes (Ramsay et *al*., 2014).

The analytical techniques most used for metabolomics studies are based on mass spectrometry system (GC-MS, LC-MS, FT-ICR-MS, EC-MS) and/or Nuclear magnetic resonance (NMR). Nowadays, NMR is considered as a powerful tool in plant metabolomics to allow structure elucidation of metabolites and detailed analysis of the biomolecular composition of a plant extract with relatively simple sample preparation. Athough NMR is less sensitive than mass spectrometry (MS), it offers major advantages by being reproducible, non-destructive, non-

specific and quantitative (Kim et *al*. 2010; Jorge et *al*., 2015; Deborde et *al*. 2017; Le Gall et *al*. 2017).

I.8.2 NMR Metabolomics

Metabolomics approaches may be classified into two main groups: foot/fingerprinting or profiling (Fig. 7). NMR foot/fingerprinting often implies rapid and simple plant matrix extraction, followed by rapid NMR acquisition of the resulting crude extract, processing and handling of NMR features (patterns of resonances or single peaks) through data reduction often named “bucketing” or “bining” (with uniform bucket/bin size across the spectra if no change of chemical shift is observed, or with variable size buckets/bins). These steps are followed by multidimensional statistical analysis such as principal component analysis (PCA). This approach does not require assignment of the NMR features (the resonance patterns); its purpose is to discriminate samples, highlight sample groups in accordance or not with the experimental design (control versus stress conditions, developmental stages, genotype screening, etc.). Nevertheless, after the observation of the PCA score plots and loadings plots, it is often necessary to assign the NMR spectral regions corresponding to the discriminant bucket-variables, in order to build hypothese on biomarkers and metabolic pathways. However, the sample extraction is very often not optimized, the metabolite recovery not checked, and the NMR acquisition parameters are usually not optimized for quantification. As a consequence, observed tendencies should be checked with orthogonal methods and sample preparation should be optimized as much as possible for the potential biomarker(s). Otherwise, over-interpretation and inaccurate conclusions in terms of plant biology may occur rapidly. Metabolite footprinting is also a kind of fingerprinting, but limited to certain types of plant samples (growth medium of plant cell suspensions, liquid sample; growth medium of callus, gel sample; root exudate collection medium, liquid sample).

NMR profiling is also used to differentiate sample groups, but based on metabolite concentrations. NMR profiling was first used for the global or broad-scope profiling of samples with relative, and sometimes absolute, quantification of metabolites. It is now also used in a targeted metabolic profiling approach, which implies optimized multiple and time-consuming extraction steps followed by a measurement of extraction recovery rate of a limited set of metabolites of interest (by checking if a given set of metabolites were properly extracted by spiking and measuring their recovery rate). In fact, the difference between profiling and fingerprinting is rather sligth. In addition, since the extraction step leads to metabolite selection,

it cannot be thus qualified as untargeted profiling. For profiling, besides a long series of multiples steps to optimize metabolite extraction and recovery, special care also has to be taken to obtain quantitative NMR spectra. The assignment of NMR features, the use of a reference and of quantitative NMR acquisition parameters are compulsory to determine accurate metabolite concentrations in extracts. The identification of metabolites is accomplished through (i) spectral comparisons with in-house databases (spectra of authentic compounds recorded under the same pH and buffer solution concentration, and the same NMR acquisition parameters) and publicly available databases (HMDB (Wishart et *al*., 2007), BMRB (Ulrich et *al*., 2008), MMCD (Cui et *al*., 2008), PRIMe (Akiyama et *al*., 2008), MeRyB (Ferry-Dumazet *et al*., 2011), Metabolights (Haug et *al*., 2013), (ii) spiking the samples with chemical standards, and (iii) 2D NMR and/or other orthogonal analytical methods.

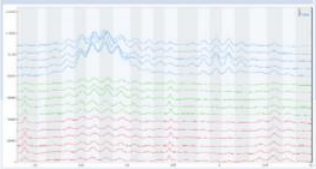
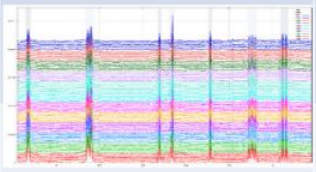
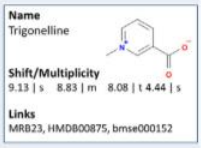
	Plant sample	Selection of specific NMR features (*i.e.* pattern of resonances or single peak)	Relative quantification or bucketing of NMR features	NMR acquisition parameters	NMR peak assignment and Metabolite identification	Relative or Absolute quantification
Footprinting	**Growth medium of plant cell suspension** or callus ; root exudate medium	No	 **Bucketing of the entire spectrum**	Rapid acquisition	Partial or complete identification	
Fingerprinting	**Crude extract** from rapid (single step) extraction	No		Rapid acquisition	Partial or complete identification	
Global or broad scope approach profiling	Optimized multiple steps time consuming extraction	Yes	 **Selection of targeted buckets**	Quantitative acquisition	Partial or complete identification	Relative quantification
Targeted Profiling	As above + Targeted metabolite recovery checked extraction	Yes		Quantitative acquisition	Metabolite identification 	Absolute quantification of targeted metabolites **Quantification (µg/g DW)** Trigonelline 294 Phenylalanine 1694 Tyrosine 424 Fumaric acid 17 Chlorogenic acid 529

Figure 7. Some characteristics of metabolomics approaches using NMR

I.8.3 Elucidation of plant response to drought by metabolomics

I.8.3.1 Primary metabolites involved in plant response

A characterization of metabolic changes induced by water depletion in the roots of *Salvia miltiorrhiza* Bunge plants was performed by using two combined analytical techniques: NMR and LC-MS. The results showed that the NMR method was effective to detect both, the primary

and secondary metabolites, whereas the LC-MS method was excellent for selectively detecting the secondary metabolites. Among the primary metabolites, five carbohydrates, galactose, glucose, sucrose, melibiose, and raffinose, were detected (Dai *et al*., 2010). In another study, nontargeted metabolomic approach by Gas Chromatography/Electron Impact-Time of Flight Mass Spectrometry (GC/EI-TOF-MS) was used to explore relative changes of the metabolite pool of gradually water-stressed Lotus japonicus ecotype Gifu plants. As a general metabolic trend in response to water stress, significant increases of fructose, glucose, galactose, and maltose were reported (Sánchez et *al*., 2012). GC-MS-based metabolite profiling was conducted to analyze the metabolites in leaf blades of 10 hybrids of maize exposed to drought (Obata et *al*., 2015). This study showed a large cluster that included several sugars and sugar alcohols, which decreased (maltose and erythritol) or did not change (raffinose and xylitol). In addition, a wide variety of amino acids, tryptophan, alanine, serine, glycine, leucine, and γ-aminobutyric acid (GABA) were accumulated in plants under drought stress (Obata et *al*., 2015). In other study, Sun et *al*. (2016) examined the metabolic responses of maize plants grown under drought through NMR metabolomic techniques. The authors found that stressed plants accumulated high levels of organic acids involved in the tricarboxylic acid (TCA) cycle (malate and citrate), amino acids such as proline and alanine, and sugars, particularly fructose, among other metabolites. In general, sucrose and particularly glucose act as osmolytes to maintain cell homeostasis. Nyarukowaa et *al*. (2016) identified and quantified metabolites in tolerant and drought-sensitive Camellia sinensis cultivars under water stress conditions using targeted analysis by GC–MS/MS and UPLC-MS. There was a significant variation in metabolite levels between drought tolerant and drought susceptible tea cultivars. Asparagine, glycine, valine, isoleucine, proline, and leucine were significantly higher in the DT (drought-tolerant) cultivars than in the DS (drought-sensitive) cultivars. The high amino acid levels observed in DT cultivars support their role as osmolytes (Shi and Chan, 2014). In addition, these elevated levels of amino acids were also attributed to a reduction in protein synthesis and an increase in the breakdown of proteins present. In regard to carbohydrates, an increase in fructose level was observed in the DS tea cultivars, whereas a decrease was detected in the DT cultivars. This could be because DT cultivars utilize fructose at a faster rate than the DS cultivars, ensuring their survival under drought stress. Under water deficit, glucose concentrations have also been documented to increase in DS plant varieties (Iordachescu and Imai, 2008). The glucose accumulation resulted in the increase of trehalose. This metabolite is a sugar reserve, supplying the plant with energy to cope with stress, as well as

a stress protectant. This carbohydrate is also responsible for protein and membrane stabilization (Yoshida and Sakamoto, 2009). Furthermore, a significant increase in the levels of polyols, e.g. mannitol, was observed. Nyarukowaa et *al*. (2016) also observed that mannitol levels were lower in the DT than in the DS tea cultivars. This compound confers stress tolerance through actively scavenging hydroxyl radicals and is also found in lower concentra- tions in DT cultivars than in DS wheat crops. The accumulation of sugars in this study was accompanied by an increase in the concentrations of the organic acid citrate, although there was no statistically significant difference between the DT and DS tea cultivars. An increase in citrate leads to an increase in other Krebs cycle metabolites. Alternative column chemistries (hydrophilic interaction chromatography, porous graphitic carbon [PGC], and anion exchange chromatography) must be applied for the target analysis of the wide range of polar metabolites typically found in the plant metabolome. Among them, a less-laborious PGC-ESI-QIT-MS/MS (porous graphitic carbon liquid chromatography–electrospray ionization mass spectrometry) method has been developed, validated, and reported for the analysis of neutral sugars in a wide range of plant metabolomics studies related to plant environment and agriculture, including the quantification of glucose, sucrose, and raffinose from Lupinus albus cortex and stele stemin response to water stress and subsequent recovery treatments (António et *al*., 2008), and raffinose family oligosaccharides (RFOs) from Haberlea rhodopensis leaves in response to severe desiccation (Gechev et *al*., 2013).

In addition, Jia et *al*. (2016) combined metabolic profiling and transcriptomics to explore the response of a Chinese cultivated herb, Astragalus membranaceus Bge. Var. mongolicus (Bge) to 14 days of progressive drought stress. Roots were harvested at 4 time-points corresponding to different stress durations. Thirty-seven compounds were identified and quantified on the NMR spectra of root methanolic extracts. Among them, the content of proline was 53-fold higher during severe stress than in control and was around 15-fold higher for trigonelline (alkaloid). The pathways most affected by the drought stress were the glycolysis, TCA cycle, glutamate-mediated proline biosynthesis, shikimate-mediated metabolism and aspartate family metabolism. The comparison of stress versus control at the molecular level showed that three of the highly elevated genes encoded P5CS (delta 1-pyrroline-5-carboxylate synthase gene), which is involved in the metabolism of the compatible solute proline.

I.8.3.2 Secondary or specialized metabolites involved in plant response

The production of reactive oxygen species (ROS) is one of the primary responses to stress following the decline in photosynthesis. Therefore plants respond to this induced oxidative stress by overproducing antioxidant compounds, such as ascorbic acid, glutathione, and polyphenols (Das and Roychoudhury, 2014). Plants produce a huge variety of secondary metabolites that have diverse physical and chemical properties. This enables to them to play a role in coordinating ecology and defense strategies of plants (Wink, 2010). Among these metabolites, polyphenols are commonly involved in the defense mechanisms toward different abiotic stress, including those related to drought (Di Ferdinando *et al*., 2014), because of their function as hydrogen donors and singlet oxygen scavengers (Labanowska *et al*., 2013). Ancillotti *et al*. (2015) analyzed metabolic changes by HPLC-MS/MS in *Nicotiana langsdorffii* plants under water stress. A decrease of antiradical activity and phenolics (represented by coumarins, such as esculetin and especially scopoletin) was observed. The complex information obtained from phenolic secondary metabolism data is in agreement with studies that reported different phenolic profiles in response to water deficit, depending on the species analyzed and even within the same species as a function of the more or less sensitive/tolerant genotype under investigation. Moreover, inconstant trends were found for different phenols in the same genotype. For example, a significant decrease of TP (polyphenols totals) was evidenced by Tejavathi *et al*. (2010) in *in vitro* plants of *Macrotyloma uniflorum* L. as a function of the increase of polyethylene glycol (PEG) percentage in the growing medium. Decreasing trends of TP and individual phenolic compounds were also observed in one tolerant and one sensitive cultivar of *Gossypium hirsutum* L. cultivated *in vivo* under water deficit conditions (Yildiz-Aktas *et al*., 2009). Conversely, TP were found to increase in other tolerant and sensitive genotypes of *G. hirsutum* cultivated *in vivo* with decreasing the watering level (Shah et *al*., 2011), which demonstrated the complex response of plant phenolic metabolism to water stress. Scalabrin et *al*. (2015) reported an untargeted metabolomic analysis using LC-MS in *Nicotiana langsdorffii* grown under water deficit, which showed an enhanced antioxidant level (polyamines) and damage of lipids, probably as a consequence of ROS production. These studies were highlighted by the results described by Sánchez-Rodríguez et *al*. (2011), who reported either increasing or decreasing trends for hydroxycinnamic acids and flavonoids in different species as observed in leaves of different *Solanum lycopersicum* cultivars in response to water deficit.

Since the response to drought and salinity stress is controlled by the hormonal network, the presence and relative concentration of phytohormones may be regarded as a good indicator of a plant's physiological condition. ABA is a phytohormone that plays an essential role in the dehydration-stress response. Studies of the molecular processes of the dehydration-stress response pathways have revealed both ABA-dependent and ABA-independent pathways (Yoshida *et al*., 2014). In Arabidopsis wild-type and knockout mutant of the NCED3 gene (nc3-2) plants subjected to dehydra- tion stress, a metabolite profiling was performed using two types of MS systems, GC/TOF-MS and CE-MS. This study revealed that a total of 64 dehydration-increased metabolites were detected in the wild type and nc3-2 mutant. The accumulation of amino acids (valine, leucine, isoleucine, tyrosine, and tryptophan, among others) depended on ABA production, but the level of RFOs, citrate, malate, and succinate (TCA cycle) were regulated by ABA independently under dehydration stress (Urano *et al*., 2009). The analysis of wild and transgenic *Nicotiana langsdorffii* plants exposed to water deficit was performed by a sensitive HPLC-HRMS (high-resolution mass spectrometry) quantitative method to assure the simultaneous and sensitive determination of two relevant plant metabolites: salicylic acid (SA) and jasmonic acid (JA), which proved to be useful in the assessment of the plant metabolic and biochemical status. Particularly in this study the levels of these two phytohormones were not directly affected by water stress; however, SA and JA could take part in the activation of early plant defenses, to be progressively depleted during stress exposure. Some studies indicate that high concentrations of SA could determine oxidative stress, thus leading to reduced abiotic stress tolerance, whereas, at moderate levels, SA is able to alleviate drought damages (Miura and Tada, 2014); in *Arabidopsis thaliana* increased SA levels were related to an enhanced tolerance toward drought stress as a consequence of the induction of H_2O_2 as signaling molecule (He *et al*., 2014). JA seems to be involved in ascorbate and glutathione metabolism, which is a key element in the cellular redox balance, and therefore could protect plants from water deficit–induced oxidative stress, as highlighted by the malondialdehyde content and electrolyte leakage (Shan and Liang, 2010). Mahouachi *et al*. (2007) observed a transient accumulation of JA in papaya seedlings after 15 days of water deficit treatment, suggesting its role as a triggering signal for ABA accumulation.

CHAPTER II: MATERIALS AND METHODS

II.1 MATERIALS

II.1.1 Plant material

Plant materials (immature flower buds and fresh seeds collected from mature pods) used (Fig. 8) were harvested at the Institute of Agricultural Research and Development (IRAD), at Nkolbisson (Yaoundé). Experiments were carried out on three cacao genotypes belonging to the Forastero family, chosen according to their embryogenic capacity observed during preliminary works done in the laboratory and to their agronomic performances:

SCA12, embryogenic genotype and productive in the field, resistant to Phytophthora pod rot caused by the infection of *Phytophthora palmivora* (Susilo *et al*., 2016);

SCA6, highly embryogenic genotype and productive in the field (Pang, 2006), resistant to Phytopthora pod rot caused by the infection of *Phytophthora megakarya* (Efombagn *et al*., 2007);

MA12, moderately embryogenic genotype and susceptible to Phytopthora pod rot caused by the infection of *Phytophthora megakarya* (Efombagn *et al*., 2007).

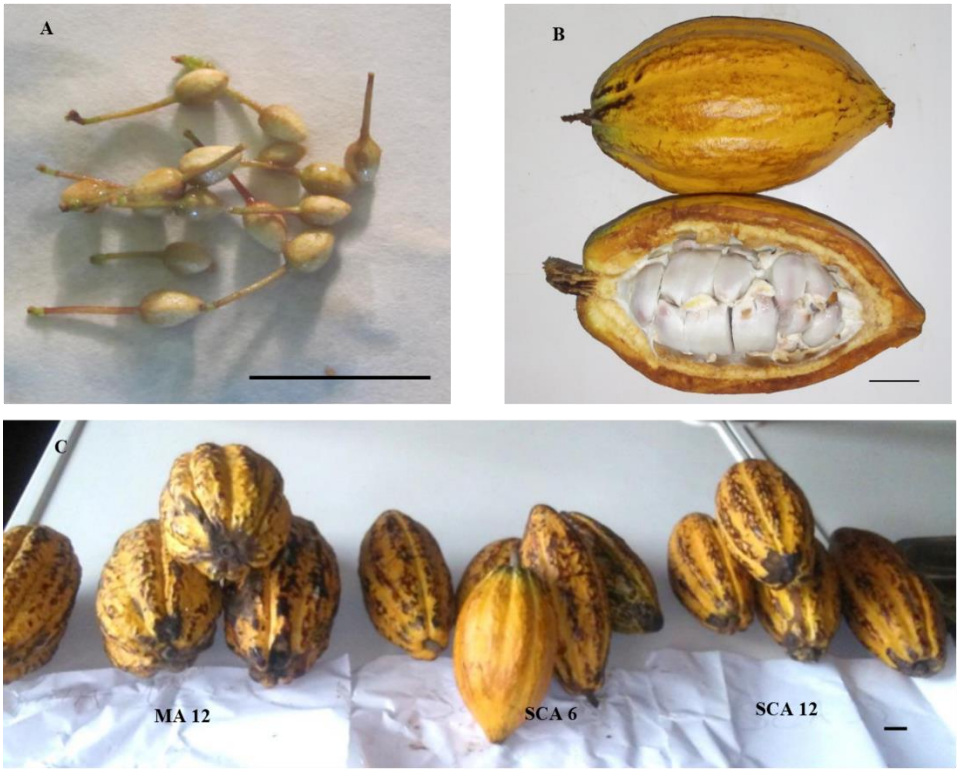


Figure 8: Plant materials of *T. cacao*: A- sterilized immature flower buds used as starting materials for primary somatic embryogenesis; B- longitudinal section of pod showing seeds covered with white mucilage and C- Mature fruits of *T. cacao* with the name of the corresponding genotype just below (Boutchouang, 2019). Bar =2 cm.

II.1.2 Culture conditions

The protocol used for somatic embryogenesis is similar to the system previously described (Maximova *et al*., 2005 and in Minyaka *et al*., 2008). Briefly, after induction in primary callus growth media, calluses were transfered in secondary callus growth media for maintenance. The expression step of tissues takes place in embryo development medium. All media were defined using DKW (Driver and Kuniyuki Walnut medium) basal salts and vitamins of Driver and Kuniyuki (1984). The plant growth regulators were supplemented in induction and maintenance steps.

The explants were first cultured in primary callus growth medium. The primary callus growth (PCG) medium was supplemented with 250 mgL^{-1} glutamine, 100 mgL^{-1} myo-inositol, 1 mLL^{-1} DKW vitamin stock (100 $mgmL^{-1}$, 2 $mgmL^{-1}$ thiamine-HCl, 1 $mgmL^{-1}$ nicotinic acid and 2 $mgmL^{-1}$ glycine), 20 gL-1 glucose, 18 μM 2,4-dichloro-phenoxyacetic acid (2,4-D) and 45.4 nM Thidiazuron (TDZ). The media were dispensed into sterilized Petri dishes after autoclaving for 20 min at 1 bar pressure and 121°C. The Petri dish contains either 35 staminodes or 35 petals. The experiments were repeated 5 times with five replicate Petri dishes at each culture initiation. The Petri dishes were incubated in dark at 25 ± 1°C for 14 days. After 14 days in PCG medium, explants were transferred to secondary callus growth (SCG) medium. SCG medium consisted of DKW basal salts, supplemented with 0.5 mLL-1 DKW vitamin, 20 gL^{-1} glucose, 9 μM 2,4-D, 250 μgL^{-1} kinetin and 0.22 % (w/v) gelrite. The cultures were also incubated at 25 ± 1°C for 14 days in darkness. The cultures from SCG medium were transferred in embryo development (ED) medium. The ED medium was made of DKW basal salts supplemented with 6.0 mM $MgSO_4$, 1 mL DKW vitamin, 30 gL^{-1} sucrose, 1 gL^{-1}, glucose and 0.22 % (w/v) gelrite. Cultures were incubated at 25 ± 1°C in darkness for 21 days. Two others sub-cultures were made every 21 days in ED medium for the full development of embryos.

The induction of secondary somatic embryos was carried out according to Maximova et *al*. (2002). Briefly, cotyledons excised from primary somatic embryos were sectioned with scalpel into thin slices and laid in petri dishes containing solidified SCG culture medium supplemented by 6-benzylaminopurine (BAP). After 14 days the explants were transferred to ED culture media. The subcultures were carried out every 21 days in fresh ED media till the full development of somatic embryos.

II.2. METHODS

II.2.1 Determination of the influence of the position of flower buds in a single tree on somatic embryogenesis of *T. cacao*

II.2.1.1 Callus and somatic embryos production

Primary somatic embryos are those obtained via somatic embryogenesis regenerated on matrix plant explants (flower pieces, leaves and zygotic embryos) whereas secondary somatic embryos are regenerated from primary somatic embryos. For the induction of primary somatic embryogenesis, the immature flowers buds of SCA6 genotype were collected at different positions on the same tree during all the experimentation, including orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII) early in the morning and kept at 4°C. They were surface sterilized in the laboratory by immersion for 20 min in 3% (w/v) sodium hypochlorite followed by three rinses in sterilized distilled water of 15, 10 and 5 min respectively. The staminodes and petals were excised with scalpels and placed on culture media into Petri dishes for regeneration through somatic embryogenesis. Callogenesis and embryogenesis frequencies were evaluated after 28^{th} and 91^{st} days, respectively. The screening diagram used for the evaluation of somatic embryogenesis using floral explants (both staminodes and petals) from different positions on the same tree is presented in Fig. 9.

II.2.1.2 Data collection

At the end (91st days) of each experience (a given culture), callus of each characteristic development stages of somatic embryogenesis, that is, callus aged 14, 28, 49, 70 and 91 days, respectively, were collected from different sources of explant and analyzed independently.

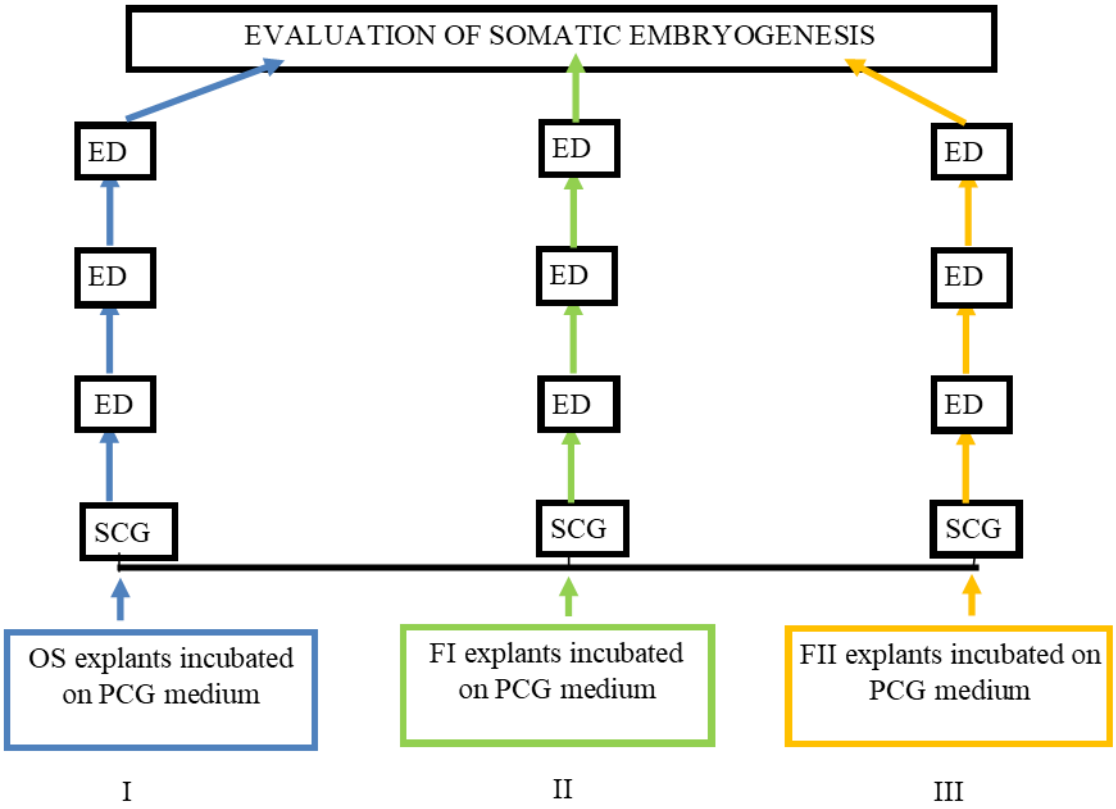


Figure 9: Screening diagram of the effect of the position of floral explant on the cacao tree on primary somatic embryogenesis (full names of abbreviations were previously indicated in section II.1.2): I (in blue) harvest of explant on orthotropic main stem and evaluation of embryogenic potential; II (in green) harvest of explant on primary plagiotropic fan branch and evaluation of embryogenic potential; III (in orange) harvest of explant on secondary plagiotropic fan branch and evaluation of embryogenic potential.

II.2.1.2 Biochemical changes in *T. cacao* callus during the developmental process using spectrophotometer

II.2.1.2.1 Estimation of protein contents

- Samples preparation

The callus (500 mg) was ground in chilled mortar with 2 mL of TAMET buffer (0.5 M Tris, 0.3 M Ascorbic acid, 0.2% (v/v) β-Mercaptoethanol, 0.01 M, EDTA and 0.02% (v/v) Triton X 100, pH 6.7); and 0.125 g polyvinylpyrrolidone (PVP) was added in the medium. The crude homogenate was centrifuged during 15 min at 20000g and 4°C (Fig. 10).

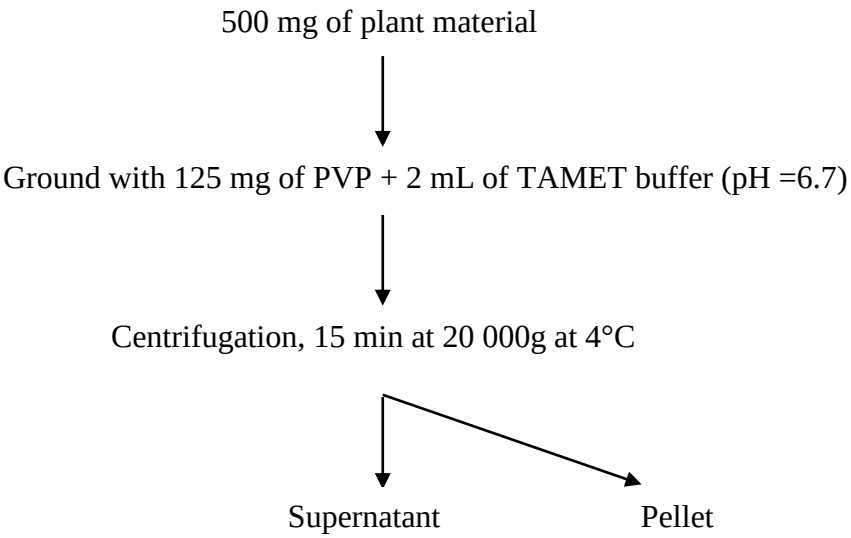


Figure 10. Diagram of soluble protein extraction's (Lecouteux, 1993).

- *Analysis of protein content*

The proteins were quantified according to the method described by Bradford (1976). It is a colorimetric method based on the variation observed on absorbance of the coomassie blue G 250, in which, in acidic medium, the brown-orange form turn into blue form which binds itself to NH_3+ functions of proteins. The fixation of the colorant to proteins stabilizes the anionic form (bleu). The increase of absorbance at 595 nm is directly proportional to the amount of colorant fixed, and to the quantity (concentration) of proteins present in the sample analyzed.

Two (2) mL of Bradford's reagent were added to 50 µl of protein extract and 185 µl of phosphate buffer. The mixture was incubated during 2 minutes. The absorbance of the blue colored complex was determined at 595 nm. The pure bovine serum albumin (BSA) ranging from 0.5 to 60 µg/µl was used as standard protein.

II.2.1.2.2 Estimation of soluble sugars contents

- *Samples preparation*

Soluble sugars were extracted according to the modified method of Babu et *al*. (2002). Biological material (400 mg) was ground in mortar with 2 mL of 80 % (v/v) ethanol and centrifuge at 6000 g for 20 min.

- *Analysis of soluble sugar content*

The total soluble sugars were evaluated using the anthrone method (Yemm and Willis, 1954). This method is based on the fact that simple monosaccharides having at least five carbon atoms are dehydrated and converted to furfural or furfural derivatives in concentrated and hot acid medium. The pentoses are transformed into furfural and hexoses into 5-hydroxymethylfurfural. These furfuralic derivatives are capable of combining with various phenolic substances having mobile H (anthron, orcinol, resorcinol).

In a series of test tubes, 50 µL of alcoholic extract was added to 5 mL of Anthron reagent, homogenized and incubated at 80°C for 20 min. After cooling in melting ice, the absorbance of the green complex formed was determined at 620 nm. Glucose was used as standard.

II.2.1.2.3 Estimation of phenolic contents

- *Samples preparation*

The extraction, embryonic mass (100 mg) were ground in chilled mortars with 2 mL of 80% (v/v) methanol at 4°C. After incubation, tubes were centrifuged thrice at 7000 g for 30 min, supernatant were recuperated each time.

- *Analysis of phenolic content*

The phenolic compounds were extracted and analyzed as described by El Hadrami (1995) and Macheix et *al*. (1990) using Folin and Ciocalteu reagent (mixture of phosphomolybdic and phosphotungstic acids). This method is based on the fact that this reagent is reduced in presence of phenols into a blue complex of molybden. This complex has a maximum absorption at 760 nm.

In a series of test tubes, 15 µl of alcoholic extract were added to Folin - Ciocalteu reagent (250 µl), 2.5 mL of distilled water and 0.5 ml of sodium carbonate (20 % (w/v)). The mixture was incubated at 40°C for 20 minutes and the blue color was determined at 760 nm. The content of soluble phenolic was expressed in mg-equivalent of gallic acid per fresh weight (FW).

II.2.1.2.4 Estimation of peroxidase (POX) activities

The peroxidase (POX) activity of crude protein extracts was measured spectrometrically at 420 nm by the guaiacol-H_2O_2 method of Erdelsky and Fric (1979). The peroxidases are

oxidoreductases that catalyze the oxidation of a substrate (proton donor) in the presence of hydrogen peroxide (H_2O_2), the reaction gives rise to tetragaiacol, a rusty red compound (absorbing light at 420 nm, approximately) and water (Fig. 11).

The peroxidase activities were measured in a reaction medium containing:
- 1 volume of hydrogen peroxide (H_2O_2) at 0.2%;
- 2 volumes of gaiacol at 1%;
- 5 volumes of phosphate buffer, 1/15 M pH 6.8.

Using gaiacol as a substrate (H^+ proton donor), the overall reaction is of the type:

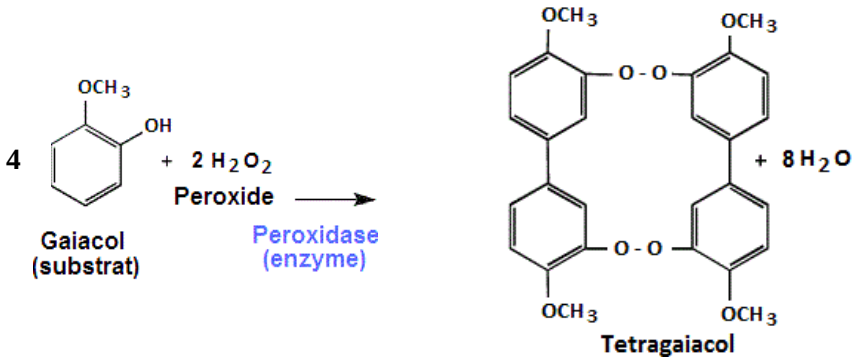


Figure 11. Peroxydase reaction using gaiacol a substrate.

In a series of test tubes, 5 ml of the reaction medium are introduced. In each tube, 50 µl of protein extract is added. The mixture was incubated for 5 minutes at room temperature and the variation of the rusty red color was determined at 420 nm on the spectrophotometer. The specific activity of the enzymes was expressed as the change in optical density per mg of proteins.

II.2.1.3 Statistical analysis

The data obtained from the experiment on the determination of the type and position of floral explants on the tree subjected to statistical analysis using SPSS software version 16.0. The analysis of variance and correlation were performed and differences between means were determined using LSD and Tukey Test.

II.2.2 Physiological responses of *T. cacao* to water stress induced by osmotic agents

II.2.2.1 Effect of PEG supply during callus differention stage on embryos formation and production

The effect of PEG supplementation in medium during callus differentiation stage on *T. cacao* somatic embryogenesis was evaluated as described in the screening diagram (Fig. 12). Briefly, plant material (callus) were regenerated from flower parts of SCA12, SCA6 and MA12 genotypes as previously described by Niemenak et *al*. (2008). For primary somatic embryogenesis expression, callus (aged of 28 days) were placed (25-35 per Petri dish) in embryo development (ED) medium supplemented with PEG at different concentrations (0.0 ; 2.5; 5.0 and 7.5 % (w/v)) to create the osmotic pressure of PEG-8000 solutions of 0.0; -0.17; -0.47 and -0.91 bars respectively. The calculation of osmotic pressure in the medium was made according to Michel (1983). After height days of incubation, callus were sub-cultured in ED medium. The Media were changed every 21 days and complete callus development was achieved after three months. All cultures were kept at 25±1°C in the darkness. Callus growth without PEG were used as control during our experimentation.

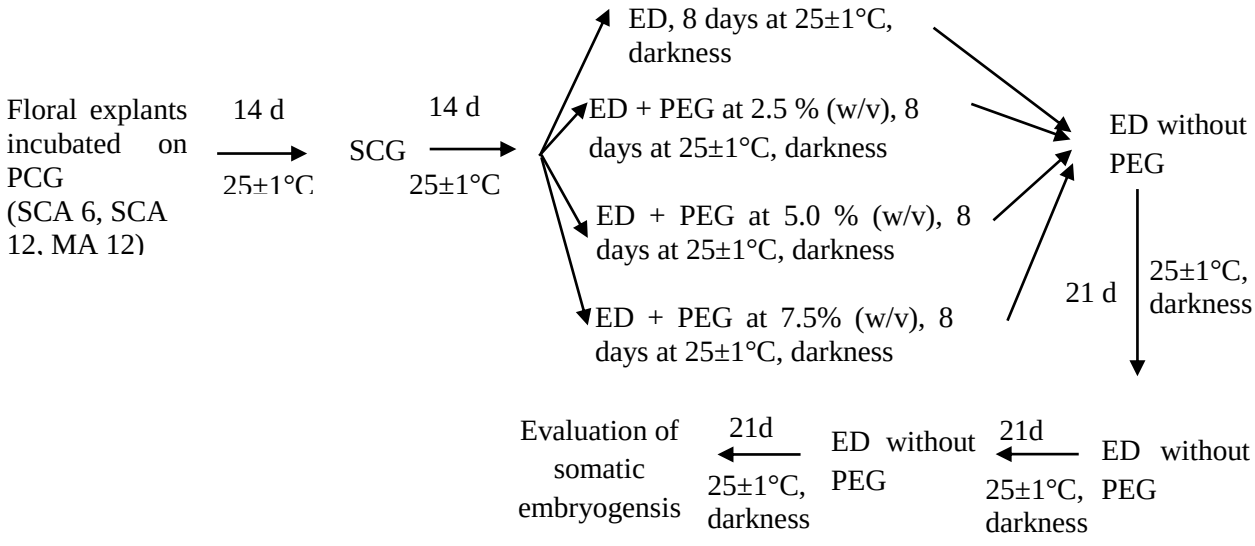


Figure 12. The screening diagram of the effect of PEG at different concentrations on primary somatic embryogenesis in *T. cacao.*

II.2.2.2 Biochemical assay during callus differentiation stage using spectrophotometer

II.2.2.2.1 Preparation of samples for analysis

The fresh and compact callus (65-100 mg) was extracted three times (25 ml each) with a mixture of CH_2Cl_2/MeOH (1:1) at room temperature. The solution was filtered through Whatman paper N°1 and then the removal of solvent under vacuum to obtain crude extracts. The crude extracs were dried, weighted and kept at 4°C for experimental used. The extract were separately dissolved in 80% methanol in a concentration of 1.5 mg/ml supplemented with a little droplets of dichloromethane, then filtrated through a syringe-filter-membrane before experimental used.

II.2.2.2.2 Analysis of phenolics and total flavonoids contents

- *Phenolics content*

The evaluation of phenolic content, Folin-ciocalteu reagent (1 ml) was added to 200 µl of alcoholic extract, 1 ml of distilled water and 1 ml of Na_2CO_3 (20% (w/v)). The mixture was incubated at 40°C for 20 min and the absorbance was determined at 760 nm. The phenolic content was expressed as mg-equivalent of catechine per fresh weight (FW).

- *Total flavonoids content*

The aluminum chloride colorimetric method is used for the determination of the total flavonoid content of the sample (Bahorun *et al*., 1996).

In a series of test tubes, 250 µl of alcoholic extract were mixed to 1 ml of distilled water and 1 ml of aluminium chloride 2% (w/v). The mixture was kept at room temperature for 10 minutes and the absorbance of the yellow complex formed was determined at 415 nm against the standard. The content of soluble flavonoid was expressed in mg-equivalent of quercetin per fresh weight (FW).

II.2.2.3 Effect of Polyethylene glycol, mannitol and sorbitol supply during maturation stage on the germination of secondary somatic embryos

The effect of exogenous osmoticum in medium during embryos maturation stage on *T. cacao* somatic embryogenesis was evaluated as shown in the screening diagram (Fig. 13). Briefly, the secondary somatic embryos (SSE) of SCA6 genotype were cultured according to Li et *al*. (1998) on ED media at a constant temperature of 26°C until they reached the early torpedo

stage. They were then transferred in ED media for maturation and germination under water stress conditions. Three chemical agents (PEG, mannitol and sorbitol) was supplemented at 5.0, 7.5 or 10.0 % (w/v) in ED medium of to create osmotic pressure of -0.47; -0.91 and -1.48 bars respectively. The calculation of osmotic pressure in the medium was made according to Michel (1983). After 14 days, the explants were sub-cultured in ED medium without chemicals agent and incubated at 25±1°C in the dark. The frequency of germination after three weeks on ED medium was calculated. Germination refers to both root and shoot development on an embryo with an intact hypocotyl (initiation of conversion to a plant).

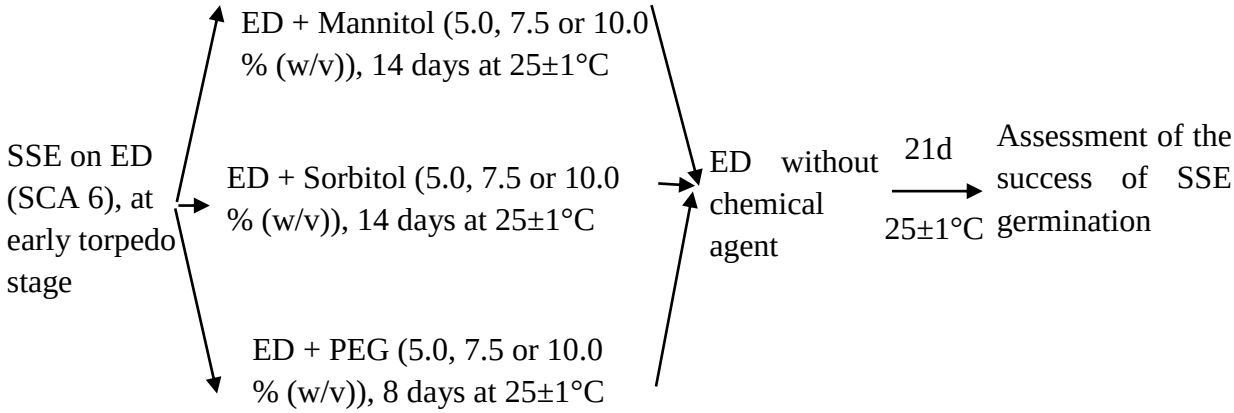


Figure 13. The screening diagram of the effect of osmoticum at different concentrations on embryos maturation and germination

II.2.2.4 Statistical analysis

The data were subjected to statistical analysis using “statistica” software version 8.0 (2007). Analysis of variance was performed where applicable and differences between means were determined using Fisher Test.

II.2.3 Metabolites involved in drought tolerance in *T. cacao*

II.2.3.1 Induction of water stress during callus differentiation stage

The twenty-eight days after introduction of explants of each genotype of *T. cacao* in embryos induction medium, a part of the callus was harvested (NS, D0) while the other part was harvested after four and eight supplementary days of growth in water stress conditions (WS, D4, D8) or in non-stressed conditions (NS, D4, D8). The osmotic stress was induced through application of middle drought stress (PEG-8000, 0.05 g/g; corresponding to -0.47 bars at 25°C) in ED medium. The evaluation of the water potential of the PEG-8000 solutions was done

according to Michel (1983). The callus of each clone were frozen at -80°C, then dried for 72 h and ground to a homogenous fine powder with a grinder before metabolites extraction.

II.2.3.2 Induction of water stress during seed germination and seedlings establishment

The seeds were germinated in the laboratory of biochemistry and plant physiology. Briefly, nine seeds were placed in dishes containing cellulose paper following a non-randomized block pattern with three replicates. A part of the seeds was harvested the first day (NS, D0) while the other part was harvested after four and eight supplementary days of growth in water stress treatment conditions (WS, D4, D8) or in control conditions (NS, D4, D8). Water stress was induced through application of middle drought stress (PEG-8000, 5% w/v; corresponding to -0.47 bars at 25°C) in incubation medium. The evaluation of the water potential of the PEG-8000 solutions was done according to Michel (1983). The seeds and seedlings of each genotype were frozen at -80°C, then dried for 72 h and ground to a homogenous fine powder with a grinder before metabolites extraction.

II.2.3.3 Biochemical characterization of metabolites using Nuclear Magnetic Resonance (^{1}H-NMR)

II.2.3.3.1 Extraction of metabolites (callus, cotyledons and hypocotyls)

The extraction was carried out according to the protocol described by Kim et al. (2010) with little modifications. Fifty (50) mg of lyophilized callus powder were transferred to 2 ml micro-tubes in which 500 µl of extraction buffer (MeOH/H_2O (50/50)) were added. The tubes were vortexed at 50°C for 10 min before ultrasonication (30 min) and centrifuged at 8000 rpm (corresponding to 14310 g) at 4°C for 10 min. The pellet was recovered in 300 µl of MeOH/H_2O mixture (50/50) vortexed at room temperature (10 min), sonicated in a bath ultrasound (20 min) and centrifuged (10 min). The pellet was recovered in 300 µl of MeOH/H_2O mixture (50/50) vortexed at room temperature (10 min), sonicated in a bath ultrasound (15 min) and centrifuged (10 min). The samples were concentrated in a SpeedVac and the pellets taken up in 800 µL of solubilization buffer (D_20/ MeOD, (50/50 (v/v)), KH_2PO4 0.1 M, TMSP-d4 0.0125% (w/v), pH 6. Tubes were vortexed for 10 min and centrifuged (10 min). Then, 800µl of the supernatant was transferred into NMR tube (Ø1.7 mm). Both deuterated methanol and water were purchased from Euriso-Top (Saint-Aubin, France).

II.2.3.3.2 NMR measurements

All spectra (^{1}H-NMR, J-resolved, COSY (correlation spectroscopy) and HSQC (heteronuclear single quantum correlation spectroscopy)) were recorded at 300 K on a Bruker Advance III 600 spectrometer operating at a frequency at 600.17 MHz for the ^{1}H using a multinuclear broadband TXI 5 mm probe. CD_3OD was used as solvent and as the internal lock. ^{1}H-NMR spectra were recorded with 128 scans, which were collected into 128 K data points with spectral width of 8403 Hz and a water suppression pulse frequence with a relaxation delay of 7.8 s. The flame ionization detector (FID) was multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation. The resulting ^{1}H-NMR spectra were automatically phased and baseline-corrected using Topspin (version 3.1, Bruker).

The two-dimensional J-resolved NMR spectra were acquired with a 3.9 relaxation delay using 16 scans per 128 increments, which were collected into 50 K data points, using a spectral width of 8417 Hz in F2 and 50 Hz in F1. The COSY NMR spectra were acquired with a 0.24 s relaxation delay using 16 scans per 512 increments, which were collected into 256 K data points, using a spectral width of 8417 Hz in F2 and 8402 Hz in F1. Similarly, the acquisition of HSQC NMR spectra were acquired with a 0.12 s relaxation delay using 128 scans per 256 increments, which were collected into 256 K data points, using a spectral of 8417 Hz in F2 and 30185 Hz in F1. All spectra were calibrated with TMSP.

II.2.3.4 Data treatment

The ^{1}H-NMR spectra were automatically reduced in ASCII files using Topspin. The procedure of alignment of spectra was carried out in several steps: noise reduction, baseline correction and alignment. The data were imported with the software Matlab (version 2013a, Mathworks Inc, Natick, MA, USA) for data processing. The baseline correction was done with the algorithm “airPLS 2.0” (Zhang *et al*., 2011). The spectra were aligned with the algorithm “icoshift” (v. 1.2), using the function average2 (Savorani *et al*., 2010); the patterns were designed automatically and corrected manually to perform the alignment of spectra. After this stage, the spectra were binned with the algorithm “dynamic adaptive binning” (DAB) (Anderson *et al*., 2010), then each bin was integrated.

The data were corrected by taking into account the noise defined in two areas (between δ 10 ppm and δ 9.5 ppm and between δ 0.5 and δ 0.3 ppm). These regions were integrated and averaged and each area half of this value was not included in the data. The proton signal

corresponding to methanol (between δ 3.34 ppm and δ 3.30 ppm) and the proton signals corresponding to TMSP (between δ 0.01 and δ 0.00 ppm) were also removed.

The metabolites were identified primarily by comparing the chemical shifts obtained on the spectra of the ^{1}H-NMR samples with the spectra of the database available in the laboratory. The identified metabolites were confirmed by comparison of 2-D spectra (J-Resolved, COSY and HSQC).

II.2.3.4 Statistical analysis

The generated data were analysed by principal component analysis (PCA) and partial lest square discriminant analysis (PLS-DA) with mean-centring were performed with SIMCA-P software (version 11.0, Umetrics, Umea, Sweden) using UV as the scaling method.

The mean area of integrated regions for each metabolite was analysed with a non-parametric statistical hypothesis test: the Wilcoxon Rank Sum test. This was performed within R's statistics base-package (v. 3.0.2) at the different p-values ($p < 0.05$; $p < 0.01$; $p < 0.001$).

CHAPTER III: RESULTS AND DISCUSSION

III.1. RESULTS

III.1.1 Influence of the position of flower buds in a single tree on somatic embryogenesis of *T. cacao*

III.1.1.1 Callus induction frequency

There were no significant differences ($p<0.05$) among the average frequency of callogenesis (after 28th days) when explants were cultivated in DKW medium containing plant growth regulators. However, callus growth was most influenced by the type and origin of explant used. The average callogenesis frequency was up to 80%, with the highest value obtained with staminodes explants collected on primary fan branch (93.97±2.43%) (Table 2).

Table 2. Average frequency of callogenesis in SCA6 genotype of *Theobroma cacao* L., 28th days after induction in Primary Callus Growth (PCG) medium.

Source	Orthotropic main Stem	Primary Fan branch	Secondary fan branch
% of staminodes-derived callus	92.50 ± 4.97^{a}	93.97 ± 2,43^{a}	81.23 ± 6.40^{a}
% of petals-derived callus	83.47 ± 9.87^{a}	90.17 ± 5.00^{a}	93.63 ± 1.78^{a}

Each value is mean ± SE of three replicates samples. Values significantly different at the 5% level of significance are indicated with different letters for the same type of explant.

III.1.1.2 Embryos induction frequency

At the end of three-weeks in embryo development medium, some callus differentiated roots or embryos. The percentage of callus producing somatic embryos after 91 days were low during all the experiment. Petals-derived callus of flower buds collected on secondary plagiotropic fan branches do not differentiate somatic embryos. No statistical differences ($p<0.05$) were found among the rate of petals-derived callus producing somatic embryos from different floral buds position. Whereas in staminodes-derived callus this rate increase significantly when moving up from orthotropic main stem (OS) to secondary fan (FII) branch. In fact, this rate of explants-derived callus producing somatic embryos were approximately 3 times higher in staminodes tissues than petals after 91st days of culture, with the highest value obtained with staminodes-derived callus from FII branch (13.70 ± 3.21%) (Fig. 14).

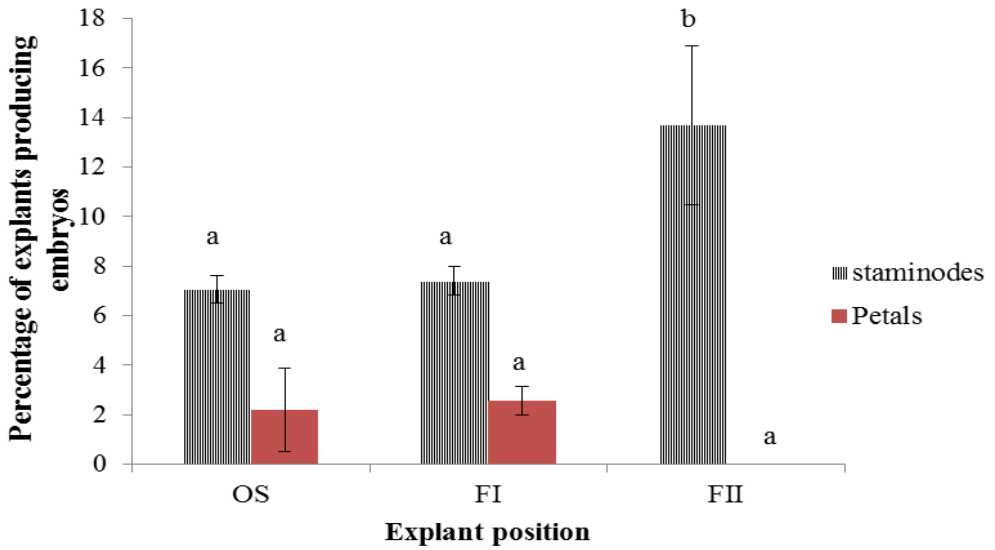


Figure 14. Influence of the position of flowers buds on the percentage of explants of *T. cacao* producing somatic embryos after 91st days. Data are presented as means of five identical experiments. Flowers buds were taken on the orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII).Values significantly different at the 5% level of significance are indicated with different letters for the same type of explant (staminode or petal).

The morphogenic structures of *T. cacao* during regeneration with floral explants are presented in Fig. 15 and shows that when petals (Fig. 15 A) of OS were used as starting material, callus were well discernable on these tissues after 14 days on PCG medium (Fig. 15 B). The transfer of this explant on secondary callus growth medium (SCG) allow a good proliferation of these tissues. Embryogenic and non-embryogenic tissues of *T. cacao* callus were discernable based on coloration; embryogenic callus were brown (phenolized) and friable, while non-embryogenic were white and rough (data not shown). Somatic embryos undergo different developmental stages as shown by the result: globular, heart shaped, torpedo and cotyledonnary stage (Fig. 15. C, D, E and F respectively). Cotyledonnary stage constituted the last development stage. At this stage, somatic embryos submitted to maturation treatment were able to germinate and generate plantlets (Fig. 15. G, H and I)

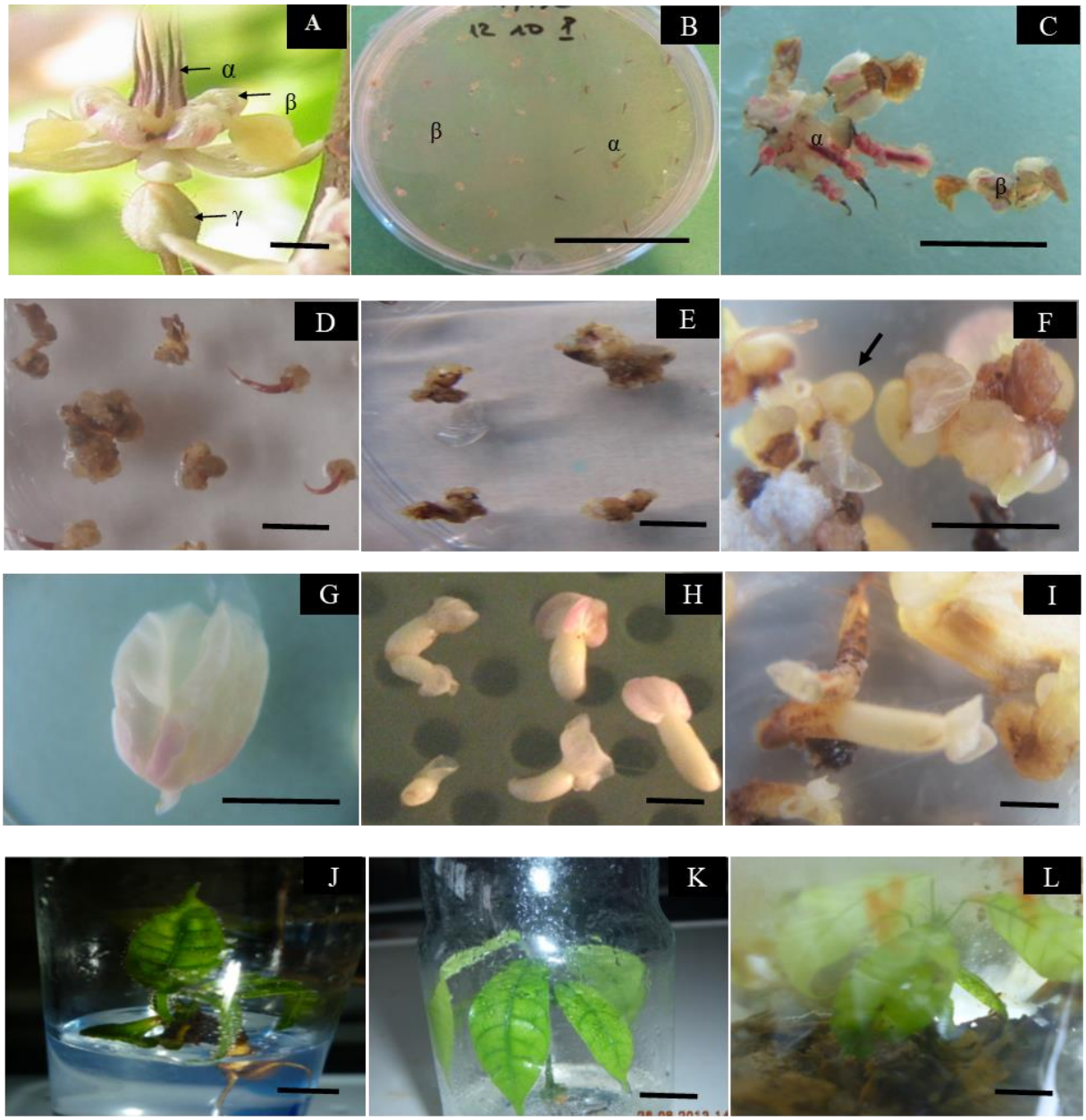


Figure 15. Morphogenic structure of *T. cacao* during somatic embryogenesis and plantlets conversion. Plant materials (A): staminodes (α), petals (β), and immature floral buds (γ). Disposition of floral explants in Petri dish (B); Explants-derived callus from orthotropic main stem growing in primary callus growth medium (PCG) (C); staminodes-derived callus (D) and petals-derived callus (E) in Secondary Callus Growth medium (SCG); Characteristic developmental stages of SE: globular (F, narrow), heart shape (G), torpedo (H) and cotyledonnary stages (I). Plantlet originated from somatic embryos matured under high concentration of sucrose, 90 (J) and 120 (K) days after transfer in root development medium. Well-developed plantlet just allocated in the substrate (L). Bar =1 cm.

III.1.1.3 Evolution of Phenolic, Sugar and Protein contents callus during somatic embryogenesis

Total phenolics compounds during somatic embryogenesis were analyzed in staminodes- and petals-derived callus from orthotropic (OS) and fan (FI and FII) branches, using SCA6 genotype. The amount of phenolic compounds was significantly higher in staminodes-derived callus from OS than FI and FII whatever the developmental stages (Fig. 16 A). The total phenolics content in callus was found to increase during secondary callus growth stage, with a maximum value (6.27 ± 6.23 mg/g of FW) obtained in OS after 28th days. However, during embryos expression stages, the amount of phenolic compounds decreased in staminodes-derived callus, leading to a maximum reduction rates of 48%, 39% and 41%, for OS, FI and FII respectively, after 49th days. In petals-derived callus, except for secondary callus growth stage (after 14th days), where the contents of phenolic compounds were found to decrease in FI and FII, the same trend was observed during our experiment (Fig. 16 B).

Constitutively, soluble sugars were low in callus from all origins during callus growth stage. The content of soluble sugars in staminodes-derived callus was found to increase during embryos induction stage, with a maximum value (7.28 ± 0.43 mg g^{-1} of FW) obtained with FI branch after 49th days; while dereasing progressively during embryos differentiation (after 49th days) and establishment stages (after 70th days) (Fig. 17 A). In petals-derived callus, phenolic compounds were found to be more accumulated in FII branch (6.36±0.18 mg g^{-1} of FW) after 49th days than in OS (5.0±0.18 mg g^{-1} of FW) and FI branch (4.61±0.18 mg g^{-1} of FW). At the contrary, petals-derived callus from FII branch displayed the lower phenolic compounds content after 91st days of culture (Fig. 17 B). But no differences were found between FII branch and OS during this period.

The protein contents in staminodes-derived callus varied from 0.54 ± 0.18 mg g^{-1} of FW (FII after 28th days) to 2.18 ± 0.37 mg g^{-1} of FW (OS after 70th days) whereas in petals-derived callus it varied from 0.72 ± 0.03 mg g^{-1} of FW (OS after 70th days) to 1.85 ± 0.24 mg g^{-1} of FW (OS after 28th days). Protein content was significantly higher in petals-derived callus from FII aged of 49th days (1.81 ± 0.42 mg g^{-1} of FW) than in OS (0.91 ± 0.18 mg g^{-1} of FW) from the same source. It appears that, cell differentiation is characterized by high protein synthesis in staminodes tissues (Fig. 18).

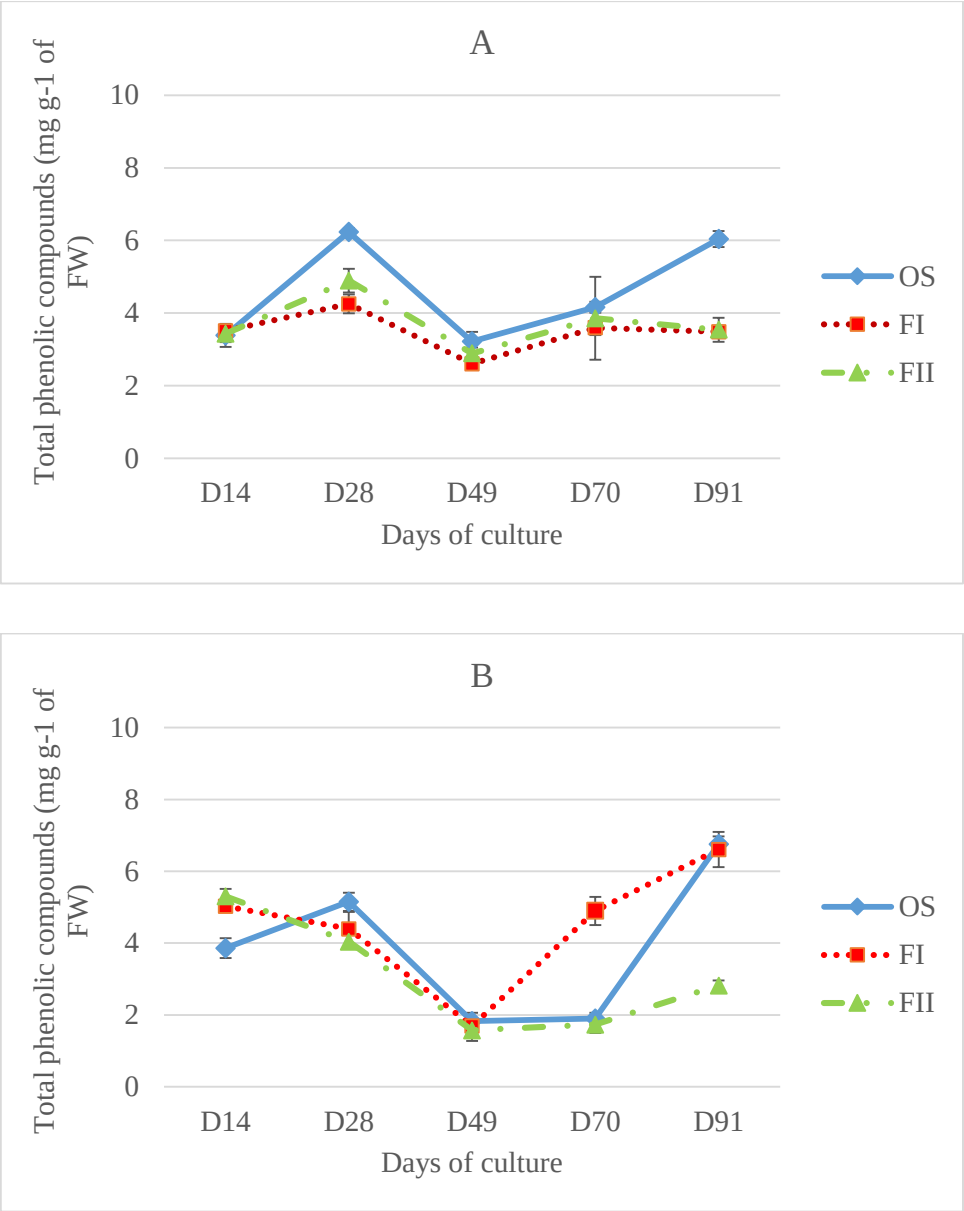


Figure 16. Influence of the position of flowers buds on the tree on total phenolics content during somatic embryogenesis of *T. cacao*. Staminodes (A) and petals (B); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (BI) and secondary plagiotropic fan branch (FII). Vertical bars represent standard error. Each plot was drawn from means of four identical experiments.

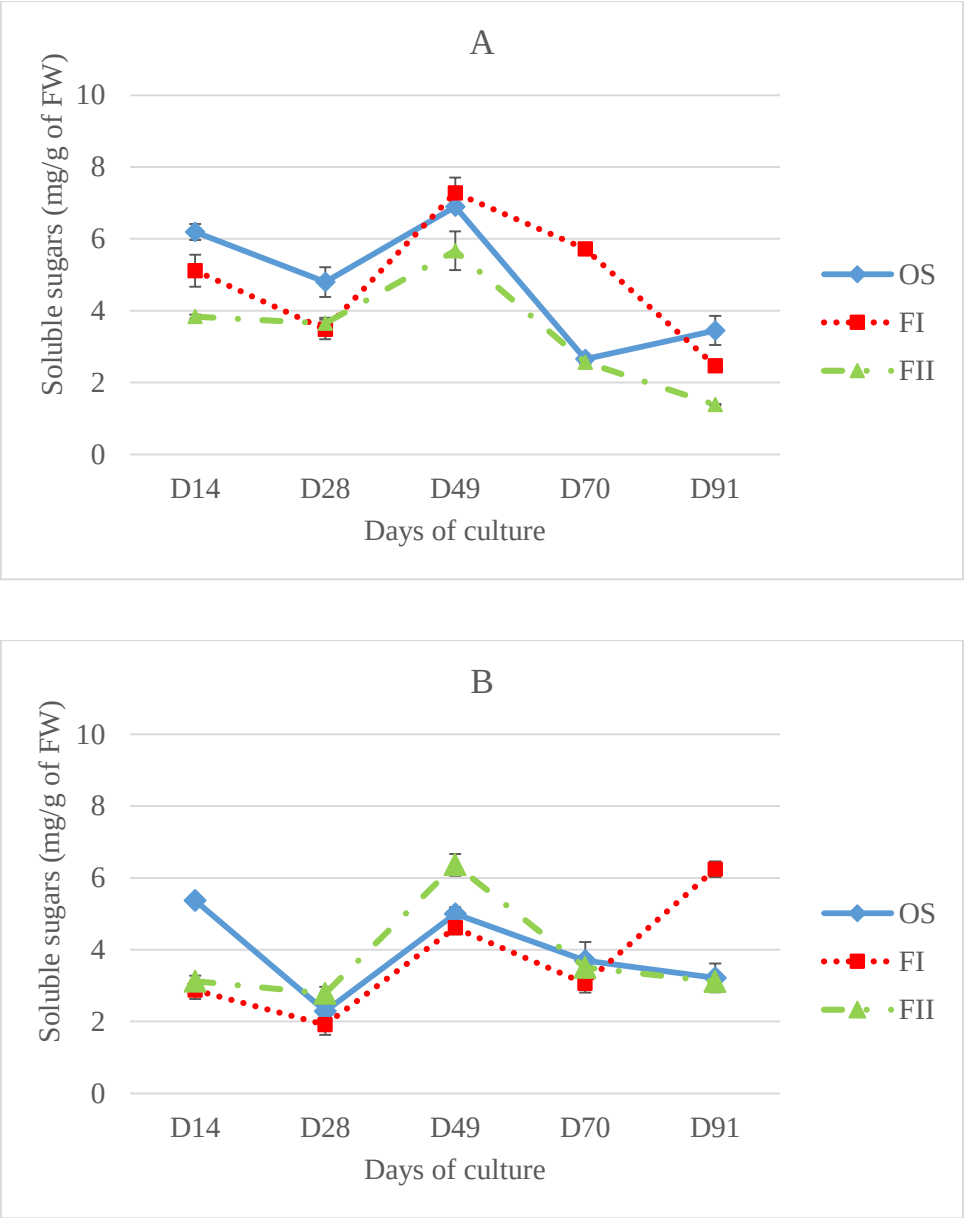


Figure 17. Influence of the position of flowers buds on the tree on total soluble sugars content during somatic embryogenesis of *T. cacao*. Staminodes (A) and petals (B); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII).Vertical bars represent standard error. Each plot was drawn from means of four identical experiments. Significance was determined at $P<0.05$ using ANOVA.

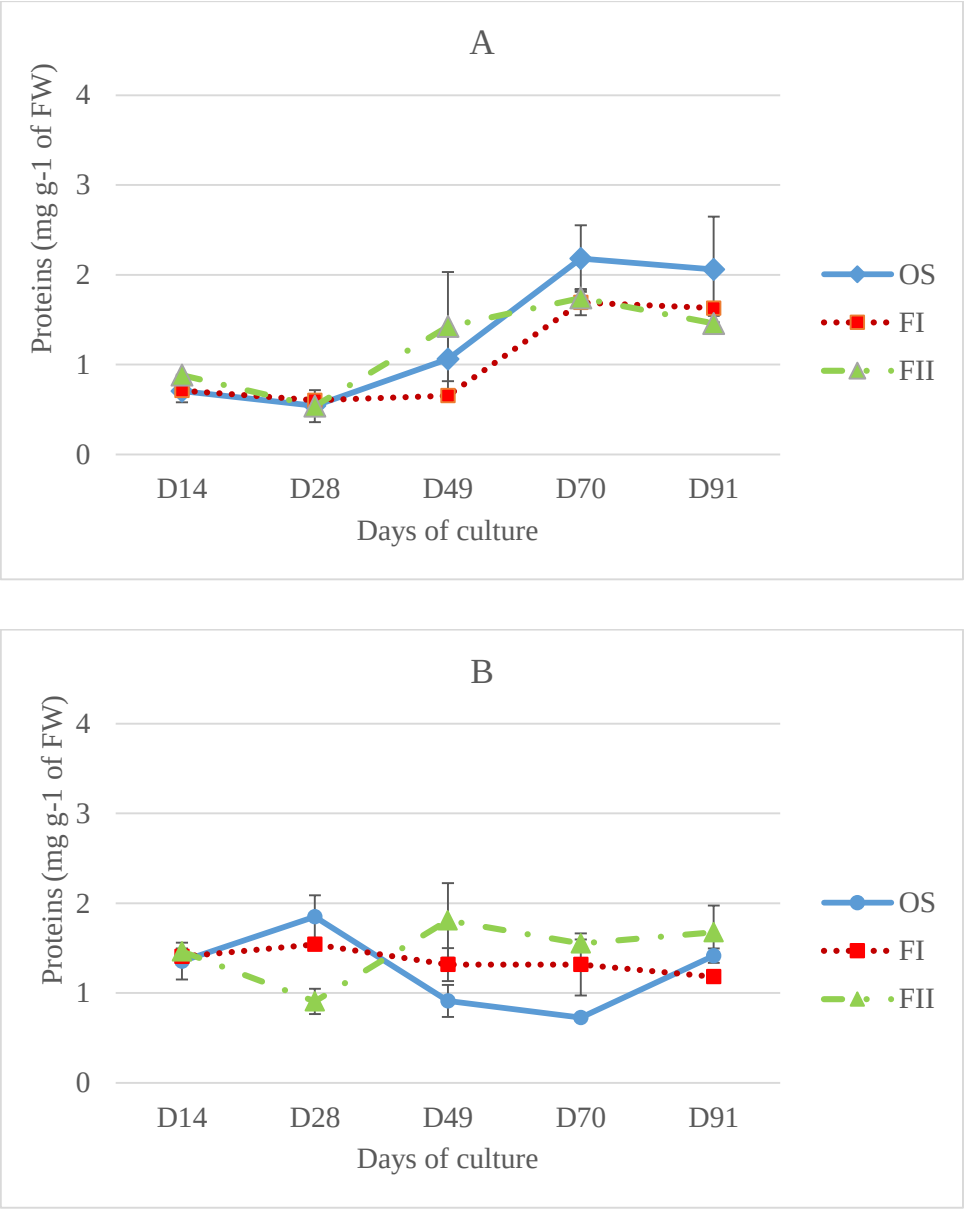


Figure 18. Influence of the position of flowers buds on the tree on proteins content during somatic embryogenesis. Staminodes (A) and petals (B); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII). Vertical bars represent standard error. Each plot was drawn from means of four identical experiments. Significance was determined at $P<0.05$ using ANOVA.

III.1.1.3 Evolution of peroxidase (POX) activities

Crude extracts of each source of tissues (at different developmental stages of callus) of SCA6 genotype were submitted to enzymatic analysis. The specific activitie of peroxidase enzymes (POX) presented pattern related to a source of morphogenetic structure. Staminodes-derived callus showed a more increased specific activities of POX during callus growth stage. The ratios measured in these callus after 28th days (corresponding to embryos induction step) between FI

and OS and between FI and FII were approximately 1.37 and 1.31, respectively. However, a reduction of these activities was observed during the following embryos expression stages (Fig. 19 A). In petals-derived callus, the activity of POX was high in tissues from both sources during callus growth stage and embryos induction step, and FII displayed the maximum value; whereas a rather low activitie was measured during embryos differentiation and establishment steps and FI displayed the lowest value (after 91st days) (Fig. 19 B).

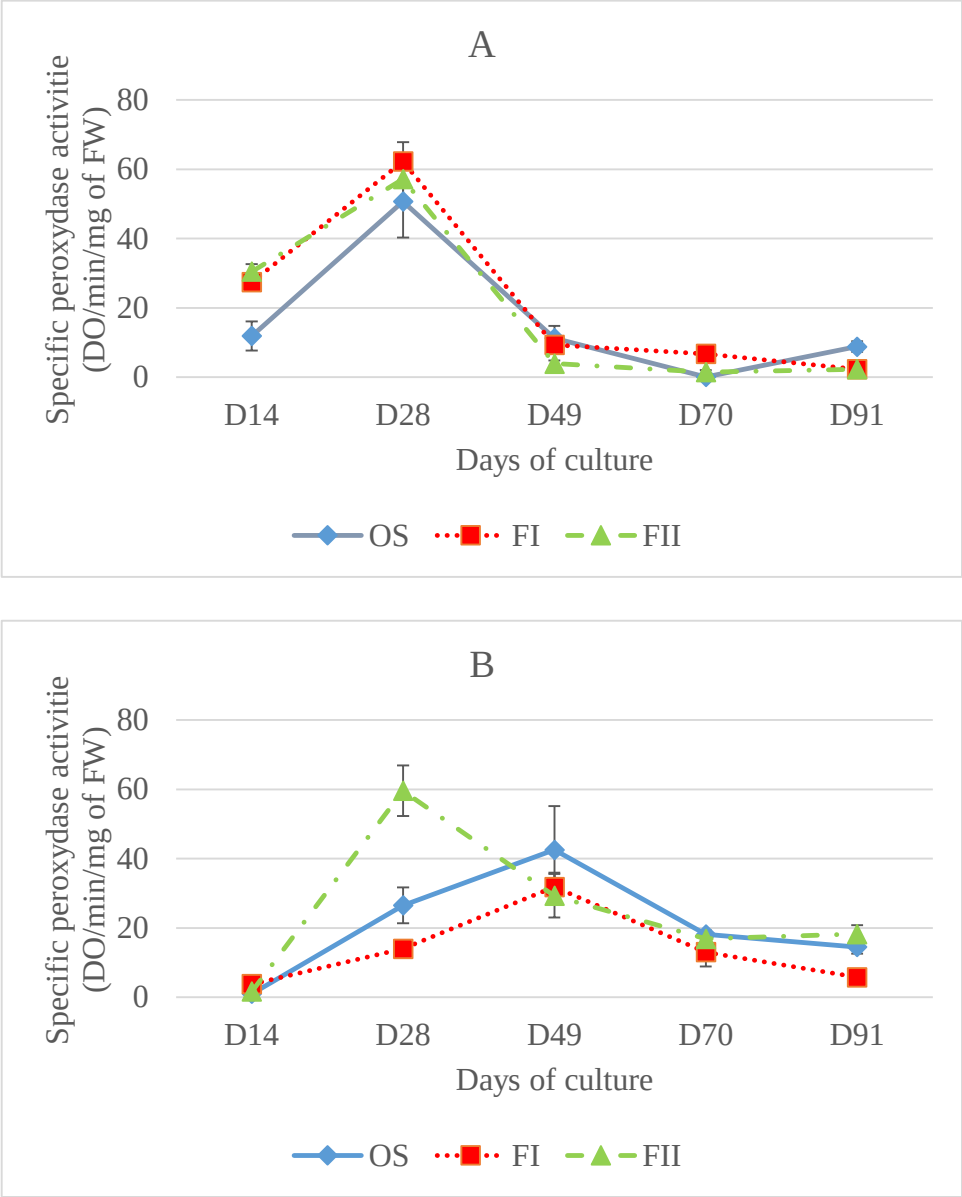


Figure 19. Influence of the position of flowers buds on the tree on peroxidases activities during somatic embryogenesis. Staminodes (A) and petals (B); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII).

Vertical bars represent standard error. Each plot was drawn from means of four identical experiments. Significance was determined at $P<0.05$ using ANOVA.

III.1.1.4 Correlations among phenolic, sugar and protein contents in orthotropic main stem, primary- and secondary plagiotropic fan branches

With staminodes-derived callus, a negative and highly significatve correlation ($r=-0.674$; $p<0.01$) was observed between proteins content and POX activities in OS. Proteins and sugars contents were also negatively and significatively correlated ($r=-0.526$; $p<0.05$) in OS. Meanwhile, a positive and significative correlation ($r=0.632$; $p<0.05$) was observed between phenolics content and POX activities on tissues from OS. In FI, a negative and highly significative correlation ($r=-0.839$; $p<0.01$) was observed between proteins content and POX activities. Whereas in the same tissue a rather positive and significative correlation ($r=0.575$; $p<0.05$) was observed between phenolics content and POX activities. In addition, in FII, a positive and highly significative correlation ($r=0.684$; $p<0.01$) was observed between phenolics contents and POX activities. Whereas, at the contrary a negative and highly significative correlation ($r=-0.642$; $p<0.01$) was observed between proteins content and POX activities. Sugars and phenolics contents were also negatively and significatively correlated in FII (Table 3).

The same analysis carried out on petals-derived callus showed not similar correlation. In OS, a negative and significative correlation ($r=-0.598$; $p<0.01$) was observed between sugars content and POX activities. While a positive and significative correlation ($r=0.770$; $p<0.05$) was observed between phenolics and proteins contents. In FI, a negative and highly significative correlation ($r=-0.894$; $p<0.01$) was observed between phenolics content and POX activities. In addition, in FII, sugars and proteins contents were positively and highly significatively correlated ($r=0.747$; $p<0.01$). Phenolics and proteins contents were also negatively and significatively correlated (Table 4).

Table 3. Pearson correlation matrix among phenolics, sugars and proteins content and peroxidase activities in staminodes-derived callus from orthotropic main stem, primary and secondary fan branches.

Source	OS				FI				FII			
Parameter	Sug	Phe	Pro	Pox	Sug	Phe	Pro	Pox	Sug	Phe	Pro	Pox
Sug	1				1				1			
Phe	0.166	1			-0.242	1			-0.635*	1		
Pro	-0.526*	-0.043	1		-0.318	-0.326	1		-0.319	0.058	1	
Pox	0.386	0.632*	-0.674**	1	0.173	0.575*	-0.839**	1	-0.260	0.684**	-0.642**	1

Sug (sugars content in tissues); Phe (phenolic content in tissues); Pro (proteins content in tissues); Pox (peroxidase activity in tissues); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII). *. Correlation is significative at p< 0.05 (bilateral);**. Correlation is significative at P< 0.01 (bilateral).

Table 4. Pearson correlation matrix among phenolics, sugars and proteins contents and peroxidase activities in petals-derived callus from orthotropic main stem, primary and secondary fan branches.

Source	OS				FI				FII			
Parameter	Sug	Phe	Pro	Pox	Sug	Phe	Pro	Pox	Sug	Phe	Pro	Pox
Sug	1				1				1			
Phe	-0.378	1			0.181	1			-0.595*	1		
Pro	-0.479	0.770**	1		-0.482	-0.073	1		0.747**	-0.602*	1	
Pox	-0.598*	-0.290	-0.190	1	0.045	-0.894**	-0.175	1	0.008	-0.070	-0.458	1

Sug (sugars content in tissues); Phe (phenolic content in tissues); Pro (proteins content in tissues); Pox (peroxidase activity in tissues); position of flowers: orthotropic main stem (OS), primary plagiotropic fan branch (FI) and secondary plagiotropic fan branch (FII).*. Correlation is significative at p< 0.05 (bilateral);**. Correlation is significative at P< 0.01 (bilateral).

III.1.2 Evaluation of the physiological responses of *T. cacao* to water stress due to osmotic agents

III.1.2.1 Effect of PEG on callus differentiation

III.1.2.1.1 Morphology and embryos production

Four-weeks-old callus regenerated from floral explants (staminodes) of secondary plagiotropic fan branches of SCA12, SCA6 and MA12 genotypes was transferred in ED medium supplemented with different concentrations (0–7.5 %) of PEG and incubated 8 days in controlled conditions. It was noticed that increasing concentration of PEG resulted in progressive changing of callus morphological aspects from whitish-brown and rought to brown and friable. In some cases, callus exhibited rhizogenic characteristics, thus reducing the regeneration abilities, but this was less common with 7.5% PEG (Fig. 20). All the callus tissues proliferated in the various PEG concentration medium with only little differences in their morphological aspects (data not shown).

The frequencies of callus producing SE is genotype- and treatment-dependent as shown by Fig. 21. In normal condition (0.0 % PEG) the frequency of callus producing SE (11.18 ± 1.37 %) is significantly higher in SCA6 as compared to SCA12 (4.97 ± 0.61 %) and MA12 (3.58 ± 0.63 %). After inclusion of 2.5 % PEG, no statistical differences were found ($p<0.05$) among the frequency of callus producing SE in the three genotypes, but the highest value was obtained with SCA6 (5.50 ± 1.56 %). However, with 5.0 % PEG, the frequency of callus producing SE was significantly higher in SCA12 (5.77 ± 0.46 %) as compared to SCA6 (5.77 ± 0.46 %) and MA12 (4.04 ± 0.67%), while no significant differences were found between SCA6 and MA12 frequencies for this treatment. In addition, inclusion of 7.5 % PEG lowered the frequency of callus producing SE in SCA12 and SCA6 (4.12 ± 0.38 % and 1.63 ± 1.96 %). However, no significant differences were found between SCA6 and MA12 values for this treatment.

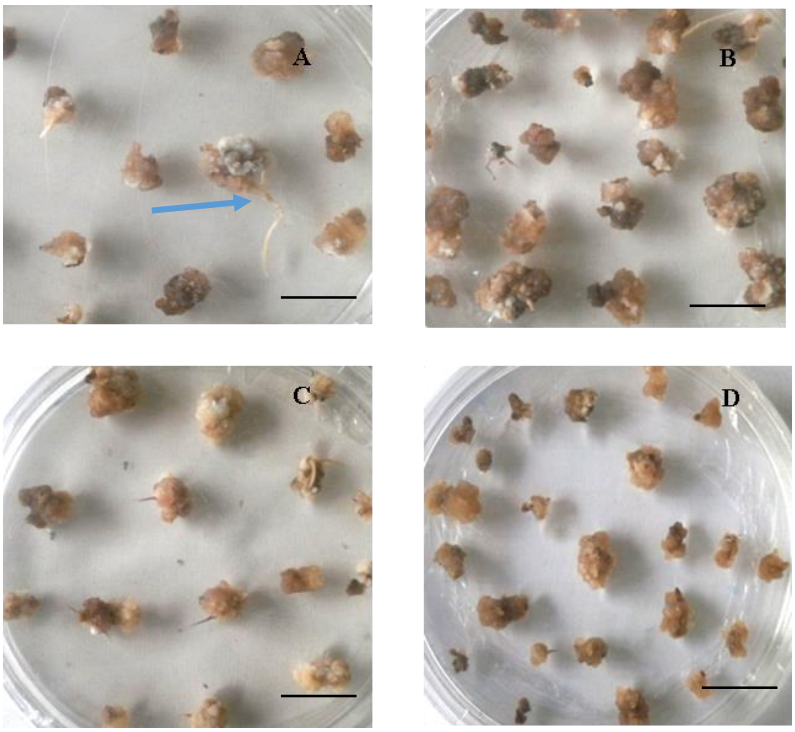


Figure 20. Morphological aspects of callus of *T. cacao* (SCA6 genotype), 8 days after incubation in PEG solutions at different: ED (A); ED + 2.5 % PEG (B); ED + 5.0 % PEG (C) and ED + 7.5 % PEG (D); rhizogenic callus (blue narrow). (Bar= 0.5 cm).

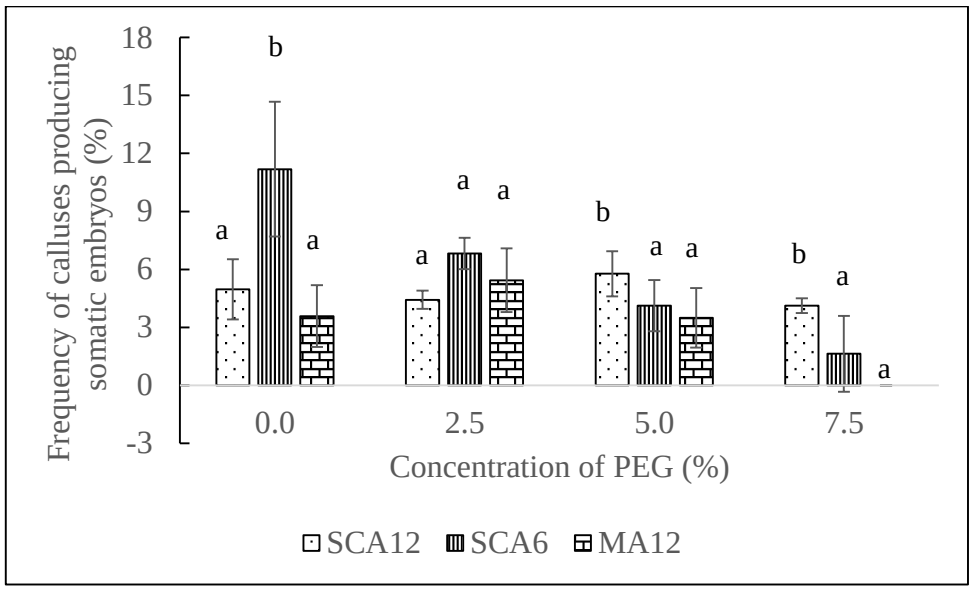


Figure 21. Comparison of the frequency of callus producing somatic embryos of SCA12, SCA6 and MA12, after supplementation of PEG during callus differentiation stage. The vertical bars represent standard error. Each plot was drawn from means of six identical experiments. The values significantly different at the 5% level of significance for the same treatment are indicated with different letters.

III.1.2.1.2 Average number of somatic embryos formed per callus

The average number of SE formed per callus is influenced by the treatment and the origin of the explant used (Fig. 22). Under normal condition (0.0 % PEG), no statistical differences were found among the average number of SE formed per callus, with the highest value (3.21 ± 0.59) obtained in SCA12 and the lowest (1.70 ± 0.32) in SCA6. After inclusion of 2.5% PEG, the average number of SE formed per callus varied from 3.0±0.31 (in SCA12) to 1.07 ± 0.07 (in SCA6), whereas with 5.0% PEG, it varied from 2.21 ± 0.31 (in SCA12) to 1.29 ± 0.18 (in MA12). No torpedo-shaped SE were formed in MA12 after inclusion of 7.5% PEG, whereas the average number of SE formed per callus was found significantly higher in SCA12 (1.57 ± 0.20) than in SCA6 (0.43 ± 0.20) for the same treatment.

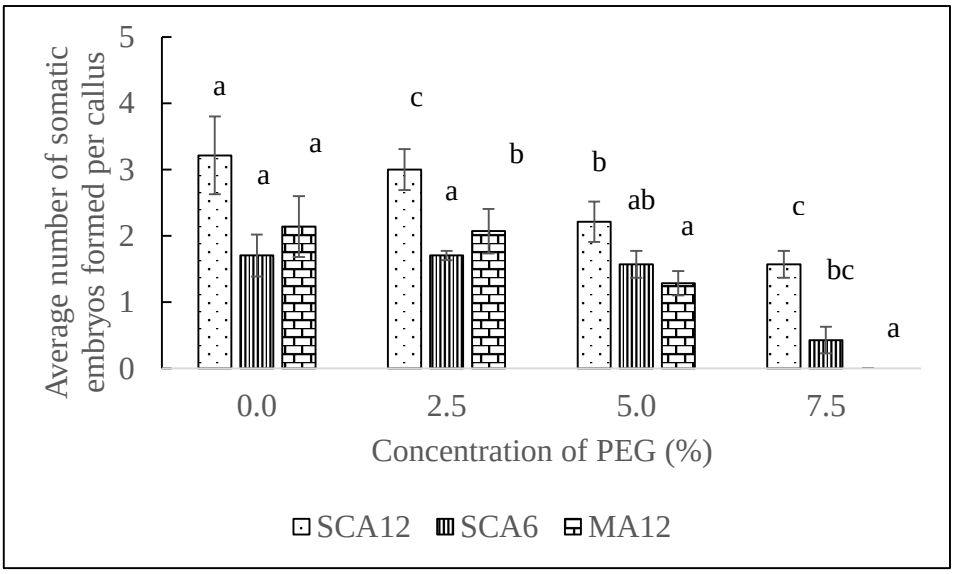


Figure 22. Influence of the concentration of PEG on the average number of somatic embryos formed in SCA12, SCA6 and MA12 genotypes, sub-cultured during 91 days in ED medium. The vertical bars represent standard error. The values significantly different at the 5% level of significance for the same treatment are indicated with different letters.

III.1.2.1.3 Evolution of phenolics and total flavonoid contents of callus

The phenolic content in callus of *T. cacao* genotypes is highly influenced by the level of PEG-induced water deficit in the medium. In SCA12, reduction of phenolics content in callus was detected. The highest phenolics content (29.69 ± 6.28 mg/g of equivalent catechine (CE) of FW) was produced in SCA12 treated with 0.0% PEG (control) as shown by Fig. 23, and this content decreased progressively with increasing concentration of PEG in the medium. There

was no regular evolution of this parameter after PEG exposure in SCA6 callus, as callus displayed a high phenolic content with 2.5% PEG (15.05 ± 1.39 mg/g CE of FW), followed by 7.5% PEG (12.06 ± 0.7 CE of FW) and 5.0% PEG (9.32 ± 1.59 CE of FW) as compared to control. The same observation was made in MA12 callus, where phenolics content was more accumulated with 2.5% PEG (18.8 ± 1.44 CE of FW).

Exposure of callus to water deficit stress led to a marked decline in flavonoid content (Fig. 24). Indeed, SCA12 callus showed a reduced flavonoid content at more than 38% with 2.5% PEG and reached a maximum rate of reduction close to 93% with 7.5% PEG, as compared to control. In addition, SCA6 callus also displayed a smaller flavonoid content with PEG than the control (9.50 ± 1.56 mg/g equivalent quercetine (QE) of FW in 2.5% PEG, 8.99 ± 0.04 mg/g QE of FW with 5.0% PEG and 2.88 ± 0.09 mg/g QE of FW with 7.5% PEG). Finally, MA12 callus was found to exhibit a reduced flavonoid content of about 33% with 2.5% PEG and reached a maximum and significattive rate of reduction close to 71% with 5.0% PEG. But this content was increased in callus treated with 7.5% PEG as compared to control. Differences were not statistically significative with 7.5% PEG medium for this genotype in relation to control.

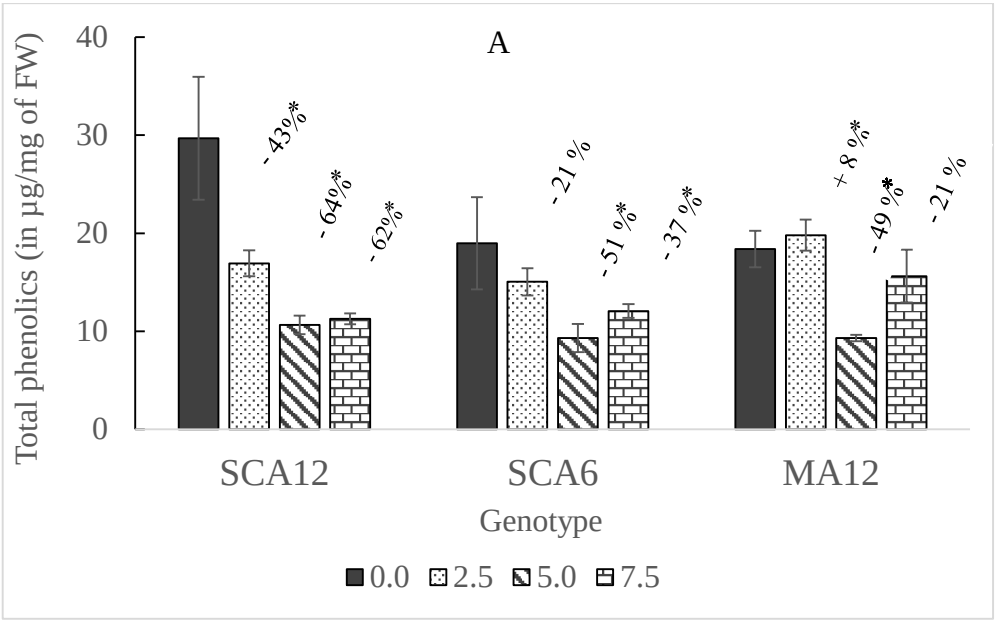


Figure 23. Content of total phenolics in callus from SCA12, SCA6 and MA12 genotypes grown in medium with PEG during callus differentiation stage. Each value is mean ± SD of three replicates samples. The increase or decrease content (in percentage) in relation to control is indicated just beside the mean±SD value. Asterisks represent diffferences statistically significant in relation to control ($p<0.05$).

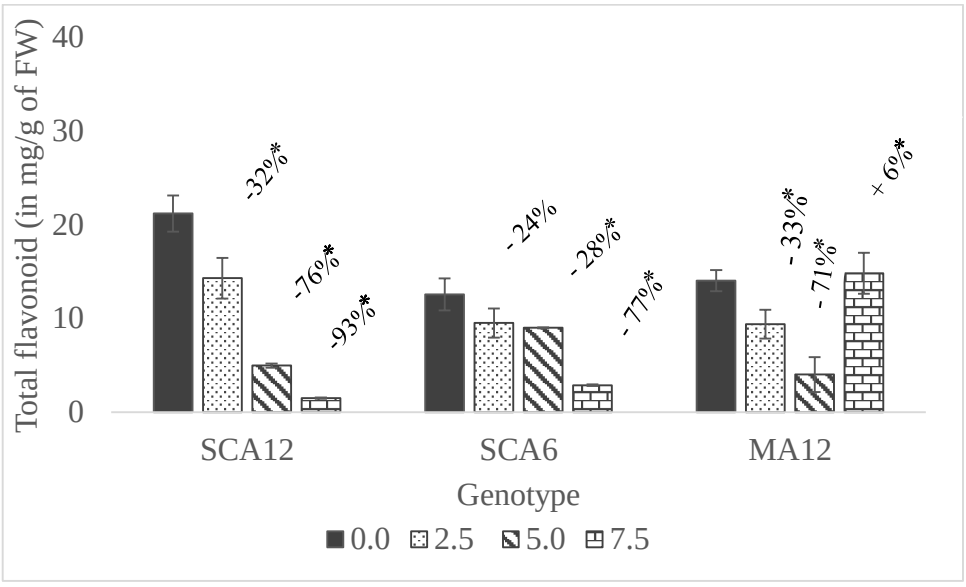


Figure 24. Content of total flavonoid in callus from SCA12, SCA6 and MA12 genotypes grown in medium with PEG during differentiation stage. Each value is mean ± SD of three replicates samples. The increase or decrease content (in percentage) in relation to control is indicated just beside the mean ±SD value. Asterisks represent diffferences statistically significant in relation to control ($p<0.05$).

III.1.2.2 Effects of mannitol, sorbitol and PEG supply during the maturation stage on the germination of secondary somatic embryos

Secondary somatic embryos (SSE) development was influenced by the type and the concentration of the chemical agent tested (mannitol, sorbitol or PEG) during maturation stage. The embryos exhibited a polar structure with an apical and basal differentiation after exposure to mannitol and PEG. In contrast, the apical-basal structure of differentiated embryos was not observe when SSE were exposed to sorbitol during maturation treatment, and in some case a bush-like architecture was observed (Fig. 25). The well-dessicated mature embryos were green in color with anthocyanin rich cotyledon and the over-dessicated embryos were brown (whole plant). These last were difficult to germinate so they were excluded from evaluations of germination frequencies.

Table 5 shows the reponses of SCA6 embryos to three different maturation media. In PEG, SCA6 embros from ED + 7.5 % (w/v) PEG exhibited the highest germination frequencies (67%), though statistical differences were found only between ED + 5.0% and ED + 10% media. In mannitol, embryos matured with 7.5% mannitol had the highest germination frequencies, and germination was reduced with 5.0% and 10%, with significant differences among these media. In a subsequent study, SCA6 embryos matured with ED + 5.0% sorbitol had the highest germination frequencies, as compared to 7.5% and 10.0%. The differences were found only between 5.0% and 10.0% sorbitol media. The combined analysis of data showed highly significant differences among the treatments at 7.5% (PEG, mannitol and sorbitol).

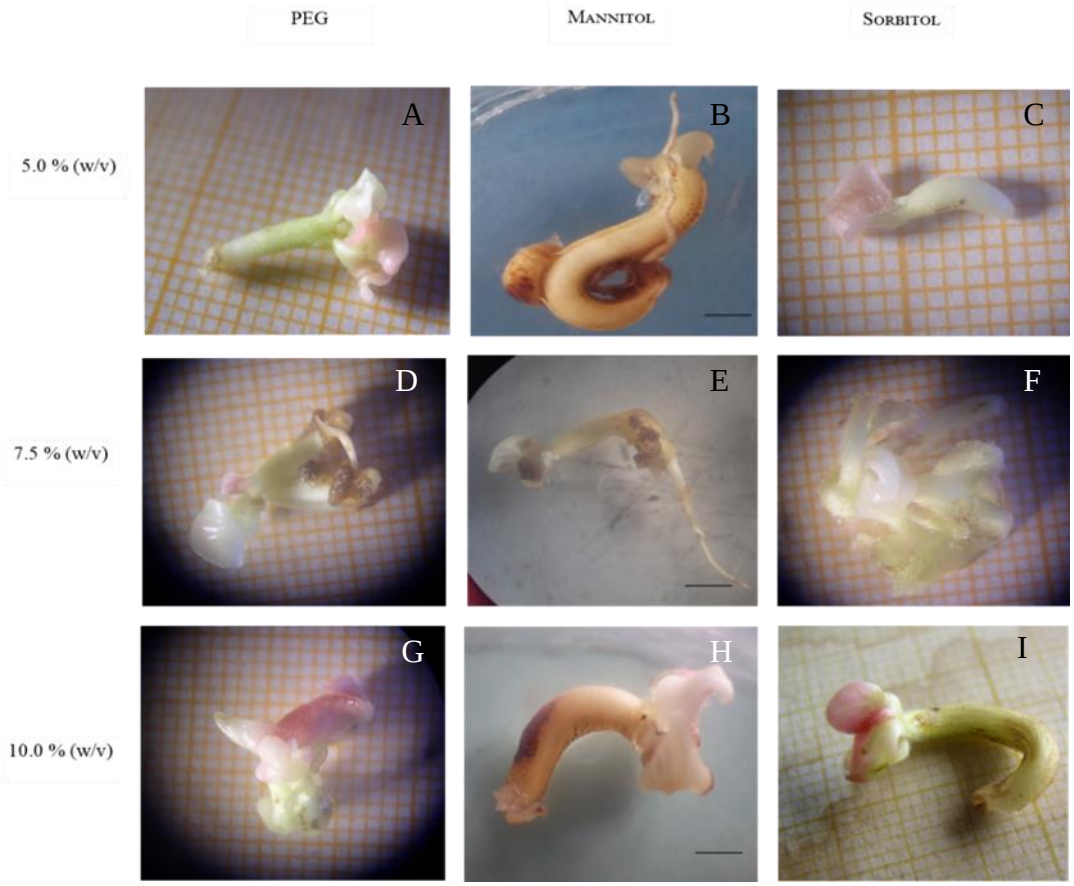


Figure 25. Morphological aspect of somatic embryos treated with different exogenous osmotica during maturation, after 21 weeks on ED medium without osmotica. Green embryos with anthocyanin rich cotyledon (A); over-dessicated embryos (B) and low-dessicated embryos (C). Embryos with an apical and basal differentiation after exposure to ED+7.5% PEG (D) and ED+7.5% mannitol (E); embryos with a bush-like architecure after exposure to ED+7.5% sorbitol (F). Embryos with only apical differentiation after exposure to ED+10.0% PEG (G), ED+10.0% mannitol (H) and ED+10.0% sorbitol (I). Bar=0.5 cm.

Table 5. Germination frequencies (%) of somatic embryos (SCA6 genotype) maturated with various osmoticum, and cultured on ED medium containing 3.0% sucrose during one month.

Treatment / Concentration	PEG	Mannitol	Sorbitol
5.0 %	20.83 ± 6.45a	33.33 ± 6.45b	16.67 ± 6.45b
7.5 %	66.67 ± 6.45b	54.17 ± 6.45c	8.33 ± 6.45b
10.0 %	8.33 ± 6.45a	4.17 ± 6.45a	0.00 ± 0.00a

Values significantly different at the 5% level of significance are indicated with different letters for the same treatment**.**

III.1.3 Characterisation of metabolites involved in water stress tolerance in *T. cacao*

III.1.3.1 Metabolomics analysis of callus under drought stress conditions

III.1.3.1.1 Metabolites identification

The metabolites present in 28-days-old callus without drought application and their corresponding chemical shifts are presented in Table 6. The metabolites identified belong to different classes: (a) amino acids, such as leucine, isoleucine, valine, asparagine, aspartic acid, glutamic acid, proline, tyrosine, gamma aminobutyric, phenylalanine and tryptophan; (b) organic acids, such as succinic acid, formic acid, aconitic acid, both the cis-isomer and the trans-isomer, and malic acid; (c) sugars, such as fructose, glucose, galactose, sucrose, maltose, raffinose and stachyose, both - α and - β anomers were identified for glucose, 1-O-ethyl-β-D-glucoside, galactose and maltose; (d) the phenolic compounds were identified in the spectrum, the flavonoids present are rutin, epicatechin, catechin and kaempferol analogues, the identified phenylpropanoids are chlorogenic acid, caffeic acid, cinnamic acid , vanilic acid, coumaric acid, shikimic acid, quinic acid, and methyl xanthines such as caffeine and theobromine. Trigonellin, a product of vitamin B3 metabolism, were identified. The peaks corresponding to choline, ethanolamine, allantoin, adenine, adenosine, uridine, hydroxybenzene derivatives, betaine and putrescine were also observed in the spectra analyzed. Among the metabolites identified on day 0 in *T. cacao* (Fig. 26, supplementary data in appendices 7 and 8), fifty-four are common to our three genotypes whereas a single metabolite, raffinose, is absent in the sugar and organic acids regions (4.0-6.0 ppm) in SCA12 and SCA6.

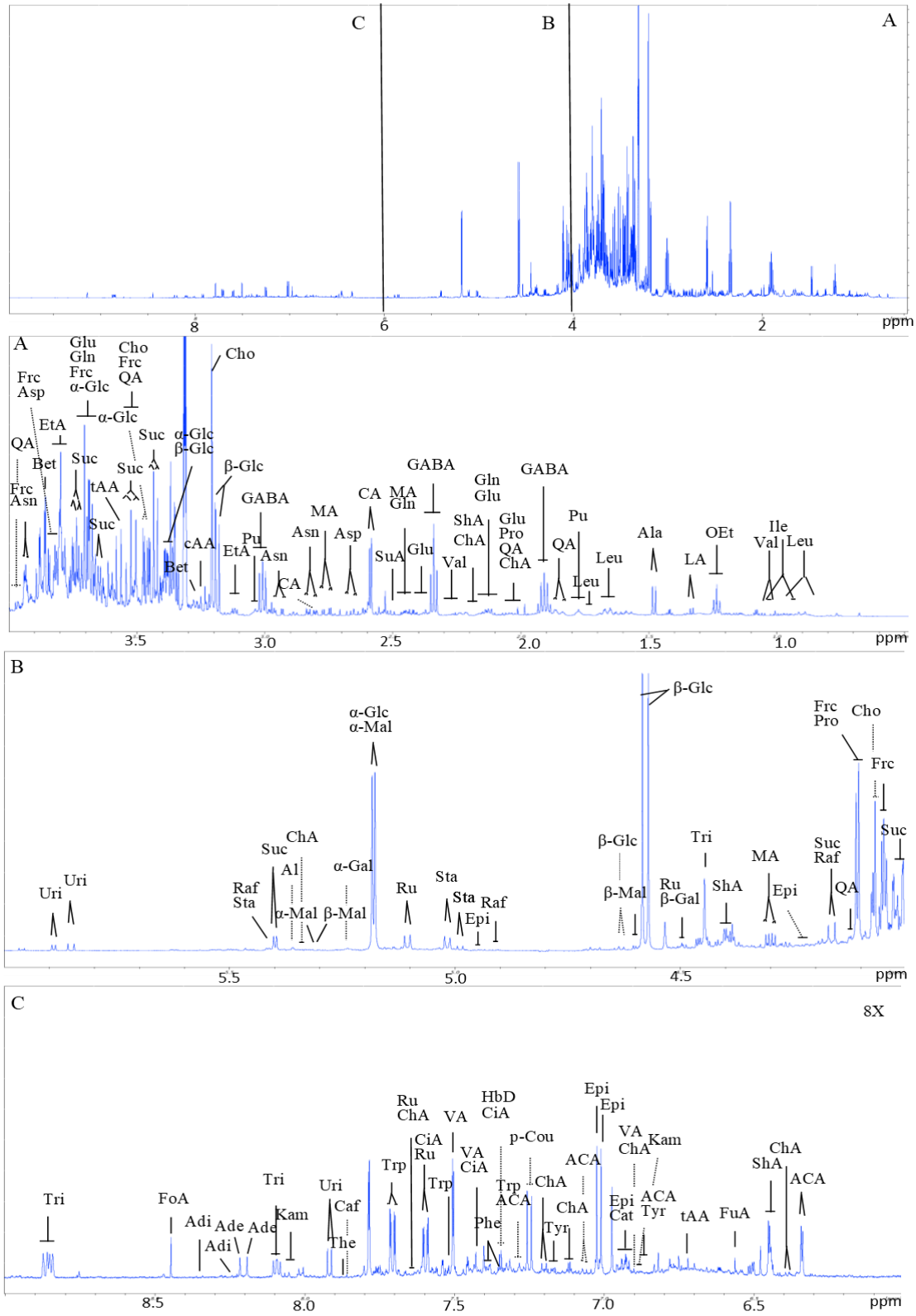


Figure 26. Characteristic NOESY ^{1}H NMR-1D spectrum of MA12 callus at D0 (aged of 28 days) with assigned peaks (full names are reported in table 8). The region δ 9.0-6.0 (C) were expanded 8 times in comparison with the regions δ 4.0-0.5 (A) and δ 6.0-4.0 (B) ppm. These three regions are shown separately (A, B and C) for the assignment.

Table 6. Principal metabolites identified in MA12 callus extracts by NMR (abbreviations used in Fig. 26 are indicated in brackets)

Metabolite	**Chemical shift (δ), coupling constant (J) and multiplicity**	**Group**
Amino acids		
Leucine (Leu)	0.97 (d) J= 6.3	5 CH_3
	0.98 (d) J= 6.6	5'CH_3
	1.66 (m)	4 CH
	1.74 (m)	3 CH_2
Isoleucine (Ile)	0.97 (t) J= 7.5	5 CH_3
	1.02 (d) J= 7.0	4'CH_3
Valine (Val)	1.00 (d) J= 7.0	4'CH_3
	1.05 (d) J = 7.1	4 CH_3
Alanine (Ala)	1.48 (d) J = 7.2	3 CH_3
Gamma-Amino Butyric Acid (GABA)	1.90 (m)	3 CH_2
	2.32 (t) J = 7.2	2 CH_2
	3.1 (t) J = 7.3	4 CH_2
Proline (Pro)	2.00 (m)	4 CH_2
	2.07 (m)	3 CH_2a
	2.33 (m)	3 CH_2b
	4.07 (dd) J = 6.4 -8.8	2 CH
Glutamic Acid (Glu)	2.03 (m)	3 CH_2a
	2.13 (m)	3 CH_2b
	2.39 (m)	4 CH_2
	3.69 (dd) J =4.3-7.4	2 CH
Aspartic Acid (Asp)	2.65 (dd) J = 9.2-17.3	3 CH_2a
	2.82 (dd) J = 3.7-17.4	3 CH_2b
	3.83 (dd) J = 3.7-9.2	2 CH
Asparagine (Asn)	2.82 (dd) J = 8.2-8.8	3 CH_2a
	2.95 (dd) J = 3.9-13.0	3 CH_2b
	3.94 (dd) J = 3.9-8.2	2 CH
Glutamine (Gln)	2.13 (m)	3 CH_2
	2.45 (m)	4 CH_2
	3.71 (t) J = 6.1	2 CH
Tyrosine (Tyr)	6.85 (m)	3, 5 CH
	7.18 (m)	2, 6 CH
Tryptophan (Trp)	7.20 (s)	2 CH
	7.53 (d) J = 8.1	1 CH
	7.72 (d) J = 7.9	4 CH
Phenylalanine (Phe)	7.36 (m)	2, 3, 4, 5, CH

Table 6 continued

Organic acids		
Lactic acid (LA)	1.32 (d) J = 6.9	3 CH_3
Shikimic acid (ShA)	2.19 (m)	6 CH2a
	2.76 (m)	6 CH2b
	4.37 (m)	3 CH
	6.44 (m)	2 CH
Malic acid (MA)	2.44 (dd) J = 9.2-15.5	3 CH2a
	2.76 (dd) J = 3.7-15.5	3 CH2b
	4.30 (dd) J = 3.7-9.2	2 CH
Citric acid (CA)	2.59 (d) J = 16.3	3 CHa
	2.77 (d) J =16.3	3 CHb
Succinic acid (SuA)	2.50 (s)	2, 3 CH2
cis-Aconitic acid (cAA)	3.27 (d)	4 CH_2
	6.24 (s)	2 CH
trans-Aconitic acid (tAA)	3.57 (s)	2 CH_2
	6.73 (s)	4 CH
para-Coumaric acid (p-Cou)	7.25 (d) J=16	2 CH
Fumaric acid (FuA)	6.54 (s)	2 CH
Vanilic acid (VA)	6.89 (d) J = 8.2	5’ CH
	7.40 (dd) J = 8.2-1.7	6’ CH
	7.52 (d) J = 1.6	2’ CH
Formic acid (FA)	8.46 (s)	1 CH
Carbohydrates		
1-O-Ethyl-β-D- glucoside (OEt)	1.20 (t) J = 7.0	
β-Glucose (β-Glc)	3.19 (dd) J = 7.9-9.4	2 CH
	3.36 (m)	4 CH
	3.87 (dd) J = 2.2-12.3	6 CH2
	4.58 (d) J = 7.9	1 CH
α-Glucose (α-Glc)	3.36 (m)	4 CH
	3.47 (dd) J = 3.8-9.8	2 CH
	3.70 (dd) J = 5.3-11.9	6 CH2
	5.18 (d) J = 3.7	1 CH
Sucrose (Suc)	3.43 (t) J = 9.7	4 CH
	3.50 (dd) J = 3.8-9.9	2 CH
	3.65 (d) J = 1.6	1’ CH2
	3.74 (t) J = 9.2	3 CH
	4.02 (t) J = 8.2	4’ CH
	4.17 (d) J = 8.7	3’ CH
	5.40 (d) J = 3.8	1 CH
Fructose (Frc)	3.52 (m)	1 CH2a
	3.78 (m)	3 CH
	3.84 (dd) J = 3.3-9.9	6 CH2a
	3.93 (m)	5 CH
	4.02 (dd) J = 1.3-12.7	6 CH2b
	4.07 (m)	4 CH

Table 6 continued

Raffinose (Raf)	4.18 (d) J = 8.7	3'' CH
	4.86 (d) J = 3.7	1 CH
	5.41 (d) J = 3.9	1' CH
β-Galactose (β-Gal)	4.52 (d) J = 7.8	1 CH
α-Galactose (α-Gal)	5.22 (d) J = 3.9	1 CH
β-Maltose (Mal)	4.59 (d) J = 7.9	1' CH
	5.29 (d) J= 3.9	1 CH
Stachyose (Sta)	4.95 (d) J = 3.6	1' CH
	4.96 (d) J = 3.6	1 CH
	5.42 (d) J = 4.0	1'' CH
α-Maltose (α-Mal)	5.18 (d) J = 3.8	1' CH
	5.30 (d) J = 3.9	1 CH
Phenolics		
Cinnamic acid (CiA)	6.47 (d) J =16.0	
	7.37 (d) J = 15.9	
	7.41(m)	
	7.62 (dd) J= 8.1-1.0	
(-)-Epicatechin (Epi)	2.74 (dd)	4 CHa
	4.3 (m)	3 CH
	4.93 (s)	2 CH
	6.05 (d) J = 1.96	8 CH
	6.92 (m)	5'CH + 6'CH
	7.02 (s)	2' CH
Rutin (Ru)	6.39 (d) J = 2.0	6 CH
	7.61 (dd) J=8.5-2.1	6' CH
	7.66 (d) J=2.1	2' CH
Hydroxybenzene derivatives (HbD)[a]	6.86 (d) J = 8.7	
	7.33 (d) J = 8.6	
Quinic acid (QA)	1.87 (dd) J = 10.2 -13.5	6 CH2a
	2.01 (ddd) J = 2.3-4.9-13.5	2 CH2a
	2.03 (dd) J= 3.5-14.4	2 CH2b
	3.52 (dd) J = 3.2-8.6	4 CH
	3.98 (ddd) J = 4.9-8.6-10.2	5 CH
	4.10 (m)	3 CH
Chlorogenic acid (ChA)	6.40 (d) J= 15.9	8' CH
	6.89 (d) J = 8.2	5 CH
	7.11 (dd)	1 CH
	7.65 (d) J = 15.9	7' CH
(+)-Catechin (Cat)	6.91 (m)	6'CH

Table 6 continued

Others compound		
Putrescine (Pu)	1.8 (m)	2, 3 CH_2
	3.04 (t) J = 7.2	1, 4 CH_2
Ethanolamine (EtA)	3.11 (m)	1 CH_2
	3.81 (m)	2 CH_2
Trigonelline (Tri)	4.45 (s)	CH_3
	8.10 (m)	5 CH
	8.86 (m)	4, 6 CH
Allantoine (Al)	5.37 (s)	4 CH
Kaempferol analogues (Kam)	8.01 (d) J = 8.8	2', 6' CH
Betaine (Bet)	3.28 (s)	CH3
	3.86 (s)	2 CH2
Choline (Cho)	3.21 (s)	CH_3
	3.51 (m)	2 CH_2
	3.86 (m)	1 CH_2
Adenine (Ade)	8.19 (s)	8 CH
	8.21 (s)	2 CH
Adenosine (Adi)	8.25 (s)	8 CH
	8.36 (s)	2 CH
Uridine (Uri)	5.85 (d) J = 8.15	5 CH
	5.90 (d) J = 4.77	1' CH
Caffeine (Caf)	3.33 (s)	N3CH3
	3.50 (s)	N1CH3
	3.93 (s)	N7CH3
	7.86 (s)	6 CH
Theobromine (The)	3.91 (s)	N7CH3
	7.88 (s)	6 CH
Caffeic Acid (ACA)	6.35 (d) J = 15.9	8 CH
	7.06 (dd) J = 8.2-1.2	9 CH
	7.15 (d) J = 1.9	7 CH
	7.29 (d) J = 15.9	2 CH
	7.56 (d) J = 15.9	7 CH
	7.92 (d) J = 8.13	6 CH

Note : ^{1}H chemical shift (δ) are expressed in ppm, coupling constant (J) are expressed in Hz and the multiplicity of the signal is indicated in the brackets. Proton assignment is indicated in the third column. NMR solvent : methanol-d4/buffer (KH_2PO_4 90 mM in D_20 Ph 6.0, containing 0.025% (w/w) TMSP-d4). [a] potentially prunin on the basis of Arisawa et *al*. (1972).

III.1.3.1.2 Metabolites changes in the genotypes in control conditions

Before studying the impact of water stress on the *Theobroma cacao* metabolome, callus of the three genotypes of cacao, were compared in control conditions during embryos induction step, using ^{1}H-NMR. Little difference was observed between the spectra of the (semi-) polar extracts of the various samples. The typical ^{1}H-NMR spectra obtained after different period (days) with callus samples grown in normal conditions is presented in Fig. 27, suggesting that a wide range of polar metabolites can be extracted by water-methanol system in cacao. After extraction, two parts, low-field signals (aromatic region) and high-field signals (aliphatic and sugar regions), were obtained in the spectra. Some signals in the first part (δ 6.0-10.0 ppm) were gradually lost or reduced between 4 and 8 days at a level which depends to the origin of the samples. Visual inspection revealed that SCA12 samples grown in non-stressed conditions contained more aliphatic compounds (amino acids and some organic acids) after 8 days than SCA6 and MA12 samples. These spectra show that there was developmental (kinetics) effect between 4 and 8 days of sampling for both *T. cacao* genotypes.

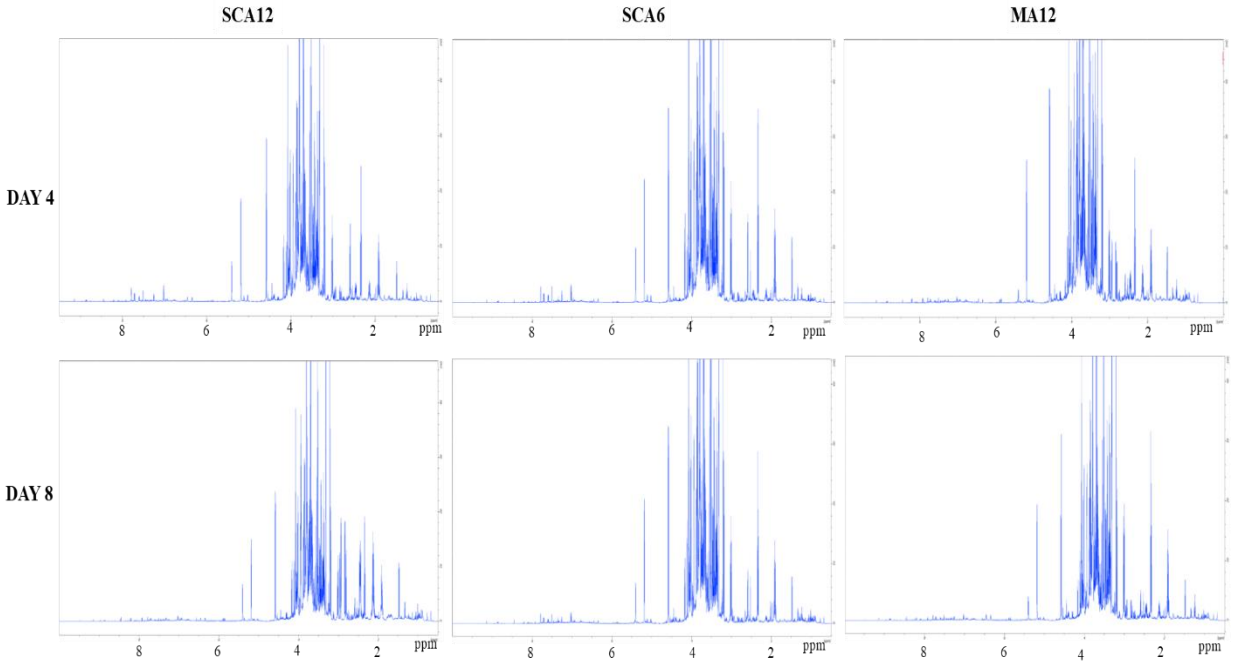


Figure 27. Characteristic NOESY ^{1}H NMR-1D spectra based on SCA12, SCA6 and MA12 data of callus samples grown in normal (control) conditions at the two different kinetic points (D4, D8).

Principal component analysis (PCA), a non-targetd analysis, was performed on the water-methanol fractions of the callus samples grown in normal conditions after 4 and 8 days. In the score plot presented in Fig. 28, the two principal components (PCs) were selected and explained 42.7% of the total variance. This score plot clearly shows that the MA12 and the SCA12 genotypes were separated from the SCA6 genotype by PC1 (29.7%), while MA12 and SCA12 could be further separated by PC2 (13%). The separation between the MA12 samples and the SCA6 samples was more marked than the one between MA12 and SCA12, and the observed separation indicated that MA12 and SCA6 were very different in their metabolic profiles. The analysis of this PCA confirms the separation of our metabolites in the absence of water stress into three distinct groups. However, PCA did not show any clear discrimination between days of sampling.

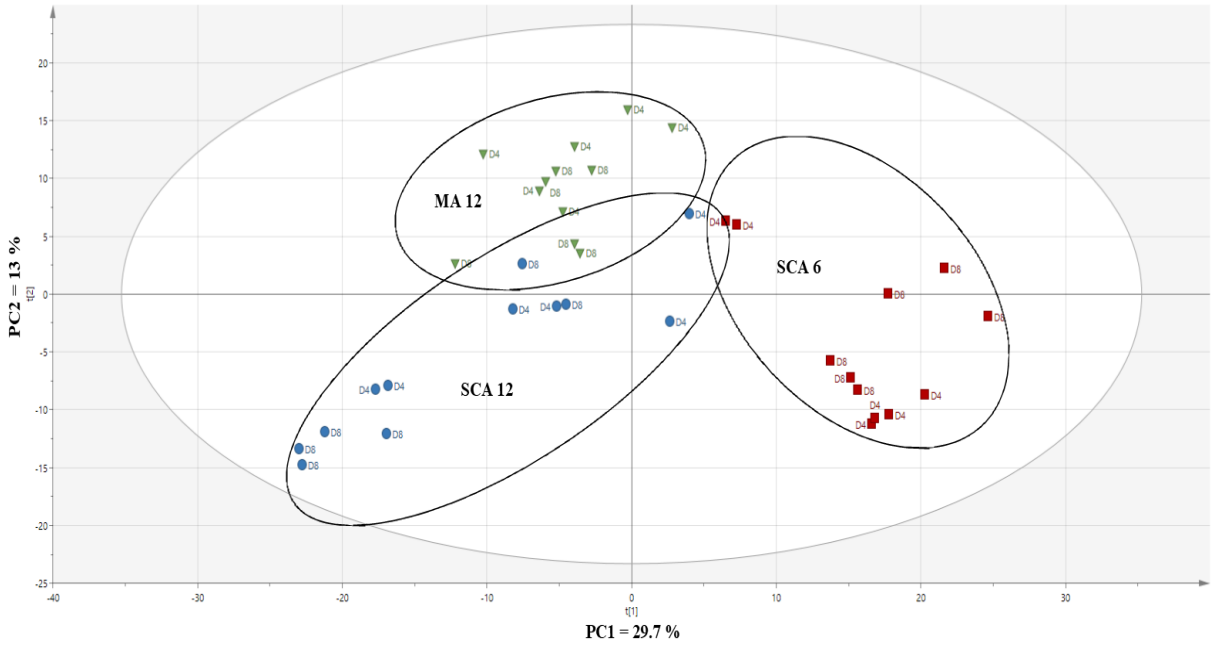


Figure 28. Principal component analysis (PCA) based on SCA12, SCA6 and MA12 NMR-^{1}H data, callus samples grown under normal conditions (D4, D8). Spectra were realigned, cut into 903 bins and integrated to perform multivariate analysis.

The loading plots of PC1 and PC2 was used for the identification of the discriminant metabolites (i.e. those that effectively separate or bring one genotype not closer to the others). All metabolites that occured in the discrimination of genotypes in PCA analyses were identified by NMR spectra (1D and 2D analysis). Mean areas of the characteristic peaks of the discriminant metabolites were compared among the three genotypes using thee Wilcoxon Rank

Sum test (Wi-test), with different p-values, $p<0.001$; $p<0.01$ and $p<0.05$. The results of statistical analysis showed that a total of 20 metabolites are responsibles for the discrimination among SCA12, SCA6 and MA12 non-stressed callus samples. SCA12, the tolerant genotype, showed a higher concentration than SCA6 and MA12 in asparagine, leucine, tryptophane, quinic acid, uridine, adenine and valine. SCA6, the less-tolerant, displayed a higher concentration in ethanolamine, sucrose, rutine, hydroxybenzen derivatives p-coumaric acid, vanilic acid and proline. Finally, MA12, the drought sensitive, displayed a higher concentration than SCA12 and SCA6 in many organic acids such as shikimic acid and cis-aconitic acid and in putrescine, betaine, trigonelline and 1-O-ethyl-β-D-glucoside. Proline was not detected in SCA12 and MA12 samples at days 4 and 8, whereas tryptophane was not detected in SCA6 sample during the same periods. In addition, one nucleotide present at day 4 in SCA6 samples was not detected after 8 days, adenine (Table 7).

Table 7. Discriminant metabolites among SCA12, SCA6 and MA12 genotypes of *T. cacao* non-stressed (NS) samples at the two kinetic points (D4 and D8).

Metabolite	**Ratio at D4**	**Wi-test**	**Ratio à D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Ratio at D8**	**Wi-test**
- discriminant for SCA12 genotype	***SCA12/SCA6***		***SCA12/MA12***		***SCA12/SCA6***		***SCA12/MA12***	
Asparagine	3.91	***	1.01	/	9.95	***	1.59	/
Leucine	2.17	***	0.93	/	1.83	***	1.27	/
Tryptophan	ND1		1.21	*	ND1		1.55	***
Quinic acid	1.51	*	0.95	/	2.69	***	1.07	/
Uridine	1.38	*	0.78	/	2.47	***	1.04	/
Adenine	1.45	*	0.75	**	ND1		1.93	***
Valine	1.91	***	1.17	*	2.64	***	1.16	**

Table 7 continued

- discriminant for SCA6 genotype	***SCA6/SCA12***		***SCA6/MA12***		***SCA6/SCA12***		***SCA6/MA12***	
Ethanolamine	1.26	*	1.22	*	1.57	**	1.25	**
Sucrose	1.33	**	1.26	**	1.68	***	1.38	***
Rutin	3.97	/	2.20	/	3.67	**	2.43	***
Hydroxybenzen derivatives	2.11	***	2.34	***	3.08	***	3.16	***
p-Coumaric acid	1.86	/	1.98	/	2.57	**	2.68	***
Vanilic acid	2.06	/	2.06	/	2.63	**	2.68	***
- discriminant for MA12 genotype	**MA12/SCA12**		**MA12/SCA6**		**MA12/SCA12**		**MA12/SCA6**	
Putrescine	1.20	**	1.98	***	1.73	***	2.16	***
Betaine	1.33	**	1.97	***	1.49	**	1.49	**
Shikimic acid	1.94	***	2.53	**	1.84	*	1.98	/
Trigonelline	1.82	*	2.0	/	1.72	/	1.17	/
cis-Aconitic acid	1.24	**	1.32	***	1.22	**	1.07	**
1-O-ethyl-β-D-glucoside	2.14	**	1.30	/	1.95	***	1.15	/
Proline	ND2		ND3		ND2		ND3	

Note : ND1, not detected in SCA6 ; ND2, not detected in SCA12 ; ND3, not detected in MA12. ***0.001, **0.01, *0.05, / not significative.

III.1.3.1.3 Metabolite changes in callus due to water deficit stress

- Comparison of metabolites of the three genotypes in drought stress conditions

The hydro-alcoholic extracts of *T. cacao* callus subjected to 4 and 8 days of water stress treatment in embryos induction medium were analyzed by ^{1}H-NMR. To verify whether there was a significant difference between the two period of water stress, the NOESY spectra of samples was investigated. The typical NOESY ^{1}H-NMR spectra based on SCA12, SCA6 and MA12 data of callus grown under water stress conditions (D4, D8) is presented in Fig. 29, suggesting that polar metabolite in samples from each genotype are influenced by the duration of the water stress. Visual inspection of spectra showed that signals are relatively constant in SCA12 samples treated with PEG after 4 and 8 days as compared with those in SCA6 and MA12 samples. Lowest peaks signals were observed in the high-frequency regions in MA12 samples after 8 days as compared to the others genotypes, suggesting a great kinetic effect.

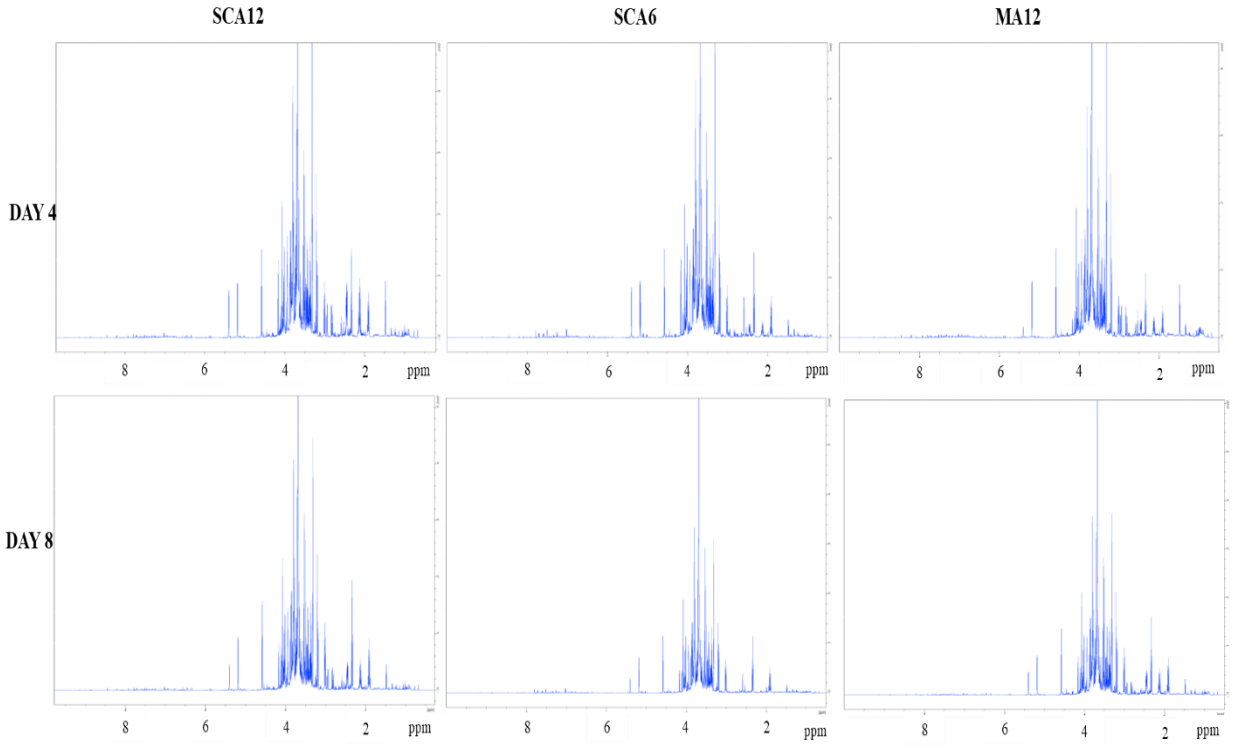


Figure 29. Characteristic NOESY ^{1}H NMR-1D spectra based on SCA12, SCA6 and MA12 data of samples grown under drought stress conditions at the two different kinetic points (D4, D8).

Metabolic analysis among our genotypes, displayed no separation with PCA. Consequently, the classification of the metabolites present in *T. cacao* callus grown under water stress conditions

was carried out by another multi-varied analysis known as Partial Least Square-Discriminant Analysis (PLS-DA). In contrast to PCA, which only uses the information from one matrix, PLS-DA also takes into account the information in another (dummy) matrix (Berrueta *et al*., 2007). When PLS-DA was applied, the separation among SCA12, SCA6 and MA12 genotypes improved considerably (Fig. 30). Indeed, PC1 and PC2 account for 42.3% of the total variance and allow the observation of three groups of samples according to the genotypes ; SCA6 samples are separated from the other two groups, by PC1 (32.7%), and the other two groups, SCA12 and MA12 could be further separated by PC2 (9.3%). However, PLS-DA did not still show any clear discrimination between days of sampling.

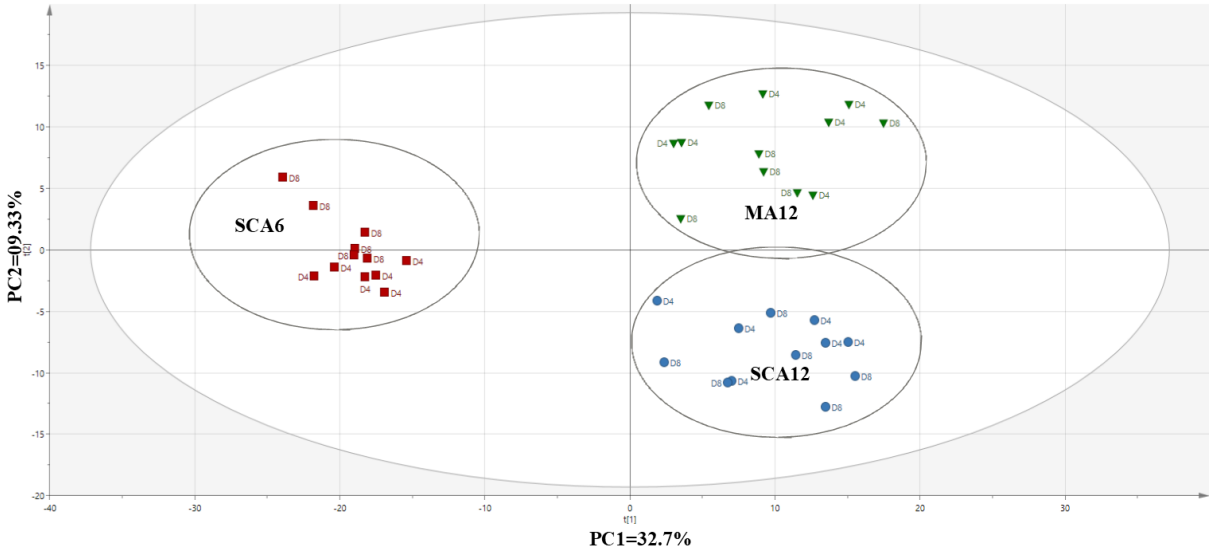


Figure 30. Partial least square – discriminant analysis (PLS-DA) based on SCA12, SCA6 and MA12 NMR-^{1}H data, callus samples grown under water stress conditions (D4, D8). Spectra were realigned, cut into 903 bins and integrated to perform multivariate analysis.

The loadings plot of PC1 and PC2 were used to find the metabolites that are responsible for the separation of our genotypes in PLS-DA. These metabolites and their respective ratios and significance are presented in table 8, which shows globally that 22 metabolites are responsible for the discrimination among SCA12, SCA6 and MA12 stressed callus after 4 and 8 days. The signals of leucine was found to be highly significative and discriminant for the genotype SCA12. Although the signals from other amino acids organic acids and sugars, including isoleucine, aspartic acid, formic acid and malic acid, and sugars, β-glucose and other compound such as allantoin were also involved in the discrimination of SCA12 genotype from the other genotypes, none of these metabolites were statistically significant. In SCA6, the discriminant

metabolites included tryptophan, rutin, vanilic acid, para-coumaric acid, hydroxybenzene derivatives, chlorogenic acid, epicatechin. All those signals in SCA6 samples were highly significative ($p<0.001$ and $p<0.01$) after 4 and 8 days. Similarly, the metabolites that discriminate the MA12 samples were phenylalanine, caffeic acid, tyrosine, stachyose, shikimic acid, proline, 1-O-ethyl-β-D-glucoside and valine. Differences were found only in caffeic acid and shikimic acid contents after 8 days. In addition, stachyose and valine were absents in the SCA12 and the SCA6 samples at both kinetic points (D4, D8), whereas proline was absent in the SCA12 samples after 4 days and malic acid was absent in the MA12 samples only after 8 days.

Table 8. Discriminant metabolites among SCA12, SCA6 and MA12 genotypes of *T. cacao* water-stressed (WS) samples at the two kinetic points (D4, D8).

Metabolite	**Ratio at D4**	**Wi-test**	**Ratio at D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Ratio at D8**	**Wi-test**
- Discriminant for SCA12 genotype	***SCA12/SCA6***		***SCA12/MA12***		***SCA12/SCA6***		***SCA12/MA12***	
Formic acid	0.90	/	1.30	/	1.27	/	1.07	/
Leucine	1.89	**	1.46	*	1.89	***	1.27	*
Isoleucine	0.90	/	1.14	/	0.90	/	1.04	/
Aspartic acid	1.27	/	1.50	*	1.36	/	1.35	/
β-Glucose	0.93	/	1.04	/	6.19	/	7.18	/
Allantoin	1.18	/	1.21	/	1.54	*	1.09	/
Malic acid	2.14	/	0.93	*	1.50	/	ND3	

Table 8 continued

- Discriminant for SCA6 genotype	***SCA6/SCA12***		***SCA6/MA12***		***SCA6/SCA12***		***SCA6/MA12***	
Tryptophan	***1.82***	***	***1.79***	***	***2.14***	***	***2.01***	***
Rutin	***2.39***	***	***2.56***	***	***2.62***	***	***2.35***	***
Vanilic acid	***2.27***	***	***2.18***	***	***2.65***	***	***2.24***	***
p-Coumaric acid	***2.65***	***	***2.82***	***	***3.03***	***	***2.56***	***
Hydroxybenzen Derivatives	***2.81***	***	***2.14***	***	***3.24***	***	***3.33***	***
Chlorogenic acid	***5.12***	***	***4.77***	***	***0.52***	***	***0.19***	***
Epicatechin	***1.52***	**	***1.64***	***	***1.69***	***	***1.66***	**
- Discriminant for MA12 genotype	***MA12/SCA12***		***MA12/SCA6***		***MA12/SCA12***		***MA12/SCA6***	
Phenylalanine	1.57	***	1.62	***	1.37	*	1.10	/
Cafeic acid	5.43	***	8.38	***	16.63	***	1.41	*
Tyrosine	9.09	***	2.42	***	ND1	/	1.35	/
Stachyose	ND1		ND2		ND1		ND2	
Shikimic acid	1.51	/	1.80	*	1.84	***	1.46	**
Proline	1.01	/	1.05	/	ND1		1.02	/
1-O-ethyl-β-D-glucoside	1.57	/	1.44	/	2.02	***	1.23	/
Valine	ND1		ND2		ND1		ND2	

Note: ND1, not-detected in SCA12; ND2, not-detected in SCA6; ND3, not-detected in MA12. ***0.001, **0.01, *0.05, / not significative.

- Comparison of metabolites in non-stressed (NS) and water-stressed (WS) conditions for each genotype

Further metabolomics analysis were performed in order to understand the intra-specific behaviour of our genotypes under water stress. For both genotypes, a water stress incubation period induced some changes at the metabolomic level : non-stressed (NS) and water-stressed (WS) samples were well distinguished (Fig. 31). For SCA12 NMR data, the two PCs (PC1=25.4% ; PC2=16.2%) interact in the separation of NS and WS samples, but with a large dispersion of samples according to PC1. While for SCA6, there is a slight dispersion of the samples, as explained here by PC1 (21.4%) and a clear separation of samples into two groups, according to PC2 (19.2%). In addition, for MA12 (PC1=21.9% ; PC2=15.9%), the PCA score plot of NMR data also shows a characteristic grouping for NS and WS samples.

Statistical analysis highlighted 17 metabolites that their content changed during water stress for SCA12, 18 for SCA6 and 17 for MA12. Table 9, presents the discriminant metabolites for SCA12, SCA6 and MA12 genotypes of *T. cacao* in WS versus NS conditions after 8 days and supplementary informations on their respective ratios in WS versus NS conditions (after 4 and 8 days) are presented in appendix 9. During water stress, stachyose, threonine, tryptophane, rutin and shikimic acid were significantly down-regulated in SCA12 whereas fructose, sucrose, aspartic acid and trans-aconitic acid were up-regulated ; in SCA6, β-maltose, leucine, γ-amino-butyric acid and cinnamic acid were down-regulated whereas sucrose, glucose, asparagin, malic acid and trigonellin were significantly up-regulated ; and finally, in MA12, aspartic acid, shikimic acid, putrescine and kaempferol were significantly down-regulated whereas sucrose, tyrosine, trans-aconitic acid and choline were up-regulated. In addition, in SCA12, β-maltose and kaempferol analogues were present only in NS samples, while hydroxybenzen derivatives were present only in WS samples ; in SCA6, leucine was detected in WS samples only after 4 days whereas hydroxybenzen derivatives was detected in NS samples at both kinetic points ; finally, in MA12, chlorogenic acid and phenylalanine were present only in NS samples, while tyrosine was detected in NS samples only after 8 days (see appendix 9).

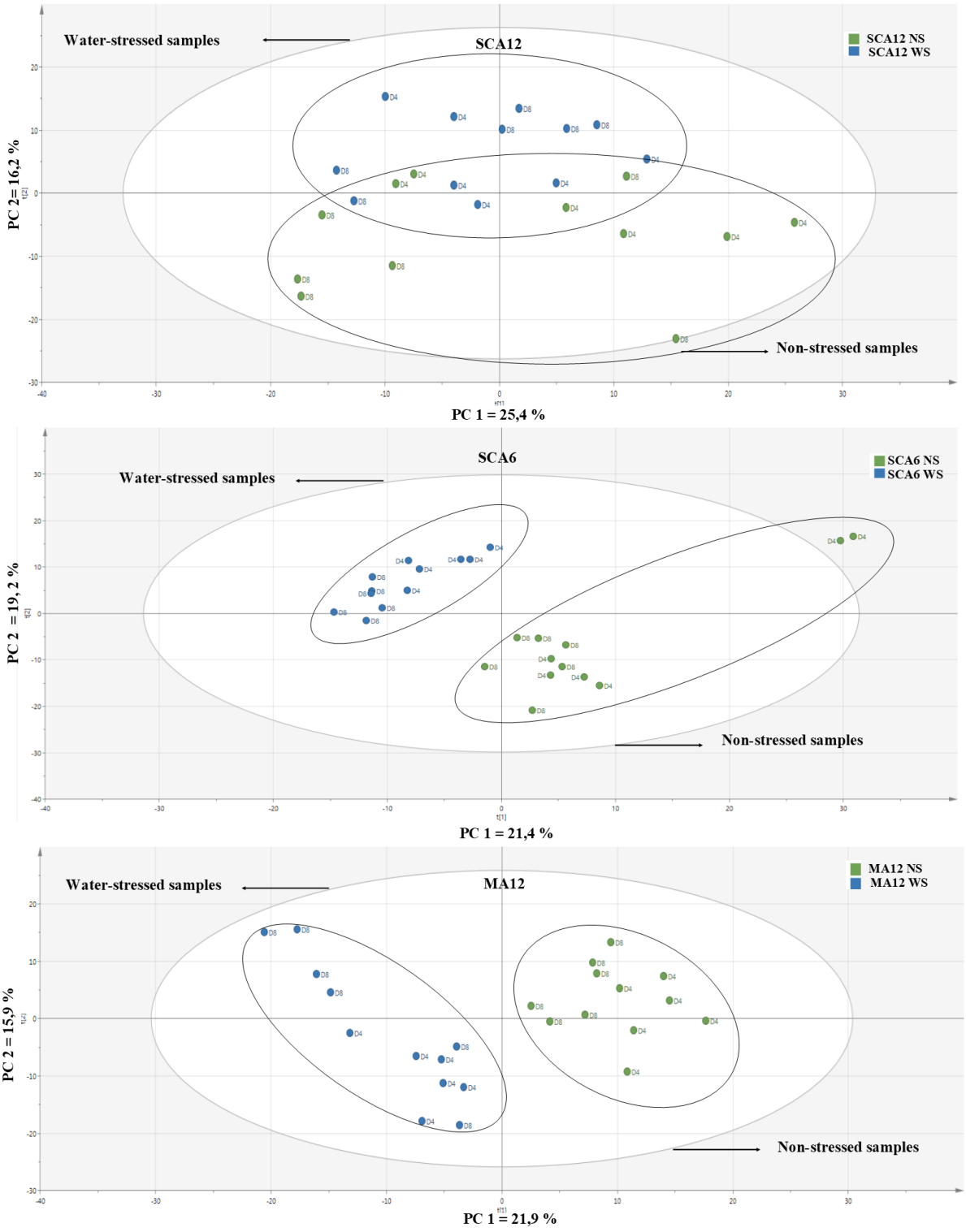


Figure 31. Principal component analysis (PCA) based on SCA12, SCA6 and MA12 ^{1}H-NMR data, comparison of non-stressed and stressed callus at the two kinetic points (D4, D8). Spectra were realigned, cut into 903 bins and integrated to perform multivariate analysis.

Table 9. Comparison of the discriminant metabolites for SCA12, SCA6 and MA12 genotypes (callus) of *T. cacao* in water-stressed (WS) versus non-stressed (NS) conditions at D8.

	Genotypes					
	SCA12		SCA6		MA12	
	NS>WS (down regulated)	WS>NS (up regulated)	NS>WS (down regulated)	WS>NS (up regulated)	NS>WS (down regulated)	WS>NS (up regulated)
Sugars metabolism	Stachyose	Fructose	β- Maltose	Sucrose		Sucrose
	β-Maltose	Sucrose	Fructose	α-Glucose		
	1-O-ethyl-β-D-glucoside		1-O-ethyl-β-D-glucoside			
Amino acids metabolism	Threonine	Aspartic acid	Leucine	Aspartic acid	Leucine	Tyrosine
	Tryptophan		Alanine	Asparagine	Aspartic acid	Proline
			Tyrosine		Phenylalanine	
			Tryptophan			
			δ-amino-butyric acid			
Phenolics metabolism	Epicatechin	Hydroxybenzen derivatives	Caffeic acid	Hydroxybenzen derivatives		

	Caffeic acid					
	Rutin					
Organic acids metabolism	Shikimic acid	trans-Aconitic acid	Cinnamic acid	Malic acid	Shikimic acid	trans-Aconitic acid
					Chlorogenic acid	
Others metabolism	Kaempferol analogues	Choline		Ethanolamine	Uridine	Choline
		Ethanolamine		Trigonelline	Putrescine	
					Kaempferol analogues	

Significant differences between ratio of NS and WS samples for SCA12, SCA6 and MA12 genotypes are indicated by the following color code:

Yellow frame: ratio < 1.5 in NS samples; Light yellow: ratio > 1.5 times in NS samples; Dark yellow: ratio > 3.0 times in NS samples; Bleue frame: ratio < 1.5 times in WS samples; Light bleue: ratio > 1.5 times in WS samples; Dark bleue: ratio > 3.0 times in WS samples.

III.1.3.2 Metabolomics analysis of seedlings

III.1.3.2.1 Morphological examination of seed germination and seedling establishment in T. cacao before ^{1}H-NMR analysis

The study was performed in cacao seeds, with regards to water stress tolerance. The elongative growth parameters of seeds (seedling fresh weigth and hypocotyl length) were measured at 0; 4 and 8 days after imbibition (DAI). The relative growths (i.e. the difference between the value at the end and at the beginning of a given stage) parameters of seedlings (fresh mass and hypocotyl length) were determined. An overview of the seedling fresh weigth and hypocotyl

length after 0; 4 and 8 days is provided in appendix 10. In the following analysis we always refer to 0-4 and 4-8 DAI to indicate the relative growth period of seed germination and seedling establishment, respectively. Fresh seed of *T. cacao* started germination two days after imbibition and completed after 4 days in normal conditions. Seeds germination and seedlings establishment were highly influenced by water stress regardless of the genotype. The average seeds germination frequency obtained for the three genotypes after 4 days of imbibition (4-DAI) of seeds grown under control conditions varied from 72.22 ± 22.77 to 94.44 ± 8.61 %, with the highest value obtained with seeds from SCA12, whereas under drought conditions, it varied from 22.22±8.61 to 62.11±8.61% (Table 10). After germination, obtained seedlings were successfully grown during early establishment stage (until 8-DAI).

Table 10. Average germination frequencies of non-stressed (control) and water-stressed seeds from SCA12, SCA6 and MA12 genotypes, 4 days after imbibition.

Génotype	SCA12	SCA6	MA12
% of germinated seed in control condition	94.44 ±8.61 %a	72.22 ± 22.77%a	83.33±14.91%a
% of germinated seed in PEG condition	62.11±8.61 %b	61.11± 22.77%b	22.22±8.61%a

Values significantly different at the 5% level of significance are indicated with different letters for the same treatment. Values are given in terms of mean ± SD (n = 9).

The statut of the three genotypes during germination and seedling establishment at three developmental stages (0; 4 and 8 days after imbibiton (DAI)) shows variations among them at the morphological level in PEG supplemented medium (Fig. 32). It is clear that SCA12 has good seed germination ability and seedling growth (elongation of the radicle at 4-DAI, visible lateral root outgrowth and growth of root system at 8-DAI) as compared to SCA6 and MA12, under drought conditions.

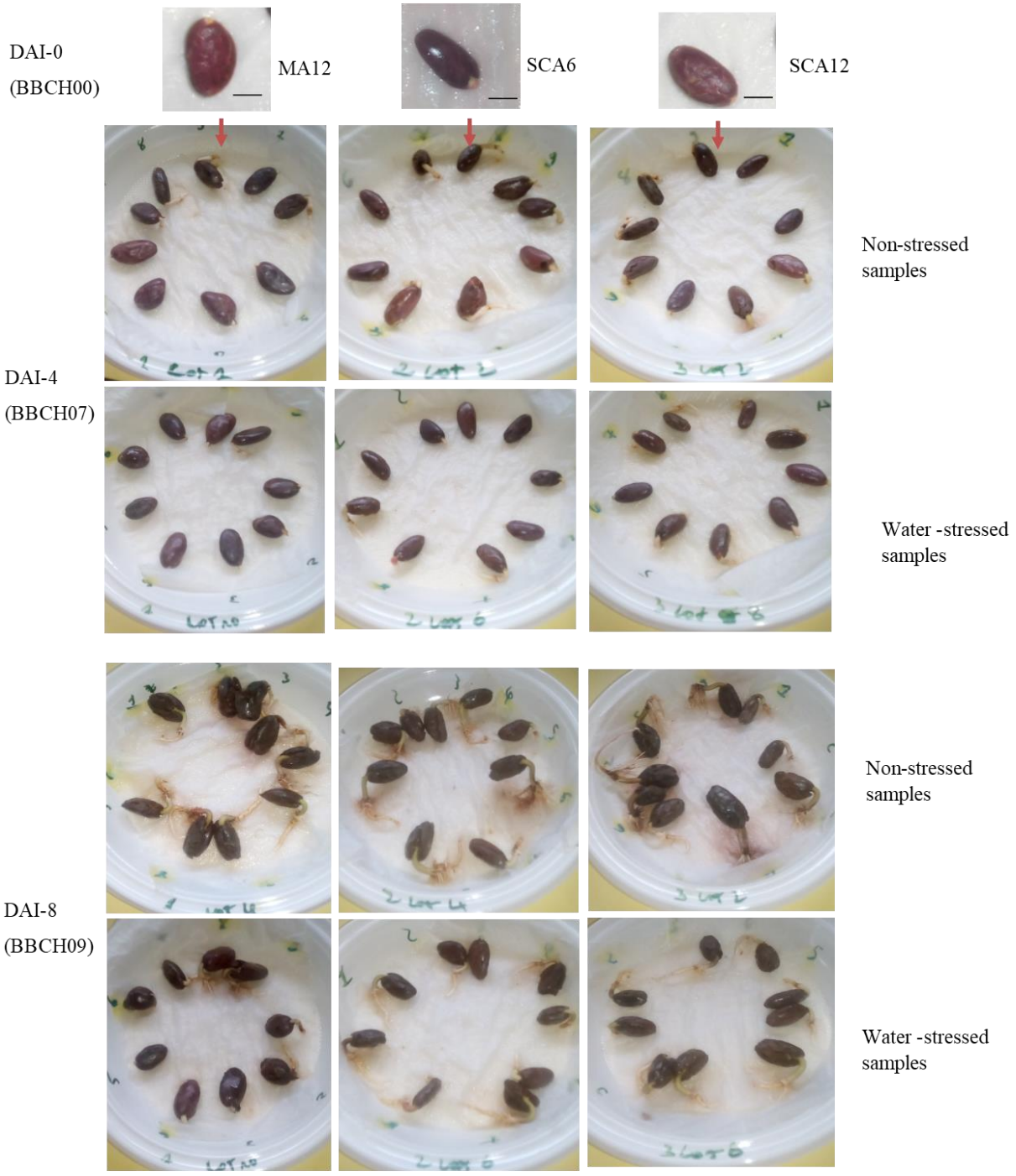


Figure 32. Morphological aspect of *T. cacao* genotypes incubated with PEG during germination and seedlings establishment. The corresponding BBCH (BASF, Bayer, Ciba-Geigy, Hoeschst) developmental codification scale is indicated just below the day after imbibition (DAI). Bar= 0.5 cm.

There is a difference in relative fresh weight growth in 0-4 DAI old SCA6 seedlings in normal condition as compared to SCA12 and MA12; whereas there is no differences between SCA12 and MA12 values. However, application of 5% PEG led to decreased relative growth of fresh

weight in these young seedlings, but no differences was found among these values. Relative growth of seedlings were lower in 4-8 DAI old seedlings of *T. cacao* (both controls and treated with PEG) than in 0-4 DAI old seedlings. SCA12 tissues had the highest relative growth of fresh weight than SCA6 and MA12 and PEG application did not change it in MA12, and decreased it in SCA12 and SCA6 tissues (Fig. 33). But no differences were found among these values.

b

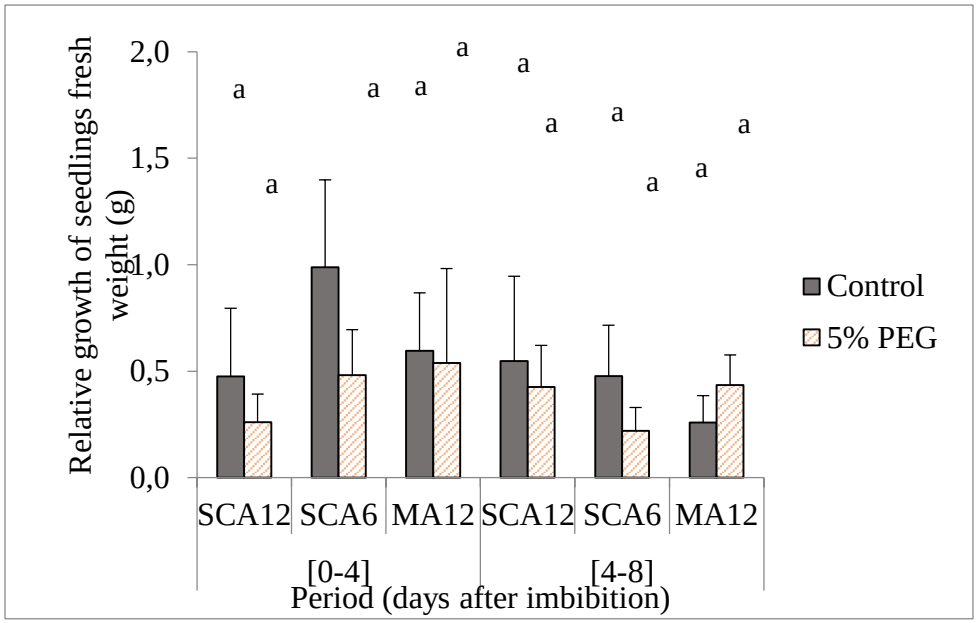


Figure 33. Influence of water stress on the evolution of the relative fresh weight of seeds during germination and seedlings establishment. Vertical bars represent standard error. Each plot was drawn from means of six identical experiments. Values significantly different at the 5% level of significance for the same treatment and for the same period are indicated with different letters.

There is no differences in relative hypocotyl length in 0-4 DAI old seedlings of SCA12, SCA6 and MA12 grown in normal conditions. However, application of 5% PEG led to reduced and rather significant differences in relative hypocotyl length in SCA12 as compared to SCA6 and MA12. Relative growth of hypocotyl was higher in 4-8 DAI old seedlings of *T. cacao* (both controls and treated with PEG) than in 0-4 DAI old seedlings; with the highest value obtained in SCA12 tissues (3.80 ± 0.24 cm) and the lowest value obtained in MA12 (3.32 ±0.18 cm) and application of PEG did not change it. However, no differences were found among these values during this period (Fig. 34).

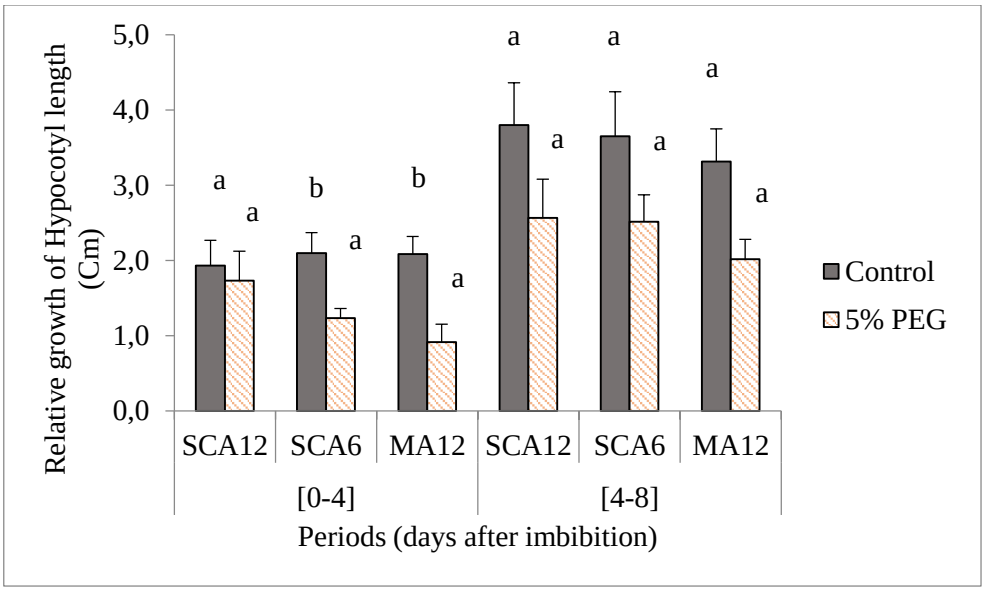


Figure 34. Influence of water deficit stress on thr evolution of relative growth of hypocotyl length seed germination and seedlings establishment. Vertical bars represent standard error. Each plot was drawn from means of six identical experiments. Values significantly different at the 5% level of significance for the same treatment and for the same period are indicated with different letters.

III.1.3.2.2 Metabolite identification

Using an analogous approach to that previously described with callus, we here unravelled seed performance under osmotic stress and specific metabolites accumulation. Fig. 35 shows 1D 600 MHz NMR spectrum of seed (cotyledons) from MA12 genotype of *T. cacao* and supplementary data on metabolic profiles of the seeds of SCA12 and SCA6 genotypes are reported (Appendices 11 and 12). The low frequency region from 0.0 to 4.0 ppm, had peaks mainly consisting of amino acids. The mid frequency region from 0.4-6.0 ppm showed peaks mainly for organic acids and carbohydrates. The high frequency region from 6.0-10.0 ppm contains mainly aromatic resonances. The assignments of the seed NMR spectrum are summarized in Table 11 with a wide range of metabolites, including amino acids, carbohydrates, phenolics and others compounds. Amino-acids founds in *T. cacao* seed include: leucine, isoleucine, valine, threonine, alanine, δ-amino-butyric acid, proline, glycine, glutamic acid, aspartic acid, asparagine, glutamine, tyrosine, tryptophane and phenylalanine. Sugars were identified by their characteristic peaks: both rafinose, sucrose and stachyose show a peak at 5.4 ppm (d), with rafinose being identified by its peak at 4.86 ppm (d, J=3.7 Hz). Others sugar included the anomeric proton of β-glucose at 4.58 ppm (d, J= 7.9 Hz) and α-glucose at 5.18 (d, J= 3.7 Hz),

the anomeric proton of α-maltose at 5.30 ppm (d, J= 3.9 Hz) and β-maltose at 5.29 ppm (d, J= 3.9 Hz), the anomeric proton of β-galactose at 4.52 ppm (d, J= 7.8 Hz), fructose at 4.07 (m) and stachyose being identified at 5.42 ppm (d, J= 4.0 Hz). Organic acids include: lactic acid, quinic acid, chlorogenic acid, shikimic acid, malic acid, citric acid, succinic acid, cis-aconitic acid, trans-aconitic acid and formic acid. The major group of metabolites present were phenylpropanoids and flavonoids. The flavonoids present included: hesperidin, (-)-epicatechin, (+)-catechin and rutin. The phenylpropanoids present included: caffeine, theobromine, caffeic acid, and hydroxybenzene derivatives were identified. Peaks corresponding to trigonelline, choline, ethanolamine, adenine, adenosine, uridine, hydroxybenzene derivatives and betaine were also observed in the spectra analyzed. Of the metabolites identified in fresh seeds (day 0), fourty-eigth were common to our three genotypes whereas a single metabolite is still absent in the sugar and organic acids region (4.0-6.0 ppm) in SCA12 and SCA6, raffinose.

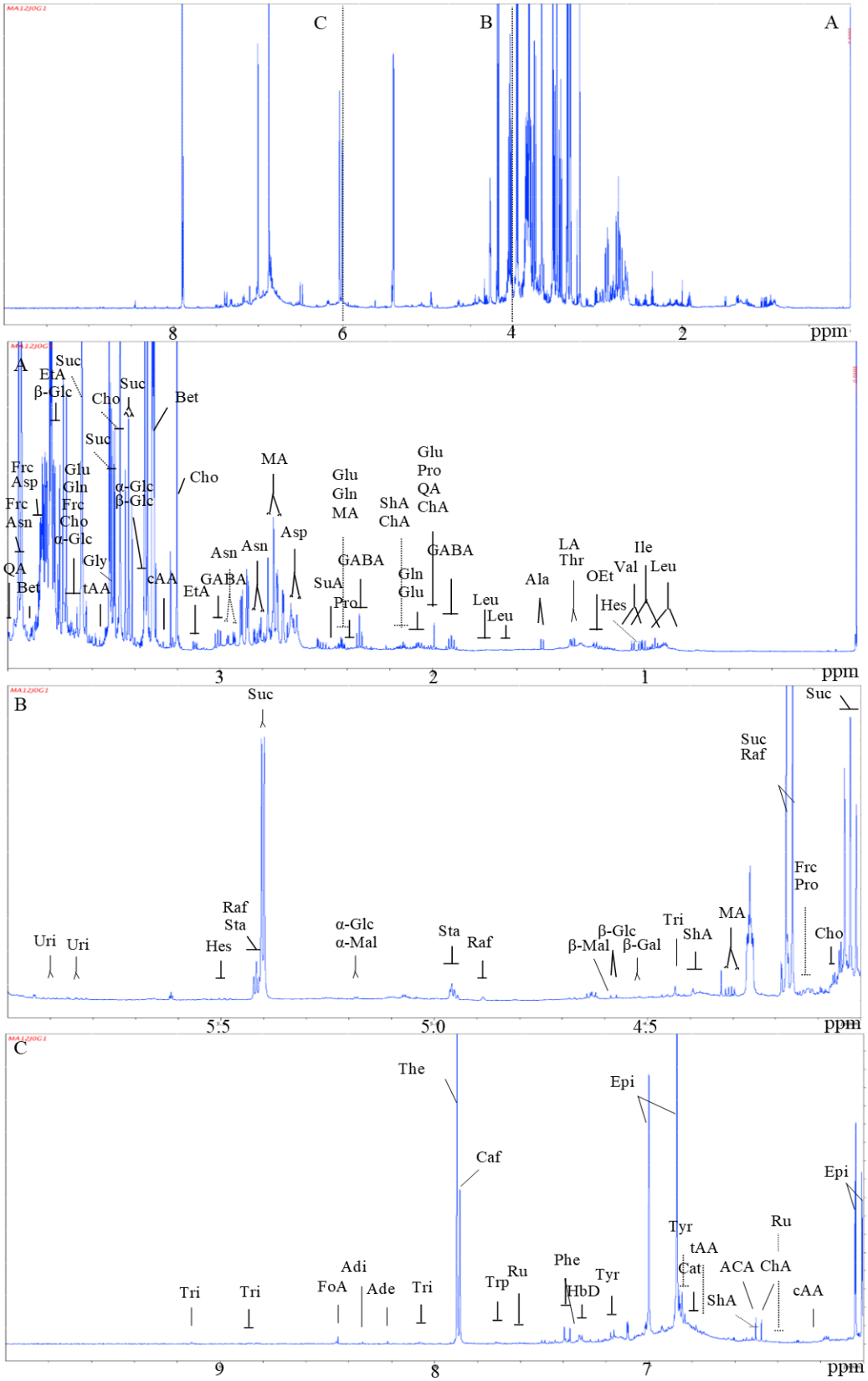


Figure 35. Characteristic NOESY ^{1}H NMR-1D spectrum of MA12 seed (coyledon) 0 DAI (collected from mature pod aged of 16 weeks) full names are reported in table 12). Three regions are shown separately A (δ 4.0-0.0), B (δ 6.0-4.0) and C (δ 9.0-6.0) for the assignment.

Table 11. Principal metabolites identified by 1H NMR in seeds extracts of MA12 (cotyledons) at day 0 (abbreviations used in Fig. 31 are indicated in brackets).

Metabolite	Chemical shift (δ), coupling constant (J) and multiplicity	Group
Amino acids		
Leucine (Leu)	0.97 (d) J= 6.3	5 CH_3
	0.98 (d) J=6.6	5'CH_3
	1.66 (m)	4 CH
	1.74 (m)	3 CH_2
Isoleucine (Ile)	0.97 (t) J=7.5	5 CH_3
	1.02 (d) J=7.0	4'CH_3
Valine (Val)	1.00 (d) J=7.0	4'CH_3
	1.05 (d) J = 7.1	4 CH_3
Threonine (Thr)	1.33 (d) J= 6.6	4 CH_3
Alanine (Ala)	1.48 (d) J = 7.2	3 CH_3
δ-amino butyric acid (GABA)	1.90 (m)	3 CH_2
	2.32 (t) J = 7.2	2 CH_2
	3.1 (t) J = 7.3	4 CH_2
Proline (Pro)	2.00 (m)	4 CH_2
	2.07 (m)	3 CH_2a
	2.33 (m)	3 CH_2b
	4.07 (dd) J = 6.4 -8.8	2 CH
Glutamic acid (Glu)	2.03 (m)	3 CH_2a
	2.13 (m)	3 CH_2b
	2.39 (m)	4 CH_2
	3.69 (dd) J =4.3-7.4	2 CH
Glutamine (Gln)	2.13 (m)	3 CH_2
	2.45 (m)	4 CH_2
	3.71 (t) J = 6.1	2 CH
Aspartic acid (Asp)	2.65 (dd) J = 9.2-17.3	3 CH_2a
	2.82 (dd) J = 3.7-17.4	3 CH_2b
	3.83 (dd) J = 3.7-9.2	2 CH
Asparagine (Asp)	2.82 (dd) J = 8.2-8.8	3 CH_2a
	2.95 (dd) J = 3.9-13.0	3 CH_2b
	3.94 (dd) J = 3.9-8.2	2 CH
Glycine (Gly)	3.50 (s)	2 CH_2
Tyrosine (Tyr)	6.85 (m)	3, 5 CH
	7.18 (m)	2, 6 CH
Tryptophane (Trp)	7.28 (s)	2 CH
	7.53 (d) J = 8.1	1 CH
	7.72 (d) J = 7.9	4 CH
Phenylalanine (Phe)	7.36 (m)	2, 3, 4, 5, CH

Table 11 continued

Organic acids		
Lactic acid (LA)	1.32 (d) J = 6.9	3 CH_3
Quinic acid (QA)	1.87 (dd) J = 10.2 -13.5	6 CH_2a
	1.92 (ddd) J = 2.6 – 4.9-14.4	6 CH_2b
	2.01 (ddd) J = 2.3-4.9-13.5	2 CH_2a
	2.03 (dd) J= 3.5-14.4	2 CH_2b
	3.52 (dd) J = 3.2-8.6	4 CH
	3.98 (ddd) J = 4.9-8.6-10.2	5 CH
	4.10 (m)	3 CH
Chlorogenic acid (ChA)	6.40 (d) J= 15.9	8’CH
	6.89 (d) J = 8.2	5 CH
	7.11 (dd)	1 CH
	7.65 (d) J = 15.9	7’ CH
Shikimic acid (ShA)	2.19 (m)	6 CH_2a
	2.76 (m)	6 CH_2b
	4.37 (m)	3 CH
	6.44 (m)	2 CH
Malic acid (MA)	2.44 (dd) J = 9.2-15.5	3 CH_2a
	2.76 (dd) J = 3.7-15.5	3 CH_2b
	4.30 (dd) J = 3.7-9.2	2 CH
Citric acid (CA)	2.59 (d) J = 16.3	3 CHa
	2.77 (d) J =16.3	3 CHb
Succinic acid (SuA)	2.50 (s)	2, 3 CH_2
cis-Aconitic acid (cAA)	3.27 (d)	4 CH_2
	6.24 (s)	2 CH
trans-Aconitic acid (tAA)	3.57 (s)	2 CH_2
	6.73 (s)	4 CH
Formic acid (FA)	8.45 (s)	CH

Table 11 continued

Carbohydrates		
1-O-Ethyl-β-D- glucoside (OEt)	1.20 (t) J = 7.0	
β-Glucose (β-Glc)	3.19 (dd) J = 7.9-9.4	2 CH
	3.36 (m)	4 CH
	3.87 (dd) J = 2.2-12.3	6 CH_2
	4.58 (d) J = 7.9	1 CH
α-Glucose (α-Glc)	3.36 (m)	4 CH
	3.47 (dd) J = 3.8-9.8	2 CH
	3.70 (dd) J = 5.3-11.9	6 CH_2
	5.18 (d) J = 3.7	1 CH
Sucrose (Suc)	3.43 (t) J = 9.7	4 CH
	3.50 (dd) J = 3.8-9.9	2 CH
	3.65 (d) J = 1.6	1’ CH_2
	3.74 (t) J = 9.2	3 CH
	4.02 (t) J = 8.2	4’ CH
	4.17 (d) J = 8.7	3’ CH
	5.40 (d) J = 3.8	1 CH
Fructose (Frc)	3.52 (m)	1 CH_2a
	3.67 (m)	1 CH_2b
	3.78 (m)	3 CH
	3.84 (dd) J = 3.3-9.9	6 CH_2a
	3.93 (m)	5 CH
	4.02 (dd) J = 1.3-12.7	6 CH_2b
	4.07 (m)	4 CH
Raffinose (Raf)	4.18 (d) J = 8.7	3’’ CH
	4.86 (d) J = 3.7	1 CH
	5.41 (d) J = 3.9	1’ CH
β-Galactose (Gal)	4.52 (d) J = 7.8	1 CH
α-Galactose (Gal)	5.22 (d) J = 3.9	1 CH
β-Maltose (Mal)	4.59 (d) J = 7.9	1’ CH
	5.29 (d) J= 3.9	1 CH
Stachyose (Sta)	4.95 (d) J = 3.6	1’ CH
	4.96 (d) J = 3.6	1 CH
	5.42 (d) J = 4.0	1’’ CH
α-Maltose (Mal)	5.18 (d) J = 3.8	1’ CH
	5.30 (d) J = 3.9	1 CH

Table 11 continued

Phenolics		
Cinnamic acid (CiA)	6.47 (d) J =16.0	
	7.37 (d) J = 15.9	
	7.41(m)	
	7.62 (dd) J= 8.1-1.0	
(-)-Epicatechin (Epi)	2.74 (dd)	4 CHa
	2.9 (dd)	4 CHb
	4.3 (m)	3 CH
	4.93 (s)	2 CH
	6.05 (d) J = 1.96	8 CH
	6.07 (d) J = 1.98	6 CH
	6.87 (m)	5’CH + 6’CH
	7.01 (d)	2’ CH
Hesperidin (Hes)	1.08 (dd)	
	2.78 (ddd)	
	5.48 (m)	
	6.90 (m)	
	6.94 (m)	
Rutin	6.39 (d) J = 2.0	6 CH
	7.61 (dd)	6’ CH
	7.66 (d)	2’ CH
Uridine (Uri)	5.85 (d) J = 8.15	5 CH
	5.90 (d) J = 4.77	1’ CH
	7.92 (d) J = 8.13	6 CH
Hydroxybenzene derivatives (HbD) [a]	6.86 (d) J = 8.7	
	7.33 (d) J = 8.6	
(+)-Catechin (Cat)	6.84 (m)	6’CH

Table 11 continued

Others compound		
Ethanolamine (EtA)	3.11 (m)	1 CH_2
	3.81 (m)	2 CH_2
Trigonelline (Tri)	4.45 (s)	CH_3
	8.10 (m)	5 CH
	8.86 (m)	4, 6 CH
Betaine (Bet)	3.28 (s)	CH3
	3.86 (s)	2 CH2
Choline (Cho)	3.21 (s)	CH_3
	3.51 (m)	2 CH_2
	3.86 (m)	1 CH_2
Adenine (Ade)	8.19 (s)	8 CH
	8.21 (s)	2 CH
Caffeine (Caf)	3.33 (s)	N_3CH_3
	3.50 (s)	N_1CH_3
	3.93 (s)	N_7CH_3
	7.86 (s)	6 CH
Theobromine (The)	3.91 (s)	N_7CH_3
	7.88 (s)	6 CH
Caffeic Acid (ACA)	6.35 (d) J = 15.9	8 CH
	7.06 (dd) J = 8.2-1.2	9 CH
	7.15 (d) J = 1.9	7 CH
	7.29 (d) J = 15.9	2 CH
Adenosine (Adi)	8.25 (s)	8 CH
	8.36 (s)	2 CH

Note : ^{1}H chemical shift (δ) are expressed in ppm, coupling constant (J) are expressed in Hz and the multiplicity of the signal is indicated in the brackets. Proton assignment is indicated in the third column. NMR solvent : methanol-d4/buffer (KH_2PO_4 90 mM in D_20 Ph 6.0, containing 0.025% (w/w) TMSP-d4). [a] potentially prunin on the basis of Arisawa et *al*. (1972).

III.1.3.2.3 Metabolites changes in the three genotypes grown in normal (control) condition

Little difference was observed between the spectra of the methanol/water extracts of the various seedlings grown under normal conditions. The ^{1}H-NMR spectra of the methanol/water extracts of SCA12, SCA6 and MA12 plants at day 4 and 8 is shown in Fig.36. Many intense peaks signals are observed in aliphatic (δ 0.0-4.0 ppm) and in sugar (δ 4.0-6.0 ppm) regions at days 4 in SCA12 samples than in SCA6 and in MA12. However, these signals were reduced at day 8 in SCA12 whereas its increased in SCA6 and MA12 in the same regions. The four major signals observed in the aromatic region were assigned to theobromine and epicatechine. Alhough there were clear visual differences between the spectra, for non biaised interpretation of the results, the samples were analysed using multivariate analysis.

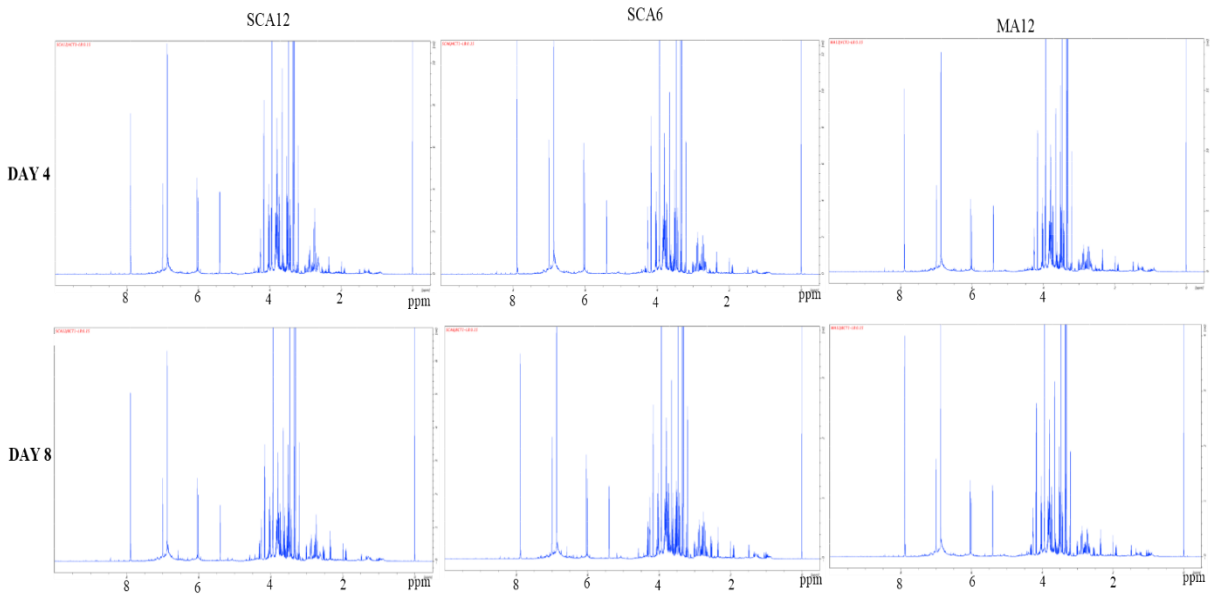


Figure 36. Characteristic NOESY ^{1}H NMR-1D spectra based on SCA12, SCA6 and MA12 data of samples grown under normal conditions at the two different kinetic points (D4, D8).

PLS-DA allowed possible discrimination among the three cacao samples but a great variability was observed among samples of the same group (Fig. 37). Consequently, the total variance (PC1 and PC2) was very low. PC1 accounted for 14.7% while PC2 accounted for 14.6% of the total variance. MA12 samples were grouped on the positive side of PC1, and SCA12 and SCA6 samples were on the negative side of PC1. There is a clear separation between SCA12 and SCA6 samples into two groups according to kinetic parameter (day 4 and day 8), explained here by PC2.

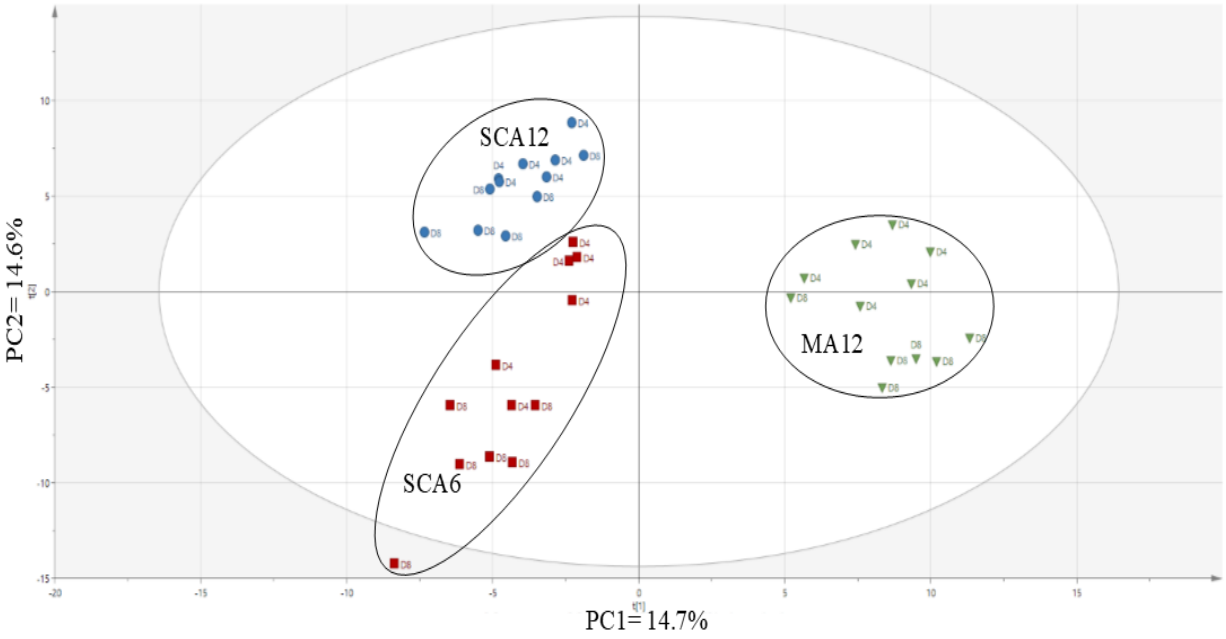


Figure 37. Partial Least Square-Discriminant Analysis (PLS-DA) of SCA12, SCA6 and MA12 NMR-^{1}H data, comparison of cultured seedlings samples under normal condition (control), at two different kinetic points (D4, D8). Spectra were realigned, cut into 267 bins and integrated to perform multivariate analysis.

Once differences is observed globally, the next step is to identify individual metabolite differences that contribute to the PLS-DA or metabolic profile separation by identifying the responsible metabolites using loadings. Mean areas of the characteristics peaks of the dicriminants metabolites were compared among the three genotypes using the Wilcoxon Rank Sum test, at different p-values: $p<0.001$, $p<0.01$, $p<0.05$. Based on statistical analysis, trigonelline, shikimic acid, glutamic acid, proline, hesperidin and epicatechin were present in higher concentration in SCA12 as compared to SCA6 and MA12. The metabolites more abundant in SCA6 were caffeic acid, sucrose, asparagine, choline and rutin as compared to SCA12 and MA12. However, rutin was absent at day 8 in SCA12. Finally, malic acid, chlorogenic acid, threonine, 1-O-ethly-β-D-glucoside, phenylalanine, tryptophane and tyrosine were present in higher concentrations in MA12 at both kinetic points as compared to SCA12 and SCA6. However, malic acid was absent in SCA12 at day 8, whereas tryptophane was not detected in SCA12 and SCA6 at both kinetic points (Table 12).

Table 12. Discriminant metabolites among SCA12, SCA6 and MA12 genotypes in non-stressed (NS) seedling samples at the two kinetic points (D4 and D8).

Metabolite	**Wi-test**	**Ratio at D4**	**Wi-test**	**Ratio à D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Ratio at D8**
- discriminant for SCA12 genotype		***SCA12/SCA6***		***SCA12/MA12***		***SCA12/SCA6***		***SCA12/MA12***
Trigonelline	***	0.71	***	1.72	***	2.35	***	2.13
Shikimic acid	/	1.04	**	1.23	/	1.11	**	1.40
Glutamic acid	*	1.32	*	1.35	/	1.08	/	1.15
Proline	*	1.40	**	1.46	*	0.83	/	1.04
Hesperidin	/	0.93	**	1.57	/	1.26	*	1.49
(+)-Epicatéchine	/	1.31	*	1.32	/	1.40	/	1.39
- discriminant for SCA6 genotype		***SCA6/SCA12***		***SCA6/MA12***		***SCA6/SCA12***		***SCA6/MA12***
Rutine		2.44		1.62		ND1		2.63
Caffeic acid	/	1.09	/	1.43	/	1.40	*	1.37
Sucrose	***	1.54	***	1.78	**	1.69	***	1.93
Asparagine	/	1.13	/	1.37	**	1.47	*	1.36
Choline	***	2.14	***	2.90	**	2.04	***	3.22

Table 12 continued

- discriminant for MA12 genotype		***MA12/SCA12***		***MA12/SCA6***		***MA12/SCA12***		***MA12/SCA6***
Malic acid	/	1.04	*	1.18	/	1.28	*	ND1
Chlorogenic acid	*	1.97	/	1.49	***	2.76	**	2.60
Threonine	***	1.68	***	1.80	**	1.40	***	1.69
1-o-ethyl-β-D-glucoside	***	1.50	***	1.40	***	1.54	**	1.32
Phenylalanine	/	1.43	/	1.35	***	2.09	**	1.82
Tryptophane		ND1		ND2	***	1.56	/	1.17
Tyrosine	*	1.54	*	1.36	**	1.69	*	1.23

ND1 : not detected in SCA12 ; ND2 : not detected in SCA6. ***0.001, **0.01, *0.05, / not significative.

III.1.3.2.3 Metabolite changes in the three genotypes due to water deficit stress

- Comparison of metabolites of the three genotypes in drought stress conditions

The typical NOESY ^{1}H-NMR spectra based on SCA12, SCA6 and MA12 data of samples grown under drought stress condition at the two kinetic points (D4, D8) is presented by Fig. 38, suggesting that polar metabolite components in samples are highly influenced by the origin and by the duration of the induced water deficit in the medium. The visual inspection of spectra showed that peak signals are relatively constant in SCA12 samples stressed with PEG at days 4 and 8 as compared to SCA6 and MA12. In the aliphatic region (δ 0.0-4.0 ppm), the signals were smaller compared to those in the sugar or aromatic region. SCA6 showed low variations of signals intensities in sugars and aliphatic regions. The sugars region was found to be most influenced in MA12 extracts, as it showed the smallest peaks signals at day 8, as compared to the others extracts.

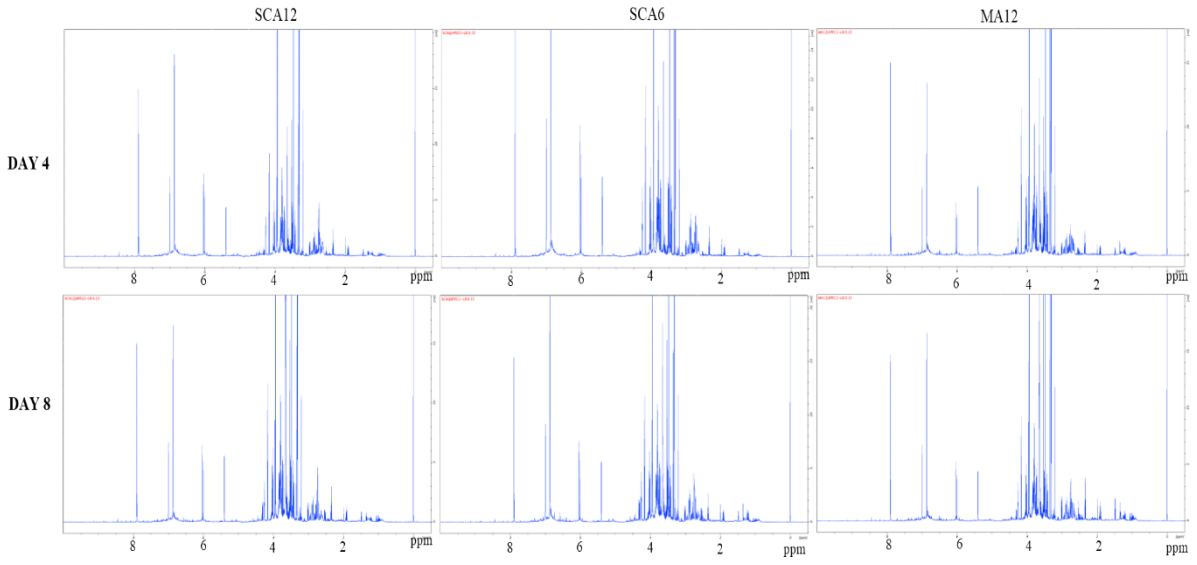


Figure 38. Characteristic NOESY ^{1}H NMR-1D spectra based on SCA12, SCA6 and MA12 data of samples grown under water stress conditions at the two different kinetic points (D4, D8).

PLS-DA analysis of the methanol fraction of the seedlings extracts of the three genotypes is showed in Fig. 39. PC1 and PC2 interact to separate the MA12 samples from the SCA6 and SCA12 samples. While SCA6 and SCA12 samples were well separated according to PC2,

with MA12 samples grouped in the positive side of PC2 and SCA12 samples grouped on the negative side of PC2. PC1 and PC2 accounted for 28.9 % of the total variance.

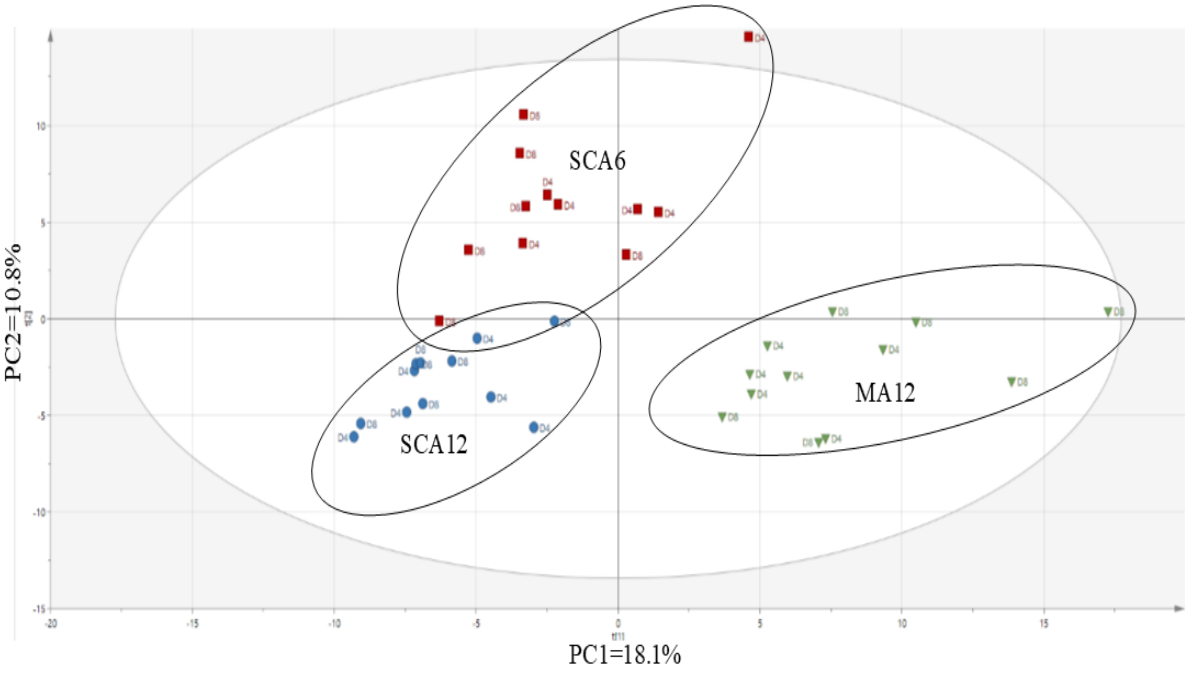


Figure 39. Partial Least Square - Discriminant Analysis (PLS-DA) of SCA12, SCA6 and MA12, comparison of cultured seedlings under drought stress conditions, at two different kinetic points (D4, D8). Spectra were realigned, cut into 267 bins and integrated to perform multivariate analysis.

Table 13 highlights the metabolites that discriminated SCA12, SCA6 and MA12 under water stress conditions, with their respective ratios and significance at the two kinetic points (D4, D8). The trigonelline, α-glucose, proline, fructose and hesperidin are more accumulated in SCA12 than in SCA6 or MA12 whereas chlorogenic acid, trans-aconitic acid, malic acid, ethanolamine and rutine accumulated more in SCA6. Formic acid, phenylalanine, shikimic acid, sucrose, choline and aspartic acid accumulated more in MA12 samples than in SCA12 and SCA6 samples. The rutin were not detected at day 4 in SCA12 and MA12.

Table 13. Discriminant metabolites among SCA12, SCA6 and MA12 genotypes in water-stressed (WS) seedling samples at the two kinetic points (D4 and D8).

Metabolite	**Ratio at D4**	**Wi-test**	**Ratio à D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Ratio at D8**	**Wi-test**
- discriminant for SCA12 genotype	***SCA12/SCA6***		***SCA12/MA12***		***SCA12/SCA6***		***SCA12/MA12***	
Trigonelline	1.28	*	1.59	***	1.27	/	1.23	/
α-Glucose	1.20	/	1.29	/	1.68	***	1.67	/
Proline	1.06	/	1.08	/	1.27	/	1.16	/
Fructose	1.32	*	1.00	/	1.43	/	1.94	*
Hesperidin	ND2		ND3		1.07	/	ND3	
- discriminant for SCA6 genotype	***SCA6/SCA12***		***SCA6/MA12***		***SCA6/SCA12***		***SCA6/MA12***	
Chlorogenic acid	1.03	/	1.19	/	1.51	*	1.43	*
trans-Aconitic Acid	1.28	/	1.68	*	1.89	*	1.81	/
Malic Acid	0.95	/	1.34	/	1.09	/	1.28	*
Ethanolamine	1.18	*	1.33	**	1.11	/	1.12	/
Rutin	ND1		ND3		1.47		2.32	
- discriminant for MA12 genotype	***MA12/SCA6***		***MA12/SCA12***		***MA12/SCA6***		***MA12/SCA12***	
Formic Acid	0.90	/	1.13	/	5.04	***	4.52	**
Phenylalanine	1.38	/	1.90	**	2.80	**	3.74	***
Shikimic Acid	1.27	/	1.64	**	1.63	**	1.84	***
Sucrose	1.04	/	1.26	***	1.13	/	1.14	/
Choline	1.02	/	1.33	**	1.28	*	1.22	/
Aspartic acid	1.03	/	1.31	**	1.20	/	1.20	**

ND1 : not-detected in SCA12 ; ND2 : not-detected in SCA6 ; ND3 : not-detected in MA12

- Comparison of metabolites in non-stressed (NS) and water-stressed (WS) conditions for each genotype

As previously done with *Theobroma cacao* callus, further metabolomics analysis were performed with seedlings samples in order to understand the intra-specific behaviour of our genotypes (SCA12, SCA6 and MA12) under water stress conditions. Unfortunatenately, multivariate analysis (PCA and PLS-DA) did not allow a clear separation of NS and WS samples for SCA12 genotype, while PCA did not allow separation of NS and WS samples for SCA6 and MA12 genotypes (data not show). Consequently, PLS-DA scores plot was performed only for the two genotypes (SCA6 and MA12) and used for further investigations. For both genotypes, a water stress period induced some changes at the metabolic level : WS and NS samples are well distinguished (Fig. 40). For SCA6 NMR data, the PCs (PC1= 14.2 % and PC2=16.1 %) interact in the separation of NS and WS samples, while for the MA12 (PC1=16.3 % and PC2= 14.9 %), the PLS-DA score plot of NMR data also shows a characteristic grouping for NS and WS samples.

Metabolites discriminating the two genotypes (SCA6 and MA12) for the WS samples were then identified and integrated. These results are presented below (Tables 14) and suplementary informations on their respective ratios in water-stressed (WS) versus non-stressed (NS) conditions are presented in appendix 12.

Statistical analysis highlighted 10 metabolites that their content changed during water stress for SCA6 and 11 for MA12. During water stress, the less-tolerant genotype SCA6 showed 6 metabolites that were more accumulated in WS samples than in NS samples after 8 days, namely fructose, 1-O-ethyl-β-D-glucoside, asparagine, proline and lactic acid. Moreover, β-glucose was not detected in WS samples after 4 days whereas it was present in NS samples of SCA6 genotype (see Appendix 13). For MA12, 7 metabolites contents, namely, fructose, sucrose, 1-O-ethyl-β-D-glucoside, rutin, chlorogenic acid, quinic acid and lactic acid were found to be significatively higher in WS samples than in NS samples after 8 days of water stress. Moerover, rutin and chlorogenic acid were not detected in NS samples after 8 days whereas they were present in WS samples (Appendix 13).

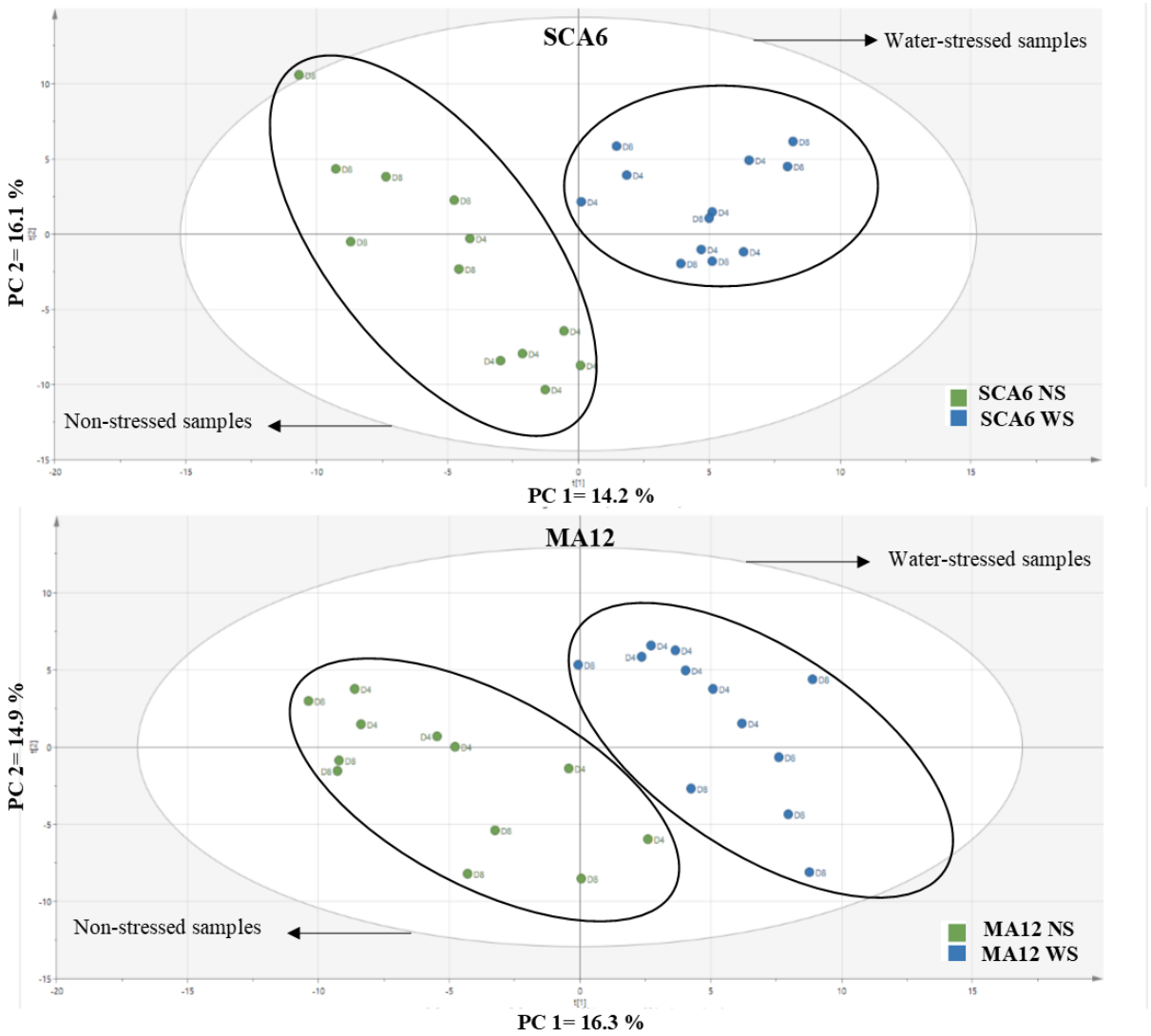


Figure 40. Partial least square discriminant analysis (PLS-DA) based on SCA6 and MA12 ^{1}H - NMR data, comparison of cultured samples under normal (non-stressed) and under middle drought (water-stressed) conditions at two different kinetic points (D4, D8). Spectra were realigned, cut into 267 bins and integrated to perform multivariate analysis.

Table 14. Comparison of the discriminant metabolites for SCA6 and MA12 genotype of *T. cacao* in water-stressed (WS) versus non-stressed (NS) conditions at D8.

Genotypes				
	SCA6		MA12	
	NS>WS (down regulated)	WS>NS (up regulated)	NS>WS (down regulated)	WS>NS (up regulated)
Sugars metabolism	β-Glucose	Fructose	α-Maltose	Fructose
	Sucrose	1-O-Ethyl-β-D-glucoside	β-Glucose	Sucrose
				1-O-ethyl-β-D-Glucoside
Amino acids metabolism	Glutamine	Proline	Asparagine	
	Leucine			
Phenolics metabolism	Hydroxybenzen derivatives			Rutin
Organic acids metabolism		Lactic acid	Malic acid	Chlorogenic acid
				Quinic acid
				Lactic acid
Others metabolism	Choline			

Significant differences between ratio of NS and WS samples of SCA6 and MA12 genotypes are indicated by the following color code:

Yellow frame: < 1.5 in NS samples; Light yellow: > 1.5 times in NS samples; Dark yellow: > 3.0 times in NS samples; Bleue frame: <1.5 times in WS samples; Light bleue: > 1.5 times in WS samples; Dark bleue: > 3.0 times in WS samples.

III.2 DISCUSSION

III.2.1 Determination of the influence of the position of flower buds in a single tree on somatic embryogenesis of *T. cacao*

The somatic embryogenesis is the process by which somatic cells, under inductive conditions, generate embryogenic cells, which undergo a series of morphological and biochemical changes resulting in the formation of somatic embryos (Komamine *et al*., 2005; Businge *et al*., 2013). The somatic embryogenesis based on the cellular totipotency which is unique to higher plants. This clonal technique is becoming a prominent *in vitro* regeneration system for cacao. The somatic embryogenesis allows rapid regeneration of elite genotypes, germplasm conservation and genetic transformation system (Maximova *et al*., 2002). However, the recalcitrance of some *T. cacao* genotypes to this technique and the numerous factors that control it limit its systematical exploration. In this study, a comparative approach was applied to study the physiological differences among *T. cacao* explants-derived callus from different origins with the hope to determine the influence of the position of flower buds in a single tree on somatic embryogenesis of *T. cacao*. The influence of the position of flowers buds on the tree on somatic embryogenesis of "SCA6" genotype, known as highly productive in farm was analyzed. The variation of phenols compounds, total soluble sugars, proteins contents and peroxidases specific activities was also performed in these conditions.

The results showed that the two types of explant used (staminodes and petals) are favorable to callogenesis with an average frequency above 80% in all callus-derived explants used in our experimentation. These results are in agreement with those previously obtained by Li *et al*. (1998) and Minyaka *et al*. (2009). These authors showed that callogenesis was effective in *T. cacao* using floral explants. The presence of 2,4- Dichloro-phenoxyacetic acid (2,4-D) and that of Thidiazuron (TDZ) in the culture medium is responsible of this callogenesis. The effect of auxin/cytokinin in the induction of somatic embryogenesis process have been reported in many species such as *Ricinodendron heudelotti* (Fotso *et al.*, 2007) and *Vitis vinifera* (Olah *et al.*, 2009). This callogenesis success could be due to the high mobilization of soluble sugars during the development step. This consolidate the fact that reducing carbohydrates are important for callus formation and cell differentiation as developed by Ana *et al.*, (1997). In fact, reducing carbohydrates regulate osmotic pressure (Blanc *et al.*, 1999) and are major components of cell wall. This study demonstrated that the use of carbohydrates depend on the position of the explants on the tree. The same observation was made for the enzymatic equipment which varies

in composition and/or function in cells of flowers buds according to their position on the tree. The study of Alemano *et al.* (2003) showed that flowers buds of cacao contain different types of phenolic compounds, and each type can be expressed qualitatively and quantitatively according to the developmental stage of the flowers, indicating the key role played by these metabolites in the regulation of cell differentiation events.

The percentage of callus producing somatic embryos was highly influenced by the origin of staminodes cultivated in culture media. In fact, the average frequency of callus producing somatic embryos increased from the orthotropic main stem (OS) to the secondary plagiotropic fan branch (FII) (with a maximum obtained in staminodes-derived callus from FII). Except for flowers buds from FII, which did not differentiate somatic embryos, the same observation was made for callus from petals. The spectral quality of light is an important signal for plants about their environment. Perception of light quality allows plants to sense the presence of other plants in their neighborhood, as well as shade, daytime and seasons (Smith, 1986 ; Ballaré *et* al., 1989 ; Wolfgang, 2007). These cues help the plants to adjust their development by increasing their aptitude to compete for resources, detect changes in daylength (Facella *et al*., 2008 ; Kidokoro *et al*., 2009 ; Nakamichi *et al*., 2011 ; Staiger *et al*., 2011) or, on a longer term, to be prepare for drastic environmental changes such as low temperatures in winter (Leduc *et al*., 2014). All of these signals can modulate the capacity of buds to grow out. This could justify the fact that flowers buds from FII, more exposed to ligth (and consequently more prepared to water stress) gave a better response to somatic embryogenesis process, as compared to those from OS, more shaded. The low rate of somatic embryos produced can be explained by the recalcitrance of this crop to somatic embryogenesis process (Minyaka *et al*., 2008).

The soluble sugars content was high in callus from all explants during embryos dedifferentiation step in FI and FII. The high content of soluble sugars may be due to the important role play by carbohydrates metabolism in the processes of organogenesis and morphogenesis. During embryos differentiation steps, sucrose content is progressively decreased. Moreover during callogenesis, there is a decrease of carbohydrates content in flowers buds suggesting an important utilization of these metabolites. Zygotic and somatic embryos highly accumulate enzymes of carbohydrate metabolism as demonstrated by several studies (Iraqi *et al*., 2001; Hendriks *et al*., 2003; Noah *et al*., 2013). The intense carbohydrate metabolism can be explained by the heavy energy demand required for processes that occur during cell division and elongation. One of the factors generally responsible of the *in vitro* recalcitrance of cacao is the high content of polyphenol compounds and their oxidation. The

high accumulation of these metabolites was particularly observed during callogenesis and embryos differentiation steps. All the explants used expressed a significant decrease in phenols compounds during the stage of embryos dedifferentiation (after 49th days) when growing in DKW medium supplemented with sulphate, with the lowest content reported in petals-derived callus from FII. There are several internal and external factors affecting the quality and/or the quantity of polyphenol compounds in plants. The quantitative differences registered within explants-derived callus from different source could be explained by the interaction of several factors including genetic, physiological and agronomic factors (like the position of flower bud on the tree) by modifying the final concentration of polyphenol compounds in each flower (Roubelakis-Angelakis and Kliewer, 1986), and by environmental factors (microclimate). Besides light and temperature, the availability of plant nutrients also has a great influence on the accumulation of polyphenol compounds (Doak and Miller, 1968; Wang and Zheng, 2001; Piccaglia *et al*., 2002). In addition, a high accumulation of polyphenol compounds in floral explants during induction steps has been demonstrated to be not favorable to somatic embryogenesis process. A similar result was obtained in date palm (*Phoenix dactylifera* L.) by Zouine and El Hadrami (2004).

The peroxidase activities were high in callus during induction steps of somatic embryogenesis and low during dedifferentiation step. The decrease activities of peroxidase during dedifferentiation have also been observed in other plant systems and was correlated with the browning of the tissue (Chun-Ping *et al*., 2015). This result underlines the implication of peroxidase in somatic embryogenesis. In fact, the higher peroxidase activity observed during dedifferentiation step in callus of petals could justify their low embryos production capacity compared to their staminodes counterpart.

In staminode explants, more suitable for somatic embryogenesis process, sugars and phenolics contents were found to be negatively related in tissues from FII. This suggests the use of sugars for the production of metabolic intermediates involved in the biosynthesis of several organic molecules, including phenolics which are essential for the control of environmental stress effects in these FII tissues. Sugars (such as disaccharides, raffinose family oligosaccharides, and fructans) are strongly related to the accumulation of reactive oxygen species under stress conditions (Keunen *et al*., 2013). However, in tissues from FI, a rather positive and significant correlation was observed between phenolics contents and the specific activities of peroxydase, suggesting that these metabolites and enzymes act together to maintain cell stability through the lowering of the amount of reactive oxygen species. A negative significant correlation was

observed between proteins and sugars contents of callus from OS. This could suggest that high proteins content are maintained among other by the absorption of sugars and their utilization for protein synthesis making sugars less available for other metabolic routes in these tissues.

III.2.2 Effect of water stress on somatic embryogenesis of *T. cacao*

Physiological analysis of the somatic embryogenesis process, using callus of SCA12, SCA6 and MA12 genotypes has been realized according to their tolerance to water stress induced by PEG during development. The total phenolics and flavonoid contents was determined in callus growing in this conditions of stress during somatic embryos dedifferentiation stage and the response of the three genotypes was compared. The frequency of germination of the secondary somatic embryos treated with three osmotic agents, namely PEG, mannitol and sorbitol at different concentrations in the maturation media was also compared for SCA6 genotype.

The results showed that cacao callus growing in medium containing the non-permeable and the non-metabolized compound polyethylene glycol (PEG) (8000 Mw) changed their morphology and progressively loose their tissues by the decrease of turgor pressure. The PEG was reported to be able to change the cell membrane stability and integrity (Lygin *et al*., 2012), which could affect tissue metabolism. The implication of turgor pressure in the conservation of cell-wall integrity and the regulation of induced responses was demonstrated in Arabidopsis seedling model system treated with cellulose biosynthesis inhibitor and hyperosmotic stress (Wormit *et al*., 2012). This study showed that carbohydrate metabolism is responsive to the changes of cellulose biosynthesis activity and turgor pressure.

Our study showed clearly the effect of PEG on callus development and somatic embryos development *in vitro* in the three genotypes studied. It appeared that callus gradually lost their potential to differentiate somatic embryos as the level of water deficit in the medium increases. Importantly, the maximum and significant values of the frequency of callus producing somatic embryos and average number of embryos formed per callus in control conditions were obtained with SCA6, while SCA12 produced the maximum and significant values in drought conditions for these parameters, as compared to others genotypes. These results suggested that a particular attention should be put on the control of SCA6 cultures in drastic environmental conditions, because this genotype has been reported to be highly productive in the fields (Pang, 2006). On the other side, SCA12 genotype can be produced and largely distributed to farmers in the case of breeding program involving drought tolerance in cacao. Our results on the reduction of

embryos production from callus under the effect of PEG are in agreement with previous works done in species such as cacao (Eliane *et al*., 2019) and rice (Wani *et al*., 2010). The 7.5 % concentration (corresponding to an osmotic pressure of -0.91 bars) appeared to be the dose that inhibit the embryos formation in the MA12 genotype; whereas the SCA12 genotype showed a superior and significant tolerance to drought at this concentration. Therefore, SCA12, SCA6 and MA12 genotypes could be considered as tolerant (to drought), middle-tolerant and sensitive respectively.

The pathway of the formation of cacao somatic embryos and their subsequent proliferation is complex and regulated by several factors. These factors include the explant type and origin, and the composition of the culture medium (Niemenak *et al*., 2015; Minyaka *et al*., 2017; 2010). In fact, the average number of somatic embryos formed per callus decreased progressively with the increase of PEG concentration in the medium. The low embryos production and development common in cacao is a major problem for the application of somatic embryogenesis in cacao breeding programs (Maximova *et al*., 2002; Minyaka *et al*., 2010). The decrease in the regeneration capacity of callus, resulting from stress due to PEG, may be explained by the slowdown in all cell physiological processes that affect growth, multiplication and therefore morphogenesis.

Cacao is known as a plant with high contents of phenolic compounds in the seeds. In plants, phenols are related to many important processes, such as defense against herbivores and pathogens and are considered as a physiological indicators of stress and act as antioxydants (Hamrouni-Sellami *et al*., 2013; John *et al*., 2014). The high production of phenols during *in vitro* culture of plant tissues, has been related to the recalcitrance and the inhibition of callus formation and plant regeneration (Alemanno *et al*., 2003; Reis *et al*., 2008). Flavonoids have been reported to play a key role in the protection of plants against biotic and abiotic stresses (Mierziak *et al*., 2014). The application of PEG during callus differentiation stage reduced the phenolics and flavonoid contents in SCA12 and SCA6 tissues, whereas, in MA12, increases (of approximately 8% and 6%, for phenolics and flavonoids respectively) was observed with 2.5% and 7.5% PEG media, as compared to controls.

The relationship between total phenolic and total flavonoid contents in SCA12 (the tolerant genotype) and SCA6 (the less-tolerant genotype) showed that under high dose of PEG (7.5 %) flavonoid contribute to about 13 % in SCA12 and 24 % in SCA6 of the total phenolic content. On the contratry, high accumulation of flavonoid was observed in MA12 with 7.5 % PEG (94

% of the total phenolic content). The rest of the proportion comes from non-flavonoid compounds such as phenylpropanoids and methylxanthines. The phenolics and flavonoids constitute a major group of compounds which act as primary antioxidants (Adesegun *et al*., 2009) and are known to react with hydroxyl radicals, superoxide anion radicals and lipid peroxy radicals in response to stress (Chandra *et al*. 2014). These results clearly support our previous statement concerning the classification of the three cacao genotypes studied in relation to their tolerance to water stress, in which the genotype SCA12, with low flavonoid content, was found to be less susceptible to stress as compared to the SCA6 (less-tolerant) and MA12 (sensitive) genotypes, with middle and high flavonoid contents, respectively.

Comparison of maturation treatments indicated that PEG (7.5 % (w/v)) treatment followed by culture on medium containing 3.0 % sucrose was the most effective to promote germination of secondary somatic embryos in SCA6 genotype, as compared to mannitol and sorbitol, regardless of the different concentrations tested. Our results are in contradiction with those reported by Walker and Parrott (2001) in soybean somatic embryos, using mannitol, sorbitol and Polyethylene glycol-4000 in maturation media. These authors found that sorbitol (3.0 %) was the best supplement for embryos germination and conversion among the different osmoticant tested. In addition, the used of 3 % mannitol was not favorable to embryos maturation in this species. The analysis of the combined data for mannitol, sorbitol and PEG treatments revealed a significatnt difference ($p \leq 0.05$) among the type of treatment for the rates of germination of embryos at the concentration of 7.5 % (w/v), with slightly high of the mean frequency of embryos (66.7±6.45 %) treated with PEG higher than those treated with mannitol (54.17 ± 6.45 %). The lowest germination percentage was obtained with 10.0 % sorbitol. A significant difference ($p \leq 0.05$) was also found among the various concentrations for mannitol, sorbitol and PEG treatments.

III.2.3 Characterisation of the metabolites involved in water stress tolerance in *T. cacao*

III.2.3.1 Metabolomics analysis of callus under drought conditions

Metabolite status of Non-Stressed and Water-Stressed genotypes

Metabolite profiling of *T. cacao* callus tissues resulted in the identification of 54 identified compounds and more than 200 peak signals detected. To our best knowledge, no metabolite profiles were available for *T. cacao* callus, consequently comparisons to litterature was only

possible for single compound. *T. cacao* calluses metabolite profiles are dominated by abundant peaks in aliphatic and sugars regions. Regarding callus status after induction (28^{th} days), difference within SCA12, SCA6 and MA12 genotypes can be observed due to a single metabolite only detected in the sugar and organic acids regions in MA12: raffinose. The accumulation of raffinose and other raffinose family oligosaccharide (RFOs) are reported to be associate with their roles as energy storage reserves and osmoprotectants (Peterbauer and Richter, 2001; Karner *et al*., 2004; Dong *et al*., 2013; El Sayed *et al*., 2014). Additional variation in development of callus can be observed depending on the genetic status of the mother plants. This most likely explains a major part of the variability in the relative abundance of peak signals in spectra. Moreover, variation between SCA12, SCA6 and MA12 genotypes during development may have derived from differences in metabolites synthesis and/or degradation during culture conditions. In fact, during our investigation, explants were exposed to several plant growth regulators (PGRs) in culture medium during 28 days for the initiation of somatic embryogenesis. During this period, MA12 tissues could have developped more protective mechanisms against stress induced by PGRs than the two other genotypes. For MA12, as regards to raffinose accumulation in tissues during callus growth, we suspect that tissues were less suitable for the embryos induction process in hormone-free medium.

The three genotypes, SCA12, SCA6 and MA12 showed differences in their metabolome profile when growing in the absence of water stress during differentiation stage. Visual inspection (indicative of the overall changes at the metabolic level) shows that several regions in the spectra, including aliphatics amino acids, sugars and aromatics compounds, show peaks with decreasing signal intensities. Asparagine, leucine and valine contents were high in SCA12 compared to the others genotypes, while sucrose, ethanolamine and hydroxybenzen derivatives contents were high in SCA6. The high content of shikimic acid was observed only in MA12. The low differences in metabolite contents in *T. cacao* tissues may be due to the small metabolic variations between SCA12, SCA6 and MA12 calluses. Moreover, calluses growth were stopped after 8 days of culture in embryos development medium, at the stage where no visible differences were observed among our genotypes; so MA12 calluses were just at an early stage of callus differentiation and possible related metabolomic changes. We can hypothesise that, in the absence of water stress, the difference in their genetic background leads to a different metabolome profile in the three cacao genotypes.

In our study, water stress induced a decrease or loss of the peak signals in the sugars region. In *Arabidopsis*, sucrose starvation induced a rapid decrease in sugar compounds with a

concomittant increase in aliphatic and aromatic amino acids in cell cultures after 12 h of culture in medium (Kim *et al*., 2007), which suggest the implication of metabolic changes in the repression of cell division during sucrose starvation. Controversely, a decrease of the peak signals in the aliphatic (amino acids and some organic acids) and aromatic regions was also observed. The dramatic effects (loss of peak signals) of water stress on the metabolic profiles of calluses tissues increased with the duration of water stress treatment in the three genotypes, but this was less perceptible on SCA12 samples, where peaks were relatively constant at Day 8 compared to Day 4. PLS-DA performed with these samples did not show any clear discrimination between days of samplings. However, according to the Wi-test ($p<0.001$; $p<0.01$) compounds with increasing or decreasing significativity between Day 4 and Day 8 were found. Our results indicated that the main metabolites implicated in the response of SCA12 calluses to water stress during differentiation were amino acids, organic acids, sugars and other compounds including leucine, isoleucine, aspartic acid, formic acid, malic acid, β-glucose and allantoin (a product of uric acid oxydation). In fact, the accumulation of free branched chain amino acids (BCAAs) such as isoleucine and leucine may serve as a substrate for the synthesis of stress-induced proteins and BCAAs may act as signaling molecules to regulate gene expression in *Arabidopsis thaliana* (Nambara *et al*., 1998). The expression of BCAA aminotransferase, involves in the last step of the biosynthesis of BCAAs, is induced in response to dehydration stress (Urano et al., 2009). Aspartic acid is the common precursor of the essential amino acids lysine, threonine, methionine and isoleucine in higher plants. In addition, aspartic acid may also be converted to asparagine, in a potentially competing reaction (Azevedo *et al*., 2006), and may be used for storage and transport of nitrogen in plants. The purine metabolite allantoin was reported to enhance abiotic stress tolerance in *Arabidopsis* through synergistic activation of abscisic acid metabolism (Watanabe *et al*., 2014). High accumulation of β-glucose in SCA12 during water stress could be the consequence of adaptation of calluses to the increase of osmotic pressure the explants are subjected to.

Numerous studies have revealed that environmental stresses often raise the accumulation of phenylpropanoids, which are believe to play a regulatory role in some metabolic processes (Janas *et al*., 2000; Wróbel *et al*., 2005; Weidner *et al*., 2009). The increases in polyphenol compounds in this case may be explained by their function as antioxidants (Nakabayashi *et al*., 2014), a function that may be less necessary in MA12 than in SCA6. In fact, the current study has demonstrated that the contents of polyphenol compounds significantly increased in SCA6 and MA12 more than in SCA12 during stress. It is possible that the combined energetic costs

of drought stress and embryos induction process reduce the availability of energy and chemical precursors for an effective stress response in MA12 samples. Therefore, determining wheither such costs are additive or synergistic should be a key goal for the verification of this hypothesis.

The measured presence of tryptophan under control conditions in *T. cacao* calluses was found significantly higher in SCA12 than other genotypes; while it was significantly higher under water stress conditions in SCA6 than other genotypes. This might be due to the fact that tryptophan acts as a key precursor for auxin (indole-3-acetic acid) biosynthesis through the tryptophan-dependent pathway, which is involved, among others, in the establishment of embryo polarity during normal embryos formation and development (Michalczuk *et al*., 1992; Sitbon *et al*., 2000; Khan *et al*., 2015). It seems that the tryptophan accumulated at high levels in SCA12 samples under non stress conditions is used immediately for the synthesis of endogenous auxin under water stress conditions. The same trend was observed for valine, more accumulated in non stress conditions in SCA12 than in other genotypes while being more accumulated under water stress condition in MA12 than other genotypes, suggesting that instead of utilizing valine, MA12 secreted or low utilized it under water stress conditions. Proline was more accumulated in MA12 under control and water stress conditions, which also points to utilization or secretion of this amino acid. The role of proline in cultures during differentiation was examined in pea plants. Free proline may act as an osmoticum as well as a nitrogen storage pool and as a source of NADP+, which is necessary for the growing of embryos. The mediation of the cellular redox potential that results from proline accumulation have a large effect on the flux of energy through redox-sensitive biochemical pathways like pentose phosphate pathway (Ghanti *et al*., 2009). These results imply that amino acids that tolerant genotype (SCA12) utilized may remain the same in sensitive genotype, or be low utilized.

Some discriminant metabolites of the three genotypes under normal conditions are always discriminant under water stress conditions. This is the case of one metabolite that accumulates more in SCA12, four metabolites that accumulate more in SCA6 and two metabolites that accumulate more in MA12.

Leucine is the metabolite that accumulates more in SCA12. In the absence of water stress, leucine was significantly more accumulated in SCA12 than in SCA6 (significant at Day 4 and Day 8; $p<0.001$) and MA12 (significant at Day 4 and Day 8; $p<0.05$) as shown by the Wi-test. Water stress induced an increase in leucine content in SCA12, a decrease in this content in

MA12, leading to an amplification of the difference in leucine content between SCA12 and MA12 at Day 4 (SCA12/MA12 ratio: 1.46) and no change at Day 8; while no change was observed between SCA12 and SCA6. Rutin, vanilic acid, hydroxybenzene derivatives and p-coumaric acid accumulate more in SCA6. However, the high rutin content in SCA12 compared to SCA6 and MA12 in control condition, decreases in water stress condition. The increased content of rutin in SCA6, indicates that part of the tolerance of SCA6 when grown under drought condition may be due to the protective effect of rutin. Vanilic acid and hydroxybenzene derivatives were both more accumulated in SCA6 than SCA12 and MA12 under both conditions (control and water stress) in the same order because they are affected by water stress in each genotype. The content of p-coumaric acid increases under controlled water stress in SCA6 whereas in the other two genotypes it remains unchanged because of the same effect of water stress in this genotype. Shikimic acid and 1-O-ethyl-β-D-glucoside accumulate more in MA12. At day 4, the high shikimic acid content in MA12 decreases under water stress while increasing in SCA12 and SCA6, which leads to a reduction of the differences in the ratios between these genotypes whereas these ratios remain unchanged at day 8 for this metabolite. The same effect is observed for 1-O-ethyl-β-D-glucoside for each genotype. One can argue that these metabolites discriminating each genotype in both conditions are different taxonomic makers of our genotypes.

Investigation of the specific metabolites changes in SCA12, SCA6 and MA12 calluses due to water stress

No significant changes in polyphenols metabolism were observed in *T. cacao* in response to water stress, except for rutin and caffeic acid contents. Several flavonoids, including rutin, protect plants from cell damage imposed by biotic and abiotic stresses (Bieza and Lois, 2001; Pourcel *et al*., 2007). Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants (Kim *et al*., 2003; Sarker and Oba, 2018). The flavonoid (rutin) showed a down-regulation in SCA12 during response to water stress with a higher ratios than the phenolic acid (caffeic acid); whereas only the phenolic acid compound (significant at Day 8) was down-regulated in SCA6 and no change in the polyphenol metabolism was observed in MA12. These results show that the metabolic pathways including the metabolism of polyphenols are very little solicited during water stress in each genotypes of *T. cacao*.

One of the most remarkable differences between the two conditions for each genotype was the 3.55 times lower content of stachyose in the WS samples of SCA12 compared to NS ones. Stachyose is a member of the Raffinose family oligosaccharides (RFOs) that plays a significant role in seed dessication (Saravitz *et al*., 1987; Castillo *et al*., 1990). RFOs were reported to be flexible molecules because their degree of polymerization may easily change and subsequently affecting the osmotic pressure (Van den Ende, 2013). Disruption of these molecules provides an alternative source of energy necessary for cellular maintenance and metabolic homeostasis during stress.

In order to improve the survival under stress conditions, the drought tolerant plants comprise osmotic adjustments mainly mediated by non-structural sugars. Sucrose, a non-reducing disaccharide, is one of the main transport sugars in plants providing the main source for carbon allocation from source to sink organs. Sucrose was up-regulated in all genotypes during water stress. Also, in this study, fructose and α-glucose were detected in higher amount in WS samples of SCA12 and SCA6, respectively. The role of sucrose, fructose and glucose is first of all to serve as osmoregulators. Their accumulation in the plant cell is known to be important not only to sustain cell turgor by osmotic adjustment and stabilize enzymes, but also to confer protection against oxidative damage by reducing levels of reactive oxygen species (ROS) to consequently help re-establish cellular redox balance. Among the metabolites up-regulated in MA12 during water stress, only sucrose seems to play a role as an osmoprotectant.

Most of the amino acids identified as significantly down-regulated during water stress in this study were aromatic amino acids (AAAs) compounds, excepted leucine. Leucine was highly down-regulated in WS samples SCA6 and low compared to down regulation in MA12. This suggests that during water stress, the amino acid leucin is more used for protein synthesis in SCA6 compared to others genotypes. Aspartic acid showed irregular pattern during water stress in *T. cacao*. Also, aromatic amino acids (AAAs) such as tryptophan and phenylalanine were down-regulated in calluses during early embryos induction phase under water stress. Tryptophan was found to be one of the top features affected by drought stress in SCA12 and SCA6. Tyrosine showed an irregular pattern in *T. cacao* calluses during water stress in MA12. AAAs are synthetized in plants through the shikimate pathway. In support to a down-regulation of shikimate pathway under withholding water conditions, SCA12 metabolome analysis revealed a marked decrease of the shikimate intermediate. AAAs are target in the process of oxidation (Mujika *et al*., 2013). The free-form of these AAAs may play a protective function

against ROS. This balance role between ROS and proteins is especially played by tryptophan inside the chloroplast (Köhl, 2016).

Trans-aconitic acid (tAA), a natural isomer of *cis*-aconitic acid (cAA), is an unusual cellular metabolite. The up-regulation of tAA during water stress, suggests that tAA acts as a drought tolerant factor. However *t*AA is not necessary for basic biological metabolisms and is known to strongly inhibit the activity of aconitase in the tricarboxylic acid (TCA) cycle in microorganisms (Du *et al*., 2017). When the intracellular medium accumulates tAA, the cell eliminates it through methylation (Cai *et al*., 1999) or the cell metabolism can move them to other place to avoid inhibition and maintain the normal operation of the TCA cycle. In this study, tAA was up-regulated in SCA12 and in MA12 during water stress and highly contributed to the discrimination between NS and WS samples. This metabolite might reduce the consumption of sugars via TCA, so they were found in high quantities in the cell and can fully play their role as osmoregulators. Also, two organic acid derivatives, including trigonelline and choline, accumulated in WS samples of SCA6 more than NS ones. Trigonelline, is the N-methyl conjugate of nicotinic acid, most likely used as precursor for NADH, NADPH and/or nucleotides (Cho *et al*., 2003). High differences ($p<0.001$) was found for this compound between NS and WS samples in SCA6 at Day 8, as it is accumulating upon stress and acting as an osmoprotectant (Alzandi and Naguib, 2020). Although choline is believe to participate in drought tolerance of plants (Tasseva *et al*., 2004), the results of our present study minor such a role of choline in WS samples of *T. cacao*, as the high levels of this organic acid derivative in MA12 and lack of significant change in SCA12 under drought were observed. One can argue that the high choline content in MA12 is due to the higher metabolic activities, as appeared low shikimic acid content and high proline content (significant at Day 4) in these tissues during water stress.

II.2.3.2 Metabolomics analysis of seedlings under drought stress

Morphological examination of seed germination and seedling establishment in T. cacao before ^{1}H-NMR analysis

The seeds from the three genotypes showed different abilities of germination. The average frequency of seed germination decrease considerably with the increase of osmotic potential of the substrat. The estimated decrease of this frequency was 35% in SCA12, 23% in SCA6 and 73% in MA12 at 5 % PEG (-0.47 bars) as compared to control (0 bars). This decrease may be

associated to the decrease of water intake and cell-division during imbibition. Similars results have been obtained in other species such as barley and Mountain Rye (Ansari and Sharif-zadeh, 2012; Thabet et *al*., 2018). Another plausible explanation is that flavonoids, which were reported by Noah (2016) to act as a possible regulators which interact with hormones for the outgrowth and development of lateral root in cacao seedling, were low accumulated in cell under drought stress.

However, the results shows important differences in seed germination abilities in control (0 bars) conditions among the three genotypes used. These differences could be explained by the genetic effects, the harvest conditions and the chemical treatment applied to the pods, which in some cases could varie.

PEG in the concentration of 5% (w/v) has demonstrated its role in reducing water absorption. This suggests that the concentration of PEG used may decrease the number of seeds germinated during incubation in both genotypes. Under drought conditions, the highest frequency of seeds germinated (62%) was obtained with SCA12 genotype whereas the lowest frequency was obtained with MA12 genotype (22%). However, no statistical difference was found for seed germination frequency between SCA12 and SCA6 genotypes. The concentration of PEG at certain osmotic values equal to the osmotic value of cacao seed is expected to inhibit the imbibition which negatively affect the germination process since imbibition is the main condition of germination (Rahardjo, 1986).

The results showed no difference during post-germination stages of the seed from the three genotypes studied. The regular decrease of the relative fresh weight and the relative hypocotyle length of the seedlings had allowed us to observe a rather significative interaction between genotype and treatment. Globally, there is a positive correlation between early seedlings establishment abilities vis-à-vis of water/osmotic stress and the origin of the seed. In fact, during the post-germination stages, no differences were observed for the relative growth of fresh weight and hypoctyl length between unstressed and water/osmotic stressed seedlings in each genotype. Therefore, it was concluded that it was the genetic origin of the seeds that was responsible of the seedlings establishment abilities during osmotic stress rather than the average fresh mass of the seeds extracted from mature pods.

Comparison of the metabolic changes among the three genotypes under normal and water stress conditions using ^{1}H-RMN

As it is well known, cacao seeds are most exploited for their nutritional values. The characterization of the huge diversity of cacao seed have mainly focused on metabolic dynamics during seed maturation and seed development (Bucheli *et al*., 2001; Wang *et al*., 2016) and on the metabolite profiling of fermented dry beans (Caliagani *et al*., 2010). In the present study, more than fifty metabolites were identified in cacao plant during seed germination and seedling establishment on the NMR plalform, comprising sugars, amino acids, oganic acids, phenolics and others compounds.

The identified metabolites during the whole germination and seedling establishment process changed with the time of incubation under control conditions. The major reasons for these changes are probably based on the differences in genetic backgrounds of the three cacao genotypes and also the accumulation of metabolites during seed germination.

The NOESY-NMR spectra of SCA12 samples showed relatively constant peaks signals during seed germination and seedling establishment under drought conditions. The most promising regions in this spectra was located at δ 10.0-6.0 ppm, where there are plenty important peaks signals variations related to drought stress among samples of the three cacao genotypes. These regions harbor several metabolites such as trigonelline, known to be involved in drought tolerance by acting as an osmoprotectant (Irankhah *et al*., 2020). Moreover, trigonelline has been found to function as a hormone involved in the control of the cell cycle in plants (Minorsky, 2002).

Besides, further investigations revealed that proline, α-glucose, fructose and hesperidin also play an important role in the adaptation to water stress in SCA12 genotype. Proline has been widely considered to be a key drought inducible metabolite because it plays an osmoprotective role in plants. As an osmotic agent, proline protects cell membranes by decreasing the cellular osmotic potential. Additionally, it has also been recognized as a regulator redox status and a ROS scavenger (Smirnoff *et al*., 1989; Szabados *et al*., 2010; Sharma *et al*., 2011). In our study, proline was more accumulated in SCA12 than in SCA6 and MA12 in non-stressed (NS) and water stressed (WS) conditions in the same range (NS ratios SCA12/SCA6: 1.40 and SCA12/MA12: 1.46 and WS ratios SCA12/SCA6: 1.06 and SCA12/MA12: 1.08) at the 4th day after imbibition. A comparable increase of this metabolite was observed at the 8th day under drought condition in all the genotypes, indicating the implication of proline in the resistance to

drought treatment in SCA12 genotype. Soluble sugars often accumulate and perform functions such as signaling and osmotic adjustment not only in cacao seeds but on a whole-plant level (Wang and Ruan, 2013; Wang *et al*., 2016). In our study, the level of monosaccharide, α-glucose and fructose was high when SCA12 genotype was growing under drought stress conditions during seed germination and seedling establishment. Sugars can indirectly affect gene expression as an energy source. SCA12 showed a stronger capability of adaptation when cultured on water stress-induced medium.

The metabolites trigonelline and hesperidine were more accumulated under normal and drought conditions in SCA12 while rutin was more accumulated in SCA6 and phenylalanine in MA12. The metabolite shikimate, a precursor of the phenylpropanoid pathway was more accumulated in SCA12 under normal condition while more accumulated in MA12 under drought condition. Choline and sucrose, more accumulated under normal condition in SCA6 were more accumulated in MA12 under drought conditions (with a highly significant level of sucrose).

The increasing levels of organic compounds under osmotic stress affect growth because of the cost of energy related to their synthesis (Lugan *et al*., 2010). It is generally accepted that there is an antagonism effect between stress tolerance and productivity (Oliver et *al*., 2000; Flowers, 2004). The genotype MA12 appears to follow this rule, because the osmotic balance maintains with the environment is due to a passive reduction in relative growth of seedling fresh weight and hypocotyl length rather than solutes/osmolytes accumulation. The shikimate and formic acid were the metabolites more accumulated in MA12 under drought condition. The formic acid level was about 5.0 and 4.5 fold higher in MA12 than SCA6 and SCA12 genotypes respectively after 8 days of culture under drought condition. The present study showed that the synthesis and the accumulation of organic compounds in MA12 under middle drought condition seems contribute to its less tolerance to stress as compared to SCA6 and SCA12.

Investigation of the specific metabolites changes in SCA12, SCA6 and MA12 seedlings due to osmotic stress

For SCA6 and MA12, as regards to the changes at the metabolic level in both unstressed and water stressed seedlings, osmotic treatment allowed to identify discriminant metabolites up- and down-regulated. Even if metabolites associated with osmotic stress remained undetected in SCA12 seedlings due to the poor separation of samples in ^{1}H-NMR during incubation under drought condition after multivariate analyses, we suspected that the level of osmotic stress used

in this case (7.5% (w/v) PEG, corresponding to -0.47 bars) had little or no significant effect on the early developmental stages of this genotype compared to other genotypes.

The amino acid proline which is a vital osmoregulator accumulated in water-stressed plants (Vyas *et al*., 1985), was significantly up-regulated in SCA6 compared to MA12. The results also showed that the high accumulation of proline in SCA6 during water stress was at the expense of other free amino acids, especially for those involved in the tricarboxylic acids (TCA) cycle. Glutamine which was down-regulated in SCA6, could be converted into γ-amino-butyric acid (GABA) and then transformed into succinic acid which enters the TCA cycle for energy production. Therefore, it can be concluded that the accumulation of proline which results from the degradation of proline-rich proteins, may be an important part of the physiological mechanism to decrease the drought damage.

The results showed that 1-O-ethyl-β-D-glucoside and lactic acid were positively related to the synthesis and the accumulation of proline in SCA6. In constrast, glucose content was significantly reduced in both genotypes during water stress treatment. The 1-O-ethyl-β-D-glucoside synthesis and its function on plant physiology especially under water deficit condition, remain unclear. Lactic acid had recently been reported to exert a polyphenol-protective effect in cacao beans during fermentation-like incubations processes (Eyamo *et al*., 2016). Glucose plays a critical role in sugar metabolism and the activation of sugar metabolism results in the decrease of relative contents of glucose due to its consumption and transformation. Sucrose may act mainly as signaling molecules in SCA6 and MA12 seedlings, because its content decreased during water stress in SCA6 while increasing in MA12. Furthermore, the decrease of choline, an important compatible solute in eucaryotes that protects cells from osmotic stress caused by dehydrating conditions (Zhang *et al*., 2010), with the decreased sucrose content in SCA6 indicated that glycine betaine induction is necessary for drought tolerance. This overall metabolic analyses help to clarify the underlying mechanism involved in the response to water stress and provides a reference for the further regulation and production of higher quality cacao plants.

CHAPTER IV: CONCLUSION AND PERSPECTIVES

IV.1 Conclusion

The general objective of this work was to evaluate the physiological and metabolomical responses of cacao subjected to water stress through somatic embryogenesis and seedling establishment**.**

The staminodes and petals explants located on the secondary fan branches (FII) were more favorable for *in vitro* regeneration through somatic embryogenesis compared to those located on the primary fan branches (FI) and to those located on the orthotropic main stem (OS) of the cacao tree. This capacity was related with the decrease of phenolics content in callus during somatic embryos establishment stage. The most pronounced difference among the tree types of explants-derived calluses concern carbohydrates metabolism: FII and FI biochemical evaluation displayed a low utilization of carbohydrates, while OS explants are characterized by intensive glycolytic activities as documented by the exceptional decreased of sugars content during somatic embryogenesis. The absence of embryos dedifferentiation on petals-derived calluses from FII has been connected to changes in specific enzymes abundances. The results suggest that stress factors (microclimate) and genetic factors affect embryogenic capacity of floral explants in *T. cacao* and thereby reduce the regeneration frequency.

Embryogenic capacities of callus was highly influenced by the variations of the osmotic potential during the treatment with PEG. Callus from SCA12 genotype exhibited the best conversion capacities under severe (7.5 % (w/v) PEG) water stress conditions and was considered as tolerant, followed by SCA6 (less-tolerant) and MA12 (sensitive) genotypes. Phenolics and flavonoids contents in callus were found to decrease in tolerant genotype under water stress. In addition, data revealed that 7.5 % PEG supplementation during maturation stage was favorable for the good conversion of somatic embryos, as compared to mannitol and sorbitol at the same concentration.

The metabolome constitution and rearrangement were found to be at the origin of the physiological responses during *in vitro* propagation of *T. cacao*, seeds germination and seedlings establishment under water stress conditions. Metabolites involved in water stress tolerance in callus include trans-aconitic acid, aspartic acid, fructose and sucrose (more accumulated) and stachyose (with reduced content). In cotyledons from seeds germinated under water stress conditions, proline, lactic acid and 1-O-ethyl-β-D-glucoside were more accumulated whereas the content of β-glucose was higly reduced.

IV.2 Perspectives

The experiment on the influence of the position of floral explant on the tree on the production of somatic embryos showed globally that somatic embryogenesis is an adapted physiological and metabolical responses to environmental stress. Based on this outcome and considering the current issue for somatic embryogenesis which is to enhance conversion of these embryos into healthy plantlets, further analysis will be necessary to investigate the flavonoids compounds that may be implicated in the adaptation of callus and embryos derived from plagiotropic fan branches to osmotic stress.

The second experiment made it possible to obtain a large part of the expected results for the identification of key metabolites involved in callus and seedlings tolerance to drought stress. More investigation by LC-MS data will allow to access the metabolic variations of the secondary metabolites accumulated in a small quantity analyzed by NMR and found in callus and seedlings. In this case, future assays may be involved on the creation of mutants plants through the culture of seedlings in the conditions that are favorable to the accumulation of key metabolites involved in water stress tolerance. The follow-up of these mutants during acclimatization in different agro-climatic conditions will help to caracterize their real agronomic performances in fields.

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APPENDIX

Appendix 1. Composition (Macronutrients, Micronutrients and Vitamins) of the culture DKW media used.

CONSTITUENTS	**mg/L**
Macronutrients-DKW A (for 1l 10×)	
Ammonium nitrate	14160
Calcium nitrate tetrahydrate	19680
Macronutrients-DKW B (for 1l 10×)	
Calcium chloride dihydrate	1490
Potassium sulfate	15590
Magnesium sulfate heptahydrate	7400
Potassium phosphate dibasic	2650
Micronutrients-DKW	
Zinc sulfate hexahydrate	1700
Manganese sulfate monohydrate	3340
Ferric sulfate heptahydrate	3380
EDTA disodium dihydrate	4540
Boric acid	480
Cupric sulfate pentahydrate	25
Sodium molybdate dihydrate	39
Vitamins DKW (for 100ml 1000×)	
Myo-inositol	l10
Thiamine hydrochloride	200
Nicotinic acid	100
Glycine	200

Appendix 2. Composition of primary callus growth medium (PCG).

	1L	2L
DKW macro A	100 ml	200 ml
DKW macro B	100 ml	200 ml
DKW micro	10 ml	20 ml
Vitamin DKW	1 ml	2 ml
Glucose	20 g	40 g
Glutamine	250 mg	500 mg
Myo-inositol	100 mg	200 mg
2.4-D (1mg/ml stock)	2 ml	4 ml
TDZ (0.2mg/l stock)	25 µl	50 µl
Gelrite	2.2 g	4.4 g
Autoclaving	120 °C during 20 mn	120 °C during 20 mn
pH	5.8	5.8

Appendix 3. Composition of secondary callus growth medium (SCG)

	1L	2L
DKW macro A	100 ml	200 ml
DKW macro B	100 ml	200 ml
DKW micro	10 ml	20 ml
Vitamine DKW	0.5 ml	1 ml
Vitamine B_5	0.5 ml	1 ml
Glucose	20 g	40 g
Myo-inositol	100 mg	200 mg
Kinetine (1mg/ml stock)	250 µl	500 µl
2.4-D (1mg/ml stock)	2 ml	4 ml
Gelrite	2.2 g	4.4 g
Autoclaving	120 °C during 20 mn	120 °C during 20 mn
pH	5.8	5.8

Appendix 4. Composition of embryos development medium (ED).

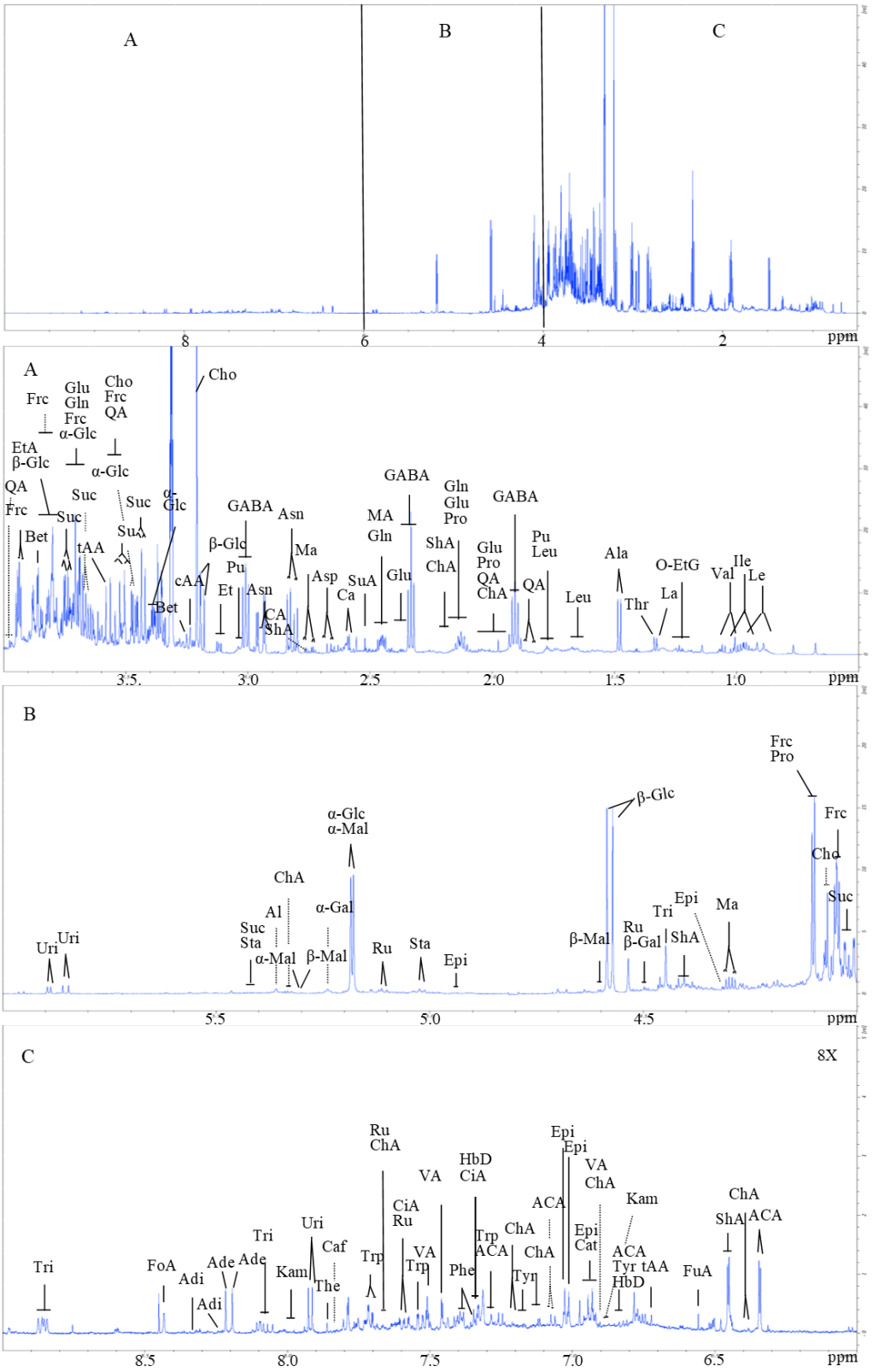
	1L	2L
DKW macro A	100 ml	200 ml
DKW macro B	100 ml	200 ml
DKW micro	10 ml	20 ml
Vitamine DKW	1 ml	2 ml
Glucose	1 g	2 g
Sucrose	30 mg	60 mg
$MgSO_4$	25 ml	50 ml
Gelrite	2.2 g	4.4 g
Autoclaving	120 °C during 20 mn	120 °C during 20 mn
pH	5.8	5.8

Appendix 5. Composition of roots development medium (RD)

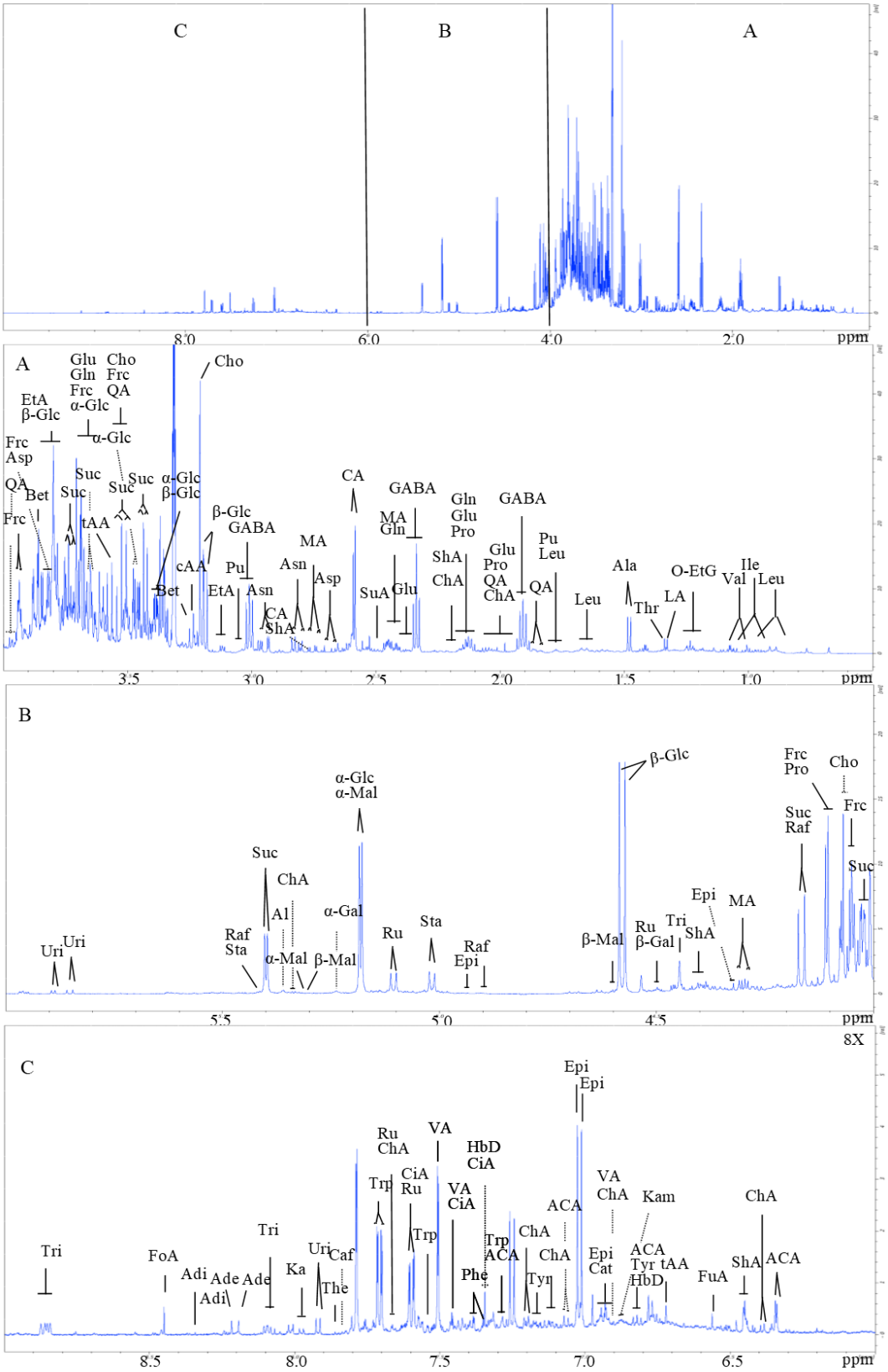
	1L	2L
DKW macro A	50 ml	100 ml
DKW macro B	50 ml	100 ml
DKW micro	5 ml	10 ml
Vitamine DKW	0.5 ml	1 ml
Glucose	10 g	20 g
Sucrose	5 mg	10 mg
KNO_3	0.3 g	0.6 g
Gelrite / phytagel	2 g ou 1.75g	4 g ou 3.5 g
Autoclaving	120 °C during 20 mn	120 °C during 20 mn
pH	5.8	5.8

Appendix 6. Composition of Anthron reagent‘s

	1L	2L
Concentrated sulfuric acid	710 ml	1420 ml
Distilled water	290 ml	580 ml
Anthrone	1 g	2 g



Appendix 7. Characteristic NOESY ^{1}H NMR -1D spectrum of SCA12 callus at D0 (aged of 28 days) with assigned peaks (full names are reported in table 8). The region δ 9.0-6.0 were expanded 8 times in comparison with the regions δ 6.0-4.0 and δ 4.0-0.0. These three regions are shown separately (A, B and C) for the assignment.



Appendix 8. Characteristic NOESY ^{1}HNMR -1D spectrum of SCA6 callus at D0 (aged of 28 days) with assigned peaks (full names are reported in table 8). The region δ 9.0-6.0 were expanded 8 times in comparison with the regions δ 6.0-4.0 and δ 4.0-0.0. These three regions are shown separately (A, B and C) for the assignment.

Appendix 9. Comparison of discriminant metabolites for SCA12, SCA6 and MA12 calluses of *T. cacao* in water-stressed (WS) versus non-stressed (NS) conditions at the two kinetic points (D4, D8).

Metabolite	**Ratio at D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Metabolite**	**Ratio at D4**	**Wi-test**	**Ratio at D8**	**Wi-test**	**Metabolite**	**Ratio at D4**	**Wi-test**	**Ratio at D8**	**Wi-test**
SCA12 NS> WS	**SCA12 NS/WS**		**SCA12 NS/WS**		**SCA6 NS> WS**	**SCA6 NS/WS**		**SCA6 NS/WS**		**MA12 NS> WS**	**MA12 NS/WS**		**MA12 NS/WS**	
Rutin	1.67	***	2.30	***	β-Maltose	2.42	***	2.61	**	Leucine	1.53	*	1.34	/
Caffeic Acid	0.92	/	1.34	**	Alanine	2.18	**	1.28	/	Putrescine	1.39	*	1.42	*
Epicatechin	0.95	/	1.16	/	Tyrosine	1.30	*	1.09	/	Uridine	1.30	**	1.16	*
Tryptophan	1.37	***	1.82	***	Tryptophan	1.31	*	1.34	***	Aspartic acid	1.37	*	1.44	**
β-Maltose	ND1		ND1		Caffeic acid	1.17	/	1.26	**	Chlorogenic acid	ND1		ND1	
Threonine	1.10	/	1.64	/	1-o-ethyl-β-D-glucoside	1.55	**	1.37	*	Kaempferol analogues	1.32	**	1.45	***
Stachyose	3.16	***	3.55	***	δ-amino-butyric acid	2.0	***	1.93	**	Phenylalanine	ND1		ND1	
1-O-ethyl-β-D-glucoside	1.04	/	1.35	/	Fructose	1.25	/	1.35	**	Shikimic acid	1.22	***	1.27	*
Shikimic Acid	1.72	***	1.80	***	Leucine	ND1		4.94	***					
Kaempferol	ND1		ND1		Cinnamic acid	1.21	/	1.31	*					

Analogues														

Appendix 9 continued

SCA12 WS > NS	**SCA12 WS/NS**		**SCA12 WS/NS**		**SCA6 WS > NS**	**SCA6 WS/NS**		**SCA6 WS/NS**		**MA12 WS > NS**	**MA12 WS/NS**		**MA12 WS/NS**	
Hydroxybenzen derivatives	ND2		ND2		Aspartic acid	1.84	***	6.64	/	Tyrosine	ND2		1.52	*
Fructose	1.69	***	1.68	***	Asparagine	1.46	**	1.42	**	Proline	1.89	***	1.83	/
Sucrose	1.23	/	1.66	**	Ethanolamine	1.21	*	1.36	**	Choline	1.68	**	1.53	**
Ethanolamine	1.18	/	1.42	*	Sucrose	1.95	*	2.41	**	trans-aconitic acid	1.26	**	1.45	***
trans-Aconitic acid	2.65	**	3.01	**	Malic acid	0.93	/	1.08	/	Sucrose	2.06	**	2.09	**
Choline	1.29	/	1.31	*	Hydroxybenzen derivatives	ND2		ND2						
Aspartic acid	1.19	/	1.68	**	Trigonelline	1.97	***	3.54	***					
					α-glucose	1.24	**	2.14	**					

ND1- metabolite not detected in stressed samples; ND2- metabolite not detected in non-stressed samples. ***0.001, **0.01, *0.05, / not significative.

Appendix 10. The evolution seedling fresh weigth and hypocotyl length after 0; 4 and 8 days during seed germination and seedlings establishment.

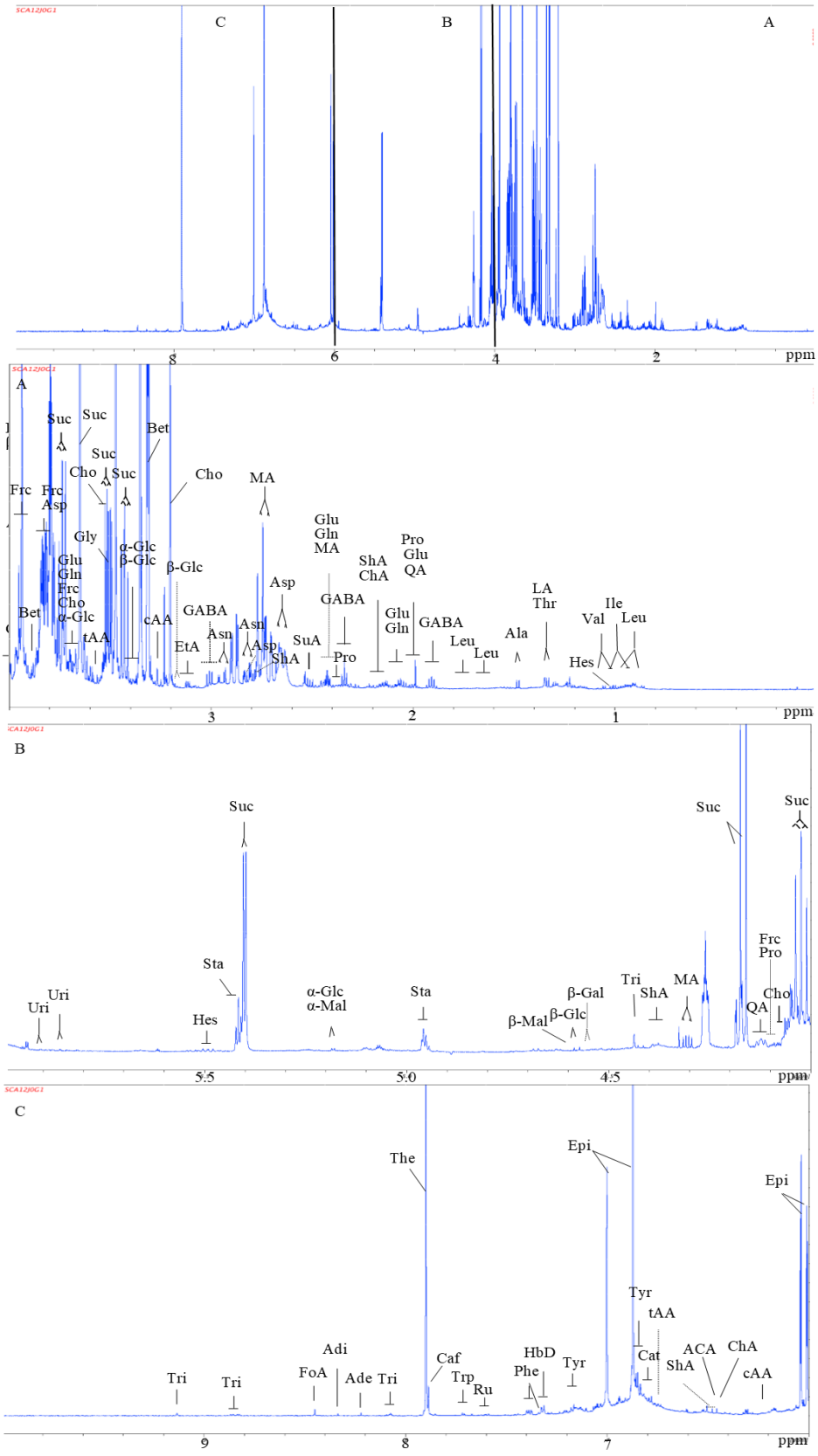
Clone	Lot N°	Treatment	Day	Repeatition N°	Fresh weight (g)	Hyooctyl length (cm)
SCA12	3lot1	Control	4	1	2.267	3.5
SCA12	3lot1	Control	4	2	2.124	2
SCA12	3lot1	Control	4	3	1.829	1
SCA12	3lot1	Control	4	4	2.302	2.3
SCA12	3lot1	Control	4	5	1.765	1.5
SCA12	3lot1	Control	4	6	1.42	0.9
SCA12	3lot1	Control	4	7	1.89	1.5
SCA12	3lot1	Control	4	8	1.787	1.1
SCA12	3lot1	Control	4	9	1.605	0
SCA12	3lot3	Control	4	1	1.462	0.9
SCA12	3lot3	Control	4	2	1.678	1
SCA12	3lot3	Control	4	3	2.126	2.1
SCA12	3lot3	Control	4	4	2.238	4
SCA12	3lot3	Control	4	5	1.428	0.9
SCA12	3lot3	Control	4	6	1.691	1.4
SCA12	3lot3	Control	4	7	1.981	1.4
SCA12	3lot3	Control	4	8	2.183	1.5
SCA12	3lot3	Control	4	9	2.165	2.2
				Mean	1.88	1.71
				SD	0.32	0.98
SCA12	3lot5	PEG	4	1	1.764	1
SCA12	3lot5	PEG	4	2	1.7	0.2
SCA12	3lot5	PEG	4	3	1.823	0.3
SCA12	3lot5	PEG	4	4	1.544	1.6
SCA12	3lot5	PEG	4	5	1.657	2.3
SCA12	3lot5	PEG	4	6	1.764	2.2
SCA12	3lot5	PEG	4	7	1.824	1.7
SCA12	3lot5	PEG	4	8	1.263	1
SCA12	3lot5	PEG	4	9	1.719	0.8
SCA12	3lot7	PEG	4	1	1.715	1.1
SCA12	3lot7	PEG	4	2	1.745	1.4
SCA12	3lot7	PEG	4	3	1.651	1.4
SCA12	3lot7	PEG	4	4	1.656	1.2
SCA12	3lot7	PEG	4	5	1.971	1.5
SCA12	3lot7	PEG	4	6	1.601	0.9
SCA12	3lot7	PEG	4	7	1.881	0.8
SCA12	3lot7	PEG	4	8	1.501	0.2
SCA12	3lot7	PEG	4	9	2.016	1.4
				Mean	1.75	1.10
				SD	0.17	0.42

Appendix 10 continued

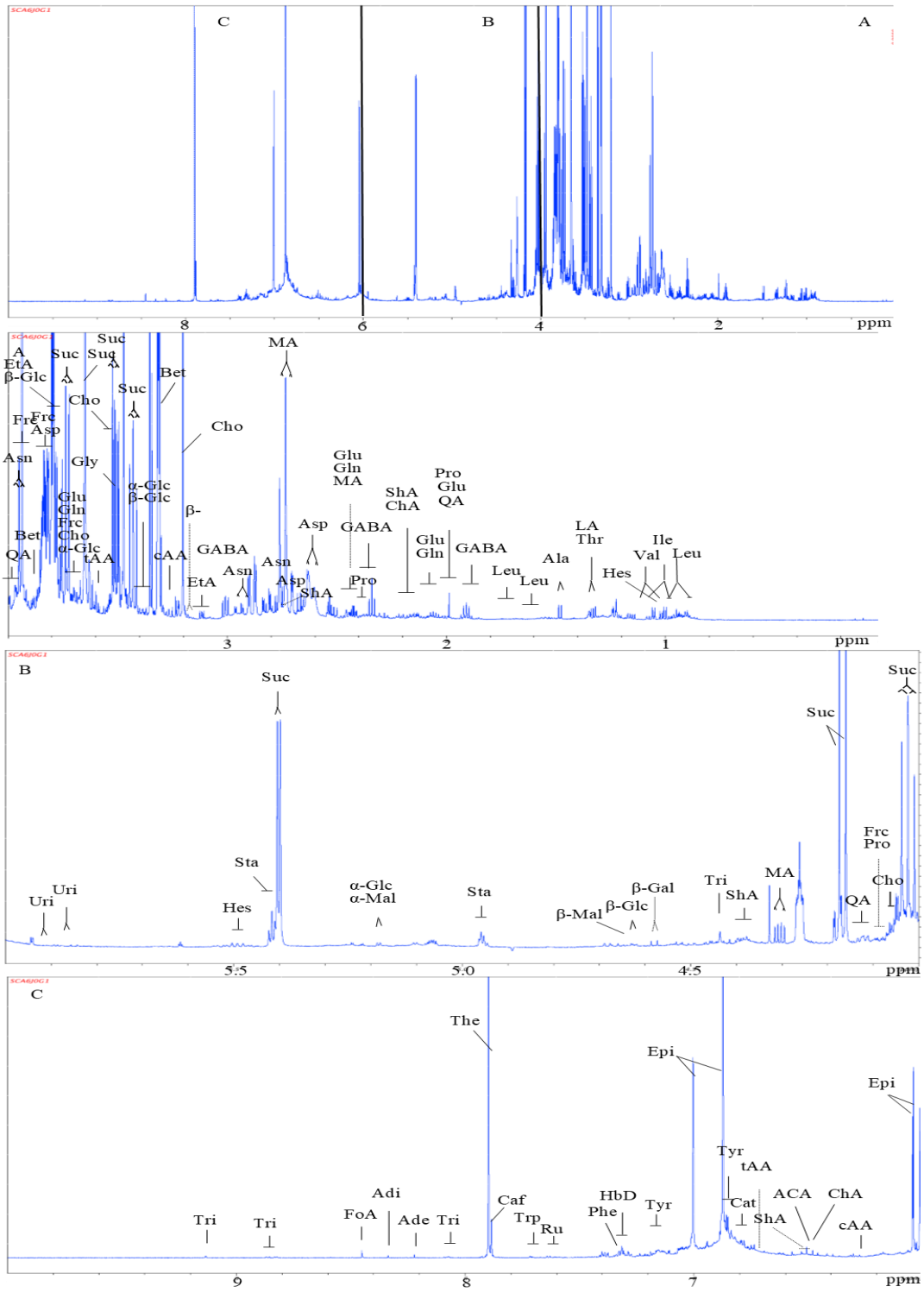
SCA6	2lot1	Control	4	1	2.064	2.1
SCA6	2lot1	Control	4	2	2.216	1.4
SCA6	2lot1	Control	4	3	1.756	2.3
SCA6	2lot1	Control	4	4	2.256	3
SCA6	2lot1	Control	4	5	1.932	1.5
SCA6	2lot1	Control	4	6	2.048	2.7
SCA6	2lot1	Control	4	7	1.82	2.1
SCA6	2lot1	Control	4	8	2.253	1.9
SCA6	2lot1	Control	4	9	1.796	1.4
SCA6	2lot3	Control	4	1	1.965	1.3
SCA6	2lot3	Control	4	2	1.419	0.6
SCA6	2lot3	Control	4	3	1.706	1.3
SCA6	2lot3	Control	4	4	1.32	0.6
SCA6	2lot3	Control	4	5	1.848	1.2
SCA6	2lot3	Control	4	6	1.478	0.4
SCA6	2lot3	Control	4	7	2.056	1.7
SCA6	2lot3	Control	4	8	2.205	2.5
SCA6	2lot3	Control	4	9	1.644	1
					1.74	1.18
					0.30	0.65
SCA6	2lot5	PEG	4	1	1.8	0.6
SCA6	2lot5	PEG	4	2	1.784	1.2
SCA6	2lot5	PEG	4	3	1.656	0.5
SCA6	2lot5	PEG	4	4	1.482	0.3
SCA6	2lot5	PEG	4	5	2.104	1.1
SCA6	2lot5	PEG	4	6	1.638	0.2
SCA6	2lot5	PEG	4	7	1.346	0
SCA6	2lot5	PEG	4	8	1.473	0.7
SCA6	2lot5	PEG	4	9	1.789	0.2
SCA6	2lot7	PEG	4	1	1.274	1.2
SCA6	2lot7	PEG	4	2	1.692	1
SCA6	2lot7	PEG	4	3	1.937	1.2
SCA6	2lot7	PEG	4	4	1.805	1.2
SCA6	2lot7	PEG	4	5	1.191	0.7
SCA6	2lot7	PEG	4	6	1.248	1.5
SCA6	2lot7	PEG	4	7	1.833	0.9
SCA6	2lot7	PEG	4	8	1.964	1.9
SCA6	2lot7	PEG	4	9	1.229	1.9
				Mean	1.57	1.28
				SD	0.33	0.42

Appendix 10 continued

MA12	1lot1	Control	4	1	2.67	2.5
MA12	1lot1	Control	4	2	2.157	1.1
MA12	1lot1	Control	4	3	2.836	1.2
MA12	1lot1	Control	4	4	2.966	1.8
MA12	1lot1	Control	4	5	2.047	0.3
MA12	1lot1	Control	4	6	3.535	0.7
MA12	1lot1	Control	4	7	2.617	1.8
MA12	1lot1	Control	4	8	2.184	2.3
MA12	1lot1	Control	4	9	2.21	0.7
MA12	1lot5	Control	4	1	2.53	1.8
MA12	1lot5	Control	4	2	2.14	1.1
MA12	1lot5	Control	4	3	2.033	2.4
MA12	1lot5	Control	4	4	2.25	2.1
MA12	1lot5	Control	4	5	3.599	1.5
MA12	1lot5	Control	4	6	1.585	0.2
MA12	1lot5	Control	4	7	4.296	1
MA12	1lot5	Control	4	8	2.517	1.8
MA12	1lot5	Control	4	9	3.043	2.1
				Mean	2.67	1.56
				SD	0.85	0.69
MA12	1lot7	PEG	4	1	3.129	0.1
MA12	1lot7	PEG	4	2	1.775	1.1
MA12	1lot7	PEG	4	3	2.019	0.2
MA12	1lot7	PEG	4	4	1.914	0.1
MA12	1lot7	PEG	4	5	2.537	0.1
MA12	1lot7	PEG	4	6	1.845	0.2
MA12	1lot7	PEG	4	7	2.449	1
MA12	1lot7	PEG	4	8	3.468	0.5
MA12	1lot7	PEG	4	9	2.239	0.6
MA12	1lot8	PEG	4	1	1.952	0.6
MA12	1lot8	PEG	4	2	2.059	1.1
MA12	1lot8	PEG	4	3	2.18	0.6
MA12	1lot8	PEG	4	4	3.115	0.6
MA12	1lot8	PEG	4	5	2.021	0
MA12	1lot8	PEG	4	6	1.624	0
MA12	1lot8	PEG	4	7	2.742	0
MA12	1lot8	PEG	4	8	1.835	0.5
MA12	1lot8	PEG	4	9	1.912	1.1
				Mean	2.16	0.50
				SD	0.47	0.43



Appendix 11. Characteristic NOESY ^{1}H NMR-1D spectrum of SCA12 fresh seed at D0 (collected from pod aged of 16 weeks) with assigned peaks. Its three regions are shown separately (A, B and C) for the assignment.



Appendix 12. Characteristic NOESY ^{1}H NMR-1D spectrum of SCA6 fresh seed at D0 (collected from pod aged of 16 weeks) with assigned peaks. Its three regions are shown separately (A, B and C) for the assignment.

Appendix 13. Discriminant metabolites among SCA6 and MA12 genotypes in unstressed and water-stressed conditions in seedlings at the two kinetic points (D4, D8).

Metabolite	**Wi-test**	**Ratio at D4**	**Wi-test**	**Ratio at D8**	**Metabolite**	**Wi-test**	**Ratio at D4**	**Wi-test**	**Ratio at D8**
SCA6 NS> WS		**SCA6 NS/WS**		**SCA6 NS/WS**	**MA12 NS>WS**		**MA12 NS/WS**		**MA12 NS/WS**
Hydroxybenzen Derivatives	/	1.11	**	1.40	α-Maltose	*	1.29	*	1.71
β-Glucose	/	ND1	**	2.25	β-Glucose	***	1.53	/	1.73
Glutamine	**	1.50	*	1.38	Asparagine	/	1.03	*	1.37
Sucrose	***	1.24	**	1.91	Malic Acid	/	0.98	**	1.76
Leucine	*	1.26	*	1.28					
Choline	/	1.15	**	2.18					
SCA6 WS> NS		**SCA6 WS/NS**		**SCA6 WS/NS**	**MA12 WS>NS**		**MA12 WS/NS**		**MA12 WS/NS**
Fructose	**	1.26	/	1.13	Rutin	/	1.00	/	ND2
Proline	/	1.19	***	2.21	Fructose	/	1.16	*	1.25
Lactic acid	/	1.12	**	2.38	Sucrose	**	1.30	*	1.16
1-O-ethyl- β- glucoside	/	1.08	*	2.13	Chlorogenic Acid	/	1.13	/	ND2
					Quinic Acid	***	2.16	/	1.52
					Lactic Acid	/	1.15	***	1.87
					1-O-ethyl- β- glucoside	*	1.34	**	1.70

ND1- metabolite not detected in stressed samples; ND2- metabolite not detected in non-stressed samples. ***0.001, **0.01, *0.05, / not significative

PUBLICATION

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