Somatic Embryogenesis of *Theobroma cacao* L.: Developmental Physiology of the Embryo and Improvement of Culture Conditions.

DISSERTATION

A thesis submitted for the degree of Dr. rer. nat (*rerum naturalium*)

to the Biology Department,

the Faculty of Mathematics, Informatics and Natural Sciences,

University of Hamburg

prepared by

Taina Soraia Muller
From Brazil

Hamburg, 2013

Genehmigt vom Fachbereich Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Professor Dr. R. LIEBEREI
Weiterer Gutachter der Dissertation:
Prof. Dr. D. SELMAR
Tag der Disputation: 3. Mai 2013

Hamburg, den 7. Mai 2013

Professor Dr. C. Lohr
Vorsitzender des Promotionsausschusses
Biologie

Nina Boeddeker
2319 Curtis St
Berkeley CA 94702

415-794-5218
nina.bo@icloud.com

April 1, 2013

To Whom It May Concern:

The quality of the English language in the doctoral thesis written by Taina S. Muller was completely reviewed by me, Nina Boeddeker. I was born and raised in the United States of America.

Nina Boeddeker

Tocando em Frente	*Voran treiben*
(Almir Sater)	*(Übersetzung: Andreas Hübner, Göttingen)*
Ando devagar	*Ich gehe langsam,*
Porque já tive pressa	*weil ich es schon eilig hatte*
E levo esse sorriso	*und nehme dieses Lachen mit,*
Porque já chorei demais	*weil ich schon zu viel geweint habe.*
Hoje me sinto mais forte,	*Heute fühle ich mich stärker,*
Mais feliz, quem sabe	*vielleicht auch glücklicher,*
Só levo a certeza	*sicher weiß ich nur,*
De que muito pouco sei,	*dass ich sehr wenig (weiß)*
Ou nada sei	*oder gar nichts weiß.*
Conhecer as manhas	*Die Tücken des Lebens zu kennen*
E as manhãs	*und jeden Morgen zu erleben,*
O sabor das massas	*wie den Geschmack des Teiges*
E das maçãs	*und der Äpfel.*
É preciso amor	*Es braucht Liebe,*
Pra poder pulsar	*um zu pulsieren,*
É preciso paz pra poder sorrir	*es braucht Frieden um zu lachen,*
É preciso a chuva para florir	*es braucht Regen zum Erblühen.*
Penso que cumprir a vida	*Ich denke, um das Leben zu meistern,*
Seja simplesmente	*muss man einfach*
Compreender a marcha	*den Weg verstehen*
E ir tocando em frente	*und alles voran treiben.*
Como um velho boiadeiro	*Wie ein alter Cowboy,*
Levando a boiada	*der die Herde treibt,*
Eu vou tocando os dias	*treibe ich die Tage.*
Pela longa estrada, eu vou	*Auf dem langen Weg gehe ich,*
Estrada eu sou.	*der Weg bin ich.*
Todo mundo ama um dia,	*Jeder liebt irgendwann,*
Todo mundo chora.	*jeder weint.*
Um dia a gente chega	*An einem Tag kommt man an*
E no outro vai embora.	*und an einem anderen geht man wieder.*
Cada um de nós compõe a sua história	*Jeder von uns schreibt seine Geschichte.*
Cada ser em si	*Jedes Wesen*
Carrega o dom de ser capaz	*trägt in sich die Gabe fähig (zu sein)*
E ser feliz	*und glücklich zu sein.*

This work is dedicated to my husband Eduardo for stay by my side and my mother Rose, who showed me how to go on.

Acknowledgements

Thank God for all forms of life on the Earth, for the life existing in a plant cell and especially for mine.
I thank my husband Eduardo Diel for supporting me in my dreams, for keeping me realistic in my ideas and for avoiding my madness. We thank our families for understanding our needs, aspiration and for being near despite the distance.
Thank you, Prof. Dr. Reinhard Lieberei for accepting me in your workgroup, for introducing me to very interesting people and situations. Thanks for finding a room for my somatic embryos among the delicious cacao beans. Thanks for showing me how to be human before to be a doctor.
My coming to Germany was only possible with the help of Dr. Douglas Steinmacher, who became a good friend. Thanks for the useful discussions and sharing ideas.
I would like to express my gratitude to Dr. Gerald Koch for turn realizable the valuable analyses by UV-microspectrophotometry and for the discussions concerning my results. In the same way, I would like to thank Karin Brandt for the help during the analyses and a friendly atmosphere during my works in the Institute of Wood Biology and Wood Protection.
Thanks Dr. Andrew Daymond from University of Reading for supply cacao flowers for part of this work.
My work in the Biocenter Klein Flottbek and Botanical Garden was possible due to the help of many special people. I want to thank Thomas Tumforde, Detlef Böhm, Dr. Helmut Kassner, Elke Woelken, Karen Dehn and Volker Ambroselli for help me in my works. Even when no words came out from my mouth, you tried to understand me and help me answering my questions. Special thanks to Karin Puttfarken for organizing my stay in Hamburg and at the University of Hamburg.
But my life did not only take place in the laboratory rooms. The integration in the society was only possible due to the good friends who I have the pleasure of having been introduced to in Germany. You were much more important for my stay in this country than you can imagine. I thank you for every new word that I learned from you. Thanks for helping me understanding and discovering your country. Julia, Piso, Janine (thanks for the precise corrections, these were very helpful), Daniel, Isaac and Elsa, you turned my days easier and made me feel like I had been born in Germany.
Very special thanks to Franziska Wülfing, who were more than a friend. You were not only a great colleague and friend but for many times also sister and mother. Thanks for your friendship and for the pieces of German culture that are attached to my heart, for the unforgettable funny long nights and weekends at the flow chamber and microscopy. Thanks also to family Wülfing for all given support to solve some difficulties.
My stay in Germany was facilitated by the preparation supported by DAAD (Deutscher Akademischer Austauschdienst) and the Goethe Institute. The time in Goettingen aroused in me the passion for this beautiful language and culture, for that my thanks.
Finally, thanks to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico-Brasil) for the financial support for this project.

Taina

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I. List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
µm	Micrometer
µmol	Micro-mol
2,4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic acid
ABS	Absorbance
ANOVA	Analysis of variance
BAP	6-Benzylaminopurine
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	Centimeter
CO_2	Carbon dioxide
cSSE	Cyclic secondary somatic embryogenesis
CSSV	*Cocoa swollen shoot virus*
DKW	Driver and Kuniyuki medium formulation (1984)
DTT	Dithiothreitol
DW	Dry weight
ED	Expression medium
FAO	Food and Agriculture Organization of the United Nations
FW	Fresh weight
g	Gram
ha	Hectare
HCl	Hydrochloric acid
HPLC	High performance liquid Chromatography
IBA	Indole butyric acid
ICCO	The international Cocoa organization
KH_2PO_4	Potassium dihydrogen phosphate
KIN	Kinetin
KNO_3	Potassium Nitrate
KOH	Potassium Hydroxide
K_2SO_4	Potassium sulphate
LUP	Laboratory Useful Plants (University of Hamburg)
M	Molar
m	Meter
mbar	Milibar
mg	Milligram
$MgSO_4$ $7H_2O$	Magnesium sulphate Heptahydrate
min	Minute
ml	Milliliter
mm	Millimeter
mM	Milimolar
MS	Murashige and Skoog medium formulation (1962)
nm	Nanometer
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Di-sodium hydrogen phosphate
OPA	O-phthalaldehyde
OsO_4	Osmium tetroxide
PCG	Primary callus growth medium

PEC	Primary embryo conversion medium
PGR	Plant growth regulator
PVPP	Polyvinyl-polypyrrolidon
pmol	Picomole
PMSF	Phenylmethylsulfonyl fluoride
PPO	Polyphenol oxydase
PSE	Primary Somatic Embryogenesis
rpm	Rotation per minute
RSE	Repetitive somatic embryogenesis
s	second
SCG	Secondary callus growth
SE	Somatic embryogenesis
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SERK	*Somatic Embryogenesis Receptor-like Kinase*
SSE	Secondary Somatic Embryogenesis
TcL1L	*Theobroma cacao leafy cotyledon1-like* homologous gene
TDZ	Thidiazuron
TF	Transcription factor
TFA	Tifluoroacetic acid
TEM	Transmission electron microscopy
TIS	Temporary immersion system
Tris	Tris(hydroxymethyl)-aminomethane
t.ton	Thousand tones
UMSP	UV-microspectrophotometry
UV	Ultra violet
w/v	Weight per volume
v/v	Volume per volume

Amino acids

ALA- Alanine
ARG- Arginine
ASN- Asparagine
ASP- Aspartic acid
CYS- Cysteine
GLU- Glutamic acid
GLN- Glutamine
GLY- Glycine
HIS- Histidine
ILE- Isoleucine
LEU- Lysine
MET- Methionine
PHE- Phenylalanine
PRO- Proline
SER- Serine
THR- Threonine
TRP- Tryptophan
TYR- Tyrosine
VAL- Valine
GABA- γ-Aminobutyric acid

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IV. Abstract

Theobroma cacao is a plant native Amazon basin region which grows naturally in understory of tropical rainforests. It is a plant of Malvaceae family and cultivated since many centuries in tropical regions around the world. The economic importance due to the source for chocolate production makes this plant one of the most studied crops in the world. *T. cacao* cultures encounter problem like attack of severe diseases, high heterogeneity and difficult propagation. Its recalcitrant seeds and complex vegetative propagation hamper the mass propagation of clones and conservation of diversity. As example of other woody plants crop, modern biotechnological approaches have been employed seeking to overcome such problems. Somatic embryogenesis is a method of *in vitro* culture successfully used for a long time as alternative of clone propagation of plants. This method allows scale up production of somatic embryos which imitate the development of zygotic embryos. The material delivered via somatic embryogenesis is both used as model for physiological studies and mass production of plantlets. Spite high production of embryos is possible some species still encounter problems regarding the maturation and conversion of the somatic embryos into plantlets. It is assumed that abnormalities during somatic embryos development as well as inefficiency in accumulating storage compounds are the main factors that affect late development and ex vitro transfer of the *in vitro* plantlets. Also methodological limitations concerning culture conditions and labor work are reported disadvantages of micropropagation of plants. The use of bioreactors or systems of temporary immersion (TIS) allowed to increase the production and to reduce the costs. Many advances have been made regarding micropropagation of cacao plantlets via somatic embryogenesis. The secondary somatic embryogenesis turned possible the establishment of cyclic cultures. Cyclic production of somatic embryos delivers high amounts of renewable plant material which can be taken from the culture cycle and submitted to physiological studies as well as for further establishment of new culture lines. This work studied the influence of various culture conditions on the production of cacao somatic embryos and on their late development up to conversion into plantlets. Cacao somatic embryos are known as showing a very poor accumulation of storage compounds. It may be the reason for high rate of somatic embryos lost observed during the steps that precedes plantlets establishment. In this work, the effect of different sucrose concentration added to the culture media as well as the different conditions for germination and conversion into plantlets were observed. Morphology and physiology of cacao secondary somatic embryos were investigated using a range of different techniques in order to provide a better understanding of the factors involved in embryos' development. It was intent to identify favorable culture conditions enable to increase the production and the quality of cacao plantlets propagated via somatic embryogenesis.

The use of TIS combined secondary somatic embryogenesis improved significantly the production of cacao somatic embryos which showed better quality than somatic embryos produced on solid medium. The production of high amounts of somatic embryos allowed the establishment of a cyclic culture of secondary somatic embryos. The heterogeneous population of somatic embryos found in the cultures allowed the morphological characterization of the seven stages of development, four stages during early developmental stages, globular, heart, torpedo and cotyledonary and 3 stages regarding growth, maturation and germination steps of the late development. The high production in a cyclic culture also permitted to carry out many physiological studies especially concerning phenolic substances as a key factor in the structural and physiological development of these embryos. UV-microspectrophotometry, a very sensitive and high specialized method allowed the topochemical investigation of phenolic compounds in the tissues of cacao somatic embryos. Distribution and accumulation of phenolic substances throughout the embryo development was observed. Lignification of cell walls of cotyledons of cacao somatic embryos was detected already in early stages of development. The content of phenolic compounds increased proportionally to embryo development and major part of phenolic substances was deposited in the cellular vacuole. Cells containing phenolic compounds in their

vacuole were found mainly under the abaxial epidermis and nearly the vascular bundles. It indicates the accumulation of phenolic compounds to be a defense mechanism of these embryos. Similar distribuition of such cells was described also in zygotic embryos of cacao (Elwers *et al*., 2010). An enzyme closely related to phenolic metabolism is polyphenol oxidase (PPO), the activity of this enzyme in cacao seeds is well documented. In somatic embryos its activity seems to be related to germination process, since significant activity was detected only in hypocotyls of germinating somatic embryos. The activity of this enzyme during germination has been reported in various plants. During late development, cacao somatic embryos demonstrated inefficiency in accumulating storage compounds in the cotyledons; it was minimized with addition of high concentration of sucrose in the maturation media. The balance among the storage compounds, starch, sugars and proteins, was obtained in embryos maturated in culture medium containing 60 g L^{-1} sucrose. It was improved when somatic embryos was cultured under this conditions for only one week. However, it was observed that the stage of embryo development play an important role in the process of plantlets establishment. Somatic embryos at stage 6 of late development without maturation treatments showed the highest content of storage compounds. These also revealed germination and conversion into plantlets similar to embryos pre-treated in maturation medium, but only when germinated in darkness. Finally, it was possible to observe that a two factorial condition seems to lead to a better conversion of cacao somatic embryos into plantlets; the timely stage of the embryo submitted to maturation conditions and the sugar concentration in the culture medium. In conclusion, the latest step of the production of cacao plantlets via somatic embryogenesis can be successfully managed when considering the importance of time response of somatic embryos at latter stages of development to changes made in the culture medium.

V. Zusammenfassung

Der aus dem Amazonasbecken stammende Kakaobaum *Theobroma cacao* L. wächst natürlicherweise im Unterholz tropischer Regenwälder. Er gehört zur Familie der Malvaceae (Malvengewächse) und wird seit vielen Jahrhunderten in tropischen Regionen rund um die Welt kultiviert. Infolge seiner großen wirtschaftlichen Bedeutung als Ausgangsstoff für die Schokoladenherstellung, zählt der Kakaobaum heute zu den am besten untersuchten Nutzpflanzen der Welt. Zu den größten Herausforderungen bei der Kultur von Kakao zählen nach wie vor der Umgang mit gefährlichen Krankheitserregern (vor allem Pilz- und Virusinfektionen), die ausgeprägte Heterogenität zwischen den Bäumen sowie deren Vermehrung. Die recalzitranten Samen des Kakaobaums und seine aufwendige vegetative Vermehrung erschweren die Massenvermehrung von Kakaoklonen und den Erhalt ihrer Vielfalt. Mit Hilfe moderner biotechnologischer Verfahren wird versucht, diese Probleme zu überwinden. Die somatische Embryogenese ist eine *in vitro* Kultur-Methode, die zur klonalen Massenvermehrung von Pflanzen eingesetzt wird. Die dabei entstandenen somatischen Embryonen weisen in ihrer Entwicklung große Ähnlichkeiten mit zygotischen Embryonen auf und dienen deshalb auch als physiologisches Versuchsmodell für die Embryonalentwicklung von Pflanzen. Bei vielen Pflanzenarten ist es bereits möglich, eine große Menge somatischer Embryonen zu erzeugen. Die Reifung dieser Embryonen und weitere Differenzierung in Jungpflanzen bereiten jedoch oftmals noch Probleme. Es wird angenommen, dass hierfür vor allem Wachstumsabnormalitäten und eine nicht ausreichende Einlagerung von Speicherstoffen verantwortlich sind. Bei vielen Pflanzen sind die für ihre *in vitro* Entwicklung optimalen Kulturbedingungen noch nicht bekannt, so dass die Mikrovermehrung häufig noch recht zeit- und arbeitsaufwendig ist. Der Einsatz von Bioreaktoren und temporären Immersionssystemen (TIS) ermöglicht eine Steigerung der Produktion somatischer Embryonen, bei gleichzeitiger Verringerung des Arbeitsaufwandes und damit der Kosten. Hinsichtlich der Mikrovermehrung von Kakao mittels somatischer Embryogenese konnten bereits beträchtliche Fortschritte erzielt werden. Die sekundäre somatische Embryogenese ermöglichte die Etablierung zyklischer Kulturen. Auf diese Weise lassen sich große Mengen nachwachsenden Pflanzenmaterials gewinnen, welches dem Kulturzyklus entnommen und für physiologische Untersuchungen oder zur Etablierung neuer Zellkulturen verwendet werden kann. In vorliegender Arbeit wurde der Einfluss verschiedener Kulturparameter auf die Produktion somatischer Kakaoembryonen sowie deren spätere Entwicklung, bis hin zur Differenzierung in Jungpflanzen untersucht. Die somatischen Embryonen von Kakao sind für ihre verminderte Einlagerung von Speicherstoffen bekannt, was auch die Ursache für die nach wie vor hohen Verlustraten während des weiteren Entwicklungsverlaufs der Embryonen sein könnte. In der vorliegenden Arbeit wurde der Effekt unterschiedlicher Saccharose-Konzentrationen im Kulturmedium auf die Reifung der Embryonen untersucht. Während der anschließenden Keimung und Differenzierung der Embryonen in Jungpflanzen wurde der Einfluss verschiedener Kulturbedingungen betrachtet. Zum besseren Verständnis der die Embryonalentwicklung steuernden Faktoren, wurden sekundäre somatische Kakaoembryonen mit Hilfe verschiedener Verfahren morphologisch und physiologisch untersucht. Ziel war es, die für die Produktivität und Qualität der somatischen Embryonen vorteilhaftesten Kulturbedingungen zu ermitteln. Die Verwendung eines TIS als Kultursystem in Kombination mit sekundärer somatischer Embryogenese führte - verglichen mit auf Festmedium gewachsenen somatischen Embryonen - zu einer signifikanten Qualitätssteigerung (weniger Wachstumsabnormalitäten und weniger Kalluswachstum). Die hohen Produktionsraten ermöglichten die Etablierung einer zyklischen Kultur von sekundären somatischen Kakaoembryonen. Im Entwicklungsverlauf der Embryonen ließen sich morphologisch sieben verschiedene Entwicklungsstadien charakterisieren: vier davon - Globuläres, Herz-, Torpedo- und Cotyledonenstadium – treten während der frühen Entwicklung auf, die übrigen drei während der späteren Entwicklung (d.h. während Wachstum, Reifung und schließlich

Keimung der Embryonen). Darüber hinaus ermöglichte die große Menge zur Verfügung stehender somatischer Embryonen die Durchführung verschiedener physiologischer Untersuchungen. Der Schwerpunkt lag dabei insbesondere auf den phenolischen Inhaltsstoffen, denen eine Schlüsselfunktion bei der strukturellen und physiologischen Entwicklung der Embryonen zukommt. Mit Hilfe der sogenannten Universal-Mikrospektralphotometrie (UMSP), einer äußerst empfindlichen, hochspezialisierten Messmethode, wurden die phenolischen Substanzen in verschiedenen Geweben somatischer Kakaoembryonen topochemisch untersucht, und ihre Verteilung und Akkumulation im Entwicklungsverlauf der Embryonen betrachtet. Bereits in frühen Entwicklungsstadien ließ sich in den Cotyledonen der somatischen Kakaoembryonen eine Lignifizierung der Zellwände beobachten. Die phenolischen Inhaltsstoffe waren hauptsächlich in die Zellvakuole eingelagert und ihr Gehalt nahm im Laufe der Entwicklung zu. Die Zellen, die in ihren Vakuolen phenolische Substanzen eingelagert hatten, befanden sich vor allem unterhalb der abaxialen (unteren) Epidermis sowie in der Nähe von Leitbündeln, was darauf hindeutet, dass die Akkumulation dieser Stoffe in den Embryonen eine Abwehrfunktion einnimmt. Eine ähnliche Verteilung phenolhaltiger Zellen ließ sich zuvor auch bei zygotischen Kakaoembryonen beobachten (Elwers *et al.*, 2010). Ein eng mit dem Metabolismus phenolischer Substanzen verbundenes Enzym ist die Polyphenoloxidase (PPO), deren Aktivität in Kakaosamen gut untersucht ist. Bei den somatischen Kakaoembryonen steht die PPO-Aktivität offenbar in Verbindung mit dem Keimungsprozess: eine signifikante Enzymaktivität konnte ausschließlich in den Hypocotylen keimender somatischer Embryonen beobachtet werden. Auch bei anderen Pflanzen gibt es Berichte über eine Aktivität der PPO während der Keimung. Während ihrer späten Entwicklungsstadien, zeigten die somatischen Kakaoembryonen eine - im Vergleich zu zygotischen Kakaoembryonen - verminderte Einlagerung von Speicherstoffen in ihre Cotyledonen. Durch den Zusatz hoher Mengen an Saccharose zum Reifungsmedium ließ sich die Akkumulation von Speicherstoffen steigern. Die größte Akkumulation von Speicherstoffen (Stärke, Zucker und Proteine) wurde nach einwöchiger Reifung der Embryonen bei einer Saccharosekonzentration von 60 g L^{-1} im Kulturmedium erzielt. Darüber hinaus ließ sich beobachten, dass das Entwicklungsstadium, in welchem die Embryonen mit dem Reifungsmedium inkubiert wurden, einen großen Einfluss auf die spätere Entwicklung der Kakaojungpflanzen hatte. Ohne Inkubation mit Reifungsmedium wiesen die Embryonen des Stadiums 6 der späten Embryonalentwicklung den höchsten Gehalt an Speicherstoffen auf. Wurden diese Embryonen im Dunkeln zum Keimen gebracht, zeigten sie morphologisch große Ähnlichkeit mit den im Reifungsmedium gewachsenen Embryonen. Die Differenzierung somatischer Kakaoembryonen zu Jungpflanzen ließ sich durch zwei Faktoren positiv beeinflussen: die Wahl des Entwicklungsstadiums, in welchem die Embryonen der Reifungsbehandlung unterzogen wurden, und die Zuckerkonzentration im Kulturmedium während der Reifung. Zusammenfassend lässt sich sagen, dass der letzte Schritt bei der Produktion junger Kakaopflanzen mittels somatischer Embryogenese erfolgreich gelingt, wenn das Zeitverhalten der somatischen Embryonen in den späteren Phasen ihrer Entwicklung berücksichtigt, und die Zusammensetzung des Kulturmediums entsprechend angepasst wird.

1.Introduction

Theobroma cacao or the chocolate tree is the main natural resource for chocolate production. It is a highly important plant with respect to a well accepted international product line (chocolate and cacao drink). It is socio-economically highly accepted because this crop is a central factor for small farmer's economy. Even in huge monocultures plantations the trees must be managed by many people due to factors like precise harvest and plant diseases control. Another important factor is the high ecological tolerance of the cacao tree. It can be combined with many other crop plants and does not really compete for nutrition and water (Lieberei and Reisdorff, 2012). Despite the enormous amounts of cacao produced around the world (more than 4 Mio tons, ICCO, accessed 03/29/2013), this production still encounters many difficulties like social conditions of the workers, infestation of the plants by severe diseases and problems concerning vegetative propagation. For one ha of cacao trees around 1000 individual plants are needed.

T. cacao is an allogamous plant with recalcitrant seeds, peculiarities that hamper the mass propagation and conservation of diversity. The propagation of cacao plants by seeds is difficult, since a high heterogeneity of genotypes is mixed in the harvested goods. For the suitable post-harvest treatment it is important to adapt the tree's traits, especially the fermentation parameters to the genotype properties, in other words, high quality raw cacao beans can only be produced on the basis of homogenous fruit characters. Propagation of homogenous plant material is possible by vegetative multiplication like rooting of stocks. In this method a developmental factor reduces mass production of plantlets, because when branches are used for rooting, the plant inherited growth dimorphism leads to two types of plant growth: branches fans rise to plagiotropic bush-like trees; cuttings from the orthopropic stems deliver typical orthotropically growing plantlets. A strategy used to overcome the behavior, which is applied to species with similar difficulties is the *in vitro* propagation via somatic

embryogenesis. This process of tissue culture is used not only as a tool for mass propagation of clonal plantlets but also as resource of genetic material in gene banks.

Cacao has been cultivated *in vitro* for many years, but the production of somatic embryos is done using different protocols. However, all these methods present a severe bottleneck at the moment of transfer of these somatic embryos to autotrophic conditions. A critical point studied by many research groups is the late development of these embryos, especially the conversion into plantlets and the establishment of these in field conditions. In the case of field transfer the major part of the somatically produced plantlets are lost. Cacao somatic embryos also show still high numbers of abnormalities and this leads to inefficiency during the production process. Efforts have been made to improve the culture conditions and to establish an ideal physiological state of the embryos which will guarantee the increase of survival rates during establishment *ex vitro* culture steps.

The efficiency of the somatic embryos in accumulating storage compounds as well as the development of morphological and physiological properties similar to zygotic counterparts are considered to be fundamental issues to reach the conversion from *in vitro* material to healthy plantlets. The main aim of this study is to work out a sequence of treatments which allow to produce high numbers of *in vitro* plantlets with high capacity to be established in *ex vitro* conditions. Temporary Immersion Systems (TIS) have been shown to be suitable for production of somatic embryos and to function as a source for clonally propagated *in vitro* plants. A well known bottleneck in *in vitro* production is the availability of competent tissue which can serve as basis for mass production of somatic embryos. In this study the use of primary somatic embryos as basis material for the scale-up production of high amounts of embryos is improved. The whole process of cyclic formation of secondary somatic embryos is explored and, finally, a protocol for mass production is formulated. As an important question the influence of growth medium composition on physiological and morphological development is investigated. It is aimed to increase the understanding of maturation

and germination occurring in cacao somatic embryos. These phases are shown to be influenced by the medium composition. The physiological responses of cacao somatic embryos to high concentrations of sucrose and further conversion into plantlets under different culture conditions are investigated. Observations concerning the morphological heterogeneity found within produced somatic embryos are carried out seeking to verify the significance of morphological features for the production of clone plantlets. The factors that influence the cacao somatic embryos production and late development in TIS are investigated with respect to long lasting effects of the tested treatments.

2. Background information

2.1 *Theobroma cacao*: Botanical peculiarities

2.1.1 Taxonomy, Morphology, Phenology

The genus *Theobroma* has evolved in the Amazon basin and was naturally dispersed through the tropical rainforest up to Southern Mexico. Recent phylogenetic analyses revealed that this genus is a member of the family Malvaceae. 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.) Schum., and *Theobroma bicolor* (Humb. & Bonpl.).

However, *Theobroma cacao* is the only species widely established in tropical regions around the world due to its economic importance as raw material for chocolate manufacturing. (Silva *et al*., 2004; Motamayor *et al*., 2002; Dias, 2001; Rehm and Espig, 1991). *T. cacao* is a perennial, allogamous and diploid plant (2n = 20). Its seeds are recalcitrant with a high level of heterozygosity as reviewed in Argout *et al.* (2010). This species is shade tolerant and drought sensitive. These ecophysiological properties enable the growth and propagation of chocolate trees in American, African and Asiatic rain forests (Almeida and Valle, 2007).

Cacao plants possess alternate large leaves with palmate venation. The leaves develop in groups of young leaves called flush (or leaf-flushing). The young leaves can be light green or red to brown, but both types turn dark green during growth. The leaves remain on the tree for 4 or 5 months, then senescence occurs (Figure 3B; Niemenak *et al*., 2010; Almeida and Valle, 2007; Greathouse *et al.,* 1971). Small flowers grow on the trunk and on main branches (cauliflory). Formally, many flowers together form an inflorescence which originates from senescent leaves axils. The cacao hermaphrodite flowers consist of 5 sepals, 5 petals, 10 stamens and a pluricarpelar ovary (5). The five fertile stamens have two anthers which are allocated in

the concave part of the petals. The style is formed by 5 fused stigmas (Figure 1; Swanson *et al.,* 2008). Cacao flowering occurs in different periods within one year depending on genotype and geographical location. In the Lower Amazon region it occurs between January and June while in the Upper Amazon cacao trees produce flowers during the whole year (Niemenak *et al*., 2010). Self-incompatibility is observed in some genotypes of cacao and is responsible for increasing allogamy in that species. The pollen tube grows normally and the mechanism of abort occurs during the fecundation of the ovule and it seems to be regulated by auxin pathways (Ford and Wilkinson, 2012; Hasenstein and Zavada, 2001; Baker *et al*., 1997; Pandey, 1960).

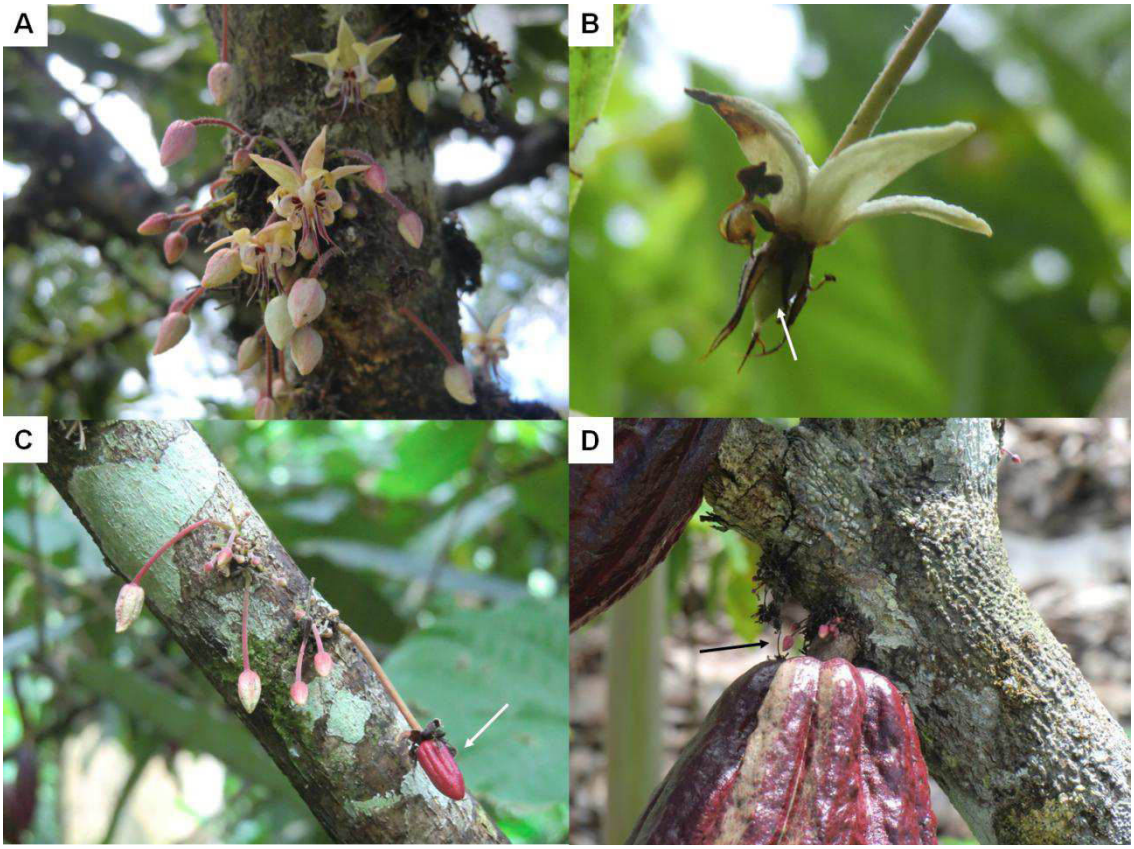

Figure 1. Cacao caulifory and fruit phenology. A- Inflorescence with flowers at different stages of development. **B-** Cacao flower after fertilization still containing sepals, some petals, sterile stamens and growing green ovary (arrow). **C-** Trunk with flowers and a not aborted cacao cherelle (arrow). **D-** Mature cacao pod and its inflorescence on the trunk (arrow). Pictures A and D were taken in the Germplasm Collection at Mars Center of Cacao Science. Pictures B and C were taken in the Fazenda Leão de Ouro (Bahia, Brazil).

After fertilization the superior ovary grows to form the cacao fruit, called pod. In cacao occurs a phenomenon called Cherelle wilt, which describes the fact that some of

the young fruits (Cherelles) stop growing and turn black, but remain attached to the trunk. It is reported that cherelle wilt is a mechanism to control the intensity of fruit set of the plant (Valle *et al.*, 1990). The normally developing pods reach maturity after approximately 130 days and can form fruits of 20 cm to 30 cm in length. Fruits differ in form, size and color. The number of seeds in a pod is variable, but up to 60 seeds can be found in one pod. In the pod, white sweet mucilage is covering the seeds which are fixed on the placenta (Figure 2; Daymond and Hadley, 2008; Nichols, 1964; Chessman, 1927).

Figure 2. Mature cacao pods. A- Mature pods of cacao on the trunks. **B-** Phenotypically diversity of cacao pods. **C-** Seeds from a mature cacao pod covered by white mucilage grouped around the placenta. Pictures A and C were taken in the Fazenda Leão de Ouro. Picture B was taken at Mars Center of Cacao Science (Bahia, Brazil).

The seeds of cacao are normally fermented and processed for chocolate fabrication. During this process the seeds die. Without this interference, about 150 days after fertilization, the seeds are mature and ready to begin the germination. After onset of germination within 30 days the seedling is established (Niemenak *et al.*, 2010).

After the cotyledons fall off, the seedling presents a determinate shoot growth. First the shoot grows orthotropically up to a hight of 80cm to 1 meter (Chupons). After that the branch dimorphism takes place. The main shoot meristem is inhibited in further growth and the apical meristem develops into 5 meristems, which form branches with indeterminate plagiotropic growth (Fan branches). These two forms of growth are alternately activated in cycles throughout the whole life time of the tree (Figure 3; Lieberei and Reisdorff, 2012; Greathouse and Laetsch, 1969).

Fruit production starts when trees are 3 years old. The highest yield is reached at the age of 20 - 40 years old. The trees produce for 100 years because of their regenerative vegetative growth (sympodial) (Niemenak *et al.*, 2010; Almeida and Valle, 2008; Rice and Greenberg, 2000).

Figure 3. Cacao branch dimorphism. A- An orthotropically growing cacao bush with the first plagiotropic branches growth. **B-** Plagiotropic branches with indeterminate growth. Red leaves belong to the actual leaf-flushing. **C-** Orthotropic shoots growing from cacao tree basis (Chupons). **D-**Typical jorquette of plagiotropic fans with an orthotropic shoot emerging from axillary shoot (Chupon-arrow) formed under the plagiotropic branches. Pictures were taken at Mars Center of Cacao Science (Bahia, Brazil).

2.1.2 *Theobroma cacao* as a genetic resource

2.1.2.1 Genetic variability

Cacao consists of many morphologically and physiologically variable populations that offer the possibility of numerous intercrosses. There are two main populations of *Theobroma cacao* according to phenotypical traits and geographical spread from the region of origin. One of those is called **Criollo,** (*Theobroma cacao* (L.) ssp. *cacao* Cuatr.), grown from Mexico to Central America as well as in Colombia and Peru. The trees belonging to this subspecies present white or light red seeds and are characterized by a very fine flavor of raw cacao beans. For this reason, they were the first domesticated cacao in Central America. **Forastero** (*T.cacao* (L.) ssp. *sphaerocarpum* Cuatr.), the much higher diversified population grown in Brazil and other countries of the Amazon basin contain trees which demonstrate high resistance to diseases established in the Amazon basin and with stable higher production, their seeds are deep violet or red due to their high content of anthocyanidin glycosides (Lieberei and Reisdorff, 2012). These populations present a high diversity and therefore have been divided into 2 geographical subgroups: Upper and Lower Amazon Forastero. Both groups are suitable for raw cacao production and up to now are important sources of mass cacao. A third group is recognized as **Trinitario,** a hybrid of criollo (matrix plant) and Forastero as pollinator (Lieberei and Reisdorff, 2012; Bhattacharjee and Kumar, 2007; Dias, 2001; Marita *et al*., 2001), which presents fairly good disease resistance and some fine flavor characteristics.

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 (Smulders *et al*., 2012; Susilo *et al*., 2011; Motamayor *et al*., 2008; Sereno *et al*., 2006). According to these studies, the cacao populations of Central and South America were subdivided into 10 genetic clusters: Amelonado, Contamana, Curaray, Guiana, Iquitos, Maranõn, Nacional, Nanay and

Purús, these occur in the Amazon basin and Bahia. Another group, number 10, is the already described Criollo which occurs also in the Amazon basin region, but is the only group occurring in Central America, due to their early distribution by humans.

2.1.2.2 Distribution

The findings on the high genetic diversity in the Amazon basin region support the theory that *T. cacao* evolved in the Upper Amazon region of Brazil, Peru and Ecuador (Ji *et al*., 2013; Zwang *et al*., 2011; Motamayor *et al*., 2008; Sereno *et al*., 2006; N'Goran *et al*., 1994). 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 the assumption (Thomas *et al*., 2012). Criollos, the only genetic group found in Central America shows a significantly lower diversity. It has probably occurred during the domestication process by selection and propagation of preferable phenotypes like sweet pulp, bigger seeds, thin pod pericarp and content of caffeine, all of which are typical characteristics of Criollos cacao disseminated throughout Central America (Thomas *et al*., 2012; Clement *et al*., 2010; Motamayor *et al*., 2002).

Thomas *et al*. (2012) suggests cacao had already been widely distributed in Western Amazon before glaciations. They describe that geographical isolation and genetic differentiation resulted in the high diversity of plants found today in this region. The establishment of other cacao populations through Mesoamerica and East Amazon was strongly affected by human intervention. The botanical characteristics of the genus are not favorable for high species dispersion observed today. Geological and climatic processes contributed to the diversification found today in that region (Clement *et al*., 2010).

During the European influence, cacao was widely domesticated throughout entire Mesoamerica and South America. The indigenous people used the pulp and seeds extensively, mainly as alcoholic drinks and the seeds as currency. The first

domestication of cacao trees is supposed to have been carried out by the Olmec populations who were known as active traders. In the region that nowadays corresponds to the capital of Olmec (Powis *et al*., 2011; Hurst 2002), residues of theobromine were found in archeological vessels dated to 1800-1000 BC. However, proper cultivation of cacao trees took place in 18th century during territorial occupation by Spanish conquests. It is known that Trinitario cacao resulted from hybridization between Criollo and Forastero cacao after devastating diseases in the Trinidad plantations. These new cacao hybrids showed higher production rates than Criollos and a greater resistance against diseases.

At the beginning of the 20th century, the groups Criollo, Amelonado, Trinitario and Nacional belonged to the typical economically used groups in Brazil and Trinidad. These were distributed to African and Asian producer countries. The objective was to increase the genetic variety in order to overcome the various diseases occurring in the respective countries. The distribution was facilitated by the strong and growing market for raw cacao beans. This was the reason for the colonial administration in African countries to establish cacao plantations and collections. Many new breeds evolved from this distribution and from the need for high propagation. Today most cacao produced around the world is based on these cultivars as reviewed in Aikpokpodion (2012).

Today cacao growth and production is an important economic factor in all countries with suitable conditions for cacao cultivation. There is a wide international activity in processing cacao production and in development of protection of this crop against diseases (Thomazella *et al*., 2012; Kilaru *et al.,* 2007; Phillips-Mora *et al*., 2007; Phillips-Mora *et al*., 2005; Scarpari *et al*., 2005; Marita *et al*., 2001).

2.1.2.3 Cultivation, propagation and conservation of diversity

Compounds like alkaloids, terpenoides and phenolics are used daily for a high percentage of population and lead the world trade of the plants-derived products. The

plants retaining such compounds are generally originated from tropical areas and became important economic resources for the developing countries, for example coffee and cacao (Lieberei and Reisdorff, 2012). The cacao tree provides the main resource for the chocolate industry. The cacao crops are established in all countries with tropical weather i.e. Indonesia, Malaysia, Côte d'Ivoire, Ghana, Brazil and other countries in South and Central America. The largest producers and exporter of cacao are Côte d'Ivoire, with a production of 1476 thousand tons (t.ton) in 2011/12, followed by Ghana (879 t.ton), Indonesia (450 t.ton), Nigeria and Brazil (220 t.ton) (ICCO Quarterly Bulletin of Cocoa Statistics, Vol. XXXVIII, No. 4, Cocoa year 2011/12). Brazil was the largest cacao producer until mid 20 century with 40% of all production in the world; it was affected by the interruption of trade during the Second World War (SWW) (Willumsen and Dutt, 1991). After the end of SWW cacao redeveloped in the commercial negotiation and Brazil cacao production was around 400.000 ton. It was interrupted in 1989 when the devastating disease, "Witches-broom" caused by the fungus *Moniliophthora perniciosa,* appeared in this country. Due to the unspecific infection and wide genetic base of the fungus, this disease devastated the cacao plantations in Brazil in beginning of the 90's. The African countries with large plantations, cheap human labor and absence of this disease are leading the production of cocoa in the international trade since the end of the 1990s. The cacao farmers in Brazil are still working against the crises expanded during the last years. However, many farmers replaced cacao plantations with other crops, especially coffee. In the course of selection resistance of cacao there was successful development of high productive and resistant new cacao clones. Today this country is the 5th largest cacao producer in the world; Bahia is the main producer region, producing 90% of total cacao beans harvested in Brazil (FAO, 2003; Griffith et al., 2003).

Cacao tree can be cultivated in three main different plantation systems: in poly-culture in Atlantic Forest areas (Cabruca), planted agroforestry systems and monoculture plantations as reviewed in Rice and Greenberg (2000) and Alvim *et al.*,

(1986). As it is known, cacao occurs naturally in shadow forest understory. Thus it is recommended to establish cacao plantations by systems that offer shade conditions. A successful example of this system is the one used in Bahia (Brazil), namely “Cabruca”. This system allows the cultivation of cacao trees under the thinned native forest where the biodiversity is considerably higher than in other systems. The inclusion of cacao trees in agroforestry systems has spread throughout West African and Latin American countries; this system consists of planted multi-crops which are sometimes combined with secondary forest (Alvim *et al.*, 1986). The species introduced in the plantations are selected according to socio-economic importance and advantages when combined to each other. The monoculture plantations commonly consist of clonally propagated plants. The maintenance and harvest in this type of system is easier but the susceptibility to diseases can be a problem. The establishment of any of these systems is much more complex. Some studies indicate implications on cacao production and health to be dependent on the forest where it is cultivated and of the trees species integrated in the agroforestry system (Isaac *et al.*, 2009; Rice and Greenberg, 2000; Wilkinson and Elevitch, 2000).

There are many factors that negatively affect the cacao cultivation: the peculiar condition of cultivation, narrow genetic base of cacao clones and the occurring diseases. These factors make the stability of cacao plantation and their conservation difficult. In America the cacao clones are strongly attacked by “Witches-broom” which has caused inestimable socioeconomics problems. In West African countries the most destructive diseases are “swollen shoot” caused by “*Cocoa swollen shoot virus*” (CSSV) and the “black pod” caused by *Phytophthora spp*. The treatments against these and other diseases are in general based on the application of pesticides. The costs of treatment and environmental damage make this practice unfeasible (Phillips-Mora *et al.*, 2005; Thevenin *et al.*, 2005; Muller and Sackey, 2004).

Some plantations are investing in the replacement by resistant cultivars, thus many efforts have been made in studies of technologies that could improve culture

conditions. Studies concerning biological control of the diseases as well as genetic interference in plants and microorganisms physiology are in progress (Thomazella *et al*., 2012; Argout *et al*., 2011; Pungartnik *et al*., 2009; Rubini *et al*., 2005; Lanaud *et al*., 2004).

Plants of cacao can be obtained by two main forms, by seeds from selected plants or by vegetative propagation. The vegetative propagation of cacao trees encounters difficulties. Generally, the vegetative propagation is made from scions from plagiotropic branches which grow and develop to a bush-like structure, but is mostly incapable to form a taproot, causing instability of the trees. Plants regenerated from rooted orthotropic chupons develop a seedling-like architecture (vertical direction). However, the farmers prefer to use scion from plagiotrophic branches, combined with extensive pruning techniques to change the bush-like architecture of the trees (Farias and Sacramento, 2003; Miller and Guiltinan, 2003; Figueira and Janick, 1995). The maintenance of the vegetative propagation process requires special work, new technologies, long time and high costs, factors that limit the use of this technique (Rehm and Espig, 1991; Figueira and Janick, 1995). Some 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 and the gene pool can only be controlled poorly (Bennet, 2003).

A representative number of studies are underway with the purpose to overcome the deficiencies found during propagation, establishment of the cacao culture and conservation of the diversity. The *in vitro* propagation is an alternative to eliminate problems imposed by plagiotropic growth. This method offers good phytosanitary conditions, saving plants from exposure to environmental hazards with genetic gain of commercial interesting individuals. The usage of *in vitro* technologies has been used as a component of breeding programs and conservations of diversity for other species and shows potential for cacao as well (Pilatti *et al*., 2011; Maximova *et al*., 2008; Miller and Guiltinan, 2003; Traore *et al*., 2003). *In vitro* culture enables genetic gain of clonal

propagated plants and conditions for germplasm conservation (Fang and Wetten, 2011; Teixeira and Marbach, 2002; Vicient and Martínez, 1998; Figueira & Janick, 1995).

2.2 Biology of somatic embryogenesis

2.2.1 Introducing totipotent cells

Many biotechnological methods have been applied to overcome the damage caused by cacao diseases, e.g. the elimination of pathogens, genetic breeding of superior clones with resistance and efficient propagation of seedlings (Argout *et al*., 2011; Kumar and Bhattacharjee, 2007; Griffith *et al*., 2003). *In vitro* propagation is an alternative for propagation of clone plantlets increasing genetic gain. The somatic embryogenesis aims to reproduce the physiology and development of the zygotic counterpart (Dodeman *et al*., 1997). For this, it is necessary to understand the physiology of plant zygotic embryogenesis.

In nature, as an alternative to the propagation via seeds, plants developed strategies like organogenesis, shoot proliferation via axillary buds and somatic embryogenesis (Zang and Ogas, 2009). Plants have evolved different approaches in order to respond to environmental signals like adjusting the metabolism and/or development traits. To guarantee continuous vegetative growth (organogenesis) during the whole life cycle and to fulfill the needs for survival without migration, plants developed special groups of cells called meristems. These are formed of stem cells capable of dividing and to renew themselves through mitotic division and additionally to differentiating into different types of specialized cells. 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; Fehér *et al*., 2003; Clark, 1997; Dodeman *et al*., 1997; Williams and Maheswaran, 1986).

These stem cells are used as a key to initiate somatic embryogenesis. *In vivo*, an example are embryos originated from somatic nucellar cells in the ovule (Zang and

Ogas, 2009; Taiz and Zeiger, 2007). The meristematic cells can also be cultured *in vitro* to regenerate embryos, in a process called somatic embryogenesis (Fehér *et al*., 2003; von Arnold *et al*., 2002; Guerra *et al*., 1999). It 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. It includes abnormal development of the embryo and low frequency of conversion into plantlets (Mondal *et al*., 2002). For *Theobroma cacao* this technique has been used to investigate critical points of the physiology of this species but mainly to improve the mass propagation (Figueira and Janick, 1995). 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).

2.2.2 Somatic embryogenesis of *Theobroma cacao*

Esan (1974) established the first *in vitro* culture of cacao tissues. Since then many researchers have tried to improve cacao *in vitro* conditions. The *in vitro* culture of cacao has been established in many different culture medium compositions, commonly based on MS media (Murashige & Skoog, 1962) and DKW media (Driver & Kuniyuki, 1984), in some cases differing in plant growth regulators and minerals concentrations (Minyaka *et al*., 2008).

Cacao presents recalcitrance in *in vitro* culture. Thus, several studies have been carried out to improve the production of somatic embryos, which showed that specific salts are required for an increase of embryogenic competence (Minyaka *et al*., 2008a and 2008b). They suggested the use of high quantities of sulphate sources during the induction of the primary somatic embryogenesis and demonstrated the importance of the balance between $MgSO_4$ and K_2SO_4 (magnesium sulphate, potassium sulphate, respectively) to promote embryogenic explants. 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 embryogenic competence of the cacao somatic cells is considered to be genotype-dependent (Li *et al*., 1998). Issali *et al.* (2008) characterized some genotypes as suitable for callogenesis and/or embryogenesis depending on culture medium and explants type. The study allowed to identify the proper explants and culture conditions for thirteen different genotypes analyzed. Such studies are fundamental for the establishment of somatic embryogenesis of different cacao genotypes (Issali *et al*., 2008; Maximova *et al*., 2002; Li *et al*., 1998; Alemanno *et al*., 1996).

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 calli 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 somatic embryos are defined as somatic embryos originated directly from matrix plant explants (leaves, flowers pieces etc.) while secondary somatic embryos are regenerated from tissues of primary somatic embryos or other secondary somatic embryos already in *in vitro* culture (Raemakers *et al*., 1995; Williams and Mahesrawan, 1985). 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). Secondary somatic embryogenesis (SSE) cultures are preferable for establishment of genetic transformation due to the rapid regenerative process and single cellular origin of the somatic embryos (Fernández and Menéndez, 2003; Maximova *et al*., 2003). Secondary somatic embryos of cacao cultivated with *Agrobacterium tumefaciens* resulted in stable genetic transformation with near-perfect segregation of the genes. However, difficulties imposed during conversion of cacao somatic embryos into plantlets allowed to investigate the introduced genes only in early stages of embryonic development (Maximova *et al*., 2002). In addition to genetic advantages, SSE allows rapid regeneration of a higher number of somatic embryos than the traditional techniques. The embryogenic competence of the cultures is preserved for longer periods of time. This is possible due to the repeated cycles of cultures permitted by the high number of somatic embryos produced (Kim *et al*., 2012; Raemakers *et al*., 1995).

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; Berleth and Chartfield, 2002; Maximova *et al*., 2002; Santos and Machado, 1989). Embryogenesis of angiosperms is divided into two main phases: an early phase that comprises the embryonic morphogenesis up to complete formation of tissues and organs. During this phase, it is possible to observe four morphological stages of embryo development: globular, heart, torpedo and cotyledonary. At this point morphogenesis is considered to be completed and the second phase, the so-called late development, begins. During this phase, embryonic organs grow and simultaneous accumulation of reserves takes place, it occurs until the achievement of physiological maturation, when embryogenesis is considered completed and the germination or desiccation of the seed takes place (Harada *et al*., 2010; Zang and Ogas, 2009; von Arnold *et al*., 2001). There are many investigations concerning morphology and physiology of the early development of

cacao somatic embryos. However, such studies on the late developmental phase are still scarce. After the establishment of a protocol for cacao somatic embryogenesis efficient for a higher number of genotypes, many other researches invested efforts to improve culture conditions of early development of cacao somatic embryos and try to overcome the genotype-dependency (Li *et al*., 1998; Alemanno *et al*., 2003; Maximova *et al*., 2002). However, difficulties in converting cacao somatic embryos into plantlets have already been reported and studied in former studies (Figueira and Janick 1995; Pence *et al*., 1980). More recently efforts have been applied on late development of cacao somatic embryos, since one of the main objectives of somatic embryogenesis is the further establishment of clone plantlets in the field (Nawrot-Chorabik, 2012; Kamle *et al*., 2011). Several media formulations are suggested to be necessary for maturation and conversion of somatic embryos of cacao into plantlets. The comparison of zygotic and somatic embryogenesis of cacao led to the identification of some factors which may limit cacao somatic embryo development. 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*., 1997). The authors suggested the need of a growth period to reach a successful embryo late development. 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*., 2008; Niemenak *et al*., 2008; Maximova *et al*., 2005; Traore *et al*., 2003). 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 were reached only in presence of abscisic acid (Alemanno *et al*., 1997). It is assumed that cacao somatic embryos require high concentrations of sucrose to accumulate lipids and other storage compounds like proteins (Pence *et al*., 1980). In contrast other studies demonstrate that there are no differences in cacao somatic embryos maturated in the presence of

different carbon sources like glucose, maltose, sucrose, fructose and sorbitol (Traore and Guiltinan, 2006). The role of these agents in cacao somatic embryos metabolism remains unclear. Investigations concerning the establishment of somatic plantlets of cacao in field conditions revealed that 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). The secondary somatic embryos seem to be also suitable for conservation of germplasm by cryopreservation (Fang *et al*., 2009; Fang *et al*., 2008). However, somaclonal variation was detected during cacao somatic embryogenesis and in plantlets regenerated from somatic embryos (Rodriguéz-Lopez *et al*., 2010; Wilkinson *et al*., 2010). Genetic mutations are supposed to occur in approximately 30% of the somatic embryos produced (Rodriguéz-Lopez *et al*., 2010). Additionally, the mutations occur depending on the explants, the time of culture and in embryos originated from older calli (Wilkinson *et al*., 2010). However, secondary somatic embryos showed a lower occurrence of mutations than primary somatic embryos (Fang *et al*., 2009; Maximova *et al*., 2002). Diminution of culture cycles and period of callogenesis can minimize the mutation rate in somatic embryos of cacao (Wilkinson *et al*., 2010).

The established systems for *in vitro* propagation of cacao are generally carried out under conventional conditions, namely on solid medium (Maximova *et al*., 2002; Li *et al*., 1998; Alemanno *et al*., 1996; Figueira and Janick, 1995). Well established micropropagation of economically important crops (*Coffea spp*, *Bactris spp, Ananas spp, Pinus spp*) are carried out in liquid media in bioreactors or systems of temporary immersion (Watt, 2012; Steinmacher *et al*., 2011; Docus *et al*., 2007; Etienne *et al*., 2006). Some advantages are presented by this kind of systems, e.g. the possibility of changing the culture media without contact with the cultured tissues as well as size of the culture vessels which can be significantly larger than those used for solid media. Furthermore, the system can be easily automated (Watt, 2012). The absence of gelling agents, gaseous exchange as well as temporary contact with culture media are the

main factors that positively influence the development of plant cells in bioreactors. Nevertheless, these systems present disadvantages like asphyxia, hyperhydricity and physiological failures supposedly caused by flooding of the tissues due to the lack of polarity (Ziv, 2000). Niemenak *et al*. (2008) applied the TIS developed at Hamburg University (Preil, 1991) to cacao somatic embryogenesis and obtained satisfactory results with respect to the production of somatic embryos. TIS significantly increased the mass of somatic embryos and allowed an improved development of the embryos. However, the occurrence of non-embryogenic calli was also higher in TIS cultures. Furthermore, the percentage of converted embryos remains low due to the abnormality of embryos formed.

The malformation of cacao somatic embryos and genetic instability also hampers strategies for cryopreservation. Cryopreservation of cacao somatic embryos was reported by Fang *et al*. (2004), they established a protocol through encapsulation-dehydration by treatments with high concentrations of sucrose combined with abscisic acid. The regenerated plantlets developed like plantlets originated from embryos that have not been cryopreserved. However, the suitability for cryopreservation seems to be genotype-dependent. Further studies demonstrated that high concentrations of sucrose in the media improve sucrose reserves in the embryo and increase the embryos survival of embryos up to 95% after cryopreservation (Fang *et al*., 2009). The loss of viability of the embryos after the cryopreservation process is related to the oxidative status of the tissue and the production of ethylene. The authors suggest the use of ethylene inhibitors or the introduction of antioxidants during the preparation of the somatic embryos for cryopreservation (Fang *et al*., 2008).

The development of technologies for propagation and conservation of cacao *in vitro* has shown positive results. However, the bottleneck is still the late phase of somatic embryos development and conversion into plantlets as well as the survival of these. The establishment of secondary somatic embryogenesis for cacao clones and the increase of production by cultivation in temporary immersion system significantly

improved the development of this protocol (Niemenak *et al*., 2008; Maximova *et al*., 2002). Nevertheless, many processes of cacao somatic embryos late development and physiology were not yet approached. The *in vitro* recalcitrance, high content of phenolic compounds (Alemanno *et al*., 2003), genotype-dependency (Li *et al*., 1998) and lack of viability of somatic embryos (Niemenak *et al*., 2008; Maximova *et al*., 2002, 2005; Alemanno *et al*., 1997) are the main characteristics of cacao embryogenic cultures described as obstacles for a successful cacao micropropagation via somatic embryogenesis. The changes in culture conditions may reveal metabolic responses which can help to understand cacao embryogenesis. The sequencing of the cacao genome allows a huge range of possibilities for studying cacao biology seeking solutions concerning field problems like diseases and production as well as molecular as physiological problems like tolerance to environmental conditions and propagation (Argout *et al*., 2011).

3. Material and Methods

3.1 Plant Material

3.1.1 Primary somatic embryogenesis (PSE)

Primary somatic embryos are those obtained via somatic embryogenesis regenerated on matrix plant explants (flower pieces, leaves and zygotic embryos).

For the induction of primary somatic embryogenesis, petals and staminoides were used as explants. Immature flower buds were excised from matrix plants of four international genotypes (CCN 51, ICS 39, ICS 95, IMC 67). The flower buds were gently provided by Intermediate Cocoa Quarantine Unit (University of Reading, Reading, England).

3.1.2 Secondary somatic embryogenesis (SSE)

Secondary somatic embryos are those originated from tissues of primary somatic embryos or other secondary somatic embryos already established in *in vitro* conditions. PSE was previously established from immature zygotic embryos obtained in a germplasm collection located in Ambanja, Madagascar. In this work, this clone is called RO3.08/1-Madagascar (RO3.08/1-Md). The PSE was regenerated before the beginnings of this work in the Laboratory for Useful Plants (Department of Applied Botany, Biocenter Klein Flottbek and Botanical Garden, University of Hamburg, Hamburg, Germany). The secondary somatic embryogenesis used in the course of this study was established from cotyledons of their primary somatic embryos. Subsequently, cyclic culture was carried out, i.e. cotyledons from secondary somatic embryos were used as explants for induction of new secondary somatic embryogenesis (SSE).

3.1.3 Further physico-chemical analyses

For morphological and biochemical analyses, secondary somatic embryos were taken from a cyclic culture regenerated on solid or in liquid medium. Only well-developed embryos were used, except when the abnormalities were in the focus of interest.

3.2 Culture Conditions

3.2.1 Primary somatic embryogenesis (PSE)

Flower buds were sent from Reading to Hamburg in tubes containing cold sterile water. The flower buds were promptly washed with sterile water containing 2 drops of Tween20®. Subsequently surface-sterilization was carried out by transferring the buds in 70% ethanol for 2 minutes, followed by the submersion in a 1% solution of sodium hypochlorite for 20 minutes. Finally, the buds were triple washed with sterile water.

The surface-sterile flowers were left in water until excision. According to the protocol described in the Protocol book of The Pennsylvania State University (Guiltinan & Maximova, version 2.1, 2010), petals and staminoids were separately placed in Petri dishes containing approximately 30 ml of primary callus growth (PCG) culture medium consisting of DKW salts and vitamins (Driver and Kuniyuki, 1984), 20 g L^{-1} glucose, 250 mg L^{-1} glutamine, 100 mg L^{-1} myo-inositol, supplemented with 9 µM L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid, Merck®) and 0.9 µM L^{-1} TDZ (Thidiazuron). After 14 days on PCG medium, the explants were transferred to secondary callus growth culture media (SCG) consisting of McCown's salts formulation and B5 vitamins (DUCHEFA®) supplemented with 20 g L^{-1} glucose (DUCHEFA®), 9 µM L^{-1} 2,4-D and 0.2 µM L^{-1} BAP (6-Benzylaminopurine). The cultures remained on SCG medium for further 14 days. Thereafter, the explants were submitted to embryo development culture medium (ED) consisting of DKW salts and vitamins (Driver and Kuniyuki, 1984), 30 g L^{-1} sucrose and 1 g L^{-1} glucose (DUCHEFA®), free of plant growth regulators (PGRs).

At end of expression phase (ED5) only genotypes CCN 51 and IMC 67 originated callus and subsequently primary somatic embryos. After induction of secondary somatic embryogenesis, these clones presented production of secondary somatic embryos. No PSE expression was observed in cultures induced in TIS. Thus, the present study was continued with the clone RO03.08/1-Md.

3.2.2 Secondary somatic embryogenesis (SSE)

LUP Protocol (Laboratory of Useful Plants-Hamburg University, Germany)

Cotyledons from somatic embryos were used as explants for initiation of the SSE cultures. The cotyledons pieces were placed in Petri dishes containing approximately 30 ml of solid medium consisting of DKW's salts and vitamins (Driver and Kuniyuki, 1984) supplemented with 20 g L^{-1} glucose, 9 µM L^{-1} 2,4-D and 0.2 µM L^{-1} BAP. After 14 days, the explants were transferred to the same medium but replacing BAP with 1.1 µM L^{-1} KIN (Kinetin). On this culture medium the explants remained for 14 days when were transferred to expression medium (ED) consisting of DKW's salts and vitamins free of PRGs and supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} glucose and 6mM $MgSO_4$ $7H_2O$ (MERCK®). The expression of the somatic embryogenesis occurred after 60 days. Subcultures were carried out every 21 days.

PennState's protocol (Pennsylvania State University, USA)

The induction of SSE was carried out according to Maximova *et al*. (2002). Briefly, cotyledons excised from somatic embryos were sectioned with scalpel into thin slices (≈2mm) and laid in Petri dishes containing solidified SCG culture medium (see section 3.2.1). After 14 days, the explants were transferred to ED culture media (see section 3.2.1). Subcultures were carried out every 21 days. The subcultures on ED culture medium were labeled as ED1, ED2, ED3 and so on.

3.2.3 Maturation of secondary somatic embryos

Maturation of cacao somatic embryos was carried out using ED culture medium (see section 3.2.1) supplemented with 40, 60 or 80 g L^{-1} sucrose. Cultivation was carried out either on solid medium or in liquid medium for 1 or 2 weeks.

3.2.4 Rooting induction culture media (RI)

The culture medium for induction of root formation in cacao somatic embryos was consisted of ½ DKW's salts and vitamins (Driver and Kuniyuki, 1984), supplemented with 300 mg L^{-1} KNO_3, 5 g L^{-1} sucrose, 10 g L^{-1} glucose (DUCHEFA®) and 7.5 µM L^{-1} IBA (indole butyric acid). The somatic embryos were cultured under this condition for 21 days (Protocol book of Pennsylvania State University, Guiltinan & Maximova, version 2.1, 2010).

3.2.5 Germination of secondary somatic embryos

Cacao secondary somatic embryos germinated in primary embryo conversion culture media (PEC) described in Protocol book of Pennsylvania State University (Guiltinan & Maximova, version 2.1, 2010). PEC medium consisted of DKW salts and vitamins (Driver and Kuniyuki, 1984) supplemented with 300 mg L^{-1} KNO_3, 1 ml L^{-1} amino acids stock solution, 10 g L^{-1} sucrose and 20 g L^{-1} glucose (DUCHEFA®).

3.2.6 Acclimatization

Somatic plantlets were transferred to a thermoplastic box containing 500 mg of sand saturated with solution consisting of ½ DKW macro and microelements (Chapter 4.2). Plantlets showed in the Chapter 4.3 were transferred to glass jars (WECK®, 2 Liter) containing a mixture of Sand, Vermiculite® and humus (1:1/2:1) saturated with ½ DKW nutrients solution. The jar was covered with glass lids. In both cases, plantlets were watered every day. Before inoculation of the plantlets, recipients with substrate were autoclaved at 121°C for 20 min 3 times, with interval of 24 hours between the autoclaving. The recipients containing the plantlets were incubated in growth chamber at 25°C±2°C under photoperiod of 12 hours.

3.2.7 Temporary immersion system (TIS, Hepfling and Preil, 2005)

The cultivation of explants or somatic embryos in liquid media was carried out by using the TIS. It consists of two flasks connected by silicon tubes, through which the culture medium is pumped from the medium bottle (SCHOTT-Duran®, 1 Liter) to the culture vessel. The latter is covered by a glass lid and sealed with rubber ring (WECK®, 1 Liter). Each of the vessels is equipped with an autoclavable sterile filter (0,2 µm-Midisart 2000, Sartorius®) through which compressed air is pumped in the system. The culture vessel contain a basket manufactured in the Laboratory of Useful Plants (Biocenter Klein Flottbek and Botanical Garden at Hamburg University- Germany) in which the plant material in cultivated. These baskets were composed of transversal sections of polypropylene bottles (KAUTEX®, 200 ml) forming rings with a diameter and height of 5 cm each. At the lower part of each ring nylon sieve (mesh size: 100 µm) was fixed with stainless steel rivets. For maturation and germination processes the baskets mentioned above were replaced by baskets consisting of stainless steel net (mesh size: 1 mm) manufactured in the same laboratory. During the cultivation period the plant material was flooded with culture medium for 1 minute 8 times a day. In maturation and germination experiments the somatic embryos were flooded for 1 minute 4 times a day. The system was installed on shelves in growth chambers at 25°C±2°C in darkness.

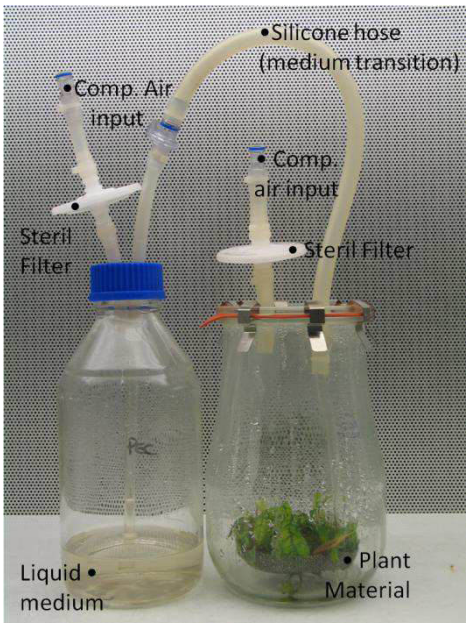

Figure 1. A unit of twin-flasks temporary immersion system used in this work. Left bottle containing liquid culture medium and right vessel containing plantlets.
The compressed air is pumped through sterile filters while the liquid medium flow through the silicone hose to the culture vessel.

3.2.8 General culture conditions

In all culture media had the pH adjusted to 5.8±0.01 using KOH solution (Potassium Hydroxide, 1M) prior to autoclaving at 121°C for 20 minutes. If required, the culture media were solidified with 2.2 g L^{-1} Gelrite (DUCHEFA®). All *in vitro* culture procedures were carried out under aseptic conditions in flow chamber. Both PSE (see section 3.2.1) and SSE (see section 3.2.2) cultures were incubated in darkness. Maturation and germination were incubated according to experimental procedures in darkness or photoperiod of 12 hours. HP-T Plus Lamps (PHILIPS®) were used as light source. The light intensity was adjusted to 80-100 µmol photon m^{-2} s^{-1}. The temperature in growth chambers was 25°C±2°C.

3.3 Analytical procedures

3.3.1 Histological approaches

3.3.1.1 Light microscopy

For light microscopy, somatic embryos were fixed overnight in a 2% paraformaldehyde solution diluted in 0,1M Phosphate Buffer (pH 7,4) at 4°C. Afterwards the samples were then washed with the same buffer twice for 15 minutes each time, and gradually dehydrated gradually in ethanol solutions (30%, 50%, 70%, 90% and 96%). The tissue was infiltrated by LR-White resin (London Resin Co Ltd, London) for 2 days. After that the samples were embedded in gelatin capsules containing resin and polymerized at 60°C overnight or until polymerization was complete. Sections (1µm) were cut transversally and longitudinally in a rotate microtome (Reichert Ultracut S, Leica®) equipped with a glass knife. The sections were collected on water drops placed on glass slides, which were dried on a hot plate at 70°C. The observation of the general tissue structure was carried out by staining the samples with 0,5% Toluidine Blue O in phosphate buffer (O‘ Brien *et al.*, 1964). For the specific staining of phenolic compounds the samples were pre-treated with 12% active chlorine (undiluted commercial sodium hypochloride solution) for 10 seconds, rinsed with distilled water,

stained with 0,5% Toluidine Blue O, rinsed again with distilled water, immediately dried with a blow of clean air and finally dried for 10 minutes at 50°C (Gutmann, 1995). The analysis of the tissue samples as well as taking photos were carried out in Olympus BH-2 microscope equipped with a ColorView IIIu camera (Soft Imaging System, GmbH).

3.3.1.2 Transmission electron microscopy (TEM)

The somatic embryos were fixed with 75 mM cacodylat buffer pH 7,0 containing 2% glutaraldehyd in for 4 hours at room temperature and washed with the same buffer 3 times. The samples were post fixed in 1% osmium tetroxide (OsO_4) in 75 mM cacodylat buffer overnight at 4°C. Thereafter, the samples were rinsed 3 times for 15 minutes with cacodylat buffer, promptly followed by dehydration in acetone gradient series (15%, 30%, 50%, 70%, 90%, 96%). The tissues were infiltrated by Spurr's epoxy resin overnight and polymerized at 70°C for either 16 hours or until polymerization of the resin was complete. Ultrathin sections (70-80nm) were cut using an ultramicrotome (Reichert-Jung, Vienna, Austria) equipped with a diamond knife. The samples were collected on cupper grids (mesh size: 150 µm), covered with Mowital in 0,3% Chloroform and stained for 10 minutes in 2% uranyl acetat and 2% lead citrate. The samples were viewed with a LEO 906Ev TEM (LEO- Zeiss, Oberkochen, Germany) equipped with Miltiscan CCD-camera (model 794) of Gatan (Munich, Germany) unsing a Digital Micrograph 3.3 software to acquire, visualize, analyse and process image data.

3.3.1.3 UV-microspectrophotometry

The samples for UV-microspectrophotometry were prepared in the same way as for TEM except for post-fixation with osmium tetroxide (see section 3.3.1.2). Semi-thin sections (2 µm) were cut on an ultramicrotome (Reichert-Jung, Vienna, Austria) equipped with a diamond knife. The sections were fixed on quartz slides, immersed in

a drop of non-UV absorbing glycerine and covered with quartz cover slips. The analyses were carried out with a ZEISS UMSP 80 microspectrophotometer (Carl Zeiss AG, Oberkochen, Germany) equipped with a scanning stage. The absorbance of the plant material was measured at a constant wavelength of 280 nm (Maximum absorbance of lignified cells walls) with a geometrical resolution of 0,25 μm^2. The images were obtained with the APAMOS scan program (Automatic- Photometric-Analysis of Microscopic Objects by Scanning, Carl Zeiss AG, Oberkochen, Germany). Absorption spectra were registered at a wavelength range from 240 to 400 nm on a spot size of 1 μm^2 using the program LAMWIN (Carl Zeiss AG, Oberkochen, Germany). Only cells that contained intact inner and outer walls were selected for UV-absorption measurements.

3.3.1.4 Scanning electron microscopy

Samples were fixed in 0.1 M phosphate buffer containing 3 % glutaraldehyde (pH 7.0) twice of 15 minutes each. The dehydration was carried out via ethanol gradient series (30%, 50%, 75% and 96 %), the samples remained for 60 minutes under the influence of each concentration followed by acetone/ethanol gradient series (30 %, 50 % and 100 %). The samples were dried to the critical point in the CPD 030 critical point dryer (Bal-TEC, Leica®) with liquid CO_2. The embryos were carefully transferred to aluminum supports and allocated in the SCD 050 Sputter Coater (Bal-TEC, Chapter II 49 Leica®) for coating with Gold palladium. The samples were observed in a LEO 1525 scanning electron microscope (Carl Zeiss SMT).

3.3.1.5 Macroscopic images capture

Images of the culture, plant tissues and somatic embryos were captured with Canon Powershot. If needed, it was coupled to a stereoscope.

3.3.2 Biochemical analysis

3.3.2.1 Quantification of polyphenols in High Performance Liquid Chromatography (HPLC)

100-500mg of cotyledons or hypocotyls of cacao somatic embryos were homogenized in 2ml of methanol (MeOH) with an Ultraturrax (Ultraturrax Typ TP 1810, 20000 rpm) for approximately 20 seconds. The homogenate was incubated on ice for 15 minutes before centrifugation for 10 minutes at 5000 rpm. After centrifugation for 10 minutes at 4000 rpm, the supernatant was collected and the extraction was repeated twice. The supernatants were combined and completely dried on rotary evaporator at 40°C/100mbar. The residual was dissolved in 2ml of methanol and filtered (0,45 µm Multoclear, CS-Chromatography) before 20µl of the extract was injected in HPLC. Chromatographic separation was performed with a LiChroCart 250-4 (Merck®) equipped with pre-column Lichrospher 100 RP-18 endcapped (5µm) at 26°C. The liquid gradient was formed by 2% acetic acid (CH_3COOH) as solvent A and acetronitril/ water/glacial acetic acid (400/90/10, v/v/v) as solvent B. Protocatechin, Cathechin and Epicatechin were used as internal standards. The value of polyphenol content was obtained with peak area of chromatograms from standard mixtures. In general, analyses were carried out with 2 biological replicates. When material was available, 3 biological replicates were analyzed.

3.3.2.2 Analysis of aminoacids in HPLC

To analyze the content of amino acids 100mg of liophilized and defatted samples were homogenized at 4°C for 1 hour in solution of PVPP (Polyvinyl-polypyrolidon) with pH adjusted to 2.5 with TFA (Tifluoroacetic acid). The homogenate was centrifuged for 10 min at 4100 rpm. The supernatant was collected and filtered through a 0,45 µm filter (Multoclear, CS-Chromatography). Samples were lyophilized and kept at -20°C until analysis. Free amino acids were derivatized with O-phthalaldehyde (OPA) prior to analysis in HPLC. Chromatographic separation was performed with a LiChroCart 250-4

(Merck®) equipped with a pre-column Li Chrospher 100 RP-18 (5 µm). Chromatographic analysis was carried out using a reverse phase binary gradient [A: 1.6 L sodium acetate solution/glacial acetic acid, 50 ml MeOH, 20 ml Tetrahydrofuran. B: 200 ml sodium acetate solution/glacial acetic acid, 800 ml MeOH at a flow rate of 1.3 ml min^{-1}. Quantification was calculated via peak area of chromatograms from standard mixtures containing 1-10 pmol μl^{-1} of each amino acid. Total amino acid concentrations were obtained by summing up of the individual amino acid concentrations. The analyses were carried out with 3 biological replicates.

3.3.2.3 Total phenols

30-100mg of lyophilized somatic embryos at different stages of development grown in TIS was grinded in a mortar with liquid nitrogen. The resulting powder was homogenized in 4ml of an acetone/water (60:40; v/v) solution for 15 minutes on ice under constant movement. After 3 minutes in ultrasonic bath, the homogenate was centrifuged for 10 minutes, 4000 rpm. The supernatant was collected and the extraction was repeated twice. When the supernatants were combined, 2 ml of acetic acid were added. Supernatants were dried in rotary evaporator at 40°C/ 60 mbar. 20 ml of water were added to the residual and the extract was stored at -20°C until quantification.

Before quantification, samples were homogenized in ultrasonic bath for 30 minutes. 200 µl of extract was combined with 2.5 % of acetic acid and 0.5 ml Folin-Ciocalteus-phenol reagent (MERCK®). After addition of 20 % Na_2CO_3 solution (sodium carbonate) the test-tube was promptly shaken and distilled water was added to complete 10 ml. The mixture was heated for 10 minutes in water bath at 70°C. After cooling at room temperature, the absorbance was obtained at a wavelength of 730nm in a spectrophotometer. Epicatechin (Sigma-Aldrich®) was used as standard.

3.3.3 Extraction and quantification of storage compounds

Before extraction of proteins, cotyledons and embryonic axis were submitted to phenol extraction. The pellet originated from phenol extractions were used for further analysis.

3.3.3.1 Quantification of Soluble proteins

Soluble proteins were extracted from somatic embryos cultured either on solid medium or in TIS in culture media containing 40, 60 or 80 g L^{-1} sucrose. The extraction method used was that described by Cangahuala-Inocente *et al*. (2009). The pellets from phenols extractions were grinded in a mortar with liquid nitrogen. The resulting powder was homogenized in 50nM extraction phosphate buffer dibasic (pH 6.8; 0.2M ß-mercaptoethanol, 2% sodium dodecyl sulfate (SDS) and 1mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was centrifuged at 4°C for 20 minutes at 13.000 rpm. The supernatants were collected for further analyses and the pellets stored at -20°C. The soluble proteins were precipitated in 4 volumes of cold methanol (100%) for 8 hours at -20°C. Thereafter, it was centrifuged at 10.000 rpm, 4°C for 20 minutes. The pellet was solubilized in 500 µl of 50mM phosphate buffer and stored at -20°C until determination of soluble protein content in spectrophotometer.

3.3.3.2 Content of total proteins

Extraction of total proteins was carried out according to the protocol described by Gallardo *et al*. (2002). Analysis of protein content was carried out for somatic embryos developed in TIS or on solid medium. Pellets from phenol extractions were grinded in a mortar with liquid nitrogen and the resulting powder was homogenized in urea/thiourea lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 50nM Tris-HCl buffer). The homogenate was stored on ice for 5 minutes. PMSF (1mM) and dithiothreitol (DDT; 14mM) were added. After homogenization, the samples were centrifuged at 13.000 rpm, 4°C for 20 minutes. The supernatant was collected and the pellet stored at -20°C. Whenever was possible 3 biological replicates were analyzed.

3.3.3.3 Dosage of proteins by Bradford Method (1976)

Content of soluble proteins and total proteins were determined with Bradford reagent (SERVA®). The absorbance in spectrophotometer was obtained at a wavelength of 595 nm. Bovine serum albumin (BSA) was used as standard. The protein concentration (µg/mg of dry weight or fresh weight) was obtained from linear equation for BSA standard curve absorbance ($y=0,0011x+0,0768$; $R^2 = 0,9802$).

3.3.3.4 Quantification Carbohydrates

A- Sugars

The method used to quantify the content of soluble sugars was the one described by Shannon (1968). The pellet obtained from protein extraction (see section 3.3.3.1 and 3.3.3.2) was homogenized with methanol: chloroform: water solution (12:5:3; v/v/v), centrifuged for 10 minutes at 2000 rpm. The supernatant was collected and the extraction repeated. The second supernatant was added to the first and stored. For each part of supernatant, 1 part of chloroform and 1,5 parts of water were added and briefly homogenized in Vortex®. The mixture was centrifuged for 10 minutes at 2000rpm. The upper aqueous phase was collected and stored at 0-4°C until analysis. The dosage was carried out in a spectrophotometer and the absorbance was obtained at a wavelength of 620nm. Previously, 2ml of a solution of 0,2% anthron in sulfuric acid (72%) were added to 200 µl of extract and 800 µl of water. The reaction mixture was heated for 3 minutes in a water bath at 100°C. The concentrations were calculated using glucose as standard.

B- Starch

The pellet residual from sugar extractions was homogenized in 1 ml of 30% perchloric acid and centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and the procedure repeated. The supernatants were combined and the determination of the starch content was carried out as described above for soluble sugar content.

3.4 Analysis of polyphenol oxidase activity

Cacao somatic embryos of different stages developed in TIS were submitted to identification of the polyphenol oxidase (PPO) activity as described by Grotkass *et al*. (1995). 300-1000 mg of cotyledons and hypocotyls were analyzed separately. Fresh plant material was homogenized in phosphate buffer 67mM (potassium dihydrogen phosphate KH_2PO_4/ di-sodium hydrogen phosphate Na_2HPO_4), pH 6.4 in Ultraturrax Typ TP 1810 (20000 rpm x10s). The homogenate was centrifuged at 13.000g for 30 min under 4°C. Promptly, an aliquot of 100µl of the supernatant was added to phosphate buffer. After 1 minute, the reaction was started by adding 100 µl of 56 mg/ 2 ml 4-methyl catechol as substrate (final concentration 7,5mM) or 100 µl of 10 % sodium dodecyl sulfate (final concentration 34 mM). The reaction mixture had a total volume of 3ml. The PPO activity was determined by the oxygen uptake at 25°C in YSI oxygen electrode 5331 (Yellow Springs, Ohio 45387, USA). The enzyme activity was measured as the initial rate of oxygen uptake, and one unit of activity was defined as the consumption of % air/g of fresh weight x h.

4. Results

4.1 Cacao somatic embryos regeneration in temporary immersion system (TIS) in comparison to standard culture conditions.

Protocols for somatic embryogenesis (SE) induction are well established for some cacao genotypes, it makes SE a potential strategy for scaling up propagation of the species. However, cacao genotype-dependency and its recalcitrance for *in vitro* culture hamper so far the inclusion of SE as standard method for micro-propagation of cacao plantlets in genetic breeding programs. Moreover, difficulties encountered in the maturation and conversion of the embryos, i.e. the long time and high costs required for plantlets production are reported by many authors as methodological limitations. The employment of the TIS for large scale propagation of healthy and vigorous plantlets *in vitro* has been described to be successful for many species (Escalona *et al*., 1999; Ducos *et al*.,2007; Steinmacher *et al*.,2011). Niemenak *et al*. (2008) reported increasing production of cacao somatic embryos in that culture system. The TIS allows the production of clones in a reduced space and its automation reduces costs. This chapter describes the effects of the TIS developed at University of Hamburg (Preil, 2005, see description in section 3.2.7, Material and Methods) on cacao somatic embryos development, germination and conversion into plantlets. It was aimed to improve *in vitro* culture conditions for production of healthy and viable somatic embryos.

4.1.1 Development of an efficient protocol for cyclic secondary somatic embryogenesis (SSE) of cacao.

4.1.1.1 Induction and expression of somatic embryogenesis (SE) on solid media.

The establishment of a cyclic secondary somatic embryogenesis (SSE) guarantees unlimited source of embryogenic cell culture and stabilize the conservation of the genetic information. Somatic embryos at different stages of development are available and can be taken from the cycle and submitted to different treatments and/or for establishment of *ex vitro* plant material. This is a highly efficient process, since the quality of the somatic embryos produced in the cycle determines the success of following steps up to the development into plantlets. In order to identify the quality and eventual lack of efficiency of cacao somatic embryos production *in vitro*, detailed observations were carried out concerning formation of embryogenic cultures formation and time necessary for expression of somatic embryogenesis under two different protocols for cacao SSE on solid media.

The first protocol used in the Laboratory for Useful Plants (Biocenter Klein Flottbek und Botanical Garden-Universität Hamburg, Germany) to obtain secondary somatic embryogenesis of cacao consists of DKW media salts and vitamins formulation in all phases of embryo development (LUP protocol, Table 1). The induction phase comprises 2 periods of 15 days each, both in the presence of the plant growth regulator (PGR) 2,4-D, 2 mg L^{-1}. Under these conditions, a high amount of callus was formed on the explants (slices of cacao somatic embryos cotyledons, see Material and Methods, section 3.2.2) still during the induction phase. These calli, which were not attached to the explants surface, were friable and of light brown color, sometimes being translucent. After the transfer of the callogenic explants to PGR-free DKW expression media, white and compact non-embryogenic masses of cells were formed on the explants, also organogenesis, like roots formation, occurred. In both cases, almost no

somatic embryos differentiated, but whenever embryogenesis occurred, the differentiation led to abnormal structures or arrest of torpedo stage (Figure 1).

Table 1. Comparison among the formulation of the two culture media employed for secondary somatic embryogenesis of cacao.

	Induction of SSE in Secondary callus growth (SCG) medium		**Expression of SSE in embryo development (ED) medium**	
Culture Media	*SCG (1°)- 15 days*	*SCG (2°)- 15 days*	*SSE-Expression medium - 21 days*	*Time till to obtain somatic embryos*
LUP Media	-*DKW's salts and vitamins - 20 g L^{-1} glucose - 2,4-D (2 mg L^{-1}) - BAP (50 µg L^{-1})	-DKW's salts and vitamins - 20 g L^{-1} glucose - 2,4-D (2 mg L^{-1}) - KIN (250 µg L^{-1})	- DKW's salts and vitamins - 30 g L^{-1} sucrose - 1 g L^{-1} glucose - 6 mM $MgSO_4$ $7H_2O$ Expression within 60 days	≈ 90 days
PennState Media	-	- McCown's salts - B5 vitamins - 20 g L^{-1} glucose - 2,4-D (2 mg L^{-1}) - BAP (50 µg L^{-1})	- DKW's salts and vitamins - 30 g L^{-1} sucrose - 1 g L^{-1} glucose Expression within 30 days	≈ 45 days

*DKW- Driver & Kuniuk, 1984.

In addition to the non-embryogenic calli formations, some explants were embryogenic. In case of embryogenesis, embryogenic clusters developed asynchronously from the callus, in other words, different stages of cacao somatic embryos grew simultaneously in the same cluster. These somatic embryos in general presented normal morphology and developed regularly up to mature cotyledonary stages. Growth of abnormal embryoid structures and disordered proliferation of non-embryogenic cells did also occur in the embryogenic cultures, but in lower quantities compared to non-embryogenic development (Figure 1F). The expression of SSE using this protocol took at least 90 days.

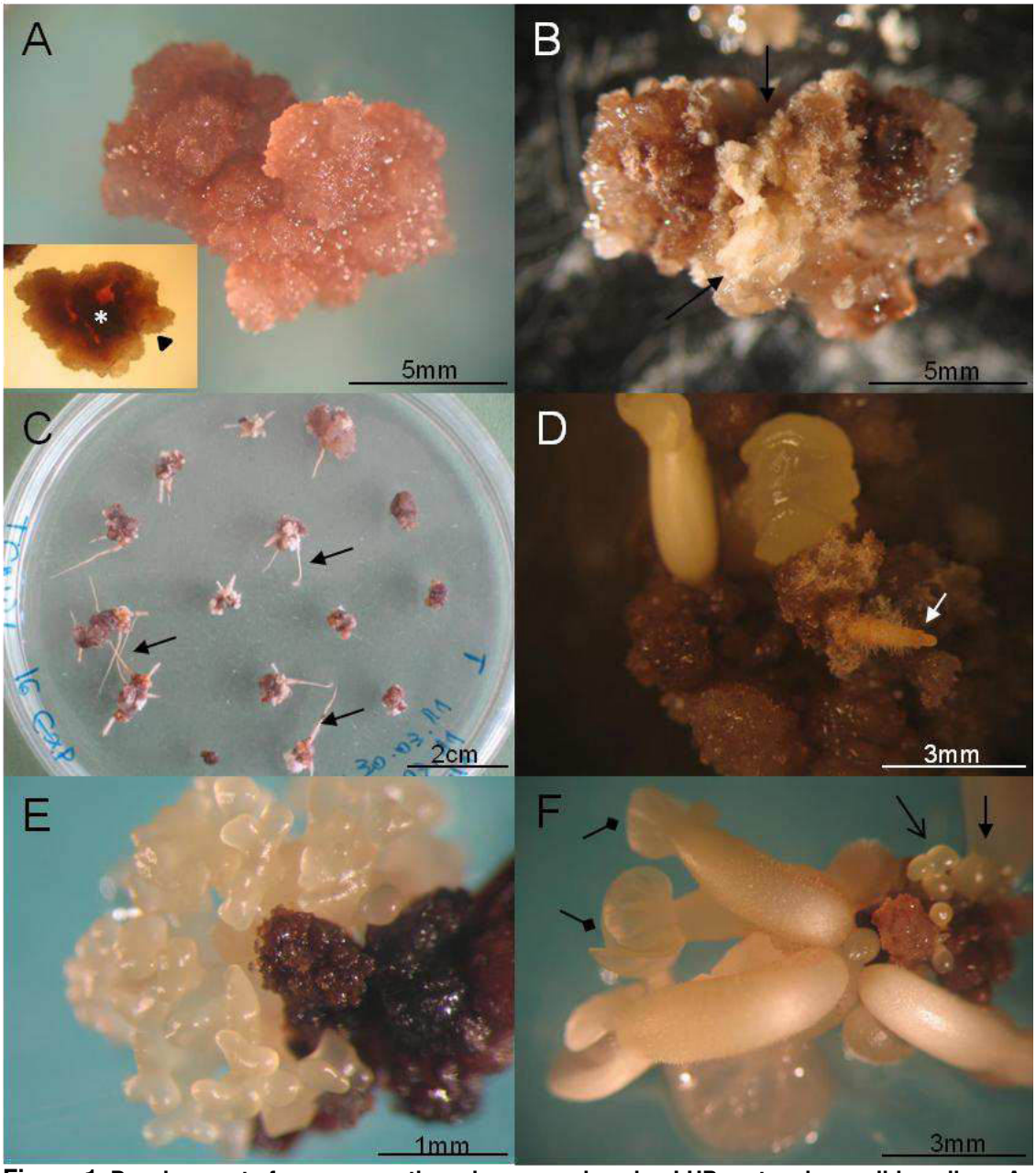

Figure 1. **Development of cacao somatic embryogenesis using LUP protocol on solid medium. A**- Callus formed during the period of SE induction. In detail, a picture of the callus under-side, the asterisk indicates the explants and the arrowhead shows the non-embryogenic mass of cells developing independently of the explants. **B-** Callus developed after transfer to PGRs free media. Arrows indicate the white non-embryogenic callus formed. **C**- Non-embryogenic cultures on solid media with organogenesis. Arrows indicate the formation of roots. **D**- Embryogenic callus with occurrence of root formation (arrow). **E**- Formation of abnormal embryoid structures (white) on embryogenic callus (brown). **F**- Asynchronous embryogenic culture presenting (→) globular somatic embryos; (➔) Heart somatic embryo; (➔) Cotyledonary somatic embryos.

The second protocol applied for SSE of cacao was that published by Penn State Cacao Research Lab (The Pennsylvania State University, 2010), which consists of only one passage of SSE induction in the presence of 2,4-D, 2 mg L^{-1}, added to the McCown's medium supplemented with B5 vitamins composition. The expression

medium is composed of DKW salts and vitamins like the first protocol (PennState's protocol, Table 1).

In comparison to the first protocol, this procedure reduced the time for somatic embryos formation (≈ 45 days), while callogenesis and organogenesis decreased or did not occur. For this study, young cotyledons of somatic embryos were used as explants for induction of SSE with the PennState's protocol. During the induction phase some morphological differences in the callogenesis could be observed. The explants remained small with poor callus growth. Sometimes the embryogenic calli were almost imperceptible or very small. When calli appear, they were of spongy consistence, dark brown in color and had a high water content among the cells. The undifferentiated cell masses, in contrast to the other protocol, remained attached to the explants (Figure 2A, B).

After transfer of the calli from induction medium to expression medium (DKW PGRs-free), the calli presented no significant increase in size, but SE expression occurred within 20 days. The complete differentiation of the somatic embryos could be observed after 2 subcultures (15 days each) on PGR-free medium. Formation of non-embryogenic masses occurred only rarely under these conditions. The embryogenesis, like in the other protocol, was asynchronous and the embryos at different stages of development were grouped in embryonic clusters (Figure 2C, D).

PennState's protocol for cacao SSE clearly improved the development of somatic embryogenesis in both induction and expression phases. The time until obtaining cacao somatic embryos is reduced and the undesired excessive callogenesis, presented in LUP protocol, can be avoided.

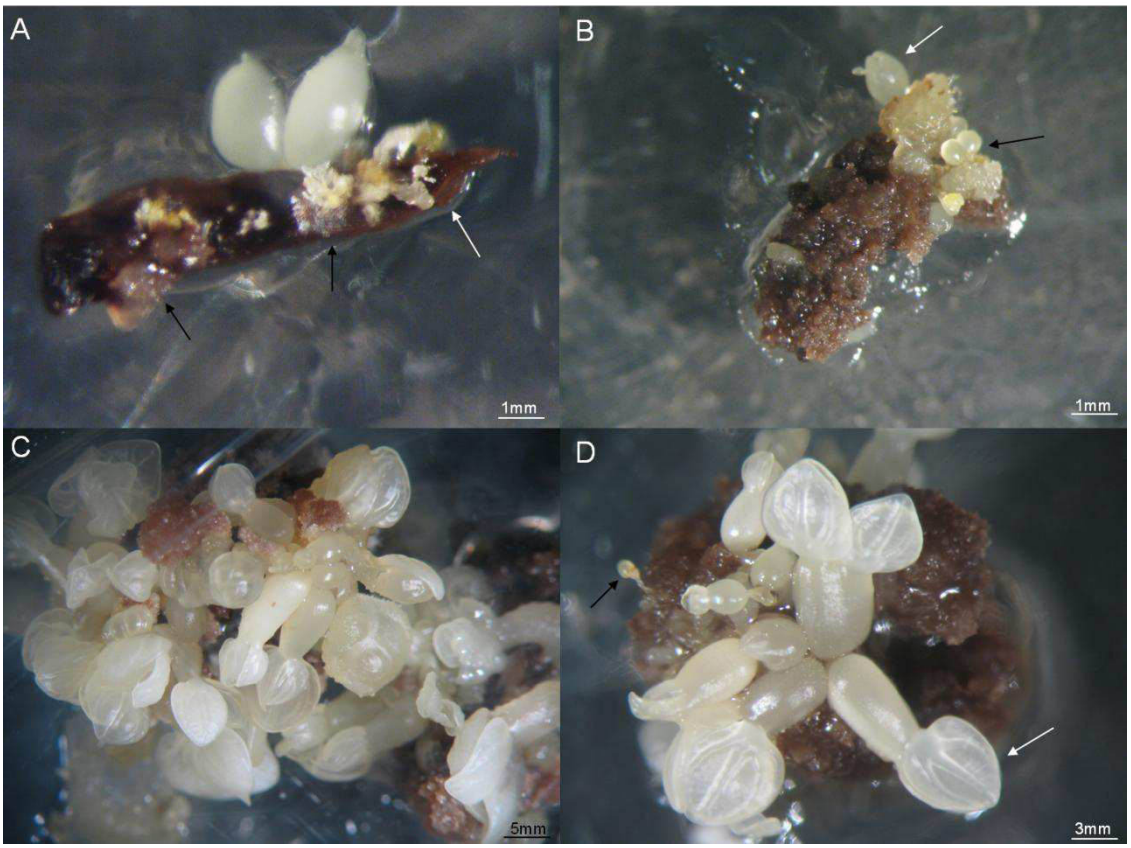

Figure 2. **Development of cacao somatic embryogenesis using PennState (The Pennsylvania State University-USA) protocol. A**- Explant (white arrow) with minimal callogenesis (black arrows) formed during the period of SE expression.. **B**- Embryogenic culture with spongy callus; black arrow indicates globular embryos and white arrow indicates heart-shaped embryo formed after 2 weeks on PGRs free medium. **C-** High production of somatic embryos obtained after 30 days in expression medium. **D**- Typical spongy embryogenic callus (brown) with asynchronous embryonic cluster containing somatic embryos from globular (black arrow) to cotyledonary stages (white arrow).

4.1.1.2 Responses of cacao embryogenic cultures to the transfer to liquid medium (TIS).

Niemenak *et al*. (2008) reported the advantages provided by the TIS (Twin flasks, Preil, 2005) for the cacao somatic embryogenesis. As the authors suggested, the embryogenic cultures regenerated on solid media using the two protocols described above (LUP and PennState, section 4.1.1.1) were transferred to liquid medium (TIS) to study the influences of liquid media on the quality of the embryogenic cultures and to access a possible increment of the production of secondary somatic embryos.

The transfer to TIS of the embryogenic calli with respective somatic embryos (produced after the LUP protocol), resulted in increase of non-embryogenic cells and

only a few of new somatic embryos were regenerated. The somatic embryos present in the culture followed the development up to maturity after transfer to TIS (Figure 3). A high frequency of abnormalities of embryos formation occurred and the proliferation of non-embryogenic calli disturbed the production of somatic embryos. Furthermore, the presence of high quantities of non-embryogenic masses requires frequent transfer and maintenance of the cultures.

In contrast to the responses presented by the cultures from LUP protocol in case of transfer to liquid medium, the embryogenic cultures from PennState's protocol, after transfer to TIS, showed regeneration of new somatic embryos, these together with existing embryos develop to maturity without any problem. The red coloration of the cotyledons after transfer to TIS happens in higher frequency in embryos originated from PennState's protocol. The calli mass remain in TIS as large as on solid media and the disaggregation of callus particles during the liquid culture seems not to disturb somatic embryos development (Figure 4).

The responses of embryogenic cultures from both protocols, LUP and PennState, were quite distinct. PennState's protocol for cacao SSE resulted in well developed somatic embryos in both, solid and liquid medium and reduces the troubles caused by disordered proliferation of non-embryogenic masses. This protocol can ideally be combined with liquid culture (TIS) for the improvement of cacao somatic embryos development.

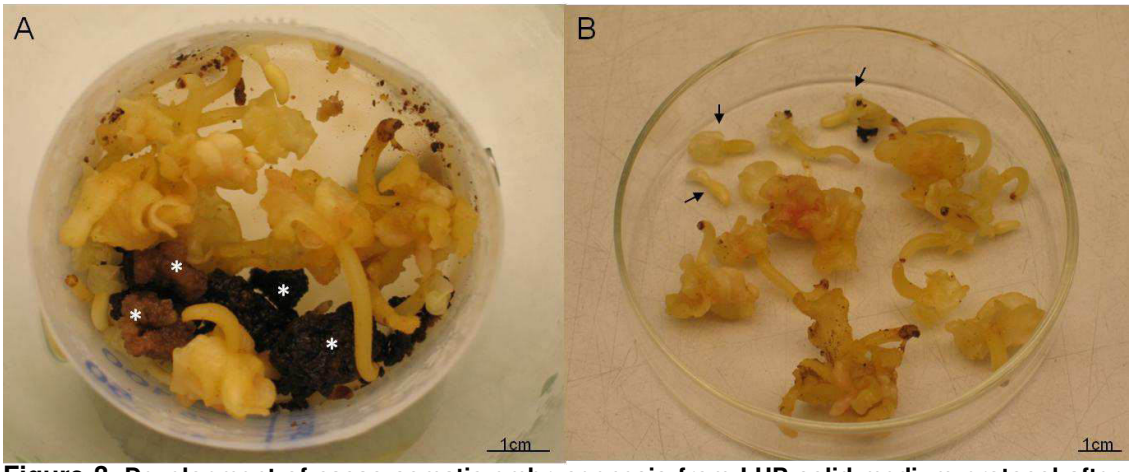

Figure 3. **Development of cacao somatic embryogenesis from LUP solid medium protocol after transfer to TIS**. **A**- Embryogenic culture after 2 subcultures in TIS. Asterisks indicate calli. **B**- Somatic embryos produced after 90 days from SE induction. Arrows indicate the new somatic embryos regenerated after transfer to liquid medium, the others were already present in the culture on solid medium before transfer to TIS.

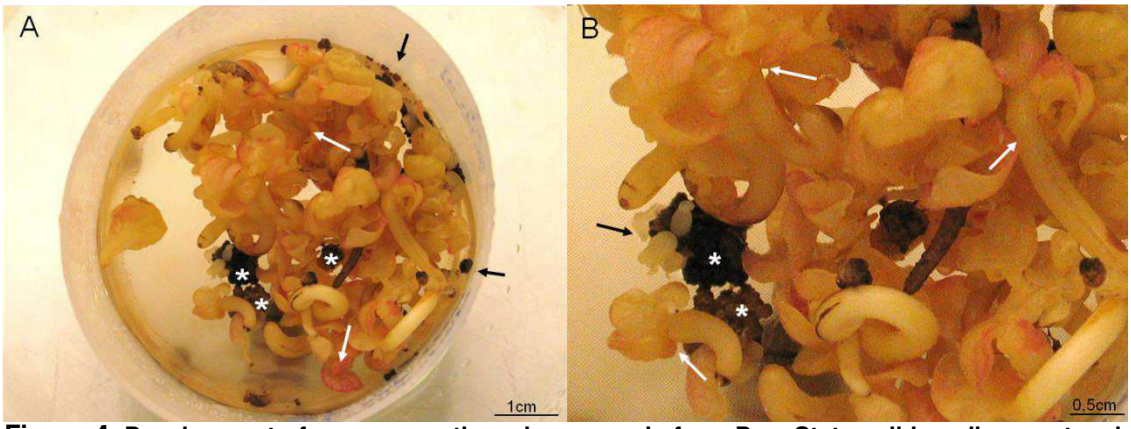

Figure 4. **Development of cacao somatic embryogenesis from PennState solid medium protocol after transfer to TIS. A**- Embryogenic culture after 3 subcultures in TIS. Asterisks indicate the calli; White arrows indicate red colored cotyledons and black arrows indicate callus particles in the culture compartment. **B**- Somatic embryos developed in TIS. Asterisks indicate the calli; Black arrow reduced embryogenic callus containing new torpedo-shaped somatic embryos regenerated after transfer to liquid medium. White arrows indicate somatic embryos maturated in TIS with red cotyledons.

4.1.1.3 Expression of SSE in TIS: a comparison between liquid culture and solidified medium culture.

The standard procedure for cacao somatic embryogenesis comprises the induction and expression phases, both on solid medium. It showed the advantages provided by TIS for somatic embryos development in relation to solid media (see 4.1.1.2). In order to study the possibility to realize the expression of SSE already in liquid culture medium instead of solid medium, new SSE was induced using

PennState's protocol and a comparison of methods was carried out. Explants of RO3.08/1 Md. (see section 3.1.2, Material and Methods), after induction on solid medium, were transferred for expression in both solid medium and TIS.

On solid expression medium (PGR-free), the calli became voluminous and nodular. Friable tissue was present in low quantities. The embryogenic culture was formed of asynchronous embryonic clusters on the calli. Because on solid media the callus is densely covered with somatic embryos and these remain connected to the callus until maturation, the somatic embryos of later stages of development compete with globular and heart-shaped embryos for space on the callus surface making it difficult to observe somatic embryos of earlier stages. The maintenance and harvest of somatic embryos from calli on solid media commonly causes injuries to the embryogenic tissue and sometimes to the other somatic embryos in the clusters (Figure 5A).

In liquid medium (TIS) the somatic embryos development was different from the solid medium growth. Globular and heart-shape embryos were commonly found on the callus, but embryos of later stages are often found unattached to the calli floating freely in the culture medium. When culture medium was pumped back into the medium vessel, the somatic embryos remained on the sieve of the culture jar (Twin-flasks system, see Material and Methods, 3.2.7). The floating and the movements caused by pumping the media led to separation of the embryos from the calli (Figure 5B).

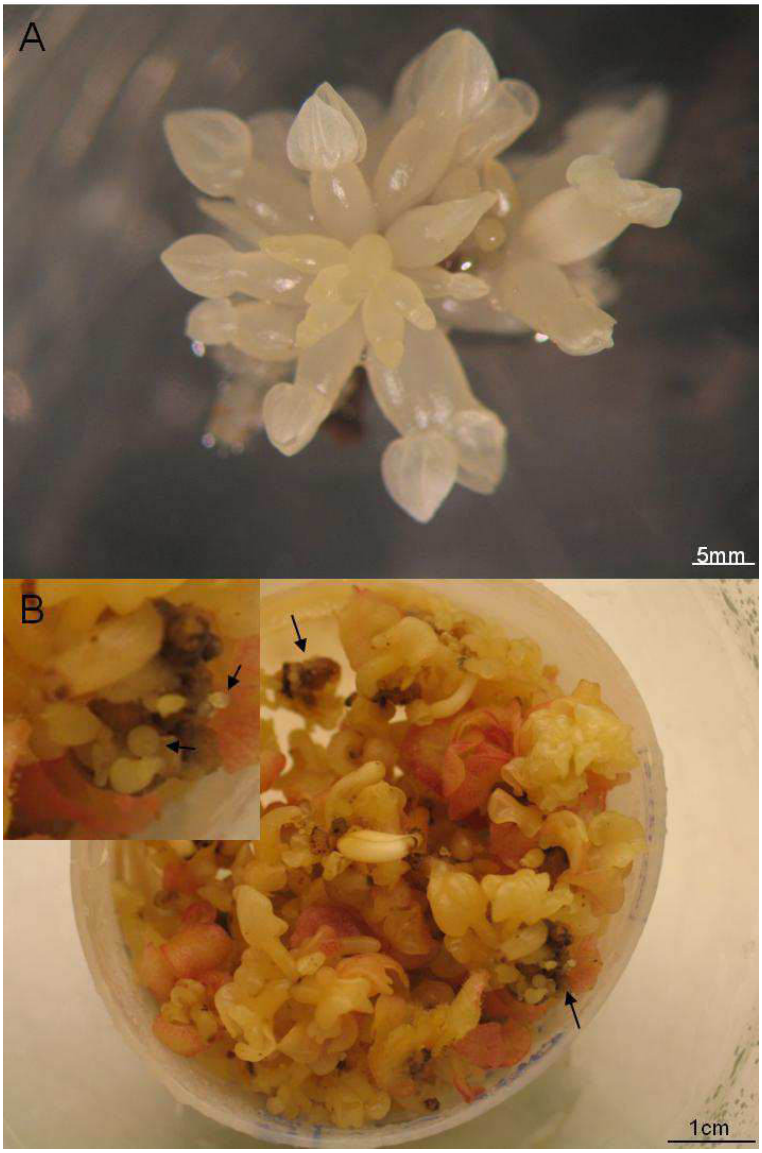

Figure 5. **Expression of cacao somatic embryogenesis in different culture conditions, solid media and TIS. A-** Typical asynchronous cluster of cacao somatic embryos formed on callus regenerated on solid media. **B-** High production of somatic embryos in culture regenerated in TIS. Arrows indicate embryogenic calli. In detail, arrows indicate globular and heart-shape somatic embryos still attached to the callus.

Over the entire growth phase of the somatic embryos a high morphological diversity was observed in the cultures. The embryos at early stages of development are very similar in both cultures, with white translucent appearance. In later developmental phases differences are evident in the morphology of the somatic embryos from solid media and TIS. The most remarkable differences between somatic embryos developed in TIS or solid medium are the color of the cotyledons, size of the mature embryos and proportion of the embryonic organs.

Somatic embryos regenerated and developed on solid media revealed whitish translucent cotyledons, which do not gain much size during the growth period and seem to be mechanically fragile. The embryonic axis at later stages is white, large, thin

and contains brown stripes (tissue oxidation). Frequently bended axes, almost in spiral form, occurred. Abnormal somatic embryos are found with high frequency on solid media, embryos with only one cotyledon or with more than 3 cotyledons, fusion of 2 or more axes and callogenesis on the meristematic regions were the most frequent structures. Early root growth (germination) was observed mainly on solid media (Figure 6A, B).

The culture regenerated in TIS allows maintenance and harvest of somatic embryos without causing injuries to the rest of the culture. The easy collection of embryos from different sizes and developmental stages provides a better overview of the morphological diversity differentiated during the expression of SSE in TIS. The embryos' axes are white and longer than the cotyledons in early phases turning yellowish during embryonic growth. The embryos at later stages of development present large, compact and undulated cotyledons, which have yellowish tissue with red regions. Somatic embryos with very small axis were frequently found in TIS, but the cotyledons showed the same characteristics as described above. Abnormalities on embryonic development, like grouped somatic embryos without cotyledons, axes fusion and pluri-cotyledonary embryos, were also observed in liquid culture. However, the frequency of these abnormalities is low considering the high amount of somatic embryos produced with this method.

The production of somatic embryos was higher in TIS. From TIS embryogenic cultures, 3 distinct groups of embryos were classified according to their morphology, together with early phase embryos at torpedo stage, they were found in high quantities detached from the calli. The groups of somatic embryos were defined as group 1: cotyledonary somatic embryos still with small and white cotyledons in their majority. Group 2: somatic embryos with small axes and red well developed cotyledons. Group 3: somatic embryos with large axis and typical proportion of cotyledons to embryo axis, found in high number in TIS culture (Figure 6C, D).

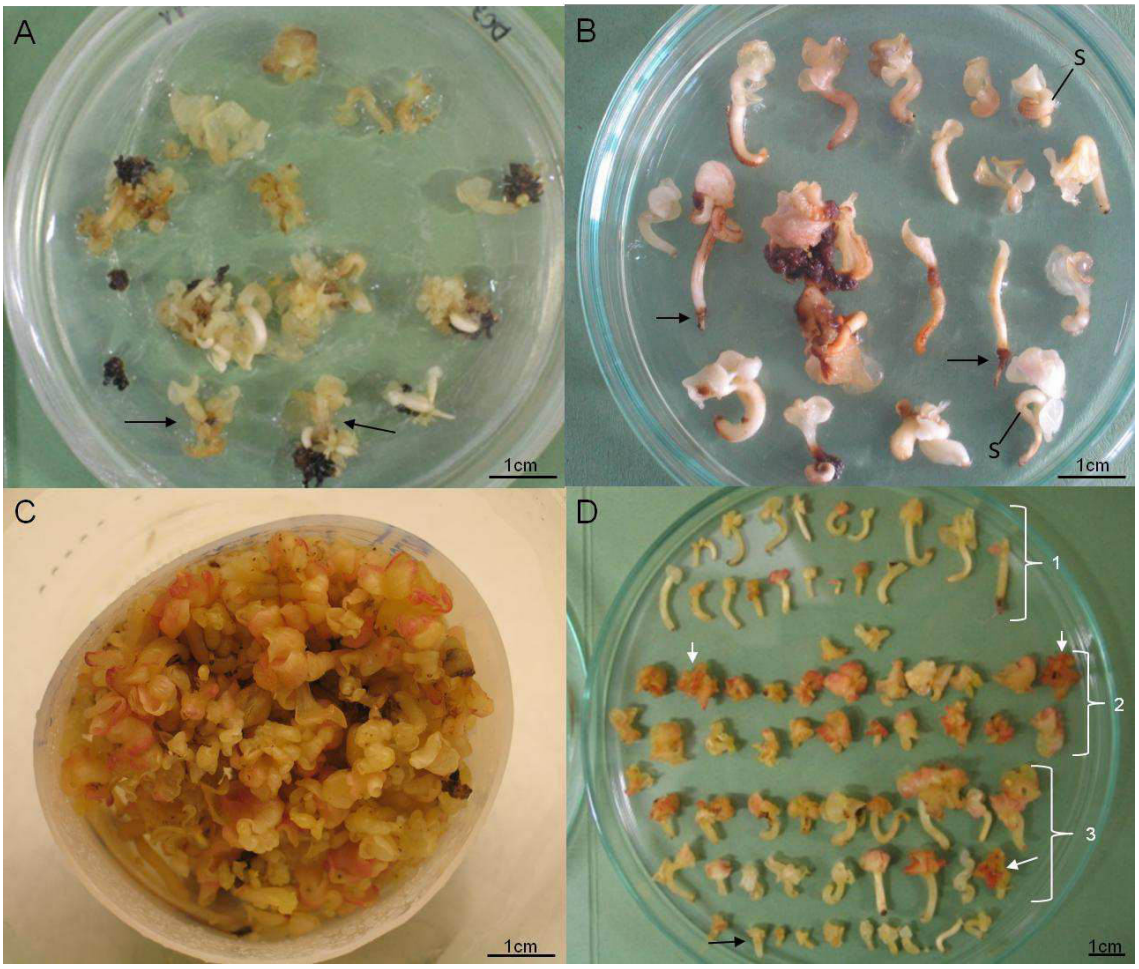

Figure 6. **Morphological diversity of cacao somatic embryos developed on solid media or TIS**. **A**- Expression of SSE on solid media. Arrows indicate mature somatic embryos still attached to the callus. **B**- Morphology of somatic embryos differentiated on solid media. Arrows indicate early germinated somatic embryos; S- spiral embryonic axis. **C**- Expression of SSE in TIS. **D**- Morphology of somatic embryos differentiated in TIS. Black arrow indicate group of Torpedo-shaped somatic embryos found unattached of the callus in TIS; White arrows indicates examples of somatic embryos with red colored cotyledons; 1- Group of cotyledonary somatic embryos; 2- Group of mature somatic embryos with smaller embryonic axis and red colored cotyledons; 3- Group of mature somatic embryos with large cotyledons and straight embryonic axis.

In the TIS culture the variation of small to large somatic embryos is continuous, and it seems to comprise different stages of temporal development. In the culture on solid medium the variety in size is for less expressed as in the TIS culture.

4.1.1.4 Cyclic secondary somatic embryogenesis (cSSE) of *T. cacao* with a Trinitario genotype from Madagascar.

The PennState's protocol for SSE of cacao on solid media combined with the TIS turned out to be advantageous for expression of secondary somatic

embryogenesis of the clone RO3.08/1-Md. However, further studies are required to observe the effects of TIS in the induction period of somatic embryogenesis. The contribution of the PennState's protocol associated with the benefits of the TIS culture in the expression phase, demonstrated to be efficient in the improvement on the culture quality and somatic embryos production. Combining these factors led us to a new protocol for the cSSE cultivation. Figure 7 represents all steps for the continuous production of cacao somatic embryos. It is necessary to observe that this protocol cannot be applied successfully to all cacao genotypes, many of the genotypes are recalcitrant and it is so far unknown which factors hamper the SE in these genotypes.

The secondary somatic embryogenesis (SSE) can be induced from cotyledonar tissue obtained from primary or secondary somatic embryos. After induction on solid medium the explants are transferred to liquid media in TIS. The cultures are transferred to fresh media every 21 days and about 30 days after introduction of the explants in the induction medium the somatic embryogenesis takes place. The secondary somatic embryos regenerated in the cycle can be harvested for conversion into plantlets, and establishment *ex vitro*, or to be used as explants source for re-induction of SSE. It characterizes a cyclic SSE protocol.

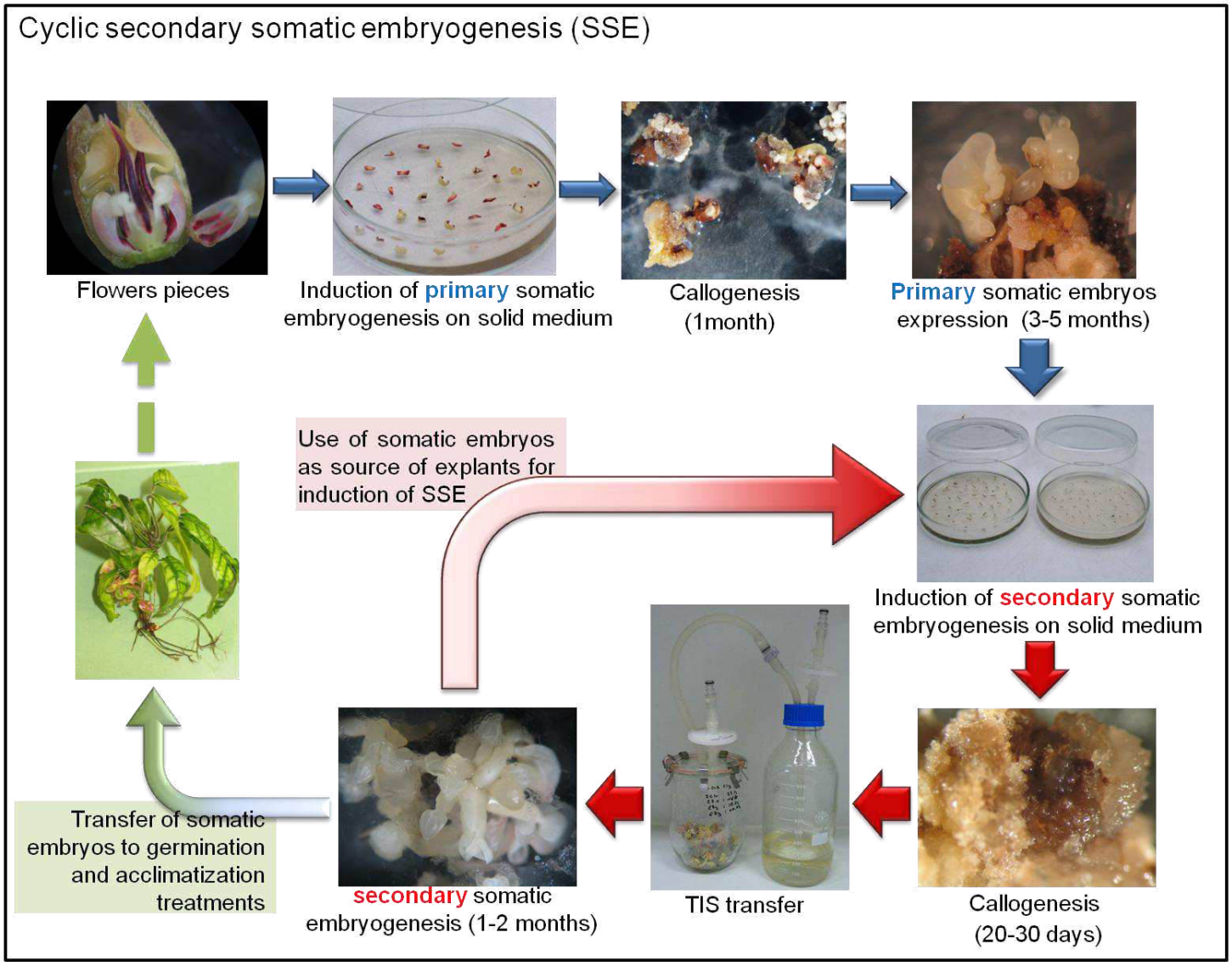

Figure 7. Cyclic secondary somatic embryogenesis defined for the propagation of the clone RO3.08/1-Md (in Red). The method combines the PennState's protocol for SSE of cacao with the technique of Temporary immersion system (TIS, Preil, 2005). **In blue**, the regeneration of primary somatic embryos, this can be used as explants source for induction of SSE. **In green**, the rescue of secondary somatic embryos from TIS and conversion into plantlets for establishment *ex vitro*.

The differentiation of somatic embryos on solid media remains stable until around seven subcultures, when the formation of abnormal structures and arrest of the torpedo stage occur constantly and the clusters become dry. Thereafter, the culture does not present morphogenic activity, neither embryogenic calli nor young somatic embryos are newly regenerated (Figure 8). For the tissue culture in TIS, the time of morphogenetic instability was not reached, almost all of the cultures presented satisfactory formation of somatic embryos at all stages during the entire period of cultivation, around 15 (fifteen) subcultures.

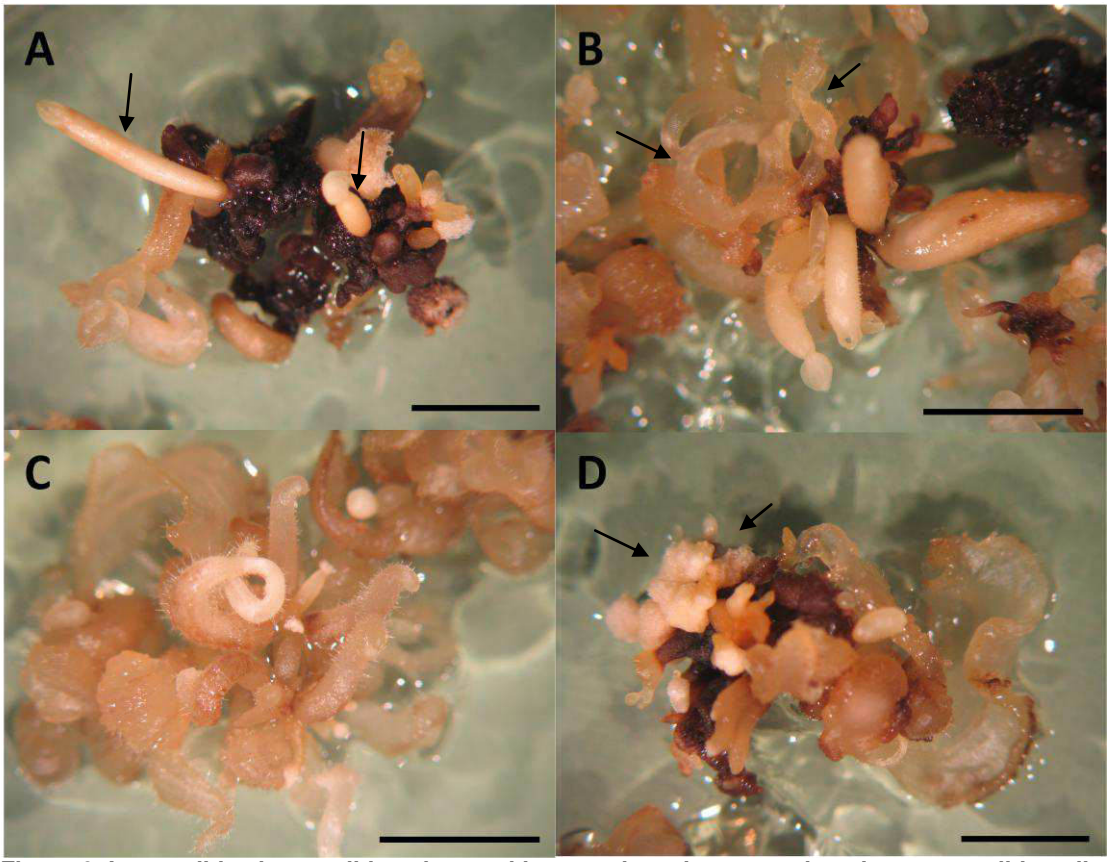

Figure 8. Irreversible abnormalities observed in somatic embryogenesis cultures on solid media after 7th subcultures. **A**- Dried callus (brown) and somatic embryos presenting arrest of torpedo stage (arrows). **B-** Regeneration of abnormal somatic embryos (arrows) and dominance of torpedo stage. **C-** Abnormal embryoid structures developed on the explants. **D**- Formation of non-embryogenic structures (arrows) and necrosis of torpedo shaped embryos. BAR: 5mm.

In both cases, solid and liquid media (TIS), a decrease of the embryogenic competence of the explants could be observed during some cycles of SE induction. Since in solid media the somatic embryos are attached to the embryogenic explants,

the non-embryogenic calli are easily identified. While in TIS just a decrease in the production of somatic embryos can be noted, it probably also occurs in explants cultured in TIS. It is not known which factors determine the stability of embryogenesis of the explants in each cycle.

4.2 Morphological and physiological characteristics of cacao somatic embryos development in Temporary immersion system (TIS).

4.2.1 Morphological and anatomical differentiation of cacao somatic embryos.

4.2.1.1 Early developmental phase

The advantages presented by an efficient cyclic SSE using PennState‘s protocol for cacao somatic embryogenesis on solid medium and the inclusion of TIS in the expression phase were appparent by the increase in production and the stable quality of the somatic embryos. Anatomical and morphological analyses were carried out with somatic embryos produced in the cyclic SSE protocol (Figure 7, section 4.1.1.4). The goal was to control the quality and to characterize the differentiation of somatic embryos developed in TIS. The fact that the somatic embryos develop detached from the callus in cultures in TIS (see section 4.1.1) facilitated the observation and collection of the embryos. The morphogenesis phase of cacao SSE delivered embryos of various morphological stages which are: Globular (Stage 1), Heart (Stage 2), Torpedo (Stage 3) and cotyledonary (Stage 4) (Figure 9). At the end of stage 4 of the embryonic differentiation, the cotyledons are accumulating reserve compounds and the transition from morphogenesis to maturation occurs. It is marked by changes in cotyledonary morphology until ripeness for germination is achieved.

Stage 1: Globular somatic embryos

Somatic embryos at globular stage are very difficult to be observed by naked eyes due to their small size ranging: from 500µm to 1mm. They remain mostly attached to the calli. By visual analysis made in this work, no suspensor was detected connecting the embryo to the callus. This was also supported in further microscopic analysis. The embryos are formed by a group of organized cells which give rise in short time to heart shaped embryos. In longitudinal histological cross sections (1µm) stained with Toluidine Blue O, it was possible to observe the anatomy and cell organization of

cacao somatic embryos at globular stages developed in TIS (Figure 10). Single-layer epidermis can be seen surrounding the globular structure; it is possible to observe the accumulation of phenolic compounds, metachromatic stained in green by Toluidine Blue O, in some layers of cells under the epidermis. Anticlinal and periclinal cellular division were present in the entire embryonic body, due to a high mitotic activity. Cells with dense cytoplasm and big nuclei often are arranged in both pole regions of the globular structure. The presence of these meristematic zones indicates the beginning of morphological polarity of the embryo.

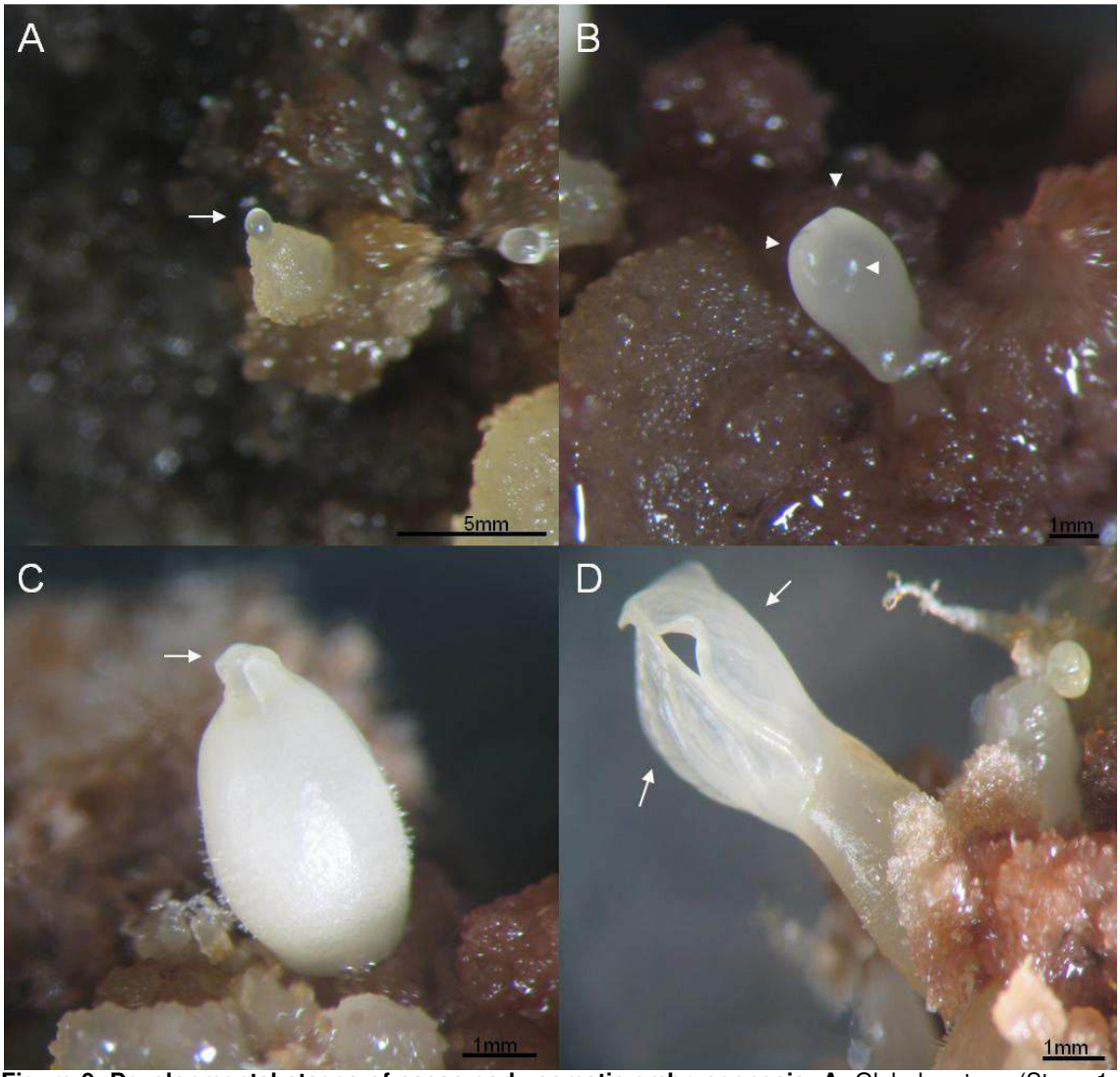

Figure 9. **Developmental stages of cacao early somatic embryogenesis. A-** Globular stage (Stage 1; arrow). **B-** Heart-shape embryo (Stage 2; arrowhead indicates expanded flanks). **C-** Torpedo-shape embryo (Stage 3; arrow indicated pre-cotyledons). **D-**Cotyledonary stage (Stage 4; arrows indicates differentiated cotyledons pair).

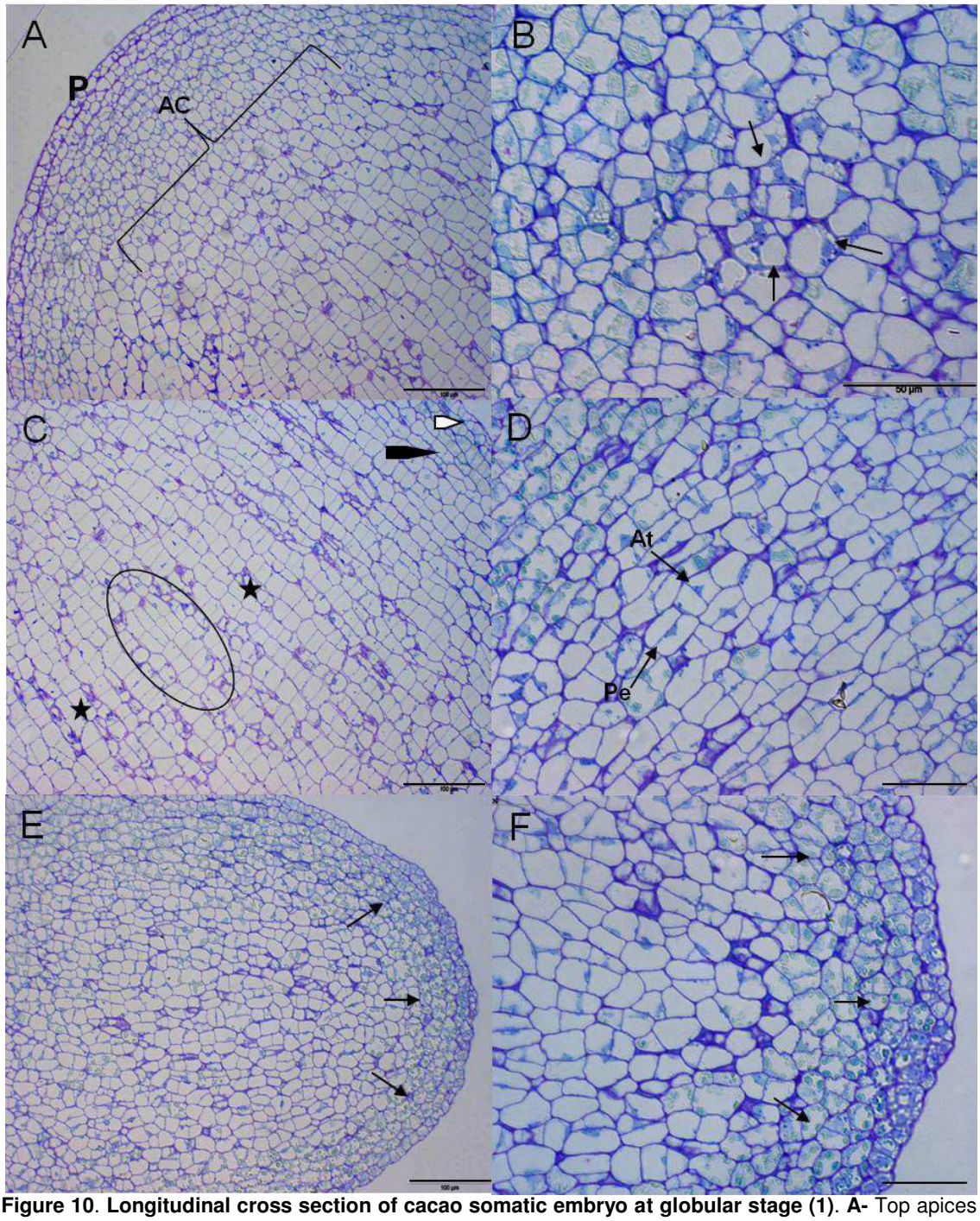

Figure 10. **Longitudinal cross section of cacao somatic embryo at globular stage (1)**. **A-** Top apices with single layer protoderm (P) and under layer zone with mitotic active cells (AC). **B-** Mitotic activity found in layers under the apical zone. **C-** Pattern of tissues primordium formation, from inner to outer layers: quiescent center (circled), strands of cells anticlinally divided suggesting organization of tissues primordium (asterisks). **D-** Typical anticlinal (At) and periclinal (Pe) cellular division found in whole extension of embryonic body. **E-** *Basal apices with multilayers of phenolic accumulating cells under the epidermis (arrow). **F-** *Close of the phenolic containing cells layers (arrows) present under the mitotically high active protoderm cells. *Phenolic compounds are green stained with Toluidine blue O. Bar: A, B, C, D, F-50µm; E-100µm.

The formation of ground meristem and procambium are presented by differentiation processes organization of inner cell layers. In the center of the embryo a small organized group of cells was observed (see circle in figure 10C). The

arrangement and form of the cells suggests a quiescent center. The polarity and cellular organization clearly supports the onset of transition to the subsequent morphological stage of embryo development, the heart stage. The differentiation of the ground meristem is followed by the definition of the apical-basal pattern of development and subsequently by the initiation of the organogenesis, represented by expansion of the cotyledonary primordium (Figure 11A and B).

Stage 2: Heart-shape somatic embryos

The definition of heart stage is marked by the differentiation of cotyledonary primordium and procambium. The high mitotic activity in apical and basal apices of the globular embryos precedes the emergence of cotyledons. Bilateral symmetry is observed in this stage after expansion of the cotyledons from the flanks of the apical meristem. The shoot meristem (SMR) is identified by the significantly higher cellular activity in the region between the cotyledonary primordium. The cells in this region contain dense cytoplasm, enlarged nuclei and a high rate of cellular divisions, more than in other regions of the embryo. These characteristics determine the apical meristematic region, which will subsequently turn into the apical shoot meristem.

From the apical region, two stripes of anticlinally divided cells are formed (Figure 11A), which extend through the complete length of the embryo until the basal region. These organized cells form the procambium which will give rise to the vascular bundle of the embryo. These patterns are markers of initiation of tissue differentiation during angiosperm embryogenesis. Within the embryonic body development at heart stage, callogenesis and meristematic zones were observed (Figure 11C). The abnormal growth is an indication for a strong morphogenetic differentiation process. The meristematic zones showed a protoderm-like cell layer, under which organized cells mitotically active were present (Figure 11).

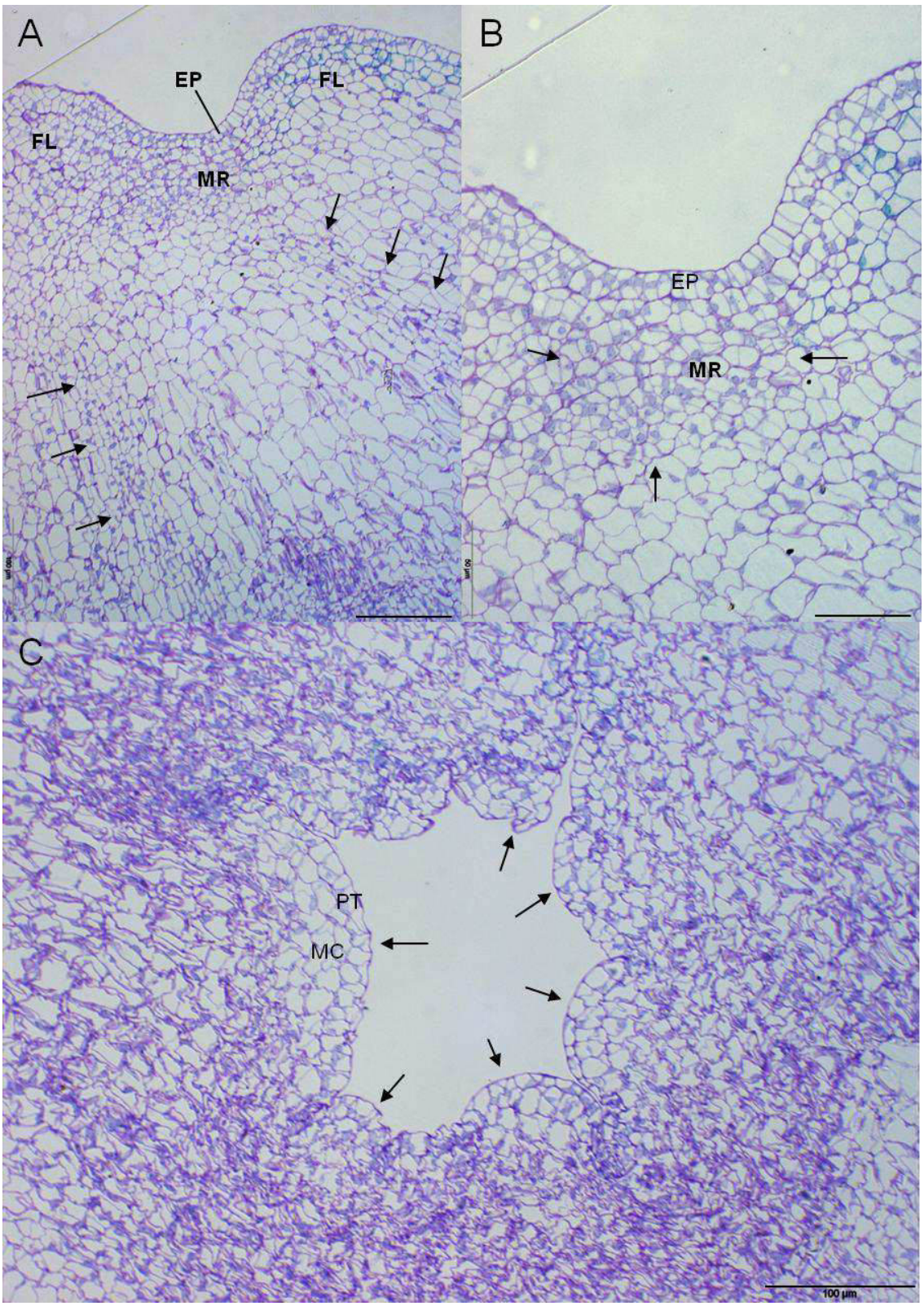

Figure 11. **Longitudinal cross section of heart-shape somatic embryo (stage 2)**. **A-** Heart-shape embryo with expanded flanks (FL) rising to cotyledons differentiation; arrows indicate strands of cells arising from meristematic region suggesting procambium formation. **B-** High cellular activity in epidermal cells (EP) and meristematic region under epidermis (MR) presenting dense cypoplam and avident nucleus (arrows). **C-** Formation of meristematic cell groups (MC) in the heart-shaped embryo body (arrows), protoderm can be observed covering all meristematic groups of cells (PT).Bar: A-B: 50µm; C-100µm.

The cacao somatic embryos at heart stage are approximately 4mm in length. The observation of these embryos on solid media is difficult due the asynchronous growth of cacao somatic embryos on the explants. In TIS they are visible but cannot exactly be distinguished from globular embryos. It is only possible with the help of optical magnification. Furthermore, the growth of the cotyledons occurs in a very short time and the embryos subsequently reach the torpedo stage.

Stage 3: Torpedo-shape somatic embryos

After initiation of tissue differentiation at heart stage, the cotyledons and axis of the somatic embryos rapidly increase in size. The torpedo-shape somatic embryos are considerably larger than heart-shape embryos (≈ 3mm to 7mm) and are easily detected in both solid media and in TIS.

The cotyledons at this phase of development are very small and three cotyledons are formed in early phases. Only two cotyledons continue to develop. At structural levels the differentiation of specific tissues and organs begins. The morphogenesis is completed with elongation of the embryonic axis and formation of the leaf-like cotyledon pair. At this point, the embryos are considered to have reached the 4^{th} stage, and are called cotyledonary somatic embryos.

Stage 4: Cotyledonary somatic embryos

The cotyledons are translucent and generally show the presence of five vascular veins branched from the cotyledonary basis. In histological transversal cross sections of a cotyledon, the cellular arrangement of this organ can be observed. It is formed of a few cell layers (from 5 to 8). Adaxial and abaxial epidermises and well differentiated ground parenchyma form the cotyledonary structure. Trichomes were observed on the adaxial side of the cotyledons. The ground parenchyma shows low occurrence of intercellular spaces. Vascular bundles are easily identified; the centrally located vascular tissue consists of xylem adaxially and phloem abaxially disposed,

surrounded by well differentiated parenchyma. The parenchymatic cells are vacuolated with thin cell walls. Only epidermal cells show some mitotic activity. Parenchymatic cells from middle and distal part of the cotyledon are morphologically distinct. It shows the high specificity in tissue differentiation already in early stages of cacao somatic embryos development (Figure 12).

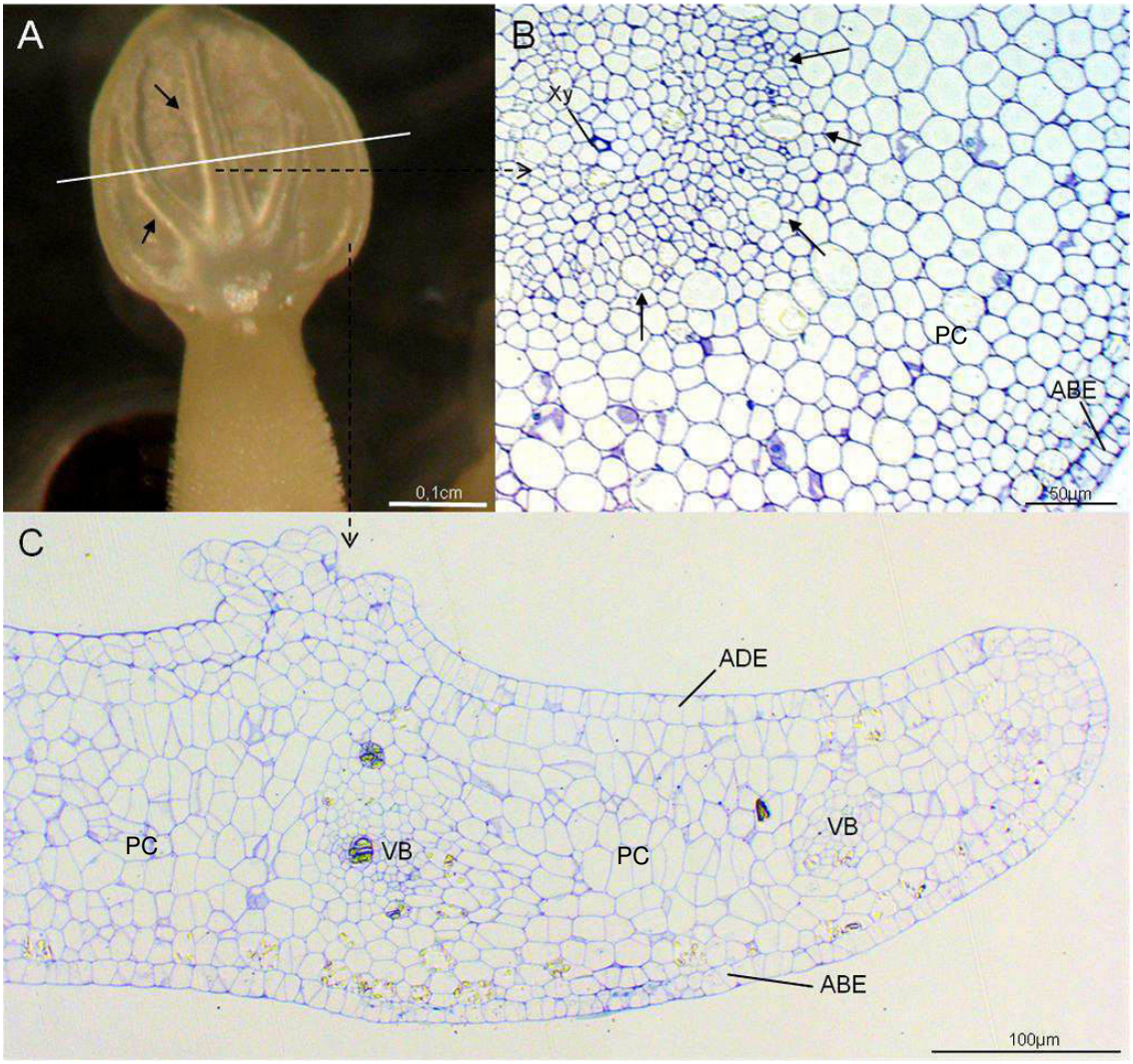

Figure 12. **Morpho-anatomy of cotyledons of cacao somatic embryos at Stage 4 of development. A-** Vascular veins in differentiated cotyledon (arrows); white line indicates transversal cross section of **B-** the main vein of the cotyledons. Central vascular tissue with phloem abaxially (arrows) and xylem (Xy) adaxially disposed. **C-** Transversal cross section of the distal part of the cotyledon. ADE- adaxial epidermis, ABE- abaxial epidermis; PC- parenchyma; VB- vascular bundle.

From the 1st until the 4th stage, the differentiation occurs in a period not longer than 15 days. At the end of this phase, the cacao somatic embryos are considered to have completed morphogenesis. With storage organs formed, the cotyledons, the

physiological maturation can take place. During this subsequent phase, the already formed organs (cotyledons and axis) will continue the tissue specification. Simultaneously, storage compounds are accumulated. These will be used later as basis for development of the radicle and growth of the shoot meristem with subsequent differentiation to form a plantlet. The morphological changes occurring during this process and related biochemical events were studied and are described in the following sections.

4.2.1.2 Histochemical study of cacao somatic embryos at the late development phase.

In order to comprehend how cacao somatic embryos are differentiated and which factors are involved in late development, the growth of cotyledons of cacao somatic embryos was investigated on a structural level by observation of the cellular organization and on a metabolic level. Especially the composition of the cells‘ deposits was characterized. The presence and role of phenolic compounds in zygotic and somatic embryogenesis has been described for several species, but especially for *T. cacao* phenolic derivatives are supposed to have a high importance in the morphogenetic process. Furthermore, the compounds are supposed to participate in formation of flavors of chocolate. These phenolics are known to be stored in the cotyledons of cacao zygotic embryos in high amounts. However, in cacao somatic embryogenesis high concentration of these metabolites so far was only reported for non-embryogenic tissues (Alemanno *et al*., 2003). Nevertheless, until present, no detailed information on the accumulation and distribution of these compounds in cotyledons of cacao somatic embryos was reported. Only the occurrence of these compounds in cacao flowers’ tissue and in undifferentiated callus has been studied (Alemanno *et al*., 2003). Description of the changes on morphology and anatomy of the cotyledons during late development until germination with respect to accumulation of phenolics is scarce.

The cotyledonary differentiation after torpedo stage (Stage 3; Figure 9) can be grouped into four morphological stages; they are mainly correlated to the growth of the embryonic organs (cotyledons, axis, root and shoot apices) (Figure 13). This chapter is aimed at characterizing this critical phase of morphogenesis by physiological markers.

The histodifferentiation was compared with accumulation and distribution of phenolic compounds in the cotyledonary tissue. Cotyledons of somatic embryos at stages 4, 5, 6 and 7 (Figure 13) were submitted to histochemical analysis under light microscopy (LM).

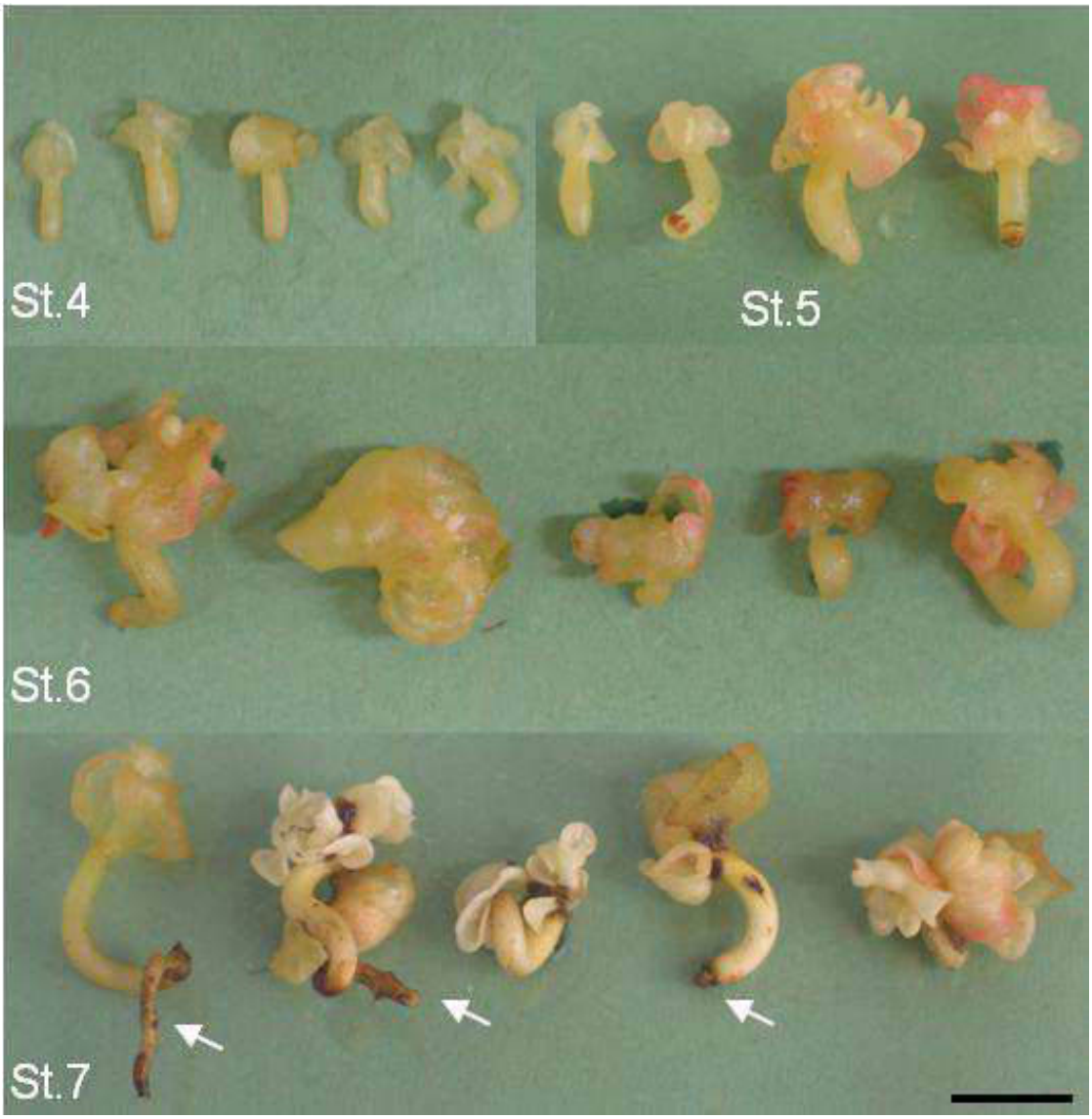

Figure 13. Morphological development of cacao somatic embryos after cotyledonary differentiation. Arrows indicates primary root of germinated somatic embryos. Bar: 1cm.

Transversal sections 2µm thick of stages 4 to 7 were stained with Toluidin Blue O after pretreatment with NaCl 12% (Guttmann, 1995) for observation of the cellular structure and identification of phenolic deposits. Difficulties were encountered to fix the tissue of cotyledons of the somatic embryo at stage 5, fixation by glutaraldehyde 4% and embedding in Spurr's resin caused collapse and distortion of the tissue. The cotyledons of this stage are in the early phase of accumulation of storage compounds. It is likely that these physiological changes in the cotyledonary tissue cause the difficulties to fix and infiltrate the resin in the tissue (Buschmann *et al.,* 2002). Similar difficulties were reported for cacao zygotic embryo at the corresponding stage in early studies by Chessman (1927). The tissue pieces analyzed at this stage presented deep dark blue filled cells which indicate high amounts of phenolic compounds deposited in addition to dense cytoplasm, swollen nuclei and accumulation of proteins and starch in considerable quantities (Figure 14 C and D). Figure 15 presents sections of cotyledons from stage 5 embedded in LR-White resin (see section 3.3.1.1, Material and Methods). This fixation is suitable for cotyledons of this stage and guarantees better maintenance of histological structures. The vascular bundles are always combined with cells filled with phenolic compounds.

In stages 4, 6 and 7 sectioned cotyledons were structurally different to stage 5. Tissue fixed in Spurr's resin delivered good structural preservation of these stages (Figure 14 A and B; Figure 16). Histological transversal cross sections of cotyledons of stage 4 present a complete overview of the relative distribution cells containing phenolic compounds in the cotyledonary tissue (Figure 14 A). These cells containing phenolic storage in the vacuole are found directly near the vascular bundles and under the epidermal layer. This type of association of phenolic storage to the bundle is also seen in stage 6 (Figure 16). Near to the adaxial epidermis of cotyledons of stage 6 some invaginations of the cell walls occur. This may be a procedural artifact, but may also be due to the current developmental growth process.

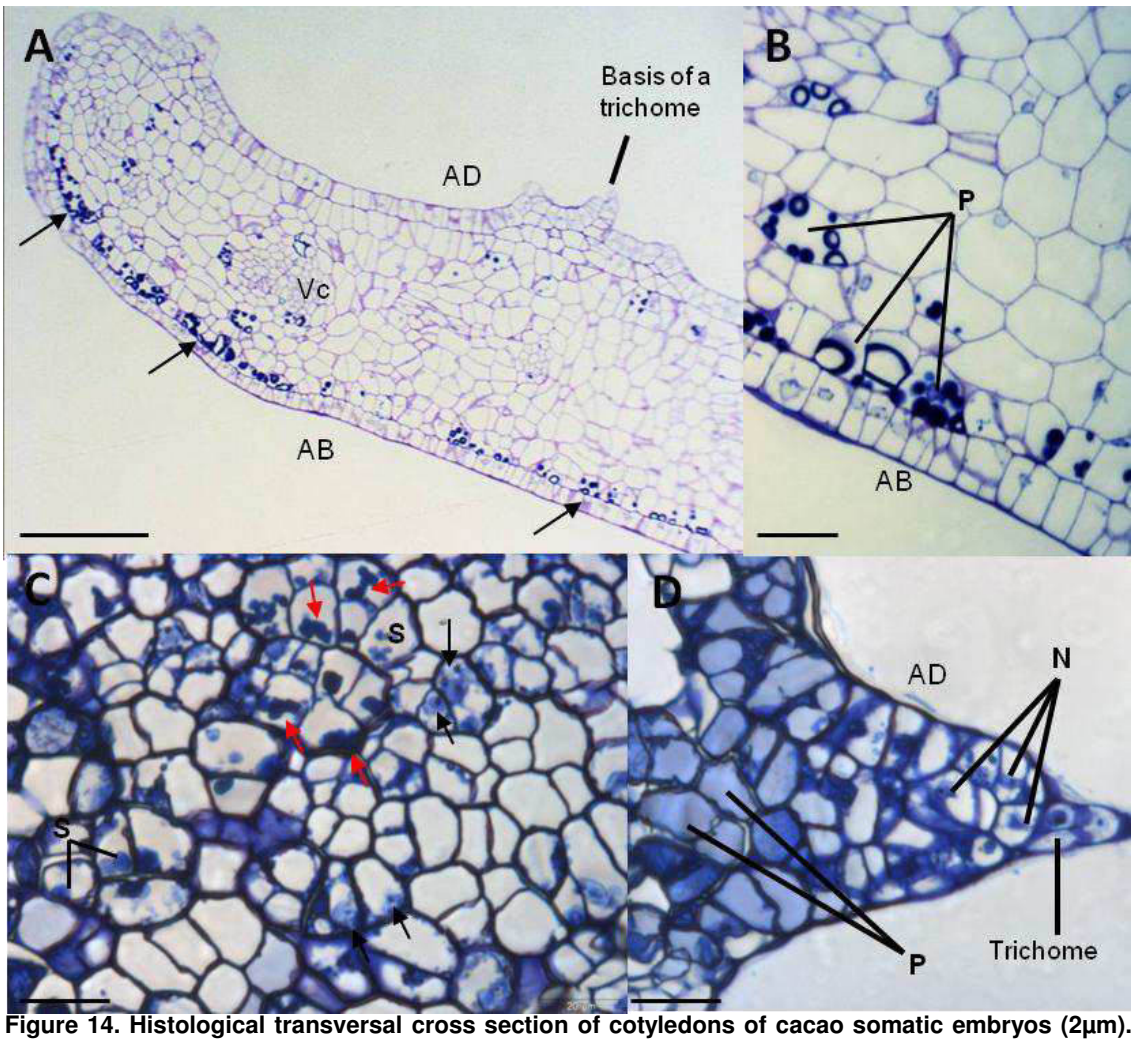

Figure 14. Histological transversal cross section of cotyledons of cacao somatic embryos (2µm). **A-** Cotyledon of cacao somatic embryo at stage 4 (AD-adaxial side; AB-abaxial side; Vc-vascular bundle; arrows indicates accumulation of phenolic compounds staining in deep blue). **B-** Detail of the tissue, note the presence of phenolic compound storage in cells under the abaxial epidermis (AB-abaxial epidermis; P-phenolic compounds). **C-** Tissue of the cotyledon of cacao somatic embryo at Stage 5 (Black arrows indicate protein storage in the cells; red arrows indicate phenolic compounds stored in the cell; S- Starch). **D-** Detail of the adaxial epidermis of cotyledons of stage 5 (AD- adaxial epidermis; N- nucleos; P- phenolic compounds stored in the cells under the epidermis). Samples stained with Toluidin blue O after pretreatment with NaOH 12% (Guttmann 1995).Bar-A:100µm; B,C and D: 20µm.

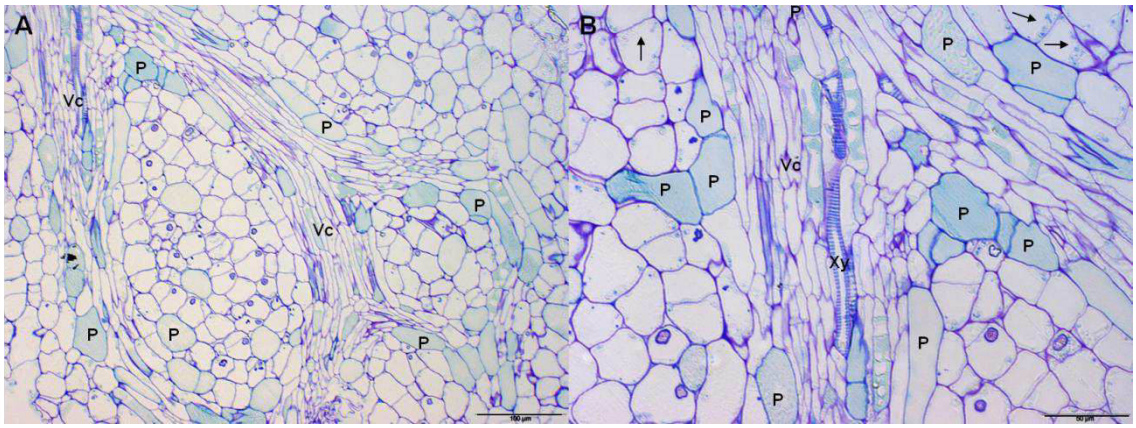

Figure 15. Histological longitudinal cross section of cotyledons of cacao somatic embryos at stage 5 embedded in LR-White resin. A- Cotyledon of cacao somatic embryo at stage 5 (Vc-vascular bundle; P- phenolic compounds stored stained in green). **B-** Intensive occurrence of cells containing phenolic compounds around the vascular bundle (Vc) (P-phenolic storage; Xy-xylem; arrows indicate cells with high accumulation of proteins).Bar: 100µm

In stage 7 cotyledons, the number of phenolic cells is higher than in the earlier stages. The cells on the abaxial side seem to be under a mechanical stress in the densely packed parenchyma (Figure 16 D), probably due to the deformation in the cotyledon seen in the late stage 7. Starch granules (S) are observed in higher quantities than in the other stages. Cells containing phenolic compounds (P) are significantly larger than in early stages and commonly form densely clustered groups. In figure 16C (cross section) the diameter of the cells containing phenolics is from 25 to 50 µm, these cells obviously are elongated up to 100µm and even more (Figure 16 C and D).

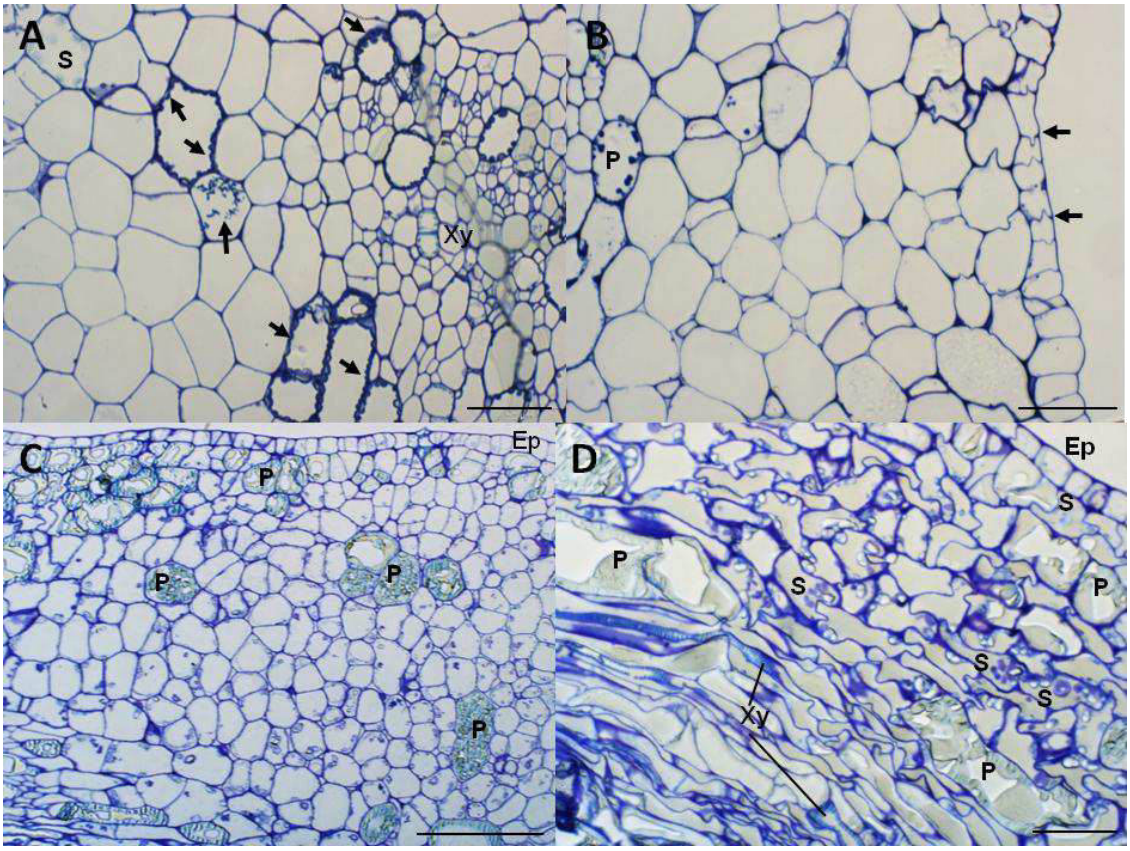

Figure 16. Histological transversal cross section of cotyledons of cacao somatic embryos (2µm). **A-** Tissue of a cotyledon at stage 6 (arrows indicate phenolic storage in the cells). **B-** Detail of the adaxial epidermis with cell wall invaginations (arrows). **C-** Cotyledon at Stage 7. **D-** Detail of deformed parenchyma cells. Samples stained with Toluidin blue O after pretreatment with NaOH 12% (Guttmann 1995). (P- cells storing phenolic compounds; Ep-epidermis; Xy-xylem; S- starch accumulation). Bar: A, B and D: 50µm; C- 100µm.

4.2.2 UV-microspectrophotometry of phenolic deposits in cotyledonary cells of cacao somatic embryos

The distribution of phenolic compounds in plant tissue can be analyzed on a cellular level and sub-cellular level by microspectrophotometry (UMSP). This equipment allows to identify the tissue and cellular distribution of phenolic compounds in microscopic samples. The method is designed to analyze the localization and quantification of substances in the absorption range between 240 and 700nm, with special sensibility in the UV-range. Two types of analyses are used in standard approaches, a field scan and a defined point scan. The field scan allows for observing the tissue distribution of phenolic compounds while the point scan analyzes the spectra of phenolic substances present in a selected point. Especially for developmental processes like embryo formation this method allows to follow the accumulation of compounds, among them cinnamic acid derivatives like lignin, but also soluble compounds like accumulated phenolics of low molecular weight.

UMSP field and point scan were carried out in cotyledons of somatic embryos at stages 4, 5, 6 and 7. The images generated from the field scanning showed the distribution of aromatic compounds throughout the tissue according to its light absorption at a constant wavelength of 278nm (maximum absorbance of hardwood lignin, Koch and Lybeer, 2005). The intensity of absorption of the compounds was given in a range from 0 to 1 represented by a color diagram, where white means absence of absorbance or underflow (zero) and black means the highest absorbance or overflow (one). The point scan resulted in values of UV-absorption of a distinct point ($1\mu m^2$) in the tissue under a wavelength range from 240nm to 600nm. Two biological replicates were used *per* stage. When possible, the field scanning was carried out in 3 different location of each analyzed part. For point scanning, each measurement was repeated in 2 different points when the values were closely similar, and up to 4 different points, when a high discrepancy within values was observed. The data was used to calculate the mean values of Absorbance.

The analysis was carried out in different cells of the tissue. In figure 17 the points of analysis are demonstrated. The cell wall of the cells from adaxial and abaxial epidermis and parenchymatic cells, phenolics compound stored in cells under both, adaxial and abaxial epidermis and intercellular spaces or middle lamella of the parenchyma.

4.2.2.1 Parenchymatic middle lamella and cell wall

The tissue of the cotyledons of somatic embryos of cacao is characterized by an early appearance of lignifications in the cell wall throughout the whole tissue. It does not seem to change along the stages studied. The UMSP profile of the parenchymatic cells shows similar distribution and concentration of lignin in the cell walls and in the middle lamellae for all four stages (Figure 18). The distribution pattern is unchanged but the color pixels indicate different intensities of UV absorbance at the wavelength of $\lambda_{278nm.}$ Cell walls of cotyledons at stage 5 already presented lignifications, however the same absorbance was detected in the cell wall of other stages. In contrast, the deposits of lignin in the middle lamella slightly increased as the stages evolved. The cotyledons with higher absorption in the middle lamella belong to stage 5. A predominance of the color magenta was observed. In the figure 18D the cutting plan is directed in the middle lamellae separating the cells. The dark blue in the cell is due to the phenolics accumulated in the vacuole of the cell.

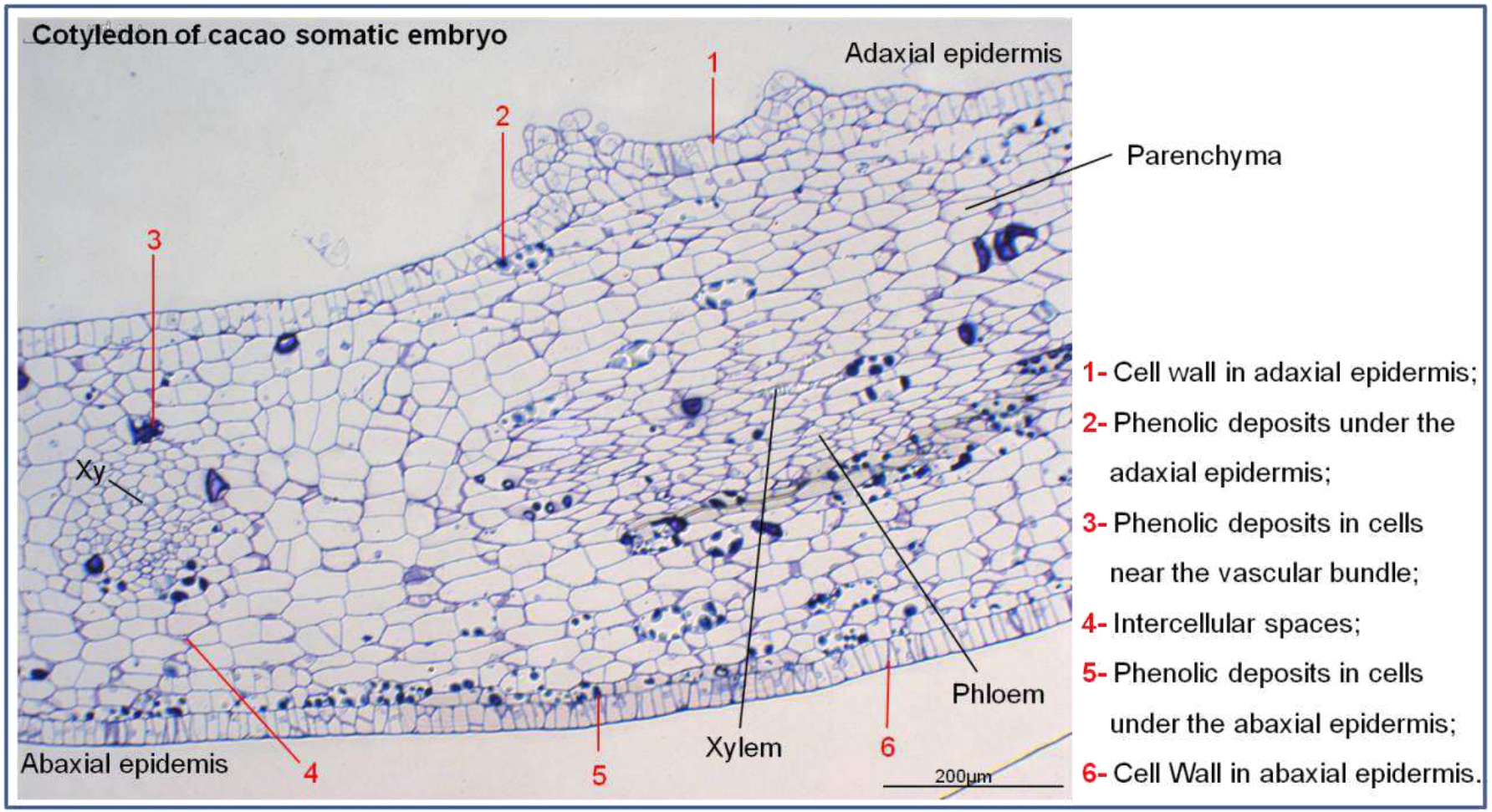

Figure 17. Representation of cells analyzed in field and point scan by UV-microspectrophometer (UMSP).

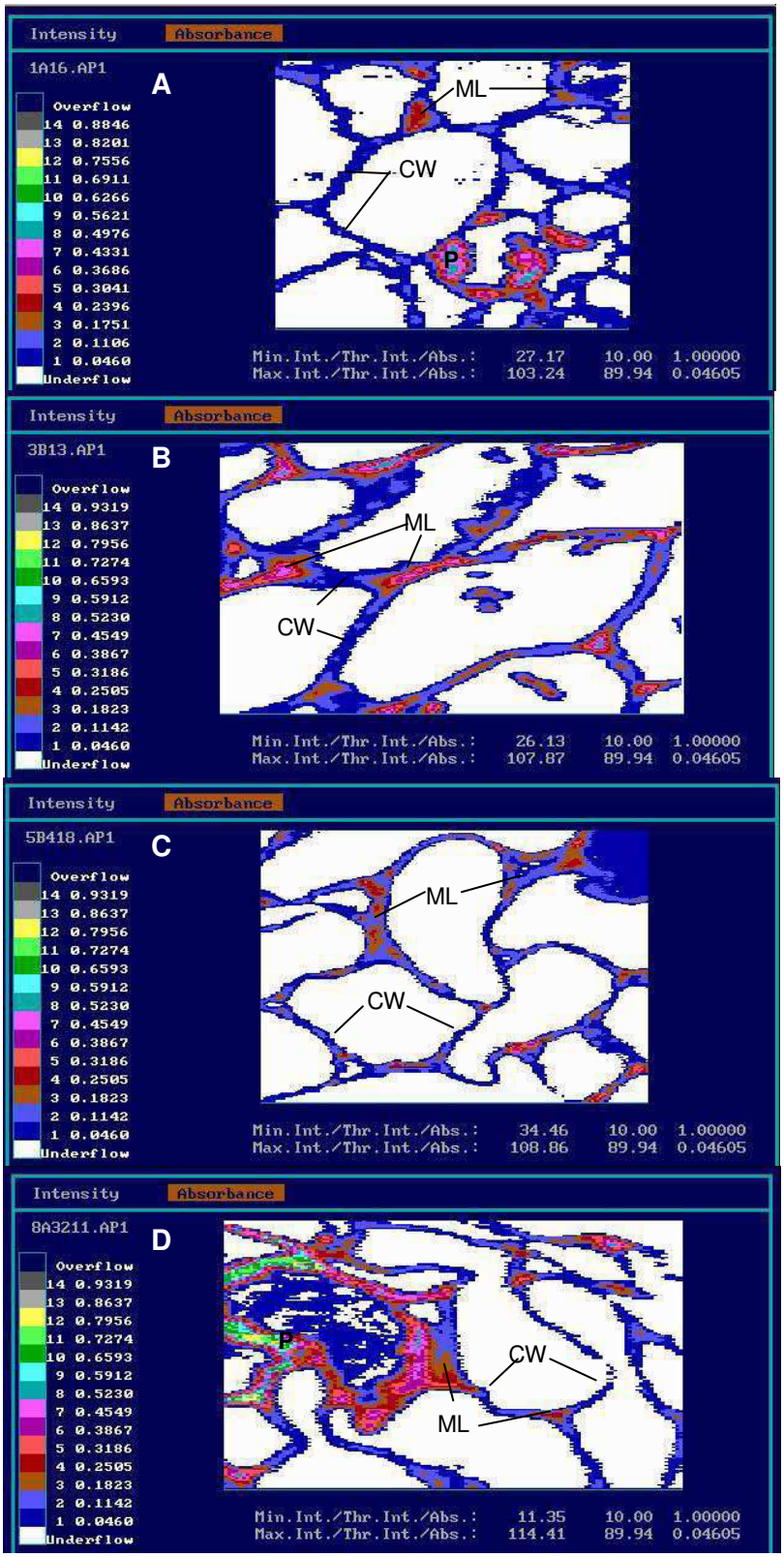

Figure 18. UV-microscopic field scann profiles of lignin distribution in cell wall and middle lamella of the parenchyma of cacao somatic embryos cotyledons at different stages of development. A- Distribution of lignin in cotyledon at stage 4. **B-** Cotyledon at stage 5. **C-** Cotyledon at stage 6. **D-** Cotyledon at stage 7. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2). CW- cell wall; ML- Middle lamella; P- phenolic stored in the cell.

Images obtained from the same cotyledons by transmission electron microscopy (TEM) revealed the accumulation of lignin in the middle lamella (Figure 19). At stage 4 (Figure 19 A), lignin is present attached to cell membrane and agglomerations of this compound are observed in the cytoplasm and in extracellular media. At stage 6, the intercellular space is covered by these compounds. Completely filled intercellular space is observed in cotyledons at stage 7. This series underlines the well known formation of secondary modification of the cell walls in growing tissues.

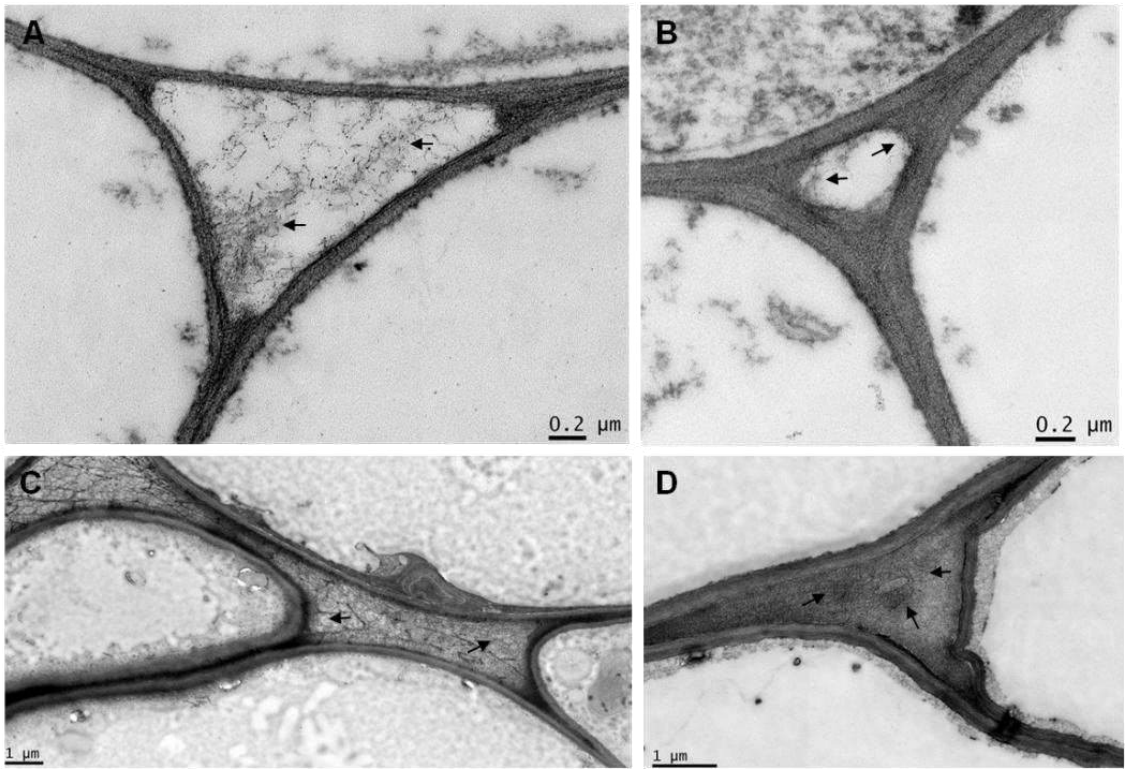

Figure 19. TEM micrographs of transversal cross section of cotyledons of cacao somatic embryos at different stages. Gradual accumulation of compounds in middle lamella of cotyledons parenchyma of embryos at (A; B) Stage 4, (C) Stage 6 and (D) Stage 7. (Arrows indicate the accumulation of compounds)

A tendency for stage-related tissue lignification was demonstrated by analysis of point scan in **middle lamella** of parenchyma, but no significant differences were found among the stages. The spectra of UV absorption of middle lamella showed a peak around 278nm, considered the peak for UV-absorption of lignin (Lybeer and Koch, 2005). For all stages the absorbance in the middle lamella varies between 0,2 and 0,3 (Figure 20). Stage 5 showed the lowest UV-absorption at this wavelength (0,21

Abs λ_{278nm}) and the cotyledons from stage 7 had the highest UV-absorption in the middle lamella of parenchyma, 0,29 Abs λ_{278nm}. The same value was detected for cotyledons at stage 6. Middle lamella of parenchyma of cotyledons at stage 4 presented 0,23 Abs λ_{278nm}. Cotyledons from stage 5 presented the lowest UV-absorption at 278nm, but revealed very high deposition in the field scan (Figure 18 B). It suggests that other aromatic compounds besides lignin are accumulated in the middle lamella.

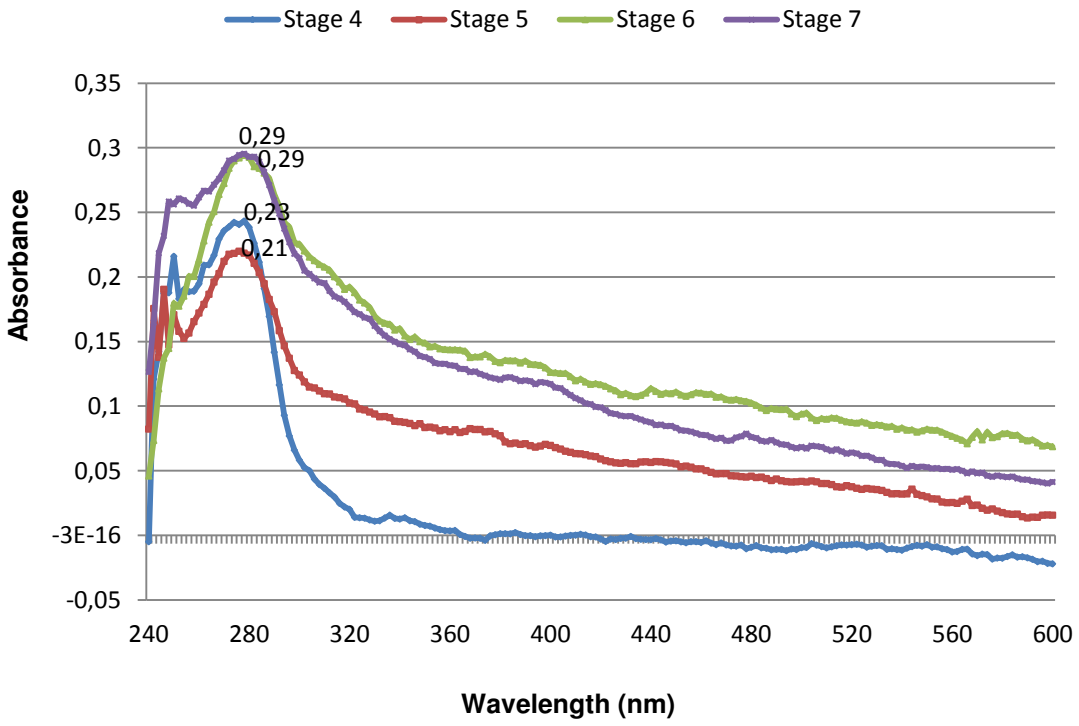

Figure 20. Middle lamella UV-absorption spectra of parenchymatic cells of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

The **cell walls** of parenchymatic cells had UV-absorption spectra with absorbance values slightly lower than observed in middle lamella. However, the intensity of UV-absorption spectra is not related to developmental process. The highest UV-absorption at 278nm was detected in cell walls of tissue of cotyledons at stage 6 (0,25 Abs λ_{278nm}), followed by cotyledons at stage 4 (0,23 Abs λ_{278nm}) and stage 7. This in contrast to the other stages presented two peaks. The first and more intense peak at 250nm (0,20 Abs λ_{278nm}) and the second peak at 278nm (0,19 Abs λ_{278nm}). The lowest

absorbance was detected also in cell walls of cotyledons at stage 5 (0,14 Abs λ_{278nm}) (Figure 21). It indicates that the high concentration of phenolic compounds observed in field scans for this stage consists of low amount of lignin. The absorbance observed in field scan may result from the accumulation of lignin precursors or conjugated forms of aromatic compounds besides lignin. It can be assumed that further secondary modifications start in this cell differentiation phase.

The results demonstrate the presence of lignin already in the cellular structure of early stage 4, and that cell wall lignification remains without great changes during cotyledons maturation. Furthermore, the low UV-absorptions of lignin peak suggest accumulation of other phenolic compounds besides lignin in middle lamella and perhaps also in the cell walls.

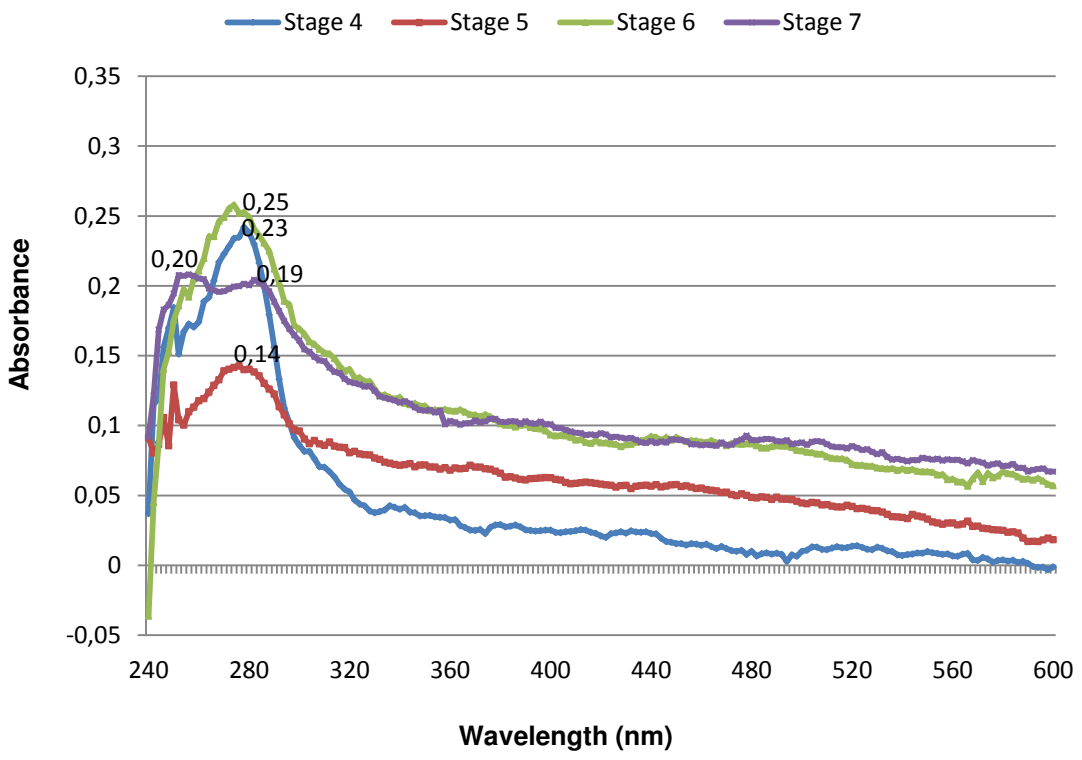

Figure 21. Cell wall UV-absorption spectra of parenchymatic cell of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

4.2.2.2 Cell wall of epidermal cells

Field scans of cell walls of epidermal cells demonstrated no significant differences in the lignifications of cell walls of adaxial and abaxial epidermal cells.

Epidermal cells of cotyledons at stage 4 showed a uniform distribution of lignin in the cell wall of both epidermises (Figure 22). However, in the field scan images it is possible to observe a high accumulation of phenolic compounds in cells directly under the abaxial epidermis. It confirms the findings of the histochemical studies (section 4.2.1) of this tissue, where cells under the abaxial epidermis stained positively for phenolic compounds in higher quantities than under the adaxial epidermis. The epidermises at stage 4 had a slightly higher absorbance in their middle lamella than in the cell walls. This is shown by a brown-red color, as also observed in parenchymatic cells (see section 4.2.2.1).

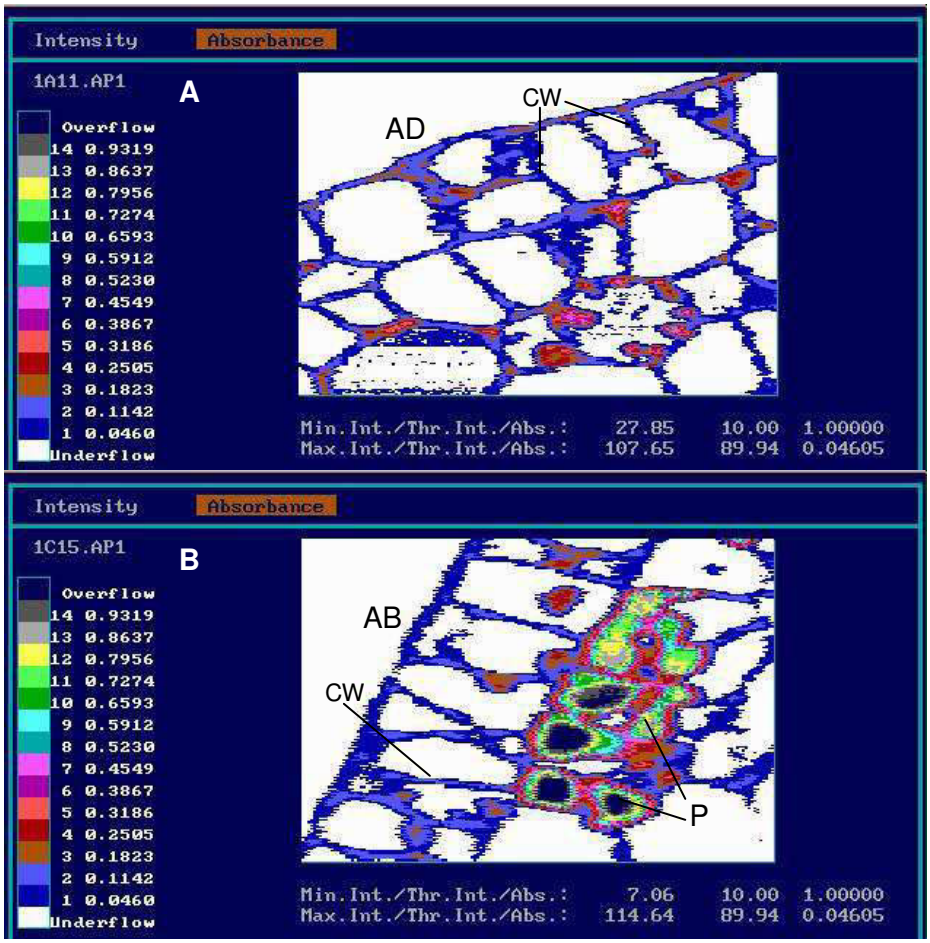

Figure 22. Adaxial and Abaxial epidermises field scanning UV-micrographs of cotyledonary tissue of cacao somatic embryos at Stage 4. A- Adaxial epidermis **B-** Abaxial epidermis. AD- adaxial epidermis; CW- Cell wall; P- phenolic compounds accumulation in cells under the epidermis. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2).

Stage 5, which presented difficulties in the process of fixation and microtome cuttings of the tissue allowed the analysis of the epidermises, which remained intact for UMSP analysis. It was the adaxial epidermis, since only this epidermis remained intact during the histochemical analysis (section 4.2.1). After field scan of this epidermis, it was possible to observe a high absorbance range in the cell wall and in the middle lamella (Figure 23). The cell wall is represented by a blue color and the middle lamellae by a brown-red color. However, the main difference in absorbance is detected in the middle lamella between epidermal cells and under epidermal cells. The absorbance varies from red to green indicating a very high absorbance of phenolic compounds, which seem to be distributed over the pectic layer of the middle lamellae.

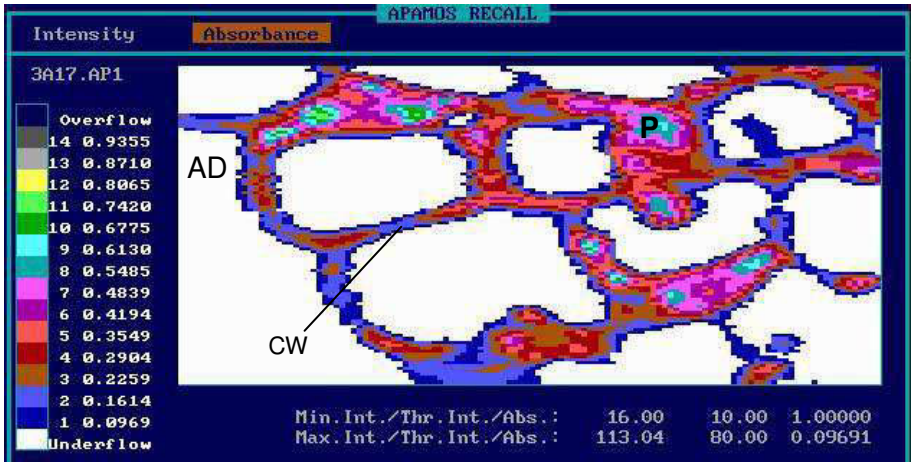

Figure 23. Adaxial epidermis field scanning UV-micrographs of cotyledonary tissue of cacao somatic embryos at Stage 5. AD- adaxial epidermis; CW- Cell wall; P- phenolic compounds accumulation in cells under the epidermis. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2).

Adaxial and abaxial epidermis of cotyledons from stage 6 did not reveale significant differences in the distribution of phenolic compounds. Both epidermises presented low absorbance in their cell wall and middle lamella. As in stage 5, the cell under the epidermis, presented the highest absorbance distributed in the middle lamella and in the cells (Figure 24). The high absorbance in the interior of the cells probably results from the accumulation of phenolic compounds in the cell vacuole as demonstrated in histochemical analysis.

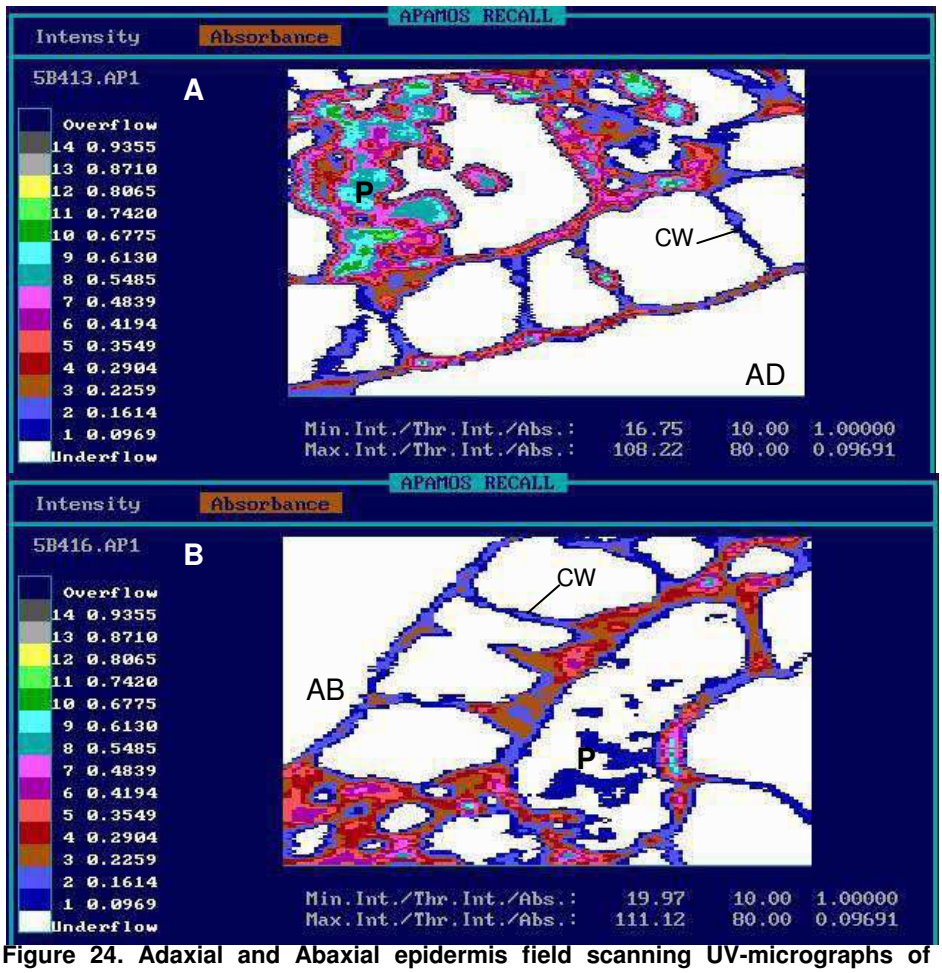

Figure 24. Adaxial and Abaxial epidermis field scanning UV-micrographs of cotyledonary tissue of cacao somatic embryos at Stage 6. AD- adaxial epidermis; AB- abaxial epidermis; CW- Cell wall; P- phenolic compounds accumulation in cells under the epidermis. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2).

Cotyledons at stage 7 presented the most significant difference between adaxial and abaxial epidermises. Cell walls of the adaxial epidermis showed the standard absorbance as in the other stages (color blue). However, the cell wall of the abaxial epidermal cells shows high absorbance (Figure 25 B). Furthermore, the cells under the abaxial epidermis revealed some spots of very high absorbance range of the experiment (Figure 25 A and B). Overflow was detected in the middle lamella of these cells. Accumulation of phenolic compounds was detected in this stage even in the interior of epidermal cells.

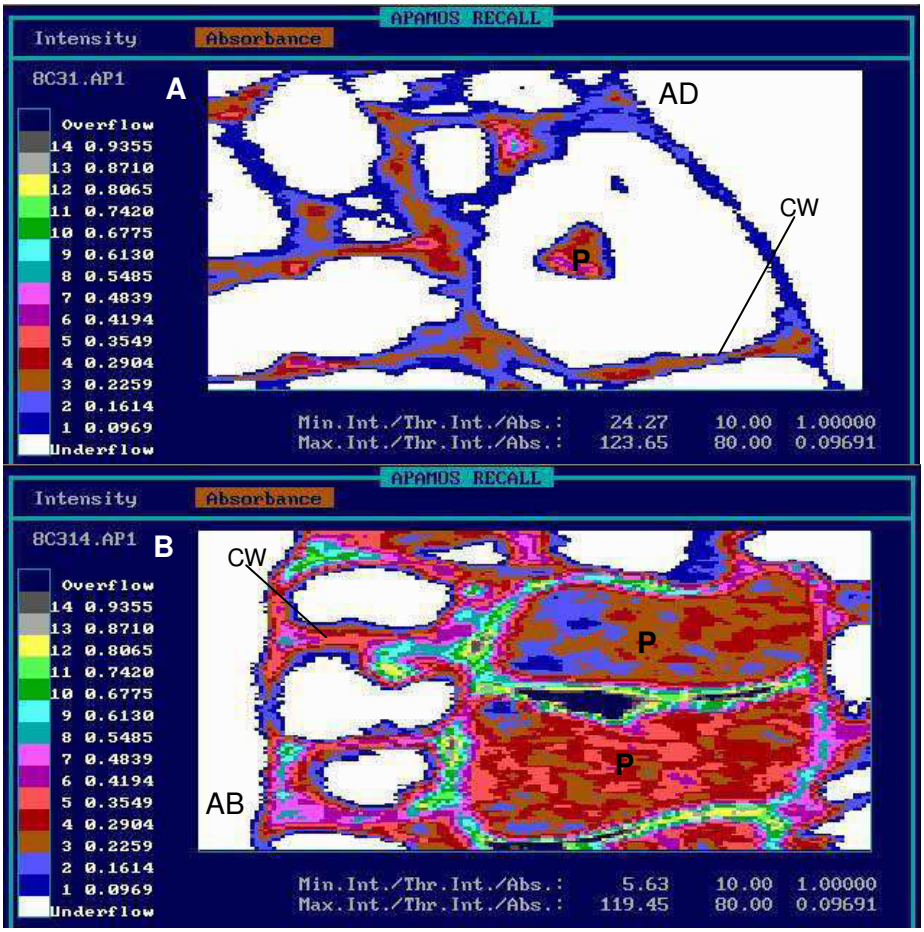

Figure 25. Adaxial and Abaxial epidermis field scanning UV-micrographs of cotyledonary tissue of cacao somatic embryos at Stage 7. AD- adaxial epidermis; AB- abaxial epidermis; CW- Cell wall; P- phenolic compounds accumulation in cells under the epidermis. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2).

Point scan analyses were carried out in the cell wall of epidermal cells to verify the presence of lignifications of these cells, and to test if other phenolic compounds have accumulated in the tissues. The results demonstrated an increase in the lignin deposition in the cell wall of cells of both epidermises, adaxial and abaxial during embryo development (Figure 26). However, no significant differences were observed in the concentration of lignin between the epidermises. The UV-absorption spectra of cell walls of cells from **adaxial epidermis** presented the typical peak at 278nm for lignin, but also had a significant peak at 250nm. This stage 7 also showed the highest UV-absorption for lignin (0,29 Abs λ_{278nm}). As observed in the field scan images, the cell wall of the epidermal cells at this stage was significantly more lignified than other stages. Stage 5 presented higher UV-absorption than stage 6, 0,27 and 0,22 Abs

λ_{278nm}, respectively. Stage 4 presented significantly lower UV-absorption than the other stages, 0,062 Abs λ_{278nm}. This difference was also observed in the field scan analysis. A slight peak was detected in all stages around 380nm.

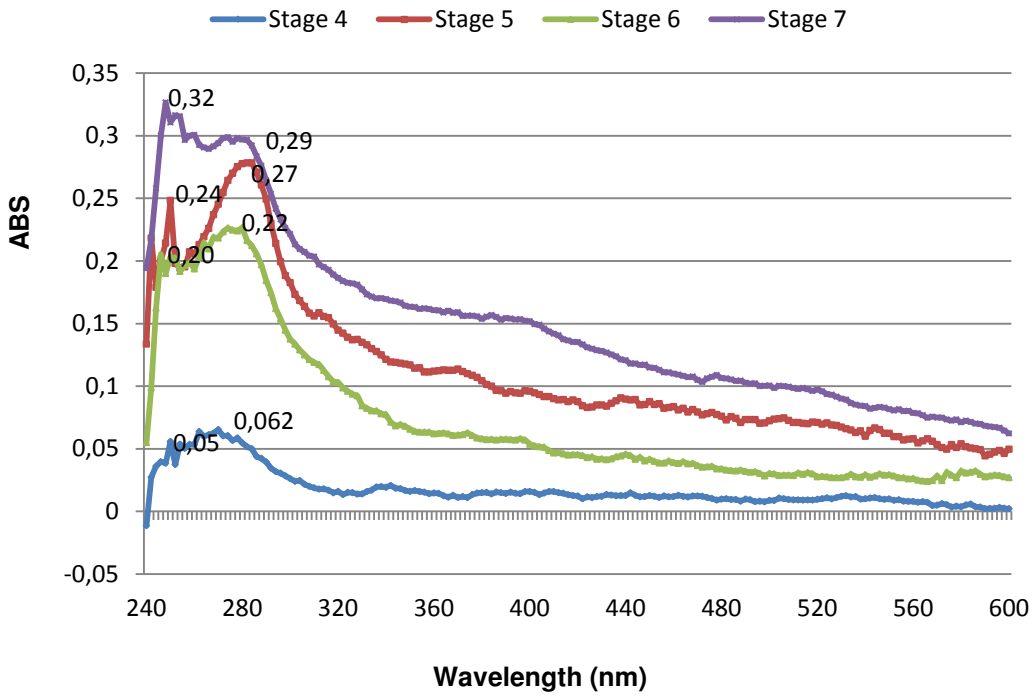

Figure 26. Cell wall UV-absorption spectra of adaxial epidermis of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

Almost the same patterns of UV-absorption and distribution was found in cell walls of adaxial and abaxial epidermal cells. The pattern of lignin absorption of cell walls of cells of the abaxial epidermis was essentially similar to that demonstrated by adaxial epidermal cells (Figure 27). The cell wall of cells of cotyledons at stage 4 showed the lowest UV-absorption, 0,037 Abs λ_{278nm} followed by cotyledons of stage 6, 0,21 Abs λ_{278nm} and stage 5, 0,22 Abs λ_{278nm}. Considerablz higher UV-absorption was detected in cell walls of abaxial epidermis of cotyledons at stage 7. These had two peaks in the adaxial epidermis, but in higher absorption. In this case, all peaks had similar UV-absorption values, the first at 250nm 0,37 Abs λ_{250nm} and the second at 278nm also 0,37 Abs λ_{278nm}.

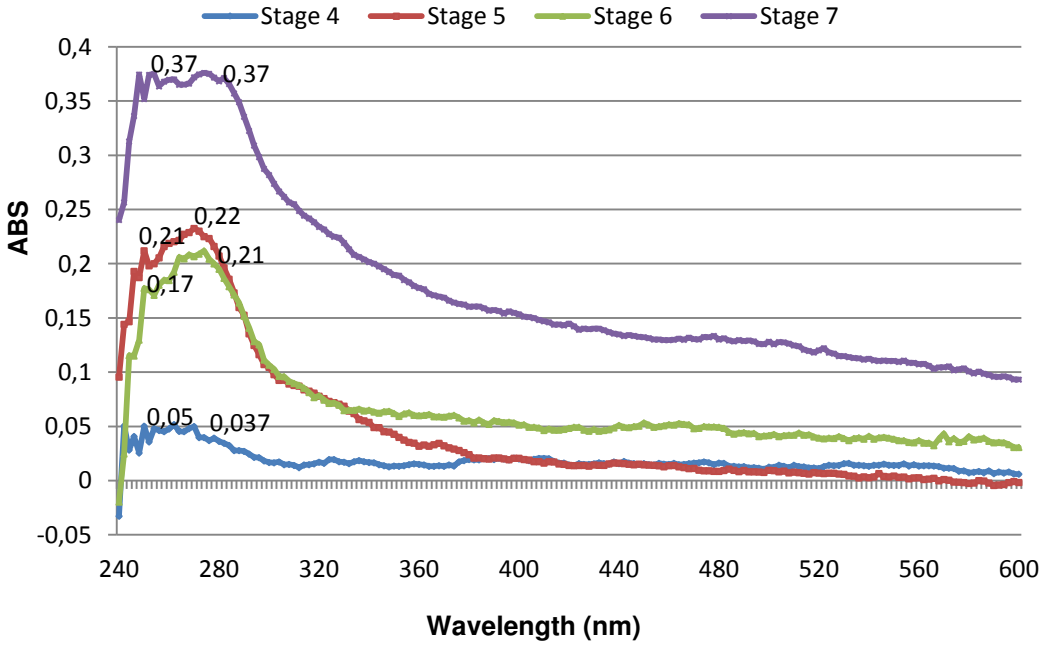

Figure 27. Cell wall UV-absorption spectra of abaxial epidermis of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

The above results demonstrated the gradual lignifications of the cellular structure of cotyledonary tissue during growth of cacao somatic embryos. The cell wall remains relatively stable during the entire period of cotyledonary growth, but a high increase of UV-absorption occurs in the middle lamella and in the vacuole. However, this may be caused by accumulation of other phenolic substances besides lignification.

4.2.2.3 Phenolic deposits in the cells.

To observe the UV- absorption spectra of phenolic compounds stored in cells, field scan and point scan were carried out in cells of different regions of the tissue containing high amount of phenolic substances (see Figure 17, section 4.2.2).

The results found by point scan analysis of phenolic deposits in cells under the adaxial epidermis revealed UV-absorption values significantly higher than those detected in cell walls and middle lamella of the same tissue (Figure 28). As in the previous analyses, the UV-absorption of phenolic compounds increased with the developmental stages. The characteristic peak for lignin UV-absorption at 278nm was seen in phenolic storage cells from all stages. The lowest lignin UV-absorption peak

was detected at stage 5 (0,51 Abs λ_{278nm}) followed by stage 4 (0,59 Abs λ_{278nm}) and stage 6 (0,73 Abs λ_{278nm}). The highest UV-absorption was 0,74 Abs λ_{278nm} in cells under the adaxial epidermis of cotyledons at stage 7. Another peak appeared at 250nm for all analyzed stages. However, all peaks showed lower UV-absorption then the lignin peak.

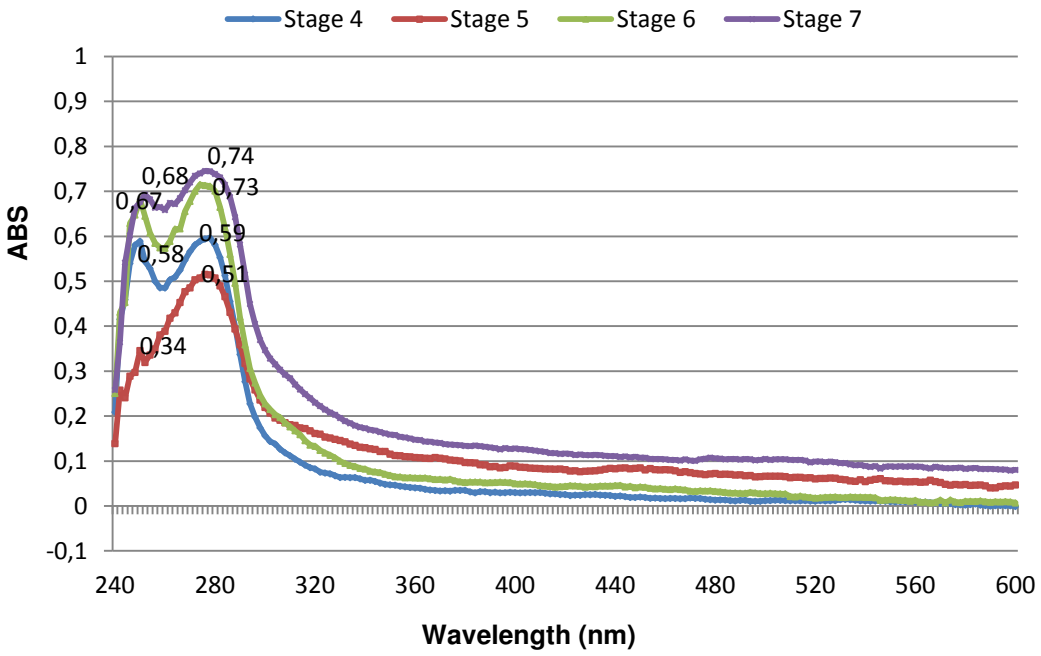

Figure 28. Storage phenolic compounds UV-absorption spectra of cell under the adaxial epidermis of cacao somatic embryos cotyledons at different stages of development. 2µm thick sections.

Analysis of the phenolic compounds stored in cells under the abaxial epidermis revealed the typical lignin peak at 278nm only in cells of cotyledons at stage 7 and 5 (Figure 29). The UV-absorption of these deposits at stage 7 was 0,936 Abs λ_{278nm} and at stage 5, 0,55 Abs λ_{278nm}. The peaks of both these stages were more accentuated than in the anterior analyses of the adaxial epidermal cells. The peak at 250nm present in the other analyses, appears here only in low intensity. In contrast to the well defined lignin peaks in these stages, the analysis of phenolic deposits in cells under the epidermis in cotyledons from stages 4 and 6 showed very low UV-absorption at 278nm. At stage 4, the maximal UV-absorption was 0,045 Abs λ_{278nm} at a wavelength of 268nm and at stage 6 it was 0,159 Abs λ_{278nm} at 254nm.

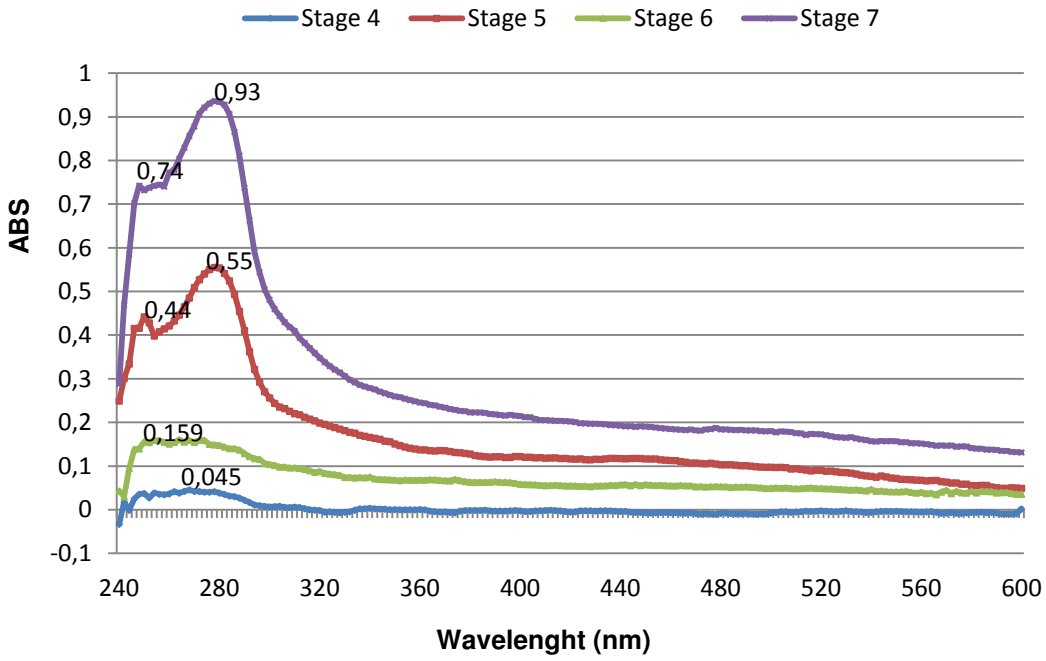

Figure 29. Storage phenolic compounds UV-absorption spectra of cell under the abaxial epidermis of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

In general, the UV-absorption of the phenolic compounds stored in the cells under both epidermises was significantly higher than observed in cell walls and middle lamellas. The lignification of the tissue observed in field scan images is confirmed by the presence of lignin peak in most of the point scan analysis. However, the detection of other peaks around 250nm and 380nm suggests the accumulation of other phenolic substances. Analysis in High Performance Liquid Chromatography (HPLC) combined with Mass spectrometry can help to identify such substances.

Images from field scan analysis with 3D projection can demonstrate the evolution of phenolic compounds accumulation in the cells under epidermis through the stages, while the lignification of cell wall remains relatively stable during the whole process (Figure 30). Every peak observed in this analysis corresponds to the absorbance of a selected area. Peaks in blue represent the lowest absorbance detected, for example in the cell wall, and peaks in yellow represent areas of high absorbance.

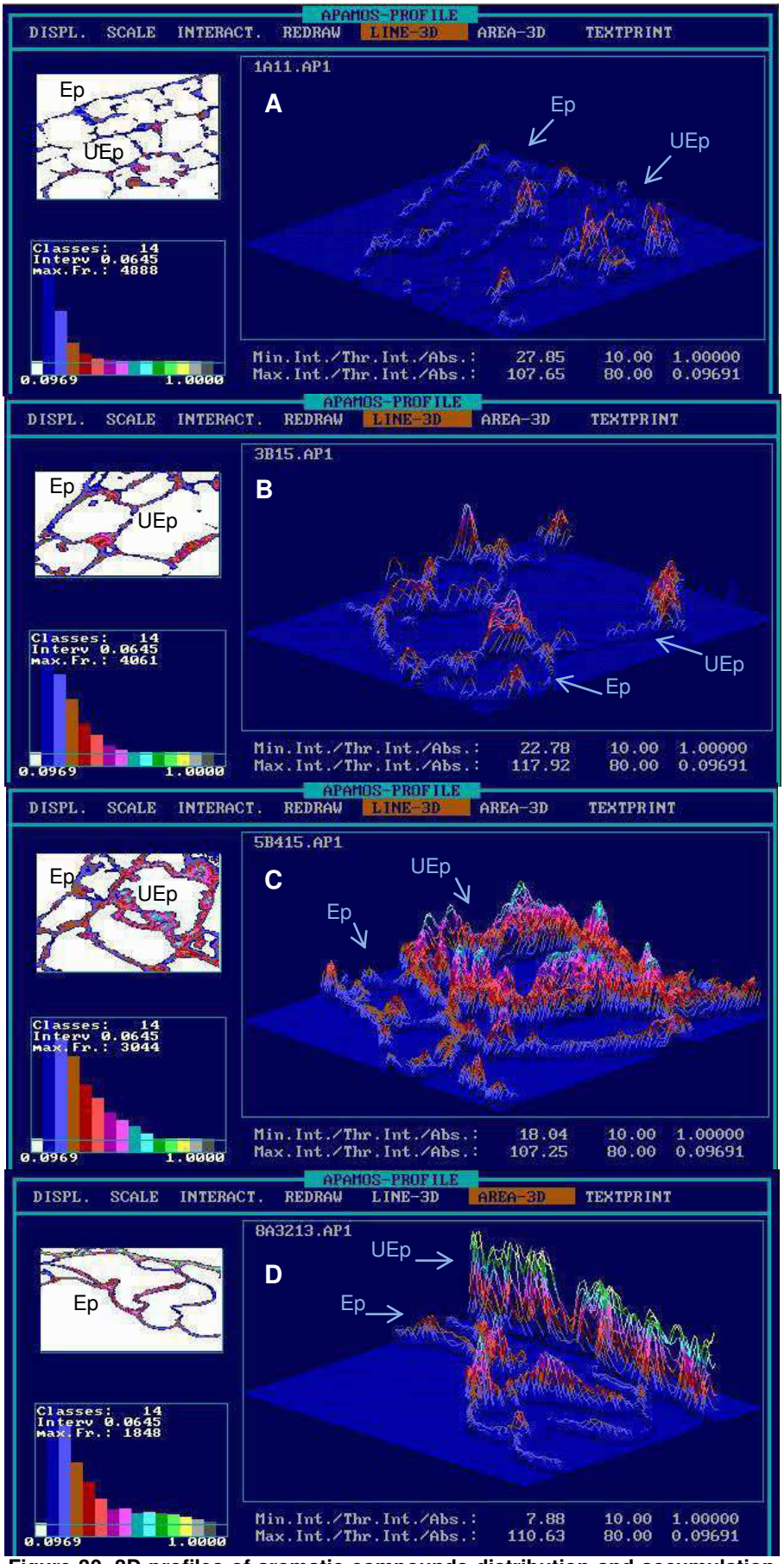

Figure 30. 3D profiles of aromatic compounds distribution and accumulation in cotyledons at different stages. A- Stage 4, **B-** Stage 5, **C-** Stage 6 and **D-** Stage 7.Ep- epidermis; UEp- parenchymatic cells under epidermis. The colour pixels represent different UV absorbance values measured at λ_{278nm} (scanning geometric resolution of 0,25µm^2).

4.2.2.4 Different forms of phenolic storage compounds in the cells.

Previous histological studies of cacao seeds have reported the presence of different forms of phenolic compound storage in the cell. It was suggested that each form corresponds to the process of phenolic compounds' accumulation and one form precedes the other (Elwers *et al.*, 2010; Anderson *et al.*, 2006). This was also observed in the cotyledons of cacao somatic embryos in histochemical and also in UMSP field scan analyses presented in the section 4.2.2.3.

Based on these observations, UMSP point scan analyses were carried out in the different phenolic deposits found during this study. The different forms observed in cacao somatic embryos cotyledons are classified as: Ring form (RF), filled cell (FC) and droplets (DF) (Figure 31). The point of analysis was determined randomly in the area of the deposits of phenolic compound in the cells.

As in the former analysis, the increase in the absorbance of the stored phenolic compounds in the cells was observed throughout the stages of cotyledonary growth (Figure 32). However, the values of UV-absorption were considerably higher than in cell walls or middle lamellae. Also in these samples two peaks were detected. The first at 250nm and the second typical lignin peak at 278nm. In contrast to the other analyses, the phenolic deposits of cotyledons at stage 6 demonstrated the highest lignin peak, 0,82 Abs λ_{278nm}. The stored phenolic compounds from stage 5 and 7 revealed similar UV-absorption for lignin, 0,71 and 0,68 Abs λ_{278nm}, respectively. Phenolic compound accumulated in cells of cotyledons at stage 4 showed an UV-absorption for lignin of 0,22 Abs λ_{278nm}.

These results support the findings of the 3D analysis shown above where the UV-absorption in stored compounds in the phenolic containing cell is significantly higher than found in the cell walls or middle lamella.

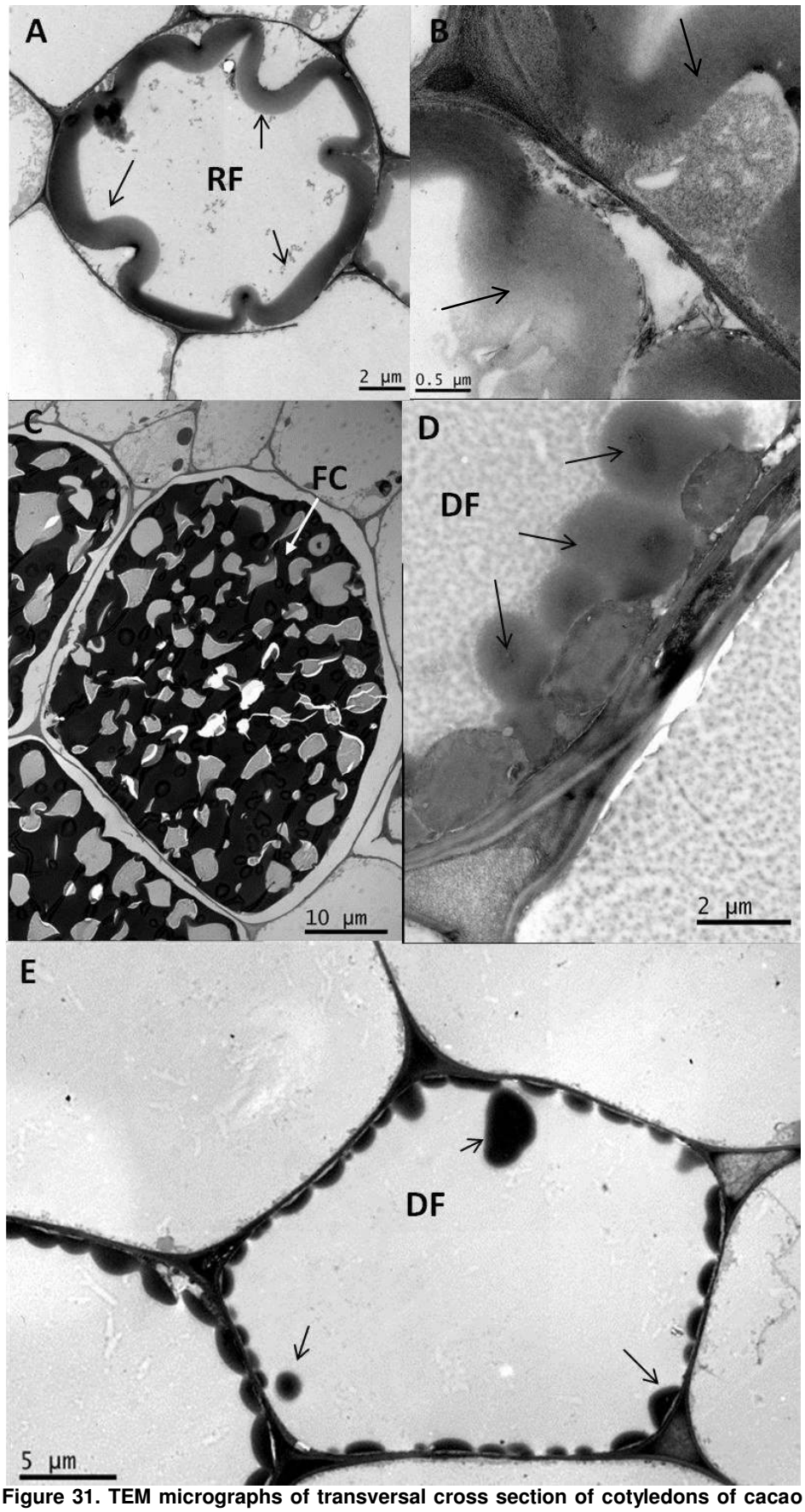

Figure 31. TEM micrographs of transversal cross section of cotyledons of cacao somatic embryos at different stages. Accumulation of phenolic compounds in cells of cotyledonary parenchyma. **A-** Ring form of phenolic compound storage (RF- arrows).**B-** Detail of phenolic compounds accumulated in ring form (arrows).**C-** Typical cell entire filled with phenolic substances (FC-arrow). **D-** Detailed image of phenolic substances accumulated in droplets form in the cells (DF- arrows).**E-** A parenchymatic cell containing phenolic substances accumulated in form of droplets (arrows).

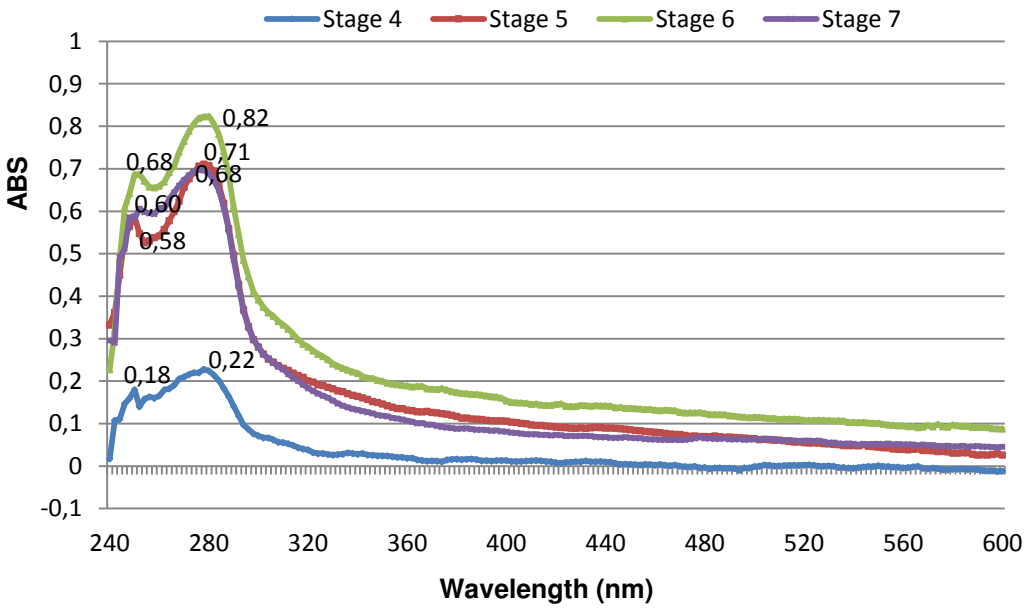

Figure 32. UV-absorption spectra of phenolic compound stored in the cells of parenchyma of cacao somatic embryos cotyledons at different stages of development. 2 µm thick sections.

When the main forms of phenolic compounds accumulated in cotyledons of cacao somatic embryos were compared, it revealed a higher accumulation of lignin in the ring form deposits, 0,47 Abs λ_{278nm} (Figure 33). This also shows another peak at 250nm, but with lower intensity, 0,39 Abs λ_{250nm}. In contrast, the UV-absorption spectra of phenolic compounds stored in the entire cell (Filled cell form) presented a peak at 250nm higher than the lignin peak at 278nm, 0,21 Abs λ_{250nm} and 0,17 Abs λ_{278nm}. The UV-absorption peaks of lignin were significantly lower in the filled cells than in the ring form stored compounds.

The results show that the phenolic compounds are accumulated in different physical or physiological stages in the cells. The peak for lignin absorption was considerably higher in the ring form deposits than in the cells which are entirely filled with phenolic substances. In the 3D images, however, high absorption and even overflow were observed in the cells entirely filled, represented by yellow and black color (Figure 34). However, this comparison was among cells of cotyledons at different stages of development (stages 4, 6 and 7). It may interfere with the result, since in this

study it was shown that the accumulation of lignin in the cells increases with the developmental stages.

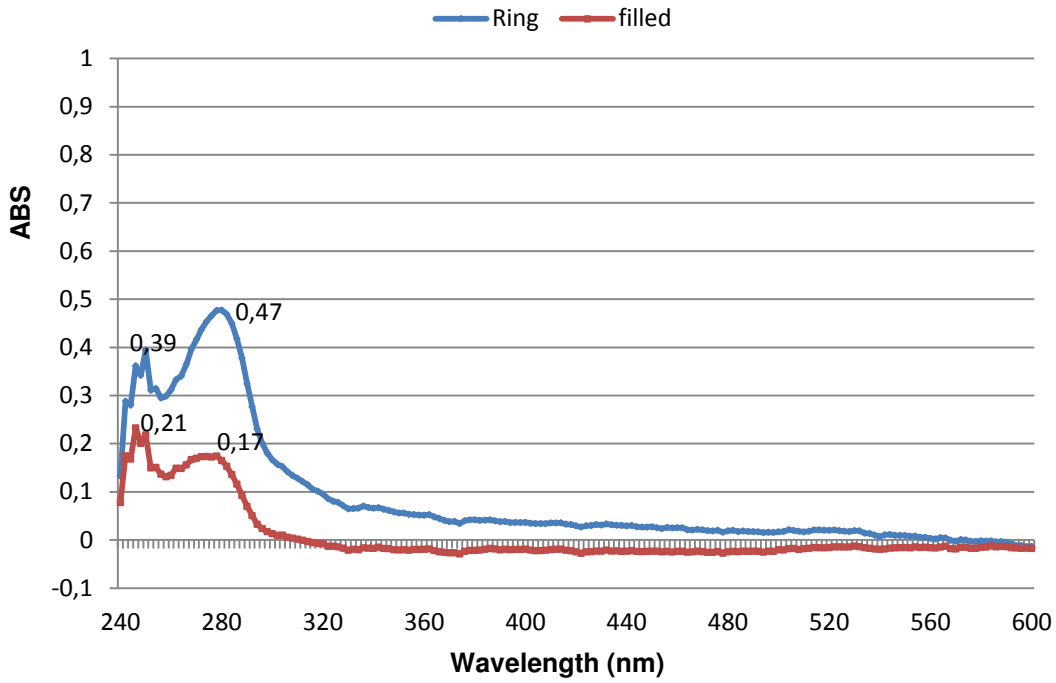

Figure 33. UV-absorption spectra of phenolic compound accumulated in different forms in cells of parenchyma of cacao somatic embryos cotyledons. 2 µm thick sections.

In order to verify the real differences among the different forms of phenolic deposits, 3D field scan images (Abs λ_{278nm}) from cotyledons at stage 4 were compared (Figure 35). In this comparison, cells containing phenolic substances deposited in ring form (RF) presented the highest absorbance range (Abs λ_{278nm}). At stage 4, the entirely filled cells presented absorbance significantly lower than the ring form. It indicates that different phenolic deposits forms in the cells of the same stage had significant differences in the absorbance and distribution of lignin. Represented by yellow and gray colors, ring form deposits had higher concentrations of lignin.

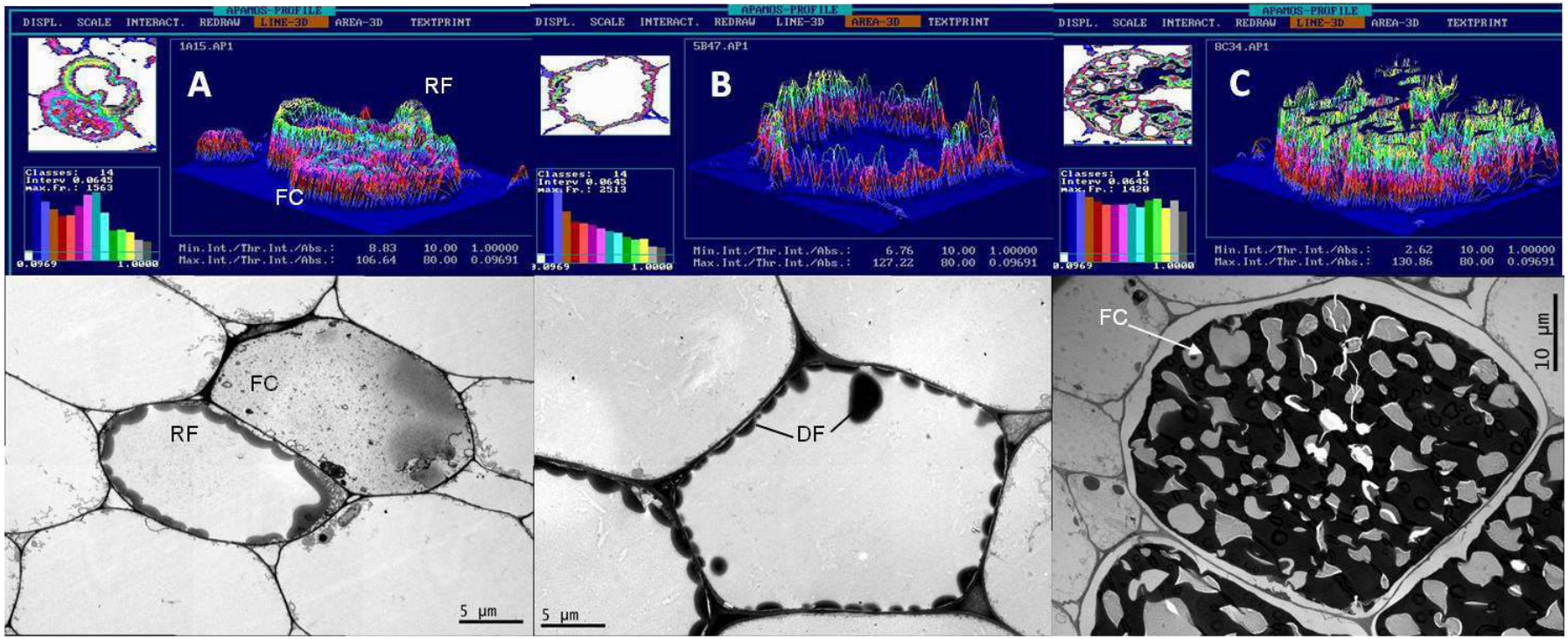

Figure 34. 3D-images of UV-micrograph and respective TEM image of the commonly found forms of phenolic deposition in cells of parenchyma of cacao somatic embryos cotyledons at different stages. A- Ring form of deposition and entire filled cell found in cotyledons at stage 4. **B-** Phenolic compounds deposited in droplets form in vacuole of cells from cotyledons at stage 6. **C-** Filled cells with phenolic compounds from tissue of somatic embryos at stage 7. RF-ring form of phenolic deposits; DF- Droplets of phenolic substances in the cells; Fc- Entirely filled cells with phenolic compounds.

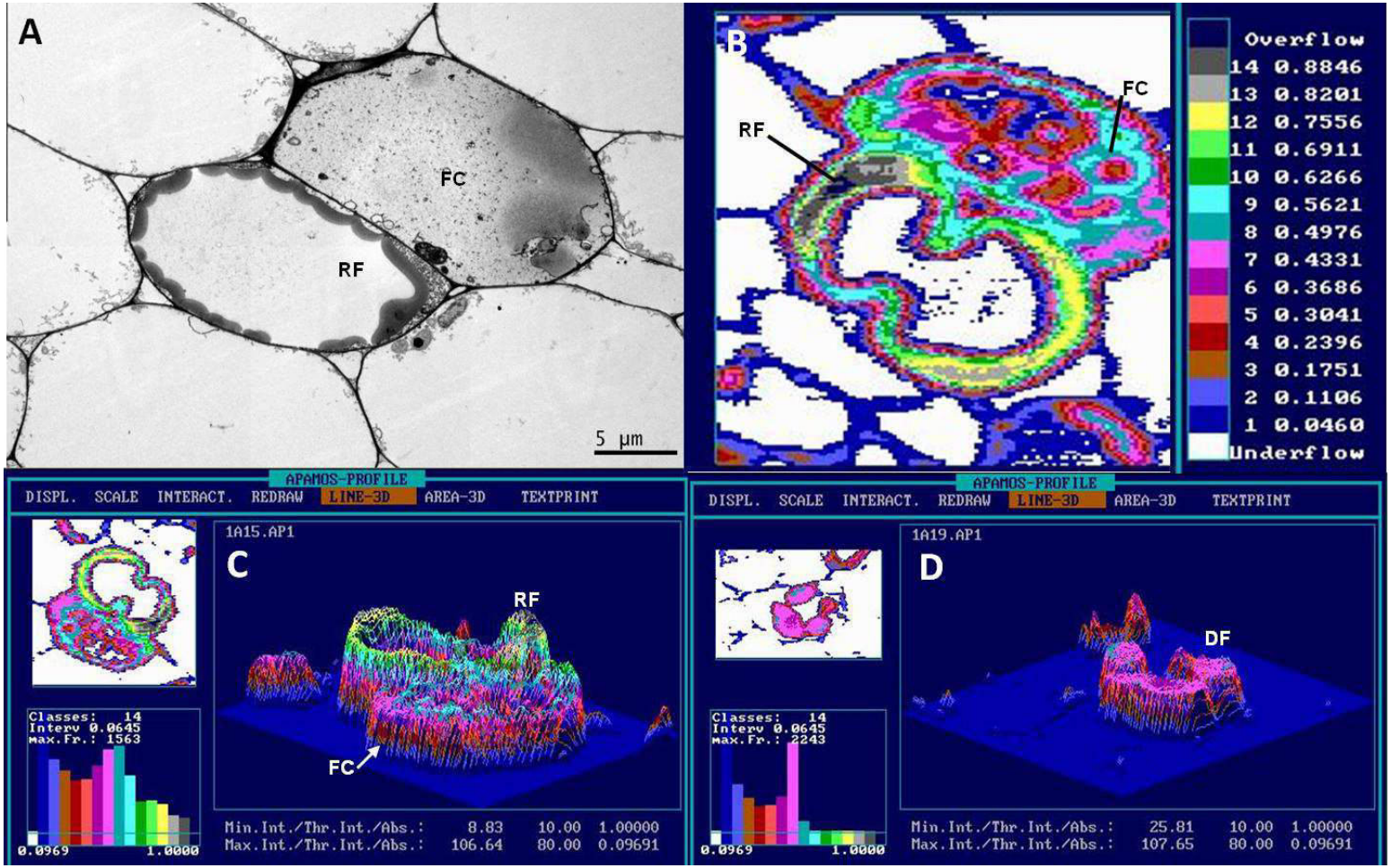

Figure 35. Different forms of phenol deposition in parenchymatic cells of cacao somatic embryos cotyledons at stage 4 of development. A- Transmission electron microscopy of cells containing phenolic deposits**. B-** UV-micrograph of the cells. **C-** 3D image of the UV-microspectrophotometry of phenolic deposits in ring form and filled cell form. **D-** UV-micrograph 3D-image of phenolic compounds deposited in form of droplets. Absorption values are color indicated, lowest absorption (0) is represented by white and highest absorption (1) is represented by black. RF- ring form; FC- entire filled cells form; DF- phenolic droplets.

In summary, the localization and distribution of phenolic compounds and lignification of the tissue was possible with the use of this technique. UMSP allowed to identify the lignification of cell walls of cotyledons of cacao somatic embryos already in early stages of development. The precision of this method demonstrated the stable lignification of the cell structure throughout the embryo development, but also demonstrated the increasing accumulation of phenolic substances in the cells other than lignin.

4.2.3 Biochemical indicators of cacao somatic embryos development.

Cacao somatic embryos regenerated in TIS or on solid medium show specific morphological characteristics like color, size of the cotyledons, axis form and vigor of the embryos. These developmental patterns seem to be determined by the culture conditions (section 4.1) and may be related to biochemical changes occurring in the embryos. The color, size of the cotyledons and structure of the axis of the embryos developed in TIS are significantly different from that of embryos developed on solid medium. Occurrence of roots and oxidation on the axes are observed mainly in somatic embryos cultured on solidified medium.

The difference in the vigor of the embryos from both cultures is evident when younger somatic embryos from TIS are compared to mature embryos cultured on solid medium. The embryos from TIS, even at an early stage, show more robust axes and voluminous cotyledons when compared to the thin axes and fragile cotyledons of mature somatic embryos formed on solidified medium cultures. To investigate if these morphological characteristics of the somatic embryos correspond to specific biochemical features, quantitative analysis of total proteins, soluble sugars and starch was carried out.

The inefficiency of cacao somatic embryos converting into plantlets has been extensively approached. Retarded development of somatic embryos after transfer to the germination medium, lack of vigor and callogenesis on the organs are some of the processes observed in this study that hamper the conversion of these somatic embryos. An efficient accumulation of storage compounds like protein, sugars and starch can determine the success of subsequent steps in the development up until plantlet's establishment. Thus, it is important to know in which concentration those compounds are accumulated in somatic embryos developed under both culture conditions, TIS and solidified medium.

4.2.3.1 Late development of cacao somatic embryos on solid medium.

When cacao somatic embryos at stage 6 (see figure 13, section 4.2.1.2) of development (around 4th subculture) are germinated on solid medium under a photoperiod of 12hrs, without any maturation pre-treatment, they remain small and cotyledons turn green within a few hours. Necrosis of the embryonic axes and hyperhydricity of the cotyledons is visible after a few days. Neither leaves nor roots develop and callogenesis is observed on the apical meristem region. Most of the embryos didi not survived and died within one week (Figure 36).

Figure 36. **Somatic embryos after 1 week on solidified germination medium. A-** Mature somatic embryo presenting shoot development (black arrow) but with necrosis on the axis (white arrow). **B-** characteristic color of somatic embryo after 1 week on germination medium. **C-** Hyperhydricity of the cotyledon, common on mature somatic embryos cultured on solid medium (white arrow), and callogenesis on the cotyledonary tissue (black arrow). **D-** Apical meristem region with callus formation (black arrow).

After 21 days of culture on solidified medium for rooting induction containing active charcoal, somatic embryos showed green cotyledons and axes, and some embryos emitted one or two leaves which were small, yellowish and sometimes translucent or abnormal. The axes did elongate and remained green during the entire

period of culture. Two forms of roots were observed: thin and long or short and thick. Curved axes, necrosis and callogenesis on apical meristem region occurred at a lower frequency (Figure 37).

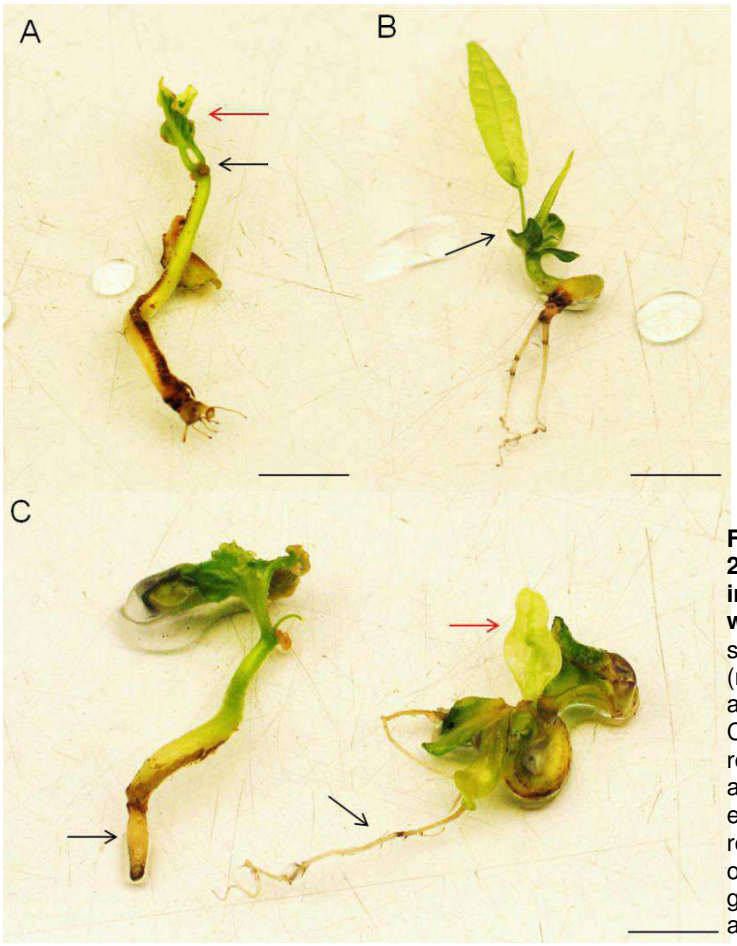

Figure 37. Somatic embryos after 21 days of culture in rooting induction medium supplemented with active charcoal. A- Germinated somatic embryo with a pair of leaves (red arrow) but with callus on the apical shoot (black arrow). **B-** Converted somatic embryos with roots and the first true leaf (black arrow). **C-** Germinated somatic embryos with two different forms of roots (black arrows), commonly observed in the experiment, and the growth of yellowish leaf (right-red arrow). Bars: 1cm.

The culture of the somatic embryos in rooting induction medium allowed the formation of roots, but these were not as well formed as the primary leaves. In both experiments the embryos did not survive until conversion and the lack of vigor was evident by the development of thin roots and fragile leaves. The results show that cacao somatic embryos growing under these conditions do not develop abilities necessary for post-embryonic development. Many molecular and physiological factors are supposed to be involved in the post-embryonic development of somatic embryos. In this study, efforts were focused on the biochemical status of the somatic embryos, in order to verify if sufficient reserves are accumulated during their development on solid

medium and whether these reserves accumulated appropriately in the cotyledons. Irregularities in the accumulation of these compounds can explain the poor germination and conversion of these embryos.

Content of proteins and carbohydrates

Cacao somatic embryos during later phases of development (Stages 5 and 6) and germinated cacao somatic embryos (Stage 7) grown on solid medium were submitted to quantification of total protein. The results showed significant differences in the content of protein in mature and germinated somatic embryos. However, the germinated somatic embryos presented higher levels of total proteins than the somatic embryos considered as mature. Furthermore, the cotyledons did not contain a significantly higher concentration of proteins than the axes (Figure 38).

In somatic embryos at stage 6, the highest content of total proteins was found in the axes, 45,53 µg/mg FW (fresh weight), while the cotyledons had 27,40 µg/mg FW. Cotyledons and axes of germinated somatic embryos contained almost the same concentration of proteins, 71,10 and 71,90 µg/mg FW, respectively. Only embryos at stage 5 presented with a higher content of protein in the cotyledons than in the axes (19,54 and 17,74 µg/mg FW, respectively), even though it is not significantly different.

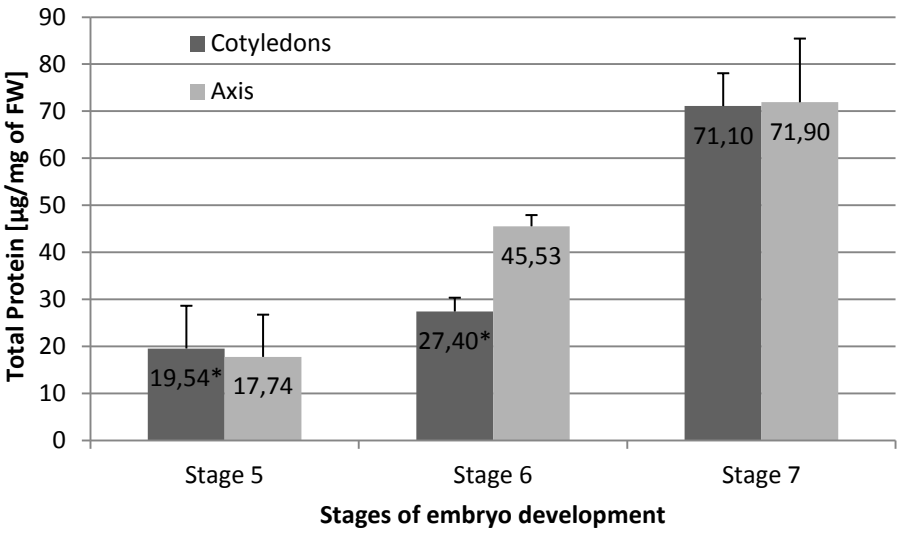

Figure 38. **Total protein content in cotyledons and axes of cacao somatic embryos grown on solid medium at different stages of late development.** Error bars represent means+standard error (n=2;*n=3).

In contrast to the proteins, the content of soluble sugars presents a different pattern of accumulation in the embryos. The soluble sugars are accumulated in the cotyledons of the mature somatic embryos at stages 5 and 6. The highest concentration was found in cotyledons of embryos at stage 5, 2,89 µg/mg FW and the lowest level was detected in the axes of embryos at the same stage, 0,62 µg/mg FW. A small increase in the concentration of sugars in the axes could be observed at stage 6, 0,81 µg/mg FW, while in the cotyledon it decreased to 1,56 µg/mg FW. In contrast, in embryos at stage 7 most of the sugar is concentrated in the axes, 2,81 µg/mg, and an increase in the cotyledon sugar levels occurs, 2,14 µg/mg FW (Figure 39).

The levels of starch are around 10 fold lower than soluble sugar levels. The stages 5 and 6 present higher concentrations of starch in the cotyledons while in the axes values are near zero. In embryos at stage 7 the concentration of starch is inverted; the axes contain the highest amount of starch, while levels in cotyledons are reduced drastically to values near zero (Figure 40). At stage 5, the cotyledons contained 0,28 µg/mg FW and the axes 0,04 µg/mg FW. The highest amount of starch in the cotyledons was detected at stage 6, 0,38 µg/mg FW, while at the same time the axes at this stage has the lowest starch content, 0,01 µg/mg FW. The opposite is true in germinated embryos (stage 7) where an increase in starch content is observed, however, most of it is concentrated in the axes, 0,49 µg/mg FW while the cotyledons contain 0,02 µg/mg FW of starch.

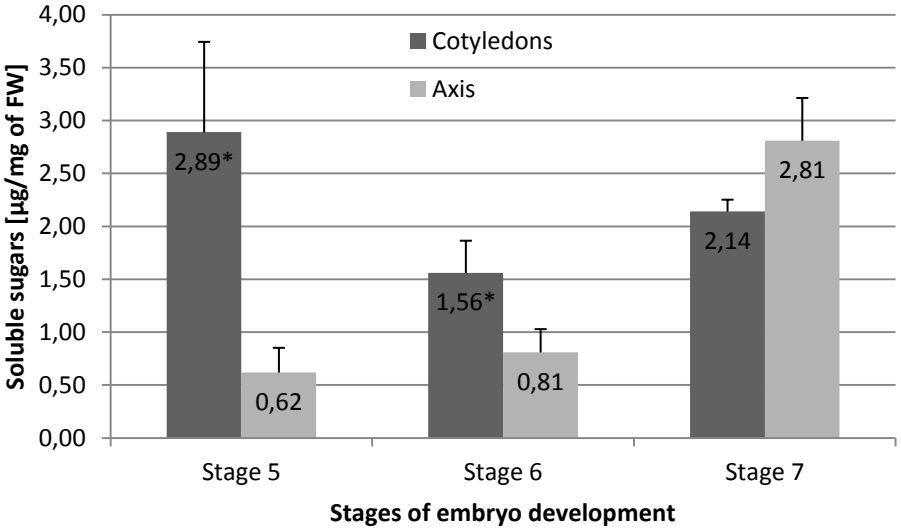

Figure 39. **Content of soluble sugar in cotyledons and axes of cacao somatic embryos grown on solid medium at different stages of late development.** Error bars represent means+standard error (n=2;*n=3).

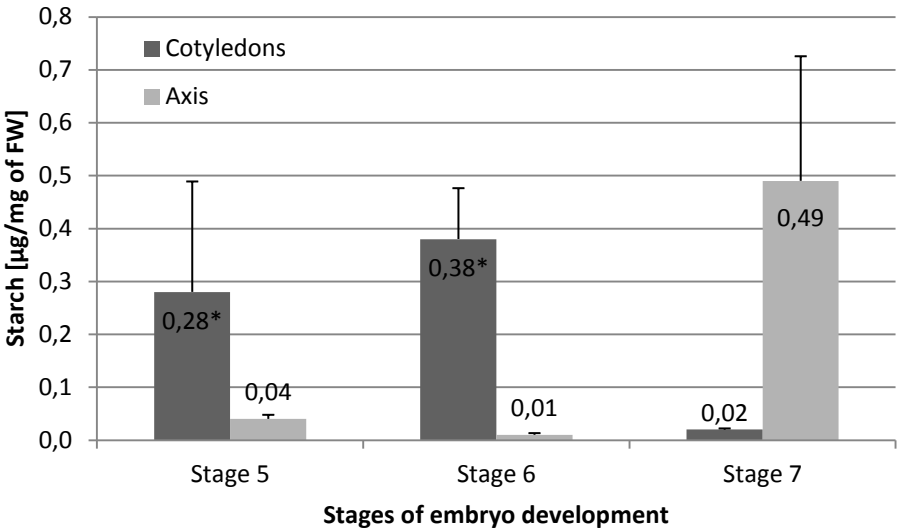

Figure 40. **Starch content in cotyledons and axes of cacao somatic embryos grown on solid medium at different stages of late development.** Error bars represent means+standard error (n=2;*n=3).

When the content of proteins and carbohydrates in the embryos is compared to each other, it is possible to observe the dynamics of reserves accumulation in somatic embryos grown on solid medium (Figure 41). The higher levels of protein and sugar in the germinated somatic embryos, mainly in the axes, may underscore the function of this organ in the physiological regulation mechanisms of somatic embryos. It is expected that germinated embryos show similar or lower concentrations of these

compounds than what is found in mature embryos. During the germination process the mobilization of reserves takes place in the storage organs. Proteolysis enhances the amount of mobile peptides which deliver new protein to the freshly growing tissues.

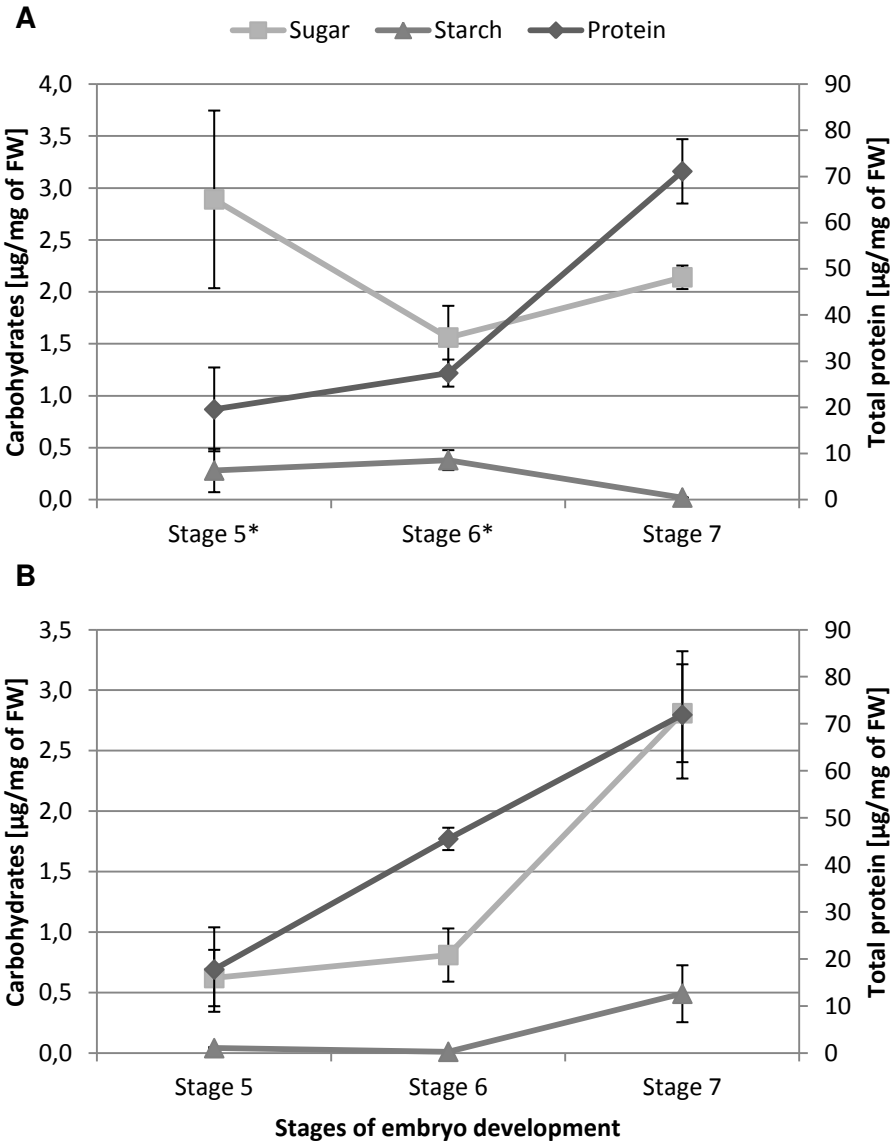

Figure 41. **Accumulation of proteins and carbohydrates in the cotyledons (A) and axes (B) of cacao somatic embryos grown on solid medium at different stages of late development**. Error bars represent means±standard error (*n=3; n=2).

4.2.3.2 Late development of cacao somatic embryos in TIS.

Cacao somatic embryos were grown and germinated in TIS for observation of morphological development. In contrast to somatic embryos germinated on solid medium, these embryos survived for longer time in culture, demonstrated low oxidation of the axes and no callogenesis was detected on the apical meristem.

All embryos turned light green after some hours of culture, but this evolved to dark green in a few days. The embryos did not lose the cotyledons and they remained attached to the embryo during the whole period of culture. The further differentiation was heterogeneous. Some somatic embryos showed significant growth of the cotyledons but did not present rooting. When later on roots were formed, these emerged abruptly from the axis apices were considerably thinner than the axis and seemed abnormal.

In another group of embryos cotyledons did not increase in size but the axes were elongated. These had a yellowish color that turned dark green in the region proximal to the cotyledon. Apparently normal germination or rooting occurred. The roots emerged continuously from axis apex and had a brownish color. Sometimes adjacent roots were observed. A third group of embryos did not show rooting nor cotyledonary growth. Only elongation of the axes and browning of its apices were noted. The emission of leaves was detected only in a few embryos and these were very small, translucent and yellowish. Supposedly these were at an earlier stage before root emission. Some embryos turned green without presenting any other morphological modifications (Figure 42).

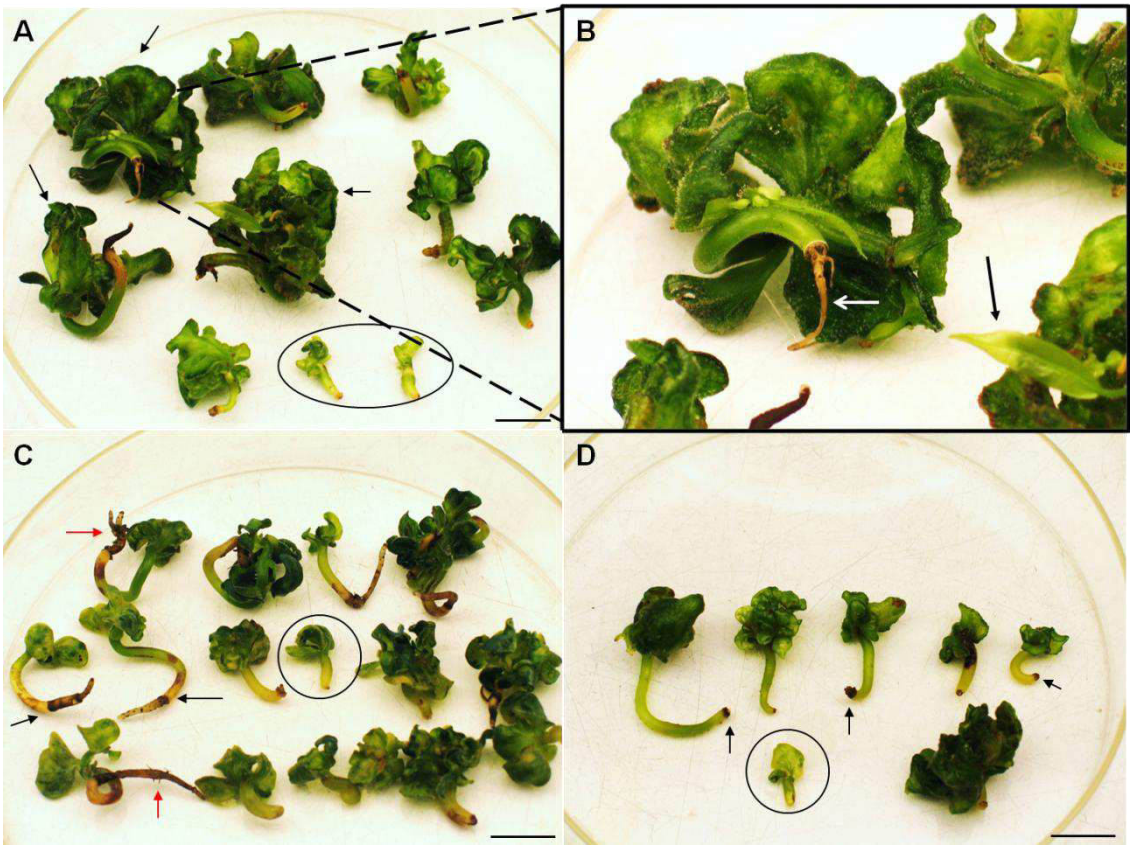

Figure 42. **Cacao somatic embryos grown and germinated in TIS. A-** Somatic embryos after 30 days in germination medium, arrows indicates the cotyledons grown during the germination period. **B-** Detail of a somatic embryo with big cotyledons and worthless root growing abruptly from the axe (white arrow); black arrow shows a small yellow translucent leaf. **C-** Somatic embryos with apparently normal rooting (black arrows), red arrows indicate roots with adjacent roots. **D-** Non-germinated somatic embryos with brownish axes apices (arrows). Circled somatic embryos did not show morphological changes during the germination.

The fact that these somatic embryos developed functional roots and displayed vigor indicates a distinct physiological development from that observed on solid medium. The embryos with roots were subsequently cultivated in germination medium in TIS while the remaining embryos were submitted to medium for rooting induction. The somatic embryos cultured in rooting induction medium developed calli in the axis and did not develop further. Most of the embryos in the germination medium survived for no more than one month. Those that developed leaves were subsequently grown in TIS. After 6 months of culture in TIS the plantlets were transferred to *ex vitro* conditions into an acclimatization box, where they survived just for a few weeks. Despite the fact that plantlets had a vigorous appearance, the internodes did not elongate and the

leaves fell off after several days (Figure 43). It is believed that contaminations and not enough humidity contributed to death of the plantlets.

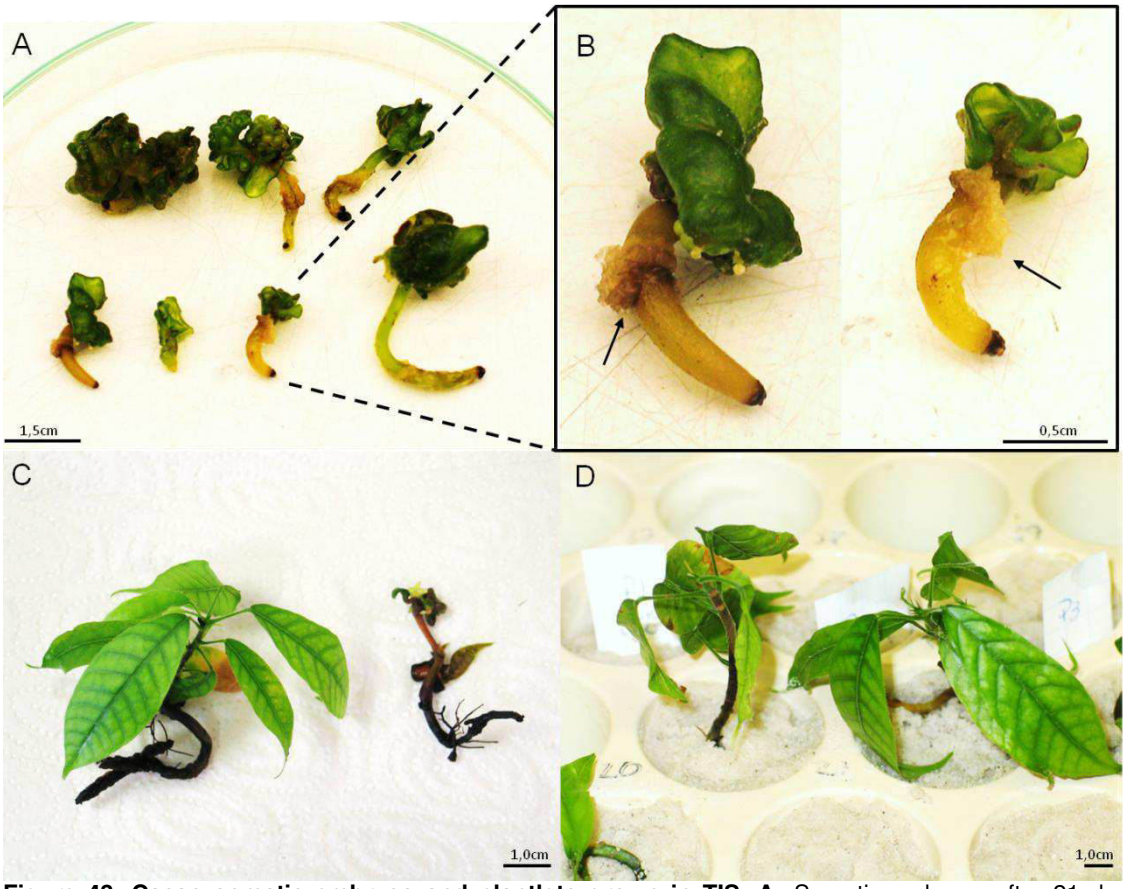

Figure 43. **Cacao somatic embryos and plantlets grown in TIS. A-** Somatic embryos after 21 days rooting treatment. **B-** Detail of somatic embryos with callus on the axis formed during culture in rooting induction medium (black arrow). **C-** Well developed (left) and abnormal (right) plantlets 6 months after germination. **D-** Well developed plantlets after transfer into acclimatization box.

Based on these observations, the contents of storage compounds were analyzed in cacao somatic embryos developed in TIS at different stages of development. The biochemical profile of cacao somatic embryos in early phases of development (torpedo shape, early cotyledonary and cotyledonary embryos or Stage 4) was compared to mature somatic embryos at Stage 6 (Figure 44). Because of the reduced size of the cotyledons, the embryos in early stages were analyzed in their entirety. Table 2 presents the values of cotyledon:axis ratio of somatic embryos at stages Torpedo, pre-cotyledonary and cotyledonary (stage 4).

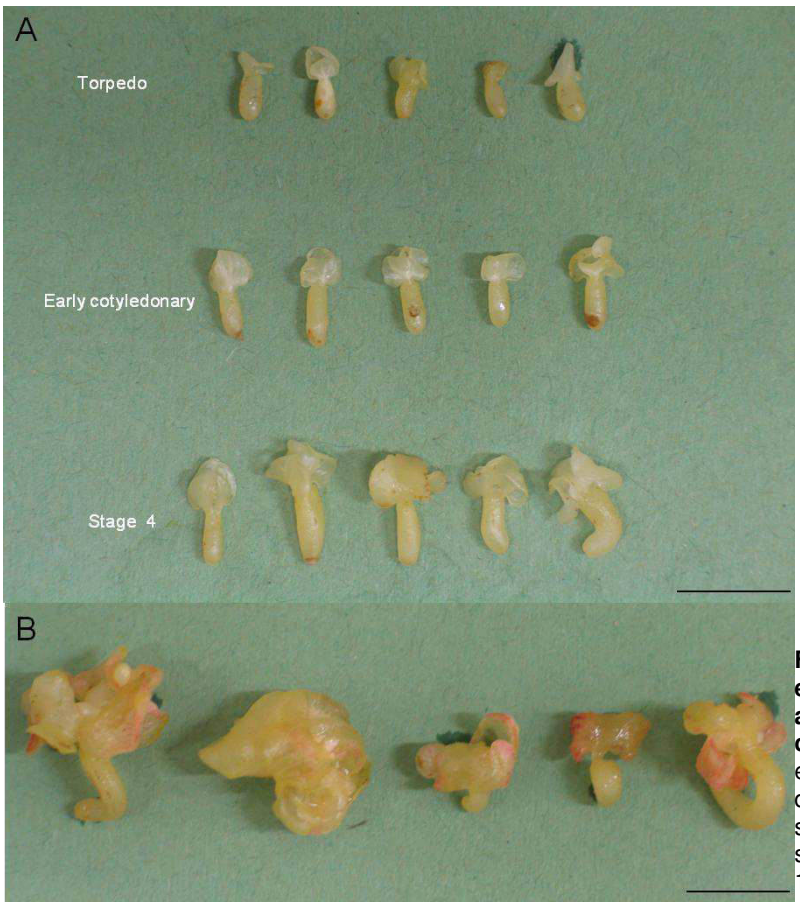

Figure 44. Cacao somatic embryos submitted to analysis of the phenolic compounds. A- Somatic embryos at early stages of development. **B-** Mature somatic embryos of cacao at stage 6 of development. Bar: 1cm.

Table 2. Proportion of cacao somatic embryos size at early stages of development.

	Size of cotyledons and axes of cacao somatic embryos		
	Torpedo Shape	Pre-cotyledonary	Cotyledonary (Stage 4)
Length of Cotyledons	0,23±0,02cm*	0,29±0,02cm	0,39±0,03cm
Length of Axes	0,36±0,02cm	0,47±0,03cm	0,63±0,06cm
Ratio cotyledon:axis	0,64<1	0,61<1	0,61<1

Content of proteins and carbohydrates

Somatic embryos developed in TIS showed considerably high amounts of total proteins already in early stages of development like torpedo and pré-cotyledonary stages (Figure 45). Small decreases in protein content were observed in somatic

embryos at stage 4, 194,81 µg/mg of dry weight (DW). This was the lowest protein content found in all stages. A significant increase in the content of total proteins was observed in cotyledons of somatic embryos at stage 6 (612,36 µg/mg DW). It was almost 3 fold higher than the level found in the axes of embryos at the same stage (245,77 µg/mg DW). The embryos at torpedo stages presented the second highest content of protein (337,84 µg/mg DW). However, it is not significantly higher than that found in pre-cotyledonary embryos, 322,12 µg/mg DW.

The high levels of proteins found in somatic embryos at stage 6 in TIS indicate that the process of storage protein accumulation takes place. At the same time, the lower concentration observed in the axes underlines that the accumulation of reserves mainly occurs in the cotyledons. It is considered to be a normal condition for accumulation of reserves in cacao embryos.

The highest concentration of soluble sugar was detected in embryos at Torpedo stage, 5,95 µg/mg DW. Levels decreased significantly during the following stages. The pre-cotyledonary embryos presented the lowest content when considering entire embryos, 0,35 µg/mg DW, but cotyledonary (stage 4) embryos had a similar concentration, 0,38 µg/mg DW. At stage 6, a slight increase was observed, and for the major part soluble sugars were concentrated in the cotyledons, 1,39 µg/mg DW, almost 4 times the amount compared to the axes, 0,23µg/mg DW (Figure 46). It indicates that cacao somatic embryos growing in TIS do not accumulate sugars during the development.

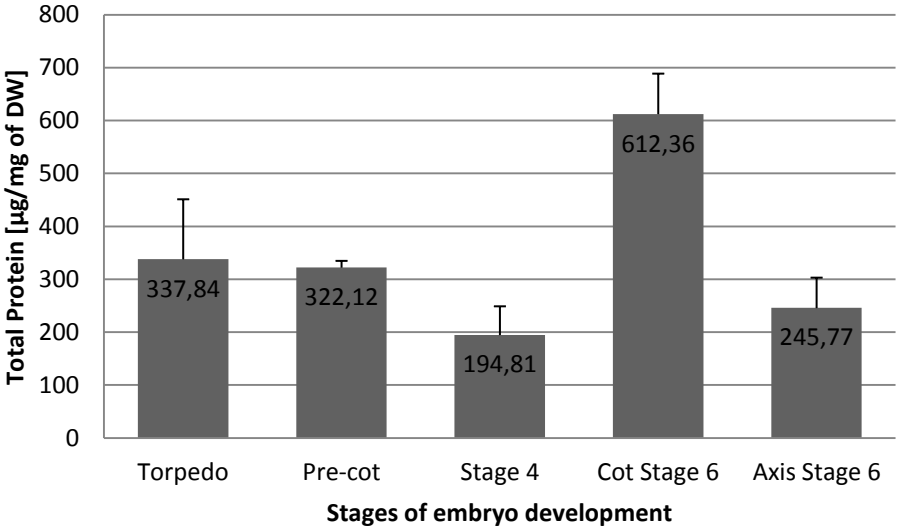

Figure 45. **Total protein content of cacao somatic embryos developed in TIS at different phases of development.** Pre-cot: pre-cotyledonary somatic embryos; Cot: cotyledons; Error bars represent means+standard error (n=3).

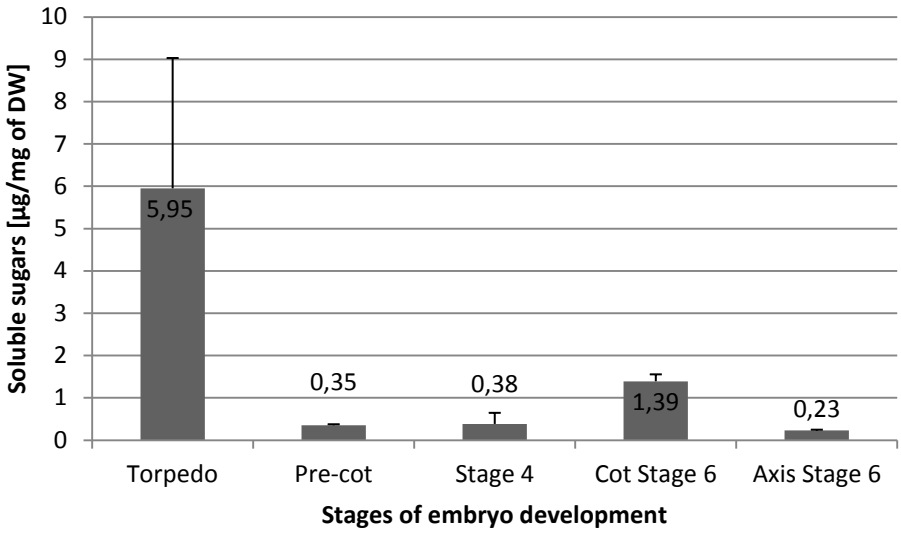

Figure 46. **Content of soluble sugars in cacao somatic embryos developed in TIS at different phases of development.** Pre-cot: pre-cotyledonary somatic embryos; Cot: cotyledons. Error bars represent means+standard error (n=3).

However, when the concentration of starch in these embryos was analyzed, a significant increase in the starch content was found at stage 6 of embryo development (Figure 47). The levels at this stage are around 10 fold higher than the levels found in embryos at early stages. Pre-cotyledonary embryos presented the lowest concentration of starch, 0,16 µg/mg DW, followed by torpedo-shaped embryos, 0,24 µg/mg DW and cotyledonary embryos, 0,39 µg/mg DW. Cotyledons and axes of embryos at stage 6

showed no significant difference in the content, but the concentration in the cotyledons was slightly higher, 3,7 µg/mg DW, than in the axes, 3,45 µg/mg DW.

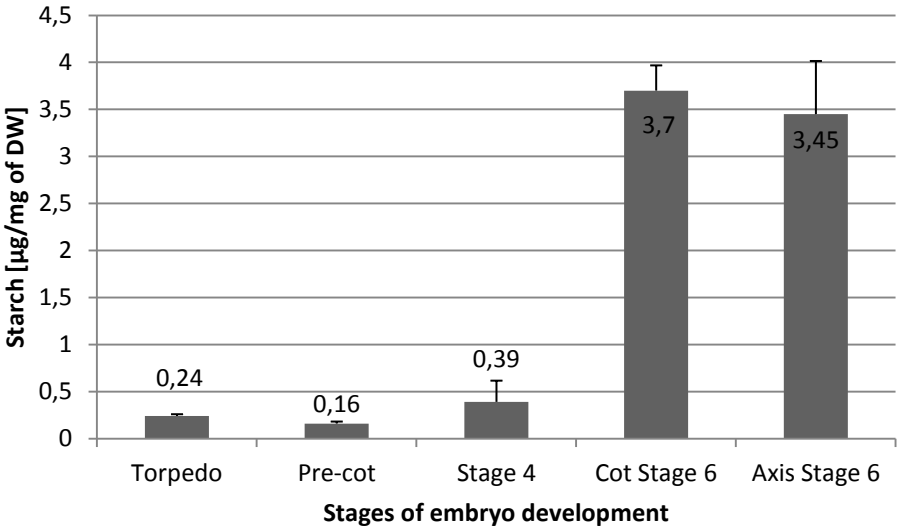

Figure 47. **Starch accumulation in cacao somatic embryos developed in TIS at different phases of development**. Pre-cot: pre-cotyledonary somatic embryos; Cot: cotyledons; Error bars represent means+standard error (n=3).

The dynamic of these compounds in the embryos throughout the developmental path shows a predominance of protein accumulation over carbohydrates (Figure 48). Despite the differences among the concentrations, it is possible to observe the increase of proteins and starch levels in the cotyledons of the mature embryos (stage 6), at the same time that sugar levels decrease. In contrast to the embryos cultured on solid medium, these embryos clearly accumulate reserves mainly in the cotyledons. At stage 6, all three compounds were present in higher concentrations in the cotyledons.

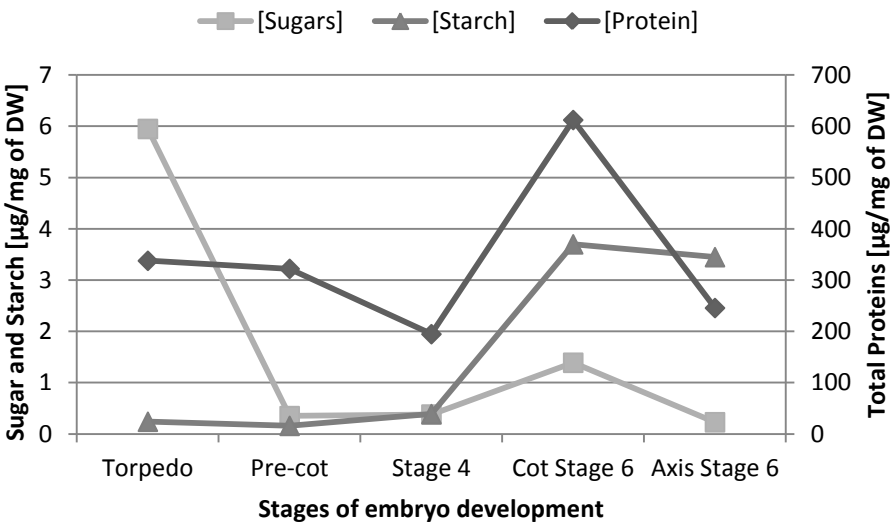

Figure 48. **Accumulation of storage compounds in cacao somatic embryos developed in TIS at different phases of development.** Pre-cot: pre-cotyledonary somatic embryos; Cot: cotyledons.

Total phenols content

Cacao is known as a plant with high contents of phenolic compounds in the seeds. In plants, phenols are related to differentiation processes, defense against herbivores and pathogens and are considered as indicators of physiological stress acting as antioxidants (Evert and Eichhorn, 2013; Taiz and Zeiger, 2007; Buchanan *et al.*, 2000). In the *in vitro* culture of plant tissues, phenols are related to recalcitrance and inhibition of callus and plant regeneration (Reis *et al.*, 2008; Alemanno *et al.*, 2003). In this work, accumulation of phenols in the cells of cotyledons of cacao somatic embryos was observed already in the early stages development (see section 4.3.1). Cacao somatic embryos at different stages of development were analyzed with regard to their total phenol contents. The HPLC spectra of polyphenols of mature somatic embryos were investigated for identification of polyphenols present in somatic embryos.

Already in torpedo stage high amounts of phenols are detected. Mature somatic embryos contain the highest concentration, as can also be observed in the histological analysis (see section 4.3.1). The phenols' levels decrease proportional to the embryonic growth during the early phase. Torpedo-shaped embryos showed levels of

47,51 µg/mg DW followed by pre-cotyledonary, 35,61 µg/mg and cotyledonary, 27,83 µg/mg DW. The somatic embryos in mature phases (Stage 6) presented phenols concentrated mainly in the cotyledons, 117,31 µg/mg DW, around twice the concentration found in the axes, 71,14 µg/mg DW (Figure 49).

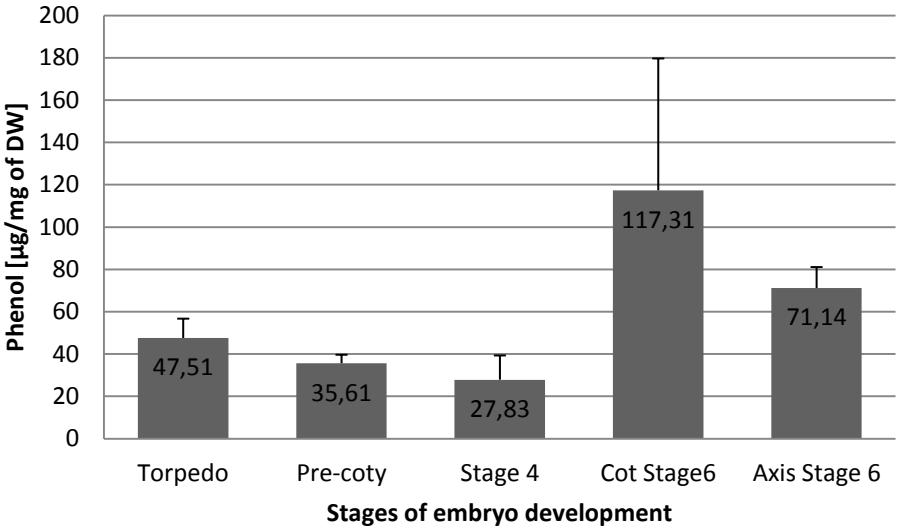

Figure 49. Content of total phenols in cacao somatic embryos developed in TIS at different stages of development. Pre-cot: pre-cotyledonary; Cot: cotyledonary. Error bars represent means+standard error (n=3).

The decrease in the concentration of relative amount of total phenols from torpedo-shaped embryos to cotyledonary embryos (Stage 4) is probably due to the rapid gain of biomass and storage compounds by the embryos at this phase of development (see above Table 2), while the endogenous levels of phenols remain stable.

Phenols of cotyledons and axes of somatic embryos were separated by HPLC for qualitative analysis. A comparison of the spectra detected differences in the peaks present in both tissues. The qualitative analysis of spectra of phenolic compounds from mature somatic embryos revealed that these compounds can be grouped into 2 major classes, based on their retention time (column Lichrospher 100 RP-18): epicatechin and respective oligomers, derivatives and conjugates of cinnamic acid and, additionally purines as typical secondary compound in *T.cacao* (Figure 50 and 52). These groups

delivered UV-spectra with several peaks. Most of the peaks were common to cotyledon and axis. These are epicatechin and the respective oligomers derivates (dimers to decamers) (peak at 231 and 278nm) and theobromin (peak at 232 and 272nm). Epicatechin and its oligomers were described by Hammerstone *et al*. (1999) in cacao seeds. Two peaks were detected only in the axis of mature and germinated embryos. These peaks presented a conformation similar to characteristic peaks of cinnamic acid amides derivates. The first peak consists of a minimum and a maximum peak ranging from 289 and 312nm characteristic for cinnamic acid amide, perhaps *p*-coumaric acid (Alemanno *et al*., 2003) (Figure 51). The second and more intensive peak had the typical conformation of cinnamic derivatives, like caffeic acid. It is characterized by a minimum and a maximum peak at 289 and 321nm, respectivelly. These peaks are similar to the peaks already described for cacao zygotic embryos and calli originated from flower pieces. However, the retention time of the peaks found in this work is different (Alemanno *et al*.,2003).

This study clearly showed the presence of considerable amounts of phenolic compounds already in very early stages of cacao embryo development. However, specific identification of these compounds is required for confirmation of the information presented here.

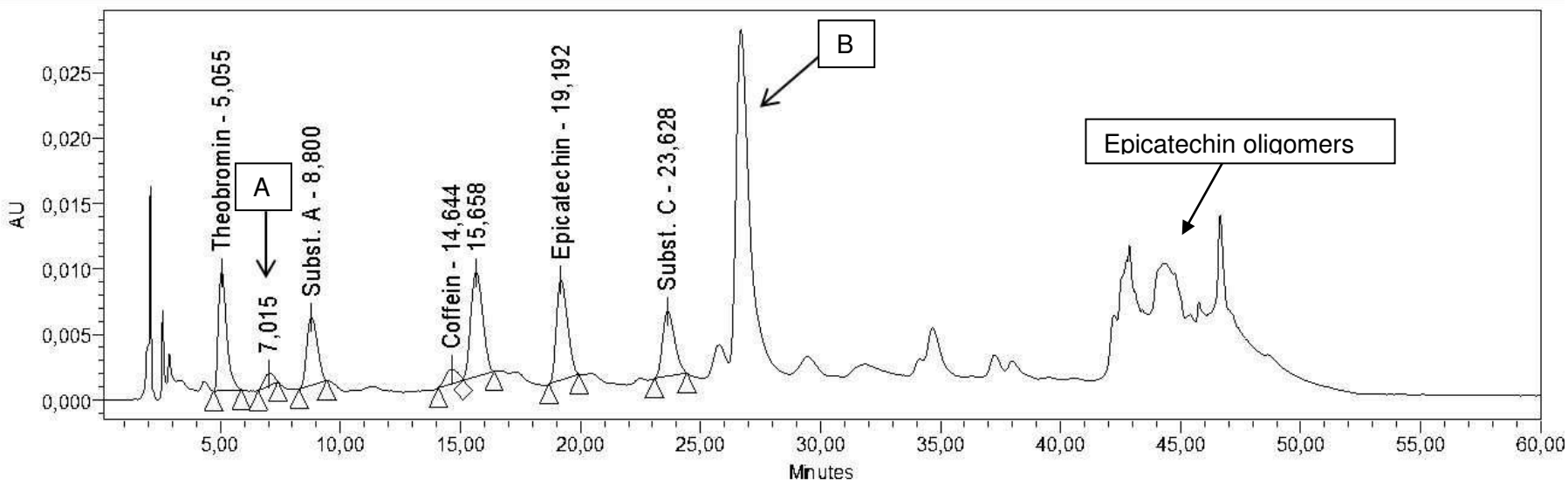

Figure 50. HPLC spectra of polyphenols in mature somatic embryos. Arrows A and B indicate peaks assumed to be cinnamic acid amides.

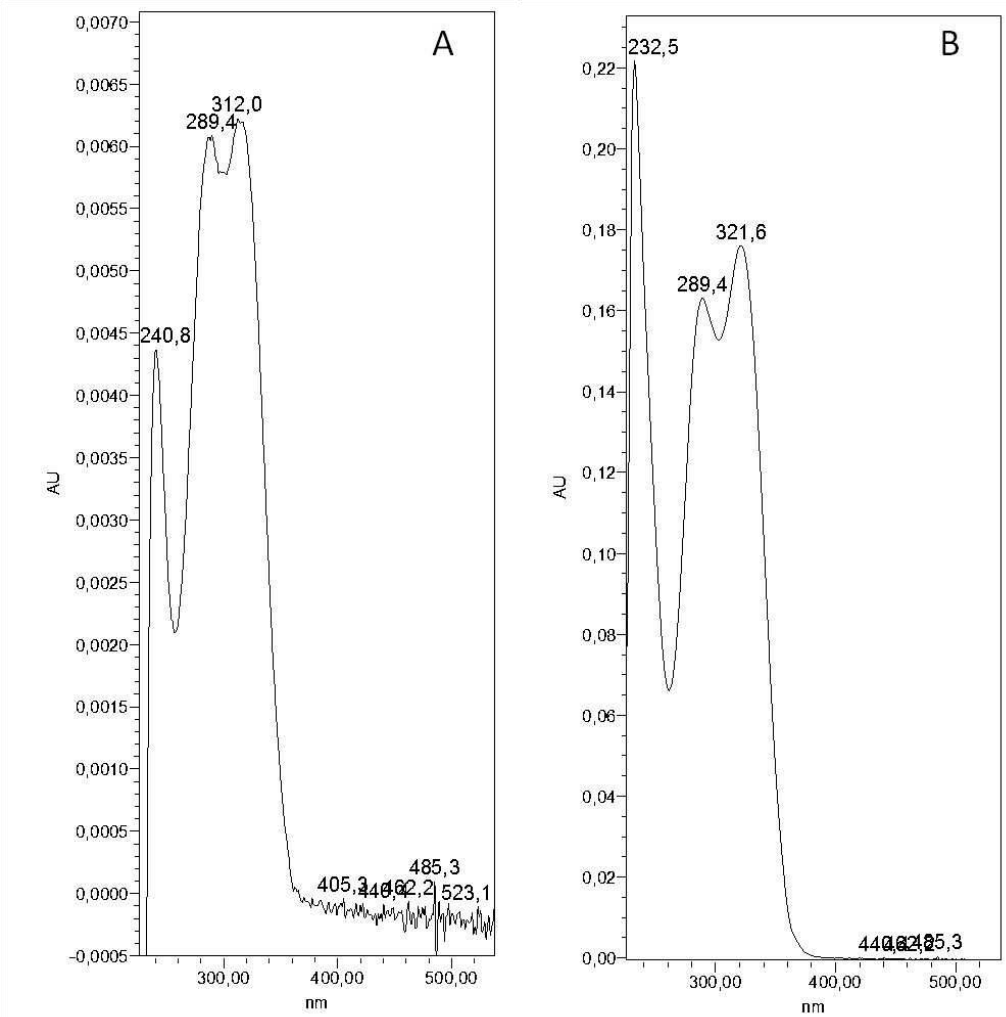

Figure 51. Spectrum peaks of polyphenols found exclusively in axes of mature somatic embryos. A- Peak at 7,015 minutes of retention. **B-** Peak at 25,13 minutes of retention.

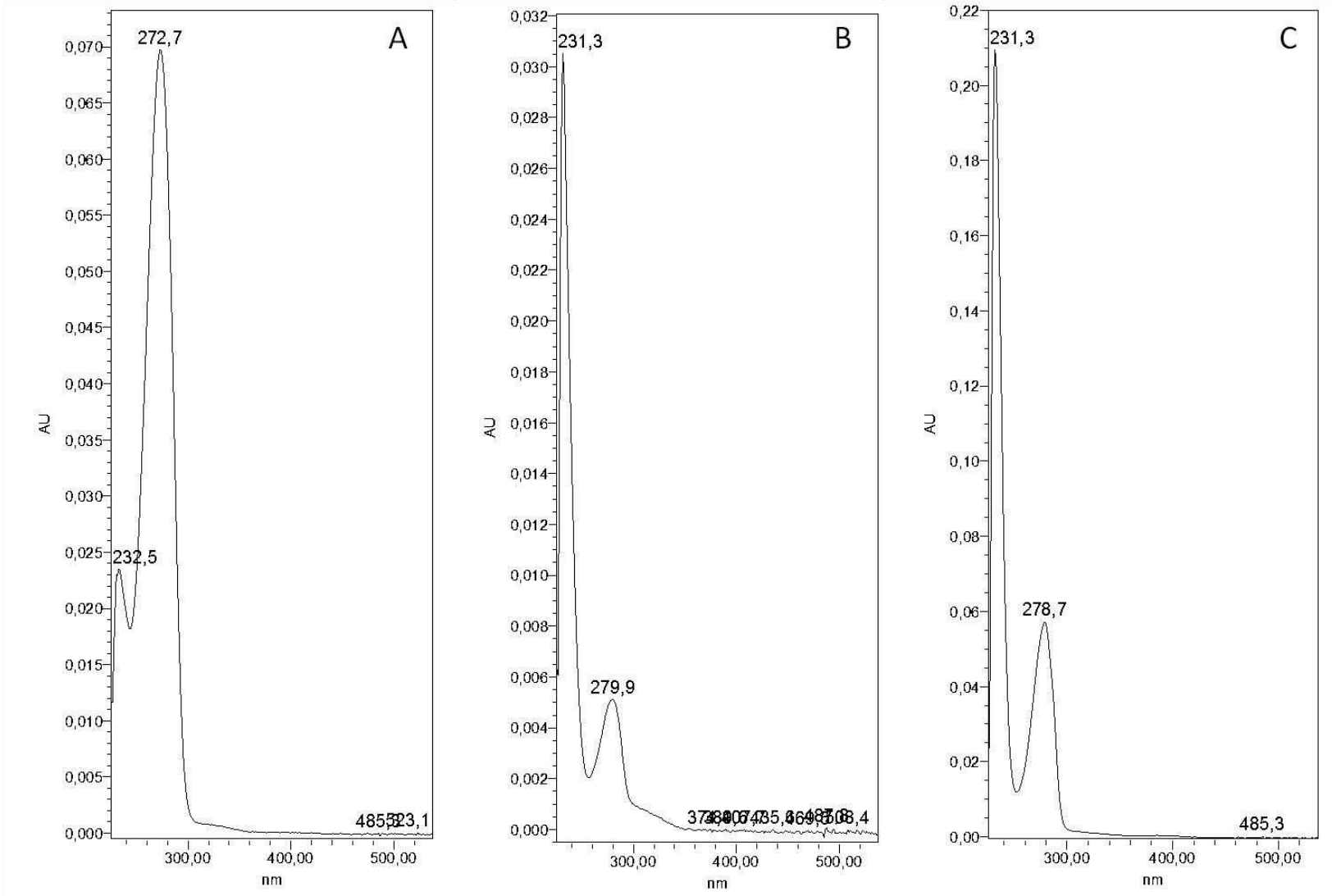

Figure 52. Typical peaks of polyphenols of cacao found mature somatic embryos . A- Theobromine peak. **B-** Coffein peak. **C-** Epicatechin peak. λ280nm.

4.2.3.3 Activity of Polyphenol oxidase in cacao somatic embryos during late development.

In addition to polyphenols present in the cacao seeds, the enzyme polyphenol oxidase (PPO) has been considered to be one of the main factors in the building of cacao flavor. Its activity and physico-chemical features in cacao seeds have been well described (Brito *et al*., 2002; Lima *et al*., 2001; Lee *et al.,* 1991; Wong *et al*., 1990). This enzyme has an important role in higher plants; however, it has rarely been analyzed in studies of somatic embryos. Cacao is known for its high content of polyphenols and high activity of PPO. Thus, it is fundamental to investigate somatic embryos of cacao *in vitro* for the comprehension of how these enzymes work in the secondary metabolism of these embryos.

Initially, somatic embryos from TIS culture at early cotyledonary stages, cotyledonary (Stage 4) and late cotyledonary (Stage 5) were submitted to analysis of O_2 uptake, to estimate the enzymatic activity during reaction with its substrate 4-methyl catechol (see Material and Methods, section 3.6). Early cotyledonary stage had the highest percentage of air consumed (3,14 µmol/gfw*h) during the reaction with substrate, followed by stage 5 (1,45 µmol/gfw*h) and stage 4 (2,26 µmol/gfw*h) (Figure 53).

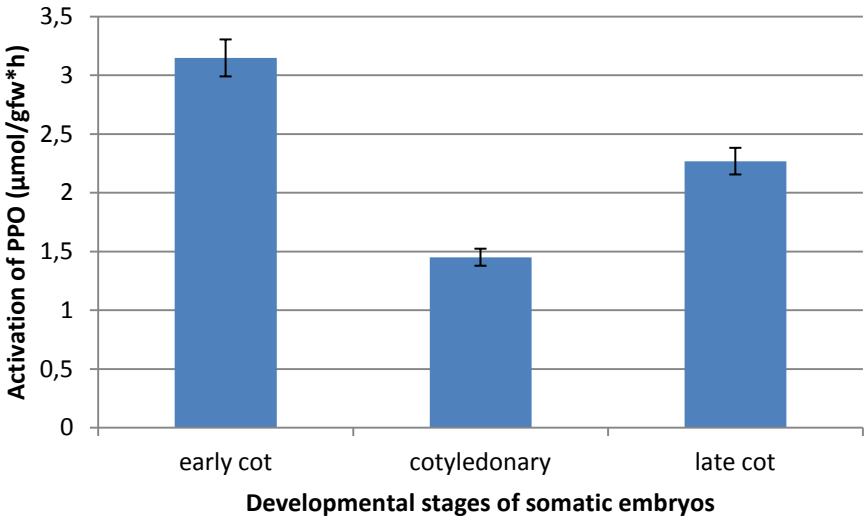

Figure 53. Polyphenol oxidase activity in somatic embryos of cacao at different stages of development. Standard error at 5%.

Based on the differences of phenol present in the cells of different stages of development described in the previous section (see section 4.3.2.2), the analysis was carried out separately for cotyledons and embryonic axes. Stages from 4 to 7 were analyzed to observe if PPO activity showed the same dynamic changes depending on developmental stages. In this analysis, the O_2 up take of the cotyledons varied from 0,007±0,26 µmol/gfw*h at stage 5 to 2,52±1,25 µmol/gfw*h at stage 4, results are presented in Table 3. No significant differences were found among the values from cotyledonary analysis. In contrast, the embryonic axis showed results dependent on the developmental stage. Embryonic axis of somatic embryos at stage 7 were unique in that they presented a different activity of PPO (158,42±19,09 O_2 µmol/gfw*h) compared to the other stages. Although the results for the embryonic axis were considerably higher than those found for cotyledons, except for stage 7, there was no significant difference in the O_2 up take of embryonic axes when combined with substrate. The values varied from 6,55±2,77 µmol/gfw*h at stage 6 to 9,23±3,16 µmol/gfw*h at stage 5a, embryonic axis at stage 5b, took 8,21±3,06 µmol/gfw*h of O_2 and at stage 4 it was 8,69±4,36 µmol/gfw*h.

Table 3. Activity of Polyphenol oxidase in cotyledons and axes of cotyledonary somatic embryos at different morphological stages. The results are given in O_2 uptake in µmol/gfw*h.

	Stage 4	*Stage 5a*	*Stage 5b*	*Stage 6*	*Stage 7*
Cotyledons	*2,52±1,25^{a*}*	*0,007±0,26^{a}*	*0,34±0,18^{a}*	*0,38±0,09^{a}*	*0,83±0,53^{a}*
Embryonic axis	*8,69±4,36^{a}*	*9,23±3,16^{a}*	*8,21±3,06^{a}*	*6,55±2,77^{a}*	*158,42±19,09^{b}*

*Means±standard error. Values followed by different letters in the lines represent significant differences determined by Tukey Test (p<0,05).

Figure 54 shows the huge difference between the embryonic axes and cotyledons among stage 7 and the others. These results demonstrate that the PPO activity is very expressed in the embryonic axes; it can be responsible for the

differences found between the values of the second and the first analysis, which was carried out with whole embryo bodies (cotyledons and embryonic axes together).

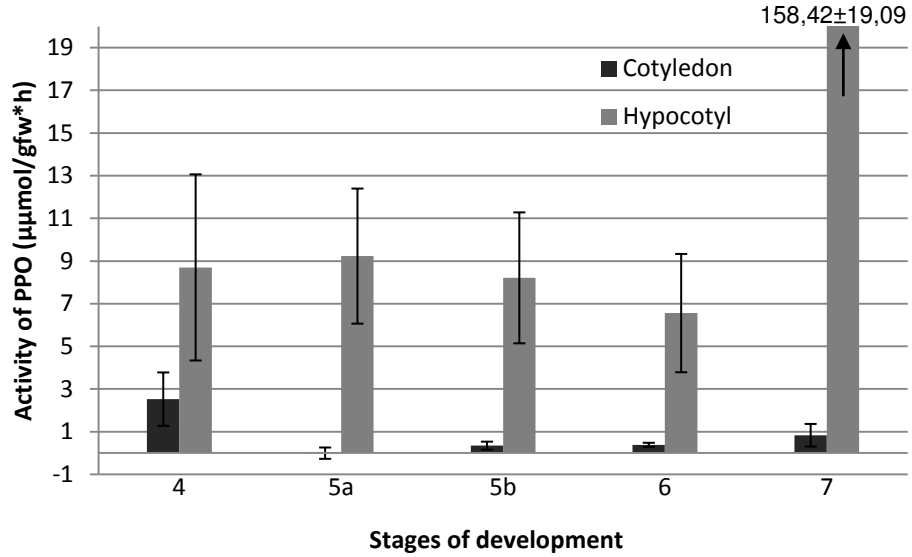

Figure 54. Polyphenol oxidase activity in somatic embryos of cacao at different stages of development. Values of means from 3 replicates and respective standard error presented in Table 3.

4.2.3.4 Amino acids profile of cacao somatic embryos in response to different culture conditions.

The quantification of amino acids has been used in plant physiology studies as an approach to metabolic status. Amino acids represent an important part of the response of somatic embryos to environmental factors, like culture conditions and media formulation. Thus, the objective of this study was to verify the profile of amino acids in somatic embryos from TIS and solid medium culture and cultured in germination medium in TIS. The dynamic synthesis of amino acids in the plants is regulated by factors like environment, development and metabolism. Because of that amino acids are considered markers of several physiological responses of the plants. The presence of different amino acids can indicate a distinct physiological status of the embryos cultured in TIS or on solid medium.

Significant differences were observed in the content of total amino acids in embryos from TIS and solid medium. Embryos cultures in germination medium, with

low concentration of sugar presented higher concentration of total amino acids than mature embryos cultured on solid medium but lower than mature somatic embryos cultured in TIS (Figure 55).

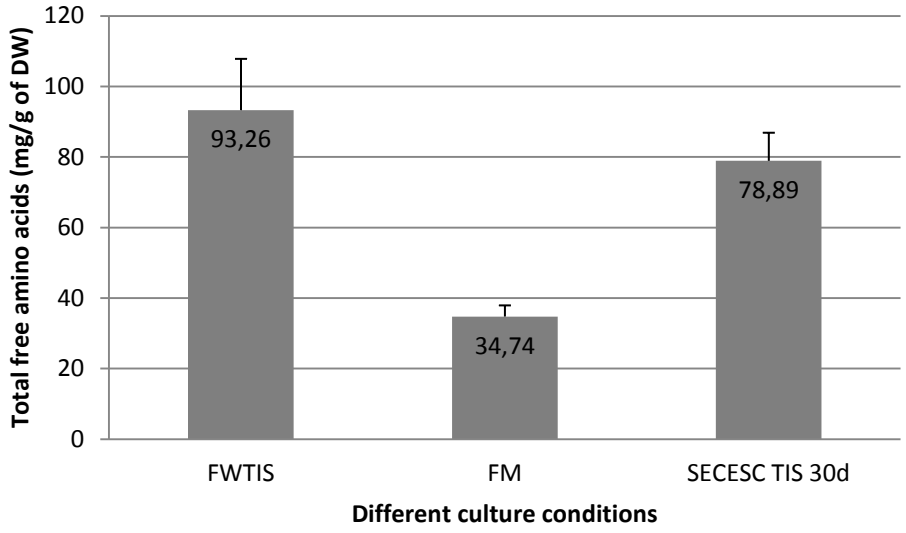

Figure 55. Concentration of total free amino acids in mature and germinated somatic embryos cultured in TIS and solid medium. FWTIS- somatic embryos matured in TIS; FM somatic embryos matured on solid medium; SECESC TIS 30 d- somatic embryos germinated in TIS after 30 days of culture. Error bars represents means±standard error (n=3).

The mature somatic embryos from TIS contained the highest concentration of total free amino acids (93,26 mg/g DW). When these somatic embryos were cultured in TIS for 30 days in culture media containing low quantities of sugars (Sucrose 10 g L^{-1}; Glucose 20 g L^{-1}) the level of total free amino acids decreased (78,89 mg/g DW). Mature somatic embryos cultured on solid medium, presented very low levels of free amino acids in comparison to the other culture conditions (34,74 mg/g DW).

The analysis of each amino acid separately showed ASN (Asparagine) as the most abundant amino acid found in maturing and germinated somatic embryos in TIS with values up to 60 mg/g DW. ARG (Arginine) and MET (Methionine) were some of the most abundant amino acids in the somatic embryos. The culture of the mature embryos in TIS influences the increase of amino acids content. Glutamine (GLN), Histidine (HIS)

and Tryptophan (TRP) were not detected in mature or germinated embryos under both culture conditions (Figure 56).

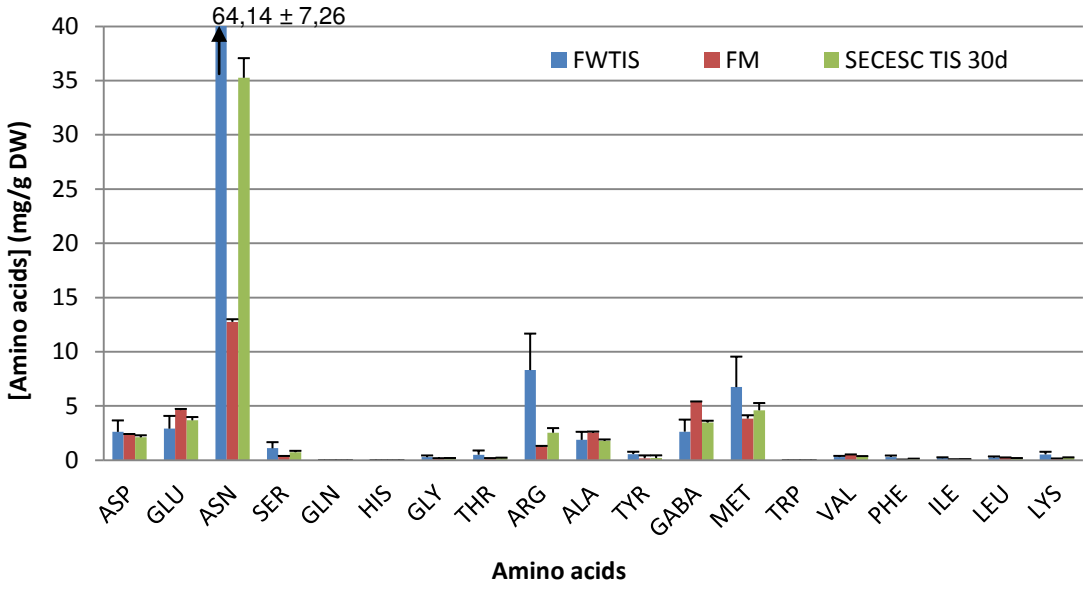

Figure 56. Concentration of free amino acids in mature and germinated somatic embryos cultured in TIS and solid medium, determined by HPLC. FWTIS- somatic embryos matured in TIS; FM somatic embryos matured on solid medium; SECESC TIS 30 d- somatic embryos germinated in TIS after 30 days of culture. Error bars represents means±standard error (n=3).

4.3 Features of post-embryonic development

4.3.1 Effect of high concentration of sucrose on the accumulation of reserve compounds in cacao somatic embryos (Stage 5).

For many species, one of the most common strategies for maturation of somatic embryos *in vitro* is to supplement the culture medium with high concentration of sugars, especially with sucrose. For cacao somatic embryos, both positive and negative effects on somatic embryo development have been described with respect to variation in sugar availability (Niemenak *et al.*, 2008; Traore & Guiltinan, 2006; Alemanno *et al.*,1996; Kononowicz and Janick, 1984; Pence *et al.*,1980). This study had the objective to identify biochemical changes which occur in cacao somatic embryos when submitted to different concentrations of sucrose for different periods of time on both solid medium as well as in TIS. Analyses were carried out concerning quantification of total protein, sugars and starch in cotyledons and axis of the embryos.

4.3.1.1 Maturation of cacao somatic embryos on solid medium supplemented with high concentrations of sucrose.

Total protein content

Cacao somatic embryos were submitted to a maturation on solid medium supplemented with 40, 60 or 80 g L^{-1} of sucrose for 2 weeks. The content of total proteins was analyzed. The figure 57 shows the significant influence of treatments with different sucrose concentration. Somatic embryos cultured under 40 g L^{-1} of sucrose showed the highest level of total proteins. However, these proteins were concentrated in the axis at 15,41 µg/mg FW (fresh weight), while in the cotyledons 0,7 µg/mg FW was detected. The higher accumulation of proteins in the axis was also observed in embryos cultured in presence of 80 g L^{-1} of sucrose, 7,52 µg/mg FW, but the cotyledons of these embryos presented a significant accumulation of proteins to 5,86 µg/mg FW. Only the somatic embryos maturated in culture medium containing 60 g L^{-1} of sucrose, had a higher level of total proteins in the cotyledons than in the axis, 7,19 and 4,25 µg/mg FW, respectively.

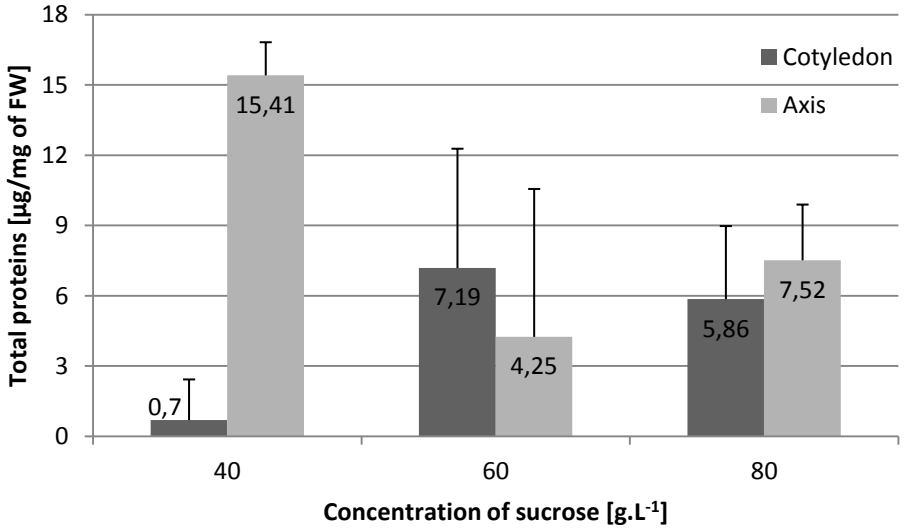

Figure 57. Total protein content in cotyledons and axis of cacao somatic embryos after 2 weeks on culture medium supplemented with different concentration of sucrose. Error bars represent means+standard error (n=2).

Considering standard conditions of accumulation of reserve compounds in cacao embryos, the results demonstrate an inefficiency of the embryos to accumulate such compounds. The treatment containing 60 g L^{-1} of sucrose in the culture medium formulation delivered the best result with respect to accumulation of proteins in the cotyledons.

Carbohydrates content

The carbohydrate levels in the somatic embryos were up to 10 fold lower compared to protein levels. Sugar levels in the cotyledons decreased with increasing sucrose concentration. The opposite occured with starch levels, which increased proportionally to sucrose concentration. In the axis sugars were positivly influenced by an increase in sucrose concentration, while starch decreased (Figure 58).

The levels of sugar in the axis of embryos were significantly higher than in cotyledons when cultured in the presence of 60 g L^{-1} and 80 g L^{-1} sucrose. In culture medium containing 6 g L^{-1} of sucrose the cotyledons contained 0,67 µg/mg FW (fresh weight), while the axis contained 0,88 µg/mg FW. At a concentration of 80 g L^{-1} of sucrose, cotyledons contained 0,38 µg/mg FW compared to 1.03 µg/mg FW in the axis. Embryos matured in culture medium containing 40 g L^{-1} of sucrose presented the highest concentration of sugars in both cotyledons and axis. Here the cotyledons had a concentration slightly higher than that of the axis, 1,20 µg/mg FW and 1,25 µg/mg FW, respectively.

In contrast, starch levels were highly concentrated in the cotyledons. Cotyledons of embryos matured in 80 g L^{-1} sucrose revealed the highest concentration of starch, 1,23 µg/mg FW. At the same time, the respective axis contained the lowest concentration, 0,29 µg/mg FW. Embryos cultured in medium supplemented with 40 g L^{-1} of sucrose accumulated 0,92 µg/mg FW in the cotyledons and 0,50 µg/mg FW in the axis. In cultures with 60 g L^{-1} of sucrose, an increase of the concentration of starch in

the cotyledons (1,12 µg/mg FW) and a decrease of the levels in axis (0,34 µg/mg FW) was observed compared to cultures containing 40 g L^{-1} of sucrose.

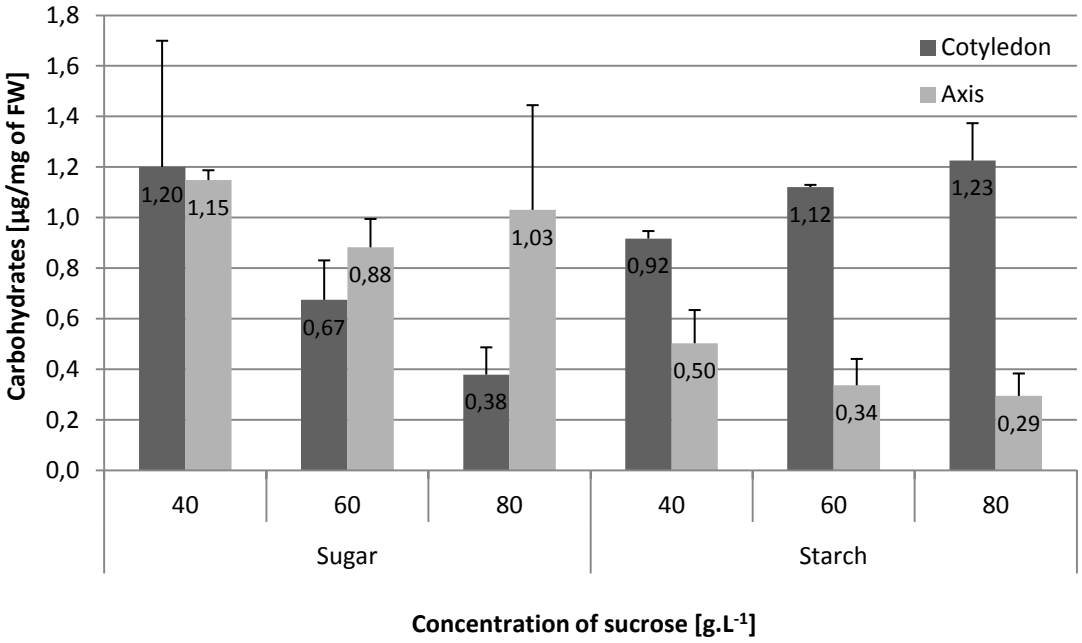

Figure 58. **Content of carbohydrates (Sugars and Starch) in cotyledons and axis of cacao somatic embryos after 2 weeks on culture medium supplemented with different concentration of sucrose.** Error bars represent means+standard error (n=2).

In summary, the concentration of sucrose in the culture medium clearly influences the accumulation of both these compounds within the embryo. In this study it was possible to observe that high concentrations of sucrose negatively affect sugar content of cotyledons, but lead to an increase in starch content. The opposite is true in the axis. The diminution of sugar in cotyledons of somatic embryos cultured in 80 g L^{-1} of sucrose may be a response to the amount of sugar available in the medium.

4.3.1.2 Maturation of cacao somatic embryos in TIS in presence of high concentration of sucrose.

Total proteins Content

Cacao somatic embryos at stage 5 were submitted to maturation in TIS. After one week of culture in TIS in the presence of high concentration of sucrose, total protein content was analyzed. The increase in the protein content of the cotyledons is proportional to the concentration of sucrose in the culture medium. However, the protein content in the embryo axis decreases when the sucrose concentration in the medium increased (Figure 59). The cotyledons of somatic embryos cultured in the presence of 40 g L^{-1} of sucrose showed the lowest levels of proteins (5,03 µg/mg FW). The highest content was found in cotyledons of somatic embryos cultured in the presence of 80 g L^{-1} of sucrose (23,4 µg/mg FW), while these also had the lowest protein amount within the axis (9,66 µg/mg FW), the axis of embryos cultured in 4 and 6 g L^{-1} of sucrose in the culture medium showed relatively high concentrations of proteins in the axis, 17,72 and 18,04 µg/mg FW, respectively. In the case of embryos cultured in 40 g L^{-1} of sucrose, the accumulation of protein was higher in the axis than in the cotyledons.

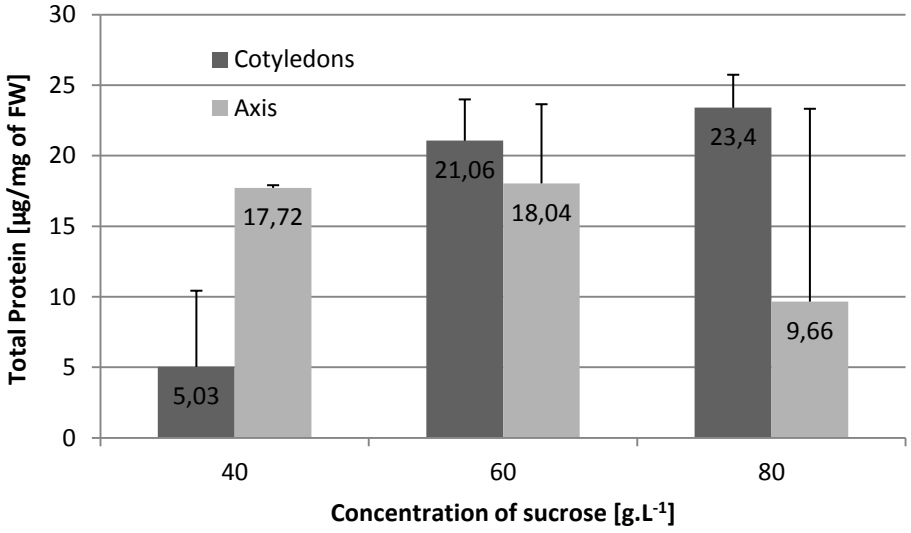

Figure 59. Total protein content in cotyledons and axis of cacao somatic embryos after 1 week in liquid culture medium (TIS) containing different concentration of sucrose. Error bars represent means+standard error (n=2).

The somatic embryos cultured for 2 weeks under the same conditions showed lower overall levels of proteins (Figure 60). For all three concentrations the axis displayed higher concentration of proteins than the cotyledons. The highest content of total protein after 2 weeks of culture was found in embryos cultured under 80 g L-1 of sucrose, where the axis contained 20,94 µg/mg FW (fresh weight) and the cotyledons 16,27 µg/mg FW. Somatic embryos maturated under 40 and 60 g L-1 of sucrose did not reveal significant differences in the protein content. However, embryos matured under 40 g L-1 of sucrose contained in the cotyledons and axis levels slightly higher, 9,84 and 13, 60 µg/mg FW, respectively. The cotyledons and axes of embryos cultured under 60 g L-1 of sucrose had a total protein concentration of 7,88 and 10,6 µg/mg FW, respectively.

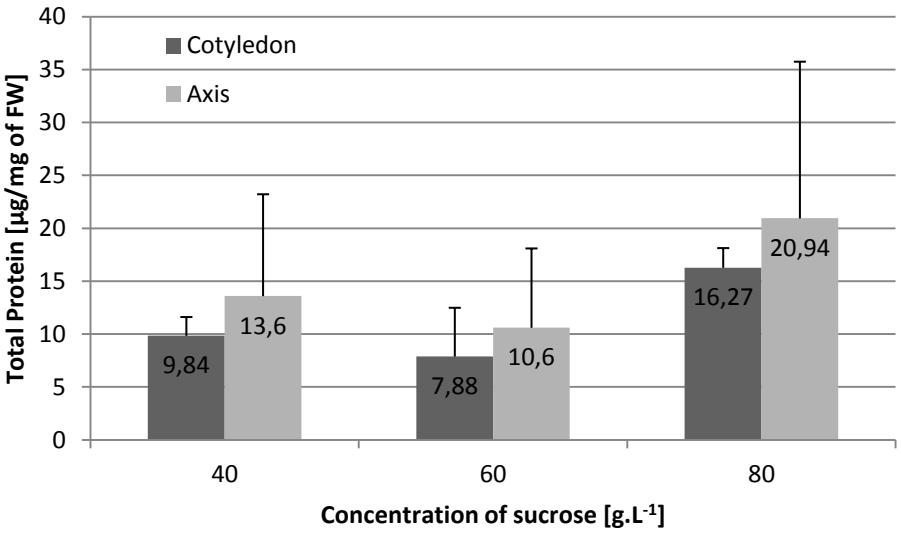

Figure 60. **Total protein content in cotyledons and axis of cacao somatic embryos after 2 weeks in liquid culture medium (TIS) containing different concentration of sucrose.** Error bars represent means+standard error (n=2).

The results show the influence of the culture period on the accumulation of proteins in the somatic embryos. Clearly, concentrations of 80 g L^{-1} of sucrose in the culture medium for 1 week resulted in highest amount of protein. The somatic embryos under this condition accumulated protein mainly in the cotyledons. Somatic embryos

cultured for 2 weeks under the same concentration of sucrose presented hyperhydricity, probably due to long exposure of the embryos to high osmotic pressure.

Content of carbohydrates

Similar to the results found on solid medium, somatic embryos cultured in TIS showed levels of sugars and starch that were around 10 fold lower than the respective protein levels. The content of sugar after 1 week of culture in the presence of high concentration of sucrose was higher than that of starch (Figure 61). The concentration of sugar in the cotyledons decreased with increasing sucrose concentration in the culture medium. This did not happen in the sugar content of the axis, in this case the sugar concentration in the embryo axis rise with the sugar concentration in the culture medium.

The highest concentration of sugars was detected in the axis of embryos cultured in the presence of 8 g L^{-1} of sucrose (2,88 µg/mg FW). The cotyledons of the same embryos presented the lowest concentration of sugars, 0,91 µg/mg FW. Embryos cultured in 60 g L^{-1} of sucrose had similar levels of sugar in cotyledons and axis, 1,73 and 1,78 µg/mg FW, respectively. Only embryos cultured in medium containing 40 g L^{-1} of sucrose revealed higher concentrations of sugar in the cotyledons than in the axis (2,36 and 1,81 µg/mg FW, respectively).

The starch content in the embryos was considerably lower than that of sugars. Higher concentration of sucrose in the culture medium caused an increase in the starch content in both axis and cotyledons, with the starch mainly concentrated in the cotyledons. An exception were embryos cultured in 60 g L^{-1} of sucrose, where similar levels were in both cotyledons and axis, 0,43 µg/mg FW. Embryos maturing in 40 g L^{-1} of sucrose in the culture medium showed the highest difference between cotyledons and axis starch content, 0,56 and 0,11 µg/mg FW, respectively. Embryos cultured in 80 g L^{-1} of sucrose showed the highest content of starch, where cotyledons contained 0,78 µg/mg FW and axis 0,50 µg/mg FW.

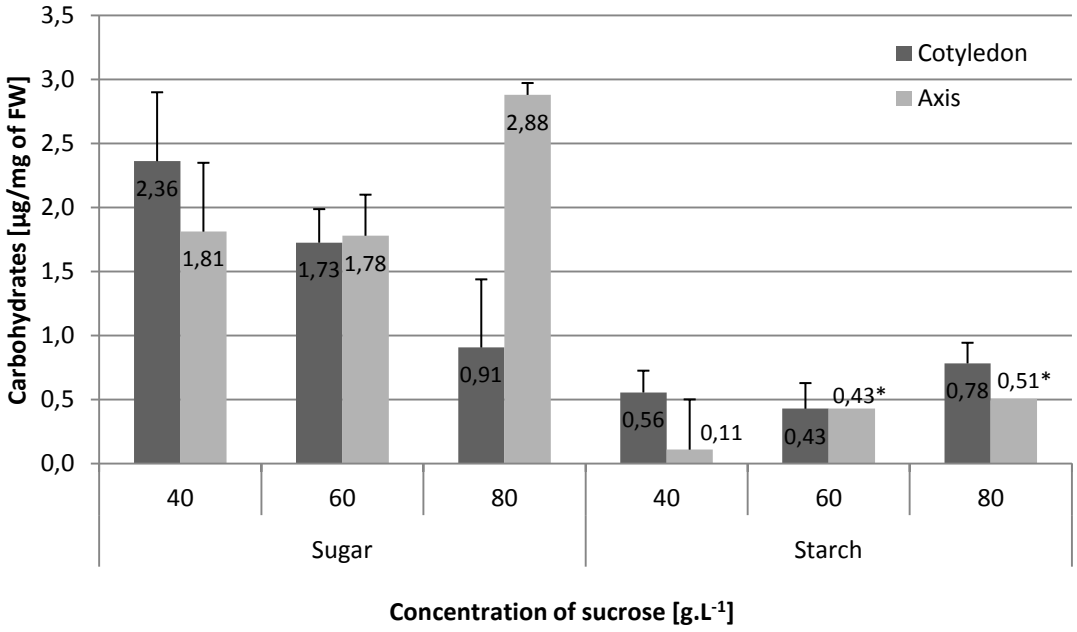

Figure 61. Content of Carbohydrates (sugars and starch) in cotyledons and axis of cacao somatic embryos after 1 week in liquid culture medium (TIS) containing different concentration of sucrose. Error bars represent means+standard error.(n=2); *(n=1).

Results for the somatic embryos cultured for 2 weeks in maturation media were similar in that they also contained higher amounts of sugars than starch (Figure 62). The content of sugar in cotyledons and axis decreases with the increase of sucrose concentration in the culture medium. In embryos cultured in the presence of 40 g L^{-1} of sucrose, the cotyledons contained higher concentrations of sugars than the axis (5,32 and 3,23 µg/mg FW, respectively). In the other treatments of 60 g L^{-1} and 80 g L^{-1} of sucrose, the axis accumulated most of the sugar. In 60 g L^{-1} sucrose, the cotyledon had 2,03 µg/mg FW and the axis 2,19 µg/mg FW. In cultures with 80 g L^{-1} of sucrose, the cotyledons had the lowest levels of sugar, 0,26 µg/mg FW. The axis contained 1,47 µg/mg FW.

The overall levels of starch in somatic embryos increased during the longer culture period. However, the starch mainly accumulated in the axis. These levels were consistent in three different concentrations of sucrose, 1,91 µg/mg FW in 40 g L^{-1} of sucrose culture, 1,88 µg/mg FW in 60 g L^{-1} of sucrose and 1,90 in 80 g L^{-1} of sucrose. The highest starch content in the cotyledons was detected in embryos cultured in 80 g

L^{-1} of sucrose (1,96 µg/mg FW). It was not significantly higher than axis content (1,90 µg/mg FW). The lowest content was observed in cotyledons of embryos from cultures with 60 g L^{-1} of sucrose, 0,60 µg/mg FW. The cotyledons from cultures with 40 g L^{-1} of sucrose presented 1,43 µg/mg FW.

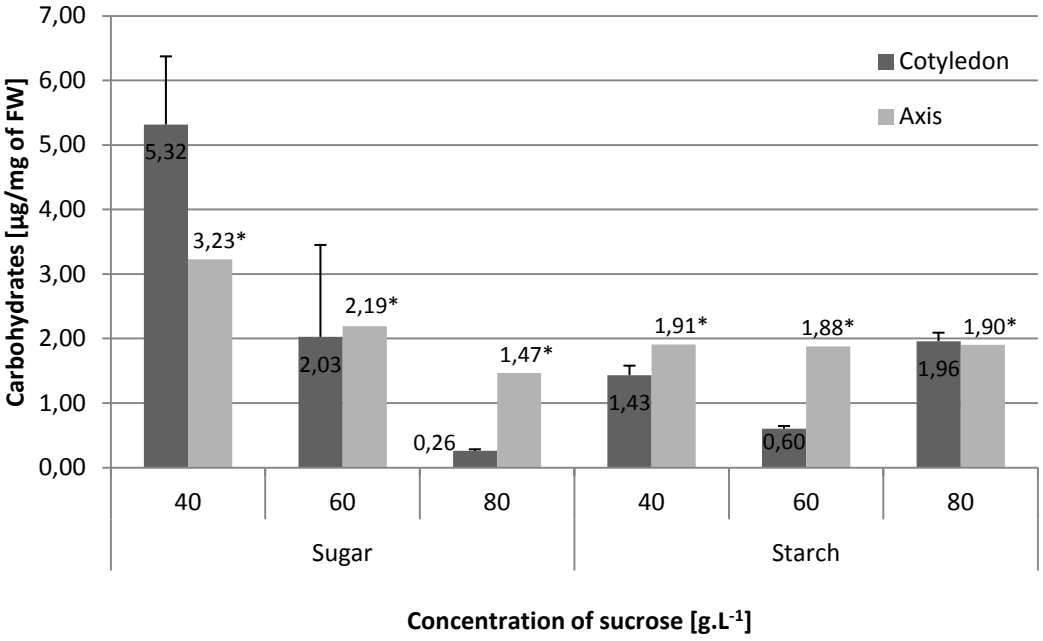

Figure 62. **Content of Carbohydrates (sugars and starch) in cotyledons and axis of cacao somatic embryos after 2 week in liquid culture medium (TIS) containing different concentration of sucrose.** Error bars represent means+standard error.*n=1.

The time of culture in the presence of high concentrations of sucrose clearly influences the accumulation of carbohydrates. In contrast to the proteins, the accumulation of carbohydrates increased when cultured for 2 weeks. The highest amounts of both, protein and carbohydrates remained in the axis, except for embryos matured in culture medium containing 40 g L^{-1} of sucrose. These embryos accumulated significantly higher amounts of sugars in the cotyledons in both one and two weeks of culture. Embryos cultured for one week also presented higher concentrations of starch in the cotyledons. The accumulation of proteins is favored by culture in liquid medium containing 60 g L^{-1} of sucrose for one week. With respect to develop as optimal condition for maturation and germination, it can be summarized that sucrose concentration and duration of the culture influence the amount and the composition of the storage compounds in the somatic embryos in the pre-germination phase. Further

observations are required to confirm the real effect of each culture conditions on somatic embryos development.

4.3.2 Germination of cacao somatic embryos and conversion into plantlets.

Fully developed seeds start to germinated and to form plantlets. For these complex differentiation processes many physiological reactions are achieved and most follow a precise developmental sequence. The pre-germination phase often is induced by external triggers. In this study on biotechnological sequences which finally end up in somatic embryos with a potential to germinate, the variation of the protein content was quantified. The objective was to identify appropriate pre-conditions to achieve good germination and conversion of the somatic embryos. The experimental conditions and the sample collection are given in the figure 63.

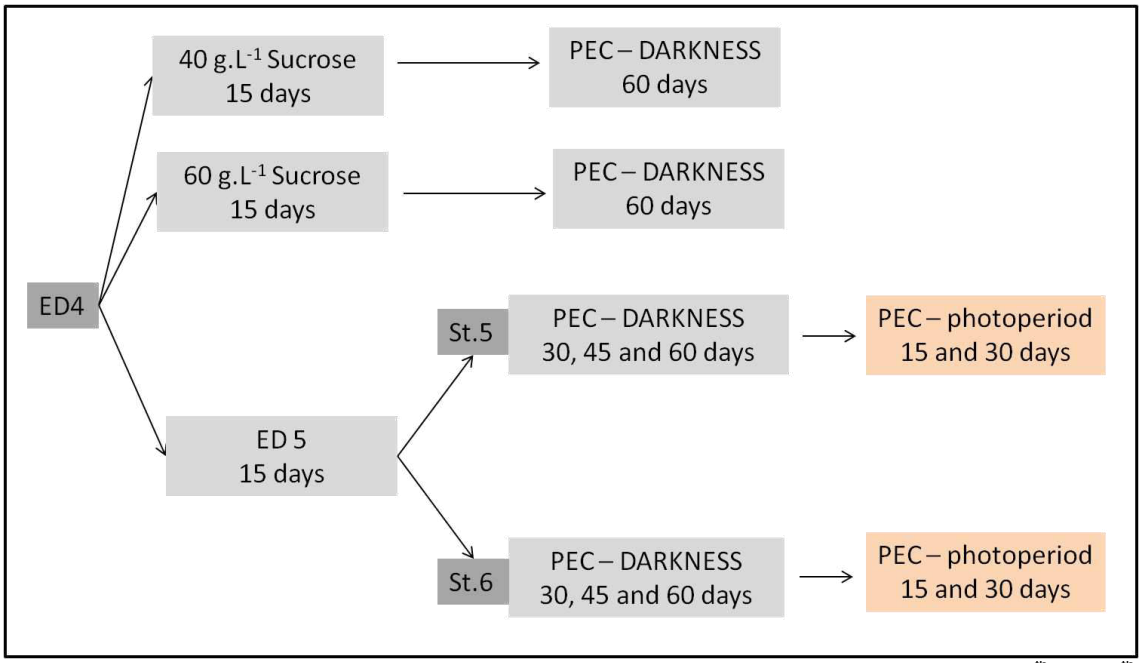

Figure 63. Experimental design presenting the times of sample collection. ED4 and 5- 4th and 5th subculture in expression media (ED), respectivelly; PEC- primary embryo conversion media; St. and St. 6- somatic embryos at stage 5 and 6 of development, respectively. (see section 3.2, Material and Methods).

4.3.2.1 Morphological differentiation during germination after maturation in presence of high concentrations of sucrose

Embryos of developmental stage 4 (Figure 64A) were used for the growth phase and maturation process with 40 or 60 g L^{-1} of sucrose. In figure 64 the embryo maturation after 15 days of incubation at 40 g L^{-1} (Figure 64 B) and 60 g L^{-1} (Figure 64C) of sucrose in absence of light is shown. The cotyledons grew considerably and first formation of roots tip was seen. The browning of the tissue in the axis apex could be observed in both cases. The cotyledons were characteristic by red regions of the tissue. It must be noticed that the genotype under study is a Trinitario (RO3.08/01-Madagascar) which in zygotic embryos red regions on cotyledons are also formed in combination with white to cream colored tissue.

After 30 days transfer to germination culture medium (PEC; see section 3.2, Material and Method) the embryos matured in presence of 40 g L^{-1} had green or white cotyledons. The hypocotyls were longer than after 15 days and the root tips were more pronounced. Some hypocotyls were also greenish, but these were partially translucent. Only a few embryos emitted roots, which were brown and as thick as the axis. Callogenesis on shoot apical meristem was rarely observed (Figure 64D). After 60 days of culture, the cotyledons took up more volume and most of the embryos revealed necrosis in former green translucent axes. Other embryos remained with white cotyledons and axes. Green cotyledons showed hyperhydricity. Only thick roots evolved and remained brown. Callogenesis was still observed but in only a few embryos. At the end of this period, most of the somatic embryos still maintained the cotyledons (Figure 64F). The number of germinated embryos did not increase after 60 days, but other embryos began root formation.

The embryos from maturation in presence of 60 g L^{-1} germinated or not, were transferred to germination culture medium with low sucrose content. After 30 days of culture in that medium, an increase in the frequency of germinated embryos was observed. Some of the cotyledons were green, but in majority white with pink/red

regions. No further increase in cotyledons or axes size was observed. The axes were still white, but the embryonic root stayed brown. Elongation of roots already existent was observed only when these were as thick as the axis. Thin roots did not evolve during this period. Many embryos at this phase showed a brownish basal apex (Figure 64 E). At the end of the germination period (60 days), the most of the embryos were germinated and other embryos had begun the root emission. Necrosis of the embryo body was observed in a few embryos, they did not survive. After 30 days of culture in germination culture medium, it was already possible to observe formation of calli on the shoot tip of some embryos. The callogenesis occurrence increased significantly after 60 days when almost all embryos presented this characteristic. The embryos which did not demonstrate callogenesis remained with their cotyledons, which were white with pink/red parts, but they had not as intense of a color as in the beginning of the culture. The axes were white and the thick roots developed normally (Figure 64 G).

In both cases of 40 and 60 g L^{-1} sucrose in the culture medium, the embryos which presented brownish root tips soon after emitted the root. These germinated embryos, in majority, had thick roots which evolved normally. Brownish root tips can be considered a marker of normal rooting during germination. Most of the embryos with roots presenting these characteristics developed into plantlets. The lower rate of germination in culture with 40 g L^{-1} sucrose in the culture medium, suggests that 60 g L^{-1} sucrose improves the germination and development of the embryos. However, embryos cultured under this condition frequently presented callogenesis on the shoot apex and a considerably high rate of germination already during the maturation period. It can lead to lack of vigor due to the early consumption of the reserves and no development of shoot apical meristem, because callus formation on this tissue interferes with apical shoot growth.

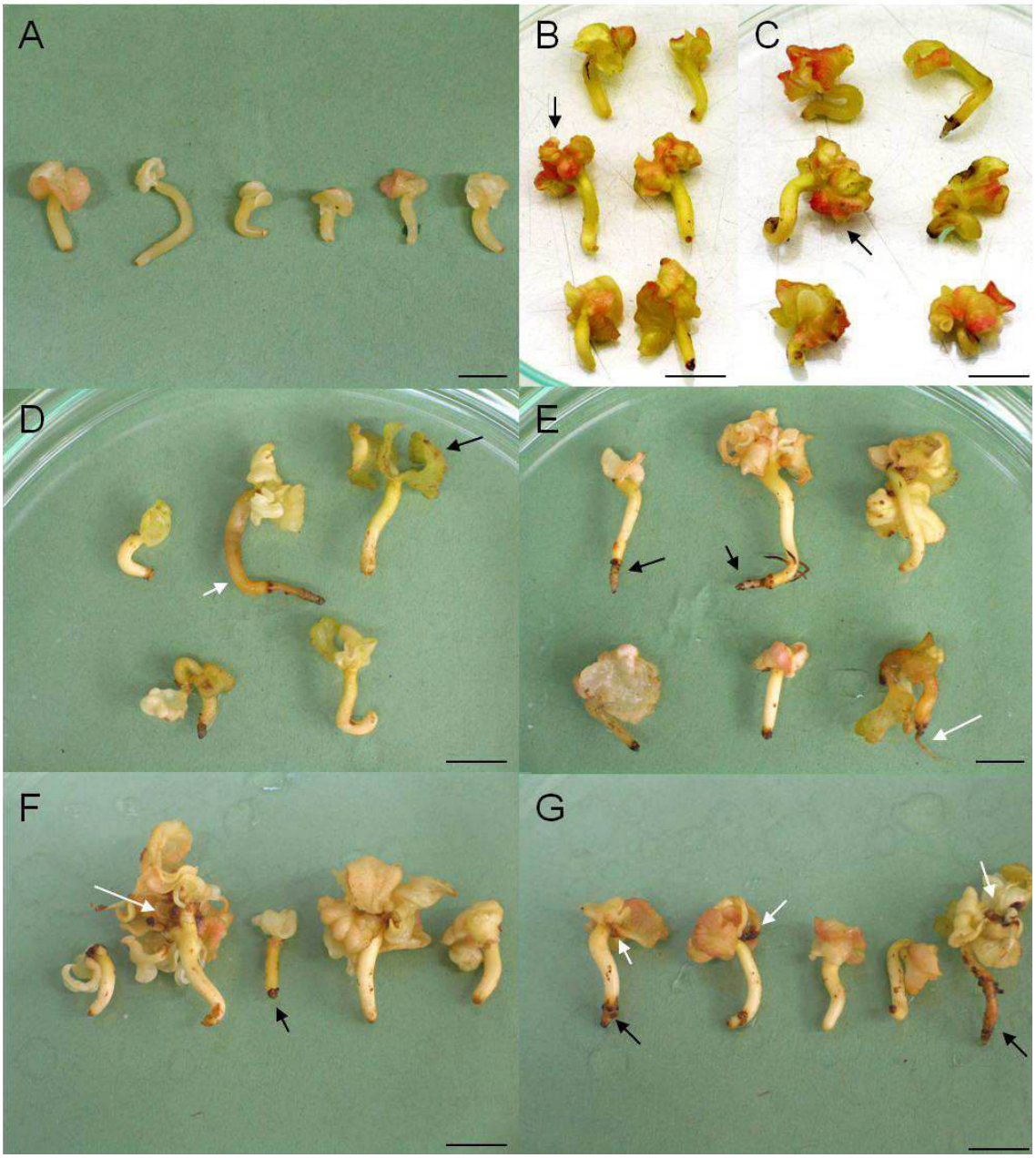

Figure 64. Morphological changes of cacao somatic embryos during germination after maturation in presence of high concentration of sucrose. A- Cacao somatic embryos submitted to maturation in culture medium containing 40 and 60 g L^{-1} of sucrose. **B-** Cacao somatic embryos after 15 days of maturation in 40 g L^{-1} of sucrose; black arrow indicates pink/red cotyledons; red arrow indicates brownish root tip. **C-** Cacao somatic embryos after 15 days of maturation in 60 g L^{-1} of sucrose; black arrow indicates pink/red cotyledons. **D-** Cacao somatic embryos matured in 40 g.L^{-1} of sucrose after 30 days of transfer to germination culture medium; Black arrow indicates green cotyledons; white arrow indicate green translucent axis. **E-** Cacao somatic embryos matured in 60 g L^{-1} of sucrose after 30 days of transfer to germination culture medium; black arrows indicate thick roots; white arrow indicates thin root. **F-** Cacao somatic embryos matured in 40 g L^{-1} of sucrose after 60 days of transfer to germination culture medium; Black arrow shows brownish root tip; white arrow shows callus on shoot apical meristem. **G-** Cacao somatic embryos matured in 60 g L^{-1} of sucrose after 60 days of transfer to germination culture medium; Black arrows show (left) new emitted root and (right) elongated root; white arrows show callogenesis on shoot apical meristem. Bar: 1cm.

4.3.2.2 Morphological aspects of cacao somatic embryos during germination without maturation pre-treatment.

After growth for 15 days in normal ED medium, the cacao somatic embryos at stage 5 and 6 were transferred to germination medium. Observations of morphological changes were carried out every 15 days.

Somatic embryos from stage 5 were characterized by slightly pink/red pigmented cotyledons, semi-translucent and with thick hypocotyls. These embryos were exclusively without brownish basal apex. Embryos at stage 6 had the characteristically well developed pink/red colored cotyledons, and many of them had a brownish basal apex (Figure 65 A and B).

After 30 days in germination medium, somatic embryos from **stage 5** showed brown stripes in the axes, turning white in some cases. A few of the embryos showed a brownish apex. The cotyledons were in majority white or translucent, but retained the pink/red parts. There were cotyledons detached from the embryos. No changes in size or volume of the embryos were detected. A few of the embryos germinated, additionally, many other embryos showed a brownish basal apex and initial root emission. Minimal callogenesis was observed only in white embryos (Figure 65 C).

After 30 days in germination culture medium, the somatic embryos from **stage 6** had predominantly greenish cotyledons, although the culture was carried out in darkness. Embryos with white and pink/red colored cotyledons were also observed. Many somatic embryos had lost their cotyledons. Callogenesis was frequent, but only in white embryos. Some of the embryos presented roots which were for the majority as thick as the axis and brown, but thin and long roots were observed as well (Figure 65 D).

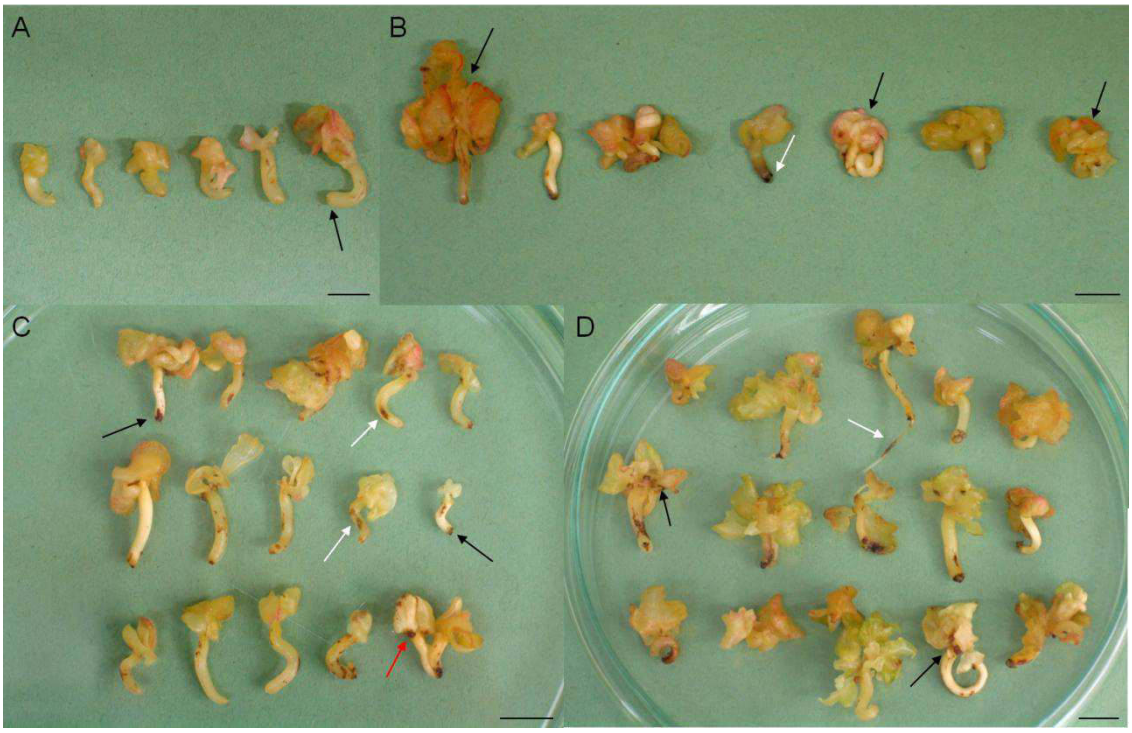

Figure 65. Morphology of cacao somatic embryos after 30 days of culture in germination medium. **A-** Cacao somatic embryos at stage 5 before incubation in germination culture medium; black arrow shows basal apex without browning. **B-** Cacao somatic embryos at stage 6 before incubation in germination culture medium; black arrows show well developed cotyledons with intense pink/red coloration; white arrow show brownish root tip. **C-** Cacao somatic embryos from stage 5; black arrow indicates brownish root tips; white arrows show brown stripes in the axis and red arrow indicates callogenesis in shoot apex. **D-** Cacao somatic embryos from stage 6; black arrows show callogenesis in white embryos; a thin and long root is indicated by a white arrow. Bar: 1cm.

After 30 days in darkness half of the embryos of both stages were transferred to culture under a photoperiod of 12 hrs (see section 3.2.8, Material and Methods). Analyses were carried out on the 15th and 30th days of culture.

For embryos of **stage 5**, no significant differences were observed in the morphology of the somatic embryos in the period from 30 to 45 days of culture in darkness. The somatic embryos, when transferred to a light regime of 12 hours at the 30st day of germination did not increase in size. All somatic embryos developed green cotyledons and axes at the proximal part. Axes which were completely white did not change their color. Root already formed presented minimal elongation. The brown stripes in the axes became prominent and most of the embryos developed brownish basal apex. No callogenesis was formed after incubation in light and already existent calli did not evolve (Figure 66A and B).

Somatic embryos of **stage 6**, after 45 days of germination, demonstrated small changes in their morphology. The browning in the axis was accentuated like in embryos of stage 5. Additionally, the roots already existent showed elongation, but all these were thin. Some embryos lost their cotyledons or presented white leaf-like structures coming from the shoot apex suggesting formation of primary leaves, although these somatic embryos still had no roots. The callogenesis did not increase, but the remaining calli became deep brown.

After 15 days of incubation in germination medium under a photoperiod of 12 hrs, the somatic embryos of stage 6 evolved similarly to embryos from stage 5. The remaining cotyledons became green as did the proximal region of the axis. White axes did not turn green during culture in light regime. The callogenesis did not change and calli also became deep brown in color. The existent roots, thick or thin, did not present any elongation. Only a few embryos presented initial root emission (Figure 66 C and D).

After 60 days of culture in darkness the embryos were analyzed. The most remarkable morphological change observed in all embryos was the color of the cotyledons. These had hyperhydricity and a dry or brown appareance. Some embryos had a necrosed axis and the cotyledons had dropped off. No changes in size or root emission were detected (Figure 67 A and C). The contrary occurred in embryos cultured under a light regime where the embryos showed some development to plantlets differentiation.

Somatic embryos of **stage 5** had deep green cotyledons, which were attached to the embryo's axis. Even previously white axes now showed a greenish color. Only previously formed thick roots showed some elongation. It was possible to observe formation of primary leaves in a few embryos. The primary true leaves were translucent and light green in color (Figure 67 B and E). It was noted that embryos with shorter axes showed consistent formation and did not develop callus.

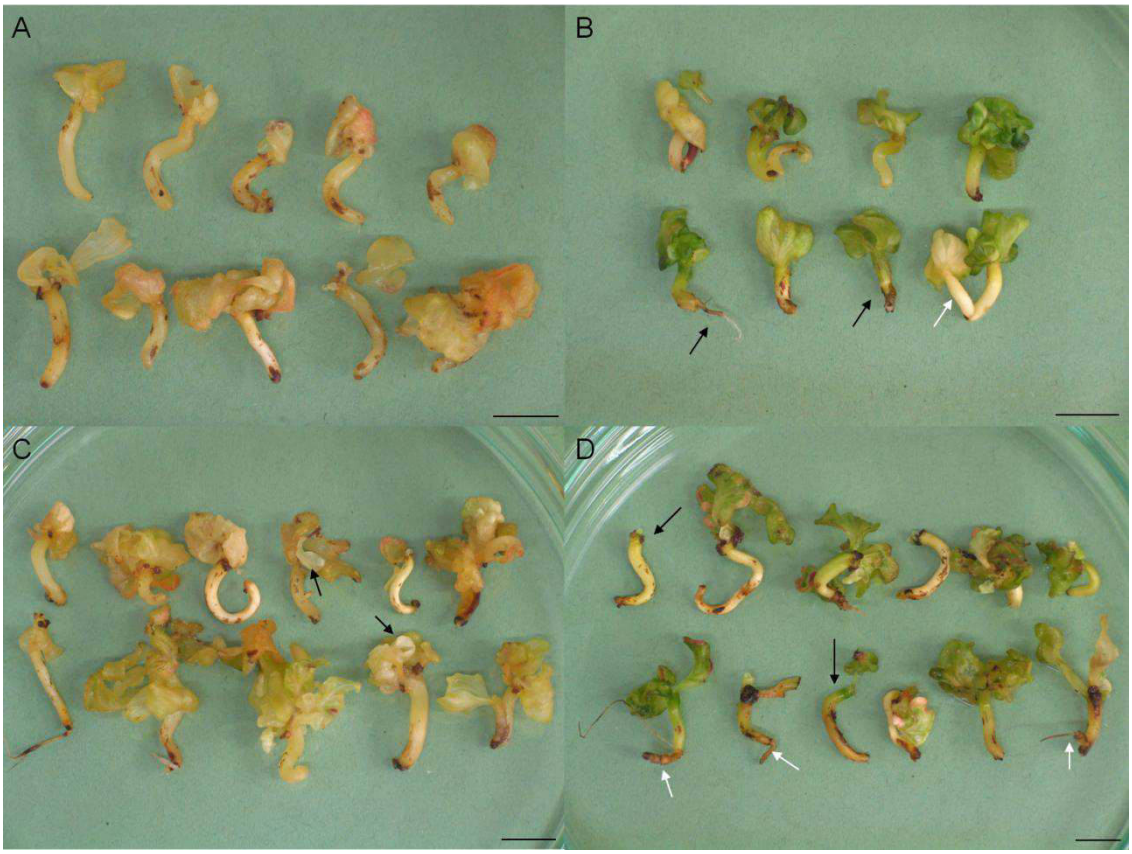

Figure 66. Morphology of cacao somatic embryos after 45 days of culture in germination medium and 15 days of incubation under photoperiod of 12 hours. A- Cacao somatic embryos of stage 5. **B-** Cacao somatic embryos of stage 5 after 15 days of incubation in light; black arrows show remaining roots; white arrow show white axes. **C-** Cacao somatic embryos from stage 6; black arrows indicates white leaf-like structures. **D-** Cacao somatic embryos from stage 6 after 15 days in photoperiod of 12 hours; black arrows show somatic embryos without cotyledons; white arrows indicates remaining roots. Bar: 1cm.

At the end of 60 days, somatic embryos from stage 6 demonstrated a considerable development in their morphology. Embryos previously containing roots evolved into plantlets developing true leaves and lateral roots. The formed lateral loots were thin and elongated, with an occasional ramification. Axes and cotyledons from other embryos necrosed and did not survive. Embryos which had no roots initiated roots emission, indicated by a brown basal apex, rupture of the embryonic root tissue and emission of a root tip. Even some somatic embryos without cotyledons demonstrated characteristics of primary roots emission (Figure 67 D and F).

Figure 67. Morphology of cacao somatic embryos after 60 days of culture in germination medium and 30 days of incubation under photoperiod of 12 hours. A- Cacao somatic embryos from stage 5 cultured in darkness. **B-** Cacao somatic embryos from stage 5 after 30 days of incubation in light regime; black arrows show primary leaves; white arrow show white axis that became green after 30 days cultured in light. **C-** Cacao somatic embryos from stage 6 cultured in darkness; black arrow indicates necrosis of embryonic body. **D-** Cacao somatic embryos from stage 6 after 30 days in light; black arrow indicate a somatic embryo without cotyledons but presenting root emission; white arrows show somatic embryos presenting necrosis; red arrows indicates plantlets with first true leaves and lateral roots. **E-** Close up of somatic embryo from stage 5 cultured 30 days in light still containing cotyledons. Black arrow indicates the emitting primary true leaf; white arrows show (upper) rupture of embryonic root tissue and (lower) thick root presenting brown points on whole its extent. **F-** Close up of plantlets formed from embryos at stage 6 cultured 30 days in light. Arrows indicate lateral roots formed between 15 and 30 days of culture in light. Bar: 1cm.

Parallel to these observations, embryos which had already germinated at the beginning 5th subculture in ED media (see section 3.2, Material and Methods) were directly incubated under a photoperiod of 12 hours. These showed complete formation of primary leaves at end of 60 days. However, the root system of these plantlets did not develop much after their transfer. The roots showed low or no elongation. Many were apparently dry and non-functional. All plantlets still contained the cotyledons. Abnormal formation of leaves was detected. These were sometimes curly and white or pink/red in color. Other plantlets had more than one pair of leaves, but the internodes, in this case,

were not elongated. Some plantlets had more than one shoot apical meristem or a deformed one from which abnormal leaf-like structures originated (Figure 68).

Figure 68. Plantlets evolved from germinated cacao somatic embryos directly transferred to light regime (12 hours-60 days). Black arrow show plantlet with more than 1 par of leaves with non-elongated internodes; white arrows indicate abnormal shoot development of somatic embryos containing dry thin roots (red arrows). Bar: 1cm.

In summary, the process of root formation during cacao somatic embryos germination was observed. It appears that this process is not synchronized with cotyledonary development. Somatic embryos showed the same characteristics of root emission during germination whether they were germinated in darkness or under light. It was observed in scanning electron microscopy (Figure 69). First, the basal apex of the embryonic axis turns brown. Thereafter, the epidermal tissue of the embryonic root suffers rupture and the emerging root tip is to be seen. This tip is brownish in color and has deep brown points on its surface. After that, elongation of the primary root takes place. The exact time needed for this process could not be verified, but it apparently is seems to work on the relation of the maturation and differentiation time, since some

embryos remained unchanged for more than 30 days without significant elongation of the root tip. The developmental interaction between root tip and shoot initial must be carefully analyzed. This developmental relation is important to carry out a successful production of plantlets.

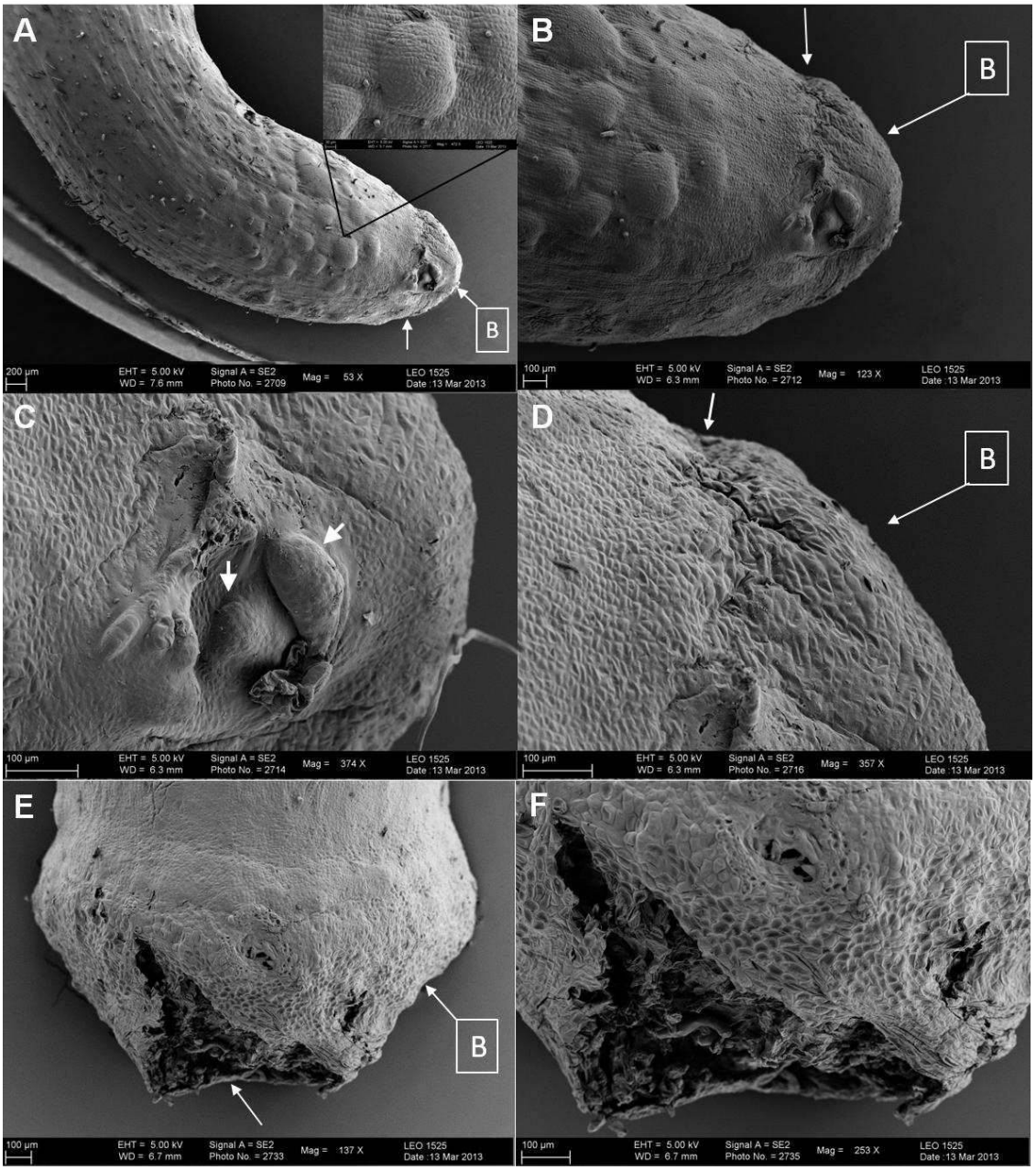

Figure 69. Morphological changes in the hypocotyls apex tissue during before emission of the primary root. A- Hypocotyl with brownish apex (arrow B), white arrow indicates the point between white and brownish tissue; in detail close up of the surface of the embryonic axe. **B-** Close up of the hypocotyls apex. White arrow indicates beginning of brownish tissue (arrow B). **C-** Structures observed in the hypocotyls apex in the region of brownish tissue. Arrows show what looks to be tissues in differentiation processes may lateral roots initiation. **D-** Close up of the point of transition (white arrow) between white and brownish tissue (arrow B) of the hypocotyls apex. **E-** Hypocotyl apex presenting tissue rupture (white arrow) in the region of brownish tissue (arrow B). **F-** Close up of hypocotyls apex after tissue rupture.

4.3.2.3 Acclimatization

At 60th day of culture, all embryos from light and darkness culture were further cultured under photoperiod of 12 hours with subcultures every 30 days. After 5 months under these conditions the development of plantlets was evaluated and these were transferred to *ex vitro* conditions.

All embryos of stage 5 did developed leaves, though the cotyledons increased significantly in volume. The primary leaves former developed were apparently inhibited by some unknown factor. Many of the cotyledons presented formation of nodular masses on their surface and high occurrence of repetitive somatic embryogenesis (R.S.E.) was observed on shoot apex and cotyledons surface. The existent roots did not show further development, but new thin roots were formed. Many embryos had necrosis of axis and cotyledons and then they died off (Figure 70 A).

The development of somatic embryos from stage 6 was totally different from that presented by embryos from stage 5. The resulting plantlets produced new leaves and increased roots ramification, but internodes remained short. The formed leaves showed normal morphology, as well the roots. The thick roots previously formed emitted lateral roots; it was possible to observe that these lateral roots emerged from prior observed brown points on surface of primary roots (Figure 70 B and E). It confirms the thick and brown primary roots as suitable for further developmental phase (conversion into plantlets). Somatic embryos which showed abnormal formation of apical shoot developed additionally R.S.E., although some embryos developed true leaves. However, these were small and apparently fragile. Embryos which did lose their cotyledons during germination formed some leaves, but these were abnormal and occurrence of R.S.E. could also be observed (Figure 70 C).

Figure 70. Cacao somatic embryos and plantlets 7 months after germination initiation. A- Cacao somatic embryos from stage 5 after at end of 7 months of culture. Black arrows indicate nodular masses formed on cotyledons surface; white arrows show torpedo shaped somatic embryos originated from R.S.E on the shoot apex. **B-** Plantlet evolved from cacao somatic embryos from stage 6 cultured 30 days in darkness and thereafter in light regime of 12 hours. **C-** Abnormal plantlet formed during culture in light regime. White arrow show abnormal pink/red curly leaves; black arrow indicates non-developed root tip and red arrow shows torpedo-shaped somatic embryos originated from R.S.E. **D-** Cacao somatic embryos from stage 6 with lateral roots (arrows) emerging from the primary root. Bar: 1cm.

Somatic embryos maturated in culture medium containing 40 or 60 g L^{-1} of sucrose revealed different forms of development at end of 5 months. Some of the embryos developed normally to plantlets as the embryos form stage 6 while other embryos showed the same characteristics as the embryos from stage 5 i.e. large

cotyledons with nodular masses and R.S.E. formation (Figure 71 A, B and C, respectively). These embryos were present in higher number. Plantlets already developed at 60th day of culture did not increase in size significantly and some plantlets died off. Also in these plantlets R.S.E was detected (Figure 71 D, E and F).

Figure 71. Cacao plantlets 7 months after germination initiation. A- Plantlet with well developed roots and leaves originated from cacao somatic embryos maturated in culture medium containing 60 gL^{-1} of sucrose. **B-** Characteristic somatic embryos maturated in high concentration of sucrose present in the culture after 5 months in light regime. White arrow indicates nodular mass formation on the cotyledonary surface. **C-** Nodular masses commonly formed on cotyledons of cacao somatic embryos after long period of culture under photoperiod; Bar-3mm. **D-** Well developed plantlets formed from somatic embryos already germinated in culture at ED4 which were directly transferred to germination conditions under photoperiod. **E-** Plantlet with abnormal development of leaves and root containing two shoot apex (white arrows) and containing R.S.E.; globular, heart-shaped and torpedo-shaped somatic embryos can be observed (black arrows). **F-** Somatic embryo with necrosis of the axis, however with formation of leaves (white arrows) and with occurrence of R.S.E (black arrows). Bar: 1cm.

All embryos and plantlets were transferred to *ex vitro* conditions, i.e. out of the TIS and with limited supply of nutrients (see section 3.2.6, Material and Methods, Figure 72). These were monitored along the development. Somatic embryos which did not showed formation of leaves or roots became infected by fungus, and necrosis occurred on whole embryonic body, these died off after 10 days. The well developed plantlets remained healthy for 30 days. However, during this period they lost the leaves and some plantlets did not showed formation of new leaves. Some other plantlets were also infected by fungus and the embryos did not survive. Plantlets that remained with the leaves were further cultivated while the glass lid was open gradually. After 3 days of lids opening the plantlets dried completely. The transfer to *ex vitro* was carried out in growth chambers with very low air humidity, what perhaps did contribute to the rapid dry of plantlets tissues.

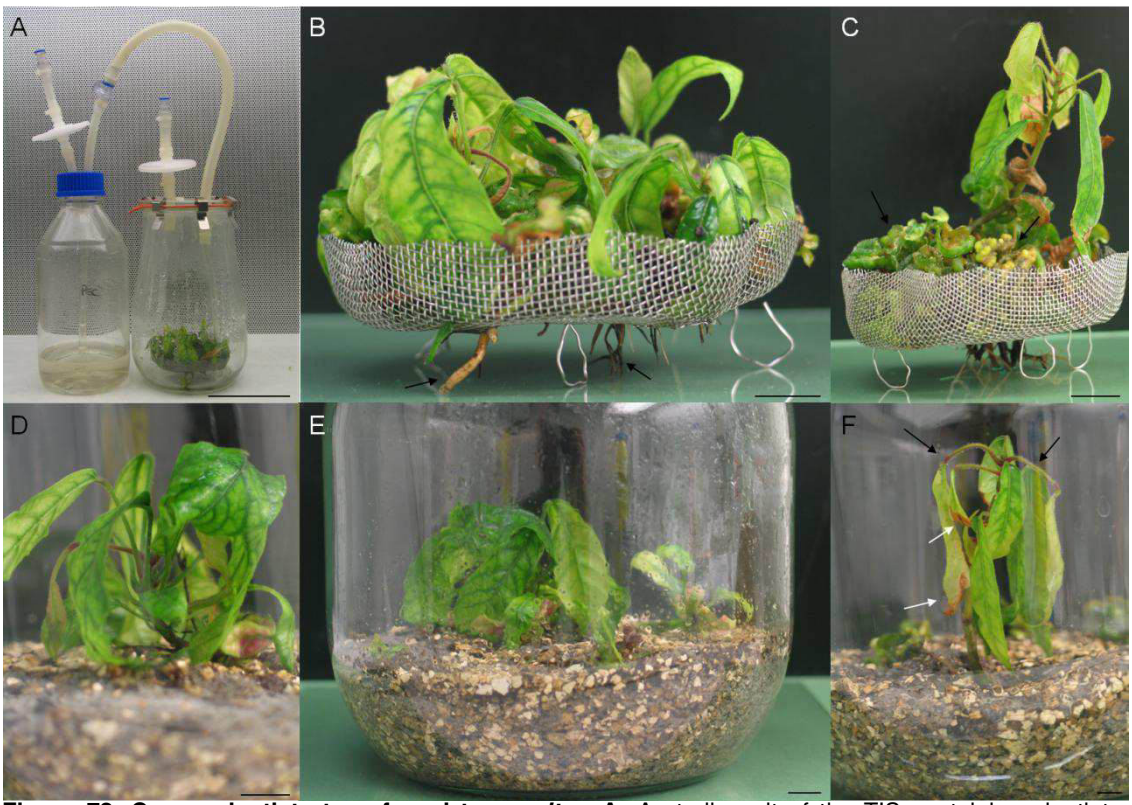

Figure 72. Cacao plantlets transferred to *ex vitro*. A- A sterile unit of the TIS containing plantlets before transfer to unsterile conditions. Bar: 10 cm. **B-** Metal sieve took from the TIS with well developed plantlets. **C-** Culture originated from embryos maturated under high concentration of sucrose containing a well developed plantlet simultaneously to abnormal developed somatic embryos (black arrows). **D-** Well developed plantlet just allocated in the substrate. **E-** Jars filled with substrate used for acclimatization. **F-** Plantlet presenting wilted leaves (black arrows) and dry leave tips (white arrows). Bar: 1cm.

In summary, the embryos from different treatments, with or without maturation pre-treatment and from different stages, showed significant morphological differences during germination processes. However, the established plantlets were very similar to each other concerning the architecture and organs morphology.

4.3.2.4 Changes in total protein content of cacao somatic embryos germinated after culture in high concentration of sucrose.

The total protein content of cacao somatic embryos were analyzed during and after maturation in culture media containing 40 or 60 g L-1 of sucrose. The results are shown in figure 73. The cotyledons revealed higher concentration of proteins than the axes. In contrast to the axes, the levels of proteins in the cotyledons do not differ significantly when maturated in 40 or 60 g L-1 of sucrose, 68,77 and 68,13 µg/mg FW , respectively. After 60 days in germination culture media the levels of total protein in cotyledons of somatic embryos maturated in 40 g L-1 sucrose decreased to 52,05 µg/mg FW and to 54,67 µg/mg FW for embryos maturated in 60 g L-1 sucrose.

Contrary to cotyledons conditions, the levels of protein in the axes increased after cultivation in germination media. Embryos maturated in 4% sucrose showed in the axes a protein concentration of 7,64 µg/mg FW and it increased to 23,29 µg/mg FW after 60 days in germination media. On other hand, axes of somatic embryos maturated in 6% sucrose contained at the end of the maturation period 24,33 µg/mg FW of protein. After 60 days cultured in germination culture media, this level increased to 43,86 µg/mg FW .

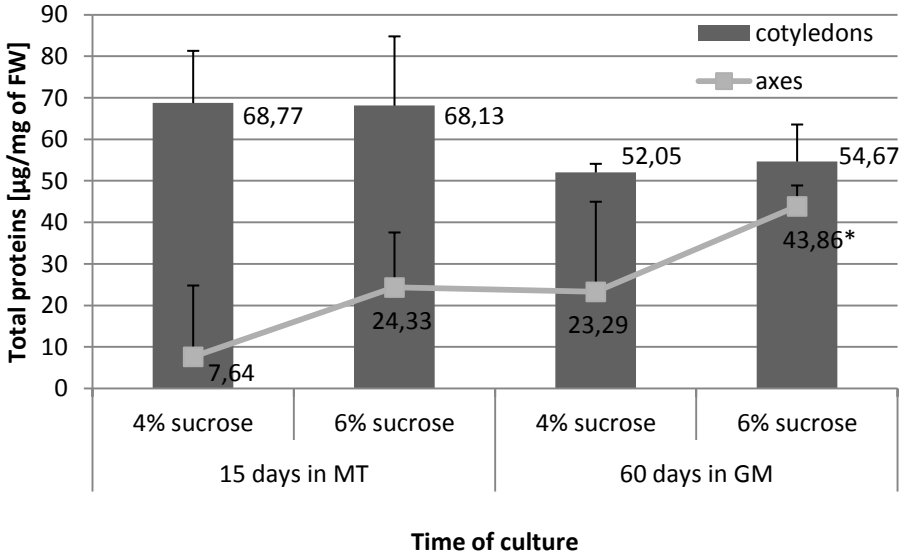

Figure 73. **Concentration of total proteins in cotyledons and axes of cacao somatic embryos matured in culture medium containing 40 or g L^{-1} sucrose after maturation (MT) and germination (GM).** Error bars represent means+standard error (n=3; *n=2).

4.3.2.5 Changes in total protein content of cacao somatic embryos after germination without maturation pre-treatment.

The concentration of protein in cotyledons and axes of somatic embryos during germination in darkness or under photoperiod of 12 hours was compared. The Table 4 show the total protein content of embryos from stage 5 and 6 after germination period.

In the figure 74A the concentration of total proteins in cotyledons and axes of somatic embryos from stage 5 submitted to germination are presented. In general, major part of proteins was concentrated in the cotyledons during whole culture period. Except the embryos cultured 15 days in light, these had higher content of protein in the axes. During germination in darkness the levels of proteins did not change significantly.

It decreased after 45 days of culture but increased at end of 60 days. Before germination, the cotyledons of embryos at stage 5 contained 43,77 µg/mg FW . After 30 days in culture media for germination, the concentration was 45,28 µg/mg FW and decreased to 32,85 µg/mg FW after 45 days of culture. After 60 days, it increased to 54,43 µg/mg FW . When the somatic embryos were transferred to culture under photoperiod of 12 hours, the concentration of proteins in the cotyledons increased

significantly. After 15 days of light regime the cotyledons contained 72,77 µg/mg FW and after 30 days it increased to 86,30 µg/mg FW .

Table 4. Concentration of total proteins in cotyledons and axes of cacao somatic embryos during germination period.

		Germination conditions					
		Darkness				*Photoperiod 12 hours*	
		T0-ED4	*T30*	*T45*	*T60*	*T15*	*T30*
Stage 5	Cot.	43,78±1,80	45,28±6,23	32,86±2,75	54,44±8,81	72,77±8,92	86,31*
	Axes	27,85±4,20**	24,40±13,77	10,15±19,95	27,72±7,19	96,84±55,49	55,62*
Stage 6	Cot.	46,84±4,32	31,51±4,20	57,27±5,72	43,06±5,11	55,00±7,23	105,90±5,26**
	Axes	34,94±7,61	27,81±3,09	15,98±17,68	24,53±2,95**	0±8,71**	13,33*

T0-ED4- somatic embryos before transfer to germination culture media; T15, T30, T45, T60- days after transfer to germination media. Values represent means±standard error µg/mg FW (*n=1; **n=2.).

Similar events occurred in the axes of those somatic embryos, although at lower levels. Before germination, the axes of the embryos at stage 5 contained 27,84 µg/mg FW . This value decreased to 24,84 µg/mg FW after 30 days of germination and to 10,14 µg/mg FW after 45 days. An increase was detected after 60 days of culture, 27,72 µg/mg FW . The concentration of proteins in axes of embryos germinated in light regime was significantly higher than found in embryos cultured in darkness, 96,84 µg/mg FW at 15th day. But it decreased after 30 days under this condition, 55,61 µg/mg FW .

Figure 74B presents the content of total protein in embryos from stage 6 along germination. The concentration of total protein in the cotyledons can be considered stabile during whole period of germination in darkness. Before germination somatic embryos at stage 6 contained 46,84 µg/mg FW of proteins. After 30 days, the concentration decreased to 31,51 µg/mg FW but increased to 57,26 µg/mg FW after 45 days. At end of the germination period (60 days), it turns to decrease to 43,06

µg/mg FW . The axes showed significant diminution in the protein content until 45 days of germination, from 34,93 µg/mg FW to 27,80 µg/mg FW at 30th day and to 15,98 at 45th day. The decrease was accentuated after 15 days of transfer to light regime. After 60 days the levels in the axes of the embryos cultured in darkness increased (24,52 µg/mg FW). Embryos cultured 30 days in light also show increase, 13,33 µg/g FW.

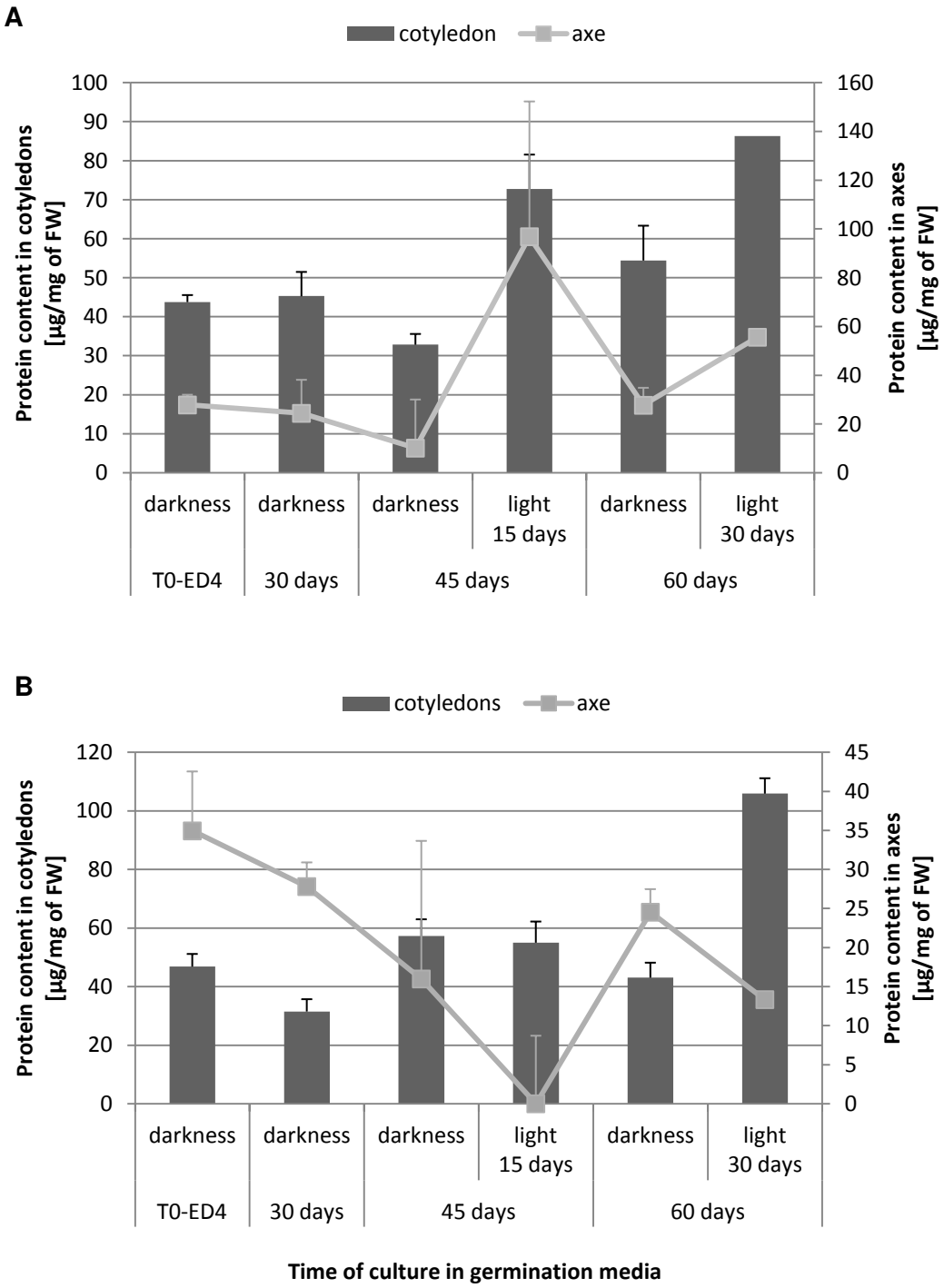

Figure 74. **Concentration of total proteins in cotyledons and axes of cacao somatic embryos at different periods and conditions of germination. A-**.Somatic embryos from stage 5. **B-** Somatic embryos from stage 6. Error bars represent means+standard error (n=3).

The results show differences in the protein content of embryos germinated in darkness or under photoperiod of 12 hours. These responses seem to be also related to stage of development of the embryos when these were submitted to germination conditions. Somatic embryos from stage 5 demonstrated increase in protein concentration in the axes when cultured in light regime, while embryos from stage 6 showed contrary results. Nevertheless, it can be considered that somatic embryos from both stages revealed levels of protein relatively similar along the germination in organs, cotyledons and axes, except for embryos cultured in light.

5. Discussion

5.1 Cacao somatic embryos regeneration in temporary immersion system (TIS) in comparison to standard culture conditions.

The formation of non-embryogenic or friable calli in cacao SE cultures was reported for various genotypes of *T.cacao* submitted to primary and secondary SE (Niemenak *et al*., 2008; Li *et al*., 1998). In this work, the application of PennState‘s protocol (The Pennsylvania State University, 2010) for SSE culture of the cacao clone RO3.08/1-Madagascar clearly improved the quality of the culture. It minimized the disordered proliferation of non-embryogenic calli. The PennState's protocol has demonstrated satisfactory results in SE induction of several cacao genotypes of all 3 main cacao genetic groups: Forastero, Criollo and Trinitario (Maximova *et al*., 2005, 2002; Li *et al*.,1998). However, cacao is an *in vitro* recalcitrant plant and modification of the culture media formulation as well as culture conditions has to be considered separately for each cacao genotype and explants (Quainoo and Dwomo, 2012; Buah, 2010; Issali *et al.,* 2008; Minyaka *et al*.,2008; Silva *et al*., 2008; Silva *et al*., 2006; Traore, 2006).

Besides PennState's protocol, other protocols are reported as efficient for induction of SE in some cacao genotypes (Figueira and Janick, 1995; Alemanno *et al*., 1993; Aguilar *et al*., 1992; Kononowicz and Janick, 1984). Nevertheless, every protocol requires an intensive usage of plant growth regulators (PGRs). The application of powerful PGRs like the synthetic auxin 2,4-D in the protocols for induction of SE is a strategy to overcome *in vitro* recalcitrance mainly for wood plants (Fehér *et al*.,2003; Benson, 1999; Dudits *et al*. 1991). However, the development of somatic embryos occurs in the absence of this PRG. The initial protocol used in the Laboratory of Useful plants (LUP-Hamburg University, Germany) exposes the tissue to culture media supplemented with 2,4-D for several weeks. A prolonged culture of plant tissues in presence of this PGR can initiate endogenous accumulation and carryover of auxin

causing instability in morphogenetic processes (Fehér *et al*., 2003; Benson, 1999; Ribnicky *et al*.,1996).

The formation of non-embryogenic callus and production of abnormal somatic embryos during SSE induced with LUP-protocol perhaps resulted from the long exposure to 2,4-D. The increase of the endogenous auxin content in the callus can be confirmed by the occurrence of roots in calli formed by using the LUP-protocol. A balance of auxin and cytokinin induces embryo formation (Guerra *et al*., 1999; Reinert *et al*., 1977). However, when exogenous or endogenous auxin is highly concentrated, roots are formed. Molecular studies concerning callus formation in *Arabidopsis* show that callus induction using 2,4-D down-regulates much more morphogenetic genes than up-regulates, additionally, the majority of up-regulated genes are related to stress responses (Karami *et al*., 2009; Long and Benfey, 2006; Che *et al*., 2006; Rhagavan *et al*., 2006). The 2,4-D is accumulated in the cells throughout the culture time. It is believed that the presence of this synthetic auxin induces the endogenous synthesis of natural auxins. Furthermore, this increase of auxin content can disturb the hormone signal transduction and transcription factors (TF) activity. MONOPTEROS, BODENLOS and LEC1 are some examples of proteins and TFs responsible for the shift of the maximum auxin content, polarity of auxin transport and meristem formation in *Arabidopsis* (Chen *et al*., 2012; Fehér, 2008; Long and Benfey, 2006). Overregulation or degradation of TFs can lead to organogenesis instead of embryogenesis. In conclusion, a SE protocol with a shorter time of 2,4-D exposure is favorable for somatic embryos regeneration and increase of the quality of the SE cultures. Furthermore, shorter periods of callogenesis minimize somaclonal variation in cacao somatic embryogenesis (Wilkinson *et al*., 2010).

Furthermore, ethylene and abscisic acid are some of the phytohormones regulated by auxin (Rhagavan *et al*., 2006). When the culture is carried out on solid medium, the tissues are cultivated in Petri dishes or small vessels with limited gas exchange. The ethylene produced cannot be efficiently disseminated. The inhibitory

effect of ethylene on callogenesis and morphogenesis has been reported for some species, but seems to be genotype specific in some cases (Rhagavan *et al*., 2006; Park *et al*., 2006; Chou and Wang, 1998; Vain *et al*., 1989).

In this study, *in vitro* tissue culture showed great improvement in the regeneration of secondary somatic embryos after cultivation in TIS. The system clearly influences the callogenesis as well as embryogenic competence. The advantages offered by this system like gas exchange, homogeneous distribution of the nutrients and less contact of the tissues with the culture media avoid processes like hyperhydricity, formation of nutrient gradients and accumulation of secondary metabolites produced by the tissues in the culture media. It is known that tissues cultured in contact with Gelrite®, the gelling agent used in LUP-protocol, showed malformation of cell wall and high water content. These factors lead to disordered proliferation of callus and deficiencies in embryo morphogenesis (Leva and Rinaldi, 2012; Etienne and Berthouly, 2002; Berthouly and Etienne, 2005).

Niemenak *et al*. (2008) introduced the TIS developed at Hamburg University in the developmental phase of cacao somatic embryos obtained from the clone Sca6 resulting in an increase of embryo production. However, the proliferation of non-embryogenic calli also increased strongly. The transfer of explants to TIS promptly after the induction phase before callogenesis or embryogenesis take place, overcomes this problem as shown in the results (section 4.1). The calli were nodular whereas friable calli were rarely observed. This observation was also reported by Ewest (2010) for SSE of the clone RO3.08/1-Madagascar developed in micro-TIS models. It was demonstrated that the formation of cacao somatic embryos took place with minimal callogenesis. However, further studies must be carried out to define the ideal TIS culture conditions for induction phase of cacao SSE, like the frequency of immersions per day.

Somatic embryogenesis of important crops like coffee (*Coffea sp*.) banana (*Musa sp*.) and peach palm (*Bactris sp*.) in bioreactors of temporary immersion has

been reported as successful (Steinmacher *et al*., 2011; Ducos *et al*., 2007; Lemos *et al*., 2001; Teisson and Alvard, 1993). Bioreactors have been applied in the clonal propagation via SE due to the following advantages: low costs, automation of the procedures and the possibility of high production rates of somatic embryos in a reduced space (Watt, 2012; Ziv, 2000; Teisson *et al*., 1995).

The performance of TIS demonstrated here for cacao SSE led to the definition of a cyclic culture. It allows the characterization of the cacao somatic embryogenesis and embryos development. Furthermore, the cyclic culture provides stability of production for a longer time than former described (Maximova et al., 2002). The early development of cacao somatic embryos under these conditions, PennState's protocol combined with TIS, is similar to that described by other authors. However, a wide range of stages and morphological traits was detected during SSE in TIS. These embryos showed singular characteristics not yet reported in other studies (Minyaka *et al*.,2008; Maximova *et al*., 2002; Li *et al.,* 1998; Alemanno *et al*.1996; Santos and Machado, 1989). This observation gives rise to the assumption that the morphological changes are induced by development of cacao somatic embryos under TIS conditions.

The phytotoxic substances accumulated in solid culture media and the permanent contact of the embryos with these substances combined with rapid decline nutrients are supposed to be the most significant factors involved in the poor quality of somatic embryos cultured on solid media (Stasolla and Yeung, 2003; Teisson and Alvard, 1995). The accumulation of phytotoxic compounds also happens in liquid media used for TIS, but the embryos are only temporally in contact with the culture media. Furthermore, the liquid media can easily be changed with more frequency without having to manipulate the tissues and embryos. It protects the somatic embryos from developing stress conditions and also insufficiency of nutrients. Additionally, the gas exchange occurring during the immersions dissipate undesired gaseous compounds formed during the process.

In conclusion, the application of the PennsState's protocol with reduced time of explants' exposure to high concentration of 2,4-D resulted in a diminution of non-embryogenic calli as well as undesired structures like roots and multicotyledonary embryos and increased the production of secondary somatic embryos. These results were improved when the SSE expression phase was carried out in TIS. The production rate and quality of the somatic embryos changed considerably which allowed the establishment of a cyclic SSE protocol for the clone RO3.08/1-Madagascar. It is important to consider that this clone has not yet been characterized molecularly. It seems to be a highly suitable clone for *in vitro* propagation of cacao. In this context, the classification of this clone would be of high interest for cacao researchers and breeders.

5.2 Morphological and physiological characteristics of cacao somatic embryos development in Temporary immersion system (TIS).

The cyclic SSE protocol applied to the clone RO3.08/1 allows for a high production rate of apparently healthy somatic embryos. However, the morphological integrity of these somatic embryos remained unclear. Hence, morpho-anatomical studies of the somatic embryos were carried out.

The variability of somatic embryos has been reported by many authors as being fundamental for the success of plantlets establishments. The identification of abnormal embryos and sorting of healthy embryos can increase significantly the success in the late stages of development (Stolla and Yeung, 2003; Etienne *et al*., 2002). Fang *et al*. (2011) showed the importance of cacao somatic embryos integrity during the cryopreservation process.

The somatic embryos produced in TIS showed a morphogenetic development similar to the one described in the literature (Minyaka *et al*.,2008; Maximova *et al*., 2002; Li *et al.,* 1998; Alemanno *et al*.1996;, Santos and Machado, 1989). The embryos developed from multicellular globular structures to cotyledonary mature embryos within

30 days. When compared to the embryogenesis of the model plant *Arabidopsis thaliana* (Arnold *et al.,* 2001; Berleth and Chartfield, 2002; Wang, 2009), cacao secondary somatic embryos show normal morphogenesis. The embryogenesis of dicotyledons presents four developmental stages during the morphogenesis: globular, heart stage, torpedo stage and cotyledonar (Kaplan and Cooke, 1997). The characteristics presented by cacao secondary somatic embryos, like a multicellular globular structure, polarity of the embryonic body, acquisition of bilateral symmetry, expansion of the cotyledon and hypocotyls, formation of ground tissues followed by shoot and root meristem development, corresponds to the stages of dicotyledons morphogenesis completed with the differentiation of cotyledons (Kaplan and Cooke, 1997). This pattern of embryonic formation was also reported during somatic embryogenesis of other dicotyledons woody plants like *Acca sellowiana, Hevea brasiliensis, Coffea arabica* (Ducos *et al*., 2007; Gangahuala-Inocent *et al*. 2004; Teisson *et al*., 1995). The development of cacao somatic embryos was compatible to the one described for zygotic embryos (Duncan and Todd, 1972; Chessmann, 1927). A high number of abnormalities were detected during the heart stage of cacao somatic embryos. However, the abnormal somatic embryos can be identified already at torpedo stage and eliminated during culture maintenance. The remaining embryos evolve to maturity and a high morphological heterogeneity was observed.

According to the traditional protocol the morphological distinct mature cacao somatic embryos are cultured equally throughout subsequent phases, meaning maturation and conversion (Alemanno *et al*., 2006; Maximova *et al*., 2005). The influence of the morphology of somatic embryos on plantlets development was observed in culture of coffee somatic embryos (Etienne *et al*., 2002). Here morphological variability was characterized by several biochemical and histological analyses by which significant differences concerning the physiological state of the embryos were detected. It is suggested that morphologically distinct cotyledonary somatic embryos require different culture conditions to reach ideal maturation and

conversion. During the cotyledonary phase (described as growth phase by Alemanno *et al*., 2006) the somatic embryos of cacao can actually be grouped into four distinct morphological stages which are also physiologically different or present individual peculiarities.

One of the main processes occurring during cacao somatic embryos development is the metabolism of phenolic substances. Cacao is known for its high phenolic content (Elwers *et al*., 2010, 2009; Stoll, 2010; Kim and Keeney, 1983). A high content of these compounds in plant tissues is related to *in vitro* recalcitrance (Müller, 2008; Reis *et al*., 2008; Umehara *et al*., 2007; Benson, 2000). Alemanno *et al*. (2003) showed the presence of phenolic compounds in tissues of cacao flowers and in non-embryogenic callus regenerated from these tissues. However, no information about these compounds in somatic embryos of cacao was reported.

Cells containing phenolic compounds are already present in early stages of cacao somatic embryos development. The distribution of such cells in the cotyledons as well as the physical form of phenolic deposits in the cells show similarities to that observed in cotyledons of cacao zygotic embryos (Elwers *et al.,*2010; Martini *et al*., 2008; Sánchez-Rabaneda *et al*., 2003). The concentration of these cells near the vascular bundles and under the epidermis probably characterizes a defense mechanism against tissue injuries and eventual infections that may occur. This hypothesis is supported by the findings concerning the activity of the polyphenol oxidase (PPO) in cacao somatic embryos demonstrated in this work. The role of this enzyme in somatic embryogenesis is discussed later in this section.

A high concentration of phenolic compounds in cacao somatic embryos was observed in different analyses. The biochemical quantification revealed a significant increase in the content of total phenols in cotyledons and axes of the mature somatic embryos. It is believed that a high amount of phenolic substances is secreted by mature and germinated somatic embryos into the culture media which affects the differentiation and development of somatic embryos in earlier stages. When mature or

germinated somatic embryos remained in culture together with the embryogenic calli and somatic embryos at earlier stages, death of major numbers of these somatic embryos as well as necrosis was observed. The transfer of the mature embryos to separate culture vessels solved this problem. In many plants, phenolic compounds were characterized mainly in hypocotyls and roots of seedlings and embryos (Haddadchi and Gerivani, 2009). The phenolic compounds accumulated in the culture media increase the auto oxidation of the tissues and death occurs (Benson, 2000). Especially in TIS, it may be aggravated by the frequent air introduction in the culture vessel due to the system mechanism. Despite of this, the process of necrosis and death of the embryos is much faster on solid media than in TIS. The somatic embryos cultured on solid media are permanently depositing substances into the culture media and at the same time in contact with phytotoxic compounds (Park *et al*., 2006). Some authors reported the positive relationship between phenolic compounds and somatic embryogenesis competence (Kouakou *et al*., 2007; Reis *et al*., 2006). However, this benefit is not extended to the morphogenesis phase of embryo development. The increase in the phenolic availability in the culture media induces activity of peroxydases which are involved in the lignification of the cell walls; these processes combined are related to growth reductions. Furthermore, the activity of these enzymes is related to differentiation of somatic embryos rather than to growth (Park *et al*., 2006). It can be overcome by addition of active charcoal to the culture media. In conifers it improved the growth of somatic embryos (Umehara *et al*., 2007). Our results demonstrated positive effects of charcoal addition in the culture media for rooting of cacao somatic embryos. It suggests that charcoal did avoid inhibitory effects of substances present in the culture media on cacao somatic embryos rooting. However, it is unknown if phenolic substances and peroxydases activity are the causes of cacao somatic embryos rooting deficiency. Further studies concerning accumulation of phenolic compounds in the culture media and its relation with enzymes activity in the embryos have to be carried out to confirm this hypothesis.

So far, the HPLC-spectra obtained from cacao somatic embryos revealed several peaks corresponding to polyphenols synthesized in mature cacao somatic embryos with differences in the axes. Based on the intensity, area and retention time of the peaks, epicatechin and its oligomers, purines like theobromine and coffein and others peaks believed to be hydroxycinnamic acid amides were characterized (Sánchez-Rabaneda *et al*., 2003; Hammerstone *et al*., 1999). Alemanno *et al*., (2003) reported the presence of such compounds in flower pieces of cacao, non-embryogenic calli and unfermented beans.

Results obtained in semi-quantitative analysis by UMSP (UV-microspectrophotometry; Koch and Kleist, 2001) indicate lignin as the main aromatic substance within phenolic compounds accumulated in the cells of cotyledons of cacao somatic embryos. This technique allowed to analyze the distribution of phenolic compounds in the tissue of cacao somatic embryos at different stages. It clearly showed the progressive lignification of the tissue throughout the development and the significant increase of phenolic deposits in the cells. It also confirmed the findings obtained by light microscopy. The spectra of UV-absorption revealed the predominance of the lignin in the composition of the cell walls, middle lamellae and deposits in the cells' vacuole. Two peaks of UV-absorption were observed in almost all parts and stages analyzed, these were at 250nm and 278nm. It characterizes the chemical composition of hardwood lignin (Koch and Lybeer, 2005).

Thickening of somatic embryos cell walls is believed to already take place during differentiation of globular embryos (Williams and Maheswaran, 1985). However, lignification at this early embryo growth phase was not reported. The results in this work demonstrated the stable lignification of cell walls throughout the cacao somatic embryo development. However, the phenolic deposits in the interior of the cells increased significantly. During lignification of a tissue monolignols are transported from the cytosol to the cell walls and lignin is synthesized in middle lamellae (Buchanan *et al*., 2000). It explains the higher UV-absorption peak at 250nm found in the phenolic

deposits in the cells of these embryos. Except for deposits in the ring form (RF, section 4.2.2.4) which presented a significant peak at 278nm, this finding suggests the presence of lignin in the composition of this kind of phenolic deposits in the cell. The UV-absorption spectrum represents lignin consisting of guaiacyl- and syringylpropane units. Some shoulders detected at 320nm can be considered to be ρ-coumaric acids (Koch and Grünwald, 2004). Hence, it is suggested that this compound may actually be present in the polyphenols profile observed by HPLC analysis carried out in this work for cacao somatic embryos.

UMSP allows a highly sensitive analysis of lignin and phenolic compounds distribution in plant tissues (Koch and Kleist, 2001). The application of this technique in analyses of tissues cultured in *in vitro* offers advantages for physiological studies of woody plants like cacao. The possibility of rapid and efficient analysis at a cellular level permits observation of physiological processes in woody plants cells impossible *in vivo* or standard histological procedures. For small somatic embryos like of cacao, chemical extraction of aromatic compounds like lignin becomes difficult due to the low amount of tissue available for the extractions. UMSP was fundamental for detection of lignin in early stages of cacao somatic embryos which are very small and provide no significant biomass yet.

Haddadchi and Gerivani (2009) observed an increase in the activity of PPO in the hypocotyls and roots of germinating soybean (*Glycine max*) seeds when being cultured in media containing phenolic extracts of root of canola (*Brassica napuse*), but the same did not occur with shoot extract. In canola, phenolic substances like cinnamic acids amides and epicatechin oligomers are found in considerable quantities. The same extracts also inhibited seed germination and seedling growth. Similar results were observed for *Brassica campestris* (Nandakumar and Rangaswamy, 1985). It is suggested that specific phenolic substrates of PPO are produced in the roots. It may explain the presence of similar hydroxycinnamic acid related peaks found only in the hypotocyl of cacao somatic embryos.

The activity of this enzyme provides one of the most efficient protection mechanisms and antifungal properties in plant cells (Mayer and Harel, 1978). This enzyme requires phenolic compounds as substrate to form cross linking substance, the quinones, which act as a precursor for physical and chemical barriers against extern external like pathogens. This process is triggered by rupture of tissue or cellular structure (Simo *et al*., 2011; Vaughn and Duke, 1984). According to this information, the accumulation of phenolic deposits in cells located directly under the epidermis and near the vascular bundle can be considered as a highly specialized system of plant defense through PPO activity. However, the analyses revealed highest activity of PPOs in hypocotyls of germinated cacao somatic embryos while in the cotyledons the activity was very low even after the a substrate was provided.

Studies demonstrated that PPO is closely related to germination events in many species, however in different ways. In some cases, it acts as protection in allelopathic interactions in emerging roots or as part of embryo respiration pathways providing quinones for ATP synthesis (Holzapfel *et al.,* 2010, Kocaçaliskan *et al*., 1995). Sabahz *et al*. (2009) also reported a high activity of PPO in hypocotyls and roots of *Astragalus cicer* during germination. They suggest that PPO activity may play a role in defense mechanisms during plantlet establishment, but also as response to tissues disruption occurring during germination and root emission. The last could be transferred to cacao somatic embryos which present disruption of the tissue around the axes tip before root emission. The activity of PPO may be responsible for the browning processes frequently observed in the apex of cacao somatic embryos axis at late stages of development. Furthermore, the periods without culture media as well as the movements during immersion in TIS could lead to drought and injuries of axes tissue that may causes PPO activation.

On the other hand, PPO activity can be related to differentiation pathways (Grotkass *et al*., 1995). PPO was associated with the rooting potential of some clones of date palms (Qaddoury and Amssa, 2003) and was detected in germinating somatic

embryos and root-forming callus of date palms (Baaziz *et al*., 1994), but only in its insoluble form. High activities of PPO were detected in hypocotyls slices of immature cacao zygotic embryos which respond positively to SE induction (Niemenak *et al*., 2012). Those authors also demonstrated the existence of a positional gradient of PPO activity throughout the embryonic axis that corresponds to embryogenic and non-embryogenic responses of this tissue. Kouakou *et al.* (2009) demonstrated the activity of two different PPO isoenzymes in embryogenic and non-embryogenic cultures of cotton (*Gossypium hirsutum*). These isoenzymes respond differently to specific activation conditions like substrate and pH value of the media. Similar results were described for suspension cultures of *Euphorbia pulcherrima* (Grotkass *et al*., 1995). Addition of antioxidants in the culture media significantly reduced the production of somatic embryos of *Pinus patuia* (Malabadi and Van Staden, 2005). According to this information, PPO can be considered as a marker for embryogenic competence and cellular differentiation (Jariteh *et al*., 2011). During germination, the differentiation of the tissue in the root tip increases. If PPO is related to differentiation processes it could be the reason for the high enzyme activity found in hypocotyls during germination of cacao somatic embryos. However, the role of PPO in morphogenetic pathways is still unclear. In *Juglans regia* L. PPO activity was strongly related to non-embryogenic cultures and death of the tissues (Jariteh *et al*., 2011). Further studies around cacao somatic embryos germination and PPO activity are required for a definitive conclusion of the role of this enzyme during the development of cacao somatic embryos.

5.3 Features for post-embryonic development

Concerning the maturation of cacao somatic embryos several problems like morphological abnormalities, lack of vigor and low rate of conversion into plantlets have been described in many studies carried out with cacao somatic embryos (Niemenak *et al*., 2008; Maximova *et al*., 2005; Alemanno *et al.,* 1997; Figueira and Janick 1995). Alemanno *et al*. (1997) characterized the morphological and physiological differences

between development of cacao zygotic and somatic embryos. These authors described four different stages of cacao zygotic embryos development after growth phase which demonstrate respective physiological development up to maturation. However, the same was not described for somatic embryos. In the present study, morphological distinctions within cacao somatic embryos development after the morphogenesis phase could be described. Morphologically distinct somatic embryos of coffee were shown to develop differentially into plantlets (Etienne *et al.*, 2002). It is suggested that the knowledge of somatic embryos development and their sorting for specific treatments can increase significantly the rate of conversion into plantlets as well as the vigor of these (Jayasankar *et al.*, 2003; Etienne *et al.*, 2002; Stasolla *et al.*, 2002).

Analysis concerning accumulation of reserves in morphologically distinct cacao somatic embryos revealed that mature appearance of the embryos, i.e. large and well developed cotyledons, is not always correlated to physiological maturity. It can indicate a deficiency of these embryos to accumulate reserves during their growth. According to the obtained results, cacao somatic embryos developed in TIS at stage 6 of development contain the major pool of reserve compounds. However, cacao somatic embryos at this stage have already begun the germination process. The fact that the embryos with the highest levels of total proteins were already germinated may indicate that an early germination occurs even before the embryos have achieved complete maturation. This advance usage of reserves leads to a lack of vigor and poor development of organs resulting in death of the tissue. On the other hand, in cacao zygotic embryos the levels of protein content as well as other reserve compounds do not show significant decrease after germination (Stoll *et al.*, 2010), since cacao seeds are recalcitrant and did not show a desiccation process before germination. It may suggest a normal state of cacao somatic embryos physiology at this developmental phase. It also demonstrates the complex biology of cacao somatic embryos development and the importance to consider each stage of late developmental phase

as individual with respect to periods and conditions of maturation, germination and conversion into plantlets.

It is well known that somatic embryos accumulate much lower concentrations of storage compounds than the zygotic counterpart (Kormuták *et al*., 2003, von Arnold *et al*., 2001; Dodeman *et al*., 1997). It was also observed for cacao somatic embryos (Alemanno *et al*., 1997), where it was demonstrated that specific maturation conditions like addition of phytohormons and agents like sucrose can improve the accumulation of storage compounds in these embryos and consequently increase the conversion rates. In this work, culture in TIS clearly led to a positive increase in the pool of reserve compounds of cacao somatic embryos. According to the results here presented, this system influenced significantly the metabolism of cacao somatic embryos when compared to those cultured on solid medium. This is indicated by the higher concentration of γ-aminobutyric acid (GABA) detected in somatic embryos cultured on solid medium. It is known that, GABA is an indicator of stress during somatic embryogenesis. GABA is related to carbon and nitrogen balance in the cells, this metabolism is in *in vitro* culture conditions unstable due to the flux of carbon resources and osmotic pressure in the cells (Bouché and Fromm, 2004). Niemenak *et al*., (2008) observed the influence of culture conditions on cacao somatic embryogenesis. In their tests higher concentrations of GABA were observed in somatic embryos cultured on solid medium or in TIS when the frequency of floating per day was low. It suggests the somatic embryos may suffer drying stress on solid medium and in TIS when immersions occur in low frequencies during the day. One of the most abundant amino acids in this work was MET, not cited by Niemenak *et al*. (2008). This can be explained because the cacao somatic embryos analyzed in that work were at an early phase of development, perhaps this amino acid is first synthesized during the late embryogenesis of cacao embryos. In both works, ASN was the most abundant amino acid found during all the development stages and culture conditions. During seed maturation ASN an important factor of amines through H^+-Co-transport of amino acid

permease, it plays a role in the cycle of Glutamate and Glutamine syntheses, precursors for most of the amino acids needed during plant development. ASN together with GLN is the main form of nitrogen arriving in legume embryos (Riebeseel *et al*., 2010). The high concentration of ASN in somatic embryos at the late phase of development may be explained by the conversion of stored protein into ASN for transport to growing organs of the plantlet, which is dependent on nitrogen (N) availability. The same N used for Glutamate and Glutamine synthase is used as source for ARG synthesis, commonly found in developing cotyledons in others species (Cánovas *et al*., 2007). The high concentration of these amino acids involved in the Glutamine/Glutamate cycle are known to be expressed during initiation of the maturation phase in Angiosperms and Gymnosperms and can be considered markers for early maturating stage (Cánovas *et al*., 2007; Sen *et al*., 2002; Tegeder *et al*., 2000). The presence or absence of specific amino acids can provide information for the understanding of cacao somatic embryos development during culture in liquid and solid medium. It allows verifying eventual deficiencies in embryonic metabolism. Based on these results, appropriate modifications in the formulation of the media formulation and culture conditions can be applied.

The incubation of cacao somatic embryos in culture medium containing agents that induce the accumulation of reserves compounds can improve the maturation of these embryos. The supplementation of the maturation culture media with 40 or 60 g L^{-1} sucrose for one week increased significantly the amounts of proteins accumulated in the cotyledons of the cacao somatic embryos. The diminution of sugars in cotyledons of somatic embryos cultured under 80 g L^{-1} sucrose may be a response to the amount of sucrose available in the medium. It is likely that the embryos take up sugar from the culture medium and reduce its synthesis in the storage organs (Gibson, 2000). This would also explain the high concentration of sugars in the axis, which serves to transport the sucrose. Starch, in contrast, showed another dynamic, in that it was highly concentrated in the cotyledons. In this case the presence of high concentrations

of sucrose being taken up from the culture medium induces the formation of starch granules in plastids to control the osmotic stress indicating a high carbon flux into starch (Wind *et al*., 2010; Weber *et al*., 2000; Buchanan *et al*., 2000).

The occurrence of callogenesis on somatic embryos matured for two weeks under 40 and 60 g L^{-1} sucrose may be caused by a stress response due the osmotic stress caused after a long period in the presence of high concentrations of sucrose.

However, it is reported that high concentration of sucrose effect the induction of embryogenic calli in *Hevea brasiliensis* tissue culture (Kouassi *et al*., 2012). On the cotyledons surface of cacao somatic embryos nodular structures could be observed which further originated somatic embryos. The exposure of *Vicia faba* to hexoses induces the formation of transfer cells under the epidermis; these regulate the transport and reallocation of sugars in the cotyledonary tissues (Mc Donald *et al*., 1996; Farley *et al*., 2000; Tegeder *et al*., 2000; Borisjuk *et al*., 2002a). However, high concentrations of hexoses inhibit the formation of such cells. A mutant of *V. faba* (*E2748*) which blocks the differentiation of epidermal cells into transfer cells develops callogenesis on the cotyledons surface at a time when sucrose increases. The callogenesis leads to the abort of the seed. The same occurs when wild-type cotyledons had the epidermis removed artificially and are cultured in culture media containing high concentrations of sucrose (Weber *et al*., 1997; Borisjuk *et al*., 2003). In *Vitis vinifera*, seed-buds cultured under high concentrations of sugars (glucose or sucrose) also resulted in formation of calli which originated somatic embryos after transfer of the calli to light, repetitive secondary somatic embryogenesis was observed (Yancheva and Roichev, 2005). Repetitive somatic embryogenesis is associated with the inability of the embryo to develop as a normal seedling (Williams and Maheswaran, 1986). This process may be occurring in cotyledons of cacao somatic embryos. The death of these embryos is also observed after formation of the nodular calli or callogenesis on the shoot meristem. The browning of the somatic embryos after several months in culture and the occurrence of repetitive somatic embryogenesis may reveal a new possibility of activation of new

embryogenic lines from senescent tissues (Jariteh *et al*., 2011) Direct somatic embryogenesis reduced somaclonal variation (Fras *et al*., 2008). It suggests that high concentration of sucrose can induce embryogenic competence of cacao somatic embryos tissues. The usage of sucrose as trigger agent for embryogenic competence may eliminate the induction of cacao somatic embryogenesis by using PGRs like 2,4-D. Detailed studies concerning ideal concentrations as well as explants are necessary to confirm this hypothesis.

Despite the advantages of increasing sucrose in the maturation media for accumulation of protein in the embryos, just a few of the embryos germinated and converted into plantlets. The action of sugars as signal molecules is very complex regulating several genes and their signaling pathways are still unclear (Gazzarini and Court, 2001; Gibson, 2000). In *Arabidopsis*, the time of exposure of the seeds to high concentrations of sucrose determines the success of seedlings development. High concentrations of sugars during maturation periods can result in arrest of post-germination development through interaction with genes related to ABA (Abscisic acid) action (Dekkers *et al*., 2008; Rognoni *et al*., 2007). However, sucrose allowed seedling development of *Arabidopsis* mutants which presents inefficiency in the catabolism of storage compounds like lipids (Pinfield-Wells *et al*., 2005).

The specific treatment of different stages of the cacao somatic embryos late development seems to be decisive to increase germination rate rather than the composition of maturation culture media. It was clearly demonstrated in this work that the moment of somatic embryos transfer to germination culture media and subsequently to culture under photoperiod plays an important role in the germination as well as in the establishment of the plantlets. In this situation it was possible to observe that somatic embryos at stage 6 are more suitable for germination corroborating with higher pool of storage compounds formerly described for somatic embryos at this stage. This work suggests that cacao somatic embryos produced in TIS are capable to germinate even without maturation pre-treatment. The quality of the somatic embryos

produced is one of the main factors of the process besides morphological heterogeneity sorting. The conversion of coffee somatic embryos into plantlets increased from 20% to 60% after selection of morphological distinct somatic embryos (Etienne *et al*., 2002). A sequence of morphological events preceding a successful germination of the somatic embryos is presented. The elongation of the primary root in darkness revealed to be fundamental for the further development of the plantlets. The behavior of cacao somatic embryos transferred to culture under light periods is documented for many plants (von Armin and Deng, 1996). The greening, non-elongation of the axis, growth of the cotyledons and ihnhibition of the apical shoot development are reported in seedling developed under light and are known as photomorphogenic development (Ang *et al*., 1998; von Armin and Wang Deng, 1996). The formation of root systems in cacao plants is known to be complex; the same is true during germination of cacao somatic embryos. The rooting process revealed a complex physiology and mainly a sensitive morphological response to modifications of culture conditions.

Further studies are required to understand which factor may trigger the successful root system formation during cacao somatic embryos germination. It seems to be possible to create a physiological status which facilitates germination. Detailed knowledge of these regulatory factors will allow to render the biotechnology of cacao in the propagation to a more successful and economically useful method. Table 1 summarizes the main founds of this work.

Table 1. Developmental processes assumed to be decisive for cacao somatic embryos post-embryonic establishment.

Culture conditions	Developmental responses
Cyclic SSE protocol (TIS + PennState's protocol)	• Improved embryogenic competence of the explants; • Increased production of somatic embryos; • Prolonged embryogenic stability of the culture; • Reduced non-embryogenic calli proliferation.
Four different developmental stages	• Confirmed the existence of four different stages during cacao somatic embryos late development; • Allowed to determine relations between morphology and physiology of somatic embryos development; • Identified tissue specific physiological processes occurring during late phase of cacao somatic embryos development; • Allowed to observe in detail morphological characteristics of embryos suitable for further conversion into plantlets.
Maturation culture media in TIS (60 g L^{-1} of sucrose/ 1 week)	• Permitted cacao somatic embryos to accumulate storage compounds in a balance that favored the embryonic development; • Improved germination and further development into plantlets in comparison to 40 or 80 g L^{-1} of sucrose.
Germination in darkness	• Allowed the embryos at stage 6 to consume storage compounds present in the storage organ more efficiently; • Resulted in growth of well developed primary roots; • Avoided death of somatic embryos and occurrence of R.S.E.; • Lead to establishment of normal healthy plantlets with root systems and well developed leaves.

Somatic embryogenesis of cacao has been improved throughout many years. Technological advances available nowadays allow detailed investigations concerning physiological and molecular aspects of cacao embryos development. The management of embryonic characteristics and methodological procedures may lead to ideal physiological conditions of the embryos. The results obtained in this work evidence a very sensible switch mechanism present in a limited period of time during cacao somatic embryos late development. Studies concerning signals transduction occurring during assimilation of nutrients present in the medium may help to identify eventual failures occurring in cacao somatic embryos physiology. Such information may clarify the reasons of somatic embryos inefficiency in accumulating storage compounds and how the usage of these compounds occurs during late phase of embryo development. So far, TIS can be considered as efficient method for production of cacao somatic

embryos increasing quality of the cultures. It seems to be beneficial for conversion of cacao somatic embryos into plantlets, the addition of sucrose in culture medium during maturation phase and the germination taking place in darkness until complete emission of the primary root.

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