Aspects of somatic embryogenesis and seed germination of peach palm (*Bactris gasipaes* Kunth)

DISSERTATION

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To Whom It May Concern

The quality of the English language in the doctoral thesis written by Douglas A. Steinmacher was completely reviewed twice by Charles R. Clement, who was born and raised in the United States of America.

Charles R. Clement, PhD
05 January 2010

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“A cada dia que vivo, mais me convenço de que o desperdício da vida está no amor que não damos, nas forças que não usamos, na prudência egoísta que nada arrisca e que, esquivando-nos do sofrimento, perdemos também a felicidade.”

Carlos Drummond de Andrade

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Douglas A. Steinmacher

Table of contents

I. List of Abbreviations

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µM	Micromolar
2,4-D	2,4-Dichlorophenoxyacetic acid
2-iP	2-isopentyladenine (6-dimethylaminopurine)
aa	Amino acids
ABA	Abscisic acid
AG	Arabinogalactan
AGPs	Arabinogalactan proteins
ANOVA	Analysis of variance
ASIL1	Arabidopsis six b-interacting protein 1-like 1
BCIP	5-bromo,4-chloro,3-indolylphosphate
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
ca.	circa
CHD3	Chromodomain-helicase-DNA-binding protein 3
CLV	Clavata
cm	Centimeter
CnSERK	*Cocos nucifera* Somatic Embryogenesis Receptor Kinase
CO_2	Carbon dioxide
DAPI	4'-6-Diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DW	Dry weight
ECMSN	Extracellular matrix surface network
EDTA	Ethylenediamine tetraacetic acid
FFM	Fat free material
FITC	Fluorescein isothiocyanate
FPLC	Fast performance liquid chromatography
g	Gram
GPI	Glycosylphosphotidylinositol
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
Jim	John Innes University monoclonal antibodies
KCl	Potassium chloride
kDa	Kilo Dalton
KDEL	CysEP - KDEL-tailed cystein endoproteinase
kgf	Kilogram force
LEC	Leafy cotyledon
LM	Leeds University monoclonal antibodies
M	Molar
m	Meter
MAb	Monoclonal antibody
mg	Milligram
$MgCl_2.6H_2O$	Magnesium chloride hexahydrate
min	Minute
ml	Milliliter
mm	Millimeter

mM	Millimolar
mRNA	Messenger ribonucleic acid
MS	Salts of Murashige and Skoog (1962)
MS/MS	Tandem mass spectrometry
MSB	Microtubule stabilizing buffer
NAA	α-Naphthaleneacetic acid
NaCl	Sodium chloride
NBB	Naphtol blue-black
NBT	Nitro blue tetrazolium chloride
NH_4HCO_3	Ammonium Bicarbonate
nm	Nanometer
nsLTP	Non-specific lipid transfer protein
OPA	o-Phthaldialdehyde
PAS	Periodic Acid-Schiff's reaction
PBS	Phosphate buffered saline
PCD	Programmed cell death
Picloram	4-amino-3,5,6-trichloropicolinic acid
PKL	Pickle
PSV	Protein storage vacuoles
PT	Primordia timing
QTOF	Quadrupole Time-of-Flight
RNA	Ribonucleic acid
rpm	Rotation per minute
S	Sedimentation Coeficient
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SNK	Student-Newman-Keuls
ßGlcY	ß-glucosyl Yariv reagent
TBS	Tris buffered saline
TEM	Transmission electron microscopy
TIR1	Transport inhibitor response 1
TIS	Temporary immersion system
TKM	Tris-KCl-$MgCl_2$ buffer
Tris	Tris(hydroxymethyl)-aminomethane
UV	Ultra-violet
w/v	Weight per volume
WUS	Wuschel
αGlcY	α-glucosyl Yariv reagent
µm	Micrometer

Amino acids

Ala	Alanine	**Gly**	Glycine	**Pro**	Proline
Arg	Arginine	**His**	Histidine	**Ser**	Serine
Asn	Asparagine	**Ile**	Isoleucine	**Thr**	Threonine
Asp	Aspartic acid	**Leu**	Leucine	**Trp**	Tryptophan
Cys	Cysteine	**Lys**	Lysine	**Tyr**	Tyrosine
Glu	Glutamic acid	**Met**	Methionine	**Val**	Valine
Gln	Glutamine	**Phe**	Phenylalanine	**GABA**	γ-Aminobutyric acid

II. List of figures

III. List of tables

IV. Abstract

Peach palm (*Bactris gasipaes* Kunth) is a member of the family Arecaceae and is the only palm species with fully domesticated populations in the Neotropics. It is a multi-purpose but underutilized species. Today, fruit production for subsistence and local markets, and heart-of-palm production for local, national and international markets are the most important uses. Conventional breeding programs of peach palm are long term efforts due to long generations, tree height, difficulties with controlled pollination and other factors. Although it is a caespitose palm tree, its propagation is currently based on seeds, as off-shoots are difficult to root. Clonal propagation is, however, extremely important. Hence, tissue culture techniques are considered to be the most likely strategy for efficient clonal plantlet regeneration of this species. Among various techniques, somatic embryogenesis offers the advantages of automated large-scale production and genetic stability of the regenerated plantlets. Similarly, understanding the morpho-histological and biochemical aspects of peach palm seed germination is important, as this is currently the main type of propagation and peach palm has recalcitrant seeds. In the present study relevant new information regarding peach palm in vitro culture as well as seed germination is reported.

The occurrence of secondary somatic embryogenesis is described and a protocol for the establishment of cycling cultures using a temporary immersion system (TIS) is presented. Cycling cultures were established and somatic embryos as explants had high embryogenic potential over the period tested. The use of TIS greatly improved the number of somatic embryos obtained, as well as subsequent plantlet growth. Histological analyses showed that starch accumulation precedes the development of somatic embryos, and that these cells presented high nucleus : cytoplasm ratios and high mitotic activity. A multicellular origin of the secondary somatic embryos is hypothesized. Plantlets were obtained and after 3 months in culture their growth was significantly better in TIS than on solid culture medium. However, during acclimatization the survival rate of TIS-grown plantlets was lower. TIS involves the use of liquid cultures and one advantage of liquid culture medium compared to solid culture media is the absence of nutrient gradients, as well as the fact that substances secreted into the culture medium with putative signaling functions are able to reach other explants. The most important secreted substances in this regard are arabinogalactan proteins (AGPs). In the present study the effect of Yariv reagent in the liquid culture medium was evaluated, and the localization of specific AGPs and pectin during induction and development of peach palm somatic embryos was demonstrated. The occurrence of an extracellular matrix surface network (ECMSN) covering globular somatic embryos is described. Somatic embryos and callus development rates were significantly affected by the presence of 30 µM Yariv reagent but no effect was observed on fresh weight increments. In the presence of Yariv reagent somatic embryos presented loose cells in the protoderm and no signs of polarization were observed. Scanning electron microscopy (SEM) analyses also confirmed protodermis mal-formation. Histological analyses from control cultures revealed a well-delimited protoderm and signs of polarization in the somatic embryos. Analyses of specific monoclonal antibodies (MAbs) against different AGP epitopes revealed a specific pattern of distribution for each epitope. MAb Jim13 had differential expression and showed intense signal on the embryogenic sector and some immediately adjacent layers. MAb Jim7 (against pectin) recognized cell walls of all cells and a specific layer over the developing somatic embryo, as well as over the shoot meristem region of mature somatic embryos. This corresponds to an

ECMSN associated with the development of somatic embryos and closely related to the expression of MAb Jim13. SEM confirmed the presence of an ECMSN covering a specific group of cells.

Morpho-histological and biochemical aspects before and during the germination of peach palm seeds were also evaluated in the present study. Histological and ultrastructural analyses of the zygotic embryo revealed its active metabolic state before germination, where numerous small vacuoles with electron-amorphous substances, endoplasmatic reticulum and Golgi complexes were observed. This active metabolic state is an important aspect related to seed recalcitrance. Histological aspects of the haustorium and endosperm were examined. A correlation between plantlet growth and endosperm breakdown was observed and a specific sequence of endosperm breakdown is described, which started with the mobilization of storage proteins. Storage proteins were extracted and partially characterized based on their buffer solubility. Three polypeptides of 45 to 67 kDa were the major bands of proteins soluble in low salt buffer. After peptide sequencing these were confirmed to be 7S vicilin-like proteins. High salt-soluble proteins were composed by two sub-units of ca. 23 kDa and ca. 32 kDa under reducing conditions; under non-reducing conditions a single protein of ca. 55 kDa was observed. These showed high homology to 11S glutelin-like proteins after peptide sequencing. Modifications in the low salt buffer-soluble protein profile were detected by SDS-PAGE and two subunits of the 7S vicilin-like globulin completely disappeared only during the final stages of germination; one subunit was still present in the final stage. Free amino acids were present at lower levels in endosperm than in haustorium throughout germination. Differences were also observed in the profile of free amino acids present in the haustorium and in the endosperm during germination.

An additional aspect not well discussed in palm seed biology is the mechanisms controlling the hydrolysis of storage compounds of endosperm during germination. In *Phoenix dactylifera*, a species with orthodox seeds, the endosperm was shown to be senescent tissue without the capacity for de novo protein synthesis. This suggests that all enzymes necessary for germination are already present in the endosperm in an inactive form or they are secreted by the haustorium. In *Bactris gasipaes*, a palm species with recalcitrant seeds, we used ultrastructural analysis and immuno-localization, and found that de novo protein synthesis occurs in the endosperm during germination and that this tissue also undergoes programmed cell death (PCD). Polysome analysis supports the observation of de novo protein synthesis. PCD is a highly regulated mechanism, which requires de novo protein synthesis, where KDEL-tailed cystein endoproteinase (KDEL-CysEP) is involved. In *B. gasipaes*, de novo synthesis of KDEL-CysEP occurred in the endosperm during germination and accumulated on endosperm cell walls. Additional PCD features included cytoplasm shrinkage and acidification. These observations may help explain the recalcitrance of *B. gasipaes* seeds, as dehydration-sensitivity is generally related to an active cell metabolism.

V. Zusammenfassung

Die Pfirsichpalme (*Bactris gasipaes* Kunth), die zur Familie der Arecaceae gehört, ist die einzige Palmenart mit vollständig domestizierten Populationen in der Neotropis. Sie ist eine vielseitig verwendbare, aber unter ihrem Potential genutzte Art. Von ökonomischer Relevanz sind heutzutage die Fruchtproduktion für die Subsistenzwirtschaft und den lokalen Markt sowie die Palmenherzenproduktion für den lokalen, nationalen und internationalen Markt. Aufgrund eines langen Generationszyklus, großer Wuchshöhe, einer nur schwer kontrolliert ablaufenden Bestäubung sowie weiterer Faktoren, benötigen konventionelle Züchtungsverfahren bei der Pfirsichpalme einen intensiven und langen Arbeitsaufwand. Obwohl es sich um eine mehrstämmig wachsende Palme handelt, basiert ihre Fortpflanzung derzeit auf Samen, da Wurzelbildung an Nebensprossen zu fördern, schwierig ist. Aus diesen Gründen kommt der klonalen Vermehrung eine entscheidende Bedeutung zu, weshalb Gewebekulturen als effizienteste Strategie für die klonale Pflanzenregeneration dieser Art gelten. Von den verschiedenen nutzbaren Möglichkeiten bietet die somatische Embryogenese den Vorteil der automatisierten Massenproduktion sowie den der genetischen Stabilität des vervielfältigten Pflanzenmaterials. Gleichzeitig ist das Verständnis der morpho-histologischen und biochemischen Vorgänge während der Samenkeimung notwendig, da diese die zur Zeit hauptsächlich verwendete Vermehrungsform darstellt und die Pfirsichpalme zudem recalcitrante Samen aufweist. In dieser Arbeit werden relevante, neue Informationen über die in vitro-Kultur sowie die Samenkeimung bei der Pfirsichpalme beschrieben, die bedeutenden Einfluss für die Arterhaltung und Züchtung dieser Art haben können.

Darüber hinaus wird das Auftreten von sekundärer somatischer Embryogenese beschrieben sowie ein Protokoll für die Etablierung von zyklischen Kulturen im temporary immersion system (TIS) vorgestellt. Zyklische Kulturen wurden etabliert und die somatischen Embryonen besaßen als Explantate über die gesamte Testperiode ein hohes embryogenetisches Potential. Die Verwendung des TIS steigerte die Anzahl der gewonnenen somatischen Embryonen sowie deren nachfolgendes Pflanzenwachstum erheblich. Histologische Analysen zeigten, dass Stärkespeicherung vor der Entwicklung der somatischen Embryonen auftritt und dass diese Zellen zudem ein hohes Kern:Cytoplasma-Verhältnis sowie einen hohes mitotisches Potential besitzen. Es wird vermutet, dass sekundäre somatische Embryonen einen vielzelligen Ursprung besitzen. Das im TIS kultivierte Pflanzenmaterial zeigte nach drei Monaten ein signifikant besseres Wachstum als die Vergleichsproben auf Festkulturmedium, während der Akklimatisierung lag die Überlebensrate von den im TIS gezogenen Pflanzen jedoch niedriger. Das TIS basiert auf Flüssigkulturmedium; dies erbringt Vorteile gegenüber dem Festmedium. Nährstoffgradienten werden nicht ausgebildet und sekretierte Substanzen mit möglicher Signalwirkung können andere Explantate erreichen. Die Arabinogalactanproteine (AGPs) sind in diesem Zusammenhang die wichtigsten sekretierten Substanzen. In der vorliegenden Studie wurde der Effekt von Yariv-Reagenz im Flüssigkulturmedium evaluiert sowie die Lage spezifischer AGPs und Pektine während Induktion und Entwicklung der somatischen Embryonen der Pfirsichpalme gezeigt. Das Auftreten eines extracellular matrix surface network (ECMSN) auf der Oberfläche von globulären, somatischen Embryonen wird beschrieben. Somatische Embryonen und Kallusentwicklung wurden in hohem Maße bei einer Zugabemenge von 30 µM Yariv-Reagenz beeinflusst. Bei Anwesenheit von Yariv-Reagenz zeigten histologische Analysen der somatischen Embryonen lose Zellen im

Protoderm und keine Anzeichen von Polarisation, während bei Kontrollkulturen ein gut entwickeltes Protoderm sowie Anzeichen einer Polarisation zu erkennen waren. Rasterelektronenmikroskopische Aufnahmen (SEM) bestätigten Fehlentwicklungen der Protodermis. Die Analyse bestimmter monoklonaler Antikörper (MAbs) gegen verschiedene AGP-Epitope zeigte ein spezifisches Verteilungsmuster für jedes Epitop. MAb Jim13 zeigte differenzierte Ausprägungen und starke Reaktionen im embryogenen Sektor sowie bei einigen direkt angrenzenden Schichten. MAb Jim7 (gegen Pektin) erkannte die Zellwände aller Zellen sowie eine spezifische Schicht an der Oberfläche des somatischen Embryos und an der Oberfläche des Apikalmeristems reifer somatischer Embryonen. Diese entspricht der ECMSN, die in Zusammenhang mit der Entwicklung somatischer Embryonen und eng in Beziehung zu der Ausprägung von MAb Jim13 steht. SEM bestätigten das Vorhandensein einer ECMSN an der Oberfläche bestimmter Zellgruppen.

Morphohistologische und biochemische Aspekte vor und während der Keimung der Pfirsichpalmensamen wurden ebenfalls in dieser Arbeit untersucht. Histologische und ultrastrukturelle Analysen des zygotischen Embryos veranschaulichten dessen aktiven Stoffwechsel vor der Keimung. Zahlreiche, kleine mit nicht-kontrastierenden Substanzen gefüllte Vakuolen, endoplasmatisches Retikulum und Golgi-Apparate wurden beobachtet. Dieses Stadium des aktiven Stoffwechsels ist ein bedeutendes Merkmal recalcitranter Samen. Untersuchungen der histologischen Eigenschaften des Haustoriums und des Endosperms wurden durchgeführt. Es konnte eine Korrelation zwischen Pflanzenwachstum und Abbau des Endosperms beobachtet werden, außerdem wird eine bestimmte Sequenz des Endospermabbaus beschrieben, die mit einer Mobilisierung von Speicherproteinen beginnt. Speicherproteine wurden extrahiert und teilweise basierend auf ihrer Pufferlöslichkeit charakterisiert. Drei Polypeptide zwischen 45 und 67 kDa bildeten die Hauptbanden von in gering konzentrierter Salzlösung löslichen Proteinen. Nach der Peptidsequenzierung wurden diese als 7S Vicilin ähnliche Proteine identifiziert. In hohen Salzkonzentrationen lösliche Proteine waren unter reduzierenden Bedingungen aus zwei Untereinheiten von ca. 23 kDa und ca. 32 kDa zusammengesetzt; unter nicht reduzierenden Bedingungen wurde ein einziges Protein von ca. 55 kDa beobachtet. Dieses zeigte nach Peptidsequenzierung starke Homologien zum 11S Glutelin ähnlichen Protein. Veränderungen im low salt buffer-soluble protein-Profil wurden mithilfe von SDS-PAGE gemessen. Die beiden Untereinheiten des 7S Vicelin ähnlichen Globulins verschwanden nur während der Endphasen der Keimung, wobei eine Untereinheit bis zur letzten Phase vorhanden war. Freie Aminosäuren waren während der Keimung im Endosperm, im Gegensatz zum Haustorium, nur in geringen Konzentrationen vorhanden. Die freien Aminosäuren im Haustorium und im Endosperm zeigten zudem unterschiedliche Zusammensetzung.

Ein zusätzlicher, in der Palmensamenbiologie wenig erforschter Aspekt ist der Mechanismus, der die Hydrolyse der Speicherstoffe im Endosperm während der Keimung kontrolliert. Bei *Phoenix dactylifera*, einer Art mit orthodoxen Samen, wurde gezeigt, dass dessen Endosperm ein seneszentes Gewebe ohne die Fähigkeit für die de novo-Proteinsynthese ist. Dieses legt die Vermutung nahe, dass alle für die Keimung notwendigen Enzyme schon im Endosperm vorhanden sind, entweder in inaktiver Form oder sekretiert durch das Haustorium. Bei *Bactris gasipaes*, eine Palme mit recalcitranten Samen, konnten wir mithilfe ultrastruktureller Analysen und Immunolokalisation nachweisen, dass während der Keimung im Endosperm die de novo-Proteinsynthese und im selben Gewebe zudem programmierter Zelltod (PCD) auftritt. Polysomale Analysen stützten die Beobachtung der de

novo-Synthese. PCD ist ein hoch regulierter Mechanismus, für den die de novo-Proteinsynthese Voraussetzung ist, bei der KDEL-tailed Cystein-Endoproteinase (KDEL-CysEP) beteiligt ist. In *B. gasipaes* findet die de novo-Synthese der KDEL-CysEP im Endosperm während der Keimung statt, um anschließend an der Endospermzellwand zu akkumulieren. Während des PCD kommt es zudem zu einer Schrumpfung des Cytoplasmas und einer Ansäuerung. Diese Beobachtungen könnten nützlich sein, um die Recalcitranz von *B. gasipaes* Samen zu erklären, da die Austrocknungssensibilität generell mit einem aktiven Zellmetabolismus verbunden ist.

1 General introduction

Peach palm (*Bactris gasipaes* Kunth) is a member of the family Arecaceae and is the only palm species with fully domesticated populations in the Neotropics. The domestication process appears to have been initiated in southwestern Amazonia, which today belongs to northern Bolivia and southeastern Peru. It is a multi-purpose but underutilized species. Today, fruit production for subsistence and local market, and heart-of-palm production for local, national and international markets are the most important uses. Ideotypes have been described for both uses, but breeding and conservation programs have suffered numerous problems, principally involving continuity. The use of in vitro techniques is an important tool that must be associated with breeding and conservation programs, although it will not resolve continuity problems.

Historically palm species, including peach palm, were considered recalcitrant to in vitro culture. During the last years, however, several advances have been achieved with in vitro culture of peach palm; nevertheless a comercial protocol does not exist as yet. Conventional breeding programs of peach palm are long term efforts due to long generations (at least 6 years), tree height, difficulties with controlled pollination and other factors. Hence, a reliable in vitro regeneration protocol for peach palm is important. Clonal propagation has the potential to reduce the time necessary for establishing elite plant seed orchards, by capturing and fixing the genetic gain expressed by selected plants. Somatic embryogenesis is the preferred in vitro regenerative route for palms, as this morphogenetic pathway may increase the number of regenerated plantlets in comparison with organogenesis. The production of somatic embryos capitalizes upon the totipotency of plant cells and involves the development of bipolar structures resembling zygotic embryos. Among other advantages, somatic embryogenesis permits the creation of cycling cultures through the use of cell suspensions or through secondary somatic embryogenesis allowing the large-scale commercial production of elite plants.

Peach palm conservation programs may also profit from the use of in vitro regeneration protocols since germplasm banks could be cloned and transferred to other institutions if necessary. Ex situ conservation of plant genetic resources usually use seed banks. However, peach palm has recalcitrant seeds and in vitro methods could contribute to long-term conservation. Regarding palm seeds biology, little is known about the biochemical aspects of palm seed germination, especially the factors involved in dehydration-sensitivity in peach palm seeds. Therefore, studies regarding peach palm seed biology are also necessary.

The present study is organized in chapters with separate specific objectives. Chapter I presents background information on peach palm and the state of art of palm in vitro research

and seed biology. In Chapter II an improved protocol for in vitro regeneration of peach palm using a temporary immersion system is described. This involves the use of liquid culture medium, in which some secreted substances could also influence the in vitro responses of peach palm. The most well studied substances are arabinogalactan proteins. The presence and possible roles of these proteoglycans were evaluated and the results are shown and discussed in Chapter III. Our results pointed to a close relationship between the presence of specific epitopes with the development of somatic embryos and the formation of an extracellular matrix network. In Chapter IV results regarding the morpho-histological and biochemical aspects of peach palm seed germination are presented. These include the partial characterization of the storage proteins, which can be a molecular marker for somatic embryo quality. However, it is clear that several mechanisms of palm seed germination biology are unknown, such as, for instance, the mechanisms of endosperm breakdown. It was thought that the palm endosperm is not able to synthesize proteins de novo. However, we show that peach palm endosperm undergoes programmed cell death during germination, a highly coordinated genetic process requiring de novo protein synthesis. These results are presented in Chapter V.

2 Aims of the research

The present study had two different main aims. The first was the improvement of the peach palm in vitro regeneration rate and the elucidation of the factors underlying somatic embryo development. The second is related to the germination of peach palm seeds, as an initiative to increase our knowledge of the biology of palm seeds. The specific objectives of the present study were:

Peach palm in vitro regeneration:

- Establish a suitable process for the in vitro regeneration of peach palm using a temporary immersion system;
- Describe the morpho-histological aspects of somatic embryo development of peach palm;
- Evaluate the possible role of arabinogalactan proteins during the development of peach palm somatic embryos;

Peach palm seed germination:

- Describe the morpho-histological aspects of the germination of peach palm seeds;
- Partially characterize peach palm globulin storage proteins;
- Show that the peach palm endosperm undergoes programmed cell death during germination.

Chapter I:

BACKGROUND INFORMATION

1 Peach palm (*Bactris gasipaes*)

1.1 Taxonomy and genetic resources

The Arecaceae family is one of the most interesting and important groups of tropical plants (Balick, 1988). It is the only botanical family of the order Arecales, constituted by approximately 2800 species subdivided into 5 subfamilies, namely calamoideae, nypoideae, coryphoideae, ceroxyloideae and arecoideae. They are distributed in most warm temperate climates and all tropical and sub-tropical parts of the world (Figure 1A-E). The subfamily Arecoideae, which *Bactris* belongs to, is well distributed in all tropical and subtropical regions of the world (Figure 1E); *Bactris* sp., however, are concentrated in Central and South America and the Caribbean (Henderson, 2000). Peach palm (*Bactris gasipaes* Kunth) also has a wide geographical distribution, from central Bolivia to northeastern Honduras and from the mouth of the Amazon River and the Guianas to the Pacific coast of Ecuador and Colombia (Figure 2; Mora-Urpí *et al.*, 1997).

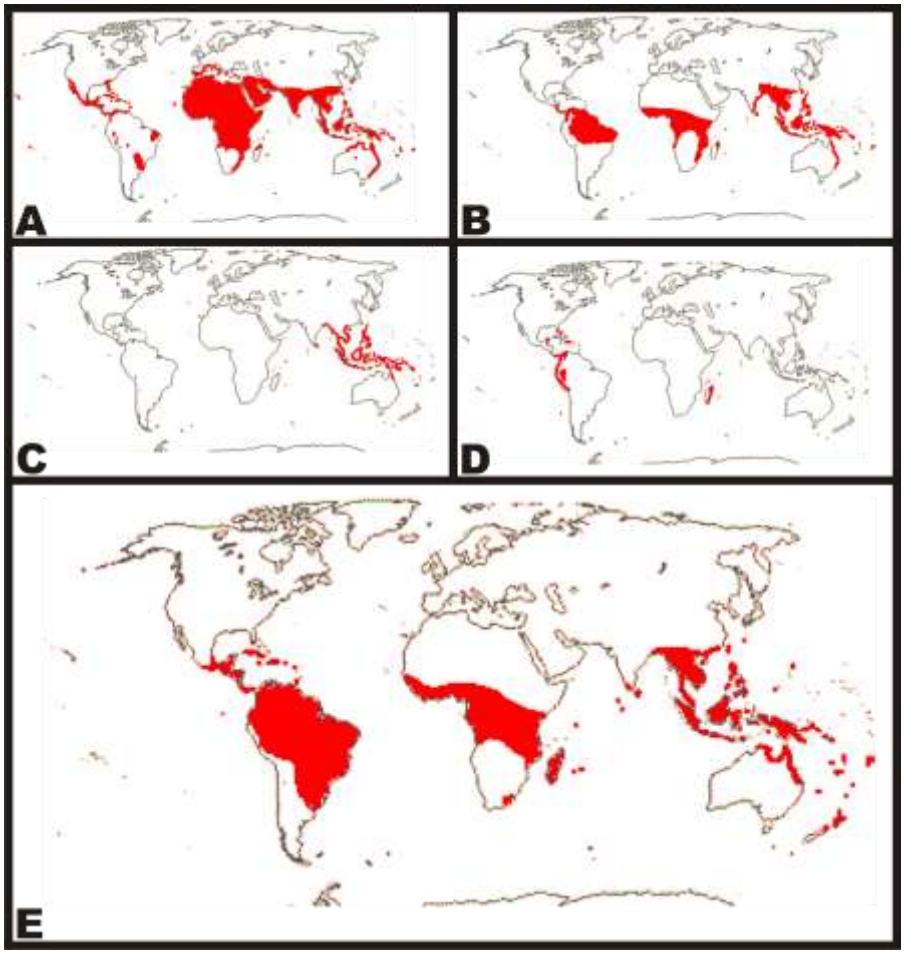

Figure 1 - Geographical distribution of orders of the family Arecaceae. A – Order Coryphoideae. B – Calamoideae. C – Nypoideae. D – Ceroxyloideae. E – Arecoideae. (Source: Missouri Botanical Garden, accessed: 25.11.2009 http://www.mobot.org/MOBOT/Research/APweb/orders/Arecalesweb.htm).

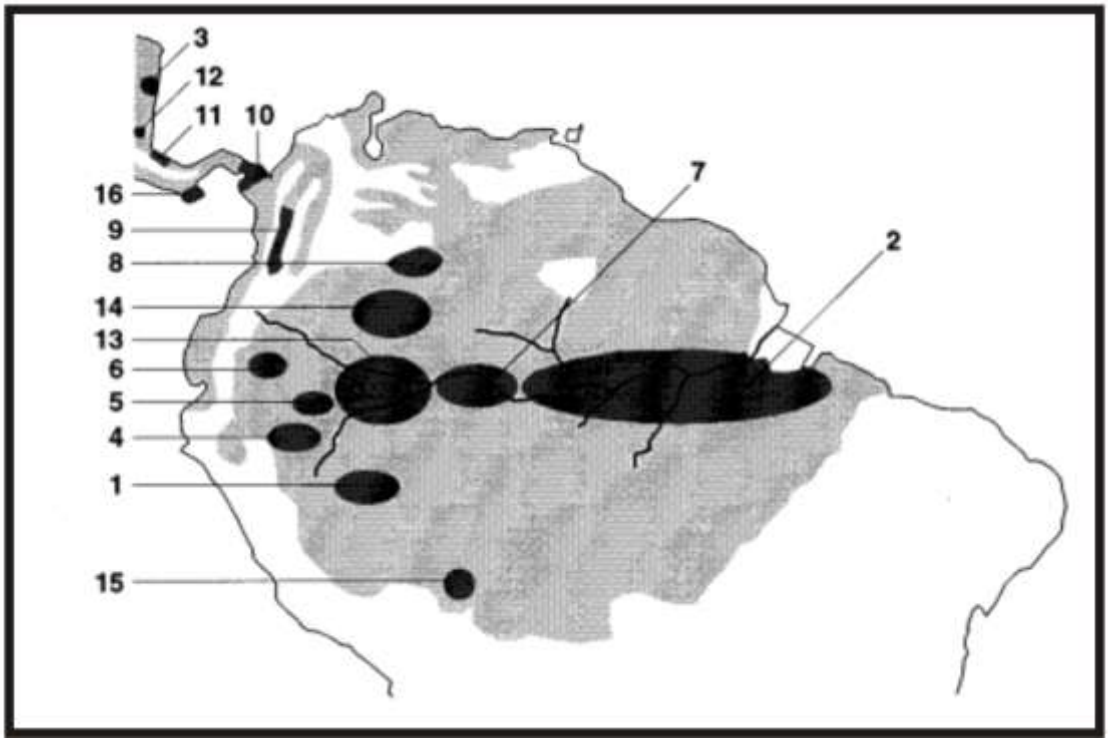

Figure 2 - Geographical distribution of *Bactris gasipaes* var. *gasipaes* and its landraces: Microcarpa -- (1) Juruá; (2) Pará; (3) Rama; (16) Azuero; (15) Tembé. Mesocarpa -- (4) Pampa Hermosa; (5) Tigre; (6) Pastaza; (7) Solimões; (8) Inirida; (9) Cauca; (10) Tuira; (11) Utilis; (12) Guatuso. Macrocarpa -- (13) Putumayo; (14) Vaupés (Source: Mora-Urpí *et al.*, 1997).

Peach palm is the only domesticated palm in tropical America and it was probably first used for its wood and later fully domesticated for its starchy-oily fruits (Clement, 2008). This species has a long history of domestication, probably 10,000 years, and became a staple food for many pre-Columbian Amerindian communities in the lowland humid neotropics (Mora-Urpí *et al.*, 1997; Clement, 2008). They valued peach palm for several reasons: it was easy to cultivate in traditional agroforestry systems, it yielded well on infertile soils, the fruits could be prepared into a variety of nutritious foods, and other plant parts could be consumed or used for construction and other household needs (Mora-Urpi *et al.*, 1997). Additionally, through western Amazonia and extending up to Costa Rica the peach palm appeared to be as important as maize (*Zea mays* L.) and cassava (*Manihot esculenta* Crantz), giving this species status as a crop plant since pre-Columbian times (Clement, 2008).

Peach palm's origin is probably in southwestern Amazonia (Clement, 2008; Clement *et al.*, 2009). Until recently it was considered a cultigen (i.e., a cultivated species with no known wild populations) but research during the last 30 years changed this (Clement *et al.*, 2009). Re-evaluation of the genus *Bactris* grouped all wild populations as *Bactris gasipaes* var. *chichagui* (H. Karsten) Henderson and all domesticated populations and landraces into *B. gasipaes* var. *gasipaes* (Henderson, 2000). The main morphological difference between wild

and cultivated forms is fruit size; wild populations have small fruits (0.5 g to 10 g), while fruits of domesticated populations vary from 10 g to 120 g (Mora-urpi *et al.*, 1997; Clement *et al.*, 2009). Additionally, significant genetic differentiation was observed at the molecular level between wild and cultivated peach palm (Couvreur *et al.*, 2006).

Cultivated peach palm is a complex of several landraces with high genetic variability (Mora-Urpí *et al.*, 1997). This ample genetic variability might be explained by different domestication stages and objectives (oil or starch) in different landscapes, as well as by the reproductive system of peach palm, which is predominantly allogamous (Mora-Urpí *et al.*, 1997). Each landrace consists as a number of populations, usually named after municipalities or communities (Adin *et al.*, 2004). An initial criterion for landraces classification was based upon fruit weight, with three different groups: Microcarpa (fruits from 10 to 20 g); Mesocarpa (20 to 70 g) and Macrocarpa (70 to 120 g) (Mora-Urpi *et al.*, 1997; Clement, 2008). However, phenotypic differences for fruit color, weight and composition, stem diameter, leaf area, disease resistance and numerous other differences have also been observed in the field and in genebanks (Martel and Clement, 1986; Clement, 1997; Farias Neto, 1999).

The gene pool of cultivated peach palm and its wild relatives is rich in diversity but also subject to genetic erosion, creating an urgent need to sample and conserve germplasm (Mora-Urpi *et al.*, 1997; Clement *et al.*, 2009). Genetic erosion is still a threat occurring in the field as well as in genebanks (Clement, 1996; Clement *et al.*, 2009). Clement *et al.* (2009) observed that in the Arc of Fire – the expanding agriculture frontier in Brazil - wild peach palm populations have become locally extinct in some parts of its original range, due to deforestation for agriculture and pasture. A difficult situation is found in field genebanks and Clement (1996) suggested that the main causes of genetic erosion in these collections were biological aspects, including diseases and pests, and lack of knowledge about the palm's biology; political-institutional aspects, such as lack of coherent research and development policies, as well as land use policies; and infra-institutional, mainly regarded to the human resources directly involved in the conservation programs. Due to several factors, obtaining resources for the maintenance of large peach palm field collections is a problem (Clement *et al.*, 2004). Mora-Urpí *et al.* (1997) also suggest a cultural aspect, as since the conquest period, Europeans promoted the culture of exotic plants (e.g., banana, rice and wheat) as substitutes of those cultivated locally, such as peach palm, which caused more genetic erosion in these species. Additionally, due to the fact that peach palm is a domesticated species, considerable genetic erosion also occurred during the Amerindian population decline after European contact (Clement, 1999).

1.2 Morphological description

Peach palm (*Bactris gasipaes* Kunth) may reach 20 m in height with stem diameters between 15 and 30 cm and internodes between 2 and 30 cm (Figure 3A). It is a caespitose species (Figure 3B), meaning that it branches at ground level forming a clump of off-shoots. The internodes have numerous rigid spines (Figure 3C), black to brown in colour. However, there are mutations presenting spineless stems (Figure 3D). The shoot apexes contain 15 to 25 pinnate leaves, with leaflets inserted in different angles. The monoecious inflorescence appears in the axils of senescent leaves. After pollination, the fruit bunch contains 50 to 1000 fruits and weighs from 1 to 25 kg (Figure 3E). The individual fruits weigh from 0.5 to 120 g (up to 250 g has been described) (Mora-Urpi *et al.*, 1997; Clement, 2008). The mature fruit is composed of a fibrous exocarp that is red, orange or yellow in color, and a mesocarp rich in starch or oil (Arkcoll and Aguiar, 1984; Mora-Urpí *et al.*, 1997; Yuyama *et al.*, 2003). During fruit development changes in exocarp color is observed, as well as alterations in the seeds, including lignification of the endocarp associated with its change in color and hardening of the gelatinous endosperm (Figure 4). The zygotic embryo is initially globular and during differentiation of the shoot meristem (Figure 5C) becomes elongated (Figure 5B). Mature zygotic embryos of peach palm are 1.5-2 mm long, with a conical shape and with the epicotyl oblique to the cotyledon blade and procambium (Figure 5C) (Steinmacher *et al.*, 2007a). Besides its morphological appearance, little is known about the physiological status of the zygotic embryos.

Peach palm shows significant growth rate even in poor soils (Mora-Urpi *et al.*, 1997), possibly due to is morphological characteristics, such as the architecture of the pinnate leaves and the root system, for the capture of sun light, rain water and soil nutrients. Adventitious roots of peach palm produce a thick superficial mat that may extend 4-5 m around the plant and most roots occupy the upper 20 cm of the soil horizon, although some roots may extend to a depth of 2 m or more, depending upon soils and presumably genotype (Mora-Urpi *et al.*, 1997; Emmerich, 2002). Palms can develop up to four root orders [i.e., oil palm (Jourdan *et al.*, 2000)], although opportunistic root development also occurs upon root disturbance. In *Bactris gasipaes*, no fourth order roots have been found in undisturbed root systems (Göllnitz *et al.*, 2000). No root hairs are observed (Figure 6A) and the exodermis consists of extraordinary large, globose slightly flatted cells and these cells are positioned in a spiral around the root cylinder (Figure 6B; Göllnitz *et al.*, 2000), resembling a corn cob under scanning electron microscopy (Figure 6C). This morphological aspect enables a significant increase on the root specific area as well as capillary strength associated with intercellular space for microorganisms (Figure 6D-E; Emmerich, 2002; Göllnitz *et al.*, 2000). At the root

apice, border-like cells were also observed (Figure 6A). These living cells are programmed to separate from the root cap and from each other as they reach the cap periphery (Hamamoto *et al.*, 2006). It has been shown that one possible role of border cells is to modulate the environment of the plant root by producing specific substances to be released into the rhizosphere, including arabinogalactan proteins (Vicre *et al.*, 2005). In peach palm the presence of this border cells has also been detected and structural analyses also suggest that these cells are released into the soil (Figure 6F-G) and are rich in arabinogalactan proteins, indicated by specific red staining (Figure 7A-B) (Steinmacher, unpublished results). These characteristics are known to attract microorganisms to the rizosphere (Vicre *et al.*, 2005). Association of peach palm with soil microorganisms has been described (Clement and Habte, 1995; Emmerich, 2002; Göllnitz *et al.*, 2000; Silva Junior and Cardoso, 2006). These aspects might contribute to our understanding of the outstanding growth of peach palm and its importance as a component of agroforestry systems (Clement, 1989; Silva Junior and Cardoso, 2006; Lieberei *et al.*, 2002).

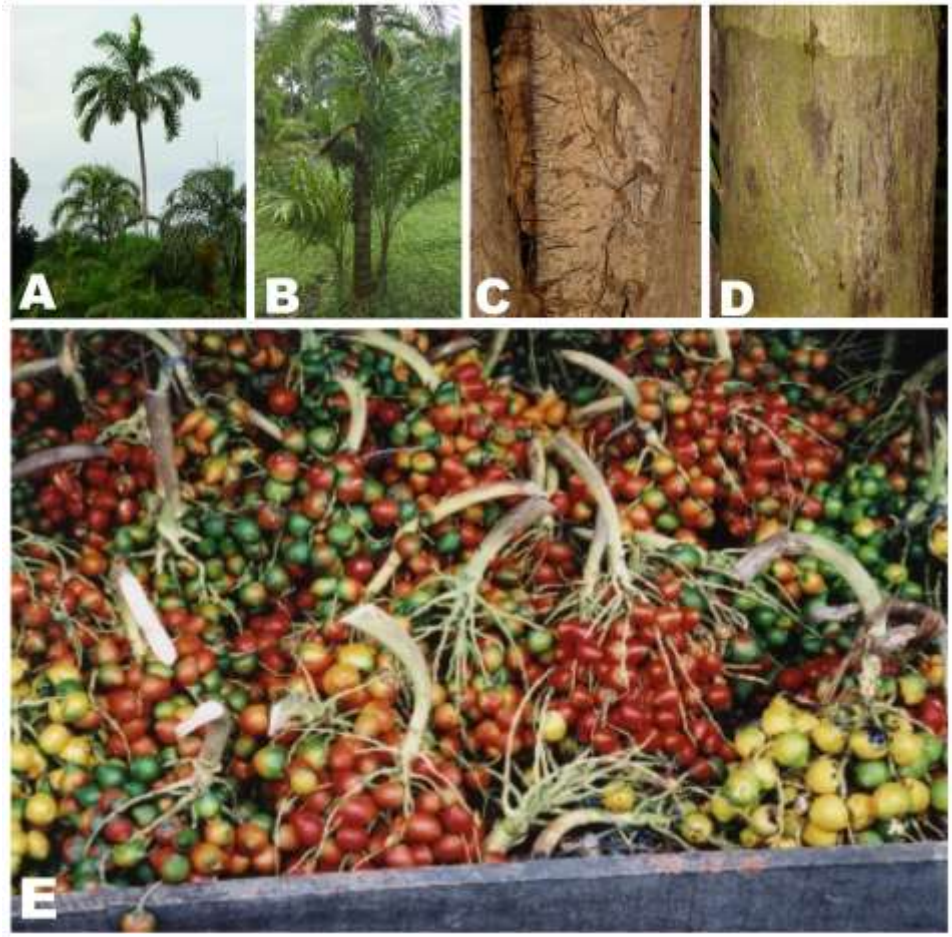

Figure 3 - Morphological aspects of peach palm. A – Adult plant. B – Plant showing the presence of off-shoots. C – Presence of rigid spines on the stem of peach palm. D – Spineless stem of peach palm. E – Fruit bunches of peach palm. (Source: Picture A and B credit John Mood Honolulu, Hawaii; Picture C-E: Credits Charles Clement, Manaus, AM)

Figure 4 - Peach palm fruit and seed development. Fruit maturation is accompanied by a change in exocarp color parallel to the lignification of the endocarp. This is represented from the upper to lower figures.

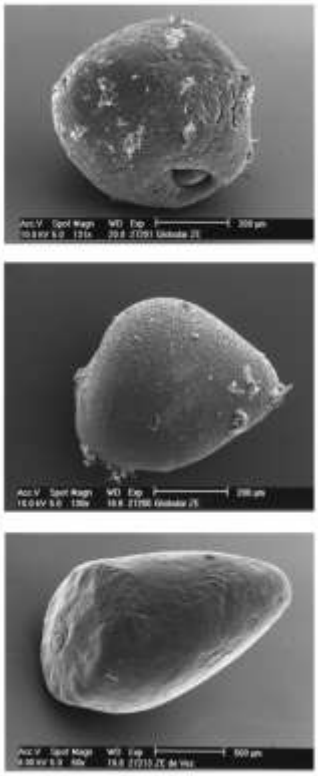

Figure 5 - Scanning electron microscopy studies showing the development of peach palm zygotic embryos from the globular to the conical form of the mature zygotic embryo.

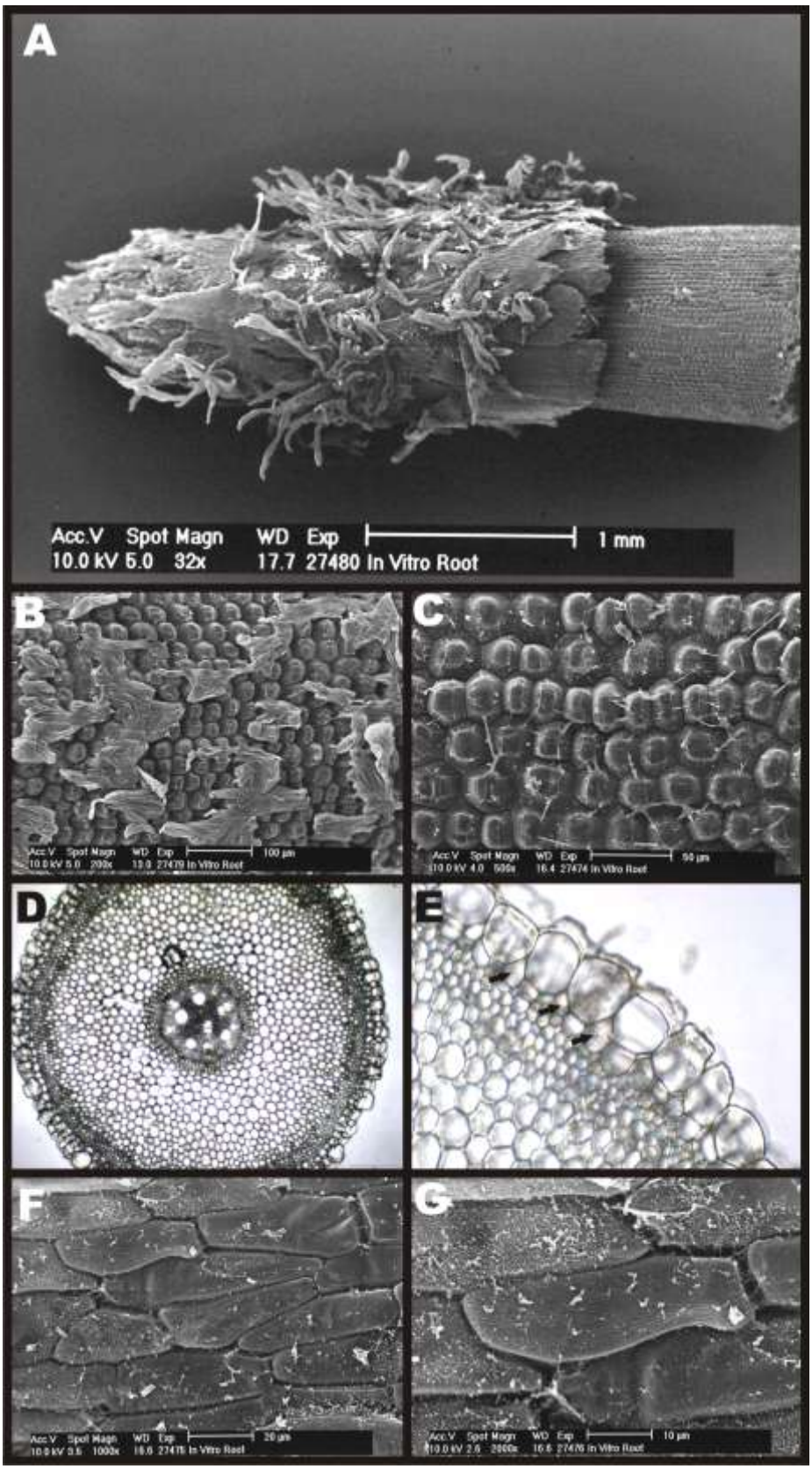

Figure 6 - Scanning electron microscopy studies revealing the peach palm root morphology. A – Root apex with the presence of border-like cells. B – Appearance of the exodermis cells of peach palm roots. C – Due to their arrangement, exodermis cells resemble a corn cob. D – Transverse section of peach palm root. E – Detailed view of the exodermis of peach palm root. Note the intercellular spaces between the cells (arrows). F – Appearance of the border-like cells. G – Detailed view of a border-like cell showing its release.

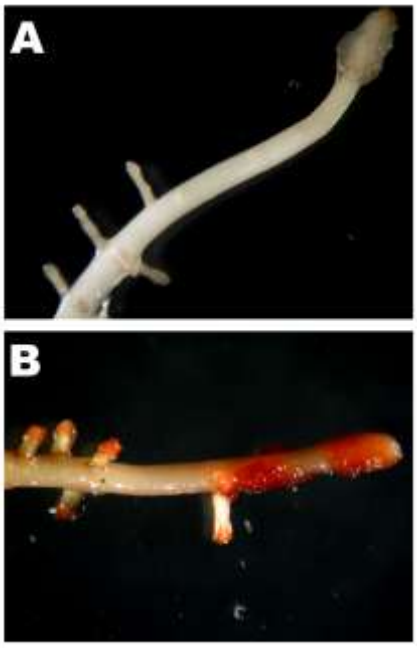

Figure 7 - Presence of arabinogalactan proteins in the border-like cells of peach palm roots. The red colour in B represents the presence of AGPs indicated by a specific staining.

1.3 Peach palm seed biology and germination

Endosperm of seeds of Arecaceae family members studied to date have shown numerous similar characteristics, such as living cells, with the main storage components stored in thick-walled cells (carbohydrates in the form of hemicellulose), lipids and proteins stored in cytoplasmatic bodies (DeMason *et al.*, 1989; Panza *et al.*, 2004). In germinating palm seeds, the storage compounds from the endosperm are transferred to the growing plantlet by a specialized organ, called the haustorium. This organ is mainly an absorptive and storage tissue that supplies the seedling with products of the endosperm. Studies with date palm (DeMason *et al.*, 1985) and *Washingtonia filifera* (DeMason, 1988) have suggested that this organ can also directly or indirectly control the breakdown of endosperm compounds.

Peach palm has recalcitrant seeds (Bovi *et al.*, 2004) but little is known about the factors imposing this dehydration sensitivity. Studies showing the effect of different substrates and treatments during its germination or providing a morphological description (Villalobos *et al.*, 1992; Bovi *et al.*, 1994; Damiao Filho *et al.*, 1999; Silva *et al.*, 2006) have been carried out, however our knowledge regarding the biochemical aspects of peach palm seed and it germination biology is limited.

It is thought that palm endosperm cells are unable to synthesize proteins de novo during germination. In coconut, this characteristic was based on enzymatic analyses associated with the absence of nuclei in the cells and with the fact that endosperm was considered non-respiring tissue (Balasubramaniam *et al.*, 1973). In date palm, detailed

ultrastructural analysis of the endosperm revealed the presence of plastids and of mitochondria with respiratory activity, but neither endoplasmatic reticulum nor Golgi complexes were found. Based on these findings, the capacity for de novo protein synthesis was excluded (DeMason *et al.*, 1983). These characteristics were further assumed to occur in *Euterpe edulis* (Panza *et al.*, 2004), although no detailed information has been shown. Therefore, studies have assumed that most enzymes necessary for the endosperm breakdown would be secreted by the growing haustorium (Verdeil *et al.*, 2002) or already present in the endosperm in an inactive form (Sekhar and DeMason, 1990).

During germination of cereal seeds, storage proteins are hydrolyzed in the endosperm resulting into a mixture of short oligopeptides and free amino acids (Higgins and Payne, 1977). These are taken up by the scutellum – partially homologous to the haustorium of palms – and the amino acids are than liberated from the peptides and are further metabolized or transferred to the growing seedling. In castor bean (*Ricinus communis* L.), a dicotyledonous species, high levels of free amino acids were found in the endosperm and then transported to the cotyledons (Robinson and Beevers, 1981). Similarly, lipids are also broken down and converted to sugar in endosperm cells during germination and then transported to the cotyledons (Robinson and Beevers, 1981). In the gymnosperm *Pinus taeda* L, the megagametophyte was able to breakdown storage proteins and export free amino acids, even in the absence of the seedling (King and Gifford, 1997).

In palms, lipids account for a large amount of storage components. In *Washingtonia filifera* and date palm (*Phoenix dactylifera*) 28 % and 18 %, respectively, of the cells'? volumes are occupied by lipid bodies (Sekhar and DeMason, 1988ab). Studies regarding lipid breakdown in palms suggested that an active lipase is localized only in the shoots of oil palm plantlets and that endosperm is essentially devoid of lipase activity (Oo and Stumpf, 1983). The haustorium is also devoid of lipase activity, but it nevertheless contains the enzymes necessary for the conversion of free fatty acids to sugar (Oo and Stumpf, 1983). This result is also supported by ultrastructural analysis of date palm haustorium, which showed the presence of glyoxysomes only in the haustorium cells (DeMason, 1985). However, if these results are correct, it remains to be discovered how lipids are transported to the plantlet shoot and back to haustorium to be converted into sugar.

In peach palm, no storage proteins have been characterized; only the presence of two high molecular weight bands present in embryogenic cultures have been hypothesized to be storage proteins (Steinmacher *et al.*, 2007b). For storage protein breakdown, proteinases are necessary (Müntz, 2007) and in date palm proteinase activity was detected first in the endosperm and only at the onset of germination (DeMason *et al.*, 1985). To the best of our

knowledge there are neither studies regarding free amino acid kinetics during palm seed germination nor detailed studies regarding proteinases in palm seeds. Therefore, further studies regarding the germination of peach palm seeds are necessary to increase our knowledge about palm seed biology, as well as to meet the growing demand of peach palm seeds, due to the palms increasing economic importance and to support its breeding and conservation programs.

1.4 Economic importance of peach palm

Peach palm has several potential and traditional uses (Clement and Mora-Urpí, 1987). The use of peach palm wood is re-appearing as an attractive market using the residue from peach palm seed-orchards (Mora-Urpí *et al.*, 1997). When manufactured, its wood might be used in the furniture industry, production of music instruments and crafts (Figure 8). The application of peach palm fibers to reinforce polyester composites is also alternative use (Santos *et al.*, 2008). Nevertheless, today peach palm is only important for its fruit, moderately popular throughout its traditional distribution, and for its heart-of-palm, a gourmet vegetable extracted from the shoot apex.

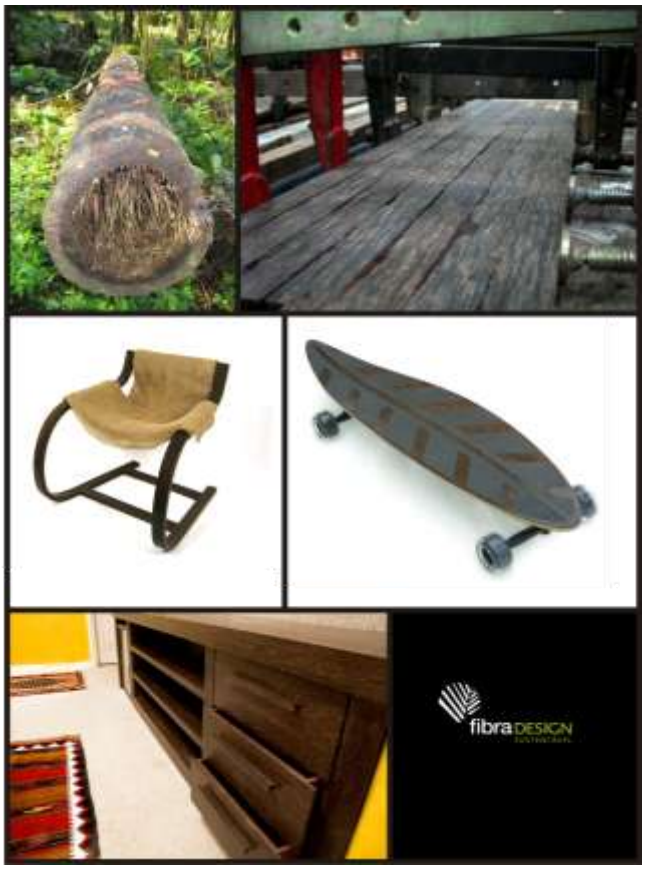

Figure 8 - Use of peach palm wood as a viable alternative use for the furniture industry and similar areas. Source: Fibra Design Sustentavel (www.fibradesign.net accessed: 25.11.2009).

Fruit

As a fruit crop, *Bactris gasipaes* is an item of broader national commerce only in Colombia, Costa Rica and Panama. Small amounts of processed fruits (Figure 9A) are produced and even exported to other countries in Central America, to the United States and Canada (Mora-Urpí *et al.*, 1997), but there is no significant international commerce. Traditionally the fruits are consumed after cooking (Figure 9B), but specialty and gourmet dishes are also prepared using peach palm fruits (Figure 9C). However, for several reasons it still hasn't attracted the attention of the private sector (Clement *et al.*, 2004) and fruit production is still designated for local markets only.

However, many farmers recognize the potential value of peach palm fruit (Mora-Urpi *et al.*, 1997). It is grown almost exclusively by smallholders in homegardens and swiddens, with a few small orchards near major consumption areas (Clement, 2008). It is suggested that 50 % of the fruit production is commercialized as fresh fruit in local markets, while the other 50 % is used for subsistence, either directly or as animal feed, or is wasted. Estimates suggest that total fruit production is about 120,000 tons per year (Clement *et al.*, 2004; Clement, 2008). For fresh fruit in local markets, usually the fruits are commercialized as bunches, each weighing from 2 to 5 kg and worth about US$ 0.50-1.00 to farmers, and in the market the bunches are sold at US$ 1.00-3.00, resulting in a market value of US$ 30 million per year (Clement, 2008).

Heart-of-palm

The heart-of-palm is composed of unexpanded juvenile leaves and sub-apical tissue (Figure 9D). It is considered a gourmet product and has a huge potential for the international market. The heart-of-palm market has an already established and important economic status in Latin America, with peach palm having advantages over other palm species used for palm heart production, such as a short life cycle, presence of off-shoots and an appreciable final product (Mora-Urpí *et al.*, 1997; Clement, 2008). Higher levels of sugar were also found in the heart-of-palm of *Bactris gasipaes* in comparison with those from *Euterpe edulis* and *E. oleracea* (Clement *et al.*, 1993). Additionally, heart-of-palm is usually sold in glass or canned as pickles (Figure 9E). Hearts of peach palm have low concentrations of the enzymes peroxidases and polyphenoloxidase, allowing *in natura* commercialization as well as processing (Clement *et al.*, 1993), attributes which might open a new market niche for peach palm (Figure 9F). Studies regarding the conservation of ready-to-use and minimally processed fresh heart have been also carried out (Clement *et al.*, 1999).

Brazil is the biggest heart-of-palm producer and consumer, and estimates revealed an increased production during the last years, from 27,031 ton in 1990 to 51,376 ton in 2003

(Rodrigues and Durigan, 2007). This includes, however, many more hearts of *E. oleracea* than of peach palm. Ecuador and Costa Rica are the major exporting countries of heart-of-palm, with most production based on peach palm; Brazil exports less than 10 % of its total heart-of-palm production (Clement, 2008).

The market for this gourmet product also attracted the attention of others producers outside Latin America, and currently peach palm is being cultivated in Hawaii/USA, Reunion Island/France, Indonesia and Malaysia.

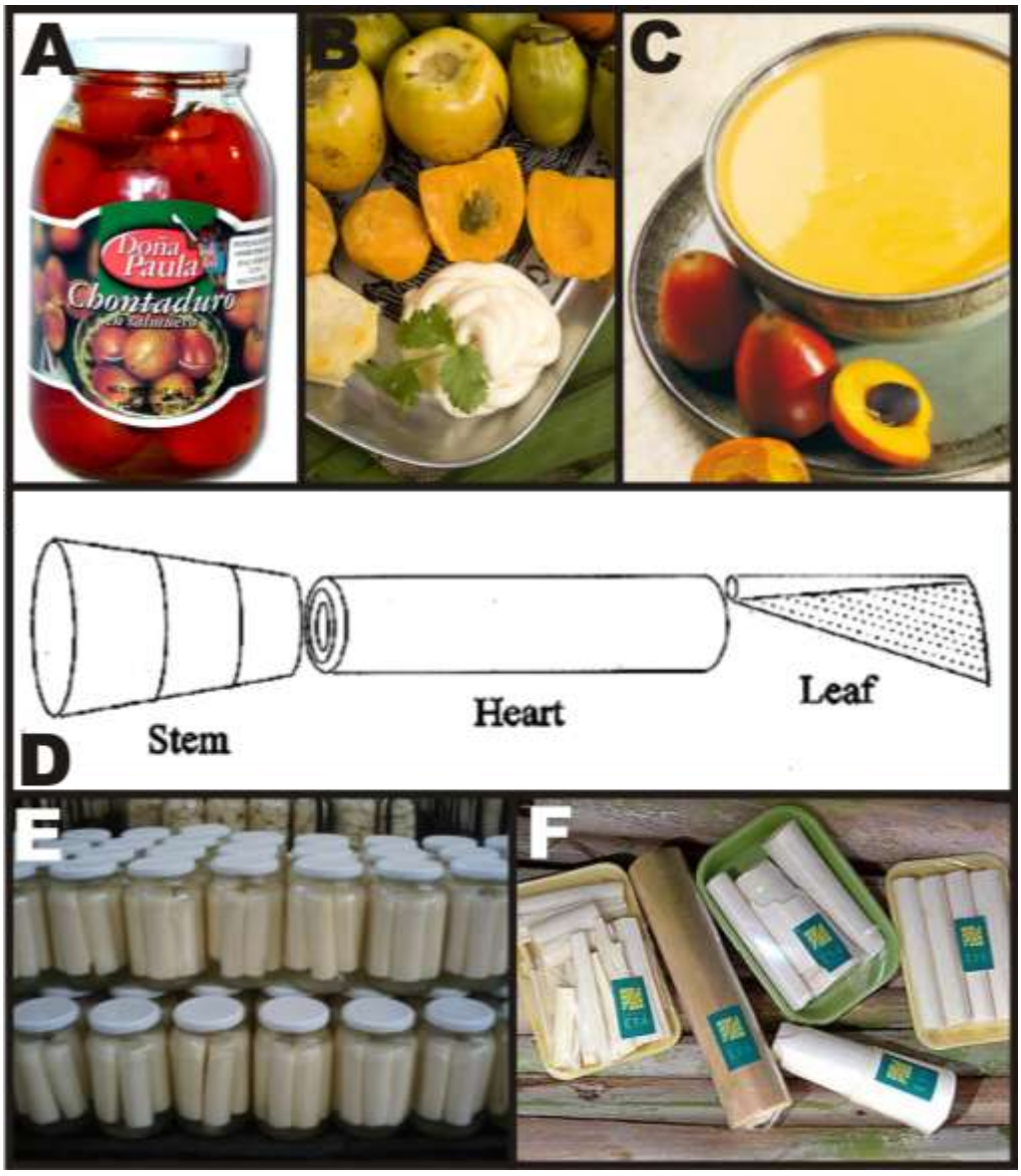

Figure 9 - Fruits and heart-of-palm of peach palm. A – Fruits sold in glass jars as pickles. B – Cooked fruits. C – Cream soup as an example of a fruit recipe for peach palm. D – Characteristics of the heart-of-palm. E – Heart-of-palms sold in glass as pickles. F – Heart-of-palm sold *in natura*. Sources (all accessed 25.11.2009):

A http://virtualtienda.com/images/chontaduro %20dona %20paula.png;
B http://www.naturelandings.com/articles.php?article=129#
C http://www.sabores.co.cr/
D http://www.hort.purdue.edu/newcrop/proceedings1996/V3-500.html
E http://www.sabordaserra.ind.br/images/empresa2.jpg
F http://www.fazendadoeta.com.br/img/Fazenda-Eta-Maio-2009-034.jpg

1.5 Breeding and conservation programs of peach palm

Peach palm is considered a multi-purpose tree (Clement and Mora-Urpi, 1987) and plays an important role as a component of agrosystems (Clement, 1989). It was listed as the number-one priority tree species for agroforestry development based on farmer-preference surveys conducted by the International Centre for Research in Agroforestry (ICRAF) (Mora-Urpí *et al.*, 1997). As previously discussed, nowadays fruit production for subsistence and market and heart-of-palm production for market are the most important uses of *Bactris gasipaes* and ideotypes for fruit and heart of palm production has been suggested (Table 1; Mora-urpi *et al.*, 1997; Clement, 2008). Therefore, conservation programs are necessary as a gene reservoir for further improvement to meet the demands of farmers and consumers for this species.

Breeding programs should start from landraces with similar characteristics to the desired final ideotype. For example, the Pampa Hermosa, Putumayo and Guatuso landraces have ideal characteristics for heart-of-palm production, such as spineless stems and presence of an adequate number of off-shoots. On the other hand, Putumayo and Vaupes are recommended for flour production as their have larger fruits with more starch content. The Pará landrace has desirable characteristics for oil production (Clement, 1997). However, up to now there is no modern cultivar for peach palm (Clement, 2008).

On farm conservation is also an alternative conservation method for *Bactris gasipaes*, and results have pointed out that farming communities in the Peruvian Amazon are maintaining a relatively broad genetic base in their peach palm populations (Adin *et al.*, 2004). This is based upon intense seed exchange, collecting seed from selected palms on the farms and on neighboring farms, as well as from selected fruits in the local markets for planting on their farms, causing ‘long-distance’ gene flow through seeds. Commercial traders from Yurimaguas also distribute fruits/seeds to other regions in the Amazon and elsewhere in Latin America where demand exists for fruit or heart-of-palm plantations (Adin *et al.*, 2004). However, further study also showed that farmers use an average of only four palms to provide seed for the establishment of their gardens (Cole *et al.*, 2007), which may cause significant inbreeding. Therefore, it has been suggested that some kind of network capable of guiding an appropriate degree of exchange of genetic material is needed for the establishment of peach palm conservation programs (Cole *et al.*, 2007).

Table 1 - Proposed ideotypes of peach palm for fruit and heart of palm production. Adapted from Mora-Urpi *et al.* (1997).

	Heart-of-palm	Fruits
Stem	• Long internodes • Spineless internodes and leaf sheaths • Soft, without much lignification	• Dwarf with low annual height increment • Spineless internodes
Leaves	• Long, tender sheath, erect blade • High net assimilation rate	• Short, erect petiole and blade • Annual production of more than 10 leaves • High net assimilation rate
Off-shoots	• Early appearance (< 6 months after field planting) • Rapid growth (< 6 months to harvest)	• Multiple offshoots developing into 5-12 stems after 12 months
General characteristics	• Early first harvest: < 10 months with 9 cm stem diameter • Annual production > 4 stems per clump • Heart of palm weighint 450 g • White colour • Good flavour • Natural peach palm odour for *in natura* or odourless for canning	• Precocious fruiting (less than 3 years) • Annual production greater than 15 bunches per year, each more than 8 kg • Fruit weight more than 85 % of total bunch weight • More than 100 fruits per bunch • Fruit weight greater than 50 g. • Fruit with exocarp waxy, no fibers or striations, red colour • Small seeds
Mesocarp composition		• Water content low (less than 50 %) • Protein content high (DW more than 14 % • High starch content (more than 60 % FW) • Fiber content low (less than 10 % DW) • Carotene content high: 20-70 mg. 100 mg^{-1} FW • Locally acceptable flavor
Agronomic aspects	• Resistant to leaf mite • Broad agronomic adaptability	• Resistant to leaf mite • Resistant to fruit borer • Broad agronomic adaptability

To date peach palm conservation programs are directed to the establishment of active field genebanks, which are suitable for use and research of the species but are not a viable approach for long-term conservation (Mora-Urpí *et al.*, 1997). However, peach palm has recalcitrant seeds (Bovi *et al.*, 2004). Therefore studies regarding in vitro regeneration for conservation purposes are necessary, as well as more studies related to palm seed biology are needed. Cryopreservation has also been tried, but a reliable protocol for peach palm must be optimized (Steinmacher *et al.*, 2007a).

The development of reliable in vitro regeneration protocols would serve as an efficient tool assisting the conservation and genetic improvement programs of this species. At the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil, for instance, a core collection is being developed. In such a collection the application of clonal propagation would ensure the multiplication of the selected palms, which could later be transferred to different Institutions for further studies or breeding programs. The formation of seed orchards with selected genotypes and also the large-scale clonal multiplication of selected palms would also benefit from an in vitro regeneration protocol bringing important improvements for farming systems.

1.6 Tissue culture of peach palm

Given the importance of peach palm for its fruit and for its heart-of-palm, various Latin American institutions have breeding programs for one or both uses, where in vitro plantlet regeneration could be an important tool. Tissue culture techniques are considered to be the most efficient strategy for clonal plantlet regeneration of this species, as well as for genetic conservation (Mora-Urpí *et al.*, 1997). However, a commercial protocol does not currently exist. The main application of in vitro regeneration techniques in breeding programs would be the fixation of the genetic gain, by the capture of the additive and non-additive components of genetic variability (Guerra *et al.*, 1999). Among various techniques, somatic embryogenesis offers the advantages of automated large-scale production and genetic stability of the regenerated plantlets (Guerra *et al.*, 1999; Steinmacher *et al.*, 2007c). Furthermore, somatic embryogenesis has the possibility to be coupled to conservation programs through, for instance, the cryopreservation of somatic embryos, as well as the production of synthetic seeds for plantlet exchange. Additionally, somatic embryogenesis can be considered as one of the most interesting pathways for scaling-up the regeneration procedure for a given species. Among other advantages, somatic embryogenesis permits creation of cycling cultures through the use of cell suspensions (Sané *et al.*, 2006; Teixeira *et al.*, 1995) or through secondary somatic embryogenesis (Perez-Nunez *et al.*, 2006).

Palm trees have been considered recalcitrant for tissue culture, although plantlet regeneration through somatic emrbyogenesis has been described for several species, including African oil palm (*Elaeis guineensis* Jacq.; Teixeira *et al.*, 1995), coconut (*Cocos nucifera* L.; Verdeil *et al.*, 1994), *Euterpe edulis* Mart. (Guerra and Handro, 1998), betel nut palm (*Areca catechu* L.; Karun *et al.*, 2004) and macauba (*Acrocomia aculeata* (Jacq.) Lood. ex. Martius.; Moura *et al.*, 2008). Numerous histological studies have been carried out and shown that cells adjacent to the vascular tissue apparently have higher morphogenetic capacity. Studies with *Cocos nucifera* (Fernando *et al.*, 2003), *Elaeis guineensis* (Schwendiman *et al.*, 1988), *Euterpe edulis* (Guerra and Handro, 1998) and *Phoenix dactylifera* (Sané *et al.*, 2006) showed that the first events of cell division were always observed in cells adjacent to the vascular tissue. The molecular aspects linked to palm somatic embryogenesis are also the subject of recent studies (Perez-Nunes *et al.*, 2009; Low *et al.*, 2008).

In vitro plantlet regeneration of peach palm was described for the first time by indirect organogenesis (Arias and Huete, 1983), later by somatic embryogenesis (Stein and Stephens, 1991; Valverde *et al.*, 1987), and by direct organogenesis (Almeida and Kerbauy, 1996). Arias (1985) described casual morphogenetic responses from different tissues and plantlet regeneration was often difficult. Thereafter, Valverde *et al.* (1987) described a protocol for in vitro regeneration of peach palm, although only ten embryogenic calli were obtained (out of 100 shoot apexes – where one explant could produce more than one callus) and each callus produced 2-8 somatic embryos. In the protocol described by Stein and Stephens (1991), only four calli cultures (out of 18 explants) producing more than 10 plantlets per culture were established. Using peach palm immature inflorescences as explants, Almeida and Kerbauy (1996) described a protocol for regeneration through organogenesis at low frequency (about 11 %), with a small number of plantlets being regenerated. Furthermore, regeneration was an apparently random response.

A complete in vitro regenerative protocol using mature zygotic embryos as explants was described for peach palm as the first step for the development of a reliable protocol for peach palm in vitro multiplication (Steinmacher *et al.*, 2007b). In this study, histological analyses showed that plantlet regeneration occurred through somatic embryogenesis and regenerated plantlets were successfully acclimatized (Steinmacher *et al.*, 2007b). However, for the clonal propagation of selected genotypes the development of protocols that allow regeneration from explants obtained from adult plants was still necessary.

Success of somatic embryogenesis in monocotyledonous species requires utilization of explants containing meristematic cells (Vasil, 1987). Inflorescences from adult palm trees contain meristematic cells and are important explant sources because they can be obtained

without damaging the donor tree (Verdeil *et al.*, 1994). Based on this premise, Steinmacher *et al.* (2007c) described the induction, development and conversion of somatic embryos of peach palm using inflorescences as the explant. These results indicated the use of the most immature inflorescence combined with a 2,4-D pre-treatment and 300 µM Picloram; however, a strong genotype effect was observed (Steinmacher *et al.* 2007c). The use of the thin cell layer technique was also described and offers promise for peach palm in vitro plant regeneration. It was observed that explant size and age has an important role in peach palm in vitro response and a complete protocol was described using young leaves and shoot apexes as explants (Steinmacher *et al.*, 2007d). This protocol has great promise in peach palm in vitro regeneration as explants can be obtained easily from adult palms since this species produces off-shoots that will allow the cloning of selected adult plants.

The formation of somatic embryos occurred in clusters, suggesting the occurrence of secondary somatic embryogenesis, although no direct evidence was shown (Steinmacher *et al.*, 2007b). The use of this morphogenetic pathway could be important to improve the regeneration rate of the whole procedure, obtaining cycling cultures. In general, cycling cultures can be obtained using cell suspension or through the induction of secondary somatic embryos, and both have been described in palms (Sané *et al.*, 2006; Teixeira *et al.*, 1995; Perez-Nunez *et al.*, 2006), however, no cycling cultures have been described in peach palm.

2 Induction and expression of somatic embryogenesis

Somatic embryogenesis capitalizes upon plant cell totipotency (Verdeil *et al.*, 2007). It is induced and triggered by a combination of factors including genotype, explant and culture conditions culminating in the development of bipolar structures without vascular connections to the mother tissue (Guerra *et al.*, 1999). It involves the acquisition of embryogenic competence, its induction and determination. Embryogenic competence of the cell is its capacity to respond to specific signals (i.e., plant growth regulators, culture conditions); induction of somatic embryogenesis occurs when the given signal produces a unique developmental response; and determination is a process where the developmental fate of the cell (or group of cells) becomes fixed and limited to a particular developmental pathway (Yeung, 1995).

The exact mechanisms by which somatic cells turn back into an embryogenic state is unknown and still an exciting question in plant biology (see Vogel, 2005). Nevertheless, resetting the whole ontogenetic program and reprogramming gene expression patterns are considered to play the main roles in the acquisition of embryogenic capacity (Dudits *et al.*, 1995; Gaj *et al.*, 2005). The reprogrammed gene expression is reflected in the synthesis of a

new transcript profile, resulting in (re)activation of cell division and dedifferentiation with a new set of development of the morphogenesis (Feher *et al.*, 2003).

Due to the plastic development of plants, cell dedifferentiation is a process by which the developmental pathway may be altered, allowing the cells to set a new developmental pattern after cell division (Feher *et al.*, 2003). Hence, in vitro recalcitrance in numerous plants, especially monocotyledons, is related with the cell's capacity to re-enter cell division (Emons, 1994). In *Cocos nucifera*, in vitro recalcitrance has been linked to the low capacity of the cells to re-enter the cell division, where approximately 90 % of the explant cells were blocked at the G0/G1 phase of the cell division cycle (Sandoval *et al.*, 2003). Although somatic embryogenesis induction might not be simply related to the cell cycle or to a certain level of mitotic activity of the cells in the explants (Doleželová *et al.*, 1992), it has been shown that the formation of callus is correlated with cell cycling and that the subsequent origin of somatic embryos initially involves cycling of cells at the margins of the callus of *Arabidopsis* (Raghavan, 2004). However, due to the complex interaction of external stimuli and cell physiological status, the re-entry of cells into the mitotic cycle may also result in the development of callus, structure without any organized growth (Dudits *et al.*, 1995). Therefore, the acquisition of embryogenic competence and embryo development are expected to be controlled by a combination of spatial and temporal regulation of specific genes.

Several genes have been correlated to somatic embryogenesis. The genes may be divided into two groups: promoters and repressors of somatic embryogenesis (Kwaaitaal and de Vries, 2007). Among the promoters of somatic embryogesis, the Somatic Embryogenesis Receptor-like Kinase (SERK) is the best characterized gene. It encodes a Leu-rich repeat transmembrane receptor-like kinase first isolated in embryogenic carrot cell cultures (Schmidt *et al.*, 1997). Its ectopic expression in *Arabidopsis* increased somatic embryogenesis initiation (Hecht *et al.*, 2001) and it has been considered as a marker for somatic embryogenesis, promoting the formation of cells competent to initiate somatic embryogenesis (Schmidt *et al.*, 1997; Kwaaitaal and de Vries, 2007). Leafy Cotyledon genes (LEC) code for transcription factors which also belong to the group of genes promoting somatic embryogenesis and are thought to play key regulatory roles in embryogenic competence acquisition (Harada, 1999; 2001). This gene was firstly isolated from *Arabidospis*, being specifically expressed in seeds; mutants presented thricomes on the cotyledons (Stone *et al.*, 2001). Its ectopic expression resulted in formation of somatic embryos in control conditions in the absence of inductive treatments (Stone *et al.*, 2001). Later it was found to encode a transcript factor containing a B3 domain and linked to auxin synthesis and sensitivity (Stone *et al.*, 2008; Chiappetta *et al.*, 2009). Similarly, the cloned sequence Wuschel (WUS) of *A. thailana* encodes a

homeodomain protein that promotes a vegetative-to-embryonic transition (Zuo *et al.*, 2002), and is also involved in specification of shoot and floral meristems (Mayer *et al.*, 1998).

In the second group of genes, related to repressors of somatic embryogenesis, are Primordia Timing (PT), Clavata (CLV) 1 and 3, and Pickle (PKL) genes (Kwaaitaal and de Vries, 2007). Mutations in the PT gene result in a phenotype characterized by a broader shoot apical meristem in the embryonic and seedling stages, by polycotyledons and by a higher number of leaves as compared to the wild type (Conway and Poethig 1997, Mordhorst *et al.*, 1998; von Recklinghausen *et al.*, 2000). CLV 1 and 3 are meristematic cell repressors and appear to play important roles in the regulation of shoot meristem development (Clark *et al.*, 1996), promoting the transition towards differentiation of cells in the shoot and floral meristems, and/or to restrict the proliferation of cells in the center of these meristems (Clark *et al.*, 1993, 1995). Double mutants (pt and clv) showed additive roles in somatic embryogenesis (Mordhorst *et al.*, 1998), as some cells of the enlarged shoot apical meristem apparently escape differentiation maintaining their embryogenic state.

PKL encodes a CHD3 protein, a chromosome remodeling factor that is ubiquitously expressed in *Arabidopsis*. The presence of this protein results in the formation of heterochromatin during post-embryonic growth and it has been shown that the presence of PKL represses embryonic traits via transcriptional repression of seed storage proteins (Ogas *et al.*, 1997) and LEC genes (Ogas *et al.*, 1999; Rider *et al.*, 2003). Similarly, a new repressor called Arabidopsis sixb-interacting protein 1-like 1 (ASIL1) was found (Gao *et al.*, 2009). This gene encodes a DNA binding protein and markedly altered the expression of LEC genes, as well as a large subset of seed maturation genes (Gao *et al.*, 2009). Therefore, the authors suggested that ASIL1 represses embryogenic genes by competing with the binding of transcriptional activators of these genes. These results together suggest that the master regulators of somatic embryogenesis would be the turning off of genes that promote embryogenic-to-vegetative growth, thus repressing embryogenic competence.

Changes in developmental pattern and acquisition of somatic embryogenesis competence might start before the exposure of the explants to a new environment or culture conditions (Yeung, 1995). Given that the tissues are explanted and cultivated usually under sub-optimal and unbalanced conditions (i.e., non-physiological concentrations of plant growth regulators), it has been proposed that somatic embryogenesis would be the last possible result of adaptation to the new environment (Dudits *et al.*, 1995; Feher *et al.*, 2003). Consequently, it has been postulated that one of the major forces modeling the transition of a somatic cell back to an embryogenic state would be the level of stress to which this cell is submitted (Dudits *et al.*, 1995; Feher *et al.*, 2003). Hence, if the stress level exceeds the cell's tolerance,

the cell will collapse and die, while the opposite would increase metabolism, triggering mechanisms of adaptation (Davletova *et al.*, 2001; Feher *et al.*, 2003).

Auxin is the most common plant growth regulator used to induce somatic embryogenesis (Dudits *et al.*, 1995). It has been shown that auxins act like a molecular glue binding to it's the TIR1 receptor and promoting ubiquitin-dependent degradation of Aux/IAA repressor proteins (Tan *et al.*, 2007; Guilfoyle, 2007). Naturally occurring auxin (indol acetic acid – IAA) and synthesized auxin analogs (NAA and 2,4-D) showed the same activity (Tan *et al.*, 2007; Guilfoyle, 2007). Among the auxin sources used to induce somatic embryogenesis, 2,4-D is the most frequently used auxin analog (Dudits *et al.*, 1995). However, other auxin analogs may also be used to induce somatic embryogenesis, such as Picloram.

Picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid) is a picolinate auxin-analog able to induce somatic embryogenesis in numerous species [i.e., cassava (Groll *et al.* 2001); tulip (*Tulipa gesneriana* L.) (Ptak and Bach, 2007); sugar cane (Garcia *et al.*, 2007)]. This auxin also induced embryogenic competence in African oil palm (Teixeira *et al.*, 1995) and was the most suitable auxin source for somatic embryogenesis in arecanut palm (*Areca catechu* L., Karun *et al.*, 2004) and macauba palm (*Acrocomia aculeata* (Jacq.), Moura *et al.*, 2008). Valverde *et al.* (1987) demonstrated that Picloram was better than 2,4-D for enhancing embryogenic callus induction in peach palm, which was later confirmed using different sources of explants (Steinmacher *et al.*, 2007[b,c,d]). Picloram is thought to have a stronger auxin effect compared to other auxin analogs. This observation is supported by the fact that germinating *Arabidopsis* seeds in Picloram-enriched culture medium could mimic an auxin over-producing mutant (*sur2*) (Delarue *et al.*, 1998). However, other differences between 2,4-D and Picloram in signaling cascades may also exist, as a mutation in one receptor altered the response for Picloram but not for 2,4-D and IAA (Walsh *et al.*, 2006).

Embryogenic cells display distinct cytological features, such small size with dense cytoplasm and small vacuoles, centrally located nucleus with prominent nucleolus and high nucleus:cytoplasm ratio (Verdeil *et al.*, 2001). Both the cytoskeleton and cell walls appear to play an essential regulatory role during the initial developmental stages of somatic embryos (Šamaj *et al.*, 2006). It is thought that for the correct development of somatic embryos symplastic isolation is required. Such isolation can occur physically through the thickening of the cell wall and closing of the plasmodesmata by deposition of callose (Dubois *et al.*, 1990; Verdeil *et al.*, 2001), as well as deposition of phenolic and lipophilic substances on cell wall (Pedroso and Pais, 1995), or also through the development of barrier cells (i.e., phenol-storing cells) between somatic embryo and mother tissue (Reis *et al.*, 2008). All these aspects may

result in the isolation of the embryogenic cells from their neighborhood cells' effects. Alteration in the molecular composition of cell walls during somatic embryogenesis was observed in coconut by Verdeil *et al.* (2001), who proposed that this might be involved in the recognition of embryogenic cells and regulation of early embryogenic states.

Another relevant factor is the development of an extracellular matrix surface network (ECMSN - also known as supraembryogenic network) over the (pre-)globular somatic embryos (Chapmann *et al.*, 2000ab; Šamaj *et al.*, 2008; Bobák *et al.*, 2003). In coconut (*Cocos nucifera* L.) and winter oilseed (*Brassica napus* L.), a fibrillar network was observed in embryogenic cells (Verdeil *et al.*, 2001; Namasivayam *et al.*, 2006). The composition of ECMSN is not completely known, but usually proteins, pectidic polysaccharides and lipophilic substances are described (Chapman *et al.*, 2000abc; Konieczny *et al.*, 2005; Namasivayam *et al.*, 2007; Popielarska-Konieczna *et al.*, 2008; Šamaj *et al.*, 2008). It is thought that the presence of lipophilic substances would in turn result in isolation, so that hydrophilic substances would not be in contact with the developing somatic embryo. This isolation is not exclusively found in somatic embryos, as zygotic embryos also present such symplastic isolation through the formation of cutin (e.g., Rodkiewicz *et al.*, 1994; Lee *et al.*, 2006).

Other components of the EMSN include proteins and proteoglycans, which during the growth of somatic embryos may be released into the culture medium and, possibly, act as signaling molecules. For instance, it is known that oligosaccharides can control the morphogenesis in tobacco (Tran Than Van *et al.*, 1974; Fry *et al.*, 1993). Additionally, it has been reported that the presence of properly glycosylated extracellular proteins is a requirement for the differentiation of carrot cells into somatic embryos (de Vries *et al.*, 1988). Therefore, for the development of somatic embryos, the conditioning of the culture medium is an important factor for induction and development of somatic embryogenesis.

3 Temporary immersion system

Temporary Immersion Systems (TIS), which involve flooding of plant tissue at regular time intervals, are often relevant for scaling-up or improving in vitro regeneration protocols and also opens possibilities to automate some culture stages. This method was firstly proposed by Alvard *et al.* (1993). The authors showed that lack of oxygenation in steady liquid culture medium was a limiting factor and among different culture methods TIS proved to be the most effective. The system uses pressure to pump the liquid medium to the plant compartment, causing forced ventilation and leading to complete renewal of the culture atmosphere during each immersion (Etienne and Berthouly, 2002). A semi-automated TIS

using the "twin flask" system was developed for pineapple (Escalona *et al.*, 1999). In this system the authors claimed that this culture system was easier to set up and use than more expensive liquid bioreactors. Later this "twin flask" system was shown to improve the regeneration rate and plantlet quality of different plant species (Etiene and Berthouly, 2002), including rubber, coffee, cacao and *Campthoteca* (Etienne *et al.*, 1997; Albarran *et al.*, 2005; Niemenak *et al.*, 2008; Sankar-Thomas *et al.*, 2008). The "twin flask" system, as suggested by the name, contains two separate flasks, one containing the explants or growing plants and the other the liquid medium. The two compartments are connected by silicone and glass tubes and air pressure is applied to the culture medium flask pumping the culture medium into the culture chamber to immerse the explants completely. After a defined time of immersion the airflow is reversed to withdraw the medium from the culture chamber (Sankar-Thomas, 2009).

TIS combines the advantages of both liquid and solid culture. These can be linked to better nutrient supply and absence of nutrient gradients expected to occur in semi-solid culture medium, absence of anoxia in the explants and lower rates of the hyperhidricity usually observed in liquid culture medium (Etienne and Berthouly, 2002; Murch *et al.*, 2004). Additionally, renewals of the headspace in TIS with surrounding air results in a supply of O_2 and the prevention of CO_2 and ethylene accumulation, and are considered to be responsible for the quality increase of shoots cultured in TIS (Aragon *et al.*, 2005; Roels *et al.*, 2006). In addition, using TIS the labour is also drastically reduced in comparison to culture and subculture in solid medium (Sankar-Thomas, 2009). Solidify agents might be considered one the major costs of in vitro culture and their elimination would drastically reduce the final cost of the plantlets. All these aspects associated with the possibility of automation of the system make TIS the best currently known system for the establishment of reliable protocol for clonal in vitro propagation. Furthermore, one advantage of liquid culture medium, besides the absence of nutrient gradients, is the fact that substances secreted into the culture medium with putative signaling functions are able to reach other explants. One of the most important secreted substances in this regard are Arabinogalactan proteins (AGPs) (Saare-Surminski *et al.*, 2001).

4 Arabinogalactan proteins

Arabinogalactan proteins (AGPs) is an umbrella term for a large class of proteoglycans widely distributed throughout the plant kingdom (Nothnagel, 1997; Seifert and Roberts, 2007). The AGPs have been associated with three fundamental cellular processes that are coordinated to produce the plant body, namely cell proliferation, cell expansion and

cell differentiation (Steele-King *et al.*, 2000). At the subcellular level AGPs are associated with the secretory pathway, plasma membrane and cell wall, as well as extracellular spaces present in the culture medium (Showalter, 2001; Šamaj *et al*., 2000).

AGPs belong to the hydroxyproline-rich glycoprotein (HRGP) super-family and have high molecular weights, usually containing 1–10 % protein as core protein (Fincher *et al.*, 1983). As AGPs are heavily glycosylated, two models for the molecular structure of AGPs have been proposed, namely the "wattle-blossom" model and the "twisted hairy rope" (Fincher *et al.* 1983; Qi *et al.*, 1991). However, based on the protein backbones, AGPs are classified into different groups (Figure 10). The first group is the classical AGPs, which contains a signal peptide at the N-terminus followed by the proline/hydroxyproline rich region and a domain responsible for attaching the protein backbone to a glycosylphosphotidylinositol (GPI) membrane anchor at the C-terminus (Showalter, 2001). Other classes of AGPs are named non-classical AGPs and include lysin-rich AGPs, cystein-rich AGPs, AG peptides, FLA (fascin-like AGPs) and chimeric AGPs (Figure 10) (Showalter, 2001; Johnson *et al.*, 2003; Motose *et al.*, 2004).

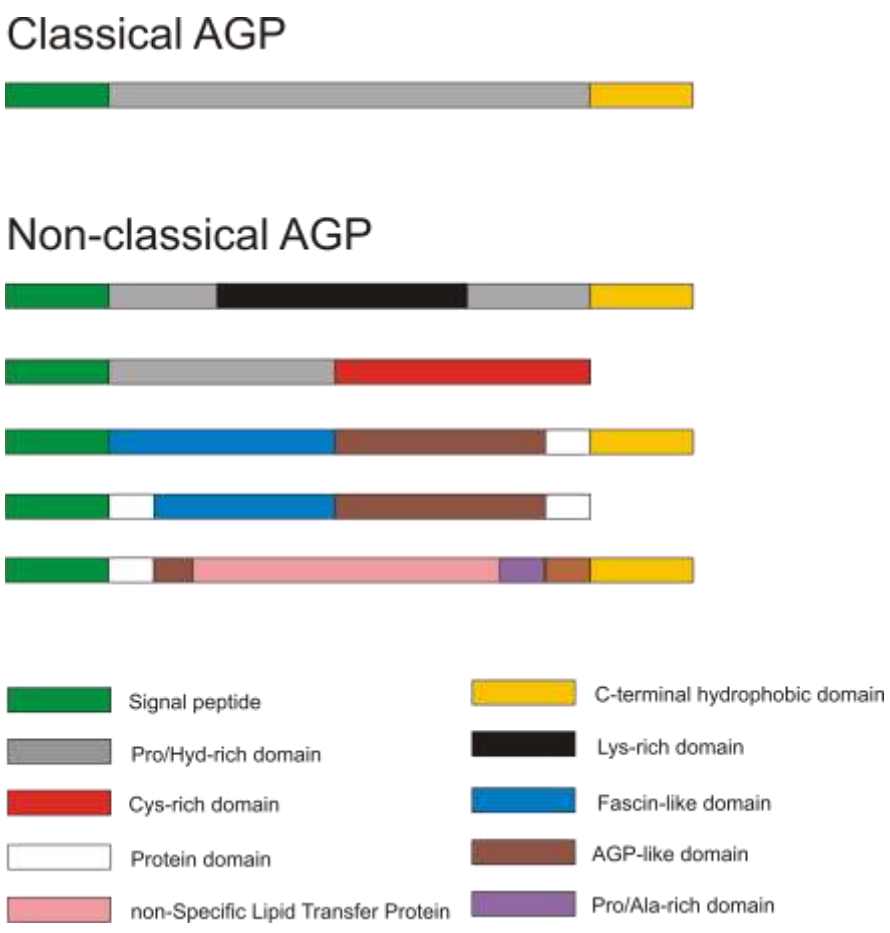

Figure 10 - Classification of arabinogalactan proteins. Adapted from Showalter (2001), Johnson *et al.* (2003), Motose *et al*. (2004).

Showalter (2001) proposed five different or concomitant models for the action mechanisms of AGPs in cellular signaling and cell adhesion, as follows: (A) signaling by the release of a carbohydrate from AGPs, which binds to a plasma membrane receptor capable of initiating an intercellular signaling cascade; (B) signaling by AGP directly or indirectly after binding ligand molecules to an appropriate plasma membrane receptor; (C) intercellular signaling by AGPs from one cell interacting with a plasma membrane receptor on an adjacent cell; (D) signaling by the GPI-anchor (glycosylphosphatidylinositol), a post-translational modification of the proteins, following release of GPI-anchored AGPs from the plasma membrane; (E) AGPs as cell adhesion molecules capable of aggregation on the plasma membrane and forming a plasma membrane-cell wall connection essential for normal growth and development. However, as more information is acquired regarding the role and structure of AGPs, additional models might be suggested. For instance, recently a chimeric AGP was described suggesting that the protein core might have a second function, for example, similar to lipid transfer proteins (Motose *et al.*, 2004). Additionally, the presence of a GPI-anchor in AGP could play an essential role in the receptor di- or polymers formation and their activation (Figure 11) (Simons and Toomre, 2000). It has also been shown that AGPs indirectly interact with the microtubule and actin in the cells (Sandar *et al.*, 2006; Driouichi and Baskin, 2008) and are also strongly bound to pectins (Immerzeel *et al.*, 2006), suggesting that AGPs might play a role in the cytoskeleton-plasma membrane-cell wall continuum (Kohorn, 2000; Baluska *et al.*, 2003). Therefore, a multifaceted biological function of AGPs is to be expected, however it is far from being completely understood. A coordinated association of different AGPs epitopes with the development of somatic embryos has been described (Saare-Surminski *et al.*, 2000), however there are so far no studies regarding the role of AGPs on palm embryogenesis.

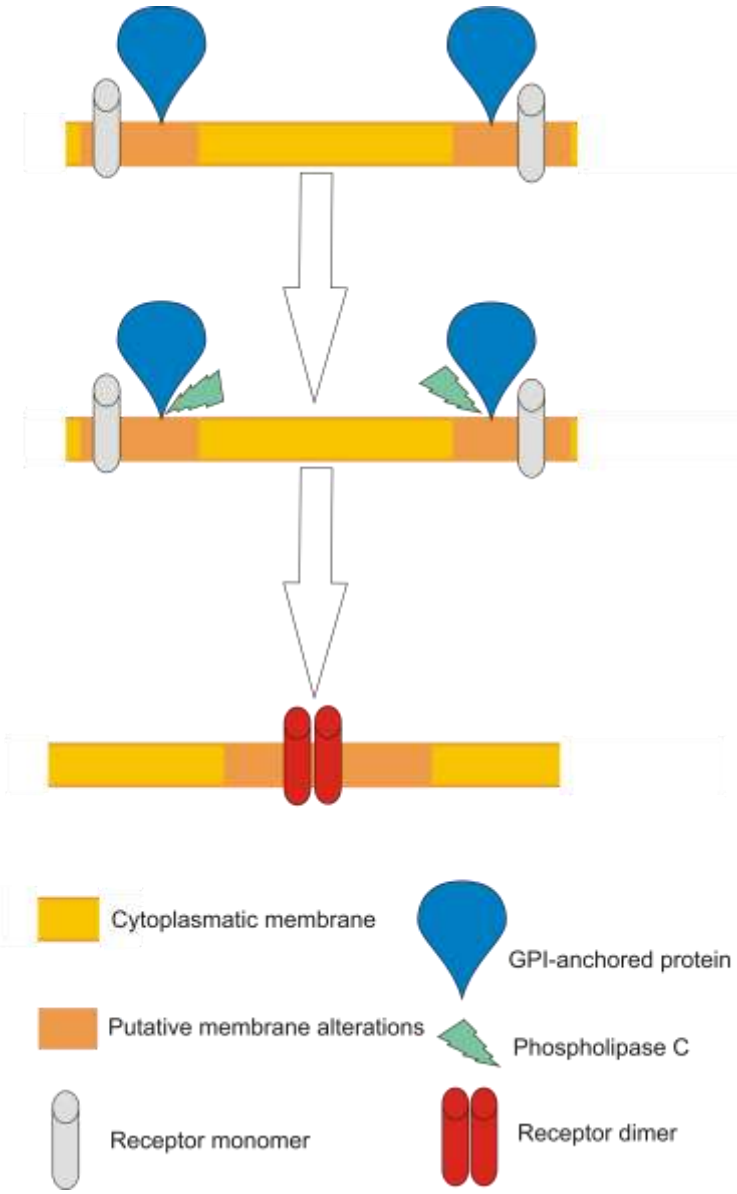

Figure 11 - Proposed mechanism of action of GPI-anchored proteins on the dimerization of membrane-bound receptors. Adapted from Simons and Toomre (2000). In this model, GPI-anchored proteins maintain the receptor monomers separated and, upon cutting of the GPI-anchor with a Phospholipase C, dimerization of the receptors may occur.

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Chapter II:

TEMPORARY IMMERSION SYSTEM IMPROVES IN VITRO REGENERATION OF PEACH PALM THROUGH SECONDARY SOMATIC EMBRYOGENESIS: INDUCTION AND MORPHO-HISTOLOGICAL ASPECTS

1 Abstract

Secondary somatic embryogenesis has been postulated to occur during induction of peach palm somatic embryogenesis. In the present study this morphogenetic pathway is described and a protocol for the establishment of cycling cultures using a temporary immersion system (TIS) is presented. Zygotic embryos were used as explants, and induction of somatic embryogenesis and plantlet growth were compared in TIS and solid culture medium. Light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used to describe *in vitro* morphogenesis and accompany morpho-histological alterations during culture. The development of secondary somatic embryos occurs early during the induction of primary somatic embryos. Secondary somatic embryos were observed to develop continually in culture, resulting in non-synchronized development of these somatic embryos. Using these somatic embryos as explants allowed development of cycling cultures. Somatic embryos had high embryogenic potential (65.8±3.0 % to 86.2±5.0 %) over the period tested. The use of TIS greatly improved the number of somatic embryos obtained, as well as subsequent plantlet growth. Histological analyses showed that starch accumulation precedes the development of somatic embryos, and that these cells presented high nucleus:cytoplasm ratios and high mitotic indices, as evidenced by 4'-6-Diamidino-2-phenylindole staining. Morphological and SEM observations revealed clusters of somatic embryos on one part of the explants, while other parts grew further, resulting in callus tissue. A multicellular origin of the secondary somatic embryos is hypothesized. Cells in the vicinity of callus accumulated large amounts of phenolic substances in their vacuoles. TEM revealed that these cells are metabolically very active, with the presence of numerous mitochondria and Golgi apparati. Light microscopy and TEM of the embryogenic sector revealed cells with numerous amyloplasts, large nuclei and nucleoli, and numerous plasmodesmata. Plantlets were obtained and after 3 months in culture their growth was significantly better in TIS than on solid culture medium. However, later during acclimatization survival rate of TIS-grown plantlets was lower. The present study confirms the occurrence of secondary somatic embryos in peach palm and developed a feasible protocol for peach palm in vitro regeneration. Further optimizations include the use of explants obtained from adult palms and improvement of somatic embryo conversion rates.

Key words: Bactris gasipaes Kunth, tissue culture, clonal propagation, Picloram.

2 Introduction

Peach palm (*Bactris gasipaes* Kunth) is a caespitose multipurpose palm tree, widely distributed in the lowland humid Neotropics (Mora-Urpí *et al.*, 1997). Although it is listed as an underutilized crop, this species is one of the most useful palms in the Neotropics and the sole palm species that was domesticated for its fruits by Amerindians during the pre-Columbian period (Clement, 1988). Currently, the two major products from peach palm are the fruit, for local consumption, and the heart-of-palm, a gourmet vegetable extracted from the shoot apex and commercialized in world markets (Clement, 2008). The heart-of-palm market is important in Latin America, and peach palm has numerous advantages for plantation production, such as perennial production from off-shoots, rapid growth rate and the possibility of fresh or minimally processed commercialization. Currently peach palm is also been cultivated in Hawaii/USA, Reunion Island/France, Indonesia and Malaysia.

Currently, major efforts for the conservation of peach palm are based on the establishment of field germplasm banks, which are constantly threatened by biotic and abiotic factors, resulting in genetic erosion (Clement, 1997; Clement *et al.*, 2004). Seed banks are not an option because this species has recalcitrant seeds (Bovi *et al.*, 2004). Hence, in vitro conservation is suggested as the most promising technique for this purpose (Mora-Urpí *et al.*, 1997). At the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM, Brazil, for instance, a core collection was created within the main collection and clonal propagation would permit its transfer to other institutions for further studies or use in breeding programs. The formation of seed orchards with selected genotypes, as well as the large-scale clonal multiplication of selected palms, would also benefit from an in vitro regeneration protocol.

Both organogenesis and somatic embryogenesis pathways were described for peach palm in vitro regeneration (Arias and Huete, 1983; Stein and Stephens, 1991), although the regeneration rate was limited. Hence, studies on somatic embryogenesis in peach palm were suggested in order to enhance the regeneration rate (Arias and Huete, 1983). Recently, the main factors affecting the induction of somatic embryogenesis from different types of explants were identified (Steinmacher *et al*., 2007abc). It was suggested that during the induction of somatic embryos, the development of clusters of somatic embryos could be due to the development of secondary somatic embryos (Steinmacher *et al.*, 2007a), but little evidence was reported. The use of this morphogenetic pathway could be important to improve the regeneration rate of the whole procedure, obtaining cycling cultures. Cycling cultures can be obtained using cell suspension or through the induction of secondary somatic embryos, and both have been described in palms (Sané *et al.*, 2006; Teixeira *et al.*, 1995; Perez-Nunez *et al.*, 2006), however, to the best of our knowledge no cycling cultures have been described in

peach palm. Embryogenic friable callus, from which cell suspension could be obtained (e.g., Teixeira *et al.*, 1995; Sané *et al.*, 2006), was observed to occur in peach palm (Steinmacher *et al.*, 2007a), but some bottlenecks, such as their apparently random induction and the loss of embryogenic potential after several subcultures, makes secondary somatic embryogenesis the most attractive pathway for scaling-up in vitro regeneration.

The use of zygotic embryos as explants in improvement and conservation programs is inappropriate, however they may serve as an interesting model for peach palm somatic embryogenesis because a relatively high induction rate was observed (Steinmacher *et al.*, 2007a) and the morpho-histological responses from zygotic embryos as explants were very similar to those observed from shoot meristem and leaf sheaths [i.e., first cell division on cells adjacent to the vascular tissue, callus growth through a meristematic zone and multicellular origin of the somatic embryos (Steinmacher *et al.*, 2007ac)].

Somatic embryogenesis is an example of plant cell totipotency. It involves the acquisition of embryogenic competence by somatic cells via their dedifferentiation and the reprogramming of their gene expression patterns (Harada, 1999; Feher *et al.*, 2003; Gaj *et al.*, 2005). Primary embryogenesis is the process by which somatic embryos are formed from plant tissue or organs used as explants, and secondary somatic embryogenesis is the process in which somatic embryos are formed from pre-existing somatic embryos or using these somatic embryos as explants (Reamakers *et al.*, 1995). Usually, somatic embryos have higher embryogenic capacity than other explants, have been shown to increase the regeneration rate in several species (Raemarkers *et al.*, 1995), and the resulting cultures maintain their morphogenic competence for several years (i.e., in *Vitis rupestris*, Martinelli *et al.*, 2001), thus increasing the potential regeneration rate several thousand-fold, as shown in coconut (Perez-Nunez *et al.*, 2006).

Temporary Immersion Systems (TIS), which involve flooding of plant tissue at regular time intervals, are often used for scaling-up or improving in vitro regeneration protocols and also offer the possibility of automating some culture stages. A semi-automated "twin flask" TIS was initially developed for pineapple (Escalona *et al.*, 1999), and later shown to improve the regeneration rate and plantlet quality for other plant species (Etienne and Berthouly, 2002). The number of regeneration protocols for other plant species using this system is increasing continually (Sankar-Thomas *et al.*, 2008; Niemenak *et al.*, 2008). Hence, an improved protocol for peach palm regeneration using TIS may be an alternative to the development of cycling cultures.

Parallel to the development of a feasible protocol for peach palm somatic embryogenesis, morpho-histological studies may also enhance our comprehension of the

process, allowing further optimizations and other uses for the protocol. Studies with *Cocos nucifera* (Fernando *et al.*, 2003), *Elaeis guineensis* (Schwendiman *et al.*, 1988), *Euterpe edulis* (Guerra and Handro, 1998), *Phoenix dactylifera* (Sané *et al.*, 2006), *Acrocomia aculeata* (Moura *et al.*, 2008) and *Bactris gasipaes* (Steinmacher *et al.*, 2007ac) showed that the first events of cell division were always observed in cells adjacent to the vascular tissue, resulting in primary calli or meristematic nodules from which somatic embryos developed.

In the present report, the occurrence of secondary somatic embryos during the induction of peach palm somatic embryogenesis was confirmed and the applicability of TIS for peach palm in vitro regeneration was studied using a twin flask system.

3 Material and Methods

3.1 Plant Material

Seeds from mature fruits, about 4 months after pollination from one selected open-pollinated tree (Mood1) of the Pampa Hermosa landrace grown in a commercial orchard in Ninole, Hawaii/USA, were used as sources of explants. The hard endocarp of the seeds was removed and the kernels (i.e., zygotic embryo enclosed in endosperm) were surface-sterilized by 1 min immersion in 70 % ethanol, followed by a 40 min immersion in sodium hypochlorite solution, provided by a solution of 40 % of commercial bleach, plus one drop of Tween 20® to each 100 ml of this solution (Steinmacher *et al.*, 2007a). Zygotic embryos were aseptically removed under a stereoscope and used in this experiment.

3.2 Culture media and conditions

The zygotic embryos were transferred to Petri dishes containing 30 ml of somatic embryogenesis induction medium. The induction medium was composed by MS (Murashige and Skoog, 1962) salts plus Morel and Wetmore (1951) vitamins, 3 % sucrose, 500 mg L^{-1} Glutamine (Duchefa®), 2.5 g L^{-1} Gelrite (Duchefa®), 1 µM $AgNO_3$ solution and 10 µM Picloram [4-amino-3,5,6-trichloropicolinic acid (Duchefa®)] (Steinmacher *et al.*, 2007a). Each Petri dish contained five zygotic embryos that were observed periodically to describe the development of the somatic embryos. After 12 weeks of culture, the somatic embryos were isolated and used as explants for the induction of secondary somatic embryos in two experimental systems: on solid medium and in a temporary immersion system. For culture on solid medium, nine somatic embryos were cultured on 30 ml induction medium as previously described for induction of primary somatic embryogenesis. The temporary immersion system (TIS) used in the present study was based on the "twin flasks" system described by Niemenak *et al.* (2008). Briefly, the plant compartment consisted of 1 L volume glass jars (Weck®)

within which 250-300 mg isolated somatic embryos were cultivated in small baskets made with 150 µm nylon sieves (Laborbedarf-Vertriebs GmbH) attached in the bottom of a 250 ml Kautex polypropylene bottle (Rotilabo®). The medium compartment was a 1 L volume Schott Duran® bottle with 250 ml liquid induction culture medium as described above, but without the gelling agent. Every 6 hours the medium was air-pumped into the plant compartment for 3 minutes of contact with the explants. The air was filter sterilized through an autoclavable 0.2 µm filter (Midisart 2000, Sartorius). All the cultures were kept in darkness at 28°C with a 6 weeks subculture interval. Callus was discarded in each subculture and isolated somatic embryos were further cultured. The induction rate was evaluated at the end of each subculture interval.

The influence of induction conditions on the maturation of somatic embryos was evaluated. After cycling cultures were established, embryogenic cultures obtained from (1) cultures cultivated continually on solid culture medium, (2) cultures cultivated in TIS for 6 weeks and then on solid culture medium for 6 weeks, and (3) from those cultivated continually in TIS were transferred to maturation conditions. In all treatments, 300-400 mg embryogenic clusters were separated into small clusters of somatic embryos and transferred to Petri dishes containing 30 ml maturation culture medium [MS salts; Morel and Wetmore vitamins; 40 µM 2,4-D (2,4-Dichlorophenoxyacetic acid); 10 µM 2-iP [2-isopentyladenine (6-dimethylaminopurine)]; 1.5 g L^{-1} activated charcoal; 1 g L^{-1} Glutamine and 500 mg L^{-1} hydrolysed casein and 2.5 g L^{-1} Gelrite (Steinmacher *et al.*, 2007a)]. The cultures were kept in the dark at 28°C for four weeks. Mature somatic embryos were than isolated and transferred to Petri dishes containing 30 mL conversion culture medium [MS salts; Morel and Wetmore vitamins; 20 µM 2-iP; 0.5 µM NAA (α-Naphthaleneacetic acid); adapted from Steinmacher *et al.* (2007a)]. The cultures were transferred to light conditions (40-60 µmol m^{-2} s^{-1} provided by TLD 58 W/840 fluorescent lamps, Philips®) and 25°C for four weeks. Thereafter, somatic embryos were transferred to Petri dishes containing 30 mL MS salts plus Morel and Wetmore vitamins and 1.5 g.L^{-1} activated charcoal for about 8 weeks until the plantlets reached 1-2 cm.

Plantlets obtained from the cultures induced in TIS and directly transferred to maturation conditions were selected, and their further growth was evaluated on solid culture medium and in TIS. The plantlets were weighed (200-250 mg each) and transferred to jars (Weck® 600 ml) containing 100 ml solid culture medium (eight plantlets per flask) and sealed with plastic film or 24 plantlets for TIS treatment containing 300 ml of the same basal culture medium, except that the active charcoal was omitted. The same TIS described for induction of secondary somatic embryogenesis was used, but without the baskets. The cultures were kept under light conditions and subcultured at intervals of 4 weeks for three

months. All the culture media were adjusted to 5.8 pH prior to adding the gelling agent and were autoclaved at 120ºC (1 kgf cm^{-2}) for 20 min.

The acclimatization procedure was adapted from Steinmacher *et al.* (2007a), keeping the plantlets in a high humidity environment for the first 3 weeks. The entire acclimatization step was carried out in a growing room at 28°C and with 16 hours 80-100 µmol m^{-2} s^{-1} provided by HP-T Plus Lamps (Philips®). To maintain the humidity the plantlets were transferred to plastic trays containing sand and watered with distilled water. The trays were placed inside of another plastic tray containing a transparent cover and this whole acclimatization apparatus was maintained in plastic bags for three weeks. After 3 weeks, the plastic bags were removed and the plantlets were watered every other day with distilled water and once a week with 5 ml per plantlet of modified Hoagland's Solution. After three months in this condition the plantlets' survival and rooting were evaluated.

3.3 Histological procedures

For light microscopy, samples were fixed in 2 % (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated in an ethanol series (30 % to 100 %) and embedded in LR White resin (London Resin Co Ltd, London). Gelatine capsules filled with resin and samples were allowed to polymerize at 65°C overnight. Specimens were cut into 1 µm thick sections with a glass-knife in a semi-automatic microtome (Reichert Ultracut S, Leica®), mounted onto glass slides with a drop of water and fixed over a hot plate (approximately 70°C). For general histology, toluidine blue O (0.5 % in phosphate buffer) was used. For protein and polysaccharide localization the double-staining technique [with NBB (Naphtol Blue-Black) and PAS (Periodic Acid-Schiff)] or only PAS was used following Fisher (1968). For DAPI (4'-6-Diamidino-2-phenylindole) staining, 100 µl of a stock solution (1 mg ml^{-1} in water) was diluted into 1 ml 0.1 M phosphate-buffer (pH 7.0). The sections were incubated in one drop of the dilute solution for five minutes and washed once with 0.1 M phosphate buffer (pH 7.0). After removing the excess washing buffer, the samples were mounted with anti-fading Citifluor (Citifluor Ltda, London) and visualized under UV excitation. All the sections were examined using an Olympus BH-2 microscope and photographed with a ColorView IIIu (Soft Imaging System, GmbH).

For scanning electron microscopy, the samples were fixed in 3 % glutardialdehyde in 0.1 M phosphate buffer (pH 7.0) and dehydrated in an ethanol series (30-100 %), followed by an acetone series (30 %, 50 % and 100 % acetone in ethanol). The samples were dried to the critical point with liquid CO_2 in an CPD 030 critical point dryer (Bal-TEC, Leica®), affixed to aluminum stubs and coated with gold palladium in a SCD 050 Sputter Coater (Bal-TEC,

Leica®). The mounted specimens were examined with a Philips XL 20 scanning electron microscope.

For transmission electron microscopy, samples were fixed in 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 2 h at room temperature. Thereafter the samples were transferred to fresh fixative solution and maintained overnight at 4°C. The samples were then rinsed in the same buffer without glutaraldehyde three times (15 min each) and postfixed in buffered 1 % OsO_4 at 4°C for 2 hours. After rinsing in cacodylate buffer, the samples were dehydrated in a graded acetone series and embedded in Spurr's resin. Ultrathin sections (80-100 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), collected over a Formvar coated copper-grid, and stained with 1 % uranyl acetate and 0.1 % lead citrate for 10 min each.

3.4 Statistical Procedure

A completely randomized design was used for all experiments. For the experiment of induction of secondary somatic embryogenesis, three repetitions composed of three Petri dishes each were evaluated for solid culture medium or three repetitions composed of two flasks for TIS. The data were subjected to analysis of variance (ANOVA) and when necessary the SNK test was used to compare means using STATISTICA v.6 (StatSoft, Inc., 2004). The variables evaluated were the percentage of callus, spongy tissue development and induction rate of secondary somatic embryos. The induction percentage was additionally separated into three embryogenic-capacity categories [low (< 5 somatic embryos), medium (5-15) and high (> 15)] because during scanning electron microscopy it was difficult to count the exact number of somatic embryos.

For plantlet growth, the experiment was conducted in a completely randomized design containing five replications, with each replication represented by one flask with solid or liquid culture medium. The evaluated variables were plantlet fresh weight, total number of plantlets and plantlet height, the latter with three classes: < 3.5 cm; 3.5-6.5 cm; > 6.5 cm. For plantlet acclimatization, the final survival rate (%), average height (cm), rooting rate (%) and average number of roots per plantlet were evaluated. The results were subjected to ANOVA as above.

4 Results

4.1 Induction of primary somatic embryogenesis

Zygotic embryos (Figure 1A) cultivated on Picloram-enriched culture medium showed swelling and callus growth during the first week of culture. Globular structures arising from callus could be observed within 4 to 6 weeks of culture (Figure 1B-C). These globular structures were whitish and usually only a few isolated globular structures could be observed on each explant at this time. Development of additional somatic embryos occurred exactly in the sectors where the globular structures first appeared (Figure 1D-E), resulting in clusters of somatic embryos at the end of 12 weeks of culture. These clusters of somatic embryos could be observed on all parts of the callus, but usually clusters in contact with the culture medium developed more somatic embryos (Figure 1F). Approximately 40 % of the explants developed somatic embryos.

Histological techniques were used to describe the sequence of development of individually selected somatic embryos. Globular structures at the onset of polarization (i.e., elongated cells) and the presence of a well-delimited protoderm were the first clearly distinguishable stages of somatic embryo development in culture (Figure 2A). Further development included their elongation, development of procambium and differentiation of the shoot meristem pole (Figure 2B). Somatic embryos transferred to maturation conditions had well-developed procambium and a developing sheathing base around the shoot meristem (Figure 2C). After the somatic embryos were transferred to conversion conditions, their development included a well-differentiated shoot meristem completely enclosed by the sheathing base (Figure 2D).

Scanning electron microscopy showed the initial development of the isolated globular structures (Figure 3A), which resulted in the development of small clusters of somatic embryos (Figure 3B). A delimited protodermis (Figure 3C) and a clear separation of the embryogenic sector and the callus sector usually could be observed (Figure 3D), where callus had loose cells while the embryogenic sector had a highly organized protodermis. In some cases the epidermis of zygotic embryos also formed a continuum with the protodermis of the somatic embryos, and the separation was visible only when the callus tissue was exposed. No suspensor-like tissue was observed and the somatic embryos had a broad basal area fused to the maternal tissue, but no vascular connections with the explant were observed through light microscopy.

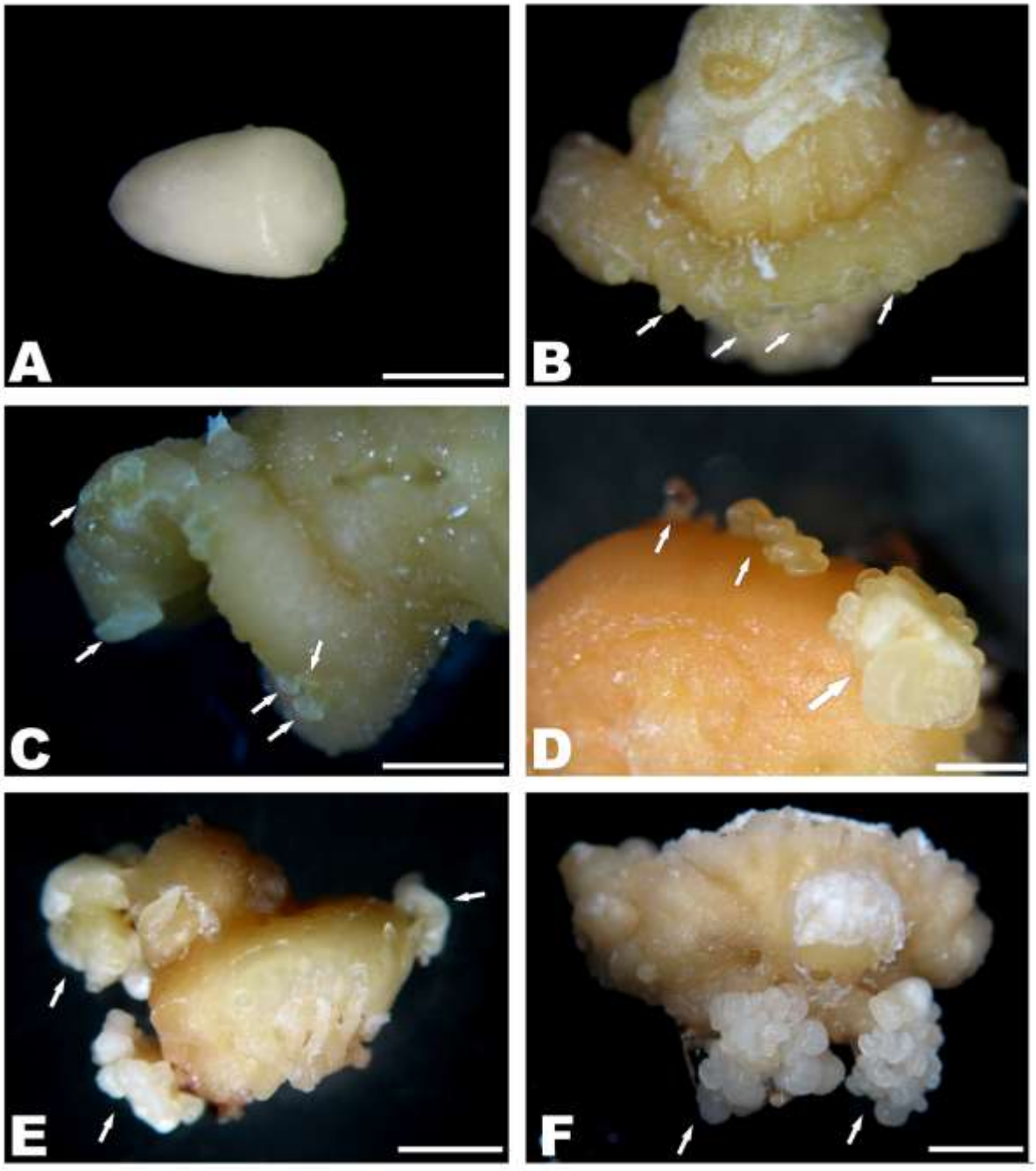

Figure 1 - Induction of somatic embryogenesis in peach palm zygotic embryos. A – Mature zygotic embryo of peach palm used as explant (bar = 1 mm). B – Initial development of globular structures resembling somatic embryos on the callus (arrows) after 4 to 6 weeks of culture (bar = 3 mm). C – Further development of globular somatic embryos (arrows) where somatic embryos had previously developed (bar = 2 mm). D – Development of a cluster of somatic embryos (arrows) on the callus after 6 weeks of culture (bar = 2 mm). E – Further development of somatic embryos on the clusters (arrow) (bar = 4 mm). F - Clusters of somatic embryos (arrows) at the end of 12 weeks of culture (bar = 4 mm).

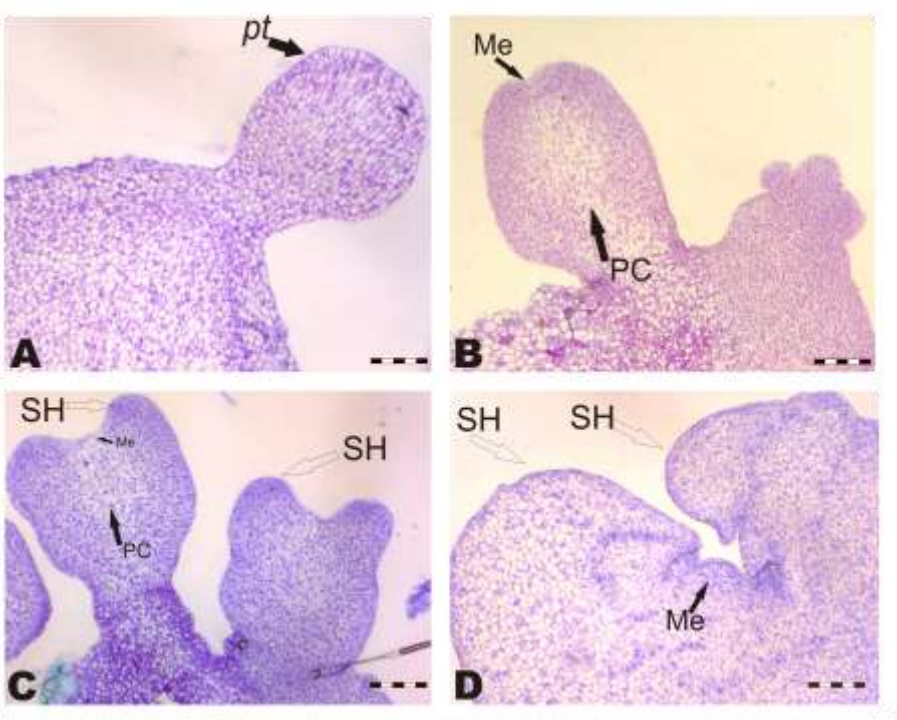

Figure 2 - Histological analyses of the development of peach palm somatic embryogenesis. A – Globular somatic embryo with well-developed protodermis (pt) (bar = 200 µm). B – Elongated somatic embryos showing the initial differentiation of the procambium (PC) and shoot meristem (Me) (bar = 200 µm). C – Mature somatic embryo revealing complete development of the procambium as well as the sheathing base (SH) (bar = 200 µm). D – Somatic embryo in conversion conditions revealing a well formed shoot meristem enclosed by the sheathing base (bar = 200 µm).

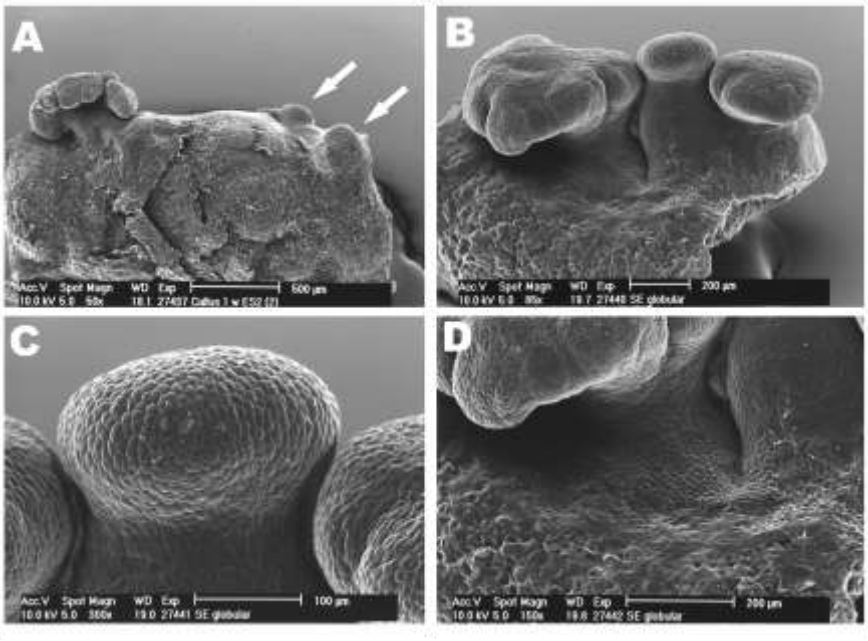

Figure 3 - Scanning electron microscopy analyses during the induction of peach palm somatic embryos. A - Initial development of the isolated globular structures (arrows) (bar = 500 µm). B - Small clusters of primary somatic embryos (bar = 200 µm). C – Globular somatic embryos with a delimited protodermis (bar = 100 µm). D – General view of the callus cells with the embryogenic sector and the callus sector (bar = 200 µm).

During the induction of somatic embryogenesis, alterations in the morphology of the somatic embryos were observed, especially, but not exclusively, in the sector where the sheathing base resulted in a mushroom-like structure (Figure 4A-B). From these somatic embryos, secondary somatic embryos started to develop, resulting in clusters of somatic embryos (Figure 4C); secondary somatic embryos arose also from globular somatic embryos (Figure 4D). These results confirm that the development of clusters of somatic embryos in peach palm is due to the development of secondary somatic embryos.

In the clusters of somatic embryos, several developmental stages could be observed, revealing non-synchronized development. This was due to the fact that secondary somatic embryos are continually produced in these conditions and that primary somatic embryos also developed at different points in a callus at different times. Histological analyses revealed that the sub-epidermal cell layers showed a more intense reaction to Toludine blue (Figure 5 A-C). As somatic embryo development progressed, intense staining was observed in the sheathing base region (Figure 5D) from where new somatic embryos arose (Figure 5E). As with SEM analysis, light histology analysis revealed the initial development of secondary somatic embryos from globular somatic embryos (Figure 5F).

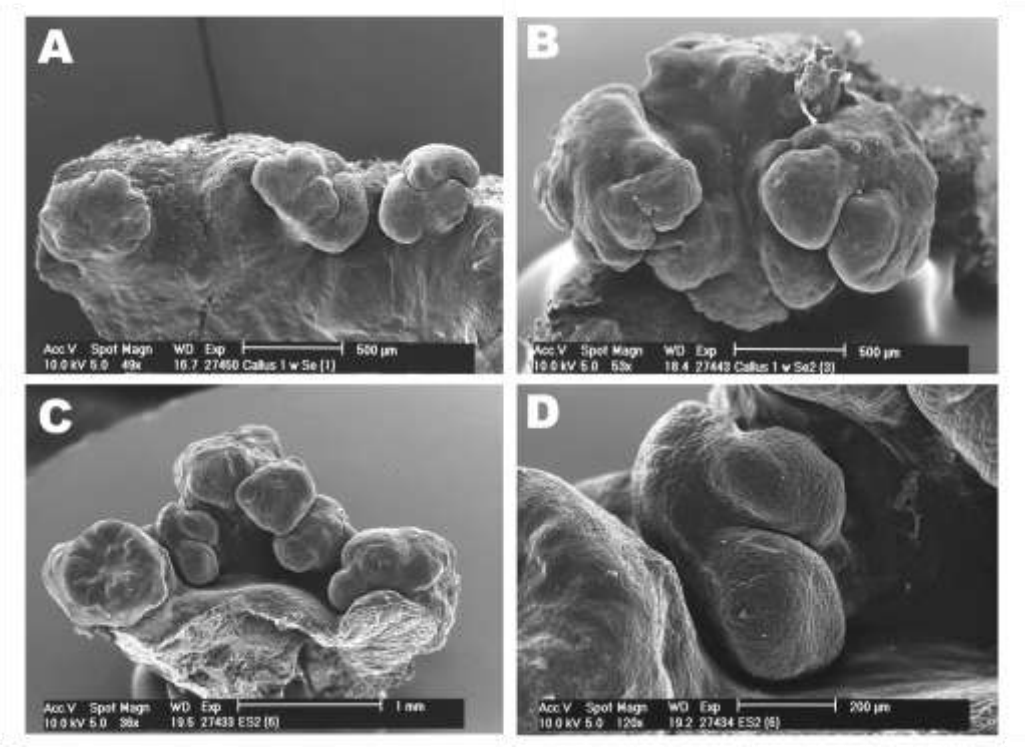

Figure 4 - Further alteration of peach palm primary somatic embryos revealed by scanning electron microscopy. A – Alteration in the meristem sheathing base resulted in a mushroom-like structure (bar = 500 µm). B – Alteration on the sheathing base of primary somatic embryos (bar = 500 µm). C – Development of secondary somatic embryos, resulting in a cluster of somatic embryos (bar = 200 µm). D – Globular somatic embryo revealing the development of secondary somatic embryos (bar = 200 µm).

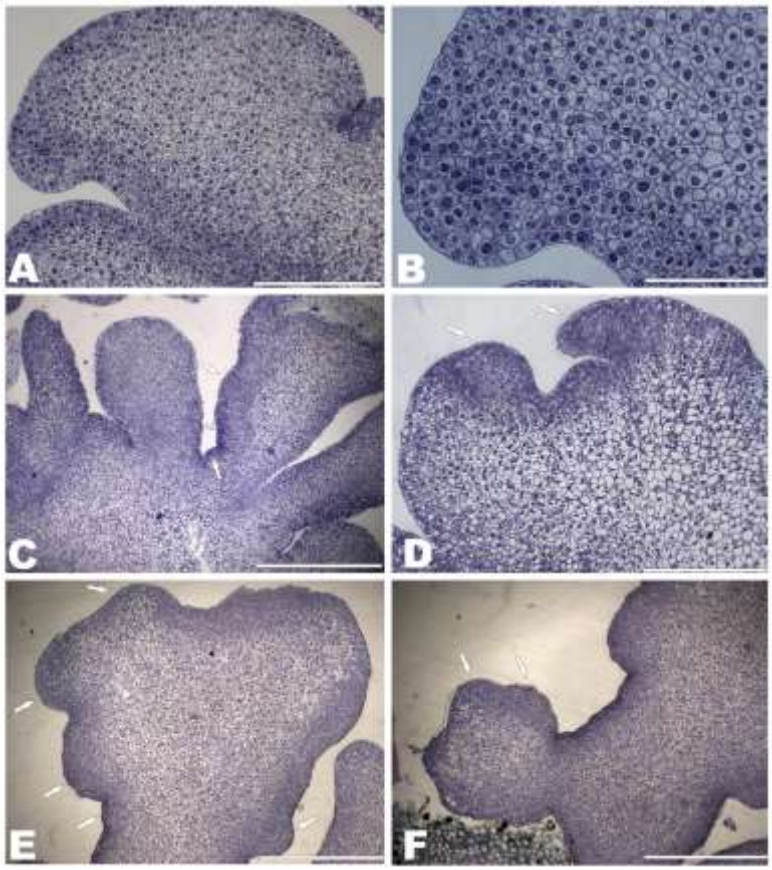

Figure 5 - Histological analyses of the development of peach palm somatic embryos stained with Toludine blue. A – Histological analysis of peach palm primary somatic embryos revealing cell division on sub-epidermal cells (bar = 200 µm). B – Detailed view of the sub-epidermal cell layers that showed a more intense reaction and several mitotic events (bar = 100 µm). C - Peach palm primary somatic embryos showing cell division in the sub-epidermal layers (arrows) (bar = 500 µm). D – Developed somatic embryo showing intense staining in the sheathing base region (arrow) (bar = 200 µm). E –Development of secondary embryos (arrows) from elongated somatic embryo (bar = 500 µm). F - Initial development of secondary somatic embryos from globular somatic embryos (arrows) (bar = 500 µm).

4.2 Induction of secondary somatic embryogenesis and plantlet regeneration

Peach palm somatic embryos had high embryogenic capacity in solid culture medium as well as in TIS, ranging from 65.8 % to 86.2 % of secondary somatic embryo development in all conditions (Figure 6 and 7). Picloram-enriched culture medium was effective in inducing secondary somatic embryogenesis. Statistical differences ($p < 0.05$) were observed for the total rate of induction only in the first cycle, not in other cycles (Table 1). However, a significantly increasing rate ($p < 0.05$) of somatic embryo induction having low embryogenic capacity was observed during the cycles on solid culture medium, while no difference was observed in TIS. On the other hand, only 2.6 to 9.7 % of the explants cultivated on solid culture medium showed high embryogenic capacity. A decreasing rate of embryogenic capacity was observed on solid culture medium for this group: 16.3 ± 2.7 % of the explants showed more than 15 somatic embryos per explant in the first cycle, while after 4 cycles (6

weeks each) only 2.6 ± 0.9 % of the explants could be classified as high capacity. On TIS, 48.8 % to 64.2 % of the explants showed high embryogenic capacity (Table 1), without statistical differences between these values, but with significant differences ($p < 0.05$) to solid culture medium. Transfer of somatic embryos from TIS to solid culture medium showed high embryogenic capacity (86.2 %), with most explants showing more than 15 somatic embryos (63.5 %), without differences with the TIS treatment. No development of spongy tissue was observed in TIS, while callus development was significantly ($p < 0.05$) higher in TIS than on solid culture medium, varying from 15.3 % to 21.4 % (Table 1).

Upon transferring somatic embryo clusters to maturation conditions for 4 weeks, differences among culture conditions were observed (Figure 8A-C). The highest increment in fresh weight was observed for clusters of somatic embryos cultivated exclusively on solid culture medium (Figure 8A; Table 2). This high increment was mainly due to the development of a spongy haustorial-like tissue (Figure 8D). Embryogenic clusters from TIS or TIS/solid showed a high number of mature somatic embryos after 4 weeks in maturation conditions (Figure 8E), without statistical differences between these treatments (Table 2). The conversion capacity of somatic embryos was around 30 % (data not shown) and plantlets were obtained (Figure 8F).

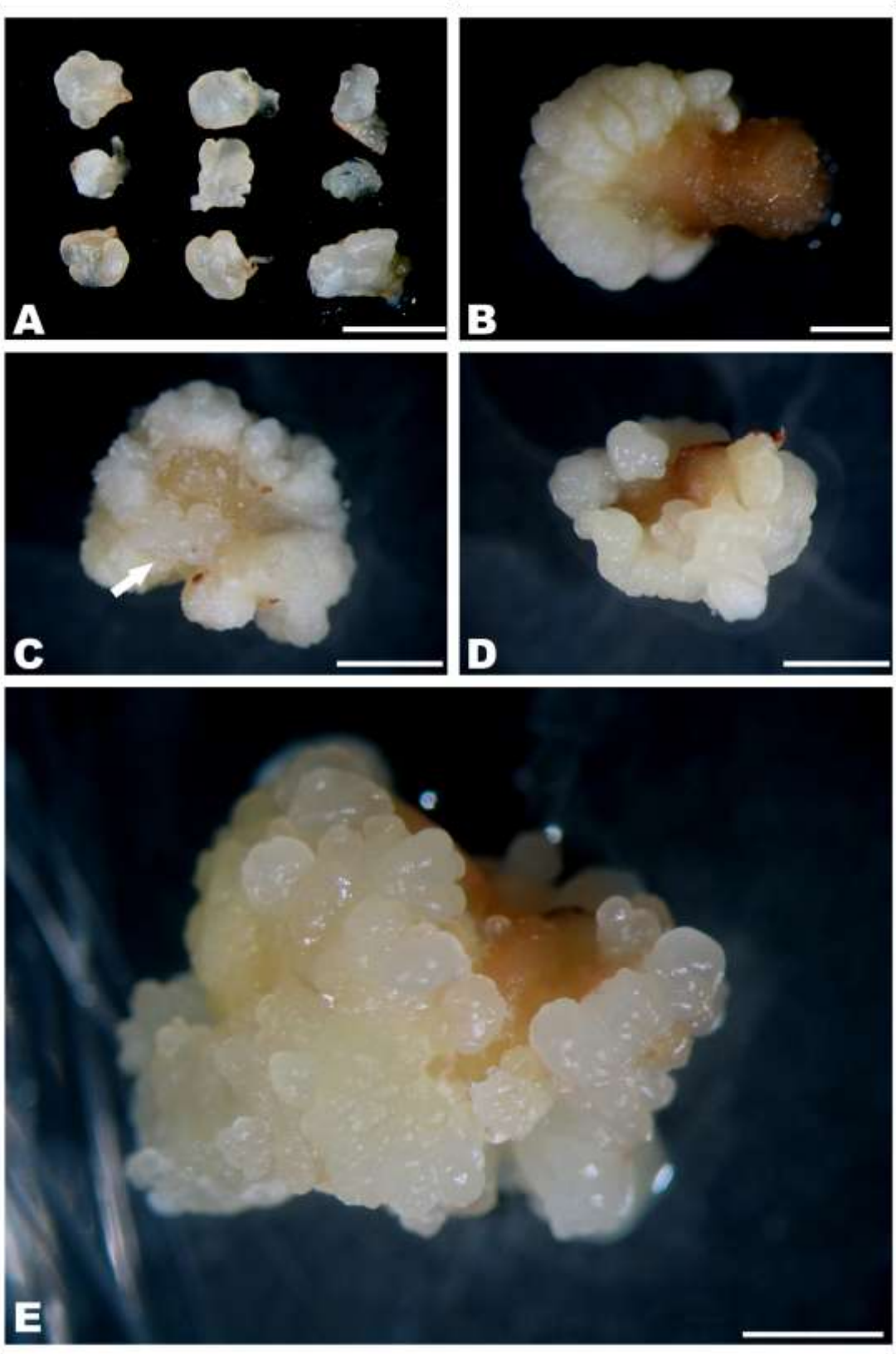

Figure 6 - Induction of secondary somatic embryos of peach palm. A – Somatic embryos used as explants (bar = 2 mm). B – Morphological aspects of the non-embryogenic spongy tissue (bar = 1 mm). C – Low embryogenic capacity class associated with the development of spongy tissue. D – Example of medium embryogenesis capacity (bar = 2 mm). E – Example of an explant showing high embryogenic capacity (bar = 2 mm).

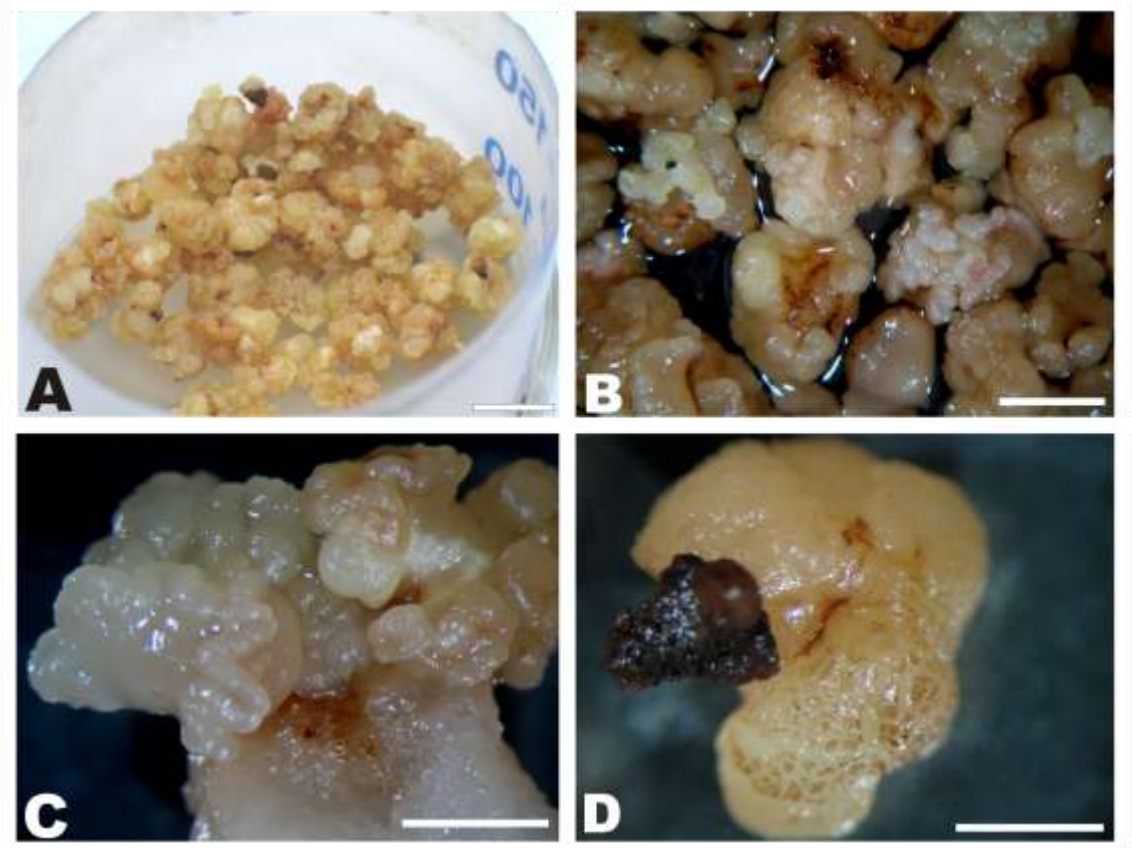

Figure 7 - Aspects of the development of peach palm secondary somatic embryogenesis in TIS (temporary immersion system). A – General aspect of the cultures (bar = 3 cm). B – Cultures showing the development of secondary somatic embryos and callus (bar = 4 mm). C – Morphological aspect of the embryogenic sector (bar = 2 mm). D – Morphological aspect of a non-embryogenic callus (bar = 2 mm).

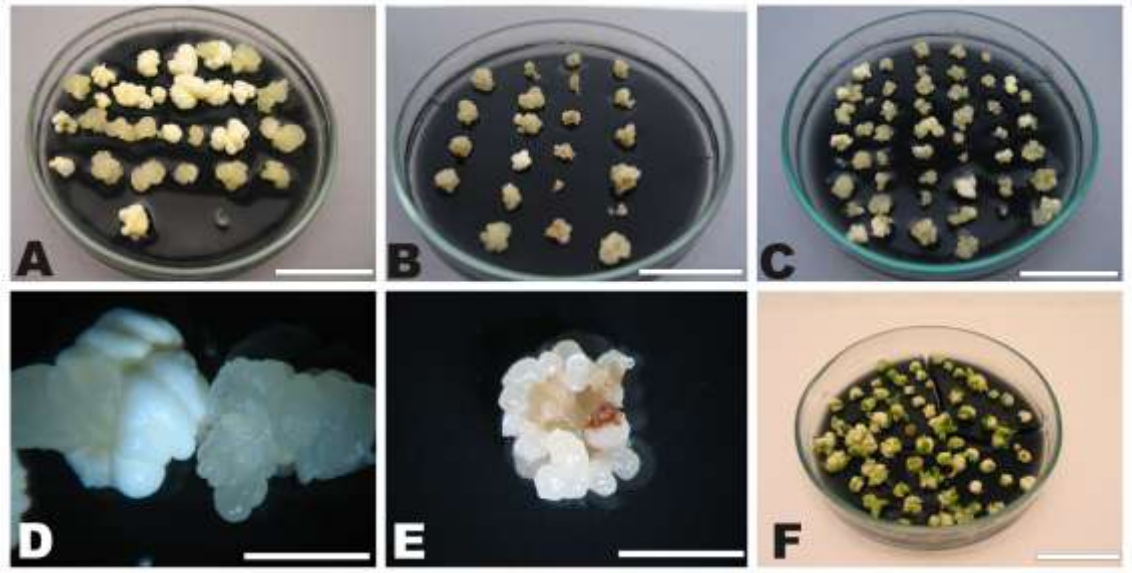

Figure 8 - Maturation of peach palm somatic embryos. A – Somatic embryos induced continually in solid culture medium (bar = 3 cm). B – Somatic embryos induced in TIS (bar = 3 cm). C – Somatic embryos in TIS cultivated for one cycle on solid culture medium (bar = 3 cm). D – Aspect of the structures developed from those cultures cultivated continually on solid culture medium (2 mm). E – Detailed view of the cultures of mature somatic embryos shown in B and C (bar = 2 mm). F – Converted somatic embryos (bar = 3 cm).

Table 1 - Percentage of induction of secondary somatic embryogenesis on peach palm in different culture conditions and during different cycles of six weeks each. Results are means ± standard error. Means followed by different letters in the column represent statistical differences identified by SNK analysis ($P < 0.05$).

	Callus	Spongy tissue	Development of secondary somatic embryos			
			Total	*Low*	*Medium*	*High*
1st solid	7.7±2.1^{bc}	26.2±2.9^{b}	66.2±3.1^{b}	14.4±1.7^{b}	35.5±2.0^{a}	16.3±2.7^{b}
2nd solid	7.6±2.8^{bc}	26.2±2.4^{b}	65.8±3.0^{b}	21.8±2.4^{b}	37.8±4.3^{a}	6.2±2.0^{bc}
3rd solid	1.3±0.5^{c}	11.1±4.2^{a}	86.6±4.1^{a}	35.5±6.0^{a}	41.3±8.6^{a}	9.7±4.5^{bc}
4th solid	0.0±0.0^{c}	31.1±1.8^{b}	70.2±2.3^{ab}	40.8±2.5^{a}	26.6±2.5^{ab}	2.6±0.9^{c}
2nd TIS	21.4±2.7^{a}	0.0±0.0^{c}	78.6±2.7^{ab}	16.5±2.2^{b}	13.3±2.9^{b}	48.8±4.5^{a}
3rd TIS	16.0±3.1^{ab}	0.0±0.0^{c}	83.9±3.0^{a}	13.4±2.3^{b}	19.5±4.2^{ab}	50.9±3.0^{a}
4th TIS	15.3±3.5^{ab}	0.0±0.0^{c}	84.6±3.5^{a}	9.7±1.0^{b}	10.7±3.7^{b}	64.2±5.8^{a}
TIS-Solid	9.3±2.4^{bc}	0.4±0.4^{c}	86.2±5.0^{a}	4.9±2.7^{b}	13.7±2.6^{b}	63.5±6.7^{a}

Table 2 - Maturation peach palm secondary somatic embryos on maturation culture medium.

Treatment	Inicial Weight (mg FW)	30 days Culture on Maturation Medium	
		Increment (g)	*Somatic embryos (100mg explant)*
Solid	343±9.3	6.89±0.74^{b}	0.0±0.0^{b}
TIS	349±13.7	3.92±1.27^{a}	62.1±16.2^{a}
TIS/Solid	370±12.2	3.96±1.03^{a}	44.9±7.8^{a}

Small plantlets (200-250 mg each) were cultivated on solid culture medium or transferred to TIS. The final fresh weight of the plantlets was higher in TIS than on solid culture medium (Figure 9; Table 3). Plantlet height was also influenced by culture conditions. After 3 months of culture on solid culture medium, no plantlet taller than 6.5 cm was observed, while in TIS 51.1±11.4 % of the plantlets were taller than 6.5 cm (Table 3). Additionally, newly formed shoots were observed in TIS as well as on solid culture medium; to date it is not clear if those shoots developed from fused somatic embryos or from the development of off-shoots, as peach palm is caespitose. Root development was observed only occasionally during the culture period (Figure 9E-F, from solid culture medium and TIS, respectively).

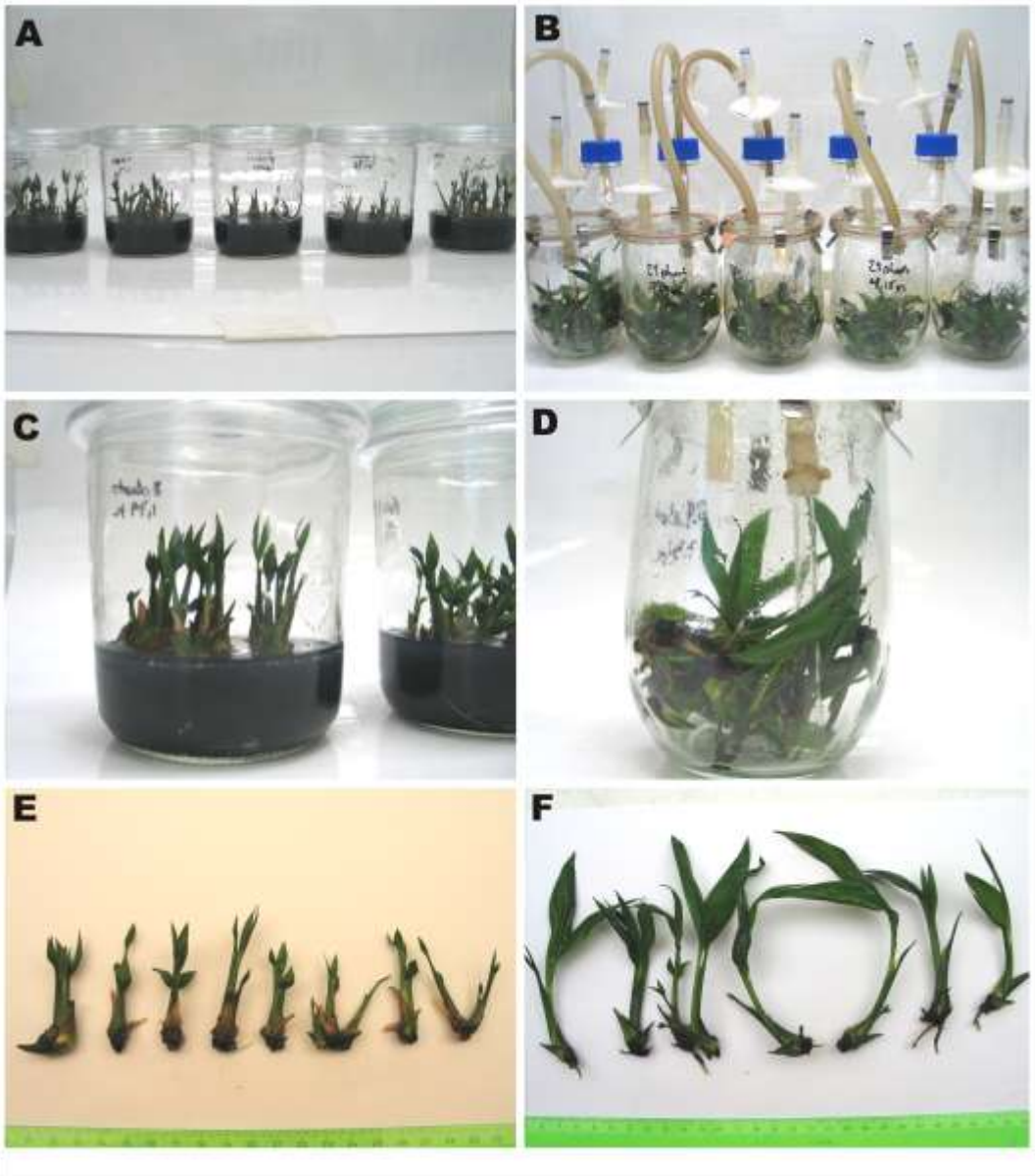

Figure 9 - Development of in vitro peach palm plantlets after three months of culture. A – General aspects of plantlets cultivated on solid culture medium. B – General aspects of plantlets cultivated in TIS. C – Aspects of plantlets cultivated on solid culture medium. D – Aspects of plantlets cultivated in TIS. E – Detailed view of the plantlets cultivated for three months on solid culture medium. F - Detailed view of the plantlets cultivated for three months in TIS.

Table 3 - Comparison of TIS and solid culture medium on subsequent peach palm plantlet growth.

Treatment	Initial (mg FW)	Final (g FW)	n Total	Class of plantlet height (%)		
				< 3.5cm	3.5 – 6.5cm	> 6.5cm
TIS	213±2	1.34±0.2	177	16.9±5.2	32.0±7.8	51.1±11.4
Solid	215±1	0.97±0.1	54	41.6±8.1	58.4±8.1	0.0±0.0

Upon transferring the regenerated plantlets to acclimatization conditions (Figure 10A-C), 65 % of TIS-grown plantlets and 97 % of solid culture medium plantlets survived after 3 months. On the other hand, plantlet height, rooting rate and number of roots per plantlet were significantly higher in plantlets from TIS than from solid culture medium (Figure 10D; Table 4). Successful rooting (Figure 10F) of TIS-grown plantlets (75.1 %) was much higher than solid culture medium grown plantlets (12.5 %) and all plantlets were allowed to grow further (Figure 10G).

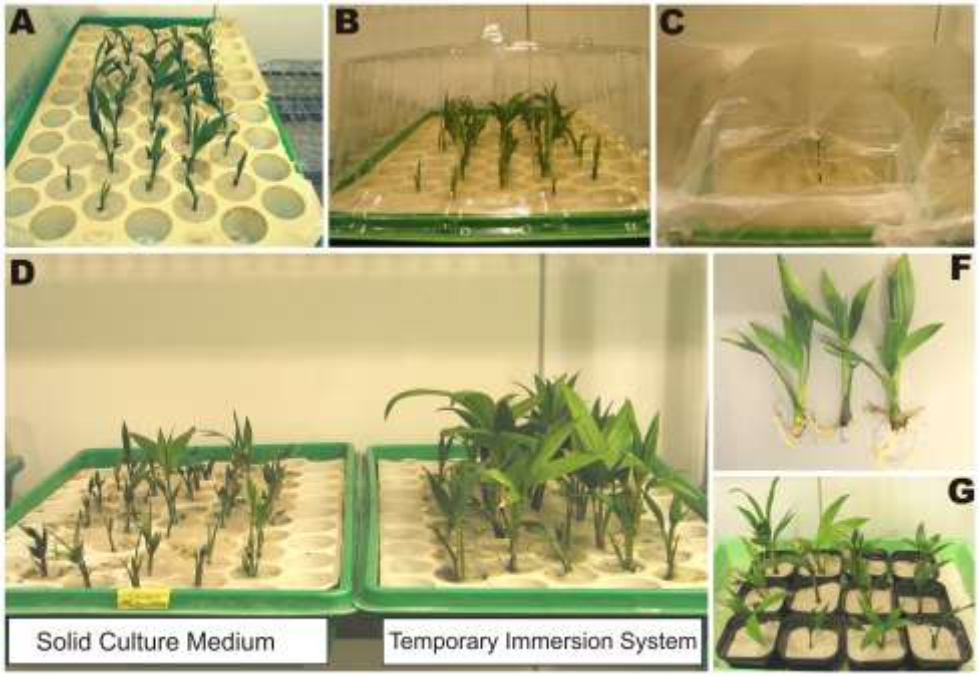

Figure 10 - Acclimatization of the in vitro regenerated peach palm plantlets. A-C – Aspects of the acclimatization apparatus utilized in the present study. D – Plantlets obtained from solid culture medium and TIS after three months of acclimatization. E – Aspect of the acclimatized plantlets obtained after successful rooting (arrow). F – Plantlets transferred to individual containers to allow further growth.

Table 4 - Peach palm plantlet growth and survival rate after 3 months of acclimatization.

Treatment	*n* plantlets	Survival (%)	Height (cm)	Rooting (%)	roots per plantlet
TIS	133	65b	11,2a	75,1a	2,3a
Solid	36	97a	6,6b	12,5b	1,3b

4.3 Morpho-histological aspects of secondary somatic embryo development

Using somatic embryos as explants (Figure 6A) resulted in secondary somatic embryos developed directly on the explants. Parallel to the development of somatic embryos, callus growth was also observed, resulting in the development of callus-like sectors and embryogenic sectors. This morphogenetic aspect was observed in both solid culture medium and TIS, however generally associated with those explants that exhibited high embryogenic

capacity (Figure 6 and 7). The low embryogenic capacity class was often associated with the development of spongy haustorial-like tissue on solid culture medium (Figure 6B and C). As observed during the induction of primary somatic embryogenesis, secondary somatic embryos developed frequently, but not exclusively, from the sheathing base and continual development of somatic embryos was observed.

SEM analyses showed the development in sectors, confirming a callus sector and an embryogenic sector (Figure 11A-B), and, once somatic embryogenesis was triggered, continual development of somatic embryos was observed (Figure 11C-D). From cultures induced in TIS, SEM analyses also revealed the presence of several small protuberances (Figure 11E), which may be considered pre-globular somatic embryos. Globular somatic embryos were also frequently seen.

Histochemical analyses revealed that starch accumulation generally precedes the development of somatic embryos (Figure 12A). Multicellular origin, involving sub-epidermic and epidermic cells (Figure 12B), is hypothesized for these somatic embryos. The subepidermic layers of cells proved to be metabolically highly active, as shown by the dense cytoplasm after Toluidine blue O staining (Figure 12C). However, in cultures on solid culture medium only a few cells showed the presence of amyloplasts (Figure 12D), while in cultures from TIS most subepidermic cells showed starch accumulation. Also from TIS, some somatic embryos showed high starch accumulation (Figure 12A). As development of somatic embryos progressed, starch accumulation was observed only in the basal area of the somatic embryos (Figure 12E) or later on those sectors where other somatic embryos would develop (Figure 12F). However, starch accumulation could not be systematically correlated with cell embryogenic capacity, as in culture some subepidermic cells also accumulated large amounts of starch without developing into somatic embryos, especially from TIS conditions. In all tested conditions, somatic embryos also appeared with a broad basal area fused to maternal tissue but without vascular connections to the explant tissue, again suggesting multicellular origin.

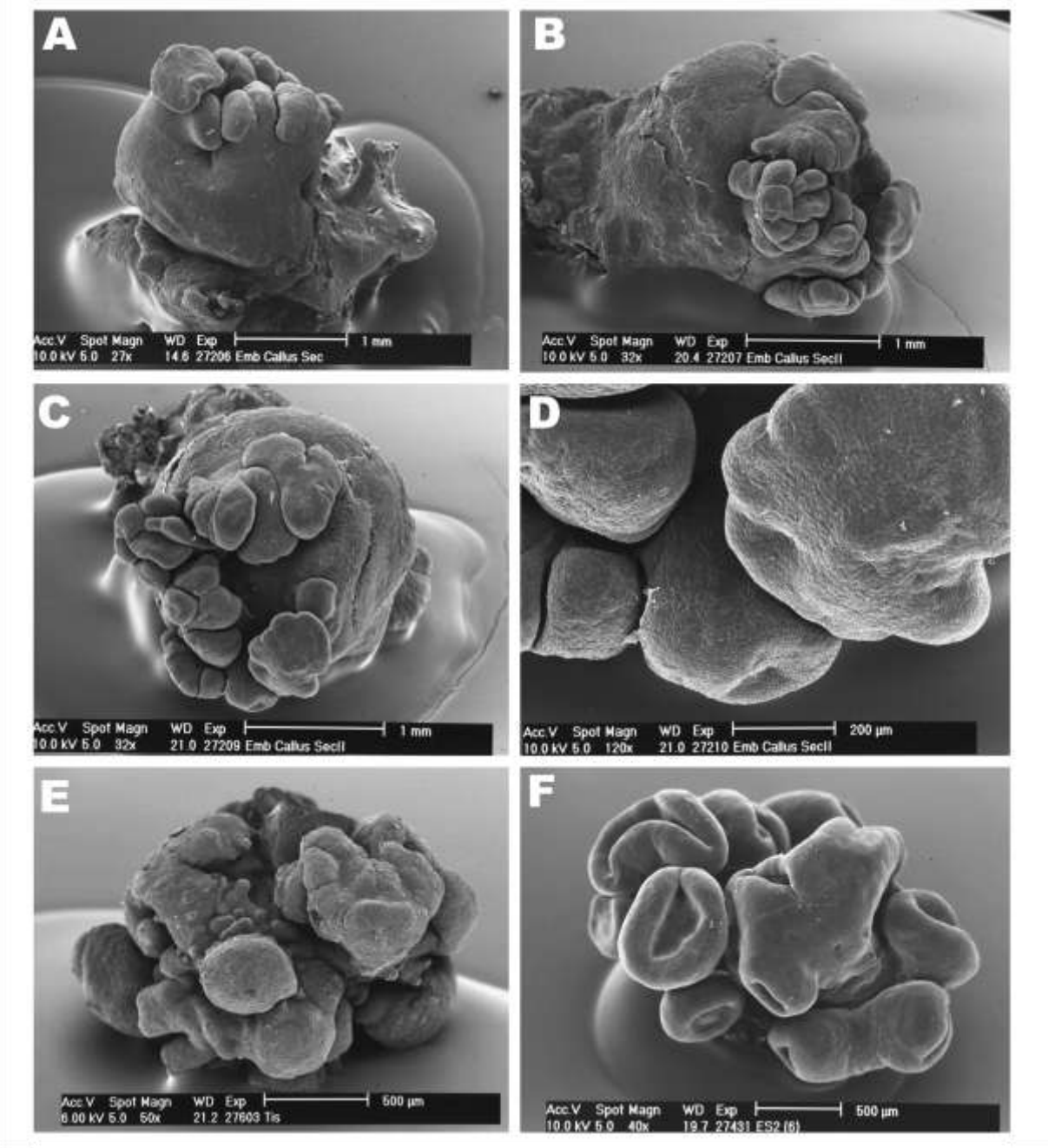

Figure 11 - Scanning electron microscopy analyses during the development of peach palm secondary somatic embryos. A-C – Development of secondary somatic embryos in sectors of the primary somatic embryos (bars = 1 mm). D – Aspect of secondary somatic embryogenesis (bar = 200 µm). E - Cultures induced in TIS revealing the presence of several small protuberances (bar = 500 µm). F – Cluster of somatic embryos (bar = 500 µm).

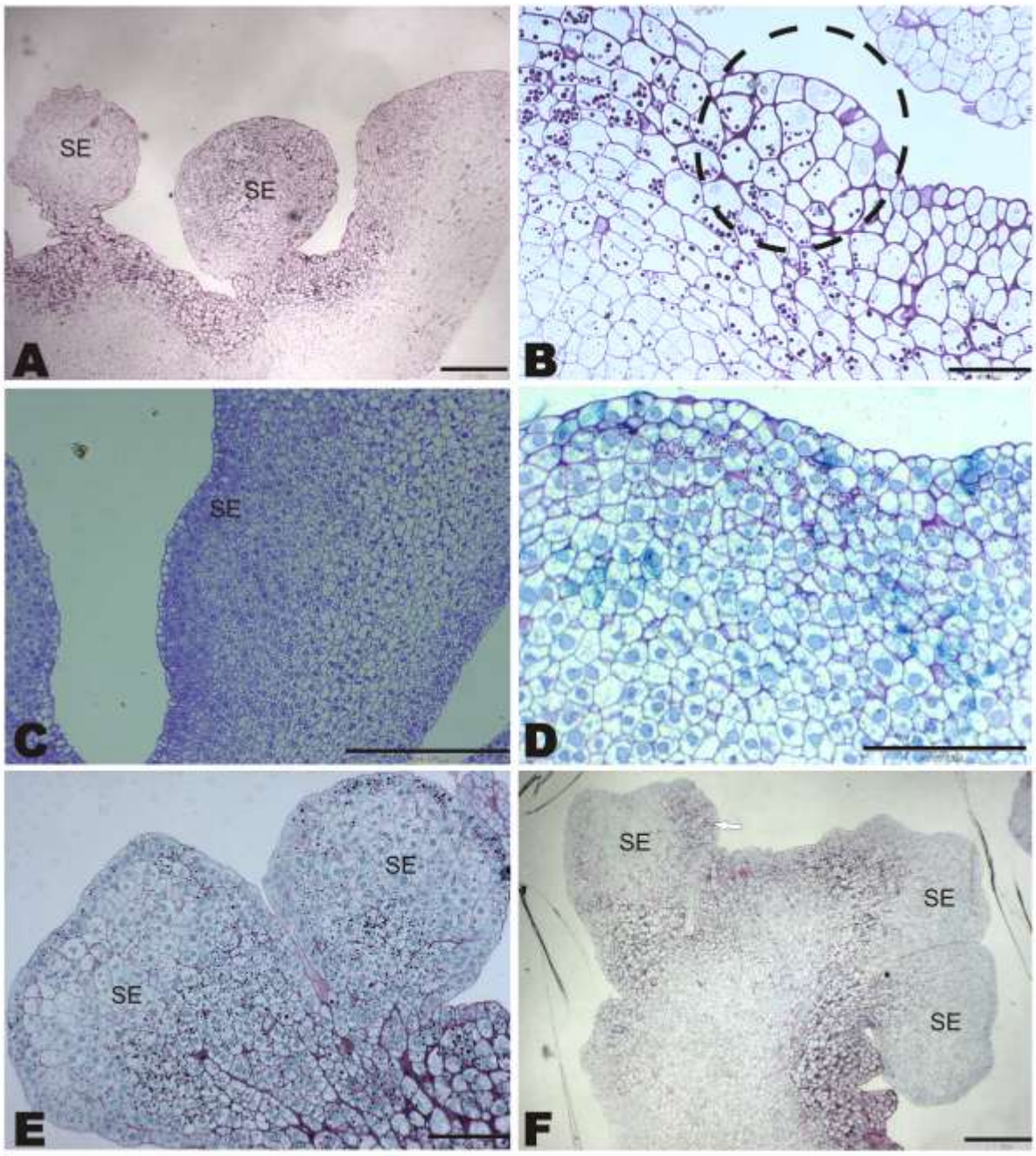

Figure 12 - Histochemical analyses during the development of peach palm secondary somatic embryos (SE). A – Cluster of somatic embryos showing starch accumulation (bar = 200 µm). B – Possible origin of somatic embryos involving sub-epidermic and epidermic cells (circle) (bar = 50 µm). C – Sample stained with Toluidine blue O revealing dense cytoplasm in the sub-epidermic cells (bar = 200 µm). D – Samples cultured only on solid culture medium (bar = 100 µm). E – Further development of somatic embryos showing starch accumulation in the basal area (bar = 100 µm). F – Specific starch accumulation in those sectors where other somatic embryos would develop (arrow) (bar = 200 µm).

Ultrastructural analyses of the embryogenic sector reveal small starch granules in the cells of the protodemis, while sub-epidermic cells had larger amyloplasts that were also more abundant and well distributed in the cells (Figure 13A). The cells contained numerous small vacuoles and a large nucleus with prominent nucleolus (Figure 13B) with no cell wall thickening, and plasmodesma was often observed connecting the cells (Figure 13C), reinforcing the multicellular origin of the somatic embryos. Also in this embryogenic sector, mitotic events could be observed by DAPI staining and a higher nucleus / cytoplasm ratio was observed in sub-epidermic cells compared with those that would result in callus growth (Figure 14 A-B). More numerous mitotic events were observed in the sub-epidermal cell layer, where secondary somatic embryos develop (Figure 14C-D).

Histological analyses of callus revealed the presence a specific zone of small cells (Figure 15A), as well as the presence of an epidermis-like layer (Figure 15B). Only a few amyloplasts were observed in some areas of the callus (Figure 15C). In the area where the embryogenic sector was in contact with the callus sector, an intense metachromatic reaction with Toluidine O was observed in the vacuoles of a specific layer of cells (Figure 15D-E). This metachromatic reaction indicates the accumulation of large amounts of phenolic substances in the vacuoles of the cells. Ultrastructural analysis of these phenol-storing cells revealed the presence of a large vacuole containing electron dense substances (Figure 13D), numerous mitochondria, some small amyloplasts, plastids also containing electron dense substances and Golgi complex (Figure 13E), all suggesting metabolically active cells.

Transferring the somatic embryo sectors from the TIS treatment to maturation conditions resulted in fully developed somatic embryos. SEM analysis revealed the presence of some fused somatic embryos (Figure 11F), which could be the origin of the multiple shoots observed during their conversion into plantlets.

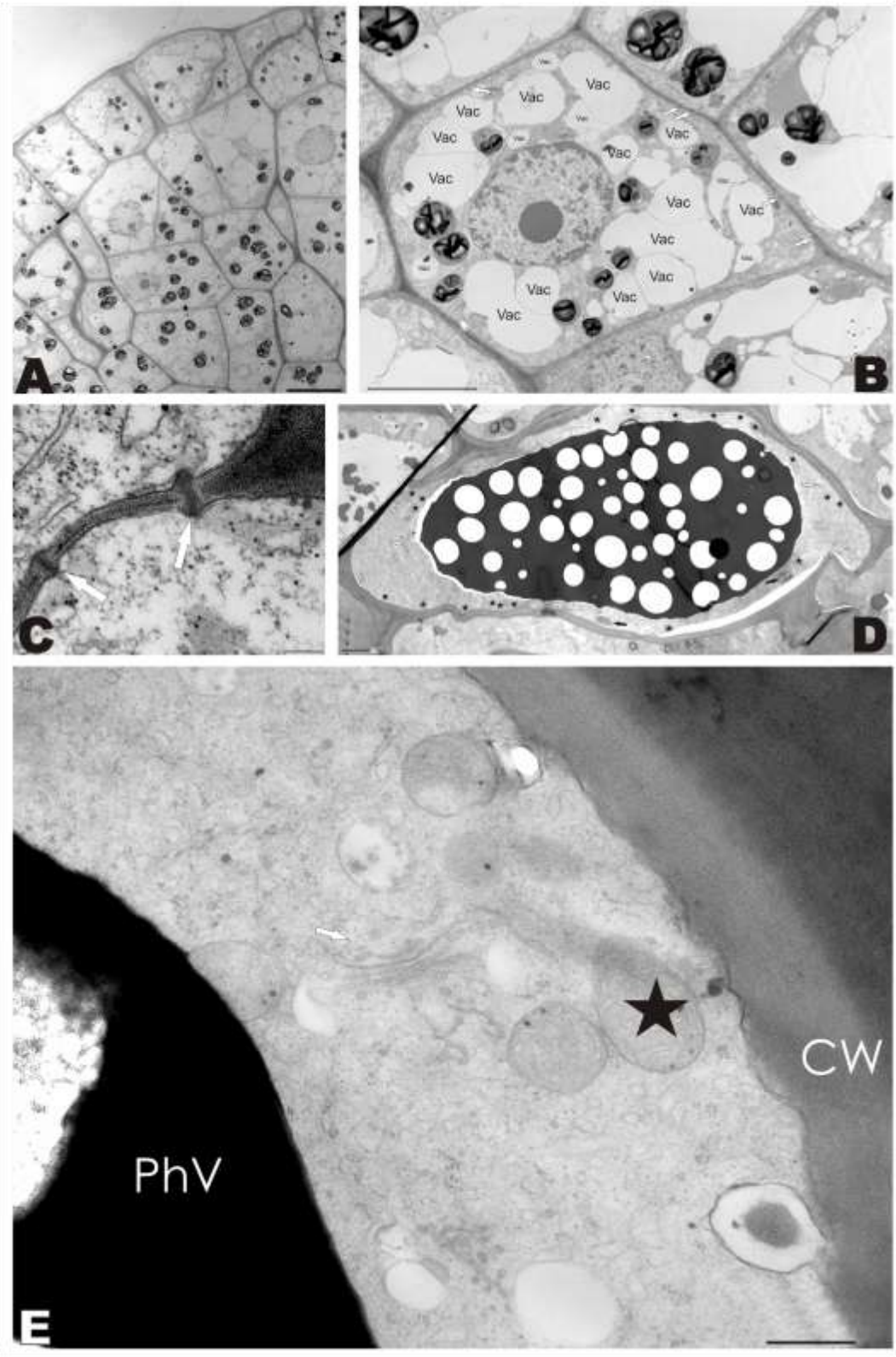

Figure 13 - Ultrastructural analyses of the embryogenic cells and phenol-storing cells. A – Embryogenic sector including epidermic and sub-epidermic cells (bar = 10 µm). B – Example of a cell of the embryogenic sector contained numerous small vacuoles (Vac) and numerous plasmodesma (arrows) (bar = 5 µm). C – Aspect of the plasmodesma (bar = 200 nm). D - Ultrastructural analysis of the phenol-storing cells (bar = 2 µm). E – Detailed view of the phenol-storing cells showing numerous mitochondria (star), and Golgi complex (arrow) (bar = 500 nm).

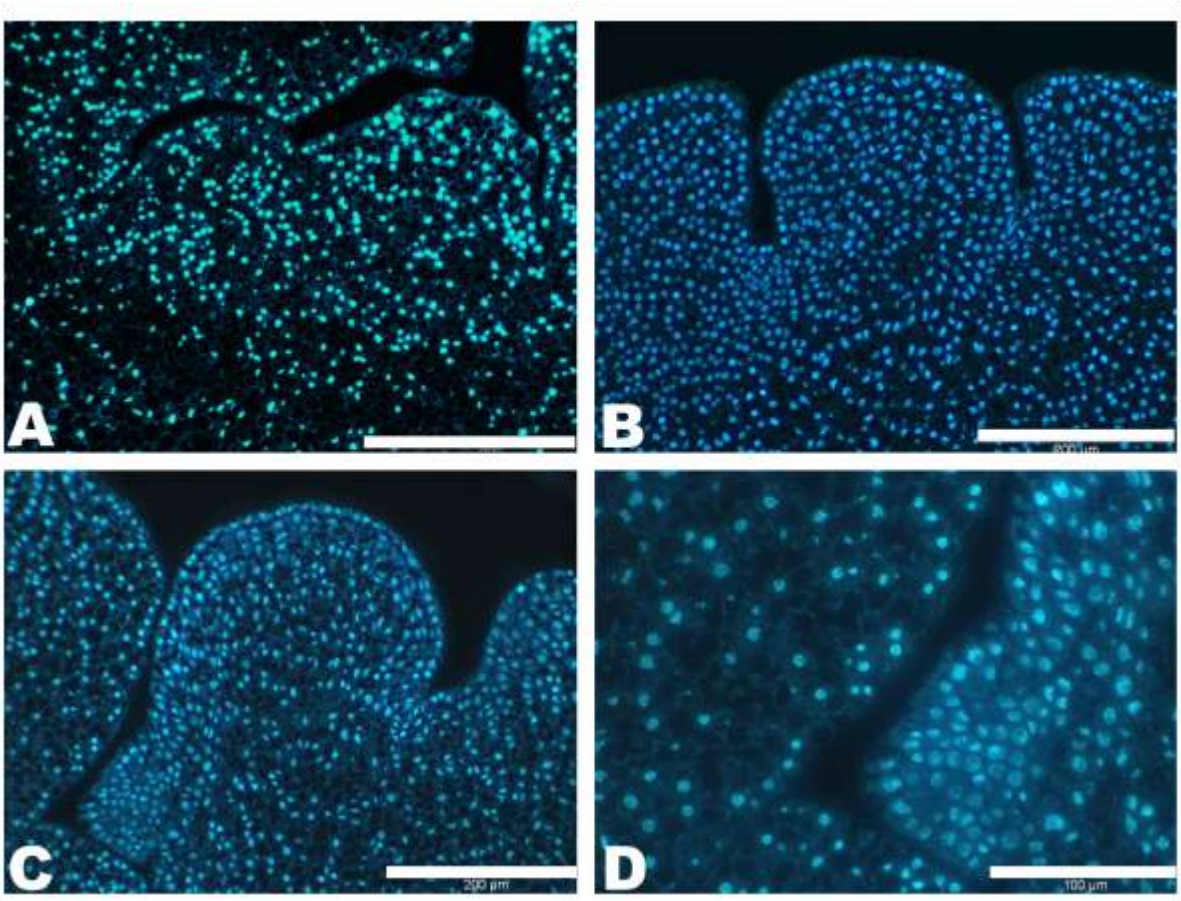

Figure 14 - Mitotic events in embryogenic sector revealed by DAPI staining. A-B - High nucleus : cytoplasm ratio observed in sub-epidermic cells (bars = 200 µm). C-D – Mitotic events during the initial development of peach palm secondary somatic embryos (bar in C = 200 µm; in D = 100 µm).

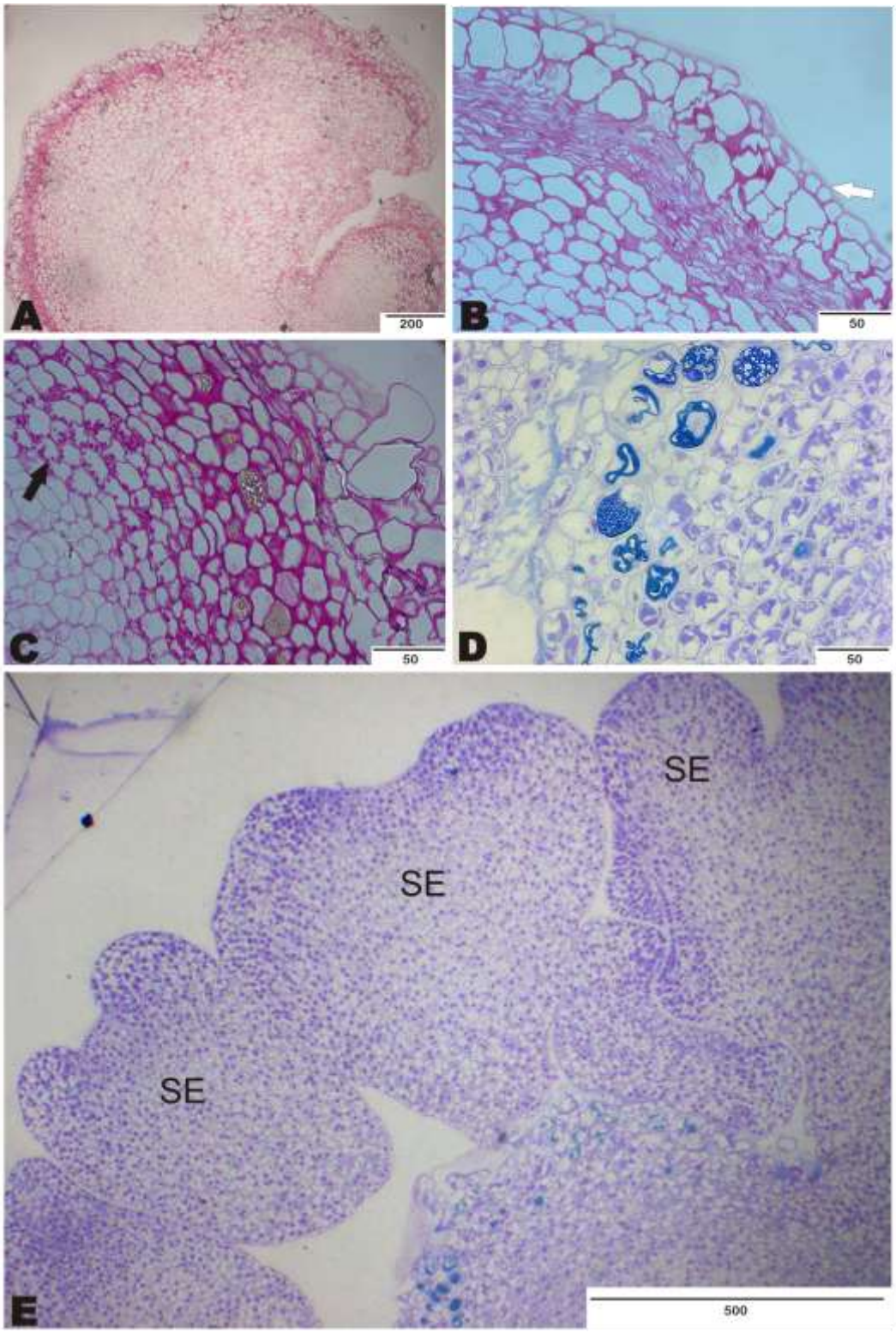

Figure 15 - Histological aspects of the callus sector. A – General view of callus stained with PAS reagent (bar = 200 µm). B – Detailed view of A showing an epidermis-like cell layer (arrow) (bar = 50 µm). C – Presence of amyloplast in the callus sector (black arrow) (bar = 50 µm). D – Histological section of the callus sector in contact with the embryogenic sector revealing the accumulation of phenolic substances in the cells (bar = 50 µm). E – General view of the callus sector and somatic embryos (SE) (bar = 500 µm).

5 Discussion

The occurrence of secondary somatic embryogenesis during the induction of peach palm somatic embryogenesis, as previously hypothesized (Steinmacher *et al.*, 2007a), was confirmed on Picloram-enriched culture medium. This was confirmed by light and scanning electron microscopy in the present study. The occurrence of secondary somatic embryos in primary culture conditions was observed in Bermuda grass (Li and Qu, 2002) and the constant requirement of plant growth regulators for the development of somatic embryos has been described in several monocot species [i.e., Banana (Khalil *et al.*, 2002); see also review by Reamakers *et al.*, 1995]. In the present study, the use of this morphogenetic pathway also allowed the establishment of an effective protocol for the induction of secondary somatic embryogenesis and permitted the development of a cycling culture.

Secondary somatic embryogenesis has already been proved to be an excellent morphogenetic process for several species, such as coconut, cassava and cacao (Perez-Nunes *et al.*, 2006; Groll *et al.*, 2001; Maximova *et al.*, 2002), and the combination of this morphogentic pathway with TIS also increases propagation efficiency in several species, e.g., rubber, coffee and cacao (Etienne *et al.*, 1997; Albarran *et al.*, 2005; Niemenak *et al.*, 2008). To the best of our knowledge, however, there are no conclusive reports on the use of TIS and secondary somatic embryogenesis in palms.

In the present study, both induction of primary and secondary somatic embryos was achieved with Picloram-enriched culture medium. The use of Picloram showed it to be an effective auxin-analog for peach palm somatic embryogenesis, as previously demonstrated (Steinmacher *et al.*, 2007abc). In the palm *Acrocomia aculeata*, the continuous maintenance of callus on Picloram-enriched medium also led to the proliferation of multicellular-origin somatic embryos (Moura *et al.*, 2008). Additionally, it is thought that auxin-analogs have a dual role during the induction of somatic embryogenesis, one related to the auxin signaling and the other to a stress component (Feher *et al.*, 2003).

Stress has been proved to be an important factor related to the acquisition of embryogenic competence (Pasternak *et al.*, 2002). In our study, efficient cycling cultures could be established only on TIS, while on solid culture medium a decreasing rate of highly embryogenic-capable cultures and an increasing rate of spongy tissue development were observed. Therefore, flooding in TIS could be seen as a type of environmental and metabolic stress, increasing the induction of somatic embryogenesis. Other advantages of TIS could be related to the nutrient uptake without hypoxia, absence of nutrient gradients as in solid culture medium and the frequent renewal of the *in vitro* atmosphere, where ethylene accumulates, for instance (Etienne and Berthouly, 2002). The presence of silver nitrate, a known ethylene

perception blocker, has been shown to greatly increase somatic embryogenesis of peach palm (Steinmacher *et al.*, 2007a).

Histological analyses revealed cells with embryogenic characteristics, such as small cells with small vacuoles and large nuclei. Such cells were usually found in subepidermic and epidermic tissue, resulting in multicellular origin somatic embryos. These cells also presented higher metabolic activity, as more starch accumulation was observed. Starch accumulation is considered a marker for embryogenic capacity in several systems, including oil palm, coconut and rattan (Schwediman *et al.*, 1988; Verdeil *et al.*, 2001; Goh *et al.*, 2000). In the present study, starch accumulation could not be systematically correlated to somatic embryogenesis, as some cells accumulated starch without developing into somatic embryos. Similar results were observed in rattan somatic embryogenesis (Goh *et al.* 2000). In coconut somatic embryogenesis, sub-epidermal cells also had a dense cytoplasm (Saenz *et al.*, 2006) and later this was correlated with the expression of the *CnSERK* gene (Perez-Nunes *et al.*, 2009), a marker-gene for somatic embryogenesis.

Histological analyses of the callus sector revealed a sector of phenol-rich cells exactly on the border of the callus, especially in the sectors where somatic embryos developed. Similar results were also observed in *Feijoa sellowiana,* and those phenol-storing cells were hypothesized to form a barrier between the somatic embryos and the mother tissue during induction and development of somatic embryos (Reis *et al.*, 2008). Blockage of symplastic transport is also thought to be a main force leading to morphological alterations (Pfluger and Zambryski, 2001) and it is possible that for the development of somatic embryos symplastic isolation is also required. Such isolation can occur physically through the thickening of the cell wall and closing of the plasmodesmata by deposition of callose (Dubois *et al.*, 1990; Verdeil *et al.*, 2001), as well as deposition of phenolic and lipophilic substances on cell walls (Pedroso *et al.*, 1995), or through the development of barrier cells (i.e., phenol-storing cells) between somatic embryos and mother tissue (Reis *et al.*, 2008), as observed in the present study. As these phenol-storing cells are metabolically very active, with the presence of numerous mitochondria and Golgi apparati, their specific roles during the development of somatic embryos remains to be elucidated.

In the present study, somatic embryos were transferred to maturation conditions prior to conversion. These steps can still be considered as a bottleneck, as some mature somatic embryos were fused or no mature somatic embryos were observed from those cultures induced only on solid culture medium. Additionally, a relatively low (around 30 %) conversion rate was observed and the regenerated plantlets had deficient root development *in vitro*. Previous studies of acclimatization of peach palm plantlets suggested that the *in vitro*-

grown roots were not functional (Arias, 1985) and they were removed, allowing new roots to develop during the acclimatization step (Steinmacher *et al.*, 2007a). Plantlets produced in TIS had lower survival rates than those from solid culture medium; however, TIS-grown plantlets showed enhanced growth and higher rooting rate, suggesting physiologically better plantlets. Among the other vantages of TIS as previously described, the ventilation of the culture containers may also result in plantlets more capable of growing in *ex vitro* conditions. In coconut, increased capacity of *in vitro*-grown plants to control water loss was related to the ventilation of the flasks (Talavera *et al.*, 2001). This suggests that TIS could also be an alternative technique for the growth of other palm species.

In conclusion, the occurrence of secondary somatic embryos during the induction of somatic embryogenesis in peach palm was confirmed. This morphological pathway allowed the development of a protocol suitable for in vitro multiplication of peach palm using a temporary immersion system and cycling cultures were established. Although zygotic embryos were used as explants, somatic embryogenesis was already obtained from inflorescences and shoot meristems (Steinmacher *et al.*, 2007bc), making this protocol useable for selected genotypes. In fact, a pilot project is already underway for the mass propagation of selected peach palm genotypes from Hawaii/USA. The maturation and conversion conditions in this protocol must also be improved, possibly using storage proteins as a quality marker. The use of a temporary immersion system may also be an interesting strategy for the scaling-up of other palm trees.

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Chapter III:

ARABINOGALACTAN PROTEINS AND CHARACTERIZATION OF THE EXTRACELLULAR MATRIX SURFACE NETWORK DURING PEACH PALM SOMATIC EMBRYOGENESIS

1 Abstract

No studies evaluating arabinogalactan proteins (AGPs) during palm embryogenesis have been carried out. In the present study the effect of Yariv reagent in the liquid culture medium was evaluated, and the localization of specific AGPs and pectin during induction and development of peach palm somatic embryos was demonstrated. The occurrence of an extracellular matrix network covering globular somatic embryos is described. Secondary somatic embryogenesis was induced in the presence of Yariv reagent (ßGlcY). Somatic embryos were collected at different developmental stages or cultivated in the presence of ßGlcY and examined under light, transmission (TEM) and scanning electron microscopy (SEM). Immunodetection using different monoclonal antibodies (MAbs) against different AGPs (Jim13, Jim8, LM2 and Jim14) and pectin epitopes was accomplished. Somatic embryos and callus development rate were significantly affected by the presence of 30 µM Yariv reagent but no effect was observed on fresh weight increment. Histological analyses from control cultures reveal a well-delimited protoderm and signs of polarization in the somatic embryos. In the presence of Yariv reagent somatic embryos presented loose cells in the protoderm and no signs of polarization could be observed. SEM analyses also confirmed protodermis mal-formation. AGPs were secreted into the culture medium and lower amounts were detected from callus culture or in the presence of 30 µM Yariv reagent. Analyses of specific MAbs against different AGPs epitopes revealed a specific pattern of distribution for each epitope. MAb Jim13 had differential expression and showed intense signal on the embryogenic sector and some immediately adjacent layers. MAb Jim7 (against pectin) recognized cell wall of all cells and a specific layer over the developing somatic embryo, as well as over the shoot meristem region of mature somatic embryos. This corresponds to an extracellular matrix surface network (ECMSN) associated with the development of somatic embryos and closely related to the expression of MAb Jim13. Scanning electron microscopy confirmed the presence of an ECMSN covering a specific group of cells and TEM analyses revealed that the ECMSN had a fibrillar and granular structure with lipophilic substances. These findings support the idea that AGPs are essential for the onset of somatic embryo induction and suggest that MAb Jim13 could be a reliable marker for peach palm somatic embryogenesis competence, as well as somatic embryos quality. Its role in ECMSN formation is discussed.

Key words: Bactris gasipaes, pectin, ultrastructure, tissue culture, liquid culture medium.

2 Introduction

Peach palm (*Bactris gasipaes* Kunth) is a caespitose palm widely distributed in the lowland humid Neotropics. This species has numerous uses, but fruit and heart-of-palm production are most important (Mora-Urpí *et al.*, 1997). Decades of research and development have yielded good results for heart-of-palm, and gave rise to numerous large germplasm collections in Latin American institutions (Clement *et al.*, 2004). This conservation effort is based on living collections in field germplasm banks, because seed banks are not possible because the species has recalcitrant seeds (Bovi *et al.*, 2004). Therefore, *in vitro* conservation is suggested as the most promising and cost effective technique for a conservation program, with clonal propagation used to backup the field genebanks (Mora-Urpí *et al.*, 1997).

An improved culture procedure recently developed for peach palm somatic embryogenesis uses liquid culture medium in a temporary immersion system (TIS) (Chapter II). One advantage of liquid culture medium compared to solid culture media is the absence of nutrient gradients, as well as the fact that substances secreted into the culture medium with putative signaling functions are able to reach other explants. The most important secreted substances in this regard are arabinogalactan proteins (AGPs).

AGPs are an umbrella term for a large class of proteoglycans widely distributed throughout the plant kingdom (Nothnagel, 1997; Seifert and Roberts, 2007). At the subcellular level these compounds are associated with secretory pathways, plasma membranes and cell walls, as well as being present in the culture medium (Showalter, 2001; Šamaj *et al*., 2000). Classical AGPs contain a domain responsible for attaching the protein backbone to a glycosylphosphotidylinositol (GPI) membrane anchor. Other classes of AGPs include lysin-rich AGPs, AG peptides, FLA (fascin-like AGPs) and chimeric AGPs. A chimeric AGP and a non-specific lipid transfer protein (nsLTP) Xylogen, with a cell-cell signaling role during xylem differentiation, have recently been described (Motose *et al.*, 2004). This chimeric AGP was recognized by the monoclonal antibody (MAb) Jim13 and was shown to play a fundamental role in xylem differentiation. Lipid transfer proteins are defined by their ability to facilitate transfer of phospholipids between membranes *in vitro* and are secreted and located in the cell wall; they have possible roles during programmed cell death, as well as in cutin formation (Kader, 1997; Eklund *et al.*, 2003).

Early in the development of somatic embryos *in vitro* polarization is established (Šamaj *et al.*, 2006). Both the cytoskeleton and cell walls appear to play an essential

regulatory role during this process (Šamaj *et al.*, 2006). It has been shown that AGPs can indirectly interact with the microtubule and actin in the cells (Sardar *et al.*, 2006; Driouchi *et al.*, 2008), playing a role during polarized growth. AGPs are also interlinked with pectins (Immerzeel *et al.*, 2006). Pectins are mostly localized in the middle lamella and primary cell wall and are, like AGP, branched molecules (Carpita *et al.*, 1993). These interactions of AGPs with pectin and microtubule support the hypothesis of a continuum between cytoskeleton, plasma membranes and cell walls (Kohorn, 2000; Baluska *et al.*, 2003). Therefore, a multifaceted biological function of AGPs is expected, although it is far from being completely understood. Their involvement has been described in distinct processes of cell differentiation, cell expansion and division, and AGPs have been proposed to be integral parts of the signaling cascade of plant growth regulators [i.e., Gibberellin (Suzuki, 2002; Mashiguchi *et al.* 2008), ABA (van Hengel *et al.*, 2004)]. Additionally, AGPs may act as molecular markers for specific cells [i.e., during reproductive development (Coimbra *et al.*, 2007)], as well as for morphogenetically competent cells (Šamaj *et al.*, 1999), with putative mechanical and physiological roles during the signaling cascade and plant cell polarization.

A more general role of AGPs as a cell-cell signaling molecule during the induction and development of somatic embryos has also been proposed. A specific set of AGPs from conditioned culture medium or from immature seeds could increase or even fully restore somatic embryo formation (McCabe *et al.*, 1997; van Hengel *et al.*, 2001). The improvement in morphogenesis was described in several species (Letarte *et al.*, 2006; Ergestdotter and von Arnold, 1995). Results pointed to a non-species-specific response, where conditioned culture medium from one species could increase the embryogenic response in other species (Kreuger and van Holst, 1993; Amar *et al.*, 2007). Furthermore it is known that a specific set of AGPs could have an inhibitory effect on somatic embryogenesis (Toonen *et al.*, 1997). The increase in somatic embryogenesis induction with the inclusion of AGPs re-isolated after an endochitinase treatment has also been described (van Hengel *et al.*, 2001).

Despite the fact that the biological functions of most AGPs are numerous and still uncertain (Chapman *et al.*, 2000; Seifert and Roberts, 2007), their characterization offers the opportunity of identifying regulatory mechanisms of somatic embryogenesis. It is known that AGPs epitopes are species-specific and are involved in different morphogenetic pathways (Seifert and Roberts, 2007). To the best of our knowledge there is no report regarding the role of AGPs during palm embryogenesis and secondary somatic embryo development.

Secondary somatic embryogenesis is associated with a few cells that escape from the coordinated development of the somatic embryo and initiate the development of a new

somatic embryo (Raemakers *et al.*, 1995; Williams and Maheswaran, 1985). In our system of peach palm *in vitro* regeneration, embryos develop from small aggregates of sub-epidermal and epidermal embryogenic cells resulting in the direct development of somatic embryos (i.e., without an intermediate callus phase). They originate from cytoplasmatic-rich, metabolically active cells, where rapid cell division associated with a drastic reduction in storage products (starch) takes place when somatic embryo development starts (Chapter II).

Somatic embryo development is frequently associated with the formation of an extracellular matrix surface network (ECMSN - also known as a supraembryogenic network) covering the (pre-)globular somatic embryos. It appears to be an evolutionarily conserved characteristic, described in gymnosperms (Šamaj *et al.*, 2008) and in angiosperms (Chapmann *et al.*, 2000ab; Bobák *et al.*, 2003), including palms (Verdeil *et al.*, 2001). The mechanisms controlling the formation of the ECMSN are largely unknown. However, it is thought that ECMSN is involved in integration and recognition of morphogenic cells within multicellular callus domains, as well as having a protective function (Popielarska-Konieczna *et al.*, 2008). The composition of ECMSN includes proteins, pectidic polysaccharides, lipophilic substances and AGPs (Popielarska-Konieczna *et al.*, 2008; Namasivayam *et al.*, 2006; Chapman *et al.*, 2000ab; Konieczny *et al.*, 2005).

The use of Yariv reagent (ßGlcY), a synthesized chemical antibody that specifically binds to and aggregates AGPs molecules causing loss-of-function, is a reliable method to study the role of AGPs during plant morphogenesis (Chapmann *et al.*, 2000). Currently, immunolocalization techniques are one of the best methods to identify and to precisely locate polymers *in situ* within complex tissues. These methods generally use monoclonal antibodies that were developed from complex cell-wall-derived materials (Knox, 2008).

In the present study the effect of ßGlcY during development of secondary somatic embryos of peach palm is described. Immunolocalization of different AGPs epitopes as well as pectins was used to describe their relationships to the development of secondary somatic embryos. In addition, the occurrence of an extracellular network rich in AGPs, lipophilic substances and pectin is presented.

3 Material and Methods

3.1 Plant material

Somatic embryogenesis was induced from three shoot meristems of a peach palm kept in the greenhouse at the University of Hamburg. The older leaf sheaths were removed and shoots with 2 leaf sheaths were treated with 1g L^{-1} streptomicyn sulphate for 1 hour. The shoots were transferred to a 70 % ethanol solution for 5 min, followed by 40 min in a 50 % solution of commercial bleach (4 - 5 % active chlorine) plus 3 drops Tween® 20 to each 100 ml of this solution. The treatment was followed by three washings of five min each with autoclaved distilled water. Thereafter, the excess of water was dried over a filter paper and two more leaf sheaths were removed. The remaining shoot tip, composed of leaf primordia and the shoot meristem, was sectioned into thin segments (approx. 1 mm thick) and transferred onto culture medium enriched with 300 µM Picloram as previously described (Steinmacher *et al.*, 2007). Primary somatic embryos were obtained, cycling secondary somatic embryos culture was established as previously described using a temporary immersion system (Chapter II) using approximately 100 mg explants, and clusters of somatic embryos from a well-established cycling culture were used in the present study.

3.2 Culture medium and conditions

About 100 mg of small clusters of somatic embryos were transferred into 250 ml Erlenmeyer flasks containing 100 ml culture medium. The culture medium was composed of MS salts (Murashige and Skoog, 1962) and Morel and Wetmore vitamins (Morel and Wetmore, 1951), plus 500 mg L^{-1} Glutamine and 10 µM Picloram. The cultures were maintained on a rotatory horizontal shaker at 100 rpm in darkness at 28 °C and cultivated for 4 weeks. Different concentrations (0, 5 and 30 µM) of ß-glucosyl Yariv-reagent, a synthetic phenylglycoside that specifically binds AGPs, was synthesized from phloroglucinol and p-aminophenyl-β-glycopyranoside according to Basile *et al.* (1989), and added to the culture medium. As the negative control, 30 µM of α-glucosyl (αGlcY) was added to the medium. This was synthesized using the same procedure but from aminophenyl-α-glycopyranoside as the precursor (Basile *et al.*, 1989). To test the effect of ßGlcY during the development of somatic embryos on solid culture medium, the same culture medium composition was gelled with 2.5 g L^{-1} gelrite. Callus culture was established using non-embryogenic callus as previously described (Steinmacher *et al.*, 2007). Maturation of somatic embryos was carried out on solid (2.5 g L^{-1} gelrite) MS culture medium, plus Morel and Wetmore vitamins and

1 g L^{-1} glutamine. 10 µM abscisic acid was filter-sterilized through 0.2 µm PET-membrane filter (Carl Roth GmbH) and added to the autoclaved culture medium. The cultures were kept in the dark at 28 °C and collected for the present study one month later. All the culture media had their pH adjusted to 5.8 and were autoclaved for 20 min.

3.3 Histological procedure and immunolocalization

Small clusters of somatic embryos were stained with a 1 mg ml^{-1} Yariv solution overnight at 4°C. The samples were examined and photographed under a stereoscope coupled to a Canon Powershot.

For fluorescence microscopy, samples were fixed with 2 % (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 24 hours at 4°C, dehydrated in an ethanol series (30 % to 100 %) twice with 15 min each step and embedded in LR White resin (London Resin Co. Ltd., London). Gelatin capsules filled with resin and the sample were allowed to polymerize at 65°C overnight. Specimens were sectioned in 1 µm thick sections with a glass-knife in a semi-automated microtome (Reichert Ultracut S, Leica®) and mounted onto glass slides with a drop of water, then fixed over a hot plate (approximately 70°C). For calcofluor staining, a solution containing 100 µg ml^{-1} was applied over the samples for 30 s and washed three times with water to remove the excess. For staining lipidic substances, Nile red was synthesized by boiling Nile blue in sulphuric acid and extracted with xylene, and observed under red fluorescence (excitation 515-560 nm; emission, greater than 590 nm) as described by Greenspan *et al.* (1985).

For fluorescence immunolocalization, semi-thick sections were blocked with 5 % BSA in PBS for one hour, followed by incubation with primary monoclonal antibodies against several AGPs epitopes (Jim13, Jim8, LM2 and Jim14) and low- or high-methylesterified pectin (Jim5 and Jim7) at 1:5 for one hour at room temperature; some samples were also incubated overnight at 4°C. Thereafter, sections were washed with 1 % BSA in PBS three times for 5 min each followed by an incubation for 2 hours with secondary antibody FITC-conjugated at 1:100 (rat Ig Sigma) in 1 % BSA in PBS. Finally, all the sections were washed with PBS three times for 5 min each. After removing the excess, the samples were mounted with antifading Citifluor (Citifluor Ltda, London) and visualized under UV excitation for calcofluor and with FITC exciter filter (BP 450-490 nm). All the sections were examined under an Olympus BH-2 microscope and photographed with a

ColorView IIIu (Soft Imaging System, GmbH). Negative control samples were obtained omitting the primary antibody.

For scanning electron microscopy, the samples were fixed with 3 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 24 hours and dehydrated in ethanol (30 % – 100 %) twice in each step for 15 min each. The samples were critical point dried with liquid CO_2 in a CPD 030 critical point dryer (Bal-TEC, Leica®), affixed to aluminum stubs and coated with gold palladium in a SCD 050 Sputter Coater (Bal-TEC, Leica®). The mounted specimens were examined with a Philips XL 20 scanning electron microscope.

For transmission electron microscopy, samples were fixed in 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for two hours at room temperature. Thereafter the samples were transferred to fresh fixative solution and maintained overnight at 4°C. The samples were then rinsed in the same buffer without glutaraldehyde with three changes (15 min each) and postfixed in buffered 1 % OsO_4 solution at 4°C for 2 hours. After rinsing in cacodylate buffer, the samples were dehydrated in a graded acetone series and embedded in Spurr's resin (Pelco®, Redding USA). Ultrathin sections (80 - 100 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), collected over a Formvar coated copper-grid and stained with 1 % uranyl acetate and 0.1 % lead citrate for 10 min each.

Samples for immunogold labeling were fixed overnight in MSB buffer (0.1 M PIPES, 10 mM EGTA, 10 mM $MgSO_4$, 0,1 % Triton X-100) plus 2 % paraformaldehyde and 0,1 % Glutardialdehyde at 4°C. After washing two times in MSB buffer, the samples were postfixed in 0.5 % OsO_4 for 1.5 hours, dehydrated in an ethanol series and embedded in LR White resin in gelatin capsules as previously described. Sections (80–100 nm) were collected on Formvar coated nickel grids. Each grid was incubated in 1 % BSA and 0.1 % BSA-fraction C in PBS for 20 min, washed and incubated in monoclonal antibodies at 1:20 for 1 h at room temperature. Grids were washed and incubated in 10 nm gold conjugated secondary antibody 1:50 in 1 % BSA and 0.1 % BSA-fraction C in PBS, after which they were washed again in PBS buffer and an additional step of 30 s in 0.5 % glutaraldehyde was applied. Thereafter the sections were stained with uranyl acetate and lead acetate. All sections were viewed with a LEO 906 E transmission electron microscope (LEO, Oberkochen, Germany) equipped with a Gatan MultiScan CCD Camera (Munich, Germany). Images were acquired using the Digital Micrograph 3.3 software.

3.4 Isolation of AGPs from culture media and quantification by radial gel diffusion

Culture media were filtered through a millipore filter (0.4 µm) and, when necessary, sodium dithionite was added. The culture medium was incubated in a water bath at 50°C until the red color disappeared. All the culture media were dialyzed with distilled water for 72 hours at 4°C. The culture media were freeze-dried and AGPs were extracted as described by Helleboid *et al.* (1998). AGPs were quantified through single radial gel diffusion, performed as described by van Holst and Clarke (1985) using gum arabic as a standard.

3.5 Statistical procedure

A completely randomized design was used. Induction of secondary somatic embryogenesis on solid culture medium was carried out with three repetitions composed of three Petri dishes each of three repetitions composed of two Erlermeyer flasks for the induction of somatic embryos in liquid culture medium. The data were subjected to analysis of variance (ANOVA) and, when necessary, the SNK test was used to compare means using the program STATISTICA v.6 (StatSoft, Inc.). The variables evaluated were the percentage of callus, development and induction rate of secondary somatic embryos, as well as fresh weight increment. The induction percentage was separated into three embryogenic-capacity categories [low (< 5 somatic embryos), medium (5-15) and high (> 15)]

4 Results

4.1 Effect of ßGlcY during the development of secondary somatic embryos

Secondary somatic embryogenesis was induced in liquid culture (Figure 1) and the fresh weight increment of explants was not affected by the addition of ßGlcY (Figure 2a). However, ßGlcY affected somatic embryogenic and callus development rates. Development of secondary somatic embryos was observed on 71 % of explants in absence of ßGlcY, while only 33 % developed in the presence of 30 µM ßGlcY. In the positive control (30 µM αGlcY), 67 % explants formed secondary somatic embryos, which was not significantly different from the absence of ßGlcY (Figure 2b). Callus development had the opposite behavior, with increasing callus development rate in the presence of ßGlcY. In the control and positive control conditions, 28 % and 32 % of the explants developed into callus, respectively, while in the presence of 30 µM ßGlcY 66 % of explants formed callus (Figure 2c).

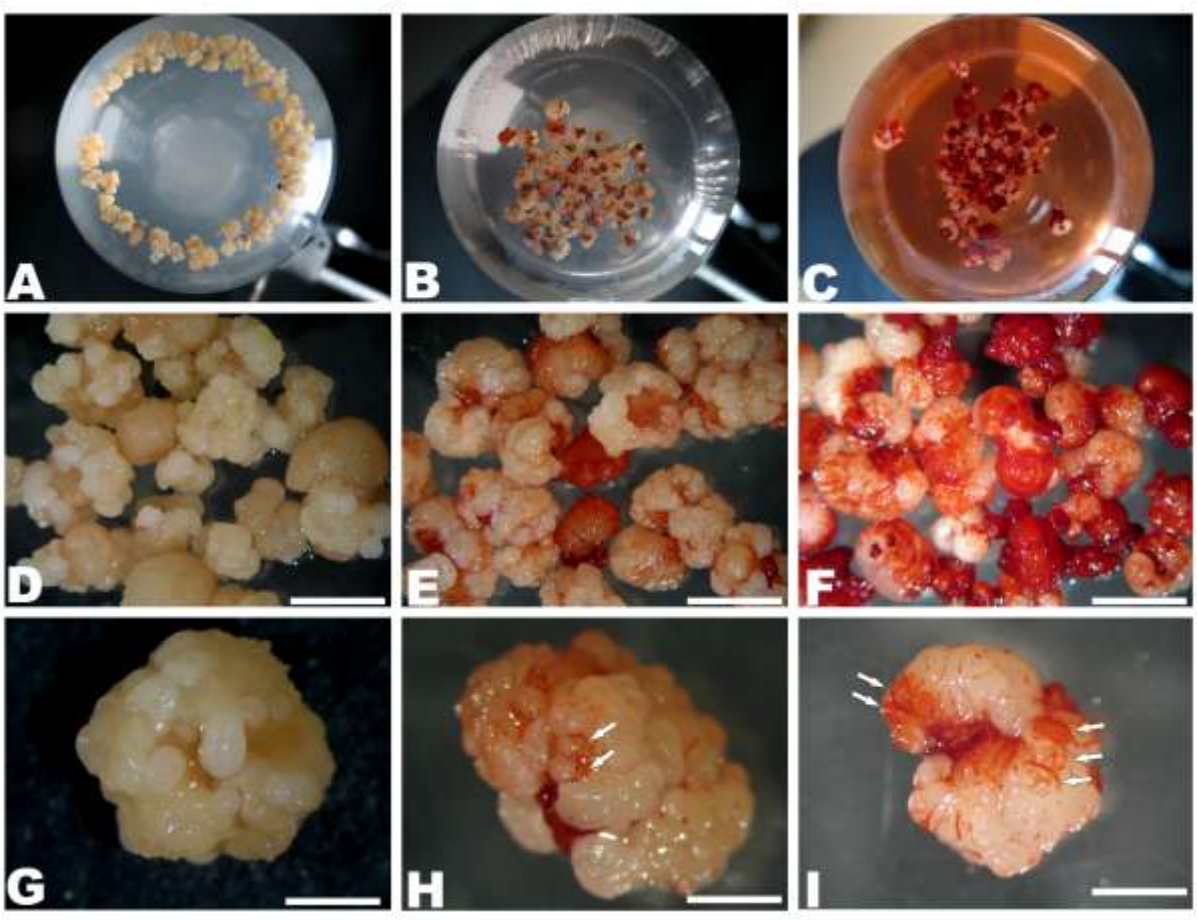

Figure 1 - Secondary somatic embryogenesis of peach palm induced in liquid culture medium. Aspects of the cultures grown in control conditions (A;D;G), in the presence of 5 µM ßGlcY (B; E; H) and in presence of 30 µM ßGlcY (C; F; I). (bars in D-F = 5 mm; G-I = 2 mm).

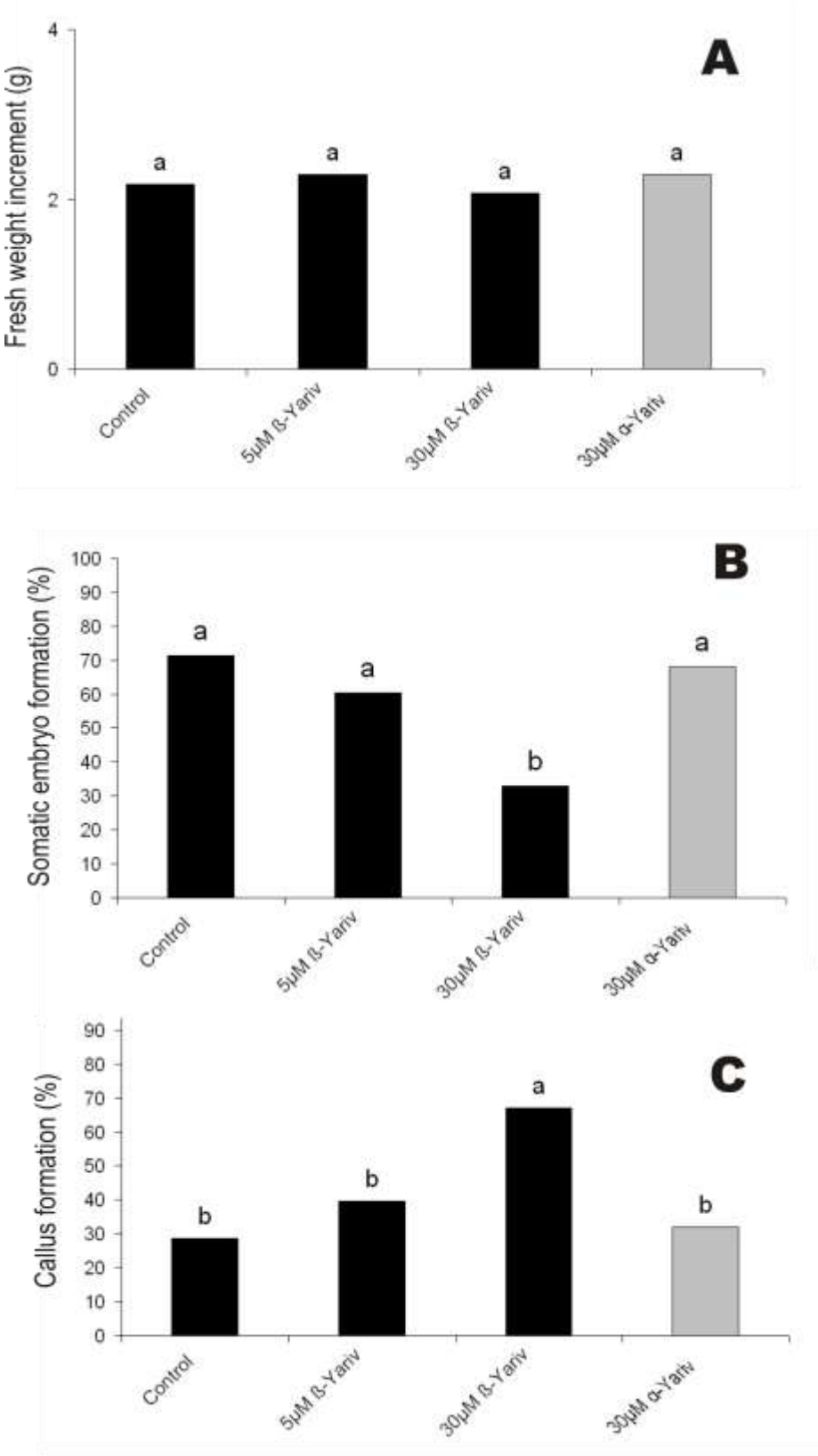

Figure 2 - Fresh weight increment and somatic embryogenesis and callus formation rates in different concentrations of ßGlcY and αGlcY after 30 days of culture. A - Fresh weight increment. B – Percentage of somatic embryo formation. C – Percentage of callus development. Different letters represent statistical differences after SNK analysis ($P < 0.05$).

Transfer of the embryogenic cultures onto solid culture medium with or without ßGlcY resulted in somatic embryo development under all conditions tested (Figure 3-4; Table 1). However, somatic embryos did not develop on those areas in contact with the culture medium in the presence of ßGlcY (Figure 5).

Table 1 - Formation of secondary somatic embryos on solid culture medium with different concentrations of ßGlcY after 30 days culture in liquid culture medium with different concentrations of ßGlcY. Values represent means ± standard error.

Liquid	Solid	Callus (%)	Somatic embryogenesis (%)			
			Total	*Low*	*Medium*	*High*
0 µM ß-Yariv	0 µM	7.4±3.7	92.6±3.7	0.0±0.0	7.4±3.7	85.2±3.7
	5 µM	7.4±3.7	92.6±3.7	11.1±6.4	11.1±0.0	70.4±7.4
	30 µM	22.2±11.1	77.8±11.1	0.0±0.0	11.1±6.4	66.7±17.0
5 µM ß-Yariv	0 µM	0.0±0.0	100.0±0.0	0.0±0.0	3.7±3.7	96.3±3.7
	5 µM	14.8±14.8	85.2±14.8	0.0±0.0	7.4±3.7	77.8±17.0
	30 µM	11.1±6.4	88.9±6.4	0.0±0.0	3.7±3.7	85.2±9.8
30 µM ß-Yariv	0 µM	33.3±11.1	66.7±11.1	0.0±0.0	0.0±0.0	66.7±11.1
	5 µM	11.1±11.1	88.9±11.1	0.0±0.0	0.0±0.0	88.9±11.1
	30 µM	0.0±0.0	100.0±0.0	0.0±0.0	0.0±0.0	100.0±0.0
Mean		11.9±3.1	88.1±3.1	1.2±0.9	4.9±1.2	81.9±3.7

4.2 Localization of AGPs through ßGlcY and its effect on somatic embryo morphology

During the development of secondary somatic embryos a callus sector and an embryogenic sector were observed (Figure 6A). The embryogenic sector was characterized by the continual development of secondary somatic embryos (Figure 6B). Two specific staining patterns could be observed after 30 days of culture in the presence of ßGlcY: the whole callus sector stained intensively (Figure 6C), while the characteristic ßGlcY stain was observed on embryogenic sectors only in the regions where secondary somatic embryos would develop (Figure 6D).

During the initial development of somatic embryos, polarized localization of AGPs was observed on pre-globular somatic embryos (Figure 6E), as well as intense staining over globular somatic embryos grown in the presence of ßGlcY (Figure 6F). Similar results were observed on clusters of somatic embryos stained overnight with ßGlcY solution, with an intense red color in specific sectors of the explant, as well as on the protoderm of the globular somatic embryos (Figure 7).

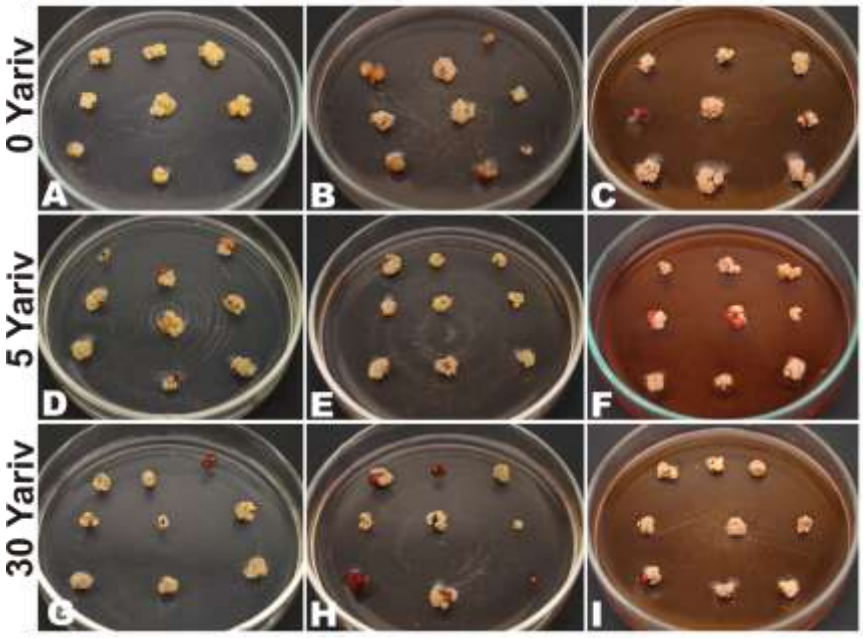

Figure 3 - Development of secondary somatic embryos on solid culture medium in the presence of different concentrations of ßGlcY [(A-D-G) control conditions; (B-E-H) 5 µM ßGlcY; (C-F-I) 30 µM ßGlcY)]. A-C – Somatic embryos from control conditions (absence of ßGlcY); D-F – Cultures cultivated in liquid culture medium containing 5 µM ßGlcY before transferring to solid culture medium. G-I – Cultures cultivated in liquid culture medium containing 30 µM ßGlcY before transferring to solid culture medium.

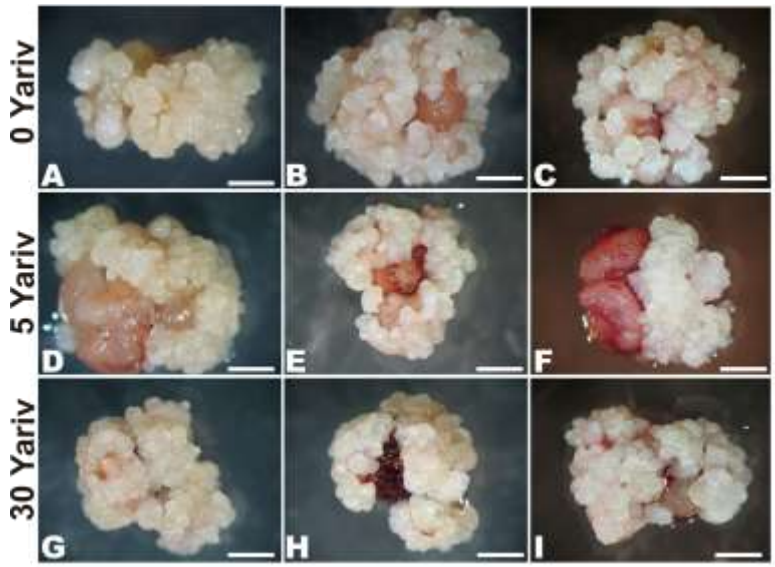

Figure 4 - Detailed view of the development of secondary somatic embryos on solid culture medium in the presence of different concentrations of ßGlcY. A-C – Somatic embryos from control conditions (absence of ßGlcY); D-F – Cultures cultivated in liquid culture medium containing 5 µM ßGlcY before transferring to solid culture medium. G-I – Cultures cultivated in liquid culture medium containing 30 µM ßGlcY before transferring to solid culture medium. All bars = 3 mm.

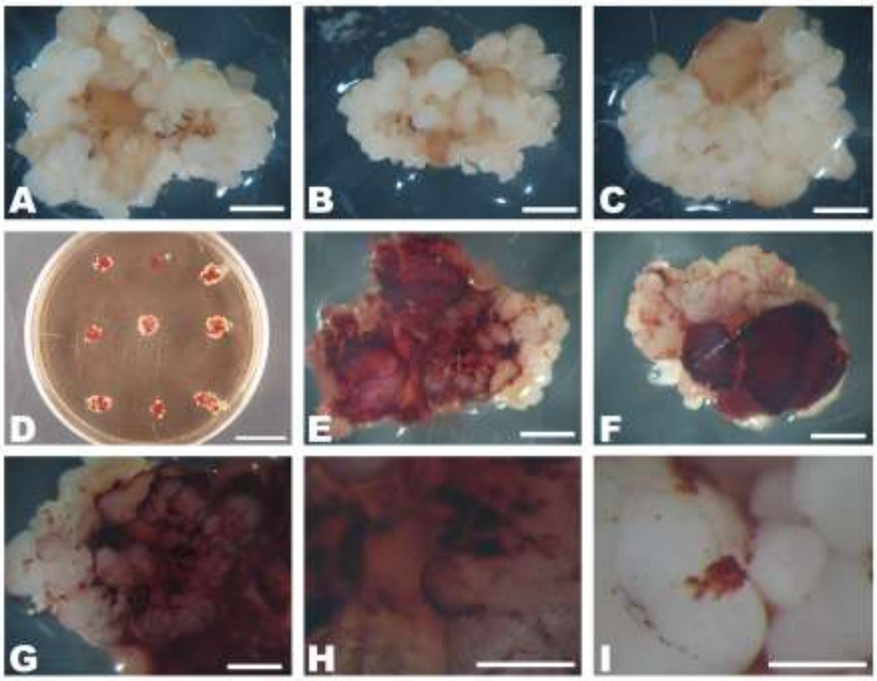

Figure 5 - Detailed view of the development of secondary somatic embryos on solid culture medium in the region of the explant in contact with the culture medium in the presence of different concentrations of ßGlcY. A-C – Somatic embryos from control conditions (absence of ßGlcY) (bars = 3 mm); D – General view of the cultures on solid culture medium containing 30 µM ßGlcY (bar = 2 cm). E-G – View of the cultures on solid culture medium containing 30 µM ßGlcY (bars = 3 mm). H-I – Detailed view of the cultures on solid culture medium containing 30 µM ßGlcY (bars = 2 mm).

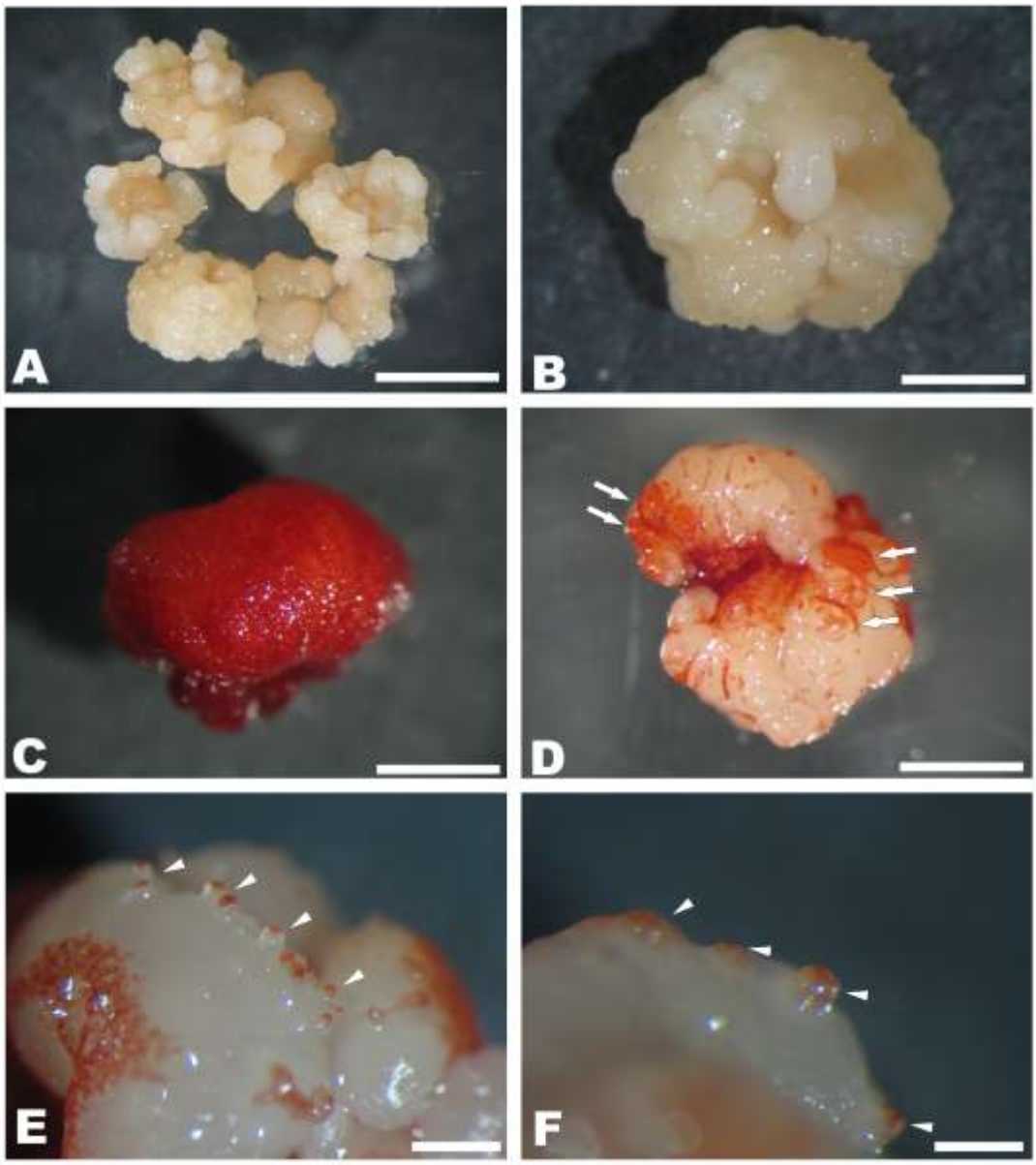

Figure 6 - Localization of AGPs on peach palm embryogenic clusters with ßGlcY. A – View of embryogenic cultures (bar = 4 mm). B – Embryogenic cluster revealing secondary somatic embryos (bar = 2 mm). C - Staining pattern in the callus (bar = 2 mm). D – Staining of embryogenic sectors showing intense reaction where secondary somatic embryos would develop (arrow) (bar = 2 mm). E – Staining pattern during the initial development of somatic embryos showing polarized localization of AGPs (arrowhead) (bar = 2 mm). F - Intense staining over globular somatic embryos (arrowhead) (bar = 1 mm).

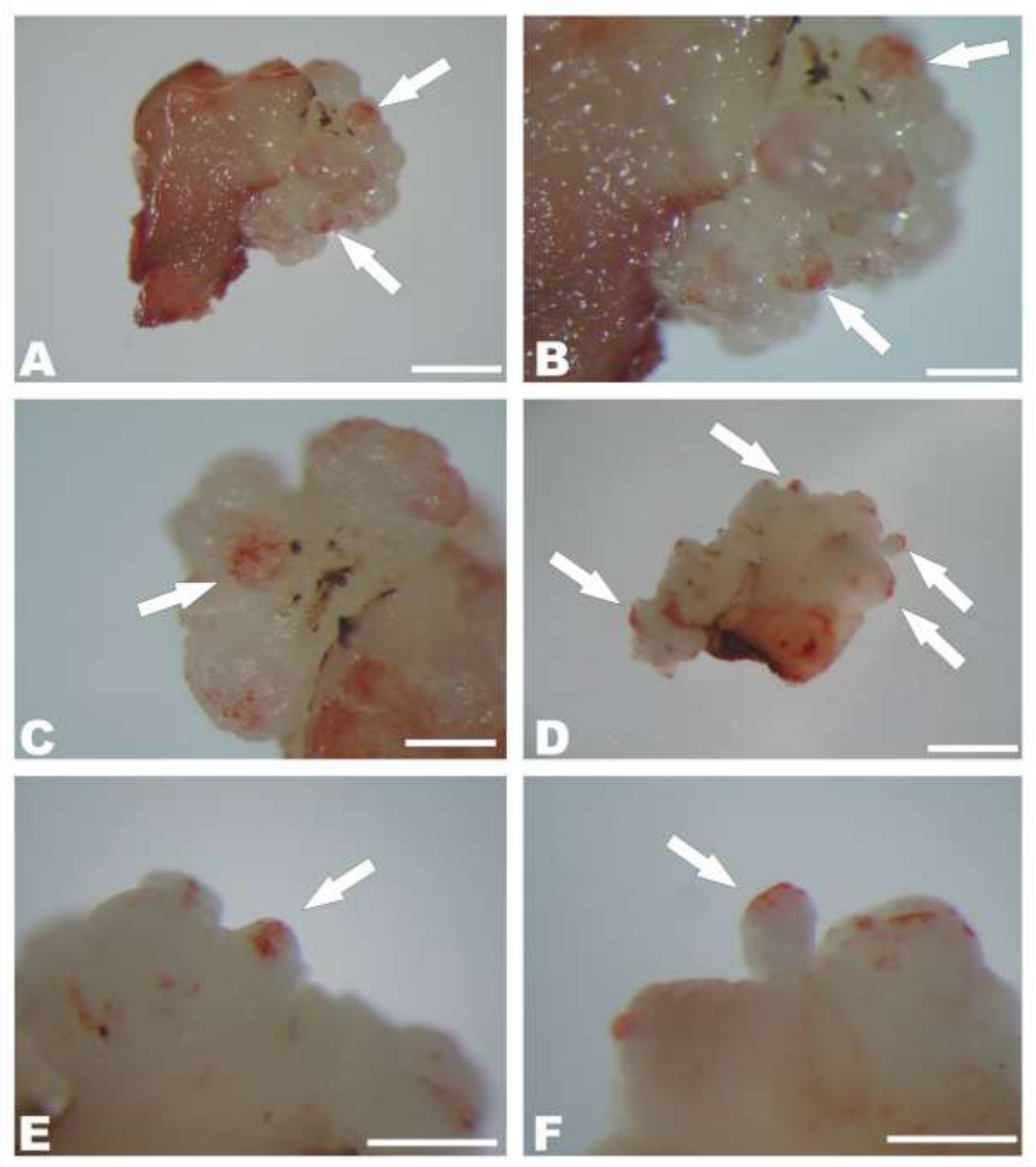

Figure 7 - Clusters of somatic embryos stained overnight with ßGlcY solution. A - F – An intense red color in specific sectors of the explant as well as on the protoderm of the globular somatic embryos was observed (arrows). (bars A and D = 5 mm; others = 2 mm).

Continuous development of secondary somatic embryos, forming clusters of somatic embryos, was also observed in scanning electron micrographs (Figure 8A). Somatic embryos at the globular stage presented well-delimited protoderm (Figure 8B). The explants with callus growth also possessed sectors of protoderm-like cells (Figure 8C-D). In the presence of ßGlcY, loose cells that grew into undelimited protoderm were observed (Figure 8E-F).

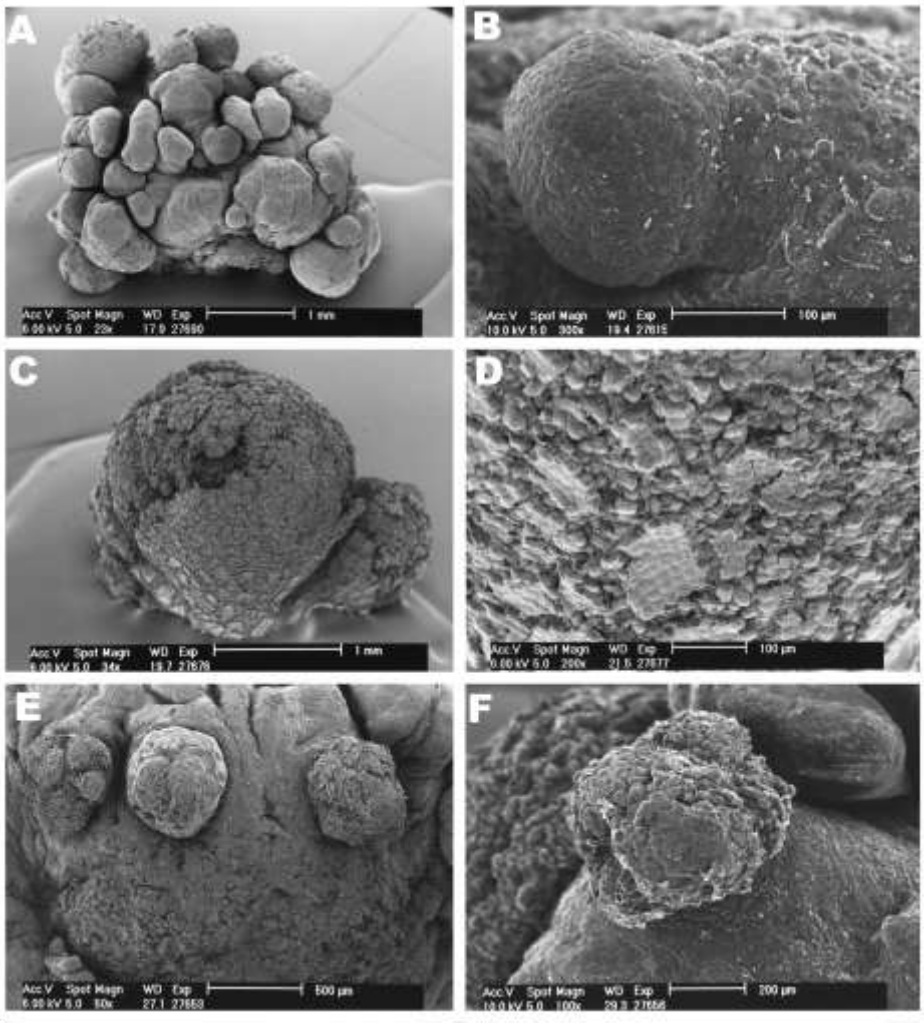

Figure 8 - Scanning electron microscopy during the development of peach palm secondary somatic embryos. A – Clusters of somatic embryos (bar = 1 mm). B – Globular somatic embryo with well-delimited protoderm (bar = 100 µm). C-D – Development of callus revealing sectors of protoderm-like cells (bar = 1 mm and 100 µm). E-F – Development of secondary somatic embryos in the presence of ßGlcY revealing loose cells that grew into undelimited protoderm (bar = 500 µm and 200 µm).

Resin embedded samples corroborate these observations, revealing that somatic embryos formed in control conditions had a single cell layer, very well delimited protoderm and some signs of polarization (Figure 9A), with elongated cells present already in globular somatic embryos (Figure 9B). In contrast, somatic embryos formed in the presence of ßGlcY had profound alterations, especially in the protoderm (Figure 9C), which had no delimited pattern and presented loose cells (Figure 9D). No sign of polarization was observed in cells in round somatic embryos with non-organized growth (Figure 9E). When combined with the localized secretion of AGPs, these observations reinforce its importance in the initial development of secondary somatic embryos, as well as for protoderm differentiation.

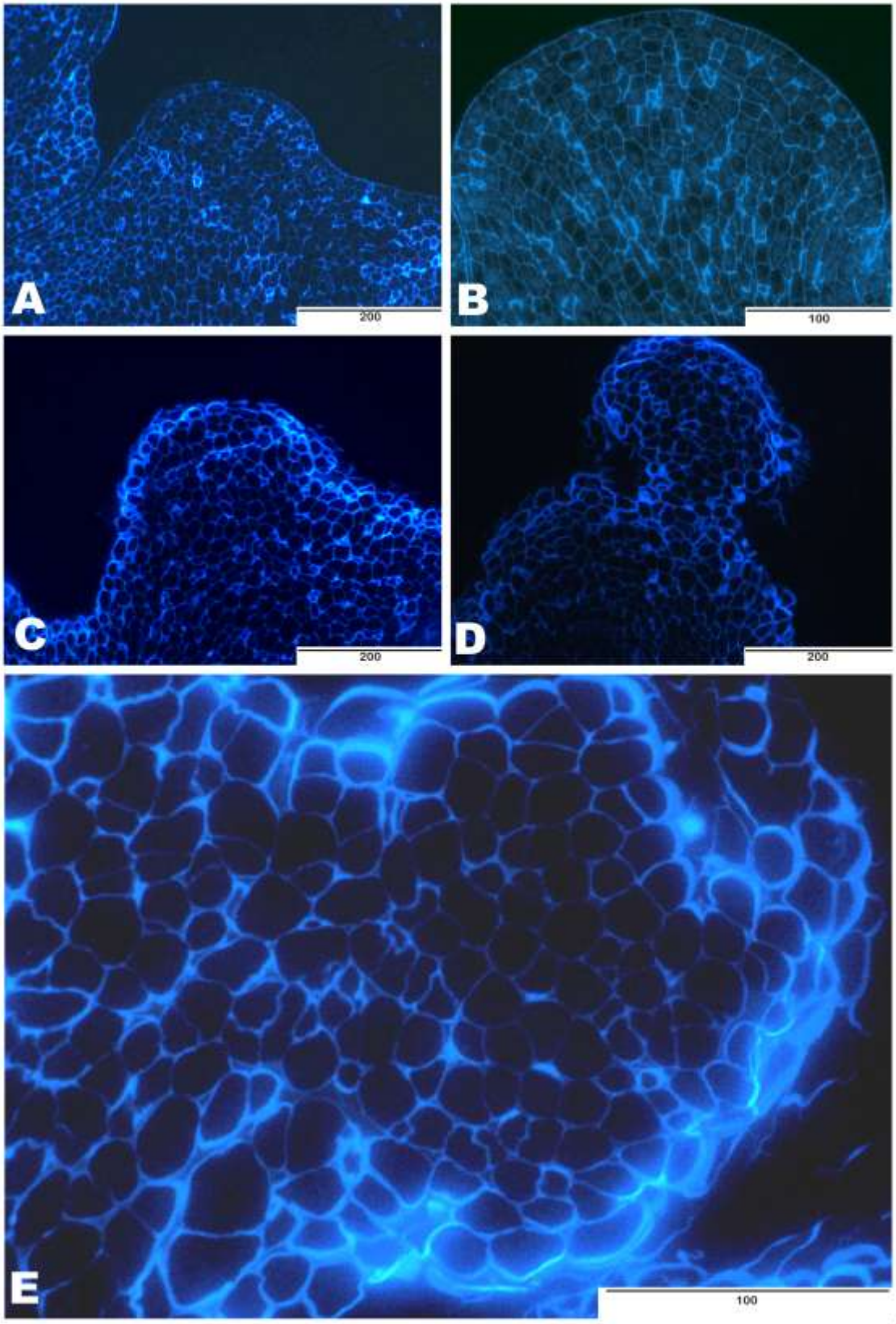

Figure 9 - Histological analyses with calcofluor staining during the development of secondary somatic embryos. A-B – Initial development of secondary somatic embryos in control conditions with a well-delimited protoderm and some signs of polarization (bar A = 200 µm; B = 100 µm). C-E - Somatic embryos formed in the presence of ßGlcY (bars C and D = 200 µm; E = 100 µm).

4.3 Quantification of AGPs secreted in the culture medium

The presence of ßGlcY drastically reduced the amount of AGPs secreted into the culture medium quantified by radial gel diffusion (Figure 10). In control culture conditions, absence of ßGlcY or presence of 30 µM αGlcY resulted, respectively, in 404 µg and 349 µg AGPs L^{-1} culture medium. In the presence of 30 µM ßGlcY, 102 µg AGPs L^{-1} culture medium was recovered, similar to the amount observed from callus culture (160 µg AGPs L^{-1}) (Table 2). In the presence of ßGlcY, a slightly greater amount of protein was obtained with Bradford´s method. Normalizing the amount of AGPs to the amount of secreted proteins showed a drastic reduction in the ratio between AGPs and proteins due the presence of ßGlcY (Table 3). Callus culture released lower amounts of proteins and a slightly higher difference was found between callus culture (88 µg AGPs mg^{-1} Protein) and the presence of 30 µM ßGlcY (21 µg AGPs mg^{-1} Protein) (Table 3). Dot-blot analysis also identified the presence of different AGPs epitopes (specifically MAb Jim13 and LM2) secreted into the culture medium (Figure 11).

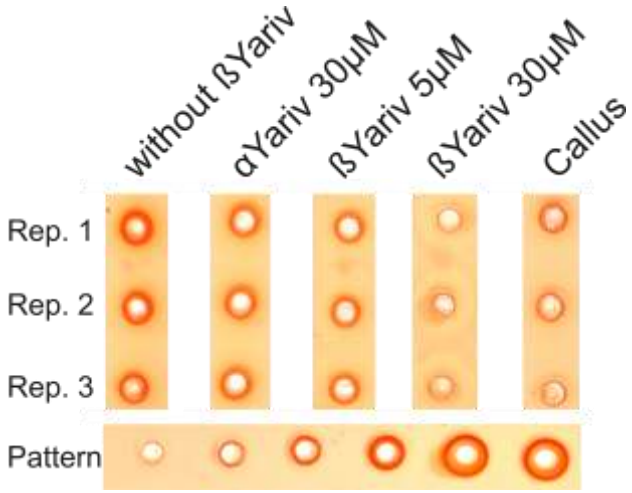

Figure 10 - Radial gel diffusion quantification of AGPs secreted into the culture medium with different concentrations of ßGlcY. Rep = repetition.

Table 2 - Quantification of AGPs and total proteins secreted into the culture medium in different culture conditions. Control cultures represent the formation of secondary somatic embryos.

	mg Protein·L^{-1} CultMedium	µg AGPs·L^{-1} CultMedium	µg AGPs·mg^{-1} Protein
Callus	2.73±1.1	160.11±23.9	58.65±35.1
Control	3.74±0.3	404.69±56.3	107.92±11.9
ßGlcY 5µM	4.62±0.1	389.74±51.5	83.92±10.1
ßGlcY 30µM	4.87±0.1	102.57±0.0	21.04±0.13
αGlcY 30µM	4.73±0.1	349.28±60.8	73.61±12.6

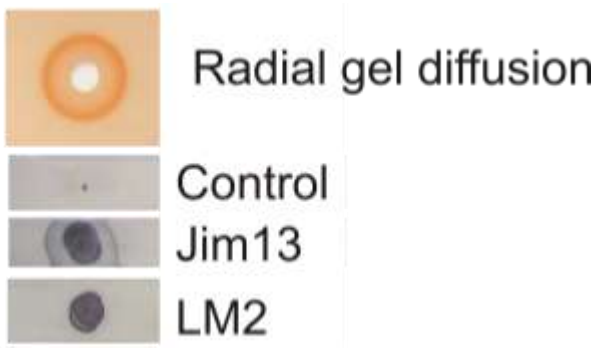

Figure 11 - Dot-blot analysis showing different AGPs epitopes (MAb Jim13 and LM2) secreted into the culture medium.

4.4 Immunolocalization of specific AGPs and pectin epitopes during the development of somatic embryos

Analyses of specific monoclonal antibodies (MAb) against different AGPs epitopes (i.e., Jim13, Jim8, LM2 and Jim14) during the development of somatic embryos revealed a specific pattern of distribution for each epitope. MAb Jim13 showed intense signal on the embryogenic sector and some immediately adjacent layers (Figure 12A); during the initial development of somatic embryos a polarized localization of MAb Jim13 was observed (Figure 12B-C). In somatic embryos transferred to solid culture medium MAb Jim13 reacted with protoderm cells and adjacent layer (Figure 12D-E). MAb Jim8 recognized the same cells but with lower intensity and was confined to the protoderm layer (Figure 12F). Arrested somatic embryos showed no reaction to either antibody (asterisk in Figure 12A). On the other hand, MAb Jim14 showed no tissue specificity, but presenting a punctuate aspect in the cell wall (Figure 13A). MAb LM2 presented no tissue specificity in somatic embryos (Figure 13B), although a strong signal was found in the endomembranes of the cells of the somatic embryos (Figure 13C). In mature somatic embryos (Figure 14A), MAb LM2 presented the same pattern as in developing somatic embryos (Figure 14B), while MAb Jim13 presented strong signal intensity in the meristem region, especially the protoderm layer and immediately adjacent cells (Figure 14C-D). MAb Jim8 was again found in the same sector, although limited to a few cells only (Figure 14E). This shows that MAb Jim13 and Jim8 have different expressions during the development of somatic embryos. Epitopes recognized by MAb Jim13 showed a stronger signal during the development of somatic embryos and were further analyzed by immunogold localization. On the callus sector, MAb Jim13 and Jim8 also recognized the protoderm-like layer, however the signal was mostly concentrated on the cell membrane (Figure 15).

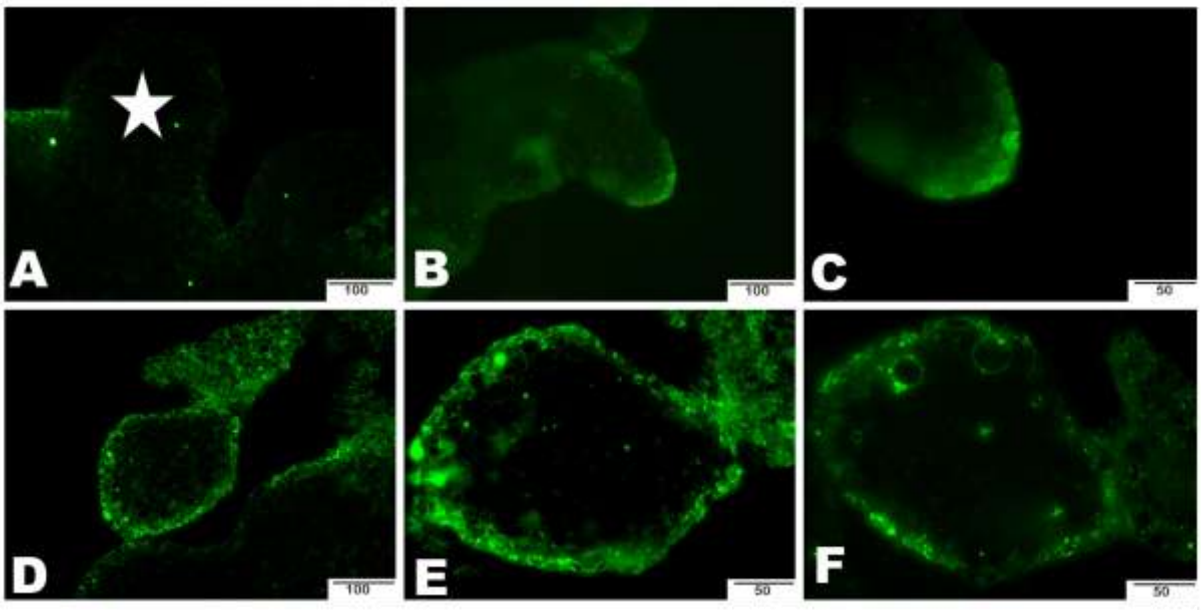

Figure 12 - Fluorescence immunolocalization of monoclonal antibodies (MAb) Jim13 and Jim8 against AGPs during the development of somatic embryos. A – MAb Jim13 showing an intense signal on the embryogenic sector and some immediately adjacent layers. Asterisk corresponds to arrested somatic embryo (bar = 100 µm). B-C- Polarized localization of MAb Jim13 during the initial development of somatic embryos (bars B = 100 µm; C = 50 µm). D-E - MAb Jim13 recognized cells of the protoderm and adjacent layer (bars D = 100 µm; E = 50 µm). F – Immunolocalization of MAb Jim8 (bar = 50 µm).

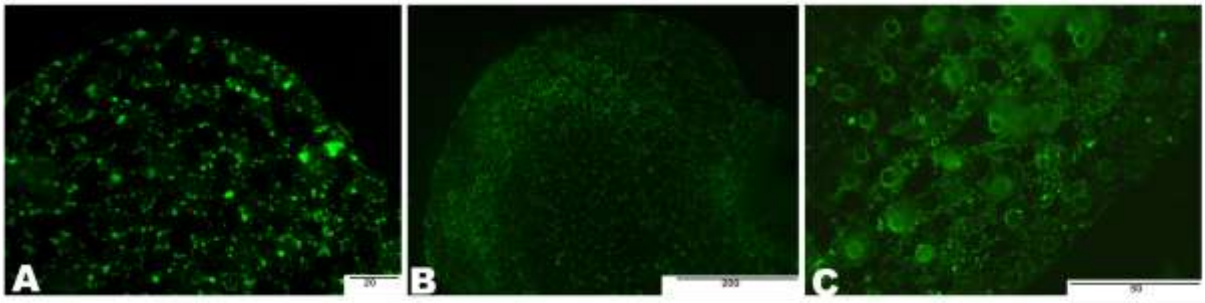

Figure 13 - Fluorescence immunolocalization of monoclonal antibodies (MAb) Jim14 and LM2 against AGPs during the development of somatic embryos A – MAb Jim14 showing punctuate aspect in the cell wall (bar = 20 µm). B – Immunolocalization of MAb LM2 revealing no tissue specificity in somatic embryos (bar = 200 µm). C – Detailed view of the immunolocalization of MAb LM2 showing endomembranes (bar = 50 µm).

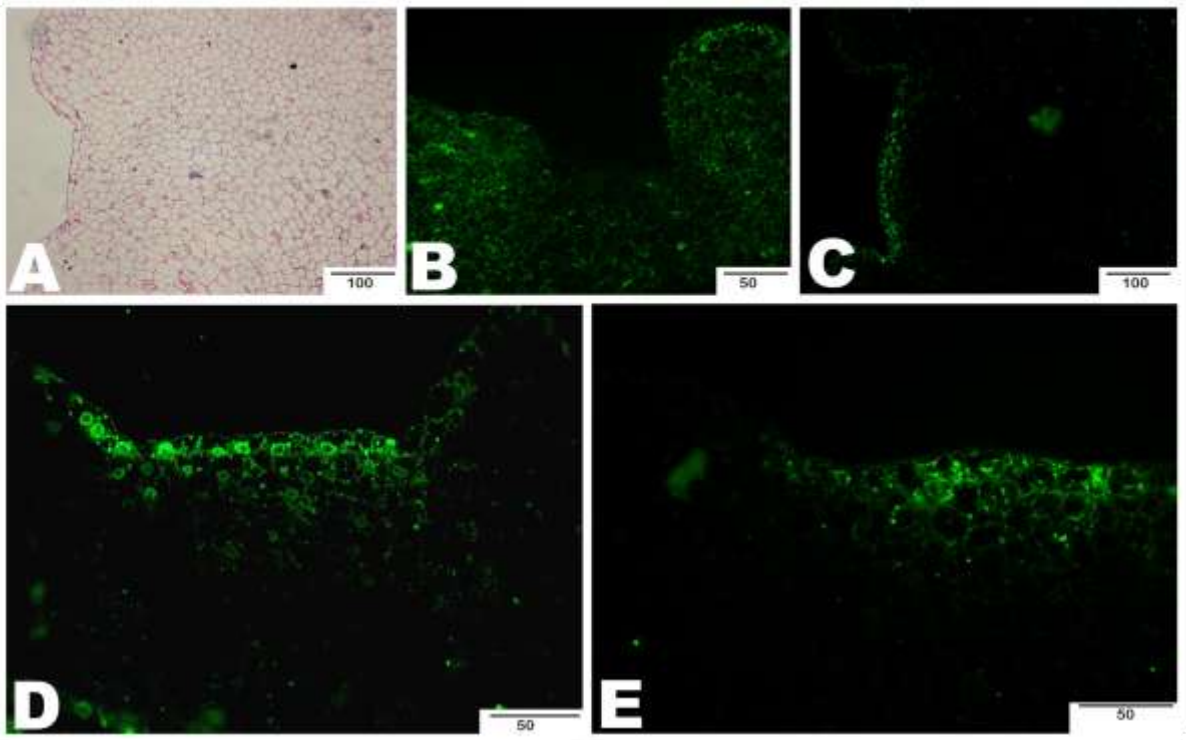

Figure 14 - Fluorescence immunolocalization of monoclonal antibodies (MAb) Jim13, Jim8 and LM2 against AGPs on mature somatic embryos. A – Morphological aspect of peach palm mature somatic embryo stained with PAS (bar = 100 µm). B - Immunolocalization of MAb LM2 revealing no tissue specificity in mature somatic embryos (bar = 50 µm). C-D – Immunolocalization of MAb Jim13 in the meristem region of mature somatic embryo (bars C = 100 µm; D = 50 µm). E – Immunolocalization of MAb Jim8 in the meristem region of mature somatic embryo (bar = 50 µm).

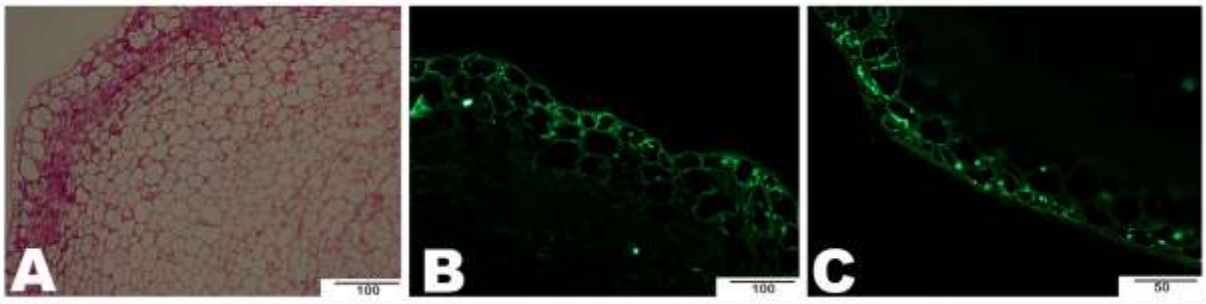

Figure 15 - Immunolocalization of AGPs in the callus sector. A - Morphological aspects of the callus after PAS staining (bar = 100 µm). B – Localization of AGPs recognized by MAb Jim13 (bar = 100 µm). C - Localization of AGPs recognized by MAb Jim8 (bar = 100 µm).

Immunogold localization of MAb Jim13 epitope confirmed that this epitope was associated with the secretory pathway. Gold particles were found associated with vesicles in the vicinity of endoplasmatic reticulum, as well as with the Golgi apparatus (Figure 16A), and were clearly associated with vesicular and cytoplasmatic membranes (Figure 16B). Although a polarized localization of MAb Jim13 epitopes within the somatic embryos was observed, a

non-polar localization of the gold particles was noticed in the cells, as gold particles were found along the plasma membrane of protoderm cells. Gold particles were also observed on the extracellular matrix surface network on the protodermis of somatic embryos (Figure 16C-D). Examination with dot-blot found that the MAb Jim13 epitope was also secreted into the culture medium.

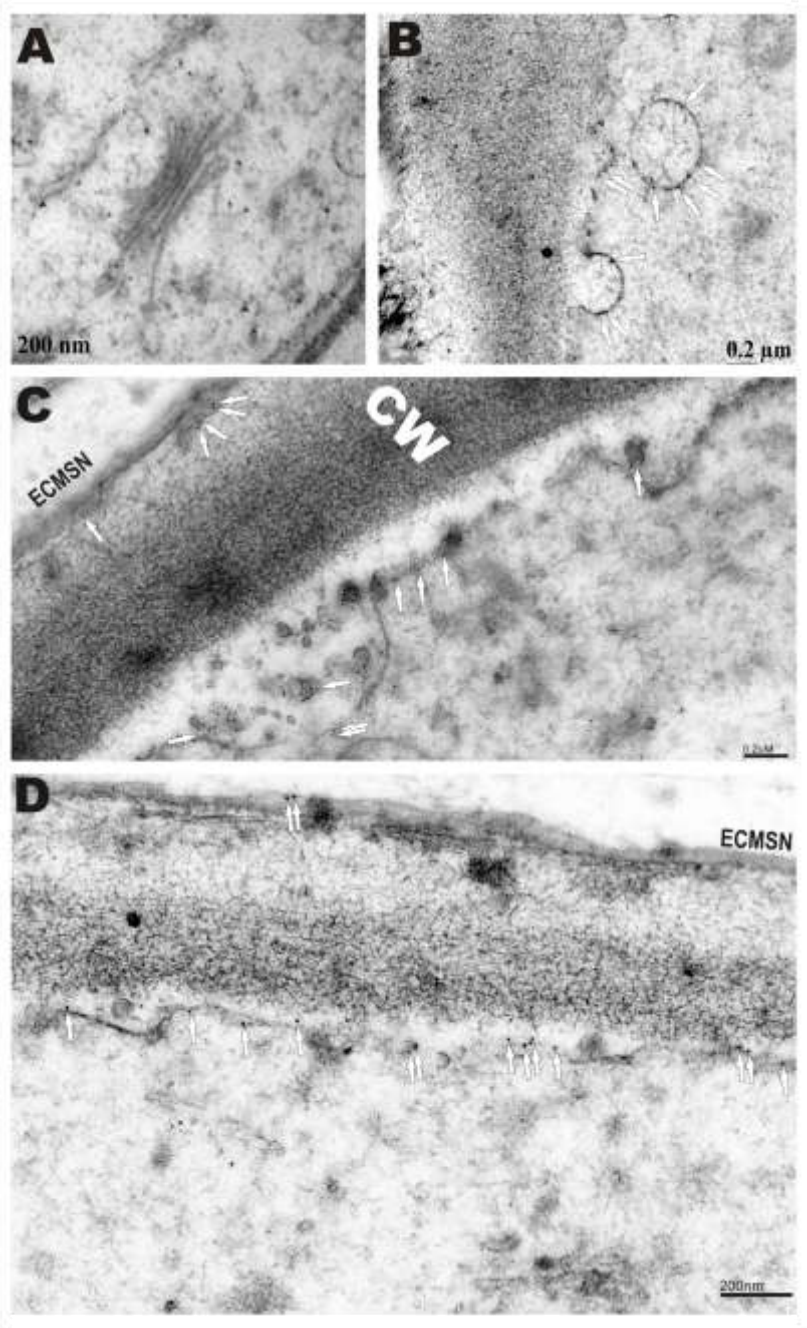

Figure 16 - Immunogold localization of MAb Jim13 epitope. A - Gold particles associated with vesicles in the vicinity of endoplasmatic reticulum and Golgi apparatus (bar = 200 nm). B – Gold particles (arrows) associated with vesicular and cytoplasmatic membranes (bar = 200 nm). C-D - Gold particles observed on the extracellular matrix surface network (ECMSN), cell wall (CW) and vesicles (arrows) (bars = 200 nm).

The presence of low- and highly-methylesterified pectin was found with immunofluorescence localization. The low-methylesterified pectins recognized by MAb Jim5 showed no tissue-specific pattern, but were observed principally on the cell junctions (Figure 17A) with weak signal intensity. MAb Jim7, which recognizes highly-methylesterified pectin, was observed in all cells throughout somatic embryo development (Figure 17B). MAb Jim7 also recognized a layer over the developing somatic embryo (Figure 17C), as well as on the meristem region of mature somatic embryos (Figure 17D-E). The presence of this extracellular layer was correlated with the cells expressing AGPs recognized by MAbs Jim13 and Jim8, and was further characterized.

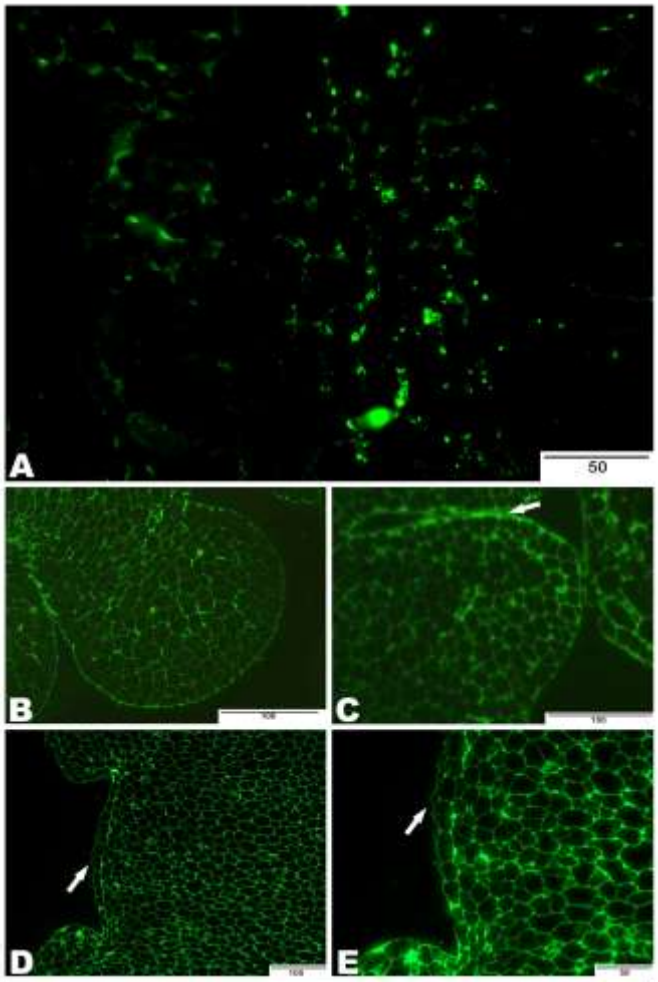

Figure 17 - Fluorescence immunolocalization of MAb Jim5 and Jim7 against pectins during the development of somatic embryos. A – MAb Jim5 on the cell wall principally on cell junctions (bar = 20 µm). B – Immunolocalization of MAb Jim7 on globular somatic embryo (bar = 100 µm). C – Immunolocalization of MAb Jim7 on developing somatic embryo recognizing an extracellular matrix surface network (arrow) (bar = 100 µm). D - Immunolocalization of MAb Jim7 on mature somatic embryo again recognizing an extracellular matrix surface network on the shoot meristem (arrow) (bar = 100 µm). E – Detailed view of the immunolocalization of MAb Jim7 showing the extracellular matrix surface network (arrow) (bar = 50 µm).

4.5 Characterization of the extracellular matrix

MAb Jim7 recognized a layer over the developing somatic embryo, as well as over the meristem region of mature somatic embryos. Scanning electron microscopy showed the presence of numerous (pre)globular somatic embryos (Figure 18A) on one explant and revealed the presence of an ECMSN covering a specific group of cells (Figure 18B). The ECMSN had a fibrillar and granular structure (Figure 18C). Secondary somatic embryos were observed to develop from these areas and initially were completely covered by the extracellular matrix (Figure 18D), but upon its further development the ECMSN was disrupted (Figure 18E). On globular somatic embryos larger than 100 µm no ECMSN was observed (Figure 18F).

Ultrastructural analyses confirmed the presence of an ECMSN covering the whole somatic embryo (Figure 19A). In protodermis cells numerous lipid bodies as well as vesicles were observed (Figure 19B). The ECMSN covering these cells could be divided into three different layers. Osmiophilic substances resembling lipid bodies attached to fibers were followed by an amorphous phase covered by a layer with fibrillar appearance (Figure 19C). Nile red staining of semi-thick sections confirmed the lipophilic nature of this substance covering the developing secondary somatic embryos (Figure 19D). Based on the immunolocalization of pectins, it is assumed that this amorphous layer is composed mainly of highly-methylesterified pectins. Cells of the explant without embryogenic capacity had no extracellular matrix (Figure 19E) and presented an amorphous layer over the cell wall (Figure 19F).

The fibrillar layer was later found again on the shoot meristem of mature somatic embryos (Figure 17D-E), corresponding to the development of an ECMSN over the shoot meristem of somatic embryos. The presence of the extracellular matrix was also confirmed through ultrastructural analyses. However, in the case of shoot meristems only a fibriliar layer was observed (Figure 20) during the late stages of somatic embryo maturation.

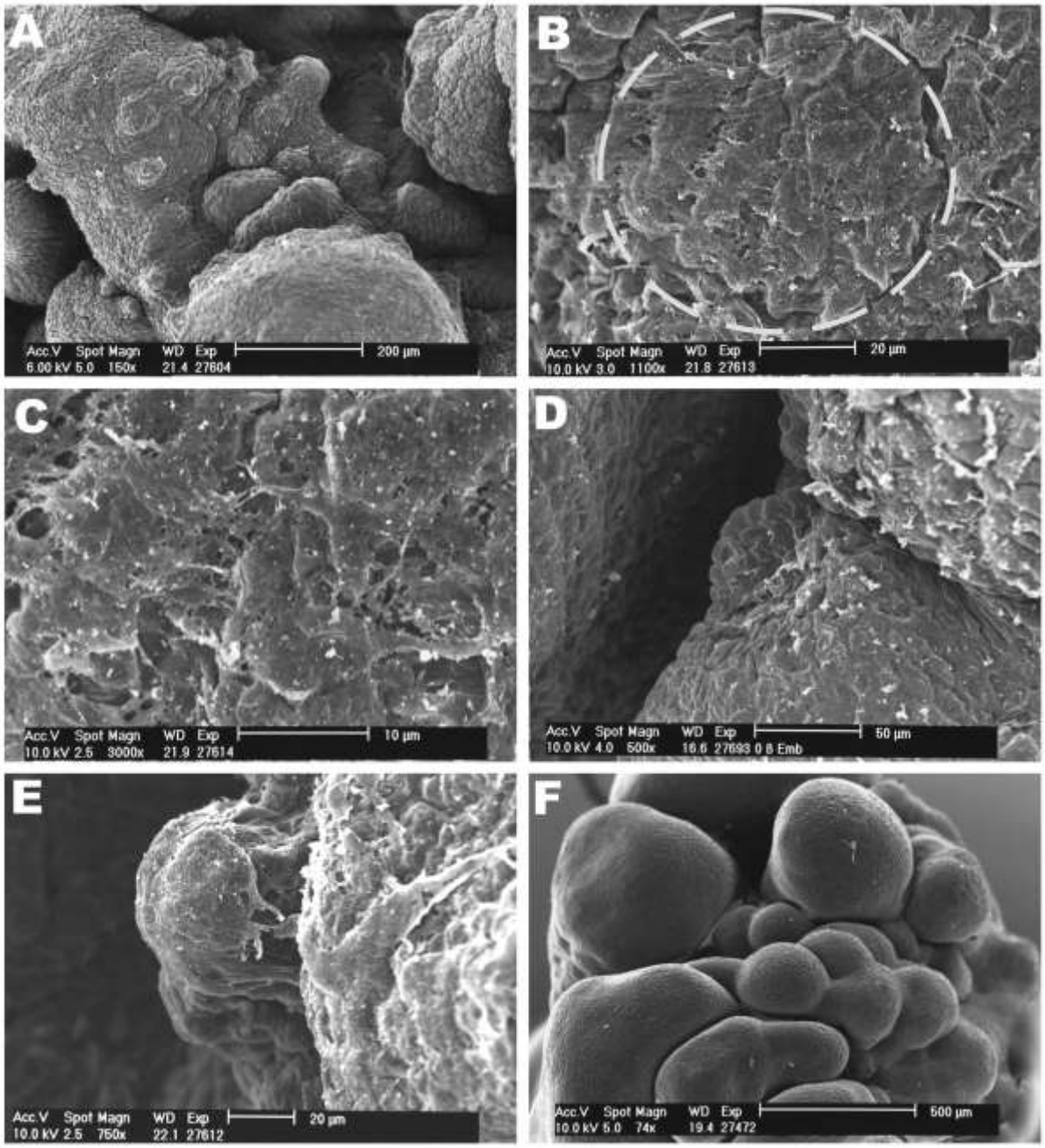

Figure 18 - Scanning electron microscopy analysis of the ECMSN. A – General view of the cultures with numerous (pre)globular somatic embryos (bar = 200 µm). B - Presence of an ECMSN covering a specific group of cells (circle) (bar = 20 µm). C - Fibrillar and granular structure of the ECMSN (bar = 10 µm). D – Secondary somatic embryos completely covered by the ECMSN (bar = 50 µm). E – Disruption of ECMSN upon somatic embryo development (bar = 20 µm). F – Globular somatic embryos (bar = 500 µm).

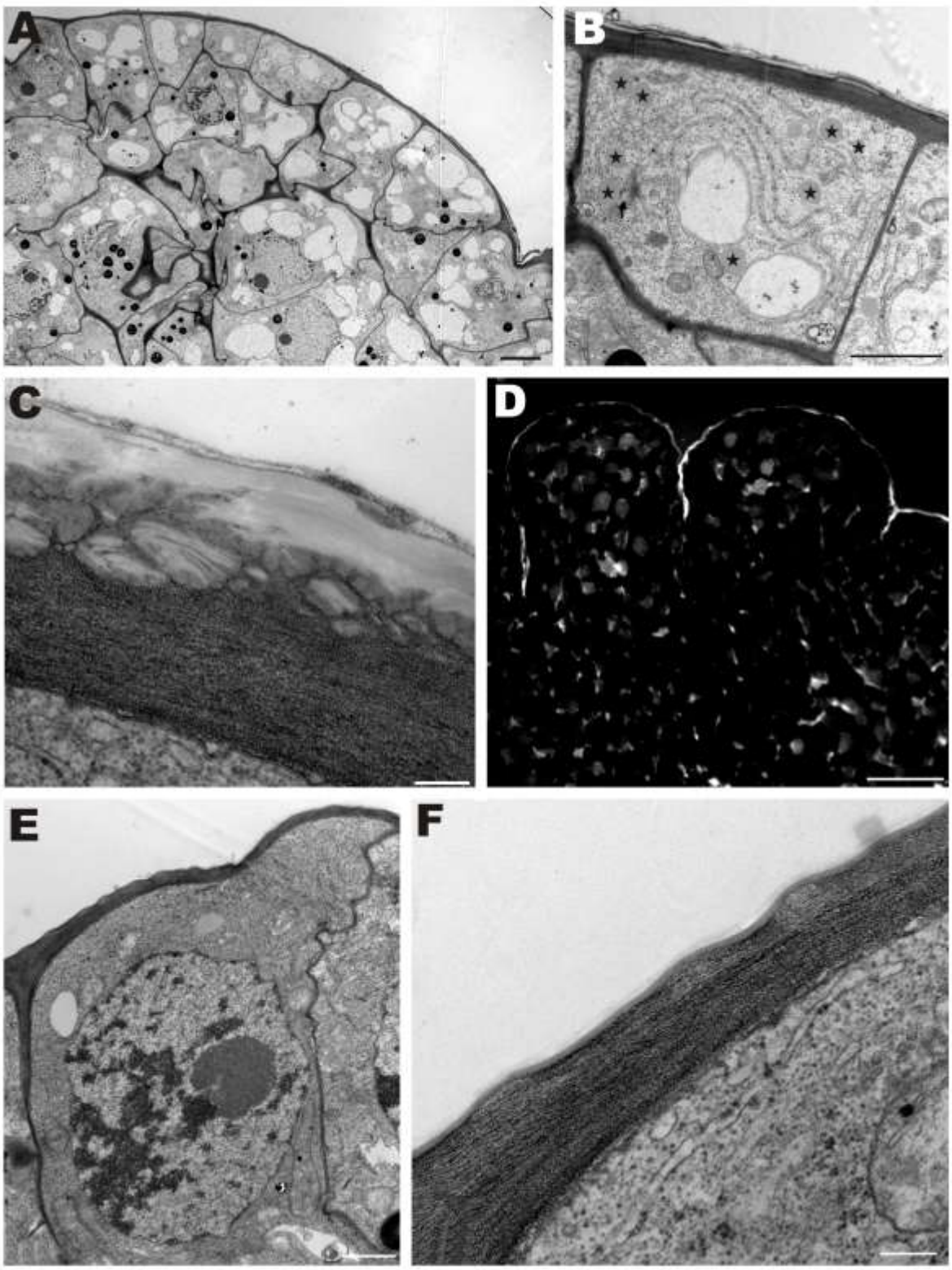

Figure 19 - Ultrastructural analyses of the ECMSN. A – ECMSN covering the whole somatic embryo (bar = 5 µm). B - Protodermic cell showing the presence of numerous lipid bodies (stars) and ECMSN (bar = 2 µm). C – Aspects of the ECMSN covering somatic embryos (bar = 200 nm). D - Nile red staining of semi-thick sections showing reaction to hydrophobic substances on the protodermis of secondary somatic embryos (bar = 100 µm). E - Cells of the explant without embryogenic capacity (bar = 1 µm). F – Detailed view of E (bar = 200 nm).

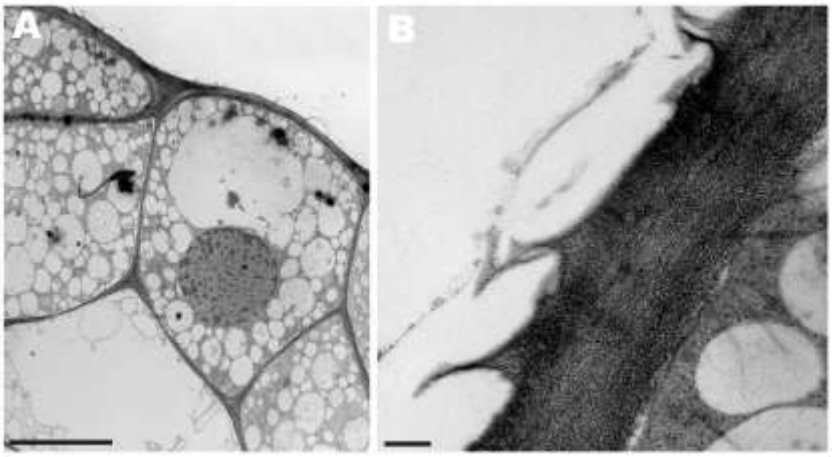

Figure 20 - Ultrastructural analysis of the ECMSN on the shoot meristem of peach palm mature somatic embryos. Bar A = 5 µm B = 200 nm.

5 Discussion

Aggregating AGPs through the inclusion of ßGlcY in the culture medium reduced secondary somatic embryo formation in peach palm, suggesting that AGPs are essential for the onset of secondary somatic embryo development. This effect was not observed in the positive control (αGlcY), proving that the responses were due to AGPs. Similar results have been observed in *Chicorium* sp. (Chapmann *et al.*, 2000).

The fresh weight increment after 30 days of culture was not affected by different concentrations of ßGlcY. A pioneering study with ßGlcY showed the inhibition of cell division in suspension-cultured rose cells in a concentration-dependent manner (Serpe and Nothnagel, 1994). In carrot, clusters transferred to maintenance medium grew into undifferentiated callus and there was little difference among the final fresh weights grown in the presence of ßGlcY (Thompson and Knox, 1998). On the other hand, 50 µM ßGlcY could completely inhibit cell division in *Brassica* sp. microspores (Tang *et al.*, 2006). However, in *Marchantia polymorpha* protoplasts, cell division and survival increased in the presence of ßGlcY (Shibaya & Sugawara, 2007).

For the formation of peach palm secondary somatic embryos a concentration-dependent response was observed, where 30 µM ßGlcY significantly reduced the somatic embryogenesis rate. The induction of primary somatic embryos in *Chicorium* sp. also followed a dose-dependent response and at 250 µM ßGlcY it was completely inhibited (Chapmann *et al.*, 2000). In carrot a stage-specific response to ßGlcY was observed, with root growth being promoted in the early stages and overall growth reduced in late stages (Thompson and Knox, 1998).

Callus formation was significantly higher in the presence of 30 µM ßGlcY. This may explain the similar final fresh weights found in the present study. Taken together, this suggests that AGPs modulates the initial development and differentiation of secondary somatic embryos without interfering in undifferentiated explant growth. Transferring the cultures to solid culture medium blocked somatic embryo development only on those areas in contact with the culture medium, demonstrating that continuous contact with ßGlcY was necessary to block the development of secondary somatic embryos. This may also be related to the properties of ßGlcY, which is a synthetic phenylglycoside with high molecular weight and low diffusion potential between cells. When applied to *Arabidopsis thaliana* seedlings, ßGlcY was not able to enter the stele, and its effect was observed only in the root epidermal cells (Willats and Knox, 1996).

In our peach palm somatic embryogenesis system, non-synchronous development was previously reported, resulting in clusters of secondary somatic embryos (Steinmacher *et al.*, 2007, Chapter II; Figure 8). Staining the somatic embryo clusters with ßGlcY showed the characteristic red color of the AGPs-ßGlcY complex over the globular somatic embryos, especially in the protoderm layer. An intense red coloration was also observed in small areas, where further secondary embryos would probably develop. AGPs were secreted in a polarized way, as intense staining was found in the apex of (pre)globular somatic embryos. A polar distribution of AGPs was also detected *in vitro*-grown tobacco zygotic embryos (Qin and Zao, 2006).

Development of somatic embryos in the presence of ßGlcY resulted in severe alterations in the protoderm. No signs of polarization in the development of somatic embryos were observed, and cells were loosened and bulged. Some indirect interaction between AGPs and microtubules occurs, and the aggregation of AGPs by ßGlcY could explain this disorder in polarization (Driouichi and Baskin, 2008). ßGlcY also depolymerizes and disorganizes cortical microtubuli, as well as affecting the organization of actin filaments (Sandar *et al.*, 2006; Driouichi and Baskin, 2008), which may in turn result in the bulged cells. In tobacco, disturbing the biological activity of AGPs by application of ßGlcY increased the symmetrical division rate in zygotes (Qin and Zao, 2006). In globular somatic embryos of *Brassica* sp., the presence of ßGlcY loosened and bulged the protoderm cells, and cells with root hair-like appearance were also observed (Tang *et al.*, 2006).

The amount of AGPs secreted into the culture medium showed a possible correlation with the development of secondary somatic embryos. Callus culture or the presence of 30 µM ßGlcY had similar results, while embryogenic cultures showed the highest amount of secreted

AGPs. The amount of AGPs detected with ßGlcY also increased in embryogenic cultures during the development of somatic embryos of *Euphorbia pulcherrima* (Saare-Surminski *et al.*, 2000). In our study, the presence of Jim13 and LM2 epitopes secreted into the culture medium was also identified by dot-blots.

MAb Jim13 and Jim8 showed different expression patterns during the onset and differentiation of peach palm somatic embryos. The expression of these epitopes was consistent with ßGlcY staining, and clearly associated with the presence of the ECMSN and its reappearance on the shoot meristem of mature somatic embryos. AGPs were previously shown to be specifically involved in the differentiation of embryos and shoot meristem formation of *A. thaliana* embryos (Hu *et al.*, 2006), as well as in the differentiation of protodermal cells during the globular stage of embryo development of carrot (Stacey *et al.* 1990). AGPs showed differential expression during protoderm development in *E. pulcherrima* (Saare-Surminski *et al.*, 2000).

Further analyses with MAb Jim13 revealed its association with the endomembranes and secretory vesicles of the protoderm cells and its presence on the ECMSN. This is in contrast to results obtained in *Chicorium* sp., where immunogold localization of different AGPs epitopes (including MAb Jim13) was found only on external cell walls of globular somatic embryos and ECMSN, but no cytoplasmatic membrane labeling was detected (Chapman *et al.*, 2000). Similar to our results, the LM2 and Jim13 epitopes were also found associated with membranes of secretory vesicles in maize, possibly being transported as a component of the membrane itself rather than their contents (Šamaj *et al.*, 2000).

The ECMSN formation in the present study was associated with an extracellular layer rich in highly-methylesterified pectin recognized by MAb Jim7. This was found over (pre)globular somatic embryos as well as on the shoot pole of mature somatic embryos. Additionally, strong signal for highly-methylesterified pectin was present on all cell walls of the somatic embryos. MAb Jim5 had very weak signal at the cell junctions. This is in agreement with others studies, which also revealed the presence of highly-methylesterified pectins (MAb Jim7) associated with the ECMSN in monocot species (Šamaj *et al.*, 2006; Konieczy *et al.*, 2007). On the other hand, the ECMSN in dicotyledon species also showed the presence of low-methylesterified pectins recognized by MAb Jim5 [i.e., chicorium (Chapman *et al.*, 2000) and kiwi (Popierlarska-Konieczna *et al.*, 2008)]. It has been proposed that such differences could be specific to monocotyledons and dicotyledons (Konieczy *et al.*, 2007). However, MAb Jim5 revealed a stronger reaction and recognized the fibrillar matrix in embryogenic cells of coconut, while Jim7 was observed at low intensity (Verdeil *et al.*, 2001).

Therefore, more studies are necessary to clarify the role and quantity of pectin methylesterification in palms.

Scanning electron microscopy revealed the presence of the ECMSN in a specific group of cells from which somatic embryos developed. During somatic embryo development, the ECMSN degraded, possibly liberating its content into the culture medium. Ultrastructural analyses confirmed the ECMSN on somatic embryos and on the shoot meristem of mature somatic embryos. It was composed of pectidic substances (localized by MAb Jim7), AGPs (localized by ßGlcY and MAbs) and lipophilic substances (shown by TEM and Nile red staining). Similar results were previously described in kiwi (Popierlarska-Konieczna *et al.*, 2008), *Triticum* (Konieczny *et al.*, 2007), *Drosera* (Bobak *et al.*, 2003) and maize (Šamaj *et al.*, 2006). Based on its composition and localization, different roles of the ECMSN have been proposed. One is related to cell adhesion and control of morphogenesis of a specific group of cells (Šamaj, 2006; Popierlarska-Konieczna *et al.*, 2008ab). This proposal is supported by the multicellular origin of the somatic embryos in our regenerative system (Steinmacher *et al.*, 2007; Chapter II). Additionally, oligosaccharides released from ECMSN might act as signaling molecules involved in the regulation of developmental processes (Erberhard *et al.*, 1989; Darvill *et al.*, 1992). A third possible role of the ECMSN is related to cuticle formation, which in turn would play a protective role (Popierlarska-Konieczna *et al.*, 2008ab).

Cuticle is formed by hydrophobic cutin monomers, which are transported from Golgi complexes and must cross the cell wall. The transport of the hydrophobic substances through the aqueous environment of the cell wall would be greatly facilitated by lipid transfer proteins. The formation of cuticle appears likely to be associated with protoderm formation and in carrot lipid transfer proteins were localized in the protoderm and later on in the shoot apex of seedlings (Sterk *et al.*, 1991). Similarly, non-specific LTP were expressed at very high levels in embryogenic cultures in oil palm and exhibited minimal expression in non-embryogenic cultures; they were suggested as molecular markers for oil palm somatic embryogenesis (Low *et al.*, 2008). In the present study, the presence of hydrophobic substances in the protoderm cells and covering somatic embryos was shown by TEM and confirmed by Nile red staining. A remarkable correlation between the presence of ECMSN and the expression of the Jim13 epitope was observed in the present study. It is very tempting to speculate that the expression of this AGP epitope has a fundamental role during ECMSN formation and not only on its composition.

The present study suggests that MAb Jim13 could be a reliable marker for peach palm somatic embryogenesis as well as somatic embryo quality, given its early expression in

sectors where somatic embryos are formed and on the shoot meristem. It remains to be determined if the AGP epitope recognized by MAb Jim13 in the present study is also a chimeric AGP with properties of a lipid transfer protein. Somatic embryogenesis of peach palm has been shown to be highly genotype-dependent (Steinmacher *et al.*, 2007). The addition of AGPs into the culture medium could be an interesting strategy to increase somatic embryogenesis induction in recalcitrant genotypes.

6 References

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Chapter IV:

MORPHO-HISTOLOGICAL AND BIOCHEMICAL ASPECTS OF PEACH PALM SEED GERMINATION

1 Abstract

The mechanisms regulating the germination of palm seeds are still largely unknown. In the present study, morpho-histological and biochemical aspects before and during the germination of peach palm seeds were evaluated. Histological and ultrastructural analyses of the zygotic embryo reveal its active metabolic state before germination, where numerous small vacuoles with electron-amorphous substances, endoplasmatic reticulum and Golgi complexes were observed. This active metabolic state is an important aspect related to seed recalcitrance. Histological aspects of the haustorium and endosperm are shown. A correlation between plantlet growth and endosperm breakdown was observed and a specific sequence of endosperm breakdown is described, which started with the mobilization of storage proteins. Storage proteins were extracted and partially characterized based on their buffer solubility. Water-soluble proteins under reducing conditions ranged from 15 kDa to 67 kDa in weight. Four low molecular weight proteins (15 kDa to 35 kDa) were especially abundant. Three polypeptides of 45 to 67 kDa were the major bands of proteins soluble in low salt buffer. After peptide sequencing these were confirmed to be 7S vicilin-like proteins. High salt-soluble proteins were composed by two sub-units of ca. 23 kDa and ca. 32 kDa under reducing conditions; under non-reducing conditions a single protein of ca. 55 kDa was observed. These showed high homology to 11S glutelin-like proteins after peptide sequencing. Modifications in the low salt buffer-soluble protein profile were detected by SDS-PAGE and two subunits of the 7S vicilin-like globulin completely disappeared only during the final stages of germination; one subunit was still present in the final stage. Free amino acids were present at lower levels in endosperm than in haustorium throughout germination. Differences were also observed in the profile of free amino acids present in the haustorium and in the endosperm during germination.

Key words: Bactris gasipaes, seed recalcitrance, ultrastructure, amino acids.

2 Introduction

Peach palm is an important, although underutilized, palm species native to the Amazon basin. This is the only palm tree that was domesticated by the Amerindians during the pre-Colombian period. Its fruits contain large amounts of starch or oil (depending on the landrace) and all essential amino acids. Its heart of palm is a gourmet vegetable already well established in internal markets in Brazil, Costa Rica and Ecuador, and with strong potential for international markets (Clement, 2008). Peach palm seeds are recalcitrant (Bovi *et al.*, 2004), losing their viability after short periods of storage, so long-term *ex situ* seed collections are not feasible.

Most studies have shown that the endosperm of palm seeds store carbohydrates (in the form of hemicellulose) in thick-walled cells, while lipids and proteins are stored in specialized cytoplasmatic oleosomes or protein storage vacuoles (PSV), respectively (DeMason *et al.*, 1989; Panza *et al.*, 2004). In germinating palm seeds, the storage compounds from the endosperm are transferred to the growing plantlet by a specialized absorptive organ, called the haustorium. Studies with date palm (DeMason *et al.*, 1985) and *Washingtonia filifera* (DeMason, 1988) suggest that this organ can also directly or indirectly control the breakdown of endosperm storage compounds. The mechanisms underlying this control are largely unknown.

Characterization of morpho-histological and biochemical aspects during the germination of peach palm is needed to better understand the species' seed biology. We are also interested in the characterization of storage proteins as an important step for the improvement of *in vitro* regeneration protocols. *In vitro* culture is an important complementary method for the conservation and improvement of peach palm (Mora-Urpi *et al.*, 1997). Recently, the main factors affecting the induction of somatic embryogenesis were identified (Steinmacher 2007abc) and an improved protocol for secondary somatic embryogenesis was established (Chapter II). However, the conversion rate and maturation of somatic embryos were considered to be bottlenecks in mass production. The accumulation of storage proteins occurs during the maturation of somatic embryos, and the quantity and quality of these storage proteins could be linked to the better conversion capacity of somatic embryos and vigor of regenerated plantlets (Merkle *et al.*, 1995; Sreedhar and Bewley, 1998; Morcillo *et al.*, 1997, 1999; Klimaszewska *et al.*, 2004). No storage proteins have been characterized in peach palm, although two high molecular-weight bands present in embryogenic cultures have been proposed to be storage proteins (Steinmacher *et al.*, 2007a).

Several aspects of palm seed biology are unknown and very little work on the characteristics of palm embryo tissues, especially those from recalcitrant seeds, has been carried out (Panza *et al.*, 2004). The breakdown of storage proteins after seed germination provides nitrogen in form of free amino acids for the development of the seedling. Studies examining storage protein mobilization kinetics or free amino acid dynamics during palm seed germination have not been published.

Therefore, in the present study, the morpho-histological aspects, major storage protein breakdown and free amino acid kinetics during peach palm seed germination are presented, and storage proteins are partially characterized.

3 Material and methods

3.1 Plant material

Seeds of peach palm from spineless populations were obtained from a commercial orchard (INACERES, Bahia, Brazil) and from ICRAF (World Agroforestry Centre). Seeds were germinated in plastic trays containing sand and watered with distilled water. The trays were kept at 28°C and 16 hours light [80-100 µmol m^{-2} s^{-1} provided by HP-T Plus Lamps (Philips®)]. Samples were collected at different stages of germination. As peach palm germination is not completely uniform, samples were collected based on germination morphology (Figure 1), including: 0 – control samples, seeds before germination; 1 – appearance of the germinative node ca. 40 days after sowing; 2 – plumule growth but still enclosed by a sheath (ligulae); 3 – appearance of first leaf sheath, with initial primary root growth; 4 – primary root growth without adventitious roots; 5 – appearance of the second leaf sheath and adventitious roots smaller than 0.5 cm; 6 – appearance of the eophyll and further growth of adventitious roots; 7 – further shoot growth and more than 3 adventitious roots; 8 – final stage evaluated, eophyll completely expanded and root system well established. The final stage occurred approximately 110 days after sowing.

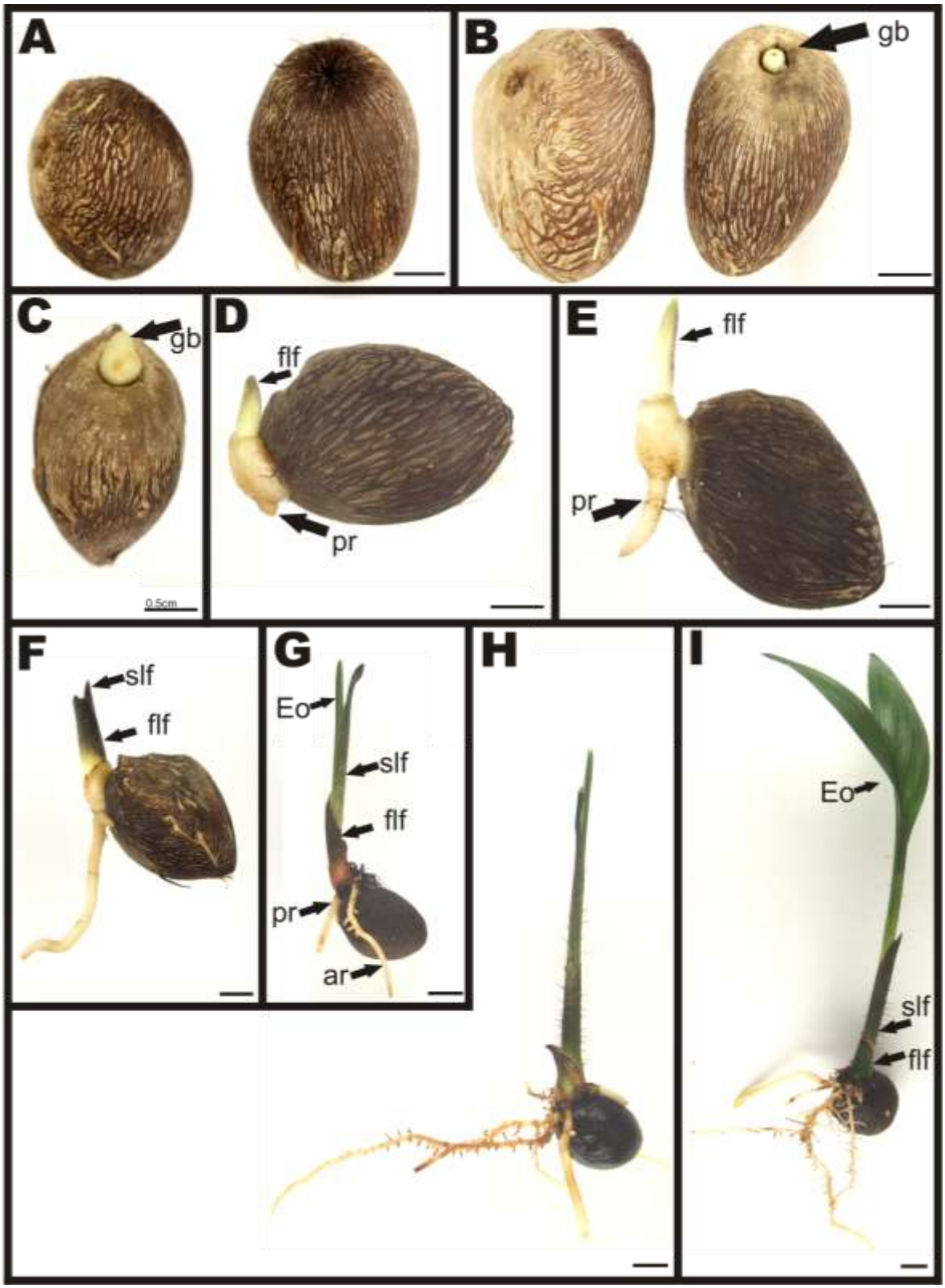

Figure 1 - Different stages of germination of peach palm seeds. A to I – Germination stages 0 to 8. gb – germinative bottom; flf – first leaf sheath; pr – primary root; slf – second leaf sheath; Eo – eophyll; ar – adventitious root. Bars A-F = 0.5 cm; G-I = 1 cm.

3.2 Histological Procedures

For light microscopy, samples were fixed with 2 % (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 24 hours at 4°C, dehydrated in an ethanol series (30 % to 100 %), embedded in LR White resin (London Resin Co Ltd, London) and allowed to polymerize according to the manufacturer's instructions. Specimens were cut 1 µm thick in a semi-automate microtome (Reichert Ultracut S, Leica®) and mounted onto glass slides. Sections were stained with Toluidine blue O (0.5 % in phosphate buffer), and for polysaccharide and protein localization PAS (Periodic Acid-Schiff's reaction) and coomasiee blue staining were used, respectively. For calcofluor staining, a solution containing 100 µg ml^{-1} was applied on the samples for 30 s and washed 3 times with water to remove the excess. The samples were mounted with antifading Citifluor (Citifluor Ltda, London) and visualized under UV excitation. All sections were examined using an Olympus BH-2 microscope and photographed with a ColorView IIIu (Soft Imaging System, GmbH).

For transmission electron microscopy, samples were fixed in 4 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) for 2 h at room temperature, transferred to fresh fixative overnight at 4°C and postfixed in buffered 1 % OsO_4 at 4°C for 2 hours. Samples were dehydrated in a graded acetone series and embedded in Spurr's resin. Ultrathin sections (80-100 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria) and stained with 1 % uranyl acetate and 0.1 % lead citrate for 10 min each.

3.3 Storage protein extraction and SDS-PAGE analysis

Samples of peach palm endosperm were freeze-dried and ground in a coffee bean grinder. Thereafter the samples were defatted with at least two volumes of n-hexane and petroleum ether in a mixer Mill (MM400, Retsch, Haan, Germany) for 10 min; samples were filtered through a 0.4 µm membrane obtaining a fine powder, and referred to hereafter as fat free material (FFM).

Proteins were extracted as described by Morcillo *et al.* (1997). Briefly, 20 mg FFM was extracted twice with 400 µl 25 mM potassium-phosphate buffer pH 7.0 with different NaCl concentrations (0.2 to 1 M). Proteins were extracted for 40 min at 4°C in a Termomixer (Thermomixer Comfort, Eppendorf, Wesseling-Berzdorf, Germany), centrifuged at 13000 rpm for 10 min. The dye-binding method of Bradford (1976) was used for protein quantification, using bovine serum albumin as standard. The 0.2 M NaCl fraction was further precipitated with 2 volumes of ethanol and the pellet was re-suspended with distilled water, obtaining the water-soluble proteins. The remaining 0.2 M NaCl fraction was also submitted

to size fractioning in a HiPrep Sephacryl™ HR S-200 column (GE Healthcare, München, Germany) coupled to a FPLC Biological DuoFlow system (Bio-Rad Laboratories GmbH, München, Deutschland).

SDS-PAGE was performed with a 12 % acrylamide separating gel and a 4 % acrylamide stacking gel (Laemmli, 1970) on a 1 mm thick mini-gel in a Mini-Protean II electrophoresis cell (Bio-Rad). Each well was loaded with approximately 25 µg proteins in SDS-loading buffer in the presence or absence of ß-mercaptoethanol. Low (14.4 to 94 kDa) and high (53 to 212 kDa) molecular mass markers (GE Healthcare, München, Germany) were used as standards. The gel was run at 64 V in the stacking gel and at 120 V for protein separation and stained with coomassie blue. Gels were run until front dye reached the end of the stacking gel or separating gel. Bands of interest were excised, the proteins reduced with DTT (dithiothreitol) (10 mM, 56°C, 30 min) , the cysteine residues modified with iodacetamid (55 mM, ambient temperature, 20 min in the dark) and the protein *in-gel* digested with trypsin [5 ng trypsin μl^{-1} (modified trypsin, Promega, Madison, USA) in 50 mM NH_4HCO_3, 37°C, overnight]. After digestion the gel pieces were repeatedly extracted (50 % acetonitrile and 5 % formic acid), the combined extracts dried down in a vacuum concentrator, redissolved in 5 % methanol and 5 % formic acid, desalted on a C18 microZipTip (Millipore, Billerica, USA), eluted with 1 µl 60 % methanol and 5 % formic acid and analysed by nano-electrospray mass spectrometry in a QTOF II instrument (Micromass, Manchester, UK). The MS/MS spectra obtained by collision-induced fragmentation of the peptides were evaluated both manually and by the Mascot MS/MS ion search algorithm (Matrix Sciences, London, UK).

3.4 Free amino acid extraction and identification by HPLC

Free amino acids were extracted from samples (20 mg lyophilized FFM) with TFA-acidified water (pH 2.5) for 1 hour on ice under constant agitation. The supernatant was filtered through a 20 µm membrane and an aliquot of 50 µl was freeze-dried and further analyzed. Amino acids were derivatizated with o-phthaldialdehyde (OPA) and identified by reverse HPLC following Rohsius *et al.* (2006). Peak areas and retention times were measured by comparison with known quantities of standard amino acids.

4 Results

4.1 Morpho-histological characterization of peach palm seed germination

Peach palm zygotic embryos are small, conical, with the epicotyl oblique to the cotyledon and are surrounded by the endosperm (Figure 2A). Histochemical analyses revealed different structures, including the apical meristem, a cotyledonary sheath, procambium and protoderm. The apical meristem was formed by small cells, with a high nucleous:cytoplasm ratio and dense cytoplasm (Figure 2B). Cells from the cotyledonary blade were vacuolated, with thin cell walls; protein storage vacuoles (PSV) were present, but with sparse contents (Figure 2C). Oblong cells with few PSV were observed in the procambium (Figure 2D).

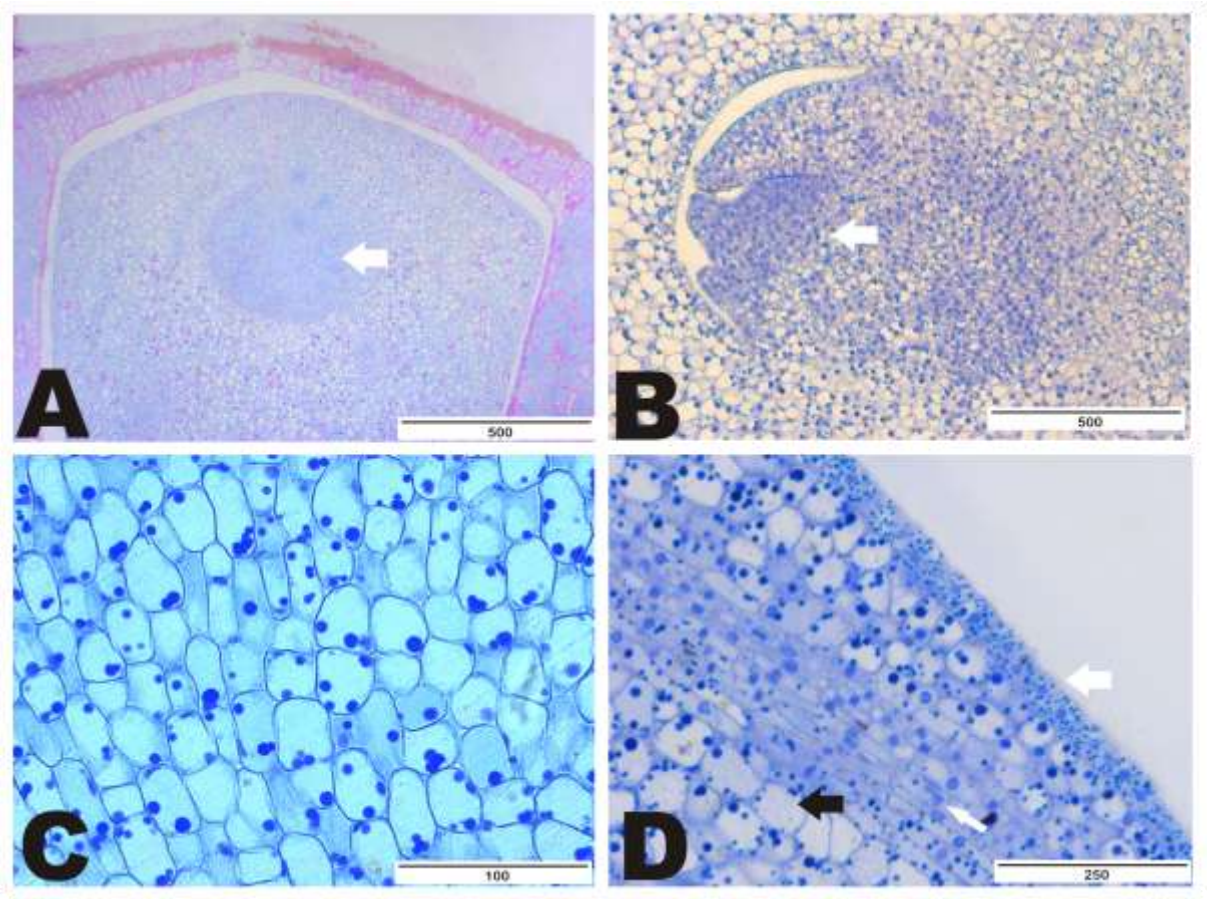

Figure 2 - Histochemical analyses of peach palm zygotic embryo. A – Zygotic embryo surrounded by the endosperm showing the shoot meristem (arrow) (bar = 500 µm). B – Detailed view of the shoot meristem (arrow) after Coomassie protein staining (bar = 500 µm). C – Parenchymatic cells of the cotyledonary blade (bar = 100 µm). D – Histological view of the cotyledonary blade (black arrow), procambium (small white arrow) and protoderm (large white arrow) (bar = 250 µm).

Ultrastructural analyses of the cotyledonary blade of the embryo confirmed the presence of numerous small vacuoles with electron-amorphous substances; electron-dense substances were scattered in the vacuoles, which are assumed to be residual proteins (Figure 3A). The presence of lipid bodies (lipid droplets) as well as amyloplasts was frequently observed (Figure 3B-C). A very active metabolic state seems to be intrinsic in the mature zygotic embryos, as numerous organelles were always observed in the parenchymatic cells of the cotyledonary blade (Figure 3D-E). These included Golgi complexes generating microvesicles (Figure 3F), numerous single microvesicles or dilated cisternae of endoplasmatic reticulum (Figure 3D-F). In some cells, fusion of vesicles with the vacuoles or cytoplasmatic membrane was observed (Figure 3E).

During germination, the peach palm zygotic embryo's cotyledonary blade elongates and forces the germinative button to protrude through the germination pore. The cotyledonary blade expands further, resulting in haustorium development (Figure 4). From the germinative button, the plumule was observed first, followed by root development (Figure 4A-B). The seedling's growth was adjacent to the seed (Figure 1). Adventitious roots then formed, and a constant increment in the fresh weight of the haustorium and shoot/root was observed during the different germination stages. This sequence was strongly correlated with endosperm breakdown (Figure 5).

The haustorium was composed by parenchymatic cells (Figure 6A), an epithelium layer and numerous vascular bundles (Figure 6B). The cytoplasm of the epithelium layer contains a denser cytoplasm than in the parenchymatic cells after Toluidine staining (Figure 6C). Epithelium cells contained small vacuoles, whereas parenchymatic cells had large central vacuoles (Figure 6C); larger intercellular spaces were also observed. Most starch accumulation is spatially localized in the inner parenchymatic cells, whereas the outer ephitelium layer contained fewer and smaller amyloplasts (Figure 6D). The haustorium had an undulating surface and residue from the endosperm was frequently observed adhered to the haustorium surface (Figure 6A-D).

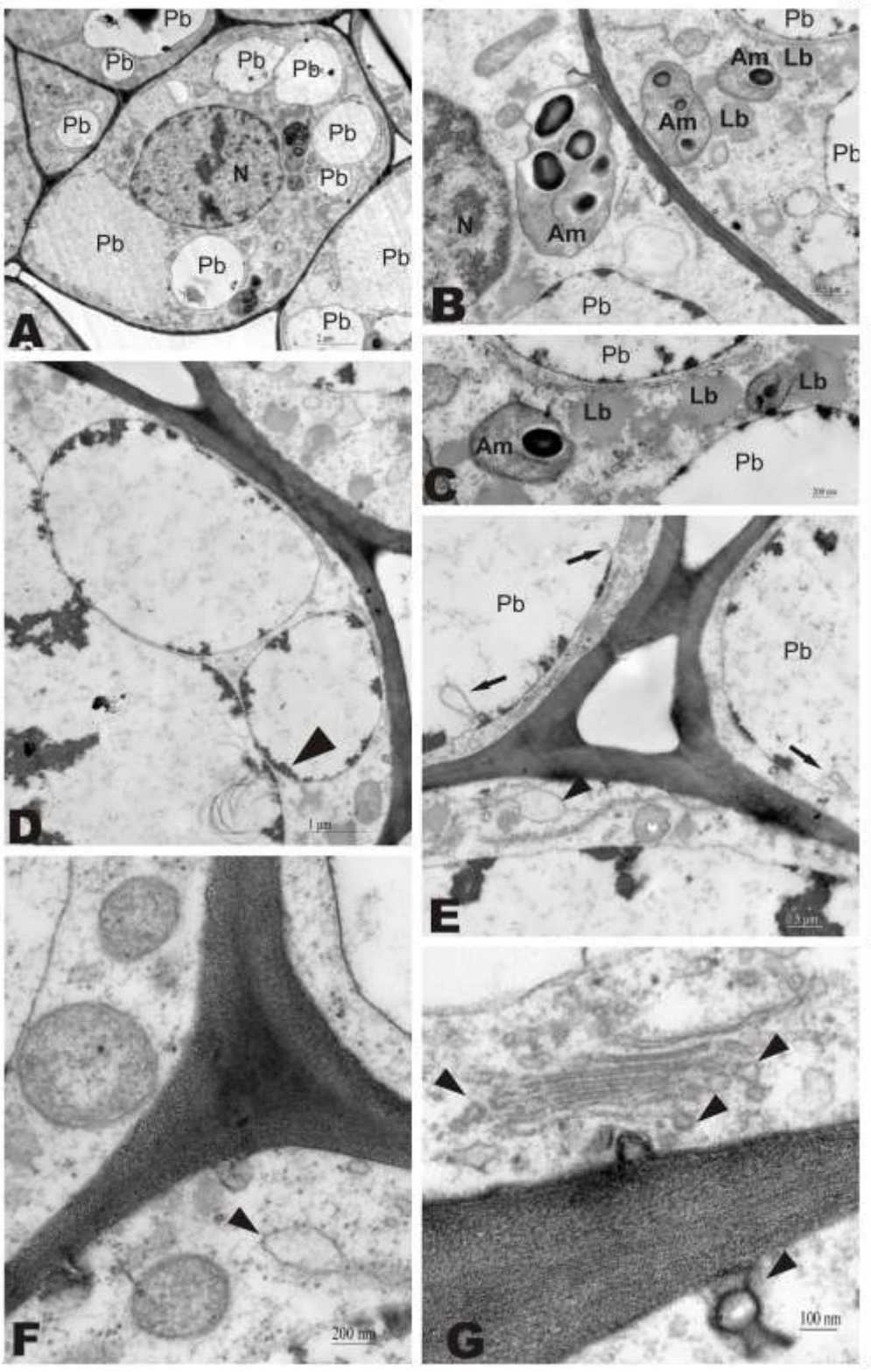

Figure 3 - TEM analyses of zygotic embryo of peach palm. A – General view of the cotyledonary blade cell (bar = 2 µm). B-C – Presence of lipid bodies and amyloplasts (bars B = 500 nm; C = 200 nm). D – Empty vacuoles with electron dense substances at their periphery (arrowhead) (bar = 1 µm). E – Fusion of vesicles with the vacuoles (arrows) as well as with dilated cisternae of endoplasmatic reticulum (arrowhead) (bar = 500 nm). F – View of a vesicle with numerous ribosomes in its vicinity (arrowhead) (bar = 200 nm). G – Evidence of the presence of Golgi complex and microvesicles (arrowhead) (bar = 100 nm).

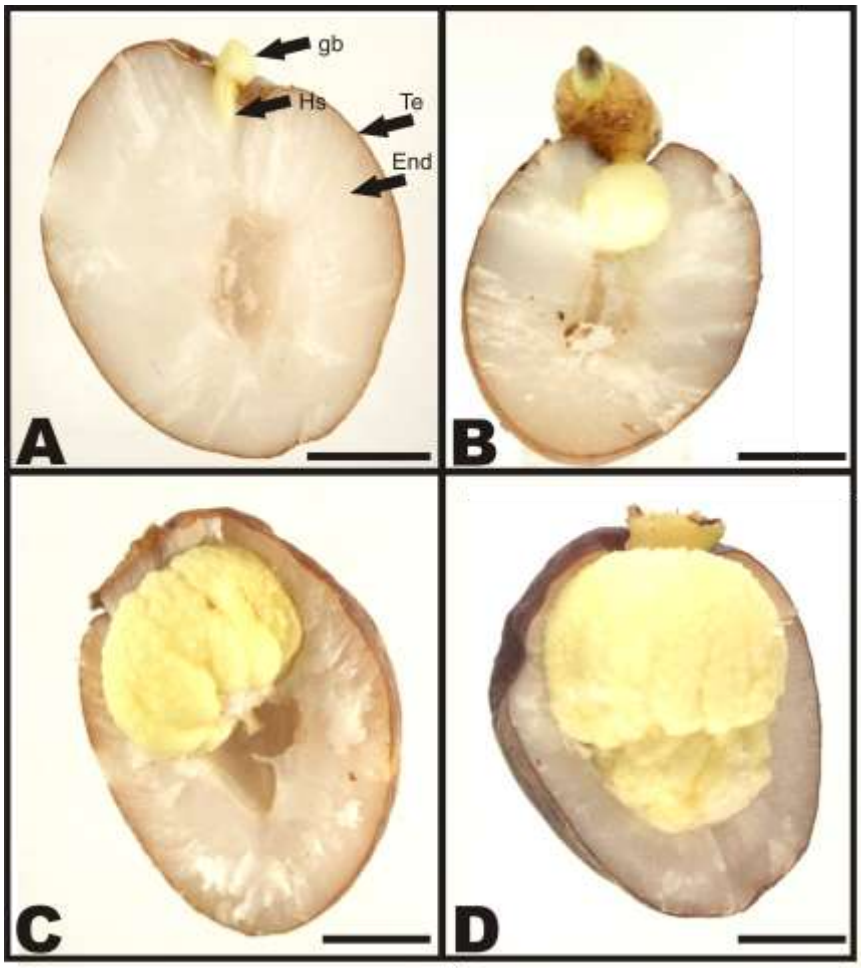

Figure 4 - Morphological view of haustorium development. Hs – haustorium; gb – germinative bottom; End – endosperm; Te – testa. Bars = 0.5 cm.

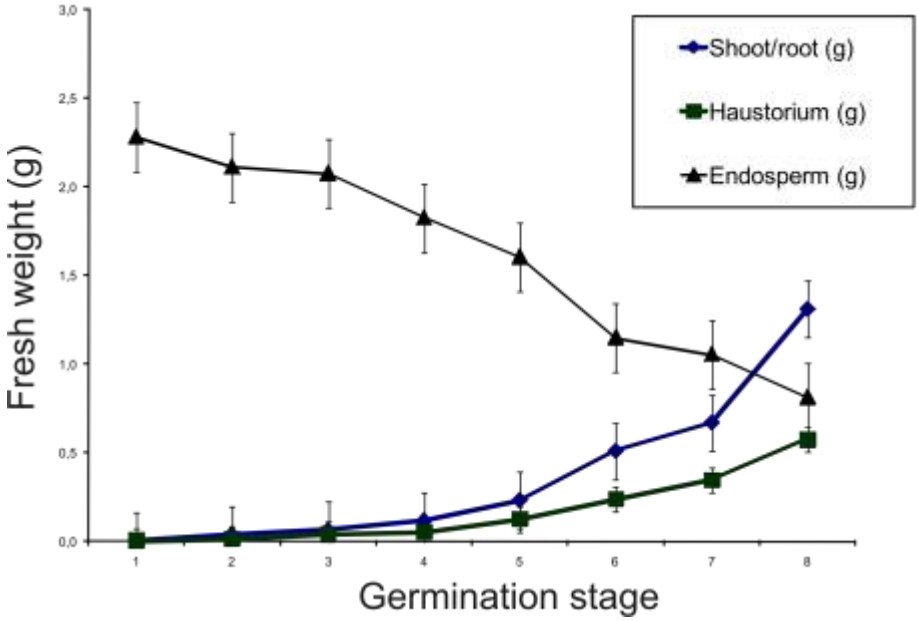

Figure 5 - Fresh weight per seed of the haustorium (g), endosperm and shoot/root (g) during different germination stages (1 to 8) of peach palm seeds. Bars represent standard errors.

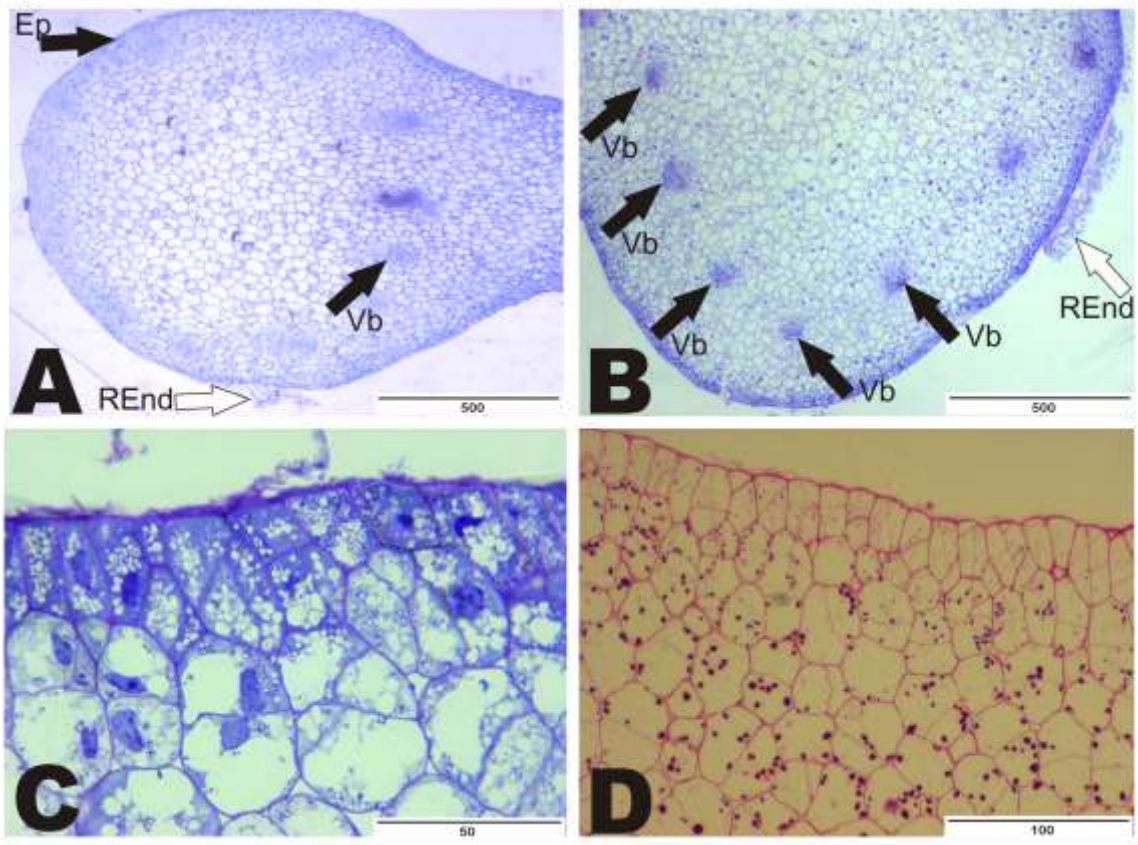

Figure 6 - Histological analyses of the haustorium of peach palm. A – Longitudinal section (bar = 500 µm). B – Transverse section revealing numerous vascular bundles (bar = 500 µm). C – View of the epithelium layer after Toluidine staining (bar = 50 µm). D – View of the epithelium and parenchymatic cells after PAS staining revealing the presence of amyloplasts (bar = 100 µm). Ep – epithelium; Vb – vascular bundles; REnd – rest of endosperm.

The zygotic embryo was enclosed in the endosperm, which in turn was covered by the testa (Figure 7A). A few cell layers composed the testa, with large amounts of phenolic deposits in vacuoles, as shown by toluidine blue (Figure 7B). The endosperm could be divided into a lateral endosperm and micropylar endosperm (Figure 7A). Cells of the micropylar endosperm had a ring of thin-walled cells with degraded content (Figure 7A), while cells composing the lateral endosperm were morphologically all similar in content and aspect (Figure 7C). Three layers of toluidine blue staining intensity could be observed: inside of the cell a layer stained more intensely, the middle lamela showed a more intense reaction, and a reasonable staining intensity was observed on the remaining cell wall (Figure 7C). Endospermic cells near the testa contained smaller PSV (Figure 7B), while cells lying immediately adjacent to the zygotic embryo were thin-walled, with sparse content or already crushed (Figure 7D). One nucleus was present in each cell, and numerous lipid bodies and two to five PSV were observed (Figure 7C-D).

Histochemical characterization upon germination revealed a specific sequence of cell breakdown. The first modification was observed in the storage protein vacuoles, resulting in a granular appearance (Figure 7D and 8A). At this point, cell walls were still intact (Figure 8B). Further degeneration resulted in cells empty of content but with still intact cell walls. Hydrolysis of the cell wall in the vicinity of the haustorium was observed after all cytoplasmatic content was degraded (Figure 8C). These cells were than crushed by the growing haustorium (Figure 8D). All cells showed reaction to calcofluor white (Figure 8E), including crushed cell walls (Figure 8F), revealing the presence of β-glucans.

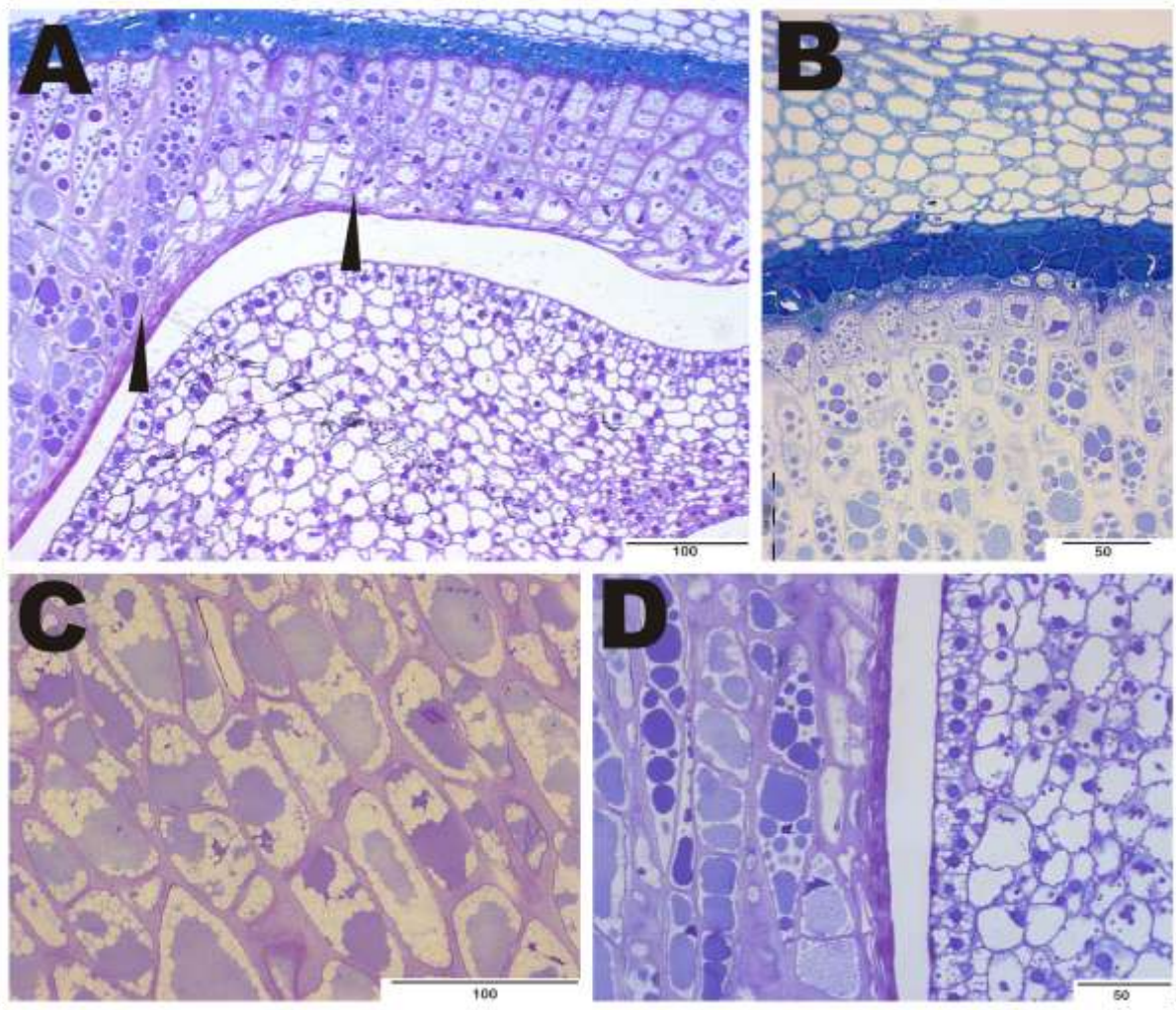

Figure 7 - Histological analyses of the endosperm of peach palm before germination. A – Zygotic embryo surrounded by the endosperm and testa. Arrows indicate micropylar endosperm (bar = 1000 µm). B – Detail of the testa after toluidine staining (bar = 50 µm). C – View of the cells composing the lateral endosperm (bar = 100 µm). D – Appearance of the cells lying immediately adjacent to the zygotic embryo (bar = 50 µm).

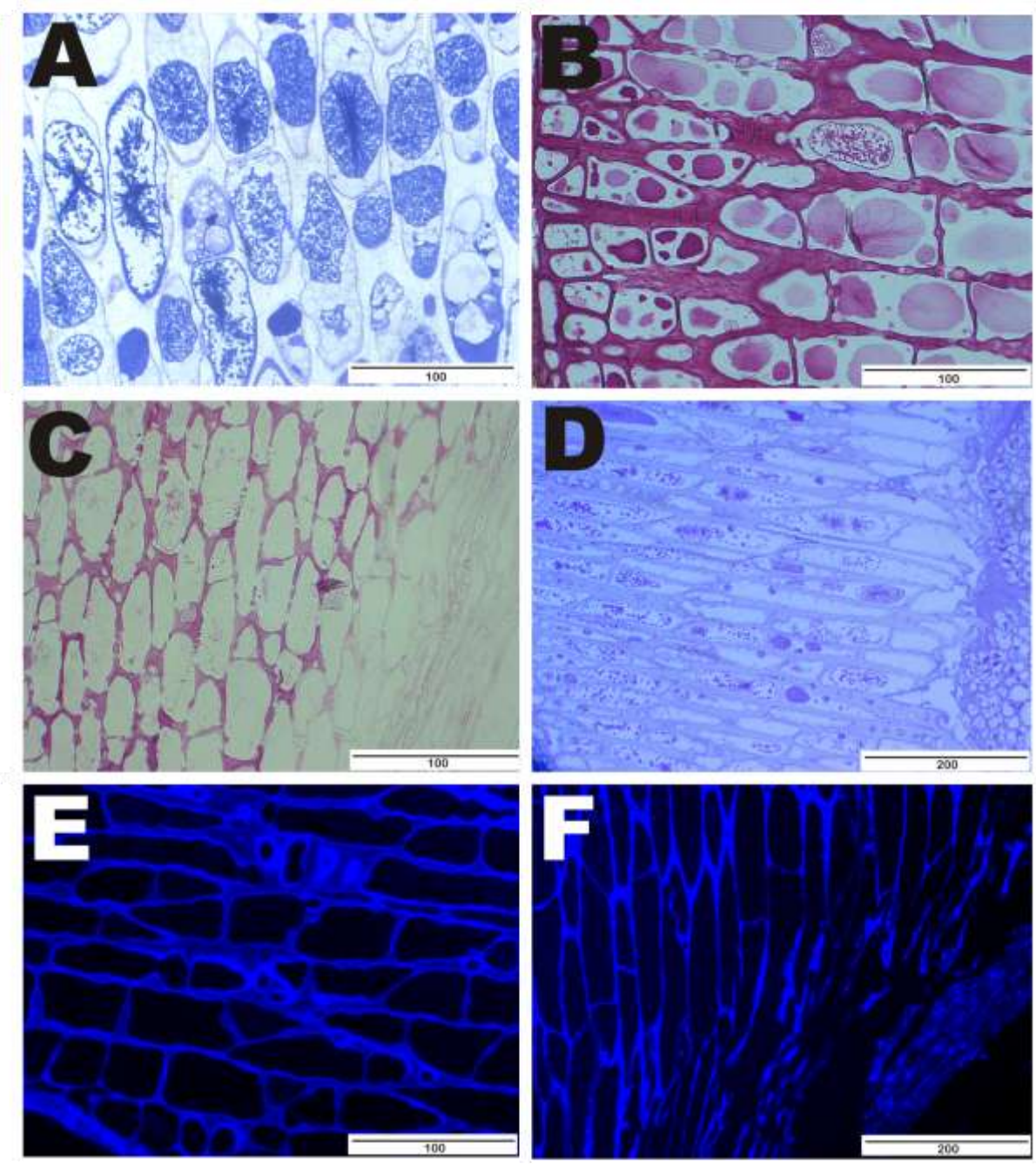

Figure 8 - Histochemical characterization of the endosperm upon germination of peach palm seeds. A – View of the protein storage vacuoles (PSV) upon germination (bar = 100 µm). B – Endosperm cells stained with PAS revealing intense reaction in the PSV and cell wall. C – Cells in the vicinity of the haustorium showing less cytoplasmatic content and abrupt cell wall hydrolysis (bar = 100 µm). D – Crushed endosperm cells caused by the growing haustorium (bar = 200 µm). E – Calcofluor staining of the endosperm cells prior to germination (bar = 100 µm). F – Calcofluor staining of the crushed endosperm cells (bar = 200 µm).

4.2 Characterization of globulins

Peach palm storage proteins extracted with different NaCl concentrations (0.2 to 1 M) showed that most proteins were 0.2 M NaCl buffer-soluble with a wide range of molecular weights (from 15 kDa up to 220 kDa). An additional fraction showed higher solubility at 1 M NaCl (Figure 9). The molecular weight of water-soluble proteins under reducing conditions ranged from 15 kDa to 67 kDa. Four low molecular weight proteins (15 kDa to 35 kDa) were especially abundant. No major modifications were observed in reducing or non-reducing conditions (Figure 10).

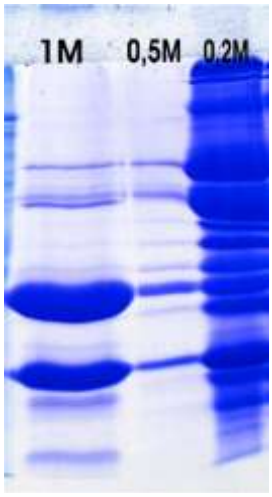

Figure 9 - Peach palm storage proteins extracted with different NaCl concentrations (0.2 to 1 M) separated by SDS-PAGE and stained with Coomassie blue.

Proteins soluble in low salt buffer were size-fractionated revealing three protein peaks. Peak 1 was composed of three proteins of 45 to 67 kDa in coomassie-stained SDS-PAGE. Peaks 2 and 3 had protein profiles similar to the crude extract, however with different band intensity of each protein. Peak 2 was enriched with the three proteins of 45 to 67 kDa present in peak 1 (Figure 10). Several high molecular weight low salt-soluble proteins (higher than 200 kDa) were found in non-reducing conditions, along with three major bands at 45 kDa and 67 kDa. Under reducing conditions, the high molecular weight proteins partially dissociated resulting in numerous bands of lower molecular weight, explaining the wide range of molecular weights mentioned above. No differences were observed under reducing or non-reducing conditions for the three major proteins with weights between 45 to 67 kDa. High salt-soluble proteins were composed by two sub-units of ca. 23 kDa and ca. 32 kDa under reducing conditions; under non-reducing conditions a single protein of ca. 55 kDa was observed (Figure 11).

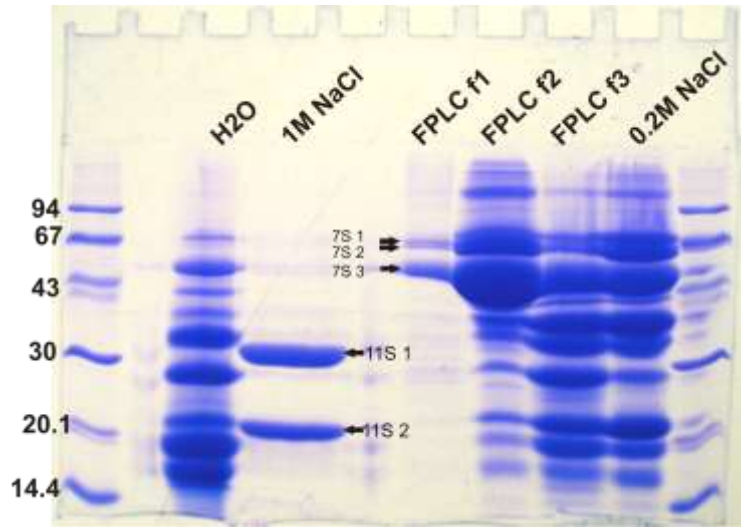

Figure 10 - Protein profile of peach palm endosperm proteins soluble in water or 1 M NaCl. Low salt-soluble proteins (0.2 M NaCl) were additionally size-fractioned through FPLC into 3 fractions (peaks). Numbers at the left correspond to molecular weight (kDa) of standard proteins.

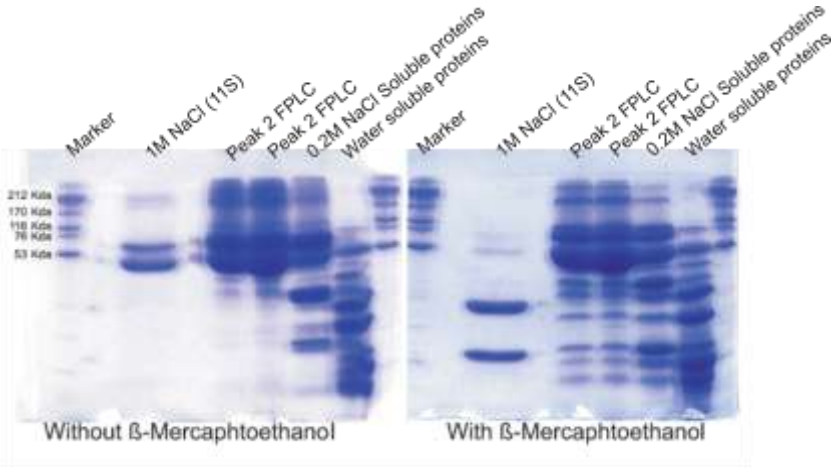

Figure 11 - Protein profiles of the different fractions under non-reducing conditions (absence of ß-mercaptoethanol) or reducing conditions (presence of ß-mercaptoethanol). Marker corresponds to the molecular weight (kDa) of standard proteins.

Based on their salt solubility, amount and molecular weight these proteins are assumed to be globulin storage proteins. Peptide sequencing of proteins confirmed that proteins from peak 1 had high homology to the 7S vicilin-like protein, while high salt-soluble proteins showed high homology to the 11S glutelin-like proteins (Table 1). Hence, the three protein bands observed correspond to the sub-units of the 7S vicilin-like protein. The high salt-soluble proteins correspond to the acidic and basic disulphide-bounded chains of the 11S glutelin-like storage globulin.

Table 1 - Peptide sequence of selected bands after SDS-PAGE and sequence analysis of the peptides evaluated both manually and by the Mascot MS/MS ion search algorithm.

Globulin	Peptide sequences	Sequence analysis[1]
11S 1	LMQALSQPR SEGFQFVSLK GMPEEVLMNSYR* LTTLNSEQLPLLR VVVADDQGNTMMDDALR	* 100 % identity with 12 aa overlap with glutelin from oil palm (AAF69015); 90 % identity with 10 aa (MPE+VLMNSYR) with legumin precursor from *Magnolia salicifolia* (CAA57847); 70 % identity in 7aa (MPEV+M +Y) with 11S globulin from *Sesamum indicum* (AAK15087); 69 % identity with 9 aa (K MPEEVL +YR) with legumin-like storage protein from *Picea glauca* (CAA44874).
11S 2	AGVTDYFDEDNEQFR	93 % identity with 14 aa (AG+TDYFDEDNEQFR) with glutelin from oil palm (AAF05770); 80 % identity with 12 aa (AG+T+YFDE NEQFR) with 11S *Avena sativa* (CAA52763); 80 % identity with 12 aa (AG+T+YFDE NEQFR) with triticin precursor from *Triticum aestivum* (AAB27108).
7S 1 and 2	VQSEVSCGTVFIVPAGHP	87 % identity with 14 aa (S+VSCGT FIVPAGHP) with 7S globulin from oil palm (AF250228); 100 % identity with 10 aa (TVFIVPAGHP) with vicilin-like protein from *Anacardium occidentale* (AF395894) ; 78 % identity with 11 aa (GT F+VPAGHP FV) with vicilin-like embryo storage protein from *Zea mays* (CAA41810); 73 % identity with 11 aa (GTVF+VP+GHP VV) with globulin-like protein from *Oryza sativa* (AAD10375).
7S 3	EADSDDYPELR* EVDEVLNAPR** GALLQATQEQLK***	* 100 % identity with 11 aa overlap with 7S globulin from oil palm (AF250228); ** 100 % identity with 10 aa overlap with 7S globulin from oil palm (AF250228); *** 66 % identity with 8 aa overlap (GA++QA+QEQ+K) with 7S globulin from oil palm (AF250228);

[1] using BLAST 2.2.22 (Altschul *et al.*, 1997).

4.3 Soluble storage protein mobilization and free amino acids during germination

The protein concentration (as mg g^{-1} FFM) in peach palm endosperm measured by the Bradford dye showed minor alterations during germination (data not shown). Modifications in the buffer-soluble protein profile were detected by SDS-PAGE and two subunits of the 7S vicilin-like globulin completely disappeared only during the final stages of germination; one subunit was still present in the final stage. As germination proceeded there was an increase in breakdown products in the range of 25 to 45 kDa (Figure 12).

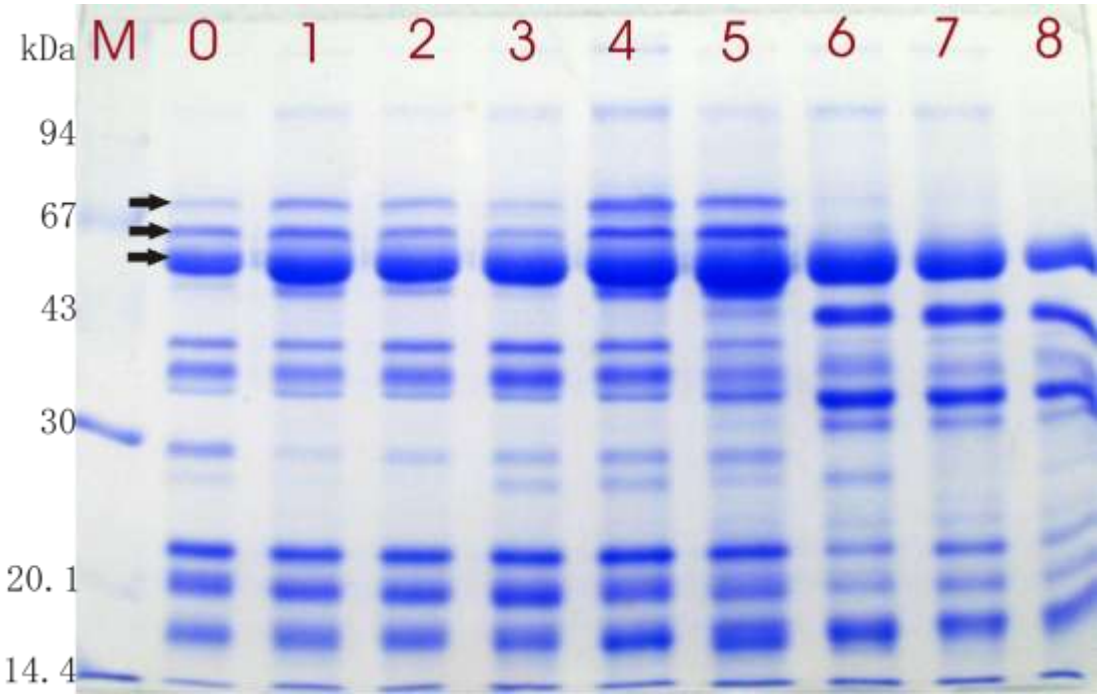

Figure 12 - Modifications in the buffer-soluble protein profile detected by SDS-PAGE during germination of peach palm proteins. Arrows correspond to 7S vicilin-like proteins. Numbers at the left correspond to molecular weight (KDa) of standard proteins.

Free amino acids were present at lower levels in endosperm than in haustorium throughout germination (Table 2 and 3). Although the endosperm is the protein storage tissue, total free amino acid content was usually 4 to 5-fold lower in endosperm compared with haustorium, ranging from 11,702 µg g^{-1} FFM to 18,272 µg g^{-1} FFM in the endosperm, and from 48,198 µg g^{-1} DW to 73,943 µg g^{-1} DW in the haustorium at different stages of germination (Table 2 and 3). Even at stage 8, where most storage protein was degraded, low levels of free amino acids were found in the endosperm (16,914 µg g^{-1} FFM).

Differences were also observed in the profile of free amino acids present in the haustorium and in the endosperm during germination. Before germination, glutamine was the main free amino acid in the endosperm with similar amounts observed in zygotic embryos; in

the zygotic embryos, however, arginine and alanine together (Arg/Ala) presented the highest amounts (12,196 μg g^{-1} DW) and a similar amount was measured in the haustorium throughout the germination period (Table 2). During germination a shift occurred and the main free amino acid in the endosperm was tyrosine, followed by GABA; in the haustorium, glutamine became the main free amino acid with increasing amounts as plantlet development progressed (Table 3).

Table 2 - Free amino acid concentrations in zygotic embryo and haustorium during different stages of peach palm seed germination. Total free amino acids correspond to the sum of all free amino acids.

	Free amino acids haustorium (µg gr^{-1})								
	0	1	2	3	4	5	6	7	8
Arg/Ala	**12196**	**12104**	**15856**	**13045**	**10609**	**12741**	**7358**	**9913**	**7260**
Asn	1931	5767	4205	5792	4459	6789	5056	2700	4613
Asp	3389	2274	2062	1009	2184	1241	1544	2031	1385
GABA	9281	2689	4552	3549	2231	1599	415	502	633
Gln	**3505**	**35421**	**14302**	**12624**	**22527**	**34764**	**38371**	**28100**	**44332**
Glu	3601	3474	2300	2157	1283	1638	1720	1582	1440
Gly	647	373	254	156	135	129	90	38	112
Ile	2231	1355	1071	853	800	574	378	418	529
Leu	1333	797	964	763	784	653	397	406	687
Lys	3300	3041	3536	2821	3289	2154	1044	1343	2257
Phe	396	536	790	462	562	500	355	362	764
Ser	3088	2678	3342	2152	1927	1408	1068	866	1174
Thr	1436	868	623	413	479	279	228	193	391
Trp	301	271	430	324	391	239	177	176	318
Tyr	1168	968	1316	953	1008	1008	655	783	1205
Val	2144	1328	1421	1126	1077	766	514	510	750
Total	**49947**	**73944**	**57025**	**48199**	**53744**	**66480**	**59369**	**49922**	**67848**

Table 3 - Free amino acid concentrations in endosperm during different stages of peach palm seed germination. Total free amino acids correspond to the sum of all free amino acids.

	Free amino acids endosperm (µg gr^{-1})								
	0	1	2	3	4	5	6	7	8
Arg/Ala	1088	1028	1398	1356	814	1021	855	638	923
Asn	1573	1298	1685	1695	1300	1170	694	723	955
Asp	1643	1340	1565	1406	1181	981	589	580	673
GABA	**2037**	**1781**	**2354**	**2064**	**1542**	**1717**	**1735**	**1798**	**2711**
Gln	3161	1482	1691	1566	1320	1501	1908	1524	2145
Glu	309	307	579	452	254	374	187	168	139
Gly	306	247	319	283	247	245	143	179	220
Ile	652	309	452	343	276	285	261	330	308
Leu	503	109	205	151	128	153	148	114	154
Lys	1498	1201	1409	1355	929	1236	906	1027	1495
Phe	298	151	273	225	157	261	177	243	359
Ser	1008	855	1113	1030	826	874	561	563	741
Thr	534	471	575	550	472	461	293	127	370
Trp	246	179	282	238	170	175	127	143	223
Tyr	**2822**	**2709**	**3488**	**3146**	**2318**	**2535**	**3112**	**3132**	**5023**
Val	596	598	792	670	501	542	372	412	477
Total	**18272**	**14066**	**18179**	**16528**	**12435**	**13530**	**11920**	**11701**	**16914**

5 Discussion

Plants can be grouped according to their seed dehydration tolerance into orthodox and recalcitrant seed types, with intermediate states. A high degree of vacuolization and an active metabolic state can be related to seed recalcitrance, and up to now in mature palm zygotic embryos the presence of vacuoles, Golgi complex and endoplasmatic reticulum has been described only in *Euterpe edulis* (Panza *et al.*, 2004). We observed a high degree of vacuolization associated with a very active metabolic state of peach palm zygotic embryos. This supports the classification of peach palm as having recalcitrant seeds (Bovi *et al.*, 2004). In addition, the cotyledonary blade of peach palm zygotic embryos was not able to grow after partial dehydration of zygotic embryos as part of a cryopreservation protocol (Steinmacher *et al.*, 2007d). Therefore, as for *E. edulis* embryos, *B. gasipaes* embryos should be considered to be a developing seedling.

In *E. edulis* the factors leading to the lack of accumulation of proteins remains to be investigated (Panza *et al.*, 2004). We assume that storage proteins present in the zygotic embryos are broken down during the post-maturation phase of the zygotic embryo to release nitrogen in the form of amino acids for the maintenance of an active metabolic state in the zygotic embryos, leaving in turn empty vacuoles in the mature zygotic embryos. In zygotic embryos of oil palm (*Elaeis guineensis* Jacq.), endoproteinases able to breakdown storage proteins were found only at the onset of germination (Aberlenc-Bertossi *et al.*, 2008). It remains to be elucidated if in recalcitrant species endoproteinases are activated already at the post-maturation phase of zygotic embryo development.

Based on the orientation of the embryo axis proposed by DeMason (1988), mature zygotic embryos of peach palm are morphologically similar to those described for *Washingtonia fillifera*, *Euterpe edulis* and *E. oleracea* (Costa el al., 2008; DeMason, 1988; Panza *et al.*, 2004), all having a axial linear type with epicotyls oblique to the cotyledon (DeMason, 1988).

Germination of peach palm follows the adjacent type. This is characterized by the extension of the cotyledonary blade extruding from the proximal part of the embryo, forming a germinative button with seedling development adjacent to the seed (Pinheiro, 2001). The cotyledonary blade expands resulting in the haustorium, which grows inside the seed and absorbs the breakdown compounds of the endosperm. The structure and ultrastructure of the haustorium in the present study revealed similarities with date palm, including the presence of epithelial cells, followed by parenchymatic cells with numerous plasmodesmata between the

cells, and large intercellular spaces (DeMason, 1985).

Starch was found specifically in the parenchymatic cells of the cotyledonary blade and haustorium, but not in the endosperm of peach palm. Starch accumulation was absent in the cotyledonary blade before germination of *E. guineensis*, *W. filifera* and *P. dactylifera* seeds, appearing only after germination (Alang, 1988; DeMason, 1988; DeMason, 1985). In *Euterpe edulis*, the parenchymatic cells frequently exhibited starch grains (Panza *et al.*, 2004). In coconut and oil palm, the glyoxylate cycle takes place specifically in the haustorium during germination (Balachandran and Arumughan, 1995; Oo and Stumpf, 1983). This enzymatic cycle converts the absorbed triacylglycerol and free fatty acids from the endosperm into sugars, which in turn may be accumulated as starch in the haustorium. In date palm, glyoxysomes were also observed in the haustorium (DeMason, 1985).

In the endosperm of peach palm seeds before germination, all cells are similar, with one nucleus, numerous lipid bodies and two to five PSV. The phenolic substances found in the seed testa can be regarded as a protection mechanism (Vermerris and Nicholson, 2006) and also have the capacity to inhibit enzymatic activities (Nicholson *et al.*, 1992). In date palm seeds, an extract of the seed coat revealed the presence of a potent hydrolase inhibitor, which remains to be identified (DeMason *et al.*, 1985).

Endosperm cells lying immediately adjacent to the zygotic embryo were thin-walled with sparse contents or already crushed. Additionally, the presence of thin-walled cells at the micropylar endosperm with degraded content resulted in a ring at the opercule. The micropylar endosperm has recently been shown to be related to germination, facilitating the embryo's emergence in hard-endosperm seeds (Gong *et al.*, 2005). In *E. edulis*, no evidence of mobilization of endosperm storage compounds before germination was observed and the endosperm appeared to be in an inactive state (Panza *et al.*, 2004). In *Asparagus officinalis* and date palm, endosperm cells in the vicinity of the embryo also failed to accumulate reserves and had thin-walled cells (Willians *et al.*, 2001; DeMason *et al.*, 1983). In the present study, it must be determined if the empty cells near the zygotic embryos result from zygotic embryo expansion or from protein mobilization before germination.

The endosperm of palms is formed by carbohydrate rich and thick-walled cells; in the present study, positive staining for calcofluor and PAS-reaction confirmed this. PAS reaction was observed uniformly over the whole cell wall. In date palm at least three different regions of the cell wall could be distinguished based on PAS-staining (DeMason *et al.*, 1983). Hence, differences in the sugar composition of the cell wall are expected to occur. Hydrolysis of cell wall components, evidenced by Schiff's reaction, occurred very abruptly after the PSV

were emptied. In date palm it has been shown that cell wall hydrolases are stored in the endosperm within the storage protein vacuoles (DeMason *et al.*, 1985), which are in turn secreted upon vacuole degradation.

Proteins from peach palm endosperm were partially characterized based on salt solubility and SDS-PAGE under reducing and non-reducing conditions, and a wide range of molecular weights was observed. Under non-reducing conditions, high molecular weight proteins were found that were broken down into numerous minor bands under reducing conditions. The presence of disulfide-bounded high molecular weight proteins was also described in coconut (DeMason and Sekhar, 1990), but their function or localization in the cells remains to be characterized.

Based on salt solubility and molecular weight of the proteins, globulins present in the endosperm of peach palm could be recognized. Similar to oil palm 7S vicilin (Morcillo *et al.*, 1997), in peach palm 7S vicilin consisted of three similar polypeptides. Sequencing protein fragments through MS/MS confirmed the high homology with vicilins from other species, notably oil palm, and showed that the three subunits were identical in their peptide sequences. The PAS-reaction of the histological sections also reacted to the protein vacuoles, suggesting glycosylation of the proteins. Different glycosylation patterns might explain different migration coefficients of the same core protein in SDS-PAGE, as suggested by the observation that the glycosylation pattern of the vicilin-like protein from *Nicotiana sylvestris* is not uniform among subunits (Gerlach *et al.*, 2009).

An 11S disulphide-bounded storage protein is also present in peach palm endosperm. This showed a high degree of sequence similarity to the glutelin storage protein found in oil palm (Chan *et al.*, 2001). Cocosin, the 11S globulin from coconut, was also found to have disulphide bonds (Garcia *et al.*, 2005). In fact, this is a conserved characteristic for 11S globulin storage proteins, which usually consist of two subunits: acidic and basic disulphide-bounded chains (Müntz, 1998). Genomic analysis revealed several copies of the glutelin gene in the oil palm genome, which were expressed in endosperm but not in zygotic embryos of this species (Rival and Parveez, 2005). At the protein level, it has also been shown in zygotic embryos of *E. guineensis* that no proteins containing disulphide-bonded subunits and no 11S storage protein exist (Morcillo *et al.*, 1997).

The PSV in the endosperm of peach palm could be divided into a matrix containing one to three protein crystalloids. Protein crystalloids were also observed in the endosperm of *Elaeis guineensis* (Alang, 1981), *Washingtonia filifera* (DeMason, 1986), *Cocos nucifera* (DeMason *et al.*, 1990) and *Euterpe edulis* (Panza *et al.*, 2004), but not in endosperm cells of

date palm (DeMason *et al.*, 1983). The role of protein crystalloids within protein PSV is still unknown, but the matrix and crystalloids appear to contain different types of proteins (Jiang *et al.*, 2000). In coconut, 7S and 11S globulins were found in both the protein matrix and the crystalloids, but in different concentrations (DeMason *et al.*, 1990).

Histological analysis of the endosperm during germination yielded results similar to those in date palm, correlating the extent of endosperm breakdown to the rate of haustorial growth (DeMason *et al.*, 1985), resulting in a gradient of endosperm breakdown. This included the presence of intact cells and degenerate cells, as well as crushed cells. The first modifications occurred in the storage protein vacuoles.

Alterations in the low salt-soluble protein profile based on SDS-PAGE were observed during germination, with the appearance of several breakdown products in the range of 25 to 45 kDa. The smallest subunit of the 7S vicilin-like protein was present during the whole period evaluated. A similar pattern of storage protein breakdown was reported for *Pisum sativum* (Basha and Beevers, 1975), where the largest subunit of the major storage protein was hydrolized first during germination. In common bean, on the other hand, the smallest subunit was degraded more rapidly than the larger subunits (Nielsen and Liener, 1984).

Differences in the free amino acid concentrations between the endosperm and haustorium were found in the present study. Although the endosperm of peach palm contains large amounts of storage proteins, this tissue had a lower amount of free amino acids in comparison with the haustorium. During germination of barley seeds, storage proteins are hydrolyzed in the endosperm, resulting into a mixture of short oligopeptides and free amino acids (Higgins and Payne, 1977). These are taken up into the scutellum – partially homologous to the haustorium of palms – and the amino acids are than liberated from the peptides, further metabolized or transferred to the growing seedling. In castor bean, a dicotyledonous species, high levels of free amino acids were found in the endosperm and then transported to the cotyledon (Robinson and Beevers, 1981). In the gymnosperm *Pinus taeda*, the megagametophyte was able to breakdown storage proteins and export free amino acids, even in the absence of the seedling (King and Gifford, 1997). More studies are needed to reveal if the endosperm of peach palm is not able to completely hydrolyze the storage proteins or if free amino acids are rapidly absorbed by the growing haustorium. The fact that tyrosine and GABA became the most common free amino acids in peach palm endosperm also deserves more attention.

The present study evaluated the morpho-histological and biochemical aspects of peach palm seed germination. Histological and ultrastructural analyses of the zygotic embryo revealed their active metabolic state, which is related to seed recalcitrance. Globulin storage proteins, which can be a marker for somatic embryo quality, were partially characterized. We suggest that several points of the biology of palm seed germination must be critically re-analyzed and several questions still remain to be answered. For example, the mechanisms controlling the breakdown of storage compounds are unknown. In the present study, the sudden degradation of the cell wall was remarkable and deserves further study. Similarly, the large difference in free amino acid concentrations in the endosperm and in haustorium, even though both tissues are in close contact, suggests that the mobilization of the storage proteins to the growing plantlet is a highly controlled mechanism, but it has not yet been well studied.

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Chapter V:

PEACH PALM ENDOSPERM CAN SYNTHESIZE PROTEINS DE NOVO DURING SEED GERMINATION AND UNDERGOES PROGRAMMED CELL DEATH

1 Abstract

Several morpho-physiological aspects of palm seed germination are unknown. In palms the embryo's cotyledonary blade develops into the haustorium, which grows inside the endosperm during germination and is thought to regulate the degradation and absorption of its storage compounds. In *Phoenix dactylifera*, a species with orthodox seeds, the endosperm was shown to be a senescent tissue without the capacity for *de novo* protein synthesis. This suggests that all enzymes necessary for germination are already present in the endosperm in an inactive form or they are secreted by the haustorium. In *Bactris gasipaes*, a palm species with recalcitrant seeds, we used ultrastructural analysis and immuno-localization and found that *de novo* protein synthesis occurs in the endosperm during germination and that this tissue also undergoes programmed cell death (PCD). Polysome analysis also supports the observation of *de novo* protein synthesis. PCD is a highly regulated mechanism, which requires *de novo* protein synthesis, where KDEL-tailed cystein endoproteinase (KDEL-CysEP) is involved. In *B. gasipaes*, *de novo* synthesis of KDEL-CysEP occurred in the endosperm during germination and accumulated on endosperm cell walls. Additional PCD features included cytoplasm shrinkage and acidification. These observations may help explain the recalcitrance of *B. gasipaes* seeds, as dehydration-sensitivity is generally related to an active cell metabolism.

Key words: Bactris gasipaes, KDEL-tailed cystein endoproteinase, PCD, acidification, Evan´s blue, ultrastrucutre.

2 Introduction

Peach palm (*Bactris gasipaes* Kunth – Arecaceae) is a Neotropical palm that was probably domesticated in southwestern Amazonia. The palm is important today for both fruit and heart-of-palm production. The fruit is an excellent food, with starches, lipids, carotene and all essential amino acids (Yuyama *et al.*, 2003). The heart-of-palm is composed of unexpanded juvenile leaves and sub-apical tissue, and has low concentrations of peroxidases and polyphenoloxidase, allowing *in natura* commercialization, as well as processing (Clement *et al.*, 1999). The peach palm for heart-of-palm agribusiness is a modern success story but it is still an almost exclusively Neotropical crop, with only experimental areas in Africa, Asia and Oceania, and no named cultivars have been brought to market (Clement, 2008).

Peach palm has recalcitrant seeds (Bovi *et al.*, 2004) and the mechanisms imposing this dehydration-sensitivity are not completely understood. The primary difference between recalcitrant and orthodox seeds is that recalcitrant seeds are actively metabolic when they are shed, in contrast to orthodox types that are quiescent (Berjak and Pammenter, 2008). Using ultrastructural analyses it has been shown that zygotic embryos of peach palm are abundantly vacuolated with numerous organelles, indicating very active metabolism (Chapter IV). Similar results were described in *Euterpe edulis*, another palm species with recalcitrant seeds (Panza *et al.*, 2004). The presence of polysomes (e.g., large cytoplasmic assemblies made up of several ribosomes spaced along a single mRNA molecule) may also be an indicative of the highly active metabolic state of seeds (Bewley, 1997).

Palm seeds have testas formed by cells rich in phenolic compounds surrounding thick-walled endosperm cells and the zygotic embryo. Neither transfer cells nor aleurone layers are observed in palm seeds (DeMason *et al.*, 1985; Panza *et al.*, 2004; Chapter IV) and endosperm cells are considered living (DeMason *et al.*, 1985; Panza *et al.*, 2004), although unable to synthesize proteins *de novo* during germination. In date palm, detailed ultrastructural analysis of the endosperm revealed the presence of plastids and mitochondria with respiratory activity, but neither endoplasmatic reticulum nor Golgi complexes were found, suggesting lack of capacity for *de novo* protein synthesis (DeMason *et al.*, 1983).

The mechanisms that palm seed endosperms exhibit to make reserves available to the growing seedlings are unknown (Alang *et al.*, 1988; Lopes and Larkins, 1993; Chapter IV). Initially it was thought that the haustorium could be responsible for the secretion of all hydrolytic enzymes responsible for the breakdown of endosperm storage compounds

(Balasubramaniam *et al.*, 1973; Verdeil *et al.*, 2001). However, in date palm and peach palm (DeMason *et al.*, 1985; Chapter IV) the first modifications in the endosperm were observed in protein storage vacuoles (PSV), and cell walls were hydrolyzed only after cytoplasm collapse. If the haustorium was responsible for enzyme secretion, the first modifications would be expected to occur in the cell wall due the presence of cell membranes (DeMason *et al.*, 1985). Alternatively, hydrolytic enzymes might already be present in the endosperm before germination, either in an inactive form or in different compartments. This is the case of the enzyme α-galactosidase of date palm, responsible for cell wall hydrolysis, which was shown to be stored in PSV and to diffuse to the cell wall upon cell collapse (Sekhar and DeMason, 1990). However, also in date palm, proteinase activity was detected first in the endosperm and only at the onset of germination (DeMason *et al.*, 1985). This suggests that different mechanisms might control the breakdown of the endosperm in palm species, but the exact mechanisms are still largely unknown. Additionally, the fact that all reports suggest a living endosperm implies a possible third mechanism in palm endosperm breakdown, namely programmed cell death (PCD).

PCD is known to play a major role in the development and/or stress responses of all eukaryotes except those in the Cercozoa group (Deponte, 2008), and is a tightly regulated mechanism requiring *de novo* protein synthesis (Lam *et al.*, 2001). To the best of our knowledge there is no report of PCD in palm seed endosperm, however some factors indirectly indicate that this tissue undergoes PCD. It is known that endosperm of species that lack an aleurone layer must remain alive and capable of producing hydrolytic enzymes to mobilize reserves during germination (Lopes and Larkins, 1993), which usually occurs through PCD. Endosperm breakdown in palms occurs only in cells in the vicinity of the haustorium (DeMason *et al.*, 1985; Chapter IV), apparently in a highly controlled manner. The fact that storage proteins are the first storage components broken down following germination (DeMason *et al.*, 1990; Chapter IV) also indicates that some signal must be perceived by the cell to trigger this process.

Plant cells undergoing PCD express papain-type cysteine endoproteases with a C-terminal KDEL sequence (KDEL-CysEP) (Schmid *et al.*, 1999; Gietl and Schmid, 2001). The amino acid sequence KDEL (Lys-Asp-Glu-Leu) is a peptide signal for retention of proteins in the endoplasmic reticulum (Munro and Pelham, 1987). KDEL-CysEP proenzyme was found to be synthesized *de novo* in the endosperm of *Ricinus communis* during germination, budding off from the endoplasmic reticulum as large vesicles called ricinosomes (Schmid *et al.*, 1998; 2001). The signal peptide is removed upon acidification of ricinosomes resulting in

enzyme activity in its mature form (Shmid *et al.*, 2001). KDEL-tailed cysteine endopeptidases are unique in being able to digest the extensins that form the basic scaffold for cell wall formation (Helm *et al.*, 2008). This endoproteinase has been identified in the nucellus of maturing *R. communis* seeds (Greenwood *et al.*, 2005), in senescing petals of day lily (*Hemerocallis* spp; Schmid *et al.*, 1999) and during *Arabidopsis thaliana* seedling, flower, and root development, especially in tissues that collapse during final stages of PCD (Helm *et al.*, 2008). During postgerminative PCD of the endosperm of tomato (*Solanum lycopersicum;* DeBono and Greenwood, 2006) and *Picea glauca* megagametophyte (He and Kermode, 2003) the presence of ricinosomes was detected and associated with PCD, resulting in anther dehiscence in tomato (Senatore *et al.*, 2009).

In the present study, numerous findings show that peach palm endosperms are able to synthesize proteins *de novo* and undergo programmed cell death during germination. Ultrastructural analyses of the cells, the presence of polysomes during germination, the *de novo* synthesis of KDEL-CysEP and its localization, cytoplasm shrinkage and its acidification are also presented. These findings are discussed with reference to seed recalcitrance of peach palm.

3 Material and methods

3.1 Plant material

Seeds of peach palm from spineless populations were obtained from a commercial orchard (INACERES, Bahia, Brazil) and ICRAF (World Agroforestry Centre/International Centre for Research in Agroforestry). Seeds were allowed to germinate in plastic trays containing sand and watered with distilled water as previously described (Chapter IV). Samples were collected at different stages of germination, including: Stage 0 – control samples, seeds before germination; Stage 1 – extrusion of the germinative button; Stage 5 – eophyll emission and development of radicle; Stage 6 – development and growth of adventitious roots; and Stage 8 – final stage evaluated, eophyll completely open with well developed root system. These stages were chosen as they cover most steps of germination from initial extrusion until leaf emission accompanied by endosperm breakdown.

3.2 Light microscopy and ultrastructural analyses

For light microscopy, samples were fixed with 2 % (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.0) for 24 hours at 4°C, dehydrated in an ethanol series (30 % to 100 %), embedded in LR White resin (London Resin Co Ltd, London) and allowed to polymerize

according to the manufacturer's instructions. Specimens were cut 1 μm thick in a semi-automate microtome (Reichert Ultracut S, Leica®) and mounted onto glass slides. Sections were double-stained with PAS (Periodic Acid-Schiff's) and naphtol-blue black (Fisher, 1969).

For fluorescence immunolocalization sections were blocked with 5 % BSA in PBS for one hour, followed by incubation with primary monoclonal antibodies against CysEP (Schmid *et al.*, 1999). The sections were mounted with antifading citifluor (Citifluor Ltd, London), examined using an Olympus BH-2 microscope and photographed with a ColorView IIIu (Soft Imaging System, GmbH). Negative control samples were obtained omitting the primary antibody. For electron microscope immunolabelling samples were chemically fixed and embedded (Šamaj *et al.*, 2000), and ultrathin sections (80-100 nm) were collected on a nickel grid and incubated with the CysEP antibody (Schmid *et al.*, 1998)

For general transmission electron microscopy, samples were fixed in 4 % glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.0) for 2 h at room temperature, then transferred to fresh fixative overnight at 4°C and postfixed in buffered 1 % OsO_4 at 4°C for 2 hours. Samples were dehydrated in a graded acetone series and embedded in Spurr's resin. Ultrathin sections (80-100 nm) were cut with an Ultracut E ultramicrotome (Reichert-Jung, Vienna, Austria), and stained with 1 % uranyl acetate and 0.1 % lead citrate for 10 min each.

3.3 Polysome analyses

Endosperm samples at different stages of germination were collected, immediately frozen in liquid nitrogen and ground to a fine powder in a mixer Mill (MM400, Retsch, Haan, Germany). Polysomes were purified and submitted to a sucrose gradient (Rogalski *et al.*, 2008). For polysome isolation, 1 ml of extraction buffer [1 x extraction solution (0.2 M Tris, 0.2 M KCl, 0.035 M $MgCl_2.6H_2O$, 0.025 M EGTA, pH 9.0 with KOH), 0.2 M sucrose, 1 % Triton X-100, 2 % polyoxyethylen-10-tridecyl-ether, 0.5 mg ml^{-1} heparin, 100 mM β-mercaptoethanol, 100 μg ml^{-1} chloramphenicol, 25 μg ml^{-1} cycloheximide] was used for each 200 mg of plant material. The samples were incubated for 10 min on ice and centrifuged for 5 min at 13200 x g at 4°C to remove nuclei and insoluble materials. Thereafter 500 μl of supernatant were layered onto sucrose step gradients [950 μl 15 %, 950 μl 30 %, 950 μl 40 % and 950 μl 55 % sucrose in TKM buffer (0.04 M Tris, 0.02 M KCl, 0.01 M $MgCl_2.6H_2O$, pH 8.5 with HCl) with addition of 50 mg ml^{-1} chloramphenicol and 100 mg ml^{-1} heparin]. The gradients were centrifuged for 80 min at 243356 x g at 4 °C and 6 fractions of 720 μl from each gradient were collected. Samples extracted with puromycin-enriched extraction buffer

were used as negative controls. Puromycin is an antibiotic that causes premature chain termination during translation in the ribosome. The collected fractions were diluted with 0.7 volume water before RNA isolation to reduce their sucrose content. RNA was extracted from individual fractions by adding EDTA (to a final concentration of 20 mM) and phenol/chloroform (0.2:1 v/v). After addition of 0.1 volume 3 M sodium acetate, pH 4.8, the RNA was precipitated with 1 volume isopropanol. The RNA pellets were redissolved in 30 µL water and aliquots of 1.5 µL were denatured for 10 min at 67°C and then loaded onto denaturing formaldehyde-containing agarose gels. The gel contained 0.1 % ethidium bromide and was visualized under UV excitation. RNA was additionally quantified with a NanoDrop® ND-1000 spectrophotometer.

3.4 SDS-PAGE and immunoblot analysis

Buffer soluble proteins were extracted as previously described (Chapter IV). SDS-PAGE was performed with a 12 % acrylamide separating gel and a 4 % acrylamide stacking gel (Laemmli, 1970) on 1 mm thick mini-gels in a Mini-Protean II electrophoresis cell (Bio-Rad Mini-Protean II). Each well was loaded with approximately 25 µg proteins. After electrophoresis, the gel was washed in methanol and prepared for electrotransfer to nitrocellulose (0.2 µm) (Schmid *et al.*, 1999) using an immunoblotting apparatus (Bio-Rad Mini Trans-Blot Module). After electrotransfer, the nitrocellulose membrane was blocked in 5 % (w/v) BSA/TBS for 1 h and incubated with CysEP antibody 1:1000 in 1 % BSA/TBS overnight at 4°C. The membranes were washed at least three times on a rotary shaker and then incubated in goat anti-mouse-alkaline phosphatase-conjugated secondary antibody 1:3000 in 1 % BSA/TBS (w/v) for 2 h and washed three times in TBS. The presence of the KDEL-CysEP was visualized by using alkaline phosphatase-buffer with BCIP/NBT as substrate.

3.5 Endosperm acidification

Two methods were used to identify *in situ* pH alterations in peach palm endosperm. For extracellular *in situ* pH, germinating seeds were cut in half and incubated for 15 min at room temperature in a solution containing the pH indicator bromocresol purple in slightly alkaline water (Dominguez and Cejudo, 1999). Samples were washed in tap water and observed under a stereomicroscope.

For cytoplasmatic pH, samples of the endosperm were cut 200 μm thick with a Vibratome® (Vibratome Series 1000 Classic) and incubated in a solution containing 50 μM acridine orange (He *et al.*, 2007) for 2 h at room temperature and then incubated in water for 1 h at room temperature with gentle shaking. Confocal microscopy was performed with a LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany).

4 Results

4.1 Endosperm characterization

Endosperm of peach palm seeds is divided into lateral endosperm and micropylar endosperm. Micropylar endosperm is constituted by a ring of thinner-walled cells (Figure 1A), while lateral endosperm cells are composed of thick cell walls, three to five PSV and numerous oleosomes (lipid bodies). All cells with cytoplasm contained a nucleus with nucleolus. During germination, the micropylar endosperm is eliminated by the extrusion of the germinative button and reserve mobilization occurred in cells of the lateral endosperm. Histochemical characterization by light microscopy upon germination revealed a specific sequence of lateral endosperm breakdown. The lateral endosperm could be divided into intact endosperm, degrading cells, empty cells and finally crushed cells in the vicinity of the haustorium (Figure 1). These aspects could be observed before germination (Figure 1B). The first modification was observed in the storage protein vacuoles, resulting in a granular appearance at the degrading region (Figure 1C). At this point, cell walls were still intact (Figure 1C). Further degeneration resulted in cells apparently empty of content but with still intact cell walls. As cell wall hydrolysis began in the lateral endosperm, a weaker PAS reaction is observed and cells are then crushed by the growing haustorium. These crushed cell walls had a more intense reaction to protein staining (Figure 1D).

Ultrastructural analyses revealed that cell walls are composed of three layers based on electron density; one dense area inside the cell wall and another at the middle lamella. The remaining cell wall showed less but uniform electron density (Figure 2A and 6A). One nucleus with a prominent nucleolus was present in each cell. The PSV were composed of the protein matrix and one to three protein crystals; PSV were surrounded by oleosomes (Figure 2B). Oleosomes and PSV occupy most of the cytoplasm volume and only small areas remained as cytosol. The presence of endoplasmatic reticulum was observed before germination (Figure 2C), as were several vesicles fusing to the cytoplasmatic membrane (Figure 2D). Mitochondria and possible plastids were also frequent.

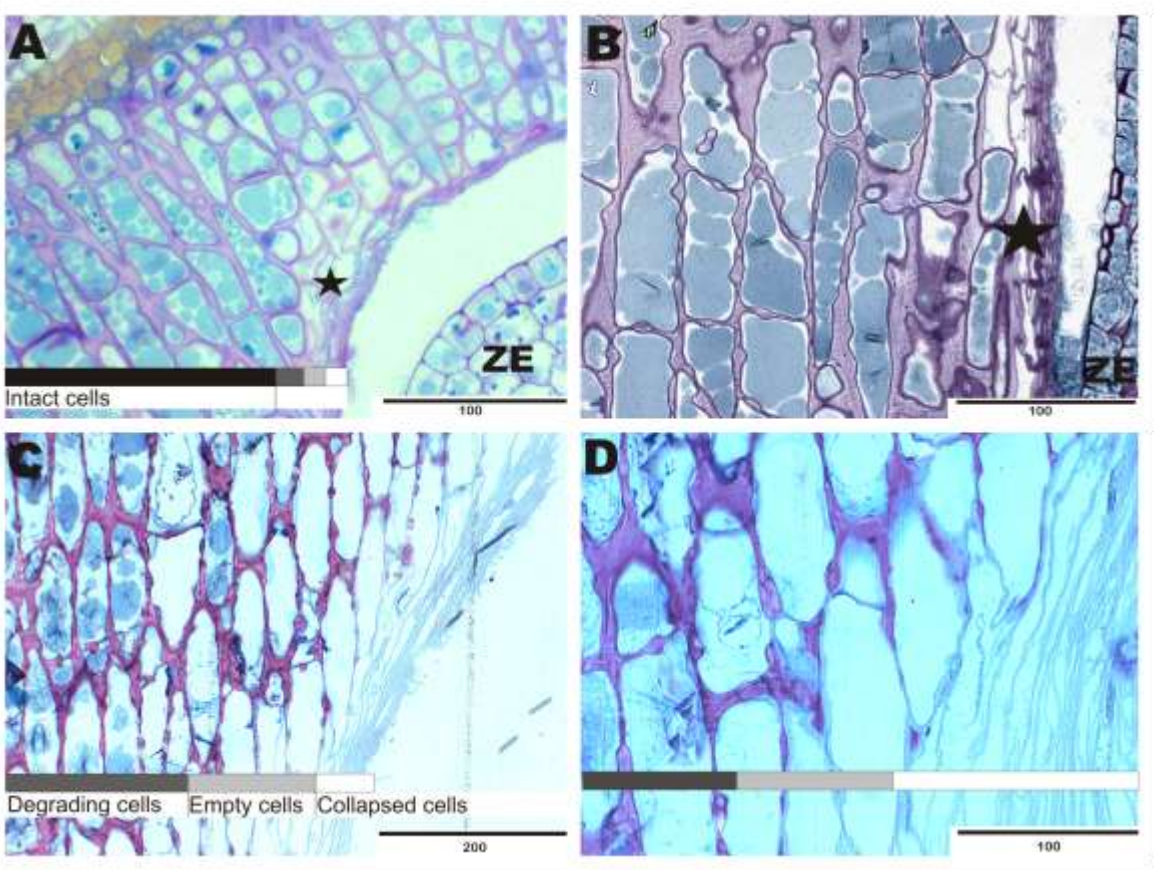

Figure 1 - Histochemical analyses of peach palm endosperm double-stained with PAS and NBB. A – Appearance of endosperm before germination showing degraded cells at the micropylar endosperm (star) and intact cells at the lateral endosperm (bar = 100 µm). B – Detailed view of the lateral endosperm near the zygotic embryo showing some degraded cells (star) (bar = 100 µm). C - Sequence of lateral endosperm breakdown in the vicinity of the haustorium. Note the sudden cell wall degradation (bar = 200 µm). D – Detailed view showing the cell wall degradation after cell content collapse (bar = 100 µm). ZE – zygotic embryos.

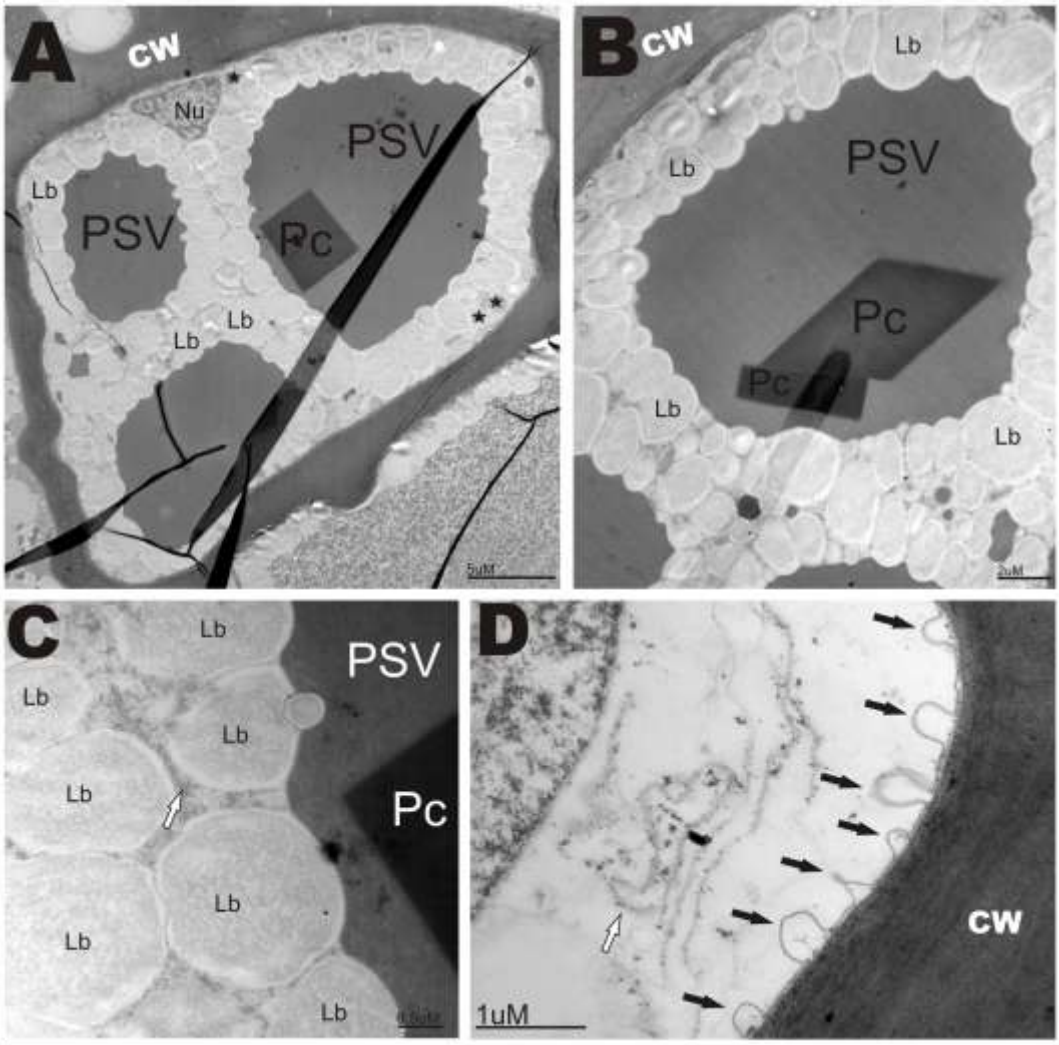

Figure 2 - Ultrastructural analyses of endosperm cells before germination. A – General view of a lateral endosperm cell, showing the cell wall (CW) and the presence of nucleus (Nu), oleosomes (Lb), protein storage vacuoles (PSV) with protein crystals (Pc) and mitochondria (stars) (bar = 5 µm). B – View of the PSV, showing the presence of two protein crystals (pc) surrounded by oleosomes (lb) (bar = 2 µm). C – Presence of endoplasmatic reticulum in the endoperm cells before germination (arrow) (bar = 0.5 µm). D – Vesicles fusing to the cytoplasmatic membrane (black arrows) as well as endoplasmatic reticulum before germination (bar = 1 µm).

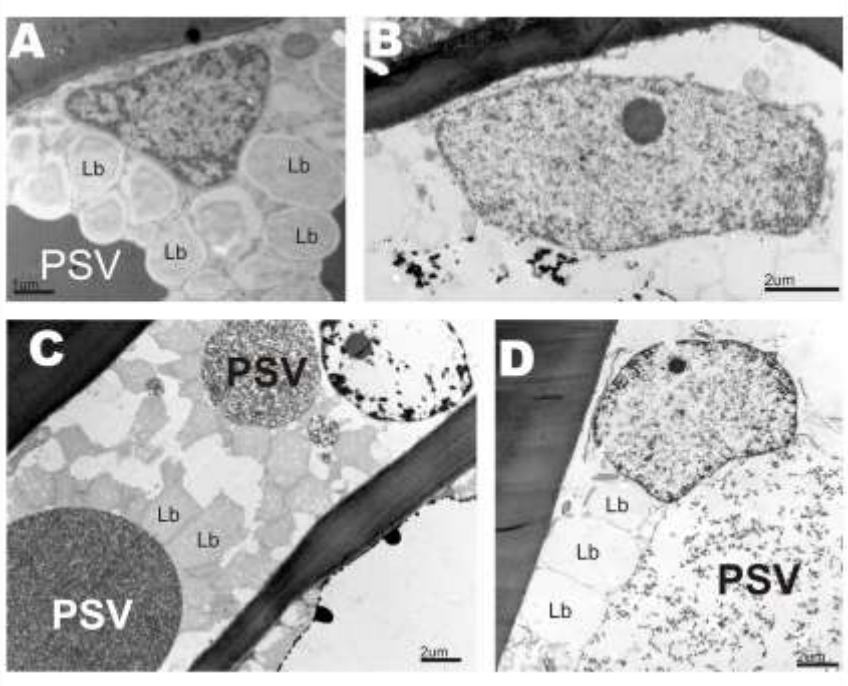

Figure 3 - Ultrastructural aspects of endosperm cells before and following germination. A – View of cell nucleus, protein storage vacuole (PSV) and oleosomes (Lb) (bar = 1 µm). B – Presence of prominent nucleolus and mitochondria (bar = 2 µm). C – Appearance of the PSV following storage protein hydrolysis. Note the chromatin condensation in the nucleus (bar = 2 µm). D – Further stages of storage protein hydrolysis and nucleus modifications. Note the presence of numerous organelles (bar = 2 µm).

Upon germination, ultrastructural alterations the cytoplasm content were observed and changes in PSV include the transition from a uniform matrix to a granular content (Figure 2 and 3). At this point several organelles were still present, including Golgi complexes and abundant endoplasmatic reticulum (Figure 3D). Thereafter, cytoplasm shrinkage was observed (Figure 4A), although cytoplasmatic content including the nucleus were still observed (Figure 4B). Thereafter, the cytoplasm shrinkage culminated in the formation of apoptotic bodies (Figure 4C), observed principally in the crushed cell area near the growing haustorium (Figure 4D). Concomitant with this, ultrastructural alterations of the nucleus were observed: chromatin was initially observed well distributed in the nucleus and later displaced to the nucleus margin (Figure 3). Evans blue staining occurred mostly in cells in the vicinity of the growing haustorium (Figure 5), confirming the occurrence of PCD in these cells. Finally, ultrastructural analysis of the cell wall also identified rapid hydrolysis based on the electron density in the cell wall (Figure 6B). Initial modification of the cell wall was observed in the inner face of the wall (Figure 6C), associated with numerous vesicles (Figure 6D). Upon its hydrolysis, weaker electron density was observed (Figure 6E).

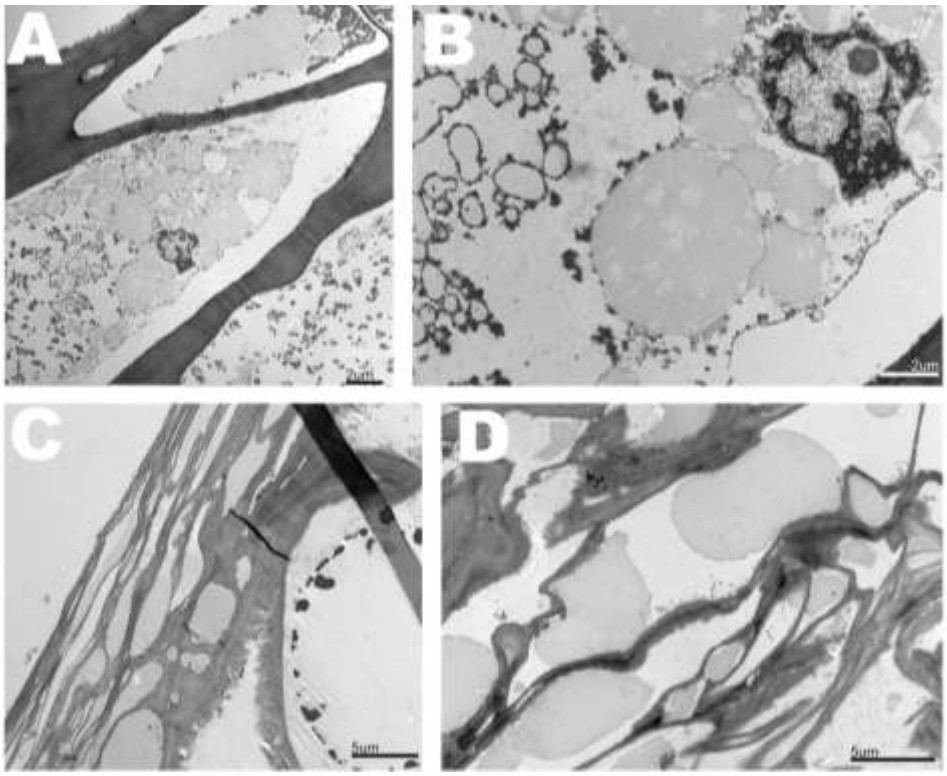

Figure 4 - Ultrastructural aspects of lateral endosperm cells in the vicinity of the growing haustorium following germination. A – Cytoplasm shrinkage resulting in the formation of apoptotic bodies (bar = 2 µm). B – Appearance of the nucleus, note intense chromatic condensation as well as the presence of several vesicles (bar = 2 µm). C – View of the crushed cells with the presence of numerous apoptotic bodies (bar = 5 µm). D – Detailed view of the apoptotic bodies within the crushed cells (bar = 5 µm).

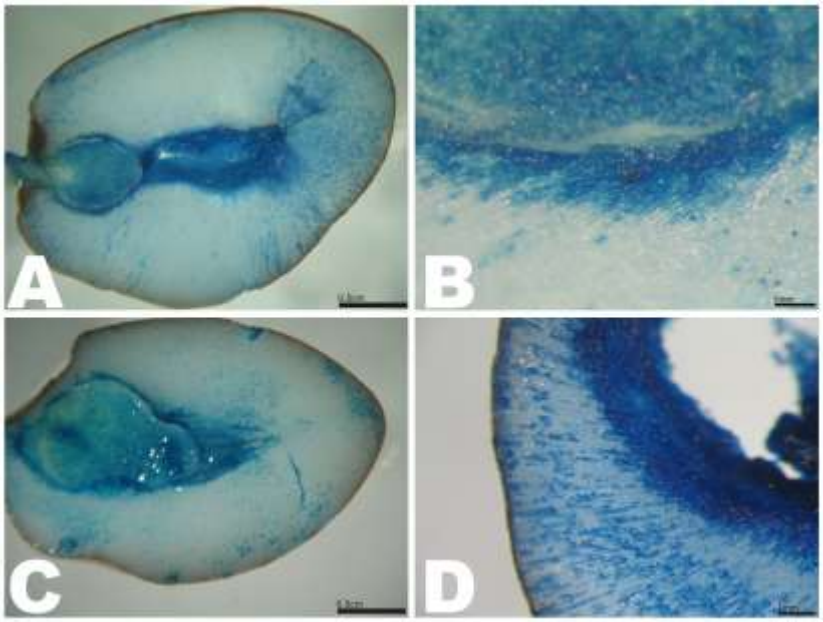

Figure 5 - Staining pattern of the endosperm with Evans blue revealing the occurrence of programmed cell death in cells in the vicinity of the growing haustorium. A – At germination stage 5 (bar = 0.5 cm). B – Detailed view of A (bar = 1 mm). C – At germination stage 6 (bar = 0.5 cm). D – Detailed view of C (bar = 1 mm).

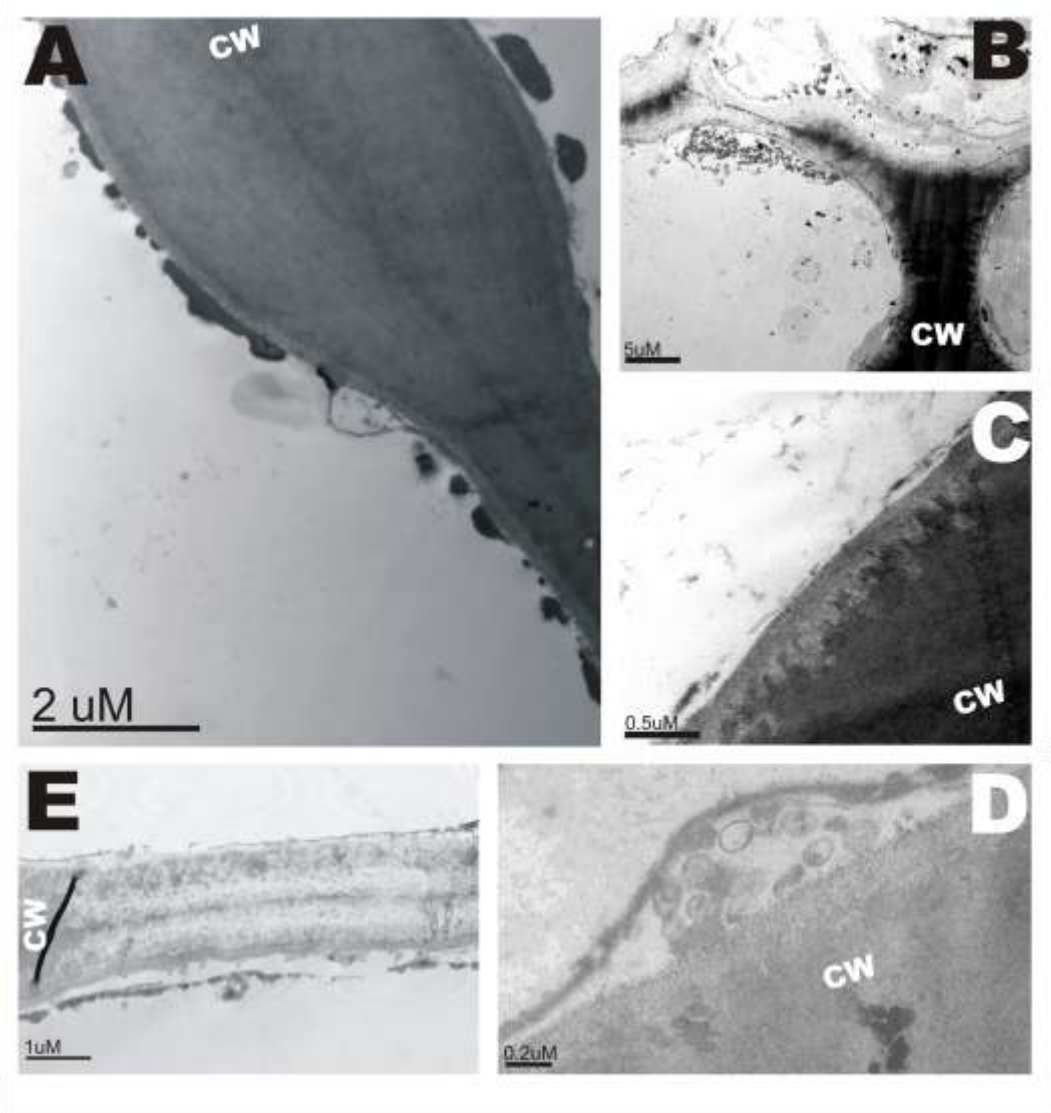

Figure 6 - Ultrastructural analyses of endosperm cell wall (cw) hydrolysis following germination of peach palm seeds. A – Appearance of the cell wall before hydrolysis, revealing three different layers of electron density (bar = 2 µm). B – Sudden hydrolysis of the cell wall (bar = 5 µm). C – Initial hydrolysis in the inner cell wall (bar = 0.5 µm). E – Low electron density of the cell wall (cw) after the hydrolysis of its components (bar = 1 µm). D – Presence of several vesicles fusing to cytoplasmatic membrane (bar = 0.2 µm).

4.2 Polysome analyses

In the present study, polysome analyses identified the active translation of mRNA molecules into proteins, observed when RNA molecules bound to ribosome sediments in the most concentrated sucrose gradient layer. Polysome associations changed during seed germination and after agarose-gel separation; RNA molecules were found in every fraction of the sucrose gradient up to germination stage 6; little signal was found in the bottom sucrose fractions of stage 8 and the negative control (Figure 7).

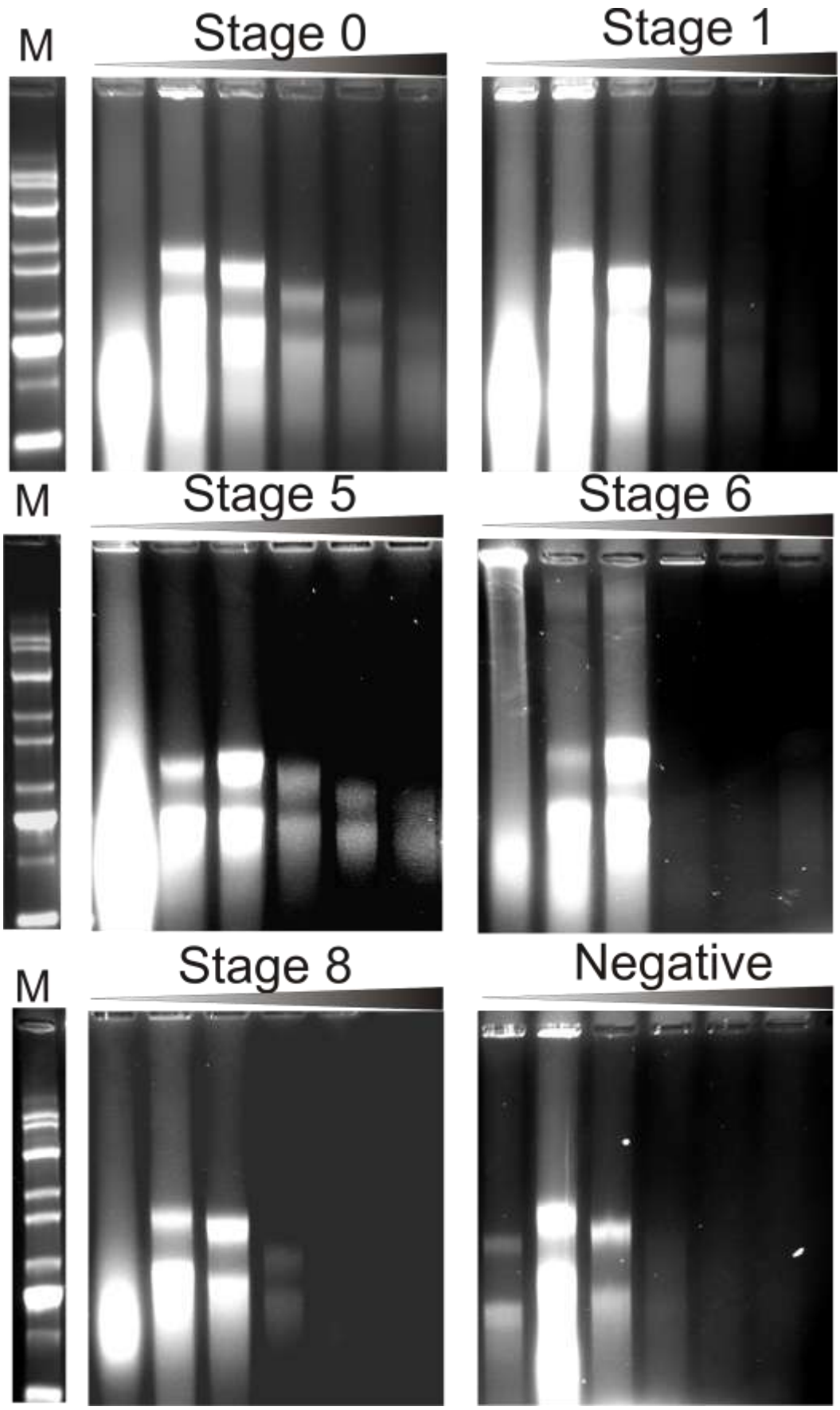

Figure 7 - Polysome analyses after sucrose gradient centrifugation on ethidium bromide-stained agarose gel from different stages of germination.

The total RNA recovered from the endosperm was lower during each successive germination stage (728 ng.µl^{-1} from stage 0 to 354 ng.µl^{-1} from stage 8) and quantifying the RNA content in each sucrose fraction showed a shift in polysome associations during germination. During the first germination stages large amounts of RNA were observed in all fractions of the sucrose gradient. During the final germination stages, reduced levels were observed in the first fractions, but significant amounts were still observed in fraction 3 (Figure 8). These results show that *de novo* protein synthesis occurs during peach palm seed germination, however the total RNA content and, consequently, gene translation were gradually down-regulated during this process.

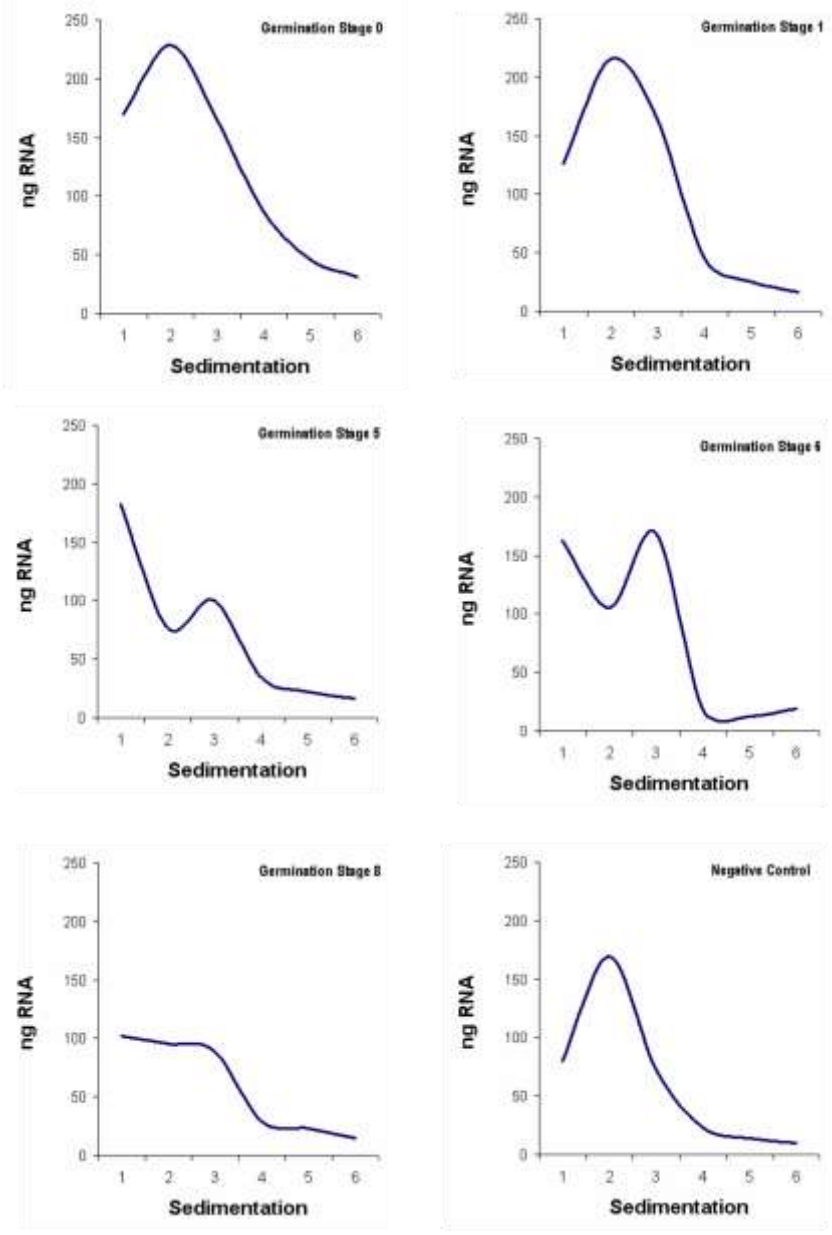

Figure 8 - Total RNA quantification after sucrose gradient centrifugation from different germination stages. RNA [ng]

4.3 The presence of cystein endoproteinase

Buffer-soluble proteins from endosperm at different germination stages were extracted and separated by SDS-PAGE and immunoblotted. The peach palm storage protein 7S vicilin-like globulin was composed by three subunits between 45 to 67 kDa. During germination two of the subunits of the 7S vicilin-like globulin completely broke down by the final stages of germination; one subunit was still present (Figure 9).

Immunoblot analysis of KDEL-CysEP identified two polypeptides, the larger with approximately 40 kDA and the smaller with approximately 35 kDa (Figure 9), which represent the proenzyme and its mature form, respectively. In the control samples, both polypeptides were already present and at the final stage of germination (Stage 8) only the mature enzyme could be detected (Figure 9). A third polypetide (approximately 43 kDa) was also identified during advanced germination stages and may be a cross-reaction product of the polyclonal antibody.

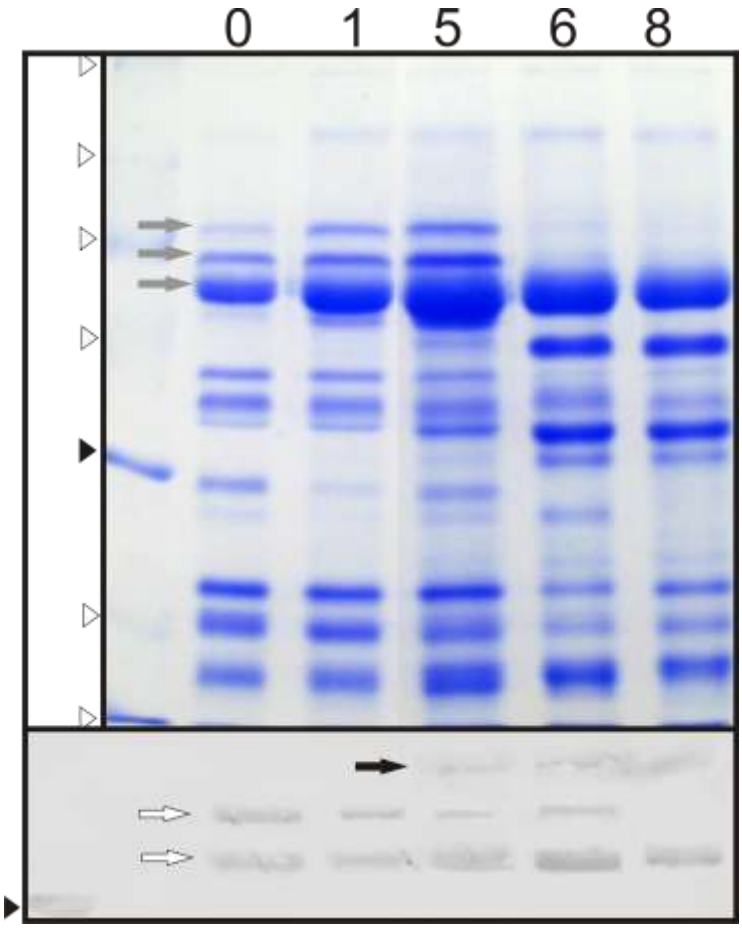

Figure 9 - SDS-PAGE of low salt buffer-soluble proteins and western-blot showing the presence of KDEL-CysEP in peach palm endosperm at different stages of germination. Grey arrows represent the three-subunits of the 7S vicilin-like storage protein. Black arrowhead corresponds to the 30 kDa molecular marker. White arrows correspond to the presence of KDEL-CysEP and black arrow to possible cross-reaction product.

The presence of KDEL-CysEP was also detected by immunofluorescence localization in the samples before germination. Signal was observed only in cells in the vicinity of the cotyledonary blade and on the crushed cell walls (Figure 10A and B). In later stages of germination the presence KDEL-CysEP was also observed in the inner cells, but always in those cells near the haustorium (Figure 10C). Large vesicles were observed in the cells but usually in low number and usually at the periphery of the cytoplasm (Figure 10D). The presence of CysEP was also detected on cell walls and an intense signal was verified on the crushed cells (Figure 10E).

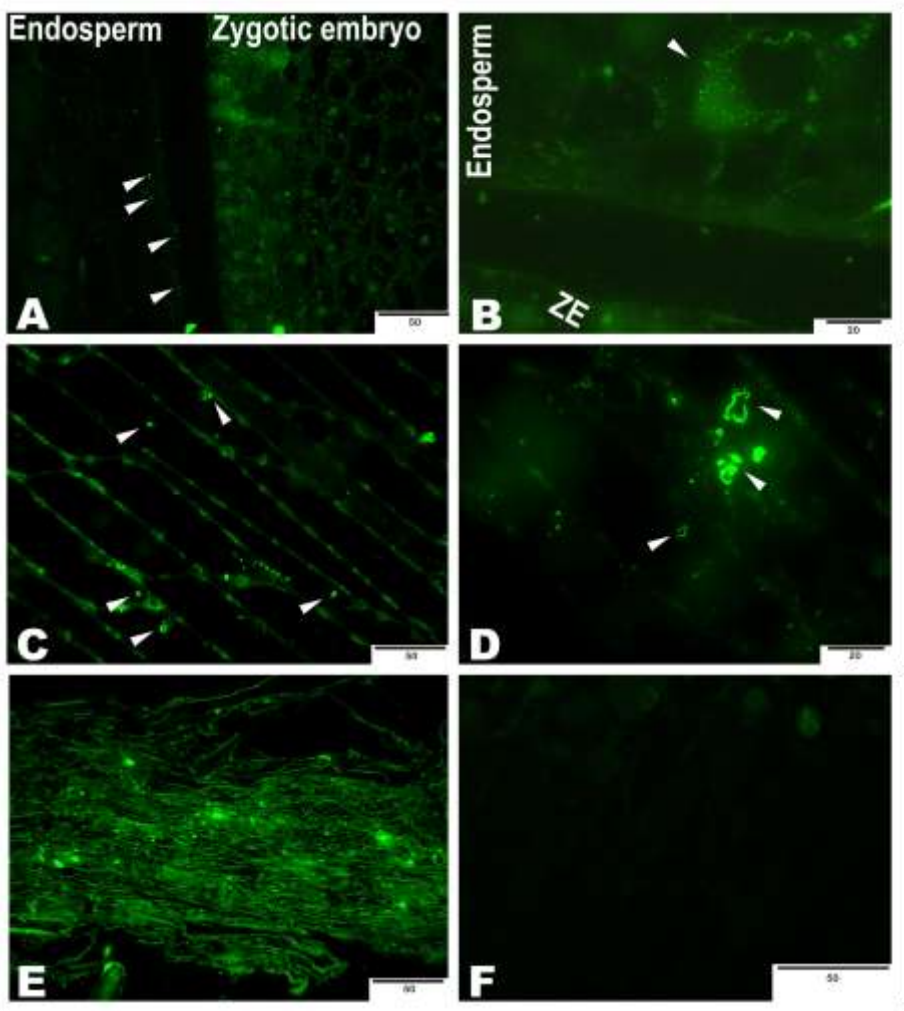

Figure 10 - Immunofluorescence localization of the KDEL-CysEP before and following peach palm seed germination. A – Presence of KDEL-CysEP in the endosperm cells before germination. Note the presence of signal only on cells near the zygotic embryo (arrowheads) (bar = 50 µm). B – Detailed view of the cells showing the presence of KDEL-CysEP (bar = 20 µm). C – Presence of KDEL-CysEP in endosperm cells following germination. Note the signal in vesicles (arrowheads) and on the cell wall (bar = 50 µm). D – Detailed view of the vesicles (arrowheads) (bar = 20 µm). E – Presence of intense fluorescent signal on the cell wall of crushed cells (bar = 50 µm). F – Negative control, where the first antibody was omitted, revealing no fluorescent signal (bar = 50 µm).

Immunogold localization identified the presence of KDEL-CysEP in the endoplasmatic reticulum of the cells (Figure 11A). This resulted in the formation of large membrane-bound vesicles (Figure 11B and C). These results show the *de novo* synthesis of KDEL-CysEP in endosperm cells. The mature enzyme accumulates later on the cell wall upon cell collapse (Figure 11D and E). In the crushed cell walls, high densities of gold particles were found (Figure 11F and G).

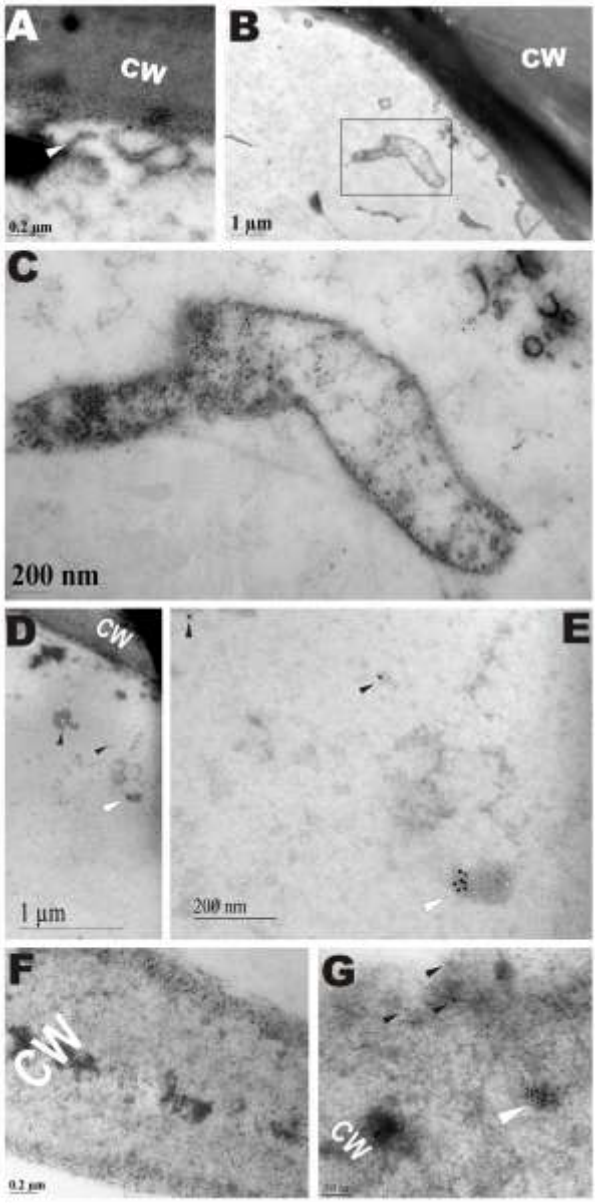

Figure 11 - Immunogold localization of KDEL-CysEP during germination of peach palm seeds. A – Presence of gold particles (arrowhead) associated with the endoplasmatic reticulum (bar = 0.2 µm). B – Presence of vesicle (ricinosome) (bar = 1 µm). C – Detailed view of the ricinosome (bar = 200 nm). D – Presence of gold particles on the cytoplasm being secreted in direction of cell wall upon collapse (bar = 1 µm). E – Detailed view of D (bar = 200 nm). F – High density of gold particles on the cell wall (cw) (bar = 0.2 µm). G – Presence of gold particles on the cell wall of crushed cells. Note the presence of agglomerates (white arrowhead) and single gold particles (black arrowhead) on the cell wall (bar = 100 nm).

4.4 Endosperm acidification

The pH indicator bromocresol purple was used to verify the extracellular *in situ* pH in peach palm endosperm. This pH indicator stains purple at pH 6.8 or above and yellow at pH 5.2 or below. The pattern of endosperm acidification started acid (yellow) before germination and during germination stage 1 in the cotyledonary blade (Figure 12A), while most of the endosperm stained purple, except in the area adjacent to the cotyledonary blade (Figure 12B). As germination progressed the pattern remained similar, with the endosperm in purple except in the vicinity of the haustorium (Figure 12C). However, extracellular pH in the haustorium showed an intense purple colour, indicating neutral pH. A yellow colour was observed only in the first cell layers of the haustorium (Figure 12D). During the final stages of germination this staining pattern continued to be observed (Figure 12E-F). To summarize, the extracellular pH of peach palm endosperm is in the neutral pH range during germination with acidification taking place only in the area with crushed cells. In the haustorium a neutral pH is established during development.

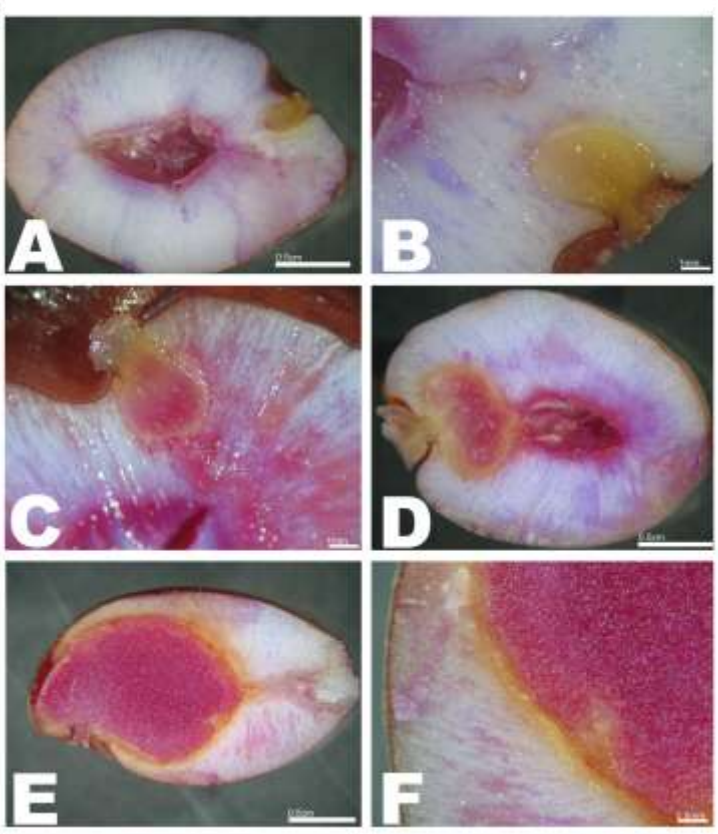

Figure 12 - Extracellular acidification of the endosperm shown by the pH indicator bromocresol purple. Yellow color represents pH 5.2 or lower, while purple color indicates pH 6.8 or above. A – At germination stage 1 (bar = 0.5 cm). B – Detailed view of A (bar = 1 mm). C – More advanced haustorium development at germination stage 1 (bar = 1 mm). D – At germination stage 5 (bar = 0.5 cm). E – At germination stage 6 bar = 0.5 cm). F – Detailed view of the acidification area in the vicinity of the haustorium (bar = 0.5 cm).

Using the pH-sensitive dye acridine orange a better picture of the pH in the endosperm of peach palm seeds during germination was obtained. This dye passes freely through membranes and accumulates in cell compartments when ionized, indicating a more acid pH than the extracellular medium. The pattern of endosperm acidification following germination is easily followed with acridine orange (Figure 13). At the onset of germination (Stage 1) acridine orange accumulates in endosperm cells only in an area adjacent to the cotyledonary blade (Figure 13A). Cells from the intact endosperm revealed low signal (Figure 13B). Major acidification remained concentrated in cells adjacent to the growing haustorium as germination progressed (Figure 13C). Our results also indicate that acidification occurred first in the protein storage vacuoles (Figure 13A and C) and later in the whole cytoplasm (Figure 13D and E); in the crushed cell area several small acidified bodies were also observed (Figure 13F).

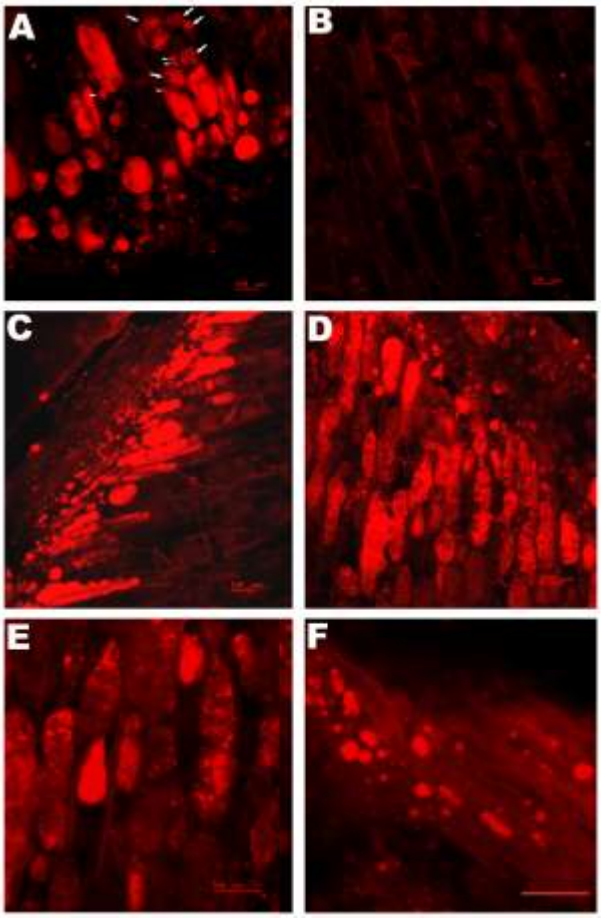

Figure 13 - Intracellular acidification of the endosperm cells indicated by acridine orange. A – Initial acidification in the protein storage vacuoles (arrows) and small vesicles (arrowhead) (bar = 50 µm). B – Low signal in the intact endosperm (bar = 50 µm). C – Acidification observed only in the vicinity of the growing haustorium (bar = 50 µm). D – Complete cytoplasm acidification; note the presence of several acidic apoptotic bodies in the crushed cells (bar = 50 µm). E – Detailed view of cytoplasm acidification (bar = 50 µm). F – Detailed view of the acidic apoptotic bodies in the crushed cells (bar = 50 µm).

5 Discussion

Previous studies considered palm endosperm as a storage tissue without the capacity of protein synthesis during germination (DeMason *et al.*, 1985; Panza *et al.*, 2004). In the present study, structural and ultrastructural analyses before germination revealed new aspects of the endosperm during germination of peach palm seeds, including the capacity of *de novo* protein synthesis as well as endosperm breakdown through PCD. Additionally, numerous vesicles and organelles, including Golgi apparati and endoplasmatic reticulum, were observed before germination. Seed recalcitrance has been widely associated with the maintenance of active cell metabolism (Berjak and Pammenter, 2008), and it has previously been associated with recalcitrance of *Euterpe edulis* and peach palm zygotic embryos (Panza *et al.*, 2004; Chapter IV). Our results expand this association to the endosperm of peach palm seeds.

In previous descriptions of date palm and peach palm the first modification in the endosperm occurs in the protein storage vacuoles, leaving nucleated cells with empty vacuoles (DeMason *et al.*, 1989; Chapter IV). These results were confirmed in the present study. A similar pattern was also described in asparagus seeds (Willians *et al.*, 2001). In these species, only after the breakdown of cytoplasmatic reserves are the cell walls hydrolyzed. In date palm it has been proposed that enzymes responsible for cell wall hydrolysis diffuse from the degenerating cytoplasm to the cell wall (DeMason *et al.*, 1985). However in the present study, ultrastructural analyses revealed the formation of apoptotic bodies that later collapse. The formation of apoptotic bodies is considered as the final stage of PCD, which was also shown by Evans blue staining to occur in the endosperm cells in the vicinity of the growing haustorium. Given the formation of apoptotic bodies instead of cytoplasm collapse, cell wall hydrolases or others proteins may also be secreted by the endosperm cells before these undergo PCD. Indeed, vesicular bodies fused to the cytoplasmatic membrane were often observed in endosperm cells of peach palm.

PCD is known to be a highly regulated mechanism requiring *de novo* cytoplasmatic protein synthesis (Lam *et al.*, 2001). Analysis of polysome associations is an attractive technique to prove active protein translation and reflects the gene expression levels that directly correlate with the total amount of protein synthesized (Kahlau and Bock, 2008). Our data show abundant polysome associations in the endosperm of peach palm seeds before and throughout germination; but their amount successively declines until the final stages of germination. This contrasts with orthodox seeds, where components necessary for protein synthesis are present in dry embryos, although polysomes are absent (Bewley, 1997). In these

seeds, rapid resumption of protein synthesis from pre-stored RNAs and single ribosomes is effected by recruitment into polysomal protein-synthesizing complexes soon after seed imbibition (Bewley, 1997). Hence, in accordance with the ultrastructural analyses, the presence of polysomes in peach palm endosperm before germination also suggests a desiccation-sensitive tissue with a very active metabolic state.

Other characteristics of PCD were observed in the endosperm of peach palm seeds during germination. The most obvious morphological change during PCD is the condensation of the protoplast away from the cell wall (Reape *et al.*, 2008), also observed to occur in peach palm endosperm. In addition, cytoplasm shrinkage, nucleus and organelle modifications, cytoplasm acidification and *de novo* synthesis of KDEL-CysEP, a marker for plant PCD (Gietl and Schmid, 2001; Helm *et al.*, 2008), were detected in the present study.

In *Ricinus communis* and *Picea glauca*, the *de novo* synthesis of KDEL-CysEP occurs during the late post-germinative phase and cleavage is completed only after complete storage protein mobilization (Schmid *et al.*, 1999; He and Kermode, 2003). In our study, SDS-PAGE and Western blot analyses indicated the presence of one sub-unit (approximatelly 45 kDa) of the 7S vicilin-like storage protein when the CysEP was already in its mature form. Immunofluorescence localization of CysEP revealed its presence in endosperm cells before germination in those areas corresponding to the degrading endosperm. In further stages of germination, the presence of cytoplasmatic vesicles as well as accumulation of CysEP on the cell wall of endosperm cells were detected. These results were confirmed by immunogold localization, which also showed the synthesis of KDEL-CysEP from endoplasmatic reticulum resulting in large cytoplasmatic membrane-bound vesicles, characteristic of ricinosomes (Gietl and Schmid, 2001). The formation of ricinosomes from ER budding off and later the accumulation of CysEP on the cell wall were described in endosperm of *R. communis* (Schimdt *et al.*, 1999; 2001). Also, during germination of *P. glauca* seeds, the megagametophyte undergoes PCD and CysEP was also found to accumulate on the cell walls (He and Kermode, 2003). It has been shown that CysEP is able to digest the cell wall protein extensin (Helm *et al.*, 2008).

An additional feature of PCD is cytoplasm acidification, important for endonuclease activity (Gottlieb *et al.*, 1996), as well as for the rapid cleavage of the KDEL-tail that occurs upon acidification of the ricinosome (Schmid *et al.*, 1999). In the present study, a narrow area of the endosperm close to the haustorium had an acid pH, seen with the bromocresol purple staining. Using acridine orange as an intracellular pH indicator, a wave-like pattern could be observed as an increasing number of cells acidified during germination. The ultrastructural

analysis revealed apoptotic bodies after cytoplasm shrinkage. Collapse of the apoptotic bodies possibly lead to the extracellular acidification in this area shown by bromocresol purple.

Besides its roles in PCD, acidification of the milieu is an important germination control mechanism involved, for instance, in storage protein hydrolysis (Dominguez and Cejudo, 1999; He *et al.*, 2007). Our results also indicate that acidification starts in the storage protein vacuoles. In addition to the hydrolysis of storage proteins, the transport of breakdown products from the endosperm of cereals to the scutellum also requires lower pH (Hardy and Payne, 1991; Dominguez and Cejudo, 1999). The activity of α-galactosidase in date palm also has a pH optimum of 4.5 (Sekhar and DeMason, 1990) and this might explain the sudden occurrence of cell wall hydrolysis in the vicinity of the haustorium. Hence, the acidification of the endosperm is a complex mechanism tightly and directly controlled by the growing haustorium. It has been suggested that mitochondria play a central role during PCD related to the liberation of cytochrome C and endonucleases (Balk *et al.*, 2003) and the loss of mitochondria's membrane stability would subsequently decrease the pH level in the cytoplasm (reviewed by Lam *et al.*, 2001). In date palm, succinate dehydrogenase activity revealing intense mitochondrial activity was detected in a crescent-shaped area adjacent to haustorium (DeMason *et al.*, 1983) similar to the wave-pattern of acidification in the present study. However, the exact mechanisms of how acidification occurs in peach palm endosperm remain to be elucidated.

In conclusion, our results show that that endosperm of peach palm seeds has an active metabolic status, shown by the presence of Golgi complexes, endoplasmatic reticulum and numerous mitochondria, as well as polysome associations. Vesicles fusing to the cytoplasm membrane and active gene translation were also observed before germination. Due to these characteristics, endosperm of peach palm seeds should be considered as dehydration-sensitive tissue, revealing its role in seed recalcitrance. The *de novo* protein synthesis capacity and presence of KDEL-CysEP associated to a tightly controlled mechanism of endosperm acidification also provide new insights into palm seed biology. There are now three mechanisms to generate enzymes for endosperm metabolism during germination: enzymes being secreted by the haustorium; activation of previously inactive enzymes in the endosperm; and *de novo* protein synthesis. Indeed, a mixture of all three might actually occur, resulting in a highly regulated mechanism for endosperm breakdown. It would be interesting to identify the presence or absence of these mechanisms in orthodox palm seeds. Further studies, including gene expression analysis associated with proteomics, would enhance our knowledge of palm seed biology.

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1 Major conclusions and future directions

In the present study relevant new information regarding peach palm in vitro culture as well as seed germination were found. These might have important impacts on the conservation and breeding programs of this species.

For the in vitro protocol a regenerative system integrating a temporary immersion system and secondary somatic embryogenesis was developed. Cyclic cultures were established and plantlets were regenerated through somatic embryogenesis. Morpho-histological analyses confirmed the occurrence of secondary somatic embryos during the induction of primary somatic embryos. Histochemical analyses showed that sub-epidermal cells were the origin of secondary somatic embryos and that these had a multicellular origin. This regenerative process still requires adjustments, especially to identify genotype effects, as well as to improve the conversion rate of somatic embryos. A pilot project is already underway for the mass propagation of selected peach palm genotypes from Hawaii/USA using the described system. The present study also showed that arabinogalactan proteins (AGPs) play an essential role during the development of peach palm somatic embryos. AGPs were secreted into the culture medium and proved to be essential for the correct development of peach palm somatic embryos. The AGPs epitopes recognized by MAb Jim13 and MAb Jim8 had different expression patterns during development of somatic embryos, with MAb Jim13 having a stronger signal. This suggests that MAb Jim13 could be a reliable marker for peach palm somatic embryogenesis as well as somatic embryo quality, given its early expression in sectors where somatic embryos developed as well as in the shoot meristem region of mature somatic embryos. The presence of these epitopes was correlated with the formation of an extracellular matrix surface network. However, the properties of the epitope recognized by the MAb Jim13 remain to be determined. In addition, analyses of other extracellular proteins might also be an important approach to better understand in vitro regeneration of peach palm.

Morpho-histological and biochemical aspects were evaluated during germination. Histological and ultrastructural analyses of the zygotic embryo revealed their active metabolic state before germination. This is an important observation related to seed recalcitrance. Globulin storage proteins, which can be markers for somatic embryo quality, were partially characterized. Further studies regarding the kinetics of storage protein accumulation during zygotic as well as somatic embryogenesis are necessary. These are important for two reasons: (i) they could confirm our hypothesis that zygotic embryos use their storage proteins right

after embryo development, leaving mature zygotic embryos with empty vacuoles; and (ii) improving storage protein accumulation in somatic embryos holds the potential to increase the regeneration rate of the developed protocol. The difference in free amino acids quantities in the endosperm and in the haustorium deserves further elucidation.

Palm seed biology is particularly interesting, given that the mechanisms of germination regulation are still largely unknown. Our findings provide a framework for continued studies to elucidate the biochemical pathways involved. The occurrence of programmed cell death in peach palm endosperms is a new factor in palm seed biology and the fact that peach palm endosperm cells are in an active metabolic state before germination also has implications for the dehydration-sensitivity of the seeds. As we have shown that the peach palm endosperm can synthesize proteins during germination, it remains to be determined which proteins and genes are active during this process. To achieve this, genomic and proteomic approaches are necessary to characterize the specific metabolic pathways underlying peach palm seed germination.

The development of biotechnological tools to assist breeding and conservation programs of peach palm is currently needed. Due to its multi-purpose characteristics, peach palm can be selected for use by smallholders, as well as for agribusiness. Understanding the biology of seed germination and in vitro culture will certainly contribute to the establishment or worldwide transfer of this species to different regions.