Physiological characteristics as analyzed by hormone profile, metabolic pathways and expression of specific induced genes of C3, C4 and CAM tropical crops propagated by Temporary Immersion Bioreactors (TIB)

Tese apresentada para obtenção do grau de Doutor em Engenharia Agronómica

Carlos Eduardo Aragón Abreu

Orientador: Profª Sara Barros Queiroz Amâncio
Co- Orientador: Drª Luísa Cristina dos Mártires Ferreira de Carvalho

JÚRI: Presidente: Reitor da Universidade de Lisboa
Vogais:

Doutora Anabela Maria Lopes Romano, Professora Associada com agregação, Faculdade de Ciências e Tecnologia da Universidade do Algarve

Doutora Sara de Barros Queiroz Amâncio, Professora Associada com agregação, Instituto Superior de Agronomia da Universidade de Lisboa (orientadora)

Doutor Jorge Manuel Pataca Leal Canhoto, Professor Auxiliar com agregação, Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Doutora Maria da Glória Calado Inglês Esquível, Professora Auxiliar, Instituto Superior de Agronomia da Universidade de Lisboa

Doutora Maria Leonor Susano Crespo da Cunha Osório, Investigadora, Centro para os Recursos Biológicos e Alimentos Mediterrânicos da Universidade do Algarve

LISBOA
2015

Index

Index of Figures……...……...……v
Index of Tables……...xi
Acknowledgments……...……...xiii
Symbols and Abbreviations……...xv
Abstract ……...……...……xvii
Resumo …...……...…xix
Resumen……xxi
Chapter 1 Introduction..... 1
1.1 Relevance and distribution of plantain, sugarcane and pineapple..... 3
1.1.1. Plantain..... 3
1.1.2. Sugarcane 4
1.1.3. Pineapple 5
1. 2. *In vitro* propagation..... 5
1.2.1. Temporary Immersion Bioreactors vs conventional propagation 6
1.2.2. TIB Functioning and gas accumulation in the headspace 8
1.3 *In vitro* propagation of plantain (C3), sugarcane (C4) and pineapple (CAM) 9
1.3.1. Plantain..... 9
1.3.2. Sugarcane 10
1.3.3. Pineapple 10
1.4. Oxidative stress 11
1.5. Photosynthesis pathways (C3, C4, CAM)..... 12
1.5.1. C3 13
1.5.2. C4 13
1.5.3. CAM..... 14
1.5.4. C4 and CAM as adaptations to environmental conditions 15
1.5.5. Comparison between C3, C4 and CAM photosynthesis pathways 16
1.6. Objectives..... 18
1.7 References 19
Chapter 2 Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization. 29
2.1 Introduction 32
2.2 Materials and Methods 34
2.2.1 Plant material and *in vitro* culture conditions..... 34
2.2.2 *Ex vitro* growth conditions 34
2.2.3 Sampling..... 35

2.2.4 Quantification of ethylene levels in the headspaces 35
2.2.5 Growth parameters 35
2.2.6 Photosynthetic rate, stomatal conductance and total transpiration 35
2.2.7 Starch quantification 36
2.2.8 Protein extraction and enzyme activity assays 36
2.2.9 Protein extraction and two-dimensional electrophoresis (2-DE) 37
2.2.10 Immunoblotting 38
2.2.11 RNA isolation and cDNA preparation 38
2.2.12 Real time PCR 39
2.2.13 Statistical analysis of the results 40
2.3 Results 40
2.3.1 Ethylene concentration in the headspaces 41
2.3.2 Growth parameters 41
2.3.3 Photosynthetic rate, stomatal conductance and transpiration 42
2.3.4 Starch 42
2.3.5 PK and PEPC enzyme activities 42
2.3.6 Gene expression of PK, PEPC and SS 42
2.3.7 Immunoblotting of peroxiredoxins 44
2.3.8 Prx gene expression 45
2.4 Discussion 46
2.5 References 50
Chapter 3 Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic characteristics and develops improved anti-oxidative response 55
3.1 Introduction 58
3.2 Materials and Methods 59
3.2.1 Plant material, *in vitro* and *ex vitro* culture 59
3.2.2 Histology and image analysis 60
3.2.3 Extraction for enzyme activity assays 60
3.2.4 Preparation of mesophyll and boundle sheath extracts 61
3.2.5 Enzyme activity assays 61
3.2.6 Native PAGE and gel activity staining 62
3.2.7 Peroxiredoxin activity and immunoblotting 63
3.2.8 RNA isolation and cDNA preparation for Real-Time PCR 64
3.2.9 Real-Time PCR and quantification of mRNA levels 64
3.2.10 Statistical analysis of the results 66

3.3 Results 66
3.3.1 Histological analyses and Imaging of ROS 66
3.3.2 Anti-oxidative response at the level of enzyme activity 68
3.3.3 Detection of different peroxiredoxins using specific antibodies 72
3.3.4 Expression of genes taking part in the anti-oxidative defense system 73
3.4 Discussion 74
3.5 References 78
Chapter 4 The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions 83
4.1 Introduction 85
4.2 Material and Methods 88
4.2.1 Plant material and *ex vitro* culture conditions 88
4.2.2 Morphological parameters 89
4.2.3 Waxes 89
4.2.4 Chlorophyll content 89
4.2.5 Succulence Index 89
4.2.6 Chlorophyll a fluorescence and photosynthetic light response curves 89
4.2.7 Soluble carbohydrates and starch 90
4.2.8 Extraction for enzyme activity and the respective assays 90
4.2.9 ABA quantification 91
4.2.10 Statistical analysis 91
4.3 Results 91
4.3.1 Plant morphology and growth parameters 91
4.3.2 Photosynthesis and chlorophyll fluorescence 94
4.3.3 Carbohydrates 98
4.3.4 PEPC and PEPCK activities 98
4.3.5 Abscisic acid 101
4.4 Discussion 101
4.5 References 105
Chapter 5 The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles 111
5.1 Introduction 114
5.2 Materials and Methods 116
5.2.1 Plant material and *ex vitro* culture conditions 116
5.2.2 Histological analyses 116

5.2.3 Succulence and pH analyses 117
5.2.4 Protein extraction and two-dimensional electrophoresis (2-DE) 117
5.2.5 Immunoblotting 118
5.2.6 Native PAGE and gel activity staining 118
5.2.7 RNA isolation and cDNA preparation 119
5.2.8 Real time PCR 120
5.2.9 Statistical analysis of the results 121
5.3 Results 121
5.3.1 Leaf anatomy, succulence and sap pH 122
5.3.2 Protein analyses and immunobloting 124
5.3.3 In gel enzyme activities 126
5.3.4 Gene expression 127
5.4 Discussion 128
5.5 References 132
Chapter 6 Final Considerations 137
6.1 Final Considerations 139
6.2 General conclusions and future perspectives 145
6.3 References 146

Figures **Page**

Figure 1.1 Schematic representation of functioning of Temporary Immersion Bioreactor (TIB) systems (A) (Escalona et al. 1999) and an image of sugarcane plants growing in TIB (B). 8

Figure 2.1 Patterns of expression of three carbon metabolism genes (Pyruvate kinase, *PK*; phosphoenol pyruvate carboxylase, *PEPC;* and starch synthase, *SS*) during acclimatization. Expression was obtained by RT-qPCR monitored during the first 14 days of acclimatization (A3, A7, A14) after *in vitro* growth in GM and TIB. Expression was normalized to that of *actin 2* and is reported to day 0 of acclimatization. Significant differences at 5% level by C-Dunnett Multiple Range Test (n=4). Positive and negative values were processed separately, for negative values the positive modular equivalent number was used. 44

Figure 2.2 Comparison of Western blots of three Peroxiredoxins (Prx E, F and Q) in plantain plants propagated in GM and TIB on day 21 of acclimatization. 2-DE gels were transferred to nitro-cellulose membranes and probed with anti-Prx E, F and Q antibodies, respectively. Arrows indicate the iso-forms detected. First dimension, IEF (left to right, pI 3.0–10.0); second dimension, SDS-PAGE (top to bottom). Protein markers (kDa) are indicated at the side of the gels. 45

Figure 2.3 Quantification of Prx gene expression by RT-qPCR (*Prx E*, *F*, *Q*) at the end of acclimatization (A21) of plantain plants previously propagated in GM and TIB. Expression was normalized to that of *actin 2* and is reported to day 0 of acclimatization. Significant differences between expression levels of each *Prx* in GM and TIB grown plants at 5% level by C-Dunnett Multiple Range Test (n=4). Positive and negative values were processed separately, for negative values the positive modular equivalent number was used. 45

Figure 3.1 Imaging of ROS accumulation in stomata (a) and trichomes (a) of sugarcane plantlets stained with NBT for superoxide radical visualization (left) and with DAB for hydrogen peroxide visualization (right). Scale bars are represented in each image. 67

Figure 3.2 Coloured stomata (a) and trichome (a) index in GM and TIB propagated plantlets prior to transfer to *ex vitro* (day 0) and after acclimatization (day 14). Values indicated by different letters are significantly different at 5 % level by C-Dunnett Multiple Range Test (n=9). 67

Figure 3.3 Total soluble enzyme activities in leaves of sugarcane plantlets propagated under GM and TIB at the moment of transfer (day 0) and during 69

acclimatization. Activities are expressed as follows: CAT, SOD, units per milligram of protein; APX, micromoles of AsA oxidized per minute per milligram of protein; DHAR, micromoles of AsA formed per minute per milligram of protein; GR, micromoles of NADPH oxidized per minute per milligram of protein; Prx, micromoles of H_2O_2 reduced per minute per milligram of protein. For clarity purposes, different scales were used. Values indicated by different letters are significantly different at 5 % level by C-Dunnett Multiple Range Test.

Figure 3.4 GR iso-enzyme in gel activity in leaves of acclimatizing sugarcane plantlets propagated under GM and TIB. Total protein extracts were subjected to native PAGE followed by activity staining. 71

Figure 3.5 Western blots of five different peroxiredoxins localized in three cellular compartments: chloroplast (2-cys Prx A; type II Prx E; Prx Q), cytosol (type II Prx C) and mitochondria (type II Prx F). Protein samples were subjected to SDS-page, transferred to a nitrocellulose membrane and probed with the specific antibodies. Western blots were performed in leaves of sugarcane plantlets propagated under both systems (GM and TIB) on day 0 of transfer to acclimatization and after 7 and 21 days of *ex vitro* growth. The numbers on the left side of each blot correspond to molecular weight (kDa). 72

Figure 3.6 Changes in the expression levels of genes of the anti-oxidative system. Quantification of mRNA levels of those sugarcane genes was performed on day 0 of *ex vitro* growth after each propagation method (GM and TIB) and after 3, 7 and 14 days. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time PCR. Relative amounts were calculated and normalized with respect to sugarcane *Act2* mRNA. Each time point in each propagation method (GM and TIB) is compared to day 0 leaves propagated in GM (control, day 0 = zero fold change). For clarity purposes, different scales were used. Values indicated by different letters are significantly different at 5 % by C-Dunnett Multiple Range Test. 73

Figure 4.1 Pineapple plants (lateral view, A; top view, B) and detail of the leaf morphology (C, arrow for thorns) at the end of *in vitro* phase (left) and at w8 of acclimatization under C3 inducing (middle) or CAM inducing (right) conditions. 92

Figure 4.2 Growth parameters of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) on w8. R, number of roots; L, number of leaves; L/W leaves length/width ratio; A, Leaf Area. Values followed by different letters between C3 and CAM induced conditions are significantly different at 5% level by t-test (n=10). 92

Figure 4.3 Fresh weight (A) and dry weight (B) of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) during acclimatization. Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=10). 93

Figure 4.4 Concentration of total chlorophyll (chl t), chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) (A) and chl a/chl b ratio (B) in leaves of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) on w8. Values followed by different letters are significantly different at 5% level by t-test (n=9). 93

Figure 4.5 Wax content on the surface of pineapple leaves (A) and Succulence Index (SI) (B) of plants under the two acclimatization treatments (C3 and CAM inducing) on w8. Values followed by different letters are significantly different at 5% level by t-test (n=9). 94

Figure 4.6 Photosynthetic rates at ambient CO_2 and increasing irradiances in pineapple plants under the two acclimatization treatments (A - C3 inducing and B – CAM inducing). Inserts in A and B show the photosynthetic rates measured at the irradiance the plants were grown, respectively C3 inducing, 40 µmol m^{-2} s^{-1} and CAM inducing, 260 µmol m^{-2} s^{-1}). Statistical analysis was performed for the values represented in the inserts. Values followed by different letters in the inserts are significantly different at 5% level by Tukey's Multiple Range Test (n=3). When no differences were found letters were withdrawn from the respective line. 95

Figure 4.7 Chlorophyll fluorescence parameters measured in leaves of plants during the two acclimatization treatments (C3 and CAM inducing). F_v/F_m represents the maximum efficiency of PSII photochemistry in dark-adapted leaves (A), F'_v/F'_m corresponds to the maximum quantum efficiency of PSII in light adapted leaves (B), ΦE equates to the operating quantum efficiency of PSII photochemistry (C), ETR, electron transport rate (D), NPQ (F_m/F'_m-1) gives an estimate of the light energy not used in photosynthesis and functions as an indicator of photoprotective processes through the dissipation of energy as heat (E) and qP, photochemical quenching (F). Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=3). When no differences were found letters were withdrawn from the respective line. 97

Figure 4.8 Carbohydrates of pineapple leaves under the two acclimatization treatments (C3 and CAM inducing). Malate (A; B), sucrose (C; D) and starch concentration (E; F) were quantified during the light period (A; C; E) and the dark period (B; D; F). Values followed by different letters are 99

significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line. For clarity of presentation the scales in A and B are different.

Figure 4.9 Total soluble PEPC (A; B) and PEPCK (C; D) activities in leaves of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) measured during the light (A; C) and the dark (B; D) periods. Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line. For clarity of presentation the scales in A, B, C and D are different. 100

Figure 4.10 Abscisic acid (ABA) concentration in pineapple sap extracted from leaves of plants under the two acclimatization treatments (C3 and CAM inducing). Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line. 101

Figure 5.1 Succulence index of leaves of pineapple plants grown under C3 and CAM induced metabolism at transfer to acclimatization (W0), at four weeks (W4) and at the end of acclimatization (W8), estimated by the Saturated Water Content (SWC). Significant differences at 5% level by C-Dunnett Multiple Range Test (n=5). 123

Figure 5.2 Determination of pH of sap extracted from leaves of pineapple plants grown under C3 (A) and CAM (B) induced metabolism at transfer to acclimatization (w0), at four weeks (w4) and at the end of acclimatization (w8), during the light and dark periods. Values on week 0 are the same on A and B, measured immediately before the beginning of acclimatization. Significant differences at 5% level by C-Dunnett Multiple Range Test (n=5). 123

Figure 5.3 Detection of pineapple proteins by 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (A) and CAM (B) induced metabolism were run on pH 3-10 IPG strips (17 cm) and then separated on 18x20 cm SDS-PAGE. Gels were stained with *Coumassie* G. Spots 1 to 5 were isolated from B and sequenced; sequences are indicated in Table 4. 124

Figure 5.4 Detection of LSU (large subunit of RuBisCO; A and B), PEPC (Phosphoenolpyruvate carboxylase; C and D) and PEPCK (phosphoenolpyruvate carboxykinase, E and F) by immunoblotting following 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (A, C and E) and CAM (B, D and F) induced metabolism were run on pH 4-7 IPG strips (7 cm) and 126

then separated on 10x8 cm SDS-PAGE. In B, LSU degradation products are confined within a rectangular box; in D, PEPC is indicated by a downward facing arrow; in E and F PEPCK is indicated by an upward facing arrow and the unspecific product of 30kDa described by the supplier of the antibody (Agrisera, Vännäs, Sweeden) is indicated by an horizontal arrow.

Figure 5.5 SOD (A), CAT (B), APX (C) and GR (D) iso-enzyme in gel activities in leaves of pineapple plants on w0, w4 and w8 of acclimatization under C3 and CAM induced metabolism, in the light and dark periods. Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD iso-forms was revealed by inhibition with KCN and H_2O_2. 127

Figure 5.6 Changes in the expression levels of *OEE 1*, *SSU*, *PEPC*, *SOD*, *CAT* and *APXt* in leaves of pineapple plants at the end of acclimatization (w8) under C3 and CAM induced metabolism, in the light and dark periods. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time quantitative PCR. Relative expressions in relation to w0 of acclimatization were calculated and values were normalized with respect to *Act2* mRNA. Comparisons of means were performed between light and dark for each induced metabolism (* above the column) and between C3 and CAM in the light and in the dark period (+ below the column). The symbols * and + indicate significant differences at 5% level by Tukey's Multiple Range Test. 128

Figure 6.1 Representation of the leaf cells of a C3 (plantain, A, B) and a C4 (sugarcane, C, D) plant after micropropagation under GM (A, C) and TIB (B, D) methods. Gene expression is represented inside the white ovals. All the other components are proteins and are represented in their most likely compartment of action: "pol" indicates polymerization of Prxs; * indicates proteins present in Bundle sheath cells of the C4 species. Protein names in bold indicate an increased enzyme activity. 141

Figure 6.2 Representation of the cells of pineapple grown under C3 (A, B) and CAM (C, D) inducing conditions in the light (A, C) and dark (B, D) periods. Gene expression is represented inside the white ovals and cell pH is shown in the cytosol. The other components are proteins and sugars and are represented in their most likely compartment of localization. Names in bold indicate an increased enzyme activity or an increased sugar concentration. 144

Tables **Page**

Table 1.1 Comparison of physiological behaviour between species showing C3, C4 and CAM pathways. 16

Table 2.1 RT-qPCR primers used for gene expression. 39

Table 2.2 Ethylene concentration in the headspace of vessels from GM and TIB systems during Elongation. 41

Table 2.3 Effect of the micropropagation system (GM or TIB) on plantain plant morphology, photosynthesis, stomata conductance, total transpiration, starch concentration and enzyme activity of Pyruvate Kinase (PK) and Phosphoenolpyruvate Carboxylase (PEPC) during Elongation. 43

Table 3.1 Real-time PCR primers used for evaluation of mRNA levels of the enzymes studied. 65

Table 3.2 SOD, CAT, APX and GR iso-enzyme in gel activities in leaves of acclimatizing sugarcane plantlets propagated under GM and TIB were quantified in relation to the value at day 0 (day 0 = 100% activity). Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD iso-forms was revealed by inhibition with H_2O_2 and KCN. Quantification was performed using the software Quantity One (Bio-Rad, Hercules, CA). 70

Table 3.3 RuBisCO, PEPC and GR enzyme activity in bundle sheath and mesophyll tissues from sugarcane leaves. Relative values of fold variation between the two tissue types. 71

Table 4.1: Environmental conditions applied in the growth chambers to induce either C3 or CAM carbon metabolism. 88

Table 4.2 Photosynthetic parameters quantified in pineapple plants under C3 and CAM inducing conditions. Results are shown for plants from w4 to w8 (CAM inducing conditions) and w6 and w8 (C3 inducing conditions). 96

Table 4.3 Stomatal conductance (gs) in pineapple plants under the two acclimatization treatments (C3 inducing and CAM inducing). C3 inducing, 40 µmol m^{-2} s^{-1} and CAM inducing, 260 µmol m^{-2} s^{-1}). 98

Table 5.1 Environmental conditions applied in the growth chambers and that induced either C3 or CAM carbon metabolism. 117

Table 5.2 Real-time PCR primers used for evaluation of mRNA levels of the 121

enzymes studied.

Table 5.3 Morphological parameters measured on pineapple leaves. 122

Table 5.4 Protein sequences obtained by Mass Spectrometry analysis of the spots represented in Fig. 5.3. 125

Table 6.1 Differences caused by the micropropagation method, GM and TIB, on the accumulation of two reactive oxygen species, hydrogen peroxide (H_2O_2) and superoxide (O_2^-) in the stotmata and trichomes of a C3 species (plantain) and a C4 species (sugarcane). 142

Table 6.2 Differences caused by the micropropagation method, GM and TIB, on several features of carbon metabolism of a C3 species (plantain). 142

Table 6.3 Differences in leaf anatomy and morphology caused by the micropropagation methods, GM and TIB, on the acclimatization of pineapple, a species displaying facultative C3-CAM carbon metabolism. 143

Acknowledgments

I would like to thank all the people and institutions that collaborated during this research project and all that were part of the collaboration between Centro de Bioplantas (Universidad de Ciego de Ávila, Cuba) and Instituto Superior de Agronomia, Centro de Botânica Aplicada à Agricultura (CBAA) (Universidade de Lisboa, Portugal).

Thanks to the financial support received from the Program Alban, the European Union Program of High Level Scholarships for Latin American, and from Fundação para a Ciência e Tecnologia (FCT) through the funding of CBAA (PEst-OE/AGR/UI0240/2011) and the PhD grant SFRH / BD / 43181 / 2008.

Thanks to all my friends, co-workers and professors that were with me through this project: Sara A, Luisa C, Silvia, Patrícia, Sara M, Ricardo, Leonor, Wanda, Glória, Maria João, Isabel, Ana Cristina, Regina, Joana, João F, Teresa, Lucas, Catarina, Rita and Luis, Joao D and Alexandre, three good friends for all time.

To other people in Lisbon: Enrique, Luisa P (her father, mother and brother), Sérgio, João A and Rodrigo.

To the Cuban friends in Lisbon: Paula (my closest family), Heslen, Olga, Joaquin, Yanni and Marta.

To the coworkers in Cuba: Maritza, Justo, Ramón, Oscar, Iris and Danilo.

Thanks to two people that were all the time by my side and that without whom this would have been impossible: Sara A, my tutor and, to me, the most high expression of scientific and professional quality and to Luisa C, my tutor, very good friend and scientist, almost a sister for me that was my support for scientific, professional and personal topics no matter what.

And in the end, the most important motivation in my professional and personal formation, my family support: To my mother and father as guides during all my formation, to my brother as example of person, to my father-in-law and mother-in-law for the support and to the people who give me love today, my wife Yanetsy, and the most lovely person of all time, my daughter Sofía.

To all of them, Thanks.

Symbols and Abbreviations

ABA, Abscisic acid
ADP, Adenosine diphosphate
Amax, Maximal photosynthesis rate
APX, Ascorbate peroxidase
AsA, Ascorbic acid / reduced ascorbate
ATP, Adenosine triphosphate
BA, 6-benzylaminopurine
CAM, Crassulacean Acid Metabolism
CAT, Catalase
CDNB, 1-chloro-2,4dinitrobenzene
CHAPS, 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate
Chl, Chlorophyll
CTAB, Hexadecyltrimethylammonium bromide
DAsA, Dehydroascorbic acid
DETC, Diethyldithiocarbamic acid silver salt
DHA-P, Dihydroxyacetone phosphate
DHAR, Dehydroascorbate reductase
DMSO, Dimetylsulfoxide
DTT, Dithiothreitol
DW, Dry weight
EDTA, Ethylenediaminetetraacetic acid
EGTA, Ethylene glycol-bis (β-aminoethyl ether)-N,N-tetraacetic acid
ETR, Electron transport rate
FAA, Formaldehyde acetic acid solution
F_v/F_m, Maximum efficiency of PSII photochemistry in dark-adapted leaves
F'_v/F'_m, Maximum quantum efficiency of PSII in light adapted leaves
FW, Fresh weight
GM, Gelled medium
GR, Glutathione reductase
gs, Stomatal conductance
GSH, reduced glutathione
GSSG, glutathione disulfide / oxidized Glutathione
HPLC, High performance liquid chromatography
IEF, Iso-electric focusing
IPG, Immoblized pH gradient
LA, Leaf area
LCP, Light compensation point
LDH, Lactate dehydrogenase

LSE, Light saturation estimate
LSU, RuBisCO large subunit
MDH, Malate dehydrogenase
MDHAR, Monodehydroascorbate reductase
MS, Murashige and Skoog medium
NADH, Nicotinamine dinucleotide reduced form
NADPH, Nicotinamine dinucleotide phosphate reduced form
NBT, Nitroblue tetrazolium
NPQ, light energy not used in photosynthesis / Stern-Volmer parameter
OAA, Oxaloacetic acid
OEE, Oxygen enhancer evolving protein
PEP, Phosphoenol pyruvate
PEPC, Phosphoenol pyruvate carboxylase
PEPCK, Phosphoenolpyruvate carboxykinase
PGA-3P, Glyceraldehyde-3 phosphate
PK, Pyruvate kinase
PMSF, Phenylmethylsulfonyl fluoride
PPF, Photosynthetic photon flux
PPFD, Photosynthetic photon flux density
Prx, Peroxiredoxin
PSII, Photosystem II
PVPP, Polyvinylpolypyrrolidone
qP, Photochemical quenching
Rd, Dark respiration
RH, Relative Humidity
ROS, Reactive Oxygen Species
RuBisCO, Ribulose.1,5.bisphosphate carboxylase oxygenase
RuBP, Ribulose-1,5-bisphosphate
SDS-PAGE, Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SI, Succulence index
SOD, Superoxide dismutase
SS, Starch synthase
SSU, RuBisCO small subunit
TCA, Trichloroacetic acid
TEMED, tetramethyl ethylene diamine
TIB, Temporary Immersion Bioreactors
ΦE, Operating quantum efficiency of PSII photochemistry / Genty parameter

Abstract

Physiology and biochemistry of *in vitro* plants can be complex and different when compared with conventional and well known plant *ex vitro* behavior. Metabolic reprogramming events that occur in a number of *in vitro* propagated plant species give rise to low *ex vitro* yields as a handicap for commercial application. Temporary Immersion Bioreactors (TIB) are a method to obtain plantlet morphology and physiology much alike that of *in vivo* grown plants. Three tropical crops were selected for their economic importance, but also because of their different photosynthetic characteristics, to compare C4 (sugarcane) and C3-CAM (pineapple) facultative photosynthetic pathways to the more common C3-type (plantain) photosynthesis. Experiments performed using plantain and sugarcane allowed to integrate the results obtained when monitoring the oxidative stress response in C3 and C4 metabolism. Pineapple plants propagated in TIB and evaluated during acclimatization under C3 and CAM inducing conditions were used to describe the facultative C3-CAM carbon metabolism and the influence of the environmental conditions on the switch from C3 to CAM. Studies based on the modulation of *in vitro* conditions which reproduce abiotic stress conditions can be used for understanding the influence of upcoming climate changes on the physiology of different species.

Key-words: carbon metabolism, CO_2 fixation, micropropagation, plant biotechnology, ROS

Resumo

Tanto a fisiologia como a bioquímica de plantas *in vitro* são complexas quando comparadas com plantas em condições naturais. A reprogramação metabólica a que cada espécie pode ser submetida em condições *in vitro* pode diminuir o rendimento da fase de crescimento *ex vitro,* consistindo na maior dificuldade para a comercialização destas plantas. Os Bioreactores de Imersão Temporária (BIT) são um método que possibilita a obtenção de plantas fisiológica e bioquimicamente competentes. Três culturas tropicais foram escolhidas pela sua importância económica e para comparação das suas diferentes características fotossintéticas, cana de açúcar (C4), ananás (C3-CAM facultativa) e bananeira (C3), a via mais comum de fixação de carono para a fotossíntese. A comparação entre bananeira e cana de açúcar permitiu caracterizar as respostas ao stresse oxidativo de plantas C3 e C4. O ananás foi micropropagado em BIT e analisado na fase *ex vitro* em condições ambientais favoráveis ao desenvolvimento de metabolismo C3 ou CAM, o que permitiu descrever o metabolismo facultativo C3-CAM e caracterizar a influência do ambiente na passagem de C3 para CAM. Estudos baseados na modulação das condições *in vitro* que reproduzam o stresse abiótico podem ser utilizados para compreender a influência das alterações climáticas na fisiologia de diferentes espécies.

Palavras chave: biotecnologia vegetal, fixação de CO_2, metabolismo do carbono, micropropagação, ROS

Resumen

La fisiología y bioquímica de plantas *in vitro* es compleja comparada con la fisiología convencional de plantas en condiciones *ex vitro.* La reprogramación metabólica que experimentan diferentes especies durante el cultivo *in vitro* puede disminuir los rendimientos en la fase *ex vitro,* como fundamental dificultad en la comercialización. Los Biorreactores de Inmersión Temporal (BIT) constituyen un método que permite obtener plantas fisiológica y bioquímicamente más competentes. Tres cultivos tropicales se seleccionaron por su importancia económica y para la comparación de sus características fotosintéticas, la caña de azúcar (C4) y la piña (C3-CAM facultativa), comparados con el plátano (C3) de rutas fotosintéticas más comunes. Los experimentos con el plátano y la caña de azúcar permitieron monitorear las respuestas al estrés oxidativo en plantas C3 y C4. Las plantas de piña propagadas en BIT y analizadas en la fase *ex vitro* bajo condiciones ambientales inductoras de metabolismo C3 y CAM, permitieron describir el metabolismo del carbono facultativo C3-CAM y la influencia del ambiente en el cambio de C3 a CAM. Estudios basados en la modulación de condiciones *in vitro* simulando estrés abiótico pueden ser utilizados para estudiar la influencia de los cambios climáticos en la fisiología de diferentes especies vegetales.

Palabras clave: biotecnología vegetal, fijación de CO_2, metabolismo del carbono, micropropagación, ROS

Chapter 1

Introduction

Introduction

Metabolic reprogramming events that occur in a number of *in vitro* propagated plant species gives rise to low *ex vitro* yields and can represent a handicap for the commercial application of these techniques. The use of Temporary Immersion Bioreactors (TIB), with plantlets developed in mixotrophic conditions by intermittent exposure to liquid medium and ventilation, has proven to be an alternative to gelled medium (GM) to obtain plantlet morphology and physiology much alike to *in vivo*, besides the lower cost, an important option for the micropropagation of tropical crops (including sugarcane, plantain and pineapple) (Lorenzo et al. 2001; Escalona et al. 2003; Aragón et al. 2005).

This technique represents an important economical resource for the massive production of plantlets for the agricultural industry but commercial application would only be undertaken if new technologies were available to automate certain procedures and if acclimatization protocols were improved (Hazarika 2003) in order to curtail losses. As a consequence, the micropropagation in TIB is one of the strategies proposed by several biotechnology companies to meet with the increasing demand. The present work selected the tropical crops sugarcane, pineapple and plantain both for their economic importance, but also because of their different photosynthetic characteristics, to compare C4 and CAM photosynthetic pathways to the more common C3-type photosynthesis.

1.1 Relevance and distribution of plantain, sugarcane and pineapple

1.1.1. Plantain

Bananas and plantains (*Musa* spp.) are among the world's most important crops. They are grown almost exclusively by small-scale subsistence farmers and play an important socioeconomic role in many developing countries of the tropics and subtropics (Panis and Thinh 2001). As crops, both are attractive to small-scale farmers for several reasons, but mostly because they are used as inexpensive food sources in small communities (De Beer and Sigawa 2010). *Musa* production is estimated to represent an important nutritional source for more than 400 million people and is a key food supplement in Caribbean and African countries. In 2011, world production of plantains reached 10.54 Mt in Uganda and 3.61 Mt in Ghana as the largest world producers. Cuba reported a production of 0.58 Mt in 2011 (FAOSTAT 2011).

Within the genus *Musa* there are several genotypes corresponding to different levels of polyploidy. *Musa* cultivars are mostly derived from hybridization between wild diploid subspecies of *Musa acuminata* (A genome) and *Musa balbisiana* (B genome), with different levels of ploidy and genomic constitution (Nunes et al. 2013). Triploid *Musa* cultivars are the most representative in feeding fruits, and plantains (CEMSA ¾) are classified as triploid AAB.

Besides their high food supply as kcal/capita/day (plantain, 9; banana, 20), *Musa* crops hold desirable qualities with relevance in the medicinal industry. They are reported as restoring normal bowl action due to high fiber content; stimulating of hemoglobin production; decreasing blood pressure and the incidence of strokes due to high potassium and low sodium content; reducing the risk of colorectal cancer, women breast cancer and renal cell carcinoma (Rashidkhani et al. 2005; Zhang 2009). Also, immunological research has identified this fruit as a possible green vaccine vector (Tripurani et al. 2003).

1.1.2. Sugarcane

Sugar crops, especially sugarcane, are projected to expand in many parts of the world in response to the rising demand for sugar, alcohol production and to the relatively high market prices. World sugarcane production attained more than 1.79 Mt in 2011 and is expected to increase by 50 Mt, by 2020-21 (FAOSTAT 2011). The bulk of the additional sugar production is expected to originate from developing countries with the main production coming from Brazil, the current leading world producer with 734.00 Mt in 2011, followed by India with 342.38 Mt. Cuba reported a production of 15.80 Mt in 2011 (FAOSTAT 2011).

In the past two decades Brazil expanded its production very rapidly, although the financial crisis of 2008 reduced the investment in new mills, slowing the overall production growth in the following years. The sugar industry in the Russian Federation has undergone a transformation in recent years and this country is expected to expand production, under the stimulus of high domestic support measures. Sugarcane is an annual crop, cultivated without rotation in five to seven cycles. The growth period takes 9 to 24 months depending on several factors, namely the variety, environmental conditions and management (Humbert 1968). Sugarcane fields should be replanted ("reformed") after five to seven consecutive cycles, requiring the elimination of stalks and soil tillage in order to place freshly cut sugarcane sprouts (Lisboa et al. 2011).

Sugarcane has several important commercial applications. Besides the traditional products, sugar and cellulose, other products are used in animal feeding and in the industry of alcoholic beverages. In the last decade, sugarcane (*Saccharum* spp.) production became pivotal in the demand for bio-ethanol. Its requirement increased considerably and is expected to increase by 3.28 fold until 2017. From 2007 to 2008, the share of ethanol in global gasoline type fuel increased from 3.7% to 5.4%. 54.6% of the sugarcane production in Brazil and 39% of the production in India are used for bioethanol production. In 2010 worldwide ethanol-derived fuel production reached 86900 million liters, with the United States as the top producer accounting for 57.5% of global production. (Lisboa et al. 2011).

1.1.3. Pineapple

After *Musa* crops pineapple is the second most important tropical fruit worldwide, its production of 21.6 Mt in 2011 contributing to over 20%. (Coveca 2002). Pineapple is cultivated mainly for fresh or canned fruit and juice, nearly 70% of being consumed as fresh fruit in the producing countries. It But it is also the single source of bromelain, a complex proteolytic enzyme used in the pharmaceutical market and as a meat tenderizing agent (Moyle et al. 2005). Its origin has been traced back to the Amazonic basin where the fruit was first domesticated. The most probable area of origin is the region comprised from upper Panama and Brazil, Paraguay and Argentina, including the northern Amazonian forest and the semi-arid regions of Brazil, Venezuela and Guyanas (Collins 1949). Worldwide production started at 1500 t when pineapple was first propagated in Europe as well as in the tropical regions of the world. The most spread variety is "*Cayena lisa*" (Smooth Cayenne) which was first introduced in Europe from French Guyana. The worldwide production in 2011 reached 2.59 Mt with Thailand as the major producer, followed by Brazil with 2.31 Mt and Costa Rica (2.26 Mt). Cuba reported a production of 76.91 kt in 2011 (FAOSTAT 2011).

1. 2. *In vitro* propagation

The use of healthy planting material complemented with an integrated pest management program is the key to obtain good crops and achieve high field production. The use of plant biotechnology, namely tissue culture techniques, allows the development of planting material free from diseases and pests and contributes to decrease the length of the cultivation cycles that, in some cases, are long. As the micropropagation established progeny is genotypically and phenotypically similar to the mother plant, usually chosen for its superior performance,

the yield is expected to be also higher (Singh et al. 2011). However, metabolic reprogramming events that occur in a number of *in vitro* propagated plant species give rise to poor *ex vitro* yields, thus posing a serious handicap for the commercial application of these techniques.

Presently, aseptic plant cultures are gaining more importance for the development of abiotic stress resistant plants and also as models to unravel the functioning of physiological and biochemical mechanisms that are triggered under stressful conditions. These mechanisms enable plants to withstand severe abiotic stresses, protecting them, at the cellular level, against drought, salinity and other abiotic stresses (Jain 2001). Also, the tight control and homogeneity of *in vitro* environmental conditions in plant tissue culture can provide a model system for the monitoring of plant behavior under specific stress conditions, giving insight into the physiological and molecular mechanisms of response (Lutts et al. 2004).

1.2.1. Temporary Immersion Bioreactors vs conventional propagation

Conventional micropropagation techniques using semisolid medium (also termed Gelled Medium, GM) are not always reliable and scaling-up is not easily achieved. Nevertheless, the use of micropropagation in horticulture, agriculture, and forestry is currently expanding worldwide, although the commercial use of micropropagation is still limited to crops with high economic value due to its relatively high production costs (Read 2007; Watt 2012).

Multiplication of plant material under *in vitro* culture is dependent on manipulation, the composition of the culture media, as well as the type of explants and the species. In several plant species, successful regeneration takes place after a number of subcultures on several successive media, which are often specific to the species. The determining factors are the combination of the concentration of plant growth regulators and other nutrients, the physiological status and competence of the plant material and its capacity to undergo morphogenetic expression (Christianson 1985; 1987).

Automated systems can be implemented only by using liquid media. Since 1976 several options of automated propagation systems have been successfully implemented (Simonton et al. 1991) but it wasn't until 1993 that Alvard et al. compared five different propagation systems in order to optimize plant growth and production. Shortly after, Teisson and Alvard (1995) defined the components for a temporal immersion system, but it took seven more years

for the properties of Temporary Immersion Bioreactors (TIB) as a plant propagation method to be correctly defined, by Etienne and Berthouly (2002).

Plant growth, gas exchange, lighting, medium agitation, temperature and pH are some of the parameters that can be controlled by using TIBs. This regulation aims at increasing multiplication rates and plant growth and at reducing space, energy and labor requirements in commercial micropropagation. In conventional micropropagation, liquid media are frequently prone to contaminations during handling of aseptic procedures. TIB systems reduce this probability of contamination due to the reduction of personal directly handling the aseptic material.

The physiological behavior of micropropagated plantlets using conventional micropropagation systems during the *in vitro* stages and during acclimatization has been well documented (Van Huylenbroeck et al. 1998; Wilson and Greenberg 1999; Van Huylenbroeck et al. 2000; Carvalho et al. 2001; Le et al. 2001; Carvalho et al. 2002; Carvalho et al. 2005; Carvalho et al. 2006). However, information on the physiological evolution of micropropagated plants under TIB conditions is scarce. Martre et al. (2001) reported on the physiology of callus cultures of *Hevea brasiliensis* grown in an automated temporary immersion system (RITA).

In pineapple, Escalona et al. (2003) showed that the maximal photosynthetic rates and the maximum quantum yield of photosystem II were low in TIB produced plantlets grown under high photosynthetic photon flux. However, those plantlets showed increases in sugar and nitrogen uptake, dry weight and leaf area, demonstrating that shoot growth is not strictly dependent on the photosynthetic process. A limitation of the previous study was that levels of key enzymes involved in carbon metabolism and their behavior during acclimatization were not evaluated, and therefore no conclusions could be drawn regarding their role during TIB growth. In general, TIB assures a higher plant yield (Teisson and Alvard 1995; Etienne and Berthouly 2002; Escalona et al. 2003; Shu-Han and Der-Ming 2008) that could be a consequence of the better aeration deriving from the periodic immersion and the renewal of the headspace, which results in reduced hyperhydricity (Roels et al. 2005; Ziv 2005).

The use of TIB, allowing plantlets to develop in mixotrophic conditions by intermittent exposure to liquid medium and ventilation, has proven to be an alternative to conventional systems in order to achieve plantlet morphology and physiology much alike to those showed

by *in vivo* plants. These advantages, together with its lower cost render TIB an important option for the micropropagation of tropical crops, such as sugarcane, plantain and pineapple (Escalona et al. 2003; Aragón et al. 2005; Arencibia et al. 2008). As a consequence, micropropagation in TIB is one of the strategies proposed by several biotechnology companies to meet with the increasing demand of material. Furthermore there appears to be a growing interest in TIB technology for the production and extraction of secondary metabolites and as a tool to study metabolic and genomic mechanisms in controlled environments (Watt 2012).

1.2.2. TIB Functioning and gas accumulation in the headspace

The TIB system used in the present research consists of two flasks, one for growing the plants and one that holds the liquid medium. The two containers are connected by tubes made of silicone and glass. The airflow is sterilized by passage through 0.2µm hydrophobic filters (Fig. 1.1). Air pressure from an air compressor pushes the medium from one container to the other, immersing the plants completely. The airflow is then reversed to withdraw the medium from the plants container. The frequency and length of the immersion period are controlled by electronic timers. Three-way solenoid valves provide the control (on/off) to let air into the system at the programmed time.

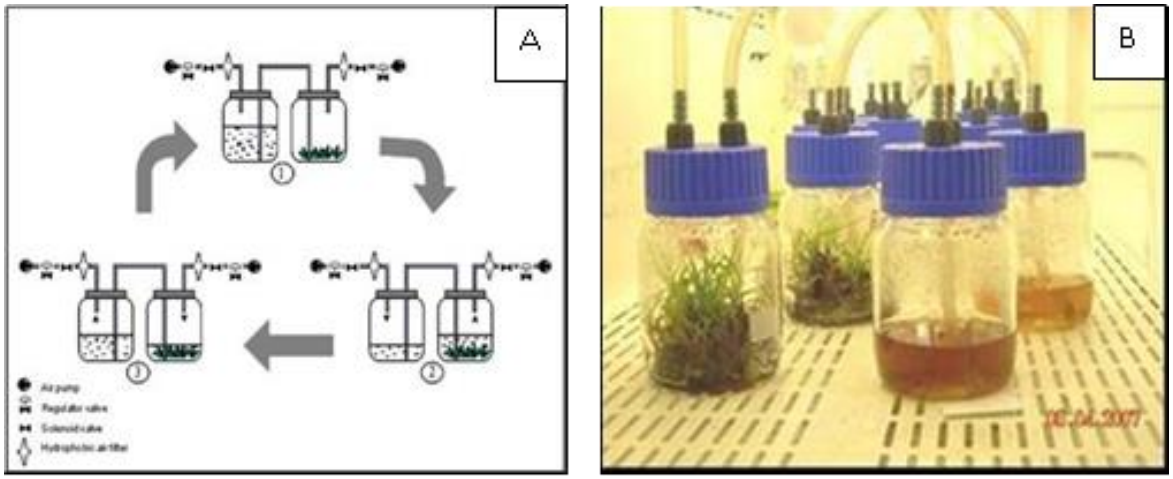

Fig. 1.1 Schematic representation of functioning of Temporary Immersion Bioreactor (TIB) systems (A) (Escalona et al. 1999) and an image of sugarcane plants growing in TIB (B).

Gas exchanges during *in vitro* propagation are regarded as a major factor affecting plant growth due to the continuous replacement of the headspace with the surrounding air (Majada et al. 2000). This prevents the accumulation of CO_2 and ethylene that occurs above the layer

of semisolid medium in closed containers and has detrimental effects on the shoots being propagated (Roels et al. 2005; 2006; Aragón et al. 2013 - Chapter 2).

The differences of TIB grown plants and semi-solid medium grown plants are based on chlorophyll content, net photosynthesis, transpiration and stomatal conductance (Jova et al. 2011). Usually, the headspaces accumulate several volatile compounds that are prone to cause oxidative stress. Leaf morphology and expansion, stomata functionality and photosynthetic rates are also affected by the presence of ethylene, aldehydes and nitric oxide associated with abscisic acid production (Woodrow et al. 1988; Jackson et al. 1991; Tanaka et al. 2005; Watt 2012). In addition, TIB headspace can be renewed by CO_2 enrichment with several advantages related to carbon metabolism and quality of the plants obtained (Aragón et al. 2009b). CO_2 enrichment has been tested during *in vitro* culture in conventional systems and during the acclimatization phase, but it is not well documented in TIB systems (Kwa et al. 1995; Amâncio et al. 1999; Nguyen and Kozai 2001; Carvalho et al. 2002; Teixeira et al. 2005; Coelho et al. 2007).

1.3 *In vitro* propagation of plantain (C3), sugarcane (C4) and pineapple (CAM)

1.3.1. Plantain

The earliest studies regarding the *in vitro* culture of *Musa* were performed in Taiwan almost forty years ago (Ma and Shii 1974). Plant biotechnology applied to plantain allows a 60-70 days shortening of the cycle that enables the farmers to grow a legume crop of short cycle that adds to the income and improves soil fertility. Using tissue culture, it is also possible to obtain planting material free from sucker borne diseases and pests (Singh et al. 2011).

Plantain propagation in TIB has been described by Aragón et al. (2005; 2009b) and Roels et al. (2005; 2006). The positive effects of TIB on shoot proliferation were thus proved for plantain. It was also demonstrated that TIB yields higher total multiplication rates when compared with semi-solid medium and TIB culture parameters such as size of the reactor, volume of medium per inoculum and immersion time and frequency were optimized (Roels et al. 2005).

1.3.2. Sugarcane

In vitro culture methods in sugarcane have had a great impact on basic research and have generated interest for commercial application. These include micropropagation of elite clones, production of disease-free planting material, screening methods for biotic and abiotic stress tolerance, and conservation of novel and useful germplasm. *In vitro* techniques for the mass propagation of healthy sugarcane plantlets through direct and indirect regeneration pathways are well established and are vital to improve sugarcane germplasm through genetic engineering (Yang et al. 2010; Snyman et al. 2011).

Sugarcane propagation in TIB has been described by Lorenzo et al. (1998), who developed a method that yielded a two fold increase in multiplication rate and a 46% reduction in cost when compared with a conventional GM protocol. The protocol applied showed that morphological and agronomic parameters of the *in vitro* propagated plants under field conditions were similar to those displayed in traditional cultivation by farmers and large companies alike. More recently Arencibia et al. (2008) used TIB in sugarcane in order to demonstrate bacteriostatic properties of phenolpropanoid compounds.

1.3.3. Pineapple

The origin of the suckers used for pineapple multiplication in the field is crucial for fruit quality (Sopie et al. 2011). Pineapple fruits derived from *in vitro* propagated plants can maintain the physical and chemical properties of the conventionally propagated ones. In fact, a panel of consumers preferred the fruits that derived from micropropagated plants, in terms of sweetness and tartness (Sopie et al. 2011).

Commercial pineapple micropropagation involves several cycles of culture in liquid medium without aeration (conventional method) for the multiplication of meristems and axillary shoots and buds (Daquinta et al. 2001). Using this approach, annual pineapple production is limited by the number of plantlets produced. In general, the commercial use of pineapple micropropagation has decreased because of the high production costs resulting foremost from high labor expenses, low multiplication rates in micropropagation and poor survival rates in acclimatization. Extensive expansion of pineapple micropropagation will not occur without the implement of new technology to provide automation and of improved acclimatization protocols (Escalona et al. 2003; Read 2007). TIB technology could allow for an efficient and rapid increase of the use of selected varieties.

1.4. Oxidative stress

Several crops show high sensitivity to abiotic stress, especially drought and high temperature. These stressful conditions can delay fructification and decrease the feasibility of the fruit production cycle, leading to severe economic losses. Evidence of oxidative stress associated to micropropagation techniques has already been put forward (Van Huylenbroeck et al. 2000; Carvalho et al. 2001). Furthermore, the transition of the plantlets to an *ex vitro* phase under high light intensity can lead the way to the production of ROS (reactive oxygen species). However, most of the studies undertaken so far have merely regarded micropropagation in semisolid medium and studies in TIB have only so far focused on carbon metabolism (*eg* Aragón et al. 2005). *In vitro* grown plants can be useful models for studying physiological processes, which otherwise would not be feasible to study in adult plants.

Moreover, ROS molecules such as O_2^- and H_2O_2 generated during oxidative stress must be kept at low levels to avoid cell damage, even though some (*eg* H_2O_2) also participate in signaling pathways (Mullineaux et al. 2006). Thus, effective anti-oxidant defense pathways must be located close to the sites of ROS formation (Shao et al. 2006; Shao et al. 2008). Several enzymes have key roles in the anti-oxidative stress response, such as superoxide dismutases and catalases and the enzymes that comprise the ascorbate-glutathione cycle (Carvalho et al. 2006).

Other strategies for the response of plant cells to stress are the differential expression of peroxiredoxins (Prxs), enzymes that reduce hydrogen peroxide (H_2O_2) and alkyl hydroperoxides, in different cellular compartments (Horling et al. 2003). These enzymes undergo oxidation during the reaction of peroxide reduction and need to be reduced by electron donors such as glutaredoxins, thioredoxins, or cyclophilins before the following catalytic cycle, rendering them slow players, but still with a determinant role in the oxidative stress defense in specific cellular compartments (Horling et al. 2003; Slesak et al. 2007). Furthermore thioredoxins are important regulators of the Calvin cycle (Besse and Buchanan 1997; Schürmann and Jacquot 2000).

Previous results obtained in plantain, studied in a previous research as a model of C3 physiology (Aragón et al. 2010), revealed an anti-oxidant enzyme response against ROS, related with peroxiredoxin polymerization, together with high levels of expression of anti-

oxidative response genes during the acclimatization of plants propagated in TIB and semisolid media (Aragón et al. 2010).

Sugarcane was selected due to its C4 physiology and plantlets micropropagated in TIB showed, in fact, better morphology and physiological behavior when compared to those propagated in GM (Aragón et al. 2009a - Chapter 3). H_2O_2 and O_2^- being produced were identified and tissue-located in leaves. The response of the anti-oxidative system was also analyzed through the monitoring of *in vitro* and *in gel* enzyme activities. The immunobloting of peroxiredoxins showed high levels of expression in TIB plantlets, some showing polymerization. The transcription of genes coding for key response enzymes was also strongly up-regulated in GM plantlets (Aragón et al. 2009a - Chapter 3).

Pineapple plants have the particularity to show a somewhat plastic morphology and physiology that can change according to the environmental conditions under which the plants are grown (Cushman 2001; 2005). In fact, several species are described as displaying a facultative C3/CAM metabolism (Chu et al. 1990; Borland et al. 2000; Luttge 2004). In pineapple plants grown under CAM inducing conditions it was possible to relate the presence of CAM metabolism associated to oxidative stress resistance characteristics (Aragón et al. 2012 - Chapter 4; Aragón et al. 2013 - Chapter 5). Pineapple plants with the capacity to shift from C3 to CAM metabolism under specific environmental conditions are an interesting model for plant biochemical studies concerning carbon metabolism and oxidative stress interactions.

1.5. Photosynthesis pathways (C3, C4, CAM)

One of the most important topics on plant physiology is gas exchange in leaves, represented by three elemental processes, photosynthesis (CO_2 fixation, O_2 released), respiration (O_2 fixation, CO_2 released) and transpiration (loss of H_2O). Photosynthesis, carried out in chloroplasts, is the process that connects carbon metabolism and oxidative stress response within the chloroplast, as both the center of high ROS production and the location of CO_2 fixation for carbohydrate synthesis. According to the photosynthetic pathway plant species are classified as C3, C4 and CAM and each has developed a specific pattern of photosynthetic CO_2 fixation, representing efficient ecophysiological adaptations to different stressing environments (Blankenship 2010).

Photosynthesis is the only biological process able to harvest sun energy. Plant physiologists and agronomists alike are currently paying attention to the impact of the environment over this process. Changes in light, ambient CO_2 concentration, and temperature can affect the photosynthetic process with significant impact on productivity, crop yield and biodiversity (Barceló et al. 2001).

1.5.1. C3

The leaf mesophyll is the most active photosynthetic tissue in higher plants (C3), its cells containing numerous chloroplasts, which in turn harbor chlorophyll molecules, the specialized light-absorbing pigments. In the thylakoids the photosynthetic electron transport chain generate the high-energy compound ATP and the reduced end product NADPH which are used for sugar synthesis in carbon fixation reactions in the stroma of the chloroplasts. In the carboxylation phase of the Calvin cycle, CO_2 and water from the environment are enzymatically combined with a five-carbon acceptor molecule, RuBP (ribulose-1,5-bisphosphate); in the reducing phase two molecules of a three-carbon intermediate (glyceraldehydes-3P, PGA or dihydroxyacetone P, DHA) are generated; next the cycle is completed with the regeneration of RuBP (Bowsher et al. 2008). These plants are called C3 plants because the first product of carbon dioxide fixation is a three-carbon compound and species such as rice, barley, wheat and potatoes have a C3 carbon metabolism (Benson 2002).

1.5.2. C4

C4 plants are called that way because their first product of CO_2 fixation is a four-carbon compound, oxaloacetic acid (OAA). This pathway evolved circa 24-35 million years ago in grass species, later some dicot species developed the C4 pathway as a response to decreasing CO_2 concentration in the atmosphere to a value as low as 180 ppm (Sage 2004; Christin et al. 2008). The evolution of the C4 pathway included the formation of distinct tissue morphology separated in mesophyll and bundle sheath (Kranz anatomy) compartments (Moore et al. 1988; Rawsthorne et al. 1988; Erhleringer and Monson 1993; Sage 2004). However, for some species it has been shown that C4 photosynthesis can actually take place in a single cell with a spatial separation of the C4 and C3 pathway within a single chlorenchyma cell, without the need for Kranz anatomy (Edwards et al. 2004).

The result of C4 photosynthesis is to concentrate CO_2 at RuBisCO level. The system that concentrates CO_2 consists in the transport of carbon products to the bundle sheath for

decarboxylation, elevating CO_2 levels around the bundle sheath chloroplasts for fixation by RuBisCO. The C4 cycle begins with the up-regulation of phosphoenolpyruvate carboxylase (PEPC) followed by the expression of the other enzymes associated to the C4 pathway (Sage 2004). C4 metabolism can be classified according to the decarboxylating enzyme and the metabolite that transiently stores the carbon molecule fixed. CO_2 is fixed by PEPC and can be transported from the mesophyll cells to the bundle-sheath cells as malic acid and return as pyruvate or it can be transported as aspartic acid and return as alanine. There are three possibilities for the decarboxylation reaction: NADP-dependent malic enzyme as it occurs in maize and sugarcane, NAD-dependent malic enzyme and phosphoenolpyruvate carboxykinase (PEPCK) (Bowsher et al. 2008).

Photorespiration is an inefficient process in what concerns carbon, energy and reducing power that often occurs in C3 species and that consists in the fixation of oxygen instead of CO_2 by RuBisCO, producing a two-carbon compound as the final product. Although photorespiration enzymes were maintained throughout evolution in organisms with the C4 photosynthetic pathway, C4 plants are able to avoid photorespiration due to the spatial separation of PSII and the Calvin cycle and the presence of the photorespiratory enzyme glycine decarboxylase in the bundle sheath (Jankovsky et al. 2001).

The C4 carbon cycle evolved from the C3 pathway, as an adaptation to grasslands and warm climates of the tropical and subtropical regions, in areas of high light availability, high temperature and/or frequent drought (Edwards et al. 2010; Gowik and Westhoff 2011).

1.5.3. CAM

The designation *Crassulacean Acid Metabolism* (CAM) derives from the observation that plants of the *Crassulacean* family accumulate large amounts of organic acids in the vacuole, mostly malic acid, in the dark. CAM metabolism evolved as an adaptation to stressful conditions in environments of severe drought, heat and high light, as a mechanism to perform photosynthesis while conserving water. In environments with severe water scarcity plants cannot afford to lose water by opening their stomata during the day to capture CO_2. CAM photosynthesis can be divided into four potential phases: phase III when plants keep stomata closed during the day and phase I opening stomata at night, thus capturing CO_2 in the dark.

Two more phases can occur, phase II when stomata open at the onset of the light period and phase IV when stomata reopen at the end of the light period. However, ATP and NAPDH are produced by light dependent reactions and thus not available at night.

CAM plants evolved to store CO_2 during the night until ATP and NADPH are available in the following day. Hence, CAM metabolism is characterized by a temporal separation of the initial phase I CO_2 fixation through phosphoenolpyruvate carboxylase (PEPC) during the night and carbon storage in a 4-carbon molecule, OAA converted to malate and stored in the vacuole as malic acid. During phase III malic acid is transferred to the cytosol as malate and decarboxylated by malic enzyme or phosphoenolpyruvate carboxylase kinase (PEPCK) then fueling Calvin cycle that takes place during the day, using the carbon stored the previous night. CAM metabolism can be classified according to the decarboxylating enzyme that releases the carbon molecule from malic acid: NADP-dependent malic enzyme, NAD-dependent malic enzyme and PEPCK as it happens in pineapple (Crayn et al. 2004; Gehrig et al. 2005; Aragón et al. 2013 - Chapter 5).

1.5.4. C4 and CAM as adaptations to environmental conditions

C3 metabolism was the first carbon fixation pathway to evolve while C4 and CAM pathways appeared as new strategies of environmental adaptation. The study of those adaptations at the level of the metabolic pathways can shed new light into those adaptations, specifically in adjusting to extreme environments.

The *circa* 7 000 species with C4 pathway are grouped in 19 families (Sage et al. 1999; Sage 2001), while CAM metabolism is present in at least 20 000 species belonging to over 30 families (Sage and Monson 1999; Winter and Holtum 2002). Both CAM and C4 pathways can be found in close relatives as is the case of species in the genus *Portulaca*. The evolution leading to CAM and to C4 metabolisms appears to have been independent of one another, because the structural changes of CAM metabolism and the changes in enzyme regulation needed to control the circadian cycle are key specific features of the CAM metabolism that evolved specifically, thus rendering the possibility of a common evolutionary origin of CAM and C4 pathways extremely unlikely (Hershkovitz and Zimmer 1997; Sage 2002).

CAM metabolism requires two major changes to the leaf structure, succulence, to facilitate the capture of night-time CO_2, and the tight packing of the mesophyll cells, to enhance CAM

performance by restricting CO_2 loss during phase III of CAM (Gibson 1982; Guralnick et al. 1986; Maxwell et al. 1997; Guralnick et al. 2001; Sage 2002; Nelson et al. 2005). Pineapple (*Ananas comosus*) is a crop that belongs to a family with species showing the full range of carbon assimilation patterns, from C3 photosynthesis to weak CAM and to strong CAM, which could be good starting points for studies focused on C3 to CAM changes in metabolism, helping to understand the mechanism of transaction probably induced by stressful environmental conditions (Crayn et al. 2004; Gehrig et al. 2005).

1.5.5. Comparison between C3, C4 and CAM photosynthesis pathways

A comparison between C3, C4 and CAM pathways is shown in Table 1, mentioning the most relevant characteristics. C4 species are able to reach at least 2-fold higher rates of carbon assimilation and biomass production than C3 plants (Xie et al. 2013). Conversely, CAM plants have lower rates of net photosynthesis, but are able to grow in conditions of extreme aridity, such as deserts (Crayn et al. 2004; Luttge 2004).

The physiological, biochemical and molecular biology features of both C4 and CAM species have been widely studied in light of the possibility to improve crop productivity or enable plant production in adverse environments of water or salt stress. Different plant models have been identified to study Calvin Cycle (*Arabidopsis thaliana*), the C4 pathway (*Flaveria* or *Cleome*) and CAM metabolism (*Mesembryamthemum crystallinum*) (Chu et al. 1990; Xie et al. 2013).

Table 1.1 Comparison of physiological behaviour between species showing C3, C4 and CAM pathways.

Feature	C3	C4	CAM	Reference
Typical species/plant groups	Wheat, barley, rice, plantain (*Musa* spp.)	Maize sugarcane (*Saccharum* spp.), millet, crab grass, Bermuda grass	Pineapple (*Bromelias* spp.), Cacti, succulents and many epiphytes	Bowsher et al. 2008
Leaf anatomy	no distinct bundle sheath, if present, non-photosynthetic; photosynthesis occurs in mesophyll cells	Kranz anatomy with photo-synthesis occu-rring in mesophyll and bundle sheath cells	Usually no palisade cells, photosynthe-sis occurs in mesophyll cells that contain large vacuoles for malic acid storage	Bowsher et al. 2008

Feature	C3	C4	CAM	Reference
Initial carboxylating enzyme	RuBisCO	PEPC active only in the light and RuBisCO	PEPC in the dark, RuBisCO in the light	Bowsher et al. 2008
Product of CO_2 fixation	PGA (C3)	OAA (C4)	OAA (C4) in the dark, PGA (C3) in the light	Bowsher et al. 2008
Chloroplasts	one type	dimorphic	one type	Barceló et al. 2001
Theoretical energy requirements (CO_2:ATP: NADPH)	1: 3: 2	1:5:2 (NADP- and NAD-malic enzyme type) 1:6:2 (PEPCK type)	1: 6.5: 2	Bowsher et al. 2008
Water use efficiency (mg dry weight produced per g H_2O lost)	1.05 - 2.22	2.85 - 4.00	8.00 - 55.0	Bowsher et al. 2008
Transpiration ratio (g H_2O/g dry wt)	450 - 950	250 - 350	18 - 125	Barceló et al. 2001
Photosynthesis rate (mg CO_2 fixed dm^{-2} h^{-1})	20 - 40	30 - 60	5 - 12 (light) 6 - 10 (dark)	Bowsher et al. 2008
Photorespiration detectable	Yes	Sometime in bundle sheath –low rates	Only in Phase IV (Net CO_2 fixation)	Bowsher et al. 2008
Chlorophyll a/b ratio	2.8	3.9	2.5 - 3.0	Barceló et al. 2001
Requirement for sodium as micronutrient	No	Yes	No	Barceló et al. 2001
CO_2 compensation point (ppm)	50 - 150 (High)	0 - 10 (low)	0 - 5 (in dark)	Barceló et al. 2001
Response to light	Light saturation easily achieved	No light saturation	-	Barceló et al. 2001

Feature	C3	C4	CAM	Reference
Photosynthesis inhibited by oxygen?	Yes	No	Yes	Barceló et al. 2001
Optimum photosynthesis temp (ºC)	15 – 25	30 – 47C	35	Barceló et al. 2001
Dry matter production (tons per hectare per year)	22 - 39	39 - 54	Low and very variable (generally less than 10)	Bowsher et al. 2008
Maximum rate of productivity (net assimila-tion rate g dry matter produ-ced per m^2 leaf area per day)	10 - 25	40 - 80	6 - 10	Bowsher et al. 2008

1.6. Objectives

The key issue addressed in the present research concerns the evaluation of TIB propagated plantlets of three major tropical species, displaying three different carbon fixation metabolisms: C3 (plantain), C4 (sugarcane) and CAM (pineapple). The analyses performed are *ex vitro* viability, physiological competence, ABA content, enzymatic characterization and expression of key genes of carbon metabolism and oxidative stress response pathways.

For that purpose, this thesis is organized into six chapters, four of which corresponding to a paper published in international scientific periodicals with referees:

Chapter 1: Introduction

Chapter 2: Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2014) Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization, Biol Plant 58:29-38.

Chapter 3: Aragón C, Carvalho L, González J, Escalona M, Amancio S (2009) Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic

characteristics and develops improved anti-oxidative response. Tropical Plant Biology 2:38-50.

Chapter 4: Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2012) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions. Plant Cell Rep 31:757-769.

Chapter 5: Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2013) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles. Plant Cell Rep 32:1807-1818.

Chapter 6: Final considerations

1.7 References

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. Plant Cell Tiss Org Cult 32:55-60.

Amâncio S, Rebordao J, Chaves M (1999) Improvement of acclimatization of micropropagated grapevine: Photosynthetic competent and carbon allocation. Plant Cell Tiss Org Cult 58:31-37.

Aragón C, Sánchez C, González-Olmedo J, Escalona M, Carvalho L, Amâncio S (2014) Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization. Biol Plant 58:29-38.

Aragón C, Pascual P, González J, Escalona M, Carvalho L, Amâncio S (2013) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles. Plant Cell Rep 32:1807-1818.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2012) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions. Plant Cell Rep 31:757-769.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2010) *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor. Biol Plant 54:237-244.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2009a) Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic

characteristics and develops improved anti-oxidative response. Tropical Plant Biol 2:38-50.

Aragón C, Escalona M, Rodriguez R, Cañal M, Capote I, Pina D, González J (2009b) Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors. *In Vitro* Cell Dev Plant 46:89-94.

Aragón C, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2005) Photosynthesis and carbon metabolism in Plantain (*Musa* AAB) growing in Temporary Immersion Bioreactor (TIB) and *ex vitro* acclimatization. *In Vitro* Cell Dev Plant 41:550-554.

Arencibia A, Bernal A, Yang L, Cortegaza L, Carmona E, Pérez A, Hu C, Li Y, Zayas C, Santana I (2008) New role of phenolpropanoid compounds during sugarcane micropropagation in Temporary Immersion Bioreactors (TIBs). Plant Sci 175:487-496.

Barceló J, Nicolás G, Sabater B, Sánchez R (2001) Fisiología vegetal. Pirámide (eds) España. pp.23-292. [In Spanish].

Benson AA (2002) Paving the Path. Annu Rev Plant Biol 53:1-25.

Besse I, Buchanan B (1997) Thioredoxin-linked plant and animal processes: The new generation. Bot Bull Acad Sinica 38:1-11.

Blankenship R (2010) Early evolution of photosynthesis. Plant Physiol 154:434-438.

Borland A, Maxwell K, Griffiths H (2000) Ecophysiology of plants with cassulacean acid metabolism. In Leegood R, Sharkey T, Von Caemmer S (eds) Photosynthesis: physiology and metabolism. Kluwer Academic Publishers, Dordrecht. pp.583-600.

Bowsher C, Steer, Tobin A (2008) Plant Biochemistry, Garland Sci, Taylor and Francis (eds), UK, Cap. 5, Photosynthetic Carbon Assimilation, pp.93-142.

Carvalho L, Vilela J, Vidigal P, Mullineaux Ph, Amâncio S (2006) Activation of the ascorbate-glutathione cycles is an early response of micropropagated Vitis vinifera L. explants transferred to *ex vitro*. Int J Plant Sci 167:759-770.

Carvalho L, Esquível M, Amâncio S (2005) Stability and activity of RuBisCO in chestnut plantlets transferred to *ex vitro* conditions under elevated CO2. *In Vitro* Cell Dev Biol Plant 41:525-531.

Carvalho L, Santos P, Amâncio S (2002) Effects of light intensity and CO_2 concentration on growth and the acquisition of in vivo characteristics during acclimatization of grapevine regenerated *in vitro*. Vitis 41:1-6.

Carvalho L, Osorio M, Chaves M, Amancio S (2001) Chlorophyll fluorescence as an indicator of photosynthetic functioning of *in vitro* grapevine and plantlets under *ex vitro* acclimatization. Plant Cell Tiss Org Cult 67:271-280.

Christianson M (1985) An embryogenic culture of soybean: towards a general theory of somatic embryogenesis In: Tissue culture in forestry and agriculture. Henke R, Hughes K, Constantin M. Hollaender (eds) Plenum Press, New York. pp.83-103.

Christianson M (1987) Causal events in morphogenesis. In: Plant tissue culture. Green C, Sommers D, Hackett W, Biesboer D (eds) Allan R. Liss, New York. pp.45-56.

Christin P, Besnard G, Samaritani E, Duvall MR, Hodkinson TR, Savolainen V, Salamin N. (2008) Oligocene CO_2 decline promoted C4 photosynthesis in grasses. Curr Biol 18:37-43.

Chu C, Dai Z, Ku MSB, Edwards GE (1990) Induction of Crassulacean Acid Metabolism in the Facultative Halophyte *Mesembryanthemum crystallinum* by Abscisic Acid. Plant Physiol 93:1253-1260.

Coelho M, Diogo M, Gonçalves J (2007) Effect of elevated CO_2 on acclimatization of *in vitro*-regenerated Chestnuts: Growth Analysis. Acta Hort 748:157-164.

Collins J (1949) History, taxonomy and culture of the pineapple. Economic Bot 3:335.

Coveca C (2002) Commission from Veracruz for agricultural commercialization. Government of Veracruz department, Mexico. [In Spanish]

Crayn D, Winter K, Smith J (2004) Multiple origin of crassulacean acid metabolism and the epiphytic habit in neotropical family *Bromeliaceae*. PNAS 101:3703-3708.

Cushman J (2001) Crassulacean acid metabolism: A plastic photosynthetic adaptation to arid environments. Plant Physiol 127:1439-1448.

Cushman J (2005) Crassulacean acid metabolism: recent advances and future opportunities. Func Plant Biol 32:375-380.

Daquinta M, Lezcano Y, Escalona M, Santos R (2001) *In vitro* multiplication of bananas (FHIA-18) with paclobutrazole plant growth regulator. INFOMUSA 10:22-24. [In Spanish].

De Beer Z, Sigawa A (2010) Banana (*Musa* spp.) Juice Production in South Africa. Acta Hort 879:233-237.

Edwards E, Osborne CP, Stromberg C, Smith S, Bond W, Christin P, Cousins A, Duvall M, Fox D, Freckleton R, et al. (2010) The origins of C4 grasslands: integrating evolutionary and ecosystem science. Science 328:587-591

Edwards G, Franceschi V, Vosnesenskaya E (2004) Singlecell C4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Biol 55:173-196.

Erhleringer J, Monson R (1993) Evolutionary and ecological aspects of photosynthetic pathway variation. Annu Rev Ecol Syst 24:411-439.

Escalona M, Samson G, Borroto C, Desjardins Y (2003) Physiology of the effects of Temporary Immersion Bioreactors on micropropagated pineapple plantlets. *In Vitro* Cell Dev Biol Plant 39:651-656.

Escalona M, Lorenzo J, González B, Daquinta M, Borroto C, González J, Desjardins Y (1999) Pineapple micropropagation in temporary immersion systems. Plant Cell Rep 18:743-748.

Etienne E, Berthouly M (2002) Temporary immersion systems in plant micropropagation. Plant Cell Tiss Org Cult 69:215-231.

Food and Agriculture Organization of the United Nations (FAOSTAT) (2011) http://faostat3.fao.org/home/index.html#DOWNLOAD. Accessed 4 August 2013.

Gehrig H, Wood J, Cushman M, Virgo A, Cushman J, Winter K (2005) Large gene family of phosphoenolpyruvate carboxylase in the crassulacean acid metabolism plant *Kalanchoe pinnata* (*Crassulaceae*) characerised by partial cDNA sequence analysis. Func Plant Biol 32:467-472.

Gibson A (1982) Anatomy of succulence. In: Ting I, Gibbs M (eds) Crassulacean acid metabolism. Proceedings of the Fifth Symposium in Botany. Baltimore, MD: Waverly Press.

Gowik U, Westhoff P (2011) The Path from C3 to C4 Photosynthesis Plant Physiol 155:56-63.

Guralnick L, Marsh C, Asp R, Karjala A (2001) Physiological and anatomical aspects of CAM-cycling in Lewisia cotyledon var cotyledon (*Portulacaceae*). Madrono 48:131-137.

Guralnick L, Ting I, Lord E (1986) Crassulacean acid metabolism in the Gesneriaceae. Amer J Bot 73:336-345.

Hazarika B (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704-1712.

Hershkovitz M, Zimmer E (1997) On the evolutionary origins of the cacti. Taxon 46:217-232.

Horling F, Lamkemeyer P, Konig J, Finkemeier I, Kandlbinder A, Baier M, Dietz K (2003) Divergent Light, Ascorbate, and Oxidative Stress-Dependent Regulation of expression of the Peroxiredoxin Gene Family in Arabidopsis. Plant Physiol 131:317-325.

Humbert R (1968) The Growing of Sugar Cane. Elsevier (eds), Amsterdam.

Jackson M, Abbott A, Belcher A, Hall K, Butler R, Cameron J (1991) Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Annal Bot 67:229-237.

Jain M (2001) Tissue culture-derived variation in crop improvement. Euphytica 118:153-166.

Jankovsky J, Smith L, Nelson T (2001) Specification of bundle sheath cell fates during maize leaf development: Roles of lineage and positional information evaluated through analysis of the tangled mutant. Develop 128:2747-2753.

Jova M, Kosky R, Cuellar E (2011). Effect of liquid media culture systems on yam plant growth (*Dioscorea alata* L. "Pacala Duclos'). Biotechnol Agron Soc Environ15:515-521.

Kwa S, Wee Y, Lim T, Kumar P (1995) Establishment and physiological analyses of phototrophic callus of the fern Platycerium coronarium (koening) under CO_2 enrichment. J Exp Bot 46:1535-1541.

Le V, Samsom G, Desjardins Y (2001) Opposite effects of exogenous sucrose on growth, photosynthesis and carbon metabolism of *in vitro* plantlets of tomato (*L. esculentum* Mill.) grown under two levels of irradiance and CO_2 concentration. Plant Physiol 158:599-605.

Lisboa C, Butterbach-bahl K, Mauder M, Kiese K (2011) Bioethanol production from sugarcane and emissions of greenhouse gases - known and unknowns GCB Bioenergy.1-16.

Lorenzo J, Blanco M, Peláez O, González A, Cid M, Iglesias A, González B, Escalona M, Espinosa P, Borroto C (2001) Sugarcane micropropagation and phenolic excretion. Plant Cell Tiss Org Cult 65:1-8.

Lorenzo J, Gonzalez B, Escalona M, Teisson C, Espinosa P, Borroto C (1998) Sugarcane shoots formation in an improved temporary immersion system. Plant Cell Tiss Org Cult 54:197-200.

Luttge U (2004) Ecophysiology of crassulacean acid metabolism (CAM). Ann Bot 93:629-652.

Lutts S, Almansouri M, Kinet J (2004) Salinity and water stress have contrasting effects on the relationship between growth and cell viability during and after stress exposure in durum wheat callus. Plant Sci 167:9-18.

Ma S, Shii C (1974) Growing banana plantlets from adventitious buds. J Chinese Soc Hort Sci 20:6-12.

Majada J, Tadeo F, Fal M, Sanchez-Tames R (2000) Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tiss Org Cult 63:207-214.

Martre P, Lacan D, Just D, Teisson C (2001) Physiological effects of temporary immersion on Hevea brasiliensis callus. Plant Cell Tiss Org Cult 67:25-35.

Maxwell K, Von Caemmerer S, Evans J (1997) Is a low conductance to CO_2 diffusion a consequence of succulence in plants with Crassulacean acid metabolism? Aust J Plant Physiol 24:777-786.

Moore B, Monson R, Ku M, Edwards G (1988) Activities of principal photosynthetic and photorespiratory enzymes in leaf mesophyll and bundle sheath protoplasts from the C3–C4 intermediate Flaveria ramossissima. Plant Cell Physiol 29:999-1006.

Moyle R, Fairbairn D, Ripi J, Crowe M, Botella J (2005) Developing pineapple fruit has a small transcriptome dominated by metallothionein. J Exp Bot 56:101-112.

Mullineaux P, Karpinski S, Baker N (2006) Spatial dependence for hydrogen peroxide-directed signalling in light stressed plants. Plant Physiol 141:346-350.

Nelson E, Sage T, Sage R (2005) Functional leaf anatomy of plants with Crassulacean acid metabolism. Func Plant Biol 32:400-419.

Nguyen Q, Kozai T (2001) Growth of *in vitro* banana (*Musa* spp.) shoots under photomixotrophic and photoautotrophic conditions. *In Vitro* Cell Dev Biol Plant 37:824-829.

Nunes O, Oliveira S, Perito E, Fortes C, Marcello J, Campos S, Gaspari G, Figueira A (2013) Genetic diversity and population structure of *Musa* accessions in ex situ conservation. BMC Plant Biol 13:41.

Panis B, Thinh N (2001) Cryopreservation of *Musa* Germplasm. INIBAP Technical Guidelines 5. INIBAP, Montpellier.

Rashidkhani B, Lindblad P, Wolk A (2005) Fruits, Vegetables and Risk of renal cell carcinoma a prospective study of Swedish women. Int J Cancer 113:451-455.

Rawsthorne S, Hylton C, Smith A, Woolhouse H (1988) Photorespiratory metabolism and immunogold localization of photorespiratory enzymes in leaves of C3 and C3-C4 intermediate species of Moricandia. Planta 173:298-308.

Read P (2007) Micropropagation: Past, present and future. Acta Hort 748:17-28.

Roels S, Escalona M, Cejas I, Noceda C, Rodríguez R, Cañal M, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tiss Org Cult 82:57-66.

Roels S, Noceda C, Escalona M, Sandoval J, Cañal M, Rodríguez R, Debergh P (2006) The effect of headspace renewal in a Temporary Immersion Bioreactor on plantain (*Musa* AAB) shoots proliferation and quality. Plant Cell Tiss Org Cult 84:155-163.

Sage R (2001) Environmental and evolutionary preconditions for the origin and diversification of the C4 photosynthetic syndrome. Plant Biol 3:202-213.

Sage R (2002) Are CAM and C4 photosynthesis incompatible? Func Plant Biol 29:775-785.

Sage R (2004) The evolution of C4 photosynthesis. New Phytol 161:341-370

Sage R, Li M, Monson R (1999) The taxonomic distribution of C4 photosynthesis. In: Sage R, Monson R (eds) C4 plant biology. New York: Academic Press. pp.551-584.

Sage R, Monson R (1999) Preface. In: Sage R, Monson R (eds) C4 plant biology. New York: Academic Press. pp.13-15.

Schürmann P, Jacquot J (2000) Plant thioredoxin systems revisited. Annu Rev Plant Physiol Plant Mol Biol 51:371-400.

Shao H, Chu L, Lu Z, Kang C (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plantlets cells. Int J Biol Sci 4:8-14.

Shao H, Chu L, Zhao Ch, Guo Q, Liu X, Ribaut J (2006) Plantlets gene regulatory network system under abiotic stress. Acta Biologica Szegediensis 50:1-9.

Shu-Han Y, Der-Ming Y (2008) *In vitro* leaf anatomy, *ex vitro* photosynthetic behaviours and growth of *Calathea orbifolia* (Linden) Kennedy plants obtained from semisolid medium and temporary immersion systems. Plant Cell Tiss Org Cult 93:201-207.

Simonton W, Robacker C, Krueger S (1991) A programmable micropropagation apparatus using cycled liquid medium. Plant Cell Tiss Org Cult 27:211-218.

Singh H, Uma S, Selvarajan R, Karihaloo J (2011) Micropropagation for Production of Quality Banana Planting Material in Asia-Pacific. Asia-Pacific Consortium on Agricultural Biotechnology (APCoAB). 92.

Slesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim Pol 54:39-50.

Snyman S, Meyer G, Koch A, Banasiak M, Watt M (2011) Applications of *in vitro* culture systems for commercial sugarcane production and improvement. *In Vitro* Cell Dev Biol Plant 47:234-249.

Sopie Y, Laurent K, Pierre Irénée B, Patrice K, Hilaire K (2011) Comparison of Pineapple Fruit Characteristics of Plants Propagated in Three Different Ways: By Suckers, Micropropagation and Somatic Embryogenesis. J Nutr Food Sci 1:110.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S (2005) Ethylene Inhibits Abscisic Acid-Induced Stomatal Closure in Arabidopsis. Plant Physiol 138:2337-2343.

Teisson C, Alvard D (1995) A new concept of plant *in vitro* cultivation liquid medium: temporary immersion. In: Terzi M, Celia R, Falavigna A (eds) Current issues in plant molecular and cellular biology. Kluwer Academic Publishers, Dordrecht. pp.105-110.

Teixeira J, Giang D, Tanaka M (2005) Novel micropropagation system for Eucalyptus uro-grandis. Bragantia Campinas 64:349-359.

Tripurani S, Reddy N, Sambasiva K (2003) Green revolution vaccines, edible vaccines. Afr. J. Biotech 2:679-683.

Van Huylenbroeck J, Piqueras A, Deberg P (2000) The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated Calathea plants. Plant Sci 135:59-66.

Van Huylenbroeck J, Piqueras A, Debergh P (1998) Photosynthesis and carbon metabolism in leaves formed prior and during *ex vitro* acclimatization of micropropagated plants. Plant Sci 134:21-30.

Watt M (2012) The status of temporary immersion system (TIS) technology for plant micropropagation. African Journal of Biotechnology 11:14025-14035.

Wilson M, Greenberg B (1999) Tryptophan photolysis leads to a UVB-induced 66 kDa photoproduct of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) *in vitro* and in vivo. Photochem. Photobiol 70:49-51.

Winter K, Holtum J (2002) How closely do the dC values of Crassulacean acid metabolism plants reflect the proportion of CO_2 fixed during the day and night? Plant Physiology 129:1843-1851.

Woodrow L, Thompson R, Grodzinski B (1988) Effects of ethylene on photosynthesis and partitioning in tomato, *L. esculentum* Mill. Journal of Experimental Botany 39:667-684.

Xie C, Li B, Xu Y, Ji D, Chen C (2013) Characterization of the global transcriptome for *Pyropia haitanensis* (Bangiales, Rhodophyta) and development of cSSR markers. BMC Genomics 14:107-120.

Yang L, Zambrano Y, Hu C, Carmona E, Bernal A, Pérez A, Zayas C, Li Y, Guerra A, Santana I, Arencibia A (2010) Sugarcane metabolites produced in CO_2-rich temporary immersion bioreactors (TIBs) induce tomato (*Solanum lycopersicum*) resistance against bacterial wilt (*Ralstonia solanacearum*). *In Vitro* Cell. Dev. Biol. Plant 46:558-568.

Zhang C (2009) Greater vegetable and fruit intake is associated with lower risk of breast cancer among Chinese women. Int. J. Cancer 124:181-188.

Ziv M (2005) Simple bioreactors for mass propagation of plantlets. Plant Cell Tiss. Organ Cult 81:277-285.

Chapter 2

Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization

Aragón C[1,2] Sánchez C[3] González J[2] Escalona M[2] Carvalho L[1] Amancio S[1]

[1]CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

[3]INIAV, Instituto Nacional de Investigação Agrária e Veterinária, 2784-505 Oeiras, Portugal

Biologia Plantarum (2014) 58:29-38

Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization

Aragón C[1,2] Sánchez C[3] González J[2] Escalona M[2] Carvalho L[1] Amancio S[1]

[1] CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2] Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

[3]INIAV, Instituto Nacional de Investigação Agrária e Veterinária, 2784-505 Oeiras, Portugal

Abstract

The current work compares the physiological and metabolic behavior of plantain plantlets micropropagated by two different techniques, Temporary Immersion Bioreactors (TIB) and Gelled Medium (GM), evaluated during the elongation phase and at the end of acclimatization. Enzyme activity and transcript levels of pyruvate kinase (PK) and phosphoenol pyruvate carboxylase (PEPC) were used as indicators for catabolic or anabolic carbon pathways, respectively, during shoot growth before acclimatization and at the end of *ex vitro* growth. TIB improved rooting and gave rise to longer shoots and higher dry weight. High levels of respiratory activity were measured at the beginning of elongation, both in TIB and GM plantlets. Photosynthesis in TIB was significantly higher than in GM from the midpoint of acclimatization on and PK-activity was lower. Starch accumulation was *circa* two fold higher in the corms than in the leaves, and always higher in corms and leaves of TIB plantlets, an indication of mixotrophic behavior. Expression levels of genes coding for carbon metabolism enzymes PK and PEPC indicated a replacement of catabolic and anaplerotic pathways by an autotrophic metabolism in TIB plantlets when compared to GM. The accumulated reserves can be used later during the first days of acclimatization, leading to higher survival rates and to better plant quality in TIB. As a whole the characteristics of TIB plantlets lead to higher survival rates and to better plant quality.

Key words: acclimatization; elongation; ethylene; PEPC; PEPCK; plantain; PRX; TIB.

2.1 Introduction

Banana and plantain are among the world's most important crops. Both are included in the *genus Musa* and most of their agronomic and physiologic properties are similar. Plantain is grown almost exclusively by small-scale-subsistence farmers and plays an important socioeconomic role in many developing countries of the tropics and subtropics (Panis and Thinh 2001). As a crop, plantain is attractive to small-scale farmers for several reasons. Production begins 14 months after planting and one plant can produce for more than 10 years. The more common uses of bananas are as a food crop; "sweet" bananas are generally eaten fresh as fruit, while "cooking" bananas and plantains are boiled, steamed, fried or roasted (Thompson 1995). Large quantities of rejected bananas are often available for animal feed, especially in banana exporting countries, and for the medicinal industry, due to their content in prot-vitamin A (Krishnan and Vijayalakshmi 2005; Weremfo et al. 2011). *Musa* is harvested throughout the year providing continuous income. In 2010 Uganda was reported as the largest *Musa* producer with 1 478 755 t (FAOSTAT 2010). Banana and plantain are usually propagated from suckers and bits (pieces of corm with attached growing points), but export-type commercial companies more commonly use tissue-cultured plantlets. The use of healthy planting material complemented with an integrated pest management program is the key to a good crop stand in the field. The use of plant biotechnology, namely tissue culture techniques, allows the development of planting material free from sucker borne diseases and pests and a reduction of the yearly cycle by 60-70 days. The interest in the use of liquid media in micropropagation (Ziv 2005), and of Temporary Immersion Bioreactors (TIB) in particular, has increased during the last decade. On average, TIB guarantees better plant quality and higher multiplication rates (Escalona et al. 1999; Lorenzo et al. 2001; Etienne and Berthouly 2002; Aragón et al. 2010).

However, conventional micropropagation systems using gelled medium (GM) are still widely used for a high number of crops. Both systems comprise specific aspects that allow good plant growth and favor acclimatization although environmental conditions associated to *in vitro* growth in either GM or TIB confer specific characteristics to the plantlets which are directly responsible for their *ex vitro* performance. TIB propagation takes place in automated systems that promote the aeration of the containers. This ventilation of the headspace is paramount to remove volatile compounds such as ethylene (Roels et al. 2006) and to promote the recirculation of the carbon dioxide necessary for photosynthesis, further enhancing

autotrophic carbon metabolism in leaves. The metabolic and physiological behavior of TIB-produced plantlets has not yet been studied in depth (Teisson and Alvard 1995; Etienne and Berthouly 2002; Escalona et al. 2003), although it is now known that TIB plantain plantlets cope better with ROS produced at the transfer from *in vitro* to *ex vitro* conditions (Aragón et al. 2010).

Once the plantlets are ready for shifting from *in vitro* conditions they must be acclimatized to adapt to the greenhouse and later to field conditions (Ma and Shii 1974; Martín et al. 2009). During this hardening period, plantlets undergo physiological adaptations to external factors like water availability, temperature and relative humidity variation, and nutrient supply. Starch accumulation in leaves of *in vitro* plantlets is vital for the acclimation to *ex vitro* conditions (Capellades et al. 1991; Cayón 2001; Bello-Pérez et al. 2002) and leaves play an important role as carbon source during the transition to autotrophy. Also, particular roles were reported for PEPC and PK during acclimatization of TIB propagated plantlets, PEPC being responsible for the mobilization of sugars through an anaplerotic route to guarantee the supply of carbon skeletons for aminoacid synthesis during *in vitro* propagation (Aragón et al. 2005). Plantain propagation in TIB was also studied under atmospheric CO_2 enrichment (1200 µg g^{-1}) (Aragón et al. 2009), that increased overall plant quality and led to a favorable balance between starch concentration in leaves and corms.

The mechanisms by which TIB grown plantlets are able to better sustain and overcome oxidative stress than GM grown plantlets, thus enhancing their propagation capacity and improving their growth, were recently elucidated, including the partial characterization and the description of the role of peroxiredoxins (Prxs) in this transition (Aragón et al. 2010). In the current study, *in vitro* elongation of plantain plantlets in TIB and GM are compared through ethylene accumulation in the headspace, photosynthetic rate and stomatal conductance, starch accumulation and PK and PEPC activities, while the expression of the genes coding for these enzymes was monitored during the acclimatization of those plantlets. Finally, to obtain a better insight in Prxs and their role in the transition to autotrophy, the gene expression and protein profile of Prxs E, F and Q were analysed at the end of acclimatization.

2.2 Materials and Methods

2.2.1 Plant material and in vitro culture conditions

Plantain shoots (*Musa* AAB cv. CEMSA 3/4) were micropropagated in gelled medium (GM; Gelrite 2.5 g dm^{-3}) containing Murashige and Skoog (1962) salts and vitamins (MS), supplemented with 30 g dm^{-3} sucrose (w/v) and 13.3µM 6-benzylaminopurine (BA) for 3 subculture cycles of 28 d. After this, only shoots with corm diameter of at least 3 mm and 3 cm length were transferred to the elongation phase. The elongation phase consisted in the preparation of the shoots for 21 d before acclimatization and was carried out using two different techniques, GM (Gelled Medium) and TIB (Temporary Immersion Bioreactors).

In both methods, the basal medium was MS supplemented with 30 g dm^{-3} sucrose, without plant growth regulators, pH adjusted to 5.8 before autoclaving at 121 ºC and 118 kPa for 20 min. In GM, plantlets were placed in glass vessels of 500 cm^3 total volume containing 150 cm^3 of media, jellified with 2.5 g dm^{-3} Gelrite, 5 shoots per vessel. TIB was performed in pairs of the same vessels, one containing 5 shoots and the other 150 cm^3 of liquid media (Escalona et al. 1999). An automatic pump system allowed the forced ventilation of TIB system with the immersion of the shoots in the media for 4min every 3h and the subsequent renewal of the headspace. Air pressure from an air compressor pushed the medium from one container to the other to immerse the plants completely. The airflow was reversed to withdraw the medium from the culture container. Atmospheric air with a CO_2 concentration of 375 µg g^{-1} was used in TIB and in GM the CO_2 concentration was variable depending of the plant metabolism, as it is a closed system. Cultures were maintained at 25 ºC under cool-white fluorescent lamps (Sylvania, Daylight F40T12/D 40 W), with a photosynthetic photon flux (PPF) of 45 ± 5 µmol m^{-2} s^{-1} under a 16h light/8h dark photoperiod.

2.2.2 Ex vitro growth conditions

After elongation, shoots produced in GM and TIB were transplanted to pots containing a sterilized mixture of hydrated peat and perlite (1:1, v/v) and placed in glass chambers with 450 dm^3 volume (500E, Aralab, Porto Salvo, PT). PPF was 200 ± 10 µmol m^{-2} s^{-1} and the photoperiod 16/8h. The initial value of relative humidity (RH) was set at 98% and was decreased until the RH in the glass chamber attained the ambient value. Temperature was kept at 25 ± 2 ºC during the light and 22 ± 1 ºC during the dark period.

2.2.3 Sampling

Plant material was harvested weekly during *in vitro* growth (Elongation: E0, E7, E14, E21) for ethylene, morphological and physiological parameters, starch, PK and PEPC activities. During *ex vitro* growth four time points were sampled (Acclimatization: A0, A3, A7; A14) for gene expression (PK, PEPC, SS). At the end of Acclimatization (A21) peroxiredoxin gene expression was quantified. One time point was coincidental between elongation and acclimatization (E21 = A0) and the whole experiment lasted 42 d (21 + 21).

2.2.4 Quantification of ethylene levels in the headspaces

Ethylene concentration in the headspaces was determined by gas chromatography. Samples of the headspace atmosphere were taken with a syringe and needle perforating the silicone tube connecting the vessel pairs in TIB systems or protruding from GM vessels. The samples were injected in sealed rubber cap vials until total atmosphere was renovated. For each time point at least three independent samples (1 cm^3) were analyzed in a gas chromatograph system (PYE UNICAM 204) equipped with aflame ionization detector (FID) and a Porapak Q column (80-100 mesh, 1.5 m length and 4 mm diameter). Nitrogen was used as carrier at a flow rate of 30 cm^3 min^{-1}, and the injector, column and detector temperatures were 25 ºC, 100 ºC and 150 ºC, respectively. An external ethylene standard (29 mm^3 dm^{-3}) was used as reference for the identification and quantification of the ethylene peak in the samples. Ethylene concentrations obtained from two independent experiments were expressed in mm^3 dm^{-3}.

2.2.5 Growth parameters

At each Elongation time point, the following growth parameters were evaluated in 15 plantlets: shoot length (cm); diameter of the base of the pseudostem (cm); number of leaves per shoot; length of the main leaf (a, cm); maximum width of the main leaf (b, cm); leaf area (LA, cm^2) calculated by the approximation to the ellipse area (LA=3.14 x (a x b) / 4) (Nakamura et al. 2005), number of roots per shoot; fresh weight (g); dry weight (g) after drying the whole plants at 50 ºC until constant weight.

2.2.6 Photosynthetic rate, stomatal conductance and total transpiration

During the Elongation phase fully expanded leaves were sampled weekly in the middle of the photoperiod. The photosynthetic rate (Amax) was recorded with a Portable CIRAS-2

Photosynthesis System (PP Systems, Herts, UK). The leaf used occupied the whole area of the cuvette (PLC6; 2.5 cm^2). The carbon dioxide concentration and the humidity of the air entering the leaf chamber were 375 µmol mol^{-1} and 80% respectively, under a controlled irradiation of 600 µmol m^{-2} s^{-1} (maximum irradiation at which photosynthesis was stable) at ambient temperature. Measurements of photosynthesis (µmol CO_2 m^{-2} s^{-1}), stomatal conductance (µmol H_2O m^{-2} s^{-1}) and total transpiration (mmol H_2O m^{-2} s^{-1}) were performed on leaves of three plantlets, with 10 measurements per plant (n=30).

2.2.7 Starch quantification

Starch content of the corm and leaves was determined in *in vitro* and *ex vitro* grown plantlets. One gram of leaf material was frozen in liquid nitrogen and ground with mortar and pestle. Soluble sugars were removed with 80% ethanol. The extract was centrifuged at 10 000 *g* at 4 ºC and the pellet was recovered on 0.2 M KOH. After the alkaline hydrolyses, an enzymatic treatment with β-amyl Glycosidase (EC 3.2.1.3 Sigma) was applied for total starch degradation (Thomas et al. 1983). Soluble carbohydrates derived from starch degradation were measured by the anthrone method (Van Handel 1968) and starch concentration was expressed in mg g^{-1} dw. Quantification was done spectrophotometrically in a Pharmacia Spectrophotometer (LKB, Ultrospec II, GE Healthcare, Little Chalfont, UK) at 620 nm using potato starch (Sigma Aldrich) to plot a standard curve.

2.2.8 Protein extraction and enzyme activity assays

Leaf material (0,25 g) was collected in the middle of the light period and immediately frozen in liquid nitrogen. The frozen samples were ground with mortar and pestle and the resulting powdered plant material was resuspended in 1 cm^3 50 mM Hepes-KOH buffer containing 12 mM $MgCl_2$, 1 mM EGTA (ethylene glycol-bis (β-aminoethyl ether)-N,N-tetraacetic acid), 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM DTT (dithiothreitol), 10% glycerol, 2 mM benzamidine, and 2 mM amino-n-caproic acid at pH 7.4 according to Siegel and Stitt (1990). The extract was filtered through cheesecloth (Miracloth) and centrifuged for 20 min at 15 000 *g* at 4 ºC. Supernatants were desalted through PD-10 columns (GE-Healthcare Life-Sciences, Buckinghamshire, UK) and used for all the determinations. Protein was quantified by the method of Bradford (1976).

Phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) was immediately measured spectrophotometrically by coupling the reaction to NADH oxidation mediated by malate

dehydrogenase. The standard assay solution contained 50 mM Tris-HCl, pH 7.6, complemented with 20 µmol $NaHCO_3$, 130 nmol NADH, 10 µmol $MgCl_2$, 5 µmol DTT, 1 U Malate Dehydrogenase (MDH) (EC 1.1.1.37) (SIGMA), 1 mM glucose-6-phospate, 50 mm^3 desalted enzyme solution in a final volume of 1 cm^3. Reactions were initiated by addition of 3.25 µmol phosphoenolpyruvate (adapted from Geigenberg and Stitt 1991). The reaction was assayed at 25 ºC by monitoring the consumption of NADH at 340 nm in a spectrophotometer (Pharmacia LKB, Ultrospec II, GE Healthcare, Little Chalfont, UK) (Hdider and Desjardins 1994).

Pyruvate kinase (PK; EC 2.7.1.40) was assayed in a reaction coupled with the Lactate Dehydrogenase (LDH) (EC 1.1.1.37) (SIGMA) reaction at 25 ºC by monitoring NADH consumption at 340 nm. The assay solution contained 50 mM imidazole-HCl, complemented with 2 mM PEP, 2 mM ADP, 10 mM $MgCl_2$, 30 mM KCl, 0.15 mM NADH, and 2.5 U cm^{-3} of desalted rabbit muscle Lactate Dehydrogenase (EC 1.1.1.27) (Sigma Aldrich) in a final volume of 1 cm^3 at pH 7.0 (Lin et al. 1989).

Both activities were expressed in units, with 1 U = 1 µmol NADH oxidized per min^{-1}. Three readings of each sample were performed.

2.2.9 Protein extraction and two-dimensional electrophoresis (2-DE)

Frozen leaf material (0.5 g) previously collected at d 21 of acclimatization in the middle of the light period was reduced to powder using a mortar and pestle in the presence of liquid nitrogen. Proteins were precipitated for 1h at -20 ºC with acetone containing 10% (w/v) TCA and 60 mM DTT, and centrifuged at 15 000 *g* for 15 min at 4 ºC. The resulting pellet was washed in acetone with 60mM DTT for 1h at -20 ºC and centrifuged again. This pellet was dried under vacuum and used as the crude extract, after being re-dissolved for 2 h at 25 ºC in a buffer containing 7 M urea, 2 M thiourea, 0.4% (v/v) Triton X-100, 4% (w/v) CHAPS ((3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate)), 60 mM DTT and 1% (v/v) IPG (Immoblized pH gradient buffer; GE Healthcare Life sciences). Protein was quantified by the method of Bradford (1976), modified by Ramagli (1999). Samples were incubated for 1 h at 25 ºC in a re-hydration buffer containing 8 M urea, 4% (w/v) CHAPS, 60mM DTT and 1% (v/v) IPG buffer.

Iso-electric focusing (IEF) was carried out using Ready-Strip-IPG-Strips (Bio-Rad, Hercules CA), 7 cm, with linear pH gradient 3-10 and 40 mg of each protein sample were loaded. The

IEF was carried out using a Protean i12 IEF cell (Bio-Rad, Hercules CA), with rehydration at 50 V for 12 h, followed by four consecutive steps in the following conditions: 250 V h^{-1}, 500 V h^{-1}, 8000 V for 2.30 h and 8000 V h^{-1} until reaching 30 000 V. After IEF strips were equilibrated for 15 min in Tris–HCl 50 mM, pH 8.8, containing 6 M urea, 30% (v/v) glycerol, 1% (w/v) SDS and 65 mM DTT. To remove DTT in excess, strips were equilibrated for 15 min in the same buffer, replacing DTT with iodoacetamide 135 mM. SDS-PAGE in the second dimension was then performed in denaturing polyacrylamide gels according to the procedure of Laemmli (1970), without stacking gel.

2.2.10 Immunoblotting

Polyclonal antibodies against chloroplast-located type II Prx E, chloroplast-located Prx Q and mitochondria-located type II Prx F were kindly supplied by Professor K-J Dietz (Bielefeld University, Germany). Western blot analysis was performed after 2-DE through the transfer of proteins to a nitrocellulose membrane (Millipore). Membranes were probed with the respective antibodies using the procedure described by Ferreira et al. (1996) and staining was performed with AP Conjugate Substrate Kit (Bio Rad, Hercules, CA).

2.2.11 RNA isolation and cDNA preparation

Total RNA from frozen leaf material collected along the acclimatization phase (A0, A3, A7 and A14) was extracted by adapting the method described by Chang et al. (1993). Extraction buffer consisted of CTAB (2%), complemented with PVPP (2%, w/w), Trizma-HCl 100 mM, 25 mM Na_2EDTA, and 2 M NaCl; pH 8.0, heated to 85 ºC prior to the addition of 400 mm^3 2-mercarptoethanol. Tissues were reduced to powder in liquid N_2 and 20 cm^3 extraction buffer were added. The same volume of chloroform:iso-amyl alcohol 24:1 was then added. This step was followed by a centrifugation at 12 000 *g* for 30 min at 20 ºC and repeated once. The aqueous phase was transferred to a new tube, and a ¼ LiCl 10M (v:v) was added. The sample was incubated at 0ºC overnight and after centrifugation at 12 000 *g* for 20 min at 4 ºC the pellet was recovered and 1.5 cm^3 of buffer was added (Trizma-HCl 10 mM, 1 mM Na_2EDTA, 1 M NaCl; 0.5% SDS (w:v), pH 8.0, previously heated to 37 ºC). Ethanol 100% (2.5 volumes) was added and the samples were incubated for 1 h at -80 ºC and then washed with ethanol 70%. After drying, the RNA was resuspended in the desired volume of distilled water.

RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI). cDNA was synthesized from 2 µg of total RNA using oligo (dT) in a 20 mm^3 reaction volume with

Revert Aid Reverse Transcriptase (Fermentas Life Science, Helsingborg, Sweden) according to the manufacturer's recommendations.

2.2.12 Real time PCR

Primer pairs used for amplification of the genes studied are presented in Table 2.1. The genomic sequences for plantain available in the GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/) were used and when plantain sequences were not available, the respective orthologs of *Vitis vinifera*, previously tested by our group, were used.

Table 2.1 RT-qPCR primers used for gene expression.

Protein name **Species**	**Accession number**	**Primers** **Name**	**Sequence**
Actin *Vitis vinifera*	AF369525.1	ACT-F ACT-R	5'- TGGATTCTGGTGATGGTGTGAGTC -3' 3´- CAATTTCCCGTTCAGCAGTAGTGG -5´
Pyruvate Kinase 1 *Musa*	Mu11M06_14	PK1-F PK1-R	5´-TTCACAACACACCTGGAGAACC´-3´ 5'- GATGTCACCTCCTCCTCGTCTG -3'
Phosphoenol pyruvate Carboxylase *Musa*	Z99987.1	PEPC-F PEPC-R	5´-GGTAGTGGAAATGTCTCGCTTGG-3´ 5´-GGCTTCTCAGGTTCATGGATTGC-3´
Starch Synthase *Musa*	JQ861709	SS-F SS-R	5´-AATCTCACGGGATATACAAGAATGC-3´ 5´-GCCTGTTAGACCCATCAGTGAAATC-3´
Type II Peroxiredoxin_E *Vitis vinifera*	JN392723	PrxE-F PrxE-R	5'- AATCTACCATAGGAATGCTCGTTGC -3' 3´- AATCAGACACAGGAAACCACAAACC -5´
Type II Peroxiredoxin_F *Vitis vinifera*	JN392724	PrxF-F PrxF-R	5'- CGAAGCATGATGATGAAATCAACGG -3' 3´- GCACCAGAAACCTTAACCTCGGATG -5´
Peroxiredoxin Q *Vitis vinifera*	JN392725	PrxQ-F PrxQ-R	5'- ACCTTCCTCACTCTTAATGGCTTCC -3' 3´- CTTTCCTCACCTTGTTTCCGTCATC -5´

The 20 mm^3 reaction mixture was composed of cDNA, 0.5 µM gene-specific primers and master mix iQ EVA Green Supermix (Bio-Rad, Hercules, CA) and an iQ5 Real Time PCR (Bio-Rad, Hercules, CA) was used. Amplification of PCR products was monitored via intercalation of EVA Green (included in the master mix). The following program was applied: initial polymerase activation, 95 ºC, 3 min; then 40 cycles at 95 ºC 15 s (denaturation), 57 ºC 30 s (annealing), 72 ºC 20 s (extension) with a single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimmers. Further, PCR products were resolved on 2% (w/v) agarose gels run at 4 V cm^{-1} in Tris-acetate-EDTA buffer (TAE), together with a 50-bp DNA-standard ladder (Invitrogen Gmb H) to confirm the presence of a single product of the desired length.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analyzed with an Rn threshold of 0.3 to obtain Cq (quantification cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). In order to compare data from different PCR runs or cDNA samples, Cq values were normalized to the Cq value of *Act2*, a housekeeping gene expressed at a relatively high and constant level (Coito et al. 2012) and the ΔΔCq method was used for the quantification of gene expression.

2.2.13 Statistical analysis of the results

Each experiment was performed twice and the combined results of both experiments were analyzed. The number of replicates performed is indicated in the legends of figures and tables. Statistical analyses were carried out using SPSS version 12 (Pérez 2005) and the treatments were compared using a non-parametric analysis by Kruskall-Wallis H. and C-Dunnett or Mann Whitney both at 5% significance level.

2.3 Results

In the present study, plantain plantlets propagated in TIB and GM conditions were compared through ethylene accumulation in the headspace, photosynthetic rate, starch accumulation and PK and PEPC activities, during *in vitro* elongation; expression of key genes in the regulation

of the transition to autotrophy during acclimatization and through the characterization of Prxs E, F and Q at the end of acclimatization.

2.3.1 Ethylene concentration in the headspaces

The ethylene concentration in the headspace of GM and TIB systems was analyzed (Table 2.2). An increase was observed in GM beginning at E14, more pronounced in the last week, reaching 0.111 mm^3 dm^{-3}. Conversely, in the headspace of TIB cultures, ethylene was only detected on E21, at significantly lower values (0.019 mm^3 dm^{-3}).

Table 2.2 Ethylene concentration in the headspace of vessels from GM and TIB systems during Elongation.

Days	Propagation Methods	Ethylene Conc. (mm^3 dm^{-3})
E0	-	Control (air)
E7	**GM**	*
	TIB	*
E14	**GM**	0.0245 b
	TIB	*
E21	**GM**	0.112 a
	TIB	0.019 b

Values followed by different letters within each column are significantly different at 5% level by C-Dunnett Multiple Range Test (n=3).
*Vestigial concentrations measured, impossible to quantify.

2.3.2 Growth parameters

Growth and morphological analyses allowed to establish that, at the end of the elongation phase, TIB propagated plantlets had significantly longer shoots and main leaves, higher number of leaves and roots, higher fresh and dry weight when compared to GM grown plantlets (Table 2.3). This rate of growth often occurs in TIB cultures, high rates of plant growth during the first weeks, slowing towards the end, when stem diameter increases by accumulation of reserves and roots protrude and expand (Table 2.3). For the same leaf area (LA), GM favored the growth of plantlets with wide and short leaves, while TIB gave rise to longer and thinner leaves. Most of the morphological parameters went through more

pronounced changes during the first 14 d of elongation, with two exceptions: the stem diameter, which increased in the last week and was not affected by the propagation method; and the number of roots per shoot, because roots protruded at d 14 in TIB and only at d 21 in GM plantlets.

2.3.3 Photosynthetic rate, stomatal conductance and transpiration

Photosynthetic and transpiration rates were measured during elongation in plantlets grown under GM and TIB (Table 2.3). Until E7 negative values of CO_2 uptake were measured, an indicator of respiratory activity. From that time point on, the plantlets were photosynthetically active with a rate of CO_2fixation 3 to 4 fold higher in TIB than in GM. Stomatal conductance of GM grown plantlets increased during the elongation phase, with a significant rise at E14. Conversely, in TIB grown plantlets, stomatal conductance decreased during elongation, attaining values of one sixth the GM plantlets levels. However the transpiration rates measured in TIB plantlets were higher, certainly due to the constant aeration of the headspace.

2.3.4 Starch

The maximum values of starch accumulation in the corms and leaves were observed on d 21 of elongation, with no differences between GM and TIB in the corms but higher values in the leaves of TIB plantlets as compared to GM (Table 2.3).

2.3.5 PK and PEPC enzyme activities

During the elongation period PK activity increased steadily in leaves of both GM and TIB plantlets, although the values were significantly higher in GM (Table 2.3). PEPC activity also increased during elongation in both TIB and GM leaf material but, conversely to PK, PEPC values were significantly higher in the leaves of TIB plantlets.

2.3.6 Gene expression of PK, PEPC and SS

Monitoring the expression of the genes that codify for phosphoenol pyruvate carboxylase (PEPC), pyruvate kinase (PK) and starch synthase (SS) during the acclimatization phase (d 0, to d 14) in GM and TIB grown plantlets (Fig. 2.1) can give an insight into the reprogramming occurring in carbon metabolism when plantlets endure the stress of transition to *ex vitro* growth. In GM, *PK* suffered an initial decrease, returning to basal levels after seven days, while in TIB the decrease was steady and values remained low until d 14 (Fig. 2.1). The trend of *PEPC* expression (Fig. 2.1) was similar in both types of plantlets except for TIB at the 7th d

of acclimatization where a significant down-regulation was observed. In accordance, TIB grown plantlets also showed a significant decrease in *SS* expression during the whole period of acclimatization, while in GM plantlets a continuous up-regulation was established after the lowest values measured on the third day (Fig. 2.1).

Table 2.3 Effect of the micropropagation system (GM or TIB) on plantain plant morphology, photosynthesis, stomata conductance, total transpiration, starch concentration and enzyme activity of Pyruvate Kinase (PK) and Phosphoenolpyruvate Carboxylase (PEPC) during Elongation.

Days	**E0**	**E7**		**E14**		**E21**	
Propagation method	**-**	**GM**	**TIB**	**GM**	**TIB**	**GM**	**TIB**
Shoot length (cm)	3.02 c	3.23 c	3.17 c	3.63 b	4.19 a	3.85 b	4.18 a
Stem diameter (cm)	0.41 c	0.51 b	0.53 b	0.57 b	0.56 b	0.73 a	0.71 a
Number of leaves per shoot	1.40 c	2.07 b	2.27 b	2.33 b	2.73 a	2.07 b	2.80 a
Length of main leaf (cm)	1.49 c	2.09 b	1.75 c	2.18 b	2.57 a	2.04 b	2.46 a
Width of main leaf (cm)	0.86 b	1.06 ab	0.79 b	1.21 a	0.93 b	1.15 a	0.97 b
Leaf Area (cm^2)	1.28 b	2.21 ab	1.38 b	2.63 a	2.39 a	2.34 a	2.38 a
Number of roots per shoot	0.00 c	0.00 c	0.00 c	0.00 c	1.80 b	1.60 b	2.93 a
Fresh weight (g)	0.67 b	0.60 b	0.49 c	0.71 b	0.86 a	0.77 b	0.86 a
Dry weigh (g)	0.035 b	0.031 b	0.026 c	0.037 b	0.045 a	0.040 b	0.043 a
Photosynthetic Rate (µmol CO_2 m^{-2} s^{-1})	-7.63 c	-10.92 c	-12.59 c	1.92 b	8.86 a	2.12 b	6.04 a
Stomata Conductance (µmol H_2O m^{-2} s^{-1})	165.10 b	169.45 b	147.06 b	331.58 a	51.68 c	392.83 a	62.84 c
Total Transpiration (mmol H_2O m^{-2} s^{-1})	1.33 b	0.81 c	2.17 a	1.34 b	1.56 b	1.29 b	1.90 a
Starch, leaves (mg.g dw^{-1})	0.00 e	371.80 c	0.00 e	388.77 c	37.26 d	896.85 b	1508.81 a
Starch, corm (mg g dw^{-1})	1150.29 b	565.16 c	1161.11 b	1059.05 b	593.01 c	3270.38 a	3591.25 a
PK (U mg $prot^{-1}$)	22.29 d	284.65 c	15.43 d	411.55 b	34.57 d	501.25 a	50.62 d
PEPC (U mg $prot^{-1}$)	6.31 c	6.33 c	15.08 a	10.34 b	18.51 a	12.06 b	17.85 a

2.3.7 Immunoblotting of peroxiredoxins

Total proteins from leaf samples collected on d 21 of acclimatization were separated by 2D electrophoresis and peroxiredoxins (Prxs) E, F and Q were identified by immunoblotting. TIB showed larger number and diversity of Prx spots (Fig. 2.2). Three spots corresponding to Prx E were identified, with a molecular weight *circa* 20 KDa and 5-6 pI (Iso-electric point), increasing in visual intensity as pI decreased. Prx F had a spot at 5.6pI, molecular weight *circa* 20 KDa and a polymerization dimmer of 40KDa, also visible in GM grown plants. Prx Q showed a spot of high intensity close to 5.4 pI and approximately 20 KDa molecular weight while spots of lower intensity were also visualized in extracts of plants grown in GM. The values of molecular weight and pI are within the expected ones for Prxs in general (Dietz 2003).

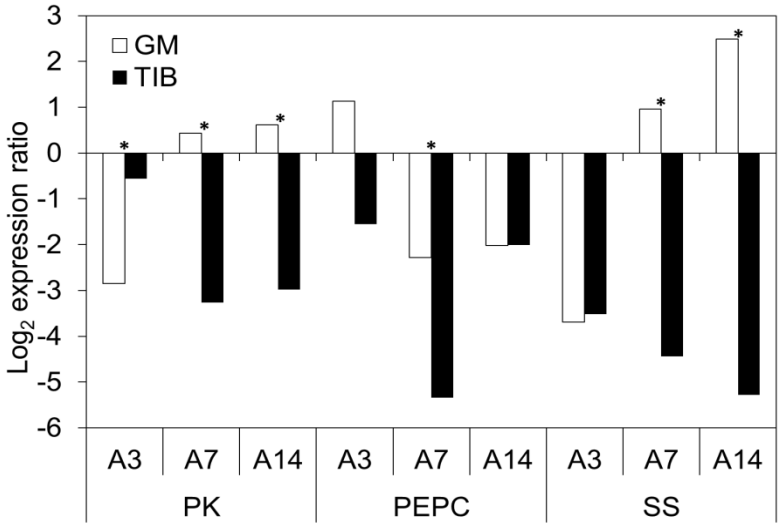

Fig. 2.1 Patterns of expression of three carbon metabolism genes (Pyruvate kinase, *PK*; phosphoenol pyruvate carboxylase, *PEPC;* and starch synthase, *SS*) during acclimatization. Expression was obtained by RT-qPCR monitored during the first 14 d of acclimatization (A3, A7, A14) after *in vitro* growth in GM and TIB. Expression was normalized to that of *actin 2* and is reported to d 0 of acclimatization. Significant differences at 5% level by C-Dunnett Multiple Range Test (n=4). Positive and negative values were processed separately, for negative values the positive modular equivalent number was used.

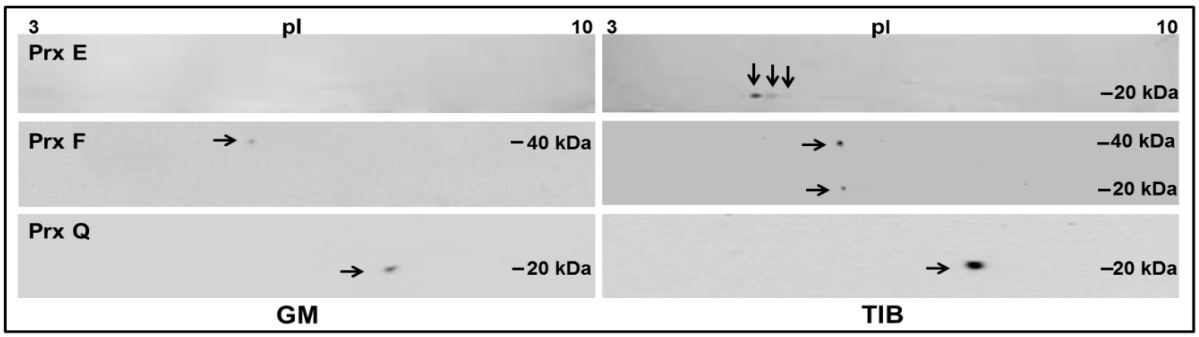

Fig. 2.2 Comparison of Western blots of three Peroxiredoxins (Prx E, F and Q) in plantain plants propagated in GM and TIB on d 21 of acclimatization. 2-DE gels were transferred to nitro-cellulose membranes and probed with anti-Prx E, F and Q antibodies, respectively. Arrows indicate the iso-forms detected. First dimension, IEF (left to right, pI 3.0–10.0); second dimension, SDS-PAGE (top to bottom). Protein markers (KDa) are indicated at the side of the gels.

2.3.8 Prx gene expression

Prx E, *Prx F* and *Prx Q* expression in plant material obtained from TIB and GM at the end of acclimatization (day 21) was monitored in relation to day 0 (Fig. 2.3). *Prx E* and *F* were moderately down-regulation in GM while *Prx Q* was strongly up-regulated in TIB propagated plants. The levels of expression of all Prxs monitored were significantly higher in TIB than in GM grown plants.

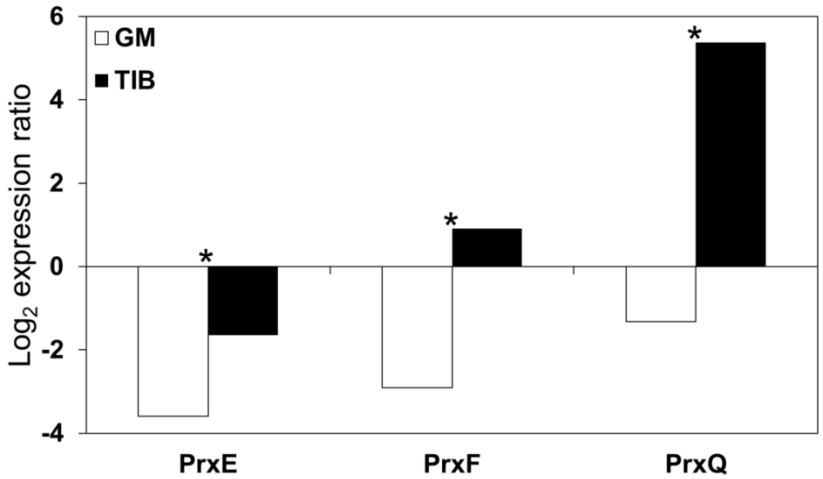

Fig. 2.3 Quantification of Prx gene expression by RT-qPCR (*Prx E, F, Q*) at the end of acclimatization (A21) of plantain plants previously propagated in GM and TIB. Expression was normalized to that of *actin 2* and is reported to d 0 of acclimatization. Significant differences between expression levels of each *Prx* in GM and TIB grown plants at 5% level by C-Dunnett Multiple Range Test (n=4). Positive and negative values were processed separately, for negative values the positive modular equivalent number was used.

2.4 Discussion

The present work aimed to assess the influence of the *in vitro* shoot elongation method, TIB or GM, on the subsequent *ex vitro* acclimatization behavior of plantain plantlets. The advantages of TIB on plantain morphology during acclimatization were previously described, focusing on physiological parameters and carbon source storage that could be used during the first days of *ex vitro* growth (Aragón et al. 2005) and also on the anti-oxidative response (Aragón et al.2010).

Plants propagated in TIB showed more favorable morphological parameters, with results similar to those obtained by Roels et al. (2005), but with a particular difference in leaf morphology, thinner and longer leaves, a feature similar to adult plantain plants. In fact, this difference in growth and also the physiological behavior observed can be considered a direct result of the propagation method applied. In TIB, there is a direct contact of the culture medium with the leaves, leading to the possibility of nutrient assimilation through them, what does not happen in GM (Escalona et al. 2003; Ziv 2005). This favors the growth of longer leaves that have a higher surface of contact to the medium. During the first period of *in vitro* culture plants are more dependent on the nutrients supplied by the culture media than upon autotrophic nutrition (Moreira et al. 2003; Larema et al. 2012). However, photosynthesis rates were higher in TIB grown plants and the transition from respiratory processes to photosynthesis was slower in GM plants. Furthermore, TIB methodology induced a decrease in stomatal conductance, a feature closely related with stomata functioning and regulation of gas exchanges. TIB grown plants have a better regulation of stomatal function and can therefore better control loss of water in the first period of acclimatization, overcoming one of the most frequent causes of plant death at that moment (Hazarika 2003).

It has been reported that the environmental conditions in TIB have a vital influence on growth, physiology and carbon metabolism of plantain leaves (Aragón et al. 2005). These features can certainly explain the significantly larger size and robustness of TIB grown plantlets. One of the main causes for this can be the continuous renewal of the atmosphere surrounding the plantlets, thus allowing the elimination of ethylene in the culture headspace, which was almost undetectable in TIB vessels, confirming the results previously obtained by Roels et al. (2006) for ethylene concentration. It is then possible to assume the effect of a renewed atmosphere on a better CO_2 assimilation *per* water molecule lost (the ratio photosynthetic rate/total transpiration) in TIB plants. Several authors have reported ethylene

as a stress-causing compound for plants, impairing normal growth, gas exchange and normal physiological functioning (Woodrow et al. 1988; Outlaw and Vlieghere-He 2001; Stearns and Glick 2003; Hall et al. 2006). The headspace composition is one of the main contributors for *in vitro* plantlet development (Buddendorf-Joosten and Woltering 1994).

Morphological and physiological changes during *in vitro* culture were supported by the analysis of carbon metabolism enzymes. The clear tendency of PK for the glycolytic pathway in GM plantlets, the lower PEPC activity (almost 50 fold) and the photosynthetic functioning confirm that free access to sugar sources from the culture media prevails over the primary synthesis by the plant (Le et al. 2001). In TIB, PEPC activity was higher, possibly due to an environment that mimics outdoor conditions, favoring RuBisCO activity that leads directly to high photosynthetic rates and also to the fixation of CO_2 by PEPC.

Starch is the main form of carbohydrate storage in plantain plantlets, with a concentration several fold higher in the corm than in leaves (Aragón et al. 2006). In plantain plantlets propagated in TIB sucrose is the less important carbohydrate storage form in leaves, further enhancing the importance of starch accumulation and sugar mobilization from corm to leaves in the first days of the *ex vitro* phase (Aragón et al. 2006), even if leaves can also be a transient starch storage organ in plants during the day, accumulating the reserves to meet the demands of the plants during night (Capellades et al. 1991). It is possible that starch accumulation in the corm depends of an endogenous metabolic regulation independent of external factors since in the present work there were no differences between GM and TIB plantlets. Nevertheless, starch concentration quantified in leaves was higher in TIB than in GM. As photosynthetic activity was higher in TIB plantlets, thus the resulting end product can be funneled to starch synthesis. During the first days of *ex vitro* growth, plantlets consume their starch reserves while adjusting to the new environmental conditions, as reported in the leaves of other plant species (Capellades et al. 1991, Van Huylenbroeck et al. 2000; Carvalho et al. 2002; Carvalho and Amâncio 2002).

GM plantlets have a higher respiratory activity than TIB plantlets, as the catabolic process that leads to ATP synthesis is through the glycolitic pathway, using the sucrose available in the culture media as the initial energy source. PK activity was 28 fold higher than PEPC activity in GM, what points to an extremely high catabolic metabolism. The glycolitic activity supported by PK also contributes to a high CO_2 concentration in the culture vessels, thus

further impairing photosynthesis in GM. In fact, the lack of ATP generation through photosynthesis could be compensated by this high glycolitic activity as another source of ATP production. It is well known that totally closed systems are higher stress sources than partially closed systems, such as TIB (Ziv 2005), thus resulting in a higher respiratory activity in GM grown plantlets and better values for physiological parameters in TIB.

PK expression during the transition to autotrophy suffered no significant changes in TIB plants, just a tendency to an initial down-regulation that recovered thereafter. The significant down-regulation of *PEPC* expression on the initial period of acclimatization in TIB could be associated with the establishment of autotrophic metabolism and with a lower involvement of PEPC in CO_2 fixation, confirming the stepwise increase of photosynthetic metabolism *ex vitro* in plants grown in TIB culture (Aragón et al. 2005). The strong down-regulation of *Starch Synthase* (*SS*) in TIB plants can be related with high starch levels in leaves of these plantlets. It has been reported that *ex vitro* plantain plants propagated in TIB primordially accumulate starch in the corm (Aragón et al. 2005; 2006) while leaves display autotrophic capacity, behaving as source and not as sink (Van Huylenbroeck and De Riek 1995; Tian et al. 2006). As a whole, TIB grown plantlets are better adjusted to an autotrophic environment.

Upon exposure to the stress of transfer to *ex vitro* plantain plants evolved an efficient defense system through which ROS are scavenged by anti-oxidant enzymes (Aragón et al. 2010). Peroxiredoxins (Prxs) are described as efficient ROS detoxification systems in the chloroplasts, mitochondria and even the nucleus, but also as key players in redox signaling during plant development and environmental acclimation (Dietz 2002; 2003). Each Prx has a distinct role with a different activation potential and cellular localization, Prx E is present in the stroma, in very low concentrations while Prxs Q and F have a more prominent role in plastids, chloroplasts and mitochondria, respectively (Dietz 2011). In a previous research we have shown that a polymerization of Prxs E and F is observed in TIB propagated plants (Aragón et al. 2010). Prx F is present in the chroroplast, an organelle where the pressure of ROS generation/scavenging is high. There, a variety of iso-forms of Prxs E, F and Q were clearly identified in TIB plants with more pI diversity in Prx E and without polymerization patterns in Prx F. Molecular weights for Prx monomers are reported to vary between 17.4-29.6KDa with some patterns of polymerization forming dimers, tetramers and octamers and their pI is reported to be between4.71 and 6.29 (Dietz 2003). The *Prxs* cellular compartmentalization between chloroplast and mitochondria was related with the pI and

polymerization pattern obtained, as chloroplast *Prxs* presented a variety of pIs (*Prxs E*) while polymerization was only evident in the mitochondria (*Prxs F*). The quantification of the expression of the respective genes showed that at the end of acclimatization GM led to the down-regulation of *Prxs E* and *F*, while *Prx Q* was up-regulated in TIB. Even though a direct relationship cannot be establish between gene expression and enzyme availability in the cell, due to the processes taking place between mature RNA formation and enzyme synthesis in the active form, the gene expression results mentioned above connect the up-regulation of the expression of *Prx Q* in TIB grown plants with the highest enzyme availability. In unstressed plants, and in plants not being able to respond to stress, *Prx Q* is only expressed in leaves, but not in roots or stems, and the level of expression is very low (Rouhier et al. 2004). Thus, it is likely that the significant up-regulation of *Prx Q* and the high levels of enzyme observed in TIB grown plants is related to the need to keep hydrogen peroxide concentrations low, in order to better overcome oxidative stress. In fact, the plant oxidative stress defense system during the *in vitro* - *ex vitro* transition was documented before (Carvalho et al. 2006, Barkova et al. 2008).

In conclusion, plantlets from TIB system, which accumulates less ethylene in the headspace, exhibited better growth and physiological capacity, showing lower stomata conductance and higher photosynthesis rates. Conversely, GM plants revealed unfavorable developmental conditions reflected in the lower photosynthetic rates and higher PK activity while in TIB grown plantlets PK activity was much lower. The physiological quality of TIB grown plantlets gave rise to a better metabolism and starch accumulation in the leaves, which can be used during the first days of acclimatization. Still in TIB plants the occurrence of different Prx iso-forms and the up-regulation of *Prx Q* are further elements that indicate a better anti-oxidative performance and thus justify the advantages of this method for producing better quality plants.

Acknowledgements

This research received financial support from Fundação para a Ciência e Tecnologia (FCT), through the PhD grant SFRH/BD/43181/2008 to C.A. and PEst-OE/AGR/UI0240/2011 to CBAA.

2.5 References

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2010) *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor. Biol.Plant 54:237-244.

Aragón C, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2005) Photosynthesis and carbon metabolism in Plantain (*Musa* AAB) growing in Temporary Immersion Bioreactor (TIB) and *ex vitro* acclimatization. *In Vitro* Cell. Dev. Plant 41:550-554.

Aragón C, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2006) Acclimatization of plantain "CEMSA ¾ (AAB)" plants propagated in Temporary Immersion System. The role of starch on the acclimatization process. INFOMUSA 1-2:32-35. [In Spanish]

Aragón C, Escalona M, Rodriguez R, Cañal M, Capote I, Pina D, González J (2009) Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors. *In Vitro* Cell. Dev. Plant 46:89-94.

Barková P, Pospísilová J, Synková H (2008) Production of reactive oxygen species and development of antioxidative systems during *in vitro* growth and *ex vitro* transfer. Biol. Plant 52:413-422.

Bello-Pérez L, Contreras-Ramos S, Romero-Manilla R, Solorza-Feria J, Jiménez-Aparicio A (2002) Propiedades químicas funcionalesdelalmidón modificado de plátano *Musa* paradisíaca L. (clon macho). Agrochemical 36:169-180.

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal.Biochem 72:248-254.

Buddendorf-Joosten J, Woltering E (1994) Components of the gaseous environment and their effects on plant growth and development *in vitro*. Plant Growth Regul 15:1-16.

Capellades M, Lemeur L, Debergh P (1991) Effects of sucrose on starch accumulation and rate of photosynthesis in rosa culture *in vitro*. Plant Cell Tiss. Organ Cult 25:21-26.

Carvalho L, Amâncio S (2002) Effect of *ex vitro* conditions on growth and acquisition of autotrophic behaviour during the acclimatization of chestnut regenerated *in vitro*. Sci. Horti 95:151-164.

Carvalho L, Santos P, Amâncio S (2002) Effects of light intensity and CO_2 concentration during the acclimatisation of *in vitro* grapevine. Vitis 41:1-6.

Carvalho L, Esquível M, Amâncio S (2005) Stability and activity of RuBisCO in chestnut plantlets transferred to *ex vitro* conditions under elevated CO_2. *In Vitro* Cell. Dev. Plant 41:525-531.

Carvalho L, Vilela B, Vidigal P, Mullineaux P, Amâncio S (2006) Activation of the ascorbate–glutathione cycle is an early response of micropropagated *Vitis vinifera* L. explants transferred to *ex vitro*. Int. J. Plant Sci 167:759-770.

Cayón G (2001) Evolution of photosynthesis, transpiration and chlorophyll during the plantain (*Musa* AAB Simmonds) leaf growth. INFOMUSA 10:12-15. [In Spanish]

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Rep 11:113-116.

Coito J, Rocheta M, Carvalho L, Amâncio S (2012) Microarray-based uncovering reference genes for quantitative real time PCR in grapevine under abiotic stress. BMC Res. Not 5:220.

Dietz K (2003) Plant peroxiredoxins. Annu. Rev. Plant Biol 54:93-107.

Dietz K (2011) Peroxiredoxins in plants and cyanobacteria. – Antioxid.Redox Sign. 15: 1129-1159.

Dietz K, Horling F, Konig J, Baier M (2002) The function of the chloroplast 2-cysteine peroxiredoxin in peroxide detoxification and its regulation. J. Exp. Bot 53:1321-1329.

Escalona M, Cejas I, Gonzalez-Olmedo J, Capote I, Roels S, Cañal M, Rodríguez R, Sandoval J, Debergh P (2003) The effect of meta-topolin on plantain propagation using a Temporary Immersion Bioreactor. INFOMUSA 12:28-30. [In Spanish]

Escalona M, Lorenzo J, González B, Daquinta M, Borroto C, González J, Desjardins Y (1999) Pineapple micropropagation in temporary immersion systems. Plant Cell. Rep 18:743-748.

Etienne E, Berthouly M (2002) Temporary immersion systems in plant micropropagation. - Plant Cell Tiss. Organ Cult 69:215-231.

Ferreira R, Franco E, Teixeira A (1996) Covalent dimerization of ribulosebisfosphate carboxylase subunits by UV radiation. Biochem. J 318:227-234.

Food and Agriculture Organization of the United Nations (FAOSTAT). FAO statistic division.http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID =567#ancor, 2010. Accessed 5th July 2012.

Geigenberger P, Stitt M (1991) A "futile" cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinuscommunis* L. seedlings when phloem transport is inhibited. Planta 185:81-90.

Hall M, Kapuya J, Sivakumaran S, John A (2006) The role of ethylene in the response of plants to stress. Pestic. Sci 8:217-223.

Hazarika B (2003) Acclimatization of tissue-cultured plants. Curr. Sci 85:1704-1712.

Hdider C, Desjardins Y (1994) Effects of sucrose on photosynthesis and phosphoenolpyruvate carboxylase activity of *in vitro* culture strawberry plantlets. Plant Cell. Tiss. Organ Cult. 36:27-33.

Jackson M, Abbott A, Belcher A, Hall K, Butler R, Cameron J (1991) Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Annal. Bot 67:229-237.

Krishnan K, Vijayalakshmi N (2005) Alterations in lipids and lipid peroxidation in rats fed with flavonoid rich fraction of banana (*Musa* paradisiaca) from high background radiation area. Indian J. Med. Res 122:540-546.

Laemmli U (1970) Cleavage of structural proteins during the heat of bacteriophage T4. Nature 227:680-685.

Larema L, Ferreira A, Witt C, Carnevalli L, Fontes R, Jardim E, Campos W (2012) Photoautotrophic propagation of Brazilian ginseng [Pfaffiaglomerata (Spreng.) Pedersen]. Plant Cell Tiss. Organ Cult 110:227-238.

Lee V, Lamaze T, Champigny M (1991) Effect of light and NO_3^- on wheat leaf phosphoenolpyruvate carboxylase activity. Evidence for covalent modulation of the C3 enzyme. Plant Physiol 97:1476-1482.

Lin M, Turpin D, Plaxton W (1989) Pyruvate Kinase isoenzymes from green algaSelenastrumminutum. I. Purification and physical and immunological characterization. Arch. Biochem. Biophys 269: 219-227.

Lorenzo J, Blanco M, Peláez O, González A, Cid M, Iglesias A, González B, Escalona M, Espinosa P, Borroto C (2001) Sugarcane micropropagation and phenolic excretion. Plant Cell Tiss. Organ Cult 65:1-8.

Ma S, Shii C (1974) Growing banana plantlets from adventitious buds. J. Chinese Soc. Hort. Sci 20:6-12.

Martín A, Casas J, Piqueras A (2009) Nitrogen assimilating enzymes during acclimatization of micropropagated *Musa* spp. plants. Acta Horti 812:421-42.

Moreira F, Borghezan M, Lima A (2003) Assessment of the carbon dissimilation methodology in the *in vitro* growth of the 'Paulsen 1103' grapevine. Crop Breed. Appl. Biotechnol 3:157-162.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays whit tobacco tissue cultures. Physiol. Plant 15:473-497.

Nakamura S, Nitta Y, Watanabe M, Goto Y (2005) Analysis of leaflet shape and area for improvement of leaf area estimation method for sago palm (Metroxylonsagu Rottb.). Plant Prod. Sci 8:27-31.

Outlaw J, Vlieghere-He X (2001) Transpiration rate. An important factor controlling the sucrose content of the guard cell apoplast of broad bean. Plant Physiol 126:1716-1724.

Panis B, Thinh N (2001) Cryopreservation of *Musa* Germplasm. INIBAP Technical Guidelines 5. INIBAP, France.

Pérez C (2005) Técnicas estadísticas con SPSS 12. Aplicaciones al análisis de datos. Pearson Educación S.A. (Ed), Spain. ISBN 84-205-4410-8.

Ramagli L (1999) Quantifying protein in 2D PAGE solubilization buffers. In: Link A.J. (ed): Methods in molecular biology-2D proteome analysis protocols. pp. 112. Humana Press, Totowa, NJ.

Roels S, Escalona M, Cejas I, Noceda C, Rodríguez R, Cañal M, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tiss. Organ Cult 82:57-66.

Roels S, Noceda C, Escalona M, Sandoval J, Cañal M, Rodríguez R, Debergh P (2006) The effects of headspace renewal in a Temporary Immersion Biorreactor on plantain (*Musa* AAB) shoot proliferation and quality. Plant Cell Tiss. Organ Cult 84:138-146.

Rouhier N, Gelhaye E, Gualberto J, Jordy M, De Fay E, Hirasawa M, Duplessis S, Lemaire S, Frey P, Martin F, Manieri W, Knaff D, Jacquot J. (2004) Poplar Peroxiredoxin Q. A Thioredoxin-Linked Chloroplast Antioxidant Functional in Pathogen Defense. Plant Physiol 134:1027-1038.

Siegel G, Stitt M (1990) Partial purification of two forms of spinach leaf sucrose-phosphate synthase which differ in their kinetic properties. Plant Sci 66:205-210.

Stearns J, Glick B (2003) Transgenic plants with altered ethylene biosynthesis or perception. Biotechnol. Adv 21:193-210.

Teisson C, Alvard D 1995) A new concept of plant *in vitro* cultivation liquid medium: temporary immersion. In: Terzi, M.; Celia, R.; Falavigna, A. (eds): Current issues in plant molecular and cellular biology. pp.105-110. Kluwer Academic Publishers, Dordrecht.

Thomas W, Rufty J, Steven C (1983) Changes in starch formation and activities of Sucrose Phospate Synthase and cytoplasmic fructose-1-6-bisphosphatase in response to source-sink alterations. Plant Physiol 72:474-480.

Thompson A (1995) Banana Processing. In: Gowen, S. (ed): Bananas and Plantains. pp. 481-492. Chapman and Hall, UK.

Tian L, Ohsugi R, Yamagishi T, Sasaki H (2006) Effects of weak light on starch accumulation and starch synthesis enzyme activities in rice at the grain filling stage. Rice Sci 13:51-58.

Van Huylenbroeck J, De Riek J (1995) Sugar and starch metabolism during *ex vitro* rooting and acclimtization of micropropagatedSpathiphyllum ´Petite´ plantlets. Plant Sci 111:19-25.

Van Huylenbroeck J, Piqueras A, Debergh P (2000) The evolution of photosyntesis capacity and the antioxidant enzymatic system during acclimatization of micropropagated Calathea plantlets. Plant Sci 155:59-66.

Weremfo M, Adinortey M, Pappoe A (2011) Haemostatic effect of the stem juice of *Musa* paradisiaca L. (*Musaceae*) in Guinea Pigs. Adv. Biol. Res 5:190-192.

Woodrow L, Thompson R, Grodzinski B (1988) Effects of Ethylene on Photosynthesis and Partitioning in Tomato, Lycopersicon esculentum Mill. J. Exp. Bot 39:667-684.

Ziv M (2005) Simple bioreactors for mass propagation of plantlets. Plant Cell Tiss. Organ Cult 81:277-285.

Chapter 3

Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic characteristics and develops improved anti-oxidative response

Aragón C[1,2] Carvalho L[1] González J[2] Escalona M[2] Amancio S[1*]

[1]CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Tropical Plant Biology (2009) 2:38-50

Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic characteristics and develops improved anti-oxidative response

Aragón C[1,2] Carvalho L[1] González J[2] Escalona M[2] Amâncio S[1]

[1] CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Abstract

Previous results have shown that sugarcane plantlets micropropagated in Temporary Immersion Bioreactors (TIB) demonstrated a better morphology and physiological behaviour when compared to plantlets propagated in Gelled Medium (GM). The present work focuses on the onset of oxidative stress symptoms at transfer to *ex vitro* and during acclimatization. The specific ROS being produced were identified and tissue-located by infiltrating leaves with specific $O_2^{-\cdot}$ and H_2O_2 staining dyes, respectively NBT and DAB. TIB plantlets showed trichomes stained with NBT and DAB, their density decreasing with time. Stomata were coloured with NBT and DAB in GM and, at the end of acclimatization, plantlets from both systems presented the lowest level of staining of both stomata and trichomes. The response of the anti-oxidative system was also analysed through *in vitro* and in gel enzyme activities and transcription levels of genes for key response enzymes. At the end of the *in vitro* phase, GM plantlets showed higher activities of APX and MDHAR, while CAT, GR, GT and DHAR activities increased in TIB. At the end of acclimatization SOD and CAT increased mainly in TIB, while GM induced the increase of APX. The immunobloting of peroxiredoxins showed that Prxs were expressed at higher levels in TIB plantlets, some showing polymerization. The transcription of genes coding for key response enzymes was strongly up-regulated in GM plantlets. In conclusion and comparing with GM, TIB produced plantlets closer to autotrophy and with improved mobilization of the anti-oxidative response.

Keywords: asc-glut cycle; *ex vitro* growth; *in vitro* propagation; oxidative stress; peroxiredoxins.

3.1 Introduction

The metabolic reprogramming event in plantlets after *in vitro* propagation is the main handicap for their *ex vitro* survival, thus conditioning the yield of the technique and representing one aspect in plant physiology which is seldom tackled with. The use of Temporary Immersion Bioreactors (TIB) has proven to be an alternative to gelled medium (GM) as an efficient propagation technique in what concerns plantlet morphology and physiology (Lorenzo et al. 2001; Escalona et al. 2003; Aragón et al. 2005). As a low cost option for the micropropagation of tropical crops (sugarcane, plantain, pineapple, etc.) this technique represents an important economical resource for the massive production of plantlets for the agricultural industry. Besides the traditional products derived from sugarcane (*Saccharum* spp.), which are sugar and cellulose, there are other products useful in animal feeding, alcoholic drink industry, etc. Recently, and due to new legislation on carbon dioxide emissions, sugarcane production became pivotal in the demand for bio-ethanol. Its requirement has already increased considerably and will be raised 3.28 fold by 2017 (Bioethanol productions 2007). As a consequence, the micropropagation of this crop in TIB is one of the strategies proposed by several biotechnology companies to meet with the increasing demand. Therefore, it is necessary to develop and improve protocols for the transition of the plantlets from mixotrophic conditions to autotrophy during the acclimatization phase. The micropropagation of sugarcane, a C_4 species, has been recently studied in TIB propagated plantlets, focusing on carbon metabolism (Rodriguez et al. 2003), but not in what concerns the oxidative stress associated with micropropagation techniques. Usually, the headspaces accumulate several volatile compounds that can cause oxidative stress (Roels et al. 2006). Leaf morphology, expansion and stomata functionality are also affected by the presence of ethylene and nitric oxide associated with abscisic acid production (Jackson et al. 1991; Tanaka et al. 2005). Moreover, the transition of the plantlets to an *ex vitro* phase under high light intensity leads the way to the production of ROS (reactive oxygen species), which seems to be another pattern of response during the acclimatization phase (Van Huylenbroeck et al. 2000; Carvalho et al. 2006). However, most of the studies have only applied GM and seldom compare it to TIB.

The use of TIB leads to an increase in the multiplication coefficient and to higher quality of the plants produced when compared with GM. TIB propagated plants show a physiology closer to autotrophy, which is favored by the possibility to recycle the air inside the vessels

thus avoiding the growth of the plantlet in a closed environment (Escalona et al. 2003; Aragón et al. 2005). Several enzymes are known to display determinant roles in the anti-oxidative stress response, such as superoxide dismutase, catalase and those responsible for the functioning of systems such as the ascorbate-glutathione cycle (Gratao et al. 2005).

Another relevant anti-oxidative response involves the differential expression of peroxiredoxins (Prxs) in different cellular compartments. These ubiquitous enzymes are unrelated to other peroxidase families but play a fundamental role in the anti-oxidant defense. They reduce hydrogen peroxide (H_2O_2) and alkyl hydroperoxides and are active against a broader spectrum of peroxides than the H_2O_2-specific ascorbate peroxidase (Noctor et al. 2007). They undergo oxidation during the reaction of peroxide reduction and need to be reduced by electron donors such as glutaredoxins, thioredoxins, or cyclophilins before the next catalytic cycle, thus rendering them as low-efficiency enzymes. However, their abundance allows them to play a determinant role in the oxidative stress defense in specific cellular parts (Horling et al. 2003).

In this paper we show the patterns of response to photo-oxidative stress during acclimatization in sugarcane plantlets propagated under Gelled Medium (GM) or Temporary Immersion Bioreactors (TIB). We have monitored ROS accumulation through imaging, and measured the activities and expression levels of anti-oxidative enzymes. We present evidence that oxidative stress during acclimatization is directly related to the propagation method applied and that TIB propagated plants suffer less from stress and are able to overcome it better than GM plants.

3.2 Materials and Methods

3.2.1 Plant material, in vitro and ex vitro culture

Sugarcane plantlets (*Saccharum* spp. hybrid) clone C91-301 were subjected to three sub-culture cycles of 21 days each in a culture medium containing Murashige and Skoog (1962) salts and vitamins, supplemented with 30 g L^{-1} sucrose (w/v) and 1.33 µM 6-benzylaminopurine (BA). The elongation phase, a preparation of the shoots before acclimatization, lasted 21 days and two different techniques were applied, GM (gelled Medium) and TIB (Temporary Immersion Bioreactors). In both techniques, the media used consisted of MS salts and vitamins supplemented with 30 g L^{-1} sucrose and without plant growth regulators. In GM, plantlets were placed in Magenta vessels (Sigma-Aldrich St.

Louis, MO) of 250 mL total volume, containing 50 mL of media and Gelrite 2.0 g L^{-1} with 5 shoots per vessel. TIB was performed in containers of 500 mL volume (Schott, Duran) containing 5 shoots per bioreactor and 200 mL of media (Escalona et al. 2003). Shoots were immersed for 4 min every 3 h. PPFD was 45 ± 5 mmol $m^{-2}s^{-1}$ and the photoperiod was 16/8h in both propagation techniques.

Microcuttings produced under TIB and GM were transplanted to pots containing a sterilised mixture of hydrated peat and perlite (1:1, v/v) and placed in glass chambers (500E, Aralab, Porto Salvo, PT) of 450 L volume. Light was provided by fluorescent lamps Gro-Lux F18W/GRO and, at plant level, photon flux density (PPFD) was 200 ± 10 µmol m^{-2} s^{-1} and the photoperiod 16/8h. The programmed relative humidity (RH) inside the glass chamber was obtained by an ultrasonic fog system controlled by a hygrometer. The initial value was set at 98% and was decreased until the RH in the glass chamber attained the ambient value at the end of acclimatization. Temperature was kept at 25 ± 2 ºC during the light and 22 ± 1 ºC in the dark.

The analyses were performed in leaves at time zero (day 0) of transfer to acclimatization, during the first seven days (day 1 to day 7) and on day 14 and 21 of *ex vitro* conditions in plantlets elongated under TIB and GM. Samples were collected in the middle of the light period.

3.2.2 Histology and image analysis

Stomatal index (%) was calculated as (SF/(SF+EF)) x 100 where SF and EF are respectively de number of stomata and epidermal cells per unit leaf area of the first leaf fully expanded (Carvalho et al. 2002). To calculate trichome index, the number of trichomes (TF) replaces SF in the above formula.

The detection of ROS was carried out as described by Fryer et al. (2002). Leaves from sugarcane plantlets were detached on day 0 and on day 14 of *ex vitro* growth in a 2 mM EDTA solution pH 5.5, incubated in 5 mM 3,3' diaminobenzidine (DAB) pH 3.8 to detect H_2O_2 or in 6 mM nitroblue tetrazolium (NBT) to detect $O_2^{-\cdot}$,and cleared in ethanol at 60 ºC.

3.2.3 Extraction for enzyme activity assays

The extracts for measuring enzyme activities were prepared at 4 ºC from 0.5 g of frozen leaf material. The material was ground using mortar and pestle, and 50% (w/w)

polyvinylpolypyrrolidone was added. The extraction buffer was Tris-HCl 0.35 M (pH 8) supplemented with EDTA 20 mM; DETC 11 mM, cystein 15 mM (Jacobs et al. 1999), and 0.2 mM PMSF. Extracts were centrifuged at 27 000 *g* for 10 min at 4 ºC and the supernatants, desalted through PD-10 columns (Amersham Pharmacia Biotech, Buckinghamshire, UK), were used for all the determinations. Protein was determined by the method of Bradford (1976) using a commercial kit (Bio-Rad, Hercules, CA).

3.2.4 Preparation of mesophyll and boundle sheath extracts

For the extraction of mesophyll tissue, leaf segments were placed between a roller and an aluminum block (both kept at 4 ºC), according to Leegood (1985). The roller was moved along the length of the leaf so that slight pressure was applied. This resulted in the displacement of mesophyll sap from the leaf blade into the extraction buffer was placed beneath the leaf. The pressure was insufficient to disturb the bundle-sheath strands that remained intact. The remaining of the process of protein extraction for both compartments and the buffer used are described above.

In all cases, the purity of the fractions was determined by measuring the bundle sheath and mesophyll marker enzymes RuBisCO and phosphoenolpyruvate carboxylase (PEPC), respectively. Maximal RuBisCO activity was determined according to Carvalho et al. (2005). PEPC activity was measured according to the method of Van Quy et al. (1991).

3.2.5 Enzyme activity assays

The activities of all enzymes were assayed using the extracts described above as samples. Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was determined at 25 ºC using a modification of the method of Hossain and Asada (1984). The reaction mixture of 1 mL contained 50 mM potassium phosphate-KOH pH 7.5, 0.5 mM AsA, 0.1 mM EDTA and the enzyme sample (*ca* 14 µg protein). The oxidation rate of AsA was estimated between 1.0 and 60 s after starting the reaction by adding 0.2 mM H_2O_2.

Dehydroascorbate reductase (DHAR) (EC 1.8.5.1) activity was determined according to the method of Dalton et al. (1993) following the increase in A_{265} due to ascorbate formation (ε_{265} = 14 $mM^{-1}cm^{-1}$). The assay mixture contained 0.1 M Hepes-KOH buffer pH 7.0, 2.5 mM GSH, 0.5 mM DAsA, 0.1 mM EDTA and the enzyme sample (*ca* 35 µg protein), in a final volume of 1 mL. The reaction rate was corrected for the nonenzymatic reduction of DAsA by

GSH. A factor of 0.98 to account for the small contribution to the absorbance by GSSG was also considered.

Monodehydroascorbate reductase (MDHAR) (EC 1.6.5.4) activity was determinated by following the decreased in A_{340} due to NADH oxidation, as described (Hossain and Asada 1984) in a coupled test system, in which the monodehydroascorbate radical is produced by ascorbate oxidase in Tris-HCl buffer, pH 7.5, containing 0.2 mM NADH, 2.5 mM AsA, 0.5 units ascorbate oxidase (from *Cucurbita* species, Sigma-Aldrich St. Louis, MO) and enzyme extract (ca 14 µg protein) in a final volume of 1 mL at 25 ºC.

Glutathione reductase (GR) (EC 1.6.4.2) was assayed by the decrease in A_{340} due to NADPH oxidation (ϵ_{340} = 6.22 mM^{-1} cm^{-1}), as described (Schaedle and Bassham 1977), in a reaction mixture of 1 mL containing 50 mM Tris-HCl pH 7.5, 0.15 mM NADPH, 0.5 mM GSSG, 3 mM $MgCl_2$ and the sample (*ca* 35 µg protein).

Superoxide dismutase (SOD) (EC 1.15.1.1) was assayed by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals (McCord and Fridovich 1969) in a 1 mL reaction mixture containing 50 mM potassium phosphate-KOH buffer pH 7.6, 0.1 mM EDTA, 0.01 mM cytochrome C, 0.05 mM xanthine, 0.03 units xanthine oxidase and the enzyme sample (*ca* 14 µg protein).

Catalase (CAT) (EC 1.11.1.6) is directly determined by the decomposition of H_2O_2 at 240 nm in a 1 mL mixture containing 50 mM potassium phosphate-KOH pH 7.0, 10 mM H_2O_2 and the sample (*ca* 35 µg protein).

Glutathione transferase (GT) (EC 2.5.1.18) was assayed as the increased of A_{340} due to the conjugation of GSH to 1-chloro-2,4dinitrobenzene (CDNB), as described (Drotar et al. 1985), in 100 mM potassium phosphate-KOH, pH 7.0, containig 2 mM CDNB, 2 mM GSH and enzyme source (ca 14-35 µg protein).

3.2.6 Native PAGE and gel activity staining

Native Polyacrylamide Gel and Gel Activity Staining Iso-forms of CAT, SOD, APX, and GR were separated in nondenaturing polyacrylamide gels by the procedure of Laemmli (1970). Equal amounts of protein extracts (25 mg) were loaded on 7% (CAT) or 10% (SOD, GR, and APX) polyacrylamide gels.

For SOD, the gel was stained according to Rao et al. (1996). Gels were incubated for 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA. To identify KCN and H_2O_2 sensitive iso-forms, this incubation solution contained 3 mM KCN or 5 mM H_2O_2, respectively. This step was followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 0.245 mM nitroblue tetrazolium, 33.2 mM riboflavin, and 0.2% tetramethyl ethylene diamine (TEMED) in darkness for 30 min. before illumination to visualize SOD iso-forms or bands (Donahue et al. 1997).

To visualize the CAT profile, gels were stained by the procedure of Anderson et al. (1995). The gels were incubated in 3.27 mM H_2O_2 for 25 min, rinsed in distilled water, and then stained in a solution containing 1% (w/v) potassium ferricyanide and 1% (w/v) ferric chloride.

Iso-forms of APX were visualized by incubating the gels for 30 min. in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate, followed by an incubation in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. Finally, gels were stained in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium for 15 min.

GR was detected by incubating the gels for 60 min in darkness in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 0.7 mM 2,6-dichlorophenolindophenol, 3.4 mM GSSG, and 0.5 mM NADPH. The staining reaction was stopped by adding 7.5% (v/v) glacial acetic acid to the staining buffer.

Relative quantification of iso-enzyme activities was determined using the software Quantity One (Bio-Rad, Hercules, CA).

3.2.7 Peroxiredoxin activity and immunoblotting

Reduction of H_2O_2 by peroxiredoxins was quantified through a non-enzymatic, DTT-dependent activity assay by measuring the decrease in H_2O_2 concentration in the assay solution. The assay contained 100 mM K-Pi buffer (pH 7.0), 0.3 to 3 µM Prx, 10 mM DTT, and 100 µM H_2O_2 in a total volume of 1 mL. The reaction was initiated with H_2O_2 and stopped with 800 µL of trichloroacetic acid (12.5% (w/v)) to an aliquot of 50 µL of assay solution. After adding 200 µL of 10 mM $Fe(NH_4)_2(SO_4)_2$ and 100 µL of 2.5 M KSCN, the

A_{480} was measured to quantify the H_2O_2 contents of the solution, and H_2O_2 reduction rates were calculated.

Polyclonal antibodies against cytosolic type II Prx C, chloroplast-located type II Prx E, chloroplast-located s-cys Prx, chloroplast-located Prx Q and mitochondria-located type II Prx F, kindly supplied by Professor K-J Dietz (Bielefeld University, Germany). Western blot analysis was performed by electrophoretic transfer of proteins, separated by SDS-PAGE, to a nitrocellulose membrane (Millipore) and probed with the respective antibodies using the procedure described by Ferreira et al. (1996).

3.2.8 RNA isolation and cDNA preparation for Real-Time PCR

Total RNA from leaves was extracted by adapting the method described by Chang et al. (1993). CTAB (2 %) was complemented with PVPP (2%, w/w), Trizma-HCl 100 mM, 25 mM Na_2EDTA, and 2 M NaCl; pH 8.0 and the buffer was heated to 85 ºC prior to the addition of 400 µL 2-mercarptoethanol. Tissues were reduced to powder in liquid N_2 and 20 mL extraction buffer were added. The same volume of chlorophorm:iso-amyl alcohol 24:1 was then added. This step was followed by a centrifugation at 12 000 *g* for 30 min at 20 ºC and repeated once. The aqueous phase was transferred to a new tube, and a ¼ LiCl 10 M (v/v) was added. The sample was incubated at 0 ºC overnight and after centrifugation at 12 000 *g* for 20 min. at 4 ºC. The pellet was recovered and 1.5 mL of buffer (Trizma-HCl 10 mM, 1 mM Na_2EDTA, 1 M NaCl; 0.5 % SDS (w/v), pH 8.0.) previously heated to 37 ºC was added. The same volume of chlorophorm:iso-amyl alcohol (24:1) was added and this step was followed by a centrifugation at 12 000 *g* for 10 min at 20 ºC and repeated once. Ethanol 100% (2.5 volumes) was added and the samples were incubated for 1 h at 80 ºC and then washed with ethanol 70%. After drying, the RNA was resuspended in the desired volume of distilled water. RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI) and reverse transcribed using random hexamers and Superscript II RNase H-reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

3.2.9 Real-Time PCR and quantification of mRNA levels

Primer pairs used for amplification of all the genes studied are presented in Table 3.1. The genomic sequences for sugarcane available in the GeneBank database were used. The real-time PCR was performed in 20 µL of reaction mixture composed of cDNA, 0.5 µM gene-

specific primers and master mix iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) using an iQ5 Real Time PCR (Bio-Rad, Hercules, CA).

Table 3.1 Real-time PCR primers used for evaluation of mRNA levels of the enzymes studied.

Protein name, intracellular localization	**RNA gene bank number**	**Primers**	
		Name	**Sequence**
Actin	AY742219.1	ACT-F	5'-GGACCTCTACGGCAACATTGTG-3'
		ACT-R	3´-CCAGACTCATCATACTCCTCCTTCG-5´
MnSOD (mitochondria)	CA242138	MSOD-F	5'-AGAAGCACCACGCCACCTAC-3'
		MSOD-R	3´-CACCCATCCAGATCCTTGTAAAGC-5´
CuZnSOD (chloroplast, cytosol)	TC48812	CZSOD-F	5'-GGCTGTTGCTGTGCTTGGTAG-3'
		CZSOD-F	3´-TGGCGGTTCTCATCTTCTGGTG-5´
CAT	EF566889.1	CAT-F	5'-GGCAAGAGCGGTTCATCAAGAG-3'
		CAT-R	3´-TGAGCGAAGCAGAGTTCAGTCC-5´
APX	CF570493	APX-F	5'-TACGGACGAGTTGATGTTACAGGAC-3'
		APX-F	3´-ACCTTCCAAGTGTATGTGCTCCAG-5´
GR (cytosol)	TC57827	GR-F	5'-TAGTGTATGGGCTGTGGGTGATG-3'
		GR-R	3´-GCTTGGATATGCTGTTCTTCATTGG-5´
Prx1 (mytochondria)	AY796053.1	Prx1-F	5'-GCCTATCTGCCGTGTCGTCTG-3'
		Prx1-R	3´-GCCTGTGTCTCAACTCGCATTTC-5´

Amplification of PCR products was monitored via intercalation of SYBR-Green (included in the master mix). The following program was applied: initial polymerase activation, 95 ºC, 3 min.; then 40 cycles at 95 ºC 15 s (denaturation), 57 ºC 30 s (annealing), 72 ºC 20 s (extension) with a single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and confirm the lack of primer dimmers. Further, RT-PCR products were resolved on 2% (w/v) agarose gels run at 4 V cm^{-1} in Tris-acetate-EDTA buffer (TAE), along with a 50-bp DNA-standard ladder (Invitrogen Gmb H), to confirm the existence of a single product of the desired length.

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analysed with an R_n threshold of 0.2 to obtain C_T (threshold cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). In order to compare data from different PCR runs or cDNA samples, C_T values were normalized to the C_T value of *Act2*, a housekeeping gene expressed at a relatively high and constant level. Gene expression was normalized to that of *Act2* by subtracting the C_T value of *Act2* from the C_T value of the gene of interest.

3.2.10 Statistical analysis of the results

All experiments were performed three times with 3 repetitions each. All statistical analyses were carried out using SPSS version 12 (Pérez 2005). All situations were analysed using non-parametric analyses, either Kruskall-Wallis H. and C-Dunnett or Mann Whitney, both at 5 % significance level.

3.3 Results

The present work compares the distinct patterns of response of sugarcane plantlets when subjected to propagation under Temporary Immersion Bioreactors (TIB) and Gelled Medium (GM). Analyses were performed at time zero (day 0) of transfer, and plantlets were monitored at intervals during the 21 days of acclimatization.

3.3.1 Histological analyses and Imaging of ROS

The histological analyses of leaf structures (stomata and trichomes) are shown in Fig. 3.1 and 3.2. Leaves were collected, infiltrated with NBT (left) or DAB (right), as described in Materials and Methods. The purple formazan deposits, which result from the reaction of NBT with O_2^- identify the regions where this molecule is produced or accumulated.

H_2O_2 was imaged in leaves infiltrated with DAB which produces a brown polymerization product from its reaction with H_2O_2, in the presence of peroxidases. The staining patterns were similar in plantlets propagated in GM and TIB, the only difference being the index of stained structures per leaf (Fig. 3.2).

Cells surrounding the stomata guard cells were stained for both ROS (Fig. 3.1). Trichomes showed a unique pattern of staining (Fig. 3.1b), with formazan deposits (NBT staining) accumulating in the glandular structure in the second cell of bicellular trichomes. When these

structures were stained with DAB the brown polymerization products accumulated preferentially in the cell wall.

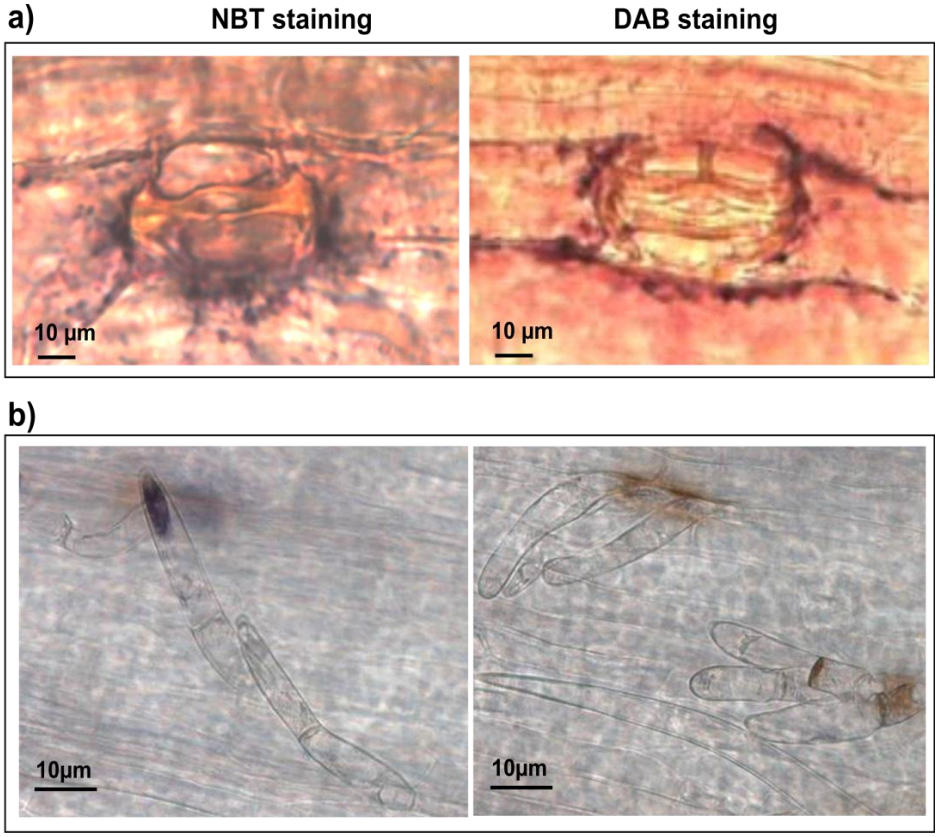

Fig. 3.1 Imaging of ROS accumulation in stomata (a) and trichomes (a) of sugarcane plantlets stained with NBT for superoxide radical visualization (left) and with DAB for hydrogen peroxide visualization (right). Scale bars are represented in each image.

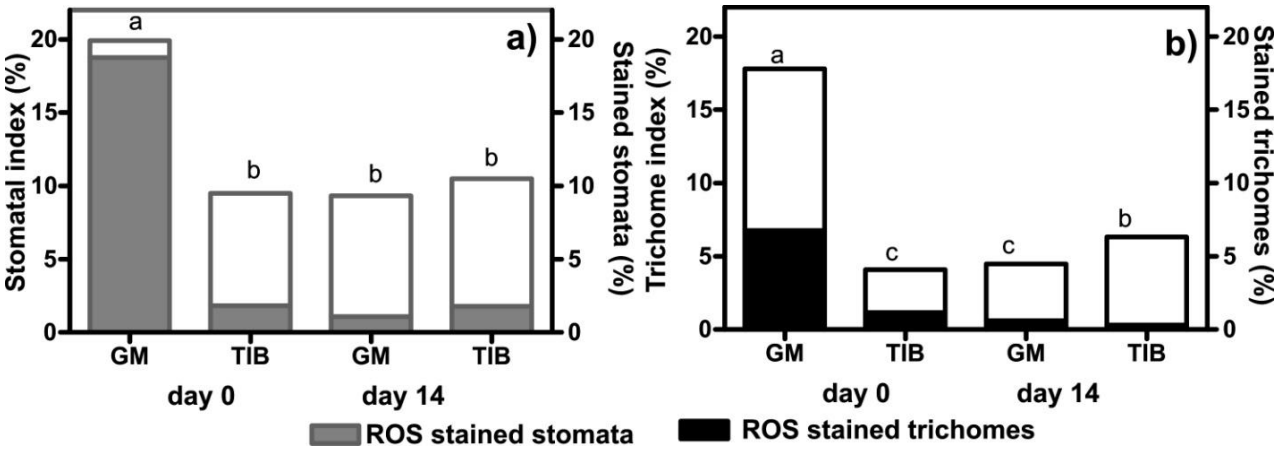

Fig. 3.2 Coloured stomata (a) and trichome (a) index in GM and TIB propagated plantlets prior to transfer to *ex vitro* (day 0) and after acclimatization (day 14). Values indicated by different letters are significantly different at 5 % level by C-Dunnett Multiple Range Test (n=9).

Fig. 3.2 shows total and stained stomatal indexes (Fig. 3.2a) and total and stained trichome indexes (Fig. 3.2b) of GM and TIB plantlets, on day 0 and day 14 of *ex vitro* growth. On day 0 both stomatal and trichome indexes were significantly higher in GM cultured plantlets as compared to TIB, and so was the percentage of colored stomata and trichomes. After 14 days *ex vitro* both indexes decreased significantly in GM plantlets while TIB plantlets maintained trichome and stomata indexes similar to day 0.

3.3.2 Anti-oxidative response at the level of enzyme activity

The total activities of several photo-oxidative stress response enzymes measured at the end of GM and TIB *in vitro* phase (day 0) and daily during the first seven days and on day 14 and 21 of acclimatization are represented in Fig. 3.3. On day 0 GM plantlets showed significantly higher activities of MDHAR and APX, TIB conditions were responsible for higher activities of CAT, GR, DHAR and GT while SOD values were similarly low in both propagation methods. The activities monitored during the acclimatization period in plantlets grown under both propagation methods showed that SOD activity increased significantly in TIB grown plantlets and was almost residual in GM plantlets. During the first seven days, enzyme activity showed a large range of values, mostly, in TIB, with peaks on days 3 and 7 for SOD, day 3 for CAT, days 3 and 5 for GR and day 4 for MDHAR. TIB plantlets maintained higher SOD, CAT, DHAR and MDHAR activities along the remaining acclimatization period, while GR, APX, Prx and GT activities were equivalent in GM and TIB plantlets on day 21. Total Prx activity decreased slowly from day 0 on in both treatments.

By applying the inhibitors described in Materials and Methods, in gel enzyme activity enabled the identification of one Mn-SOD and three CuZn-SOD, whose relative activity was quantified (Table 3.2). Mn-SOD (SOD-D) increased slightly from day 0 to day 7 and remained high until day 21 in both GM and TIB. Two of the three CuZn-SOD iso-enzymes were constitutive (SOD-A and B) and showed constant activity levels in both propagation methods and through time while SOD-C was present on day 0 in both treatments and also on day 21 in TIB. In TIB, while the difference in total activity between day 0 and day 21 is six fold, in gel SOD activities increase between 10 and 17%. Apparently, high activities in the extracts were not reproduced proportionally in gel. CAT showed a single band with similar levels of activity only on day 0 in GM and TIB. Once again, the activities in the extracts were not reproduced in gel.

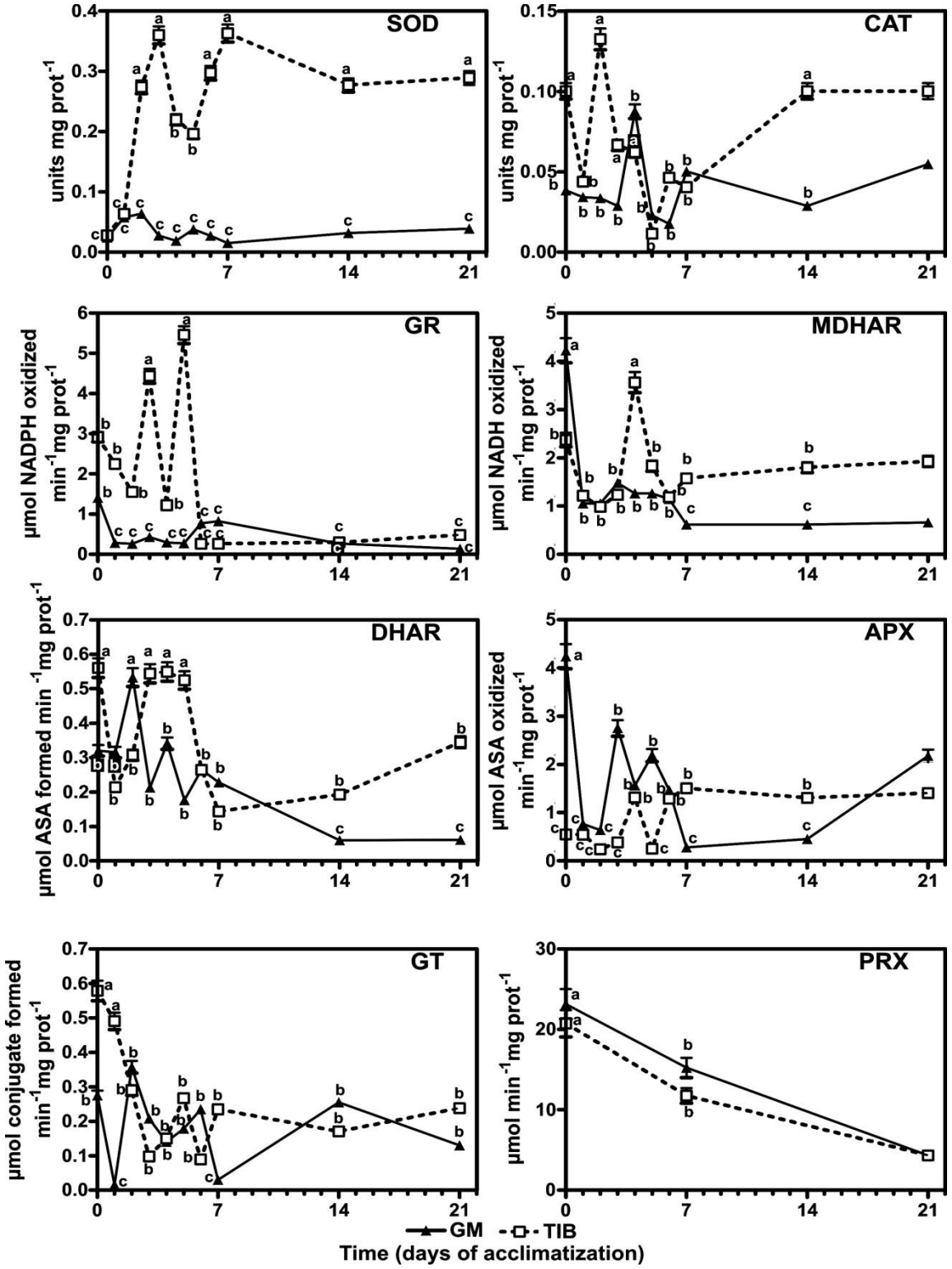

Fig. 3.3 Total soluble enzyme activities in leaves of sugarcane plantlets propagated under GM and TIB at the moment of transfer (day 0) and during acclimatization. Activities are expressed as follows: CAT, SOD, units per milligram of protein; APX, micromoles of AsA oxidized per minute per milligram of protein; DHAR, micromoles of AsA formed per minute per milligram of protein; GR, micromoles of NADPH oxidized per minute per milligram of protein; Prx, micromoles of H_2O_2 reduced per minute per milligram of protein. For clarity purposes, different scales were used. Values indicated by different letters are significantly different at 5 % level by C-Dunnett Multiple Range Test.

Table 3.2 SOD, CAT, APX and GR iso-enzyme in gel activities in leaves of acclimatizing sugarcane plantlets propagated under GM and TIB were quantified in relation to the value at day 0 (day 0 = 100% activity). Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD iso-forms was revealed by inhibition with H_2O_2 and KCN. Quantification was performed using the software Quantity One (Bio-Rad, Hercules, CA).

	GM:			**TIB:**		
	0	**7**	**21**	**0**	**7**	**21**
APX-A	100	99	95	96	98	96
APX-B	100	97	93	97	94	94
GR-A	100	83	89	95	88	92
GR-B	100	80	122	86	85	89
GR-C	100	104	121	106	109	111
GR-D	100	92	106	92	94	101
CAT	100	-	-	101	-	-
SOD-A	100	109	105	99	101	97
SOD-B	100	104	103	98	101	101
SOD-C	100	-	-	113	-	100
SOD-D	100	110	111	104	117	107

APX in gel activity revealed two iso-enzymes, neither showing significantly different activities than *in vitro*. Also, the two fold difference in activity between days 0 and 21 in TIB was not visible in gel. Four GR iso-enzymes were detected, respectively of 55, 54, 46 and 45 kDa (GR-A through D; Table 3.2 and Fig. 3.4). To elucidate whether GR iso-enzymes are related to a possible distinction of iso-forms between bundle sheath and mesophyll cells, we designed an experiment enabling the physical separation of proteins between those two types of cells (see Material and Methods).

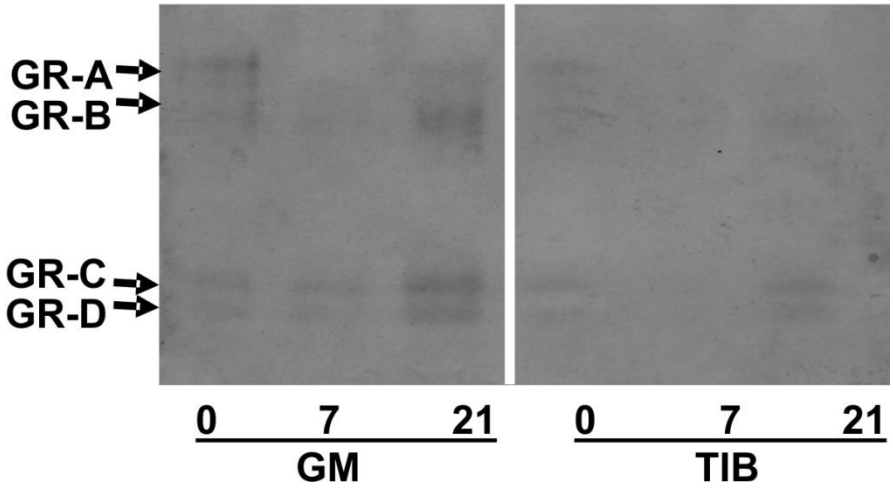

Fig. 3.4 GR iso-enzyme in gel activity in leaves of acclimatizing sugarcane plantlets propagated under GM and TIB. Total protein extracts were subjected to native PAGE followed by activity staining.

To confirm the tissue separation we measured RuBisCO and PEPC enzyme activities in both cell types. In a successful separation, there should be a significantly higher activity of RuBisCO in the bundle sheath and of PEPC in the mesophyl (Pastori et al. 2000). RuBisCO activity in the bundle sheath cells was 3.5 fold higher than in the mesophyl (Table 3.3), while the opposite was observed for PEPC activity, 2.23 fold higher in mesophyl cells. Using these extracts, *in vitro* GR enzyme activity was 1.5 fold higher in bundle sheath cells (Table 3.3). Further, in gel GR activity performed with extracts of the two separate tissues showed that GR-A and GR-C are present in the bundle sheath and GR-B and GR-D in the mesophyl (data not shown). Only the bundle sheath iso-form GR-C shows a tendency for higher activity at the end of *ex vitro* growth. In GM plantlets, in gel isozymes and total activitiy of GR (Fig. 3.4) follow an equivalent trend; in TIB, the decrease in total activity from day 0 to day 21 (Fig. 3.3) is not apparent in Fig. 3.4.

Table 3.3 RuBisCO, PEPC and GR enzyme activity in bundle sheath and mesophyll tissues from sugarcane leaves. Relative values of fold variation between the two tissue types.

	RuBisCO	**PEPC**	**GR**
Bundle sheath / Mesophyll	3,5	0,44	1,5

3.3.3 Detection of different peroxiredoxins using specific antibodies

The immunodetection of five different Prx (Fig. 3.5) revealed the response of Prx iso-enzymes present in specific organelles (chloroplast, mitochondria and cytosol). Chloroplast Prxs showed different patterns of response: 2-Cys Prx was present in the highest concentration as a single band of *circa* 20 kDa, while Type II E and Prx Q were present in lower amounts and revealed polymerization bands, particularly in TIB (*circa* 60 kDa in Prx Q and 40 kDa in type II E). Type II Prx C (cytosol) was detected in low amount while the mitochondria-located type II Prx F was present in high levels, with higher intensity in TIB as compared to GM and evidencing a 40 kDa polymerization band.

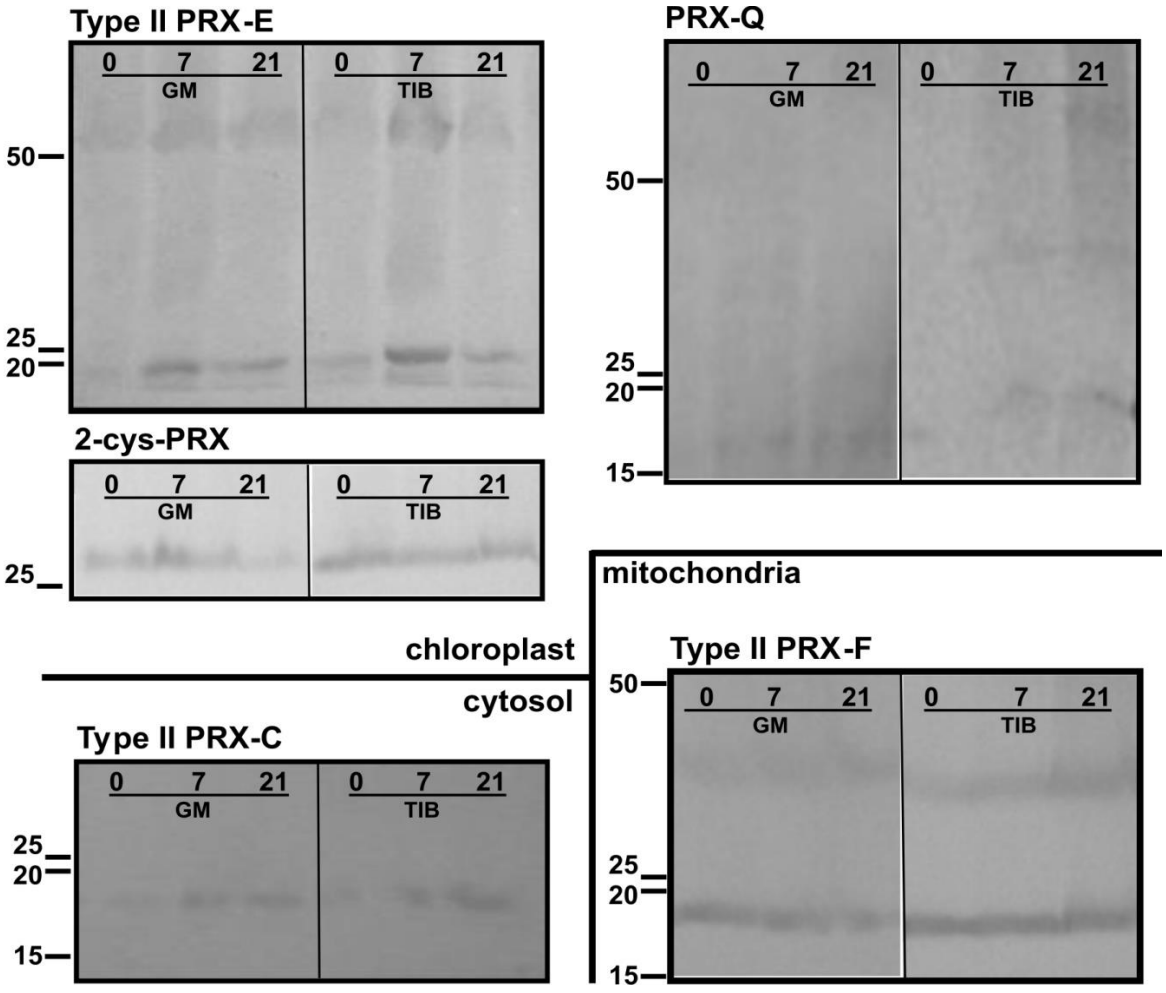

Fig. 3.5 Western blots of five different peroxiredoxins localized in three cellular compartments: chloroplast (2-cys Prx A; type II Prx E; Prx Q), cytosol (type II Prx C) and mitochondria (type II Prx F). Protein samples were subjected to SDS-page, transferred to a nitrocellulose membrane and probed with the specific antibodies. Western blots were performed in leaves of sugarcane plantlets propagated under both systems (GM and TIB) on day 0 of transfer to acclimatization and after 7 and 21 days of *ex vitro* growth. The numbers on the left side of each blot correspond to molecular weight (kDa).

3.3.4 Expression of genes taking part in the anti-oxidative defense system

The expression of sugarcane *APX*, *CAT*, *MnSOD*, *CuZnSOD*, *GR* and *Prx1* genes was monitored on day 0, 3, 7 and 14 of *ex vitro* growth, by real time RT-PCR (Fig. 3.6) with primer design based on sugarcane sequences available on NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez).

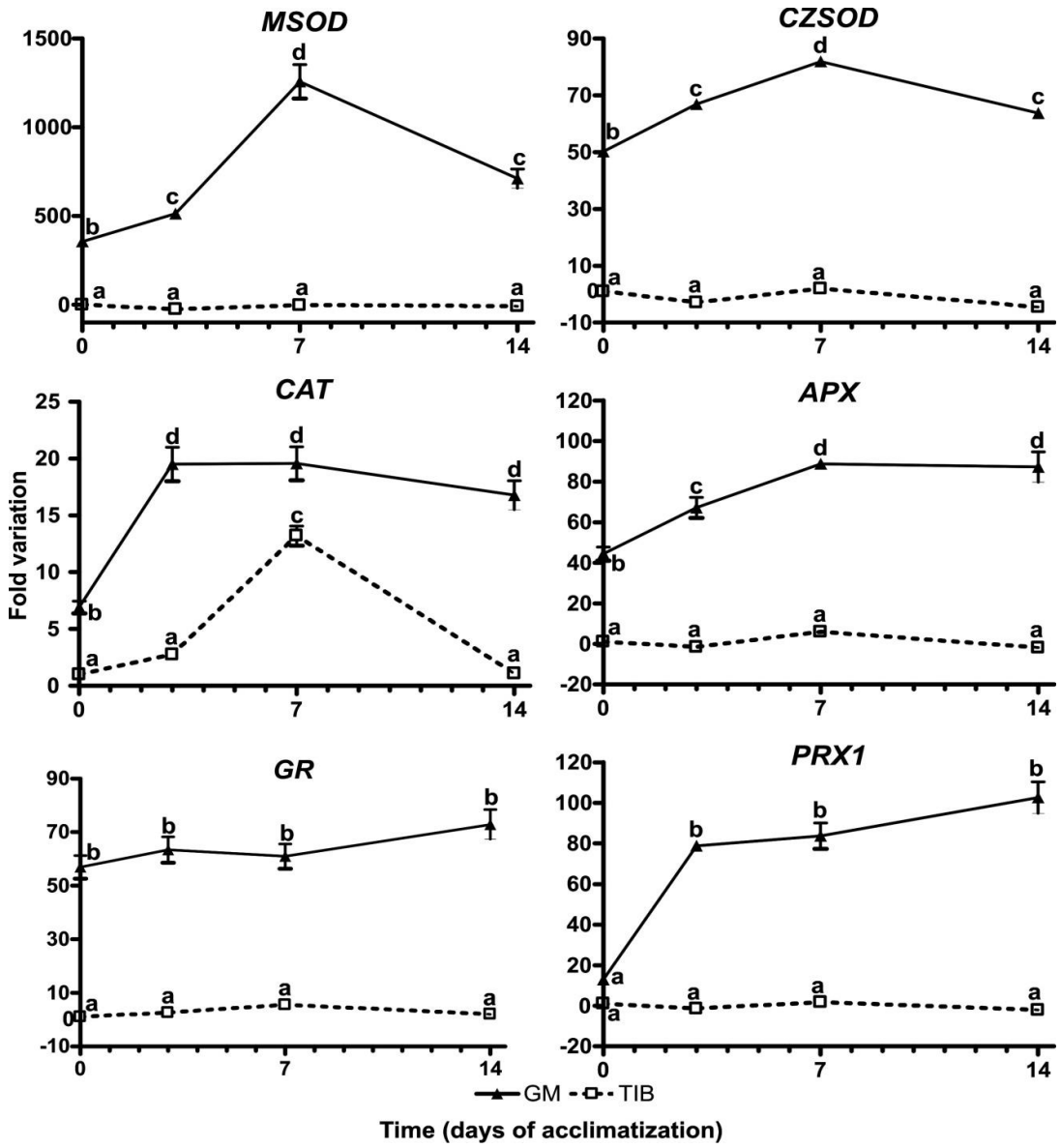

Fig. 3.6 Changes in the expression levels of genes of the anti-oxidative system. Quantification of mRNA levels of those sugarcane genes was performed on day 0 of *ex vitro* growth after each propagation method (GM and TIB) and after 3, 7 and 14 days. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time PCR. Relative amounts were calculated and normalized with respect to sugarcane *Act2* mRNA. Each time point in each propagation method (GM and TIB) is compared to day 0 leaves propagated in GM (control, day 0 = zero fold change). For clarity purposes, different scales were used. Values indicated by different letters are significantly different at 5 % by C-Dunnett Multiple Range Test.

On day 0, all the studied genes were up-regulated in GM as compared to TIB and most of them maintained higher levels of expression for the entire period of acclimatization. On day 0 *Prx1* expression in GM was only 10 fold higher than in TIB, increasing sharply to *circa* 80 fold on day 7. *APX, CAT, CuZnSOD and GR* transcripts were expressed more than 50 fold higher in GM on day 0, and further increased their expression during acclimatization. Extreme responses were measured in *MnSOD* and *CAT*, the first with an increase in expression levels reaching 1200 fold in GM and the second with the lowest levels of up-regulation in GM, between 7 and 20 fold and accounting for the only up-regulation in TIB, on day 7.

3.4 Discussion

The behaviour of sugarcane plantlets propagated *in vitro* using two distinct methods, GM and TIB, was compared during acclimatization to ascertain the efficiency of both micropropagation methods. Symptoms of photo-oxidative stress and the resulting response of the anti-oxidative system were monitored by ROS imaging, enzyme activities and gene expression of pathways affected by photo-oxidative stress and associated with recovery mechanisms.

In GM and TIB propagated plantlets, stomatal structures evidenced functional behaviour shown by the similar patterns of coloration with ROS staining dyes emerging in the cells surrounding the stomata. The major differences between the two treatments were observed in the stomatal and trichome indexes on day 0 of acclimatization, which corresponds to the last day in each of the propagation systems. Leaves formed in GM showed significantly higher stomatal index corresponding to a lower number of mesophyll cells and to larger intercellular spaces, as a direct consequence of lack of headspace ventilation (Majada et al. 2000), differing from those produced in TIB, whose indexes result from the presence of smaller cells and an anatomy similar to autotrophic leaves, observed on day 14 of *ex vitro* growth. Also, the much lower percentage of coloured stomata in TIB propagated plantlets on day 0 is probably related to a more advanced stomatal regulation provided by this propagation method. In GM propagated plantlets, almost 100% of the stomatal cells were stained on day 0. The gain of function of functional stomata is recognizable by DAB staining since it has been shown that H_2O_2 triggers the activation of a signalling pathway over functional stomatal cells (Vilela et al. 2007). Later on, normal regulation of stomatal differentiation and functioning can proceed following the development of roots, new buds and the increase in

ABA content (Vilela et al. 2007), and DAB staining decreases, as was the case in GM propagated sugarcane.

The glandular capacity of trichome structures was apparent in the second cell of the bicellular structure. This can be related with the maturation of trichomes as a response to light stress. Trichomes have the capacity to refract light and reduce the damage brought upon by leaf surface exposure to high light (Wagner 1991). Glandular trichomes are modified epidermal cells that can also confer resistance to biotic stress (Martin and Glover 2007), e.g. by entrapping insects in the polymerized trichome exudates (Kowalski et al. 1992). Trichome index showed a pattern similar to the stomata one, with an additional factor: the death of trichomes during *ex vitro* growth of GM plantlets. Overall, the low stomatal and trichome numerical and staining indexes displayed by the leaves of plantlets under TIB on day 0, places them closer to authotrophic behaviour.

In vitro propagated plants can attain measurable photosynthetic rates, TIB giving rise to higher rates than GM (Aragón et al. unpublished data). However, at least in GM propagated plants, the CO_2 fixation capacity does not curtail the symptoms of photoinhibition and oxidative stress that appear upon the higher light intensities applied at transfer (Carvalho et al. 2002; 2006). The anti-oxidative enzyme response triggered in the cells is a powerful tool to avoid the negative collateral effects of photo-oxidative stress. At the end of the *in vitro* phase (day 0) GM plants evidenced an activation of the H_2O_2 processing branch of the ascorbate-glutathione cycle (higher APX and MDHAR activity), while TIB plants induced not only the DHAR/GR branch but also the activities of other response systems that contribute to curtail oxidative stress damage (CAT and GT). This may explain why TIB plants transferred to *ex vitro*, evidencing high SOD activity, a scavenging strategy that seems to confer a more efficient response to the imposed photooxidative stress, still presented the expected sensitivity of APX to H_2O_2 (Changas et al. 2008). Two of the Cu-Zn SOD iso-forms identified did not change their levels of activity while the inducible iso-form responded to the acclimatization conditions. However, while in GM the variation in SOD activity matched the pattern of MnSOD, in TIB the increase in SOD activity was much higher than the iso-form variation. Sugarcane leaves exposed to paraquat-induced oxidative stress also showed differences between the intensity of activity bands and total SOD activity, which can be explained by the lower sensitivity of the former method and/or the lack of detection of further iso-forms (Chagas et al. 2008). In fact, seven Cu-Zn iso-forms were found in sugarcane

seedlings under the effect of cadmium, although SOD activity remained constant (Fornazier et al. 2002). In GM, particularly *MSOD* but also *CZSOD*, were significantly up-regulated *in vitro*, with the transcript of MnSOD still increasing significantly thereafter. Apparently, TIB plantlets were able to keep high levels of SOD activity without the need to synthesize new enzyme molecules, since the levels of *CZSOD* and *MSOD* did not vary during the whole period. The level of expression and activity of MnSOD in GM plantlets reflects a sound development of the mitochondria, the cellular location of this iso-enzyme (Ruth et al. 2002). However, heterotrophic behavior of GM plantlets, in favor of the anti-oxidative response at the mitochondrial level (Noctor et al. 2007), is in addition an intrinsic characteristic of C_4 metabolism, where mitochondria play an important role in the CO_2 concentration cycle (Leegood 2002).

In gel GR activity revealed four iso-forms, two located in the bundle sheath, and two from the mesophyll, all showing activity, 50% higher in bundle sheath cells., GR activity was not detected in the bundle sheath in a study designed to localize anti-oxidants in maize leaves (Doulis et al. 1997), suggesting that the lack of NADPH in these tissues could explain the absence of GR activity. Also in maize, GR transcripts were present in the bundle sheath but no enzyme activity could be measured, probably due to postranscriptional regulation (Pastori et al. 2000). One difference between these studies and ours, where the co-existence of activity in the four iso-enzymes of GR was found, is that they were performed in adult plants. The specialization of bundle sheath cells is achieved as a result of a combination of the procambial lineage with vein cells in adjacent position (Smith et al. 1996; Jankovsky et al. 2001). It is, therefore, expected to find differences between an adult plant and an *in vitro* propagated one, where specialized tissues are not yet fully developed. It is, in fact, interesting to notice that the bands for GR bundle sheath iso-forms were fainter in TIB plants, probably due to a more advanced specialization than in GM.

Peroxiredoxins (Prxs) decompose ROS and lipid peroxides and tune them in signalling events. Their total activity decreased throughout acclimatization in both treatments. All higher plant Prxs are nuclear-encoded proteins (Dietz 2003) and to distinguish the presence of Prxs targeted to different cellular compartments, we used specific antibodies for five Prxs. Chloroplast located 2-cys Prx A showed a defined pattern with significantly higher quantities in TIB propagated plantlets. Also, chloroplast type II Prx E and Prx Q were almost absent in GM and showed polymerization in TIB propagated plantlets. This shows that TIB not only

induced a pronounced anti-oxidative response at the level of the chloroplast, but also induced the polymerization of enzymes that, in GM plantlets were poorly represented. Mitochondria revealed a pattern similar to the one observed in chloroplasts, with clear polymerization of type II Prx F, and a stronger signal in TIB propagated plantlets. This Prx, the only one targeted to the mitochondria in Arabidopsis, is an important component of the mitochondrial defense system against peroxide stress (Finkemeier et al. 2005). As a whole, GM propagated plantlets evidenced low Prx activity, consistent with the low quantities of the enzymes, which did not show polymerization, although higher levels of the correspondent transcripts were measured, while TIB propagated plantlets also showed low enzyme activity, together with basal expression levels and higher quantity of protein, which displayed polymerization. Prx polymerization is a consequence of an oxidative environment in the chloroplast and mitochondria, and can be used as a marker for oxidative stress (Heiber et al. 2007). These results, once more, point to the conclusion that the anti-oxidative response of GM plantlets was strongly up-regulated at the transcription level without the corresponding activity of translated proteins, due to post-transcriptional or post-translation regulation. The hypothesis that H_2O_2 can behave as a signal for triggering the transcription of anti-oxidant genes (Mullineaux et al. 2006) seems to be confirmed in GM plants.

The overall up-regulation of transcripts in GM plantlets must indicate a much higher induction of the anti-oxidative genes than in TIB propagated plantlets. The lack of up-regulation reported in TIB can be explained by the previous acclimation to patterns of gas exchange much closer to *ex vitro* environment (Kozai and Zobayed 2000).

Until the present work, no studies had been performed to unravel the pattern of oxidative stress response and recovery responsible for the consensual empirical opinion that TIB techniques prove to be better propagation systems than GM. Previous studies supporting that opinion studied the morphology (Lorenzo et al. 2001; Rodriguez et al. 2003) and the photosynthetic capacity (Aragón et al. 2005). With the present work we showed evidence that TIB propagated sugarcane plants present, at the moment of transfer to *ex vitro* conditions, functional stomata and trichome structures with characteristics similar to *ex vitro* plants, helping them cope with the stress imposed upon by acclimatization. Contrary to what happened in GM, TIB plantlets maintained the anti-oxidative system activated from the *in vitro* phase, and the major ROS scavenger, SOD, was present at high levels of activity until the end of acclimatization. In the mesophyll and bundle sheath, and within the different cell

compartments, H_2O_2 produced by high SOD activity can be processed by the GR branch of the ascorbate-glutathione cycle and/or by CAT and Prx. The polymerization of Prx is, apparently, a transient solution for the oxidative environment in chloroplast and mitochondria. All these data put in evidence the better preparation of TIB plantlets to cope with the stress of acclimatization and the adjustment of their autotrophic behaviour to the anti-oxidative response to *ex vitro* environment.

Acknowledgments

To the Program Alban, the European Union Program of High Level Scholarships for Latin American, scholarship number E06D100217CU to C.A; "*Fundação para a Ciência e Tecnologia"*, for the post-doc grant SFRH/BPD/5707/2001 to L.C. and Plurianual funds to CBAA.

3.5 References

Anderson M, Prasad T, Stewart C (1995) Changes in isozyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. Plant Physiol 109:1247-1257.

Aragón C, Escalona M, Capote I, Pina P, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2005) Photosynthesis and carbon metabolism in Plantain (*Musa* AAB) growing in Temporary Immersion Bioreactor (TIB) and *ex vitro* acclimatization. *In Vitro* Cell Dev Biol Plant 41:550-554.

Bioethanol productions (2007) www.bioethanol.com.ph

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-254.

Carvalho L, Santos P, Amâncio S (2002) Effects of light intensity and CO_2 concentration on growth and the acquisition of *in vivo* characteristics during acclimatization of grapevine regenerated *in vitro*. Vitis 41:1-6.

Carvalho L, Esquível MG, Amâncio S (2005) Stability and activity of RuBisCO in chestnut plantlets transferred to *ex vitro* under elevated CO_2. *In Vitro* Plant. 41:523-531.

Carvalho L, Vilela J, Vidigal P, Mullineaux P, Amâncio S (2006) Activation of the ascorbate-glutathione cycles is an early response of micropropagated *Vitis vinifera* L. explantlets transferred to *ex vitro*. Int J Plant Sci 167:759-770.

Chagas R, Silveira J, Ribeiro R, Vitorello V, Carrer H (2008) Photochemical damage and comparative performance of superoxide dismutase and ascorbate peroxidase in sugarcane leaves exposed to paraquat-induced oxidative stress. Pesticide Biochem Physiol 90:181-188.

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Reptr 11:113-116.

Dalton D, Baird L, Langeberg L, Taughet C, Anyan W, Vance C, Sarath G (1993) Subcellular localization of oxygen defence 21 enzymes in soybean (Glycine max L. Merr.) root nodules. Plant Physiol 102:481-489.

Dietz K (2003) Plant peroxiredoxins. Annu Rev Plant Biol 54:93-107.

Donahue J, Okpodu C, Cramer C, Grabau E, Alscher R (1997) Responses of antioxidants to paraquat in pea leaves: relationships to resistance. Plant Physiol 113:249-257.

Doulis A, Debian N, Kingston-Smith A, Foyer C (1997) Differential localization of antioxidants in maize leaves. Plant Physiol 114:1031-1037.

Drotar A, Phelbs P, Fall R (1985) Evidence for glutathione peroxidase activities in culture plant cells. Plant Sci 42:35-40.

Escalona M, Samson G, Borroto C, Desjardins Y (2003) Physiology of the effects of Temporary Immersion Bioreactors on micropropagated pineapple plantlets. *In Vitro* Cell Dev Biol Plant 39:651-656.

Ferreira R, Franco E, Teixeira A (1996) Covalent dimerization of ribulose bisfosphate carboxylase subunits by UV radiation. Biochem J 318:227-234.

Finkemeier I, Goodman M, Lamkemeyer P, Kandlbinder A, Sweetlove LJ, Dietz KJ (2005) The mitochondrial type II peroxiredoxin F is essential for redox homeostasis and root growth of Arabidopsis thaliana under stress. J Biol Chem 280:12168-12180.

Fornazier RF, Ferreira RR, Vitória AP, Molina SMG, Lea PJ, Azevedo RA (2002) Effects of cadmium on antioxidant enzyme activities in sugar cane. Biol Plant 45:91-97.

Fryer M, Oxborough K, Mullineaux P, Baker N (2002) Imaging of photoxidative stress responses in leaves. J Exp Bot 53:1249-1254.

Gratao P, Polle A, Lee P, Azevedo R (2005) Making the life of heavy-metal stressed plants a little easier. Func Plant Biol 32:481-494.

Heiber I, Ströher E, Raatz B, Busse I, Kahmann U, Bevan M, Dietz KJ, Baier M (2007) The redox imbalanced Mutants of Arabidopsis Differentiate Signaling Pathways for Redox Regulation of chloroplast antioxidant enzymes. Plant Physiol 143:1774-1788.

Horling F, Lamkemeyer P, Konig J, Finkemeier I, Kandlbinder A, Baier M, Dietz KJ (2003) Divergent Light, Ascorbate, and Oxidative Stress-Dependent Regulation of expression of the Peroxiredoxin Gene Family in Arabidopsis. Plant Physiol 131:317-325.

Hossain M, Asada A (1984) Inactivation of ascorbate peroxidase in spinach chloroplasts on dark addition of hydrogen peroxide: its protection by ascorbate. Plant Cell Physiol 25:1285-1295.

Jackson M, Abbott A, Belcher A, Hall K, Butler R, Cameron J (1991) Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Annal Bot 67:229-237.

Jankovsky JP, Smith LG, Nelson T (2001) Specification of bundle sheath cell fates during maize leaf development: Roles of lineage and positional information evaluated through analysis of the tangled mutant. Develop 128:2747-2753.

Kozai T, Zobayed S (2000) Acclimatization. In: Spier RE (ed) Encyclopedia of Cell Technology, John Wiley and Sons, Inc pp1-12.

Kowalski S, Eannetta N, Hirzel A, Steffens J (1992) Purification and Characterization of Polyphenol Oxidase from Glandular Trichomes of Solanum berthaultii. Plant Physiol 100:677-684.

Laemmli UK (1970) Cleavage of structural proteins during the heat of bacteriophage T4. Nature 227:680-685.

Leegood R (1985) The intercellular compartmentation of metabolites in leaves of Zea mays L. Planta 164:163-171.

Leegood R (2002) C4 photosynthesis: principles of CO2 concentration and prospects for its introduction into C3 plants. J Exp Bot 53:581-590.

Lorenzo J, Blanco M, Peláez O, González A, Cid M, Iglesias A, González B, Escalona M, Espinosa P, Borroto C (2001) Sugarcane micropropagation and phenolic excretion. Plant Cell Tiss Org Cult 65:1-8.

Majada JP, Tadeo F, Fal MA, Sánchez-Tamés R (2000) Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation Plant Cell Tiss Org Cult 63:207-214.

Martin C, Glover BJ (2007) Functional aspects of cell patterning in aerial epidermis. Curr Opin Plant Biol 10:70-82.

McCord J, Fridovich I (1969) Superoxide dismutase: an enzymic function for erythrocuprein. J Inorg Biochem 244:6049-6055.

Mullineaux P, Karpinski S, Baker N (2006) Spatial dependence for hydrogen peroxide-directed signalling in light stressed plants. Plant Physiol 141:346-350.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473-497.

Noctor G, Paepe R, Foyer CH (2007) Mitochondrial redox biology and homeostasis in plants. TRENDS Plant Sci 12:125-134.

Pastori G, Mullineaux P, Foyer CH (2000) Post-Transcriptional Regulation Prevents Accumulation of Glutathione Reductase Protein and Activity in the Bundle Sheath Cells of Maize. Plant Physiol 122:667-675.

Pérez C (2005) Técnicas estadísticas con SPSS 12. Aplicaciones al análisis de datos. ISBN 84-205-4410-8. Pearson Educación SA (ed), Spain. ISBN 84-205-4410-8.

Rao M, Paliyath G, Ormrod D (1996) Ultraviolet-B- and ozoneinduced biochemical changes in antioxidant enzymes of Arabidopsis thaliana. Plant Physiol 110:125-136.

Rodriguez R, Cid M, Pina D, Gonzalez-Olmedo J, Desjardins Y (2003) Growth and photosynthetic activity during acclimatization of sugarcane plantlets cultivated in Temporary Immersion bioreactors. *In Vitro* Cell Dev Biol Plant 39:657-662.

Roels S, Noceda C, Escalona M, Sandoval J, Canal MJ, Rodriguez R, Debergh PC (2006) The effect of headspace renewal in a temporary immersion bioreactor on plantain (*Musa* AAB) shoot proliferation and quality. Plant Cell Tiss Org Cult 84:155-163.

Ruth A, Neval E, Lendwood H (2002) Role of superoxide dismutase (SODs) in controlling oxidative stress in plants. J Exp Bot 53:1331-1341.

Schaedle M, Bassham J (1977) Chloroplast glutathione reductase. Plant Physiol 59:1011-1012.

Smith LG, Hake S, Sylvester AW (1996) The tangled-1 mutation alters cell division orientations throughout maize leaf development without altering leaf shape. Develop 122:481-489.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S (2005) Ethylene Inhibits Abscisic Acid-Induced Stomatal Closure in Arabidopsis. Plant Physiol 138:2337-2343.

Van Huylenbroeck JM, Piqueras A, Deberg PC (2000) The evolution of photosynthetic capacity and the antioxidant enzymatic system during acclimatization of micropropagated Calathea plants. Plant Sci 135:59-66.

Van Quy L, Lamaze T, Champigny ML (1991) Effect of light and NO_3^- on wheat leaf phosphoenolpyruvate carboxylase activity. Evidence for covalent modulation of the C3 enzyme. Physiol Plant 97:1476-1482.

Vilela J, Carvalho L, Ferreira J, Amâncio S (2007) Gain of function of stomatal movements in rooting *Vitis vinifera* L plantlets: regulation by H_2O_2 is independent of ABA before the protruding of roots. Plant Cell Rep 26:2149-2157.

Wagner G (1991) Secreting Glandular Trichomes: More than Just Hairs. Plant Physiol 96:675-679.

Chapter 4

The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions

Aragón C[1,2] Carvalho L[1] González J[2] Escalona M[2] Amancio S[1*]

[1]CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Plant Cell Reports (2012) 31:757-769

The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions

Aragón C[1,2] Carvalho L[1] González J[2] Escalona M[2] Amâncio S[1]

[1] CBAA/DRAT, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Abstract

Many plant species grown under *in vitro* controlled conditions can be used as models for the study of physiological processes. Adult pineapple can display CAM physiology while *in vitro* it functions as a C3 plant. *Ex vitro A. comosus* has plastic morphology and physiology, both easy to modify from C3 to CAM by changing the environmental conditions. The yield of survival for a rentable propagation protocol of pineapple is closely related with the C3/CAM shift and the associated physiological characteristics. In the present work *ex vitro* pineapple plants were divided in two sets and subjected to C3 and CAM inducing environmental conditions, determined by light intensity and relative humidity, respectively, 40 $\mu mol.m^{-2}.s^{-1}$/85% and 260 $\mu mol.m^{-2}.s^{-1}$/ 50%. The results demonstrated that the stress imposed by the environmental conditions switched pineapple plants from C3 to CAM behaviour. Comparing to CAM induced, C3 induced pineapple plants showed substandard growth parameters and morphological leaf characteristics but a better rooting process and a higher ABA production, a phenotype closer to adult plants, which are expected to produce fruits in a normal production cycle. We conclude that the upholding of these characteristics is conditioned by low light intensity plus high relative humidity, especially during the first 8 weeks of *ex vitro* growth. It is expected that the better understanding of pineapple acclimatization will contribute to the design of a protocol to apply as a rentable tool in the pineapple agronomic industry.

Keywords: ABA; CAM; carbon metabolism; malic acid; oxidative stress.

4.1 Introduction

Plants under *ex vitro* controlled conditions can be used as models for the study of physiological processes that cannot be easily monitored in adult plants exposed to a natural environment. In what concerns pineapple, *ex vitro* plants have a somewhat plastic morphology and physiology that can change according to the environmental conditions under

which the plants are grown. As a consequence, this species is described as displaying a possible facultative C3/CAM metabolism, mainly in relation to malic acid accumulation. Beside of the high versatility of CAM and CAM-like behaviour plants alluded to is certainly also related to the fact that there is nothing specific in the enzymatic complement of CAM metabolism (Borland and Griffiths 1989; Cushman 2001; Luttge 2004).

The ice plant *Mesembryanthemum crystallinum* performs C3 or CAM photosynthesis in response to the presence or absence of drought or salt stress, respectively and is thus referred as a facultative C3/CAM metabolism plant (Cushman et al. 2008b). For understanding the C3/CAM switch, the transfer of a CAM metabolism gene into C3 plants, for example the PPDK gene from *M. crystallinum* into tobacco was performed by Sheriff et al. (1998) and Matsuoka et al. (2001). Currently, a challenging research is being undertaken in order to try to change the carbon metabolism of some C3 crops into C4 photosynthesis in order to increase the yield, the most representative example being the attempt to obtain C4 rice (Doubnerova and Ryslava 2011).

The attempt at the re-fixation of CO_2 released by respiration through the introduction of the dark activated enzyme PEPC of CAM plants into C4 transgenic rice is also well documented and its practical application may lie in the near future, by obtaining rice plants performing the canonical reactions of C4 CO_2 fixation (Bianjiang and Demao 2009). It is worth to refer that the attempt to adjust CAM metabolism must avoid changing the mesophyll and bundle sheath architecture, because CAM metabolism is more associated to the temporal separation of the processes than to the spatially separated reactions as it happens in C4 photosynthesis plants (Edwards et al. 2004; Christin et al. 2009).

The main purpose of acclimatization of *in vitro* plants is to obtain competent material to be transferred to field conditions at the lowest time span. However, yield is one of the most important economic aspects to be considered, in particular in generally low yield recalcitrant species, (Read et al. 2007; Arora et al. 2010). Whether *ex vitro* pineapple plants develop C3 or CAM metabolism is certainly the key to obtain successful adult plants. Several metabolic parameters can distinguish between C3 and CAM metabolism, *e.g.* malic acid and starch concentration (Dodd et al. 2003). However, to date, the knowledge of what exactly triggers the shift in the physiology of pineapple from C3 *in vitro* plants to CAM *ex vitro* plants is still

far from complete, several studies point to a photoperiod induction (Brulfert et al. 1982) others to environmental parameters such as light, relative humidity and temperature.

Pineapple is considered the third most important tropical crop worldwide, after bananas and mangoes. This crop is cultivated mainly for fresh or canned fruit and juice, but is also the only source of bromelain, a complex proteolytic enzyme used in the pharmaceutical market and as a meat tenderizing agent (Moyle et al. 2005). Brazil for example, has an annual production of 1471000 t per year (FAOSTAT 2010). Pineapple plants in field conditions are well characterized (Dopazo et al. 2009) showing high sensitivity to abiotic stress and a need for a tight control of water availability and temperature, rather that light intensity (Bartholomew et al. 2003). Abiotic stress can delay the fructification and decrease the economic revenue of the production cycle.

Nievola et al. (2005) conducted an experiment with *in vitro* pineapple plants in order to characterize the influence of temperature in carbon metabolism. The results put in evidence morphological and biochemical adjustments, but it is difficult to extrapolate to the processes occurring under *ex vitro* conditions. It is necessary to take into account that the *ex vitro* setting puts plants in autotrophic nutrition conditions, lacking the benefits provided by the media culture during the *in vitro* phase responsible for unique morphology and physiology characteristics.

Plants with facultative CAM carbon metabolism can be classified in three groups according to the enzyme that releases CO_2 directly or not from malic acid: plants with NAD-dependent malic enzyme, plants with NADP-dependent malic enzyme and plants with PEPCK enzyme, pineapple being included in the last group (Weise et al. 2011). Several physiological processes can be affected by the transition from C3 to CAM metabolism, such as gas exchange, efficiency of the functioning of PSII, as well as PEPC and PEPCK activities which are closely dependent of light and dark regulation (Cushman and Bohnert 1999).

The main objective of the present research is to better understand the process of acclimatization of pineapple to develop a protocol for the micropropagation industry that enables the production of economically viable plants. With that purpose we tested two *ex vitro* conditions, one similar to the *in vitro* environment, supposed to maintain C3 characteristics and a second one, more similar to a CAM inducing environment, and

monitored morphological, physiological and biochemical parameters in plants growing in both conditions.

4.2 Material and Methods

4.2.1 Plant material and ex vitro culture conditions

Pineapple plants (*Ananas comosus* (L.) Merr. var MD-2) micro-propagated in Temporary Immersion Bioreactors (TIB; Escalona et al. 1999) were transplanted to pots containing a sterilized mixture of hydrated peat and perlite (1:1, v/v) and placed in glass chambers with 450 L volume(500E, Aralab, Porto Salvo, PT). Light was provided by fluorescent lamps (Gro-Lux F18W/GRO) and the photoperiod was 16/8h. Plants were divided into two sets of 120 plants each and placed in two glass chambers with different environmental conditions determined by the light intensity and relative humidity (RH) that produce changes in range temperature day and night (Table 4.1). The programmed RH inside the glass chambers was obtained by an ultrasonic fog system controlled by a hygrometer. Temperature was variable depending of the light and RH conditions. Irrigation was also differential between treatments, on C3 inducing conditions the saturation of substrate was guaranteed while on CAM inducing conditions the plants were irrigated once at the beginning of the light period every other day.

Table 4.1 Environmental conditions applied in the growth chambers to induce either C3 or CAM carbon metabolism.

Treatments	**C3**	**CAM**
Light (µmol.m^{-2}.s^{-1})	40	260
Humidity (RH) (%)	85	50
Temperature (ºC)	(22±2/20±1)	(28±2/20±1)
Irrigation	saturation	deficit

Measurements were taken at time zero (w0) of transfer to acclimatization and weekly during the eight weeks of acclimatization. Samples for all further analyses were collected in the middle of the light period and, in the case of carbohydrates and enzyme activity, also in the middle of the night period, always from a D leaf, defined as a leaf forming a 45^{o} angle with the vertical axis.

4.2.2 Morphological parameters

The following parameters were quantified in 10 plants: number of leaves, length of D leaf (cm), width of D leaf (cm), number of roots, plant fresh weight (g), and plant dry weight (g; after drying the shoots at 70^{o}C until constant weight was attained).

4.2.3 Waxes

Wax extraction was performed in 100 mL chloroform per leaf. Leaves were immersed in chloroform and shaken gently. The solution containing the waxes was completely evaporated and the wax content was quantified by gravimetric difference (Hamilton 1995).

4.2.4 Chlorophyll content

Chlorophyll was extracted from five leaf disks. Disks were incubated in 3 mL DMSO at 65ºC for 1 hour, and absorbance was measured at 645 and 663 nm (adapted from Hiscox and Israelstam 1979). The chlorophyll concentration of the extracts was calculated using the equations described by Porra et al. (2002): Chl *a* (µg mL^{-1}) = 12,00xA_{663} – 3,11xA_{645}; Chl *b* (µg mL^{-1}) = 20,78xA_{645} – 4,88xA_{663}; Chl t (µg mL^{-1}) = 17,67xA_{645} + 7,12xA_{663}; and converted to mg Chl cm^{-2} leaf area (Richardson et al. 2002).

4.2.5 Succulence Index

The Succulence Index (SI) was determined using the values of chlorophyll content and fresh and dry weight with the formula: SI= (FW-DW)/Chl t, where FW - fresh weigh, DW - dry weight; Chl t – total chlorophyll content (Kluge and Ting 1978).

4.2.6 Chlorophyll a fluorescence and photosynthetic light response curves

Chlorophyll *a* fluorescence was measured with a Mini-PAM Photosynthesis Yield Analyzer (Walz Mess- und Regeltechnik, Effeltrich, Germany) in dark-adapted (15 min) and light adapted leaves. Photosynthetic light response curves and stomatal conductance (gs) were obtained using the Photosynthesis Measurement System (Li-COR biosciences LI6400 Lincoln, Nebraska), with increasing irradiances ranging from 0 to 1850 µmol quanta m^{-2}s^{-1}. Several parameters were calculated using the Photosynthesis Assistant 1.1 software (Dundee Scientific, United Kingdom) for data analysis. Abbreviations: Amax, Maximal photosynthesis; LCP, Light compensation point; LSE, Light saturation estimate; Rd, Dark respiration; ΦE, Quantum efficiency.

4.2.7 Soluble carbohydrates and starch

Extraction for soluble carbohydrates and starch was performed according to Stitt et al. (1978). The quantification of soluble carbohydrates (malic acid, sucrose, glucose, and fructose) was done by HPLC chromatography (Pharmacia LKB equipment with an IR detector (35ºC) and an HPX-87 H (BIORAD, Hercules, CA 300×7.8 mm (60ºC)). The solution containing the carbohydrates was filtered through 0.2 µm filters (Sartorius) previously stabilized in distilled water. Sulfuric acid (25 mM) was used as the mobile phase at 0.6 mL min^{-1}. The volume injected was 20 µL. All the standards (malic acid, sucrose, glucose, and fructose, obtained from SIGMA-Aldrich) were prepared at 1 mg/mL and used for calibration. Starch quantification was performed by the method of Stitt et al. (1978).

4.2.8 Extraction for enzyme activity and the respective assays

The extractions for measuring enzyme activities were performed at 4 ºC from 0.5 g of frozen leaf material. The material was ground using mortar and pestle in the presence of liquid nitrogen, and 50% (w/w) polyvinylpolypyrrolidone was added. The extraction buffer was Tris-HCl 0.35 M (pH 8) supplemented with EDTA 20 mM; DETC 11 mM, cysteine 15 mM (Jacobs et al. 1999), and 0.2 mM PMSF. Extracts were centrifuged at 27 000 *g* for 20 min at 4 ºC and supernatants, desalted through PD-10 columns (GE-Healthcare Life-Sciences, Buckinghamshire, UK), were used for all the determinations. Protein was quantified by the method of Bradford (1976) using a commercial kit (Bio-Rad, Hercules, CA). The reaction catalyzed by phosphoenolpyruvate carboxylase (PEPC) was performed in 1 mL of 50 mM HEPES–KOH buffer at pH 7.4 according to Siegl and Stitt (1990). The catalyzed PEPC reaction was coupled with the L-malate dehydrogenase (EC 1.1.1.37) (SIGMA-Aldrich) reaction and assayed at 25°C by monitoring NADH consumption at 340 nm (Le et al. 1991). PEPC activity was expressed as µmol min mg^{-1} Prot and the protein content used was *circa* 20-40 mg mL^{-1}. Phosphoenolpyruvate carboxykinase (PEPCK) was assayed after the incubation of the extract in the standard assay solution containing 40 mM Hepes-KOH, pH 8.0, 0.25 mM ATP, 0.5 mM $MnCl_2$ and 0.1 mM oxaloacetate, for 10 min at 25ºC. HPLC chromatography (as described previously for soluble carbohydrates) was used for the quantification of PEP. A standard curve was obtained with PEP purchased from SIGMA-Aldrich. PEPCK activity was expressed as µmol min mg^{-1} Prot and the protein content used was *circa* 20-40 mg mL^{-1}.

4.2.9 ABA quantification

ABA concentration was determined in pineapple leaves collected during *ex vitro* growth. The sap fluid from leaf tissue was extracted by mechanical pressure. The sap collected (*circa* 0.5 mL) was clarified with 0.1 g of activated charcoal. Fifty microliters of this solution were used as sample to quantify ABA through immunoassay by indirect enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, using a commercial kit (Olchemim Enzyme Immunoassay, Olomouc, Czech Republic), according to the manufacturer's recommendations.

4.2.10 Statistical analysis

All experiments were performed three times: morphological analyses were performed with 10 repetitions, photosynthesis and chlorophyll fluorescence were performed with 3 repetitions and all other analyses with 9. All statistical analyses were carried out using SPSS version 12 (Pérez 2005). All situations were analysed using one-way ANOVA followed by Tukey's Multiple Range Test or t-test for the comparison of two conditions, both at 5 % significance level.

4.3 Results

The present work compares the distinct patterns of pineapple plants when subjected to acclimatization under the environmental conditions described in Table 4.1. Morphology and growth parameters were assessed at the final point of the experiment (w8). Physiological and biochemical parameters were analyzed at time zero (w0) of transfer, and during the eight weeks of acclimatization except for photosynthesis rates and ABA concentration that were obtained every two weeks (w2, w4, w6, w8).

4.3.1 Plant morphology and growth parameters

At the end of acclimatization (w8) plants under CAM inducing conditions (Fig. 4.1, right) were taller, with leaves displaying a less intense green hue and thorns in the margins (Fig. 4.1C, arrow). Root number was significantly higher in plants under C3 inducing conditions while leaf area was higher in plants under CAM inducing conditions due to their larger width, since the length/width ratio was higher in the leaves of plants under C3 inducing conditions.

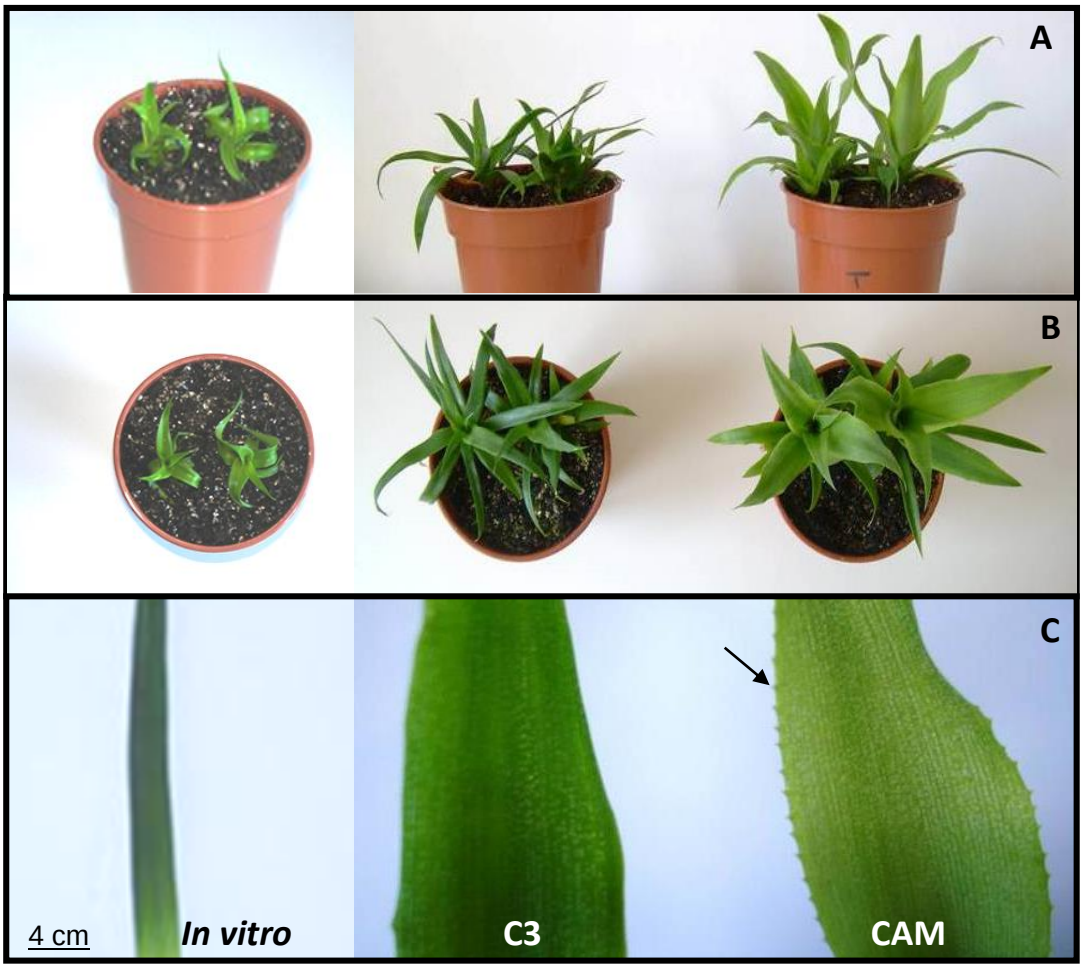

Fig. 4.1 Pineapple plants (lateral view, A; top view, B) and detail of the leaf morphology (C, arrow for thorns) at the end of *in vitro* phase (left) and at w8 of acclimatization under C3 inducing (middle) or CAM inducing (right) conditions.

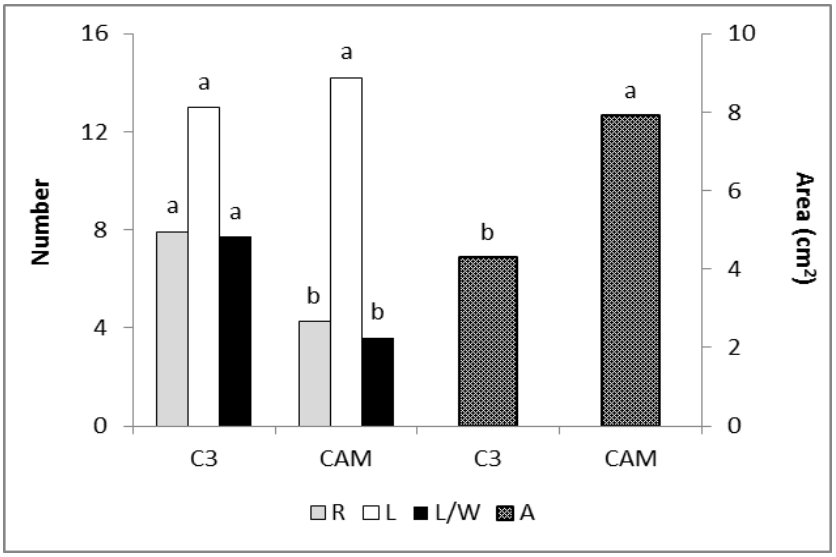

Fig. 4.2 Growth parameters of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) on w8. R, number of roots; L, number of leaves; L/W leaves length/width ratio; A, Leaf Area. Values followed by different letters between C3 and CAM induced conditions are significantly different at 5% level by t-test (n=10).

The number of leaves was almost equivalent in plants under both conditions (Fig. 4.2). The evolution of fresh weight (Fig. 4.3A) was identical under both treatments with a net decrease on w4, followed by a steady increase thereafter. On the contrary, the dry weight (Fig. 4.3B) was distinct between plants under the two treatments with the dry weight of plants under CAM inducing conditions increasing significantly from w2 on while the plants under the C3 inducing environment mimicked the pattern reported for fresh weight.

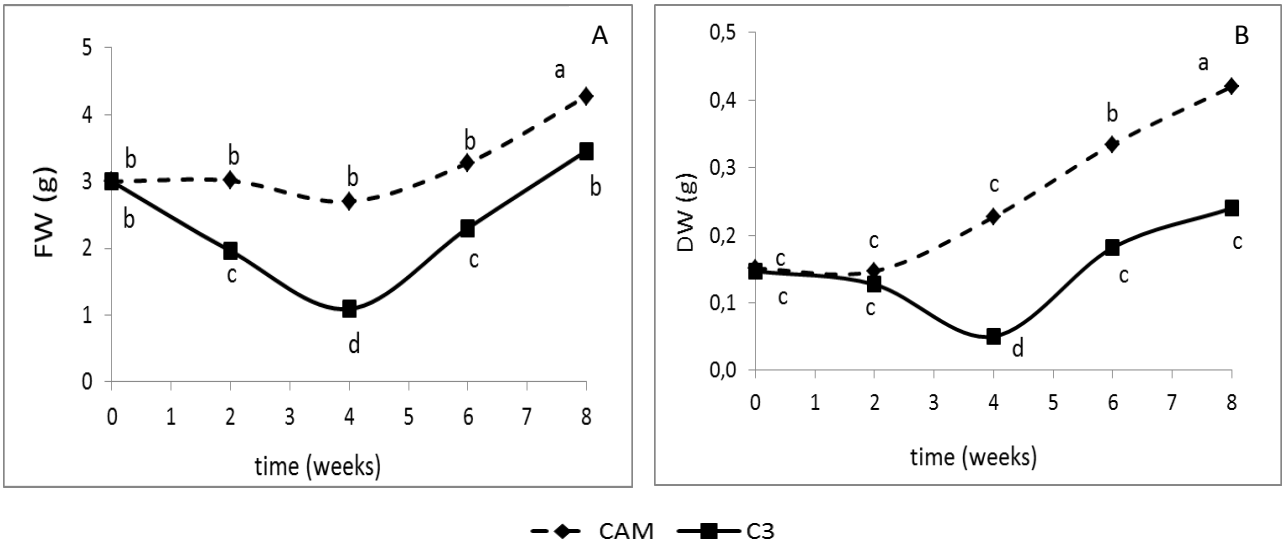

Fig. 4.3 Fresh weight (A) and dry weight (B) of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) during acclimatization. Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=10).

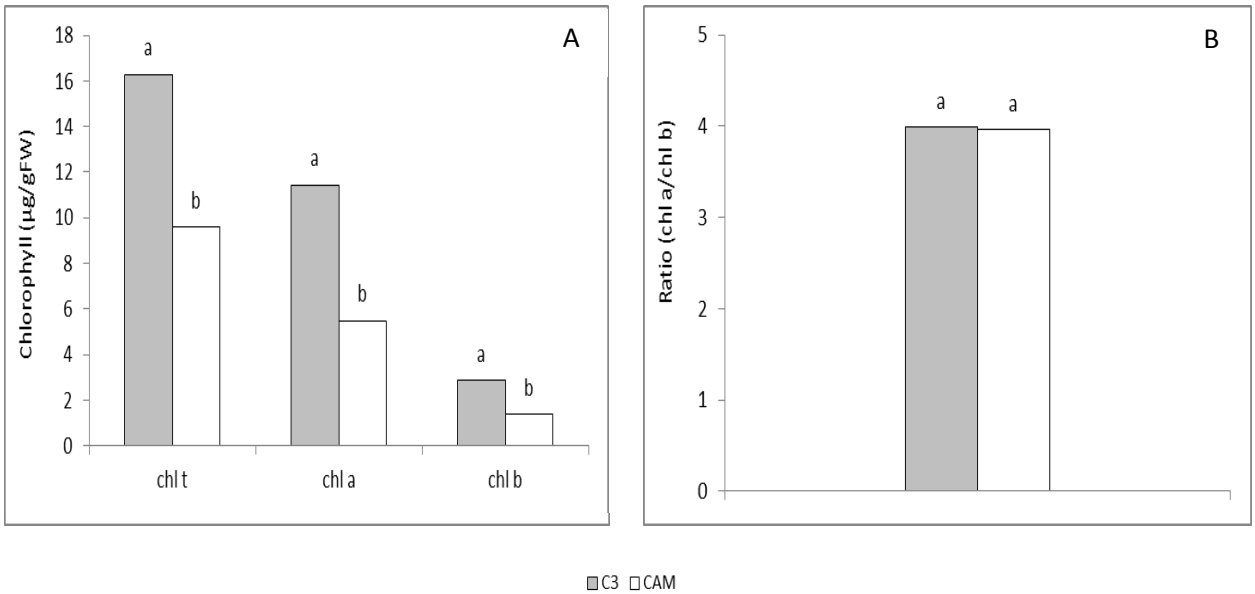

Fig. 4.4 Concentration of total chlorophyll (chl t), chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) (A) and chl a/chl b ratio (B) in leaves of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) on w8. Values followed by different letters are significantly different at 5% level by t-test (n=9).

In accordance with the shade of the leaves, the concentration of *a*, *b* and total chlorophyll was higher in plants under C3 inducing conditions (Fig. 4.4). Another interesting feature of CAM induced plants was the higher deposition of waxes on their surface (Fig. 4.5A). The Succulence Index (SI) is related to the water holding ability during drought and thus can give an important insight into CAM metabolism. Plants under this condition presented significantly higher SI values than plants growing in C3 conditions (Fig. 4.5B).

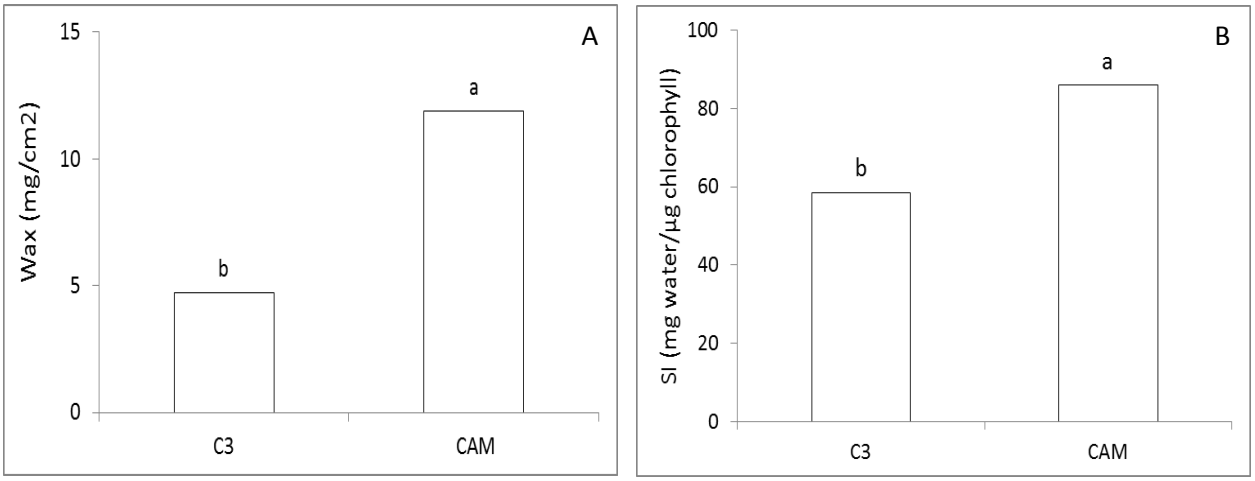

Fig. 4.5 Wax content on the surface of pineapple leaves (A) and Succulence Index (SI) (B) of plants under the two acclimatization treatments (C3 and CAM inducing) on w8. Values followed by different letters are significantly different at 5% level by t-test (n=9).

4.3.2 Photosynthesis and chlorophyll fluorescence

The curves of photosynthesis rates at ambient CO_2 and increasing light intensities obtained at w0 and w2 in plants under C3 and CAM inducing conditions (Fig. 4.6A and B, respectively) follow a similar trend with significantly higher values on w0 at the highest light intensity (1800 µmol quanta $m^{-2}s^{-1}$) and a tendency for higher CO_2 release (respiration) on w2, even at high light intensities. On w4, PAR increase resulted in higher photosynthetic rates in plants under C3 inducing conditions when compared with CAM grown plants. On w6 and w8 the values of photosynthetic rates were significantly higher in both sets of plants, reaching the highest values at 750 µmol quanta $m^{-2}s^{-1}$, and decreasing slowly for higher light intensities. The apparent photosynthesis rates measured at the light intensity the plants were grown (50 and 200 µmol quanta $m^{-2}s^{-1}$ for C3 and CAM inducing conditions, respectively) are shown in the inserts of Fig. 4.6 and are significantly higher in CAM induced plants (Fig. 4.6B, insert) from w4 on.

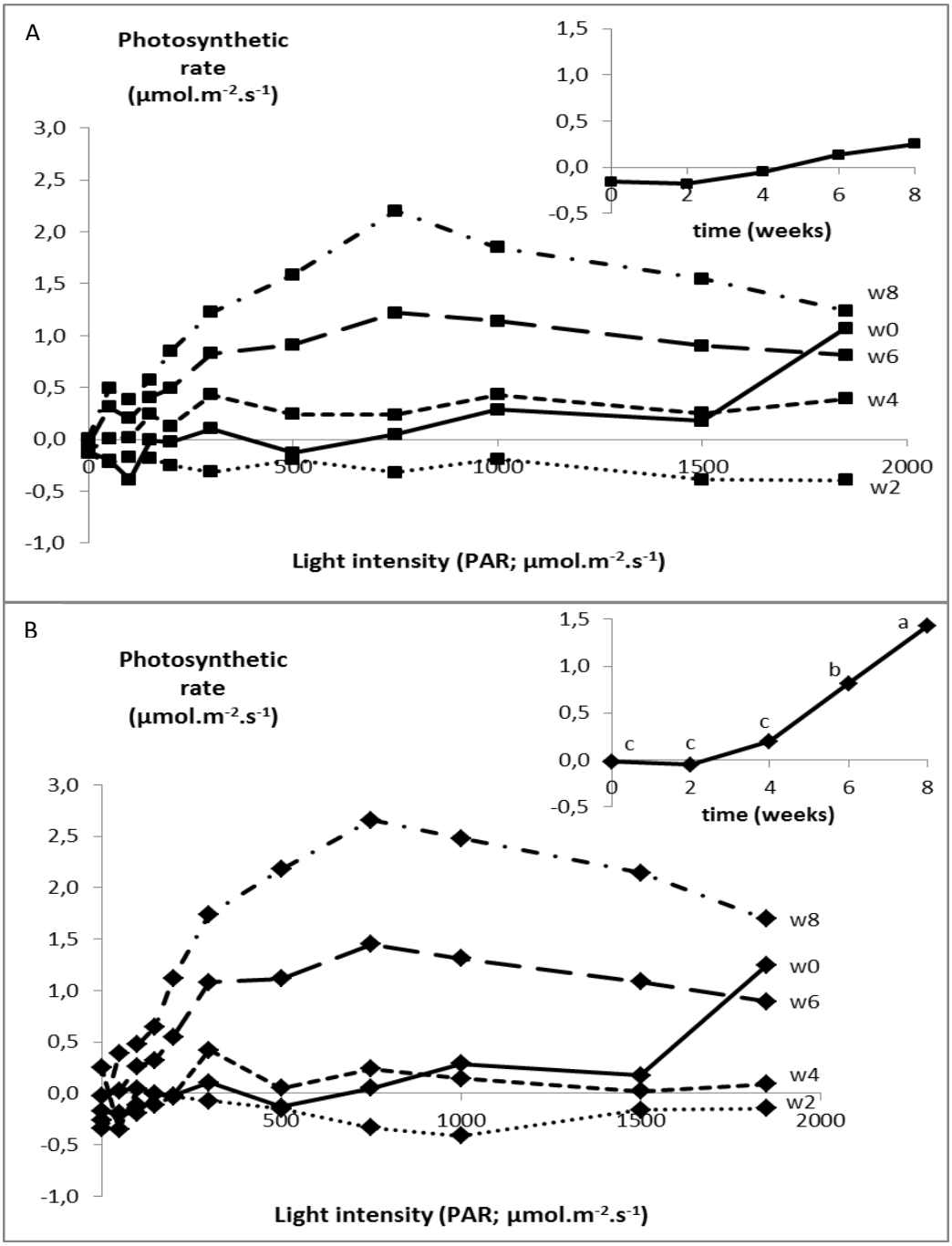

Fig. 4.6 Photosynthetic rates at ambient CO_2 and increasing irradiances in pineapple plants under the two acclimatization treatments (A - C3 inducing and B – CAM inducing). Inserts in A and B show the photosynthetic rates measured at the irradiance the plants were grown, respectively C3 inducing, 40 µmol m^{-2} s^{-1} and CAM inducing, 260 µmol m^{-2} s^{-1}). Statistical analysis was performed for the values represented in the inserts. Values followed by different letters in the inserts are significantly different at 5% level by Tukey's Multiple Range Test (n=3). When no differences were found letters were withdrawn from the respective line.

The light response curves were processed with Photosynthesis Assistant 1.1 (Dundee Scientific, UK) to calculate the maximal photosynthetic rate (Amax), the light compensation point (LCP), the light saturation estimate (LSE), the photosynthetic quantum efficiency (ΦE,

expressed x1000) and the dark respiration (Rd). Before w4 and in C3 induced plants on w4 the values had correlation coefficients lower than 0.90, thus without physiological significance and therefore, these values are not shown on Table 4.2. In CAM induced plants LCP and LSE showed significantly higher values and, on w8 Rd followed the same trend. Interestingly, no significant differences were monitored for ΦE in plants under both acclimatization treatments. Stomatal conductance values (gs) measured at the light intensities of growth (Table 4.3) showed a similar pattern in both treatments except that stomata opening in C3 inducing plants was delayed from w4 to w6.

Table 4.2 Photosynthetic parameters quantified in pineapple plants under C3 and CAM inducing conditions. Results are shown for plants from w4 to w8 (CAM inducing conditions) and w6 and w8 (C3 inducing conditions).
Amax, maximal photosynthetic rate; LCP, light compensation point; LSE, light saturation estimate; ΦE, Quantum efficiency (expressed x1000) and Rd, dark respiration.
*: Significantly different at 5% level by t-test (n=3); ns: Not significantly different at the same level; na: not available, measured values were impossible to process with the available software because the correlation coefficients were lower than 0.90, thus without physiological significance.

Induction condition	**Week**	**Parameter**				
		Amax	**LCP**	**LSE**	**ΦE**	**Rd**
C3	**4**	na	na	na	na	na
CAM		0,44±0,11	47,01±0,31	78,21±0,58	2,64±0,26	0,13±0,09
C3	**6**	1,33±0,21ns	23,32±0,26	343,84±2,51	2,23±0,11*	0,05±0,001ns
CAM		1,49±0,31ns	39,28±0,18*	550,37±4,12*	1,37±0,16	0,05±0,001ns
C3	**8**	1,54±0,21	2,95±0,31	321,35±2,41	4,15±1,07ns	0,012±0,002
CAM		2,46±0,27*	19,17±0,41*	505,82±3,79*	6,78±2,31ns	0,13±0,01*

The efficiency of PSII functioning was influenced by the stress imposed upon the transfer of plants to the *ex vitro* treatments (Fig. 4.7). The majority of the chlorophyll fluorescence parameters decreased significantly after transfer to *ex vitro* in C3 induced plants except non photochemical quenching which increased until w4 and stabilized thereafter. The results obtained in CAM induced plants were less predictable, with all light dependent parameters suffering significant decreases from w4 on. On w8 only Fv/Fm did not differ between C3 and CAM induced plants.

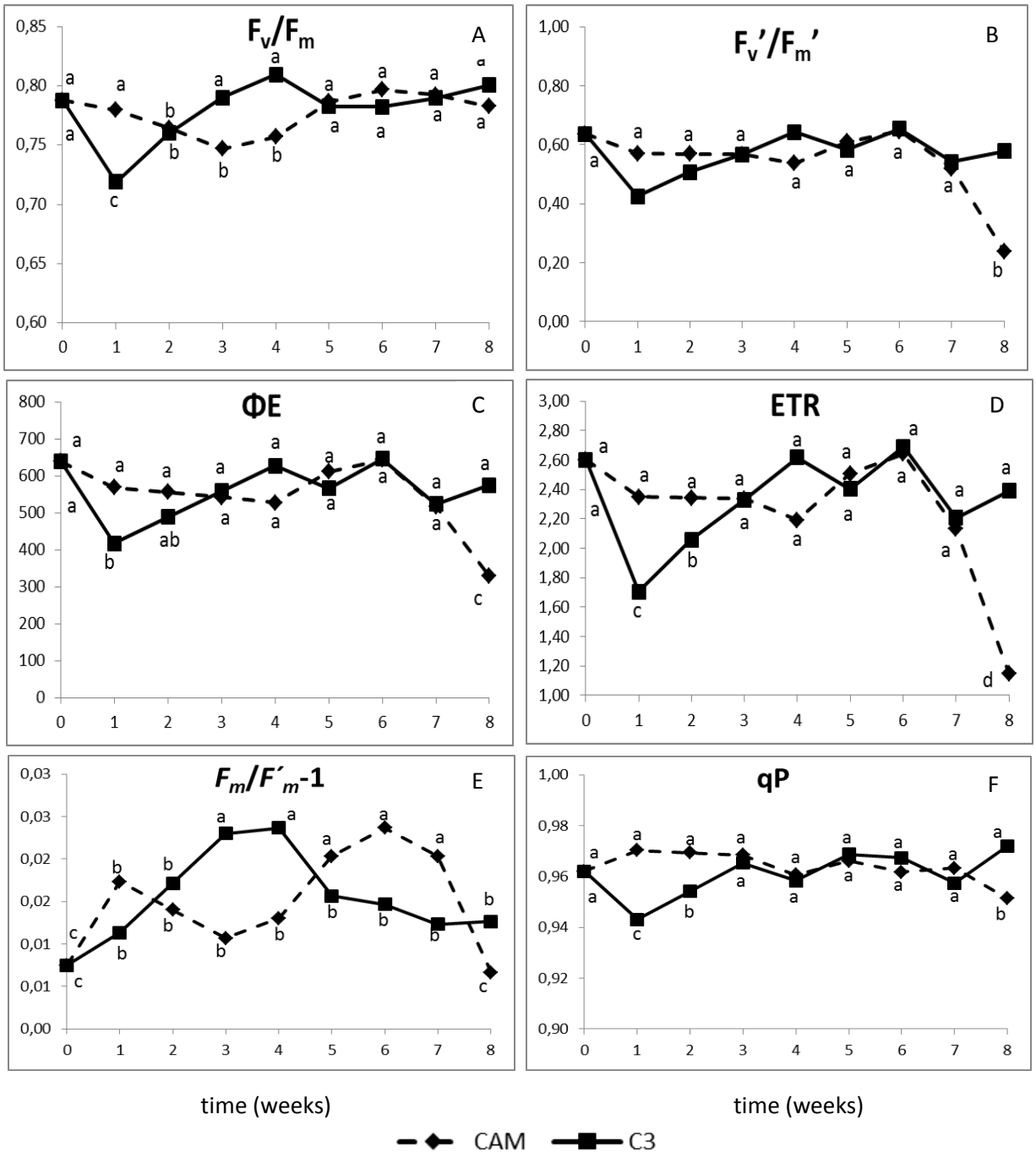

Fig. 4.7 Chlorophyll fluorescence parameters measured in leaves of plants during the two acclimatization treatments (C3 and CAM inducing). F_v/F_m represents the maximum efficiency of PSII photochemistry in dark-adapted leaves (A), F'_v/F'_m corresponds to the maximum quantum efficiency of PSII in light adapted leaves (B), ΦE equates to the operating quantum efficiency of PSII photochemistry (C), ETR, electron transport rate (D), NPQ (F_m/F'_m-1) gives an estimate of the light energy not used in photosynthesis and functions as an indicator of photoprotective processes through the dissipation of energy as heat (E) and qP, photochemical quenching (F). Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=3). When no differences were found letters were withdrawn from the respective line.

Table 4.3 Stomatal conductance (gs) in pineapple plants under the two acclimatization treatments (C3 inducing and CAM inducing). C3 inducing, 40 µmol m^{-2} s^{-1} and CAM inducing, 260 µmol m^{-2} s^{-1}).
* Significantly different at 5% level by t-test (n=3); ns: Not significantly different at the same level; na: not available, the values measured had no physiological significance and correspond to totally closed stomata.

Induction condition	**Week**				
	0	**2**	**4**	**6**	**8**
C3	0,126±0.001ns	na	0,024±0.001	0,126±0.003ns	0,126±0.002ns
CAM	0,126±0.001ns	na	0,141±0.003*	0,131±0.002ns	0,127±0.001ns

4.3.3 Carbohydrates

Carbohydrate quantifications were performed in the middle of the light and of the dark period. The concentration of malic acid measured during the dark was higher than during the light (Fig. 4.8A), a difference more evident in plants under CAM inducing conditions after w5. Sucrose concentration presented oscillating values in both treatments, especially during the light period (Fig. 4.8C). Significantly higher values were obtained in plants under CAM inducing environment on w5 and w4 respectively, in the light and in dark period. In what concerns starch, from w0 until w3 (Fig. 4.8E) during the day its concentration was typical of plants with CAM metabolism, with a steady increase, similar in both treatments. From then on and until w8, starch concentration decreased sharply in plants under C3 inducing conditions while in CAM induced plants that decrease was slow and justified their significantly higher starch concentration. Showing a contrasting response, the values measured in the middle of the dark period were much lower (Fig. 4.8F) and although they increased slightly in both treatments, starch concentration in CAM inducing conditions was one half as compared to C3 inducing plants.

4.3.4 PEPC and PEPCK activities

Higher activities of PEPC were measured during the dark (Fig. 4.9B) as compared with the values obtained during the light (Fig. 4.9A). Plants under both treatments showed a peak of enzyme activity, earlier (w1) in CAM induced plants. That peak was observed on w2 in plants under C3 inducing conditions and the activities decreased slowly thereafter, diverging from activities in CAM induced plants that, after a minimum on w4, increased steadily. During the light period PEPC activities attained in average one third the values measured

during the dark, without significant differences between plants under the two treatments except a peak on w1 in the plants under CAM inducing environment and a small peak on w4 in plants of the other treatment.

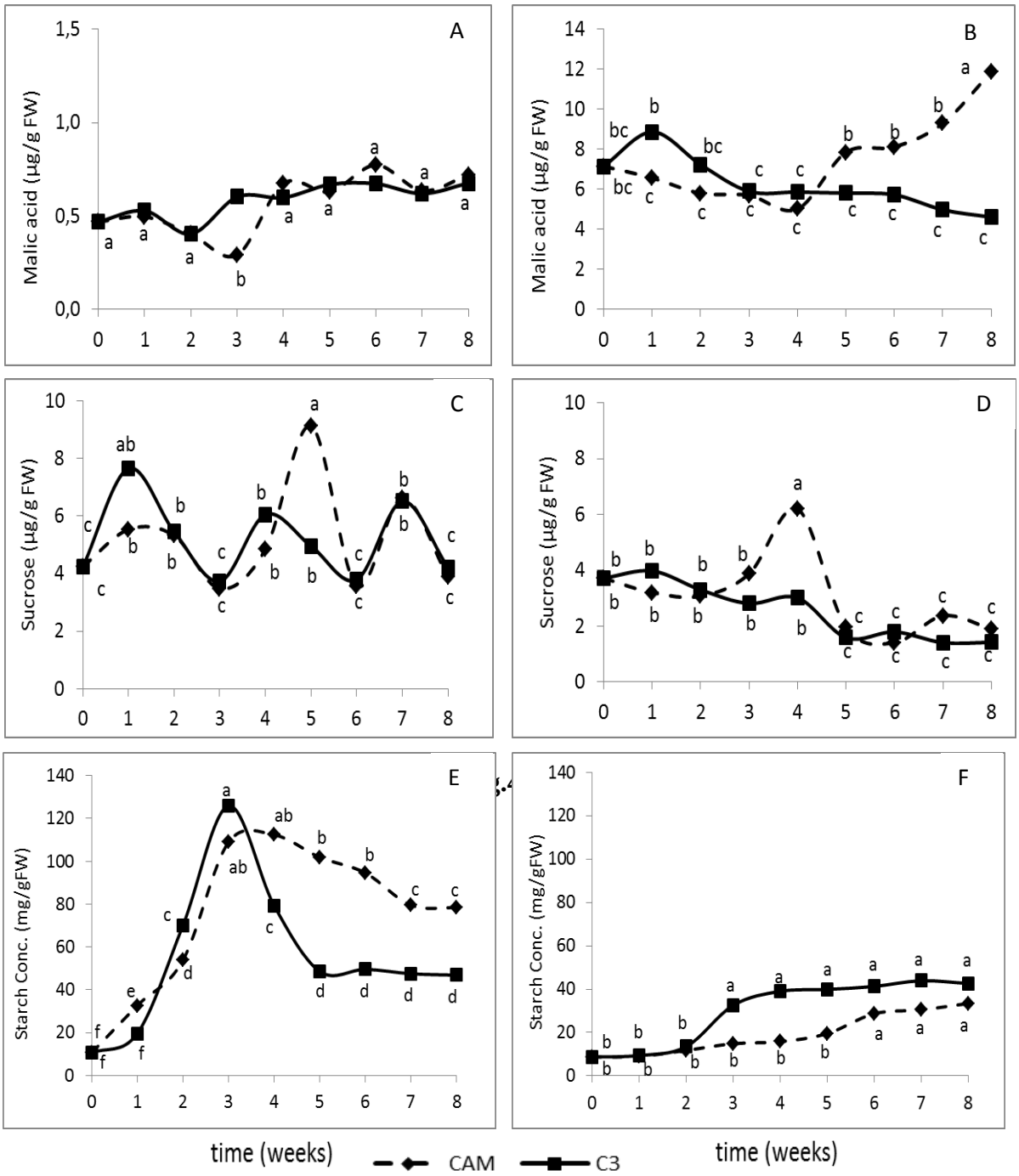

Fig. 4.8 Carbohydrates of pineapple leaves under the two acclimatization treatments (C3 and CAM inducing). Malate (A; B), sucrose (C; D) and starch concentration (E; F) were quantified during the light period (A; C; E) and the dark period (B; D; F). Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line. For clarity of presentation the scales in A and B are different.

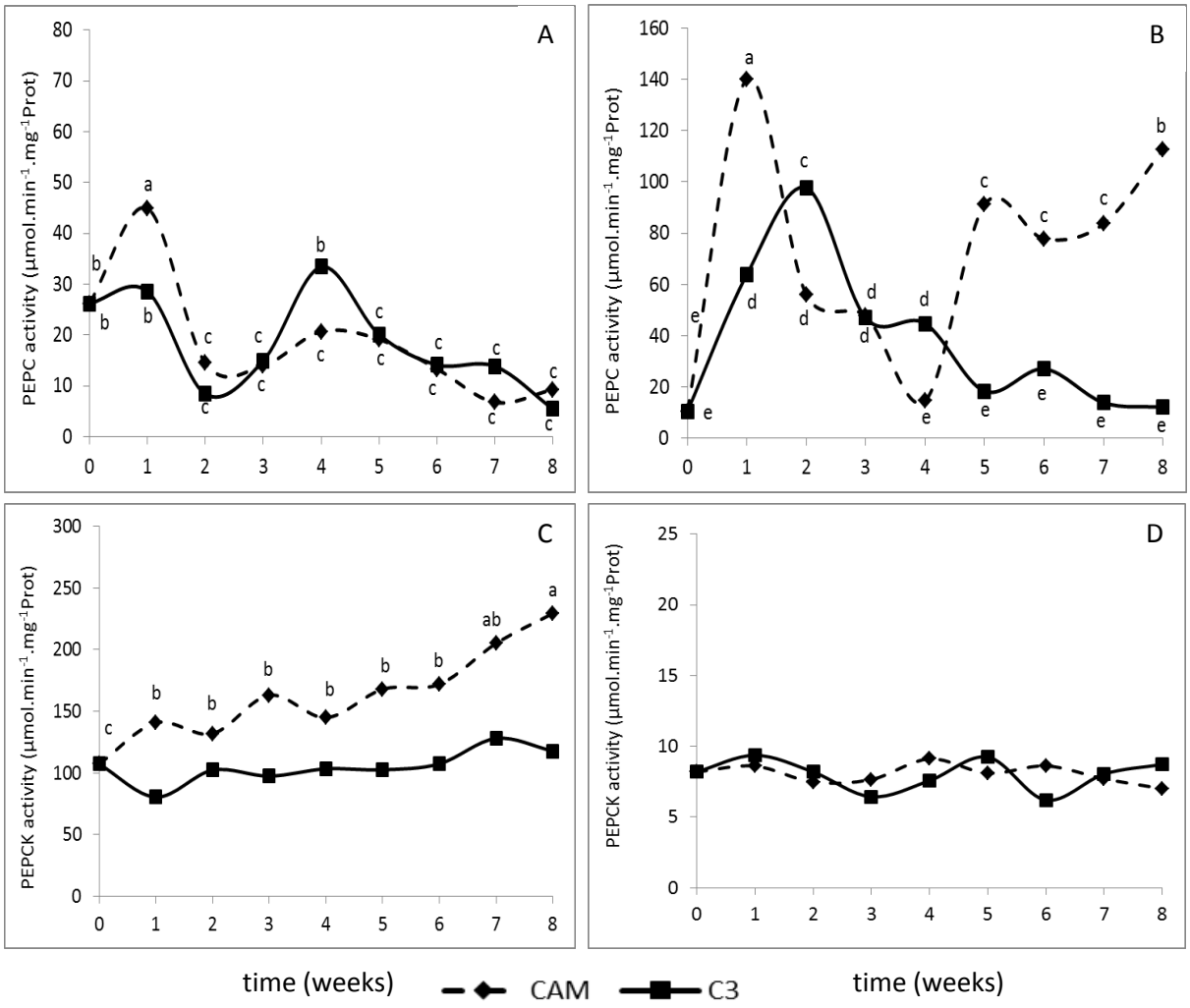

Fig. 4.9 Total soluble PEPC (A; B) and PEPCK (C; D) activities in leaves of pineapple plants under the two acclimatization treatments (C3 and CAM inducing) measured during the light (A; C) and the dark (B; D) periods. Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line. For clarity of presentation the scales in A, B, C and D are different.

The activity of PEPCK is crucial to characterize CAM metabolism in pineapple plants. In contrast with PEPC activity, the values of PEPCK activity were tenfold higher during the light (Fig. 4.9C) as compared with the values obtained during the dark (Fig. 4.9D). The values were stable during the whole acclimatization period, with the exception of plants under CAM inducing conditions where, in the light, a steady increase of activity was measured, which on w8 reached twice the value measured on w0 as well as the activity measured in C3 inducing plants during the entire treatment.

4.3.5 Abscisic acid

The ABA concentration measured in the sap collected from pineapple plants showed significantly different values in both treatments, (Fig. 4.10) with significantly higher concentration in plants under C3 inducing conditions from w4 on, attaining on w8 20 fold the ABA concentration measured in plants under CAM inducing conditions, which kept constantly low values during the whole period of acclimatization.

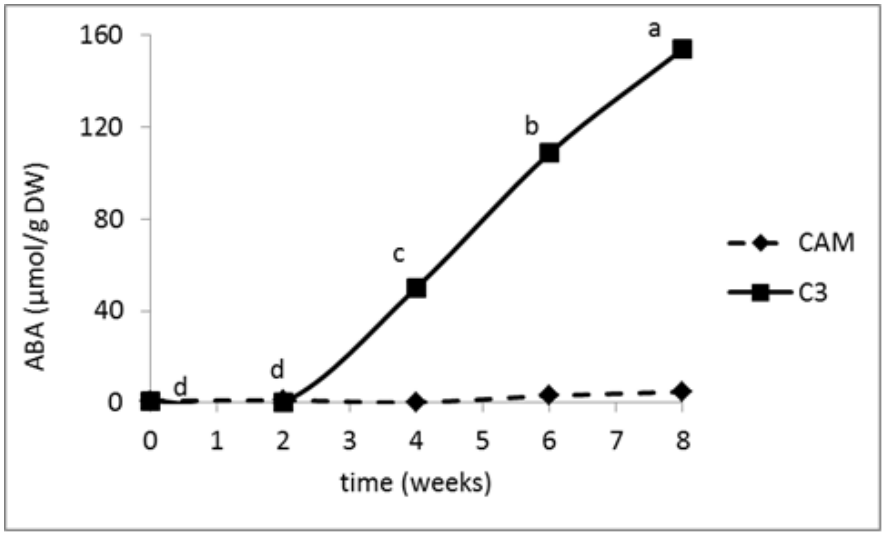

Fig. 4.10 Abscisic acid (ABA) concentration in pineapple sap extracted from leaves of plants under the two acclimatization treatments (C3 and CAM inducing). Values followed by different letters are significantly different at 5% level by Tukey's Multiple Range Test (n=9). When no differences were found letters were withdrawn from the respective line.

4.4 Discussion

Previous results on *in vitro* pineapple have shown that it is possible to alter the carbon metabolism C3/CAM, by changing the environmental temperature during the light and the dark period (Nievola et al. 2005). However, this process is extremely expensive and out of the budget for greenhouse production in third world countries; instead, a change in light intensity and relative humidly is cheaper and can by itself control ambient temperature. The behavior of micropropagated C3 pineapple plants, during acclimatization using two distinct treatments, C3 and CAM inducing conditions, was compared to ascertain the efficiency of promotion of both types of metabolism. Plant morphology, physiology, key metabolites and enzyme activities were monitored in order to get an insight into when and how, during the acclimatization phase, the shift from C3 to CAM metabolism occurred.

The environmental conditions applied caused obvious phenotypic divergence in the pineapple plants studied. These changes were certainly in accordance with the higher values of succulence index (SI) and malic acid concentration during the dark period in CAM induced plants, where the pre-defined environmental conditions were able to switch plant morphology from C3 to CAM by w4. The so-called "typical" pineapple plant presents wide leaves with a succulent appearance (Bartholomew and Kadzimin 1977). The water usage pattern of CAM plants represents a physiological adaptation to a specific environment and was the main contributor to the higher SI and dry weight what can be explained by the high starch concentration of these plants as compared with plants under C3 conditions. Although CAM induced plants presented better carbon assimilation, higher dry weight and leaf area, the rooting process was enhanced in plants under C3 inducing conditions. The *ex vitro* rooting process can be vital for a normal water transport from roots to leaves delivering water needed for transpiration. The lower growth and carbon assimilation rates of plants with C3 metabolism can be compensated by the emission of roots and represent a crucial factor to the final yield of plants with this type of metabolism. A fundamental mechanism to circumvent the harmful effects of water balance during the first weeks of acclimatization of CAM growing plants is the wax concentration of leaves. Plants under CAM inducing conditions have a better capacity to hold water while the stomata are closed due to the wax protection of the cuticle. These two aspects, rooting and leaf wax, can be considered positive strategies to control water balance, set apart by the environmental conditions: wax deposition in leaves of CAM induced plants and a better rooting process in C3 induced plants.

Photosynthesis rates and the functioning efficiency of PSII were used to characterize the stringent relationship between morphology and physiology of these plants. At the end of the acclimatization period, CAM induced plants showed a decrease in the parameters associated with the functioning efficiency of PSII. Conversely, the highest values of photosynthetic rates measured in CAM plants on w8 were similar to values obtained in adult pineapple plants under greenhouse conditions (Keller and Luttge 2005) and to those of other CAM plants (Bartholomew et al. 2003), but are low as compared with the range of 8-25 µmol m^{-2} s^{-1} reported for C3 plants. The low CO_2 fixation rates measured in pineapple are in part due to low gas diffusion, hindering leaf conductance. The transpiration rates of pineapple can range from 0.05-0.23 $mmolm^{-2}s^{-1}$ (Bartholomew et al. 2003).

The photosynthesis rates equivalent to adult plants in parallel with chlorophyll fluorescence parameters typical of *in vitro* plants (Carvalho and Amancio 2002) can result in oxidative stress symptoms that take place in immature *in vitro* cultured plants, due to the saturation of the still not fully functioning electron transport system of thylakoid membranes. In fact, indications of oxidative stress can appear in chloroplasts of immature leaves from *in vitro* plants when carbon fixation attains the levels of adult leaves as measured through photosynthetic rates (Carvalho et al. 2006). Photosynthetic rates are directly associated with the production of sucrose during the light period, as a possible final destination for the fixated CO_2, although starch can be an alternative final carbon storage form. Plants with CAM metabolism presented a concentration of sucrose that was stable between day and night (Antony et al. 2008). In CAM induced plants the balance between sucrose and starch shifted to starch storage during the light period. A known feature of C3 plants is the oscillation of starch concentration between day and night (Cushman et al. 2008a) but in species with C3/CAM facultative metabolism, such as *Mesembryanthenum crystallinum,* that oscillation is 20% higher and the authors advanced that it represents a key feature to maintain CAM metabolism (Dodd et al. 2003). Comparing the oscillation of starch levels in pineapple plants under CAM and C3 inducing conditions we verified that it is more than 6 fold higher in CAM induced plants. It is well documented that the concentration of starch in leaves is important to define a good metabolic balance in C3, C4 or CAM species (Kotting et al. 2010; Weise et al. 2011). New insights into sugar signaling in plants are unraveling its importance and providing conclusive results that demonstrate its contribution to growing and physiological improvement (Smeekens et al. 2010). Here, a direct correspondence between photosynthetic rate and diurnal starch synthesis was shown in pineapple plants with CAM induced metabolism. Dark respiration (Rd) can reflect a process of carbon loss; significant differences were only measured on w4, the moment when several parameters point to a differentiation between both types of metabolism.

Pineapple plants are classified as CAM plants that use PEPCK to decarboxylate oxaloacetic acid; this is the most challenging sub-group of CAM plants because the regulation of PEPCK is not yet well established. It is well established that, in the PEPC subgroup of CAM plants, the induction of CAM metabolism requires PEPC protein synthesis (Taybi and Cushman, 2002). On the contrary, in pineapple the common function of the enzyme pair PEPC/PEPCK shows low PEPC and high PEPCK activity during the light period and the reverse during the

dark (Theng et al. 2008). The present experiment seems to confirm that assumption, since in dark conditions PEPC activity decreased in C3 induced plants when the same enzyme was activated in the CAM induced ones. Week four seems to be the crucial moment for the switch of CAM induced pineapple plants to CAM metabolism; these plants are characterized by this decrease in the dark activation of PEPC activity triggered by the prevailing CAM environmental conditions, and that can be due to protein phosphorylation at that time point. Up until now, PEPC regulation by the PEPC kinase has not been clearly described in pineapple plants due to difficulties during the attempt at cloning this gene (Theng et al. 2008). The regulation and metabolic changes between light and dark carried out by different protein kinases appear to be rather uncommon in this species.

In what concerns ABA concentration w4 was also a turning point with a drastic increase in plants under C3 inducing conditions. Root emission could be closely related with ABA levels in the leaves of these plants as ABA is considered and important element for shoot and root growth by preventing an excess of ethylene, a growth inhibitor hormone (Nair et al. 2009). Typically, in drought conditions, ABA helps protect against stress by promoting the expression of CAM in the PEPC subgroup (Chu et al 1990, Taybi and Cushman, 2002). However, a feature of ABA, still not well characterized, is its capacity to control shoot growth under water sufficient conditions (Sharp and Lenoble 2002), exactly the growth conditions of C3 induced pineapple plants. It must be noted that stomatal opening was independent of ABA sap concentration per dry weight.

W4 is the decisive moment during the acclimatization of pineapple under both environmental conditions, with several parameters changing their trends, among which fresh and dry weight, NPQ, dark malic acid and sucrose concentration, light starch concentration and dark PEPC activity.

The results taken as a whole allow to conclude that oxidative stress caused by the abiotic stress conditions imposed exerted a key influence over plant metabolism (Mullineaux et al. 2006; Batkova et al. 2008), helping to provide pineapple plants with the ability to switch from C3 to CAM metabolism (Borland and Dodd 2002). Pineapple plants maintaining C3 metabolism showed a better rooting capacity and a higher production of ABA, what can be associated with an adult phenotype. The upholding of these characteristics was conditioned by the high relative humidity and low light intensity during the two first two months of *ex vitro* growth.

Acknowledgments

Fundação para a Ciência e Tecnologia (FCT), for PhD grants SFRH/BD/43181/2008 to C.A. and "Plurianual" PEst-OE/AGR/UI0240/2011 financial support to CBAA.

4.5 References

Antony E, Taybi T, Courbot M, Mugford S, Smith A, Borland A (2008) Cloning, localization and expression analysis of vacuolar sugar transporters in the CAM plant *Ananas comosus* (pineapple). J. Exp. Bot. 59:1895-1908.

Arora K, Sharma M, Srivastava J, Ranade S, Sharma A (2010) Rapid *in vitro* cloning of a 40-year-old tree of Azadirachta indica A. Juss. (Neem) employing nodal stem segments. Agroforest Syst. 78:53-63.

Bartholomew D, Kadzimin S (1977) Pineapple. In: Alvin, P.; Kozeowski, T.; eds. Ecophysiology of tropical crops. New York, NY: Academic Press, pp 113-156.

Bartholomew D, Paul R, Rohrbach K (2003) Crop environment, plant grothand physiology. In: Bartholomew D, Paul R, Rohrbach K The pineapple: botany, production and uses. (eds.) CABI Publishing, Wallingford. UK. pp 69-108.

Batkova P, Pospisilova J, Synkova H (2008) Production of reactive oxygen species and development of antioxidative systems during *in vitro* growth and *ex vitro* transfer. Biologia Plantarum 52: 413-422.

Bianjiang Z, Demao J (2009) Constructing C4 rice - the challenge of new green revolution. African Journal of Food Science 3:434-438.

Borland A, Dodd A (2002) Carbohydrate partitioning in CAM plants: reconciling potential conflicts of interest. Functional Plant Biology 29:707-716.

Borland A, Griffiths H (1989) The regulation of citric acid accumulation and carbon recycling during CAM in *Ananas comosus*. J. Exp. Bot. 40:53-60. doi:10.1093/jxb/40.1.53

Borland A, Griffiths H, Hartwell J, Smith J (2009) Exploiting the potential of plants with crassulacean acid metabolism bioenergy producing on marginal lands. J. Exp. Bot. 60:2879-2896.

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-254.

Brulfert J, Muller D, Kluge M, Queiroz O (1982) Photoperiodism and crassulacean acid metabolism. Planta. 154:326-331.

Carvalho L, Vilela J, Vidigal P, Mullineaux P, Amancio S (2006) Activation of the ascorbate-glutathione cycles is an early response of micropropagated *Vitis vinifera* L. explants transferred to *ex vitro*. Int. J. Plant Sci. 167:759-770.

Carvalho LC, Amancio S (2002) Antioxidant defence system in plantlets transferred from *in vitro* to *ex vitro:* effects of increasing light intensity and CO_2 concentration. Plant Sci. 162:33-40.

Christin P, Petitpierre B, Salamin N, Buchi L, Besnard G (2009) Evolution of C4 Phosphoenolpyruvate Carboxykinase in Grasses, from Genotype to Phenotype. Mol. Biol. Evol. 26:357-365.

Chu C, Dai Z, Ku MSB, Edwards GE (1990) Induction of Crassulacean Acid Metabolism in the Facultative Halophyte Mesembryanthemum crystallinum by Abscisic Acid. Plant Physiol. 93:1253-1260.

Cushman J (2001) Crassulacean Acid Metabolism. A Plastic Photosynthetic Adaptation to Arid Environments. Plant Physiology 127:1439.1448.

Cushman J, Agarie S, Albion R, Elliot S, Taybi T, Borland A (2008a) Isolation and characterization of mutants of common ice plant deficient in crassulacean acid metabolism. Plant Physiology 147:228-238.

Cushman J, Bohnert H (1999) Crassulacean acid metabolism: Molecular Genetics. Annu. Rev. Plant Physiol. Plant Mol. Biol. 50:305-32. doi:10.1146/annurev.arplant.50.1.305

Cushman J, Tillet R, Wood J, Branco M, Schlauch K (2008b) Large-scale mRNA expression profiling in the common ice plant, *Mesembryanthemum crystallinum*, performing C3 photosyntheesis and Crassulacean acid metabolis, (CAM). J. Exp. Bot. 59:1875-1894.

Dodd A, Griffiths H, Taybi T, Cushman J, Borland A (2003) Integrating diel starch metabolism with the circadian and environmental regulation of Crassulacean acid metabolism in *Mesembryanthemum crystallinum*. Planta 216:789-797.

Dopazo AR, Armenteros EF, Gafas JP, Peña O, Fornaris LM, Mulen L (2009) Manejo del cultivo de la piña (*Ananas comosus* (L.) Merr.) cv. Española Roja, en Cuba. Rev. Citri. Frut. 26:71-75.

Doubnerova V, Ryslava H (2011) What can enzymes of C4 photosynthesis do for C3 plants under stress? Plant Science 180:575-583.

Edwards G, Franceschi V, Voznesenskaya E (2004) Single cell C4 photosynthesis versus the dual-cell (Kranz) paradigm. Annu Rev Plant Biol. 55:173-196.

Escalona M, Lorenzo J, González B, Daquinta M, Borroto C, González J, Desjardins Y (1999) Pineapple micropropagation in temporary immersion systems. Plant Cell Rep. 18:743-748.

Food and Agriculture Organization of the United Nations (FAOSTAT) (2010) FAO statistic division. IOP Publishing PhysicsWeb. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567. Accessed 2 June 2011.

Hamilton R (1995) Waxes: chemistry, molecular biology and functions. Edinburgh: Orly Press, pp 149.

Hiscox JD, Israelstam GF (1978) A method for the extraction of chlorophyll from leaf tissue without maceration. Can J Bot 57:1332-1334.

Jacobs A, Dry I, Robinson, S (1999) Induction of different pathogenesis-related cDNAs in grapevine infected with powdery mildew and treated with ethephon. Plant Pathol 48:325-336.

Keller P, Luttge U (2005) Photosynthetic light-use by three bromeliads originating from shaded sites (*Ananas ananassoides, Ananas comosus* cv. *Panare*) and exposed sites (*Pitcairnia pruinosa*) in the medium Orinoco basin, Venezuela. Biologia Plantarum 49:73-79.

Kluge M, Ting I (1978) Crassulacean acid metabolism: analysis of an ecological adaptation. Berlin: Springer-Verlag, pp 1-105.

Kotting O, Kossmann J, Zeeman S, Lloyd J (2010) Regulation of starch metabolism: the age of enlightenment? Current Opinion in Plant Biology 13:321-329.

Le V, Lamaze T, Champigny M (1991) Effect of light and NO^{3-} on wheat leaf phosphoenolpyruvate carboxylase activity. Evidence for covalent modulation of the C3 enzyme. Plant Physiol. 97:1476-1482.

Lee M, Hebda C, Nowak T (1981) The role of cations in avian liver phosphoenolpyruvate carboxykinase catalysis. J Biol Chem. 256:12793-12801.

Luttge U (2004) Ecophysiology of Crassulacean Acid Metabolism (CAM). Ann Bot 93:629-652.

Matsuoka M, Furbank R, Fukayama H, Miyao M (2001) Molecular engineering of c4 photosynthesis. Annu. Rev. Plant physiol. Plant Mol. Biol. 52:297-314.

Moyle R, Fairbairn D, Ripi J, Crowe M, Botella J (2005) Developing pineapple fruit has a small transcriptome dominated by metallothionein. J. Exp. Bot. 56:101-112.

Mullineaux PM, Karpinski S, Baker N (2006) Spatial dependence for hydrogen peroxide-directed signaling in light stressed plants. Plant Physiol 141:346-350.

Nair V, Jaleel C, Gopi R, Panneerselvam R (2009) Changes in growth and photosynthetic characteristics of *Ocimum sanctum* under growth regulator treatments. Front. Biol. China 4:192-199.

Nievola C, Kraus J, Freschi L, Souza B, Mercier H (2005) Temperature determines the occurrence of CAM or C3 photosynthesis in pineapple plantlets grown *in vitro. In Vitro* Cell. Dev. Biol.-Plant 41:832–837.

Pérez C (2005) Técnicas estadísticas con SPSS 12. Aplicaciones al análisis de datos. Pearson Educación S. A. España. pp. 10-85.

Porra J (2002) The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b. Photosynthesis Research 73:149-156.

Read P (2007) Micropropagation: Past, present and future. Acta Hort. 748:17-28.

Richardson AD, Duigan SP, Berlyn GP (2002) An evaluation of noninvasive methods to estimate foliar chlorophyll content. New Phytologist 153:185-194.

Sharp R, Lenoble M (2002) ABA, ethylene and the control of shoot and root growth under water stress. J. Exp. Bot. 53:33-37.

Sheriff A, Meyer H, Riedel E, Schmitt J, Lapke C (1998) The influence of plant pyruvate, orthophosphate dikinase on a C3 plant with respect to the intracellular location of the enzyme. Plant Sci. 136:43-57.

Siegl G, Stitt M (1990) Partial purification of two forms of spinach leaf sucrose-phosphate synthase which differ in their kinetic properties. Plant Sci. 66:205-210.

Smeekens S, Ma J, Hanson J, Rolland F (2010) Sugar signals and molecular networks controlling plant growth. Current Opinion in Plant Biology 13:273-278.

Stitt M, Müller C, Matt P, Gibon Y, Carillo P, Morcuende R, Scheible W, Krapp A (2002) Steps towards an integrated view of N metabolism. J. Exp. Bot.53: 959-970.

Taybi T, Cushman JC (2002) Abscisic acid signaling and protein synthesis requirements for phosphoenolpyruvate carboxylase transcript induction in the common ice plant J. Plant Physiol. 159: 1235-1243.

Theng V, Agarie S, Nose A (2008) Regulatory phosphorylation of phosphoenolpyruvate carboxylase in the leaves of *Kalanchoe pinnata*, *K. daigremontiana* and *Ananas comosus*. Biologia Plantarum 52:281-290.

Weise S,Wijk K, Sharkey T (2011) The role of transitory starch in C3, CAM, and C4 metabolism and opportunities for engineering leaf starch accumulation. J. Exp. Bot 62:3109–3118.

Chapter 5

The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles.

Aragón C[1,2] Pascual P[1] González J[2] Escalona M[2] Carvalho L[1] Amâncio S[1]

[1]CBAA/DRAT, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Plant Cell Reports (2013) 32:1807-1818

The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles

Aragón C[1,2] Pascual P[1] González J[2] Escalona M[2] Carvalho L[1] Amâncio S[1]

[1]CBAA/DRAT, Instituto Superior de Agronomia, Universidade de Lisboa, Portugal

[2]Laboratorio de Células y Cultivo Tejidos, Centro de Bioplantas, Universidad de Ciego de Ávila, Cuba

Abstract

Pineapple is one of the most important tropical crops worldwide. The use of Temporary Immersion Bioreactors (TIB) for the first stages of pineapple propagation enables precise control of plant growth, increases the rate of plant multiplication, decreases space, energy and labor requirements for pineapple plants in commercial micropropagation. Once the plantlets are ready to be taken from the reactors, they are carefully acclimatized to adapt to natural environmental conditions, and a facultative C3/CAM metabolism in the first two months of growth is characteristic of pineapple plants, depending on environmental conditions. We subjected two sets of micropropagated pineapple plants to C3 and CAM-inducing environmental conditions, determined by light intensity/relative humidity (respectively 40µmol.m^{-2}s^{-1}/85% and 260µmol.m^{-2}.s^{-1}/50%). Leaves of pineapple plants grown under CAM inducing conditions showed higher leaf thickness and more developed cuticles and hypodermic tissue. Proteomic profiles of several proteins, iso-enzyme patterns and transcriptomic profiles were also measured. Five major spots were isolated and identified, two of them for the first time in *Ananas comosus* (OEE 1; OEE 2) and the other three corresponding to small fragments of the large subunit of RuBisCO (LSU). PEPC and PEPCK were also detected by immunobloting of 2DE at the end of both *ex vitro* treatments (C3/CAM) during the dark period. Iso-enzymes of SOD and CAT were identified by electrophoresis and the transcript levels of OEE 1 and CAT were associated with CAM metabolism in pineapple plants.

Keywords: CAM; carbon metabolism; OEE; oxidative stress; PEPC; PEPCK; RT-qPCR; RuBisCO.

Key message: Proteomic and transcriptomic profiles of key enzymes were monitored in pineapple plants propagated under C3 and CAM inducing metabolisms to obtain insight into the CAM-facultative metabolism and the relationship of CAM plants with oxidative stress.

5.1 Introduction

Pineapple is the second most important harvest crop worldwide, after banana, contributing to over 20% of the production of tropical fruits (Coveca 2002). Nearly 70% of the pineapple produced is consumed as fresh fruit in producing countries. Global production began at *circa* 1500 t when, in addition to tropical regions, pineapple propagation started in Europe (Coppens et al. 1993). In 2011 the international production surpassed two million tons with Brazil as the major producer, followed by The Philippines and Costa Rica (FAOSTAT 2011). The most widely used variety is Smooth Cayenne which was first introduced in Europe from French Guyana. However, several new varieties have been introduced to improve the quality of the fruit that reaches international markets, such as MD2 (Golden ripe, Extra sweet and Maya gold) (Sopie et al. 2011).

The use of byproducts of pineapple culture in feed production, canning and juice extraction has been encouraged. In animal food, leaves can be used in three forms: fresh, dried and in silage (Coppens and Leal 2003). Pineapple may offer additional advantages, such as its relevance as fiber source. Pineapple fiber has numerous qualities, such as its texture, its length (up to 60cm), high water and dye holding capacity, high whiteness, brightness, resistance to salt and tension strength. Bromelin has been produced for use as meat tenderizer and as a component of pharmaceuticals. A major problem that both large scale commercial production of pineapple and the expansion of the existing small farms face is the difficulty in obtaining uniform planting material in large quantity due to the low rate of multiplication by conventional methods and the lack of high quality propagules. Pineapple propagation is performed asexually, using shoots from different parts of the plant, such as bulbs, crowns and axilary buds. Recent advances in plant biotechnology methods applied to pineapple crops enhanced their potential application, both for basic studies and for direct application in agriculture (Read 2007; Aragón et al. 2009; 2010; 2012). The use of bioreactors enables more precise control of plant growth, gas exchange, illumination, medium agitation, temperature and pH than the conventional culture vessels (Escalona et al. 1999). Temporary Immersion Bioreactors have been used since 1999 (Escalona et al. 1999) and increase considerably the number of plantlets produced in comparison with conventional vessels. In fact, this method not only increases the rate of plant multiplication, but also reduces space, energy and labor requirement, concerns that are paramount to commercial micropropagation.

Once the plantlets are ready for shifting outside the reactors, they have to be carefully acclimatized to adapt to natural environmental conditions. During hardening, the plantlets undergo physiological adaptations to changing external factors like water, temperature, relative humidity and nutrient supply. In the first two months of growth pineapple plants display a facultative C3/CAM (Crassulacean Acid Metabolism), depending on environmental conditions (Aragón et al. 2012). CAM metabolism developed as a response to a stressful environment and gave rise to more succulent leaves, higher starch concentration during the light period and higher photosynthetic rates than C3 metabolism (Aragón et al. 2012). However, direct results of the possible damage or other metabolic changes caused by these environmental conditions are still unclear.

CAM photosynthesis appears to have originated as a means to scavenge respiratory CO_2 under conditions where the carbon balance is restricted, in environments where water availability becomes temporarily or seasonally constrained, such as deserts or rock outcrops. CAM metabolism causes major changes to leaf structure, succulence being the most obvious innovation as it facilitates the capture of night-time CO_2 released by respiration (Guralnick et al. 2001; Sage 2002; Nelson et al. 2005). CAM metabolism is found in a wide range of species with distinct phylogenetic lineages and habitats. All these species developed diverse structures and adaptations to stressful environments but they all share anatomical and functional traits (Kore-eda et al. 2005; Nelson et al. 2005). Plants with CAM metabolism have a complex balance of carbon flux inside the cell. The soluble sugars resulting from the assimilation of CO_2 in the dark are essential for the light period that follows. PEPC catalyzes the reaction of carboxylation of phosphoenolpyruvate, with oxaloacetate and organic phosphate as products and has several functions in plants, such as an anapleurotic function in leaves and non-photosynthetic tissues (Gehrig et al. 2005).

C3, C4 and CAM photosynthetic pathways are well characterized but there are still a few metabolic details not well understood (Willert et al. 2005). The interpretation of the C3/CAM transition as a complex interaction between environment and metabolism, in opposition to a basic molecular interpretation of the circadian cycle linked to carbon metabolism are still elements to be clarified on pineapple plants. In plants showing facultative C3/CAM metabolism the CAM state can be induced as a response to stressful conditions, showing different levels of transition from C3 to complete CAM metabolism (Luttge 2004). We have extensively investigated the *ex vitro* growth conditions needed to shift pineapple plants from

C3 to CAM metabolism and performed a partial physiological characterization (Aragón et al. 2012), but more details of the molecular processes taking place will contribute to the interpretation of the events as a whole. Transport activities and the control of intracellular compartmentalization, organic acid and carbohydrate flux are some of the most challenging processes to consider on CAM metabolism. Their knowledge could be the key to understand the temporal separation of carbon metabolism and the control of enzymes such as pyruvate orthophosphate dikinase (PPDK), phosphoenolpyruvate carboxylase (PEPC) and starch synthase (Kore-eda et al. 2005).

Previous studies with pineapple plants micropropagated in TIB assessed the environmental conditions that induce C3 and CAM metabolism and demonstrated that the CAM inducing condition is the most stressful (Aragón et al. 2012). The main objective of the current research was to obtain more insight into the facultative metabolism of pineapple TIB grown plants under C3 and CAM inducing conditions, with the main focus on the relationship with oxidative stress, more in evidence in the plants showing CAM metabolism.

5.2 Materials and Methods

5.2.1 Plant material and ex vitro culture conditions

Pineapple plants (*Ananas comosus* (L.) Merr. var MD-2) micropropagated in Temporary Immersion Bioreactors (TIB; Escalona et al. 1999) were grown in glass chambers as described in Aragón et al. (2012). Two sets of 120 plants each were placed in two glass chambers with different regulation of light intensity, relative humidity (RH) and irrigation (Table 5.1). Measurements were taken at the moment of transfer to acclimatization (week zero, w0), at w4 and at w8 of acclimatization. Samples were collected in the middle of the light period and in the middle of the dark period, always from leaf D, defined as the leaf forming a 45^{o} angle with the vertical axis.

5.2.2 Histological analyses

To analyse the distribution of tissues in the leaf transverse cross sections of pineapple leaves were performed. The leaves were fixed in FAA reagent (solution of: 90% ethanol (50%), 5% acetic acid, 5% formaldehyde) and included in polyethylene glycol 20% at 60^{o}C for 24h. The inclusions were then cut by microtome (SM 2400, Leica Microsystems) and the cross sections were carefully stretched on glass slides for observation and measurements in an optical microscope (Axioskop 2, Zeiss®) with camera (AxioCam HRM, Zeiss®). Using the

software AxioVision 4.8 (Zeiss®) the following parameters were measured: total leaf, mesophyll, epidermis and hypodermic tissue thickness.

Table 5.1 Environmental conditions applied in the growth chambers and that induced either C3 or CAM carbon metabolism.

Treatments	C3	CAM
Light (µmol m^{-2} s^{-1})	40	260
Humidity (RH) (%)	85	50
Temperature (ºC)	(22 ± 2/20 ± 2)	(28 ± 2/20 ± 2)
Irrigation	Saturation	Deficit

5.2.3 Succulence and pH analyses

Succulence analyses were performed following the Saturated Water Content (SWC) protocol, a dimensionless measure of water mass held by a given tissue or organ at full hydration normalized to the tissue's dry mass: SWC = (Leaf mass at full hydration (g) – dried leaf mass (g)) / dried leaf mass (g). To obtain leaf mass at full hydration the leaves were soaked in distilled water overnight. When leaves gained < 5 % in mass between subsequent weighings they were considered saturated. To obtain the dried leaf mass leaves were transferred to an oven at 60 ºC until constant weight was attained.

To quantify pH in sap extracted from leaves, they were crushed until 0.5 mL of sap was obtained. The sap was clarified though the addition of activated charcoal to eliminate pigments and pH was immediately quantified with pH indicator strips (Merck KGaA 64271).

5.2.4 Protein extraction and two-dimensional electrophoresis (2-DE)

Frozen leaf material (0.5 g) previously collected in the middle of the light period was reduced to powder using a mortar and pestle in the presence of liquid nitrogen. Proteins were precipitated for 1 h at -20 ºC with acetone containing 10 % (w/v) TCA (trichloroacetic acid) and 60 mM DTT (dithiothreitol), and centrifuged at 15 000 *g* for 15 min at 4 ºC. The resulting pellet was washed in acetone with 60 mM DTT for 1 h at -20 ºC and centrifuged again. This pellet was dried under vacuum and used as the crude extract, after being re-

dissolved for 2 h at 25 ºC in a buffer containing 7 M urea, 2 M thiourea, 0.4 % (v/v) Triton X-100, 4 % (w/v) CHAPS ((3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane sulfonate)), 60 mM DTT and 1 % (v/v) IPG (Immoblized pH gradient buffer; GE Healthcare Lifesciences). Protein was quantified by the method of Bradford (1976), modified by Ramagli (1999). Samples were incubated for 1h at 25 ºC in a re-hydration buffer containing 8 M urea, 4 % (w/v) CHAPS, 60 mM DTT and 1 % (v/v) IPG buffer.

Iso-electric focusing (IEF) was carried out using Ready-Strip-IPG-Strips (Bio-Rad, Hercules CA), 11 cm or 17 cm, with linear pH gradients 3-10 or 4-7 and 40 mg of each protein sample were loaded. The specific conditions of each IEF are specified in the respective Figure. IEFs were carried out using a Protean i12 IEF cell (Bio-Rad, Hercules CA), with rehydration at 50 V for 12 h, followed by four consecutive steps in the following conditions: 250 V/h, 500 V/h, 8 000 V for 2.30 h and 8 000 V/h until 30 000 V were attained. After IEF, strips were equilibrated for 15 min in Tris–HCl 50 mM, pH 8.8, containing 6 M urea, 30 % (v/v) glycerol, 1 % (w/v) SDS (sodium dodecyl sulfate*)* and 65 mM DTT. To remove DTT in excess, strips were equilibrated for 15 min in the same buffer, replacing DTT with 135 mM iodoacetamide. SDS-PAGE in the second dimension was then performed in denaturing polyacrylamide gels according to the procedure of Laemmli (1970), without stacking gel.

5.2.5 Immunoblotting

Polyclonal antibodies against RuBisCO were obtained as described in Esquivel et al. (1998), and polyclonal antibodies against PEPC and PEPCK were purchased from Agrisera (Vännäs, Sweeden). Western blot analysis was performed after 2-DE through the transfer of proteins to a nitrocellulose membrane (Millipore). Membranes were probed with the respective antibodies using the procedure described by Ferreira et al. (1996) and staining was performed with AP Conjugate Substrate Kit (Bio Rad, Hercules, CA).

5.2.6 Native PAGE and gel activity staining

Native Polyacrylamide Gel and Gel Activity Staining Iso-forms of CAT, SOD, APX and GR were separated in nondenaturing polyacrylamide gels by the procedure of Laemmli (1970). Equal amounts of protein extracts (25 mg) were loaded on 7 % (CAT) or 10 % (SOD, GR and APX) polyacrylamide gels.

For SOD, the gel was stained according to Rao et al. (1996). Gels were incubated for 30 min in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA (ethylenediamine

tetraacetic acid). To identify H_2O_2 and KCN (potassium cyanide) sensitive iso-forms, this incubation solution contained 3 mM KCN or 5 mM H_2O_2, respectively. This step was followed by incubation in 50 mM potassium phosphate buffer (pH 7.8) containing 245 µM NBT (nitroblue tetrazolium), 33.2 mM riboflavin and 0.2 % TEMED (tetramethyl ethylene diamine) in darkness for 30 min before illumination to visualize SOD iso-forms or bands (Donahue et al. 1997).

To visualize the CAT profile, gels were stained using the procedure described by Anderson et al. (1995). The gels were incubated in 3.27 mM H_2O_2 for 25 min, rinsed in distilled water, and then stained in a solution containing 1 % (w/v) potassium ferricyanide and 1 % (w/v) ferric chloride.

Iso-forms of APX were visualized by incubating the gels for 30 min in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate, followed by an incubation in the same buffer containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min, as in Carvalho et al. (2005). Finally, gels were stained in 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT for 15 min.

GR was detected according to Carvalho et al. (2005), by incubating the gels for 60 min in darkness in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM 3-(4,5-dimethylthiazol-2-4)-2,5-diphenyl tetrazolium bromide, 0.7 mM 2,6-dichlorophenolindophenol, 3 mM GSSG and 0.5 mM NADPH. The staining reaction was stopped by adding 7.5% (v/v) glacial acetic acid to the staining buffer.

5.2.7 RNA isolation and cDNA preparation

Total RNA from leaves was extracted by adapting the method described by Chang et al. (1993). Extraction buffer consisted of CTAB (2 %), complemented with PVPP (2 %, w/w), Trizma-HCl 100 mM, 25 mM Na_2EDTA, and 2 M NaCl; pH 8.0, heated to 85 ºC prior to the addition of 400 µL 2-mercarptoethanol. Tissues were reduced to powder in liquid N_2 and 20 mL extraction buffer were added. The same volume of chlorophorm:iso-amyl alcohol 24:1 was then added. This step was followed by a centrifugation at 12 000 *g* for 30 min at 20 ºC and repeated once. The aqueous phase was transferred to a new tube, and a ¼ LiCl 10 M (v:v) was added. Samples were incubated at 0 ºC overnight and after centrifugation at 12 000 *g* for 20 min at 4 ºC the pellet was recovered and 1.5 mL of buffer was added (Trizma-HCl 10 mM, 1 mM Na_2EDTA, 1 M NaCl; 0.5 % SDS (w:v), pH 8.0, previously heated to 37 ºC).

Ethanol 100 % (2.5 volumes) was added and the samples were incubated for 1 h at -80 ºC and then washed with ethanol 70 %. After drying, the RNA was resuspended in the desired volume of distilled water.

RNA samples were treated with RQ1 RNase-Free DNase (Promega, Madison, WI). cDNA was synthesized from 2 µg of total RNA using oligo (dT) in a 20 µL reaction volume with RevertAid Reverse Transcriptase (Fermentas Life Science, Helsingborg, Sweden) according to the manufacturer's recommendations.

5.2.8 Real time PCR

Primer pairs used for amplification of the genes studied are presented in Table 5.2. The genomic sequences for pineapple available in the GeneBank database (http://www.ncbi.nlm.nih.gov/genbank/) were used and when pineapple sequences were unavailable, the respective orthologs of *Arabidopsis thaliana, Agave tequilana or Leymus chinensis* were used.

The 20 µL reaction mixture was composed of cDNA, 0.5µM gene-specific primers and master mix iQ EVAGreen Supermix (Bio-Rad, Hercules, CA) and an iQ5 Real Time PCR (Bio-Rad, Hercules, CA) was used. Amplification of PCR products was monitored via intercalation of EVAGreen (included in the master mix). The following program was applied: initial polymerase activation, 95 ºC, 3 min, followed by 40 cycles at 95 ºC 15 s (denaturation), 57 ºC 30 s (annealing), 72 ºC 20 s (extension) with a single fluorescence reading taken at the end of each cycle. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimmers. Further, PCR products were resolved on 2 % (w/v) agarose gels run at 4 $V.cm^{-1}$ in Tris-acetate-EDTA buffer (TAE), together with a 50-bp DNA-standard ladder (Invitrogen Gmb H), to confirm the existence of a single product of the desired length.

Table 5.2 Real-time PCR primers used for evaluation of mRNA levels of the enzymes studied.

Protein name **Species**	**Gene bank accession**	**Primer Sequences**
Actin *Ananas comosus*	CO731409.1	5'- GGCACCACACCTTCTACAAC -3' 3´- CTCACACCATCACCAGAATCC-5´
PEPC *Ananas comosus*	AJ312631.1	5'- AGGTCATTGAGCAGTCTTTCG-3' 3´- CCACAACAGCGATCTCATCC -5´
Catalase *Ananas comosus*	GU266543.1	5'- GACTAACCATAGCCACGCAACT -3' 3´- GCCAGGTCTTGGTCACATCA -5´
CuZnSOD *Ananas comosus*	AJ250667.1	5'- CGCACCTGAAGATGAGACC -3' 3´- CCGATAATTCCACAAGCAACTC -5´
APX-thyl *Ananas comosus*	GU266541.1	5'- CTCCTCCTCGTCTTCATCTTCC -3' 3´- ATGCCAGCCTAAGCGAACC -5´
SSU *Arabidopsis thaliana*	GW667494.1	5'- GGGTCCAGTGCATGAAGG -3' 3´- TTCCACATCGTCCAGTACC -5´
OEE 1 *Leymus chinensis*	ABQ52657.1	5'- CCAAGAGGCTGACCTTCGA -3' 3´- CGGTGTGGTAGGAGCAGAACG -5´

To generate a baseline-subtracted plot of the logarithmic increase in fluorescence signal (ΔRn) versus cycle number, baseline data were collected between the cycles 5 and 17. All amplification plots were analyzed with an Rn threshold of 0.3 to obtain Cq (quantification cycle) and the data obtained were exported into a MS Excel workbook (Microsoft Inc.). Relative expressions were calculated in relation to w0 of acclimatization. In order to compare data from different PCR runs or cDNA samples, Cq values were normalized to the Cq value of *Act2*, a housekeeping gene expressed at a relatively high and constant level (Coito et al. 2012) and the ΔΔCq method was used for the quantification of gene expression.

5.2.9 Statistical analysis of the results

Statistical analyses were carried out using SPSS version 12 (Pérez 2005) and the treatments were analyzed using a non-parametric analysis Kruskall-Wallis H. and C-Dunnett and Tukey's multiple range test for gene expression, both at 5% significance level.

5.3 Results

The present work compares the distinct features of pineapple plants when subjected to acclimatization under environmental conditions that promote the development of either CAM or C3 metabolism (Table 5.1). Leaf anatomy, proteomic analyzes, patterns of in gel iso-

enzyme activities and gene expression were assessed in order to characterize the two metabolism pathways and the moment of shifting from C3 to CAM, in CAM inducing conditions.

5.3.1 Leaf anatomy, succulence and sap pH

Using leaf cross sections it was possible to measure total leaf, mesophyll, epidermis and hypodermic tissue thickness and identify the differences between the two acclimatization treatments (Table 5.3).

Table 5.3 Morphological parameters measured on pineapple leaves.

	Leaf	**Mesophyll**	**Cuticle (ax)**	**Cuticle (ab)**	**HT(ab)**
			Thickness (μm)		
C3	444.24 ± 30.21	315.64 ± 25.21	58.12 ± 2.85	64.82 ± 4.67	32.52 ± 2.61
CAM	550.49 ± 35.53	364.81 ± 28.12	52.43 ± 3.28	146.15 ± 13.39	133.79 ± 10.75
	*	-	-	*	*

HT: Hypodermic Tissue; ax: Axial; ab: Abaxial

*Significant differences at 5% level by C-Dunnett multiple range test (n=30).

Leaves of pineapple plants grown under CAM inducing conditions showed higher leaf thickness and more developed epidermic and hypodermic tissue, especially the abaxial epidermal and hypodermal cell layers.

Succulence (S) was measured at the end of the *in vitro* phase (w0) and on w4 and w8 of acclimatization (Fig. 5.1). S values increased in leaves under both acclimatization treatments from w0 to w4 but on w8 they were significantly higher in plants grown in CAM inducing metabolism when compared with C3 plants. The pH was measured in leaf sap of C3 and CAM induced plants on the same time points, in the middle of the light or the dark period (Fig. 5.2). On w0, the sap pH was significantly higher in the light period when compared to the dark and on w4 no significant difference could be detected. On w8, under CAM inducing conditions the pH decreased significantly during the dark period, while plants under C3 inducing conditions had significantly higher pH in the dark period.

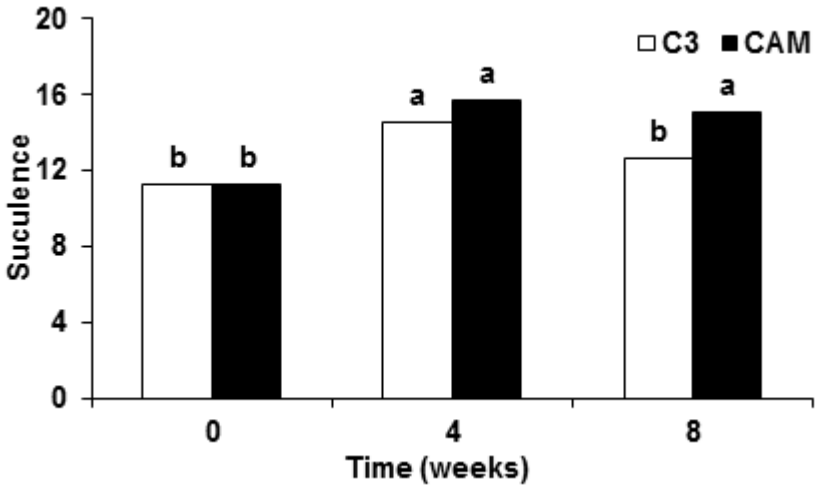

Fig. 5.1 Succulence index of leaves of pineapple plants grown under C3 and CAM induced metabolism at transfer to acclimatization (w0), at four weeks (w4) and at the end of acclimatization (W8), estimated by the Saturated Water Content (SWC). Significant differences at 5% level by C-Dunnett multiple range test (n=5).

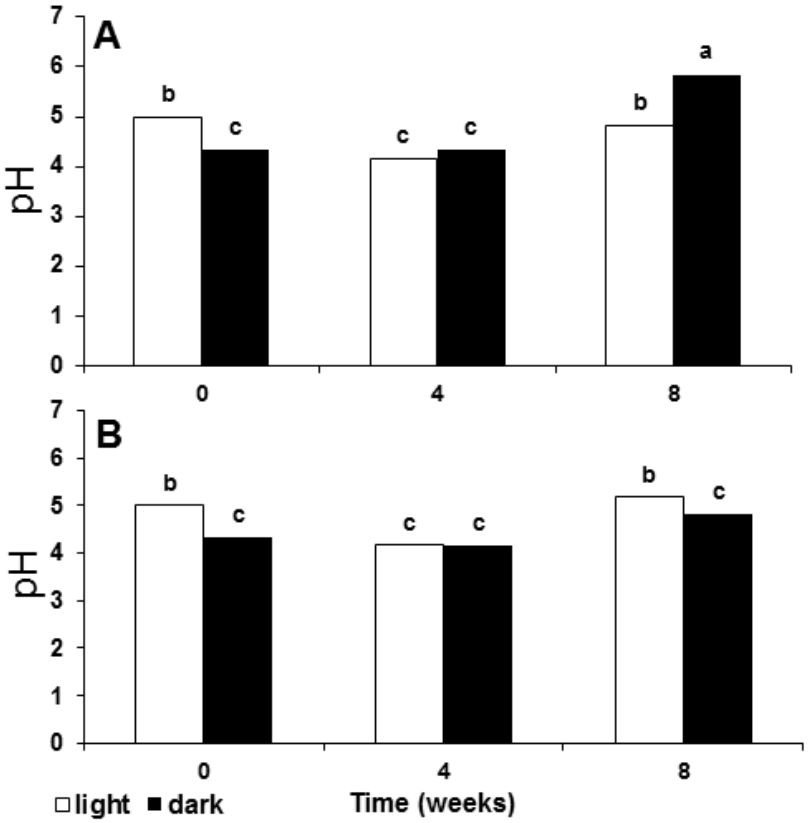

Fig. 5.2 Determination of pH of sap extracted from leaves of pineapple plants grown under C3 (A) and CAM (B) induced metabolism at transfer to acclimatization (w0), at four weeks (w4) and at the end of acclimatization (w8), during the light and dark periods. Values on week 0 are the same on A and B, measured immediately before the beginning of acclimatization. Significant differences at 5% level by C-Dunnett Multiple Range Test (n=5).

5.3.2 Protein analyses and immunobloting

Two dimensional electrophoresis (2DE) applied to protein extracts put in evidence several differential spots. On w8 we focused on those that were absent or only vestigial in C3 conditions and that were present in high quantity in CAM grown plants (Fig. 5.3). Five spots were isolated and identified (Fig. 5.3 and Table 5.4), the two iso-forms of Oxygen Evolving Enhancers, OEE 1 and OEE 2, identified for the first time in *Ananas comosus* by comparison of peptide (mass/charge) profiles after digestion with trypsin (Spots 1, 2 and 3; sequences in Table 5.4) and small fragments of the large subunit of RuBisCO (LSU, Spots 4 and 5), already annotated in Swiss-Prot, were also identified.

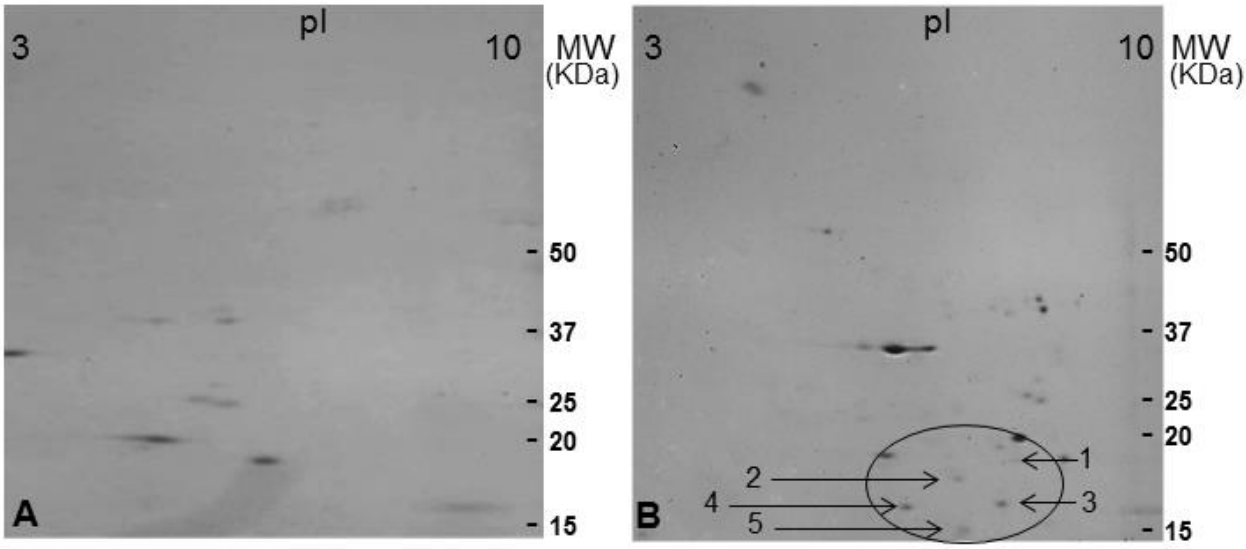

Fig. 5.3 Detection of pineapple proteins by 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (A) and CAM (B) induced metabolism were run on pH 3-10 IPG strips (17 cm) and then separated on 18x20 cm SDS-PAGE. Gels were stained with *Coomassie* G. Spots 1 to 5 were isolated from B and sequenced; sequences are indicated in Table 5.4.

As LSU fragments were identified in CAM grown plants, immunoblotings with anti-LSU antibodies were performed following 2DE to identify all existing LSU fragments and assess the influence of the environmental conditions over LSU degradation patterns (Fig. 5.4A and B). Under both inducing conditions the iso-electric focusing (IEF) separation revealed populations of spots at iso-electric point (pI) 4 to 5 and *circa* 9, at 50kDa, and smaller spots corresponding to LSU degradation, with peptides of low molecular weight and several pI, all higher than 5, almost exclusively found in CAM induced material (Fig. 5.4B).

Table 5.4 Protein sequences obtained by Mass Spectrometry analysis of the spots represented in Fig. 5.3.

Spot	Name	Annotation	Amino acid sequence (aa)
1, 2	**OEE 2** *Ananas comosus*	P12302	--------MASATPAIVFRSKSPSSSSQRYVSISK-------LVCK----------TNTDFLPYNGDGF------ KWNPSKEVEYPGQVLRALG------IT---------- KDYGAPEEFLSKVDYLLGKQ---------- YYFLSVLTRTADGDEGG----------KAQAGDKR----------
3	**OEE 1** *Ananas comosus*	P12359	--RLTYDEIQSKIGGRASSARPSSHVARAFGVDAG--- ARELAQKCTDAAK MEGAAGPFKARLATS ALLVSGATAEGAPKRLTYDEIQSKTYMEVK----------KFCLEPTSFTVK---------- TKLMTRLTYTLDEMEGPLEVFGEE------ ERKDGIDYAAVTVQLPGGERVPFLFTIK---------- GSSFLDPK----------
4	**RuBisCO** *Ananas comosus*	P48683	RBL_ANACO
5	**RuBisCO** *Ananas comosus*	P48683	RBL_ANACO

OEE: Oxygen Evolving Enhancer protein http://www.ebi.ac.uk/interpro/potm/2004_11/Table.htm

PEPC was also detected by immunobloting of 2DE at the end of both *ex vitro* treatments (C3 or CAM inducing) during the dark period (Fig. 5.4C and D). One single spot was visible in plants under CAM inducing conditions, at *circa* pI 4.95 and 100kDa (Fig. 5.4D), characteristics of the intact PEPC. In C3 conditions no corresponding spot was detected (Fig. 5.4C). Conversely to PEPC, PEPCK was also detected by immunobloting of 2DE in both *ex vitro* treatments, but during the light period (Fig. 5.4E and F). Two spots were visible in both treatments, one corresponding to the intact PEPCK, at pI 7.57 and 80kDa and the other corresponding to an unspecific reaction with a lower molecular weight peptide (30kDa), well described by the supplier of the antibody (Agrisera, Vännäs, Sweeden). Under CAM inducing conditions (Fig. 5.4F) the spot corresponding to PEPCK was more intense than in C3 plants where the spot corresponding to the unspecific reaction was predominant (Fig. 5.4E).

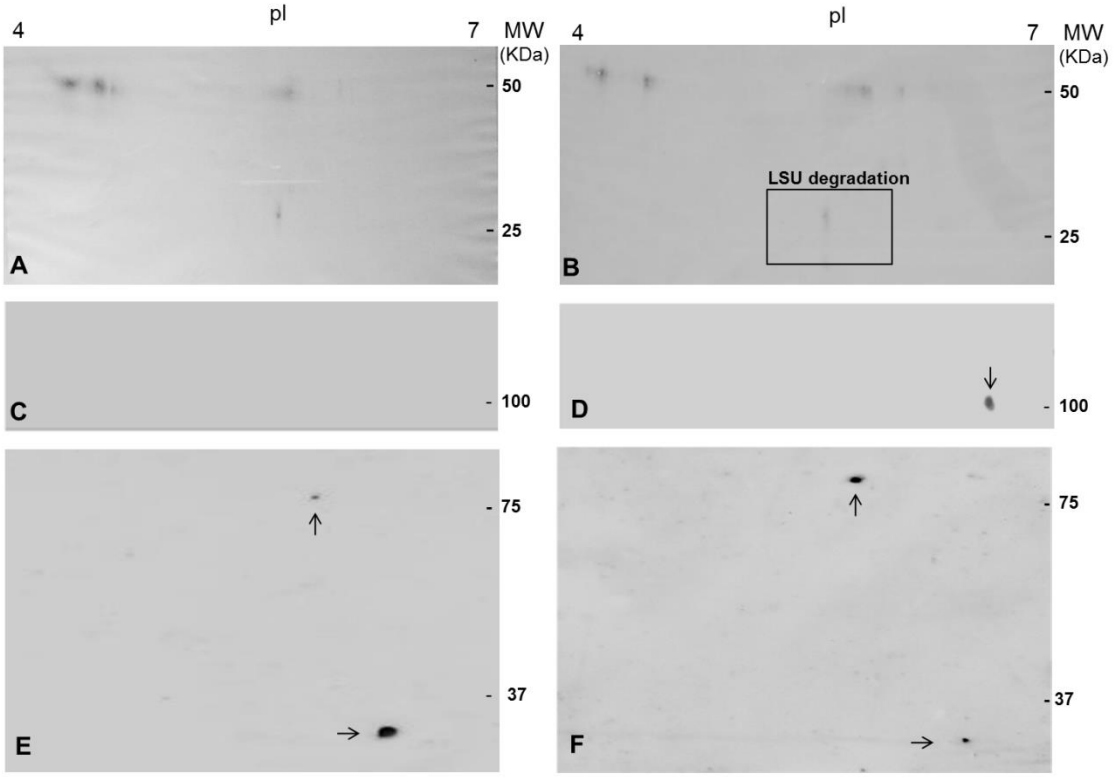

Fig. 5.4 Detection of LSU (large subunit of RuBisCO; A and B), PEPC (Phosphoenolpyruvate carboxylase; C and D) and PEPCK (phosphoenolpyruvate carboxykinase, E and F) by immunoblotting following 2-dimentional electrophoresis. Soluble protein extracts of pineapple leaves at w8 of acclimatization under C3 (A, C and E) and CAM (B, D and F) induced metabolism were run on pH 4-7 IPG strips (7 cm) and then separated on 10x8 cm SDS-PAGE. In B, LSU degradation products are confined within a rectangular box; in D, PEPC is indicated by a downward facing arrow; in E and F PEPCK is indicated by an upward facing arrow and the unspecific product of 30kDa described by the supplier of the antibody (Agrisera, Vännäs, Sweeden) is indicated by an horizontal arrow.

5.3.3 In gel enzyme activities

To assess the effects of the induced carbon metabolism on the anti-oxidative response, the activities of the different iso-forms of four key enzymes of the anti-oxidative system, SOD, CAT, APX and GR were analysed by in gel activity staining (Fig. 5.5). It was possible to identify one CuZnSOD iso-form, using specific reactions with inhibitors (Fig. 5.5A), one CAT (Fig. 5.5B), one APX (Fig. 5.5C) and two GRs (Fig. 5.5D). During the light period SOD activity was present in C3 and CAM induced material, the latter more intensely all over the experiment except on w0 while in and dark period only CAM induced plants showed SOD activity, mostly on w4. CAT was faintly detected on w0 and during the light in C3 plants had high activity on w4 that declined on w8 and in CAM plants was undetected. In the dark period CAT activity was higher in C3 but was present in both treatments and time

moments. In C3 plants APX was active during the first part of the experiment while in CAM induced plants the activities were much lower, even vestigial on w8 in the dark. The two iso-forms of GR were only clearly visible on w0 both in the light and in the dark period.

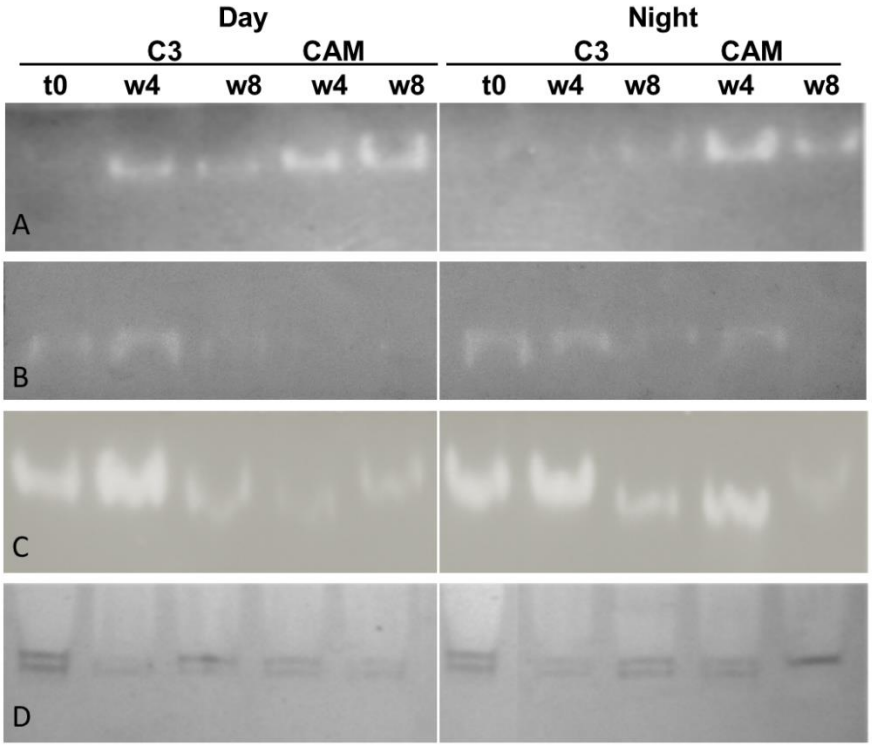

Fig. 5.5 SOD (A), CAT (B), APX (C) and GR (D) iso-enzyme in gel activities in leaves of pineapple plants on w0, w4 and w8 of acclimatization under C3 and CAM induced metabolism, in the light and dark periods. Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD iso-forms was revealed by inhibition with KCN and H_2O_2.

5.3.4 Gene expression

The transcripts of genes coding for the proteins and enzymes referred above were quantified by RT-qPCR on w0 and w8 and their expression recorded on w8 in relation to w0, in C3 and CAM grown plants after light and dark periods (Fig. 5.6). *OEE 1 and SSU* were moderately down-regulated in C3 and CAM induced plants in the light and slightly up-regulated in the dark. *APXt* was down-regulated during the light and the dark periods, in either C3 or CAM induced plants. *PEPC* and *SOD* showed no changes in response to the inducing regimes and to the light conditions. *CAT* was significantly up-regulated in both C3 and CAM inducing conditions in the dark while with significantly lower values in the latter.

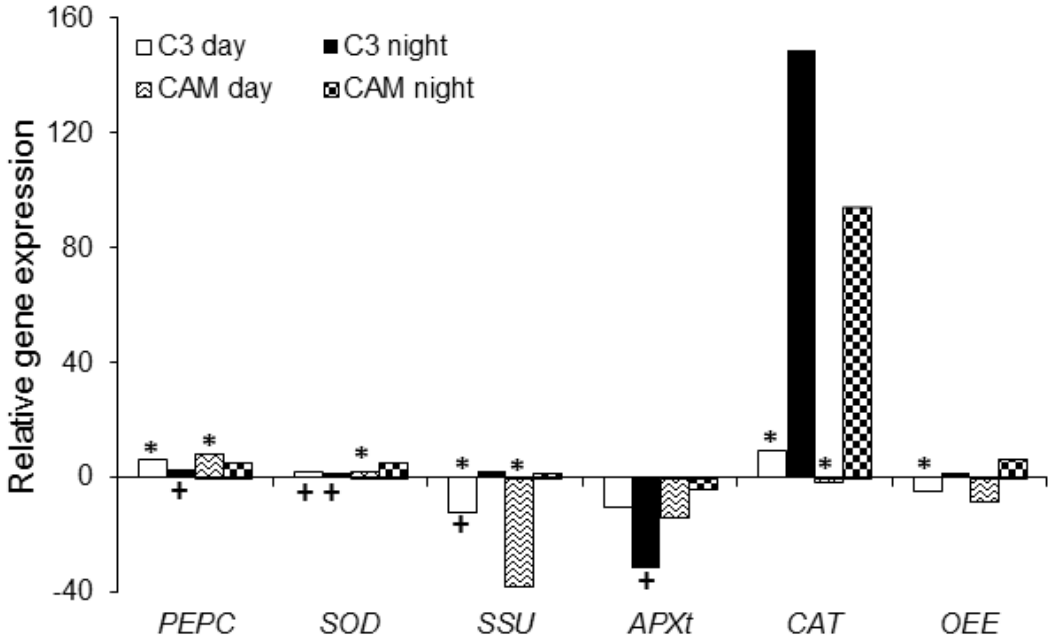

Fig. 5.6 Changes in the expression levels of *OEE 1, SSU, PEPC, SOD, CAT* and *APXt* in leaves of pineapple plants at the end of acclimatization (w8) under C3 and CAM induced metabolism, in the light and dark periods. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time quantitative PCR. Relative expressions in relation to w0 of acclimatization were calculated and values were normalized with respect to *Act2* mRNA. Comparisons of means were performed between light and dark for each induced metabolism (* above the column) and between C3 and CAM in the light and in the dark period (+ below the column). The symbols * and + indicate significant differences at 5% level by Tukey's multiple range test.

5.4 Discussion

Although the designation Crassulacean Acid Metabolism (CAM) derives from the *Crassulaceae* family, the *Bromeliaceae Ananas comosus* is the CAM species with the highest commercial value. CAM physiological and biochemical features are deeply associated to leaf anatomy. In opposition to the majority of non CAM plants, the anatomy of adult pineapple leaves is characterized by the presence of aerating canals, fiber strands, water storage tissue and hypodermis as described in adult pineapple plants by D´Eeckenbrugge and Leal (2003) and more recently, by Pérez et al. (2011; 2012) in new pineapple somaclonal variants. Anatomy of *in vitro* and *ex vitro* pineapple leaves has also been compared (Barboza et al. 2006). Leaf anatomy of *in vitro* propagated pineapple plants also shows canals, fibers, an aquifer parenchyma, a chlorenchyma and hypodermic tissue, mostly maintained during the initial *ex vitro* stages. In the current study leaf sections were significantly thicker in CAM induced *ex vitro* plants due to the thickness of the epidermis, hypodermis and water storage tissues. However, the leaf anatomy of CAM plants has a high degree of plasticity in response

to the environmental conditions. For example, in different CAM plants the formation of water storage tissues could be observed after exposure to high light intensity, eventually as a heat dissipation strategy (Espírito Santo and Pugialli 1998). The large vacuoles of the cells of those tissues are also associated to malic acid storage, an aspect described in a number of CAM species

In agreement with previous results obtained in the same experimental system (Aragón et al. 2012), succulence, normally associated to leaf anatomy of CAM plants of different families (Madison 1977), was also verified. Mesophyll tight cells with highly enlarged vacuoles contribute not only to water but also to malic acid storage (Winter and Smith 1996). Tight packed large cells with less intercellular air space than in C3 plants (Nelson et al. 2005) can also be responsible for an enhanced CO_2 fixation efficiency due to the restriction of CO_2 efflux (Borland et al. 2000). The daily variations of mesophyll pH in CAM plants usually follow the trend of values decreasing in the dark period due to the accumulation of malic acid and increasing in the light period when malic acid released from the vacuole is decarboxylated by cytoplasmic malic enzyme (Antony et al. 2008). In fact, it is possible to establish a direct relationship between the low pH values in the dark in *in vitro* and *ex vitro* CAM induced plants and the high malic acid concentrations measured (Aragón et al. 2012; quantifications obtained in the same experimental system).

The differential protein profile of C3 and CAM grown plants revealed a prominent expression of two iso-forms of the chloroplastic Oxygen-Evolving Enhancer protein 1-1 (OEE1 and OEE2), a 33kDa subunit of the oxygen evolving system of photosystem II (PSII), proteins that are responsible for the protection, repair and dissipation of excess energy in the PSII and that are essential for photosynthesis (Murakami et al. 2002). Due to their function of stabilizing the manganese cluster associated to the primary site of water splitting, these proteins are also known as Manganese-Stabilizing Proteins (MSP-1) (Yi et al. 2005). In *Arabidopsis thaliana* MSP-1 is encoded by *psbO-1* and MSP-2 by *psbO-2* (Yi et al. 2005). It is interesting to note that, conversely to protein expression, the transcriptional rates of *Ananas comosus psbO-1* were higher in the dark period. CAM inducing conditions, with high light and low water availability, are highly prone to oxidative stress (Aragón et al. 2012), and thus, CAM induced plants appear to have developed mechanisms to avoid damage in the PSII.

Monitoring both the enzyme activities and the gene expression profiles of major ROS scavengers and enzymes of the Asc-glut cycle give insight into the overall response of the plants to oxidative stress and whether they are able to cope with the demanding environment of acclimatization (Aragón et al. 2009; 2010). A particular adaptation of pineapple CAM induced plants to oxidative stress was revealed by their ROS (reactive oxygen species) scavenging profile, CuZnSOD and CAT activity during the whole photoperiod and SOD activity during the dark period. Among these two key players in the ROS detoxification pathways SOD is responsible for the superoxide radical dismutation while CAT operates the rapid and efficient removal of hydrogen peroxide and its conversion to water in all cell compartments with the exception of chloroplasts (Shao et al. 2008). There seems to be a time shift between *CAT* expression and CAT enzyme activity in both C3 and CAM induced plants, with high levels of *CAT* expression in the dark period and high CAT activity in the light period.

A strict regulation and coordination between the response to oxidative stress and carbon metabolism should exist in plants with CAM metabolism. The presence of several fragments of RuBisCO's Large Subunit (LSU) in leaves of CAM induced plants suggests a higher turnover of this protein than in C3 plants. Previous results showed polymerization patterns of LSU in plants under stress conditions, namely under UV light (Ferreira et al. 1996; Wilson and Greenberg 1999). RuBisCO is only able to fulfill its function of CO_2 fixation when LSU subunits are coupled with SSU subunits (Small Subunit of RuBisCO), which also regulate enzyme activity (Ferreira et al. 2000). The significant down-regulation of *SSU* in the light period and its up-regulation in the dark period are not in agreement with the normal CO_2 fixation by RuBisCO in CAM plants. Also, the differences between the accumulation of *APXt* transcripts and enzyme activity, either in C3 or CAM plants also point to the important role of circadian rhythm in the adaptation of this species to particular environmental conditions and in the regulation of mRNA transcription. The genes that are synchronized to the circadian-clock suggest a relationship between mRNA abundance and proteins associated to C3/CAM facultative photosynthesis (Cushman 2005; Shao et al. 2006).

In CAM plants PEPC catalyzes the fixation of atmospheric CO_2 in the dark and is also responsible, as in C3 plants, for anapleurotic roles in leaves and non-photosynthetic tissues, as well as for specific functions in stomatal guard cells (Kopka et al. 1997; Gehrig et al. 2005). Furthermore, PEPC is reported to be induced under forced stressing conditions in

plants with CAM metabolism (Borland and Taybi 2004). In leaves of pineapple plants grown under CAM inducing conditions PEPC had a lower iso-electric point (pI: 4.95), a characteristic usually reported in species displaying facultative C4/CAM metabolism (pI: 5.4; Lara et al. 2003). The differences in pI could be related to the patterns of regulation by phosphorylation in C4/CAM facultative plants compared with C3/CAM ones (Vidal and Chollet 1997). Higher levels of PEPC phosphorylation reduce the sensitivity of the enzyme to its inhibitor, malate, and increase its sensitivity to its activator, Glucose-6-Phosphate. PEPC is phosphorylated in the dark in CAM plants and in response to light in C4 plants (Chollet et al. 1996; Nimmo 2000) and this post-translational modification decreases the pI of proteins (Lara et al. 2003). The only PEPC iso-enzyme detected in CAM grown pineapple had a low value of pI (4.95) what can thus be associated with a high pattern of phosphorylation as well as a high functional capacity for CO_2 fixation. In our pineapple system, the expression of *PEPC* was continuously measured both in the light and in the dark and in C3 and CAM induced plants. As so, the major role in the control of the physiological function of this protein must be assigned to the post-translational regulation by phosphorylation. In the CAM species *Kalanchoe pinnata*, PEPC is described as having seven iso-forms (Gehrig et al. 2005). Two of these iso-forms (A and D) are present in pineapple leaves, iso-form D being the most abundant, suggesting that it is likely to serve a dedicated CAM-specific role. The systematic characterization of PEPC iso-forms in families with species that contain the full range of carbon assimilation patterns, from C3 photosynthesis to weak CAM and to strong CAM, as is the case of *Bromeliaceae,* could shed more light to PEPC regulation (Crayn et al. 2004; Gehrig et al. 2005).

PEPCK is responsible for a dual function, the decarboxylation of oxaloacetate with the concomitant phosphorylation of pyruvate to phosphoenolpyruvate and the release of a CO_2 molecule. In some CAM species including pineapple, PEPCK supplies CO_2 for the fixation by RuBisCO during the light period (Weise et al. 2011). The presence of this enzyme in an active form in CAM grown plants confirms the typical CAM metabolism functioning of these plants, further supporting the results of succulence index, pH balance and PEPC behavior.

As a whole, pineapple plants grown under CAM inducing conditions presented a typical CAM metabolism associated to oxidative stress resistance characteristics, as demonstrated by in gel activities of anti-oxidative enzymes, protein degradation patterns and gene expression of key players in the oxidative stress network, mainly *CAT*.

The induction of C3 or CAM metabolisms by direct manipulation of environmental temperature has such high costs that it is almost impossible to be used commercially. In the present work, as in Aragón et al. (2012), the metabolism shift from C3 to CAM was achieved by temperature variation between day and night, by changing light intensity, relative humidity and water supply, whereas a difference of 2°C in the C3 condition and of 8°C in the CAM condition was obtained. This is a considerable advantage for commercial micropropagation and acclimatization procedures, as it is easy and cost-effective. Pineapple plants with the capacity to shift from C3 to CAM metabolism under specific environmental conditions become well adapted for further growth under natural environmental conditions and are also an interesting model for plant biochemical studies concerning carbon metabolism and oxidative stress interactions.

Acknowledgements

Acknowledgments to Fundação para a Ciência e Tecnologia (FCT) for the financial support to CBAA (PEst-OE/AGR/UI0240/2011) and the PhD grant SFRH/BD/43181/2008 to CA.

5.5 References

Anderson M, Prasad T, Stewart C (1995) Changes in isoenzyme profiles of catalase, peroxidase and glutathione reductase during acclimation to chilling in mesocotyls of maize seedlings. Plant Physiol 109:1247-1257.

Antony E, Taybi T, Courbot M, Mugford S, Smith A, Borland A (2008) Cloning, localization and expression analysis of vacuolar sugar transporters in the CAM plant *Ananas comosus* (pineapple). J Exp Bot 59:1895-1908.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2009) Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic characteristics and develops improved anti-oxidative response. Tropical Plant Biol 2:38-50.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2010) *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor. Biol Plant 54:237-244.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2012) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions. Plant Cell Rep 31:757-769.

Barboza S, Ribeiro D, Teixeira J, Portes T, Souza L (2006) (*in portuguese*) Anatomia foliar de plantas micropropagadas de abacaxi. Pesquiza Agropecuaria Brasileira 41:185-194 (in portuguese)

Borland A, Maxwell K, Griffiths H (2000) Ecophysiology of plants with cassulacean acid metabolism. In Leegood R, Sharkey T, von Caemmer S (eds) Photosynthesis: physiology and metabolism. Kluwer Academic Publishers, Dordrecht, pp.583-600.

Borland A, Taybi T (2004) Synchronization of metabolic processes in plants with Crassulacean acid metabolism. J Exp Bot 55:1255-1265.

Bradford M (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-254.

Carvalho L, Esquível M, Amâncio S (2005) Stability and activity of RuBisCO in chestnut plantlets transferred to *ex vitro* conditions under elevated CO_2. *In Vitro* Cell Dev Biol - Plant 41:525-531.

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113-116.

Chollet R, Vidal J, O'Leary M (1996) Phosphoenolpyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. Annu Rev Plant Physiol Plant Mol Biol 47:273-298.

Coito J, Rocheta M, Carvalho L, Amancio S (2012) Microarray-based uncovering reference genes for quantitative real time PCR in grapevine under abiotic stress. BMC Res Not 5:220.

Coppens G, Leal F (2003) Morphology, anatomy and taxonomy. In: Bartholomew D, Paull R, Rohrbach K (eds) The pineapple: botany, production and uses. CABI, Wallingford, Oxford, UK, pp 13-32.

Coveca C (2002) Commission from Veracruz for agricultural commercialization. Government of Veracruz department, Mexico. (*Original in Spanish*).

Crayn D, Winter K, Smith J (2004) Multiple origin of crassulacean acid metabolism and the epiphytic habit in neotropical family Bromeliaceae. PNAS 101:3703-3708.

Cushman J (2005) Crassulacean acid metabolism: recent advances and future opportunities. Func Plant Biol 32:375-380.

Coppens G, Duval M, Van Miegroet, F (1993) Fertility and self incompatibility in the genus *Ananas.* Acta Horti 334:45-51.

D´Eeckenbrugge GC, Leal F (2003) Morphology, Anatomy and Taxonomy. In Bartholomew R, Paull R, Rohrbach K (Eds.) The Pineapple. Botany, Productions and Uses. CABI Wallingford, Oxford, UK, pp.13-166.

Donahue J, Okpodu C, Cramer C, Grabau E, Alscher R (1997) Responses of antioxidants to paraquat in pea leaves: relationships to resistance. Plant Physiol 113:249-257.

Escalona M, Lorenzo J, González B, Daquinta M, Borroto C, González J, Desjardins Y (1999) Pineapple micropropagation in temporary immersion systems. Plant Cell Rep 18:743-748.

Espírito Santo A, Pugialli HRL (1998) Estudo da plasticidade anatômica foliar de *Stromanthe thalia* (Vell.) J.M.A. Braga (Marantaceae) em dois ambientes de Mata Atlântica. Rodriguésia 50:107-122 (in portuguese)

Esquível M, Ferreira R, Teixeira A (1998) Protein degradation in C3 and C4 plants with particular reference to ribulose bisphosphate carboxylase and glycolate oxidase. J Exp Bot 322:807-16.

FAOSTAT (Food and Agriculture Organization of the United Nations) 2011. FAO statistic division. http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID =567#ancor Accessed May 20, 2013.

Ferreira R, Esquível M, Teixeira A (2000) Catabolism of ribulose bisphosphate carboxylase from higher plants. CurrTop Phytochemistry 3:130-165.

Ferreira R, Franco E, Teixeira A (1996) Covalent dimerization of ribulose bisphosphate carboxylase subunits by UV radiation. Biochem J 318:227-234.

Gehrig H, Wood J, Cushman M, Virgo A, Cushman J, Winter K (2005) Large gene family of phosphoenolpyruvate carboxylase in the crassulacean acid metabolism plant *Kalanchoe pinnata* (*Crassulaceae*) characerised by partial cDNA sequence analysis. Func Plant Biol 32:467-472.

Guralnick L, Ku M, Edwards G, Strand D, Hockema B, Earnest J (2001) Induction of PEP carboxylase and Crassulacean acid metabolism with Gibberellic acid in Mesembryanthemum crystallinum. Plant Cell Physiol 42:236-239.

Holtum J, Smith A, Neuhaus E (2005) Intracellular transport and pathways carbon flow in plants with crassulacean acid metabolism. Func Plant Biol 32: 429-449.

Kopka J, Provart N, Muller-Rober B (1997) Potato ward cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation. Plant J 11:871-882.

Kore-eda S, Noake C, Ohishi M, Ohishi J, Cushman J (2005) Transcriptional profiles of organellar metabolite transporters during induction of crassulacean acid metabolism in mesembryanthenum crystallinum. Func Plant Biol 32:451-466.

Laemmli U (1970) Cleavage of structural proteins during the heat of bacteriophage T4. Nature 227:680-685.

Lara M, Disante K, Podestá F, Andreo C, Drincovich M (2003) Induction of a Crassulacean acid like metabolism in the C4 succulent plant, *Portulaca oleracea* L.: physiological and morphological changes are accompanied by specific modifications in phosphoenolpyruvate carboxylase. Photosynthesis Res 77:241-254.

Luttge U (2004) Ecophysiology of crassulacean acid metabolism (CAM). Ann Bot 93:629-652.

Madison M (1977) A revision of *Monstera* (*Araceae*). In Contributions of the Gray Herbarium Harvard University, Cambridge, Mass 207:3-100.

Murakami R, Ifuku K, Takabayashi A, Shikanai T, Endo T, Sato F (2002) Characterization of an Arabidopsis thaliana mutant with impaired psbO, one of two genes encoding extrinsic 33-kDa proteins in photosystem II. FEBS Lett 523:138-142.

Nelson E, Sage T, Sage R (2005) Functional leaf anatomy of plants with crassulacean acid metabolism. Func Plant Biol 32:409-419.

Nimmo H (2000) The regulation of phosphoenolpyruvate carboxylase in CAM plants. Trends Plant Sci 5:75-80.

Pérez C (2005) Técnicas estadísticas con SPSS 12. Aplicaciones al análisis de datos. ISBN 84-205-4410-8. Pearson Educación S.A. (Ed), España. pp.1-120

Pérez G, Mbogholi A, Sagarra F, Aragón C, González J, Isidrón M, Lorenzo J (2011) Morphological and physiological characterization of two new pineapple somaclones derived from *in vitro* culture. *In Vitro* Cell Dev Biol - Plant 47:428-433.

Pérez G, Yanez E, Mbogholi A, Valle B, Sagarra F, Yabor L, Aragón C, González J, Isidrón M, Lorenzo J (2012) New Pineapple Somaclonal Variants: P3R5 and Dwarf. Amer J Plant Sci 3:1-11.

Ramagli L (1999) Quantifying protein in 2D PAGE solubilization buffers. In: Link AJ (ed) Methods in molecular biology: 2D proteome analysis protocols. Totowa, NJ: Humana Press; pp.112.

Rao M, Paliyath G, Ormrod D (1996) Ultraviolet-B- and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. Plant Physiol 110:125-136.

Read P (2007) Micropropagation: past, present and future. Acta Horti 748:17-28.

Sage R (2002) Are CAM and C4 photosynthesis incompatible? Func Plant Biol 29:775-785.

Shao H, Chu L, Lu Z, Kang C (2008) Primary antioxidant free radical scavenging and redox signaling pathways in higher plantlets cells. Int J Biol Sci 4:8-14.

Shao H, Chu L, Zhao Ch, Guo Q, Liu X, Ribaut J (2006) Plantlets gene regulatory network system under abiotic stress. Acta Biologica Szegediensis 50:1-9.

Sopie E, Tanoh H, Kouakou L, Yatty J, Kouamé P, Mérillon J (2011) Phenolic profiles of pineapple fruits (*Ananas comosus* L. Merrill) Influence of the origin of suckers. AJBAS 5:1372-1378.

Vidal J, Chollet R (1997) Regulatory phosphorylation of C4 PEP carboxylase. Trends Plant Sci 2:230-237.

Weise S, van Wijk K, Sharkey T (2011) The role of transitory starch in C3, CAM, and C4 metabolism and opportunities for engineering leaf starch accumulation. J Exp Bot 62:3109-3118.

Willert D, Armbrüster N, Drees T, Zaborowski M (2005) *Welwitschia mirabilis*: CAM or not CAM - what is the answer? Func Plant Biol 32:389-395.

Wilson M, Greenberg B (1999) Tryptophan photolysis leads to a UVB-induced 66 kDa photoproduct of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) *in vitro* and *in vivo.* Photochem Photobiol 70:49-51.

Winter K, Smith J (1996) An introduction to crassulacean acid metabolism: biochemical principles and ecological diversity. In Winter K, Smith J (eds) Crassulacean acid metabolism, ecophysiology and evolution. Springer Verlag: Berlin, pp.1-10.

Yapo E, Kouakou K, Bognonkpe J, Kouame P, Kouakou T (2011) Comparison of Pineapple Fruit Characteristics of Plants Propagated in Three Different Ways: By Suckers, Micropropagation and Somatic Embryogenesis. J Nutr Food Sci 1:110.

Yi X, McChargue M, Laborde S, Frankel LK, Bricker TM (2005) The manganese-stabilizing protein is required for photosystem II assembly/stability and photoautotrophy in higher plants. J Biol Chem 280:16170-16174.

Chapter 6

Final Considerations

6.1 Final Considerations

Plantain (*Musa* spp.), sugarcane (*Saccharum officinarum*) and pineapple (*Ananas comosus*) were the crop species selected for the current research due to their different carbon fixation metabolism (C3, C4, C3-CAM facultative, respectively). Experiments performed using plantain and sugarcane propagated in Temporary Immersion Bioreactors (TIB) or using gelled medium (GM) and later acclimatized with the same protocol allowed to integrate the results obtained when monitoring the oxidative stress response in C3 and C4 metabolism (Aragón et al. 2014 - Chapter 2; Aragón et al. 2009a - Chapter 3; Aragón et al. 2012 - Chapter 4; Aragón et al. 2013 - Chapter 5). Also, pineapple plants propagated in TIB and evaluated during acclimatization under C3 and CAM inducing conditions were used to describe the facultative C3-CAM carbon metabolism displayed by these species and the influence of the environmental conditions on the switch from C3 to CAM (Chapter 4 and 5).

When attempting to apply new technologies of *in vitro* propagation such as TIB to plant species with distinct physiological characteristics (C3, C4, CAM), we aimed to find common physiological and biochemical pathways as well as identify the specific characteristics of each species, in order to obtain adequate and balanced micropropagation and acclimatization protocols. C3, C4 and CAM metabolism in *in vitro* plants can be reflected differently in *ex vitro* plants. However, the efficiency to control the *in vitro* environment and the juvenile plasticity of *in vitro* cultured plants render these systems good experimental models for basic plant physiology studies. Furthermore, studies based on the modulation of *in vitro* conditions which reproduce abiotic stress conditions can be used for understanding the influence of upcoming climatic changes on the physiology of different species.

TIB system has multiple advantages over conventional micropropagation methods due to the use of liquid medium that allows an automation of the propagation process (Teisson and Alvard 1995). This technology leads to good overall results, all of them influenced by TIB properties of, namely the reduction of plant material manipulation by lab personal thus decreasing considerably both the probability of contamination and the costs of production (Cabasson et al. 1997; Escalona et al. 1999; Escalona 2006; Hanhineva and Karenlampi 2007).

It has been reported that TIB conditions are able to induce a response closer to the behavior of *ex vitro* plants. This occurs because TIB provides the renewal of the headspace thus

eliminating harmful volatile compounds like ethylene, nitrogen monoxide (NO) and aldehydes. These harmful volatile compounds could affect leaf morphology, growth of stomata and also impair gas exchanges (water loss and CO_2 fixation) with consequences at the level of photosynthesis (Woodrow et al. 1988; Jackson et al. 1991; Buddendorf-Joosten and Woltering 1994; Tanaka et al. 2005). Conversely, liquid medium allows the formation of thicker cuticles due to the elimination of low molecular weight waxes, avoiding water loss in the first days of acclimatization due to thin cuticles (Ziv 2005; Shu-Han and Der-Ming 2008). Furthermore, a better nutrition is obtained in TIB systems due to the contact of the liquid medium with the whole surface of the leaves, allowing a better absorption of nutrients (Roels et al. 2005). Additionally, forced ventilation reduces the harmful high relative humidity typical of micropropagation environments, leading to well developed and more oxygenated tissues (Majada et al. 2000; Nguyen and Kozai 2001).

Assuming that TIB propagated plants display a response to oxidative stress similar to *ex vitro* plants, acclimatization will have better chances of being successful, originating better quality plants than those propagated in GM medium. This quality can be reflected on physiological parameters, functioning of the anti-oxidative stress metabolism (Slesak et al. 2007) and faster growth in acclimatization.

The advantages directly related to the plant's quality are the increase of plant multiplication rates and the production of plants displaying better morphological and physiological characteristics (Aragón et al. 2005; Aragón et al. 2006; Hanhineva and Karenlampi 2007; Rodríguez et al. 2008; Shu-Han and Der-Ming 2008; Watt 2012). Recent studies show the positive effects of TIB when combined with increased CO_2 concentrations in *in vitro* plant physiology (Aragón et al. 2009b) and the effects of TIB technology in the response of plantain plants to oxidative stress (Aragón et al. 2010).

In the present work new insight into the improvement of plant physiology was made available, especially on the oxidative stress metabolism. Plantain and sugarcane plants grown on TIB showed higher activity of several key anti-oxidant enzymes (SOD, CAT, APX and GR) (Aragón et al. 2014 - Chapter 2; Aragón et al. 2009a - Chapter 3). The values of enzyme activities in TIB plants were species dependent but always higher when compared with those displayed by plants grown in GM conditions (Table 6.1; Fig. 6.1). Furthermore, TIB plants of both species presented a metabolic reprogramming favoring the transcription of genes coding for anti-oxidant proteins thus facilitating the adaptation to acclimatization.

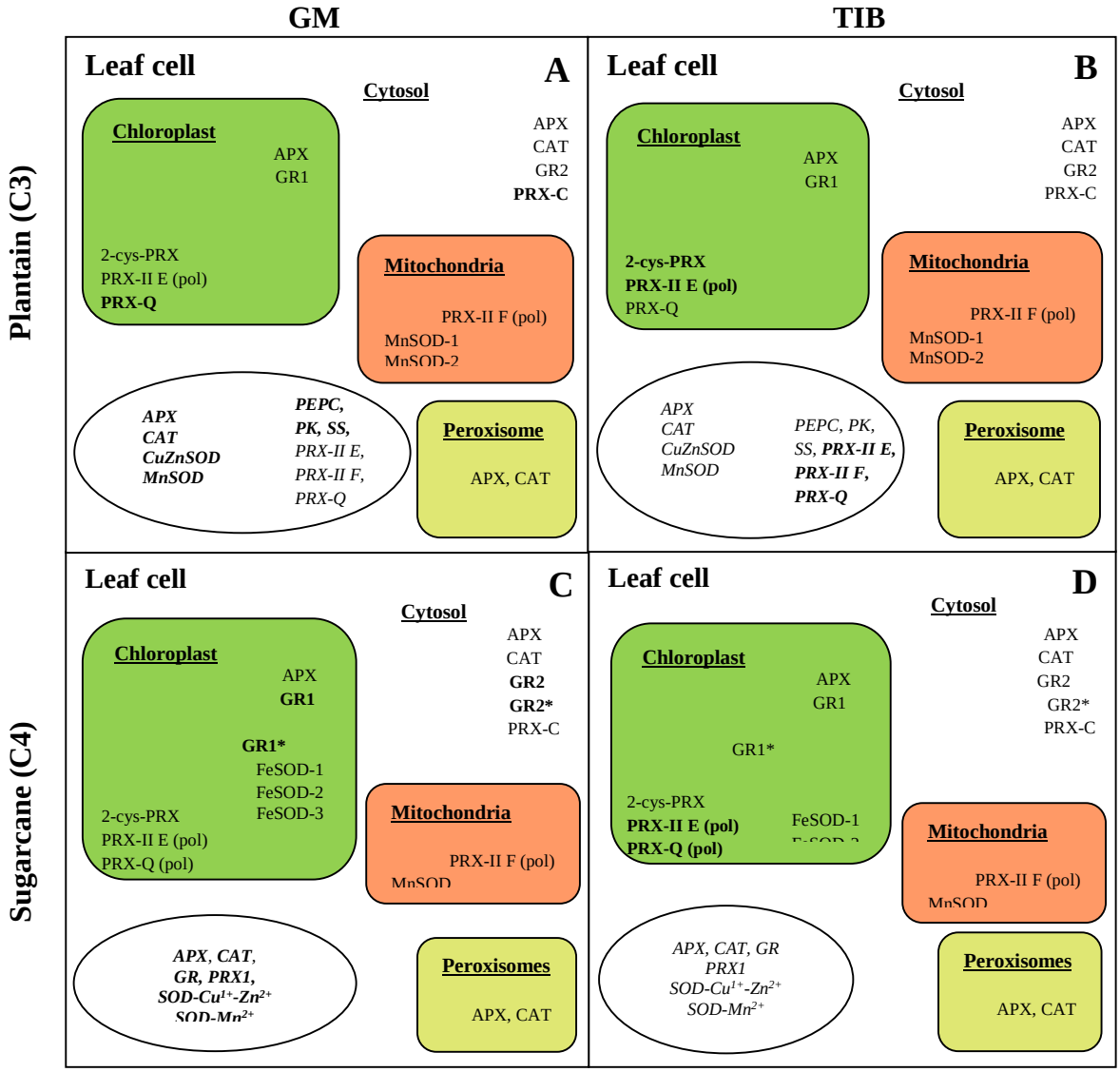

Fig. 6.1 Representation of the leaf cells of a C3 (plantain, A, B) and a C4 (sugarcane, C, D) plant after micropropagation under GM (A, C) and TIB (B, D) methods. Gene expression is represented inside the white ovals. All the other components are proteins and are represented in their most likely compartment of action: "pol" indicates polymerization of Prxs; * indicates proteins present in Bundle sheath cells of the C4 species. Protein names in bold indicate an increased enzyme activity.

Peroxiredoxins (Prxs) decompose ROS and lipid peroxides, avoiding oxidative stress damage, with a simultaneous role in signaling events (Dietz 2011). Prxs are widespread in the cell, with specific members in the chloroplast, the mitochondria, the cytosol and the nucleus, showing a variety of iso-forms defined by several iso-electric points and molecular weights that include polymerization aggregates, formed as a consequence of an oxidative environment. These polymerization aggregates could be responsible for other functions such as chaperone and signaling (Dietz 2011). The results presented in Chapters 2 and 3 confirm

that the function of Prxs at the anti-oxidant defense level was higher in plants propagated in TIB.

Table 6.1 Differences caused by the micropropagation method, GM and TIB, on the accumulation of two reactive oxygen species, hydrogen peroxide (H_2O_2) and superoxide (O_2^{-}) in the stotmata and trichomes of a C3 species (plantain) and a C4 species (sugarcane).

Feature		**C3**		**C4**	
		GM	**TIB**	**GM**	**TIB**
Plant species		**Plantain**		**Sugarcane**	
Stomata	**H_2O_2 accumulation**	(+)	(-)	(+)	(-)
	$O_2^{-\cdot}$ accumulation	(+)	(-)	(+)	(-)
Trichomes	**H_2O_2 accumulation**	n.p.	n.p.	(+)	(-)
	$O_2^{-\cdot}$ accumulation	n.p.	n.p.	(+)	(-)

n.p., not present; + increase; - decrease.

Plantain plants were also compared on carbon metabolism (Table 6.2) attending to the micropropagation technique GM or TIB. Several indicators were improved on plants propagated in TIB giving better plant morphological quality with better physiological characteristics.

Table 6.2 Differences caused by the micropropagation method, GM and TIB, on several features of carbon metabolism of a C3 species (plantain).

Feature	**C3**	
	GM	**TIB**
Ethylene	(+)	(-)
Shoot length/ Stem diameter	(-)/(=)	(+)/(=)
Number of leaves/roots	(-)/(-)	(+)/(+)
Length/Width/ Area of main leaf	(-)/(+)/(=)	(+)/(-)/(=)
Fresh/Dry weight	(-)/(-)	(+)/(+)
Starch, leaves/corm	(-)/(=)	(+)/(=)
Photosynthetic Rate	(-)	(+)
Stomata Conductance	(+)	(-)
Total Transpiration	(-)	(+)

+ increase; - decrease; =, equivalent.

On pineapple plants carbon metabolism and oxidative stress response are well connected since these plants showed a facultative C3-CAM metabolism, and the trigger for the development of CAM metabolism was related to stressful environmental conditions (Table 6.3; Fig. 6.2). As reported in Aragón et al. 2012 (Chapter 4) and Aragón et al. 2013 (Chapter 5) it was shown for the first time that environmental conditions induced TIB grown pineapple plants to develop a differential metabolism (C3 or CAM) in a relative short period of time (4 to 8 weeks) of acclimatization.

Table 6.3 Differences in leaf anatomy and morphology caused by the micropropagation methods, GM and TIB, on the acclimatization of pineapple, a species displaying facultative C3-CAM carbon metabolism.

Feature	**Facultative**			
	C3		**CAM**	
Leaf thickness	(-)		(+)	
Cuticle	Axial	Abaxial	Axial	Abaxial
	(=)	(-)	(=)	(+)
Hypodermic tissue	Axial	Abaxial	Axial	Abaxial
	(=)	(-)	(=)	(+)
Thorns	n.p.		(+)	
Roots	(+)		(-)	
Leaves length/width ratio	(+)		(-)	
Leaf area	(-)		(+)	
Fresh weight	(-)		(+)	
Dry weight	(-)		(+)	
ABA	(+)		(-)	
Chlorophylls *a*	(+)		(-)	
Chlorophylls *b*	(+)		(-)	
Totals chlorophylls	(+)		(-)	
Chlorophylls ratio *a*/*b*	(=)		(=)	
Waxes	(-)		(+)	
Succulence index	(-)		(+)	
Stomatal conductance	(=)		(=)	

n.p., not present; + increase; - decrease; =, equivalent.

Gs values are not shown on chapter 4, but results were obtained in the same plant material.

The characterization of C3 or CAM metabolism was obtained by anatomical and morphological characteristics (leaf area, number of leaves and roots), succulence index, quantification of waxes, photosynthesis parameters and metabolites, malic acid, activity of carbon metabolism enzymes (PEPC, PEPCK) and ABA balance. C3-CAM plant metabolism was also characterized at the molecular level (Fig. 6.2) through the identification and sequencing of differentially abundant proteins, immunological detection of PECP and

PEPCK and quantification of transcripts of carbon metabolism and oxidative stress associated genes.

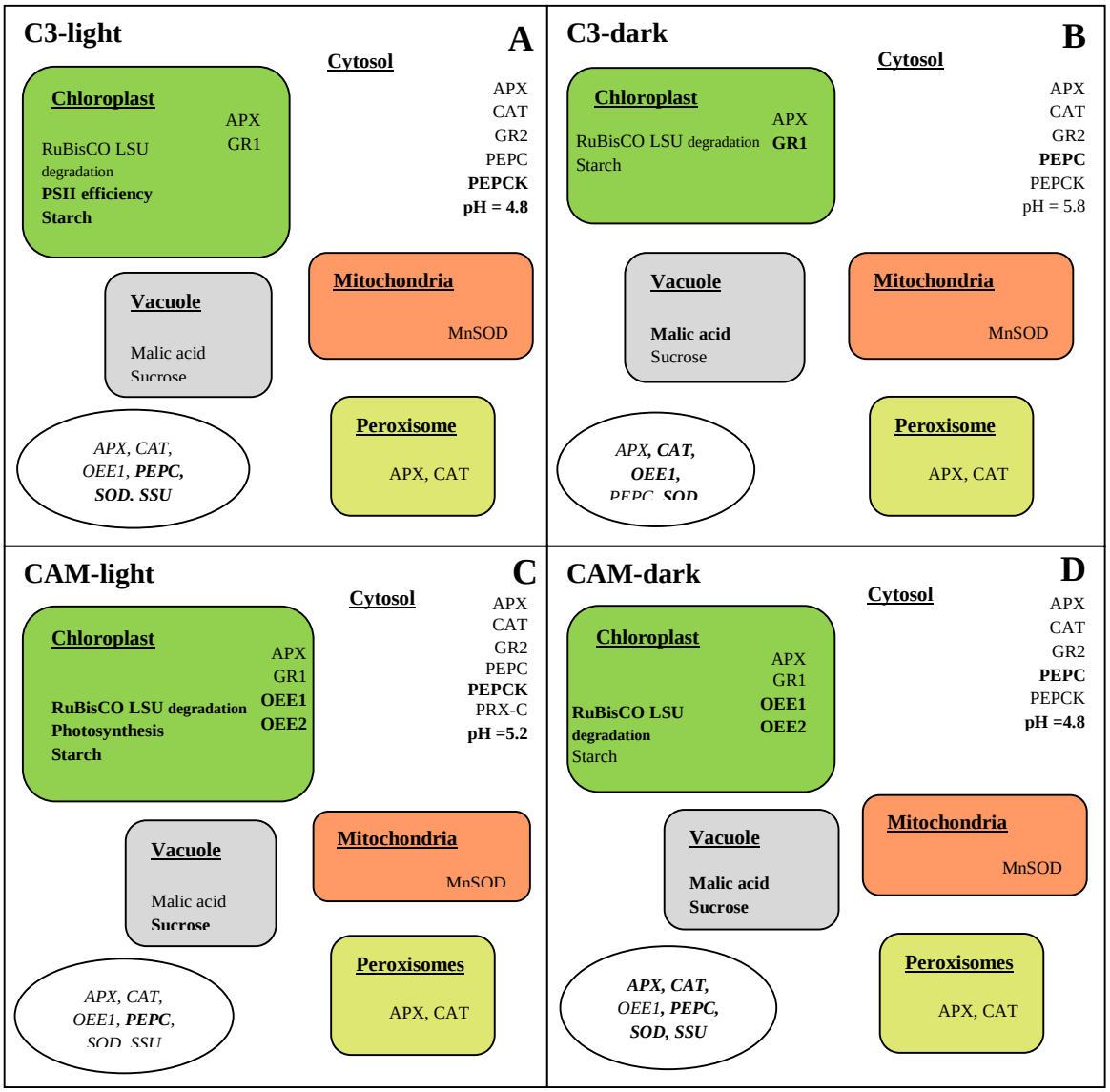

Fig. 6.2 Representation of the cells of pineapple grown under C3 (A, B) and CAM (C, D) inducing conditions in the light (A, C) and dark (B, D) periods. Gene expression is represented inside the white ovals and cell pH is shown in the cytosol. The other components are proteins and sugars and are represented in their most likely compartment of localization. Names in bold indicate an increased enzyme activity or an increased sugar concentration.

The metabolism shift from C3 to CAM is based on oxidative stress signals generated by temperature variation between day and night. Nievola et al. (2005) reported the variation of C3 and CAM phenotype on pineapple plants by directly changing the temperature of the lab environment of *in vitro* plants. However, in micropropagation and acclimatization procedures it is almost impossible to induce C3 or CAM metabolisms by changing the environmental

temperature, due to the high costs associated. Here we defined the establishment of distinct C3 or CAM metabolism by changing three environmental variables, light intensity, relative humidity and water supply, thus obtaining a 2°C difference between day/night in the C3 inducing condition and an 8°C in the CAM inducing condition.

Knowledge on C3-CAM facultative carbon metabolism allowed us to understand the specificity of each pathway in order to shift plants with C3 physiology to CAM, taking advantage of their better behavior in facing environmental stress. Pineapple plants showing C3 metabolism had a better rooting capacity and a higher production of ABA, what can be associated with an adult phenotype appropriated for fruit production.

6.2 General conclusions and future perspectives

In vitro environment is associated with several stressful conditions such as low temperature, closed environment, culture media with high concentrations of plant growth regulators and abundance of nutrients such as sucrose, leading to mixotrophic metabolisms (Nguyen et al. 1999; Joyce et al. 2003; Roels et al. 2006; Dubuc and Desjardins 2007). When plants are shifted to *ex vitro* conditions abiotic stress is prone to occur, in close relation with high light intensity, low humidity and the change to autotrophic nutrition due to the absence of available nutrients. All these factors can affect plant growth and even survival in acclimatization contributing to the high risk of the whole micropropagation process (Grene 2002; Hazarika 2003; Puthur 2005; Read 2007). Van Huylenbroeck et al. (1998; 2000), Le et al. (2001) and Carvalho et al. (2006) described several metabolic changes when C3 plants were transferred from *in vitro* to *ex vitro* conditions.

The present work aimed to compare the acclimatization outcome C3, C4, C3-CAM-facultative plants after microprapagation in GM or TIB systems. As a whole the research allowed to draw three general conclusions: i) each species behaved according to its photosynthetic characteristics (C3, C4, C3-CAM-facultative); ii) the oxidative stress response and carbon metabolism were closely connected in the C3-CAM facultative species (pineapple) and iii) TIB system was more advantageous as compared to GM.

From the results reported and discussed two main future lines of research dealing with the acclimatization of *in vitro* plants can be proposed: i) Unravel the role of anti-oxidative stress response using as model the onset of abiotic stress; ii) Elucidating facultative carbon

metabolism in an attempt to contribute to the achievements of current international goals of plant breeding concerning the integration of C3, C4 and CAM physiologies, such as developing C4-rice.

6.3 References

Aragón C, Sánchez C, González-Olmedo J, Escalona M, Carvalho L, Amâncio S (2014) Comparison of plantain plantlets propagated in temporary immersion bioreactors and gelled medium during *in vitro* growth and acclimatization. Biol Plant 58:29-38.

Aragón C, Pascual P, González J, Escalona M, Carvalho L, Amâncio S (2013) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions: Proteomic and transcriptomic profiles. Plant Cell Rep 32:1807-1818.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2012) The physiology of *ex vitro* pineapple (*Ananas comosus* L. Merr. var MD-2) as CAM or C3 is regulated by the environmental conditions. Plant Cell Rep 31:757-769.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2010) *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor. Biol Plant 54:237-244.

Aragón C, Carvalho L, González J, Escalona M, Amâncio S (2009a) Sugarcane (*Saccharum* spp. Hybrid) propagated in headspace renovating systems shows autotrophic characteristics and develops improved anti-oxidative response. Tropical Plant Biol 2:38-50.

Aragón C, Escalona M, Rodriguez R, Cañal M, Capote I, Pina D, González J (2009b) Effect of sucrose, light, and carbon dioxide on plantain micropropagation in temporary immersion bioreactors. *In Vitro* Cell Dev Biol Plant 46:89-94.

Aragón C, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2006) Acclimatization of plantain "CEMSA ¾ (AAB)" plants propagated in Temporary Immersion System. The role of starch on the acclimatization process. INFOMUSA 1-2:32-35. [In Spanish].

Aragón C, Escalona M, Capote I, Pina D, Cejas I, Rodríguez R, Cañal M, Sandoval J, Roels S, Debergh P, González-Olmedo J (2005) Photosynthesis and carbon metabolism in Plantain (*Musa* AAB) growing in Temporary Immersion Bioreactor (TIB) and *ex vitro* acclimatization. *In Vitro* Cell Dev Biol Plant 41:550-554.

Buddendorf-Joosten J, Woltering E (1994) Components of the gaseous environment and their effects on plant growth and development *in vitro*. Plant Growth Reg 15:1-16.

Cabasson C, Alvard D, Dambier D, Ollitrault P, Teisson C (1997) Improved Citrus somatic embryo development by temporary immersion. Plant Cell Tiss Org Cult 50:33-37.

Carvalho L, Vilela J, Vidigal P, Mullineaux Ph, Amâncio S (2006) Activation of the ascorbate-glutathione cycles is an early response of micropropagated Vitis vinifera L. explants transferred to *ex vitro*. Int. J Plant Sci 167:759-770.

Dietz K (2011) Peroxiredoxins in plants and cyanobacteria. – Antioxid.Redox Sign 15:1129-1159.

Dubuc J, Desjardins Y (2007) Effect of autotrophic and mixotrophic tissue culture conditions on the expression of primary metabolism genes of tomato plantlets. Acta Hort 748:165-172.

Escalona M (2006) Temporary immersion beats traditional techniques on all fronts. Prophyta Annual. 48-49.

Escalona M, Lorenzo J, González B, Daquinta M, Borroto C, González J, Desjardins Y (1999) Pineapple micropropagation in temporary immersion systems. Plant Cell Rep 18:743-748.

Grene R (2002) Oxidative Stress and Acclimation Mechanisms in Plants. The Arabidopsis Book. American Society of Plant Biologists (eds) USA. pp.1-20.

Hanhineva K, Karenlampi S (2007) Production of transgenic strawberries by temporary immersion bioreactor system and verification by TAIL-PCR. BMC Biotechnol 7:11.

Hazarika B (2003) Acclimatization of tissue-cultured plants. Curr Sci 85:1704-1712.

Jackson M, Abbott A, Belcher A, Hall K, Butler R, Cameron J (1991) Ventilation in plant tissue cultures and effects of poor aeration on ethylene and carbon dioxide accumulation, oxygen depletion and explant development. Annal Bot 67:229-237.

Joyce S, Cassells A, Mohan S (2003) Stress and aberrant phenotypes in *in vitro* culture. Plant Cell Tiss Org Cult 74:103-121.

Le V, Samsom G, Desjardins Y (2001) Opposite effects of exogenous sucrose on growth, photosynthesis and carbon metabolism of *in vitro* plantlets of tomato (*L. esculentum* Mill.) grown under two levels of irradiance and CO_2 concentration. Plant Physiol 158:599-605.

Majada J, Tadeo F, Fal M, Sanchez-Tames R (2000) Impact of culture vessel ventilation on the anatomy and morphology of micropropagated carnation. Plant Cell Tiss Org Cult 63:207-214.

Nguyen Q, Kozai T (2001) Growth of *in vitro* banana (*Musa* spp.) shoots under photomixotrophic and photoautotrophic conditions. *In Vitro* Cell Dev Biol Plant 37:824-829.

Nguyen Q, Kozai T, Niu G, Van Nguyen U (1999) Photosynthetic characteristic of coffee (*Coffea arabusta*) plantlets *in vitro* in response to different CO_2 concentration and light intensities. Plant Cell Tiss Org Cult 55:133-139.

Nievola C, Kraus J, Freschi L, Souza B, Mercier H (2005) Temperature determines the occurrence of CAM or C3 photosynthesis in pineapple plantlets grown *in vitro. In Vitro* Cell Dev Biol Plant 41:832-837.

Puthur J (2005) Influence of light intensity on growth and crop productivity of *Vanilla planifolia* Andr. Gen. Appl. Plant Physiol 31:215-224.

Read P (2007) Micropropagation: Past, present and future. Acta Hort 748:17-28.

Rodríguez R, Aragón C, Escalona M, González-Olmedo J, Desjardins Y (2008) Carbon metabolism in leves of micropropagated sugarcane during acclimatization phase. *In Vitro* Cell Dev Biol Plant 44:533-539.

Roels S, Escalona M, Cejas I, Noceda C, Rodríguez R, Cañal M, Sandoval J, Debergh P (2005) Optimization of plantain (*Musa* AAB) micropropagation by temporary immersion system. Plant Cell Tiss Org Cult 82:57-66.

Roels S, Noceda C, Escalona M, Sandoval J, Cañal M, Rodríguez R, Debergh P (2006) The effect of headspace renewal in a Temporary Immersion Bioreactor on plantain (*Musa* AAB) shoots proliferation and quality. Plant Cell Tiss Org Cult 84:155-163.

Shu-Han Y, Der-Ming Y (2008) *In vitro* leaf anatomy, *ex vitro* photosynthetic behaviours and growth of *Calathea orbifolia* (Linden) Kennedy plants obtained from semisolid medium and temporary immersion systems. Plant Cell Tiss Org Cult 93:201-207.

Slesak I, Libik M, Karpinska B, Karpinski S, Miszalski Z (2007) The role of hydrogen peroxide in regulation of plant metabolism and cellular signalling in response to environmental stresses. Acta Biochim Pol 54:39-50.

Tanaka Y, Sano T, Tamaoki M, Nakajima N, Kondo N, Hasezawa S (2005) Ethylene Inhibits Abscisic Acid-Induced Stomatal Closure in Arabidopsis. Plant Physiol 138:2337-2343.

Teisson C, Alvard D (1995) A new concept of plant *in vitro* cultivation liquid medium: temporary immersion. In: Terzi M, Celia R, Falavigna A (eds) Current issues in plant molecular and cellular biology. Kluwer Academic Publishers, Dordrecht. pp.105-110.

Van Huylenbroeck J, Piqueras A, Deberg P (2000) The evolution of photosynthetic capacity and the anti-oxidant enzymatic system during acclimatization of micropropagated Calathea plants. Plant Sci 135:59-66.

Van Huylenbroeck J, Piqueras A, Debergh P (1998) Photosynthesis and carbon metabolism in leaves formed prior and during *ex vitro* acclimatization of micropropagated plants. Plant Sci 134:21-30.

Watt M (2012) The status of temporary immersion system (TIS) technology for plant micropropagation. African J Biotechnol 11:14025-14035.

Woodrow L, Thompson R, Grodzinski B (1988) Effects of ethylene on photosynthesis and partitioning in tomato, *L. esculentum* Mill. J Exp Bot 39:667-684.

Ziv M (2005) Simple bioreactors for mass propagation of plantlets. Plant Cell Tiss Org Cult 81:277-285.