# *Ex vitro* acclimatization of plantain plantlets micropropagated in temporary immersion bioreactor

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## Abstract

Plantain (*Musa* ABB CEMSA 3/4) plantlets were micropropagated in temporary immersion bioreactors (TIB) or in gelled medium (GM). After *ex vitro* transfer ROS accumulation was determined by infiltrating leaves with nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB). Stomatal cells were more stained with NBT and DAB in GM plants than in TIB plants, but the difference disappeared at the end of acclimatization. At the end of the *in vitro* phase, GM plantlets showed higher activities of ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR), while activities of catalase (CAT), superoxide dismutase (SOD) and glutathione transferase (GT) were higher in TIB grown plantlets. At the end of acclimatization GT, SOD, CAT and MDHAR stabilized at low values of activity in plantlets derived from both treatments. Concerning the correspondent genes, GM plantlets showed higher expression of all transcripts with the exception of *CuZnSOD*. The immunobloting of peroxiredoxins (PRXs) showed that chloroplast-located PRXs were expressed at higher levels in TIB plantlets, some showing polymerization. In conclusion, TIB grown plantlets had an improved anti-oxidative response when compared with GM.

*Additional key words*: ascorbate-glutathione cycle, *ex vitro* growth, oxidative stress, TIB culture.

## Introduction

The commercial application of micropropagation for a broader range of species would only take place if new technologies were available to automate certain procedures and if acclimatization protocols were improved (Kitto 1997). The use of temporary immersion bioreactors (TIB) has proven to be an alternative to standard gelled medium (GM). TIB is an efficient propagation technique, that improves plantlet morphology and physiology and it represents a low cost option for the massive production of plantlets for the tropical agricultural industry (*e.g.* sugarcane, plantain, pineapple) (Lorenzo *et al.* 2001, Escalona *et al.* 2003, Aragón *et al.* 2005).

Evidence of oxidative stress during micropropagation has often been reported (Van Huylenbroeck *et al.* 2000, Carvalho *et al.* 2001). Photosynthesis can be down regulated either due to a lack of $CO_2$ or ethylene production in closed vessels or due to the feedback inhibition of Calvin cycle enzymes by the sucrose in the medium (Jackson *et al.* 1991, Tanaka *et al.* 2005). Moreover, the transition of the plantlets to an *ex vitro* conditions under high irradiance can lead to the production of reactive oxygen species (ROS; for review see Baťková *et al.* 2008). However, comparisons of GM with TIB have so far focused only on carbon metabolism (*e.g.* Aragon *et al.* 2005).

The aim of this paper is to show the differences in response to oxidative stress during acclimatization in plantain plantlets propagated on GM or in TIB by monitoring ROS accumulation, the activities of antioxidative enzymes and expression of corresponding genes.

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*Abbreviations*: APX - ascorbate peroxidase (EC 1.11.1.11); BA - 6-benzylaminopurine; CAT - catalase (EC 1.11.1.6); DAB - 3,3'-diaminobenzidine; DHAR - dehydroascorbate reductase (EC 1.8.5.1); GM - gelled medium; GR - glutathione reductase (EC 1.6.4.2); GT - glutathione transferase (EC 2.5.1.18); MDHAR - monodehydroascorbate reductase (EC 1.6.5.4); MS - Murashige and Skoog medium; NBT - nitroblue tetrazolium; PPFD - photosynthetic photon flux density; PRX - peroxiredoxin; ROS - reactive oxygen species; SOD - superoxide dismutase (EC 1.15.1.1); TIB - temporary immersion bioreactor.
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## Materials and methods

**Plants:** Plantain plantlets (*Musa* AAB, CEMSA ¾) were subjected to three sub-culture cycles of 21 d each, in culture media containing Murashige and Skoog (1962) salts and vitamins, supplemented with 30 g $dm^{-3}$ sucrose (m/v) and 1.33 µM 6-benzylaminopurine (BA). Two different techniques, GM (gelled medium) and TIB (temporary immersion bioreactors) were applied for the last subculture (21 d). In both techniques, the media used consisted of MS salts and vitamins supplemented with 30 g $dm^{-3}$ sucrose and without plant growth regulators. In GM, plantlets were placed in *Magenta* vessels (*Sigma-Aldrich*, St. Louis, MO, USA) containing 50 $cm^3$ of media gelled with 2.0 g $dm^{-3}$ *Gelrite* (5 shoots per vessel). TIB was performed in containers (*Schott*, Mainz, Germany) with 200 $cm^3$ of media and 5 shoots per bioreactor (Escalona *et al.* 2003). Shoots were immersed for 4 min every 3 h. Photosynthetic photon flux density (PPFD) was 45 ± 5 µmol $m^{-2}$ $s^{-1}$ and the photoperiod was 16 h in both propagation techniques.

Microcuttings produced under TIB and GM were transplanted to pots containing a sterilised mixture of moistened peat and *Perlite* (1:1, v/v) and placed in glass chambers (*500E*, *Aralab*, Porto Salvo, Portugal). PPFD was 200 ± 10 µmol $m^{-2}$ $s^{-1}$ and the photoperiod 16 h. The initial value of air humidity 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 during the dark period.

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 at days 14 and 21. Samples were collected in the middle of the light period.

Stomatal index, SI [%], was calculated as SF/(SF+EF) × 100 where SF and EF are number of stomata and number of epidermal cells, respectively, per unit leaf area of the first fully expanded leaf (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 were detached and incubated in 5 mM 3,3' diaminobenzidine (DAB) at pH 3.8 to detect $H_2O_2$ or in 6 mM nitroblue tetrazolium (NBT) to detect $O_2^{\cdot-}$.

**Enzyme activities:** The extracts for measuring enzyme activities were prepared at 4 ºC from 0.5 g of frozen leaf tissue ground with 50 % (m/m) polyvinylpolypyrrolidone (PVPP). The extraction buffer was 0.35 M Tris-HCl (pH 8) supplemented with 20 mM EDTA; 11 mM sodium diethyldithiocarbamate (DETC), 15 mM cystein (Jacobs *et al.* 1999), and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Extracts were centrifuged at 27 000 *g* for 10 min at 4 ºC and the supernatants, desalted through *PD-10* columns (*GE Healthcare Lifesciences*, Little Chalfont, UK), were used for all the determinations. Total protein was quantified by the method of Bradford (1976) using a commercial kit (*Bio-Rad*, Hercules, CA, USA).

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 $cm^3$ 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 absorbance ($A_{265}$) due to ascorbate formation (coefficient of absorbance = 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 $cm^3$. 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 by Arrigoni *et al.* (1981), 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*) and enzyme extract (*ca* 14 µg protein) in a final volume of 1 $cm^3$ at 25 ºC.

Glutathione reductase (GR; EC 1.6.4.2) was assayed by the decrease in $A_{340}$ due to NADPH oxidation (coefficient of absorbance = 6.22 $mM^{-1}$ $cm^{-1}$), as described by Schaedle and Bassham (1977), in a reaction mixture 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 $cm^3$ 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 $cm^3$ 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 by Drotar *et al.* (1985), in 100 mM potassium phosphate-KOH, pH 7.0, containing 2 mM CDNB, 2 mM GSH and enzyme source (*ca.* 14 - 35 µg protein).

Reduction of $H_2O_2$ by peroxiredoxins (PRX) 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 peroxiredoxin, 10 mM DTT, and 100 µM $H_2O_2$. The reaction was initiated with $H_2O_2$ and stopped with trichloroacetic acid. After adding 10 mM $Fe(NH_4)_2(SO_4)_2$ and 2.5 M potassium thiocyanate (KSCN), the $A_{480}$ was measured to quantify the $H_2O_2$ contents of the solution, and $H_2O_2$ reduction rates were calculated.

**Native PAGE and gel activity staining:** Isoforms of CAT, SOD, APX, and GR were separated in nondenaturing polyacrylamide gels by the procedure of Laemmli (1970). Equal amounts of protein extracts (25 µg) 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 isoforms, 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 % tetramethylethylene diamine (TEMED) in darkness for 30 min before transfer to PPFD of 200 µmol $m^{-2}$ $s^{-1}$ to visualize SOD isoform 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 % (m/v) potassium ferricyanide and 1 % (m/v) ferric chloride.

Isoforms 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 the 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-dichloro-phenolindophenol, 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 isoenzyme activities was determined using the software *Quantity One* (*Bio-Rad*, Hercules, CA, USA).

**Peroxiredoxin immunoblotting:** Polyclonal antibodies against cytosolic type II Prx C, chloroplast-located type II Prx E, chloroplast-located 2-cys Prx, chloroplast-located Prx Q and mitochondria-located type II Prx F, kindly given by Prof. K.-J. Dietz (Bielefeld University, GE, USA). 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).

**RNA isolation and real-time PCR:** Total RNA from leaves was extracted by adapting the method described by Chang *et al.* (1993). 2 % (m/v) cetyltrimethylammonium bromide (CTAB) was complemented with 2 % PVPP (m/v), 100 mM Trizma-HCl, 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 $mm^3$ β-mercaptoethanol. Tissues were reduced to powder in liquid $N_2$ and 20 $cm^3$ extraction buffer were added. The same volume of chlorophorm + isoamyl 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 10 M LiCl (v/v) was added. The sample was incubated at 0 ºC overnight and then centrifuged at 12 000 *g* for 20 min at 4 ºC. The pellet was recovered and 1.5 $cm^3$ 10 mM Trizma-HCl pH 8.0, complemented with 1 mM $Na_2EDTA$, 1 M NaCl and 0.5 % SDS (m/v), previously heated to 37 ºC was added. The same volume of chlorophorm + isoamyl 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, USA) and reverse transcribed using random hexamers and *Superscript II* RNase H-reverse transcriptase (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's recommendations.

Primer pairs used for amplification of all the genes studied are presented in Table 1. The genomic sequences for plantain available in the GeneBank database were retrieved and the program *Beacon Designer 6* (*Premier Biosoft*) was used to design the primers pairs. Real-time RT-PCR reactions contained 20 $mm^3$ reaction mixture composed of cDNA, 0.5 µM gene-specific primers and master mix *iQ SYBR Green Supermix* (*Bio-Rad*) using an *iQ5* real time PCR (*Bio-Rad*). 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 30 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 dimers. Further, RT-PCR products were resolved on 2 % (m/v) agarose gels and run at 4 V $cm^{-1}$ in Tris-acetate-EDTA buffer (TAE) with a 50-bp DNA-standard ladder (*Invitrogen*) 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 10 and 27. 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*. 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.

**Statistical analysis:** All experiments were performed three times with 3 repetitions. Statistical analyses were carried out using *SPSS version 12*. All situations were analyzed using non-parametric analysis (Kruskall-Wallis or Mann-Whitney), both at 5 % significance level.

Table 1. Real-time PCR primers used for evaluation of mRNA levels of the enzymes studied. Accession numbers correspond to sequences available in the *GeneBank* database (http://www.ncbi.nlm.nih.gov/sites/entrez) and in the *Global Musa Genomics Consortium* database (http://www.musagenomics.org/).

| Protein | Accession No. | Primer name | Primer sequence |
|---|---|---|---|
| Actin | EF672732.1 | Ma-actin | 5'-TTGATTCTGGTGATGGTGTGAGCC-3'<br>3´-CTACCACTGCTGAACGGGAAATTG-5´ |
| MnSOD | AF510071.1 | Ma-MSOD | 5'-ACGTCACCAACTACAACAATGCC-3'<br>3´-GCCCAACCAAGTGTGCTGTG-5´ |
| Cu-ZnSOD | DQ866814 | Ma-CZSOD | 5'-TGGCAACGGGAGGCAATCTG-3'<br>3´-AGGAGAGAATGAGGGAGGAGAGG-5´ |
| CAT | EU139298.1 | Ma-CAT | 5'-CGCTCACCACAACAATCACTACG-3'<br>3´-AATCACATTCTTCTCACGCTTGCC-5´ |
| APX | AF146521 | Ma-APX | 5'-GTTCGGACCACCTCAGGGATG-3'<br>3´-TCGTCTTTCTCGCTGCTCAGG-5´ |

## Results

The histological analyses of plantain stomatal guard cells stained with NBT or DAB evidenced brown polymerization products, resulting from DAB reacting with $H_2O_2$ in the presence of peroxidases and purple formazan deposits, which result from the reaction of NBT with $O_2^-$ (Fig. 1). The stomatal index and staining pattern was analysed in the adaxial and abaxial side of the leaves (Fig. 2A,B). The adaxial side showed the highest stomatal index in *in vitro* GM propagated plantlets, however, at the end of acclimatization the difference between treatments was not observed. No marked changes were observed in stomatal index on the abaxial side. The staining pattern was mostly similar in GM and TIB propagated plantlets with the exception of high staining percentage in guard cell on the abaxial side of *in vitro* GM plantlets.

The activities of the ascorbate-glutathione cycle enzymes APX, DHAR, MDHAR and GR were higher in GM plantlets on day 0 while TIB plantlets evidenced higher SOD, CAT, PRX and GT activities (Fig. 3). With the exception of GR and PRX, all the enzymes studied reached their maximum activity between days 3 and 5 in GM propagated plantlets, decreasing to minimum values on day 7. Conversely, in TIB plantlets only GR and APX activities increased in the first 3 d. On day 21, GT, SOD, CAT and MDHAR stabilized at low activities in plantlets grown under both treatments. Two exceptions were APX and DHAR, showing rather high activity in GM and TIB, respectively.

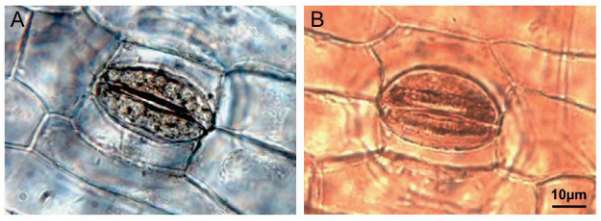


Fig. 1. Imaging of ROS accumulation in stomatal cells of plantain plantlets stained with NBT for superoxide radical (*A*) and with DAB for hydrogen peroxide (*B*).

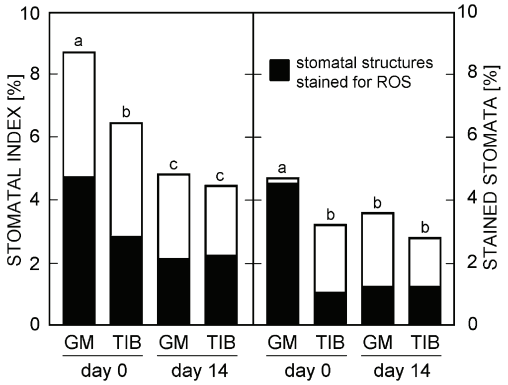


Fig. 2. Stomatal index and percentage of stained stomata in the adaxial (*on the left*) and abaxial (*on the right*) side of the leaves prior to transfer to *ex vitro* (day 0) and after acclimatization (day 14). Values indicated by different letters are significantly different at 5 % using C-Dunnett multiple range test ($n$ = 9).

Two SOD isoenzymes, SOD A and SOD B, were detected upon treatment with KCN and $H_2O_2$, confirming that they are Mn isoforms. Both were present at higher levels in GM than in TIB on day 0, decreasing slightly with time. CAT showed one isoenzyme with higher activity in TIB, and it was unaltered with time. It was possible to detect two isoenzymes of APX; APX-A, whose activity decreased with time while the activity of APX-B increased slightly in both treatments. The two GR isoenzymes detected showed the highest activity in GM on day 0 (Table 2).

Western blot analysis of five peroxiredoxins allowed the characterization of the response in different cell compartments (Fig. 4). From three PRX in chloroplasts Type II E was the most abundant in both treatments with maximum on day 7. Also, PRX Q showed maximum values on day 7 in both treatments while 2-cys PRX was hardly detected in GM grown plantlets and showed the highest quantity on day 7 in TIB. Cytosol located type II PRX C was hardly detected during acclimatization. In fact, in GM grown plantlets this PRX was not even

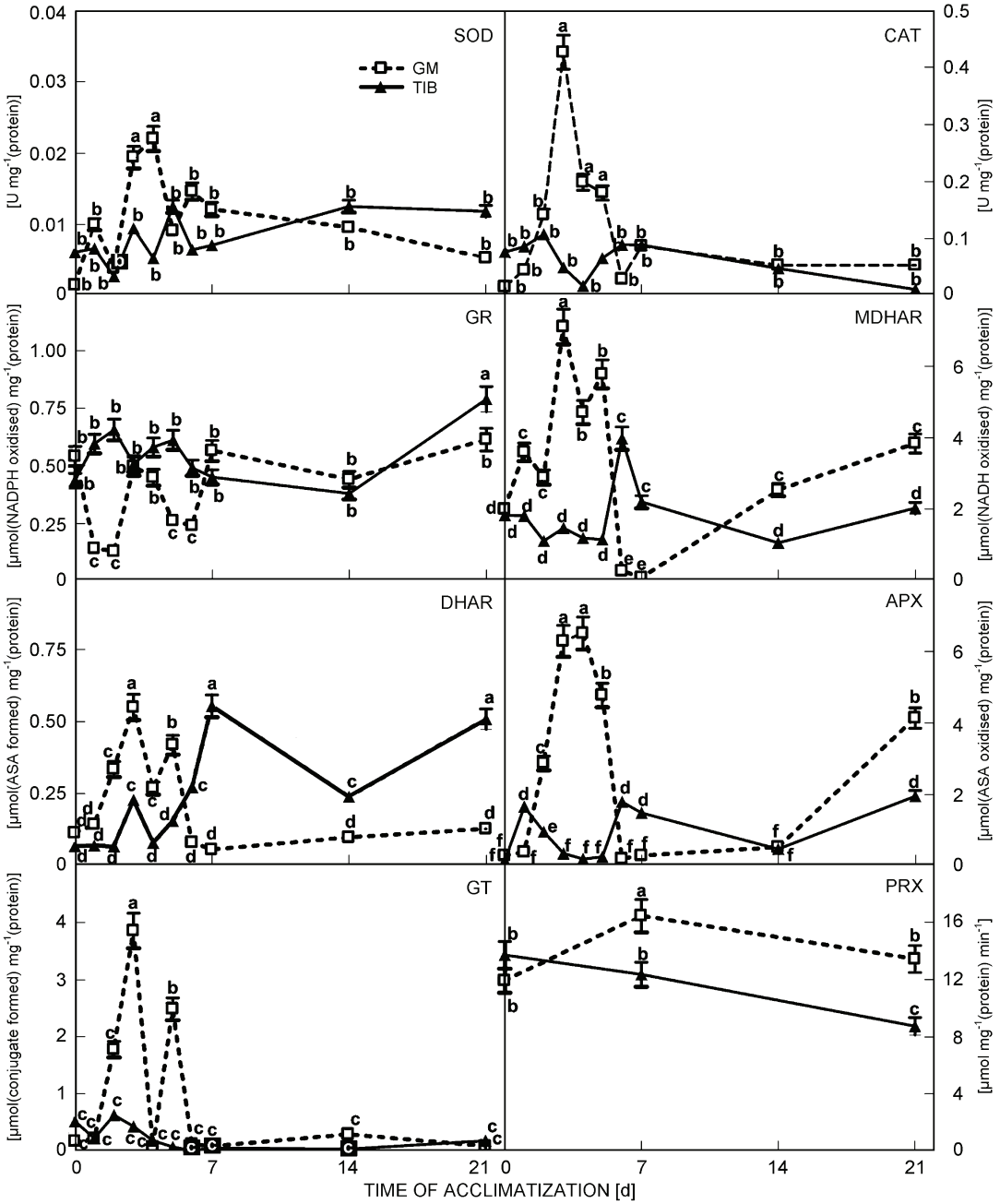


Fig. 3. Total soluble enzyme activities in leaves of plantain plantlets propagated under both systems (GM and TIB). Different letters are used when values differ significantly ($P < 0.05$; Mann-Whitney test, $n = 9$).

Table 2. Activities of SOD, CAT, APX and GR isoenzymes in leaves of acclimatizing plantain plantlets propagated under GM and TIB were quantified in relation to the value for GM at day 0 (100 % activity). Total protein extracts were subjected to native PAGE followed by activity staining for the four enzymes. Discrimination between SOD isoforms was revealed by inhibition with $H_2O_2$ and KCN.

| | GM | | | TIB | | |
|---|---|---|---|---|---|---|
| | 0 | 7 | 21 | 0 | 7 | 21 |
| SOD-A | 100 | 98 | 96 | 94 | 93 | 94 |
| SOD-B | 100 | 77 | 75 | 72 | 71 | 70 |
| CAT | 100 | 108 | 102 | 116 | 116 | 117 |
| APX-A | 100 | 93 | 96 | 94 | 92 | 89 |
| APX-B | 100 | 87 | 103 | 98 | 105 | 109 |
| GR-A | 100 | 97 | 87 | 84 | 83 | 78 |
| GR-B | 100 | 96 | 89 | 87 | 85 | 81 |

detected on day 0. Conversely, type II PRX F, located in mitochondria was very abundant in both treatments from day 0 until the end of acclimatization.

The expression of key genes of the anti-oxidative defence system was monitored using real time RT-PCR (Fig. 5). The expression of all genes was higher in GM plantlets all along the experiment with the exception of *CuZnSOD* which only increased significantly on day 21. *CAT* and *MnSOD* showed peaks of expression on day 3 while the expression of *APX* was constantly high. In TIB plantlets, both *APX* and *MnSOD* showed the lowest values on day 7.

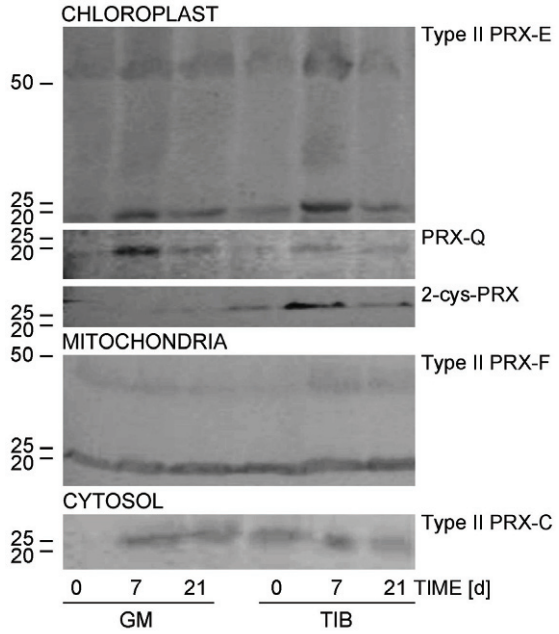


Fig. 4. Western blots of five different peroxiredoxins localized in three cellular compartments: chloroplast (2-cys Prx; type II Prx E; Prx Q), mitochondria (type II Prx F) and cytosol (type II Prx C). Protein samples were subjected to SDS-page, transferred to a nitro-cellulose membrane and probed with the specific antibodies. Western blots were performed in leaves of plantain plantlets propagated under both systems (GM and TIB) on day 0 of transfer to acclimatization and after 7 and 21 d of *ex vitro* growth.

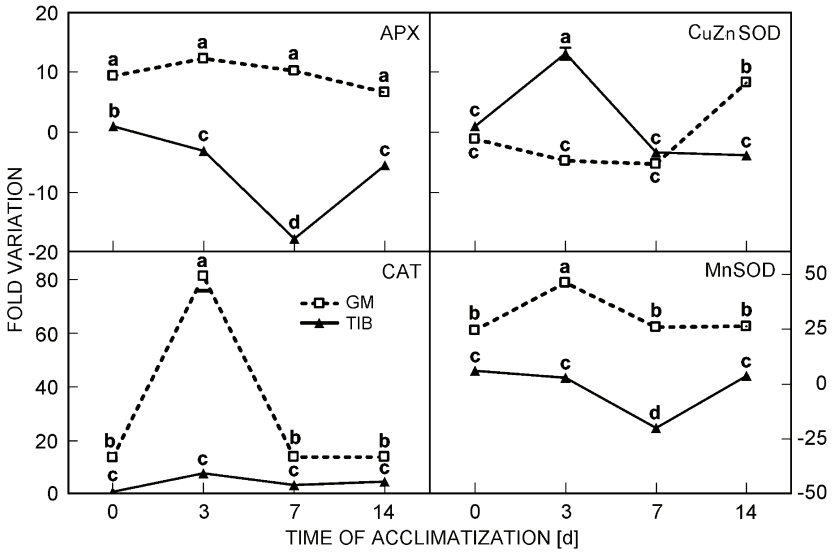


Fig. 5. Expression levels of genes of the anti-oxidative system. Quantification of mRNA levels was performed on day 0 of each propagation method and after 3, 7 and 14 d of *ex vitro* growth. mRNA was isolated from leaves, converted to cDNA, and subjected to real-time PCR. Relative amounts were calculated and normalized with respect to plantain *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.

## Discussion

Plantain plantlets propagated in TIB had a better capacity to sustain the stress imposed upon transfer to *ex vitro* conditions (Aragon *et al.* 2005). The present work aims to get an insight into the cellular features that support the quality of TIB plantlets at the level of anti-oxidative stress response. For that purpose *in situ* ROS deposition, the activity of anti-oxidative stress enzymes and the expression of the correspondent genes were compared in *ex vitro* plantain plantlets propagated *in vitro* through GM and TIB.

DAB staining of the stomatal guard cells revealed the deposition of $H_2O_2$ associated with organelles engaged in electron transport (Desikan *et al.* 2004, Kwak *et al.* 2006, Vilela *et al.* 2007). The similar distribution of stomata staining pattern of *in vitro* TIB plantlets as compared to those 14 d after transfer to *ex vitro* conditions demonstrated that plantain grown in this system developed leaves morphologically close to autotrophic leaves formed *ex vitro*.

Most plants display an efficient defence system able to scavenge ROS, keeping their concentration at levels adjusted to cellular signaling (Mullineaux *et al.* 2006, Ślesak *et al.* 2007). SOD, CAT and the ascorbate-glutathione cycle enzymes are the key players of that defence system (Baťková *et al.* 2008). It was possible to identify specific characteristics of the anti-oxidant efficiency in GM and TIB plantlets during *ex vitro* growth. The primary response to oxidative stress is provided by SOD, the enzyme that scavenges $O_2^-$ into $H_2O_2$ (Alscher *et al*. 2002, Shao *et al.* 2008), which was transiently activated in GM plantlets. During the first week *ex vitro* two features were observed in GM plantlets: the increased activity of ascorbate-glutathione cycle enzymes APX, DHAR and MDHAR, and the activation of PRXs, acting against a broader spectrum of peroxides than APX (Dietz 2003). We can infer that these plantlets were subjected to higher levels of stress, and this response must be related to the elimination of excess $H_2O_2$, to be kept at levels adjusted for signalling. The high activity of GT during the first week can help repair cell structures damaged by ROS (Wangwattana *et al*. 2008). As a whole, the up-regulation of the anti-oxidant system during the first 7 d after *ex vitro* transfer suggest that GM plantlets are less suited to cope with the stress at the moment of transfer than TIB plantlets. However, at the end of acclimatization antioxidant system in GM and TIB plantlets behaved similarly.

Transcript levels of *MnSOD*, *APX* and *CAT* showed a stronger up-regulation in GM than in TIB plantlets. Two Mn isoforms of SOD were identified while three MnSODs were described in *Lycopersicon esculentum* propagated under GM. In *Nicotiana benthamiana* (Carvalho *et al.* 2008) and *Vitis vinifera* (Carvalho *et al.* 2006) also FeSODs were observed. Although total CAT activity was low under both propagation techniques, one isoform with rather high activity was observed, as in *L. esculentum* and *V. vinifera*, while *N. benthamiana* displayed two isoforms (Carvalho *et al.* 2006, 2008). APX revealed two isoforms, while in *L. esculentum* and *V. vinifera* three isoforms were detected (Carvalho *et al.* 2006, 2008). The heterogeneity of the isoenzyme set present in each plant species, influences the effectiveness of the response mechanism to the damage caused by the excess of ROS, due to different substrate affinities and cellular localizations.

Immunoblotting of PRXs revealed specific band patterns. Chloroplast isoforms, more abundant in TIB plantlets, could play the lead role in the better adjustment of these plantlets, *e.g*. 2-Cys-PRX might early eliminate $H_2O_2$ (Konig *et al*. 2002). The polymerization strategy of Type II PRX-E on day 7 is a response to stress conditions (Konig *et al.* 2002). Mitochondria Type II PRX-F showed similar patterns under both propagation techniques, confirming that the major differences in PRX metabolism occur in the chloroplasts. The cytosol PRX II-C was present in TIB plantlets from day 0 while in GM it was only detected after some days after *ex vitro* transfer. This again suggests that TIB plantlets are better prepared to withstand *ex vitro* conditions. *A. thaliana* revealed very low levels of PRX II-C mRNA under optimal conditions, but increased under high irradiance or salinity stress (Dietz 2003).

*In vitro* propagated banana and plantain have been described as displaying a good anti-oxidant capacity (Mokbel and Hashinaga 2005). The present study elucidates 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.

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