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Sugarcane (Saccharum sp. Hybrid) Propagated in Headspace Renovating Systems Shows Autotrophic Characteristics and Develops Improved Anti-oxidative Response

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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

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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 [2, 15, 31]. 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 (*Sacharum* sp.), 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 [3]. 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 [41], but not in what concerns the oxidative stress associated with micropropagation techniques. Usually, the headspaces accumulate several volatile compounds that can cause oxidative stress [42]. Leaf morphology, expansion and stomata functionality are also affected by the presence of ethylene and nitric oxide associated with abscisic acid production [24, 46]. 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 [7; 47]. 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 [2, 15]. 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 [20].

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 [37]. 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 [22].

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.

Methods

Plant Material, *In Vitro* and *Ex Vitro* Culture

Sugarcane plantlets (*Sacharum* sp. hybrid) clone C91-301 were subjected to three sub-culture cycles of 21 days each in a culture medium containing Murashige and Skoog [36] 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 [15]. Shoots were immersed for 4 min every 3 h. PPFD was 45±5 mmol m^{-2} s^{-1} and the photoperiod was 16/8 h 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 F18 W/GRO and, at plant level, photon flux density (PPFD) was 200± 10 μmol m^{-2} s^{-1} and the photoperiod 16/8 h. 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°C during the light and 22± 1°C 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.

Histology and Image Analysis

Stomatal index (%) was calculated as (SF/(SF+EF)) × 100 where SF and EF are respectively de number of stomata and epidermal cells per unit leaf area of the first leaf fully expanded [5]. 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. [19]. 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°C.

Extraction for Enzyme Activity Assays

The extracts for measuring enzyme activities were prepared at 4°C 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, and 0.2 mM PMSF. Extracts were centrifuged at 27,000 *g* for 10 min at 4°C 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 [4] using a commercial kit (Bio-Rad, Hercules, CA).

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°C), according to Leegood [29]. 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. [6]. PEPC activity was measured according to the method of Van Quy et al. [48]

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°C using a modification of the method of Hossain and Asada [23]. 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. [10] 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 [23] 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°C.

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 [44], 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 [34] 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 [14], in 100 mM potassium phosphate-KOH, pH 7.0, containig 2 mM CDNB, 2 mM GSH and enzyme source (ca 14-35 µg protein).

Native PAGE and Gel Activity Staining

Native Polyacrylamide Gel and Gel Activity Staining Isoforms of CAT, SOD, APX, and GR were separated in nondenaturing polyacrylamide gels by the procedure of Laemmli [28]. 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. [40]. 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% tetramethyl ethylene diamine (TEMED) in darkness for 30 min. before illumination to visualize SOD isoforms or bands [12].

To visualize the CAT profile, gels were stained by the procedure of Anderson et al. [1]. 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.

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 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 isoenzyme activities was determined using the software Quantity One (Bio-Rad, Hercules, CA).

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), 0.3–3 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 2-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. [16].

RNA Isolation and cDNA Preparation for Real-Time PCR

Total RNA from leaves was extracted by adapting the method described by Chang et al. [9]. 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°C 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:isoamyl alcohol 24:1 was then added. This step was followed by a centrifugation at 12,000 *g* for 30 min at 20°C and repeated once. The aqueous phase was transferred to a new tube, and a 1/4 LiCl 10 M (V/V) was added. The sample was incubated at 0°C overnight and after centrifugation at 12,000 *g* for 20 min. at 4°C. The pellet was recovered and 1.5 mL of SSTE buffer (Trizma-HCl 10 mM, 1 mM Na_2EDTA, 1 M NaCl; 0.5 % SDS (w/v), pH 8.0.) previously heated to 37°C 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°C and repeated once. Ethanol 100% (2.5 volumes) was added and the samples were incubated for 1 h at 80°C 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.

Real-Time PCR and Quantification of mRNA Levels

Primer pairs used for amplification of all the genes studied are presented in Table 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). 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°C, 3 min.; then 40 cycles at 95°C 15 s (denaturation), 57°C 30 s (annealing), 72°C 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.

Table 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-R	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′

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.

Statistical Analysis of the Results

All experiments were performed three times with three repetitions each. All statistical analyses were carried out using SPSS version 12 [39]. All situations were analysed using non-parametric analyses, either Kruskall-Wallis H. and C-Dunnett or Mann Whitney, both at 5 % significance level.

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.

Histological Analyses and Imaging of ROS

The histological analyses of leaf structures (stomata and trichomes) are shown in Figs. 1 and 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. 2). Cells surrounding the stomata guard cells were stained for both ROS (Fig. 1). Trichomes showed a unique pattern of staining (Fig. 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. 2 shows total and stained stomatal indexes (Fig. 2a) and total and stained trichome indexes (Fig. 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.

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. On day 0 GM plantlets showed significantly higher activities of MDHAR and APX, TIB conditions were

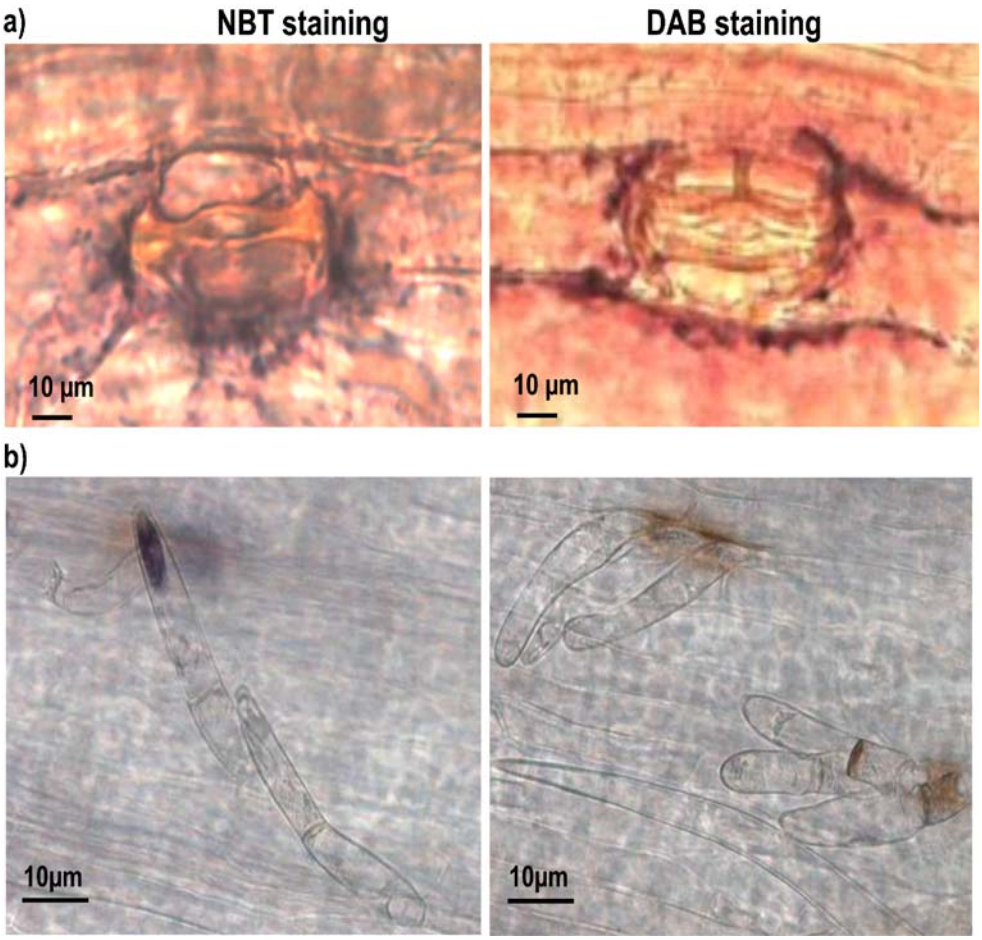

Fig. 1 Imaging of ROS accumulation in stomata (**a**) and trichomes (**b**) of sugarcane plantlets stained with NBT for superoxide radical visualization (left) and with DAB for hydrogen peroxide visualization (right). Scale bars are represented in each image

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 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 isoenzymes were constitutive (SOD-A and B) and showed constant activity levels in both propagation methods and through time while SOD-C was present on

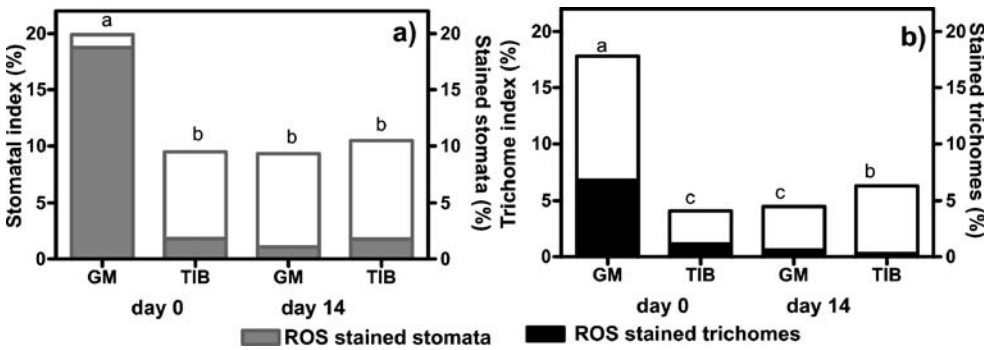

Fig. 2 Coloured stomata (**a**) and trichome (**b**) index in GM and TIB propagated plantlets prior to transfer to *ex vitro* (day 0) and after acclimatization (day 14). Values indicated by different letters are significantly different at 5 % level by C-Dunnett Multiple Range Test (n=9)

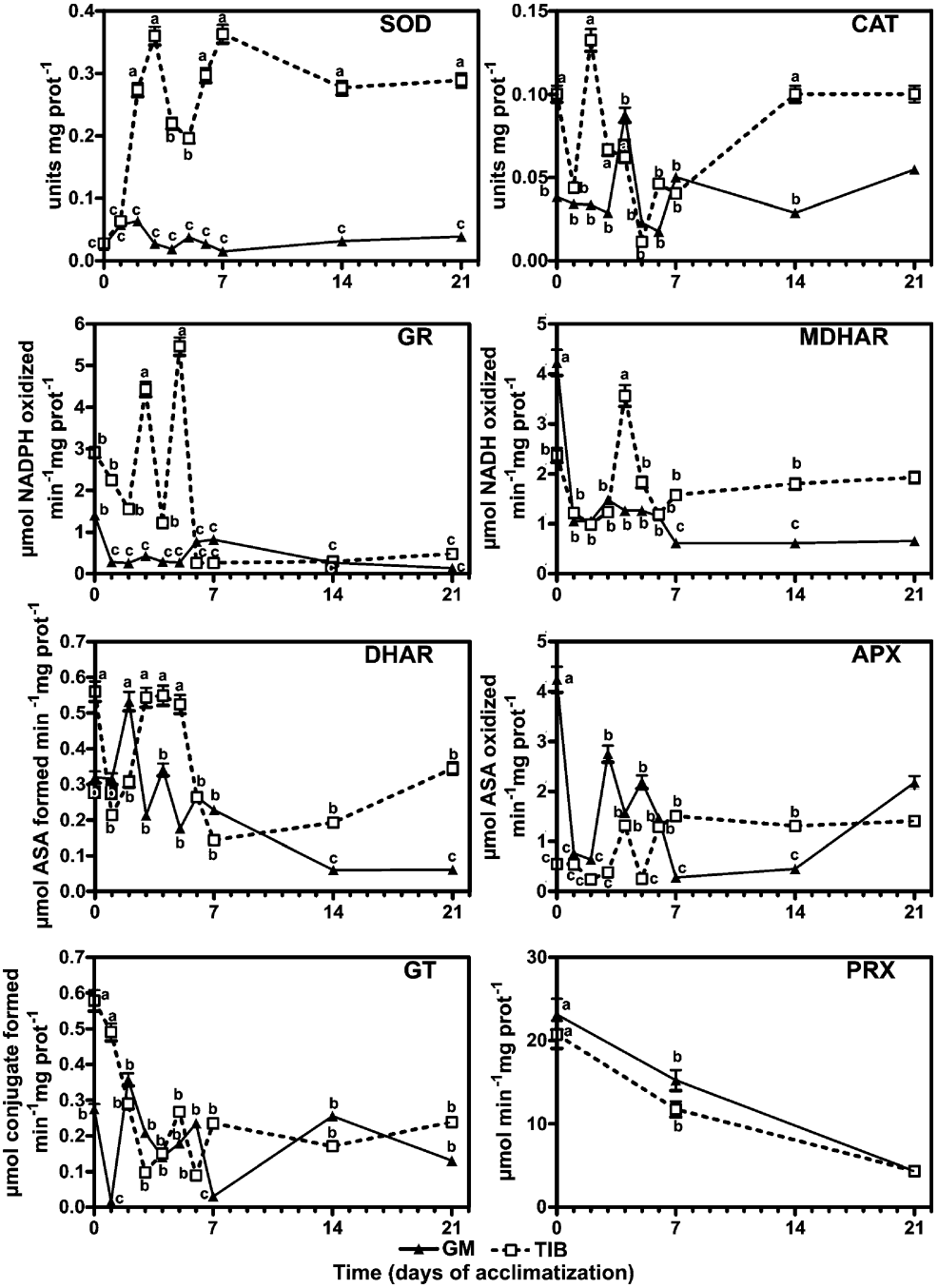

Fig. 3 Total soluble enzyme activities in leaves of sugarcane plantlets propagated under GM and TIB at the moment of transfer (day 0) and during acclimatization. Activities are expressed as follows: CAT, SOD, units per milligram of protein; APX, micromoles of AsA oxidized per minute per milligram of 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

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. APX in gel activity revealed two isoenzymes, 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 isoenzymes were detected, respectively of 55, 54, 46 and 45 kDa (GR-A through D; Table 2 and Fig. 4). To elucidate whether GR isoenzymes are related to a possible distinction of isoforms 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). 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

Table 2 SOD, CAT, APX and GR isoenzyme 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)

	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

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. Quantification was performed using the software Quantity One (Bio-Rad, Hercules, CA)

et al. 2000). RuBisCO activity in the bundle sheath cells was 3.5 fold higher than in the mesophyl (Table 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). 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 isoform 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) follow an equivalent trend; in TIB, the decrease in total activity from day 0 to day 21 (Fig. 3) is not apparent in Fig. 4.

Detection of Different Peroxiredoxins Using Specific Antibodies

The immunodetection of five different Prx (Fig. 5) revealed the response of Prx isoenzymes 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.

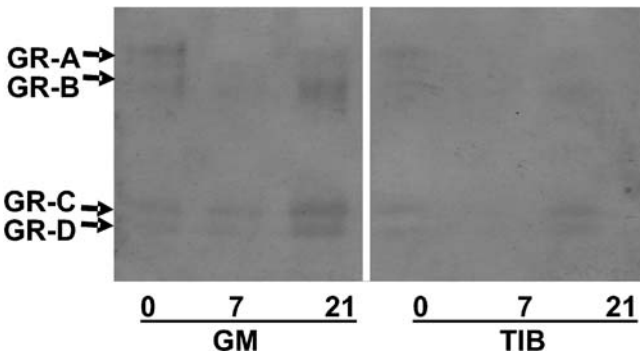

Fig. 4 GR isoenzyme in gel activity in leaves of acclimatizing sugarcane plantlets propagated under GM and TIB. Total protein extracts were subjected to native PAGE followed by activity staining

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. 6) with primer design based on sugarcane sequences available on NCBI (http://www.ncbi.nlm.nih.gov/sites/entrez). 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.

Table 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

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 (Fig. 1) 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 (Fig. 2). 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 [32], 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 [49]. Later on, normal regulation of stomatal differentiation and functioning can proceed following the development of roots, new buds and the increase in ABA content [49], and DAB staining decreases, as was the case in GM propagated sugarcane.

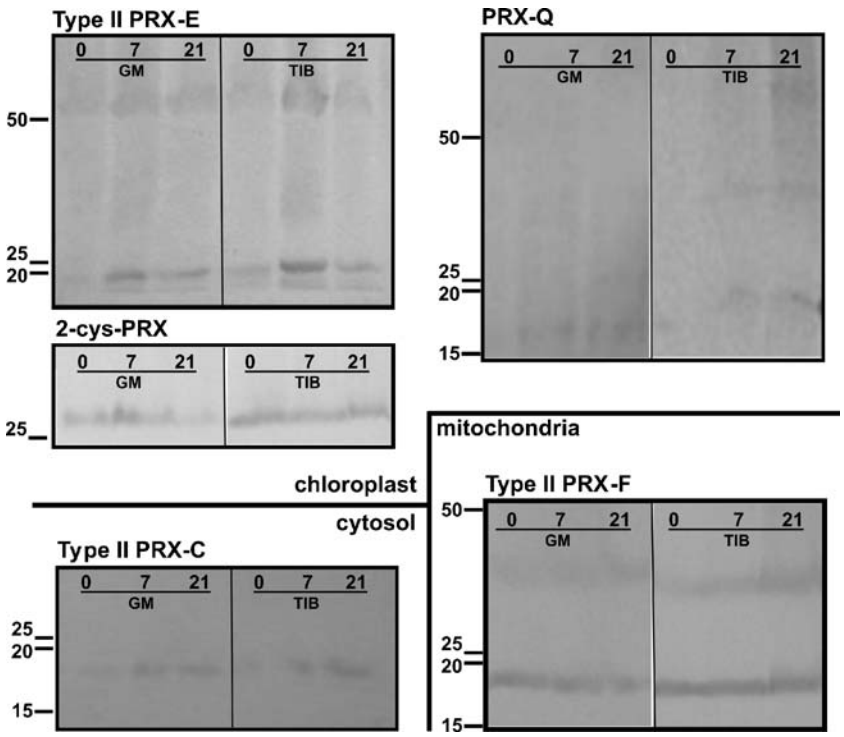

Fig. 5 Western blots of five different peroxiredoxins localized in three cellular compartments: chloroplast (2-cys Prx A; type II Prx E; Prx Q), cytosol (type II Prx C) and mitochondria (type II Prx F). Protein samples were subjected to SDS-page, transferred to a nitrocellulose membrane and probed with 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)

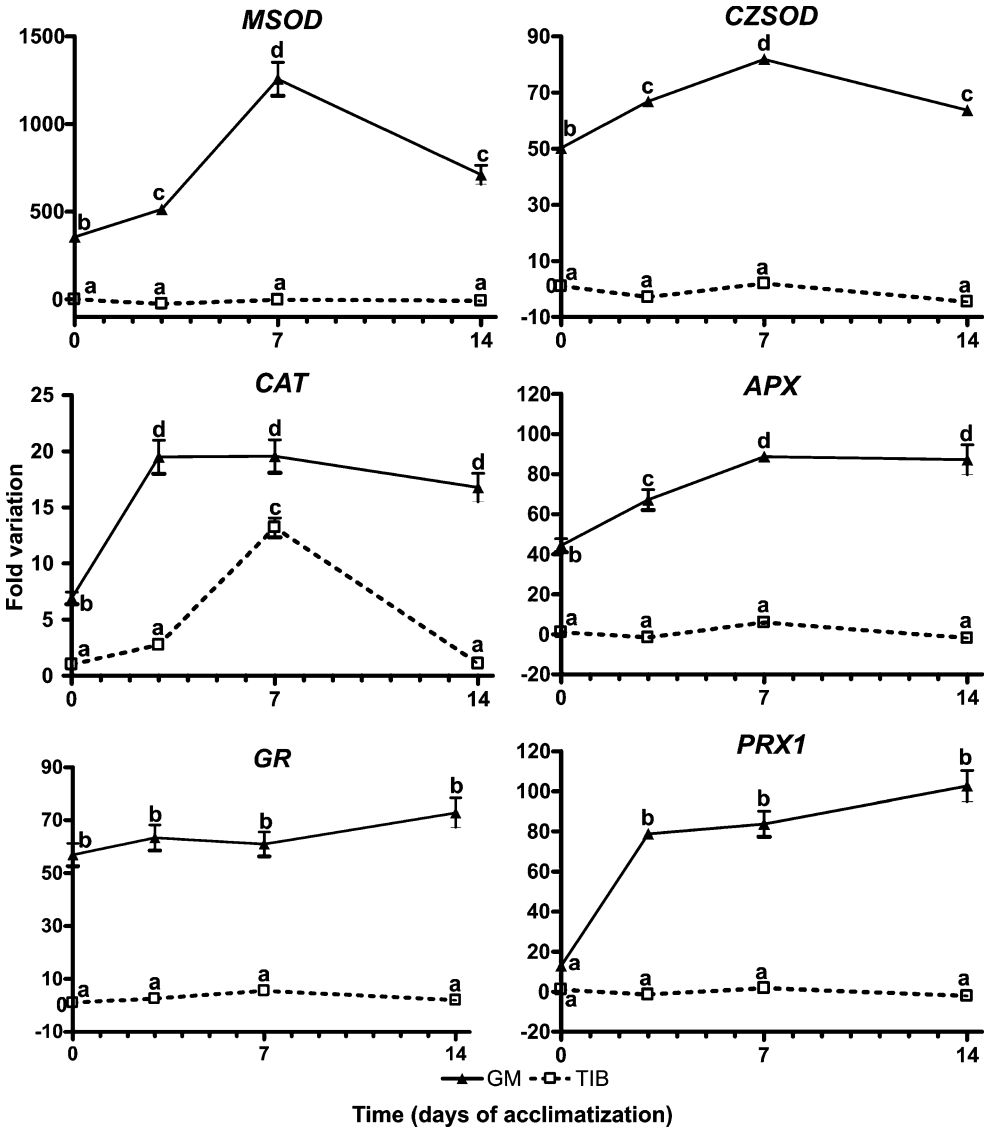

Fig. 6 Changes in the expression levels of genes of the anti-oxidative system. Quantification of mRNA levels of those sugarcane genes was performed on day 0 of *ex vitro* growth after each propagation method (GM and TIB) and after 3, 7 and 14 days. mRNA was isolated from leaves, converted to cDNA, and 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

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 [50]. Glandular trichomes are modified epidermal cells that can also confer resistance to biotic stress [33], e.g. by entrapping insects in the polymerized trichome exudates [27]. 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 [5, 7]. 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, Fig. 3). 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 [8]. Two of the Cu-Zn SOD isoforms identified did not change their levels of

activity while the inducible isoform 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 isoform 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 isoforms [8]. In fact, seven Cu-Zn isoforms were found in sugarcane seedlings under the effect of cadmium, although SOD activity remained constant [18]. In GM, particularly *MSOD* but also *CZSOD*, were significantly up-regulated *in vitro*, with the transcript of MnSOD still increasing significantly thereafter (Fig. 6). 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 isoenzyme [43]. However, heterotrophic behavior of GM plantlets, in favor of the anti-oxidative response at the mitochondrial level [37], is in addition an intrinsic characteristic of C_4 metabolism, where mitochondria play an important role in the CO_2 concentration cycle [30].

In gel GR activity revealed four isoforms, 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 antioxidants in maize leaves [13], 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 [38]. One difference between these studies and ours, where the co-existence of activity in the four isoenzymes 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 [25, 45]. 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 isoforms 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 (Fig. 3). All higher plant Prxs are nuclear-encoded proteins [11] 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 [17]. 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 [21]. 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 [35] seems to be confirmed in GM plants.

The overall upregulation of transcripts in GM plantlets (Fig. 6) must indicate a much higher induction of the anti-oxidative genes than in TIB propagated plantlets. The lack of upregulation reported in TIB can be explained by the previous acclimation to patterns of gas exchange much closer to *ex vitro* environment [26].

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 [31, 41] and the photosynthetic capacity [2]. 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.

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