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Change in sugar, sterol and fatty acid composition in banana meristems caused by sucrose-induced acclimation and its effects on cryopreservation

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To understand the mechanisms of sucrose-induced acclimation in relation to plant cryopreservation, sugars, sterols, fatty acids of different lipid fractions (neutral lipids, glycolipids and sphingolipids and phospholipids), as well as free fatty acids were analyzed in proliferating meristem cultures of different banana varieties. The four banana varieties that were selected show different post-thaw shoot regeneration rates (0–53.4%). All mentioned parameters were analyzed using (1) control meristems that were cultured on a normal sucrose concentration (0.09 *M*), which resulted in low survival after cryopreservation; and (2) 2-week sucrose precultured meristems (0.4 *M*). This sucrose preculture, essential for regeneration after cryopreservation, resulted in a significant increase of each of seven sugars detected. The ratio of stigmasterol/sitosterol (St/Si) in sucrose-pretreated meristems significantly increased. The sucrose pretreatment also resulted in a significant increase of total fatty acid content of the neutral lipid fraction and of the glycolipid and sphingolipid fraction, as well as the total free fatty acid content. The individual fatty acid content of the phospholipids was differently changed by the sucrose pretreatment for the given varieties studied. In most cases, sucrose pretreatment resulted in an increase of the double bond index (DBI) in the neutral lipids and a decrease of DBI in the glycolipids and sphingolipids, in phospholipids as well as in free fatty acids. Principal component analysis of all collected data revealed that (1) for the control material, sucrose and total sugar contents were closely linked to the post-thaw shoot regeneration, suggesting that sucrose and total sugar may be main limiting factors to survive cryopreservation; (2) accumulation of large quantities of sugars (glucose, fructose, sucrose and total sugar) in sucrose-pretreated material cannot explain the differences in survival after cryopreservation of the four banana varieties. We assume that a minimal amount of sugars is needed in meristem cultures to survive cryopreservation. Still, other limiting factors do influence the survival following the sucrose

Abbreviations – BA, benzyladenine; C14:0, myristic acid; C16:0, palmitic acid; C16:1, palmitoleic acid; C18:0, stearic acid; C18:1, oleic acid; C18:1c11, *cis*-vaccenic acid; C18:2, linoleic acid; C18:3, linolenic acid; $CHCl_3$, chloroform; DBI, double bond index; FAMEs, fatty acid methyl esters; FID, flame ionization detector; FW, fresh weight; IS, internal standard; iso-PrOH, iso-propanol; PCA, principal component analysis; PVS, plant vitrification solution; Si, sitosterol; St, stigmasterol; WCOT, wall-coated open-tubular.

pretreatment. We observed that the parameters which are closely linked to the post-thaw shoot regeneration are a minimal change in the ratios of St/Si, the minimal change of the DBI of phospholipids and free fatty acids, as well as linoleic acid content (C18:2); and (3) inositol, raffinose, myristic acid (C14:0) and oleic acid (C18:1) were present in small quantities; however, they could be correlated to survival after cryopreservation, suggesting that they may be also involved in cryopreservation process.

Introduction

It is estimated that up to 100 000 plants, representing more than one-third of all the world's plant species, are currently threatened or face extinction in the wild (BGCI 2005). Their preservation is essential for classical and modern (genetic engineering) plant breeding programmes. Moreover, biodiversity provides a source of compounds to the pharmaceutical, food and crop protection industries. Cryopreservation or freeze-preservation at ultra-low temperature (−196°C) is the method of choice for the long-term conservation of plant genetic resources, because under these conditions, biochemical and most physical processes are arrested. Besides its traditional use for the conservation of genetic resources, cryopreservation is now more and more applied for the safe long-term storage of plant tissues with specific characteristics like medicinal and alkaloid-producing cell lines and hairy root cultures, genetically transformed tissues (Elleuch et al. 1998), and transformation-competent tissues (Gordon-Kamm et al. 1990). Recently, it was also proven that cryotherapy can be successfully applied to eradicate viruses from plum, banana and grape (Brison et al. 1997, Helliot et al. 2002, Wang et al. 2003). However, despite the fact that cryogenic procedures are now being developed for an increasing number of recalcitrant seeds and in vitro tissues/organs, the routine utilization of cryopreservation for the preservation of plant biodiversity is still limited.

All cryopreservation protocols are developed through 'trial and error' and depend on natural freezing resistance of the species, explant size and type, and water content. Care is thereby taken to avoid ice intracellular crystallization during the freezing process causing physical damage to the tissue (Sakai 2000, Panis et al. 2001). The only way to prevent ice crystal formation at ultra-low temperatures without an extreme reduction in moisture content is through vitrification, i.e. non-crystalline solidification of water (Sakai et al. 1990). To obtain a vitrified solution, it needs to be sufficiently concentrated and cooling rates need to be high (Fahy et al. 1984). The existing cryogenic strategies rely on freeze-dehydration, addition of cryoprotective substances including the recently developed plant vitrification solutions (PVS), desiccation and acclimatization or combinations of these processes (Panis et al. 2001). It is now generally accepted that the critical step to achieve post-thaw survival lies in the induction of tolerance towards dehydration/desiccation. In practice, sucrose pretreatment is often used as one of the methods to induce such tolerance depending on the plant species, tissue type and research group.

The induction of freezing and dehydration tolerance in nature is now more and more subject to intensive investigations. A wide range of studies indicates that the cell membrane systems are the primary site of injury in plants (Fujikawa et al. 1999, Piotrowska et al. 2000, Cyril et al. 2002, Yamada et al. 2002, Campos et al. 2003, Aroca et al. 2005, Lindberg et al. 2005, Taulavuori et al. 2005). Tolerance mechanisms depend on membrane stabilization (through changes in lipid composition, accumulation of sugars and production of membrane protecting polypeptides), the induction of anti-oxidative mechanisms, increase of sugar levels in the apoplastic space and the transcription of genes coding molecular chaperones (Thomashow 1999).

Besides proteins, sterols and phospholipids are the major components of plant membranes. Plant sterols not only are able to regulate membrane fluidity and permeability but also can modulate the activity of membrane-bound enzymes (Grandmougin-Ferjani et al. 1997, Hartmann 1998). It is now also recognized that phospholipids are not only just structure components of membranes, but they can also act as cofactors for membrane enzymes, signal precursors or signalling molecules themselves (Laxalt and Munnik 2002).

Fatty acids of phospholipids influence membrane flexibility and permeability. The unsaturation degree of phospholipid fatty acids is closely associated with abiotic stress resistance in plants (Graham and Patterson 1982, Hugly and Somerville 1992, Miquel et al. 1993, Kodama et al. 1994). In recent years, there is increasing evidence that lipids also function as mediators in many plant processes including signal transduction, cytoskeleton rearrangements and membrane trafficking (Wang 2004). For example, free fatty acids (more specifically oleic acid) are considered as stimulators of the signalling enzyme PLDδ,

which has an anti-cell death function (Zhang et al. 2003) suggesting that free oleic acid is a signalling messenger and that PLDδ is one of its potential targets. In addition, it was also proven that the saturated fatty acids, such as palmitic and stearic acid, were involved in cell regulation (Bonaventure et al. 2003).

In banana, cryopreservation protocols have been developed for different tissues (Panis et al. 1990, 1996, 2002, 2005, Abdelnour-Esquivel et al. 1992, Chin 1996, Thinh et al. 1999). Using the proliferating meristem cultures as starting material, two cryopreservation protocols were developed: a simple freezing method and a vitrification method, both of which are based on a sucrose pretreatment. These two methods have been applied to a wide range of banana varieties belonging to different genomic groups and resulted in different post-thaw regeneration rates (Panis and Thinh 2001, Panis et al. 2002).

In the present paper, changes of soluble sugars, sterols, fatty acids of different lipid fractions and free fatty acids in banana-proliferating meristems induced by a 2-week sucrose pretreatment are investigated, and possible consequences for such sucrose-induced acclimation in relation to cryopreservation ability are discussed.

Materials and methods

Plant material

In vitro plants of four banana varieties were provided by the International Network for the Improvement of Banana and Plantain transit centre, Belgium. The selected varieties, Cachaco (ABB cooking banana), Williams (AAA dessert banana), Obino L'Ewai (AAB plantain) and Mbwazirume (AAA East African Highland banana) belong to four different genomic groups, of which is known that they behave differently towards cryopreservation (Panis et al. 2002). Proliferating meristem cultures of these four varieties were initiated as described previously (Panis et al. 2002) and subsequently maintained on P4 medium. This P4 medium contains high concentrations (100 μ*M*) of benzyladenine and is the standard medium for 'highly' proliferating meristem cultures of banana.

Cryopreservation

Cryopreservation has been carried out according to an optimized 'combined cryopreservation' method that is routinely applied for the conservation of banana germplasm at the Laboratory of Tropical Crop Improvement, K.U. Leuven, Belgium (Panis and Thinh 2001, Agrawal et al. 2004). Two tissue types have been subjected to the cryopreservation protocol: (1) meristem clumps directly collected on the standard P4 medium; and (2) meristems collected on the sucrose preculture medium. The latter medium is similar to P4 medium but contains an elevated concentration of sucrose (0.4 *M* instead of 0.09 *M*), which is known to significantly increase the post-thaw recovery rates depending on the banana varieties (Panis et al. 1996, 2002). Hereinafter, the P4 and sucrose preculture mediums are, respectively, referred as 'control' and 'pretreated' medium. Consequently, meristems which were collected on the P4 medium or on the sucrose preculture medium are referred as 'control' or 'pretreated' meristems, respectively. Fourteen days after subculture (for control meristems) or after sucrose preculture (for pretreated meristems), meristem clumps of 1.5–3 mm diameter (containing three to five meristematic domes) were excised and transferred to a loading solution and kept for 20 min at room temperature and subsequently treated with the PVS2 (Sakai et al. 1990) for 2 h at 0°C. Then, 10 meristem clumps were transferred to a droplet of PVS2 solution on a strip of aluminium foil (5 × 20 mm). The aluminium strip together with the meristem clumps was plunged into liquid nitrogen with a fine forceps. After a minimal storage (1 h), thawing took place by rinsing the strips in a recovery solution at room temperature. Recovery of the meristem clumps took place according to the method and growth conditions described by Panis et al. (2005). After 5 weeks, shoot regeneration was determined under a binocular microscope. Clumps with at least one regenerating shoot are considered as regenerated clumps.

Analysis of sugars, sterols, bound fatty acids and free fatty acids

Fourteen days after subculture (for control meristems) or after sucrose preculture (for pretreated meristems) as mentioned above, fresh meristem clumps from the four varieties were excised. After determination of their fresh weight (FW), the meristem clumps were freeze-dried, and dry weight was recorded and used for analysis.

Sugars

Approximately 2 mg (exact dry weight was recorded) of the freeze-dried powdered meristem clumps were homogenized in a 15 ml of polypropylene centrifuge tube containing 3 ml of 80% (v/v) ethanol containing lactose (250 mg l^{-1}) as internal standard (IS). The tube was closed with a polyethylene screw cap, and the mixture was incubated at 80°C for 20 min during which period it was vortexed five times. After cooling in an ice bath, the mixture was centrifuged at 4000 *g* for

10 min at 4°C. A volume of 2.5 ml of the supernatant was collected and freeze-dried under reduced pressure in a speed-vac concentrator. The dried extract was dissolved in 10 ml of distilled water and filtered (0.22 μm pore diameter). Analysis of soluble sugar was performed by High Performance Anion Exchange chromatography coupled with Pulsed Amperometric Detection (Dionex Chromatography Co., Sunnyvale, California). The Dionex DX-500 BiolC system consisted of a GP50 Gradient Pump with on-line Degas, an E01 Eluent organizer, an ED40 Electrochemical Detector (gold electrode) and an AS50 Autosampler. Sugar separation on a CarboPac PA-1 (Dionex) column was achieved by using an isocratic 150 m*M* NaOH (J. T. BAKER, Phillipsburg, NJ) elution at 1 ml min^{-1}. Sugar identification and measurement were achieved by comparison with retention times (relative to that of the IS) and calibration curves obtained with sugar standards (glucose, fructose, inositol, lactose, raffinose, sorbitol, stachyose and sucrose were obtained from Sigma-Aldrich, St. Louis, MO).

Sterols and fatty acids bound to different lipid fractions

A modified procedure of Geuns et al. (1997b) was used for small amount of banana meristem tissues. Approximately 10 mg (exact dry weight was recorded) of the freeze-dried powdered meristem clumps were homogenized in 1 ml of iso-PrOH containing cholestanol (50 mg l^{-1}) as IS. The mixture was incubated for 30 min at 0°C with continuous shaking. After centrifugation at 10 000*g* for 10 min, the supernatant was collected (first collection) and the residue was extracted again with 1 ml of $CHCl_3$: MeOH (2:1) for another 30 min at the same conditions. After centrifugation at 10 000 g for 10 min, the supernatants were combined, and 2 ml of $CHCl_3$ and 1 ml of a 0.88% KCl solution were added. The mixture was thoroughly shaken in a Teflon-lined screw cap centrifuge tube and centrifuged at 10 000 g for 10 min. The upper aqueous layer was aspired, and 1 ml of MeOH : saline solution (1:1) was added. After shaking and centrifugation at 10 000 *g* for 10 min, the upper phase was aspired together with the interphase. The lower phase was evaporated at 40°C under a stream of N_2. The residue (lipid extracts) was dissolved in 2 ml of $CHCl_3$: acetic acid (100:1) and applied to a silica gel column which is made in a 5-ml pipette tip plugged with glass wool and containing 500 mg silica gel (MN-silicagel 60 for column chromatography, 0.063–0.2 mm, Machery-Nagel, Düren, Germany) wetted with $CHCl_3$: acetic acid (100:1). Ten millilitre of $CHCl_3$ was applied to the column for eluting neutral lipids such as sterols, glycerides, etc. (fraction 1). Five millilitre of acetone, followed by 5 ml of acetone : MeOH : acetic acid (100:5:1) were applied to the column for eluting glycolipids and sphingolipids (fraction 2). Then, 7.5 ml of MeOH : $CHCl_3$: H_2O mixture (10:5:4) was applied to the column for eluting the phospholipids. To this fraction, 2.25 ml of $CHCl_3$ and 3 ml of H_2O were added. The mixture was vortexed and centrifuged. The upper water phase was pipetted off, and the lower $CHCl_3$ phase containing the phospholipids was used for further analysis (fraction 3). Fraction 1 was evaporated under a stream of N_2 at 40°C. The residue was dissolved in 100 μl of dichloroethane of which 0.2 μl was injected into the GC^2 (Fisons Instruments GC8000 Series; Fisons Instruments, Milan, Italy, with a Shimadzu C-R5A printer-plotter; Shimadzu Corp., Kyoto, Japan) equipped with a home-made on-column injector (Geuns 1983). The residues from parts of fraction 1, 2 or 3 were dissolved in 100 μl of toluene containing heptadecanoic acid methyl ester as an IS. Then, 100 μl of 5% KOH in MeOH was added, and the mixture was allowed to stand exactly 4 min at room temperature. After adding 200 μl of H_2O, the fatty acid methyl esters (FAMEs) were extracted in 200 μl of pentane. After washing by adding 100 μl of H_2O, the purified pentane fraction containing the FAMEs was collected, of which 3 μl was injected into the GC^2 (Shimadzu GC-14 A with a Shimadzu C-R5A printer-plotter).

The GC^2 conditions for sterol analysis: column – wall-coated open-tubular fused silica, 25 m × 0.32 mm, CP-Sil-8 CB, d.f. = 0.12 μm; oven at 250°C; carrier – H2 at 0.7 bar; Flame Ionization Detector (FID) at 290°C; injection port: 285°C. The GC^2 conditions for fatty acid analysis: column: Varian 50 m × 0.25 mm CP-SIL88, d.f. = 0.25 μm; carrier – H2 at 1 bar, gradient from 170°C (10 min) to 190°C; detector – FID at 220°C; split injector (1:100) at 190°C.

Free fatty acids

Approximately 10 mg (exact dry weight was recorded) of the freeze-dried powdered meristem clumps were extracted with 300 μl of dry acetone containing free heptadecanoic acid (33.3 mg l^{-1}) as IS. The residue was again extracted two times with 300 μl of dry acetone (without IS). The pooled acetone fractions were evaporated under a gentle stream of N_2. The residue was dissolved in 300 μl of 2% KOH, and the neutral fraction was removed by extraction with three equal volumes of peroxide free diethyl ether. The KOH fraction was acidified to pH $\leq$ 3 with 3 N HCl. The acidified mixture was extracted three times with peroxide-free diethyl ether. The pooled ether was evaporated under a gentle stream of N_2. The residue was dissolved in 100 μl of ether-diazomethane : MeOH (9:1), and the

mixture was allowed to stand for 5 min at room temperature. Then, 200 µl of H_2O and 250 µl of pentane were added. After vortexing and centrifugation (10 000 *g* for 10 min), the pentane fraction was collected of which 3 µl was injected into the GC^2 for free fatty acid determination. The GC^2 conditions were as described above.

Double bond index

Double bond index (DBI) of fatty acids was calculated according to Quartacci et al. (2001) as follows:

$$\mathrm{DBI} = \frac{[(1 \times \%\,\text{monoenes}) + (2 \times \%\,\text{dienes}) + (3 \times \%\,\text{trienes})]}{\sum \%\,\text{saturated fatty acids}}$$

Data analysis

Data collected from at least three independent experiments were subjected to analysis of variance (ANOVA). Where necessary, the original data were log transformed in order to keep the homogeneity of the variance before ANOVA. The Newman and Keuls' method was used for mean comparison. Principal component analysis (PCA) was performed via the correlation matrix after standardizing the data. Statistics process was performed on a program of STATISTICA 6.1 (StatSoft, Inc. Tulsa, Oklahoma).

Results

Cryopreservation

Post-thaw regeneration rates of both control and pretreated meristems are linked with the varieties (Fig. 1). Shoot regeneration rates after cryopreservation were very low for meristems collected after a 2-week period of culture on the control medium. For most varieties, shoot regeneration rates were close to zero, while only for Cachaco this amounted up to 8.1%. Sucrose pretreatment resulted in significantly higher post-thaw shoot regeneration rates that were linked with the variety: high rates for Cachaco (53.4%), followed by Williams and Obino L'Ewai (23.8 and 15.6%, respectively). The East African highland banana variety Mbwazirume did not respond irrespective of the preculture treatment. This differential variety response to the sucrose pretreatment allowed us to correlate post-thaw regeneration rates with changes of sugars, sterols, fatty acids of different lipid fractions and free fatty acids in meristems induced by a sucrose pretreatment.

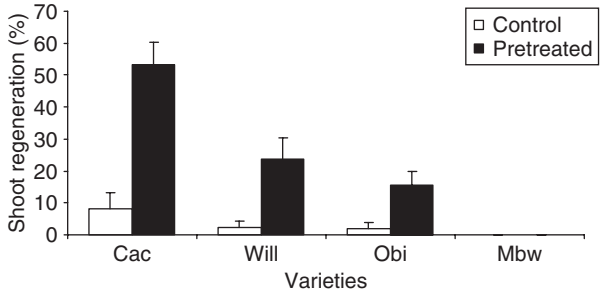

Fig. 1. Shoot regeneration (%) of meristems after cryopreservation for four banana varieties. Control = meristems collected on the control medium; pretreated = meristems collected on the sucrose-pretreated medium. Cac = Cachaco (ABB cooking banana), Will = Williams (AAA dessert banana), Obi = Obino L'Ewai (AAB plantain) and Mbw = Mbwazirume (AAA East African Highland banana). Vertical bars correspond to SE, n = 5–7.

Sugar

Sucrose pretreatment induced a decrease of moisture content in sucrose-pretreated meristems (77.1–78.6%) compared with that in the control ones (92.9–94.7%) (data not shown). Such decrease was mainly due to a significant increase of total soluble sugar in the pretreated materials (see below). Therefore, all the results are expressed on a FW basis. In both control and pretreated meristems, seven soluble sugars could be detected, i.e. glucose, fructose, sucrose, raffinose, stachyose, inositol and sorbitol (Table 1). In each variety, a 14-day sucrose pretreatment resulted in a significant increase of all individual sugars. Most abundant sugars in banana meristems were glucose, fructose and sucrose. Less abundant sugars were raffinose, stachyose, inositol and sorbitol. The total amount of sugars in the meristem cultures after a sucrose pretreatment (8.8–11.4%) almost reached the sugar concentration (0.4 M = 13.6%) of the medium. For the control meristems, the sugar content is 0.2–0.5%. After sucrose pretreatment, the total amount of sugars in meristems increased 26.5-fold, mainly due to significant increases of glucose, fructose and sucrose (21.4–31.4-fold). The increases of the less abundant sugars were 4.5–9.2-fold. Total and individual sugar contents significantly differed between the varieties for the control meristems. After the sucrose pretreatment, differences between varieties were less pronounced as all the varieties absorbed huge amounts of sugar (Table 1).

Sterols

The most abundant sterols that were detected in banana meristems are sitosterol (Si) and stigmasterol (St), followed by campesterol. The cholesterol content was generally very low and less influenced by the variety or sucrose pretreatment (Table 2). Campesterol content was not significantly changed by the sucrose

Table 1. Soluble sugar content (% fresh weight) in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Values represent the means of five independent experiments. Means followed by the same letter in the same column and for each sugar were not significantly different at $P = 0.05$ according to Newman and Keuls' method. ND, Not detected.

Medium	Variety	Glucose	Fructose	Sucrose	Raffinose
Control	Cachaco	0.076 b	0.049 c	0.341 c	0.017 c
	Williams	0.049 c	0.038 d	0.083 f	ND
	Obino l'Ewai	0.058 c	0.035 d	0.243 d	0.006 d
	Mbwazirume	0.090 b	0.076 b	0.149 e	0.007 d
Pretreated	Cachaco	1.449 a	1.362 a	7.239 a	0.037 b
	Williams	1.539 a	1.440 a	5.974 b	0.086 b
	Obino l'Ewai	1.590 a	1.746 a	7.793 a	0.041 b
	Mbwazirume	1.542 a	1.318 a	5.462 b	0.219 a
Medium	Variety	Stachyose	Inositol	Sorbitol	Total
Control	Cachaco	0.019 c	0.022 c	0.011 b	0.529 d
	Williams	0.003 e	0.008 d	0.003 c	0.182 f
	Obino l'Ewai	0.033 b	0.010 d	0.011 b	0.397 e
	Mbwazirume	0.006 d	0.009 d	0.003 c	0.337 e
Preteated	Cachaco	0.059 a	0.139 a	0.045 a	10.293 ab
	Williams	0.067 a	0.136 a	0.047 a	9.246 bc
	Obino l'Ewai	0.074 a	0.137 a	0.025 a	11.361 a
	Mbwazirume	0.133 a	0.066 b	0.099 a	8.776 c

pretreatment. In all varieties, sucrose pretreatment resulted in an increase of the St/Si ratio. Only for the varieties Obino L'Ewai and Mbwazirume, a significant increase of St concentration was recorded. The change of the St/Si ratio by the sucrose pretreatment, given as the ratio of the third over the second column of Table 3, depended on the variety used. The lowest value, implicating the smallest change, was observed in the best survivor after cryopreservation (Cachaco, 2.09) followed by Williams and Obino l'Ewai (2.36 and 2.40, respectively). This value was relatively high for Mbwazirume (3.55), the worst survivor after cryopreservation (Table 3, Fig. 1).

Bound fatty acids

Lipids that were extracted from banana meristems were fractionated into three major fractions: (1) neutral lipids; (2) glycolipids and sphingolipids; and (3) phospholipids. After transmethylation, FAMEs from the different lipid fractions were analyzed by GC[2]. Saturated fatty acids of the three lipid fractions are myristic acid (C14:0), pal-

Table 2. Sterol content in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Values represent the mean of three independent experiments. Means followed by the same letter in each column are not significantly different at $P = 0.05$ according to Newman and Keuls' method.

		Sterol content (ng mg^{-1} fresh weight)			
Medium	Variety	Cholesterol	Campesterol	Sitosterol	Stigmasterol
Control	Cachaco	5.3 b	36.7 a	166.3 a	170.9 c
	Williams	5.5 b	47.7 a	135.2 ab	245.3 ab
	Obino l'Ewai	6.9 ab	45.1 a	109.7 ab	160.0 c
	Mbwarizirume	5.5 b	24.4 a	153.1 ab	80.6 d
Pretreated	Cachaco	6.1 b	30.9 a	99.2 ab	213.1 bc
	Williams	7.4 ab	48.7 a	69.2 b	296.7 a
	Obino l'Ewai	9.9 a	41.2 a	80.0 b	279.6 a
	Mbwazirume	6.6 ab	29.2 a	109.7 ab	205.1 bc

Table 3. Stigmasterol to sitosterol (St/Si) ratio in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Means followed by the same letter irrespective of the column are not significantly different at $P = 0.05$ according to Newman and Keuls' method.

Variety	Control	Pretreated	Ratio of pretreated/control
Cachaco	1.03 de	2.15 bc	2.09
Williams	1.81 cd	4.28 a	2.36
Obino l'Ewai	1.46 cd	3.49 ab	2.40
Mbwazirume	0.53 e	1.87 bcd	3.55

mitic acid (C16:0) and stearic acid (C18:0). Unsaturated fatty acids are palmitoleic acid (C16:1), oleic acid (C18:1), *cis*-vaccenic acid (C18:1c11), linoleic acid (C18:2) and linolenic acid (C18:3) (Table 4). The majority of the fatty acids in banana meristems occurred in the phospholipid fraction, followed by the glycolipid and sphingolipid fraction. The amount of the fatty acids bound to the neutral lipid fraction is relatively low. In each lipid fraction, palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3) were present in large quantities.

In all varieties, sucrose pretreatment significantly increased the total fatty acid content of the neutral lipid fraction and of the glycolipid and sphingolipid fraction. Especially, the total fatty acid level of neutral lipid fraction increased three-fold (for Mbwazirume) to six-fold (for Obino L'Ewai) while this was only 1.5–2-fold for the glycolipids and sphingolipids. For the neutral lipids, we observed a substantial increase for most of the fatty acids except for stearic acid (C18:0), palmitoleic acid (C16:1) and *cis*-vaccenic acid (C18:1c11), while for the glycolipids and sphingolipids the increase was minimal for palmitoleic acid (C16:1), oleic acid (C18:1) and linolenic acid (C18:3). The changes in total fatty acids of phospholipids are less pronounced. Only in Obino L'Ewai, a

Table 4. Fatty acid content of different lipid fractions in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Values represent the mean of three independent experiments. Means followed by the same letter in each column and in each lipid fraction are not significant at $P = 0.05$ according to Newman and Keuls' method.

		Fatty acid content (ng mg^{-1} fresh weight)								
Medium	Variety	C14:0	C16:0	C18:0	C16:1	C18:1	C18:1c11	C18:2	C18:3	Total
Neutral lipid fraction										
Control	Cachaco	1.3 c	10.7 b	4.4 ab	4.5 a	7.9 c	1.1 abc	8.3 c	4.9 c	43.2 c
	Williams	2.1 bc	11.8 b	3.1 ab	2.7 a	5.6 c	0.3 c	8.4 c	7.1 c	41.1 c
	Obino l'Ewai	1.4 c	8.6 b	6.4 ab	3.6 a	3.1 c	1.1 abc	4.7 c	4.3 c	33.3 c
	Mbwazirume	1.4 c	9.3 b	2.2 b	2.8 a	4.1 c	0.1 c	5.3 c	4.6 c	29.8 c
Pretreated	Cachaco	3.0 ab	47.1 a	7.4 a	10.7 a	29.7 a	2.7 a	84.1 a	50.2 a	234.9 a
	William	3.3 ab	37.7 a	7.7 a	8.5 a	19.3 b	1.9 ab	48.2 ab	37.6 a	164.1 ab
	Obino l'Ewai	3.9 a	41.3 a	8.5 a	11.0 a	29.5 a	1.9 ab	55.4 a	40.3 a	191.8 a
	Mbwazirume	4.5 a	27.3 a	6.9 a	5.6 a	15.6 b	1.0 bc	23.8 b	15.5 b	100.1 b
Glycolipid and sphingolipid fraction										
Control	Cachaco	1.1 c	72.4 c	8.3 c	1.0 cd	25.2 bc	3.9 cd	59.7 c	137.7 b	309.3 c
	Williams	1.0 c	52.8 c	10.5 c	1.3 bc	8.3 ef	3.5 de	37.3 d	143.3 b	258.1 d
	Obino l'Ewai	1.0 c	66.3 c	11.7 c	1.0 cd	11.2 de	3.5 de	59.8 c	181.1 a	335.5 c
	Mbwazirume	0.8 c	63.6 c	9.3 c	0.7 d	5.8 f	2.6 e	56.4 c	105.4 c	244.5 d
Pretreated	Cachaco	1.7 b	140.1 a	18.6 b	1.6 ab	29.8 b	6.6 ab	107.7 a	197.9 a	503.9 a
	William	1.9 b	138.4 a	22.9 ab	1.7 ab	20.8 bc	8.0 a	94.6 a	200.1 a	488.4 ab
	Obino l'Ewai	1.9 b	117.7 b	26.2 a	1.9 ab	46.7 a	5.2 bc	76.9 b	154.2 b	430.7 b
	Mbwazirume	2.5 a	129.4 ab	26.7 a	2.2 a	17.2 cd	6.9 ab	80.2 b	191.2 a	456.4 ab
Phospholipid fraction										
Control	Cachaco	2.3 bc	523.8 def	25.4 d	2.8 b	157.0 b	12.3 c	763.9 ab	244.3 c	1731.8 bc
	Williams	1.6 c	462.1 ef	39.7 bcd	2.8 b	71.7 cd	13.6 bc	609.7 bc	502.9 a	1704.1 bc
	Obino l'Ewai	2.5 bc	429.8 f	33.1 d	1.6 b	32.3 d	9.2 d	623.5 bc	411.8 ab	1544.0 c
	Mbwazirume	3.4 bc	595.7 cde	48.3 bc	2.9 b	67.4 cd	13.2 bc	902.1 a	457.5 ab	2090.4 ab
Pretreated	Cachaco	3.1 bc	698.0 abc	52.7 b	3.4 b	111.6 bc	18.2 a	792.8 ab	393.3 bc	2073.2 ab
	William	3.0 bc	649.6 bcd	36.7 cd	3.7 b	149.8 b	13.7 bc	742.3 ab	299.1 bc	1897.9 bc
	Obino l'Ewai	5.7 ab	803.9 a	69.9 a	2.6 b	319.5 a	17.0 ab	744.4 ab	354.7 bc	2317.6 a
	Mbwazirume	8.6 a	748.9 ab	64.0 a	7.2 a	87.7 cd	19.0 a	527.1 c	399.0 bc	1861.5 bc

Table 5. Free fatty acid content in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Values represent the mean of four independent experiments. Means followed by the same letter in each column are not significant at $P = 0.05$ according to Newman and Keuls' method.

		Free fatty acid content (ng mg^{-1} fresh weight)								
Medium	Variety	C14:0	C16:0	C18:0	C16:1	C18:1	C18:1c11	C18:2	C18:3	Total
Control	Cachaco	2.0 ab	129.7 c	21.0 d	1.9 ab	41.0 cd	5.1 b	186.8 d	173.9 c	561.5 c
	Williams	1.3 b	124.3 c	26.6 cd	2.0 ab	26.0 de	5.3 b	198.7 d	226.7 bc	610.9 c
	Obino l'Ewai	1.1 b	92.1 c	19.7 d	1.4 b	22.5 e	5.1 b	165.8 d	162.7 c	470.5 c
	Mbwazirume	2.6 ab	239.2 b	32.7 bcd	2.6 ab	25.9 de	8.5 b	425.6 bc	335.4 ab	1072.5 b
Pretreated	Cachaco	4.2 ab	360.2 a	48.2 abc	4.8 ab	163.4 a	22.2 a	623.0 a	422.3 a	1648.2 a
	William	2.6 ab	264.4 b	64.8 a	1.8 ab	86.2 b	10.9 b	417.3 bc	405.5 a	1253.5 ab
	Obino l'Ewai	3.9 ab	361.1 a	52.3 ab	5.4 a	237.7 a	24.5 a	527.1 ab	408.9 a	1620.8 a
	Mbwazirume	4.8 a	239.0 b	49.5 abc	2.1 ab	61.2 bc	12.4 b	304.0 cd	278.0 abc	951.0 bc

significant increase was recorded following the sucrose pretreatment.

Free fatty acids

The composition of free fatty acids of banana meristems was the same as the bound ones (Table 5). The major free fatty acids in banana meristems were palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3). Sucrose pretreatment resulted in a significant increase of these free fatty acids as well as of stearic acid (C18:0) for three of the four varieties. The total free fatty acid content significantly increased two-fold to three-fold in sucrose-pretreated meristems in three out of the four varieties.

DBI

Changes of DBI are summarized in Table 6. For most varieties, sucrose pretreatment resulted in a significant increase of DBI in the neutral lipid fraction and a significant decrease of DBI in the glycolipid and sphingolipid and the phospholipid fractions (Table 6). Significant decrease of DBI of free fatty acids was recorded in the varieties Obino L'Ewai and Mbwazirume.

PCA

As shown in Tables 1–6, there are 53 (for the control material) and 58 (for the pretreated material) parameters

Table 6. Double bond index of bound and free fatty acids in meristems of four banana varieties after 2 weeks of culture on control or on sucrose-pretreated medium. Means followed by the same letter in each lipid fraction irrespective of the column are not significant at $P = 0.05$ according to Newman and Keuls' method. NL, neutral lipid; GSL, glyco and sphingolipid; PL, phospholipid.

	Variety	Control	Pretreated	Ratio of pretreated/control
NL fraction	Cachaco	2.81 c	6.25 a	2.22
	Williams	2.80 c	4.67 b	1.67
	Obino l'Ewai	2.04 c	5.07 b	2.48
	Mbwazirume	2.54 c	2.94 c	1.16
GSL fraction	Cachaco	6.89 b	5.29 cd	0.77
	Williams	8.10 a	5.02 d	0.62
	Obino l'Ewai	8.61 a	4.60 d	0.53
	Mbwazirume	5.92 c	4.84 d	0.82
PL fraction	Cachaco	4.42 c	3.85 d	0.87
	Williams	5.58 a	3.69 d	0.66
	Obino l'Ewai	5.44 a	3.29 e	0.61
	Mbwazirume	5.03 b	2.91 f	0.58
Free fatty acids	Cachaco	6.33 ab	6.62 ab	1.05
	Williams	7.32 ab	6.46 ab	0.88
	Obino l'Ewai	7.59 a	6.09 c	0.80
	Mbwazirume	6.95 ab	5.24 c	0.75

that were measured in this work. In order to identify possible parameters that may be closely associated with cryopreservation abilities, a PCA method was employed. PCA is a classical multivariate method, generally used to reduce the number of variables and to detect the structure of the relationships between variables. It creates a limited number of informative factors, called principal components, designed to extract the maximum of the initial total variance of the data set. The projection of the variables on the first factorial plane, according to their factorial coordinates, is a powerful way to investigate the relationships between variables. Through this PCA method, two graphics were drawn by using data collected from the control or the sucrose-pretreated materials, respectively (Fig. 2). Two significant factors accounting for 44.2 and 32.8% of the variation were identified for the control material (Fig. 2A), and two significant factors accounting for 53.2 and 28.3% of the variation were identified for the pretreated material (Fig. 2B).

It is noted that in Fig. 2, for sake of clarity, only names of sugars and of shoot regeneration percentage are given. Names of other parameters which are represented as circle points in the graphics are mentioned below or given in Fig. 3. For the control material, the sucrose and the total sugar contents were found to be closely linked to the post-thaw shoot regeneration (Fig. 2A). Inositol, raffinose and sorbitol could also be correlated to the shoot regeneration. However, the contents of these individual sugars were relatively low (see Table 1). Only oleic acid (C18:1) of all lipid fractions as well as some fatty acids in the neutral and the glycolipid and sphingolipid fractions could be closely linked to the shoot regeneration rates (data not shown). For the sucrose-pretreated material, the large quantity of sugars including sucrose and total sugar content was not closely correlated to the post-thaw shoot regeneration. Only inositol could be positively linked to the cryopreservation ability (Fig. 2B).

Fig. 3A–E represent the same graphic as Fig. 2B, each figure showing the positions of a different set of variables. As shown in Fig. 3, the parameters which are closely linked to the cryopreservation are the changes in the St/Si ratio by sucrose pretreatment (Fig. 3A), the DBI of phospholipids and free fatty acids observed in the sucrose-pretreated meristems and the change of DBI by sucrose pretreatment (Fig. 3D, E). Also high proportions of individual fatty acids could be linked to cryopreservation ability: linoleic acid (C18:2) of all lipid fractions (Fig. 3B–E), linolenic acid (C18:3) of the neutral lipid fraction and the free fatty acids and *cis*-vaccenic acid (C18:1c11) and palmitic acid (C16:0) of the neutral lipid fraction. Among the different lipid fractions, the total fatty acid level in the neutral lipid

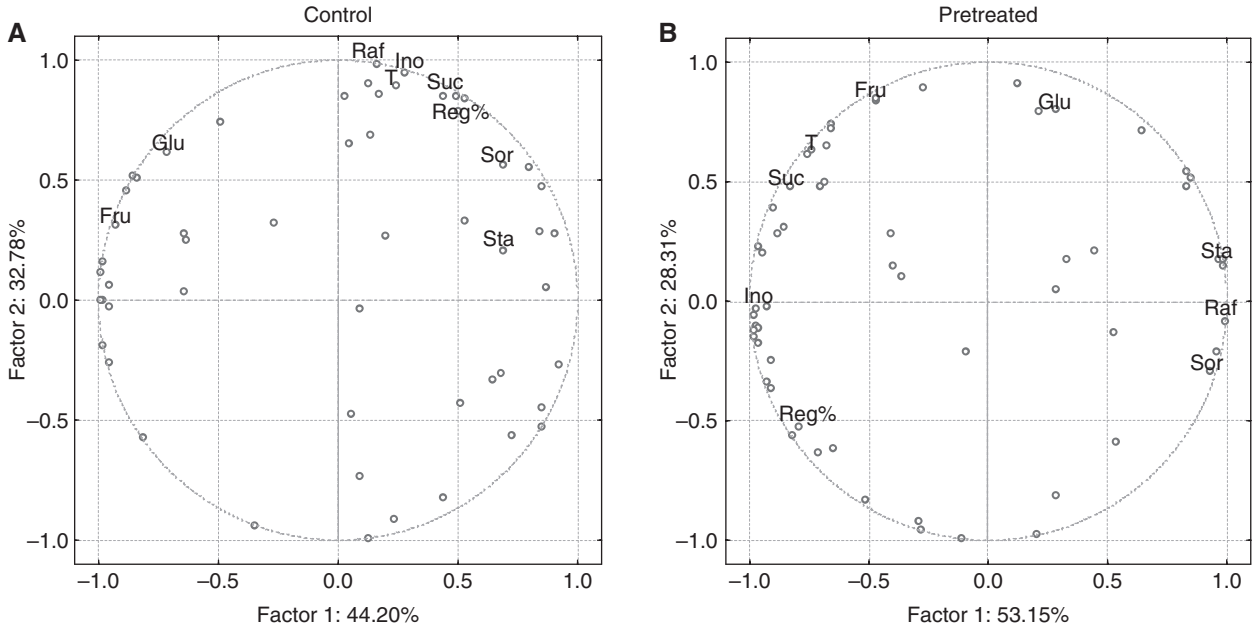

Fig. 2. Result of principal component analysis by using the data collected from the control (A) or the sucrose-pretreated banana meristems (B) as mentioned in Fig. 1 and Tables 1–6. For the sake of clarity, only names of sugar and of shoot regeneration percentages are given. Names of other parameters which are represented as cycle points in the graphics are mentioned in the text for the control or given in Fig. 3 for the pretreated materials. Reg% = shoot regeneration (%); Glu = glucose; Fru = fructose, Suc = sucrose; Raf = raffinose; Sta = stachyose; Ino = inositol; Sor = sorbitol and T = total sugar.

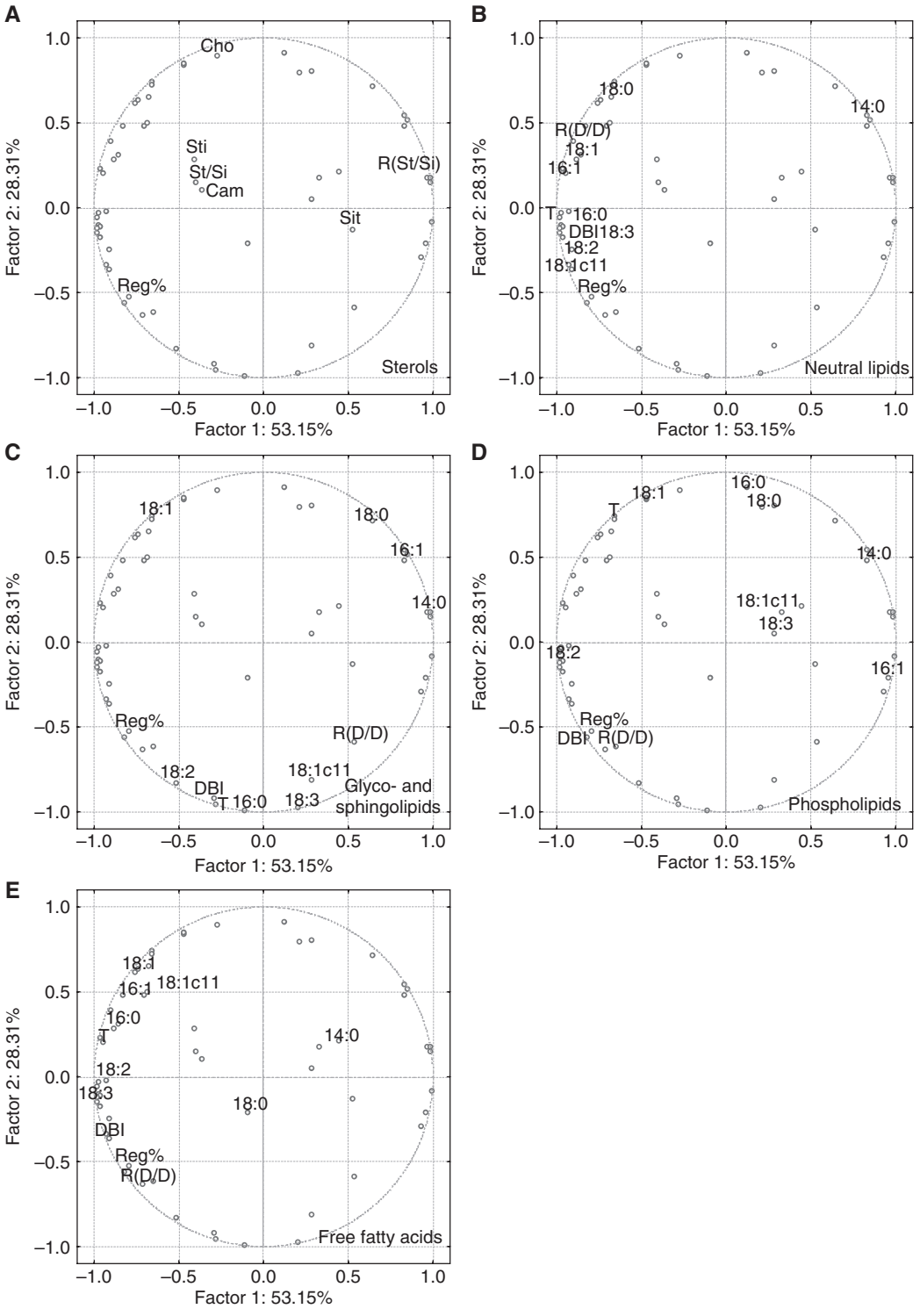

Fig. 3. Result of principal component analysis by using the data collected from sucrose-pretreated banana meristems as mentioned in Fig. 1 and Tables 1–6. All graphics are the same to Fig. 2B, only names of different parameters are separately given, for the sake of clarity, according to sterol and different lipid fractions. Reg% = shoot regeneration (%); Cho = cholesterol; Cam = campesterol; Sti = stigmasterol; Sit = sitosterol; St/Si = stigmasterol/sitosterol; R (St/Si) = ratio of St/Si in sucrose-pretreated meristems to St/Si in control ones; DBI = double bond index; R(D/D) = ratio of DBI in sucrose-pretreated meristems to DBI in control ones; T = total fatty acids. 14:0 = myristic acid; 16:0 = palmitic acid; 16:1 = palmitoleic acid; 18:0 = stearic acid; 18:1 = oleic acid; 18:1c11 = *cis*-vaccenic acid; 18:2 = linoleic acid and 18:3 = linolenic acid.

fraction was more closely linked to the cryopreservation ability. The myristic acid (C14:0) content in all lipid fractions was negatively correlated with post-thaw regeneration (Fig. 3B–E).

Discussion

Previous work showed that 14 days of pretreatment with a high sucrose concentration (0.4 *M*) was optimal to obtain high regeneration rates after cryopreservation of banana-proliferating meristems belonging to different varieties (Panis et al. 1996, 2002). This was confirmed with the experimental material in this study; post-thaw shoot regeneration rates of meristems following such sucrose pretreatment increased for three of the four given banana varieties (Fig. 1). As summarized in Table 1, sucrose pretreatment resulted in a considerable increase of both the individual and total soluble sugar contents in banana meristems. PCA showed that for the control meristems only, total sugar and particularly the sucrose content was closely linked to post-thaw shoot regeneration (Fig. 2A). This might indicate that sucrose and total sugar are main limiting factors to survive cryopreservation of the control meristems (see Fig. 1) and at least partially explain why sucrose pretreatment could increase the post-thaw shoot regeneration of some banana varieties. Some less abundant sugars and fatty acids such as oleic acid (C18:1) could also be linked to the post-thaw shoot regeneration of control meristems, suggesting that they may also be associated with the cryopreservation process.

After sucrose pretreatment, of all sugars detected, only inositol could be positively correlated to the post-thaw shoot regeneration (Fig. 2B). Indeed, whereas inositol accumulated to about 0.14% in the varieties that could survive cryopreservation, its accumulation was only 0.07% in Mbwazirume having no survival after cryopreservation (Table 1). It was already reported before that inositol (1,4,5) trisphosphate production is a common response to salt and hyperosmotic stresses in plants and that it may play an important role in processes leading to stress tolerance (Drobak and Watkins 2000). However, the accumulation of most other sugars (glucose, fructose, sucrose, stachyose, sorbitol or total sugar) could not explain the variability observed between the banana varieties in their response towards cryopreservation (Table 1, Fig. 2B). We can thus assume that a minimal amount of sugars in meristem cultures is needed to survive cryopreservation and that this level is reached for all varieties after sucrose preculture.

The protective role of sugars in plants is not completely understood. Sugar accumulation in plant tissues is a known strategy for plants to tolerate freezing stress. Moreover, sugars and more specifically sucrose were found to be powerful cryoprotectants in plants. A sugar treatment results in a slow reduction in moisture content (Uragami 1991), due to its osmotic effect and sucrose uptake, thus depressing the freezing point and the amount of freezable water. Sugars can also maintain the liquid crystalline state of the membrane bilayers and stabilize proteins under frozen conditions (Kendall et al. 1993). Especially disaccharides like sucrose seem to be very effective membrane protectors (Crowe et al. 1984). The protective effect of soluble sugars on membrane stability during drought and freeze-induced desiccation relies on their ability to form hydrogen bonds with the polar headgroups of membrane phospholipids and thus to replace water molecules bound to phospholipids in the hydrated state. Recently, it was reported that exogenous sucrose at low concentrations serves as a metabolic substrate for low-temperature-induced metabolic alterations, while at higher concentration, it has a direct cryoprotective effect on cellular membranes (Uemura and Steponkus 2003). Moreover, sugars are also known to have important functions as signalling molecules that control gene expression and developmental processes in plants (Sheen et al. 1999, Rolland et al. 2002, Atanassova et al. 2003).

Due to the lack of correlation between sugar concentrations and post-thaw regeneration, the limiting factor deciding on survival following sucrose pretreatment should be related to other parameters. In this work, we therefore focused further on sucrose-induced changes of membrane components such as sterols, fatty acids bound to different lipid fractions as well as free fatty acids in relation to cryopreservation.

Proteins, sterols and phospholipids are major membrane components. Plant sterols are crucial components of the cellular membranes in which they regulate their fluidity and permeability. Sterols with a small side chain (cholesterol and campesterol) are more effective in the stabilization of membranes and in the reduction of the membrane permeability than sterols with a bulky side chain (Si and St) (Grunwald 1974, Douglas and Walker 1983). In this study, we observed that sucrose pretreatment did not result in significant changes of cholesterol and campesterol levels for the four banana varieties under investigation (Table 2). However, we observed an increase in the St /Si ratio in banana-proliferating meristems as a result of the sucrose pretreatment (Table 3). Furthermore, such increase of St/Si ratio as a result of the sucrose pretreatment seemed to be related to the cryopreservation ability: the increase is less for the best survivor Chachaco and more for the bad survivor Mbwazirume (Table 3). The importance of the change

in St/Si ratio for cryopreservation is also revealed by the PCA (Fig. 3A). The results of the present work are in consistence with those in bean plants (Guye 1989). Here, a low St/Si ratio was recorded in the most chill-tolerant genotype (*Phaseolus coccineus cv.* Prizewinner), while an increase in this sterol ratio was recorded in the most chill-sensitive genotype (*Phaseolus aureus cv. Berken*). Experiments with soybean phosphatidylcholine bilayers indicated that all the plant sterols are able to regulate membrane fluidity, but with different efficiency (Schuler et al. 1990, 1991, Krajewski-Bertrand et al. 1992): Si and campesterol are the most efficient sterols for restricting the mobility of phospholipid fatty acyl chains, while St significantly reduces its ordering ability. An appropriate composition of sterols in the cell membrane is crucial for an optimal enzymatic activity, ion and metabolite transport or channelling, protein–protein and protein–lipid interactions, signal transduction and moreover essential to face fluctuating environmental conditions (Stallaert and Geuns 1994, Hartmann 1998, Laggner et al. 2003, Schaller 2003, Berglund et al. 2004). The present work suggests that a strong increase of the St/Si ratio in proliferating-banana meristems induced by a sucrose pretreatment is unfavourable to obtain high recovery following cryopreservation. An increase of the St/Si ratio has been correlated with the ageing of tissues of mung bean and tobacco (Geuns et al. 1997b) and different stress conditions (Geuns et al. 1997a). Moreover, it was proven that the increase of the St/Si ratio was correlated with an increase of membrane permeability of mung bean hypocotyl segments (De Clerck and Geuns 1988).

The composition of the phospholipid bilayer also plays a crucial role in the unique functional characteristics of plant cellular membranes. The majority of the fatty acids bound to lipids (between 80 and 88% for the control and between 73 and 79% for the pretreated meristems) were present in the phospholipid fraction (Table 4). Total fatty acid content of the neutral lipid and of the glycolipid and sphingolipid fractions significantly increased due to sucrose pretreatment for all four banana varieties (Table 4). However, a significant increase of the total fatty acids of the phospholipids was only observed in Obino L'Ewai. The 2-week treatment with a high concentration (0.4 *M*) of sucrose has thus, besides a direct effect on lowering of the freezing point of the cell solution and protection of membranes, also an indirect abiotic stress effect. Lipid changes under stress conditions were already observed for many plant species. For example, an increase in neutral lipid content and a decrease in polar lipid content were observed in safflower (Hamrouni et al. 2001) and in rape plants (Dakhma et al. 1995) under water-deficient conditions. Increase of neutral lipid content was observed in maize seedlings subjected to water deficits under field conditions (Navariizzo et al. 1989). These authors suggested that the accumulation of neutral lipid (triacylglycerols) may afford some adaptive advantages, because it represents a means of storing fixed carbon as a substantial energy reserve which can be readily utilized when drought stress is relieved. Indeed, in this study, we observed that the total fatty acid content of the neutral lipid fraction was more closely linked to the post-thaw shoot regeneration than those of the glycolipids and sphingolipids and phospholipids (Fig. 3). This may imply that accumulation of this neutral lipid can contribute to the improvement of post-thaw recovery following the cryopreservation in banana.

Complementary to the bound fatty acids, also the free fatty acid composition was determined. As summarized in Table 5, the total free fatty acid content significantly increased by the sucrose pretreatment in the three best survivors after cryopreservation. It was already suggested that free fatty acids, produced during water stress by action of lipases on polar lipids, could be stored in neutral lipid (triacylglycerols) in order to avoid oxidation by free radicals and reactive oxygen species (Dakhma et al. 1995). Although, in the present work, we could not prove a direct link between the different lipid fractions, we observed that fatty acids in the neutral lipid fraction and free fatty acids follow a similar pattern: (1) in the PCA, almost all fatty acids, except C14:0, are loaded on the left-hand side of the graphics, meaning that they are linked to cryopreservation ability in both cases (Fig. 3B, E) and (2) total fatty acids of the neutral lipid fraction and free fatty acids follow similar increases after a sucrose pretreatment, except for the free fatty acids of Mbwazirume which remain almost constant.

Changes of the fatty acid contents in banana meristems after sucrose pretreatment are linked to the different lipid fractions (Tables 4 and 5). Such changes have already been described in various plants that were subjected to stress conditions. It is now generally accepted that an increase of the proportion of unsaturated fatty acids in membrane lipids is favourable for tolerating abiotic stress (Graham and Patterson 1982, Hugly and Somerville 1992, Miquel et al. 1993, Kodama et al. 1994, Nishida and Murata 1996, Murata and Los 1997, Cyril et al. 2002, Orlova et al. 2003, Sakamoto et al. 2004). In our experiments, a high linoleic acid (C18:2) content after sucrose pretreatment could be linked to post-thaw regeneration. On the contrary, high amounts of myristic acid (C14:0) are negatively correlated with survival, whatever the lipid fraction (Fig. 3B–E).

In general, sucrose pretreatment increased the DBI of the neutral lipid fraction and decreased that of the other lipid fractions as well as that of free fatty acids. In the sucrose-pretreated meristems, a high DBI was recorded in Cachaco, the best survivor, while a low DBI was found in Mbwazirume, the worst survivor, whatever the lipid fraction. This is also revealed by the PCA: the DBI measured in the sucrose-pretreated meristems was indeed positively correlated to cryopreservation ability (Fig. 3B–E). This is especially the case for the phospholipid fraction and free fatty acids (Fig. 3D, E), which were both present in large quantities (Tables 4 and 5).

Although the sucrose pretreatment is required for a high survival after cryopreservation (effects on water contents of the cells and its possible protecting effects on proteins and membranes), it also induces stress in the banana meristems. The membrane properties observed in control tissues changed following sucrose pretreatment as seen by a change in sterol composition (increase of St/Si) and a decrease of the DBI of membranous fatty acids. The consequences of these two changes provoke a less optimal functioning of the membranes. The varieties in which the membrane changes were minimal were characterized as the best survivors after cryopreservation. This proves that maintenance of membrane integrity is of utmost importance in cryopreservation experiments. These results suggest that the application of specific sterols or fatty acids (preferably unsaturated ones) in addition to high-sucrose concentrations may be used to establishing new, more efficient plant cryopreservation protocols. Experiments are now executed with banana meristems as well with tissues derived from other plant species to confirm this hypothesis.

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