Rangel-Fajardo, M.A., Delgado-Alvarado, A., Córdova-Tellez, L., Zavaleta-Mancera, A., Villegas-Monter, A., López-Andrade, A.P. and Vidales-Fernandez, I. (2011), *Seed Sci. & Technol.,* **39**, 1-11

Accumulation of sugars in cacao (*Theobroma cacao* L.) seeds of three genetic origins and its relationship to desiccation tolerance

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(Accepted January 2011)

Summary

The main characteristic of recalcitrant seeds is their sensitivity to desiccation, but this sensitivity may vary, depending on genetic and environmental factors, and physiological and biochemical parameters, such as the presence of oligosaccharides of the storage tissue. In this study, tolerance of different cacao genotypes to desiccation was assessed and related to the concentrations of oligosaccharides in their seeds. Fruits of Pound 7 (Forastero), UF668 (Trinitario) and Carmelo (Criollo) were collected from a field plantation of the National Institute of Research in Forestry, Agriculture and Livestock, state of Tabasco, Mexico at 5, 6 and 7 months after flowering (maf). The seeds were dried to 300 g H_2O kg^{-1} fw, and seeds recently extracted from fruits (646 g $H_2O \cdot kg^{-1}$ fw) were used as controls. Sensitivity to desiccation was measured as seed germination (%) and raffinose, stachyose, sucrose, glucose, fructose, arabinose, sorbitol, galactose and mannitol were quantified in the embryonic axes and cotyledons using high precision liquid chromatography. Initial germination was 93%, which decreased 50% on average at 300 g $H_2O \cdot kg^{-1}$fw. The proportions of raffinose : sucrose differed; the genotypes UF668 and Pound 7, and seeds from the second harvest (six maf) had higher proportions of 0.25, 0.22 and 0.28 respectively, which were directly related to higher germination. We conclude that the proportion of raffinose : sucrose has a positive influence in sensitivity to desiccation.

Introduction

Most angiosperm seeds acquire the ability to tolerate severe reductions in moisture content at the end of their development; this allows them to remain viable and the embryos

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quiescent for long periods (Bewley, 1995). However seeds differ in their tolerance to dehydration and have been classified as orthodox, recalcitrant and intermediate (Roberts, 1973). Orthodox seeds tolerate losses in the water content of up to 90% and 95%, intermediate 90% to 88%, and recalcitrant seeds from 85% to 50% (Farrant *et al*., 1993; Gentil, 2001).

The ability to survive dehydration depends on diverse adaptation mechanisms that prevent cells from damage during water loss. In orthodox seeds, Blackman *et al*. (1992) suggest that maturation and acquisition of tolerance to dehydration may be related to the accumulation of sugars, proteins, lipids and other similar compounds. The accumulation of trehalose and sucrose (disaccharides) and raffinose (oligosaccharide) have been associated with membrane stabilization (Buitink *et al*., 2000) and cytoplasm vitrification during seed drying (Blackman *et al*., 1992; Sun *et al*., 1994). High concentrations of oligosaccharides and the formation of the viscose vitreous phase delays the crystallization of solutes, inhibits molecular mobility and restricts biochemical reactions (Koster, 1991), thus preventing deterioration of macromolecular structures during dehydration.

In recalcitrant seeds, the concentration of some monosaccharides such as glucose, fructose and galactose, can be higher than those reported in orthodox seeds (Steadman, 1996; Farnsworth, 2000), as in the case of *Landolphia kirkii* Dyer (Berjak *et al*., 1992) and *Avecina marina* L. (Farrant *et al*., 1993). These sugars are involved as promoters of Maillard reaction products (Koster and Leopold, 1988), which stimulate respiration and favour the formation of free radicals (Leprince *et al*., 1995). The presence of these sugars at high concentrations in tissues tends to favour sensitivity to desiccation (Koster and Leopold, 1988; Hoekstra *et al*., 1994). It has been stated that for recalcitrant seeds to be less sensitive to dehydration, the sole presence of specific sugars such as raffinose in proportion with sucrose is not sufficient (Koster and Leopold, 1988; Bruin and Leopold, 1991). However the mechanisms involved in seed protection against desiccation are still not completely understood. In this study we quantified the accumulation of sugars (raffinose, stachyose, sucrose, glucose, fructose, arabinose, sorbitol, galactose and mannitol) in seeds of three populations of cacao representing phenotypically contrasting genotypes from the state of Tabasco, Mexico. The relationship of these sugars to the degree of tolerance to desiccation was determined.

Materials and methods

Fruits of three cacao clones of three genetic origins were used: (1) criollo (local Mexican race) Carmelo, endemic (state of Tabasco, México) material selected by growers, (2) forastero Pound 7 (Peruvian origin, selected by J.F. Pound in 1938), and (3) trinitario hybrid UF 668, (Costa Rican origin, selected by United Fruit Co.). All the materials are protected in the germplasm bank (field plantation) at the National Institute of Research in Forestry, Agriculture and Livestock (INIFAP), Huimanguillo-Cárdenas, state of Tabasco, Mexico.

Fruits were harvested approximately five, six and seven months after flowering (maf), using the indicators colour and size. The fruits were washed and disinfected with 5%

sodium hypochlorite for 1 h. Seeds were immediately extracted and washed with abundant water to remove the mucilage. At each harvest, once the seeds of the genotypes were washed, they were dried in the laboratory on paper towels (Marquis Georgia-Pacific) at 20 to 26°C with 70-75% relative humidity. Seeds were monitored until they contained 300 g $H_2O \cdot kg^{-1}$fw. The water content of recently harvested seed (from 763 and 580 g $H_2O \cdot kg^{-1}$fw) was used as the reference. Water content of 10 seeds of each harvest date was measured according with Changrun and Sun (1999) then the samples were frozen with liquid nitrogen and stored at -40°C for sugar analysis.

Sugar extraction. Sugar was extracted from 50 and 250 mg of tissue from embryonic axes and cotyledons respectively, using three replicates for each sugar per tissue. The sugar levels in the embryo (scutellum + radicle hypocotyl axis) of the HS2 hybrid maize, an orthodox seed, were used as a reference. The tissue was placed in 15 mL tubes with 15 mL of 80% methanol (v/v), then they were boiled at 37°C for 10 min three times. The supernatant was recovered and 100 µL mannitol was added as an internal standard. The tubes were shaken in an orbital action shaker at 300 rpm for 20 min, and centrifuged at 3000 rpm at 4°C for 10 min. The supernatant was separated and the pellet was washed twice with 1 mL of 80% ethanol (v/v) and discarded, conserving the ethanol used for washing. An aliquot of 5 mL was mixed with 3 mL of chloroform; the mixture was shaken and centrifuged at 2000 rpm at 4°C for 3 min. The aqueous phase was separated and 50 mg of polyvinylpolypyrrolidone (PVPP, Sigma) was added and centrifuged for 10 min at 3000 rpm at 20°C. The supernatant was decanted into a centrifuge tube and 1 mL of distilled water was added and centrifuged again, recovering the supernatant. The obtained extracts were dried in a rotary evaporator (R-114 Büchi, Switzerland) at 37°C. The residues were re-suspended with 1 mL distilled water and filtered into columns (5 mL) containing 2 mL Dowex-1 and 3 mL Dowex-50 resin. The extract was stored at -20°C until analysis.

Sugar quantification. The extracts were analyzed in a high precision liquid chromatograph (HPLC, HP 1100, Hewlett Packard) with a Supelcogel Ca (Supelco 59305-U 30 cm × 7.8 mm, 9 µm) column and an Aminex (HPX-87C, 300 mm × 7.8 mm, 5 µm, Bio-Rad, 417467) column with a de-ionized water mobile phase (18 mohm) and flow of 0.6 mL min^{-1}, and a refraction index detector. Injection volume was 20 µL with analysis time of 30 min per sample.

Sugar concentration of the samples was determined with pattern curves, which were prepared with standard solutions of 0, 50, 100 and 150 mg mL^{-1} raffinose, stachyose, sucrose, glucose, fructose, arabinose, sorbitol, galactose and mannitol (All SIGMA brand).

Accumulation of sugars was expressed in $mg \cdot g^{-1}$fw and all of the samples were standardized in their weight at 300 g H_2O kg^{-1}fw using the equation proposed by Moreno (1996).

Desiccation tolerance. Measurement of sensitivity was based on germination tests on cacao seeds from each treatment (maf) at the moment of extraction and after drying to 300 g $H_2O \cdot kg^{-1}$fw. For the germination tests, 60 seeds were distributed into four replications of 15 seeds each and placed between paper towels 24 × 23.5 cm (Marquis Georgia-Pacific) moistened with distilled water, which were rolled up then placed in a germination

chamber at 30 ± 1°C (Mumbord and Brett, 1982). Germinated seeds (protruded radicle) were counted at 3 d and 7 d (King and Roberts, 1982).

Statistical analysis. The data were subjected to analysis of variance with SAS for Windows 6.12 software, using a complete randomized model with a factorial array of treatments, and means were compared with the Tukey test ($\alpha = 0.05$).

Results

Accumulation of sugars. Accumulation of monosaccharide, disaccharide and oligosaccharide groups in cacao seeds was a function of their maturity, genotype, level of dryness and seed tissue, as well as of their interaction (table 1). The seeds harvested 5 and 6 months after flowering (maf), showed higher levels of stachyose (9.9 $mg \cdot g^{-1}fw$), while raffinose increased after 6 months (0.27 $mg \cdot g^{-1}fw$) and then decreased. Accumulation of sucrose was highest (1.25 $mg \cdot g^{-1}fw$) at 5 maf, decreased after 6 months and increased again to 1.17 $mg \cdot g^{-1}fw$ after 7 months. The monosaccharides all showed a significant increase after 7 months, except for mannose, which had a decrease of up to 0.37 $mg \cdot g^{-1}fw$ with seed maturation.

The genotype UF668 accumulated 47.8 and 66.1% more stachyose and 29.6 and 70.4% more raffinose than Pound 7 and Carmelo, respectively. However, both UF668 and Pound 7 accumulated significantly more sucrose than Carmelo. Accumulation of monosaccharides varied among genotypes: UF668 was notable in the increase of mannitol, Pound 7 in fructose and sorbitol, and Carmelo in glucose. No significant differences among genotypes were found for accumulation of the monosaccharides galactose and arabinose. The effect of dehydration from 646 to 300 g H_2O $kg^{-1}fw$ was reflected in significant decreases (37, 5.6 and 3.7%) in stachyose, raffinose and sucrose, respectively, while galactose, mannitol and sorbitol increased 51, 24 and 25.6%. No significant changes in the accumulation of glucose, fructose and arabinose were detected as the result of seed dehydration (table 1).

Accumulation of sugars is also function of the seed tissue: the embryonic axes had 18.5, 70.4, 54.8, 56.5, 80 and 43.8% more stachyose, raffinose, sucrose, glucose, mannose and sorbitol, respectively, than the cotyledon, while there were no significant differences between the two tissues in content of galactose, fructose and arabinose (table 1). The cacao tissue had higher levels of stachyose, galactose, arabinose, mannose and sorbitol than did the maize tissue (table 1). In contrast, the maize tissue had higher raffinose, sucrose and glucose concentrations than did cacao.

The factors months after harvest, genotype and dryness had an interactive effect on the accumulation of the sugars evaluated, but in most of the cases no clear trends were detected. Thus, the interactions discussed in this section are those in which accumulation of the sugars showed greater differences. In the interaction harvest × genotype (figure 1), UF668 seeds maintained the highest accumulation of stachyose in all three harvests, compared with Pound 7 and Carmelo, but there was no defined pattern of changes in relation to degree of maturity. In Pound 7 stachyose concentration tended to increase, while in UF668 and Carmelo it decreased (figure 1 A). The genotypes UF668 and Carmelo achieved the highest accumulation of raffinose when harvested 6 maf, followed

Table 1. Averages of sugars determined in cacao seeds, results of the principal factors of variation.

Factor	Sugars (mg·g^{-1}fw)								
	Oligosaccharides		Disaccharides	Monosaccharides					
	St	Raf	Suc	Glu	Gal	Fru	Ara	Man	Sor
Harvest* (maf)									
5	9.9 a	0.07 c	1.25 a	0.37 b	0.13 b	0.04 b	0.13 b	1.31 a	0.31 b
6	9.31 a	0.27 a	0.76 c	0.46 b	0.17 b	0.03 b	0.09 b	0.75 c	0.28 b
7	6.39 b	0.19 b	1.17 b	1.49 a	0.51 a	0.13 a	0.21 a	0.94 b	0.52 a
MSD	2.12	0.02	0.06	0.14	0.27	0.04	0.08	0.16	0.1
Genotype**									
UF668	13.8 a	0.27 a	1.27 a	0.81 b	0.21 a	0.04 b	0.11 a	1.30 a	0.32 b
Pound 7	7.21 b	0.19 b	1.30 a	0.44 c	0.40 a	0.08 a	0.13 a	1.17 a	0.48 a
Carmelo	4.68 c	0.08 c	0.59 b	1.08 a	0.22 a	0.07 ab	0.18 a	0.52 b	0.31 b
MSD	1.45	0.02	0.06	0.14	0.27	0.04	0.08	0.16	0.1
Drying*									
Initial	10.5 a	0.18 a	1.08 a	0.77 a	0.18 b	0.08 a	0.16 a	0.86 b	0.32 b
300	6.61 b	0.17 b	1.04 b	0.77 a	0.37 a	0.06 a	0.13 a	1.13 a	0.43 a
MSD	1.45	0.009	0.04	0.096	0.19	0.03	0.05	0.11	0.07
Tissue*									
Axis	9.43 a	0.27 a	1.46 a	1.08 a	0.29 a	0.07 a	0.15 a	1.66 a	0.48 a
Cotyledon	7.69 b	0.08 b	0.66 b	0.47 b	0.26 a	0.06 a	0.13 a	0.33 b	0.27 b
MSD	1.45	0.009	0.04	0.096	0.19	0.03	0.05	0.11	0.07
Maize									
Axis	3.39	0.47	8.29	0.54	0.16	ND	0.04	1.88	0.07
Scutelum	ND	0.49	2.89	0.60	0.07	ND	ND	0.44	0.05

Means with the same letter in each variable and for each factor are not statistically different (Tukey, 0.05). maf: months after flowering. St: stachyose, Raf: raffinose, Suc: sucrose, Glu: glucose, Gal: galactose, Fru: fructose, Ara: arabinose, Man.: mannitol, Sor: sorbitol. *Means of all genotypes; **Mean of all other variables per genotype

by a decrease at 7 months even though at this time the raffinose concentration in UF668 surpassed that of Pound 7 and Carmelo by 61 and 62% (figure 1 B). In UF668 the concentration of sucrose and glucose increased at 6 and 7 maf (figure 1 C and D), while the concentration of sucrose in Pound 7 and Carmelo decreased in seeds harvested 6 maf, but increased in those harvested 7 maf. This trend was particularly clear for Pound 7. In Carmelo, the glucose concentration increased markedly after 7 months to give concentrations 84.6 and 44.5% higher than Pound 7 and UF668 (figure 1 D).

The interaction harvest × drying resulted in contrasting patterns of change in stachyose and raffinose when water content decreased to 300 g H_2O kg^{-1} fw (figure 2 A and B), with a reduction in concentration after 6 months followed by an increase, in contrast to the increase and then decrease in concentration seen in the fresh seeds.

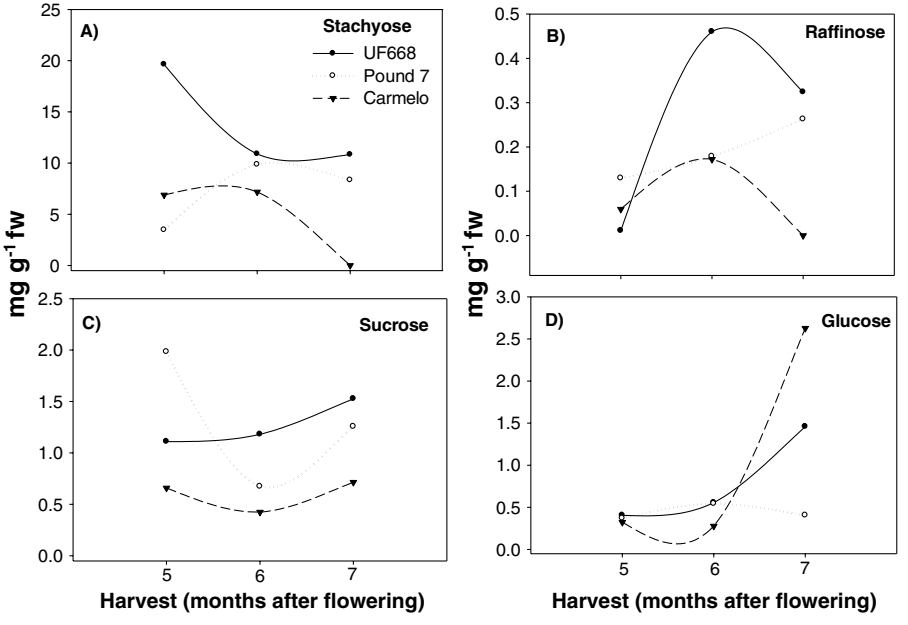

Figure 1. Accumulation of A) stachyose, B) raffinose, C) sucrose and D) glucose in cacao seeds, results of the interaction harvest × genotype. Each data point is the mean for each sugar per genotype. n = 3.

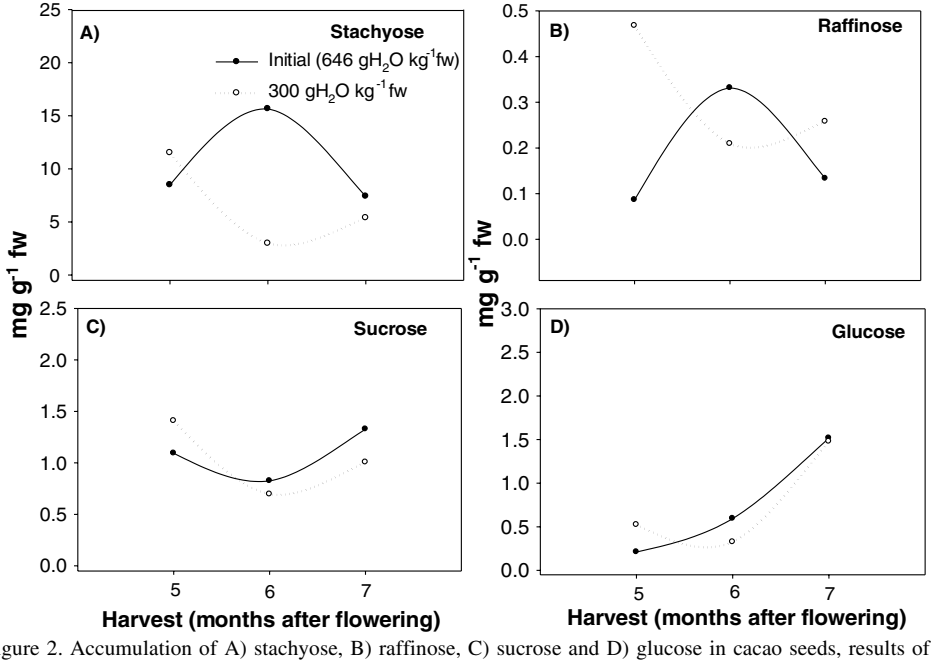

Figure 2. Accumulation of A) stachyose, B) raffinose, C) sucrose and D) glucose in cacao seeds, results of the interaction harvest × drying. Each data point is the mean of all genotypes. n = 3.

As a result the dried seeds had lower stachyose concentrations after 6 and 7 months, lower raffinose at 6 months and higher raffinose at 7 months (figure 2 A and B). The most conspicuous differences were obtained at 6 maf with decreases of 74.2 and 55.3% in the oligosaccharides stachyose and raffinose. Changes in sucrose and glucose showed similar patterns of change in both the fresh and dried seeds (figure 2 C and D) and the dried seeds tended to have lower concentrations of both sugars.

In the interaction genotype × drying, a decrease to 300 H_2O kg^{-1} fw resulted in no significant change in stachyose content in Carmelo; in Pound 7 it increased 4.4%, while in UF668 it decreased 60% (figure 3 A). The concentration of raffinose in UF668 increased 19% with drying, while in Pound 7 and Carmelo it decreased 9 and 65% (figure 3 B). Sucrose decreased by 20 and 42% in UF668 and Carmelo, while it increased by 30.33% in Pound 7. Glucose increased 35% in Carmelo and decreased 45% in UF668 but there was no marked change in Pound 7 (figure 3 C and D).

Desiccation tolerance of cacao

Sensitivity to desiccation was quantified by measuring germination response after reducing seed water content to 300 g $H_2O \cdot kg^{-1}$fw. Sensitivity to drying depend on seed maturity; seeds harvested 6 maf were most tolerant having a germination consistently up to 30% higher than those harvested at other times (table 2). The genotypes UF668 and Pound 7 were less sensitive to desiccation and germinated up to 30% more than Carmelo, the most sensitive genotype. Drying did, however, cause an overall 59% reduction in germination (table 2).

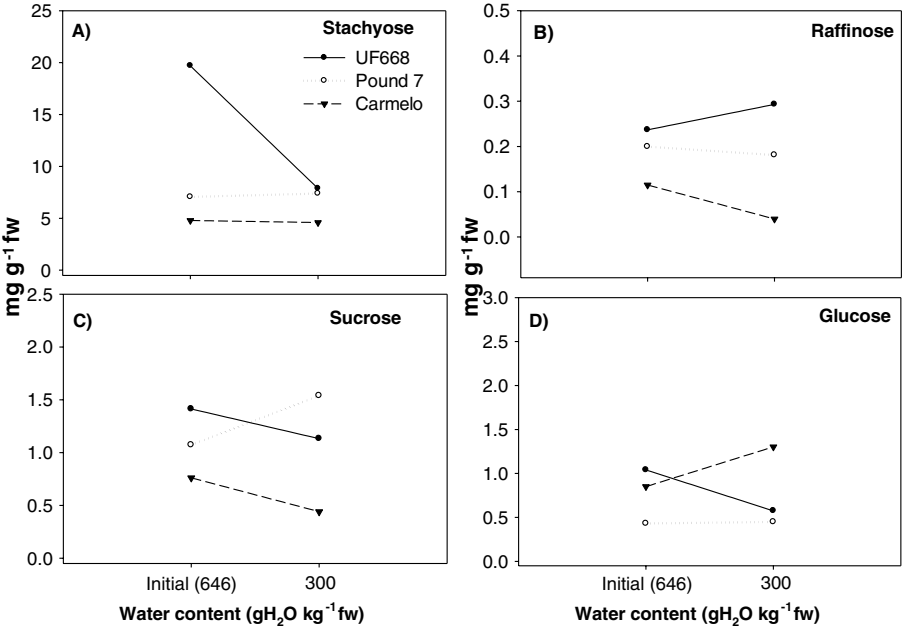

Figure 3. Accumulation of A) stachyose, B) raffinose, C) sucrose and D) glucose in cacao seeds, results of the interaction genotype × drying. Each data point is the mean for each sugar per genotype. n = 3.

Table 2. Single factors analyzed for the variable germination and proportions of raffinose : sucrose in cacao seeds.

Factors	Germination (%)	Raffinose : sucrose
Harvest (maf)*		
5	56 b	0.055 c
6	83 a	0.282 a
7	50 b	0.156 b
MSD	7.29	0.017
Genotype**		
UF668 (Trinitario)	74 a	0.251 a
Pound 7 (Forastero)	71 a	0.221 b
Carmelo (Criollo)	44 b	0.021 c
MSD	7.29	0.017
Drying*		
Initial (646 g $H_2O \cdot kg^{-1}$fw)	93 a	0.174 a
300 g $H_2O \cdot kg^{-1}$fw	34 b	0.155 b
MSD	4.95	0.012

Means with the same letter in each variable and for each factor are not statistically different (Tukey, 0.05). maf: months after flowering. *Means of all genotypes; **Mean of all other variables per genotype.

Discussion

The role of sugars in tolerance or sensitivity to desiccation has been amply discussed by several authors. It has been documented that oligosaccharides (stachyose and raffinose) and disaccharides are essential for cell cytoplasmic vitrification, which prevents damage to cell walls. This is due not so much to the presence of a particular sugar as to the proportion in which they are found (Brenac *et al*., 1997). The accumulation of some of the sugars quantified here has been determined in orthodox, intermediate and recalcitrant seeds. Sucrose, stachyose and raffinose increase considerably in orthodox seeds at the end of seed maturation and have been associated with desiccation tolerance. Bucheli *et al*. (2001) detected the presence of raffinose and stachyose in the axis of two cacao clones, in which maximums were quantified four and five maf. This fluctuation in accumulation was also detected in the present study. Accumulation of stachyose was 97% higher than raffinose, a finding that does not agree with the report of Steadman *et al*. (1996), who found values of up to 13.4 $mg \cdot g^{-1}$ dw for raffinose and 56.2 $mg \cdot g^{-1}$dw for sucrose. This difference may be due to different techniques of extraction and analysis used. Most of the monosaccharides reached their peaks 7 maf, coinciding with the trend of dry weight accumulation observed in the present study (data not shown). We detected that the highest accumulation of raffinose in seeds harvested 6 maf coincided with a higher rate of germination. Likewise, the highest accumulation of stachyose, raffinose and sucrose concurred with the highest rate of germination of UF668.

Differences in accumulation of sugars among genotypes have been reported by several authors (Dussert *et al*., 1998; Hong *et al*., 2000), but there are few studies that assess

intraspecific differences. Our study, particularly, shows differences in accumulation of sugars in genotypes of the same species, with UF668 surpassing other genotypes especially in oligosaccharides and disaccharides, which are associated with tolerance to drying. The greater accumulation of these sugars in the axes compared with the cotyledons coincides with that reported by Steadman *et al*. (1996). However, in orthodox-type species, such *Acer platanoides* L.*,* the concentrations of stachyose, raffinose and sucrose increased when water content decreased (from 520 g $H_2O \cdot k^{-1}$fw to 70 g $H_2O \cdot k^{-1}$fw) (Hong *et al*., 2000), while our study found that the concentrations of these sugars decreased (37% in the case of stachyose, 5.6% in raffinose and 3.7% in sucrose) when water content was reducing. This is possibly associated with the recalcitrant behaviour of the seed species analyzed.

No relationship was found between accumulation of monosaccharides and the tolerance to drying (300 g $H_2O \cdot kg^{-1}$fw) detected in the cacao seeds of three genetic origins. Since some studies conducted on recalcitrant seeds have demonstrated that the concentrations of sugars can surpass those reported in orthodox seeds (Steadman, 1996; Farnsworth, 2000), the role of sugars in desiccation tolerance has been questioned. The proportion of raffinose : sucrose, however, has been found to correlate positively with tolerance to desiccation (Koster and Leopold, 1988; Kuo *et al*., 1988). In our study, it is clear that the seeds harvested 6 maf surpassed those harvested 5 and 7 maf with 80.5 and 44.7% more raffinose and sucrose, and these seeds also had the highest germination. In terms of genotypes, UF668 surpassed Pound 7 (12%) and Carmelo (92%) the proportion of raffinose : sucrose (table 2). Also, before drying, the highest proportion of raffinose : sucrose obtained coincided with the highest germination, confirming the role of the proportion of raffinose to sucrose in desiccation tolerance. Nevertheless, the levels of accumulation in cacao are individually low compared with the results obtained from the maize axis, which had 43% more raffinose and 84% more sucrose than the cacao genotype UF668 (table 1), corroborating the importance of these sugars in tolerance to drying to which maize is characteristically subjected.

It has been found that the ratio oligosaccharides : sucrose is 1.04 in beans (Bailly *et al*., 2001), 0.6 in wheat (Black *et al*., 1996), and 1.0 in peas (Corbineau *et al*., 2000). However, some authors (Bruni and Leopold, 1992; Sun *et al*., 1994) believe that the presence of these sugars is not sufficient to confer desiccation tolerance to recalcitrant seeds, arriving to the conclusion that the mechanisms of protection against drying are still not entirely clear. Bailly *et al*. (2001) indicate that the proportion of oligosaccharides : sucrose correlates highly with tolerance to drying in beans, while Lin and Huang (1994), who analyzed sugar contents in 11 recalcitrant species, concluded that the concentrations of oligosaccharides and their proportions in its relation to sucrose are not determinant insensitivity to desiccation for these recalcitrant seeds. On the other hand recently Fang *et al*. (2009) pointed out that cocoa somatic embryos could survive desiccation up to 260 g H_2O kg^{-1} dw without loss of viability, which was remarkably enhanced by treatment with sucrose; it was hypothesized that this disaccharide works as an extracellular cryoprotectant. The results of our study, however, suggest that both the quantity and the proportions of sugars such as raffinose and sucrose may be involved in tolerance to drying and seed germination of *T. cacao*.

Conclusions

The accumulation of sugars varied among seeds harvested at different stages of maturity. Concentrations were higher in the genotypes UF668 and Pound 7. Drying caused a reduction in the accumulation of raffinose, stachyose and sucrose. The highest concentrations of raffinose, stachyose and sucrose, as well as the highest raffinose : sucrose ratio coincided with lower sensitivity to desiccation and higher rates of germination found in seeds harvested six maf, in the genotypes UF668 and Pound 7, and in seeds before drying. No relationship was detected between sensitivity to desiccation and accumulation of the monosaccharides studied.

Acknowledgements

The authors wish to thank Dr Victor Gonzalez Hernandez and Dra Ana Bertha Cruz Pérez for giving the facilities of equipment and the excellent technical assistance in the analysis of sugars.

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