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Acquisition of desiccation tolerance in developing oil palm (*Elaeis guineensis* Jacq.) embryos *in planta* and *in vitro* in relation to sugar content

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Abstract

Relationships between desiccation tolerance and dry matter, water and sugar contents were studied throughout the development of oil palm (*Elaeis guineensis* Jacq.) zygotic embryos and in immature embryos cultured on a sucrose-enriched medium. Embryo dry weight during *in planta* development increased between 80 and 140 d after pollination (DAP) and was then stable until maturity. Embryos underwent dehydration until 120 DAP, but their moisture content remained high at maturity (*c.* 2 g H_2O g^{-1} DW). Desiccation tolerance was acquired between 83 and 104 DAP, and was positively correlated with embryo age and dry weight, and negatively correlated with initial water content during this period. Sucrose, the main soluble sugar present throughout embryo development, accounted for an average of 24% of the dry weight. Glucose and fructose contents decreased to less than 1 mg g^{-1} DW in embryos at maturity. At 117 DAP, as embryos became tolerant to desiccation, the monosaccharides/sucrose ratio fell to 0.015 and raffinose was detected. Stachyose appeared later in 147-day-old embryos and accumulated until shedding. *In vitro* culture of immature embryos in the presence of high sucrose concentrations (350 and 700 mM) resulted in an increase in their dry weight and a decrease in their water content, and induced the acquisition of desiccation tolerance. Under these conditions, sucrose accumulated in embryos to 30–40% on a dry weight basis, but neither raffinose nor stachyose was detected. Acquisition of desiccation tolerance by oil palm immature embryos was associated both *in planta* and *in vitro* with an accumulation of dry matter, a reduction of moisture content, and a fall in the monosaccharides/sucrose ratio. *In planta*, survival to dehydration was also related with the deposition of oligosaccharides whereas *in vitro*, it was related with high sucrose accumulation. The role of sugars in the acquisition of desiccation tolerance in oil palm embryos is discussed.

Keywords: dehydration tolerance, *Elaeis guineensis* Jacq., raffinose, seed development, stachyose, sucrose

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Introduction

Desiccation is a natural event occurring at the end of development of orthodox seeds, i.e. seeds that tolerate dry and cold storage. In contrast, recalcitrant seeds are generally damaged by dehydration and cannot be stored for extended periods (Roberts, 1973). Oil palm seeds can be stored for 2–3 years, and isolated oil palm embryos can tolerate desiccation (Grout *et al.*, 1983). However, given the reduced viability at cool storage temperatures (0 and –20°C) and at low moisture content for some cultivars, oil palm seeds appear to display an intermediate storage behaviour, being neither orthodox nor recalcitrant (Ellis *et al.*, 1991).

Various processes have been mentioned in connection with the acquisition of desiccation tolerance, including modifications of intracellular characteristics, such as reduction of the degree of physical vacuolation, intracellular de-differentiation, 'switching off' of metabolism, presence of free-radical scavenging systems and oleosine, and repair mechanisms during rehydration (reviewed by Pammenter and Berjak, 1999; Oliver *et al.*, 2001). Specific compounds, such as heat-stable and late-embryogenesis abundant (LEA) proteins (Kermode, 1995; Wolkers *et al.*, 2001), amphipathic molecules (Hoekstra *et al.*, 1997) and soluble sugars and cyclitols (Koster and Leopold, 1988; Crowe *et al.*, 1992; Horbowicz and Obendorf, 1994), have been proposed as protective substances, especially in the dehydrated

state. Acquisition of desiccation tolerance has been associated with accumulation of sucrose and oligosaccharides during seed development of various orthodox species, i.e. *Brassica campestris* (Leprince *et al.*, 1990), maize (Chen and Burris, 1990; Brenac *et al*., 1997), wheat (Black *et al*., 1996), lupin (Gorecki *et al.*, 1997) and pea (Corbineau *et al*., 2000). However, sucrose and oligosaccharides have also been detected in recalcitrant seeds of *Avicenia marina* (Farrant *et al*., 1993) and *Quercus robur* (Finch-Savage *et al*., 1993; Finch-Savage and Blake, 1994).

During seed drying, the formation of a cytoplasmic glassy matrix, in which molecular mobility is reduced, is an important process in the protection of membranes and macromolecules (Williams and Leopold, 1989; Buitink *et al*., 1999). Soluble sugars, sucrose and oligosaccharides, may form a glass during drying, resulting in molecular stabilization of the aqueous state during desiccation (Williams and Leopold, 1989; Wolkers *et al.*, 1998). Moreover, vitrification of the intermembrane sugar solution could enhance the resistance of the lipid phase transition in membranes during dehydration (Bryant *et al*., 2001). In contrast, monosaccharide contents correlate with desiccation sensitivity (Hoekstra *et al*., 2001). Fructose and glucose could be involved in Maillard reactions and would be a source of hydroxyl radicals in dehydrated tissues, thus favouring the appearance of browning (Koster and Leopold, 1988; Van der Toorn and McKersie, 1995). In contrast to orthodox seeds, monosaccharide levels are not reduced in the later stages of development in recalcitrant seeds (Farrant *et al*., 1993; Finch-Savage *et al*., 1993).

Desiccation tolerance is acquired during late embryogenesis in seeds still on the mother plant and is lost after germination (Vertucci and Farrant, 1995). The main regulatory factors through which the seed environment controls embryo development are thought to be abscisic acid (ABA) and restricted water uptake (Kermode, 1995). Artificial induction of desiccation tolerance has also been achieved after slow or fast drying of immature seeds or embryos of maize and wheat (Bochicchio *et al*., 1994; Brenac *et al*., 1997; Black *et al.*, 1999) and of somatic embryos of carrot and alfalfa (Tetteroo *et al*., 1994). Osmotic stress, using polyethylene glycol, coupled with ABA, promoted maturation and desiccation tolerance of white spruce somatic embryos (Attree *et al.*, 1992). High sucrose concentrations in the maturation medium of alfalfa somatic embryos delayed germination, allowed accumulation of dry matter to continue and induced tolerance to drying (Anandarajah and McKersie, 1990).

The relationships between the onset of desiccation tolerance and changes in dry weight, water and sugar contents have not been reported in developing oil palm embryo. The aims of the present work were: (1) to investigate the change in desiccation tolerance of the oil palm embryo throughout development *in planta*; (2) to study the changes in soluble sugars during seed development; and (3) to evaluate the effect of sucrose concentration during culture of immature embryos on acquisition of desiccation tolerance in relation to carbohydrate content.

Materials and methods

Plant materials and in vitro *culture of immature embryos*

Oil palm (*Elaeis guineensis* Jacq.) zygotic embryos were excised from hybrid *dura* × *pisifera* seeds (category C1001) obtained after controlled pollination. Seeds, harvested between 1995 and 1998, were supplied by INRAB (Institut National de Recherche Agronomique du Bénin, Station de Recherche sur le Palmier à Huile) located in Pobé, Benin. For each developmental stage studied, embryos originated from groups of different trees. Seeds were collected after endosperm solidification, i.e. approximately 80 d after pollination (DAP), until fruit shedding at *c*. 180 DAP. Embryos were isolated from seeds after endosperm sterilization for 5 min in 4.8% sodium hypochlorite.

Immature embryos, harvested at 91 DAP, were cultivated *in vitro* for 14 d in Petri dishes (55 mm diameter) (one or two embryos per dish) containing 10 ml basal tissue culture medium (Aberlenc Bertossi *et al*., 1999) supplemented with casein hydrolysate (500 mg l^{-1}), agar (8 g l^{-1}) and sucrose at three different concentrations: 88 mM, (standard culture medium), 350 or 700 mM, at 27°C in the dark.

Drying conditions and water content determination

Isolated embryos (three samples of 10–20 embryos) were dried for 4 h in glass containers (120 ml) in the presence of 40 g of silica gel. Embryos were freeze-dried for determination of water content, which was calculated on a dry weight basis. Results are expressed as the mean moisture content obtained from three replicates of 10–20 embryos.

Determination of embryo survival

After drying, the embryos were placed in 55 mm diameter Petri dishes (one or two embryos per dish) containing 10 ml basal culture medium containing sucrose (88 mM), naphthaleneacetic acid (0.054 µM), kinetin (0.46 µM) and Phytagel (2 g l^{-1}). Survival was determined as the mean percentage of embryos

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resuming elongation (increase in size) after 14 d of culture at 27°C in the dark.

Soluble carbohydrate measurements

Soluble sugars were extracted according to Ky *et al.* (2000). Freeze-dried embryos were ground, and 10 mg of the powder was homogenized in 10 ml distilled water containing lactose (500 mg l^{-1}) as an internal standard; the extract was then heated for 15 min at 60°C. Colloidal material in aqueous extracts was precipitated with the Carrez solutions, as described by Trugo and Macrae (1984). Samples were then centrifuged for 10 min at 2600 *g*, and the supernatant was filtered (0.2 µm pore, Nalgene, Rochester, NY, USA) before being analysed using anion exchange chromatography coupled with pulsed amperometric detection (Dionex S.A., Voisins Le Bretonneux, France). After dilution, 25 µl samples were injected on to a Carbo-Pac PA1 precolumn and column, and eluted with sodium hydroxide (150 mM) at a flow rate of 1 ml min^{-1}, using a GP 40 pump (Dionex). Identification and quantification of the soluble sugars were performed by comparison of retention times and peak areas with standards (Sigma-Aldrich, Saint-Quentin Fallavier, France), relative to those of the lactose internal standard. Results were expressed as mg of sugars g^{-1} of dry weight of embryos and correspond to the means of measurements carried out with three extracts.

Statistical analysis

Lines representing least-squares adjustments were applied to data concerning the changes in dry weight and water content throughout embryo development (Fig. 1), using Statistica software (version 5.1, Statsoft, Maisons-Alfort, France). Linear regressions were applied to data from embryo desiccation survival during development *in planta* (Fig. 2). The effects of embryo age and sucrose concentration in the medium on dry weight, water content, survival and sugar contents (Tables 1–4) were analysed by ANOVA, and the significance of the means was compared by the Newman–Keuls test (Newman, 1939; Keuls, 1952).

Results

Embryo development in planta

Figure 1 shows the changes in dry weight and moisture content of the oil palm embryo during seed development *in planta*. Embryos were very small (about 0.5 mg DW) at 80–85 DAP and their mean moisture content was 4.5 g H_2O g^{-1} DW. Dry weight increased approximately linearly from 80 to 120 DAP, reached 1.6 mg $embryo^{-1}$ at 140 DAP, and then remained stable until fruit shedding at about 180 DAP (Fig. 1A). Reserve accumulation in the embryo was associated with a decrease in moisture content to 2 g g^{-1} DW at 120 DAP (Fig. 1B). Water content then fluctuated around this value until maturity. Despite variability in embryo dry weight and water content among the different seed batches, a significant negative linear relationship exists ($r = -0.92$) between the two parameters throughout embryo development.

Acquisition of desiccation tolerance and changes in soluble carbohydrates during embryo development

All freshly isolated embryos from seeds harvested from 83 to 180 DAP were able to germinate on standard culture medium (data not shown). Effects of a 4 h dehydration in the presence of silica gel on embryo desiccation tolerance, evaluated by survival rate after 14 d of culture, depended on their age (Fig. 2). Between 83 and 104 DAP, embryos progressively acquired desiccation tolerance. After dehydration for 4 h, embryo water content averaged 0.06 g H_2O g^{-1} DW. From 83 to 104 DAP, significant linear relationships existed between desiccation tolerance of oil palm embryos and their age ($r = 0.79$, Fig. 2), their dry weight ($r = 0.90$) and their initial water content ($r = -0.97$). Beyond 110 DAP, embryo water content after 4 h of dehydration was 0.11 g H_2O g^{-1} DW, and embryos were almost completely tolerant (Fig. 2). The onset of desiccation tolerance preceded the end of reserve accumulation (cf. Fig. 1A) and the cessation of water loss of the embryo (cf. Fig. 1B) by a few days.

From 89 to 179 DAP, soluble carbohydrate contents accounted for 25–28% dry weight of the embryo (Table 1) and sucrose was the main sugar. Sucrose varied between 224 and 248 mg g^{-1} DW throughout embryo development. At 89 DAP, embryos contained no detectable raffinose or stachyose. Glucose and fructose contents declined between 89 and 147 DAP and the monosaccharides/sucrose ratio reached 0.015 in the desiccation-tolerant embryos (Table 1). Raffinose was detected in embryos at 117 DAP, after they became tolerant to desiccation, and gradually accumulated to reach 35 mg g^{-1} DW at maturity. Stachyose appeared by 147 DAP and increased until 179 DAP. The oligosaccharides/sucrose ratio increased during seed development, reaching 0.074 at the onset of desiccation tolerance and 0.20 at maturity (Table 1).

Induction of desiccation tolerance of immature embryos during in vitro *culture as related to soluble carbohydrates*

At 91 DAP, embryos had a mean dry weight of 0.7 mg and a mean water content of 3.7 g H_2O g^{-1} DW (Fig. 1). *In vitro* culture of such immature embryos in the

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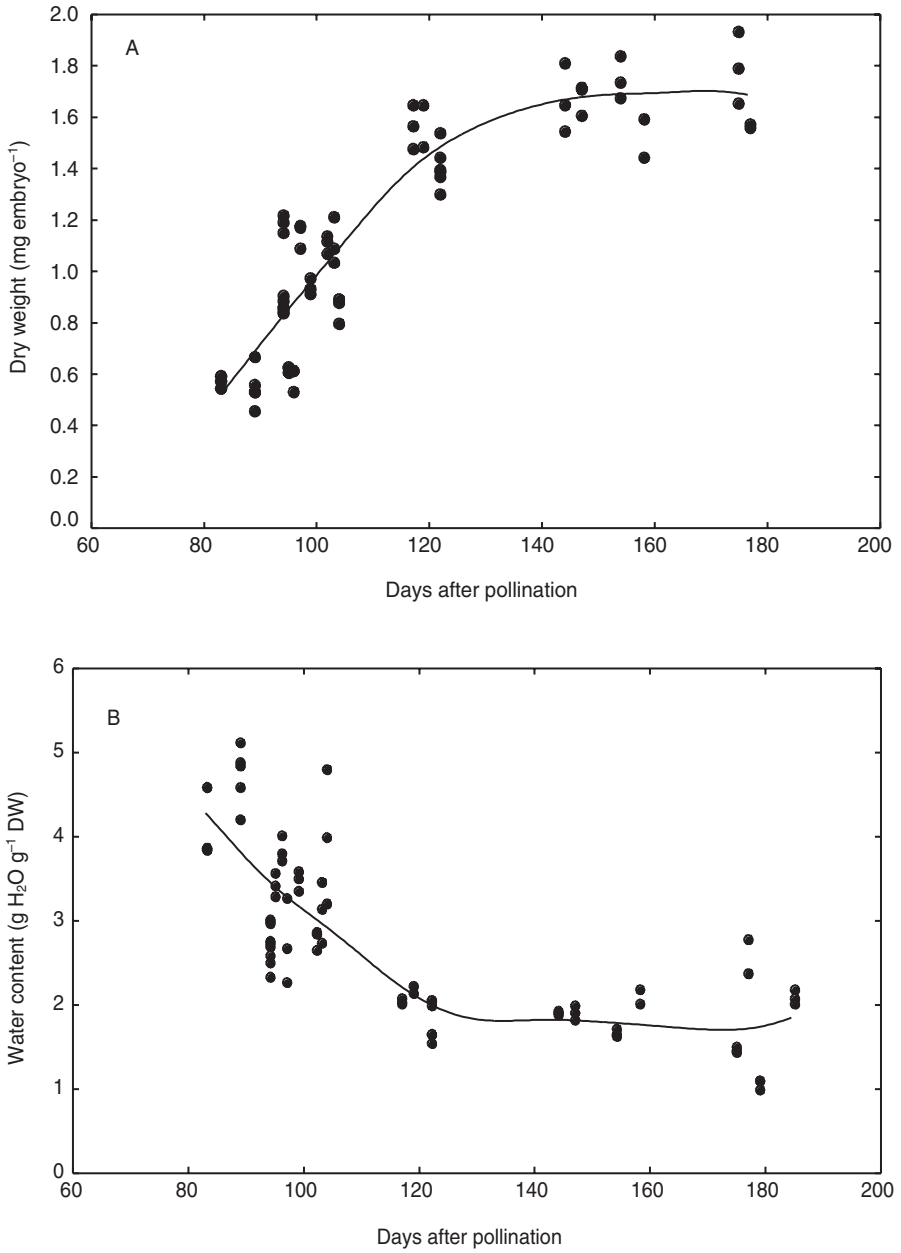

Figure 1. Changes in dry weight (A) and water content (B) of oil palm embryos during seed development. Each point corresponds to measurements carried out with 10–20 embryos excised from seeds harvested between 1995 and 1998. Lines represent least-squares adjustments.

standard medium containing low sucrose (88 mM) for 14 d resulted in an increase in dry weight and water content (Table 2). These culture conditions favoured embryo hydration and allowed them to elongate (data not shown), suggesting that increased dry weight was probably due to growth rather than to dry matter accumulation.

When 91-day-old embryos were cultured for 14 d on higher sucrose concentrations (350 and 700 mM), their dry weights increased from 0.7 mg at 91 DAP to 2.08 and 1.93 mg, respectively (Table 2), and water contents decreased from 3.7 g H_2O g^{-1} DW at 91 DAP to 2.43 and 1.61 g H_2O g^{-1} DW, respectively. In this case, embryos were dehydrated and did not elongate (data not shown), which suggests that high sucrose favours dry matter accumulation and maturation, rather than growth.

After 4 h of desiccation in the presence of silica

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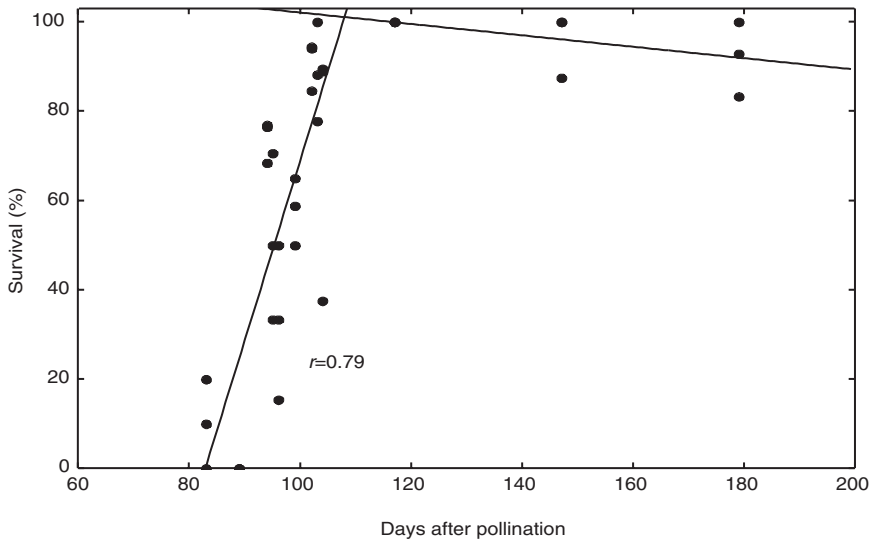

Figure 2. Changes in desiccation tolerance of embryos of oil palm during seed development. Survival was expressed as the percentage of viable embryos after 4 h of drying in the presence of silica gel as a function of their age. Each measurement was performed on 10–20 embryos. Lines represent linear regressions, and *r* is the coefficient of correlation. Embryo survival was scored after culture for 14 d at 27°C.

Table 1. Soluble carbohydrate contents, monosaccharides/sucrose and oligosaccharides/sucrose ratios in oil palm embryos at various stages of development. Means of three measurements are presented

	Soluble carbohydrate (mg g^{-1} DW)						
Age (DPA)	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Monosaccharides/ sucrose	Oligosaccharides/ sucrose
89	2.60 b	6.05 c	240 c	0 a	0 a	0.036	0
117	1.05 a	2.48 b	233 b	17 b	0 a	0.015	0.074
147	0.62 a	0.54 a	248 d	31 c	9.6 b	0.005	0.162
179	0.82 a	0.61 a	224 a	35 d	10.9 b	0.006	0.204

Letters indicate homogeneous groups, $P < 0.05$ (Newman–Keuls test).

Table 2. Dry weight and water content of oil palm embryos harvested at 91 DAP and cultivated *in vitro* for 14 d at 27°C on a medium containing 88, 350 or 700 mM of sucrose. Means of three measurements are presented

Sucrose (mM)	Dry weight (mg)	Water content (g g^{-1} DW)
88	2.72 b	5.00 c
350	2.08 a	2.43 b
700	1.93 a	1.61 a

Letters indicate homogeneous groups, $P < 0.05$ (Newman–Keuls test).

gel, embryos cultured on standard medium containing 88 mM sucrose were 84% viable but not totally dehydrated (0.53 g g^{-1} DW; Table 3). Because of incomplete dehydration of embryos, no conclusions can be made about the acquisition of desiccation tolerance in such conditions.

In contrast, after 4 h of desiccation, embryos supplied with high sucrose (350 or 700 mM) were more dehydrated (0.17–0.18 g g^{-1} DW, Table 3), and embryo water contents were close to those of 110–180 DAP embryos developed *in planta*, dehydrated in the same conditions (0.11 g H_2O g^{-1} DW). Immature embryos cultured *in vitro* for 14 d on 350–700 mM sucrose exhibited high survival (85–100%; Table 3).

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Table 3. Water content and percentage survival after dehydration on silica gel for 4 h, soluble carbohydrate contents, and monosaccharides/sucrose and oligosaccharides/sucrose ratios in oil palm embryos harvested 91 DAP and cultivated *in vitro* for 14 d at 27°C on a medium containing 88, 350 or 700 mM of sucrose. Means of three measurements are presented

			Soluble carbohydrate (mg g^{-1} DW)						
Sucrose (mM)	Water content (g g^{-1} DW)	Survival (%)	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Monosaccharides/sucrose	Oligosaccharides/sucrose
88	0.53 b	84 a	3.97 b	3.22 a	225 a	0 a	0 a	0.032	0
350	0.18 a	100 a	3.24 b	1.34 a	303 b	0 a	0 a	0.015	0
700	0.17 a	85 a	1.75 a	1.12 a	402 c	0 a	0 a	0.007	0

Letters indicate homogeneous groups, $P < 0.05$ (Newman–Keuls test).

Therefore, the high sucrose supply induced the maturation programme and acquisition of desiccation tolerance.

Some differences in glucose, fructose and sucrose contents were observed between 89-day-old embryos developed *in planta* (Table 1) and 91-day-old- embryos cultured for 14 d *in vitro* on a medium containing 88 mM sucrose (Table 3), but the monosaccharides/sucrose ratio was similar (0.032–0.036) and oligosaccharides were not detected. As compared to 91 DAP immature embryos (Table 1), culture on high-sucrose media (350 and 700 mM) resulted in a decrease in glucose content and an accumulation of sucrose (Table 3). In sucrose-supplied embryos (350 and 700 mM), the sucrose content increased to 30 and 40% of the dry weight, respectively, and the monosaccharides/sucrose ratio decreased to 0.015 and 0.007, respectively. These two latter values were equivalent to those observed in embryos developed *in planta* after the acquisition of desiccation tolerance and at maturity (117 and 179 DPA, respectively, Table 1). Raffinose and stachyose were not detected during *in vitro* culture of the immature embryos (Table 3).

Discussion and conclusion

The maturation phase of oil palm embryo development is characterized by a filling phase until approximately 120 DAP, followed by a cessation of the accumulation of storage products, as reported for most orthodox seed species (Bewley and Black, 1983). During the filling phase, the decline in embryo water content may be due to the progressive accumulation of storage products, lipids, proteins (Ferdinando *et al*., 1985; Morcillo *et al*., 1998) and soluble sugars (mainly sucrose). By 120 DAP, seed water loss ceased and mature embryos subsequently contained 1.8–2 g H_2O g^{-1} DW. This feature is generally not encountered in seeds of orthodox species, in which the final stage of development is characterized by marked dehydration, with shedding from the mother plant at a low water content (Bewley and Black, 1983). In their natural habitat of origin in West Africa, oil palm seeds remain dormant after fruit shedding until the rainy season (Hartley, 1988). During their development *in planta*, oil palm embryos acquire tolerance to desiccation (Fig. 2). These results are in agreement with those obtained by Grout *et al.* (1983) with mature excised embryos desiccated in a sterile air-flow. Desiccation tolerance was acquired by 110 DAP, before the end of the filling phase. Acquisition of desiccation tolerance *in planta* and *in vitro* was associated with an accumulation of dry matter and a reduction of water content. The slow dehydration of the embryos may induce the synthesis of specific compounds (ABA, LEA proteins or antioxidants, reviewed by Vertucci and Farrant, 1995; Pammenter and Berjak, 1999) involved in desiccation tolerance.

Desiccation tolerance was associated with a reduction in monosaccharide contents and a decrease in the (monosaccharides/sucrose) ratio, both *in planta* (Table 1) and *in vitro* with high sucrose supply (Table 3). Sinniah *et al.* (1998) also found a negative correlation between monosaccharide content and the ability of *Brassica* seeds to tolerate desiccation. The decrease in monosaccharide substrate might limit respiratory metabolism, which has the potential for uncontrolled free-radical processes under conditions of water stress (Pammenter and Berjak, 1999). However, high monosaccharide contents might be an indicator of a highly active metabolism rather than being detrimental *per se* (Hoekstra *et al*., 2001).

The assumption of the role of sugars in the protection of cellular components during drying was largely supported (Koster and Leopold, 1988; Hoekstra *et al.*, 2001). Carbohydrates would be important factors contributing to the glassy state (Wolkers *et al*., 1998). Throughout oil palm embryo development *in planta*, sucrose accumulated in embryos from day 89 onwards, and accounted for an average 24% of the dry weight (Table 1). Sucrose was detected in embryos before they became tolerant, and may be necessary, but not sufficient, to confer desiccation tolerance. *In vitro*, desiccation tolerance induced in immature oil palm embryos by culture in the presence of high sucrose was associated with substantial accumulation of this sugar, which

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represented 30–40% of the dry weight (Table 3). In maize and wheat, the acquisition of desiccation tolerance throughout embryo development was associated with the appearance of raffinose and an increased oligosaccharides/sucrose ratio (Bochicchio *et al*., 1994; Black *et al*., 1996; Brenac *et al*., 1997). Similar results were obtained in oil palm (Table 1). Acquisition of desiccation *in planta* between 89 and 117 DAP was associated with the appearance of raffinose, but this sugar represented only 1.7% of the dry weight. *In vitro,* desiccation tolerance induced in immature oil palm embryos by culture in the presence of high sucrose was not related to oligosaccharide accumulation (Table 3). Likewise, the induction of tolerance to desiccation in immature maize embryos by an application of ABA (Bochicchio *et al*., 1994), or in wheat by slow dehydration (Black *et al*., 1999), was not associated with oligosaccharide synthesis. Recent observations do not support the hypothesis that oligosaccharides affect stability of the intracellular glasses, but they might be considered as markers of seed maturity or serve as storage reserves (Buitink *et al*., 2000).

In the developmental programme set up *in planta*, desiccation tolerance was associated with the presence of sucrose and oligosaccharides, whereas in the *in vitro* cultivated embryos, this phenomenon was associated with a large deposition of sucrose and the absence of oligosaccharides. Therefore, neither sucrose accumulation nor oligosaccharide synthesis was consistently associated with desiccation tolerance. In addition to the presence of carbohydrates, several other mechanisms of protection may be involved in close coordination, allowing survival in the dehydrated the state (Oliver *et al*., 2001).

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