In Vitro Cell. Dev. Biol.—Plant 33:163–172, July–August–September 1997

1071-2690/97 $05.00 + 0.00

A COMPARISON BETWEEN *Theobroma cacao* L. ZYGOTIC EMBRYOGENESIS AND SOMATIC EMBRYOGENESIS FROM FLORAL EXPLANTS

L. ALEMANNO, M. BERTHOULY, AND N. MICHAUX-FERRIERE

CIRAD-CP, BP 5035, 34032 Montpellier Cedex 1, France (L. A., M. B.), and CIRAD-GERDAT, BP 5035, 34032 Montpellier Cedex 1, France (N. M.-F.)

(Received 31 December 1996; accepted 2 April 1997; editor L. C. Fowke)

SUMMARY

In order to improve the late phases of *Theobroma cacao* L. embryogenesis from tissues of maternal origin, zygotic embryogenesis and somatic embryogenesis were compared, with respect to morphological, histological, and physiological parameters. Zygotic embryogenesis could be divided into three steps: (a) embryogenesis *sensu stricto*, (b) a growth period in which cotyledonary embryos reached their final dimensions, and (c) a maturation period in which embryos accumulated protein and starch reserves, dehydrated to a water content equal to 30%, and underwent a modification in soluble sugar composition. Monosaccharides and sucrose contents decreased to the benefit of the oligosaccharides raffinose and stachyose. The formation of somatic embryos by use of basic protocols was studied to define the limiting factors that could lie behind their poor development. Morphological abnormalities of somatic embryos, which represented 80% of the total population, were described. A histological study showed that somatic embryos lacked starch and protein reserves; moreover, their water content was much higher than that of their zygotic counterparts. Introducing a growth period into the culture protocol made for better embryo development. Adding sucrose and abscisic acid to the maturation medium was effective in increasing reserve synthesis and resulted in higher germination, conversion, and acclimatization rates.

Key words: maturation; reserve accumulation; embryogenesis late phases; ABA (abscisic acid); recalcitrant seeds.

INTRODUCTION

Theobroma cacao L. is an important source of income for several developing countries. Because of its allogamy, somatic embryogenesis from tissues of maternal origin would be of great use for the multiplication of selected clones. The first reports on somatic embryogenesis of cocoa (Esan, 1977; Pence et al., 1980) were restricted to immature zygotic embryo tissues and therefore had limited value for propagation. The first reports of successful production of somatic embryos from nonsexual tissues and the conversion of these embryos into plants came from Lopez-Baez et al. (1993) and Söndahl et al. (1993). Whatever their origin, the maturation and conversion of cocoa somatic embryos into plants has always been a problem (Wang and Janick, 1984; Duhem et al., 1989; Söndahl et al., 1993; Figueira and Janick, 1995). For the first time, Lopez-Baez et al. (1993) obtained very good germination, conversion, and acclimatization rates. Somatic embryos from floral explants (Alemanno et al., 1996) were obtained according to Lopez-Baez et al. (1993) protocols, but the late phases of somatic embryogenesis did not enable plant production. One strategy often described in the literature for improving these late phases consists of using zygotic embryogenesis as a reference. This approach is based on observations of the morphological, anatomical, and biochemical changes in zygotic embryos. Such observations can help to define new culture conditions for somatic embryo maturation. In several instances it has resulted in the optimization of media, hence of somatic embryo maturation, germination, and conversion rates (Carman, 1988; Faure, 1990; Hakman et al., 1990; Attree and Fowke, 1993; Etienne et al., 1993; Krochko et al., 1994). We first studied *Theobroma cacao* L. zygotic embryogenesis considering several parameters: morphological, histological, and physiological, namely water status and soluble sugar content, two important parameters in embryogenesis (Leprince et al., 1993). A comparison between zygotic and somatic embryogenesis was used to determine factors that could be limiting and could explain the poor germination and conversion rates of the somatic embryos. Thereafter, experiments were designed to improve these factors, especially reserve synthesis (starch and proteins) by modifying sugar and protein contents of the medium (Lai and McKersie, 1994), addition of abscisic acid (ABA) (Etienne et al., 1993; Misra et al., 1993; Brisibe et al., 1994) and an increase of the osmotic potential of the medium (Misra et al., 1993; Brisibe et al., 1994).

MATERIALS AND METHODS

Zygotic embryogenesis. Pods from hand-pollinated IMC 67 (Upper-Amazonian Forastero) by UF 613 (Trinitario) were provided by the CIRAD trials at Paracou-Combi in French Guiana. Pods were harvested every week and zygotic embryos were divided into 8 stages of maturity (Iz–VIIIz) according to size and color.

Somatic embryogenesis. Somatic embryos were obtained according to Lopez-Baez et al. (1993). One genotype was used: NA 79, an Upper-Amazonian Forastero. Flower buds 6–8 mm long were taken from *Theobroma cacao* L. trees grown in the greenhouse and sterilized for 20 min in a 10% sodium hypochlorite solution. After the buds were dissected, only fused staminodes and stamens were cultured on a callogenesis medium consisting of Murashige and Skoog's (1962) medium supplemented with glycine (3 mg l^{-1}, 40 µmol l^{-1}), lysine (0.4 mg l^{-1}, 3 µmol l^{-1}), leucine (0.4 mg l^{-1}, 3 µmol l^{-1}), arginine (0.4 mg l^{-1}, 1.9 µmol l^{-1}), tryptophan (0.2 mg l^{-1}, 1 µmol l^{-1}), 2,4-dichlorophenoxyacetic acid (2 mg l^{-1}, 9 µmol l^{-1}), kinetin (0.25 mg l^{-1}, 1.16 µmol

TABLE 1

COMPOSITION OF NEWLY DEFINED MATURATION, GERMINATION AND CONVERSION MEDIA FOR COCOA SOMATIC EMBRYOS.[a]

Media[b]	Casein hydrolysate g l^{-1}	ABA (μmol l^{-1})	PEG 4000 (%)	Sucrose g l^{-1} (mmol l_{-1})	Gibberellic acid mg l^{-1} (μmol l^{-1})
S_{40}	—	—	—	40 (117)	—
S_{80}	—	—	—	80 (234)	—
S_{80} CH	2	—	—	80 (234)	—
S_{40} ABA 10	—	(10)	—	40 (117)	—
S_{80} ABA 10	—	(10)	—	80 (234)	—
S_{80} ABA 10 CH	2	(10)	—	80 (234)	—
S_{40} PEG 2.5	—	—	2.5	40 (117)	—
S_{40} PEG 5	—	—	5	40 (117)	—
S_{40} PEG 7.5	—	—	7.5	40 (117)	—
S_{40}- PEG 2.5 ABA 20	—	(20)	2.5	40 (117)	—
S_{40} PEG 5 ABA 10	—	(10)	5	40 (117)	—
S_{40} PEG 5 ABA 20	—	(20)	5	40 (117)	—
S_{40} PEG 7.5 ABA 10	—	(10)	7.5	40 (117)	—
S_{40} PEG 7.5 ABA 20	—	(20)	7.5	40 (117)	—
GM	—	—	—	20 (58.5)	3(8.6)
CM	—	—	—	20 (58.5)	—

[a]Basal medium: Murashige and Skoog (1962).
[b]S, sucrose; CH, casein hydrolysate; ABA, abscisic acid; PEG, polyethylene glycol; GM, germination medium; CM, conversion medium.

l^{-1}), coconut water (50 ml l^{-1}), and sucrose (40 g l^{-1}, 117 mmol l^{-1}). After 3 wk on this medium, the tissues were transferred to a hormone-free expression medium supplemented with glycine (1 mg l^{-1}, 14 μmol l^{-1}), lysine (0.2 mg l^{-1}, 1.5 μmol l^{-1}), leucine (0.2 mg l^{-1}, 1.5 μmol l^{-1}), arginine (0.2 mg l^{-1}, 0.95 μmol l^{-1}), tryptophan (0.1 mg l^{-1}, 0.5 μmol l^{-1}), coconut water (100 ml l^{-1}), and sucrose (40 g l^{-1}, 117 mmol l^{-1}).

Somatic embryo maturation. Preliminary experiments (data not shown) were carried out according to Lopez-Baez et al. (1993) and Alemanno et al. (1996). Standard maturation was performed under low intensity light (7 μE m^{-2} s^{-1}) with a basal medium MS/2 containing amino acids in the same proportion as the expression medium, growth regulators (indole-3-acetic acid 0.05 mg l^{-1}, 0.28 μmol l^{-1}; indole-3-butyric acid 0.05 mg l^{-1}, 0.25 μmol l^{-1}; gibberellic acid 0.02 mg l^{-1}, 0,057 μmol l^{-1}; adenine sulfate 0.5 mg l^{-1}, 2.7 μmol l^{-1}), and maltose (40 g l^{-1}, 111 μmol l^{-1}). Petri dishes 55 mm in diameter were used, each containing six somatic embryos. New maturation media were defined (Table 1). Maturation took place in total darkness. The numbers of somatic embryos used for each treatment are indicated in the results tables. In these experiments, only embryos defined as normal (category 1) were used (*see* Results). The data sets were tested with the generalized linear model followed by the contrast method.

Germination and conversion. Preliminary experiments (data not shown) were carried out according to Lopez-Baez et al. (1993) described earlier. Along with the modifications to the maturation media, new germination and conversion media (respectively GM and CM) were designed (Table 1). The numbers of somatic embryos used for each treatment are indicated in the results tables. Germination and conversion rates were determined after 5 wk on GM and CM. Embryos were considered as germinated when there was hypocotyl elongation and radicle extension. They were considered to be converted when the shoot apex developed and formed a shoot with leaves. The set of data were tested with the generalized linear model followed by the contrast method.

Acclimatization. Plantlets were transferred to a substrate of vermiculite/sand/perlite (2/2/1) watered with a MS/4 solution inside a small greenhouse where the relative humidity was regulated between 80 and 98% and the temperature between 25 and 30° C. The numbers of somatic embryos used for each treatment are indicated in the results tables. The data sets were tested with the generalized linear model followed by the contrast method.

Morphology. The zygotic embryos were measured every week up to full maturation on six representative samples; the standard deviation was calculated.

Histology. Histological observations were carried out every week on zygotic embryos and after 5 wk on maturation media for the somatic embryos. For each treatment, an average of five explants were fixed in 1% glutaraldehyde solution in a phosphate buffer (0.2 *M*, pH 7.2), 4% acrolein, and 1% wt/vol caffeine. After explants were dehydrated and embedded in Kulzer resin (Leica, Wehrheim, Germany), sections 3 μm thick were cut with an LKB Historange microtome. A double stain PAS (periodic acid-Schiff)-naphthol blue-black was used. PAS stains polysaccharides red (Martoja and Martoja, 1967), and naphthol blue-black specifically stains soluble and insoluble proteins blue-black (Fisher, 1968).

Measurement of water parameters. Fresh weight (FW) and water content (WC) were determined every week from stage IIIz for zygotic embryos (the two first stages corresponding to embryos too small to be used), and before and after the maturation period for somatic embryos. Each measurement was taken on six representative samples, and the standard deviation was calculated. Water content (WC) represented the ratio FW − DW / FW × 100, where DW was the dry weight obtained after the embryos were left 24 h at 50° C. Osmotic potentials (Ψ_s) were measured with a dewpoint microvoltmeter (Wescor HR 33, Logan, UT). Frozen embryo samples were placed in the measurement chamber (thermocouple hygrometer sample chamber C51, Wescor, Logan, UT) for 1 ½ h at 21° C. Microvolt readings were recorded for Ψ_s (Lang, 1967). The Ψ_s were measured from stage IIIz to VIIIz for zygotic embryos and after the maturation period for somatic embryos. The values shown were the average of five readings and the standard error was calculated. The data sets were treated with Student–Newmann–Keuls test at the 5% level of significance.

Analysis of soluble sugars. Sugars were extracted twice in boiling 80% ethanol. They were then separated by ionic chromatography and detected by pulse amperometry as described in Peschet and Giacalone (1991). The soluble sugar contents were measured from stage IIIz to VIIIz for the zygotic embryos, and only before maturation for the somatic embryos. Each measurement was taken once, with a standard deviation from 1% to 3%.

RESULTS

Zygotic embryogenesis. Morphological study. The elongation of cocoa ovules and embryos from the 9th to the 21st wk after pollination is shown in Fig. 1. The embryo growth curve can be divided into three periods. The first lasted for 10 wk after pollination (Iz) when the egg remained at a unicellular stage (Bouharmont, 1960). During the second period, the length of the embryos increased until 14 wk after pollination (Vz) when they reached the same length as the ovules. This period began with embryogenesis *sensu stricto*: globular (Iz), heart, and cotyledonary stages (IIz), then a true growth period took place (stage IIz to stage Vz). During the third period, stages Vz to VIIIz, embryo length remained constant, color became darker, and weight increased (Fig. 2).

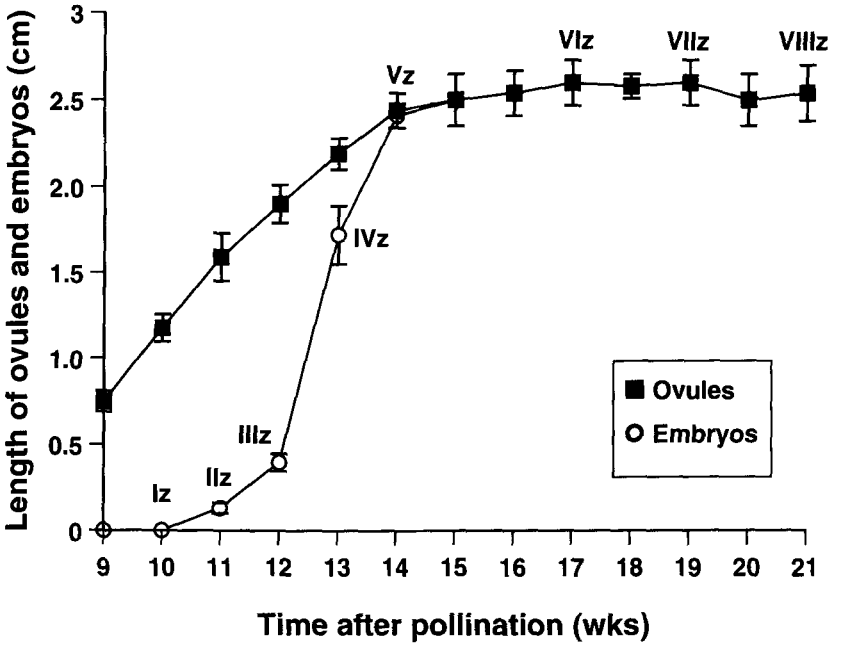

FIG. 1. Elongation of cocoa ovules and embryos from 9 wk after pollination to maturity.

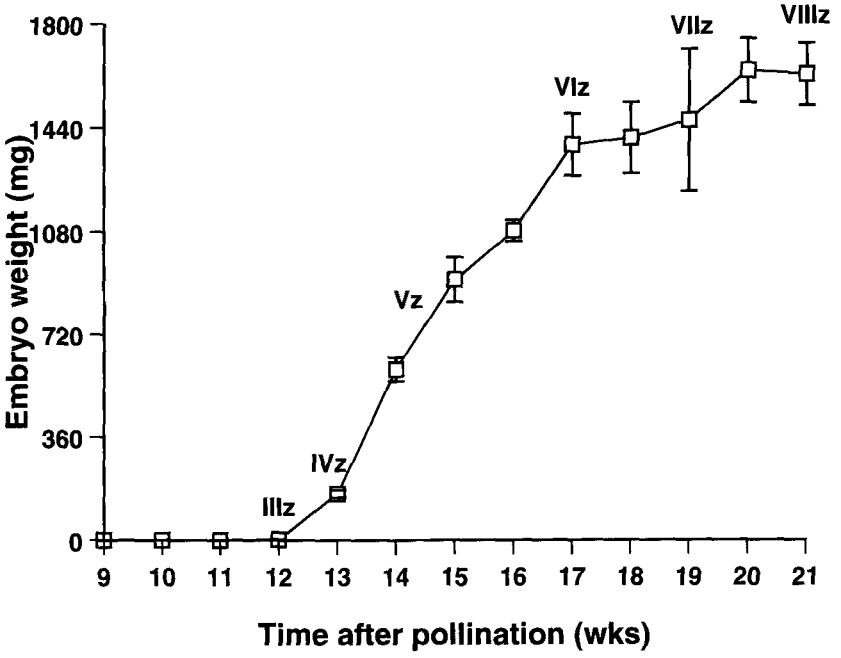

FIG. 2. The weight of cocoa zygotic embryos from 9 wk after pollination to maturity.

Histological study. At the end of the growth period (Vz), cells from embryonic axes and cotyledons had large empty vacuoles, a small nucleus, and a reduced cytoplasm (Fig. 3 *A*). During the maturation period, the most important fact was progressive synthesis of storage molecules. At the beginning of this step, a small number of globules could be seen inside the vacuoles. These globules were made of proteins as shown by specific coloration. The cytoplasm contained small starch grains (Fig. 3 *B*). The cells continued to accumulate reserves, and around the 17th wk after pollination (VIz), the number of starch grains increased as well as their volume. Protein reserves were much more numerous and tended to form granular units (Fig. 3 *C*). At full maturity, cells from axes and cotyledons contained several large starch grains and large protein inclusions that occupied the main part of the cellular volume (Fig. 3 *D*).

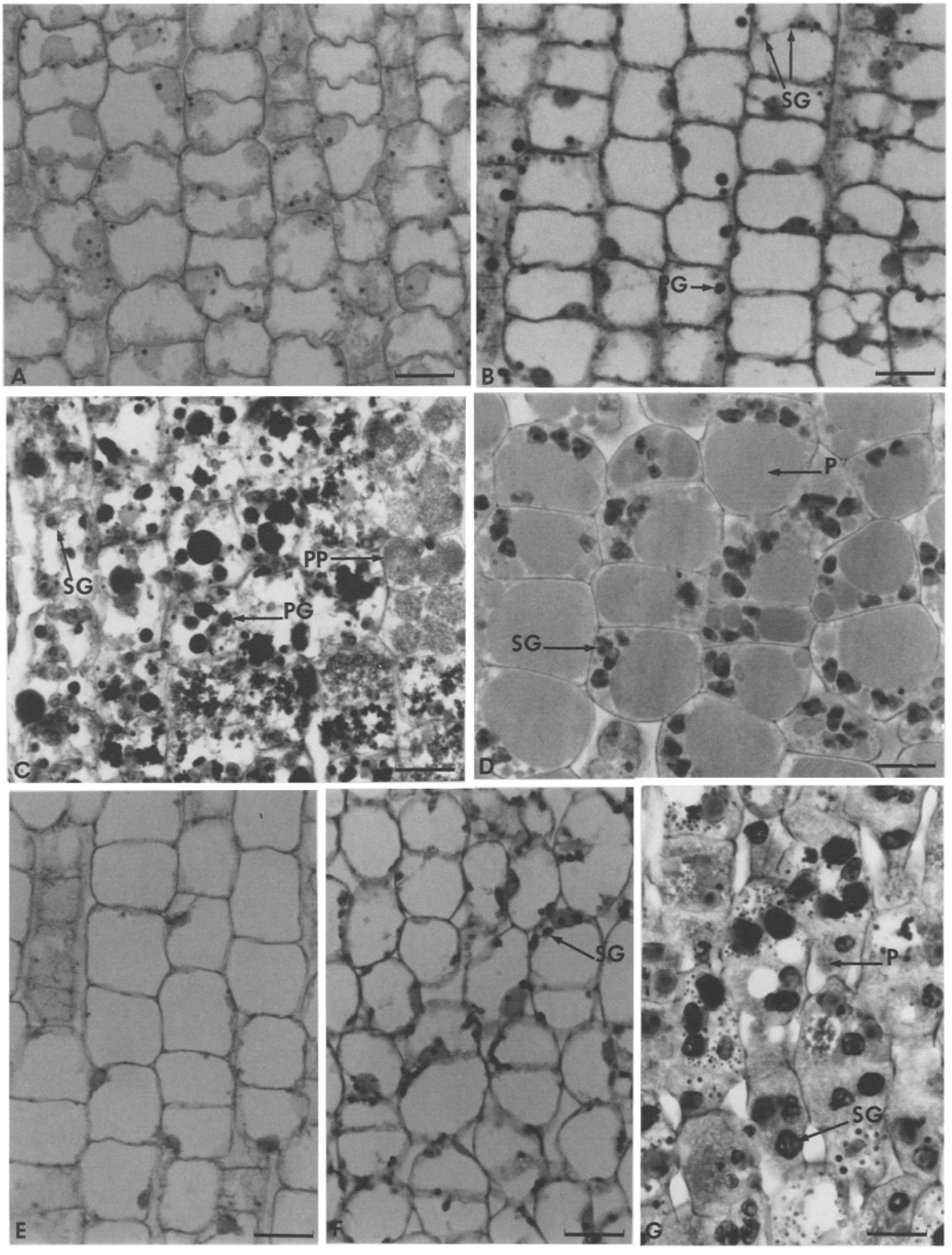

FIG. 3. Microscopic observations of the reserves of cocoa zygotic and somatic embryo cells. *A*, Cells of a zygotic embryo at the end of the growth period. *B*, Beginning of storage molecule synthesis by a zygotic embryo; small starch grains (SG) and protein globules (PG) are visible. *C*, Zygotic embryo 17 wk after pollination. Starch grains (SG); protein globules (PG); protein precipitates (PP). *D*, Fully mature zygotic embryo. Large starch grains (SG) and proteins (P) are present in the cells. *E*, Cells of a somatic embryo, completely devoid of reserves, after the maturation period of the standard protocol. *F*, Cells of a somatic embryo cultured on maturation medium S_{80}. Small starch grains (SG) are visible. *G*, Cells of a somatic embryo cultured on maturation medium S_{80} ABA 10. Starch grains (SG) and proteins (P) are present. *Bar* = 15 μm.

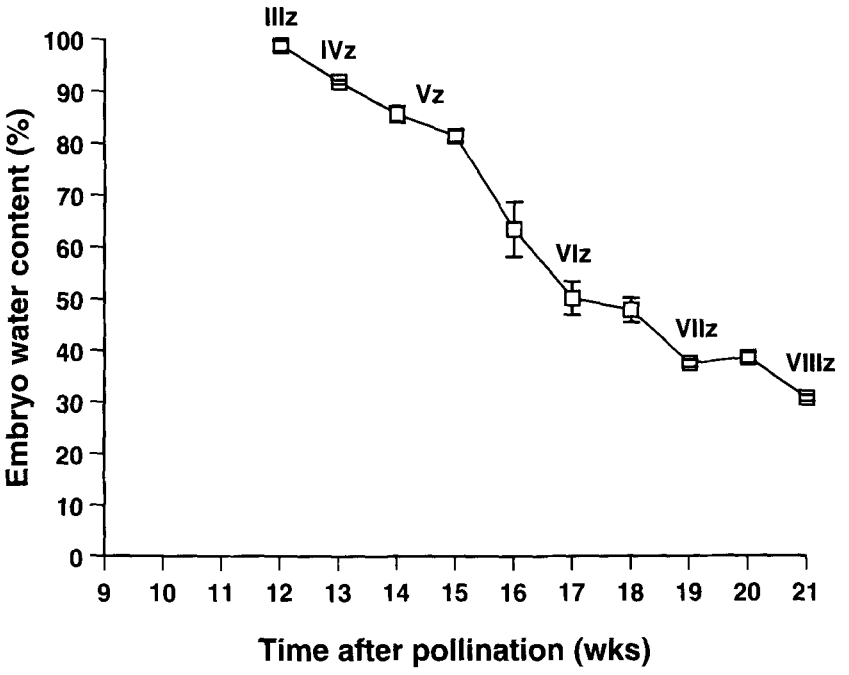

FIG. 4. The water content of cocoa zygotic embryos up to maturity.

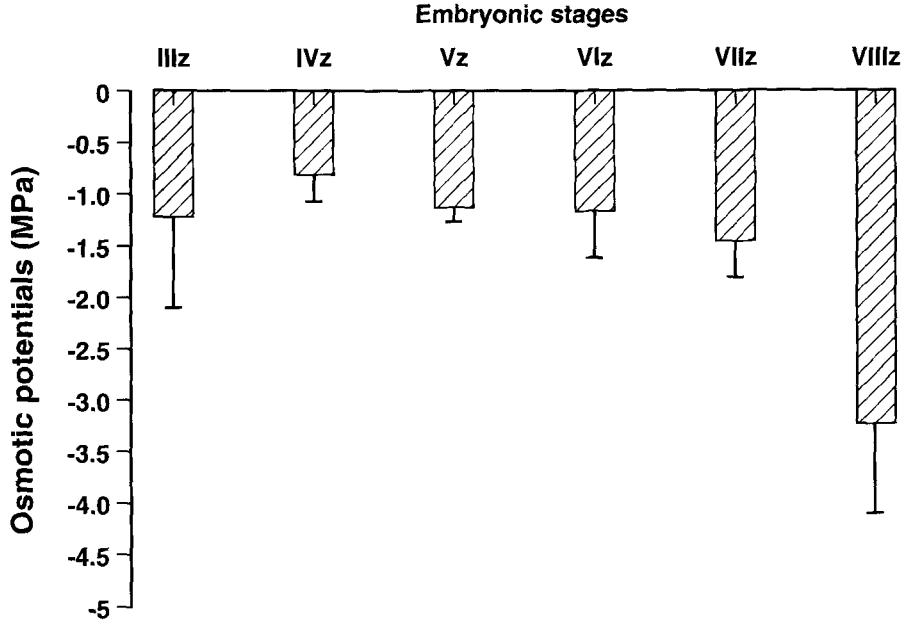

FIG. 5. The osmotic potential of cocoa zygotic embryos from stage IIIz to VIIIz.

Physiological study. At the beginning of growth (IIIz), the zygotic embryos had a water content close to 98% (Fig. 4). During the growth period, it steadily decreased to 80% 14 wk after pollination (Vz). Finally, the maturation period was characterized by substantial dehydration; when fully mature (VIIIz), embryos had a water content of around 30%. Osmotic potentials were relatively constant (around −1 MPa) up to stage VIz (Fig. 5); thereafter, they became more negative and reached −3.3 MPa at maturity (VIIIz). Stages IIIz and IVz, growing embryos, were characterized by high glucose, fructose, and sucrose content (Table 2). The oligosaccharides stachyose and raffinose were absent or in very low quantities. Dehydration came with a modification of the sugar composition: the concentrations of glucose, fructose, and sucrose sharply decreased; conversely, raffinose and stachyose levels significantly increased. Rhamnose, ara-

TABLE 2

VARIATIONS IN THE SOLUBLE SUGAR CONTENT OF COCOA ZYGOTIC EMBRYOS FROM STAGE IIIZ TO THEIR MATURITY, AND OF SOMATIC EMBRYOS BEFORE MATURATION

Soluble sugars[b]	Age and stage of embryos[a]						Somatic embryos before maturation
	12 wk Stage IIIz	13 wk Stage IVz	15 wk Stage Vz	17 wk Stage VIz	19 wk Stage VIIz	21 wk Stage VIIIz	
Rhamnose	0.00	0.00	0.00	0.00	0.00	0.00	0.39
Arabinose	0.00	0.00	0.00	0.00	0.01	0.01	0.00
Galactose	0.00	0.00	0.07	0.06	0.05	0.03	0.00
Glucose	3.12	1.07	0.27	0.70	0.23	0.11	2.51
Xylose	0.00	0.07	0.08	0.00	0.00	0.00	0.08
Fructose	3.25	1.61	0.30	0.57	0.25	0.10	2.60
Sucrose	4.04	5.50	2.42	1.30	0.87	1.05	7.99
Raffinose	0.05	0.04	0.07	0.06	0.05	0.14	0.01
Stachyose	0.00	0.00	0.02	0.02	0.04	0.17	0.00

[a]After pollination.
[b]Quantities are expressed in g per 100 g of dry matter.

binose, and galactose, which were absent during growth, were synthesized during maturation.

The steps of cocoa zygotic embryogenesis are diagrammatically represented in Fig. 6 *A*.

Somatic embryogenesis: Comparison with zygotic embryogenesis. Morphological aspects. When compared to their zygotic counterparts, somatic embryos could be classified into three main categories. Normal embryos (category 1) exhibited clear bipolarity, well-defined shoot and root apices, and cotyledons. This most closely resembled zygotic embryo morphology except for size, which was smaller. Particularly, somatic embryos did not have large, purple cotyledons. The most developed part of the somatic embryos was the embryonic axis, which could reach the same length as that seen in zygotic embryos. Embryos belonging to category 2 frequently resulted from the fusion of two or more embryos at the axis or cotyledons. Another category (category 3) of embryos had a protoderm but did not show any bipolar organization, lacking apices and cotyledons. Such structures could not evolve. Under standard conditions, somatic embryos of the genotype NA 79 could be classified as follows; category 1: 21%, category 2: 6%, and category 3: 73%.

Culture stages. A diagram of the somatic embryogenesis stages is shown in Fig. 6 *B*. When compared to Fig. 6 *A*, in the protocol described by Lopez-Baez et al. (1993), somatic embryos were subcultured on maturation medium before they had time to grow.

Histological study. At the end of the maturation period, embryonic axis and cotyledon cells of somatic embryos drastically lacked storage reserves (Fig. 3 *E*). Moreover, as maturation took place under light, many of the embryos revealed chlorophyll synthesis as well as vitrification. During the germination and conversion phases, many of the cells of the embryos showed degeneration and oxidation, leading to somatic embryo death. No plantlets were obtained. In conclusion, compared to their zygotic counterparts, somatic embryos lacked distinct growth and reserve accumulation stages.

Improvement of the late phases of somatic embryogenesis in reference to zygotic embryogenesis. Introduction of a growth phase. If somatic embryos formed on the expression medium were subcultured on the same medium for another 3 wk, they were able to grow. Indeed, 30 somatic embryos measuring less than 5 mm when transferred to a fresh expression medium measured 8.2 ± 1.7 mm at the end of the subculture. In all the following experiments, this expression medium was used as a growth medium for 3 wk.

Improvement of reserve accumulation. Several maturation media modifications were made. Two criteria were used to monitor somatic embryo maturation: opacity of the embryos and the existence or absence of reserves, checked histologically. The results are given in Table 3. Maturation in the dark prevented chlorophyll synthesis but did not change the translucent appearance of the embryos. The lack of reserve synthesis confirmed our preliminary observations (Fig. 3 *E*). Two factors seemed to be essential in opacity and reserve synthesis. The replacement of maltose by sucrose increased starch synthesis and the rate at which embryos became opaque (Fig. 3 *F*). The introduction of ABA clearly induced protein synthesis (Fig. 3 *G*). Polyethylene glycol (PEG) alone had no effect on reserve synthesis.

Physiological study. Water parameters (water content and osmotic potential) were measured for somatic embryos before and after the maturation period, for five of the treatments previously described (Table 4). Embryos grown on standard medium had a water content close to that of embryos at the end of the expression medium. The other four treatments enabled a significant decrease in somatic embryo water content. Osmotic potential was not significantly different between maturation treatments, though a tendency did exist. Moreover, their values were not as negative as those of fully matured zygotic embryos. As regards the soluble sugar content of the somatic embryos, it was possible to determine it only before maturation (cf Table 2). The composition was close to that of growing zygotic embryos, but the sucrose, xylose, and rhamnose concentrations were much higher. Raffinose and stachyose content was very low compared to that of zygotic embryos.

Information provided by these different studies enabled us to define new media and culture sequences for somatic embryogenesis which came closer to zygotic embryogenesis; they are shown in Fig. 6 *C* and *D*.

Germination, conversion and acclimatization. The replacement of maltose by sucrose improved germination and conversion rates (Table 3). When casein hydrolysate was added, no improvement could be seen. ABA significantly increased germination and conversion rates when associated with a high sucrose concentration. For sucrose concentrations equal to 40 g l^{-1}, adding PEG did not affect germination and conversion rates, except for a concentration of 2.5%. Adding

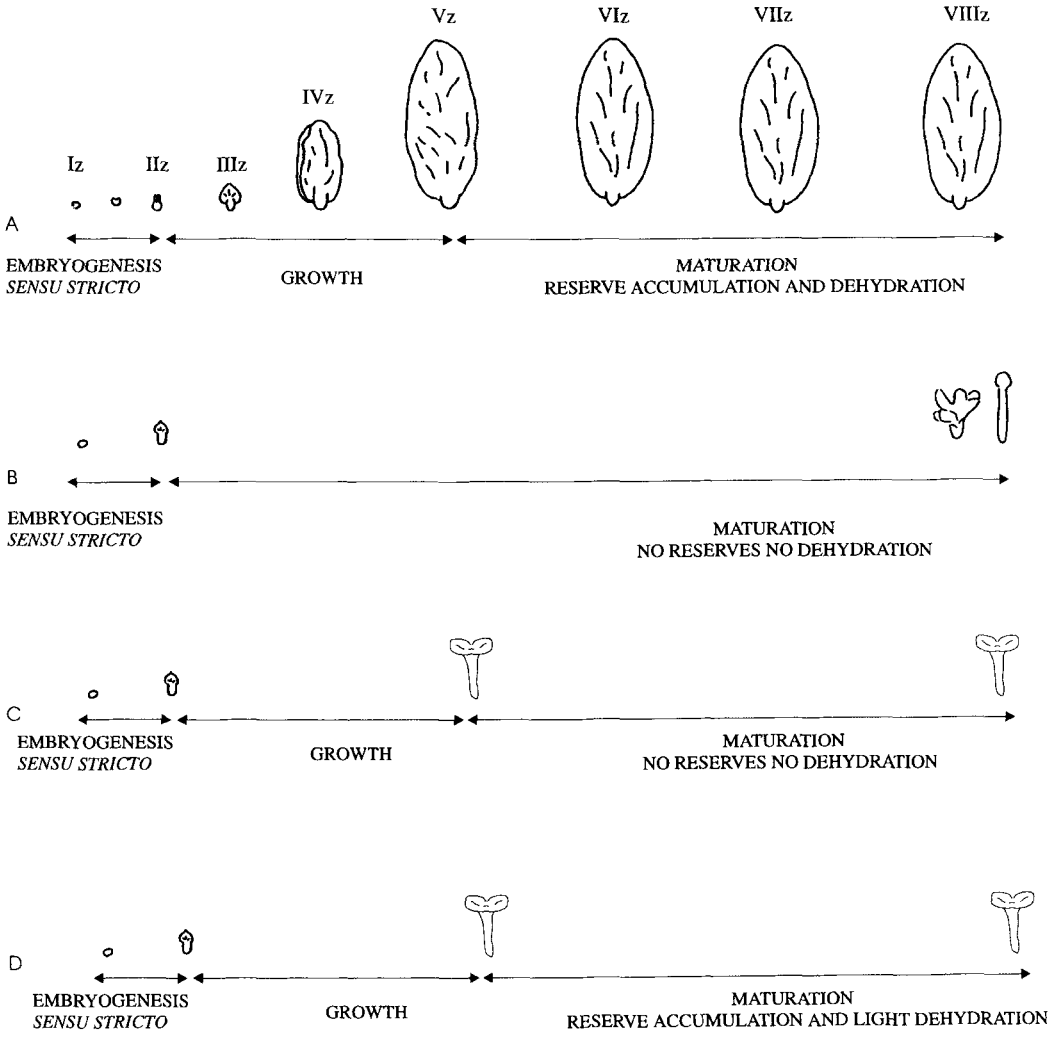

FIG. 6. Comparison of the zygotic embryogenesis stages with three different somatic embryogenesis protocols. *A*, Zygotic embryogenesis. *B*, Standard somatic embryogenesis. *C*, Standard somatic embryogenesis with introduction of a growth phase. *D*, Standard somatic embryogenesis with introduction of a growth phase and maturation medium S_{80} ABA 10.

both PEG and ABA gave variable results. Germination rates were improved when ABA was present at a concentration of 20 μmol l^{-1} for high PEG contents. Nevertheless, conversion rates were very low. PEG may have been toxic to somatic embryos. Only one treatment resulted in a significant increase of the acclimatization rate: S_{80} ABA 10 (sucrose: 80 g l^{-1}; ABA: 10 μmol l^{-1}).

DISCUSSION

It is still not easy to obtain somatic embryos from maternal tissues of *Theobroma cacao*. An additional difficulty lies in the lack of successful conversion of the somatic embryos into plants (Figueira and Janick, 1995; Pence, 1995). Several factors have been studied to improve maturation and to increase conversion into plants, such as soaking embryos in distilled water or renewing liquid medium (Wang and Janick, 1984), cotyledonary excision (Novak et al., 1986), or micrografting of somatic embryos (Aguilar et al., 1992); these experiments were always performed with somatic embryos of zygotic origin. Very few studies are available on embryogenesis late phases with somatic embryos of maternal origin. Figueira and Janick (1993) showed the beneficial effect of increased concentrations of atmospheric CO_2 on the conversion of nucellar somatic embryos. But the number of plants recovered was still very low, as was also reported by Söndahl et al. (1993) with somatic embryos of petal and nucellar origin. Conversely, Lopez-Baez et al. (1993) reported extremely high rates of germination, conversion, and acclimatization. Our results were intermediate and still unsatisfactory if this technique is to be routinely used.

The morphological abnormalities observed with cocoa somatic embryos have already been observed for other plants: peanuts (Wetzstein and Baker, 1993), grapevine (Goebel-Tourand et al., 1993), and soybean (Buchheim et al., 1989). A histological study of cocoa somatic embryos at the end of the maturation period as defined by the initial protocol clearly demonstrated their deficit in reserve compounds of any nature, compared to that of zygotic embryos. Indeed, such somatic embryos resembled zygotic embryos at the end of the growth period before reserve synthesis. This difference between somatic and

TABLE 3

EFFECT OF SEVERAL MATURATION MEDIA ON SOMATIC EMBRYO OPACITY, RESERVE SYNTHESIS, GERMINATION, CONVERSION, AND ACCLIMATIZATION RATES

Maturation treatment[a]	Number of somatic embryos[b]	Opaque embryos (%)	Accumulation and nature of reserves	Germinated embryos (% compared to total embryos)	Converted embryos (% compared to total embryos)	Converted embryos (% compared to germinated embryos)	Acclimatized embryos (% compared to germinated embryos)
Standard	39	0z	—	20.5z	0.0z	0.0z	0.0
Standard and darkness	38	0z	—	31.6z	0.0z	0.0z	0.0
S_{40}	90	45.5wx	Starch	48.8y	11.1y	22.7y	0.0
S_{80}	63	93.6u	Starch	42.8y	20.6xy	48.1y	0.0
S_{80} CH	53	83.0u	Starch/protein	15.1z	1.9z	12.5yz	0.0
S_{40} ABA 10	119	61.3u	Starch	25.2z	10.1y	40.0y	0.0
S_{80} ABA 10	50	100t	Starch/proteins	64.0y	28.0y	43.8y	21.4 (6.0)
S_{80} ABA 10 CH	106	64.1v	Starch/proteins	24.5z	0.0	0.0z	0.0
S_{40} PEG 2.5	126	39.7x	—	34.1y	1.6z	4.7z	0.0
S_{40} PEG 5	84	21.4y	—	17.8z	2.4z	13.3yz	0.0
S_{40} PEG 7.5	97	16.5y	—	11.3z	0.0z	0.0z	0.0
S_4 PEG 2.5 ABA 20	113	39.8x	Starch/proteins	20.4z	0.0z	0.0z	0.0
S_{40} PEG 5 ABA 10	90	51.1vwx	Starch/proteins	22.2z	5.5z	25.0y	0.0
S_{40} PEG 5 ABA 20	148	40.5x	Starch/proteins	46.6y	1.3z	2.9z	0.0
S_{40} PEG 7.5 ABA 10	93	63.4v	Starch/proteins	12.9z	0.0z	0.0z	0.0
S_{40} PEG 7.5 ABA 20	111	46.8wx	Starch/proteins	45.9y	2.7z	5.9z	0.0

[a]For treatment abbreviations, refer to Table 1.

[b]Observations were made after 5 weeks' culture on maturation medium.

[c]Acclimatization rates compared to total number of embryos. Values followed by the same letter are not significantly different (test carried out by generalized linear model followed by the contrast method).

TABLE 4

WATER STATUS AND OSMOTIC POTENTIAL OF SOMATIC EMBRYOS AT THE END OF THE MATURATION PERIOD ON DIFFERENT MEDIA

Treatments	Water content of somatic embryos[a] (%)	Osmotic potential[a] (MPa)
Expression medium	89.9 ± 0.7z	nd[b]
Standard	89.8 ± 1.0z	−1.38 ± 0.11z
S_{80}	83.2 ± 2.6z	−2.11 ± 0.72z
S_{80} CH	81.3 ± 2.5y	−2.26 ± 0.40z
S_{80} ABA 10	79.4 ± 0.8y	−2.16 ± 0.72z
S_{80} ABA 10 CH	82.9 ± 2.2y	−1.85 ± 0.47z

[a]Values followed by the same letter are not significantly different at the 5% level with the Student–Newman–Keuls test. For treatment abbreviations, refer to Table 1.

[b]nd = not determined.

zygotic embryos is very common (Crouch, 1982; Attree and Fowke, 1993; Krochko et al., 1994). Lai and McKersie (1994) showed a positive correlation between the quality of *Medicago* somatic embryos and their protein and amino acid content.

The control of reserve accumulation seems to be different according to the type of seed (recalcitrant or orthodox). In orthodox seeds, abscisic acid and high osmoticum have been implicated in storage reserve accumulation. The ABA concentration is elevated during the maturation phase in embryonic tissues of tomato (Hocher et al., 1991), *Picea glauca* (Attree and Fowke, 1993), carrot (Liu et al., 1994) and *Picea taeda* (Kapik et al., 1995). The relationship between ABA and reserve synthesis is shown in several plants such as *Picea glauca* somatic embryos (Attree and Fowke, 1993), sugarcane somatic embryos (Brisibe et al., 1994), and hybrid larch somatic embryos (Gutmann et al., 1996). Leal et al. (1995) demonstrated the induction of reserve protein (coniferin) transcripts after ABA treatment of somatic embryos of white spruce. But, in *Medicago sativa* L., Lai and McKersie (1994) established that the presence of ABA in the maturation medium had no effect on reserve synthesis by somatic embryos. In zygotic embryos of the same species, the use of an ABA synthesis inhibitor affected the endogenous ABA content of the embryos but had no effect on storage protein synthesis (Xu and Bewley, 1995). However in *Avicennia marina*, a recalcitrant species, ABA embryo content is very low during reserve synthesis (Farrant et al., 1993). In cocoa, Pence (1991) showed a substantial increase in ABA synthesis in embryo stages where their water content was about 70 to 80%. This stage could correspond, from our study of zygotic embryogenesis, to the very beginning of the maturation phase. Our study demonstrated that there is a relationship between the presence of ABA in the maturation medium and the synthesis of protein bodies in somatic embryo cells. The combination of the ABA factor with a high sucrose content enabled a significant improvement of embryo germination and conversion rates.

At full maturity, cocoa zygotic embryos had a water content close to 30%. It is equal to 50% in cork oak (Finch-Savage and Blake, 1994) and 45% in litchee, another plant with recalcitrant seeds (Fu et al., 1994). Water status enables a distinction to be made between recalcitrant and orthodox seeds whose water content can be as low as a few percent: 6.9%, for example, in *Ricinus* (Kermode and Bewley, 1985). The definition of new maturation media for cocoa somatic embryos has resulted in a 10% decrease in their water content compared to the initial concentration (90%). But this reduction is largely inadequate compared to the situation encountered in zygotic embryos. Orthodox seeds are capable of surviving desiccation, but only after a particular stage of development. If they are desiccated before

that stage, they will not germinate (Adams et al., 1983). In soybean, pea, and maize, acquisition of desiccation tolerance was associated with a decrease in monosaccharide and sucrose contents and with an increase in oligosaccharide (stachyose and raffinose) content (Koster and Leopold, 1988). In cocoa zygotic embryogenesis, we found the same type of evolution even if such embryos do not exhibit desiccation tolerance. In the highly recalcitrant seeds of *Avicennia marina*, large quantities of soluble sugars, stachyose in particular, were present (Farrant et al., 1993).

Our work helps to extend knowledge about the physiology of recalcitrant cocoa seeds and provides good indications about some of the reasons for the poor development and conversion of somatic embryos into plants. (a) Morphological abnormalities were frequent; one of the major factors that could improve this aspect is the auxin type and concentration in the culture media (Rodriguez and Wetzstein, 1994). (b) Introducing a growth phase before maturation into the somatic embryogenesis protocol improved somatic embryo development. (c) Modifying certain maturation medium parameters, especially increasing sucrose concentration and adding ABA, favored synthesis of somatic embryo protein and starch reserves. (d) Variations in the maturation medium also resulted in greater embryo dehydration but it was still not as great as in the zygotic counterparts. To achieve this, somatic embryos could be submitted to gradual drying as is commonly the case in somatic embryogenesis protocols, as it is beneficial for germination (McKersie and Bowley, 1993; Misra et al., 1993; Attree et al., 1995; Etienne et al., 1997).

Acknowledgments

We thank Philippe Lachenaud for the pollination experiments in French Guiana and Christian Cilas for statistical analysis.

References

Adams, C. A.; Fjerstad, M. C.; Rinne, R. W. Characteristics of soybean seed maturation: necessity for slow dehydration. Crop Sci. 23:265–267; 1983.

Aguilar, M. E.; Villalobos, V. M.; Vasquez, N. Production of cocoa plants (*Theobroma cacao* L.) via micrografting of somatic embryos. In Vitro Cell. Dev. Biol. 28P:15–19; 1992.

Alemanno, L.; Berthouly, M.; Michaux-Ferrière, N. Histology of somatic embryogenesis from floral tissues in *Theobroma cacao* L. Plant Cell Tissue Organ Cult. 46:187–194; 1996.

Attree, S. M.; Fowke, L. C. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tissue Organ Cult. 35:1–35; 1993.

Attree, S. M.; Pomeroy, M. K.; Fowke, L. C. Development of white spruce (*Picea glauca* (Moench) Voss) somatic embryos during culture with abscisic acid and osmoticum, and their tolerance to drying and frozen storage. J. Exp. Bot. 46:433–439; 1995.

Bouharmont, J. Recherches cytologiques sur la fructification et l'incompatibilité chez *Theobroma cacao* L. Publ. Inst. Natl. l'Etude Agron. Congo. Série Scientifique. 89:18–39; 1960.

Brisibe, E. A.; Miyake, H.; Taniguchi, T., et al. Abscisic acid and high osmoticum regulation of development and storage reserve accumulation in sugarcane somatic embryos. Jpn. J. Crop Sci. 63:689–698; 1994.

Buchheim, J. A.; Colburn, S. M.; Ranch, J. P. Maturation of soybean somatic embryos and the transition to plantlet growth. Plant Physiol. 89:768–775; 1989.

Carman, J. G. Improved somatic embryogenesis in wheat by partial simulation of the in-ovulo oxygen growth regulator and desiccation environments. Planta 175:417–424; 1988.

Crouch, M. L. Non-zygotic embryos of *Brassica napus* L. contain embryo-specific storage proteins. Planta 156:520–524; 1982.

Duhem, K.; Le Mercier, N.; Boxus, P. H. Données nouvelles sur l'induction et le développement d'embryons somatiques chez *Theobroma cacao* L. Café, Cacao, Thé 33(1):9–14; 1989.

Esan, E. B. Tissue culture studies on cocoa (*Theobroma cacao* L.). A supplementation of current research. In: Proceedings, Fifth International Conference on Cacao Research Idaban, Nigeria; 1977:116–125.

Etienne, H.; Sotta, B.; Montoro, P., et al. Comparison of endogenous ABA and IAA contents in somatic and zygotic embryos of *Hevea brasiliensis*. Plant Sci. 92:111–119; 1993.

Etienne, H.; Lartaud, M.; Michaux-Ferrière, N., et al. Improvement of somatic embryogenesis in *Hevea brasiliensis* (Müll. Arg.) using the temporary immersion technique. In Vitro Cell. Dev. Biol. Plant (in press) 1997.

Farrant, M.; Pammenter, W.; Berjak, P. Seed development in relation to desiccation tolerance: a comparison between desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* and desiccation-tolerant types. Seed Sci. Res. 3:1–13; 1993.

Faure, O. Embryons somatiques de *Vitis rupestris* et embryons zygotiques de *Vitis* sp.: morphologie, histologie, histochimie et développement. Can. J. Bot. 68:2305–2315; 1990.

Figueira, A.; Janick, J. Development of nucellar somatic embryos of *Theobroma cacao*. Acta Hortic. 336:231–236; 1993.

Figueira, A.; Janick, J. Somatic embryogenesis in cacao (*Theobroma cacao* L.). In: Jain, S. M.; Gupta, P. K.; Newton, R. J., ed. Somatic embryogenesis in woody plants. Vol. 2. Angiosperms. Dordrecht, Boston, London: Kluwer Academic Publishers; 1995:291–310.

Finch-Savage, W. E.; Blake, P. S. Indeterminate development in desiccation-sensitive seeds of *Quercus robur* L. Seed Sci. Res. 4:127–133; 1994.

Fisher, D. B. Protein staining of ribboned epon sections for light microscopy. Histochemie 16:92–96; 1968.

Fu, J. R.; Jin, J. P.; Peng, Y. F., et al. Desiccation tolerance in two species with recalcitrant seeds *Clausenia lansium* (Lour.) and *Litchi chinensis* (Sonn.). Seed Sci. Res. 4:257–261; 1994.

Goebel-Tourand, I.; Mauro, M. C.; Sossountzov, L., et al. Arrest of somatic embryo development in grapevine: histological characterization and the effect of ABA, BAP and zeatin in stimulating plantlet development. Plant Cell Tissue Organ Cult. 33:91–103; 1993.

Gutmann, M.; von Aderkas, P.; Label, P., et al. Effects of abscisic acid on somatic embryo maturation of hybrid larch. J. Exp. Bot. 47:1905–1918; 1996.

Hakman, I.; Stabel, P.; Engström, P., et al. Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). Physiol. Plant. 80:441–445; 1990.

Hocher, V.; Sotta, B.; Maldiney, R., et al. Changes in abscisic acid and its β-D-glucopyranosyl ester levels during tomato (*Lycopersicon esculentum* Mill.) seed development. Plant Cell Rep. 10:444–447; 1991.

Kapik, R. H.; Dinus, R. J.; Dean, J. F. D. Abscisic acid and zygotic embryogenesis in *Pinus taeda*. Tree Physiol. 7–8:485–490; 1995.

Kermode, A. R.; Bewley, J. D. The role of maturation drying in the transition from seed development to germination. I. Acquisition of desiccation-tolerance and germinability during development of *Ricinus communis* L. seeds. J. Exp. Bot. 36:1906–1915; 1985.

Koster, K. L.; Leopold, A. C. Sugars and desiccation tolerance in seeds. Plant Physiol. 88:829–832; 1988.

Krochko, J. E.; Bantroch, D. J.; Greenwood, J. S., et al. Seed storage proteins in developing somatic embryos of alfalfa: defects in accumulation compared to zygotic embryos. J. Exp. Bot. 45:699–708; 1994.

Lai, F. M.; McKersie, B. D. Regulation of starch and protein accumulation in alfalfa (*Medicago sativa* L.) somatic embryos. Plant Sci. 100:211–219; 1994.

Lang, A. R. G. Osmotic coefficients and water potentials of sodium chloride solutions from 0 to 40° C. Aust. J. Chem. 20:2017–2023; 1967.

Leal, I.; Misra, S.; Attree, S. M., et al. Effect of abscisic acid, osmoticum and desiccation on 11S storage protein gene expression in somatic embryos of white spruce. Plant Sci. 106:121–128; 1995.

Leprince, O.; Hendry, G. A. F.; McKersie, B. D. The mechanisms of desiccation tolerance in developing seeds. Seed Sci. Res. 3:231–246; 1993.

Liu, L.; Hammond, E. G.; Wurtele, E. S. Accumulation of petroselinic acid in developing somatic carrot embryos. Phytochemistry 3:749–753; 1994.

Lopez-Baez, O.; Bollon, H.; Eskes, A., et al. Embryogenèse somatique de cacaoyer *Theobroma cacao* L. à partir de pièces florales. C. R. Acad. Sci. Paris 316:579–584; 1993.

Martoja, R.; Martoja, M. Initiation aux techniques de l'histologie animale. Paris: Masson et Cie; 1967.

McKersie, B. D.; Bowley, S. R. Synthetic seeds of alfalfa. In: Synseeds. Boca Raton, FL: CRC Press; 1993:231–255.

Misra, S.; Attree, S. M.; Leal, I., et al. Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann. Bot. 71:11–22; 1993.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–479; 1962.

Novak, F. J.; Donini, B.; Owusu, G. Somatic embryogenesis and in vitro plant development of cocoa (*Theobroma cacao*). In: Proceedings of the International Symposium on Nuclear Techniques and In Vitro Culture for Plant Improvement. International Atomic Energy Association, Vienna; 1986:443–449.

Pence, V. C. Abscisic acid in developing zygotic embryos of *Theobroma cacao*. Plant Physiol. 95:1291–1293; 1991.

Pence, V. C. Somatic embryogenesis in cacao (*Theobroma cacao*). In: Bajaj, Y. P. S., ed. Biotechnology in agriculture and forestry. Vol. 30. Somatic embryogenesis and synthetic seed I; 1995:455–467.

Pence, V. C.; Hasegawa, P. M.; Janick, J. Initiation and development of asexual embryos of *Theobroma cacao* in vitro. Zeitschrift Pflanzenphysiol. 98:1–4; 1980.

Peschet, J. L.; Giacalone, A. Un nouveau concept en analyse des sucres: la chromatographie ionique couplée à l'ampèrométrie pulsée. Industrie Agricole Alimentaire 108:1–4; 1991.

Rodriguez, A. P. M.; Wetzstein, H. Y. The effect of auxin type and concentration on pecan (*Carya illinoinensis*) somatic embryo morphology and subsequent conversion into plants. Plant Cell Rep. 13:607–611; 1994.

Söndahl, M. R.; Liu, S.; Bellato, C. M., et al. Cacao somatic embryogenesis. Acta Hortic. 336:245–248; 1993.

Wang, Y. C.; Janick, J. Inducing precocious germination in asexual embryos of cacao. HortScience 19:839–841; 1984.

Wetzstein, H. Y.; Baker, C. M. The relationship between somatic embryo morphology and conversion in peanut (*Arachis hypogaea* L.). Plant Sci. 92:81–89; 1993.

Xu, N.; Bewley, J. D. The role of abscisic acid in germination, storage protein synthesis and desiccation tolerance in alfalfa (*Medicago sativa* L.) seeds, as shown by inhibition of its synthesis by fluridone during development. J. Exp. Bot. 287:687–694; 1995.