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SOMATIC EMBRYOGENESIS IN *CITRUS* SPP.: CARBOHYDRATE STIMULATION AND HISTODIFFERENTIATION

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Summary

Somatic embryogenesis from nucellus-derived callus cultures of five cultivars, including three (Caipira, Seleta Vermelha, and Valencia) of sweet oranges (*C. sinensis* L. Osbeck), Rangpur lime (*C. limonia* L. Osbeck), and Cleopatra mandarin (*C. reticulata* Blanco) (lines I and II), were studied. Callus lines maintained on MT medium supplemented with 50 g l^{-1} sucrose were transferred to MT medium supplemented with different carbohydrate sources: galactose, glucose, lactose, maltose, or sucrose at 18, 37, 75, 110, or 150 m*M*, or glycerol at 6, 12, 24, 36, or 50 m*M*. Globular embryos were observed after approximately 4 wk, in several treatments. Cultures of Valencia and Caipira sweet oranges and Cleopatra mandarin (line I) showed high numbers of embryos on medium containing galactose, lactose, and maltose. Histological studies showed somatic embryos in all developmental stages with a normal histodifferentiation pattern. The other two cultivars (Rangpur lime and Cleopatra mandarin, line II) formed very few embryos, which did not develop further following the globular stage. Some of the abnormalities observed were lack or dedifferentiation of protoderm and absence of apical meristems and procambial strands. Embryos that followed the normal sequence of development were easily converted into plants. Non-embryogenic cultures continued as proliferating callus cultures, eventually forming a few embryos which did not convert into plants. Statistical analyses of the callus response to carbohydrate treatments was done using an overdispersion Poisson model.

Key words: galactose; generalized linear models; histology; maltose; overdispersion.

Introduction

Tissue culture techniques were first used in citrus for the development of somatic embryos from nucellar tissue for obtaining virus-free plants (Ranga Swamy, 1961; Rangan et al., 1968, 1969). The morphogenic characteristic of citrus cultures stimulated the use of tissue culture techniques for breeding purposes by somatic hybridization (Grosser and Gmitter, 1990; Jumin and Nito, 1996) and genetic transformation by the introduction of exogenous genes (Kobayashi and Uchimiya, 1989; Peña et al., 1995a,b).

Callus cultures are commonly induced from citrus nucellar tissue obtained from immature seeds (Gmitter and Moore, 1986; Kunitake et al., 1991; Vu et al., 1993; Oliveira et al., 1994; Mendes-da-Glória et al., 1999). These calluses become independent of exogenous plant growth regulators (Kochba et al., 1982; Spiegel-Roy and Saad, 1986), being maintained on MT medium (Murashige and Tucker, 1969). Callus subculture onto medium with high levels of sucrose (Murashige and Tucker, 1969; Kochba and Button, 1974; Hidaka and Omura, 1989), or a high concentration of benzylaminopurine (Kobayashi et al., 1988; Ling and Iwamasa, 1997) stimulates callus proliferation. After 6–10 subcultures such callus cultures are usually considered habituated, continuing to proliferate in the absence of growth regulators.

Citrus plant regeneration can be obtained from callus cultures by somatic embryogenesis (Kochba et al., 1978b; Cabasson et al., 1995; Ling and Iwamasa, 1997). Although somatic embryogenesis is considered widespread in citrus polyembryonic varieties (Ranga Swamy, 1958; Kochba and Spiegel-Roy, 1977; Kunitake and Mii, 1995), several factors influence this process.

In many species, somatic embryogenesis is induced by the presence of auxins in the culture medium, and somatic embryos develop after subculture to a medium containing reduced or no auxin levels (Merkle et al., 1995). In citrus, however, the presence of auxin or cytokinin suppresses somatic embryogenesis (Kochba et al., 1978a; Kunitake and Mii, 1995). The autonomous growth of callus indicates an auxin:cytokinin balance that favors habituation. In citrus, somatic embryogenesis from callus has been obtained by different treatments, including reduction of sucrose in the culture medium (Kochba and Button, 1974); a change in the carbohydrate source to galactose instead of sucrose (Kochba et al., 1978b, 1982; Hidaka and Omura, 1989; Cabasson et al., 1995; Kunitake and Mii, 1995), or to carbohydrates that produce galactose in their metabolism, such as lactose or raffinose (Kochba et al., 1978b;

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Ling and Iwamasa, 1997); or by using auxin (Kochba et al., 1978a) or gibberellic acid synthesis inhibitors (Spiegel-Roy and Saad, 1986).

Somatic embryogenesis in citrus is one of the techniques that makes genetic advances possible for this genus, enabling the recovery of plants from genetically transformed callus, protoplast-derived cultures and somatic hybridization experiments (Gmitter et al., 1992). Efficiency of plant recovery is thus very important and should be well defined for different cultivars, since it has been shown to be cultivar-dependent.

The objective of the present report is to evaluate the effect of six carbohydrate sources and levels in the culture medium on the stimulation of somatic embryos from callus cultures of five citrus cultivars, including sweet oranges, mandarins and one lime cultivar. Histological evaluation of embryo developmental stages was performed for the determination of embryo quality.

Materials and Methods

Plant material. Callus cultures of three sweet orange varieties (*Citrus sinensis* L. Osbeck, cvs. Caipira, Seleta Vermelha and Valencia), two mandarin lines (*C. reticulata* Blanco, cv. Cleopatra, line I and Cleopatra, line II) and one lime variety (*C. limonia* L. Osbeck, cv. Rangpur lime) were used for the experiments. These cultures were initially obtained from nucellar tissue (Oliveira et al., 1994) and maintained for approximately 4 yr (Cleopatra lines I and II, Seleta Vermelha, Valencia and Rangpur lime) or 1 yr (Caipira) on MT medium (Murashige and Tucker, 1969) supplemented with 500 mg l^{-1} malt extract and 50 g l^{-1} sucrose (callus proliferation medium), in the dark, at 27 ± 1°C, with monthly subcultures.

Somatic embryogenesis. Approximately 50 mg of callus of all the callus lines, except Seleta Vermelha sweet orange, were transferred to Petri dishes (100 × 15 mm) containing MT medium supplemented with one of the following carbohydrates: galactose, glucose, lactose, maltose, or sucrose, at 18, 37, 75, 110, or 150 m*M*, or glycerol at 6, 12, 24, 36, or 50 m*M*. Cultures were maintained at 27 ± 1°C, 16-h photoperiod and a light intensity of 4 μmol m^{-2} s^{-1} for 4 wk.

Experimental design. The experiment followed a completely randomized design, with three replications per treatment. Each plot consisted, initially, of 50 mg of callus in one Petri dish with 20 ml of culture medium. The evaluation was done by counting the globular-stage somatic embryos formed in each plot, with the aid of a stereo microscope.

Statistical analyses. Data from responsive treatments (callus line×sugar) were analyzed using Poisson distribution and also by modeling over-dispersion, in order to define the best model for the analysis of count data (Hinde and Demétrio, 1998a, b).

Histological analyses. Somatic embryos in different stages of development were fixed in paraformaldehyde (3%) and glutaraldehyde (2%) in cacodylate buffer (0.2 *M*, pH 7.2) under refrigeration for 5 d. Dehydration was done at room temperature in a series of 100% methyl celosolve (ethylene glycol monomethyl ether), ethanol, propanol, and butanol, followed by infiltration with Historesin at 4°C overnight (Rodriguez and Wetzstein, 1998). Polymerization was done at room temperature, with Historesin (Leica, Heidelberg). Serial sections (5 μm) were prepared in a rotary microtome with a tungsten-carbide knife, the sections floated in water drops and dried on a hot plate (40°C). Sections were stained with acid fuchsin (1%), rinsed in distilled water and counter stained with toluidine blue (0.5%) (Feder and O'Brien, 1968).

Results

Somatic embryogenesis. Cultures of Seleta Vermelha sweet orange obtained from nucellus were different from all the others, in that very small amounts of callus were obtained, followed by the development of numerous somatic embryos. Subcultures of callus into proliferation medium did not stimulate callus proliferation, showing instead continuous and repetitive somatic embryo formation associated with very small amounts of callus. Consequently, it was not possible nor necessary to use these cultures in the carbohydrate experiments, since embryo development occurred prior to any stimulation treatment.

After 4 wk in culture with different carbohydrate sources and concentrations, calluses from the other four cultivars showed differences in overall response, confirming the cultivar influence in callus growth and embryo development. The callus lines could be divided in two groups, according to their response to the carbohydrate treatments (Table 1). Valencia and Caipira sweet oranges and Cleopatra mandarin, line I, were considered highly embryogenic when cultured in medium with galactose, lactose, or maltose. Cultures maintained in some combinations of source and concentration of carbohydrate produced from more than 100 to more than 400 embryos per plate. On the other hand, Cleopatra mandarin, line II, and Rangpur lime were considered as poor embryogenic cultures, with very few embryos obtained in some treatments only. The number of embryos obtained varied not only with cultivar, but also with type and concentration of carbohydrate in the culture medium. The use of glucose, glycerol and sucrose was not effective, with very few or no embryos formed independent of callus line and concentration (Table 1).

Somatic embryo development was non-synchronized, with embryos in different developmental stages in the same culture. Embryo conversion was obtained in medium containing gibberellic acid.

Statistical analyses. Count data in general are analyzed using Poisson distribution. If we suppose that the number of embryos are random variables, Y_i, $i = 1, 2, \ldots n$, with means μ_i, the standard Poisson model assumes that $Y_i \sim P(\mu_i)$ with variance function $Var(Y_i) = \mu_i$ and $\log(\mu_i) = x_i'\beta$, where x_i is a line of the design matrix (treatment effects and covariates) and β is the parameter vector. For a well-fitting model we would expect that residual deviance (a measurement of fitness of the model) would be approximately equal to the residual degrees of freedom. When this does not happen one explanation is that the variation may simply be greater than that predicted by the model and this phenomenon is described as *overdispersion* (Hinde and Demétrio, 1998a, b). One way of modeling overdispersion is to replace the variance function of the original model by the more general form $Var(Y_i) = \phi\mu_i$ and use a quasi-likelihood method for estimating β and the additional parameter ϕ (called heterogeneity factor). Another approach would be to assume a negative binomial distribution for the number of embryos.

Fitting the standard Poisson model to these data gave residual deviances of 1643.2 on 66 degrees of freedom (df), 684.6 on 35 df, and 575.5 on 36 df, respectively, for Caipira and Valencia sweet oranges, and Cleopatra mandarin, line I, giving strong evidence of possible overdispersion. Using half-normal plots (Demétrio and Hinde, 1997) it was shown that the constant overdispersion model with a quadratic linear predictor fits the data well. Table 2 shows the results of the ratio statistics for linear and quadratic regression models, suggesting that the quadratic regression model is statistically significant for all three cultivars. The expected number of embryos can be calculated using the following equation $\hat{Y}_i = \exp(\hat{a} + \hat{b}x + \hat{c}x^2)$, where $\hat{a}$, $\hat{b}$ and $\hat{c}$ are the parameter estimates and x corresponds to the concentration of carbohydrates given in Table 1.

TABLE 1

NUMBER OF *CITRUS* SOMATIC EMBRYOS OBTAINED IN SIX CARBOHYDRATE SOURCES EACH AT FIVE DIFFERENT CONCENTRATIONS (MEAN OF THREE REPLICATIONS)[a]

		Callus line				
Carbohydrate	m*M*	Valencia	Caipira	Cleopatra line I	Cleopatra line II	Rangpur lime
Galactose	18	21	25	17	7	0
	37	14	39	87	2	0
	75	18	67	133	3	0
	110	4	194	122	0	0
	150	75	124	114	0	0
Glucose	18	8	50	4	5	0
	37	3	78	4	6	0
	75	0	17	3	3	0
	110	0	61	1	8	0
	150	0	39	0	0	0
Lactose	18	47	32	3	6	0
	37	219	30	25	37	0
	75	239	102	77	2	0
	110	174	99	145	31	0
	150	173	22	146	6	0
Maltose	18	376	62	127	0	13
	37	245	65	175	26	0
	75	369	25	57	11	2
	110	406	24	164	35	1
	150	424	13	60	57	0
Sucrose	18	1	14	9	0	0
	37	1	23	1	0	0
	75	1	10	0	0	0
	110	2	3	0	0	0
	150	15	10	0	0	0
Glycerol	6	5	3	3	0	0
	12	3	0	1	0	0
	24	31	9	3	0	0
	36	48	39	26	0	5
	50	37	48	19	0	10

[a] Each replication consisted of one Petri dish with 50 mg of callus.

TABLE 2

ESTIMATES OF THE OVERDISPERSION PARAMETER ($\hat{\phi}$), THE RATIO STATISTICS AND THE PARAMETER ESTIMATES (STANDARD ERRORS) FOR CAIPIRA AND VALENCIA SWEET ORANGES, AND CLEOPATRA MANDARIN, LINE I

Terms	df	Caipira	Valencia	Cleopatra, line I
Linear	5	5.56	52.38	12.90
Quadratic	3	16.30	3.72	7.70
$\hat{\phi}$		24.40	19.97	14.59
df		66	35	36
Galactose	$\hat{a}$	1.456 (0.6195)	3.934 (0.8154)	2.931 (0.4401)
	$\hat{b}$	0.7361 (0.0144)	−0.0482 (0.0264)	0.0390 (0.0108)
	$\hat{c}$	−0.0003863 (0.000079)	0.0003369 (0.000149)	−0.0001838 (0.000059)
Lactose	$\hat{a}$	2.056 (0.5717)	4.294 (0.3274)	0.8424 (0.7966)
	$\hat{b}$	0.0609 (0.0146)	0.0215 (0.0088)	0.0661 (0.0162)
	$\hat{c}$	−0.0003522 (0.000086)	−0.0000954 (0.000050)	−0.0002573 (0.000078)
Maltose	$\hat{a}$	4.473 (0.4507)	5.189 (0.2298)	4.898 (0.2793)
	$\hat{b}$	−0.0135 (0.0162)	0.0125 (0.0062)	0.0022 (0.0087)
	$\hat{c}$	0.0000055 (0.000105)	−0.0000449 (0.000035)	−0.0000397 (0.000053)

$\hat{a}$, $\hat{b}$ and $\hat{c}$ are parameter estimates of the equation $\hat{Y}_i = \exp(\hat{a} + \hat{b}x + \hat{c}x^2)$, using Poisson distribution for Y_i.

The corresponding charts (Fig. 1) show that independent of the callus line, the higher number of somatic embryos in low concentrations of carbohydrates was obtained with maltose. For callus lines Caipira and Cleopatra, line I (Fig. 1a, c), the number of somatic embryos decreased with increase in maltose concentration. The opposite was observed for Valencia (Fig. 1b), where the best response was obtained with maltose, and the number of embryos was higher with increasing doses of maltose and lactose. However, few Valencia embryos were obtained in galactose, while both galactose and lactose gave good results for Caipira and Cleopatra,

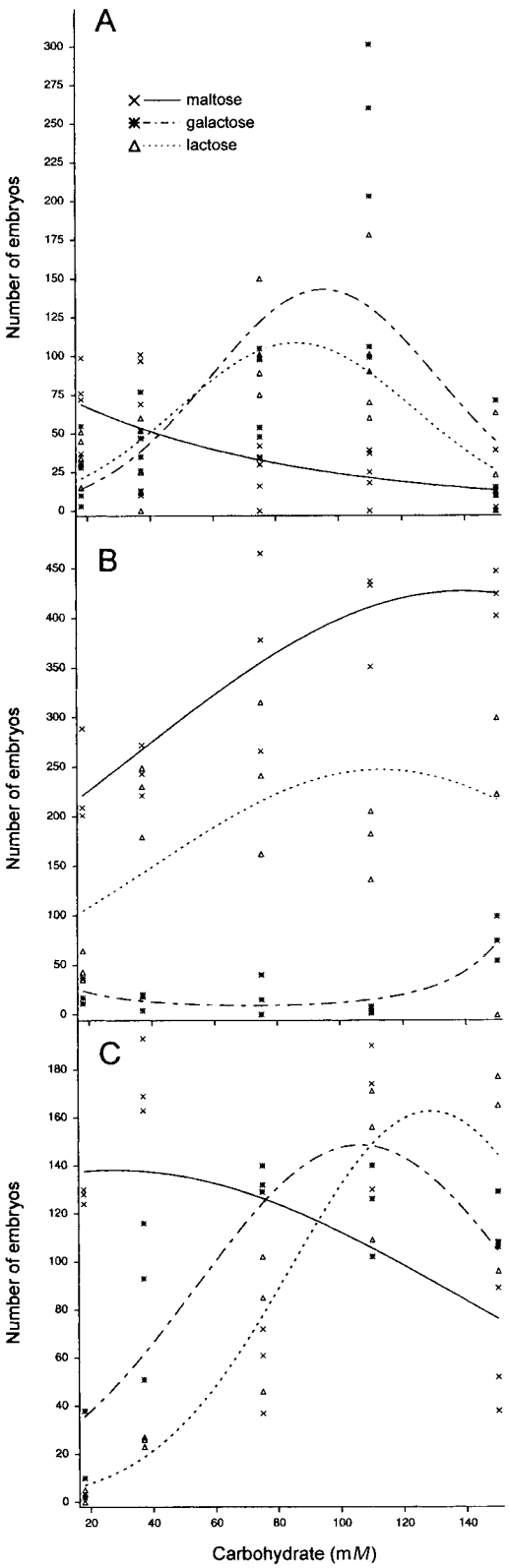

FIG. 1. Observed (*symbols*) and estimated (*lines*) values of embryogenic response of callus lines of citrus cultivars to different carbohydrates. *A*, Caipira sweet orange (*Citrus sinensis*); *B*, Valencia sweet orange (*Citrus sinensis*); *C*, Cleopatra mandarin (*C. reticulata*).

line I, with a similar trend, i.e., the number of embryos increased with increase in carbohydrate concentration up to a maximum, followed by a decrease in the number of embryos formed.

Histological analyses. Morphological and histological observations of somatic embryos in different stages of development showed that in the embryogenic callus lines Caipira, Seleta Vermelha and Valencia sweet oranges, somatic embryos (Fig. 2a) followed the normal developmental sequence – globular, heart shape, torpedo and cotyledonary stages – and conversion into plants occurred naturally and at high rates. Histological sections of these embryos revealed globular embryos with well-developed protoderm and in some cases the beginning of differentiation of a procambial region (Fig. 2b). In heart-shape embryos (Fig. 2c) the establishment of polarity was visible with initial development of cotyledons and procambial strands leading to torpedo- (Fig. 2d, e) and cotyledonary-stage embryos (Fig. 2f) with a continuous protoderm, well-defined cotyledons and connecting procambial strands. Apical meristems were not completely defined in early cotyledonary stages, but regions with slightly smaller cells could be observed at the future shoot apical region and root subapical meristem region, where the procambial strands converge (Fig. 2f).

Embryos formed in cultures of Cleopatra mandarin, lines I and II, and Rangpur lime, when observed under the dissecting microscope, were initially globular, but developed to form structures with various forms, not resembling the normal embryo developmental stages (Fig. 3a). These structures rarely converted into plantlets. Histological sections showed globular embryos, in some cases with a central mitotically active region (Fig. 3b). Protoderm was present and proliferation of callus could be observed on the surface of the embryos. Sections of the structures shown in Fig. 3a reveal several abnormalities. These were somatic embryos which did not continue their normal development, and instead abnormal somatic embryos were produced, probably related to a change in their embryogenic program. Cell proliferation was observed in the shoot apical region (Fig. 3c, e) as well as precocious elongation of the embryo axis (Fig. 3d), and discontinuity or de-differentiation of the protoderm (Fig. 3c, f), probably leading to proliferation of callus and cessation of embryo development.

DISCUSSION

For all combinations tested (cultivar, carbohydrate source and concentration), sucrose did not stimulate somatic embryogenesis, except in Seleta Vermelha sweet orange nucellus-derived callus. In callus cultures of this cultivar, well-formed somatic embryos developed in medium containing sucrose as the carbohydrate source, and the proliferation of callus was prevented. Seleta Vermelha sweet orange cultures were embryogenic and highly repetitive, when maintained on MT medium containing 500 mg l^{-1} malt extract and 50 g l^{-1} sucrose.

For the other embryogenic cultivars, galactose, lactose, and maltose were the best carbohydrate sources for somatic embryogenesis induction. Kochba et al. (1978a, b) related the galactose efficiency in promoting somatic embryogenesis to its ability to inhibit auxin synthesis, modifying the endogenous auxin balance (Cabasson et al., 1995). This indicates that somatic embryogenesis in citrus is favored by conditions that lower the endogenous auxin concentration (Kochba and Spiegel-Roy, 1977). Maltose, a carbohydrate that produces glucose in its metabolism, has also

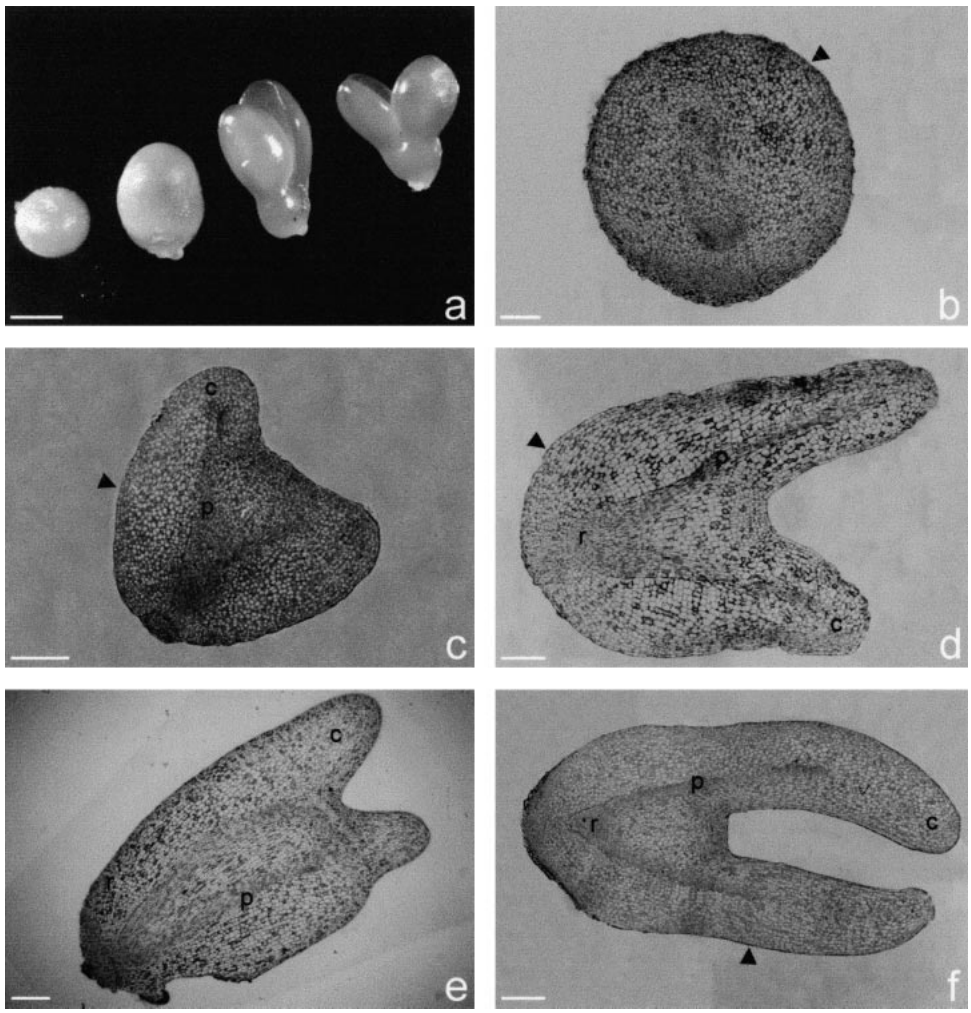

FIG. 2. Somatic embryogenesis from embryogenic callus of Caipira (*e*), Seleta Vermelha (*a, c, d, f*) and Valencia (*b*) sweet oranges. *a*, Normal developmental sequence – globular, heart-shape and cotyledonary stages of somatic embryos (from left to right). *b–f*, Histological section of globular-stage somatic embryo (*b*) showing well-developed protoderm and beginning of differentiation of a procambial region; heart shape-stage (*c*) somatic embryo with cotyledons and procambial strands; torpedo-stage (*d, e*) somatic embryo; cotyledonary-stage (*f*) somatic embryo showing well-developed cotyledons and procambial strands connecting the apical meristems. Abbreviations: c, cotyledon; p, procambial strands; r, root meristem; arrowhead, protoderm (*bars* = 1 mm (a), 200 μm (*b–f*)).

been shown to be effective in stimulating somatic embryogenesis from citrus callus (Hidaka and Omura, 1989; Pérez et al., 1998). The actual effect of glucose-producing carbohydrates in the embryogenic process, however, is not known (Strickland et al., 1987; Kunitake and Mii, 1995). Maltose was recently tested in citrus (Pérez et al., 1999) and also showed positive results in the induction of grape (Coutos-Thevenot et al., 1992) and alfalfa somatic embryos (Strickland et al., 1987). These carbohydrates probably affect the process physiologically, rather than as an energy supply, since their effects occur at low concentrations (Kochba et al., 1978b).

Glucose and fructose have not been efficient for citrus somatic embryogenesis (Kochba et al., 1982; Pérez et al., 1999), although other species show a positive response to these carbohydrates (Komai et al., 1996). Some authors reported that a subculture to medium with glycerol can release citrus somatic embryos from embryogenic arrest (Hidaka and Omura, 1989; Gavish et al., 1992; Singh et al., 1992). However, in the present experiment, only a low number of somatic embryos formed on medium containing this carbohydrate.

Poisson distribution, which is recommended for the statistical analysis of count data, was modified since high deviance values were obtained indicating the occurrence of overdispersion. Possible explanations for overdispersion would be the intrinsic variability in the experimental material (callus cultures) or a correlation among individual responses, which would give an additional component of variability, not accounted for by the basic model. Citrus embryogenic callus is, indeed, a mass of different cell types which could be responsible for the occurrence of overdispersion. Cabasson et al. (1995) described two types of starch-rich cells in cell clusters of *Citrus deliciosa*. The most abundant type had vacuolated cytoplasm with large starch grains, while the less

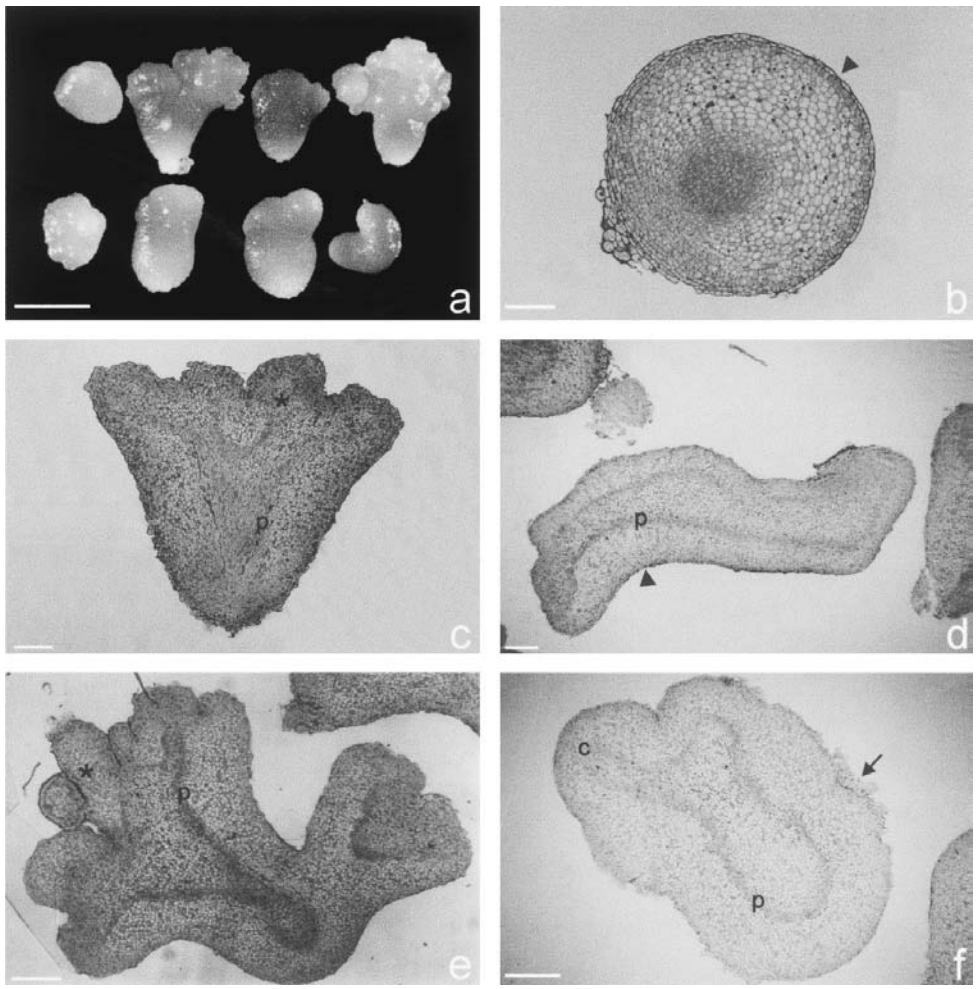

FIG. 3. Somatic embryogenesis from callus lines of Cleopatra mandarin, line I (*b*) and line II (*f*), and Rangpur lime (*a*, *c–e*). *a*, Various types of abnormal somatic embryos; *b*, histological section of globular-stage somatic embryo with a central mitotically active region; *c–f*, histological sections of somatic embryos showing various types of malformations, including proliferation of cells (*) in the shoot apical region (*c*, *e*), elongated embryo axis (*d*) or lack of protoderm continuity (*arrow*) (*f*). Abbreviations: *c*, cotyledon; p, procambium; *arrowhead*, protoderm (*bars* = 1 mm (a), 200 μm (*b–f*)).

common type had small starch granules and a high nucleo-cytoplasmic ratio and were considered embryogenic. In tissue culture systems, every time an explant source is subdivided it is likely that explants with individual characteristics will be created and these characteristics are not accounted for by parameters of variation of statistical models, generating overdispersion.

Abnormalities in somatic embryogenic development are described in many other systems, probably caused by inadequate culture conditions, which are often cultivar-specific. Different degrees of morphological alterations, such as embryo fusion, formation of more than two cotyledons, or lack of proper apical meristem formation have been described (Alemanno et al., 1996; Rodriguez and Wetzstein, 1998). Padmanabhan et al. (1998) described five types of morphological variants in sweet potato cultures, mainly related to the formation of the shoot apex. Lack or low conversion of somatic embryos can be related to abnormalities in the shoot apical meristem (Merkle et al., 1990; Rodriguez and Wetzstein, 1994; Padmanabhan et al., 1998). Abnormal formation of the protoderm has also been described as an important factor, which can lead to the arrest of development of the somatic embryo (Yeung, 1995).

In the present work we reported differences in the response of citrus callus of different cultivars to treatments with six carbohydrates in different concentrations, confirming that the cultivar is indeed an important factor influencing somatic embryogenesis efficiency in citrus. Callus subculture from sucrose-containing medium to a fresh medium containing either galactose, lactose, or maltose, stimulated the embryogenic process in responsive cultures. Histological characterization of the process allowed observation of a change in the embryo developmental pathway in some cultivars, leading to abnormalities of development. In highly embryogenic cultures, the normal embryo developmental sequence was confirmed with anatomical observations. This type of information is important to define the normal embryo developmental pathway and to find possible reasons for low recovery of plants from less embryogenic callus cultures. Studies regarding development and maturation of citrus somatic embryos are important for higher rates of conversion into plants.

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