SOMATIC EMBRYOGENESIS IN GOIABEIRA SERRANA: GENOTYPE RESPONSE, AUXINIC SHOCK AND SYNTHETIC SEEDS

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ABSTRACT –The induction and control of *in vitro* somatic embryogenesis is dependent of a number of factors. The genotype of the mother plant donor of explants and the kind of auxin play essential roles in conferring embryogenic competence. Once obtained somatic embryos may be encapsulated in alginate originating synthetic seeds that can be stored at low temperatures. Considering this the present work aimed at the assessment of five different genotypes, five periods of 2,4-D shock (20 µM for 1, 2, 4, 8 and 16 weeks) (), and different substances supplemented to sodium alginate to obtain synthetic seeds. Zygotic embryos of *Feijoa sellowiana* were inoculated in basal LP medium (von Arnold and Erikson, 1981) supplemented with Morel's vitamins, sucrose (3%) and phytagel (0.2%). The highest rates of somatic embryogenesis induction were observed in the genotypes 50-4 and 101. Shock of two weeks in 2,4-D conferred embryogenic competence in the same levels obtained with the shock of 8 weeks. Histological evaluations revealed the direct origin of the somatic embryos from the epidermic surface of cotyledons. Synthetic seeds containing MS salts and sucrose resulted in higher rates of contamination than capsules free of these substances. KNO_3 (100 mM) resulted in the opening of 81.2% of the capsules as compared to 0% of opening in the treatment with water.

ADDITIONAL INDEX TERMS: *Acca sellowiana*, micropropagation, pineapple-guava, encapsulation.

ABBREVIATIONS: CH - Casein Hydrolysate; 2,4-D - 2,4- dichlorophenoxyacetic acid; GA_3 - gibberellic acid; Gln - L-glutamine; LPm – von Arnold and Eriksson (1981) modified medium; Kin – Kinetin; ½MS - half strength Murashige and Skoog's; SE - somatic embryos; somatic embryogenesis; ZE -zygotic embryos.

EMBRIOGÊNESE SOMÁTICA EM GOIABEIRA SERRANA: EFEITO DO GENÓTIPO, CHOQUE AUXÍNICO E SEMENTES SINTÉTICAS

RESUMO – A embriogênese somática *in vitro* é dependente de uma série de fatores, dentre os quais o genótipo da planta matriz doadora de explantes e a fonte de auxina para conferir competência embriogênica são considerados como determinativos. Uma vez obtidos, os embriões somáticos podem ser encapsulados em alginato para a obtenção de sementes sintéticas, permitindo assim o armazenamento em baixas temperaturas e o posterior cultivo de maneira semelhante à semente verdadeira. Os aspectos cita-

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dos foram estudados no presente trabalho visando elucidar pontos de controle da embriogênese somática. Assim, embriões zigóticos excisados de frutos maduros de cinco acessos de *Feijoa sellowiana* Berg, foram avaliados quanto ao seu potencial embriogenético. Choques auxínicos de 2,4-D (20 μM) em períodos de 1, 2, 4, 8 e 16 semanas foram utilizados para estabelecer o período mínimo de aquisição de competência embriogênica dos explantes e o posterior efeito deste fitorregulador sobre o desenvolvimento e qualidade dos embriões somáticos. Diferentes composições de cápsulas de alginato foram empregadas para avaliar a polimerização das cápsulas e a contaminação das sementes sintéticas. A maior percentagem de indução da embriogênese somática e o maior número de embriões ocorreram nos acessos 101 e 50-4. Choques de 2,4-D de duas semanas foram suficientes para proporcionar valores similares de indução e produção de embriões somáticos, àqueles obtidos com choques de 4 e 8 semanas. Estudos histológicos demonstraram a origem direta da embriogênese somática a partir da epiderme cotiledonar. A adição dos sais de MS e sacarose ao alginato de sódio resultou em altos valores de contaminação das sementes sintéticas, quando comparados às capsulas isentas destas substâncias. O emprego do KNO_3 (100 mM) permitiu a despolimerização e a abertura de 81,2% das sementes sintéticas em comparação aos valores de 0% para o tratamento com água.

TERMOS ADICIONAIS PARA INDEXAÇÃO: *Acca sellowiana*, micropropagação, goiabeira serrana, encapsulamento.

INTRODUCTION

Somatic embryogenesis (SE) recapitulates morphologic and developmental events that occur in the zygotic embryogenesis. *In vitro* SE takes place in the absence of vascular connections with the mother-plant (Zimmerman, 1993). Following appropriate induction, somatic cells can generate a complete plant, since they contain a complete set of genetic information (Merkle *et al.*, 1995). The competence of cells, tissues, and organs in response to specific environmental or chemical signals, may be the starting point to new developmental programs leading to *in vitro* SE induction (Yeung, 1995).

The induction and control of somatic embryogenesis are dependent of the explant source, the genotype of the mother plant, and the type and level of growth regulators supplemented to culture medium with 2,4-**D** as the auxin normally employed for the induction of embryogenic competence (Guerra *et al.*, 1999).

Once obtained a somatic embryo can be stored as a synthetic seed. The synthetic seed technology based on the encapsulation of somatic embryos in a hydrogel capsule was proposed by Redenbaugh *et al.*, (1986). The main advantages of this technology is are protection of the somatic embryos and ease in storage, conservation, transport and conversion to plantelts (Onishi *et al.*, 1994, Guerra *et al.*, 1999).

The 'goiabeira serrana' (*Feijoa sellowiana* Berg, synonymous of *Acca sellowiana* (Berg.) Burr.) is a fruit species native of the South of Brazil and North of Uruguay, where it is not yet commercially cultivated. The naturally occurring populations in Santa Catarina State show great variability in the fruit size, color and other features (Nodari *et al.*, 1997). In New Zealand, Australia, USA and some countries of Europe it has been cultivated in commercial orchards since the begining of the 20th century (Ducroquet and Hickel, 1997).

Conventional techniques of vegetative propagation of *F. sellowiana* based on cuttings are difficult because of the low rates of success. Apparently, the phenol oxidation negatively affects the percentage of rooting (Duarte *et al.*, 1992; Fachinello *et al.*, 1992).

Micropropagation techniques have been used to overcome such problems. Various reports of the application of tissue culture techniques based on organogenesis in this species have been published elsewhere (Bhojwani *et al.*, 1987; Canhoto & Cruz, 1996a; Dal Vesco and Guerra,

1999; Oltramari *et al.*, 2000). These protocols have been based on the culture of nodal segments, apical and axillary bud, and microcuttings, generally showing low efficiency in terms of regenerative potential. In parallel, research efforts have been alternatively directed to the induction and control of somatic embryogenesis. Thus, protocols concerning this morphogenetic pathway were developed by Cruz *et al.*, (1990), Canhoto and Cruz (1994, 1996b), and Guerra *et al.*, (1997), Dal Vesco and Guerra (2001) aiming at the mass propagation of superior genotypes.

Therefore, the purposes of the present work were to study (a) the role of shocks of 2,4-**D** in the induction of *F. sellowiana* embryogenic competence; (b) the influence of the mother plant genotype in the induction and control of *in vitro* somatic embryogenesis of *F. sellowiana*, and; (c) the use of the synthetic seed technology for the encapsulation and handling of somatic embryos of this species.

MATERIAL AND METHODS

Plant material

Mature zygotic embryos were collected from fruits of selected mother plants maintained at the germplasm collection of the Experimental Station of São Joaquim (EPAGRI). These fruits were transported to the Laboratory of Developmental Physiology and Plant Genetics, Depto. de Fitotecnia, Universidade Federal de Santa Catarina, Brazil. The seeds, collected 135 days after anthesis were extracted and disinfected with ethanol (70%) for 1 min; commercial bleach (2.0% NaClO) for 20 min and then rinsed three times with sterile water. The zygotic embryos were excised in aseptic chamber and innoculated in test tubes (25 x 150 mm) containing 15 mL of basal medium LPm (von Arnold and Eriksson, 1981) supplemented with Morel's vitamins (Morel and Wetmore, 1951), sucrose (3%), and Phytagel® (0.2%). The pH was adjusted to 5.8 prior to autoclaving. The cultures were maintained in culture room in the dark at 25 ºC during the induction phase. For somatic embryo conversion the cultures were kept at the photosynthetic photon flux of 40 μmol m^{-2} s^{-1} provided by cool white lamps and 16 h d^{-1} photoperiod

Effect of Genotypes

Zygotic embryos of different genotypes of *F. sellowiana* were employed to evaluate somatic embryogenesis induction. The basal LPm medium was supplemented with 2,4-**D** (20 μM) and L-Gln (4 mM). The experiment was arranged in a completely randomized design (CRD) with five *F. sellowiana* genotypes: 50-4, 53**B**-7, 101, 529, and 152-12x458 from the germplasm collection. Each experimental unit was consisted of ten explants, replicated four times. The percentages of explants having embryos and the number of viable somatic embryos per explant was recorded after 5, 10 and 15 weeks in culture, and submitted to Analysis of Variance (ANOVA).

Shock of 2,4-D

Zygotic embryos of the genotype 101 were excised and then inoculated in the basal LPm medium supplemented with 2,4-**D** (20 μM) and Gln (4 mM). The experiment was arranged in a CRD with 1, 2, 4, 8 and 16 weeks intervals of incubation time in culture medium supplemented with 2,4-**D**. Each experimental unit was consisted of 10 explants replicated four times. After the shock period the cultures were transferred to the LPm basal medium. The percentage of induction and the number of somatic embryos per explant were recorded after 5, 10, 15 and 20 weeks in culture, and the percentage of explants with somatic embryos were submitted to regression analysis.

Histology

Samples were selected and fixed in solution 2.5% glutaraldheyde in phosphate buffer (0.1 M, pH 6.8) and dehydrated in an ethanol ascending series, xilol and embedded in paraffin wax (Sass, 1951). Serial sections (8-12 μm) were

obtained using a rotary microtome Slee Technik® and stained with safranin and fast-green.

Synthetic seeds

Pre-germinated torpedo and cotyledonary somatic embryos after two weeks in basal culture medium containing Kin (0.5 μM) and GA_3 (0.5 μM), were encapsulated in sodium alginate 1% (Carlo Erba®) and immediately complexed in $CaCl_2$ (50 mM) during 20 min. The capsules were then stored at 4ºC for different periods of times. A treatment combination of three factors (4x4x2) was employed. In the first factor, four types of artificial endosperm added to sodium alginate were tested: (i) GA_3 (0.05 μM) + Kin (0.05 μM); (ii) ½MS + GA_3 (0.05 μM) + Kin (0.05 μM); (iii) ½MS + sucrose (2%) + GA_3 (0.05 μM) + Kin (0.05 μM); and (iv) ½MS + sucrose (2%) + GA_3 (0.05 μM) + Kin (0.05 μM) + CH (500 mg/l). As the second factor, different periods of storage at 4ºC (one, two, three, and four weeks), were tested. Two treatments of capsule opening, with immersion in 100 mM of KNO_3 or distilled water during 20 min were also employed as levels of the third factor. Each experimental unit was constituted by five capsules, replicated three times and arranged as a CRD. Five weeks after the transference to petri dishes containing distilled water the conversion rate and the frequencies of contamination were recorded.

Statistical analysis

Data on percentage, number and conversion of somatic embryos were submitted to the test of F-maximum. When necessary, the data were transformed to log (x + 2) or $(x + 0.5)^{0.5}$. The data were then submitted to Analysis of Variance, to the Student-Newman-Kuels (SNK) test, and to the Regression Analysis, according to Sokal and Rohlf (1995).

RESULTS AND DISCUSSION

Effect of Genotypes

Out of five cultured genotypes three of them revealed significant ($P<0.01$) rate of somatic embryogenesis and yield of SE ($P<0.05$) after 10 weeks in culture (Figure 1a, b). Visual evaluations in stereomicroscope showed the presence of globular somatic embryos after four weeks in culture. This embryogenic induction occurred in at a low frequency and asynchronously. After five weeks in culture, heart and torpedo shaped somatic embryos were observed. Finally, after ten weeks in culture a high frequency of torpedo and cotyledonary somatic embryos was observed (Figure 2a). An increase in the rates of somatic embryogenesis induction was observed after ten weeks in culture as compared to the level recorded after five weeks in culture (Figure 1a, b). After 15 weeks in culture, a lower number of somatic embryos, some of them abnormal and whitish-opaque, in comparison with those formed in shorter period treatments.

While the highest frequency and number of somatic embryos were observed in the genotypes 50-4 and 101, the genotype 53B-7 revealed the lowest induction rates and production of somatic embryos (Figure 1 and Figure 2b).

The induction and control of somatic embryogenesis in *F. sellowiana* has been associated with an interaction between the genotype of the mother plant and the constitution of the culture medium (Canhoto and Cruz, 1996a and Guerra *et al.*, 1997). Genotype specificity for embryogenic induction was also reported in other embryogenic systems such as in conifers (Attree and Fowke, 1993), *Vitis spp* (Gray, 1995), *Juglans spp* (Preece *et al.*, 1995), *Theobroma cacao* (Alemanno *et al.*, 1996), and *Gossypium hirsutum* (González-Benito *et al.*, 1997).

2,4-D Shock

The transfer of explants after two weeks in the culture medium containing 2,4-**D** to the culture medium free of growth regulators resulted in early production of somatic embryos in a large percentage of the explants (Figure 3a, b). However, when the cultures were submitted to a one-week shock of 2,4-**D**, then subcultured in a culture medium free of growth regulators, a low percentage of induction and a low number of

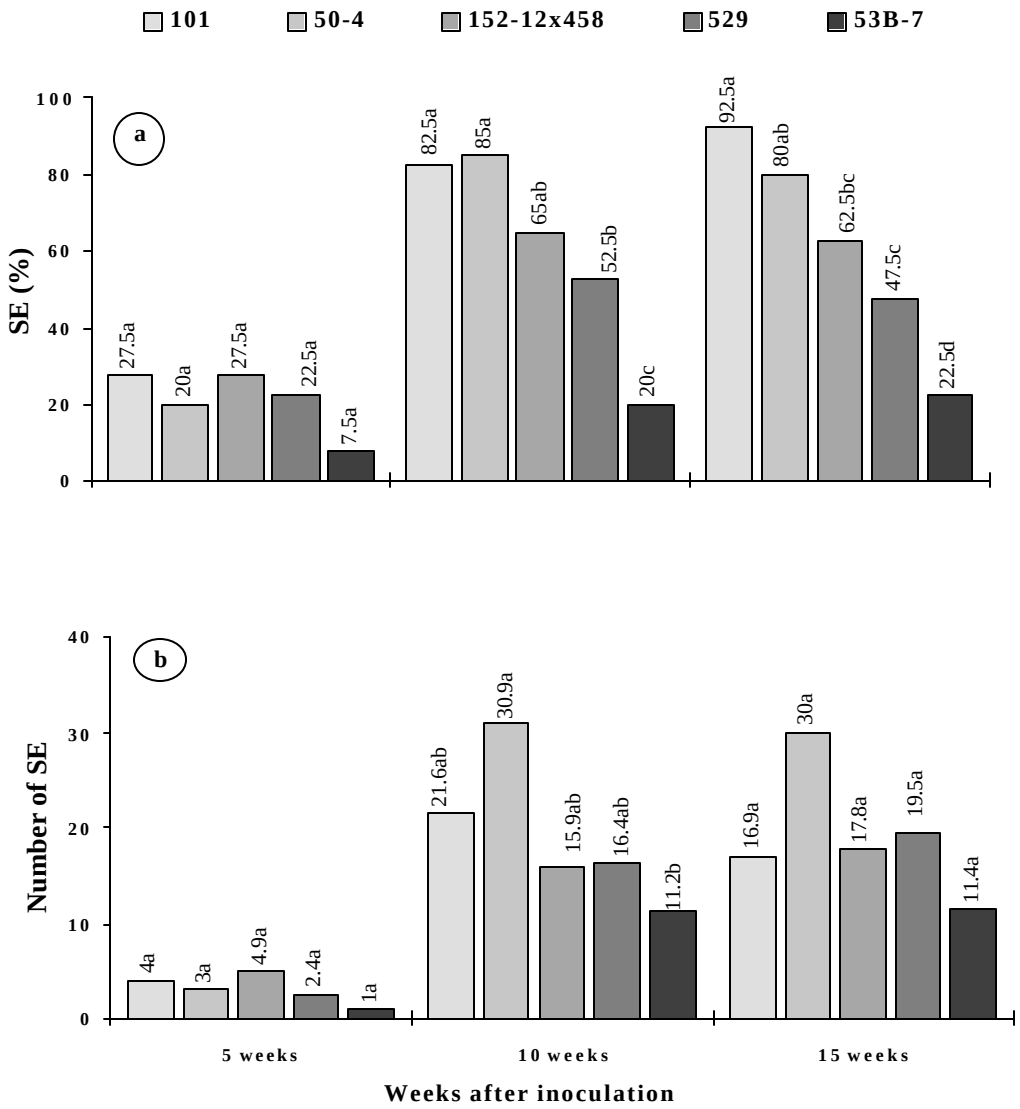


FIGURE 1 - Induction of somatic embryos (SE): **a)** percentage of SE[a] e **b)** number of SE[b] per embryogenic explant, in five genotypes of *F. sellowiana* in LPm culture medium supplemented with 2,4-D (20 μM), after different periods (weeks) of culture.

Means of four replicates. Values followed by same letters are not significantly different at the 5% level by SNK test.

[a] CV (%) for 5 wk = 76.8; 10 wk = 20.5 and 15 wk = 25.7

[b] (CV%) in 5 wk = 91.3; 10 wk = 23.0 [y] and 15 wk = 51.2.

[y] data transformed for analysis using $(x + 0.5)^{0.5}$

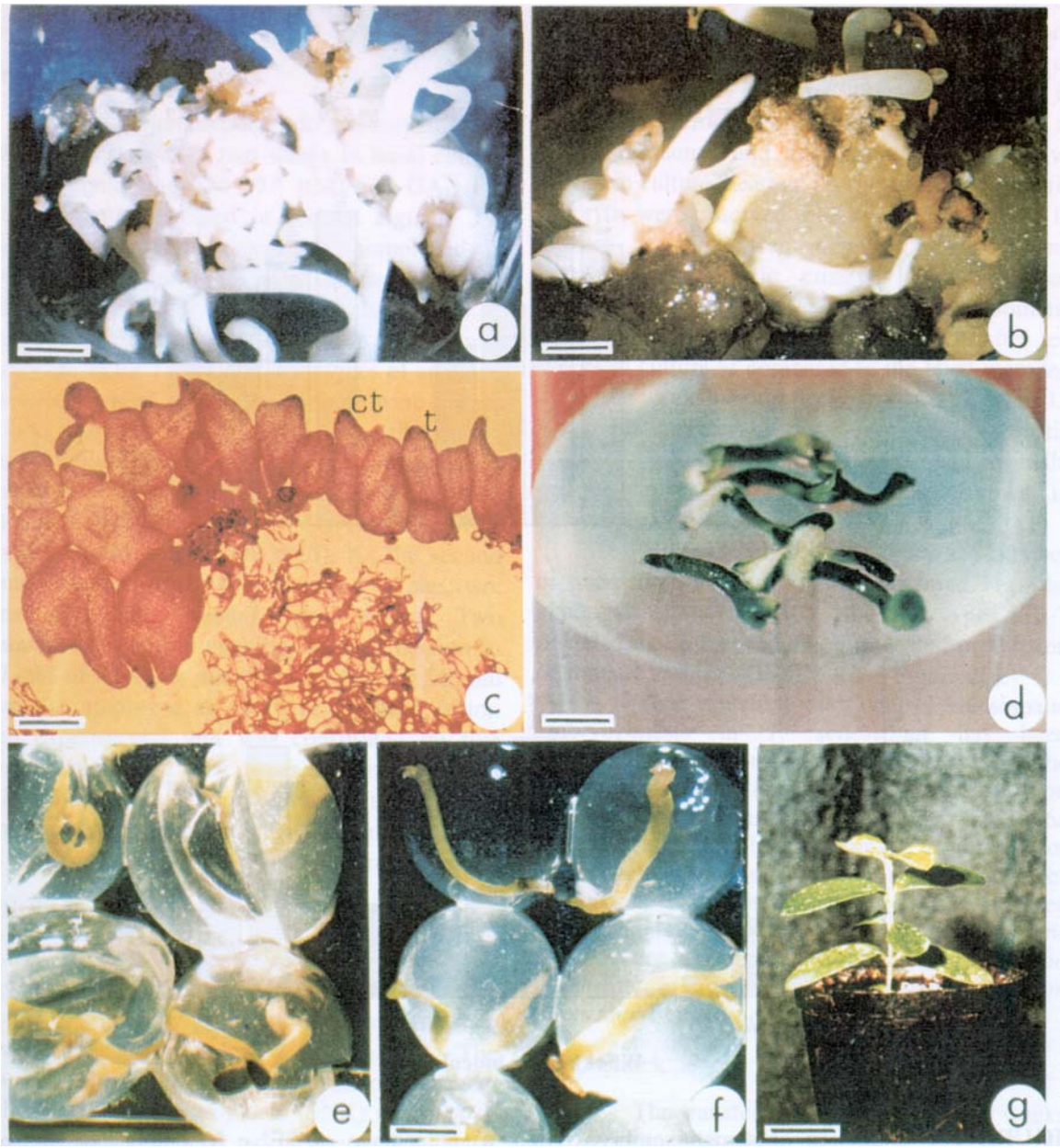


FIGURE 2 – **a**) High frequency and asynochronous somatic embryos (SE) of *F. sellowiana* (bar: 2.6 mm), **b**) low frequency SE (bar: 2.6 mm); **c**) Transection of embryogenic culture showing different stages of SE, after 12 weeks in culture (t-torpedo stage; ct-cotyledonary stage; bar: 0.1 mm) **d**) Pre-germinated SE, after 2 weeks on basal culture medium (bar: 2.6 mm); **e**) Synthetic seeds treated with KNO_3 (100 mM) during 20 min (bar: 1.8 mm); **f**) Synthetic seeds treated with water (bar: 1.8 mm); **g**) Plantlets growing in soil after 10 weeks in greenhouse (bar: 3.2 cm).

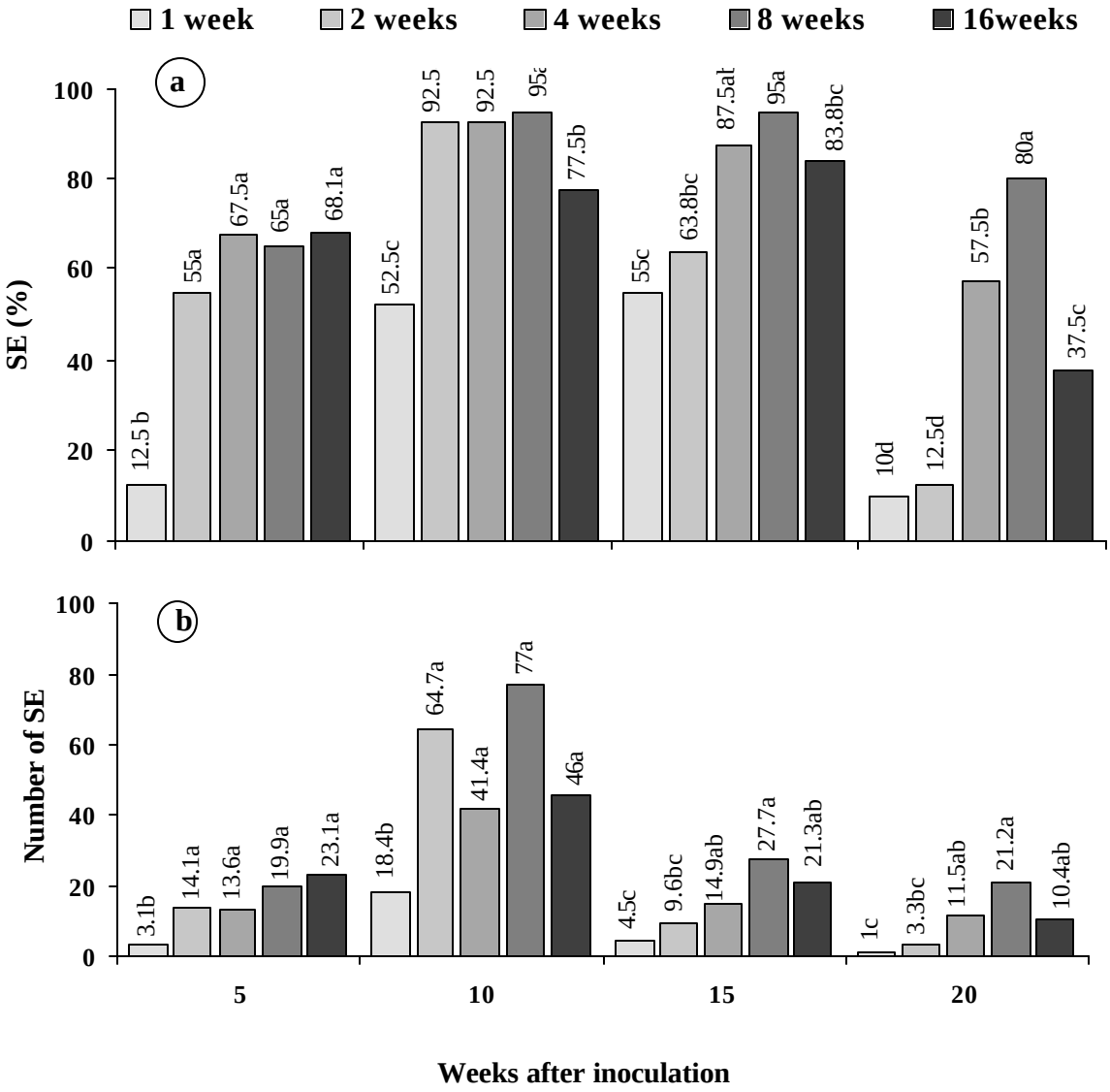


FIGURE 3 - Effect of shock of 1, 2, 4, 8 and 16 weeks of 2,4-D (20 µM), **a)** percentage of somatic embryos (SE)[a], and **b)** number of SE per explant[b, y] of genotype 101 of *F. sellowiana*, after different periods (week) of culture.

Means of four replicates. Values followed by the same letters are not significantly different at the 5% level by SNK test.

[a] CV (%) in 5 wk = 28.9; 10 wk = 9.7; 15 wk = 19.0 and 20 wk = 33.5

[b] CV (%) in 5 wk = 24.4; 10 wk = 18.2; 15 wk = 22.0 and 20 wk = 35.5.

[y] data transformed for analysis using $(x + 0.5)^{0.5}$

somatic embryos were observed after ten weeks in culture. In these two treatments, the induction of somatic embryogenesis culture. In these two treatments, the induction of somatic embryogenesis occurred directly from the cotyledonary surface of the zygotic embryos used as explants and evolved rapidly to the cotyledorary stage. This was confirmed by anatomic and histological studies showing that the globular somatic embryos originated directly from the epidermic cell layers of cotyledon, after five weeks in culture (Figure 2c).

Previously, Cruz *et al.*, (1990) showed that the culture of explants during five days in medium culture supplemented with 2,4-**D**, was sufficient to promote the somatic embryogenesis in *F. sellowiana*. Thus, the present work supports the role of 2,4-D in the promotion of Feijoa somatic embryogenesis.

The group of cells present in the cotyledonary surface of zygotic embryos of *F. sellowiana* was termed meristematic cells (Canhoto and Cruz, 1996b), or embryogenic cells, since they do not need long exposition time to auxins to induce somatic embryogenesis (de Jong *et al.*, 1993; Guerra *et al.*, 1999). In adequate culture conditions these cells are competent to launch an embryogenetic developmental program, resulting in the production of somatic embryos, mimicking the developmental pattern of zygotic embryogenesis (Zimmerman, 1993; Toonen *et al.*, 1996). These results suggest that, under certain conditions, these cells may be competent to originate synchronously high frequency of somatic embryos (Osuga and Komamine, 1994).

A shock of 16 weeks with 2,4-**D** negatively affected the induction rate and the development of somatic embryos (Figure 3a, b), suggesting that the maintenance of primary cultures of *F. sellowiana* in the presence of this growth regulator may originate some abnormalities in somatic embryo development. However, the permanence during eight weeks of the somatic embryos in the culture medium containing 2,4-**D**, resulted in a decrease in the frequency of induction of somatic embryos after 15 weeks in culture after shock (Figure 4). According with these data the quadratic adjustment showed high and significant r^2 and P-values. The adjusted model allowed us to estimate that the highest induction rate (98.7% in 13.5 weeks in culture after shock) was obtained with eight weeks 2,4-D shock. Four and two weeks 2,4-D shock resulted in 93.7% and 86.4% of embryogenic induction after 11.9 and 10.7 weeks in culture after shock, respectively. Cruz *et al.*, (1990) also observed in *F. sellowiana* that long periods in the presence of 2,4-D did not improve the embryogenic response. In *Carica pubescens* the best induction and formation of somatic embryos occurred with shocks of six days in culture medium supplemented with 2,4-D plus BAP (Jordan and Velozo, 1996).

The results of the present work showed that low exposition time in 2,4-D was associated with a very rapid differentiation and development of somatic embryos, which showed increased germination rates. These results suggest that the correct manipulation of this growth regulator in the induction phase of *F. sellowiana* somatic embryogenesis is a key factor to the establishment of its regenerative protocol and the commercial viability of these micropropagation system.

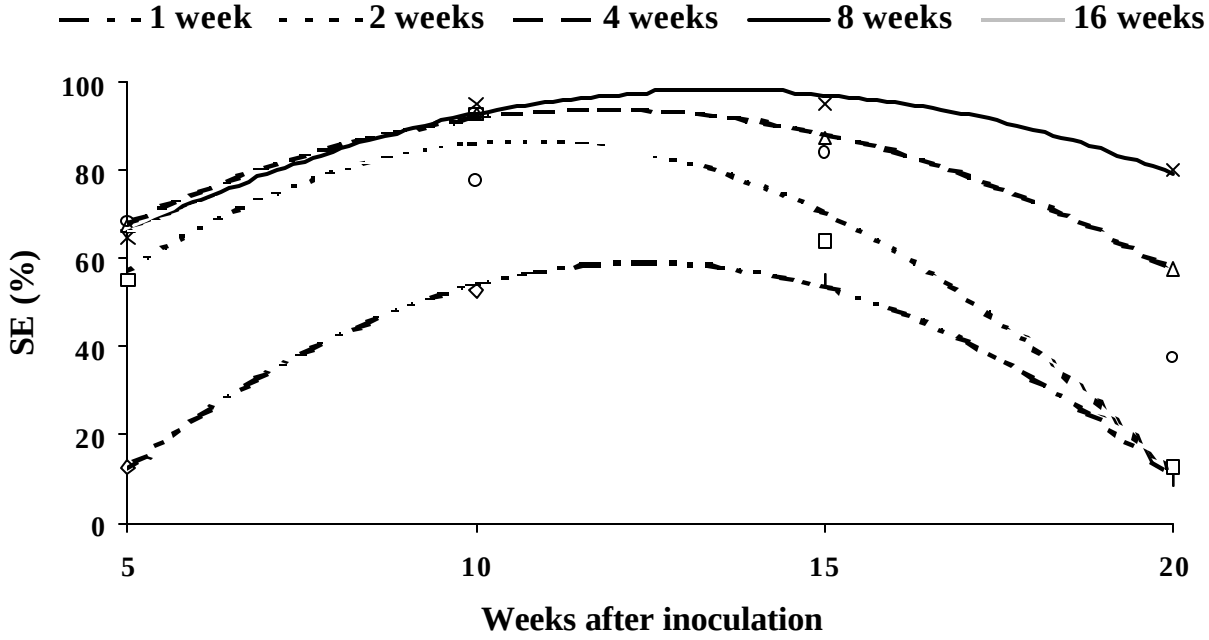
Synthetic seeds

Pre-germinated torpedo shaped somatic embryos (Figure 2d) were utilized for encapsulation. Polymerization of sodium alginate (1%) in $CaCl_2$ (50 mM) resulted in the formation of hydrogel capsules with somatic embryos inside. The treatment with KNO_3 (100 mM) during 20 min resulted in high rate of capsules opening (Table 1) of the synthetic seeds (Figure 2e) as compared to the treatment with distilled water (Figure 2f). Increasing levels of nutrients and sucrose for the reconstitution of a synthetic endosperm resulted in high rates of capsule contamination during the incubation period.

In the technology of synthetic seeds, the use KNO_3 has been proposed in order to soften the

hidrogel capsule. Since the sodium alginate is complexed by Ca^{+2} ions, the resulting synthetic seed is stored in the form of a hard capsule. The mechanical resistance of the capsule in association with the low temperature of storage prevents the germination of the encapsulated somatic embryo. The results of the present work (Table 1) showed that the germination of the encapsulated somatic embryo is facilitated by a pre-treatment of the synthetic seed with a solution of KNO_3 (100 mM), resulting in 81.2% of opened capsules as compared with 0% in the treatment with water. The best KNO_3 concentration resulted from previous experiments (data not shown). According to Onishi *et al.*, (1994) the K^+ ions from KNO_3 replaces the Ca^{+2} of the calcium alginate capsule thus allowing the softening and opening of the synthetic seed and the subsequent germination of the somatic embryos. Dal Vesco & Guerra (2001) showed that the conversion of *F. sellowiana* somatic embryos to plantlets was enhanced by culturing them in half-strenght MS culture medium supplemented with BAP (0.5 μM).

The conversion of somatic embryos was possible in autoclaved soil and the derived plantlets were successfully acclimatizated (Figure 2g).



$Y_{(1wk)} = - 72.5 + 21.15x - 0.85x^2 \quad r^2 = 0.997$

$Y_{(2wk)} = - 16 + 19.08x - 0.89x^2 \quad r^2 = 0.971$

$Y_{(4wk)} = 16.25 + 13.05x - 0.55x^2 \quad r^2 = 0.998$

$Y_{(8wk)} = 16.25 + 12.15x - 0.45x^2 \quad r^2 = 0.982$

$Y_{(16wk)} = 18.47 + 12.22x - 0.56x^2 \quad r^2 = 0.903$

FIGURE 4 - Effects of 2,4-D (20 μM) shock (1, 2, 4, 8 and 16 weeks) on percentage of somatic embryos (SE) of the genotype 101 of *F. sellowiana* as a function of time (weeks) in LPm culture medium. Each value represents the means of four replicates.

TABLE 1 - Effect of different artificial endosperm and time of storage at 4ºC in the contamination of synthetic seeds and effect of KNO_3 on the opening of capsules, five weeks after the transference to incubation.

Artificial Endosperm	
Sodium alginate plus:	Contamination (%)
GA_3 (0.05 μM) + Kin (0.05 μM)	36.6 a
½MS + GA_3 (0.05 μM) + Kin (0.05 μM)	72.4 b
½MS + sucrose (2%) + GA_3 (0.05 μM) + Kin (0.05 μM)	77.6 b
½MS + sucrose (2%) + GA_3 (0.05 μM) + Kin (0.05 μM) + Casein (500 mg/l)	80.8 b
Storage in 4 ºC	
Number of weeks	Contamination (%)
1	54.2 a
2	60.8 a
4	75.0 b
3	77.6 b
Opening Capsule	
Treatments	Opened Capsule (%)
KNO_3	81.2 a
Control (H_2O)	0.0 b

Means of three replicates. Values followed by same letters are not significantly different at the 5% level by SNK test.

In conclusion, the results obtained in the present work showed that somatic embryogenesis in *Feijoa sellowiana* is dependent, among other factors on the genotype of the mother plant. A single shock of two weeks in 2,4-**D** conferred embryogenic competence and allowed the development of morphological normal somatic embryos. Anatomic and histological studies showed that the somatic embryos originated directly from the epidermic cell layers of cotyledon of zygotic embryos used as explant. Those somatic embryos once encapsulated in synthetic seeds containing MS salts and sucrose resulted in higher rates of contamination than capsules free of these

substances. To complete the cycle, the treatment of synthetic seeds with KNO_3 (100 mM) allowed the opening of 81.2% of the capsules as compared to 0% of opening in the treatment with water.

Besides elucidating some of the control points of the somatic embryogenesis in this species towards the establishment of a mass clonal micropropagation system, the results obtained in the present work provided additional support for this species to be used as a reference system to the somatic embryogenesis in woody dicots.

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