

# Recovering polyploid papaya *in vitro* regenerants as screened by flow cytometry

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**Abstract** Several protocols have been proposed for *in vitro* propagation of papaya, either based on somatic embryogenesis or shoot organogenesis. It is well-known that tissue culture-based approaches are frequently associated with somaclonal variation. Whether on the one hand this phenomenon can preclude further stages of *in vitro* culture, on the other hand it can generate useful genetic variability for crop improvement. However, somaclonal variation analyses are limited in papaya tissue culture. The DNA ploidy level of 250 papaya somatic embryogenesis-derived plantlets from immature zygotic embryos was analyzed by flow cytometry. *In vitro*-grown and greenhouse seed-derived plantlets were used as diploid standards. Flow cytometry unambiguously evidenced euploid (diploid, mixoploid, triploid and tetraploid) and aneuploid papaya plantlets, indicating that *in vitro* culture conditions can lead the occurrence of somaclonal variation. Additionally, the two subsequent flow cytometry analyses

showed that the DNA ploidy level remained stable in all cloned papaya plantlets during the successive subcultures in the multiplication medium.

**Keywords** Aneuploid · *Carica papaya* · DNA ploidy · Euploid · Polyploidy · Somaclonal variation · Somatic embryogenesis · Tissue culture

## Abbreviations

ABA Abscisic acid  
BAP 6-Benzylaminopurine  
2,4-D Dichlorophenoxyacetic acid  
NAA  $\alpha$ -Naphthaleneacetic acid  
DAPI 4',6'-diamidino-2-phenylindole

## Introduction

Papaya (*Carica papaya* L.), a member of Caricaceae family, is a plant of which popularity and the easiness that it can be propagated have made it ubiquitous in tropical and subtropical regions of the world (Chen and Chen 1992; Manshardt 1992; Yang and Ye 1992; Yang et al. 1996; Lai et al. 2000; Bhattacharya and Khuspe 2001). Papaya fruit is a nutritious source of vitamins C and A, primarily consumed as a fresh dessert fruit, whereas unripe fruits synthesize significant amount of the proteolytic enzyme papain (Manshardt 1992; Bhattacharya and Khuspe 2001).

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The majority of the commercial plantations of *C. papaya* have been established by seed propagation (Bhattacharya and Khuspe 2001; Fernando et al. 2001); however, this practice hinders the cultivar progress and agricultural profitability owing to sexual factors, heterogeneity as a result of cross-pollination and susceptibility to the *Papaya ringspot virus* (Chen et al. 1987; Fitch 1993; Yang et al. 1996; Bhattacharya and Khuspe 2001).

Biotechnological-based techniques are relatively advanced in papaya, including micropropagation, somatic embryogenesis, embryo rescue, genetic transformation and molecular markers (Manshardt 1992; Fitch 1993; Ashmore and Drew 2006). Hence, different protocols have been proposed for in vitro propagation of important papaya cultivars. Particularly, somatic embryogenesis is the most used procedure to obtain asexual embryos, aiming to scale up the clonal propagation (Fitch 1993; Castillo et al. 1998a) and to supply synthetic seeds (Castillo et al. 1998b), and to the production of transgenic plants (Yang et al. 1996).

In vitro clonal propagation techniques provide the ability to efficiently multiply and maintain large numbers of elite genotypes, including putative transformants. Nevertheless, genotypic instability is commonly observed in plants derived from tissue culture and is at least partly due to in vitro-induced stress (Larkin and Scowcroft 1981; Evans et al. 1984; Karp 1995; Chen et al. 2006). These variations are unpredictable in nature and characterized by the occurrence of genomic alterations, heritable (genetic) and non-heritable (epigenetic), in plant cells, embryos, tissue and organs, induced by in vitro conditions (De Klerk 1990; Wyman et al. 1992; Henry et al. 1996; Tremblay et al. 1999; Jain 2001; Polanco and Ruiz 2002; Bennici et al. 2004; Smýkal et al. 2007).

Somaclonal variation may be of great interest in breeding programs and chromosome engineering because it can generate novelties for crop improvement (Al-Zahim et al. 1999; Do et al. 1999; Jain 2001; Polanco and Ruiz 2002; Rakoczy-Trojanowska 2002). It may be useful in screening and selecting papaya somaclonal variants with resistance to fungal diseases (Manshardt 1992). On the other hand, if in vitro clonal propagation or genetic transformation is the main goal, it becomes an undesired phenomenon.

Somaclonal variation may be partly circumvented by culture procedure modulation (Evans et al. 1984); however, there is no fundamental understanding of the cause or methods to prevent or diagnose it. Thus, it is important to know the frequency, genomic distribution, mechanisms, and factors influencing somaclonal variation (Polanco and Ruiz 2002). Direct methods, as molecular (Al-Zahim et al. 1999; Fourré 2000; Gesteira et al. 2002; Polanco and Ruiz 2002; Encheva et al. 2003; Renau-Morata et al. 2005; Chen et al. 2006), cytometric (Brutovská et al. 1998; Thiem and Sliwinska 2003; Alan et al. 2007; Parc et al. 2007), and cytogenetic tools (Kochevenko et al. 1996; Fourré et al. 1997; Brutovská et al. 1998; Al-Zahim et al. 1999; Do et al. 1999; Tremblay et al. 1999; Menéndes-Yuffá et al. 2000) have been used to detect and select somaclonal variants.

The dark-side of the somaclonal variation has been somehow threatening researchers throughout the years, though recurrently discarding promising and potentially useful material for genetic manipulations, including the chromosome engineering.

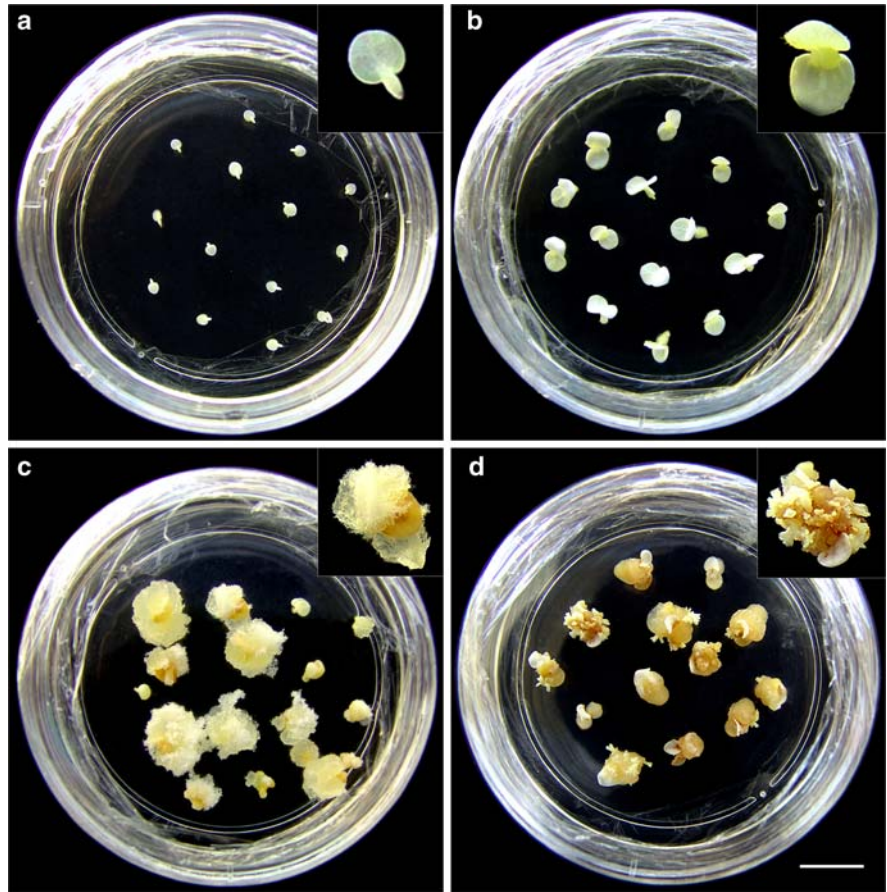
Our work aimed to use this approach in order to verify its potential application to recover polyploid papaya plantlets derived from somatic embryogenesis as screened by flow cytometry.

## Materials and methods

### Plant material and tissue culture

Hermaphroditic *C. papaya* ‘Golden’ fruits were supplied by the Caliman Agrícola S.A. (Linhares, Espírito Santo, Brazil). Harvested fruits from 90 to 114 days post-anthesis (Fitch and Manshardt 1990) were surface-sterilized as described by Koehler (2004). Fruits were air-dried in a laminar flow hood for 10 min prior to dissection and embryo excision. Ten immature zygotic embryos (Fig. 1a) were placed in 60 × 15 mm Petri dishes (J. Prolab<sup>®</sup>, Brazil) with embryogenic induction medium containing half-strength MS basal salts supplemented with MS vitamins (Murashige and Skoog 1962), 9.05 μM 2,4-D, 0.55 mM myo-inositol, 2.75 mM L-glutamine, 6% (w/v) sucrose and 0.28% (w/v) Phytigel (Sigma<sup>®</sup>, USA), as recommended by Castillo et al. (1998a, b), Almeida et al. (2000, 2001) and Koehler (2004). The pH of this medium was adjusted to 5.7

**Fig. 1** Somatic embryogenesis in *Carica papaya* from immature zygotic embryo cultivated in 2,4-D-supplemented induction medium. **(a)** Immature zygotic embryos at first day of culture. **(b)** Cultures showing initial callogenic response at the apical meristem after 10 days. **(c)** Differentiated callus at 20 days of culture. **(d)** Embryogenic induction medium with somatic embryos in distinct development stages arising from callus. Note at each up right corner an inset to highlight details of the embryogenic culture stages. Bar = 1 cm



prior autoclaving. Petri dishes were sealed with PVC film (Goodyear<sup>®</sup>, Brazil), and cultures were kept in the dark at 27°C.

After 60 to 70 days, embryogenic callus were transferred to maturation medium of similar composition with regard to the embryogenic induction medium, however devoid of 2,4-D, and supplemented with 0.5 µM of ABA (Castillo et al. 1998a, b). Cultures were maintained at 27°C under a 16/8 h light/dark regime with 36 µmol m<sup>-2</sup> s<sup>-1</sup> light radiation provided by two fluorescent lamps (20 W, Osram<sup>®</sup>, Brazil) for 20 days.

Subsequently, heart embryos were isolated and placed onto germination medium [MS basal medium salts and vitamins supplemented with 0.55 mM myo-inositol, 3% (w/v) sucrose and 0.7% (w/v) agar (Merck<sup>®</sup>, USA)] in the dark.

Regenerated plantlets selected by flow cytometry were further propagated in vitro as recommended by Lai et al. (2000). Shoot tips were excised and

cultured into multiplication medium [MS-based medium supplemented with MS vitamins, 0.55 mM myo-inositol, 3% (w/v) sucrose, 0.88 µM BAP, 0.11 µM NAA and 0.7% (w/v) agar (Merck<sup>®</sup>, USA)]. Cultures were maintained at 27°C under a 16/8 h light/dark regime with 36 µmol m<sup>-2</sup> s<sup>-1</sup> light radiation. The proliferative shoot cultures were subcultured on a monthly basis.

#### Flow cytometry

Leaf samples were collected from regenerated and cloned *C. papaya* plantlets by subcultures on a monthly basis. In vitro germinated and greenhouse seed-raised plantlets were used as external diploid standards.

The flow cytometry analyses were accomplished as described by CyStain UV Ploidy Partec<sup>®</sup> protocol. Leaves were placed in distilled water at 4°C and cut

into 2 cm<sup>2</sup> fragments. Suspension of nucleus was extracted by chopping (Galbraith et al. 1983) in 0.5 ml of Partec<sup>®</sup> solution buffer containing DAPI (excitation/emission wavelengths: 320–385/415–520) (Shapiro 2003), soon after, was added 1.5 ml of same solution. Suspension was filtered in nylon filter (Partec<sup>®</sup>) with 30 µm mesh diameter after 2 min. After 15 min in the dark, the nuclear suspensions were analyzed with a Partec-PAS<sup>®</sup> flow cytometer (Partec<sup>®</sup> GmbH, Munster, Germany), equipped with an UV lamp emitting at 388 nm and a TK 420 filter. The equipment was carefully calibrated and aligned using microbeads and standard solutions according to the manufacturer's recommendations and nuclei suspension of the standard papaya plants. FlowMax<sup>®</sup> software (Partec<sup>®</sup>) was used for data analyses. More than 5,000 nuclei were analyzed and three independent replications were used for DNA ploidy level determinations.

Initially, the DNA ploidy level of all plants was measured by flow cytometry to select the polyploid plantlets. These plantlets were maintained in multiplication medium and the stability of the DNA ploidy level was assessed twice, every 30 days in the multiplication phase, coincidentally to each subculture step.

## Results and discussion

Embryogenic cultures were established from immature zygotic embryos of *C. papaya* (Fig. 1a). This explant source supplied friable and compact callus after approximately 5 days on embryogenic induction medium. The differentiation of the globular somatic embryos was observed after 20–25 days, from the friable callus formed mainly in the shoot apical meristem (Fig. 1b–d). Similar results were also obtained by Fitch and Manshardt (1990), Fernando et al. (2001) and Koehler (2004). In accordance with Koehler (2004), the majority of the globular embryos developed asynchronously (Fig. 1d).

As a result of the high rate of somatic embryogenesis (~35 somatic embryos per callus) and suitable tissue culture medium used for maturation (Koehler 2004), 320 papaya somatic embryos were placed in germination medium and 250 (~78%) papaya plantlets were obtained. Interestingly, 35 (14%) of the recovered plantlets displayed low

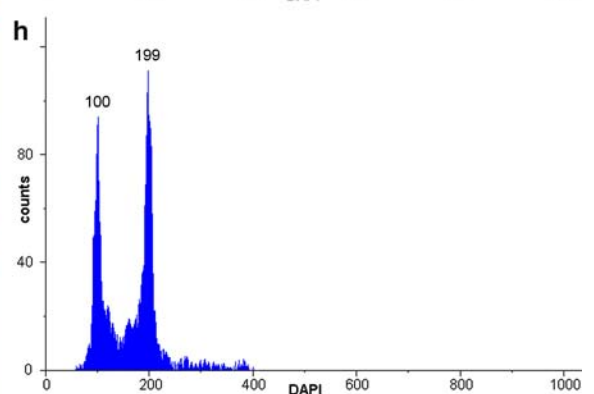
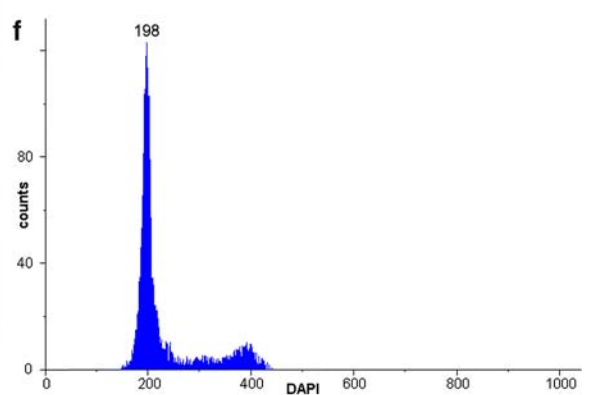
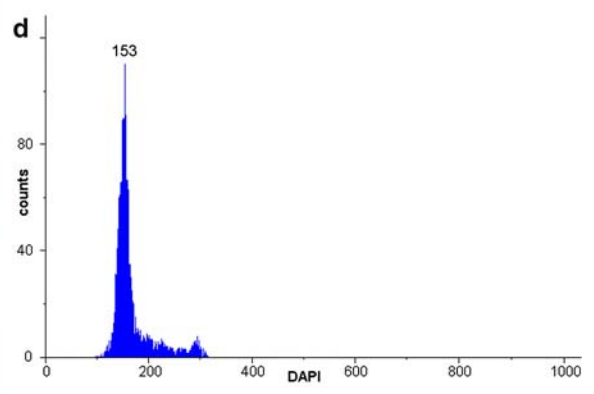
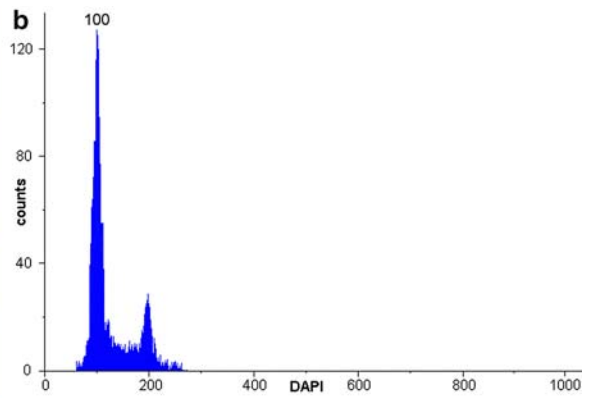
**Fig. 2** Papaya plantlets obtained by somatic embryogenesis and cultivated in BAP and NAA-supplemented multiplication medium, and respective flow cytometry histograms of G<sub>1</sub>/G<sub>0</sub> peak of the relative 2C DNA content nuclei stained with DAPI. **(a)** Diploid plantlet representing the 215 (86%) regenerants that showed the same DNA ploidy level of the standard plants (2X). **(b)** Histogram showing G<sub>1</sub>/G<sub>0</sub> peak (channel 100) of the diploid (2C = 2X) samples. **(c)** Triploid plantlet representing the 11 (4.4%) regenerants that showed DNA ploidy level 2C = 3X. **(d)** Histogram showing G<sub>1</sub>/G<sub>0</sub> peak (channel 153) of the triploid (2C = 3X) samples. **(e)** Tetraploid plantlet representing the 5 (2%) regenerants that showed DNA ploidy level 2C = 4X. **(f)** Histogram showing G<sub>1</sub>/G<sub>0</sub> peak (channel 198) of the tetraploid (2C = 4X) samples. **(g)** Mixoploid plantlet representing the 14 (5.6%) regenerants that showed DNA ploidy level 2C = 2X and 2C = 4X. **(h)** Histogram showing G<sub>1</sub>/G<sub>0</sub> peaks (channels 100 and 199) of the mixoploid (2C = 2X and 2C = 4X, respectively) samples. Note that the polyploid samples presented similar morphological development with regard to diploid papaya plantlets. Bar = 1 cm

growth rates, while initially cultured in the germination medium, with poor development of the roots and leaves.

Flow cytometry analysis evidenced that 215 (86%) of the papaya plantlets displayed the same DNA ploidy level of the standard plants (2C = 2X), whereas 35 (14%) plantlets showed different DNA heteroploidy levels: 5 (2%) aneuploid plantlets (data not shown); 14 (5.6%) mixoploid plantlets with G<sub>0</sub>/G<sub>1</sub> cells 2C = 2X and 2C = 4X; 11 (4.4%) triploid plantlets with G<sub>0</sub>/G<sub>1</sub> cells 2C = 3X; and 5 (2%) tetraploid plantlets with G<sub>0</sub>/G<sub>1</sub> cells 2C = 4X (Fig. 2a–h).

Due to carefully calibration of the flow cytometry equipment, the channel of the G<sub>0</sub>/G<sub>1</sub> peak for the standard and diploid plantlets was 100 ± 3, triploid plantlets 153 ± 2, tetraploid plantlets 198 ± 2 and mixoploid plantlets 100 ± 1. In addition, the high quality of the nuclear suspensions, with intact nuclei isolated in sufficient quantity, allowed the obtention of histograms showing coefficients of variation lower than 3.5%.

In the present work, the somaclonal variation occurred in the papaya plantlets regenerated by means of indirect somatic embryogenesis. Accordingly, dedifferentiated callus culture is reported to be particularly susceptible to somaclonal variation (Larkin and Scowcroft 1981; Karp 1995) and ploidy variations are generally observed in callus culture (Thiem and Sliwinska 2003). However, the DNA ploidy level remained stable in all cloned papaya plantlets during the successive subcultures in the multiplication stage.



Ploidy alterations are the most common genetic instability in tissue cultures (Phillips et al. 1994). Aneuploid, euploid (3X and 4X) and mixoploid regenerated plantlets were also observed in *Hypericum perforatum* tissue culture (Brutovská et al. 1998). The altered karyotypes in somaclones included chromosomal rearrangements as well as aneuploidy and euploidy. Aneuploidy may be caused by non-disjunction, aberrant spindles, lagging chromosomes and/or chromosome breakage (Jain 2001).

The aneuploid papaya plantlets continued to display a weak and inadequate development, though becoming senescent and dying after transfer to multiplication medium (data not shown). The reduction of the regeneration potential has been correlated with a change of ploidy of the cells (Wyman et al. 1992; Alan et al. 2007).

Concurrently, abnormal papaya plantlets derived from somatic embryos were also found elsewhere (Fernando et al. 2001; Koehler 2004). This condition did not allow the propagation of these plantlets and further analysis of the DNA ploidy stability. In this study, the euploid papaya plantlets showed similar morphological development with regard to diploid counterparts after transfer to multiplication medium (Fig. 2a, c, e, g). In accordance with Wyman et al. (1992) plants with aberrant chromosome numbers may exhibit normal phenotypes.

Currently available flow cytometry analysis was also applied to verify the stability of the DNA ploidy level of these plantlets. The two subsequent flow cytometric analyses evidenced that the DNA ploidy level remained stable throughout all cloned polyploids. The ploidy level can be detected by conventional chromosomic counting (Kochevenko et al. 1996; Fourré et al. 1997; Brutovská et al. 1998; Al-Zahim et al. 1999; Do et al. 1999; Tremblay et al. 1999; Menéndez-Yuffá et al. 2000). Though, this technique is laborious and lengthy, especially when the number of individuals analyzed is high (Roux et al. 2003). Therefore, we have used flow cytometry, which is suitable for reliable (Bennett and Leitch 1995; Lee et al. 2002; Doležel and Bartoš 2005) and rapid DNA ploidy screening (Brutovská et al. 1998; Roux et al. 2003; Loureiro et al. 2005, 2007), specially when the relationship between chromosome complement and DNA content is not directly verified (Suda et al. 2007).

The polyploidization is the most common change in chromosome number and it generally results from endopolyploidization (endoreduplication or endomitosis) or nuclear fusion (Brutovská et al. 1998; Jain 2001; Chen and Gao 2007). However, there is not a single mechanism or a factor that controls the occurrence of somaclonal variation. The plant genotype is one of the major factors that determines the type and frequency of genetic alterations (Jain 2001). In addition, the number of subcultures, the chemical and physical characteristics of the culture medium, the type and age of explant can also lead to somaclonal variation (Tremblay et al. 1999; Jain 2001; Etienne and Bertrand 2003; Bennici et al. 2004). Also, the use of 2,4-D in the induction of somatic embryogenesis may have a direct or indirect effect in the occurrence of somaclonal variation (Gesteira et al. 2002; Rakoczy-Trojanowska 2002; Pontaroli and Camadro 2005), due to its mutagenic potential (Pontaroli and Camadro 2005). Likewise the present work, this growth regulator has been widely used for papaya tissue culture (Fitch and Manshardt 1990; Chen and Chen 1992; Fitch 1993; Castillo et al. 1998a, b; Almeida et al. 2000, 2001; Koehler 2004). In spite of that, so far no cytometric-based works have been reported to assess DNA ploidy level in the recovered papaya plantlets.

Based on the results, we can conclude that the somatic embryogenesis induction protocol using 9.05  $\mu\text{M}$  2,4-D is suitable for inducing somatic embryogenesis and for obtaining genetically modified papaya plants. Therefore it cannot be neglected that in vitro stress condition, during somatic embryogenesis, may induce genetic variation. As reported by Fourré (2000), in tissue culture, the combination of a high rate of mitotic division, and the occurrence of unusual combinations of nutrients and plant growth regulators active in both the production and repair of mutational levels, may explain, at least in part, the high rate of mutations.

This study also evidenced the occurrence of somaclonal variation in the somatic embryogenesis-raised plantlets of papaya. We identified mixoploid, triploid and tetraploid papaya plantlets with stable DNA ploidy levels, indicating that the in vitro culture conditions induced the occurrence of somaclonal variation. In addition, these results confirmed that the flow cytometry is a practical, reliable and rapid tool for DNA ploidy screening. It is expected that the

papaya somaclonal lines recovered will enrich our breeding program with novel genetic variability resources.

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## References

- Alan AR, Zeng H, Assani A et al (2007) Assessment of genetic stability of the germplasm lines of medicinal plant *Scutellaria baicalensis* Georgi (Huang-qin) in long-term in vitro maintained cultures. *Plant Cell Rep* 26: 1345–1355
- Almeida EP, Oliveira RP, Dantas JLL (2000) Protocolo para a embriogênese somática do mamoeiro. *Pesq Agrop Bras* 35:2017–2024
- Almeida EP, Oliveira RP, Dantas JLL (2001) Indução e desenvolvimento de calos e embriões somáticos em mamoeiro. *Sci Agric* 58:51–54
- Al-Zahim MA, Ford-Lloyd BV, Newbury HJ (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Rep* 18: 473–477
- Ashmore SE, Drew RA (2006) The application of biotechnology in an integrated project of conservation and utilization of papaya and its wild relatives. *Acta Hortic* 725:89–94
- Bennett MD, Leitch IJ (1995) Nuclear DNA amounts in angiosperms. *Ann Bot* 76:113–176
- Bennici A, Anzidei M, Vendramin GG (2004) Genetic stability and uniformity of *Foeniculum vulgare* Mill. regenerated plants through organogenesis and somatic embryogenesis. *Plant Sci* 166:221–227
- Bhattacharya J, Khuspe SS (2001) In vitro and in vivo germination of papaya (*Carica papaya* L.) seeds. *Sci Hortic* 91:39–49
- Brutovská R, Čellárová E, Doležel J (1998) Cytogenetic variability of in vitro regenerated *Hypericum perforatum* L. plants and their seed progenies. *Plant Sci* 133:221–229
- Castillo B, Smith MAL, Yadava UL (1998a) Liquid system scale up of *Carica papaya* L. somatic embryogenesis. *J Hortic Sci Biotech* 73:307–311
- Castillo B, Smith MAL, Yadava UL (1998b) Plant regeneration from encapsulated somatic embryos of *Carica papaya* L. *Plant Cell Rep* 17:172–176
- Chen J, Henny RJ, Devanand PS, Chao CT (2006) AFLP analysis of nephthytis (*Syngonium podophyllum* Schott) selected from somaclonal variants. *Plant Cell Rep* 24:743–749
- Chen MH, Chen CC (1992) Plant regeneration from *Carica* protoplasts. *Plant Cell Rep* 11:404–407
- Chen LL, Gao SL (2007) In vitro tetraploid induction and generation of tetraploids from mixoploids in *Astragalus membranaceus*. *Sci Hortic* 112:339–344
- Chen MH, Wang PJ, Maeda E (1987) Somatic embryogenesis and plant regeneration in *Carica papaya* L. tissue culture derived from root explants. *Plant Cell Rep* 11:348–351
- De Klerk GJ (1990) How to measure somaclonal variation. *Acta Bot Neerl* 39:129–144
- Do G, Seo B, Ko J et al (1999) Analysis of somaclonal variation through tissue culture and chromosomal localization of rDNA sites by fluorescent in situ hybridization in wild *Allium tuberosum* and a regenerated variant. *Plant Cell Tiss Org Cult* 57:113–119
- Doležel J, Bartoš J (2005) Plant DNA flow cytometry and estimation of nuclear genome size. *Ann Bot* 95:99–110
- Encheva J, Köhler H, Friedt W et al (2003) Field evaluation of somaclonal variation in sunflower (*Helianthus annuus* L.) and its application for crop improvement. *Euphytica* 130:167–175
- Etienne H, Bertrand B (2003) Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. *Tree Physiol* 23:419–426
- Evans DA, Sharp WR, Medina-Filho HP (1984) Somaclonal and gametoclonal variation. *Am J Bot* 77:759–774
- Fernando JA, Melo M, Soares MKM et al (2001) Anatomy of somatic embryogenesis in *Carica papaya* L. *Braz Arch Biol Techn* 44:247–255
- Fitch MMM (1993) High frequency somatic embryogenesis and plant regeneration from papaya hypocotyls callus. *Plant Cell Tiss Org Cult* 32:205–212
- Fitch MMM, Manshardt RM (1990) Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). *Plant Cell Rep* 9:320–324
- Fourré JL (2000) Somaclonal variation and genetic molecular markers in woody plants. In: Jain SM, Minocha SC (eds) *Molecular biology of woody plants*. Kluwer Academic Publishers, Dordrecht
- Fourré JL, Berger P, Niquet L et al (1997) Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. *Theor Appl Genet* 94:159–169
- Galbraith DW, Harkins KR, Maddox JM et al (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science* 220:1049–1051
- Gesteira AS, Otoni WC, Barros EG, Moreira MA (2002) RAPD-based detection of genomic instability in soybean plants derived from somatic embryogenesis. *Plant Breed* 121:269–271
- Henry Y, Marcotte JL, Buysier J (1996) The effect of aneuploidy on karyotype abnormalities in wheat plants regenerated from short- and long-term somatic embryogenesis. *Plant Sci* 114:101–109
- Jain SM (2001) Tissue culture-derived variation in crop improvement. *Euphytica* 118:153–166
- Karp A (1995) Somaclonal variation as a tool for crop improvement. *Euphytica* 85:295–302
- Kochevenco AS, Ratushnyak YI, Gleba YY (1996) Protoplast culture and somaclonal variability of species of series *Junglandifolia*. *Plant Cell Tiss Org Cult* 44:103–110
- Koehler AD (2004) Embriogênese somática em mamoeiro (*Carica papaya* L.): anatomia, histoquímica e influência de ACC, AVG e STS e de pulsos de 2,4-D. Dissertation, Universidade Federal de Viçosa

- Lai C, Yeh S, Yang J (2000) Enhancement of papaya axillary shoot proliferation in vitro by controlling the available ethylene. *Bot Bull Acad Sinica* 41:203–212
- Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* 60:197–214
- Lee JH, Arumuganathan K, Kaepler SM et al (2002) Variability of chromosomal DNA contents in maize (*Zea mays* L.) inbred and hybrid lines. *Planta* 215:666–671
- Loureiro J, Capelo A, Brito G et al (2007) Micropropagation of *Juniperus phoenicea* from adult plants explants and analysis of ploidy stability using flow cytometry. *Biol Plant* 51:7–14
- Loureiro J, Pinto G, Lopes T et al (2005) Assessment of genetic stability of *Quercus suber* L. somatic embryogenesis process using flow cytometry. *Planta* 221:815–822
- Manshardt RM (1992) Papaya. In: Hammerschlag FA, Litz RE (eds) *Biotechnology of perennial fruit crops*. CAB International, Wallingford
- Menéndez-Yuffá A, Silva RF, Rios L et al (2000) Mitotic aberrations in coffee (*Coffea arabica* cv. ‘Catimor’) leaf explants and their derived embryogenic calli. *Electron J Biotechnol* 3:161–166
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Parc G, Rembur J, Rech P, Chriqui D (2007) In vitro culture of tobacco callus on medium containing peptone and phytate leads to growth improvement and higher genetic stability. *Plant Cell Rep* 26:145–152
- Phillips RL, Kaepler SM, Olhoft P (1994) Genetic instability of plant tissue culture: breakdown if normal controls. *Proc Natl Acad Sci USA* 91:5222–5226
- Polanco C, Ruiz ML (2002) AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants. *Plant Sci* 162:817–824
- Pontaroli AC, Camadro EL (2005) Somaclonal variation in *Asparagus officinalis* plants regenerated by organogenesis from long-term callus cultures. *Genet Mol Biol* 28:423–430
- Rakoczy-Trojanowska M (2002) The effects of growth regulators on somaclonal variation in rye (*Secale cereale* L.) and selection of somaclonal variants with increased agronomic traits. *Cell Mol Biol Lett* 7:1111–1120
- Renau-Morata B, Nebauer SG, Arrillaga I et al (2005) Assessments of somaclonal variation in micropropagated shoots of *Cedrus*: consequences of axillary bud breaking. *Tree Genet Genomes* 1:3–10
- Roux N, Toloza A, Radecki Z et al (2003) Rapid detection of aneuploidy using flow cytometry. *Plant Cell Rep* 21:483–490
- Shapiro HM (2003) *Practical flow cytometry*. Wiley-Liss, New Jersey
- Smykal P, Valledor L, Rodríguez R, Griga M (2007) Assessment of genetic and epigenetic stability in long-term in vitro culture of pea (*Pisum sativum* L.). *Plant Cell Rep* 26:1985–1998
- Suda J, Kron P, Husband BC et al (2007) Flow cytometry and ploidy: applications in plant systematics, ecology and evolutionary biology. In: Doležel J, Greilhuber J, Suda J (eds) *Flow cytometry with plant cells*. Wiley-VCH, Germany
- Thiem B, Sliwiska E (2003) Flow cytometric analysis of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) in vitro cultures. *Plant Sci* 164:129–134
- Tremblay L, Levasseur C, Tremblay FM (1999) Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *Am J Bot* 86:1373–1381
- Wyman J, Brassard N, Flipo D et al (1992) Ploidy level stability of callus tissue, axillary and adventitious shoots of *Larix X eurolepis* Henry regenerated in vitro. *Plant Sci* 85:189–196
- Yang J, Ye C (1992) Plant regeneration from petioles of in vitro regenerated papaya (*Carica papaya* L.) shoots. *Bot Bull Acad Sinica* 33:375–381
- Yang J, Yu T, Cheng Y et al (1996) Transgenic papaya plants from *Agrobacterium*-mediated transformation of petioles of in vitro propagated multishoots. *Plant Cell Rep* 15:459–464