Somatic embryogenesis from leaf explants of hermaphrodite *Carica papaya*: A new approach for clonal propagation

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Carica papaya L. is an economically relevant fruit crop in some tropical and subtropical countries. Though this species shows three polygamous sexual types, commercial production of the fresh fruit is mainly established from hermaphrodite lines. As a result of the cross-pollinating reproductive mechanism of hermaphrodite *C. papaya*, cultivation areas also show unwanted female plants. Comparatively, hermaphrodite plants exhibit considerable variation in regards to yield, fruit quality, and susceptibility to pathogens. In this context, the present work aimed at establishing a somatic embryogenesis protocol to provide regenerants from leaves of hermaphrodite *C. papaya* plants. Leaf explants, collected in the rainy season, provided high frequency of friable embryogenic calli (FEC). In culture medium supplemented with 2,4-dichlorophenoxyacetic, FEC overgrew into a yellowish friable mass that fully covered the leaf explants. The somatic embryogenesis process occurred asynchronously, with new globular embryos continuously forming from the FEC. Torpedo and early cotyledonary somatic embryos matured in medium containing polyethylene glycol, activated charcoal and abscisic acid. These embryos were germinated, and normal seedlings were recovered. Based on these outcomes, the tissue culture protocol presented here may be considered a successful alternative for large-scale and clonal propagation of adult hermaphrodite plants of *C. papaya*.

Key words: *Carica papaya*, clonal propagation, hermaphrodite plants, leaf explant, somatic embryogenesis.

INTRODUCTION

Carica papaya L. belongs to the family Caricaceae, which is found in tropical and subtropical regions of the world (Silva et al., 2007). This species shows three polygamous sexual types: male, female, and hermaphrodite plants. Further intermediate sexual types have also been described (Hofmeyr, 1938; Storey, 1953; Chan, 1996; Rimberia et al., 2006). This variation occurs because genders in *C. papaya* are determined by association of genetic (Liu et al., 2004; Ming et al., 2007) and

environmental (Storey, 1941, 1953; Yu et al., 2008) factors. Papaya is an economically important fruit, owing to its high nutritional value, as well as medicinal and industrial applications (Drew, 2003; Silva et al., 2007; Zhang et al., 2011). Commercial production of the fresh dessert fruit is mainly based on hermaphrodite lines, since its pyriform fruits are generally preferred over the spherical females ones (Urasaki et al., 2002; Yu et al., 2008).

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Most crops of hermaphrodite *C. papaya* have been established by seed propagation (Bhattacharya and Khuspe, 2001; Silva et al., 2007). This sexual type is mainly allogamous, segregating in hermaphrodite and female plants in a 2:1 ratio. Identification and removal of unwanted female plants only takes place after reproductive maturity that is five to eight months from planting. Consequently, seed propagation leads to waste of resources, and increases production cost, space demand, planting time, and management (Urasaki et al., 2002; Chaves-Bedoya and Nuñez, 2007).

Owing to the cross-pollinating reproductive mechanism of hermaphrodite *C. papaya*, its commercial plantations also exhibit great heterozygosity and genetic variability (Drew, 2003; Bhattacharya and Khuspe, 2001). As a result, plants exhibit considerable variation in regard to yield, fruit quality and susceptibility to pathogens (Fitch, 1993; Yang et al., 1996), which is undesirable for commercial purposes.

Given the problems caused by spread-seminiferous, researchers have developed tissue culture techniques to propagate a large number of *C. papaya* elite lines (Manshardt, 1992; Fitch, 1993; Drew, 2003; Ashomore and Drew, 2006; Silva et al., 2007). Significant progress has been achieved in somatic embryogenesis systems (Silva et al., 2007), mainly starting from immature zygotic embryos (Fitch and Manshardt, 1990; Castillo et al., 1998a; Renukdas et al., 2003; Ascencio-Cabral et al., 2008; Clarindo et al., 2008; Malabadi et al., 2011; Anandan et al., 2012). For *C. papaya*, this is the most used tissue culture system to scale up propagation (Fitch, 1993; Castillo et al., 1998b), supply synthetic seeds (Castillo et al., 1998a), and produce transgenic plants (Yang et al., 1996). However, the sexual issue remains unsolved.

Considering the problems associated with seed propagation of hermaphrodite *C. papaya*, the present work aimed at adapting a somatic embryogenesis protocol to provide plantlets from leaves of this sexual type, and ultimately improve commercial production.

MATERIALS AND METHODS

During one year, young leaves were collected from hermaphrodite *C. papaya* plants, cultivated at the Universidade Federal do Espírito Santo (ES - Brazil), and Universidade Federal de Viçosa (MG - Brazil). The leaves were sprayed with solution containing 2.0 g l^{-1} Agrimycin[®] PM (Pfizer, Brazil), 0.5 ml l^{-1} Ethion 500 (Bayer CropScience[®], Brazil), 10.0 ml l^{-1} Assist[®] (Basf, Brazil), and 1.0 g l^{-1} Curathane (Dow AgroSciences, Brazil). After 24 h, the leaves were surface-washed by rinsing with liquid detergent, then kept under running water for 2 h (Clarindo et al., 2008).

In a laminar flow hood, the leaves were disinfected by immersion in 70% ethanol (Merck[®], USA) for 20 s, then in 1.5% sodium hypochlorite (Merck[®], USA) solution containing 10 drops l^{-1} of Tween 20 (Sigma[®], USA), for 20 min. Subsequently, the leaves were rinsed four times with sterile dH_2O, air-dried, and the primary nervure, margins, apical, and basal portions were cut off (Clarindo et al., 2008).

Tissue culture conditions

Five leaf fragments (1 cm^2 each) were excised and placed, with the abaxial surface upwards, onto 60 × 15 mm Petri dishes (J. Prolab[®], Brazil) containing embryogenic induction medium, which consisted of half-strength MS basal salts (Sigma[®], USA), 10 ml l^{-1} MS vitamins (Murashige and Skoog, 1962), 9.05 µM 2,4-dichlorophenoxyacetic acid (2,4-D - Sigma[®], USA), 0.55 mM myo-inositol (Sigma[®], USA), 2.75 mM L-glutamine (Sigma[®], USA), 87.6 mM sucrose (Sigma[®], USA), and 0.28% (w/v) Phytagel (Sigma[®], USA) (Castillo et al., 1998a, b; Almeida et al., 2000; Clarindo et al., 2008; Anandan et al., 2012). The pH of the medium was adjusted to 5.7 prior to autoclaving. The Petri dishes were sealed with PVC film (Goodyear[®], Brazil), and cultures were kept in the dark at 27°C.

At intervals of up to one month, the explants showing friable embryogenic calli (FEC) were transferred to fresh tissue culture medium, with 2,4-D (Sigma[®], USA) molarity being decreased by 50% on a monthly basis (4.53, 2.26, 1.13 µM, until 0.57 µM). Further physical conditions were maintained (Monmarson et al., 1995).

FEC exhibiting somatic embryo clusters at torpedo and early cotyledonary stages were transferred to maturation medium. Composition of this medium was similar to embryogenic induction medium, but was devoid of 2,4-D (Sigma[®], USA) and supplemented with 100 mg l^{-1} malt extract (Acumedia Manufactuires[®], USA), 5% polyethylene glycol 2000 (PEG - Merck[®], USA), 2 g l^{-1} activated charcoal (Merck[®], USA) and 5 µM abscisic acid (ABA - Sigma[®], USA); pH was 5.7 (Castillo et al., 1998a, b; Almeida et al., 2000; Clarindo et al., 2008; Anandan et al., 2012). In this phase, the cultures were maintained at 27°C under a 16/8 h light/dark photoperiod, with 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram[®], Brazil), for three months.

Subsequently, mature cotyledonary embryos were screened, placed into germination medium [MS basal medium salts and vitamins (Sigma[®], USA), supplemented with 0.55 mM myo-inositol (Sigma[®], USA), 58.4 mM sucrose (Sigma[®], USA), 0.65% (w/v) agar (Sigma[®], USA); ph = 5.7], and cultured at 27°C under a 16/8 h light/dark photoperiod, with 36 µmol m^{-2} s^{-1} light radiation provided by two fluorescent lamps (20 W, Osram[®], Brazil). Finally, germinated embryos were transferred to glass flasks containing the same medium. The flasks were maintained under the same environment conditions.

RESULTS AND DISCUSSION

As a result of disinfestation and handling of explants under aseptic conditions, no contamination was detected during the embryogenic process. Consequently, the young leaves showed no endogenous contamination. Other approaches for *C. papaya in vitro* clonal propagation were accomplished from shoot tips (Litz and Conover, 1979; Lai et al., 2000; Agnihotri et al., 2004), or axillary buds (Litz and Conover, 1977; Drew, 1988, 1992; Reuveni et al., 1990; Lai et al., 1998). However, the establishment of aseptic cultures from these explants is often hindered by high incidence of endogenous pathogens (Silva et al., 2007). Considering this fact and the absence of contamination in the present work, young leaves can be considered a successful alternative explant for clonal propagation of adult *C. papaya* elite plants.

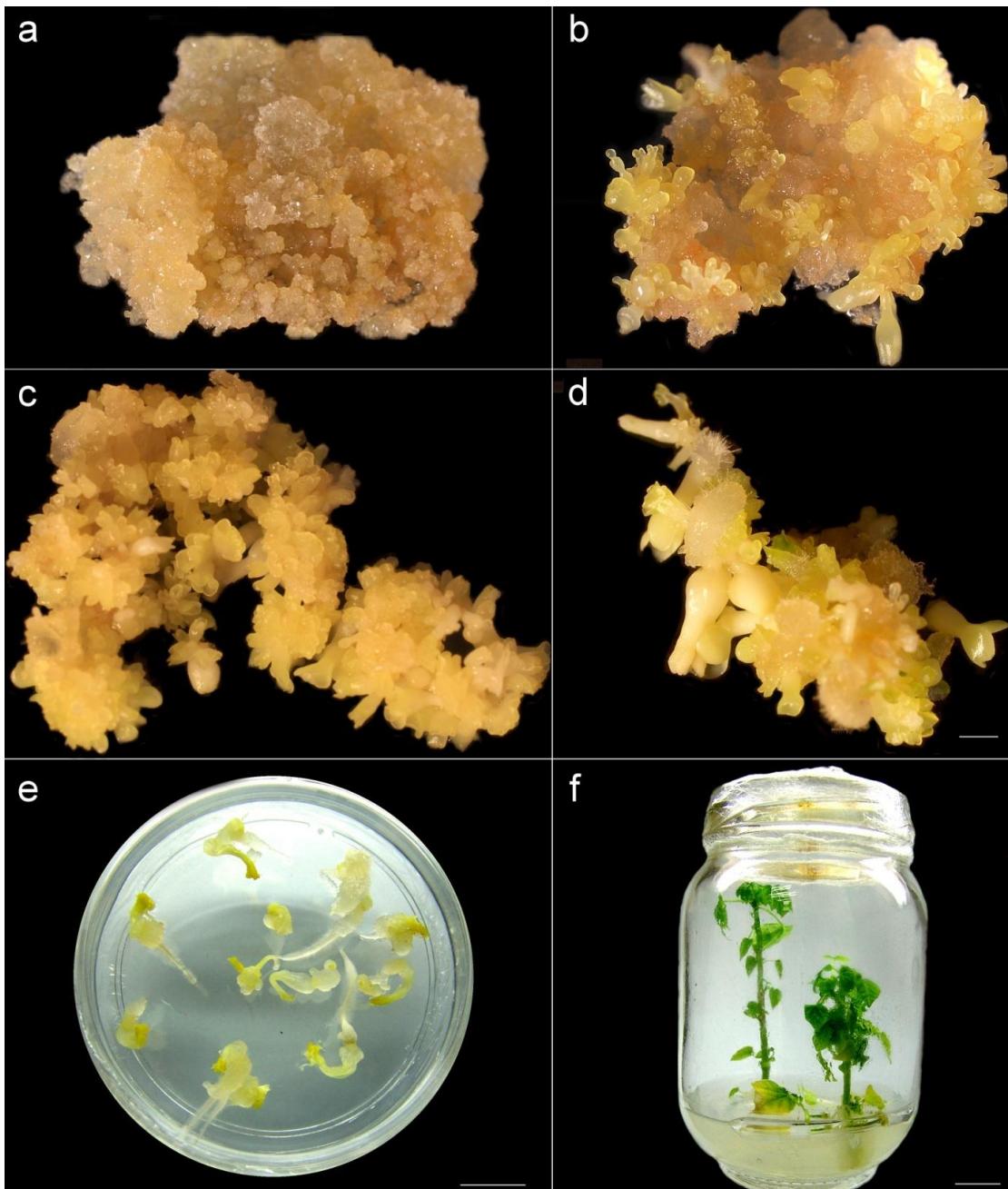Callogenic response was observed in leaf explants collected in the rainy season (period between October

Figure 1. Somatic embryogenesis from leaf explants of hermaphrodite *Carica papaya* 'Golden'. **a)** FEC overgrew into a yellowish friable mass that fully covered the leaf explants. **b)** Globular somatic embryos emerged as small yellowish structures from the surface of the FEC mass. **b - d)** Asynchronized development of somatic embryos. FEC exhibiting somatic embryos at distinct development stages: globular, heart-shaped, torpedo, and early cotyledonary. **d)** Mature cotyledonary somatic embryos showing whitish to pale yellow color. **a - d,** Bar = 0.2 cm. **e)** Germinated somatic embryos. **f)** Well-developed *C. papaya* plantlets recovered from somatic embryos. **e- f**, Bar = 1 cm.

and January in the areas of explant collection). These explants were highly responsive to the tissue culture conditions, providing FEC (Figure 1a) and non-embryogenic calli. According to this observation, *in vitro* response was strongly influenced by climatic seasonality,

which was associated with the rainy period. As reported by some authors (Berthouly and Michaux-Ferrière, 1996), the concentration of endogenous growth regulators is directly related to high frequency of callogenic response from leaf explants. Therefore, we suggest collecting *C.*

papaya leaves during the rainy season for establishment of somatic embryogenesis systems.

As observed by other authors (Clarindo et al., 2008; Anandan et al., 2012), FEC and non-embryogenic calli were grown under the same tissue culture conditions (data not show). FEC were clearly identifiable by pale yellow color and friable aspects (Figure 1a). The calli initiated on the nervure portion, suggesting that embryogenic competence is confined to these areas. After approximately one month of culture, 74% of the leaf explants showed FEC. This value is similar in relation to the immature zygotic embryo explants, which show high-embryogenic potential. Malabadi et al. (2011) observed the FEC proliferation in 85% of the zygotic embryos and Anandan et al. (2012) in 75.12%.

During five months of culture, only explants exhibiting FEC were subcultured into embryogenic induction medium, with 2,4-D molarity being reduced by 50% on a monthly basis. As a result, FEC overgrew into a yellowish friable mass that fully covered the leaf explants (Figure 1a).

Globular somatic embryos emerged as small yellowish structures from the surface of the FEC mass (Figure 1b). Throughout five months in embryogenic induction medium, somatic embryos were observed at distinct development stages (globular, heart-shaped, torpedo and early cotyledonary; Figures 1b and c). Regarding this result, the process of somatic embryogenesis was asynchronous, with new globular embryos continually forming throughout embryogenic callus cultivation (Figures 1b and c). This result was also observed in other embryogenic systems, using distinct *C. papaya* explants, such as petiole sections (Yang and Ye, 1992), hypocotyl segments (Fitch, 1993; Castillo et al., 1998b), zygotic embryos (Fitch and Manshardt, 1990; Castillo et al., 1998a; Clarindo et al., 2008; Malabadi et al., 2011; Anandan et al., 2012) and endosperm (Sun et al., 2011).

The embryogenic induction medium was supplemented with 2,4-D, a synthetic growth regulator largely used in *C. papaya* somatic embryogenesis (Castillo et al., 1998a; Bhattacharya et al., 2002; Clarindo et al., 2008; Malabadi et al., 2011; Sun et al., 2011; Anandan et al., 2012). 2,4-D has been considered fundamental for pro-embryogenic mass induction (Malabadi et al., 2011), because it stimulates an increase in endogenous levels of the natural auxin hormone (Michalczuk et al., 1992), resulting in cell proliferation and somatic embryo formation (Pasternak et al., 2002).

FEC of *C. papaya* exhibiting torpedo and early cotyledonary somatic embryos (Figure 1c) were transferred to maturation medium. After three months, apparently well-formed, mature cotyledonary somatic embryos were obtained, showing whitish to pale yellow color (Figure 1d).

Maturation medium was supplemented with the plant growth regulator ABA, which has been widely used for regulating the course of this process *in vitro* (Chen and Chen, 1992; Castillo et al., 1998b). This phytohormone plays an important role in inhibiting the secondary embryogenic process, preventing early germination (von Arnold et al., 2002), and stimulating deposition of reserve products (von Aderkas et al., 2002a; Sholi et al., 2009).

Activated charcoal is commonly added to tissue culture media to absorb inhibitory, toxic substances, as well as plant growth regulators, including 2,4-D (von Aderkas et al., 2002b; Aboshama, 2011). As *C. papaya* explants were initially inoculated into embryogenic induction medium supplemented with 2,4-D at relative high concentrations, the influence of this auxin was minimized by the presence of charcoal, contributing to somatic embryo development, conversion, and maturation.

PEG is a known non-plasmolyzing osmoticum that mimics the water stress naturally occurring in seeds during the late maturation stages (Ćalić-Dragosavac et al., 2010). It has been reported that variables such as carbon source, ABA and osmotic agents are used to increase conversion rates and germination of somatic embryos in several species (Attree et al., 1995; Capuana and Debergh, 1997; Robichaud et al., 2004; Krajňáková et al., 2009; Ćalić-Dragosavac et al., 2010). In the current study, combined application of all three compounds in the maturation medium contributed to improve the quality of somatic embryos, subsequently evidenced by high germination rates (Figure 1e).

Apparently well-formed, mature cotyledonary somatic embryos were selected, inoculated into germination medium and converted into plantlets (Figure 1f). Seedlings exhibiting abnormal development (12%) showed hyperhydric leaves and roots, callus formation, hypocotyl thickening and/or absence of apical meristem. Abnormal seedlings were also found by Clarindo et al. (2008), using zygotic embryos as explants. By measuring the DNA ploidy level of the plantlets, those authors demonstrated the occurrence of somaclonal variation during somatic embryogenesis of *C. papaya* in tissue culture medium supplemented with 2,4-D.

The present study reports, for the first time, a tissue culture procedure established from leaves of adult hermaphrodite *C. papaya* plants. Considering the problems associated with seed propagation and the cross-pollinating reproductive mechanism of hermaphrodite *C. papaya*, this protocol and explant source may be considered a potential alternative for large-scale and clonal propagation of this commercial type of *C. papaya*.

ACKNOWLEDGEMENTS

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