

Regular Article

Induction of somatic embryogenesis in Papaya (*Carica papaya*)

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This study highlights the induction of somatic embryogenesis using immature zygotic embryos of *Carica papaya* commercial varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, and Co-3 respectively. Somatic embryos formed in the presence of thidiazuron (TDZ) in combination with 2, 4-dichlorophenoxy acetic acid (2, 4-D), but never on explants cultured on control medium lacking plant growth regulators. Embryogenic callus could be induced from immature zygotic embryos after 4-6 weeks of culture on full-strength Murashige and Skoog inorganic salts, which served as the basal medium supplemented with 4.52 μM 2, 4-D and 2.27 μM TDZ (induction and maintenance medium). The ability to induce embryogenic tissue varied for different papaya tested varieties, and there was a mixed genotypic response on the induction of embryogenic cultures. The highest percentage of somatic embryogenesis was noticed in a papaya variety Taiwan-786 ($87.0 \pm 4.2a$), followed by Taiwan-785 ($85.0 \pm 3.0a$), and Coorg Honey dew ($81.0 \pm 3.2a$) respectively. This protocol is simple and reproducible, and could be useful for regenerating large number of plants as well as provide a target tissue for genetic transformation experiments.

Key words: *Carica papaya*, Karnataka, micropropagation, somatic embryogenesis

Abbreviations: ABA-Absciscic acid; MS-Murashige and Skoog medium; TDZ-thidiazuron (N-phenyl-N-1, 2, 3-thidiazuron-5'-ylurea); 2,4-D-2,4-di-chloro-phenoxy-acetic acid.

Papaya (*Carica papaya* L.) belongs to family *Caricaceae* is one of the economically important fruit crops in many tropical and subtropical countries. Papaya (*Carica papaya*) fruit is known for its high nutritive and medicinal value. Papaya cultivation had its origin in South Mexico and Costa Rica. Total annual world production of papaya is estimated over 6.8 million tonnes of fruits. India leads the world in papaya production with an annual output of about 4.5 million tones in 2010-2011. Some of the commercial

varieties of *Carica papaya* grown in Belgaum and surroundings of Dharwad district in Karnataka state, India are Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, and Co-3 respectively. Because of the high superior quality and nutritional as well as medicinal value of the fruit, Indian papaya has a great demand in African countries, Middle East and European market. Other leading producers are Brazil, Mexico, Nigeria, Indonesia, China, Peru,

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Thailand and Philippines. Domestic demand for fresh fruits of papaya in India has been on the increase in line with rising incomes, population growth, and increased health consciousness among consumers (Anonymous, 2005). In addition, growth in demand for Indian fresh and processed tropical fruits of papaya has been strong in recent years, and this trend is likely to continue in the medium term (Anonymous, 2005).

The somatic chromosome number in the dicotyledonous genus *Carica*, is $2n=18$ (Teixeira da Silva et al. 2007). Most *Carica* spp. are dioecious, except for *C. papaya* which is characterized by various flower types and three primary, polygamous sexual types, viz. pistillate (female; mm), staminate (male; M1m) and hermaphrodite (M2m) (Teixeira da Silva et al. 2007). The fruit is consumed world-wide as fresh fruit and as a vegetable or used as processed products (Teixeira da Silva et al. 2007). Papaya fruits consist mostly of water and carbohydrate, low in calories and rich in natural vitamins and minerals, particularly in vitamins A and C, ascorbic acid and potassium (Teixeira da Silva et al. 2007). Papaya plants are also produced for papain and chymopapain, two industrially important proteolytic enzymes found in the milky white latex exuded by fruits (Teixeira da Silva et al. 2007). In general, female fruits tend to exude more papain than hermaphrodite fruits (Teixeira da Silva et al. 2007). In India, unripe and semi-ripe papaya fruits are ingested or applied on the uterus to cause abortion (Teixeira da Silva et al. 2007).

Papaya (*Carica papaya* L.) conventionally propagated by seeds, and therefore, cultivation is hindered by problems due to its inherent heterozygosity and dioecious nature. Plants grown from seeds of open pollinated flowers result in a mixture of genotypes, with a considerable variation in disease susceptibility, fruit quality and yield (Saker et al. 1999). Although conventional vegetative propagation methods such as

grafting and rooted cuttings exist, they are often tedious and impractical when carried out on a large scale (Saker et al. 1999). The application of plant tissue culture techniques is one of the most frequently used strategies for commercial micropropagation of plants. Somatic embryogenesis is the most widely adopted regeneration system for many plant species (Malabadi et al. 2004c; Malabadi and Nataraja, 2006a, 2006b; Malabadi and van Staden, 2005a, 2005b, 2005c; Malabadi and van Staden, 2006; Malabadi et al. 2008a, 2009; Aronen et al. 2007, 2008; Park et al. 2009; Malabadi and Teixeira da Silva, 2011; Malabadi et al. 2011a, 2011b, 2011c). The present study was carried out to establish a somatic embryogenesis system for commercial varieties of *Carica papaya* viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, Co-7, and Co-3 respectively for large-scale clonal propagation. This protocol is reproducible and could be used for the genetic improvement of the papaya crop.

Materials and methods

Initiation of embryogenic tissue

Immature zygotic embryos of *Carica papaya* L. varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, Co-7, Co-3 were dissected out from unripe fruits of field grown mother plants from Belgaum, and Dharwad, Karnataka state, India. The unripe fruits were first surface washed and immersed (5min) in sterile distilled water for 3 to 4 times to get rid of dust particles. They were surface decontaminated sequentially with 70% (v/v) ethanol (5 min) and 0.1% (w/v) $HgCl_2$ (2 min) (Sigma-Aldrich, St. Louis, USA), and thoroughly rinsed for 3 times with sterilized double distilled water. Unripe fruits of all the varieties of *Carica papaya* were cut longitudinally with the sterilized blade for the extraction of immature zygotic embryos.

Prior to dissection of the embryos, the seeds were observed microscopically for the stage specificity of the embryos. Immature zygotic embryos were dissected out under sterile conditions, and subsequently were cultured under sterile conditions on Murashige and Skoog, (1962) basal medium with 3.0% sucrose, 0.7% agar, 0.5 g l^{-1} myo-inositol, 1.0 g l^{-1} casein hydrosylate, 0.5 g l^{-1} L-glutamine, 250 mg l^{-1} peptone, 0.2 g l^{-1} *p*-aminobenzoic acid, and 0.1 g l^{-1} biotin, all purchased from Sigma. The medium was supplemented with a range of thidiazuron (TDZ) concentrations (0.45, 2.27, 4.54, 9.08 and 11.35 μM), and 2, 4-dichlorophenoxy acetic acid (2, 4-D) at a concentration of 4.52 μM singly and in combination without any other growth hormones. The cultures were raised in 25 mm \times 145 mm glass culture tubes (Borosil, Mumbai, India) containing 15 ml of the above basal medium under cool white fluorescent light (Mysore lamps, India) at 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and $25 \pm 3^\circ\text{C}$ with a relative humidity of 55-60%. The pH of the media was adjusted to 5.8 with 1 N NaOH or HCl before agar was added. Nutrient media without TDZ and 2, 4-D served as the control. The media were then sterilized by autoclaving at 121°C at 1.04 Kg cm^{-2} for 15 min. Both L-glutamine, and TDZ were filter sterilized (Whatman filter paper, pore size = 0.45 μm ; diameter of paper = 25 mm), and added to the media after autoclaving when the medium had cooled to below 50°C .

All the tissue cultures of *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, Co-3 were examined for the presence of different developmental stages of somatic embryogenesis by morphological and cytological observations of callus. The cultures showing different stages of cell division were identified by microscopic observation. The callus was subcultured on the initiation medium for further 2 weeks for the better development of embryogenesis.

The full strength inorganic salts Murashige and Skoog, (1962) basal medium supplemented with 4.52 μM 2, 4-D and 2.27 μM TDZ (induction medium) was used as an effective induction medium for producing the embryogenic tissue. On the other hand the callus without pro-embryonic cell divisions was considered as non-embryogenic. Non-embryogenic tissue was separated immediately from the rest of the tissue to avoid the overgrowth of the tissue. The efficiency of plant growth regulators and their concentrations were analyzed on the basis of visual observation (callusing percentage, percentage of explants forming embryogenic tissue, callus growth and callus necrosis). The ineffective treatments were discontinued.

Maintenance of embryogenic tissue

The embryogenic tissue of *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, Co-3 showing various developmental stages of somatic embryogenesis were maintained on full strength inorganic salts MS (Murashige and Skoog, 1962) basal medium supplemented with 4.52 μM 2, 4-D and 2.27 μM TDZ for the proliferation of callus (maintenance medium). The embryogenic tissue was subcultured for every 2 weeks. All the cultures were maintained under a cool white fluorescent light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at $25 \pm 3^\circ\text{C}$ with a relative humidity of 55-60%. The percentage of cultures showing somatic embryogenesis has been recorded.

Partial desiccation

For maturation, embryogenic tissue clumps of each of *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, Co-3 were partially desiccated. One gram fresh weight of tissue of each embryogenic line

were transferred to sterile empty Petri dishes (60 mm diam.) containing two sterile Whatman filter paper disks (50 mm) (Schleicher and Schuell, qualitative circles) (Malabadi and Nataraja, 2006). The Petri dishes were sealed with Parafilm, and kept at $25 \pm 2^\circ\text{C}$ in the dark for 24 hr to obtain the desired extent of desiccation (Malabadi et al. 2004c; Malabadi and van Staden, 2005abc; Malabadi and Nataraja, 2006). After desiccation, the partially desiccated tissue of each embryogenic line belongs to different varieties of *Carica papaya* were transferred to maturation medium to induce further embryo development.

Somatic embryo maturation

The partially desiccated embryogenic tissue of *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, Solo, Co-1, C0-7, Co-3 were transferred to maturation medium to induce further embryo development. The full strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium supplemented with 3.0% sucrose, 5 μM ABA, and 0.8% agar (maturation medium) was adopted for this purpose. All the cultures were again maintained in the dark for 4 weeks. Microscopic observation was conducted to ensure the development of somatic embryos. The total number of somatic embryos produced after 4 weeks on maturation medium per one gram fresh weight of embryogenic tissue was recorded.

Germination and recovery of plantlets

After maturation, the somatic embryos were taken from the cultures for germination. The germination medium used was half strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium with 0.7% agar without any growth regulators (germination medium). Somatic embryos were considered germinated as soon as radical elongation occurred and conversion

to plantlet was based on the presence of an epicotyl. After 4 weeks on germination medium, the plantlets were directly transferred to vermiculite. Plantlets were placed in a growth room under a 16 hr photoperiod ($50\mu \text{ mol m}^{-2} \text{ s}^{-1}$) for hardening. Somatic embryo proliferation in terms of root, shoot development, plant conversion was recorded.

Statistical analysis

In all the above experiments, each culture tube received a single immature zygotic embryo of papaya as one explant. Each replicate contained 25 cultures, and one set of experiment is made up of 2 replicates. Therefore, 50 immature zygotic embryos were cultured for one set of experiment for each *Carica papaya* variety viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, solo, Co-1, C0-7, and Co-3. All the experiments were repeated 3 times. Data presented in the tables were arcsine transformed before being analyzed for significance using ANOVA (analysis of variance, $p < 0.05$) or evaluated for independence using Chi-square test. Further, the differences contrasted using a Duncan's multiple range test ($\alpha = 0.05$) following ANOVA. All statistical analysis was performed using the SPSS statistical software package version 13.0.1.1 Microsoft Windows.

Results and Discussion

In the present investigation, the immature zygotic embryos induced callus after 4 weeks of culture on the full strength inorganic salts Murashige and Skoog, (1962) basal medium supplemented with 4.52 μM 2, 4-D and 2.27 μM TDZ (induction medium) in all the tested *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, solo, Co-1, C0-7, and Co-3 respectively (Table-1 and 2). Embryo-genic areas were clearly visible from the rest of the

callus by their globular and glazy appearance and emerged as distinct white structures. The embryogenic cells were small, richly cytoplasmic and actively dividing with a prominent nucleus (Figure. 1-B). These cells were generally present in small, compact aggregates and showed competence for embryogenesis (Figure. 1B). On the other hand non-embryogenic cells were large, vacuolated and often elongated with sparse cytoplasm and few starch grains. Such cells did not show morphogenetic competence as described previously in sugarcane by Ho and Vasil (1983). In a control study, the immature zygotic embryos did not promote callus formation (Table-1 and 2). Immature zygotic embryos survived for three weeks and eventually turned brown and dead. Table-1 and 2 shows the embryogenic response of *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, solo, Co-1, C0-7, and Co-3 scored after 8 weeks on the initiation medium. The frequency of embryogenic tissue formation of all the tested *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, solo, Co-1, C0-7, and Co-3 was varied from each other

(Table-1 and 2). There was a clear mixed response of different varieties of the *Carica papaya* cultured on the full strength inorganic salts Murashige and Skoog, (1962) basal medium supplemented with 4.52 μ M 2, 4-D and 2.27 μ M TDZ (induction medium) (Table-1 and 2). The highest percentage of somatic embryogenesis was noticed in a variety Taiwan 786 (87.0 \pm 4.2a), followed by Taiwan 785 (85.0 \pm 3.0a), and Coorg Honey dew (81.0 \pm 3.2a) respectively (Table-1 and 2). The lowest percentage of somatic embryogenesis was recorded in *Carica papaya* variety Co-1 (2.0 \pm 0.1b) followed by sunrise (9.0 \pm 1.0b), and Co-7 (13.0 \pm 0.1a) respectively (Table-2). All the tested *Carica papaya* varieties viz. Coorg Honey dew, Washington, Honey dew, Pusa delicious, Pusa nanha, Taiwan 786, Taiwan 785, Sunrise, solo, Co-1, C0-7, and Co-3 failed to induce embryogenic tissue on the higher concentrations (9.08 and 11.35 μ M) of TDZ (Table-2). On the other hand lower concentration of TDZ (2.27 μ M) has induced the highest percentage of somatic embryogenesis in all the tested commercial varieties of *Carica papaya* (Table-1 and 2). Therefore, 4.52 μ M 2, 4-D and 2.27 μ M TDZ are the optimum concentrations for the induction of embryogenic tissue in all the tested varieties of *Carica papaya* (Table-1and 2).

Table 1: The effect of various concentrations of TDZ on initiation of embryogenic tissue in different varieties of *Carica papaya* cultured on full strength MS basal medium supplemented with 4.52 μ M 2, 4-D.

TDZ (μ M)	Embryogenic tissue initiation in different varieties of <i>Carica papaya</i> * (%)						
	Coorg* Honey dew	Washing -ton	Honey* dew	Pusa* delicious	Pusa* nanha	Taiwan* 786	Taiwan* 785
Control	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0 c	0.0 \pm 0.0c
0.45	21.0 \pm 1.2b	0.0 \pm 0.0c	8.0 \pm 0.4b	8.0 \pm 0.5b	16.0 \pm 0.2b	4.0 \pm 0.0b	2.0 \pm 0.1b
2.27	81.0 \pm 3.2a	31.0 \pm 1.2b	28.0 \pm 3.0b	48.0 \pm 3.5b	26.0 \pm 3.4b	87.0 \pm 4.2a	85.0 \pm 3.0a
4.54	11.0 \pm 0.2b	0.0 \pm 0.0c	2.0 \pm 0.1b	3.0 \pm 0.1b	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c
9.08	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c
11.35	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c

Control=MS basal medium without growth regulators such as 2, 4-D and TDZ; Data scored after 8 weeks and represents the mean \pm SE of at least 3 different experiments. In each column, the values with different letters are significantly different (P<0. 5).

*Different *Carica papaya* varieties.

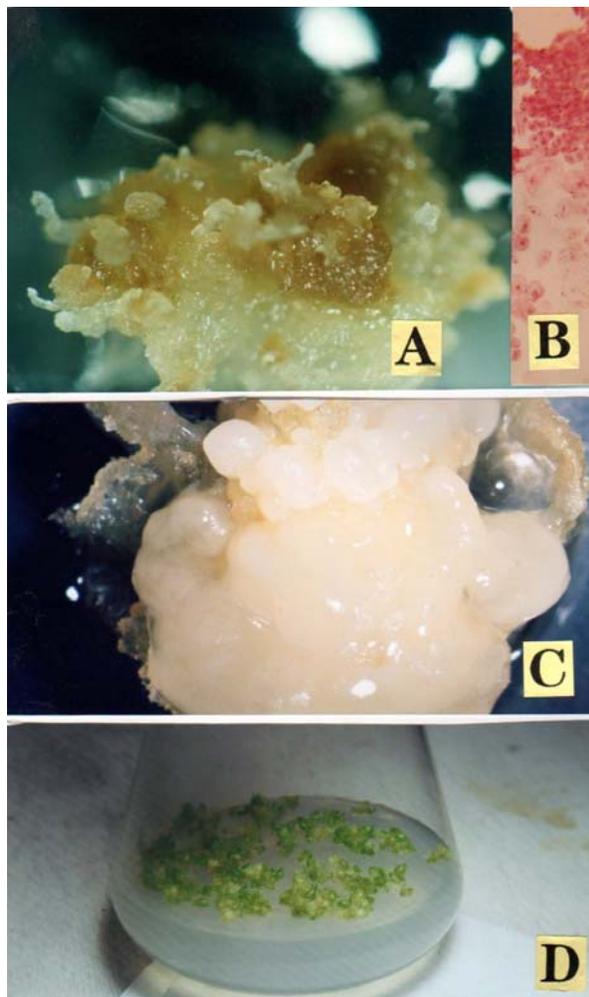


Figure 1: Induction of somatic embryogenesis using immature zygotic embryos in *Carica papaya* variety viz. Taiwan 786. A: Proliferation of embryogenic tissue on MS (Murashige and Skoog, 1962) basal medium supplemented with 4.52 μ M 2, 4-D and 2.27 μ M TDZ (maintenance medium). B. Aceto carmine stained different cell division stages (2-8 celled stages) of somatic embryo-genesis as seen under the microscope. C. Various developmental stages of somatic embryos (oval, round, globular and heart shaped) as seen under a microscope on maturation medium. D. Germination of somatic embryos on MS (Murashige and Skoog, 1962) basal medium with 0.7% agar without any growth regulators (germination medium).

The embryogenic tissue was well maintained on the maintenance medium (Figure-1A). Microscopic observation of callus revealed different stages (2, 4, and 8 celled) of active cell division confirming the somatic embryogenesis on the maintenance medium in all the tested *Carica papaya* varieties (Figure-1B). Cells were stained with the acetocarmine for the microscopic observation of developmental stages of somatic embryogenesis (Figure-1B). Somatic embryogenesis occurred in the presence of TDZ in combination with 2,4-D, whereas it was never observed in explants cultured on control lacking hormones (Table-1). The partially desiccated embryogenic tissue was subcultured on maturation medium for further development of somatic embryos. In our present study, partial desiccation treatment has improved and enhanced the somatic embryo formation as reported earlier in many plant species (Malabadi *et al.* 2004c; Malabadi and Nataraja, 2006; Malabadi and van Staden, 2005ab). On the other hand, the embryogenic tissue without partial desiccation resulted in the delayed maturation of somatic embryos in all the tested varieties of *Carica papaya* lines. Therefore, partial desiccation treatment has boosted the formation of somatic embryos in all the tested varieties of *Carica papaya* in our present study. All the cultures were again maintained in the dark for 4 weeks. The full strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium supplemented with 3.0% sucrose, 5 μ M ABA and 0.8% agar (maturation medium) was adopted for this purpose. The total number of somatic embryos produced after 4 weeks on maturation medium per one gram fresh weight of embryogenic tissue is summarized in Table-3. At first, somatic embryos appeared as small white to yellow protuberances on the surface of the callus (Figure. 1C). Furthermore, we never observed embryogenesis without callus

formation. The embryos passed through recognizable globular, heart, torpedo and early cotyledonary stages, finally resulting in germinated embryos on the germination medium (Figure 1D). The callus developed somatic embryos on maturation medium after a period of 4 weeks (Fig-1). The percentage of somatic embryogenesis was not similar in all the tested varieties of *Carica papaya* (Table-3). Genotypic influence on somatic embryogenesis has been observed previously and a similar explanation holds

for the differential response between the *Carica papaya* varieties tested by us. After maturation, somatic embryos were picked from the cultures for germination (Fig-1D). The germination medium used was half strength (inorganic salts) MS (Murashige and Skoog, 1962) basal medium with 0.7% agar without any growth regulators (germination medium). A large number of somatic embryos were continuously developed and germinated with a distinct shoot meristem and radicular end (Figure-1D).

Table 2: The effect of various concentrations of TDZ on initiation of embryogenic tissue in different varieties of *Carica papaya* cultured on full strength MS basal medium supplemented with 4.52 μ M 2, 4-D.

TDZ (μ M)	Embryogenic tissue initiation in different varieties of <i>Carica papaya</i> * (%)				
	Sunrise*	Solo*	Co-1*	Co-7*	Co-3*
Control	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c
0.45	0.0 \pm 0.0c	0.0 \pm 0.0c	2.0 \pm 0.1b	0.0 \pm 0.0c	0.0 \pm 0.0c
2.27	9.0 \pm 1.0b	19.0 \pm 1.4a	2.0 \pm 0.1b	13.0 \pm 0.1a	41.0 \pm 1.9a
4.54	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c
9.08	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c
11.35	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c	0.0 \pm 0.0c

Control=MS basal medium without growth regulators such as 2, 4-D and TDZ; Data scored after 8 weeks and represents the mean \pm SE of at least 3 different experiments. In each column, the values with different letters are significantly different ($P < 0.05$).

*Different *Carica papaya* varieties.

De Bruijne *et al.* (1974) first induced somatic embryos from papaya callus using seedling petiole segments but no plants were regenerated (Teixeira da Silva *et al.* 2007). On the other hand Mehdi and Hogan (1979) could regenerate somatic embryos on MS medium containing coconut water (CW), IAA, IBA, NAA and kinetin, although the concentrations were not specified (Teixeira da Silva *et al.* 2007). Bhattacharya and Khuspe (2000) induced somatic embryos in 'Honey Dew' and 'CO2' following the culture of immature zygotic embryos on MS + 3 mg/L 2,4,5-T in the dark for 3-6 weeks (Teixeira da Silva *et al.* 2007). Maturation of embryos was achieved in medium supplemented with ABA at 0.1 mg/L or on PGR-free medium (71% in 'Honey Dew' and 59% in 'CO2') (Teixeira da Silva *et al.* 2007).

Lin and Yang (2001) also generated somatic embryos from adventitious roots within four months (Teixeira da Silva *et al.* 2007). Immature zygotic embryo explants of *Carica papaya* were cultured on MS medium supplemented with 2,4-D (2.0 mg/l) and formed globular embryos on explants without callus formation in 4-6 weeks (Bhattacharya *et al.* 2002). Maturation and conversion of somatic embryos was also achieved on the same medium (Bhattacharya *et al.* 2002). Cotyledonary stage embryos germinated to 63.66 and 68.33% in cv. honey dew and washington respectively in MS basal medium supplemented ABA (0.5 microm/l). Robust development and proliferation of plantlet roots *in vitro* was obtained on MS basal medium. Hardened plantlets have 60% survival rate

(Bhattacharya et al. 2002). In contrast, Yie and Liaw (1977) when using the internode stem of seedlings, first induced callus on MS containing 5.0 μ M NAA and 0.5 μ M kinetin, then somatic embryos on MS containing 0-0.25 μ M IAA and 5.0-10 μ M kinetin, and subsequently regenerated plantlets (Teixeira da Silva et al. 2007). Arora and Singh (1978a) advanced this finding by also inducing roots *in vitro* from shoots derived from somatic embryos (Arora and Singh, 1978b; Teixeira da Silva et al. 2007). The authors showed that auxin was critical for the initiation and

subsequent growth of callus and that out of the 3 auxins tested, NAA was most effective, followed by 2,4-D and IAA (Arora and Singh, 1978ab; Teixeira da Silva et al. 2007). Addition of 1.0 mg/L NAA was sufficient for good callus growth, occasionally assisted by the addition of GA3 up to 1.0 mg/L (Arora and Singh, 1978b; Teixeira da Silva et al. 2007). These authors claimed that the milky latex inhibited the establishment of *in vitro* cultures from mature tissues of both male and female plants (Arora and Singh, 1978b; Teixeira da Silva et al. 2007).

Table 3: Somatic embryogenesis and seedling recovery in different varieties of *Carica papaya*

Papaya* varieties	Somatic embryogenesis (%)	Somatic embryos recovered per gram fresh wt of embryogenic tissue	Seedlings recovered per gram fresh wt of embryogenic tissue
Coorg Honey dew control	81.0 \pm 3.2a 0.0 \pm 0.0c	31.0 \pm 4.0b 0.0 \pm 0.0c	17.0 \pm 1.5b 0.0 \pm 0.0c
Washington control	31.0 \pm 1.2b 0.0 \pm 0.0c	11.0 \pm 0.2b 0.0 \pm 0.0c	8.0 \pm 1.0b 0.0 \pm 0.0c
Honey dew control	28.0 \pm 3.0b 0.0 \pm 0.0c	5.0 \pm 0.3b 0.0 \pm 0.0c	1.0 \pm 0.2b 0.0 \pm 0.0c
Pusa delicious control	48.0 \pm 3.5b 0.0 \pm 0.0c	6.0 \pm 0.4b 0.0 \pm 0.0c	3.0 \pm 0.1b 0.0 \pm 0.0c
Pusa nanha control	26.0 \pm 3.4b 0.0 \pm 0.0c	16.0 \pm 2.0b 0.0 \pm 0.0c	7.0 \pm 1.4b 0.0 \pm 0.0c
Taiwan 786 control	87.0 \pm 4.2a 0.0 \pm 0.0c	27.0 \pm 0.2b 0.0 \pm 0.0c	10.0 \pm 1.0b 0.0 \pm 0.0c
Taiwan 785 control	85.0 \pm 3.0a 0.0 \pm 0.0c	31.0 \pm 2.0b 0.0 \pm 0.0c	19.0 \pm 1.0b 0.0 \pm 0.0c
Sunrise control	9.0 \pm 1.0b 0.0 \pm 0.0c	2.0 \pm 1.0b 0.0 \pm 0.0c	1.0 \pm 0.1b 0.0 \pm 0.0c
Solo control	19.0 \pm 1.4b 0.0 \pm 0.0c	12.0 \pm 1.0b 0.0 \pm 0.0c	6.0 \pm 1.0b 0.0 \pm 0.0c
Co-1 control	2.0 \pm 0.1b 0.0 \pm 0.0c	0.0 \pm 0.0c 0.0 \pm 0.0c	0.0 \pm 0.0c 0.0 \pm 0.0c
Co-7 control	13.0 \pm 0.1b 0.0 \pm 0.0c	0.0 \pm 0.0c 0.0 \pm 0.0c	0.0 \pm 0.0c 0.0 \pm 0.0c
Co-3 control	41.0 \pm 1.9b 0.0 \pm 0.0c	18.0 \pm 3.0b 0.0 \pm 0.0c	10.0 \pm 2.5b 0.0 \pm 0.0c

Control=MS basal medium without growth regulators such as 2, 4-D and TDZ; Data scored after 6 weeks and represents the mean \pm SE of at least 3 different experiments. In each column, the values with different letters are significantly different ($P < 0.5$).

*Different *Carica papaya* varieties.

Although this problem was not encountered by Litz and Conover (1980) who induced somatic embryos from the peduncles of adult *C. stipulata* plants. *C. stipulata* is not important as a fruit crop, but is important germplasm since it is resistant to PRSV (Teixeira da Silva et al. 2007). Litz and Conover (1982) furthered their own findings by inducing callus from ovules, and somatic embryos that subsequently formed germinated in 10-20% of the cultured ovules both solid and liquid White's medium supplemented with 60 g/L sucrose, 400 mg/L glutamine, 20% (v/v) filter-sterilized coconut milk and 8 g/L agar (Litz and Conover, 1982; Teixeira da Silva et al. 2007). Chen et al. (1987) regenerated somatic embryos in three months from 'Sunrise Solo' seedling root explants cultured on ½MS containing 5.4 µM NAA, 2.3 µM kinetin and 2.6 µM GA3, and finally 100 plants per explant (Teixeira da Silva et al. 2007). Litz et al. (1983) induced callus from the midrib (0.3-2.0 mg/L BA with 0.5-3.0 mg/L NAA) and lamina (0.6-3.0 mg/L BA with 1.2-5.0 mg/L NAA) of cotyledons of axenically-grown *C. papaya* seedlings when cultured on MS basal medium (Teixeira da Silva et al. 2007). Fitch (1993) and Fitch et al. (1998) induced somatic embryosis 'Kamiya Solo' from an initial callus phase when hypocotyl sections were cultured on ½MS with modified MS vitamins, 2.3 to 112.5 mM 2,4-D, 400 mg/L glutamine, and 6% sucrose (Teixeira da Silva et al. 2007). Cônsoli et al. (1995) also claimed success with the use of hypocotyls, epicotyls and leaves, although no details of the medium were defined, nor was the cultivar used mentioned (Teixeira da Silva et al. 2007). Similar generalizations were made by Neupane et al. (1998) when using 'Sunrise Solo', 'Kapoho Solo' and 'Sunset Solo' (Teixeira da Silva et al. 2007). Yamamoto and Tabata (1989) also induced hypocotyl somatic embryos using 0.1-1.0 µM 2,4-D (Teixeira da Silva et al. 2007). One-cm long explants of an

unspecified age were cultured on Linsmaier and Skoog (1965) medium containing 10 µM 2,4-D (Yamamoto et al. 1986; Teixeira da Silva et al. 2007). Pale yellow, friable embryogenic calli were produced but plantlets were not regenerated since the focus of their studies was on laticifer development in papaya somatic embryos (Teixeira da Silva et al. 2007). Monmarson et al. (1995) produced embryogenic calli from the integuments of immature seeds at a high-frequency (Teixeira da Silva et al. 2007). Romyanon et al. (2007) found that somatic embryos cultured in half-strength liquid MS medium containing 22.5 µM 2,4-D and 2.5 µM ABA yielded higher cell mass (dry-weight basis) than parallel treatments with other combinations of PGRs (Teixeira da Silva et al. 2007). Induction of somatic embryogenesis in *C. papaya* was extensively studied by Litz and Conover and other researchers (Litz, 1984, 1986; Litz and Conover, 1977, 1978a, 1978b, 1979, 1980, 1981, 1982, 1983; Litz et al. 1983; Drew, 1987, 1988, 1992). Ovules are an excellent source of regenerable papaya cultures via somatic embryogenesis. 'Ovular' somatic embryos are mainly derived from nucellar tissue (Litz and Conover, 1981, 1982, 1983), but also from highly embryogenic zygotes produced in interspecific crosses between papaya and *C. cauliflora* (Moore and Litz, 1984; Manshardt and Wenslaff, 1989; Teixeira da Silva et al. 2007). Litz and Conover (1981) also reported that occasionally cultured ovules from self-pollinated papayas also became embryogenic, although the zygotic or maternal origin was not specified (Teixeira da Silva et al. 2007). Gonsalves et al. (1998), based on earlier work by Fitch et al. (1990) induced somatic embryogenesis in 'Sunrise Solo' immature zygotic embryos (Teixeira da Silva et al. 2007). Davis and Ying (2004) induced somatic embryos from immature seeds, placed aseptically on Fitch's liquid medium, ½MS and vitamins, 50 mg/L myo-inositol, 6% sucrose, 10 mg/L 2,4-D and 400 mg/L

glutamine; two months thereafter, they were transferred to a similar medium, the difference being the inclusion of 2% sucrose, 0.1 mg/L BAP and 0.01 mg/L NAA (Teixeira da Silva *et al.* 2007). Magdalita *et al.* (2002) were able to induce 7730 somatic embryos from a initial culture of 11,900 zygotic embryos of 'Solo' (i.e. 65% conversion) on de Fossard medium with 0.25 μ M each of BAP and NAA and 10.0 μ M GA3 (Teixeira da Silva *et al.* 2007).

Fitch (1993) found that an increase in osmoticum up to 7% sucrose resulted in a simultaneous increase in the percentage somatic embryogenesis of 'Kapoho Solo' hypocotyls (Teixeira da Silva *et al.* 2007). Similar findings were reported by Litz (1986). Genotype also played a role in the success of somatic embryogenesis, with 'Kapoho' > 'Sunset' > 'Sunrise' > 'Waimanalo' (Fitch 1993; Teixeira da Silva *et al.* 2007). Fitch and Manshardt (1990) had previously found, however that the order was 'Waimanalo' > 'Sunrise' > 'Kapoho' > 'Sunset', although this order varied depending on the medium constituents and concentration of phytohormones added (Teixeira da Silva *et al.* 2007). For example 'Waimalo' showed the lowest (57%) embryogenic yield compared to 'Sunset' (93%) when 5 mg/L 2, 4-D was included in the medium (Teixeira da Silva *et al.* 2007). In their study, CW, BA, TDZ, 2,4-D or picloram could induce somatic embryos, singly, or in combination (Teixeira da Silva *et al.* 2007). Litz and Conover (1982, 1983) also found 20% (v/v) CW to be efficient on either MS or White's medium for the induction of somatic embryos (Teixeira da Silva *et al.* 2007). Jordan *et al.* (1982) could induce somatic embryogenesis in 'mountain papaya' or *C. candamarcensis* (i.e. *C. pubescens*) using hypocotyl calluses induced from greenhouse-grown seedlings on a medium containing 5-25 μ M NAA and 5 μ M kinetin produce and grow 14,000 elite female clones generated from micro-cuttings of nodes of apically dominant plants, using a method

established earlier by Drew (1992) (Teixeira da Silva *et al.* 2007). Lai *et al.* (1998) could mass produce plants when papaya plantlets were repeatedly subcultured on MS medium supplemented with 0.88 μ M BA and 0.1 μ M NAA, and this method is currently used to mass produce papaya in Taiwan (Teixeira da Silva *et al.* 2007). Similar propagation medium (MSNB) for multiple shoot formation was devised by Yang and Ye (1996) in which shoots were induced from petioles on MS supplemented with Gamborg's B5 vitamins (Gamborg *et al.* 1968), 0.8 μ M BA and 0.1 μ M NAA (Teixeira da Silva *et al.* 2007). Castillo *et al.* (1997) claimed the importance of an equal concentration (100 μ M) of FeNa₂EDTA and FeNa EDDHA (Sequestrene®) in producing the highest shoot proliferation. Chan and Teo (1994) used a 10-week solid proliferation medium followed by a 10-week liquid proliferation medium to mass produce shoots (116 plants per explant) (Teixeira da Silva *et al.* 2007). The proliferation medium consisted of MS + 0.1 mg/L BA + 500 mg/L casein hydrolysate + 0.38 mg/L riboflavin (Teixeira da Silva *et al.* 2007). Suksa- Ard *et al.* (1998) showed how elongation of shoot masses, initiated on an MS medium with BA, could be achieved with the application of 2.5 μ M 2-iP on medium containing 3% sucrose and 12 g/L agar (Teixeira da Silva *et al.* 2007). Drew (1992) found that 1% fructose resulted in better plant production, especially over repeated sub-cultures, than when 2% sucrose was used (Teixeira da Silva *et al.* 2007).

Thidiazuron (TDZ) is a substituted phenyl urea with cytokinin like activity (Mok *et al.* 1982). Very recently TDZ induction of somatic embryogenesis using nucellar tissue of mango (*Mangifera indica* L.) var. Ratnagiri has been reported (Malabadi *et al.* 2011a). In this study somatic embryos formed in the presence of thidiazuron (TDZ) in combination with 2,4-dichlorophenoxy acetic acid (2,4-D), but never on explants cultured on control medium lacking plant growth

regulators. Embryogenic callus could be induced from nucellar tissue (collected over 3 different years from the same source) after 4-9 weeks of culture on full-strength Murashige and Skoog inorganic salts - which served as the basal medium - supplemented with 4.52 μM 2,4-D and 2.27 μM TDZ (induction medium) (Malabadi et al. 2011a). An average of 35 somatic embryos per gram of fresh wt of tissue could be developed after 12 weeks and germinated, each with a distinct shoot meristem and radicular end (Malabadi et al. 2011a). The morphology and growth of seedlings derived from somatic embryos was normal. The high embryogenic potential of nucellar explants of var. Ratnagiri in this study creates the possibility for large-scale clonal propagation of mango (Malabadi et al. 2011a). The potentiality of TDZ to stimulate shoot formation is very common in many plants including orchids (Massimo et al. 1996; Eapen et al. 1998; Kanyand et al. 1994; Singh et al. 2001; Malabadi et al. 2004a, 2004b, 2008, 2009a; Earnst, 1994; Chen and Piluek, 1995; Nayak et al. 1997; Chen and Chang, 2000ab; Hong et al. 2008; Chen et al. 2002; Malik and Saxena, 1992; Thomas and Katterman, 1986). A protocol for induction of direct somatic embryogenesis, secondary embryogenesis and plant regeneration of *Dendrobium* cv. *Chiengmai* Pink was developed using TDZ (Chung et al. 2007). 5-25% of leaf tip segments of *in vitro* grown plants *Dendrobium* cv. *Chiengmai* Pink directly formed somatic embryos on half strength MS medium supplemented with 0.3, 1 and 3.0mg/l TDZ (Chung et al. 2007). TDZ is effective in induction of *in vitro* morphogenesis in shoot regeneration and direct somatic embryogenesis (Chen et al. 1999; Chen and Chang, 2000ab) of several orchids (*Phalaenopsis*, *Doritaenopsis*, *Cymbidium aloifolium*, *Dendrobium aphyllum*, *Dendrobium moschatum*, *Oncidium*). Moreover, TDZ combined with 2,4-D are required for callus induction in *Cymbidium ensifolium* var. *misericors*. (Chang and Chang, 1998),

Oncidium (Chen and Chang, 2000a, 2000b), *Phalaenopsis* (Chen et al. 2000) and *Paphiopedilum* (Huang et al. 2001). Wilhelm, (1999) reported successful micropropagation of juvenile Sycamore maple (*Acer pseudoplatanus*) via adventitious shoot formation by the use of 0.04 μM TDZ. In case of pea cvs Sugar Ann and Patriot, an average of 20 shoots formed on MS basal medium supplemented with 0.5 or 1.0 μM TDZ (Massimo et al. 1996). TDZ either alone (4.54 or 9.08 μM) or in combination with IAA (5.71 μM) on MS supplemented medium induced high frequency of shoot regeneration from primary leaf segments of 3 pigeonpea (*Cajanus cajan* L.) cultivars (Eapen et al. 1998). These investigations are in conformity with the present findings.

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