International Journal of Agricultural Research

ISSN 1816-4897

Academic Journals Inc.

www.academicjournals.com

International Journal of Agricultural Research 1 (2): 151-160, 2006
ISSN 1816-4897

Influence of Phytohormones, Culture Conditions and Ethylene Antagonists on Somatic Embryo Maturation and Plant Regeneration in Papaya

N.N. Renukdas, M.L. Mohan, S.S. Khuspe and S.K. Rawal
Plant Tissue Culture Division, National Chemical Laboratory,
Pune 411 008, India

Abstract: Growth regulators, picloram, 2,4,5-T, 2,4-D and dicamba, were incorporated at concentrations ranging from 0.40-60 μM in a modified MS medium to study their influence on induction of somatic embryogenesis in two Indian papaya cultivars viz. Honey Dew and Washington. Somatic embryos were induced from the meristematic region of the intact immature zygotic embryos in the presence of all the growth regulators tested. The response, however, varied with the concentration of the growth regulator used and the incubation conditions. The somatic embryogenesis response was in the order: picloram (4.0 μM) > dicamba (20.00 μM) > 2,4-D (10.00 μM) > 2,4,5-T (4.00 μM). The incubation of the cultured explants in dark or in light also played a significant role on induction of somatic embryogenesis induction. Somatic embryo maturation was achieved upon transfer of the cotyledonary stage somatic embryos to the hormone free modified MS basal medium supplemented with ethylene antagonists Spermidine, ABA and $AgNO_3$. A maximum of 96.67% embryos matured in presence of 1 μM Spermidine, 90.0% matured in presence of 0.1 μM ABA and 86.67% in presence of 0.5 μM $AgNO_3$. The matured somatic embryos germinated upon transfer to a modified basal medium containing MS basal salts and B_5 vitamins. The regenerated plantlets were transferred to soil and hardened under green house conditions and later transferred to the field.

Key words: Abscisic acid, 2,4-dichlorophenoxyacetic acid, 2,4,5-T, dicamba, papaya, picloram, Silver nitrate, somatic embryogenesis, Spermidine

Introduction

Papaya (*Carica papaya* L.) is an important fruit crop, widely cultivated in the tropical and sub-tropical regions of the world. It is valued for its fruit and the proteolytic enzymes papain and chymopapain (Medora *et al*., 1979). It is also a rich source of vitamins A and C. Commercial losses due to the papaya ring spot virus disease have acted as a disincentive to the cultivators and consequently the area under papaya plantation is shrinking every year. However, *in vitro* cultural techniques can contribute to increased crop productivity. Somatic embryogenesis, organogenesis and plantlet regeneration of papaya has been reported (Litz and Conover, 1981; Rajeevan and Pandey, 1983, 1986; Chen *et al*., 1987; Drew, 1987; Winnaar, 1988; Fitch 1990; Fitch and Manshardt, 1990; Zou and Euo, 1992; Fitch *et al*., 1993; Hossain *et al*., 1993; Bhattacharya *et al*., 2002, 2003/4; Renukdas *et al*., 2003/4). Callus culture (DeBruijne *et al*., 1974; Medhi and Hogan, 1976; Arora and Singh, 1978; Kumar *et al*., 1992) and plantlet production (Mondal *et al*., 1990, 1994) in *Carica papaya* has also been reported.

Corresponding Author: Dr. S.K. Rawal, Head, Plant Tissue Culture Division, National Chemical Laboratory, Pune 411 008, India Tel: +91 020 25902220/25902219 Fax: +91020 5893438

The requirement of 2,4-D and sucrose for induction of callus and somatic embryogenesis was demonstrated by Suska-Ard *et al*. (1999).

Both mature and immature embryos from papaya undergo de-differentiation and the callus tissues regenerate somatic embryos in response to the phytohormones present in the culture media. In the present communication, influence of phytohormones and culture conditions on the induction of somatic embryogenesis from mature and immature embryo explants were studied in two cultivars of papaya (*Carica papaya* L.).

Polyamines play a major role in cell division and growth in both prokaryotes and eukaryotes (Evans and Malmberg, 1989). Polyamines have been related with the induction of somatic embryos in *Daucus carota* (Feirer *et al*., 1985; Fienberg *et al*., 1984; Montague *et al*., 1979), *Medicago sativa* (Meijer and Simmonds, 1980) and *Nicotiana tabacum* (Torrigiani *et al*., 1987). Metabolism of polyamines is connected with several pathways. Their biosynthesis is closely related to ethylene biosynthesis, since both compete for methionine as the precursor. Ethylene is a gaseous plant hormone, involved in regulation of many physiological responses in plants (Feirer *et al*., 1985; Reid, 1987). While ABA and spermidine inhibit ethylene production, silver nitrate blocks its action. Silver nitrate is known to enhance somatic embryogenesis and shoot regeneration responses in sugarcane, cucumber, carrot, maize, sunflower and wheat tissue culture systems (Purnhauser *et al*., 1987). In alfalfa (*Medicago sativa*) ABA dramatically enhances somatic embryo quality (Lecouteux *et al*., 1993). Spermidine has also been implicated in enhancing regeneration responses in *Brassica campestris* (Chi *et al*., 1994).

The objective of the present study was to evaluate the influence of ethylene antagonists ABA, $AgNO_3$ and spermidine on maturation of papaya somatic embryos *in vitro* and to develop an effective protocol for fast regeneration.

Materials and Methods

Explant Preparation

Immature (90-115 days post-anthesis) and mature (130-145 days post-anthesis) fruits of *Carica papaya* L. cv. Honey Dew and Washington were collected from the field. Fruits were washed with running water for 1 h, immersed for 10 min in 1% solution of a commercial disinfectant containing Chlorhexidine Gluconate and Cetrimide (Savlon™, Johnson and Johnson, Hyderabad, India) and rinsed with running water. The fruits were then rinsed with 70% ethanol for 30s, washed with sterile distilled water in a laminar airflow cabinet and the seeds excised under aseptic conditions. From these seeds, zygotic embryos were taken out and used as culture explants.

Media and Culture Conditions

A modified MS medium containing MS salts (Murashige and Skoog, 1962), B_5 vitamins (Gamborg *et al*., 1968) and 30 g L^{-1} sucrose was used as the basal medium. The medium was further supplemented with growth regulators and gelled with 7.5 g L^{-1} agar (Hi-Media Laboratories Pvt. Ltd., Mumbai, India). The growth regulators picloram, 2,4,5-T, 2,4-D and dicamba were used at concentrations ranging from 0.40-60.00 μM and added to the basal medium before autoclaving. The pH of the medium was adjusted to 5.8 before autoclaving. The medium was autoclaved at 18 psi and 121°C for 20 min. The cultures were incubated at 25±2°C either in continuous dark or in continuous irradiance using cool-white fluorescent light (25 μ mol m^{-2} sec^{-1}) at 25±2°C.

Induction of Embryogenesis

Ten mature or immature zygotic embryos each, with cotyledons intact and in triplicate were cultured per petri dish (55×15 mm) containing 30 mL of the medium for the induction of somatic embryogenesis. The experiments were repeated three times.

Development of Somatic Embryos

For further development, regenerated embryos at the globular stage were separated from the mother explant after 4-6 weeks, transferred to fresh medium and incubated for a further two weeks. The modified MS basal medium devoid of any growth regulator served as the control. Thirty explants (10 in each petri dish) per treatment were used and the experiments were repeated three times.

Maturation of Cotyledonary Somatic Embryos

For maturation of cotyledonary stage embryos, these were transferred to MS basal medium supplemented with either ABA or spermidine or $AgNO_3$ (each at 0.05, 0.1, 0.5, 1.0, 5.0, 10.0 and 20.0 μM concentrations). The embryos were maintained for 1 week on these media.

Conversion of Cotyledonary Stage Somatic Embryos

Matured torpedo and cotyledonary stage embryos developed on the above media were transferred to modified MS basal medium for conversion into plantlets. The embryos were incubated for two weeks with continuous illumination under cool white fluorescent light (25 μ mol m^{-2} sec^{-1}) at 25±2°C. Somatic embryos developing shoots and roots were then transferred to test tubes containing modified MS basal medium for plantlet development.

Transfer of Plantlets to Soil

Regenerated plantlets were dipped in 0.5% Carbendazim (Bavistin®, BASF, Bombay, India) for 5-10 min and then washed with sterile distilled water. The treated shoots were transferred to pots containing autoclaved potting mixes of soil and sand (1:2). The pots were covered with polypropylene film. Plants were hardened in a greenhouse under diffused light conditions. The plants were transferred to the field after 3-4 weeks.

Statistical Analyses

Somatic embryogenesis rate (%) data were subjected to an analysis of variance (ANOVA). The data was statistically analyzed by ANOVA and the treatment means were compared with Student's t-test (Snedecor *et al*., 1967; Sokal and Rohlf, 1973; Chandel, 1993). In the two-way ANOVA of growth regulator and the two papaya varieties, among all the growth regulators tested. The three-way ANOVA was used to study the effects of growth regulator, culture condition and variety.

Scanning Electron Microscopy (SEM)

The somatic embryos were fixed in 2% Gluteraldehyde (Sigma, St. Louis, USA) for 48 h at room temperature and then rinsed in phosphate buffer (NaH_2PO_4 27.6 g L^{-1} and Na_2HPO_4 28.4 g L^{-1}, pH 7.2-7.4). Somatic embryos were dehydrated by stepwise passage through ethanol series and dried in a Bio-Rad E3000 critical point drying apparatus (VG Microtech, Oxford, UK). The samples were mounted on SEM stubs with double-sided adhesive tape and sputter-coated with gold-palladium (20-30 nm) using Bio-Rad E5200 (VG Microtech, Oxford, UK). The samples were examined under a Stereoscan S120 (Cambridge, England) scanning electron microscope.

Results and Discussion

Immature (90-115 days post-anthesis) and mature (130-145 days post-anthesis) fruits of Carica *papaya* L. cv. Honey Dew and Washington were collected from the field and zygotic embryos excised from these were used as explants. The embryogenesis response from mature zygotic embryo was rather low (30%) as compare to the response elicited from immature zygotic embryos (97%). Hence, immature zygotic embryos were used for all further studies (Fig. 1A). The efficiency of highest somatic embryogenesis for the treatments was defined as the percentage of explants forming somatic embryos at different concentrations of the phytohormones used (Table 1). The response of the explants on the modified MS basal medium without phytohormones served as the control.

An unorganized mass of callus formed from the responsive immature embryo explants within two weeks of inoculation. Globular embryos were seen to form after four to six weeks of incubation (Fig. 1B). Morphological and scanning electron microscopy observations of the regenerated somatic embryos revealed formation of somatic embryos in the presence of low phytohormone concentrations (0.40-4.00 μM) in the medium. At relatively higher phytohormone concentrations (20.00-40.00 μM) watery callus formed from the explants. At very high concentrations of 2,4-D and 2,4,5-T (40.00-60.00 μM) the explants turned necrotic. Incorporation of dicamba in the medium elicited a diametrically opposite response. While callus formed at low concentrations (0.40-20.00 μM), higher concentrations (20.00-60.00 μM) induced somatic embryos from the immature embryo explants. The extent of the morphogenetic response elicited from the two cultivars of papaya, varied with the growth regulator used and its concentration in the medium under light and dark incubation culture conditions (Fig. 2A and 2B).

The data analysis of the growth regulators showed significant difference in the induction of somatic embryogenesis (Table 1). Picloram 4.00 μM elicited the highest embryogenesis response that was significantly (97%) different compared to the other growth regulators. The somatic embryogenesis

Table 1: Percentage of highest somatic embryogenesis response elicited in the various phytohormones used

	Dark incubation				Light incubation			
	Immature ZE explant		Mature ZE explant		Immature ZE explant		Mature ZE explant	
Phytohormone	HD	W	HD	W	HD	W	HD	W
Picloram (4.14 μM)	97±6	93±6	30±0	27±6	70±6	63±6	23±15	13±6
2,4,5-T (3.91 μM)	93±6	90±0	27±15	27±6	43±6	47±6	13±6	7±6
2,4-D (9.05 μM)	93±6	90±6	23±0	10±0	73±6	70±6	13±6	7±6
Dicamba (22.62 μM)	93±6	90±6	26±0	23±6	83±6	83±6	20±10	16±6
Significance	NS	NS	NS	NS	NS	NS	NS	NS
Replicates								
Phytohormone (P)	*	*	*	*	*	*	*	*
Variety (V)	*	*	*	*	*	*	*	*
Culture Conditions (CC)	*	*	*	*	*	*	*	*
P X V	*	*	*	*	*	*	*	*
V X CC	*	*	*	*	*	*	*	*
PXVXCC	*	*	*	*	*	*	*	*

Data scored after 6 weeks of incubation, average of three replicates of experiments±SD. NS *: Non-significant or significant at p = 0.05%, respectively, ZE: Zygotic embryo, HD: Honey Dew, W: Washington variety

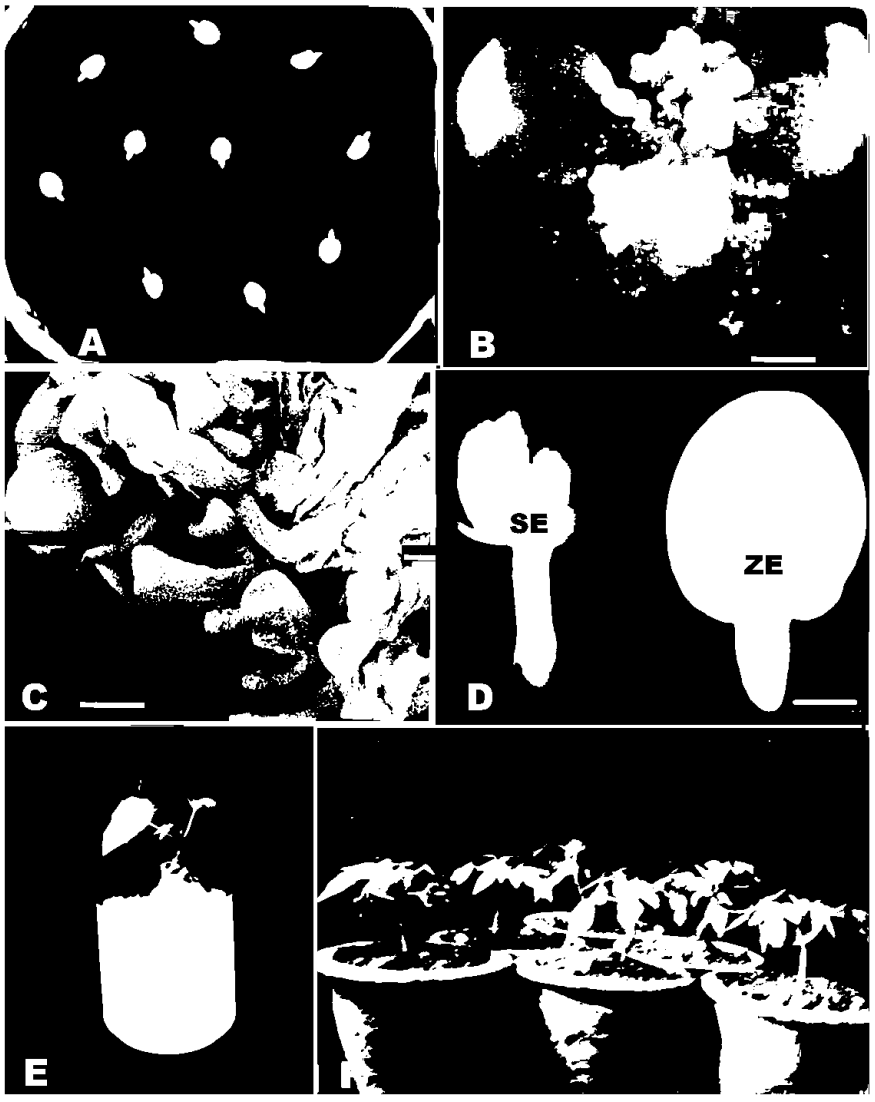

Fig. 1A-F: Stages of papaya somatic embryo induction, maturation and regeneration in papaya from immature embryo explant

A: Immature zygotic embryo explants used for induction of somatic embryogenesis.

B: Induction of somatic embryos induced from the meristimatic regions from zygotic embryos of 4-6 weeks old explant. Bar = 200 μM

C: Scanning electron micrograph of 8 weeks old mature somatic embryos. Bar = 400 μM

D: Somatic embryo is similar to zygotic embryo. Bar = 300 μM. E. Plantlet formation from single somatic embryo (16 weeks old) on MS basal medium. Bar = 200 μM F. Somatic embryo derived plantlet in soil after hardening

SE: somatic embryo. ZE: zygotic embryo

Table 2: Duration required for the induction, maturation and conversion of somatic embryo

	Mean time required (weeks)		
Stages of somatic embryo	Honey Dew	Washington	Earlier reports
Induction	4-6	4-6	14 (Fitch and Manshardt, 1990; Fitch, 1993) 12 (Litz and Conover, 1982) 12 (Castillo *et al*., 1998)
Maturation	4-6	6-8	4-8 (Fitch and Manshardt, 1990; Fitch, 1993) 16 (Litz and Conover, 1982) 2 (Castillo *et al.*, 1998)
Conversion	2-4	2-4	1-4 (Fitch and Manshardt, 1990; Fitch, 1993)

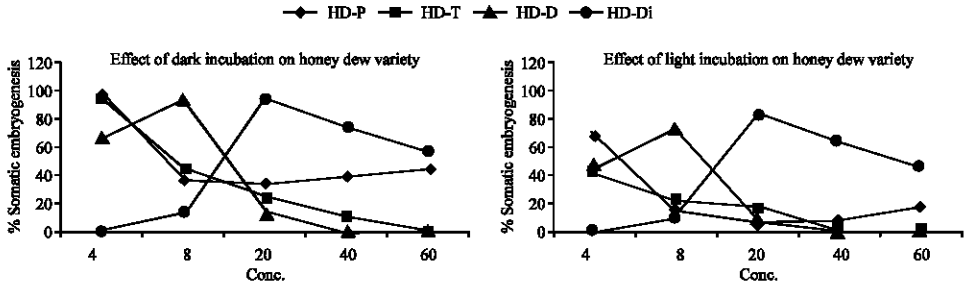

Fig. 2A: Effect of phytohormones on somatic embryogenesis in papaya

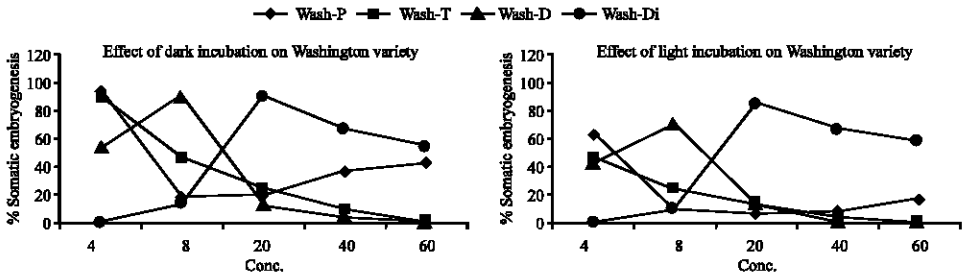

Fig. 2B: Effect of phytohormones on somatic embryogenesis in papaya
HD: Honey Dew variety, Wash: Washington variety, P: Picloram, T: 2,4,5-T, D: 2,4-D, Di: Dicamba, Conc.: Concentration in µM

response of the papaya cv. Honey Dew and Washington varied with the concentration of the hormones used.

In the two-way ANOVA of growth regulator and the two papaya varieties, among all the growth regulators tested, picloram at 4.00 µM induced significantly higher somatic embryogenesis response (97%) in variety Honey Dew under dark incubation than in light incubation. Both Honey Dew and Washington varieties showed no significant difference with respect to somatic embryogenesis frequency under light incubation condition. However, Honey Dew variety performed significantly better under dark incubation than the Washington variety.

The three way ANOVA effects of growth regulator, culture condition and variety are found to be significant (Table 2).

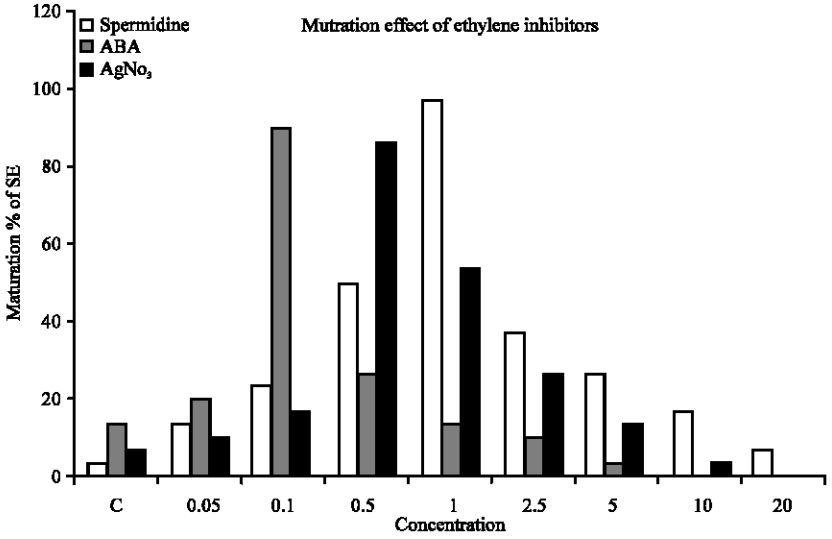

Fig . 3: Effect of ethylene antagonists in µM concentrations on maturation of somatic embryos

Some concentrations of picloram, 2,4,5-T, 2,4-D and dicamba were evaluated for their potential to induce somatic embryogenesis. Best embryogenesis responses were obtained in presence of picloram at 4.00 µM (97%), 2,4,5-T at 4.00 µM (93%) 2, 4-D at 10.00 µM (93%) and dicamba at 20.00 µM (93%) respectively upon incorporation in the basal medium. Somatic embryos regenerated from the meristematic regions (Fig. 1B) of the explant. Differentiation of embryos was continuous and non-synchronous and embryos at various developmental stages were observed. For embryo maturation cotyledonary stage somatic embryos were transferred to the modified MS medium supplemented individually with spermidine, ABA or $AgNO_3$ in concentrations ranging from 0.05 µM to 20 µM (Fig. 3). The respective percent embryo maturation was 96.67, 90.0 and 86.67% on these media. On the control medium somatic embryos showed 13.3, 3.3, 6.7%, respectively. Somatic embryos were visible after 4-6 weeks of incubation on the regeneration media. While both morphologically normal and abnormal embryos were regenerated, abnormal structures were partially or completely fused and failed to develop into whole plantlet. Normal somatic embryos were morphologically similar to the zygotic embryo (Fig. 1D). Maturation of somatic embryos was adverse by higher (10 µM) concentration of ethylene antagonists. Explants in all the treatments with the three ethylene antagonists viz. spermidine, ABA and $AgNO_3$ used at 1.0, 0.1 and 0.5 µM, respectively. Higher concentrations of all the three ethylene antagonists inhibited the maturation of somatic embryos (Fig. 3). The mature cotyledonary embryos (Fig. 1C) were transferred to MS basal medium for conversion into plantlets. The normal cotyledonary embryos germinated and grew into whole plantlets (Fig. 1E). The formation of torpedo/cotyledonary structures (8-10 per explant) was achieved in 12-14 weeks starting from the culture of the zygotic embryos on induction medium and it took another 6-8 weeks for regeneration. The plantlets were transferred to 8 cm earthen pots and hardened in green house (Fig. 1F). The regenerated somatic embryos showed all the stages of development. The normal structures showed the presence of well-developed shoot and root meristem, which germinated and grew into whole plantlets. Adventitious embryogenesis as well as secondary embryogenesis was also observed. At higher phytohormone concentrations the embryogenesis frequency was reduced in both the varieties.

The highest percentage of embryogenesis induced in both the varieties was in presence of picloram at 4.00 μM in the culture medium (97±6 and 93±6%, respectively for the varieties Honey Dew and Washington, respectively).

The role of light in the induction of somatic embryogenesis was quiet contradictory. Halperin (1970) suggested that light quality had no effect on carrot somatic embryogenesis; while Ekiz and Konzak (1997) reported that the light intensity rather than light quality was important for callus induction in wheat anther cultures. Callus induced in light and in darkness revealed a wide range of morphogenetic responses (density of cells as well as the ability for organogenesis). In the present study we found that the highest embryogenesis frequency occurred in dark incubation rather than in light incubation. After the initiation of embryogenesis, the cultures were shifted to light for further development, chlorophyll synthesis and chloroplast formation.

Somatic embryos at the cotyledonary stage were transferred to the modified MS basal medium for conversion. These normal converted cotyledonary structures were germinated on basal modified MS medium. These germinated structures grew into whole plantlets (Fig. 1F).

Torpedo/cotyledonary stage somatic embryo developed within 6-8 weeks, which took further 4-6 weeks for maturation. However, embryo production continued even after eight months of culture. The embryos converted and developed into plantlets within 12-16 weeks. These plantlets were transferred to 8-cm pots containing soil and sand mixture (3:1) and hardened in green house. The novelty of this study was short duration (Table 2) for regeneration of papaya plantlets compared to the earlier reports by Fitch and Manshardt, (1990) and Fitch (1993), which required 14 weeks on induction medium and a further 4-8 weeks of maturation. Chen *et al*. (1987) observed root derived somatic embryogenesis after 3 months of culture. Litz and Conover (1982) obtained somatic embryogenesis from *C. papaya* × *C. cauliflora* hybrid ovules culture after 3 months on induction medium and 4 months on maturation medium. DeBruijene *et al*. (1974) and Yie and Liaw, (1977) did not mention time required for producing their somatic embryos.

This study demonstrates that very high frequency somatic embryogenesis in papaya cultivars can be achieved with the incorporation of 4.00 μM picloram in the modified MS medium and maturation of somatic embryos in different ethylene antagonists significantly reduced the time required for the entire process of plantlet formation.

Acknowledgements

This study was supported by a research fellowship from C.S.I.R, Govt. of India to N.N. Renukdas.

References

Arora, I.K. and R.N. Singh, 1978. *In vitro* plant regeneration in papaya. Curr. Sci., 47: 867-868.

Bhattacharya, J., S.S. Khuspe, N.N. Renukdas and S.K. Rawal, 2002. Somatic embryogenesis and plant regeneration from immature embryo explant of papaya cv. Washington and honey dew. Ind. J. Exp. Biol., 40: 624-627.

Bhattacharya, J., N.N. Renukdas, S.S. Khuspe and S.K. Rawal, 2003/4. Multiple shoot regeneration from immature embryo explants of papaya. Biol. Plant., 47: 327-331.

Chandel, S.R.S., 1993. Handbook of Agricultural statistics. Achal Prakashan Mandir, New Delhi, India, B18-B63.

Chen, M.H., P.J. Wang and E. Maeda, 1987. Somatic embryogenesis and plant regeneration in *Carica papaya* L. tissue culture derived from root explants. Plant Cell Rep., 6: 348-351.

Chi, G.L., Wen-Shu, Lin, Justin, E.E. Lee and Eng-Chong Pua, 1994. Role of polyamines on *de novo* shoot morphogenesis from cotyledons of *Brassica campestris* ssp. Pekinesis (Lour) Olsson *in vitro*. Plant Cell Rep., 13: 323-329.

DeBruijne, E., E. Delanghe and R. van Rijack, 1974. Action of hormones and embryoid formation in callus cultures of *Carica papaya* L. Int. Symp. Fytofarm. Fytiata, 26: 637-645.

Drew, R.A., 1988. Rapid Clonal propagation of papaya *in vitro* from mature field-grown trees. Hortic. Sci., 23: 609-611.

Ekiz, H. and C.F. Konzak, 1997. Effects of light regimes on anther culture response in bread wheat. Plant Cell Tissue Org. Cult., 50: 7-12.

Evans, P.T. and R.L. Malmberg, 1989. Do polyamines have role in plant development? Annu. Rev. Plant Physiol. Plant Mol.Biol., 40: 235-269.

Feirer, R.P., S.R. Wann and D.W. Einspair, 1985. The effects of spermidine synthesis inhibitors on *in vitro* plant devlopement. Plant Growth Reg., 3: 319-327.

Fienberg, A.A., H.H. Choi, W.P. Libich and Z.R. Sung, 1984. Developmental regulation and polyamine metabolism and differentiation of carrot culture. Planta, 162: 532-539.

Fitch, M.M.M., 1993. High frequency somatic embryogenesis and plant regeneration from papaya hypocotyl callus. Plant Cell Tissue Organ Cult., 32: 205-212.

Fitch, M.M.M. and R.M. Manshardt, 1990. Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.) Plant Cell Rep., 9: 320-324.

Gamborg, O.L., R.A. Miller and K. Ojima, 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res., 50: 151-158.

Halperin, W., 1970. Embryos from Somatic Plant Cells. In: Padykula, H.A. (Ed.), Control Mechanisms in the Expression of Cellular Phenotypes. Symp. Int. Soc. Cell Biol. Acad Press, New York, pp: 169-191.

Hossain, M., S.M. Rehman, R. Islam and O.I. Jorder, 1993. High efficiency plant regeneration from petiole explants of *Carica papaya* L. through organogenesis. Plant Cell Rep., 13: 99-102.

Kumar, S.S., S. Singh, H.P. Singh, A.K. Singh and A. Singh, 1992. Growth regulator studies in tissue culture of three species of papaya. Biol. Zentralbl., 111: 21-26.

Lecouteux, G., F.M. Lai and B.D. Mckersie, 1993. Maturation of alfalfa (*Medicago sativa*) somatic embryos by absisic acid, sucrose and chilling stress. Plant Sci., 94: 207-213.

Litz, R.E. and R.A. Conover, 1981. Effect of sex type, season and other factors on *in vitro* establishment and culture of *Carica papaya* L. explants. J. Am. Soc. Hortic. Sci., 106: 792-794.

Litz, R.E. and R.A. Conover, 1982. *In vitro* somatic embryogenesis and plant regeneration from *Carica papaya* L. Ovular callus. Plant Sci. Lett., 26: 153-158.

Medhi, A.A. and L. Hogan, 1976. Tissue culture of *Carica papaya*. Hortic. Sci., 11: 311.

Medora, R.S., D.E. Bilderback and G.P. Mell, 1979. Effect of media on growth of papaya callus cultures. Z. Pflanzenphysiol., 91: 79-82.

Meijer, E.G.M. and J. Simmonds, 1988. Polyamine levels in relation to growth and somatic embryogenesis of *Medicago sativa*. J. Exp. Bot., 203: 787-794.

Mondal. M., S. Gupta and B.B. Mukherjee, 1994. Callus culture and plantlet production in *Carica papaya* (Var. Honey Dew). Plant Cell Rep., 13: 390-3.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Purnhauser, L., P. Medgyesy, M. Czako P.J. Dix and L. Marton, 1987. Stimulation of shoot regeneration in *Triticum aestivum* and *Nicotiana plumbaginifolia* Viv. tissue cultures using ethylene inhibitor silver nitrate. Plant cell Rep., 6: 89-95.

Rajeevan, M.S. and R.M. Pandey, 1983. Propagation of papaya through tissue culture. Acta. Hortic., 131: 131-139.

Rajeevan, M.S. and R.M. Pandey, 1986. Lateral bud culture of papaya (*Carica papaya* L.) for clonal propagation. Plant Cell Tiss. Org. Cult., 6: 181-188.

Reid, M.S., 1987. Ethylene in Plant Growth and Devlopement and Scenescence. In: Davies, Pj (Ed.) Plant Hormones and Their role in Plant Growth and Devlopement, Martinus Nijhoff Publication Dordrecht, The Netherlands, pp: 267-279.

Renukdas, N.N., M.L. Mohan, S.S. Khuspe and S.K. Rawal, 2003. Influence of boron on the induction of somatic embryos in papaya (*Carica papaya* L.) cv. Honey Dew. Biol. Plant., 47: 129-132.

Snedecor, G.W. and W.G. Cochran, 1967. Statistical Methods, Oxford and IBH Publishing Co., New Delhi, pp: 258-498.

Sokal, R.R. and F.J. Rohlf, 1973. Introduction to Biostatistics. W.H. Freeman and Company, pp: 134-184.

Suska-Ard, P., I. Kataoka, Y. Fujime and S. Subhadrabandhu, 1999. Requirement of 2,4-D and sucrose for somatic embryogenesis of papaya. Neppon. Nogyo, 43: 1-4.

Torrigiani, P., M.M. Altamura, G. Pasqua, B. Monacelli, F.D. Serafini and N. Bagni, 1987. Free and conjugated polyamines during de novo floral vegetative bud formation in thin cell layers of tobacco. Physiol. Plant., 70: 453-460.

Winnar, W.D., 1988. Clonal propagation of papaya *in vitro*. Plant Cell Tissue Org. Cult., pp: 305-310.

Yie, S. and S.I. Liaw, 1977. Plant regeneration from shoot tips and callus of papaya. *In vitro*, 13: 564-568.

Zou, Y. and H. Guo, 1992. High frequency somatic embryogenesis of *Carica papaya* L. and plant regeneration. Zhongshan Daxue Xuebao, Ziran Kexueban, 31: 60-65.