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POLYAMINE MEDIATED GENOTYPE INDEPENDENT SOMATIC EMBRYOGENESIS IN PAPAYA (*CARICA PAPAYA* L.)

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Abstract

A highly repeatable genotype independent, two-steps somatic embryogenesis protocol was developed. Immature zygotic embryos excised from 90 to 120 days old fruit of Pusa Delicious, CO 7 and Red Lady induced somatic embryos in half strength MS medium fortified with 10 mgl^{-1} 2,4-D and polyamine spermidine at 100 µM. Embryos were matured with 45 mgl^{-1} polyethylene glycol (PEG) and germinated on hormone free medium. Embryogenesis frequency among different cultivars ranged from 5.7 to 72 per cent. Rooted plantlets were successfully acclimatized in autoclaved coco peat fortified with MS salt mixture under shade net condition. The plants were established successfully in field.

Key words : Genotype, *Carica papaya* L., polyamine, somatic embryogenesis, PEG.

Introduction

Papaya (*Carica papaya* L.) belongs to family Caricaceae (Badillo, 2000; Van Droogenbroeck *et al*., 2002). Major papaya producing countries are Brazil, Mexico, Nigeria and India (Jayavalli *et al*., 2011). Papaya has become an important fruit crop having immense nutritive and commercial value. It is a fair source of iron and calcium, a good source of vitamins A and B, and an excellent source of vitamin C. The latex of papaya plant has commercial use, as it is the source of proteolytic enzymes, papain and chymopapain.

Papaya suffers serious losses due to Papaya Ring Spot Virus (PRSV) disease (Kumari *et al*., 2015) across the country and Papaya Leaf Curl Virus (PaLCuV) disease in northern as well as southern part of India. There is an urgent need to develop transgenic papaya conferring resistance against these viruses in India. PRSV resistant transgenic papaya has been successfully developed and commercialized in Hawaii, USA (Gonsalves and Ferreira, 2003 and Gonsalves *et al*., 2004). A robust *in vitro* regeneration system which holds good for major genotypes of papaya is pre-requisite for development of transgenic variety in papaya. Earlier *in vitro* regeneration protocols have been proposed but they were all genotype-dependent with low regeneration frequency rate (Fitch, 1993, Anandan *et al.,* 2010; Cabral *et al*., 2008; Farzana *et al*., 2008 and Cai *et al*., 1999). Present paper, therefore, describes a genotype-independent and highly repetitive polyamine mediated *in vitro* regeneration system of papaya.

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Materials and Methods

Cultivars and explant

Three cultivars of papaya were chosen for the study; Red Lady, being the most popular cultivar in the country; Pusa Delicious, being the leading gynodioecious cultivar from north India and CO 7, being the most popular south Indian cultivar. The trees were grown inside a shade net house. Each tree was selfed to harvest seeds in homozygous state.

Three types of explants were chosen for the study *viz*., immature zygotic embryos excised from unripe fruit; hypocotyls and root apices from *in vitro* grown seedlings. All the plants were subjected to different bioregulants in order to induce embryogenesis.

Preparation of explants

Sterilization and inoculation

The fruits were washed in running tap water and then soaked in 1.0 per cent Sodium Hypochlorite (NaOCl)

solution containing 1 drop of Tween-20 for surface sterilization for 1 hour and then washed with autoclaved distilled water 5 times.

Immature zygotic embryo

The fruits were bisected under aseptic conditions; the testa of seeds was removed using forceps and scalpel and then white and plump immature seeds were scooped out. Excised immature zygotic embryos were inoculated onto induction medium; under dark condition for 10-12 weeks for somatic embryogenesis.

Hypocotyl

The hypocotyl portion of *in vitro* grown plantlets was bisected and cut into small pieces and inoculated onto the culture media.

Root apices

Similarly, root apices of *in vitro* grown papaya plants were also cut into small pieces and inoculated onto inoculation medium.

Nutrient formulations for somatic embryogenesis

Explants were inoculated on to induction medium containing half strength MS media supplemented with differential regime of 2,4-D (2,4-dichlorophenoxyacetic acid) (5, 10, 15 and 20 mgl-^{1}), 400 mgl^{-1} glutamine, additional 1 mgl^{-1} MS vitamin with 6 per cent sucrose, 0.08 per cent agar. The pre-embryonic calli (PEC) were subjected to proliferation medium fortified with different polyamines *viz*., spermidine, spermine and putrescine at concentrations ranging from 50, 100 and 200 µM for 48 hours under dark conditions and then retracted from the media and again kept onto fresh induction medium devoid of any polyamine in dark, for observing the impact of polyamine in augmenting embryogenesis. The somatic embryos were removed from proliferation medium after 90-120 days and inoculated on MS medium fortified with different osmoticums *viz*., poly ethylene glycol (PEG), sorbitol and mannitol (15, 30 and 45 mgl^{-1}) along with 500 mgl^{-1} activated charcoal for a week. The dehydrated embryos were further subjected to regeneration medium containing 100 mgl^{-1} IAA (indole-3-acetic acid), 4 mgl^{-1} BAP (6-benzylaminopurine), 100 mgl^{-1} glutamine and 100 mgl^{-1} casein hydrolysate for 30-60 days. 1 cm long papaya shootlets with 4 true-leaves stage were withdrawn from regeneration media and inoculated on rooting medium containing half strength MS medium with 500 mgl^{-1} activated charcoal, 2 per cent sucrose, 0.08 per cent agar and different IBA (indole-3-butyric acid) concentrations (2, 3 and 4 mgl^{-1}) for 7 days. After the emergence of root primordial, the shootlets were removed from rooting medium and incubated in light on basal MS medium for growth and proliferation.

Acclimatization

Healthy rooted plantlets having 4-6 open leaves were inoculated on autoclaved coco peat fortified with 4.31 gml^{-1} MS salt. When leaves start touching the cap of bottles, they were removed and gradually shifted to shade net house with 90 per cent humidity.

Culture conditions and experimental layout

All the experiments have been laid down in Completely Randomized Design (CRD) and each treatment was replicated thrice. The culture conditions comprised of 25±2°C temperature, 50-55% RH (relative humidity) and 3000 lux light/dark regime of 16/8 hours.

Statistical analyses

The experiments were repeated at least thrice with minimum of 10 explants in each set of experiment. Analysis of variance (ANOVA), CD (critical difference) value and SEm (standard error mean) were computed using OP-STAT software.

Results and Discussion

Embryogenic potential of different papaya cultivars

For assessment of different cultivars' response for somatic embryogenesis, a total of 100 immature zygotic embryos of each cultivar have been inoculated per treatment and each treatment was replicated thrice.

All the cultivars of papaya responded to somatic embryogenesis with differential frequencies. Although Pusa Delicious was found to be the most responsive cultivar wherein callus formation took comparatively lesser time (41.6 days) with more number of explants (72 per cent) inducing callus. Each explant produced 19 globular embryos in 12 weeks' time (fig. 1). Not only the embryogenic clumps were heavier (257.1 mg/clump) but also the weight of individual embryo was higher (234.2 SE/clump) as compared to the other cultivars. Growth value (197.7) and Growth index (196.7) were also very high in Pusa Delicious over Red Lady and CO 7. Over all, Pusa Delicious was found to be a highly responsive cultivar. The zygotic embryo cotyledons of Pusa Delicious opened within 7 days after transfer to induction medium exposing the meristem, where a cluster of somatic embryos, about 2-3 mm in diameter, developed in 8 weeks. Whereas Red Lady cultivar was late in showing embryogenesis, the somatic embryos regenerated from this tissue within 4 weeks were non-friable and loose layered, unlike the previous clusters of early stage embryos that resulted in increased mass of tissue. Approximately 19.6 per cent explants of Red Lady formed

callus which was followed by 5.7 per cent recorded in CO 7, which was found to be the least responsive cultivar in terms of somatic embryogenesis (table 1).

Regeneration potential of cultivars depends upon the endogenous level of plant growth hormone. Sahi, which is an established cultivar of papaya in Bangladesh, was found to be highly regenerative under *in vitro* condition (Azad and Rabbani, 2005). Tainung No. 2 which is popular cultivar in Taiwan has also been found highly regenerative under *in vitro* condition. Thus, it could be concluded from the data that somatic embryogenesis can be induced in all papaya cultivars irrespective of their genotype following the current study.

Effect of explant on embryogenesis

Among three explants used (immature zygotic embryo, hypocoyl and root apices), the immature zygotic embryos took least time for callusing (45 days) and gave the highest number of embryos/ explant (19). A total of 9 embryos regenerated into plantlets using immature zygotic embryos (fig. 2). Hypocotyl demonstrated low frequency embryogenesis (16 embryos/ explant) which lead to development of 7 plants. Root apices were swollen and formed loose yellowish callus at the cut end. However, it failed to form any embryo (table 2).

Cotyledons, leaves and hypocotyls are found to be the best explant for embryogenesis in tomato (Madhulata *et. al.,* 2006). However, immature zygotic embryo is the most preferred explant in papaya (Cai *et al*., 1999; Fitch, 1993 and Kumari *et al*., 2015). Shoot tips and leaves were also employed as explant (Fitch *et al*., 1990) with little success. Litz and Conover (1978 a, b) reported micropropagation of papaya through shoot explants as well.

Effect of 2,4-D on somatic embryogenesis

The results showed 10 mgl^{-1} 2,4-D coupled with 400 mgl^{-1} glutamine fortified on half strength MS medium induced maximum embryos in minimum time (fig. 3). It also reduced the total time taken for callusing. The weight

Table 1 : Response of different papaya cultivars for somatic embryogenesis.

Variety	Days taken for callusing	% callusing	No. of SE	Weight of each embryonic clump	Weight of SE/Clump	Growth Value	Growth Index
Pusa Delicious	41.6	72.0	19.0	257.1	234.2	197.7	196.7
Red lady	77.0	19.6	4.6	37.7	27.7	28.9	27.9
CO-7	67.6	5.7	3.6	14.5	10.4	11.2	10.2
SEm±	0.858	2.327	0.576	2.838	2.870	1.861	1.038
C.D. (p 0.05)	3.027	8.208	2.031	10.012	10.125	6.565	NS

Table 2 : Effect of explant on embryogenesis.

Type of Explant	Days taken for callusing	No. of embryos formed	No. of embryos/ explant	No. of plant regenerated
Immature zygotic embryos	45	21	19	9
Hypocotyl	156	16	13	7
Root apices	143	0	0	0
SEm±	1.414	2.944	2.804	2.236
C.D. (p 0.05)	4.989	10.385	9.174	NS

Table 3 : Effect of 2,4-D on somatic embryogenesis of papaya.

2,4-D(mgl^{-1})	Days taken for callusing	% callusing	No. of somatic embryos	Weight of embryonic clump (mg)	Weight of somatic embryo/clump (mg)	Growth value	Growth index
5	56.33	33.61	7.33	0.18	0.14	138.10	137.10
10	41.35	71.03	18.66	0.32	0.29	253.15	252.15
15	66.00	32.08	12.33	0.21	0.18	161.79	160.79
20	77.00	19.63	4.66	0.038	0.031	29.00	28.00
Control	41.35	71.03	18.66	0.32	0.29	253.15	252.15
SEm±	0.98	6.68	0.83	0.015	0.009	11.23	11.23
C.D. (p 0.05)	3.26	22.13	2.76	0.048	0.030	37.19	37.19

Fig. 1 : Somatic embryogenesis in papaya cv. Pusa Delicious: (a) Immature zygotic embryo, (b) Somatic embryo, (c) germination of embryos, (d) Rooting of shootlet, (e) Acclimatization of plantlets, (f) Acclimatized plants in poly bags and (g) Establishment of plants in field.

of embryos and embryonic clump was also significantly higher at this concentration (table 3). The results are in accordance with Fitch (1993) and Cai *et al.* (1999), who also found that 2,4-D at 10 mgl^{-1} is prerequisite for embryo induction in papaya. The observations made during embryogenesis of papaya cv. Pusa Delicious revealed that 7-8 explants implanted on petriplate without dipping their radicle induced faster embryogenesis. The fused dicotyledons of embryos unfolded in 5-6 days and callusing started from the radicle. Within 3 weeks the explant was overlapped with callus forming a slimy layer of tissues beneath. Morphogenesis started from 4^{th} week leading to production of numerous threads-like yellowish-white structures called somatic embryos. The rate of formation of embryos increased when embryogenic calli cut into pieces and subcultured on fresh medium. The embryos emerging from the slimy growth of tissues beneath the embryogenic calli were soft and non brittle as compared with those emerging on the embryogenic calli.

Lad *et al.* (1997) reported that embryogenic nucellar cultures of mango were established on 4.5 µM 2,4-D. Somatic embryogenesis occurred directly from the nucellar explants at low frequencies. 2,4-D in combination with glutamine leads to induction and proliferation of adventive embryoni. Zouine and El Hadrami (2006) found that glutamine with 2,4-D proved better for embryo production. The proliferation of embryogenic suspension culture in two cultivars (Jihel and Bousthami Noir) of *Phoenix dactylifera* L. was tested on liquid media with or without 2,4-D and with different glutamine concentrations (3.35×10^{-4}, 6.7×10^{-4} and 13.4×10^{-4} M). The liquid medium with 0.1 mg l^{-1} 2,4-D and 6.7×10^{-4} M glutamine has clearly improved the proliferation of somatic embryos.

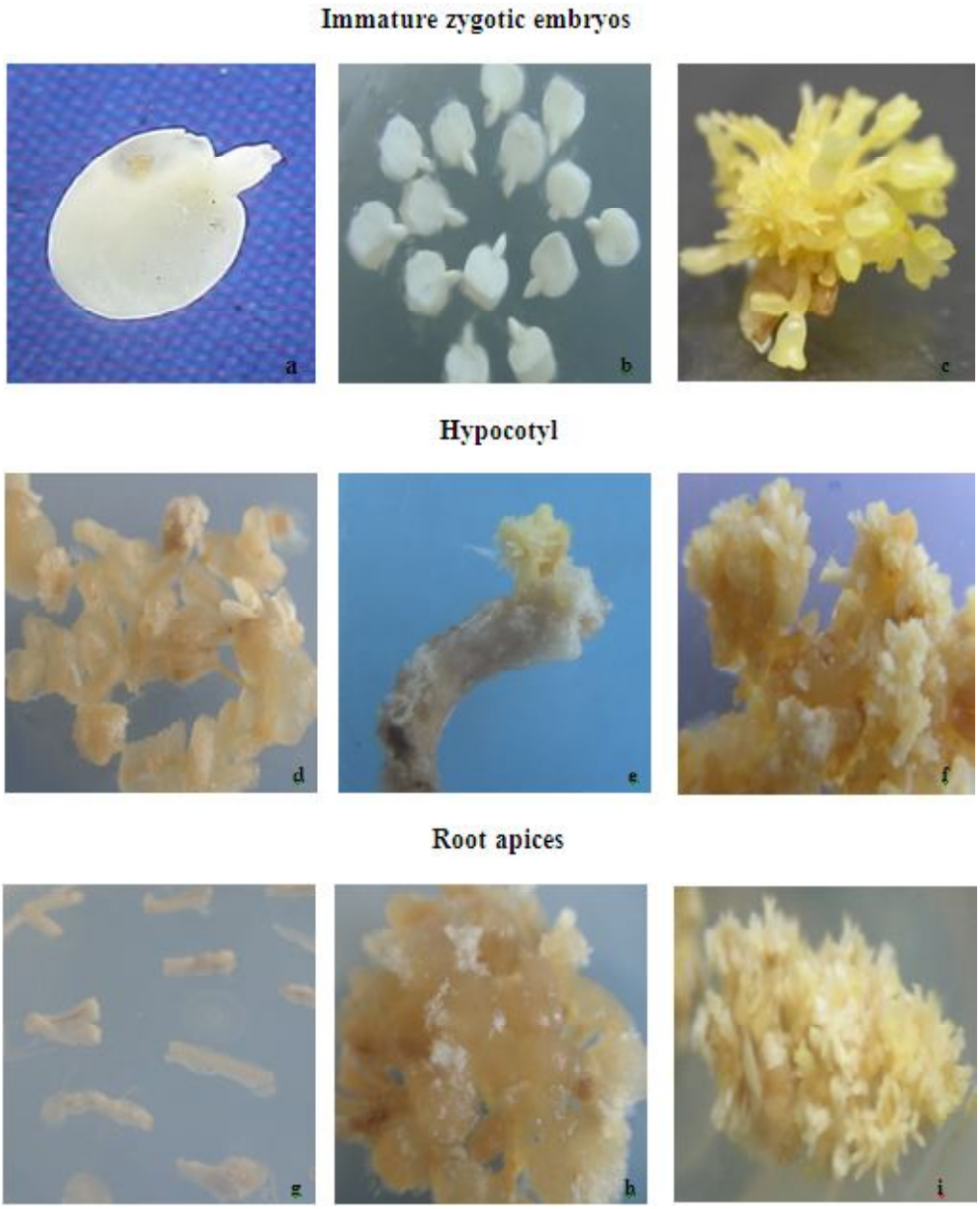

Fig. 2 : Embryogenesis in papaya using different explants: (a,b,c) Imature zygotic embryo; (d,e,f) Hypocotyl and (g,h,i) Root apices.

Effect of polyamine on proliferation of somatic embryos

Quick somatic embryogenesis was induced on PEC (pre embryonic calli) of papaya inoculated on ½ MS medium fortified with 10 mgl^{-1} 2,4-D, 400 mg l^{-1} glutamine, 1 mgl^{-1} MS vitamin, 6 per cent sucrose, 0.8 per cent agar and 100 µM spermidine. It is clear from the data (table 4) that threefold increase in embryogenesis was observed under the influence of 100 µM spermidine (70.6 embryos/ explant) with significant increase in weight of embryonic clump (899 mg) followed by 75 µM spermidine (53.3 embryos/explant). All three polyamines (PAs) *viz*., spermine, spermidine and putrescine augmented production of embryos over control. Increasing concentration (50, 75, 100 µM) of polyamines enhanced embryo production as well (fig. 4). However, it is interesting to note that callusing was reduced in explants treated with polyamine suggesting direct embryogenesis. Explants exposed to 100 µM spermidine were least (7.03 per cent) callused whereas maximum callusing was observed in tissues devoid of any polyamine during the entire course of investigation.

PAs play a pivotal role in *in vitro* morphogenesis and improved shoot and root induction (Bajaj and Rajam,

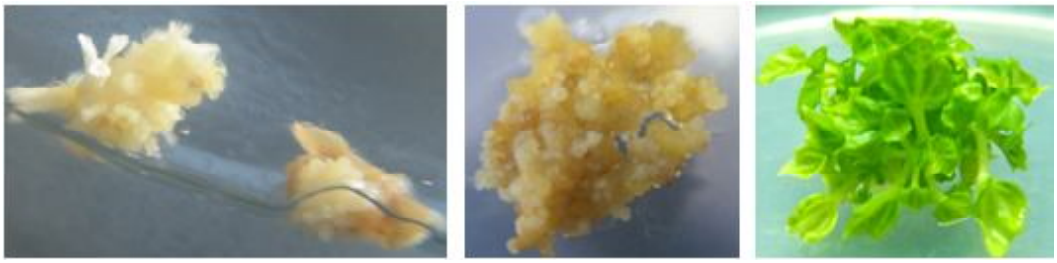

Fig. 3 : Somatic embryogenesis of papaya under the influence of 2,4-D.

Fig. 4 : Effect of polyamine on enhanced embryogenesis over control.

Fig. 5 : Somatic embryogenesis under the influence of PEG: (a) Somatic embryo, (b) Desiccated embryo and (c) Regenerating embryo.

Fig. 6 : *In vitro* rhizogenesis in papaya: (a) Inoculation of 1-2 cm long *in vitro* shoot (b) Induction of primary root, (c) Fully developed roots of papaya in coco peat and (d) Acclimatized papaya plant in polyhouse.

1996 and Rajam *et al*., 1998). PAs have never been used to augment regeneration frequency in papaya; as current study is the first report of the same. However, PAs have been reported as an important determinant for regulation of somatic embryogenesis (Minocha and Minocha, 1995, Minocha *et al.*, 1999 and Rajam *et al*., 2004).

Effect of osmoticum on conversion of embryos

Somatic embryos do not germinate unless they are mature enough. Various chemicals and stresses have been suggested to enable somatic embryos to mature and thereby germinate at high frequencies. The data revealed that polyethylene glycol (PEG) at 45 mgl^{-1} (table 5) was found to be the most suitable osmoticum to convert globular embryos into cotyledonary embryos and their subsequent germination and regeneration into plantlets (28 per cent) (fig. 5).

It seems that dehydration of embryos leads to conversion in papaya Zhang *et al.* (2005) found that somatic embryos of *Eruca sativa* developed into mature embryos on MS medium in the presence of 45 mgl^{-1} PEG.

Table4 : Influence of polyamines on induction of embryogenesis.

Polyamine	Concentration (uM)	% Explant callused	No. of somatic embryos/ explant	Weight of embryonic clump (mg)
Putrescine	0	72.66	18.60	328
	50	25.70	22.0	363
	75	19.20	25.0	380
	100	11.50	26.6	400
Spermine	0	72.66	18.6	328
	50	20.00	27.0	376
	75	14.30	35.3	425
	100	10.60	40.6	484
Spermidine	0	72.66	18.6	328
	50	16.00	45.3	463
	75	11.60	53.3	600
	100	7.03	70.6	899
SEm±		6.042	6.924	6.664
C.D. (p 0.05)		17.741	20.329	19.567

Table 5 : Effect of different osmoticums on conversion of embryos.

Osmoticum	Concentration (mgl^{-1})	No. of cotyledons	% embryos matured	% plants regenerated
Poly Ethylene Glycol (PEG)	15	158.00	47.00	12.66
	30	181.66	58.66	17.66
	45	216.66	81.00	28.33
Mannitol	15	49.00	8.33	1.00
	30	59.33	8.00	0.33
	45	77.33	8.66	0.66
Sorbitol	15	46.33	6.33	0.00
	30	60.33	6.00	0.00
	45	75.00	6.66	0.33
Control	0	20.22	2.89	0.00
	SEm±	5.14	1.55	0.63
	C.D. (p 0.05)	15.30	4.63	1.89

Table 6 : Effect of IBA on *in vitro* root induction in papaya.

Auxin (mgl^{-1})	No. of roots	No. of secondary roots	Length of roots (cm)	Fresh weight of roots (gm)	Dry weight of roots (gm)
½ MS + IBA 1.0	1.667	3.667	4.55	0.083	0.004
½ MS + IBA 2.0	3.0	2.667	4.63	0.061	0.015
½ MS + IBA 3.0	1.33	5.0	5.48	0.133	0.007
Control (½ MS)	0.00	0.00	0.00	0.00	0.00
SEm±	1.232	1.622	0.772	0.018	0.006
C.D. (p 0.05)	NS	NS	NS	NS	NS

After desiccation, somatic embryos developed into plantlets by culturing the mature somatic embryos on half strength MS medium containing 0.24 ìM IBA. Partial desiccation of mature somatic embryos improves conversion frequencies (Hammatt and Davey, 1987) but efforts to duplicate the developmental environment of zygotic embryos should further improve the maturation of somatic embryos (Finkelstein and Crouch, 1986). Factors which determine the ability of embryos to convert into plants include the synthesis and accumulation of storage compounds, especially storage proteins and the acquisition of desiccation tolerance (Blackman *et al.,* 1992; Kermode, 1995). The importance of water relations in controlling embryo maturation was proposed by Fischer *et al.* (1987) and has been supported by evidence from both embryo culture experiments (Xu *et al.,* 1990) and *in situ* studies (Saab and Obendorf, 1989).

***In vitro* rhizogenesis and acclimatization**

2 cm long microshoots having 4 healthy leaves were subjected to half strength MS medium fortified with 2 mgl^{-1} IBA + 500 mgl^{-1} activated charcoal, produced 3 primary roots/shoot (fig. 6). *In vitro* rhizogenesis reduced with decreasing or increasing IBA concentration. The fresh and dry weights for all the treatments ranged from 0.061 to 0.13 and 0.004 to 0.015, respectively. Hence, this is apparent from the data that 2 mgl^{-1} IBA is optimum for profuse rooting in papaya (table 6).

Yu *et al*. (2001) reported 2.5 µM IBA is optimum for rooting under dark condition as the photooxidative nature of IBA causes delayed growth of plant. Many authors have reported IBA or NAA (1-naphthaleneacetic acid) as suitable auxins for inducing roots in papaya. Higher frequency of rooting occured when papaya plantlets were subcultured on media containing IBA and NAA (Winnaar, 1988).

Conclusion

Micropropagation in papaya has been attempted through shoot tip, somatic embryos and callus culture. However, somatic embryogenesis remains a preferred pathway for genetic manipulation. Several authors have reported somatic embryogenesis in papaya with low frequency regeneration system. Polyamines have been demonstrated to be useful in regeneration in few crops. The current study reports a three-fold increase in somatic embryogenesis in papaya with use of 100 µM spermidine.

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