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In-Vitro androgenesis in papaya (*Carica papaya* L.) *cv.* Pusa Nanha

Gyanchand[1], Manoj Kumar Sharma[1], Sandeep Kumar[1], Sushma Sagar[1], Vinay Kumar[2] and Mukesh Kumar[1*]

[1]Department of Agriculture Biotechnology, S. V. P. University of Agriculture & Technology, Meerut-250110 (U. P.), INDIA

[2]National Institute of Biotic Stress Management, Raipur (Chhatisgarh), INDIA

*Corresponding author. E-mail: mukeshbt06@gmail.com

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Abstract: Papaya (*Carica papaya* L.) is an economically important fruit crop of tropics and subtropics. It has high nutritional value, as well as medicinal and industrial applications. Papaya is a polygamous species with three sex types male, female, and hermaphrodite. Conventional methods of papaya breeding are time consuming and needs advent of anther culture which may be effective for shortening of breeding cycles. The present study on *in vitro* androgenesis in papaya *cv.* Pusa Nanha observed the highest embryo induction rate (8.0%) when anthers were cultured on agar medium with 0.1 mg/L BA and 0.1 mg/L NAA after incubation in liquid MS medium with 2.0% sucrose for 7 days at 35ºC. The high temperature (35ºC) was more suitable for embryo induction in papaya than slightly low temperature (25ºC). At these both temperatures longer incubation of anthers in water reduced embryo induction rate. Sugar starvation results were ambiguous. Shoots were also developed in the media when used in liquid form. The highest rooting (75.0%) was observed at 2.0 mg/L IBA. Increasing IBA concentration reduced rooting. All well rooted plants were hardened in hardening chamber and successfully transferred to field. The present findings indicated that anther culture can be efficiently contributed for the direct micro-propagation of papaya plants. This study would also be helpful to the researchers to develop more efficient anther culture protocols for further improvement of papaya through *in vitro* androgenesis.

Keywords: Anther culture, embryogenesis, MS media, papaya, plant growth regulators

INTRODUCTION

Papaya (*Carica papaya* L.), a member of family Caricaceae, is the most economically valuable fruit crop, owing to its high nutritional value, as well as medicinal and industrial applications (Drew, 2003; Silva *et al.,* 2007; Zhang *et al.,* 2011). It consists 55 species placed under four genera, namely- *Carica, Cyclimorpha, Jacaratia* and *Jarilla* (Badillo, 1971; Dallwitz, 1980). Papaya is a rich source of antioxidants, vitamins (A, B, C and E), minerals (magnesium and potassium) and fibers. It contains a digestive enzyme papain that effectively treats causes of trauma, allergies and spots injuries. It is also used as a tonic to improve cardiovascular system, to treat dyspepsia, hyperacidity, dysentery, constipation, heart diseases and attacks, colon cancer and all types of digestive and abdominal disorders. Papaya is grown in around sixty countries, with the bulk of production in developing economies. Papaya is a major tropical fruit grown commercially in India, Brazil, Mexico, Australia, Hawaii, Thailand, South Africa, Philippines, Indonesia and Taiwan. India is the largest producer of papaya contributing more than 38.00% of the total world's production (Indian Horticulture database, 2011). Papaya is conventionally propagated by seeds, grafting and rooted cuttings but these methods often tedious and impractical when carried out on a large scale due to considerable variation in disease susceptibility, fruit quality and yield (Rajeevan and Pandey, 1986). Conventional methods of papaya breeding requires at least six to eight generations developing a homozygous line (Ray, 2002). Tissue culture has the possibility for overcoming problems of lack of efficient preparing materials associated with breeding programmes for cultivar improvement. These techniques could offer a valuable alternative and reliable procedure for mass propagation of homogenous and uniform plants for both commercial and research purposes. Anther culture is a promising technique for shortening the breeding cycle via haploid production. For the first time, the anther culture was reported by Guha and Maheshwari (1964) in *Datura innoxia* plant at the Botany Department, South Campus, University of Delhi. To date, androgenic haploids have been produced in over 170 species; several good reviews provide lists of these species (Maheshwari *et al.,* 1982; Bajaj, 1983; Heberle-Bors, 1985; Dunwell, 1996). The first attempt to utilize anther culture in papaya breeding was made by Litz and Conover (1978, 1979). Beside this, *in vitro* androgenesis in papaya has also been reported recently by several other researchers (Tsay and Su, 1985; Rimberia *et al.,* 2005; Rimberia *et al.,* 2006; Azad *et al.,* 2013). In

anther culture, it has been generally known that many factors (such as genotype and physiological state of the donor plant, physical and chemical factors of media, and developmental stages of pollen, pre-culture treatments) influence haploid induction efficiency (Sopory and Munshi, 1996). Therefore, the present research work was undertaken to investigate *In vitro* androgenesis in papaya (*Carica papaya* L.) *cv.* Pusa Nanha.

MATERIALS AND METHODS

Plant materials: In the present study, flower buds from papaya variety named 'Pusa Nanha' were used as plant material. The present work was carried out during 2012-2013 in Plant Tissue Culture and Molecular Biology laboratories at the Department of Agriculture Biotechnology, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut (U.P.) India.

Collection of anthers and surface sterilization: The healthy flower buds were collected from field grown plants during September - October, 2012 from Horticulture Research Centre, Sardar Vallabhbhai Patel University of Agriculture & Technology, Meerut (U.P.). The flower buds were kept in 250 mL conical flask (Borosil, India) with mouth covered by muslin cloth under running tap water for 20 min followed by a by a 3 min treatment with a 5.0% (v/v) aqueous solution of Laboline (Qualigens, India) and rinsed 5-6 times with sterilized distilled water. In a laminar flow hood, the flower buds were surface sterilized in 70% ethanol (Merck, USA) for 20 sec, then in 1.0% sodium hypochlorite (Merck, USA) solution containing 10 drops/L of Laboline (Qualigens, India), for 7-8 min and rinsed three times with sterilized distilled water.

***In-vitro* embryo induction and plant regeneration:** After sterilization, anthers were aseptically removed under laminar air flow from the buds and pre-treated in liquid MS media (Murashige and Skoog, 1962) for 1, 4 and 7 days at 20, 25 and 35ºC. For pre-treatment anthers were placed in test tubes containing: (1) water (distilled water without sucrose); (2) MS nutrients without sucrose; (3) MS nutrients with 2.0% sucrose. The pre-treated, anthers were transferred on MS [19] agar medium supplemented with 0.5 mg/L BA (Sigma, USA), 0.1 mg/L NAA (Sigma, USA), 3.0% sucrose (Qualigens, India) and 0.6% agar (Qualigens, India). The pH of the medium was adjusted to 5.8 with 1.0N NaOH/HCl before adding the gelling agents. Following autoclaving at 121°C and 15 psi pressure for 20 min, the media were poured into 90-mm-diameter Petri dishes, each containing 25 mL of culture medium. The number of anthers per petridish was 50. The dishes were sealed with Parafilm and incubated in the dark at 25 ± 2°C. Three replicated plates were used per treatment for each experiment.

To hasten the growth of shoots derived from embryos were transferred into the culture tubes (25×150 mm; Borosil, India) containing 10 ml of liquid MS medium supplemented with 0.5 mg/L BA, 0.1 mg/L NAA and 3.0% sucrose and then sequentially sub-cultured on the same media at fifteen days intervals up to forty five days. The developed shoots were treated with different concentrations (0.5-4.0 mg/L) of indole-3-butyric acid (IBA) to promote rooting. All plantlets were maintained for ten to twelve weeks at 25 ± 2ºC and 60–65 % relative humidity under a 16/8 h (light/dark) photoperiod with light supplied by cool-white fluorescent tubes (Philips, India) at an intensity of 48μmol/m^{2}s^2. In the present investigation, the effects of high temperature, starvation and physical state of media on embryo induction were observed.

Hardening and acclimatization of plantlets: The well rooted tissue cultured plantlets were carefully transferred to the vermiculite pots supplemented with full strength Hyponex solution (N:7, P:6, K:19). These were hardened in a hardening chamber with gradually controlling temperature, humidity and light as the ambient conditions. The established plantlets after acclimatization were transferred in polythene bags under ambient conditions. After one month of acclimatization, the anther cultured plantlets were ready to transplant into the field.

Statistical analysis: Data on embryo induction and *in vitro* rooting were recorded at regular time intervals for all treatments. All the experiments were carried out in a complete randomized design maintained with three replicates and each experiment was repeated thrice. The effect of different treatments on *in vitro* androgenesis in papaya was quantified and the data was statistically analyzed by using software OPSTAT 1.0.

RESULTS

In the present investigation, the experiments were conducted to investigate the effects of high temperature, starvation and physical state of media along with exogenous hormones on *in-vitro* androgenesis in papaya *cv.* Pusa Nanha. In the present investigation, following results were observed.

Effects of temperature, starvation and physical state of media on embryo induction: In the present investigation, fifty anthers of papaya *cv.* Pusa Nanha were used for each treatment. No embryo formation was observed from anthers pre-treated in water and of MS nutrients medium with or without sucrose at 20ºC. On the other hand, embryos were induced on anthers pre-treated in liquid medium at both 25 ºC and 35ºC as shown in table 1. At 25 ºC, embryos were formed at a rate of 2.0-4.0 % when anthers were pre-treated in MS liquid + sucrose medium for four and seven days. At 25ºC embryo induction was also observed at a rate of 4.0 % when anthers were incubated in water for one day. At 35ºC, embryos were induced at the rate of 6.0 % upon incubation of anthers in water for one day and at the rate of 4.0% in MS liquid medium for four days and at the rate of 8.0% in MS liquid medium with 2.0% sucrose for seven days. Starvation effects were clearly indicated that the presence of sucrose in the media was essential for optimum embryo induction from anthers (Table 1).

Table 1. Embryo induction on anthers at agar medium with 0.1 mg/L BA and 0.1 mg/L NAA after pre-treated in water and MS liquid medium with or without sucrose at 20 ºC, 25 ºC and 35ºC.

Temperature (ºC)	Medium	Duration (Days)	No. of anthers used	No. of anthers formed embryos (%)
20	Water	1	50	0
		4	50	0
		7	50	0
	MS	1	50	0
		4	50	0
		7	50	0
	MS+2.0 % Sucrose	1	50	0
		4	50	0
		7	50	0
25	Water	1	50	2 (4.0)
		4	50	1 (2.0)
		7	50	0
	MS	1	50	0
		4	50	1 (2.0)
		7	50	2(4.0)
	MS+2.0 % Sucrose	1	50	0
		4	50	2 (4.0)
		7	50	3 (6.0)
35	Water	1	50	3 (6.0)
		4	50	2 (4.0)
		7	50	1 (2.0)
	MS	1	50	1 (2.0)
		4	50	2 (4.0)
		7	50	1 (2.0)
	MS+2.0 % Sucrose	1	50	1 (2.0)
		4	50	3 (6.0)
		7	50	4 (8.0)

The highest embryo induction rate (8.0%) was observed when anthers were cultured on agar medium supplemented with 0.1 mg/L BA and 0.1 mg/L NAA after incubation in liquid MS medium supplemented with 2.0% sucrose for seven days at 35ºC. These results indicated that the pre-treatment of anthers in liquid MS medium are effective for embryo induction at 35ºC than at 25ºC. The present investigation suggested that high temperature (35ºC) was more suitable for embryo induction in papaya than slightly low temperature (25ºC) as shown in table 1. At both temperatures (25ºC and 35ºC) longer incubation of anthers in water reduced embryo induction rate (Table 1). The effect of sugar starvation treatment on embryo induction was ambiguous.

Effects of different concentrations of IBA on rooting: In the present study, well developed shoots on liquid MS medium supplemented with 0.5 mg/L BA, 0.1 mg/ L NAA and 3.0% sucrose were used for *in vitro* rooting experiments. Twenty shoots were used for *in vitro* rooting in each treatment. An auxin-IBA (0.0-4.0 mg/ L) was used for rooting of *in-vitro* developed shoots (Table 2). The results indicated that ½ MS supplemented with 2.0 mg/L IBA produced highest rooting (75.0%). Increasing IBA concentrations reduced rooting percentage. Rooting (15.0 %) was also obtained on ½ MS media without IBA. In the present study, no callus formation was observed during the rooting of shoots.

Hardening and field acclimatization of plantlets: Out of 140 plantlets used for rooting only 44 plantlets showed vigorous rooting while 10 plantlets showed weak rooting. These well rooted plantlets were transferred to hardening chamber for hardening. After hardening, only 30 plants survived. These plants were carefully transplanted to field for further investigation.

DISCUSSION

Successful *in-vitro* androgenesis depends on various factors, *e.g.,* genotype and physiological state of the donor plant, microspore developmental stages, culture medium, and pre-culture treatments (Palmer and Keller, 1997; Rimberia *et al*., 2005 and 2006; Azad *et al.,* 2013). Growth regulators in culture media are one of the most important components for pollen embryogenesis *via* anther culture (Zhang and Lespinasse, 1992; Sopory and Munshi, 1996; Rimberia *et al*., 2005 & 2006; Azad *et al.,* 2013). In some plant species, an auxin or a cytokinin alone is required for inducing pollen embryogenesis (Sopory and Munshi, 1996), while in others, such as cereals and fruit crops, a combination of both auxins and cytokinins are necessary (Bajaj, 1990; Zhang and Lespinasse, 1992; Ochatt and Zhang, 1996; Rimberia *et al.,* 2005 and 2006; Azad *et al.,*

Table 2. Effects of different IBA concentrations on *in vitro* rooting of papaya shoots (Mean±SD of three replicates).

IBA Concentration (mg/L)	Number of shoots used for root induction	Number and % of shoots showing root induction	Number of roots/shoot (Mean±SD)	Root length (cm) (Mean±SD)	Callus induction
½ MS+0.00	20	3 (15.0)	1.20±0.15	0.75±0.20	++
½-MS+ 0.25	20	5 (25.0)	1.65±0.32	1.35±0.26	+
½-MS +0.50	20	7 (35.0)	1.90±0.55	1.58±0.37	+
½-MS +1.00	20	10 (50.0)	2.35±0.48	2.05±0.47	---
½-MS +2.00	20	15 (75.0)	3.70±0.75	3.40±0.76	---
½-MS +3.00	20	8 (40.0)	3.20±0.84	2.80±0.45	+++
½-MS +4.00	20	6 (30.0)	1.86±0.62	1.28±0.34	+++

Here, +++ High, ++ Medium, + Low, --- None

2013).

In the present study, the effects of different temperatures, starvation and physical state of media on embryo induction were analyzed. Highest embryo induction rate (8.0%) was observed when anthers were cultured on agar medium with 0.1 mg/L BA and 0.1 mg/L NAA after incubation in liquid MS medium with 2.0% sucrose for 7 days at 35ºC. These results are supported from the results of Rimberia *et al.* (2005). In their experiments, highest embryo induction rate (rate of anthers forming embryos) was 4.2% on agar medium with 0.1 mg/L BA and 0.1 mg/L NAA after pre-treating anthers in water for 1 day at 35ºC. The results of the present study indicated that the pre-treatment of anthers in liquid medium 1-7 days was effective for embryo induction, and that the pre-treatment at 35ºC tends to be more efficient for embryo induction than that at 25ºC. Therefore, it seems that liquid medium and high temperature are important for inducing embryos from anthers. At these both temperatures longer incubation of anthers in water reduced embryo induction rate. The pre-treatment of anthers at low and high temperatures is also effective for embryo induction in anther culture of many plant species including cereals *e.g.* rice (Ying, 1986; Sopory and Munshi, 1996).

The effect of nutrients and sugar starvation on embryo induction during pre-treatment was ambiguous. Moreover, the starvation treatments are known to be efficient for embryo induction (Sangwan and Sangwan-Norreel, 1996; Rimberia *et al.*, 2005). In the present study, the

Fig. 1. *In vitro androgenesis in papaya (A) Papaya flower buds showing anthers (B) Embryogenic callus induction and shoot regeneration (C) Shoots ready for rooting (D) Hardening of rooted plants in hardening chamber (F) Acclimatized plants transferred to vermiculate pots.*

effects of IBA on rooting were also observed. The results showed that 2.0 mg/L IBA gave highest rooting (75.0%). Increasing in IBA concentration reduced rooting percentage. Callus formation was also observed through the basal ends of plantlets on basal ½-MS media and with slightly lower as well as higher IBA concentrations. The rooting results were closer to those of reported by earlier workers in papaya (Rajeevan and Pandey, 1983 and 1986; Drew, 1988; Mondal *et al.,* 1990; Kataoka and Inoue, 1991; Rimberia *et al.*, 2005). However, Wilna (1988) reported that, 4.0 mg/L of IBA was more effective on *in vitro* rooting of papaya. On the other hand, Litz and Conover (1978) found that NAA at 1.0 mg/L concentration was best for papaya *in vitro* rooting. In this study, after hardening 30 plants were successfully transplanted to the field.

Conclusion

In the present study, it was concluded that higher temperature was more suitable for embryo induction in papaya than low temperature. At these temperatures longer incubation of anthers in water reduced embryo induction rate in anthers. Sugar starvation results were ambiguous. Shoots were also developed on the above media while used in liquid form. Best rooting (75.0%) was observed at 2.0 mg/L IBA. Increasing in IBA concentration adversely affected rooting in shoots. All well rooted plants were hardened and successfully transferred to field. In this study, the embryo induction rate was generally low, and a few embryos developed directly into plantlets but most of them produced multiple embryos or calli. To overcome these problems, there is need to established optimal cultural conditions for normal development and acclimatization of plantlets, *i.e.* many plantlets were also died during these processes. For the development of an efficient haploid production system in papaya, the standardization of *in vitro* cultural conditions and their effects on normal embryo induction is an essential but critical step. Therefore, the present study would be helpful to the researchers to develop more efficient anther culture protocols for further improvement of papaya through *in vitro* androgenesis.

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