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Somatic Embryogenesis and Plantlet Regeneration from Immature Embryos of Papaya (*Carica papaya* L.)

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Keywords: Papaya, Somatic embryogenesis, Immature embryos, Plantlet regeneration

Abstract

The experiment was carried out in the Laboratory of the Biotechnology Division, Bangladesh Agricultural Research Institute (BARI) to find out embryogenic responses and plantlets regeneration from immature embryos of papaya. The explants were inoculated on Murashige and Skoog (MS) medium supplemented with 0, 36, 45, 54 & 63 μM 2, 4-D and incubated in complete darkness at 25°C ± 1°C. Calli were obtained within 3 weeks of culture period. The highest percentage of explants (46.67) produced callus using 2, 4-D at a concentration of 45 μM. Calli were then transferred to somatic embryogenesis medium where embryogenic calli were developed, turned into greenish color and these somatic embryos were noticed within 10 days. The highest number of somatic embryos (86.33) was produced per explant on MS containing 54 μM 2, 4-D. Somatic embryos were regenerated on MS supplemented with different concentrations and combinations of BAP and NAA including GA_3. The maximum regeneration percentage of somatic embryos (intact rooted plantlets) (54.17) was recorded in the medium supplemented with 0.4 μM each of BAP and NAA in combination with 10 μM GA_3. The somatic embryo-derived plantlets were successfully established in small plastic pots with prepared soil and finally transplanted in the field. The plants were grown well and produced fruit under their natural environment.

Introduction

Papaya (*Carica papaya* L.) is a member of the family Caricaceae. It is an important fruit of the tropics and subtropics which is used for consumption as a fresh fruit, used in drinks, jams, candies and as dried or crystallised fruit (Villegas 1997). Green fruit is also used as culinary preparations (Malabadi et al. 2011). Nutritionally, papaya is a good source of calcium and an excellent source of vitamins A and C (Nakasone and Paull 1998). Papaya

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leaf juice has been used for treatment of malaria and dengue (Ahmad et al. 2011). The propagation of papaya through seeds causes considerable variability in a commercial population (Litz and Conover 1978). Papaya production is also affected by a range of diseases but the most important is caused by papaya ring spot virus (PRSV) (Mahon et al. 1996), which has become the limiting factor for commercial papaya production in many areas of the world (Nakasone and Paull 1998). Globally, papaya ringspot disease (PRSD) is the most devastating disease affecting papaya production in almost every region where it is grown, which is a major constraint for the papaya industry (Premchand et al. 2023). Development of resistant varieties through hybridization is very difficult due to lack of natural sources of resistance. Improvement of papaya through genetic transformation is one of the important tools which was otherwise difficult by conventional breeding techniques. But success in gene transfer techniques requires an efficient regeneration of plants from *in vitro* culture.

Previously, considerable interest has been made towards genetic improvement by using *in vitro* techniques, such as, protoplast culture (Chen and Chen 1992), somatic embryogenesis (Chen et al. 1987, Yamamoto and Tabata 1989, Manshardt and Wenslaff 1989a & 1989b, Fitch and Manshardt 1990, Chen et al. 1991, Fitch 1993, Fernando et al. 2001, Yu et al. 2001, Minh & Thu 2001) and plant transformation using *Agrobacterium* or microprojectile bombardment (Fitch et al. 1990, Yang et al. 1996, Mahon et al. 1996, Cheng et al. 1996, Cabrera-Ponce et al. 1996, Manshardt 1999, Magdalita et al. 2000, Ferreira et al. 2002). However, transformation frequency is strongly influenced by the way shoots are regenerated *in vitro* (Mukhopadhyay et al. 1992, Detrez et al. 1994). Somatic embryos are good source materials for genetic transformation. It acts as a powerful tool for genetic improvement of any plant species because of its single cell origin (Bhansali 1990, Jha et al. 2007) as well as the somatic cell containing genetic information to form complete and fertile plants (Merkle et al. 1987).

Several reports are available on somatic embryogenesis of *Carica papaya* L. on varieties, like Sunset, Kapoho, Honey Dew, Solo, Sunrise etc. which are not commonly cultivated in Bangladesh. The frequency of responses to somatic embryogenesis within a species may vary considerably from one genotype to another (Chaudhary and Prakash 2019). Therefore, the study was undertaken to develop an efficient plant regeneration system via somatic embryogenesis for further genetic improvement of papaya.

Materials and Methods

The experiment was conducted in the Laboratory of Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur. Local papaya varieties were selected from Bangladesh Agricultural Research Institute Campus. The selection criteria were based on plant height, fruit size, yield, fruit quality and sex of mother plant. Green good quality papayas were collected, and immature seeds were taken from that fruit. The seeds were washed with running tap water with jet and trix for 30 min. and the seeds

were put in Rifampicin (300 mg/l) solution and shaken. After that seeds were washed 2-3 times with sterilized distilled water. Then the seeds were sterilized with 60% Clorox solution with two drops of Tween-20 for 20 min. and washed 3-4 times with sterilized distilled water. The embryos were isolated in a laminar air flow cabinet which was used as explants for callus induction.

These explants were inoculated in MS basal medium with 3% sucrose and 0.8% agar. The medium for callus formation included different concentrations of 2, 4-D at 0, 36, 45, 54, 63 μM. Somatic embryogenesis media included 0.1 μM α-naphthaleneacetic acid (NAA) and 0.9 μM BAP (Yu et al. 2001). The medium for regeneration of somatic embryos was supplemented with T_0 (control), T_1 (0.2 μM each of BAP and NAA + 10 μM GA_3), T_2 (0.3 μM each of BAP and NAA + 10 μM GA_3), T_3 (0.4 μM each of BAP and NAA + 10 μM GA_3), T_4 (0.5 μM each of BAP and NAA + 10 μM GA_3). The pH of all the media was adjusted to 5.8 before autoclaving. The media were autoclaved at 121°C and 1.06 kg cm^{-2} for 20 min.

All initial cultures were incubated in complete darkness at 25 ± 1°C for callus induction. After callus formation, they were cultured on somatic embryogenesis medium and kept in 16/8 hours light/dark photoperiod with a light intensity of 2000 lux under cool white, fluorescent tubes. After 20 days they were again transferred to embryo regeneration medium. The well-developed embryo-derived plantlets with good rooting were removed from the culture vessels/tubes and all the adhering media were washed carefully so that the root damage was the least. The plantlets were then planted into small plastic pots containing sand, soil and decomposed cow dung at the ratio of 1:1:1 and covered with plastic bags for one or two weeks. The bags were perforated, and the holes were gradually enlarged to acclimate the plantlets to normal net house conditions. The plantlets were maintained in the net house with proper care and watered whenever necessary. Experiments were carried out in a completely randomized design with three replications. Data collected were subjected to analysis of variance and means were separated by DMRT using MSTATC statistical program.

Results and Discussion

Embryogenic callus formation, development of somatic embryos and plantlets regeneration are shown in Figs 1-3. Calli were noticed within 3 weeks after induction with 2, 4-D. Significant differences were observed in different treatments of 2, 4-D. The highest percentage of explants produced callus (46.67) in 45 μM 2, 4-D concentration followed by 54 μM 2, 4-D concentration (37.67%). The lowest percentage of callus (15) was observed in control treatment. On the other hand, very good quality callus was found in 54 μM and 63 μM 2, 4-D concentrations (Table 1).

Calli were then transferred to somatic embryogenesis medium (0.1 μM NAA and 0.9 μM BAP). In this medium, embryogenic calli were developed and turned into greenish color and somatic embryos were noticed within 10 days (Fig. 3a). In the present study, it

has been observed that from callus initiation to embryo formation, it took seven weeks. Chaudhary and Prakash (2019) also reported similar observations in their study. They found embryos within six weeks of culture. This is due to varietal difference. High frequency somatic embryogenesis was reported previously in different types of papaya tissues. Somatic embryos were appeared after three to six weeks using immature zygotic embryos (Fitch and Manshardt 1990). On the other hand, somatic embryos were appeared ten to fourteen weeks on hypocotyls sections (Fitch 1993), three months on root explants (Chen et al. 1987) and seven weeks in mature zygotic embryos (Fernando et al. 2001). The present study supported the result of Fernando et al. 2001).

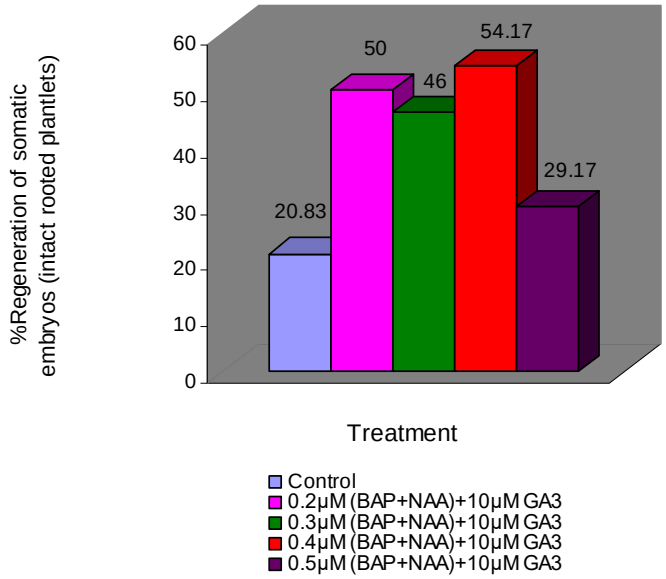

Fig.1. Regeneration percentage of somatic embryos (intact rooted plantlets) in different treatment combination

A significant difference was observed in the number of somatic embryos per explants. The highest number of somatic embryos per explant (86.33) was produced when explant was induced with 54 µM 2, 4-D concentration. On the other hand, lowest number of somatic embryos per explant (14.67) was recorded from 36 µM 2, 4-D concentration. Results showed that in control treatment, no embryo was formed although they were produced some calli (Table 1). Result also showed that explant induced with 54 µM 2, 4-D contained more somatic embryo clusters (Fig. 3b). Above that level, fewer embryos were observed (Table 1). In the present study, it was observed that 54 µM 2, 4-D concentration is optimum for embryogenic callus growth and somatic embryo formation. In general, high frequencies of somatic embryogenesis induction are obtained in response to the auxins (NAA) and 2, 4-D. Minh and Thu (2001) reported good somatic embryogenesis and high embryogenic cell multiplication rate using auxin (2, 4-D) and

NAA. Yamamoto and Tabata (1989) also used 2, 4-D for inducing papaya hypocotyl somatic embryos. Furthermore, 2, 4-D was also important for inducing embryogenesis in immature zygotic embryos (Fitch and Manshardt 1990). On the other hand, the shorter period of time that cultures are exposed to 2, 4-D may help decrease the incidence of abnormalities that

Table 1. Embryogenic response of papaya in different concentrations of 2, 4-D.

Concentration (μM)	Percent of explant producing callus	Number of embryo/explant	Quality of callus
MS + 0.0 2, 4-D	15.00 c	0.00 e	+
MS + 36.0 2, 4-D	24.67 c	14.67 d	+
MS + 45.0 2, 4-D	46.67 a	23.00 c	++
MS + 54.0 2, 4-D	37.67 b	86.33 a	+++
MS + 63.0 2, 4-D	33.33 b	40.00 b	+++
CV%	18.99	9.89	
LSD (0.05)	11.25	6.11	

Means bearing same letters do not differ significantly at 5% level of probability + poor, ++ good, +++ very good.

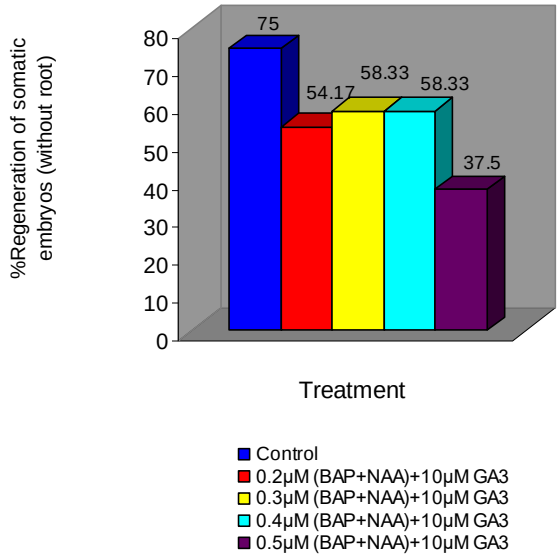

Fig. 2. Regeneration percentage of somatic embryos (without root) in different treatment combination.

could arise from 2, 4-D treatment. Therefore, embryogenic calli should be removed from 2, 4-D as soon as they are observed. Studies on the morphological aspects of somatic embryogenesis have been performed in several species and showed that somatic

embryos can have different origins according to the species and the explant type. For example, in geranium and tomato somatic embryos are formed from the epidermal cells of hypocotyl explants. In eggplant, somatic embryos induced by NAA are formed from perivascular parenchymatic cells originating indeterminate meristematic masses, which can either give rise to adventitious roots or pro-embryogenic masses (Magioli and Mansur 2005). Somatic embryos were originated from the superficial cells of the pre-embryonic complexes located in the peripheral and internal regions of the callus (Fernando et al. 2001).

When the embryos were transferred to regeneration medium, plantlets were developed *in vitro* (Fig. 3c & d). Results indicated that in regeneration media, some somatic embryos were regenerated intact rooted plantlets and some regenerated without root. The highest regeneration percentage of somatic embryo (intact rooted plantlets (54.17) was recorded in 0.4 μM each of BAP and NAA including 10 μM GA3 followed by 0.2 μM each of BAP and NAA including 10 μM GA3 and 0.5 μM each of BAP and NAA including 10 μM GA3 (50%). On the other hand, the lowest intact rooted somatic embryo regeneration percentage (20.83) was observed in control treatment (Fig.1). Furthermore, the maximum regeneration percentage of somatic embryos (without rooted plantlets) was recorded from control treatment (75) followed by 0.3 μM each of BAP and NAA including 10 μM GA3 and 0.4 μM each of BAP and NAA including 10 μM GA3 (58.33%). The lowest regeneration percentage (37.5) of somatic embryos (without root) was recorded from 0.5 μM each of BAP and NAA including 10 μM GA3 (Fig. 2). Although there were no significant differences among the regeneration treatment combination. The present study supported with the result of Magdalita et al. (2000) where they used the same hormonal combinations for somatic embryo regeneration. *In vitro* germination of somatic embryos is controlled by exogenous auxin (0.5-11.0 μM NAA) and cytokinin (0.2-0.9 μM BA). Higher concentrations of cytokinin caused excessive swelling of the hypocotyl and inhibited shoot and tri-lobed leaf development. Higher auxin concentrations also inhibited hypocotyl development as well as causing excessive callus formation form the surface of the germinating embryos. In this study, it was also observed that among the somatic embryos, some abnormal embryos were produced (Fig. 3e) where aberrant cotyledons were noticed and shoot apex became stunted. Result showed that about 14-17% abnormal somatic embryos were found among the regeneration treatments. But at higher rate was observed in higher concentrations of auxin and cytokinin induced treatments. For example, in 0.5 μM each of BAP and NAA including GA3 treated medium produced 17% abnormal somatic embryos. Similar observations were reported by Fernando et al. (2001) and Yu et al. (2001). Fernando et al. (2001) showed that cotyledonary somatic embryos without shoot apex, cylindrical somatic embryos without shoot apex and somatic embryos with multiple foliar structures. Yu et al. (2001) reported aberrant cotyledons and shoot apex disappeared or became stunted.

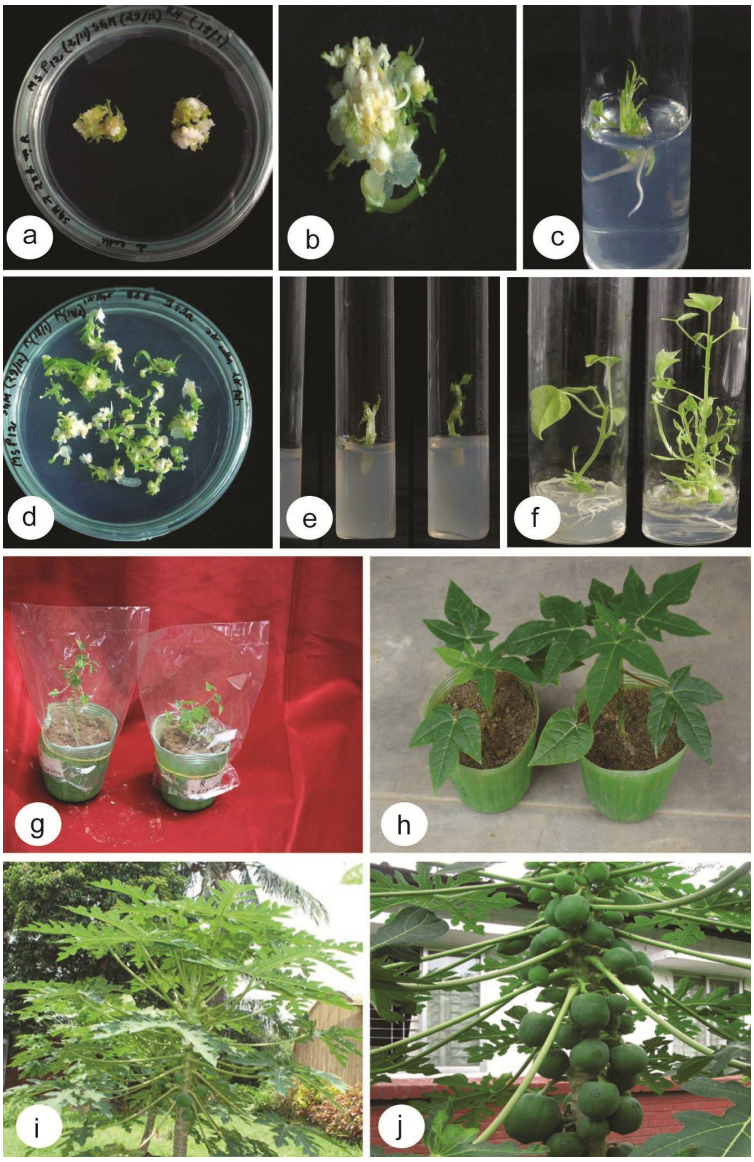

Fig. 3. Somatic embryogenesis in papaya: Somatic embryos derived from callus in the medium treated with 54µM 2,4-D after culturing 11 weeks (a) and 12 weeks (b). Regenerated somatic embryos on MS medium supplemented with 0.1µM NAA and 0.9µM BAP (c) and (d). Regenerated abnormal somatic embryos on MS medium containing 0.5µM each of BAP and NAA + 10µM GA3 which were noted aberrant cotyledons and shoot apex became stunted (e). Well rooted somatic embryo derived plantlets on MS medium supplemented with 0.4µM each of BAP and NAA + 10µM GA3 (f). Hardening of plantlets in *ex vitro* condition (g). Established plantlets in pot (h). Established plant in the field (one year old) (i). Established plant with fruit (one year and one month old) (j).

The well-developed embryo-derived plantlets with good rooting (Fig. 3f) in test tubes were kept at room temperature from three to five days for hardening. They were then planted in prepared potting soil and about 50% of the plantlets survived. After one and half months they were again transferred to bigger pots with prepared soil and kept in net house with proper management. There the plants grew normally and were found well established within three months (Fig. 3h). The plantlets were ready for planting in the field. They were then transplanted in the field in front of Biotechnology Division, Bangladesh Agricultural Research Institute (BARI), Gazipur. The somatic embryo derived plants were successfully established in field, and they were grown well (Fig. 3i). The plants were produced flower within three months from planting in the field and beared fruit within three months after flowering. A good number of fruits were produced, and the size of individual fruit was also acceptable (Fig. 3j).

Results from the study suggested that the 2, 4-D plays an important role in somatic embryogenesis and 54 μM 2, 4-D concentration is optimum for somatic embryo formation. The germination of somatic embryos and plantlet formation was enhanced significantly when the 2, 4-D treated calli transferred to MS medium fortified with BAP, NAA and GA_3. The treatment combination with 0.4 μM BAP + 0.4 μM NAA + 10 μM GA_3 produced the highest number of regenerated plantlets. The results also suggested that the somatic embryogenesis protocol can play an important role for the development of disease resistant transgenic papaya variety(s) in future. This is the first study in Bangladesh that the somatic embryogenesis in papaya has been included all the steps of regeneration from explant inoculation in the medium, regeneration of plantlets *in vitro* and *ex vitro* survivability up to the field establishment of crop which was not done earlier.

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