Journal of Applied Horticulture, 11(1): 50-53, January-June, 2009

Effect of growth regulators on *in vitro* plant regeneration of female papaya using axillary bud as an explant

Renu Singh, Ram C. Yadav* and Neelam R. Yadav

Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar. 125004, India
**E-mail*: *renuboora@gmail.com*

Abstract

A study was carried out on mature female papaya (*Carica papaya* L.) plant of Selection 1 cultivar by using axillary bud as an explant and media supplementation with the main aim to assess the effect of growth regulators (auxins, cytokinins) and silver nitrate on *in vitro* regeneration of female papaya plant. Total of 28 media were used for shoot regeneration while for root regeneration total of eight media were tested supplemented with different growth hormones. Based on the results of this study, for shoot proliferation, MS basal medium supplemented with BAP (1.0 mg L^{-1}) and BAP (2.0 mg L^{-1}) + NAA (0.1 mg L^{-1}) was found to give the best results while MS medium supplemented with IBA (2.0 mg L^{-1}) gave best rooting percentage. Besides, auxins and cytokinins, effect of silver nitrate ($AgNO_3$) on plant regeneration from axillary buds taken from mature female papaya plant was also carried out.

Key words: Auxins, axillary bud, benzylaminopurine (BAP), cytokinins, indole acetic acid (IAA), *Carica papaya* L., α-naphthalene acetic acid (NAA), proliferation, silver nitrate ($AgNO_3$)

Introduction

Papaya (*Carica papaya* L.) is a native of tropical America and belongs to family *Caricacea*. The plant starts fruiting in just one year and gives economically high yield per acre next to banana. The total area under papaya cultivation in India was 57,000 ha (FAO, 2000). Papaya is very nutritious and has much therapeutic value. The fruit contains fair amount of vitamin A, B_1, B_{12} and C. The tremendous yielding potential of this crop is left economically unexploited due to several problems associated with its cultivation. The main problem associated is lack of clonal multiplication methods and in tissue culture bacterial contaminants are the limiting factors (Litz and Conover, 1981).

It is well known that vegetative propagation of papaya through conventional methods has not been successful with commercial viability. Thus, there is ample scope to overcome the above limitations through tissue culture. The plant tissue culture technique has been successfully employed in the regeneration of various fruit crops like almonds, strawberry, citrus, etc (Bajaj, 1986). However, papaya is a problematic crop for micropropagation from grown up plants as it contains a good amount of latex interfering in proliferation. Therefore, the present study was undertaken with a view to evolve a tissue culture technique in papaya and to evaluate the effect of different growth regulators on the vegetative regeneration of female papaya plant using axillary buds.

Materials and methods

The present investigation was carried out in Tissue Culture Laboratory of the Department of Biotechnology and Molecular Biology, CCS Haryana Agricultural University, Hisar, India. Axillary buds as explants were excised from the field grown sexually differentiated female plants. The chemicals of high purity were used throughout the course of investigation. The excised explants *i.e.* axillary buds were cut (6 mm) and washed with single glass distilled water containing few drops of teepol. The explants were then treated with 0.5 per cent Bavistin (fungicide) for 45 minutes. The disinfections steps after bavistin treatment were carried out in the horizontal laminar flow cabinet. Explants were further rinsed 4-5 times with distilled water followed by 70 per cent alcohol treatment for 30 seconds and by 50 per cent sodium hypochlorite for 3 minutes. After this treatment, they were rinsed 4-5 times with double distilled water finally, given 0.1% $HgCl_2$ treatment for 4 minutes and then the explants were again rinsed 5-6 times with autoclaved double distilled water. In the present study, MS basal medium supplemented with different auxins and cytokinins were initially tried for culture establishment (Murashige and Skoog, 1962). The medium supplemented with kinetin, NAA, BAP and $AgNO_3$ giving better response were further used for regeneration and those which don't gave better regeneration were discarded in the initial of the experiment. All the cultures were maintained at 25 ± 1°C under 16/8 hours cycles of light (light intensity 50 µmol m^{-2} s^{-1}) and dark. The regenerated shoots were further cultured on regeneration medium with variable concentration of growth hormones. The multiple shoots formed were then transferred to rooting media in order to get root formation. The data was recorded time to time and presented as the mean of three repeats. Data were analyzed statistically using one-way analysis of variance (ANOVA).

Results and discussion

In general, poor response for shoot regeneration on all the media was observed when axillary bud was used as explant (Fig. 1A). Maximum (70.1%) response was observed on medium supplemented with BAP (Table 1) while very poor response was observed on the media supplemented with kinetin (Table 2). No shoot regeneration was observed on most of the Media supplemented with kinetin.

Maximum (70.1%) response (Fig. 1B) was observed on medium supplemented with and moderate response was observed on MS supplemented with BAP (2.0) + NAA (0.1), BAP (1.0) + IAA (0.2) and BAP (2.0) + NAA (0.5) (65.0, 51.1 and 45.8%, respectively), while rest of media gave poor response (Table 1). In case of kinetin maximum shoot regeneration (21.4%) response was observed on medium supplemented with Kinetin (2.5) and minimum response was observed on medium supplemented with Kinetin (5.0) + NAA (0.2) (11.7%).

When $AgNO_3$ was added in the media (Table 3) supplemented with other growth hormones, the regeneration response was observed maximum (59.0%) on MS medium supplemented with BAP (1.0) + $AgNO_3$ (2.0) + Zeatin (3.0). Moderate (50.0%) regeneration response was observed on MS + BAP (2.0) + $AgNO_3$ (2.0) + Zeatin (2.5) medium, while MS medium supplemented with $AgNO_3$ (2.0) and $AgNO_3$ (1.0) gave comparatively poor response (24.8 and 13.2%, respectively). No response was observed on MS medium without any growth regulators.

The regenerated plantlets from micropropagation experiments grown *in vitro* were transferred to different rooting media for carrying out their rooting *in vitro* (Fig. 1C). Roots formed were thick and strong (Fig. 1D). No response was observed when medium was devoid of any auxin. Maximum rooting frequency (67.2%) was observed on MS medium supplemented with IBA (2.0); however, it was minimum (22.2 and 27.8%) on medium supplemented with IBA (0.1) and IBA (0.5), respectively (Table 4).

In vitro micropropagation has been successfully used for many horticultural fruit trees (Das *et al.*, 1996). Multiple shoot production from axillary buds obtained from mature trees is now recognized as a better alternative of micropropagation in fruit trees where fidelity of the propagules is of prime importance (Quarashi and Mitra, 1998; Tavares *et al.*, 1996).

The overall conclusion from the above regeneration experiment emerged that the media comprised of MS basal salt + BAP (2.0 mg L^{-1}) + NAA (0.1 mg L^{-1}) gave the best regeneration results in *C. papaya*. As outlined by Skoog and Miller (1957), root and shoot initiation is basically regulated by the interaction between auxins and cytokinins. This combination of both the hormones was equally effective when regeneration was done with the shoot tips therefore, we can conclude that a proper ratio of cytokinins and auxins helps in both the shoot and root formation from different explants. Axillary buds were associated with relative high level of endogenous growth substances in juvenile tissues in comparison to the adult tissues. This implies the poor regeneration of axillary buds from mature plants than the explants taken from the juvenile plant. The un-branched character of the papaya plant also offers a serious limitation in using axillary buds as explants in the experiment.

Effect of cytokinins: The most striking influence on bud-break and shoot multiplication has been found with the use of auxins and cytokinins (Normanly, 1995). The most commonly used cytokinins are BAP, kinetin, 2ip and zeatin, the latter two being natural cytokinin. Superiority of BAP over other cytokinins has been reported and discussed in relation to shoot proliferation in culture of trees (Bonga and Jvon Aderkas, 1992). In the present study, lower level (1.0 mg L^{-1}) of BAP and of kinetin (2.5 mg L^{-1}) induced highest frequency of shoot regeneration. Regeneration response of BAP was superior over kinetin by taking less time for induction of shoot regeneration. In the present study, highest frequency of shoot regeneration and axillary bud proliferation could be achieved on BAP (1.0 mg L^{-1}) without the need of subculture. BAP is reported to have favoured axillary shoot proliferation in several tree species (Eeswara, 1998; Purohit and Dave, 1996; Purohit and Singhvi, 1998). In the present study, it was observed that an increase in the level of cytokinin from 1.0 to 5.0 mg L^{-1} produced a negative effect on all the parameters except shoot number (Tables 1, 2).

Table 1. Shoot induction response from axillary bud explants on media supplemented with BAP in papaya

Medium Composition (mg L^{-1})	Average number of cultured explants	Average number of responding explants	Mean (%) response (Mean ±S.E.)*
MSP	50	0	00.0 (04.05 ± 0.01)
MS + BAP (1.0)	100	70	70.1 (57.14 ± 0.06)
MS + BAP (2.5)	70	24	34.4 (36.14 ± 0.31)
MS + BAP (5.0)	74	19	25.4 (30.56 ± 1.10)
MS + BAP (1.0) + IAA (0.2)	74	38	51.1 (45.90 ±0.63)
MS + BAP (2.5) + IAA (0.2)	80	15	18.7 (26.01 ± 0.70)
MS + BAP (5.0) + IAA (0.2)	80	10	12.4 (21.01 ± 0.25)
MS + BAP (2.0) + NAA (0.5)	100	46	45.8 (42.88 ± 0.74)
MS + BAP (2.0) + NAA (0.1)	100	65	65.0 (53.99 ± 0.22)
MS + BAP (1.0) + NAA (0.2)	58	12	20.6 (27.32 ± 0.75)
MS + BAP (2.5) + NAA (0.2)	56	10	17.8 (25.32 ± 0.15)
MS + BAP (5.0) + NAA (0.2)	78	8	10.2 (19.04 ± 0.50)

LSD (P=0.05)=1.641, *Transformed value

Table 2. Shoot induction response from axillary bud explants on media supplemented with kinetin in papaya

Medium Composition (mg L^{-1})	Average number of cultured explants	Average number of responding explants	Mean (%) response (Mean ±S.E.)*
MSP	50	00	00.0 (04.05 ± 0.01)
MS + Kin (1.0)	54	00	00.0 (04.05 ± 0.01)
MS + Kin (2.5)	74	16	21.4 (27.91 ± 0.53)
MS + Kin (5.0)	58	10	16.9 (24. 61 ± 0.68)
MS + Kin (1.0) + IAA (0.2)	84	00	00.0 (04.05 ± 0.01)
MS + Kin (2.5) + IAA (0.2)	48	00	00.0 (04.05 ± 0.01)
MS + Kin (5.0) + IAA (0.2)	48	00	0.00 (04.05 ± 0.01)
MS + Kin (1.0) + NAA (0.2)	60	00	00.0 (04.05 ± 0.01)
MS + Kin (2.5) + NAA (0.2)	80	14	17.5 (25.06 ± 0.55)
MS + Kin (5.0) + NAA (0.2)	84	10	11.7 (21.07 ± 1.24)
MS + Kin (0.5) + 2,4-D (2.5)	74	00	00.0 (04.05 ± 0.01)
MS + Kin (1.0) + 2,4-D (2.5)	100	13	12.0 (20.67 ± 0.28)

LSD (P=0.05)=1.379, *Transformed value

Table 3. Shoot induction response from axillary bud explants on media supplemented with $AgNO_3$ in papaya

Medium composition (mg L^{-1})	Average number of cultured explants	Average number of responding explants	Mean (%) response (Mean ±S.E.)*
MS	60	0	00.0 (04.05 ± 0.01)
MS + $AgNO_3$ (2.0)	120	30	24.8 (30.16 ± 0.52)
MS + $AgNO_3$ (1.0)	106	14	13.2 (21.71 ± 0.32)
MS + BAP (1.0) + $AgNO_3$ (2.0) + Zeatin (3.0)	100	59	59.0 (50. 48 ± 0.29)
MS + BAP (2.0) + $AgNO_3$ (2.0) + Zeatin (2.5)	100	50	50.0 (45.27 ± 0.01)

LSD (*P*=0.05)=1.120, *Transformed

Effect of cytokinins (BAP and kinetin) in combination with auxins (NAA): In the present study combined effect of cytokinin and auxin was promotive on shoot regeneration from nodal explant, however, an inhibitory effect was observed in the combination where levels of cytokinins were high (5.0 mg L^{-1}). Auxins (NAA, 2, 4-D and IAA) were also examined for their influence on multiple shoot production and growth of papaya axillary bud cultures. Auxins were found to affect only the growth of the cultures rather than proliferation rate. NAA (0.1-0.2 mg L^{-1}) resulted in better growth of the cultures and hence included in the medium. Similar response by shoot and axillary bud cultures with regard to auxin application were reported in papaya (Buriken *et al.*, 1988; Renveni, 1990). As a promotive effect on shoot regeneration in combination of BAP/kinetin with NAA, maximum shoot regeneration frequency in axillary bud explant (65.0%) was observed on basal medium with BAP (2.0 mg L^{-1}) and NAA (0.1 mg L^{-1}) while kinetin was found to have less promotive effect in combination with NAA. The complementary effect of cytokinin and auxin has been observed by Miller and Drew (1990) during shoot proliferation in papaya. Highest shoot production was achieved on medium containing BAP (2 µM) and NAA (0.5 µM). A combination of BAP (2.0 mg L^{-1}) and NAA (1.0 mg L^{-1}) was found to enhance shoot bud proliferation in cultured shoot tip in papaya (Litz and Conover, 1981). Combination of cytokinin and auxin thus has been found to promote shoot bud proliferation effectively.

Effect of auxins: Various auxins were tried with the objective to induce roots in shoots. In this study, medium containing MS basal + IBA (2.0 mg L^{-1}) was found most appropriate for root induction followed by MS basal + IBA (1.0 mg L^{-1}). However, the induction of rooting in the regenerated shoots of papaya was found to be very difficult. Plantlets produced with IBA were normal with regard to the shoot and root growth. IBA has been found to be effective for root induction in papaya-regenerated shoots by Bhattacharya *et al.*, (2002), Buriken *et al.*, (1988), Fitch (1993), Rajeevan and Pandey (1983), Singh *et al.*, (2000). Although IAA also induced root formation but the root development was poor as compared to IBA. The strength of the basic medium employed is reported to influence root initiation in several papaya cultivars. However, better root induction has been obtained in papaya with half strength MS medium (Renveni *et al.*, 1990).

Table 4. Root induction response in shoots regenerated from axillary bud explant on various rooting media in papaya

IAA (mg L^{-1})	NAA (mg L^{-1})	IBA (mg L^{-1})	Number of cultured explants	Number of responding explants	Mean (%) response (Mean ±S.E.)*
-	-	-	6	00	00.0 (04.05 ± 0.01)
2.0	-	-	7	00	00.0 (04.05 ± 0.01)
-	-	0.1	12	2	15.0 (20.43 ± 8.25)
-	-	0.5	9	2	19.4 (23.30 ± 9.74)
-	-	1.0	9	4	44.4 (42.02 ± 3.25)
-	-	2.0	11	7	63.9 (52.46 ± 3.65)
-	0.5	0.1	10	3	30.5 (33.79 ± 1.74)
-	0.5	0.5	9	3	33.3 (35.53 ± 0.01)

LSD (*P*=0.05)=1.431, *Transformed value

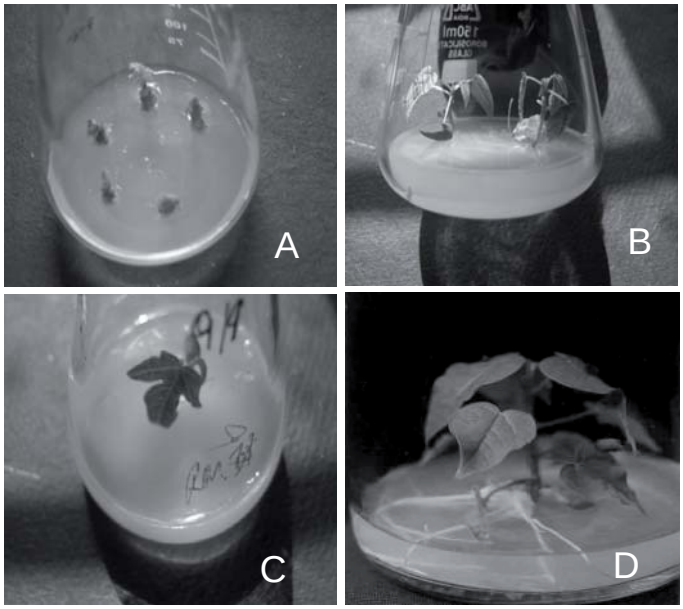

Fig. 1. A. Nodal explants cultured on regeneration medium; B. Multiple shoot formation from axillary explants; C. Regenerated shoot on rooting medium; D. Regenerated shoots with roots on rooting medium

Effect of silver nitrate ($AgNO_3$): $AgNO_3$ is an ethylene inhibitor in plants which is reported to help in somatic embryogenesis (Songstad *et al.*, 1991). $AgNO_3$ may also serve as stress agent inducing endogenous ABA accumulation, Ag+ being a metallic ion may also promote somatic embryo production via an increase in the endogenous ABA levels (Kong and Yeung, 1994, 1995). But in our experiment when $AgNO_3$ was added in the media (Table 3) supplemented with other growth hormones, the regeneration response was observed maximum (59.0%) on MS supplemented with BAP (1.0) + $AgNO_2$ (2.0) + Zeatin (3.0). Thus, overall in conclusion media containing $AgNO_3$ has no promotive effect on shoot formation.

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