



Differential Influence of Growth Regulators during Somatic Embryogenesis of Gynodioecious Papaya Varieties 'CO.7' and 'Red Lady'

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Authors' contributions

This work was done in collaboration among all authors. Authors KS and KKK designed the study. Author CKR performed the experiment, statistical analysis and wrote the first draft of the manuscript. Authors KS, KKK, CK and GK supervised the study. Authors KS, KKK and CK corrected the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

The study involved two auxins viz., 2,4-D (2,4-Dichlorophenoxyacetic acid) and picloram at three different concentrations (1,2, 3 mg/L) in full strength MS media to study their comparative influence on induction of somatic embryogenesis from immature zygotic embryos of two gynodioecious varieties of papaya 'CO.7' and 'Red Lady'. In papaya cultivar 'CO.7', 2,4-D at 2 mg/L gave the highest callus induction frequency of 90.93%, whereas comparatively higher concentration of 3 mg/L 2,4-D was found suitable for 'Red Lady' (87.26%). Although there was profuse callus formation, 2

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mg/L 2,4-D recorded comparatively higher frequency of embryogenic calli in 'Red Lady' (51.67%) when compared to 'CO.7' (30.00%). Somatic embryo maturation was achieved upon transfer of embryogenic calli exhibiting globular stage embryos on to maturation medium (MS medium + ABA (Abscisic acid) and BAP (Benzyl amino purine) in different concentrations + glutamine 400 mg/L). In the maturation medium, the combination of 1.5 mg/L ABA and 0.4 mg/L BAP registered better conversion of the globular embryo to cotyledonary embryos than other levels. The frequency of somatic embryo germination was higher in 'Red Lady' (50.00%) as compared to 'CO.7' (31.67%) on half-strength MS medium devoid of growth regulators.

Keywords: Somatic embryogenesis; papaya; picloram; 2,4-Dichlorophenoxyacetic acid; CO.7; Red Lady; immature zygotic embryos.

1. INTRODUCTION

Papaya (*Carica papaya* L.) belongs to the family Caricaceae and it is commercially cultivated in the tropical regions of the world for its nutritionally rich fruits characterized by high carotene and vitamin C content [1]. Although papaya is cross-pollinated and highly heterozygous, it is commercially propagated through seeds. Unplanned seed propagation often results in higher genetic variability with further difficulties in maintaining genetic purity [2] as plants show variations for yield, fruit quality and susceptibility to pathogens [3,4]. Based on sex forms, papaya cultivars can be classified broadly as dioecious which segregate into male and female [5] when seedlings are raised and as gynodioecious varieties which normally segregate to female and bisexual sex forms. Since cultivation of dioecious varieties requires thinning of excess males during early growth phase, the gynodioecious varieties are cultivated in large acreages as compared to dioecious varieties. In the early 1980s and 1990s mainly the gynodioecious 'Solo' papayas which were introduced from USA were cultivated in India. Crop improvement efforts at TNAU resulted in the high yielding red pulped gynodioecious varieties 'CO.3' and 'CO.7'. Another gynodioecious introduction 'Red Lady' became popular among growers owing to its better skin traits which helped in long distance transport. It is also high yielding and productive (120-125 t/ha) with red pulp. All the gynodioecious varieties of papaya are highly susceptible to the viral disease caused by Papaya Ring Spot Virus (PRSV). Somatic embryogenesis offers wider scope to generate true to type clonal plants with very high multiplication rate and further it also offers scope for synthetic seed production and in genetic transformation studies to impart resistance to diseases such as PRSV. If successful somatic embryogenesis could be achieved, then genetic transformation could be attempted in these two varieties to impart PRSV resistance.

In papaya also, as an alternative to seed propagation, somatic embryogenesis have been attempted by many researchers [6,7,8]. Different papaya varieties respond differently when subjected to *in vitro* cultures. The maturity of explants also influences the response due to their endogenous hormonal and physiological status. So the protocols have to be standardized for both cv. 'CO.7' and 'Red Lady'. The present studies were therefore taken up.

Auxins are the essential growth regulators for initiation and continuous growth of callus [9]. Earlier reports showed that 2,4-D and picloram (a synthetic auxin) promotes callus induction and somatic embryogenesis in papaya [10,11,12,13]. However, after initiation further embryogenesis requires ABA, which acts as an ethylene antagonist during maturation of somatic embryos and enhances the quality of somatic embryos [11]. The present study was carried out to find out the effect of 2,4-D, picloram and ABA on somatic embryogenesis of papaya (*Carica papaya* L) in the two gynodioecious cultivars 'CO.7' and 'Red Lady'.

2. MATERIALS AND METHODS

The immature fruits (90 - 100 days aged) of the papaya cultivars 'CO.7' and 'Red Lady' were collected from the Orchard maintained by the Department of Fruit Science, Horticultural College and Research Institute, TNAU, Coimbatore (Plate.1a). The unripe fruits were washed rigorously with distilled water thrice, surface sterilized with 70% (v/v) ethanol for two minutes and soaked in 1.25% sodium hypochlorite (w/v) for an hour and then further washed thrice with sterile distilled water. The immature seeds were collected from unripe fruits under the laminar air-flow chamber (Plate.1b).

The collected seeds were initially washed with sterile distilled water, followed by distilled water

(added with 2-3 drops Tween 20 per 100 ml) for a minute and again washed with sterile distilled water to remove excess traces of surfactants. Then these seeds were surface sterilized using 70% (v/v) ethanol for 30 sec and 1.25% sodium hypochlorite (w/v) for 3 min and finally washed

thrice with sterile distilled water to remove the traces of chlorine. Immature zygotic embryos were excised under stereo-microscope (Zeiss Stemi DV4) by removing the sarcotesta layer of the seed and giving a gentle cut on the embryo sac with a sterilized blade and used as explants.

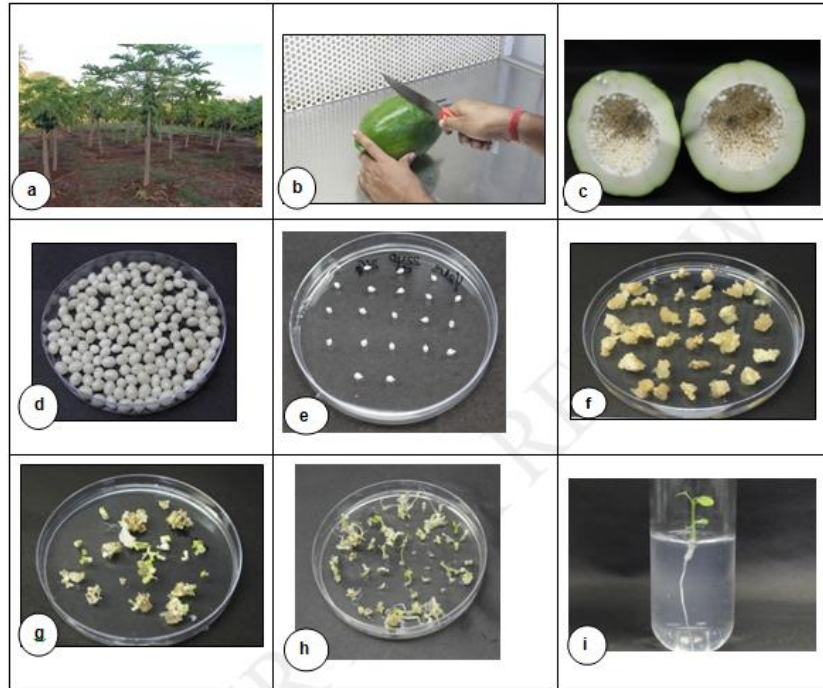


Plate. 1. Schematic representation of steps involved in somatic embryogenesis of papaya
a. Papaya field, b. 90-100 days old selfed fruit cutting in aseptic condition, c. Transverse section of fruit, d. Seeds isolated from immature fruit, e. Excised immature zygotic embryo, f. Callus formation after 45 days of culturing in CIM containing 2,4-D, g. Matured calli, h. Germination of cotyledonary embryos, i. Developed plantlet with root and shoot

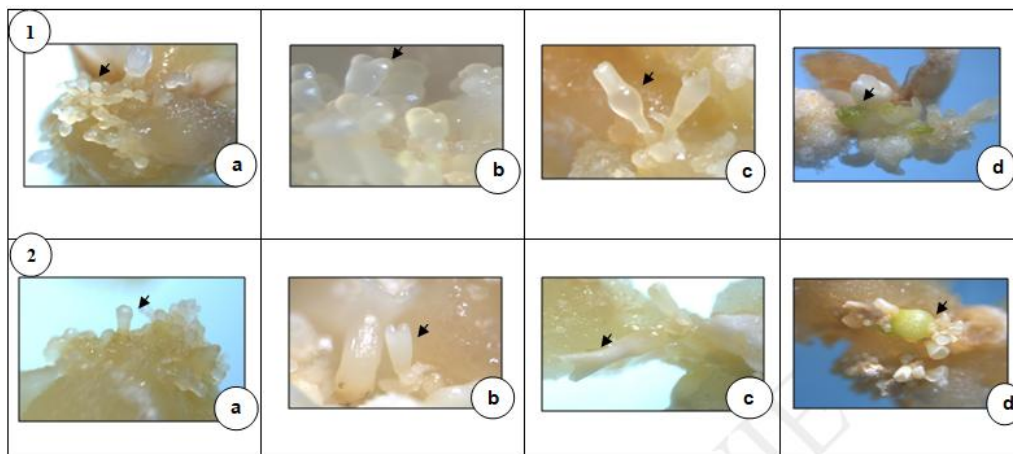


Plate. 2. Different stages of somatic embryo development in papaya cultivars 'CO.7' (1) and 'Red Lady' (2)
a. Globular stage, b. Heart stage, c. Torpedo stage and d. Cotyledonary stage

The Callus induction media (CIM) was formulated with full-strength MS [14] with 500 mg/L of proline, 400 mg/L of glutamine, 300 mg/L of casein hydrolysate, 30 g sucrose, 4 g/L of gelrite and supplemented with 2,4-D (1, 2, 3 mg/L) or picloram (1, 2, 3 mg/L). Before adding gelrite the pH was normalized to 5.8 using 0.1 N NaOH. The sterilization of media was done by autoclaving at 121°C for 15 min. 25 ml of medium was poured in each petri plate and allowed to solidify. Twenty immature zygotic embryos excised from the immature papaya seeds were inoculated on the CIM (Plate.1e), containing either 2,4-D or picloram. The culture plates were kept under darkness at 25 ± 2°C for 45 days to induce callus and sub-cultured on the same CIM at every fortnightly interval up to the somatic embryo induction. The percentage of embryogenesis was calculated by determining the ratio of number of embryogenic calli observed to the total number of explants inoculated and expressed in percentage.

To induce embryo maturation, embryogenic calli with globular shape embryos were placed on the maturation media containing full-strength MS, 30 g sucrose, 4 g/L of gelrite supplemented with different concentrations of ABA (1.5 mg/L and 2 mg/L) and BAP (0.4 mg/L and 0.6 mg /L). The cultures were maintained for two weeks under dark and then incubated under the light with a 16/8 hours photoperiod for four weeks (sub-cultured on fresh media at fortnightly interval). The somatic embryo maturation was ensured by observing under the microscope. Later matured cotyledonary embryos were transformed on to the regeneration medium *i.e.*, half strength MS basal medium, 0.4% gelrite, 3% sucrose supplemented with different combinations and concentrations of growth regulators viz., BAP (0.2, 0.4 mg/L), naphthalene acetic acid (NAA) (0.1 mg/L) and phloridzin (3 mg/L). The cultures were kept under light with a 16/8 hours photoperiod for two weeks. For shoot formation, 2-3 subcultures were done at the fortnightly interval. Plantlets were allowed to grow in regeneration media for about 45- 60 days. Later the well-developed plantlets with roots and shoots were transferred to hardening. The experiment was done using completely randomized design (CRD) with three replications. Observations on callus induction, maturation of somatic embryos and regeneration of shoots were taken up periodically. The data were analyzed computing ANOVA with critical difference at 5% probability.

3. RESULTS AND DISCUSSION

To optimize the somatic embryogenesis protocol for two gynodioecious papaya varieties 'Red Lady' and 'CO.7', different media compositions with two different auxins 2,4-D and picloram were employed based on the earlier reports [10,11,12]. Initially, the number of days taken for callus initiation was recorded (Table 1). Callus initiation was observed to be earlier in 'CO.7' (9.24 days) with 2 mg/L 2,4-D, whereas for 'Red Lady' the least number of days (9.28 days) for callus initiation was observed with 3 mg/L 2,4-D. In contrast, the treatments with picloram exhibited delayed callus initiation. Similar results have been observed in papaya when immature zygotic embryos were cultured on initiation medium containing 2,4-D and picloram [15,13].

Callus formation was observed in all the treatments after 4-6 weeks of inoculation. The callus induction was higher in 'CO.7' (90.93%) and 'Red Lady' (87.22%) with 2 mg/L and 3 mg/L of 2,4-D respectively. Differential response of different papaya varieties during embryogenesis with same concentrations of 2,4-D was also reported earlier [10]. In both the varieties of papaya, all three concentrations of picloram resulted in a lower rate of callus induction compared to 2,4-D (Table 1). Among the two, auxins selected for callus initiation, 2 mg/L 2,4-D performed better by recording the highest callus induction frequency compared to other treatments and a further increase in the concentration of 2,4-D resulted in browning of calli. Earlier reports showed that callus induction medium supplemented with 2,4-D has given successes across wide range of papaya cultivars [16,12,17]. Low levels of 2,4-D was also reported to substantially influence the initiation of somatic embryos in papaya, especially from immature zygotic embryos [8]. Similar to the present study, Chaudhary and Prakash. Chaudhary and Prakash [13] also reported that 2,4-D was the most responsive for promoting callus induction rate in papaya compared to picloram. Reduced response with picloram may be attributed to the genotype or the physiological status of the explant.

Creamy white, friable and compact calli were produced in MS medium supplemented with 2 mg/L 2,4-D in 'Red Lady' and yellowish-white friable calli in 'CO.7'. However MS media supplemented with the other levels of 2,4-D and picloram resulted in non-embryogenic calli (Table 1). Embryogenic calli formation was

Table1. Effect of 2,4 D and picloram on somatic embryogenesis of papaya varieties Red Lady and CO.7

Sl. No	Treatment	Days taken callus initiation		Percentage of callus induction		Per cent of embryogenesis		Morphological nature of callus	
		Red Lady	CO.7	Red Lady	CO.7	Red Lady	CO.7	Red Lady	CO.7
1	MS alone (control)	0.00	0.00	0 (0.62)	0 (0.62)	0 (0.62)	0(0.62)	No callus formation	No callus formation
2	2,4 D @ 1mg/L	11.33 ^b	11.50 ^b	71.66 (57.86) ^b	76.10 (60.89) ^b	21.67 (27.70) ^c	13.30 (21.33) ^c	Yellowish white and smaller	Creamy white and smaller
3	2,4 D @ 2mg/L	9.41 ^d	9.24 ^c	85.55 (67.68) ^a	90.93 (75.74) ^a	51.67 (45.95) ^a	30.00 (33.16) ^a	Slightly yellowish-white friable and spongy	Creamy white friable
4	2,4 D @ 3mg/L	9.28 ^d	9.37 ^c	87.22 (69.11) ^a	86.10 (68.22) ^a	35.00 (36.23) ^b	20.00 (26.44) ^b	Slightly yellowish-white friable	Creamy white with slightly spongy
5	Picloram @ 1mg/L	13.95 ^a	12.80 ^a	46.66 (43.08) ^d	48.30 (44.04) ^d	16.67 (24.04) ^d	11.70 (19.88) ^d	Whitish brown and small	Brownish white nonfriable and small
6	Picloram @ 2 mg/L	11.01 ^b	11.08 ^b	66.11 (54.75) ^c	66.67 (54.75) ^c	31.67 (34.23) ^b	23.30 (28.85) ^b	Brownish small and nonfriable	Brownish white nonfriable and small
7	Picloram @ 3 mg/L	10.45 ^c	10.70 ^b	70.55 (57.13) ^b	72.22 (58.24) ^c	23.33 (28.85) ^c	18.30 (28.85) ^b	Brownish and watery	Brownish mass of callus
	SEd	0.21	0.57	1.54	4.52	1.62	2.05		
	CD @ 5 %	0.45	1.23	3.31	9.70	3.47	4.40		

Figures in parenthesis are arcsin transformed values; values followed by similar alphabets within a column are not significantly different at 0.05 probability. Where SEd- Standard error deviation, CD- Critical difference

observed under a stereo microscope after 45-60 days of callus culturing. In both varieties of papaya, MS media fortified with 2 mg/L 2,4-D was found suitable for embryogenic callus formation. Higher embryogenic calli formation was registered in 'Red Lady' (63.33%), as compared to 'CO.7' (30.00%) (Table 1). Malabadi et al. [12] reported that 2,4-D stimulates an increase in endogenous levels of natural auxins, which have been fundamentally responsible for pro-embryogenic mass induction, resulting in cell proliferation and somatic embryos formation [18]. The difference in embryogenic calli formation between the two varieties may be attributed to their genetic differences.

Maturation is a crucial step for the success of somatic embryogenesis. Essential processes of this stage include cell expansion, differentiation and accumulation of reserve substances necessary for germination and regeneration of somatic embryos [19]. In the present study, embryogenic calli with globular stage somatic embryos were observed under stereomicroscope after 60-70 days of culturing (Plate. 2. Aa).

The growth regulator, ABA has been widely used in somatic embryo maturation medium because it induces the final development of the embryo through the accumulation of LEA (late embryogenesis abundant) proteins related to the

Table 2. Effect of different concentrations of ABA and BAP on maturation of somatic embryos in papaya cultivars CO.7 and Red Lady

SL. NO	Treatment	Red Lady	CO.7
1	MS (control)	0.00 ^d (0.62)	0.00 ^d (0.62)
2	ABA 1.5 mg/L+ Glutamine 400 mg/L	16.67 ^c (24.10)	18.33 ^c (25.35)
3	ABA 1.5 mg/L+ 0.4 mg/L BAP +Glutamine 400 mg/L	56.67 ^a (48.83)	58.33 ^a (49.80)
4	ABA 1.5 mg/L+ 0.6 mg/L BAP + Glutamine 400 mg/L	33.33 ^b (35.26)	41.67 ^b (40.21)
5	ABA 2 mg/L+ Glutamine 400 mg/L	1.67 ^d (7.43)	3.33 ^d (10.51)
6	ABA 2 mg/L+ 0.4 mg/L BAP +Glutamine 400 mg/L	0.00 ^d (0.62)	0.00 ^d (0.62)
7	ABA 2 mg/L+ 0.6 mg/L BAP +Glutamine 400 mg/L	0.00 ^d (0.62)	0.00 ^d (0.62)
	SED	2.82	1.78
	CD @ 5 %	6.04	3.82

- Figures in parenthesis are arcsin transformed values; values followed by similar alphabets within a column are not significantly different at 0.05 probability. Where SED- Standard error deviation, CD- Critical difference

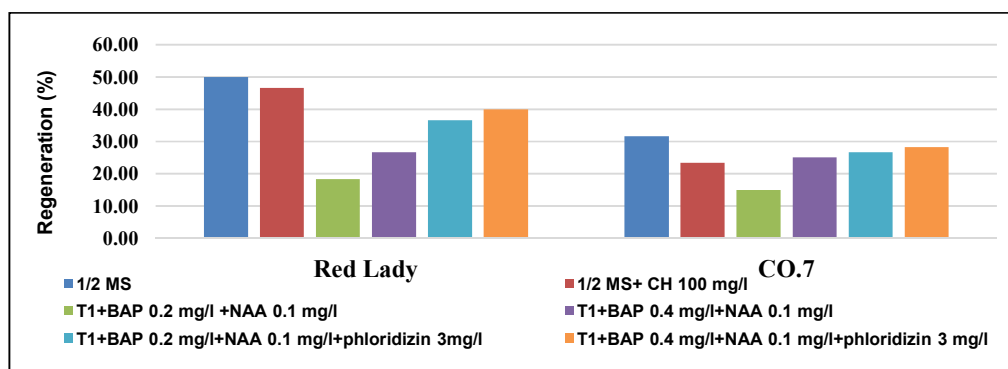


Fig. 1. Effect of Half strength MS, NAA, BAP and phloridizin on regeneration of papaya cvs. 'Red Lady' and 'CO.7'

Where MS- Murashige & Skoog, NAA- Naphthalene Acetic Acid, BAP- Benzyl Amino Purine

maintenance of desiccation tolerance [20,21], stimulate the storage of reserve substances [22,23], prevent precocious germination [24] and essential for the normal development and maturation of somatic embryos in papaya [25]. Hence, the induced embryogenic calli were transferred on to somatic embryo maturation medium containing MS medium fortified with different concentrations of ABA and in combination with varying levels of BAP to find out the best combination for hastening maturity. It was earlier reported that ABA allows embryogenesis induction, improves embryo maturation and prevents early germination in papaya [26]. The results revealed that in 'CO.7' and 'Red Lady', the maturation of globular embryos to cotyledonary embryos was 58.33 & 56.67 percentage respectively on MS media fortified with 1.5 mg/L ABA in combination with 0.4 mg/L BAP (Table 2). In the present study, drying and browning of embryogenic calli were observed with a further increase in ABA concentration.

During somatic embryo maturation, different developmental stages of somatic embryogenesis viz., heart, torpedo and cotyledonary stages were observed under a stereomicroscope in both the cultivars (Plate. 2). In the different treatments studied, the regeneration of cotyledonary embryos ranged from 18.33-50.00 per cent in 'Red Lady' and 15.00-31.67 per cent in 'CO.7' respectively. The regeneration of mature somatic embryos of 'Red Lady' was better compared to 'CO.7' generally and in both the varieties higher regeneration was observed in half-strength MS media devoid of any growth regulator (Fig. 1). The results obtained in the present study is in contrast to the earlier reports indicating that papaya somatic embryogenesis was dependent on presence of growth regulators viz., BAP, NAA and IAA [27,28,29,13]. Only lower response was observed in the other combinations BAP (0.4 mg/L) and NAA (0.1 mg/L) even with phloridzin (3 mg/L) in the regeneration medium lower regeneration was observed when compared to medium free of growth regulators in both varieties. Further callusing was observed at the base of shoots. This result is in contraction to the report of Ascencio-Cabral et al. [30] who observed better germination and growth of somatic embryos in papaya in the presence of phloridzin. The endogenous synthesis of growth hormones in sufficient levels could have been the reason for higher regeneration from mature embryos in half MS media devoid of any supplementation of growth regulators.

4. CONCLUSION

The present study proved that 2,4-D can comparatively influence higher rate of induction of somatic embryogenesis in both papaya cultivars 'CO.7' and 'Red Lady' than picloram. 2,4-D at 2 mg/L is the optimum concentration for high frequency of embryogenic callus induction. Ms medium supplemented with 1.5 mg/L ABA in combination with BAP 0.4 mg/L was found to enhance maturation of somatic embryos. Half-strength ms medium devoid of growth regulators was found sufficient to influence satisfactory regeneration of matured somatic embryos.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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