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RESEARCH ARTICLE

Somatic embryogenesis in papaya (*Carica papaya* L. cv. Rathna)

A.R.F. Farzana[1], P.G.V.N. Palkadapala[1], K.M.M.N. Meddegoda[1], P.K. Samarajeewa[2] and J.P. Eeswara[1*]

[1] *Department of Crop Science, Faculty of Agriculture, University of Peradeniya, Peradeniya.*

[2] *Biotechnology Division, Plant Genetic Resource Centre, Gannoruwa.*

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Abstract: A protocol was developed for somatic embryogenesis from zygotic embryos, hypocotyl explants from *in vitro* seedlings and leaf explants from greenhouse grown plants of *Carica papaya* L. cv. Rathna. Seeds in which the sarcotesta membrane is removed, were germinated in conical flasks with sterilized water and maintained on an orbitral shaker, and within 2-4 week were used to excise hypocotyls explants. Zygotic embryos were isolated directly from sterilized seeds.

Embryogenic calli from zygotic embryos and hypocotyl explants were initiated successfully on Murashige and Skoog (MS) medium containing 3 mgL^{-1} Naphthalene Acetic Acid (NAA) when compared with MS medium containing combination of 3 mgL^{-1} NAA and 2 mgL^{-1} 2,4 Dichlorophenoxyacetic acid (2,4-D). Induction of embryogenic calli from mature and immature leaf explants were evaluated on media supplemented with 3 mgL^{-1} NAA, 3 mgL^{-1} Indole-3-butyric acid (IBA) and 1 mgL^{-1} 2,4-D. Immature leaf explants cultured on 1 mgL^{-1} 2,4-D was found to be the most suitable for callusing.

Effect of 100 mgL^{-1} Casien hydrolysate, 0.1 mgL^{-1} Abscisic acid (ABA), 0.5 mgL^{-1} 6-Benzylaminopurine (BAP) and hormone-free MS media were tested for the maturation of calli and for the formation of somatic embryos. Casein hydrolysate was found to be the most suitable for maturation of calli. Matured embryogenic calli could be proliferated on MS medium supplemented with 0.5 mgL^{-1} BAP. Germination of embryos was evaluated in 0.1 mgL^{-1} BAP and 0.1 mgL^{-1} NAA combination, 0.02 mgL^{-1} NAA and 0.5 mgL^{-1} BAP as well as in hormone-free MS medium. The medium supplemented with 0.02 mgL^{-1} NAA and 0.5 mgL^{-1} BAP combination gave significantly higher germination. Further development of plantlets was achieved through germination of somatic embryos on hormone-free MS medium. Sixty percent of the plantlets could be successfully acclimatized on coir dust: sand=1:1 medium in a propagator.

Keywords: Leaf, hypocotyls, papaya, somatic embryogenesis, *Carica papaya* L. cv. Rathna, zygotic embryo.

INTRODUCTION

Carica papaya L. (papaya or papaw), native to tropical America, is a fruit crop which is widely distributed throughout tropical and warmer subtropical regions of the world.

This crop was introduced to Sri Lanka in the 16th century. Compared to other perennials it is a fast growing crop with a short gestation period. Flowers are produced four months after germination of seeds and bear fruits 7-8 months after planting. A tree is capable of producing 30-40 fruits (2 kg/fruit) annually. A papaya plantation at full bearing age is capable of producing 75-100 mt fruit/ha/yr. It is a highly domesticated and common home garden fruit tree in most parts of Sri Lanka[1]. Even though papaya can be grown in all agro climatic zones of Sri Lanka, dry and intermediate zones are the best regions for papaya cultivation. The current extent of land under papaya is about 800 ha and the annual production is approximately 11000 mt. The production is primarily consumed locally while a small quantity (around 2 mt) is exported[2].

The consumption of papaya is growing steadily in parallel with the increase in the number of health conscious food consumers, as the fruit is low in calories and sodium but high in dietary fibre, calcium, potassium and vitamins A and C. Papain, an enzyme produced by papaya, is used in meat tenderizers, face and hair care products and in various manufacturing applications such as leather, wool, rayon and beer[3].

Papaya is easily cultivated and has good agronomic features such as rapid growth, requirement of minimum-

*Corresponding author

growing space, early production and high yields. Papaya has multiple uses, gives prompt returns and adapts to diverse climatic and soil conditions. Breeding research conducted so far have resulted in significant increase in development of characters such as dwarfing, uniform fruiting, increase in fruit size and improvement of quality, amount of flesh, cold tolerance, fruiting precocity, and tolerance to various stress conditions[3].

There is a good market for papaya in the European Union (UK, Germany and Switzerland), Maldives and Middle East countries[2]. The unavailability of high quality fruit throughout the year is a major problem in the export of papaya. The lack of true to type varieties is one of the major constraints faced by the papaya growers in Sri Lanka. In addition the large number of unproductive male plants, great genetic variability resulting in fruit of varying shape, size, appearance, taste and high incidence of disease (ring spot virus disease) are some of the problems in papaya cultivation[4]. Therefore, the profit margin of the local papaya industry is very low[2]. Hence, it is necessary to develop improved high yielding varieties, which are suitable for the export market.

Papaya is essentially cross-pollinated and seed propagation is not able to maintain even some of the parental characteristics in the offspring[5]. Seed propagation results in variations in the quality and size of the fruit as well as productivity. The various forms of papaya, which present a special problem both to the breeder and grower, also limits commercial cultivation. To develop true to type plants through selection or to develop new varieties, an efficient vegetative propagation technique is required[6]. Even though grafting and budding techniques have been successful, they are not commercially practised, since the number of plants produced per mother plant is limited. Thus, the use of micro propagation techniques, which can be used to produce a large number of true to type high quality planting material is an essential requirement in papaya cultivation. Furthermore, tissue culture techniques can be used as an important tool in crop improvement programmes since they help to overcome the problems experienced in conventional breeding methods and in rapid clonal production of crops.

Although the success of tissue cultured papaya clones has been reported, rooting of micropropagated plantlets is a major problem that is encountered[7]. Therefore, somatic embryogenesis offers an attractive alternative, as the embryos are bipolar structures bearing both root and shoot apices.

In addition, embryogenic cultures can optimally produce relatively large numbers of embryos per culture flask. When grown in liquid medium, embryos usually float freely in the medium and do not need manual separation and mechanical handling (e.g. fluid drilling) is easy.

Once dormancy is induced in somatic embryos and incorporated into artificial seeds which can be handled like normal seeds, stored, shipped and planted. Due to their properties, somatic embryos may also prove useful in long-term storage methods such as cryopreservation. Somatic embryos have also proved to be excellent material for genetic transformation studies due to their competency in expressing incorporated DNA[4]. The regeneration system is of prime importance for genetic transformation. Those F1 hybrids produced through genetic transformation can be used for *in vitro* propagation and to obtain uniform planting material with desired characters.

Although a large number of reports are available from other countries with regard to the production of somatic embryos of papaya, no information has been reported to date in Sri Lanka. The frequency of response to somatic embryogenesis within a species may vary considerably from one genotype to another[8] and different conditions may be required for each genotype[9]. Therefore, suitable protocols have to be established for different papaya varieties available in Sri Lanka.

Hence, this project was conducted with the ultimate objective of establishing a suitable protocol for somatic embryogenesis in papaya, cultivar Rathna.

METHODS AND MATERIALS

General tissue culture methods: Murashige and Skoog[10] (MS) medium, containing 3 % sucrose, and phytagel (1.75 gL^{-1}) or agar (8 gL^{-1}) was used throughout the experiments. Different levels of plant growth regulators were added to the media prior to sterilization, as defined in each experiment. The pH of the media was adjusted to 5.8. Unless otherwise stated, culture tubes (75 mm x 25 mm) containing 10 mL of medium were used throughout the study. The cultures were incubated under aseptic conditions with light intensity of 55 $\mu ECmu^{-2}s^{-1}$ at 25°C and a photoperiod of 16 light /8 h darkness or in complete darkness in the incubator. Cultures in liquid media were maintained on an orbital shaker at 100 rpm.

Visual observations of the cultures were made weekly and data were taken as defined in each experiment. The data were analyzed using the CATMOD procedure in SAS.

Experiment I: *In vitro germination of papaya seeds:* Papaya seeds of cultivar Rathna were sterilized by shaking in 10% Clorox (5.25% Sodium hypochlorite) with 2-3

Table 1: Effects of medium and splitting on germination papaya seeds.

Culture medium		No. of replicates Treatment	
		Split	Non-split
nutrient	full MS	60	60
medium	½ MS (macro-nutrient)	60	60
	¼ MS (macro-nutrient)	60	60
	water agar	60	60
wet filter paper		250	-
sterile water (on orbitral shaker)		75	75

drops of Tween 20 for 10 min followed by rinsing with sterile water and by shaking again in 10% Clorox alone for another 10 min. Thereafter the seeds were thoroughly washed three times with sterile distilled water. Sterilized seeds were divided into two batches. In one batch the seed coats were split under aseptic conditions using a sterile scalpel and cultured single seed per tube on different media as specified in Table 1, while the remaining batch was cultured on the same media without splitting the seed coats. All cultures were maintained under 16 h light/ 8 h darkness photoperiod. Sterile water culture was established in three 250 mL conical flasks containing 100 mL of sterile water and 25 seeds in each flask and maintained on an orbital shaker at 100 rpm.

Seed germination in each treatment was recorded fortnightly and the results were analyzed using the CATMOD procedure in the SAS statistical package. The seeds germinated on wet filter paper and in sterile water were transferred to MS basal medium for further growth, and they were maintained under a photoperiod regime of 16 h light/8 h darkness.

Experiment II: *Effect of 2,4-D and NAA on callus initiation from zygotic embryo, hypocotyl and leaf explants*: Hypocotyl segments from *in vitro* seedlings and zygotic embryos were excised directly from sterilized seeds and cultured on MS medium supplemented with 3 mg L^{-1} Naphthalene Acetic Acid (NAA) alone or a combination of 3 mg L^{-1} NAA and 2 mg L^{-1} 2,4 Dichlorophenoxyacetic acid (2,4-D.)

Leaf explants were from greenhouse-grown papaya plants (3 months old) of cultivar Rathna, which were sprayed with fungicide 2 days prior to the excision. The first two (immature) as well as the 3-5th (mature) leaves were taken from the plant separately, rinsed under running tap water and then in liquid soap followed by distilled water. They were surface sterilized with 5 % Clorox for 5 min with 2-3 drops of Tween 20 followed by rinsing with sterile water and again with 5% Clorox alone for another 5 min. Then, the pieces of lamina (approximately 1cm x 1cm) containing at least one vein were excised both from immature and mature leaf explants and were cultured separately on MS medium supplemented either with 3 mg L^{-1} NAA, 3 mg L^{-1} Indole butyric acid (IBA) or 1 mg L^{-1} 2,4-D. Each culture tube contained 1 piece of leaf explant and each treatment was carried out in tubes. All cultures were incubated at 25ºC under darkners and callus induction was recorded at the time of subculturing using the following scores:

Level of callus production	Score
no callus	0
callus, just initiated	1
low callus	2
medium callus	3
good callus	4

Experiment III: *Effect of Casien hydrolysate, ABA and BAP on production of somatic embryos*: All calli from zygotic embryos, hypocotyl explants and leaves, which were isolated 4-8 wks after establishment were transferred to MS medium containing either 100 mg L^{-1} casein hydrolysate, 0.1 mg L^{-1} Abscisic acid (ABA), 0.5 mg L^{-1} 6-Benzylaminopurine (BAP) or without hormone. Each treatment was replicated 20 times. The cultures were maintained under a photoperiod of 16 h light/8 h darkners. Observations were made after 4 wks of culturing and the data analyzed using the SAS computer package.

After 4 wks, the calli obtained from hypocotyls, zygotic and leaf explants cultured on media containing casein hydrolysate, ABA, BAP or without hormones, were transferred to 0.5 mg L^{-1} BAP supplemented MS medium and half of them were maintained under complete darkness and the rest under 16 h light/8 h dark condition. Responses of calli were recorded after 4 wks.

Experiment IV: *Identification of a suitable medium for germination of somatic embryos*:

The embryos initiated from zygotic, hypocotyl and leaf originated calli were isolated and transferred for germination to MS medium without hormone, with 0.1 mg L^{-1} BAP and 0.1 mg L^{-1} NAA combination or 0.02 mg L^{-1} NAA and 0.5 mg L^{-1} BAP combination. The cultures were maintained under a light intensity of about 55 $\mu Em^{-2}s^{-1}$ at 25ºC and 16 h light and 8 h dark condition. Each treatment consisted of 10 replicates. Fourteen days after establishment, the number germinated embryos were counted.

Experiment V: *Development of plantlets and acclimatization*: After 4 wks of establishment, the emerged plantlets were transferred to hormone–free MS medium with 3% sucrose, and maintained under 16 h light/8 h dark condition and subcultured at 4 weekly intervals. At the time of acclimatization, plants with well developed root systems were removed from the media and the root system was washed thoroughly in lukewarm water to remove agar**.** The plants were next dipped in 1% fungicide (Redomyl) solution for few minutes and kept on a newspaper to drain. Small clean pots filled with sterilized medium of sand: coir dust (1:1) were used for planting. The plants were then maintained in the greenhouse inside a small propagator under 100% relative humidity (RH). After 1 wk, RH was reduced by opening the valve gradually. Since then, watering was done as required by regular checking of the media.

RESULTS

Experiment I: *In Vitro* germination of papaya seeds

Two and four weeks after establishment, seeds cultured on sterile water on orbitral shaker showed significantly higher germination compared to wet filter paper and agar media at α= 0.05 probability level (Figure 1), whereas there was no germination observed in any other media.

No significant differences were observed between germination of split and non-split seeds. However, from sixth week onwards, split seeds showed a significantly higher percentage of germination compared to the non-split seeds (Figure 1) at α= 0.05 probability level.

Experiment II: Effects of 2,4-D, NAA and IBA on callus initiation from zygotic embryo, hypocotyl and leaf explants

Zygotic embryos and hypocotyls explants produced calli after two weeks of culture. Those calli were initially cream white (ice crystal-like) in colour and increased in size continuously. The results revealed that zygotic embryos produced more calli compared to those of hypocotyl explants and this was significantly different at α = 0.05 probability level. Furthermore, MS medium containing 3 mg L^{-1} NAA was better for callus production from zygotic and hypocotyl explants compared to the medium with both NAA and 2,4-D (Figure 2) and was significantly different at α= 0.05 probability level. One of the cultures containing 3 mg L^{-1} NAA produced an embryo even before transference to the maturation medium.

Calli initiated from immature leaf explants within 1 week were creamy white and continued to grow rapidly. With time, some parts of the callus turned yellowish brown. It took around three weeks for the initiation of calli from mature leaf explants and their growth was very slow. There was a significant difference (at α= 0.05) in the production of calli from leaves of different ages. The first two leaves from the shoot apex (or immature leaves), produced significantly higher amount of calli (α= 0.05) than the mature leaves (3-5 leaves from the shoot apex).

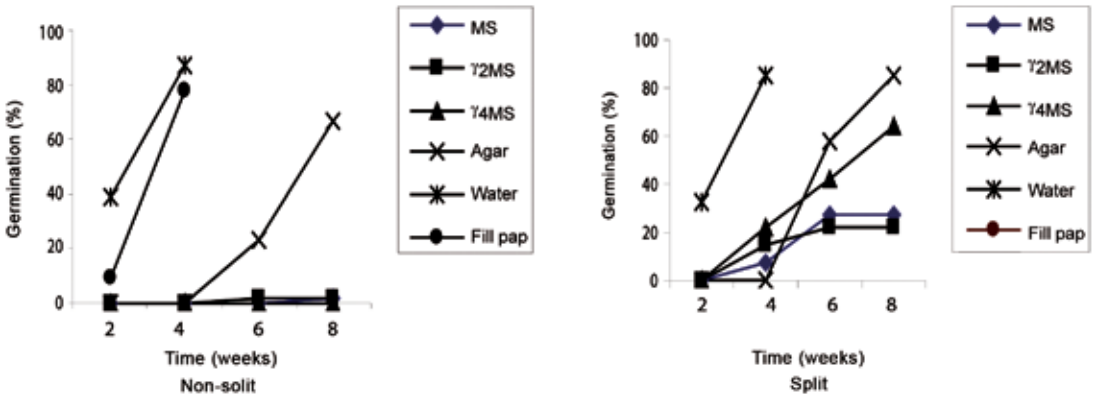

Figure 1: *In vitro* seed germination

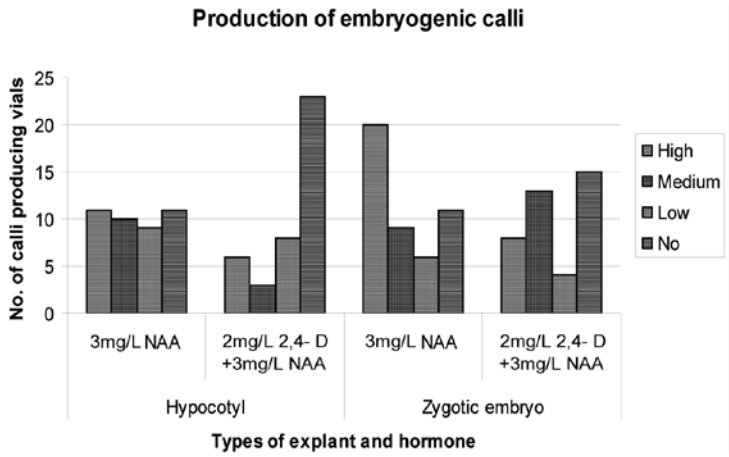

Figure 2: Effect of NAA and combination of NAA and 2,4-D on the production of callus from zygotic embryo and hypocotyl explants (Legend: High = callus with score 4; Medium = callus with score 3; Low = callus with score 2; No = callus not formed or score 0).

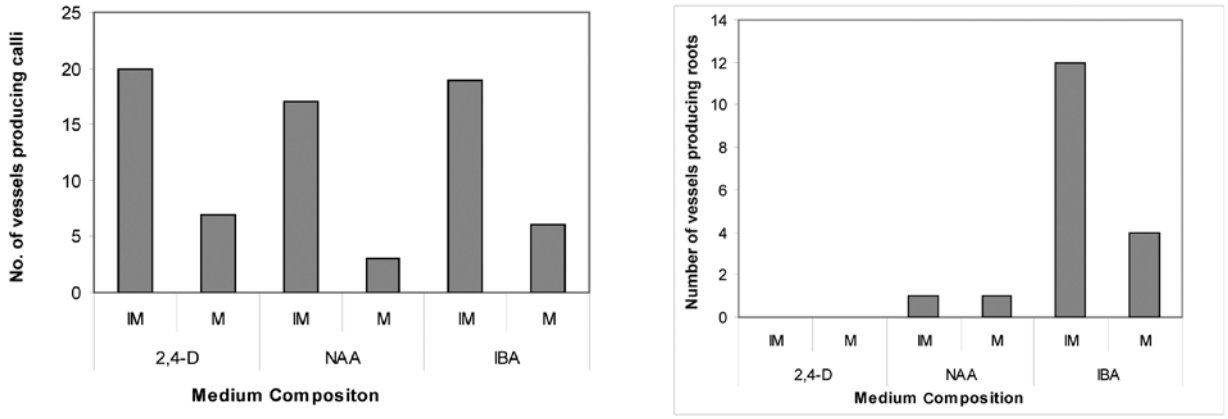

Figure 3: Callogenesis from leaf explants (a) level of callus production (b) fraction of root-forming callus (IM-first two leaves, M-3-5 leaves from the apex).

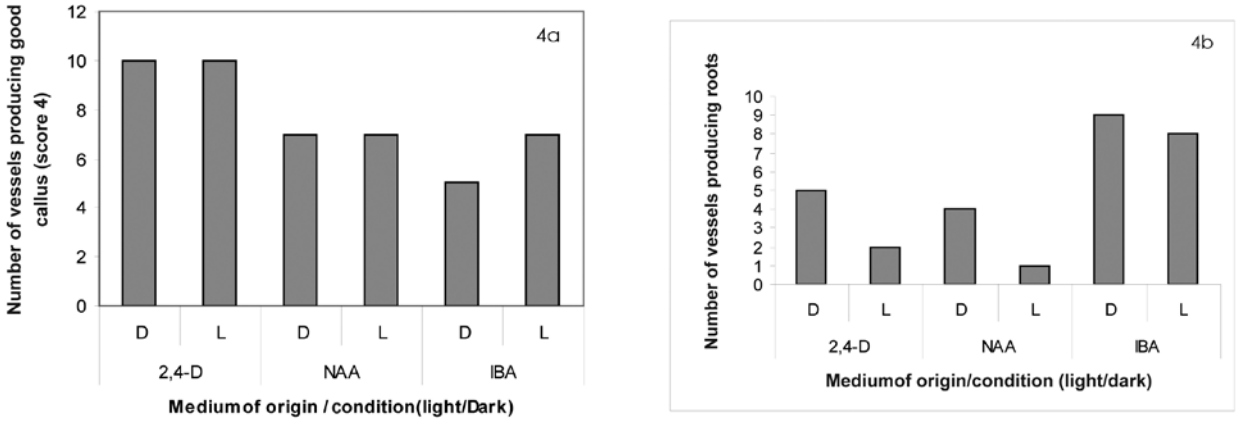

Figure 4: Performance of leaf callus on maturation medium (M3) (a) growth of the callus (b) proportion of root-forming callus (D-dark, L-light condition)

At the time of subculturing, the level of callus production in different media was as shown in Figure 3a. It was observed that, rate of callus initiation varied among media and the age of leaves, and this was significant at the probability level of $\alpha = 0.05$ (Figure 3b and 3c). Medium containing 2,4-D produced significantly higher amount of calli compared to the NAA and IBA containing media. Furthermore, MS medium containing IBA and NAA produced a significantly higher number of calli with roots compared to the medium with 1 mg L^{-1} 2,4-D.

Experiment III: Effect of Casien hydrolysate, ABA and BAP on production of somatic embryos

There are significant differences ($\alpha = 0.05$) in the growth of calli in casein hydolysate, ABA growth regulator free and BAP containing media. Calli grown in a medium containing casein hydrolysate continuously increased in size and turned dark brown in color than those in the ABA medium. During the early stages, calli in the medium containing ABA grew at a low rate. Growth of the calli completely stopped after three weeks of transferring to the media and calli turned into compact hard structures. Calli grown in MS medium without growth regulators became yellow to cream in colour and swelled into a compact mass. After 3-4 weeks of transferring to the growth regulator free medium, calli started to produce roots.

Calli in BAP supplemented medium grew more rapidly than those without BAP, and the calli became more friable and nodular in nature. Therefore, it was decided to transfer all the calli to MS medium supplemented with 0.5 mg L^{-1} BAP for further multiplication.

Two weeks after transferring to BAP supplemented medium (M_3), the calli which originated in casein supplemented medium appeared as white to pale yellow calli, that were opaque, with nodular and convoluted surfaces and appeared organized. [Figure 6 A(i)].

All calli from leaf explants continued to grow in the maturation medium (M_3), maintained under both light and dark conditions. Growth rate was relatively higher in calli originated in media with and 2,4-D subcultured on to MS medium with 0.5 mgL^{-1} BAP [Figure 6A (ii)]. The number of calli with roots were much higher in the cultures maintained under dark conditions (Figure 4a) and also in those of IBA origin (Figure 4b). The growth rate of calli originated in media with 2,4-D was significantly higher (at $\alpha = 0.05$) than those originated in media with IBA and NAA. Although growth of calli maintained under light condition was higher than the ones under dark condition (Figure 4a), this difference was not significant at $\alpha = 0.05$ probability level. However, formation of roots on calli was significantly higher in IBA induced calli, compared to those induced by the other two hormones (Figure 4b).

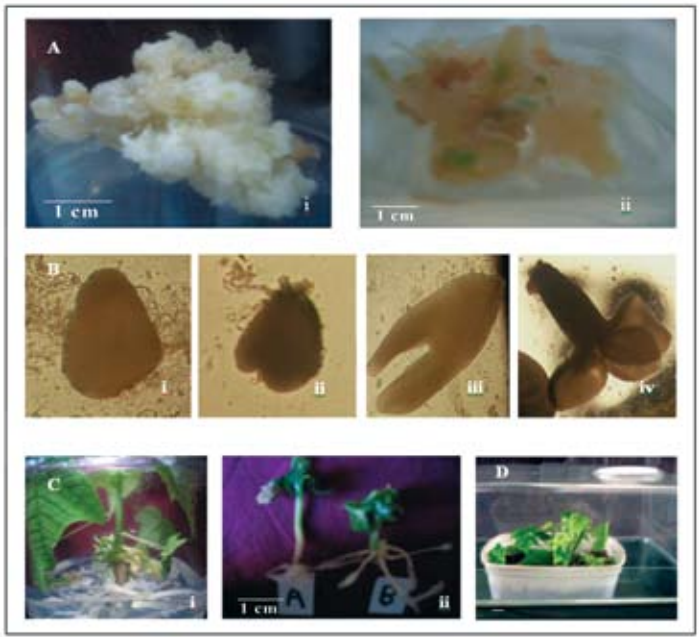

Figure 5: *In vitro* cultures of papaya

A. Callus on maturation medium **A(i)**-from hypocotyl explants, **A(ii)**-from leaf explants; **B**-somatic embryos at different stages (10×4) **B(i)**-globular, **B(ii)**-heart-shape, **B(iii)**-torpedo and **B(iv)**-cotyledonary stage; **C(i)**-a -plantlet produced from somatic embryo, **C(ii)**-Comparison of plantlets from natural seed (A) and somatic embryo (B); **D**- Plantlets produced from somatic embryos placed in the propagator for acclimatization.

Eight weeks after transferring of calli on to MS medium supplemented with 0.5 mg L^{-1} BAP, different stages of somatic embryos were observed under the microscope (Table 2 and Figure 5B).

Experiment IV: Identification of a suitable medium for germination of somatic embryos

None of the embryos germinated in 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} BAP supplemented or growth regulator–free MS medium even after 8 weeks. Germination of somatic embryos were observed within 4 weeks in the 0.02 mg L^{-1} NAA and 0.5 mg L^{-1} BAP supplemented media (Figure 5C). Leaves were prominent in the plantlets and both shoots and roots emerged and developed simultaneously. Thus, it is possible that the high cytokinin concentration (0.5 mg L^{-1} BAP) could have contributed to the germination of somatic embryos.

Experiment V: Development of plantlets and acclimatization

After transferring to the hormone – free nutrient medium, the plantlets continued to grow (Figure 5D). Soon after acclimatization, the plants looked wilted, but recovered with time. However, only 60% of the plants could be acclimatized successfully.

DISCUSSION

In the present study, more than 120 days were taken for the completion of somatic embryogenesis whereas somatic embryos have been obtained in a very short period of 49 days in 'Solo' type papaya[11]. This variation in response may be due to the difference in genotypes.

Seedlings grown *in vitro* represent a particularly convenient source of explant for somatic embryo induction[12]. In agreement with the results of Fitch[11], sterilized water was the most suitable medium for *in-vitro* germination (>80%) of seeds.

Lange[13] reported that intact papaya seeds show a very low germination, or do not germinate at all and germination can be enhanced by removal of the aril. It is believed that the aril membrane acts as a barrier against the liberation of water-soluble inhibitors, which retard germination. Therefore, in the present study, the aril of the seeds was removed before culturing for germination. Removal of the aril and leaching of the seeds prior to planting of papaya has been suggested for higher and faster germination[14]. This effect was evaluated in the present study using seeds of the local variety, by splitting the seed coat of half of the seed lot. This treatment was significantly effective.

Seed extracts were found to contain growth regulating substances including a gibberellins-like substance, cytokinin-like substances, and both acidic and neutral inhibitors[14]. This may be the reason for the significantly high germination in both sterilized water and sterile wet filter papers, where, the seeds were in contact with water. Therefore, these inhibitors may have diffused and diluted throughout the medium rather than concentrate in the immediate environment of the seeds, on the semi-solid media. Furthermore, the enhanced germination observed in water and on wet filter paper may be due to the good aeration provided to them. It was also shown through this experiment that water is necessary for germination while nutrients were not required for germination of papaya seeds.

In agreement with earlier reports[11], zygotic embryos and hypocotyls from aseptically grown seedlings of the local cultivar, were well suited for the induction of embryogenic calli and somatic embryos in papaya.

Carica papaya trees exhibit sexual dimorphism and identification of sex at the nursery stage is a major problem in papaya. Tissues taken from the mature trees may offer a solution to obtain true-to-type plants. Hence, in this experiment, leaf explants from greenhouse grown plants were also evaluated to develop a suitable protocol for somatic embryogenesis in papaya with the objective of adapting it to field grown plants. Leaves from field grown plants were not used due to difficulties of obtaining sterile cultures.

Mature tissues of the explant may have an inhibitory effect on the capacity on the meristematic tissues to form embryogenic callus[15]. Irrespective of the type of

Table 2: Production of embryos from leaf explants in maturation medium.

Maturity of explant	Media of origin	Count of embryos (stage)		
		Globular/ Heart-shaped	Torpedo	Cotyledonary
Immature	2,4-D	3	11	12
Mature	2,4-D	1	4	5
	NAA	2	3	5

explant used, age plays a critical role in determining the response *in vitro*. Older explants produce either root-forming or non-regenerable calli[16]. This was investigated in the present study, where immature (first two leaves from the top) and mature leaves (3-5 leaves from the top) were compared for induction of calli and further development. The immature leaves were significantly better in calli induction and mature leaves, produced more root-producing calli, which showed relatively slower regeneration.

In contrast to Jordan *et al.* [17], the leaf explants taken from the greenhouse-grown plants in this study showed a negligible rate of contamination. Therefore, even field grown plants can be used, by proper surface sterilization procedures. The age of the plant was not important; young leaves even from mature plants were used successfully[18, 19]. Therefore, in future studies, explants taken from field-grown plants can be used to initiate embroygenic calli using the protocol developed by the present study.

Most of the explants were cultured on MS or a modified MS medium[20]. A key element of the MS medium is the presence of high levels of nitrogen in the form of ammonium nitrate. The benefits of reduced nitrogen, in addition to nitrate, for both embryo initiation and maturation have been well established[21].

Of all the auxins, 2,4-D has proven to be the most efficient in producing calli[22]. However, in the present study 2,4-D was not so effective on hypocotyl and zygotic explants, whereas leaf explants incubated in the dark with 2,4-D (1 mg L^{-1}) produced significantly better calli.

The presence of auxin or auxin-like substances was critical for embryo initiation and lowering of auxin or its complete absence fostered maturation[23-25]. Somatic embryogenesis is induced by transferring embryogenic cell clusters to auxin-free medium[26]. Therefore, in the present study, calli were transferred to hormone-free MS medium. Growth of calli in hormone-free medium indicated that they were embryogenic and, quite a large number of embryos were also formed in this media from mature leaf explants. In a number of species, cytokinins were important in fostering somatic embryo maturation[27] and especially cotyledon development[28]. This may be the reason, for the good performance of the medium supplemented with BAP in the present study.

Although the callus induced in the IBA supplemented medium grew rapidly on maturation medium (M_3:MS medium supplemented with 0.5 mgL^{-1} BAP), this treatment cannot be recommended, as the bulk of callus from IBA treated medium contained more of root-forming callus. In most cases, somatic embryos of papaya germinated readily in the absence of growth regulators[29] or on media containing relatively low concentrations of auxins and cytokinins[18, 30-34]. As in the study of de Bruijne, *et al.*[35] and Ammirato[36], in the present study it was not possible to germinate embryos on MS medium without hormone and was necessary to treat with cytokinins. Cytokinins are required for growth of embryos into plantlets and for cotyledon development[37]. Although most of the obtained plantlets showed normal development, in a few of them, secondary embryogenesis was visible. For controlling repetitive embryogenesis, ABA has proven effective[38, 39]. However, ABA in maturation medium has been evaluated in the present study, for the maturation of embryogenic calli, and it was proved to be ineffective. As an alternative, it is shown[40] that by adding inositol while simultaneously reducing the sucrose concentration and maintaining the cultures in darkness, somatic embryos matured free of extraneous proliferation and germinate precociously.

CONCLUSION

In the present study, a method has been developed for somatic embryogenesis of papaya (cultivar: Rathna). It was found that both hypocotyl explants and zygotic embryos should be incubated on MS medium supplemented with 3 mg L^{-1} NAA for the induction of embryogenic calli while 1 mg L^{-1} 2,4-D is suitable for callus induction from immature leaf explants.

The initiated calli has to be transferred to MS medium supplemented with 100 mg L^{-1} casiein hydrolysate followed by media supplemented with 0.5 mg L^{-1} BAP for the maturation of embryos. Matured embryos have to be germinated on MS medium supplemented with the combination of 0.5 mg L^{-1} BAP and 0.02 mg L^{-1} NAA. It is anticipated that the developed protocols, which could help to satisfy the increased demand for plant material of papaya in future.

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