In vitro cloning of female and male *Carica papaya* through tips of shoots and inflorescences


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Raising cultures of proliferating shoots of female and male plants of *Carica papaya* through shoot apices taken from mature plants was difficult because of high incidence of endogenous bacterial contamination. Whilst they were easily raised through culture of young inflorescences tips of female and male plants. There was no difference in the requirement of nutrients and growth regulators for proliferation of shoots raised from shoot tips or inflorescence tips, but at the initial stage, the different explants differed in their requirement of growth regulators for induction of shoot bud differentiation. BM1, a modification of MS medium was used as the basal medium in all the cases. There was a pronounced callusing tendency in tender shoots initially used for rooting, for which well-developed shoots (< 4 cm long) were found suitable. Rooting was achieved in developed shoots through a 4-step procedure: Step 1-An initial pulse treatment with a high concentration of IBA (10 mg l\(^{-1}\)) using BM3 medium, a modified Knop medium with trace elements and disodium-ferric-ethylene-diaminetetra-acetate (Na-Fe-EDTA) of MS medium and 2% sucrose for 24 h; Step 2- Their subculture in medium BM2 differing from BM1 in having 50 mg l\(^{-1}\) m-inositol and supplemented with IAA (0.25 mg l\(^{-1}\)) along with AdS (15 mg l\(^{-1}\)) and 2% sucrose for 7 days; Step 3- Roots at the cut end of about 95% shoots were visible, after about 10 days, in the same medium as used in Step 1, but having supplements of 3 vitamins, 2 amino acids and 0.25 mg l\(^{-1}\) IAA, while sucrose was removed; and Step 4- The just rooted shoots when finally transferred to the semi-sterilized moist Soilrite contained in culture tubes, formed healthy roots without intervening callusing, while the shoots also remained healthy. Such plantlets when ultimately transplanted along with Soilrite plugs to the potted soil showed about 80% transplant success. *In vitro*-raised plants appeared normal and fruited under field conditions after about 6 months of *ex vitro* growth.

**Keywords:** Dioecious, endogenous infection, *ex vitro* growth, intervening callusing, *in vitro* rooting, long-term culture, shoot proliferation

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**Introduction**

*Carica papaya* L. (papaya), the fifth important fruit of India, is valued for the nutritional qualities of its fruit as a source of provitamin A and calcium as well as for the pharmaceutical industry as a rich source of commercial papain, a proteolytic enzyme. The plant starts fruiting in just one year and gives economically high yield per acre next to banana. However, a big disadvantage regarding its commercial cultivation is its dioecious character and heterozygosity coupled with cross pollination. Thus, the conventional method of its propagation through seeds results in great variability in fruit quality. Since colour and flavour of its fruit are controlled by pollen grains, cloning through tissue culture of female and male elite plants is warranted to ensure uniform quality and yield of fruit. There are several reports on tissue culture multiplication of this plant, including genetic transformation. It has been micropropagated mostly by using seedling explants, but also through shoot tips taken from mature plants. There are quite a few reports on efficient micropropagation of papaya, they are in alien varieties. As expected, such protocols are not equally applicable in case of indigenous varieties in view of the wide variation existing in the *in vitro* responses of different varieties and clones in general. The poor transplant success of the *in vitro*-raised plantlets, which in turn depends on difficulty in obtaining desirable rooting of microshoots is a big hindrance in its commercial propagation. Furthermore, establishment of aseptic cultures through shoot apices of mature plants is intractable because of high incidence of endogenous infection. This paper reports cloning of mature plants of *C. papaya* var. Honey Dew of known sexuality employing tips of their shoots and young inflorescences. There appears to be no earlier report on the latter aspect.

**Materials and Methods**

**Preparation of Explants**

For initiation of cultures, the explants were collected from about 1-year-old mature elite female...
and male plants of Carica papaya L. var. Honey Dew grown in the Experimental Plot of the Tissue Culture Laboratory, National Botanical Research Institute, Lucknow. Shoot apices (2 cm long) were excised from young axillary shoots of both female and male plants in the late Summer season, while tips of young female and male inflorescences (~1-cm-long) were collected during Spring season.

Tips of shoots and inflorescences were washed with filtered running tap water for 15 min, treated with 5% neutral liquid detergent solution of ‘Labolene’ (Glaxo Smith Kline Pharmaceuticals, Ltd., Mumbai) for 10 min, washed again with distilled water and surface-sterilized in 0.1% HgCl$_2$ (w/v) solution for 15 min, washed again with distilled water and surface-sterilized in 0.1% HgCl$_2$ (w/v) solution for 15 min followed by their thorough washing with sterile distilled water. Tips of shoots (1 cm) and inflorescences (0.3-0.5 cm) were trimmed by cutting off the basal exposed portions before their inoculation in nutrient medium.

Composition of Media, their Sterilization and Incubation of Cultures

Composition of BM1 medium, where it differed from MS$^{14}$ medium, was: KNO$_3$, 1000; NH$_4$NO$_3$, 1500; CaCl$_2$.2H$_2$O, 400; Na$_2$SO$_4$, 100; (NH$_4$)$_2$SO$_4$, 100; thiamine-HCl, 1; and L-arginine-HCl, 10 mg l$^{-1}$. Medium BM2 was similar to BM1 except m-inositol (50 mg l$^{-1}$), while BM3 comprised half-strength concentrations of salts of Knop$^{15}$ medium with MS trace elements and disodium-ferric-ethylene-diaminetetra acetate (Na-Fe-EDTA, 3 ml l$^{-1}$), prepared after the manner of MS.

All the media were adjusted to pH 5.8, before adding agar, and sterilized by autoclaving at 1.08 kg/cm$^2$ for 15 min. Cultures, including those of root induction, were incubated under 25 $\mu$mol m$^{-2}$s$^{-1}$ quantum flux density for 15 hrs a day at 27±1°C and 70±4% RH maintained in culture room.

Establishment of Cultures and Shoot Proliferation

The shoot tip explants were initially cultured in the liquid medium BM1 containing different combinations of antibiotics, of which some of the effective ones were chloramphenicol (100, 250 and 500 mg l$^{-1}$), streptomycin (100, 250 and 500 mg l$^{-1}$) and penicillin (100, 250 and 500 mg l$^{-1}$) for 24 hrs in view of the endogenous bacterial infection. After antibiotic treatment, infection-free explants were transferred to semisolid medium supplemented with: 6-benzylaminopurine (BAP), 0.2; indole-3-acetic acid (IAA), 0.5; gibberellic acid (GA), 25; adenine sulphate (AdS), 40; and L-glutamine, 25 mg l$^{-1}$. For further growth and proliferation, the explants were cultured in the same nutrient medium, but having reduced concentrations of IAA (0.25 mg l$^{-1}$), GA(1 mg l$^{-1}$) and AdS (25 mg l$^{-1}$). Surface-sterilized young tips of female and male inflorescences were cultured on the nutrient agar medium comprising BM1 supplemented with: BAP, 0.25; IAA, 1; GA, 1; 2-(chloroethyl) trimethylammonium chloride (CCC), 2.5; AdS, 40; and L-glutamine, 50 mg l$^{-1}$. Using medium BM1, the effects of different concentrations of IAA (0.1, 0.25, 0.5 and 1 mg l$^{-1}$) and BAP (0.1, 0.25, 0.5 and 1 mg l$^{-1}$) along with CCC (0.1, 0.25, 0.5, 1, 2.5 and 5 mg l$^{-1}$) and AdS (15, 25, 35 and 40 mg l$^{-1}$) were seen on shoot proliferation.

Nurturing of Isolated Shoots and their Rooting

Well-developed shoots were excised from cultures of proliferating shoots and nurtured using BM2 medium supplemented with: BAP, 0.01; IAA, 0.5; and AdS, 15 mg l$^{-1}$. The nurtured shoots having stout stem with good foliage (~ 4 cm long) were subjected to rooting treatments, using medium BM2 supplemented with: d-biotin, 0.1; CCC, 0.1; and AdS, 15 mg l$^{-1}$. Initially, effects of different concentrations of IAA (0.25, 0.5, 1, 2.5 and 3 mg l$^{-1}$), indole-3-butyric acid (IBA; 0.25, 0.5, 1, 2, 2.5 and 3 mg l$^{-1}$) and $\alpha$-naphthaleneacetic acid (NAA; 0.25, 0.5, 1, 2, 2.5 and 3 mg l$^{-1}$) in combination with riboflavin (4 mg l$^{-1}$) with or without phloroglucinol (1 mg l$^{-1}$) or chlorogenic acid (1 mg l$^{-1}$) were seen.

In another strategy, which was adopted in view of the excessive intervening callusing, shoots were subjected to the following sequence of rooting media: Step 1 - Culture of nurtured shoots in the liquid medium BM3 + 10 mg l$^{-1}$ IBA + 2% sucrose for 24 h; Step 2 - Transfer of shoots to the semisolid medium BM2 supplemented with 0.1 mg l$^{-1}$ d-biotin + 0.1 mg l$^{-1}$ CCC + 0.25 mg l$^{-1}$ IAA + 15 mg l$^{-1}$ AdS + 2% sucrose for 7 days; Step 3 - Transfer of shoots to the semisolid medium BM3 + 1 mg l$^{-1}$ thiamine-HCl + 0.5 mg l$^{-1}$ pyridoxine-HCl + 0.5 mg l$^{-1}$ nicotinic acid + 10 mg l$^{-1}$ L-arginine-HCl + 50 mg l$^{-1}$ L-glutamine + 0.25 mg l$^{-1}$ IAA; and Step 4 - Transfer of just rooted shoots to the semi-sterilized Soilrite (M/s Karnataka Explosive Ltd., Bangalore, India) contained in culture tubes (aseptic).

Acclimatization of In Vitro-raised Plants

In vitro-raised plantlets were acclimatized mainly by controlling the RH from 95% to 60% under high light intensity (37.5 $\mu$mol m$^{-2}$s$^{-1}$ quantum flux...
density) received from fluorescent tubes supplemented with incandescent bulbs. Plantlets were directly transferred to the various potting mixtures comprising garden soil, garden soil + leaf mould (3:1), garden soil + Soilrite (1:1) and Soilrite. In another approach, the just rooted shoots were incubated under aseptic conditions in semi-sterilized Soilrite (0.72 kg/cm² for 10 min) contained in cotton plugged tubes and hardened by gradually removing the plugs during a period of 7 days, after which the plantlets along with the Soilrite plugs were transplanted to potted garden soil+Soilrite (1:1) for ex vitro growth.

Results and Discussion

Establishment of Cultures and Shoot Proliferation

There was 100% contamination in cultures of shoot apices taken from mature plants in medium BM1 supplemented with: BAP, 0.25; IAA, 0.5; GA, 25; AdS, 40; and L-glutamine, 25 mg l⁻¹. In this medium, the contamination showed up after 10 to 25 days of incubation. In the same basal medium, supplemented with 0.25 mg l⁻¹ BAP, 1 mg l⁻¹ IAA, 2.5 mg l⁻¹ CCC, 40 mg l⁻¹ AdS and 50 mg l⁻¹ L-glutamine, the explants of young inflorescences were infection-free. The incidence of high bacterial infection in cultures, employing shoot apices of field-grown plants, has been a serious impediment in the clonal multiplication of *C. papaya*. Thus, the infection-free cultures obtained with the use of inflorescence explants is a great headway made in the process of in vitro cloning of this dioecious plant. The high incidence of endogenous infection may be assigned to the occurrence of well-developed laticiferous ducts in the stem as compared to their meagre development in young inflorescence. Regeneration from inflorescence explants has been found practicable in a number of other plant species, including intractable-to-regenerate trees, like, *Elaeis guineensis*, *Phoenix dactylifera* and *Cocos nucifera*.

The bacterial contamination associated with shoot tip explants was alleviated (up to 90%) with the incorporation of 250 mg l⁻¹ each of penicillin and streptomycin during the initial incubation for 24 hrs. The infection-free cultures of shoot apices as well as tips of inflorescences of both female and male plants were regenerative in the presence of 0.1 mg l⁻¹ BAP, 0.25 mg l⁻¹ IAA, 40 mg l⁻¹ AdS and 50 mg l⁻¹ L-glutamine, while the incorporation of 0.25 mg l⁻¹ CCC not only improved shoot proliferation, but also inhibited intervening callusing, which comprised the optimum treatment. The twin effects of CCC can be interpreted due to its antigibberellicin action. Gibberellic acid suppresses shoot bud differentiation as in tobacco stem callus and somatic embryo formation in callused hypocotyl of cantaloupe. A definite effect of CCC in suppression of callusing of shoots has been reported in *Vigna mungo* and in tomato. Similarly, with the suppression of endogenous levels of GA by antigibberellins (CCC and ancymidol), promotion of shoot regeneration has been reported in cultures of *Hibiscus acetosella*, *Cucumis melo*, *Citrullus lanatus* and *Psophocarpus tetragonolobus*. Particularly, regeneration of sturdy shoots was obtained in cultures of *V. mungo* in the presence of CCC and in *Asparagus* with the use of another antigibberellin, ancymidol. The rate of proliferation of offshoots increased up to 4th subculture of 20-day-duration each, after which it was stabilized. Initially, the transformation of young flower buds comprising tips of inflorescences took much time for their transformation into green shoot buds. However, once this stage was reached in the 2nd subculture, the rate of proliferation levelled up with that of shoots produced from shoot tip culture. The regenerative potentiality of cultures did not decline during long-term culture of 4 years, when the well-developed shoots with green foliage were obtained (Fig. 1). A group of an average 4 young shoots proliferated by a factor of 5 shoots per culture during 20-day-incubation. There was hardly any difference in the rate of multiplication or vigour of proliferating shoots in cultures raised from female or male plants as also from shoot apices of mature plants or from young inflorescence tips.

Root Induction in Isolated Shoots

Young tender shoots (~2 cm long), when subjected to rooting treatments, invariably callused at base from where the adventitious roots were formed. Such rooted shoots were unfit for ex vitro transplantation as they did not survive. Similarly, the sturdy shoots with well-developed stem (<4 cm long), showed excessive callusing of basal cut end preceding formation of abnormally swelled and fuzzy roots when subjected to different concentrations and combinations of various auxins with or without a phenolic acid. The effective concentrations of the three auxins (IAA, IBA and NAA) ranged between 0.5 to 2 mg l⁻¹ and IBA was found most effective. However, the concentrations of auxins, which were most effective for root induction in microshoots, were equally more potent to induce callusing preceding root induction. A combination of
IBA (0.1 mg l\(^{-1}\)) and NAA (0.5 mg l\(^{-1}\)) did induce about 80% rooting within 20 days, but the roots were not normal and had fuzzy and swelled appearance with excessive intervening callusing (Fig. 2 Lt). Addition of phloroglucinol or chlorogenic acid to the auxin containing treatment did not improve rooting but increased callusing. The use of riboflavin along with auxin did neither improve the condition of roots nor the percentage of rooting, which is contrary to the exclusive beneficial role assigned to it in root induction of isolated shoots of *C. papaya*\(^9,10\). Thus, the rooted shoots with excessive intervening callusing were also found unfit for ex vitro transplantation as all the plants died. This situation could be compared with the formation of multiple roots from the callused base of shoots of Citrus grandis and *C. aurantifolia*\(^26,27\) and of *Simmondsia chinensis*\(^28,29\). In all cases, *in vitro* rooted shoots died after transplantation to soil unless the intervening callusing was eliminated\(^27,29\), attributed to non-alignment of the conducting tissues of shoot and root.

The nurtured shoots, when processed through the improved rooting strategy using high concentration of auxin as a pulse treatment, while the basal medium was of purely inorganic salt composition, virtually resulted in elimination of callus formation at the cut end of shoots before their rooting. The incubation of shoots in the pulse treatment of high IBA (10 mg l\(^{-1}\)) gave optimum results at 24 hrs, far less effective at 12 hrs and promoted excessive callusing at 36 hrs at the cut end of shoots (Table 1). Subsequently the shoots, which had been subjected to pulse treatment for 24 hrs in *Step 1*, when transferred to the medium BM2 with specific supplements in *Step 2* for 7 days and then cultured in medium BM3 with the specific supplements in *Step 3*, produced young root primordia in about 95% shoots within 10 days. Such shoots with just visible roots, on transfer to the same agarified medium devoid of any supplements, (medium BM3), produced healthy and fast-growing roots (Fig. 2 Rt), but even such rooted shoots did not survive *ex vitro* transplantation. However, such shoots with just visible roots when transferred to the semi-sterilized Soilrite in *Step 4*, the roots grew, while shoots also remained healthy (Fig. 3). This strategy of involving the *Step 4* in the rooting process of the isolated shoots, in which auxin, vitamins and sucrose were absent from the substratum, is comparable with the normal rooting procedure. It is well known that an auxin is essential for induction of root primordia, but for their further growth it is not required as also that sugar is essentially required during the initial phase of root induction\(^30\).

**Hardening of Rooted Shoots and their Ex Vitro Growth**

During the *Step 4* of the *in vitro* cloning procedure, the rooted shoots had also been prepared for their subsequent growth in a potting mixture devoid of any carbohydrate source, thus, allowing growth of only autotrophic plantlets. During the *Step 4*, the plantlets, while still growing under aseptic condition, had been encouraged for restoration of normal photosynthetic process\(^31\). The process of rooting in the *Step 4* resembles with the *ex vitro* procedure reported for propagation of *papaya*\(^32\), but is free from its limitations resulting due to seasonal factors. Subsequent to *Step 4*, when conditions were created so as to have a humidity regime gradually from about 95% RH to about 60% RH during 10 days by gradually loosening the cotton plugs before their
complete removal, the plantlets continued to remain healthy with turgid leaves. Such hardened plantlets when finally transferred from culture tubes along with the Soilrite plugs to the mottled mixture of garden soil and Soilrite (1:1), showed about 80% transplant success. Again, prevention of plantlets from desiccation during the initial 7 days of ex vitro growth was crucial so also the scarce watering of the potted plants. Foliar feeding with mineral salts during initial 2 to 3 new leaves, were transferred to glasshouse conditions, where they grew normally (Fig. 4). It took about 6 months from establishment of aseptic culture conditions, where they grew normally (Fig. 4). It took about 6 months from establishment of aseptic culture from an elite field-grown plant to obtain a plant of an average height of 12 cm. The plants grown under field conditions were true-to-mother type and bore fruits about 6 months from establishment of aseptic culture from an elite field-grown plant to obtain a plant of an average height of 12 cm. The plants grown under field conditions were true-to-mother type and bore fruits.

### Acknowledgement

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### References


### Table 1—Effects of duration of pulse treatment of IBA 10 mg l⁻¹ on subsequent rooting of isolated shoots of *Carica papaya* processed through a 4-step procedure

<table>
<thead>
<tr>
<th>Length of exposure (h)</th>
<th>Time taken in root induction (days)</th>
<th>Rooting during step 3 (%)</th>
<th>Number of roots per shoot during step 4</th>
<th>Root length during step 4 (cm)</th>
<th>Intervening callusing</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>13-19</td>
<td>46.66 ± 6.66</td>
<td>2.11 ± 0.11</td>
<td>3.66 ± 0.08</td>
<td>Nil</td>
</tr>
<tr>
<td>24</td>
<td>9-13</td>
<td>95.00 ± 6.66</td>
<td>2.5 ± 0.10</td>
<td>2.78 ± 0.11</td>
<td>Nil</td>
</tr>
<tr>
<td>36</td>
<td>10-16</td>
<td>86.66 ± 6.66</td>
<td>5.06 ± 0.15</td>
<td>2.63 ± 0.18</td>
<td>Excessive</td>
</tr>
</tbody>
</table>

The data are based on 5 replicate cultures ±SE, while the experiment was repeated thrice.

*Details given in the Materials and Methods*


