A modified technique for in vitro propagation of papaya (*Carica papaya* L.)

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*Carica papaya* L. is an important crop in many subtropical and tropical countries. Many problem areas still exist with the commercial *in vitro* propagation of papaya. These include leaf senescence, stunted plants produced as a result of cytokinin toxicity, reduced rooting ability and poor root quality. A technique using activated charcoal at 3g l⁻¹ as an intermediary culture step before rooting, improved the overall condition and maturity of the microplants thus reducing the cytokinin-toxicity effects resulting from long term use of this phytohormone. Rooting was achieved by soaking the bases of the plantlets in a 5mg l⁻¹ IBA solution for one hour and transferring them to a sucrose-free vermiculite medium. This method improved on a double inoculation method previously used where plants were inoculated into an IBA containing medium and then transferred to an IBA-free medium three days later. To reduce ethylene build-up within flasks, the lids were modified to contain small openings plugged with cotton wool. These were removed during initial acclimatisation before transfer to the greenhouse.

**Abbreviations**: BA = benzyladenine, IBA = indole butyric acid, NAA = 1-naphthaleneacetic acid, PEG = polyethylene glycol

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**Introduction**

Papaya is grown commercially in many subtropical and tropical countries. Tissue culture of papaya has aided in rapid clonal propagation of selected lines. Methods for regeneration of *in vitro* papaya plants have been described using callus cultures (Debruijne *et al.* 1974, Litz and Conover 1977, Arora and Singh 1978, Jordan *et al.* 1983, Pandey and Rajeevan 1983, Fitch 1993), somatic embryos (Cheng *et al.* 1996, Ernawati *et al.* 1997, Castillo *et al.* 1998) and apical and axillary bud explants (Medhi and Hogan 1976, Drew and Smith 1986, Rajeevan and Pandey 1986, Drew 1992, Singh *et al.* 1997).

Many problem areas and limitations still hamper the commercial production of papaya *in vitro*. Callus production may lead to off-types (Larkin and Scowcroft 1981) while problems such as contamination at initiation and endophyte contamination after several subcultures remain major problems. Other problem areas include the reduction of proliferation rates and thus the inability to subculture indefinitely due to cytokinin toxicity (Drew 1988), leaf senescence and poor rooting and its effect on subsequent acclimatisation (Magdalita *et al.* 1997).

Methods used to promote rooting include the transfer of papaya plantlets from an IBA medium to an agar medium free of plant growth regulators after three days in the dark (Drew 1988). This method (Drew 1988) involves two subcultures within three days which, if not strictly managed, would result in roots being exposed to IBA for too long. Exposure of papaya roots to IBA for longer periods resulted in damaged roots (Magdalita *et al.* 1997). Single node sections of rooted cuttings were later used as an alternative explant source (Drew 1992). Although high rooting percentages were achieved with the above nodal culture technique, the plants had small shoots and callused roots (Drew *et al.* 1993). Plants with poor shoot growth (length and vigour) were difficult to acclimatise. Roots formed on agar also did not produce root hairs and sometimes there was little vascular continuity between the shoot and root system (Drew 1999 pers. comm.). A one-step method using riboflavin injection into the plantlets at the rooting phase, instead of transfer to hormone-free medium from the dark to light, saved time and cost (Drew *et al.* 1993).

The addition of activated charcoal to plant growth media is known to have many beneficial effects. These include the release of naturally present or previously adsorbed compounds into the media and the creation of a dark environment, thus facilitating the accumulation of photosensitive auxins or co-factors and encouraging a rooting response (Pan and Van Staden 1998). Other beneficial effects include the adsorption of inhibitory compounds such as phenolics (Weatherhead *et al.* 1978) and plant growth regulators such as cytokinins, auxins and ethylene (Ebert *et al.* 1993). The effect of activated charcoal on the micropropagation of...
papaya was studied in an attempt to reduce cytokinin toxicity. Premature leaf senescence during the culture period remains a major practical problem preventing this technique from becoming established commercially. Closed conditions are known to cause the accumulation of gases in the headspace of the vessel. Compounds such as ethylene have been identified. Silver thiosulphate (STS) at 0.3mM, increases leaf area production and reduces leaf senescence (Magdalita et al. 1997) when using a nodal culture system (Drew 1988). The incorporation of a 0.02μm filter disc into the vessel lid to regulate ethylene exchange has been suggested (Yang et al. 1997), however, this option is costly. Considering all the problem areas, innovative research is necessary to shorten the culture cycle and improve on current techniques so that they may be applied commercially to papaya.

Materials and Methods

A Sunrise Solo selection of Carica papaya L. called AF1 was used in all trials. Greenhouse grown plants were sprayed with a solution of 5ml l–1 Promalin® (mixture 1:1 v/v of BA at 19g l–1 and GA3 + GA4 at 19g l–1). Elongated axillary buds/shoots were removed one month later. The shoots were rinsed in soapy water, soaked and agitated in the fungicides Captan® (active ingredient captab, 2g l–1) and Benlate® (active ingredient benomyl, 1g l–1) for 1h. The explants were decontaminated in a 1% sodium hypochlorite solution with three times in sterile distilled water, immersed into a 50mg l–1 Rifampicin solution for 20min, dipped in 70% ethanol for 10s and finally rinsed twice in sterile water.

The plants were initiated on Drew and Smith (1986) (DS) vitamins and salts, 0.5mg l–1 BA and 0.1mg l–1 NAA, 2% sucrose and 6.5g l–1 agar.

**Multiplication of papaya — the effect of charcoal and gelrite on multiplication**

Media were prepared containing 20g sucrose, DS salts and vitamins, 1mg l–1 NAA and 1mg l–1 BA with the following treatments: A, 2g l–1 gelrite; B, 7g l–1 agar (Unilab — Saarchem, No. 1065000); C, 1.5g l–1 activated charcoal (Sigma) and 2g l–1 gelrite; and D, 3g l–1 activated charcoal (Sigma) and 2g l–1 gelrite. The pH of the media were adjusted to 5.7 with 1M KOH or HCl and hot dispensed into glass jars (70mm high, volume capacity 100ml) with polycarbonate screw lids and autoclaved at 121°C for 22min at a pressure of 110kPa. Shoots were multiplied from actively growing excised side shoots 5–10mm long. Plants were transferred aseptically into the jars and incubated at 25 ± 2°C at 16:8h light/dark cycles (45μmol m–2 s–1) using cool white fluorescent tubes. Multiplication rate (calculated as the number of shoots removed from the culture after the four week growing period) and hyperhydricity were recorded after four weeks.

The effect of charcoal on appearance and multiplication rate of papaya

Media were prepared containing 20g sucrose, DS salts and vitamins, 1mg l–1 NAA and 1mg l–1 BA and solidified with 6g l–1 agar. Activated charcoal was added to the media in the following concentrations: 0g l–1, 1g l–1, 2g l–1 and 3g l–1. The pH of the media were adjusted to 5.7 with1M KOH and HCl before autoclaving. Plants were grown as described in the first experiment. Observations regarding appearance and senescence were recorded.

The effect of vermiculite as a supporting medium on root production

Glass jars containing 40ml dry vermiculite and 40ml of the following:

1. DS salts and vitamins — control;
2. 1g l–1 PEG 6000 with DS salts and vitamins;
3. 3g l–1 PEG 6000 with DS salts and vitamins;
4. L-cysteine (60mg l–1) with DS salts and vitamins; and
5. 3g l–1 PEG-6000 with 3g l–1 activated charcoal and DS salts and vitamins were autoclaved at 121°C for 22min. Papaya plantlets were pulsed in a solution containing 5mg l–1 IBA for 1h under sterile conditions and grown for four weeks under conditions described earlier. The number and appearance of roots were recorded.

The effect of cotton wool plugs on senescence and acclimatisation of papaya

Uniform plantlets from multiplication media containing 20g l–1 sucrose, DS salts and vitamins, 1mg l–1 NAA and 1mg l–1 BA, solidified with 6g l–1 agar were pulsed aseptically with 5mg l–1 IBA for 1h, then placed aseptically into vermiculite media. The flasks were sealed with lids modified by cutting a 12mm diameter hole with a cotton-wool plug inserted into it. Plants were grown in a growth chamber until rooted (4 weeks) under the same conditions as outlined earlier in this report, after which the cotton-wool plugs were removed to observe the effect on acclimatisation in a growth chamber.

Results and Discussion

Papaya multiplication rate was affected by the type of gelling agent used, with agar it was significantly reduced (Figure 1). Hyperhydricity on gelrite alone was severe and all plantlets had to be discarded. The multiplication rate for plantlets grown on gelrite with activated charcoal was significantly reduced when 3g l–1 activated charcoal was used. The multiplication rate for plantlets grown on 1.5g l–1 activated charcoal and gelrite was significantly higher than when grown on agar alone and almost equaled the multiplication rate of those grown on gelrite alone. However, the addition of 1.5g l–1 activated charcoal to the media was not sufficient to eliminate hyperhydricity completely. Drew et al. (1993) also demonstrated that 2g l–1 activated charcoal added to the media gave finer roots and smaller shoots compared to those produced on a riboflavin treatment.

The addition of activated charcoal to growth media is known to have various effects on plants such as the adsorption of inhibitory compounds such as phenolics (Weatherhead et al. 1978) and plant growth regulators such as cytokinins, auxins and ethylene (Ebert et al. 1993). It is possible that activated charcoal played a role in reducing hyperhydricity in papaya by the adsorption of such inhibitory substances.
An experiment using agar as a gelling agent with various concentrations of activated charcoal showed that activated charcoal applied at a concentration of 3 g l⁻¹ had a marked effect on overall appearance of the micro plants (Table 1). The leaves were more mature, stems darker in colour and petioles red, all indications of a more mature, healthier plant. Smooth white roots were formed on plantlets grown on a medium supplemented with 2 g l⁻¹ activated charcoal (Figure 2A).

Since the repeated use of cytokinin in the multiplication media could lead to toxicity effects, it is recommended that 3 g l⁻¹ charcoal be added into the medium as an additional preconditioning step prior to rooting. Although results show that the multiplication rate is lowered with the addition of this amount of activated charcoal, the overall growth of the plant was improved. This increases the chances for successful acclimatisation.

Plants placed into a pure vermiculite medium plus DS salts and vitamins and one with PEG-6000 plus activated charcoal had the highest number of roots (Table 2). The roots (Figure 2B) formed in these treatments were stronger than the roots formed on vermiculite medium with PEG-6000 alone and L-cysteine alone. None of the treatments were significantly different in terms of rooting percentage. PEG is known to assist in the acclimatisation of micropropagated plants (Zaid 1981) by contributing to better stomatal closure and uniform growth of the plants (Yu et al. 2000). However, instead of improving root development on an agar medium for a week, an IBA pulse was also effective, reducing handling, making the process more economical.

No leaf senescence occurred in cultures with cotton-wool plugged lids (Figure 2C) compared to where the containers were sealed. Once the plants had rooted, the plugs were removed. The initial response in sealed containers was to shed most of the leaves. Following this, new leaves started to form. Since no sugars were placed into the vermiculite medium, micro-organism growth was minimal. After six weeks, the hardened plant was ready for transplanting.

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Table 1: The effect of activated charcoal concentration on appearance and senescence of papaya in vitro

| Charcoal conc. (g l⁻¹) | Appearance Senescence |
|-----------------------|-----------------------|
| 0                     | Veined, spindly, yellow leaves | Yes |
| 1                     | Light yellow leaves | No |
| 2                     | Green leaves | No |
| 3                     | Mature palmate leaves, dark green | No |

Table 2: The effect of vermiculite as a supporting medium with various acclimatisation treatments on papaya rooting in vitro. LSD at 0.05 alpha level = 46.081 (ANOVA). NS in table indicates non-significance at the 0.05 level on rooting percentage

| Treatment | % rooting | Root appearance |
|-----------|-----------|-----------------|
| 1         | 80 NS     | Strong, attached |
| 2         | 40 NS     | Many roots, root hairs visible |
| 3         | 80 NS     | Strong, attached |
| 4         | 75 NS     | Longer roots, root hairs visible |
| 5         | 80 NS     | Highest number of roots, root hairs visible |
Figure 2: A: Papaya plantlet with smooth roots formed on medium supplemented with 2g l⁻¹ charcoal; B: Papaya roots formed on medium in vitro; C: Papaya plantlet grown on vermiculite medium with a lid containing a cotton-wool plug
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