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An alternative *in vitro* plant regeneration system in papaya (*Carica papaya* L.) through callus derived nodular cultures

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ABSTRACT

In vitro regeneration systems either based on organogenesis or somatic embryogenesis has been well established in *Carica papaya* L. Whereas a third morphogenetic pathway involving plant regeneration system through nodular cultures have not been reported. Here, we report an efficient and rapid *in vitro* regeneration system through callus derived nodular culture in papaya cultivar CO7. Excised zygotic embryos were cultured onto Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of auxins and cytokinins. Maximum response from explants producing callus (82.30%) was observed on MS medium with 2.0 mg/l of 2, 4, 5 trichlorophenoxyacetic acid (2, 4, 5-T), whereas a high frequency of nodulation (62.30%) was induced on MS medium containing 1.0 mg/l of 2, 4, 5-T. Nodules were separated from callus cultures and transferred onto MS medium fortified with 0.4 mg/l BAP (6-benzylaminopurine) and 0.04 mg/l NAA (α-naphthaleneacetic acid) showed regeneration efficiency of up to 62.22%. The healthy elongated shoots with 2–3 trilobed leaves were successfully rooted (72.12%) on a half-strength MS medium containing 1.0 mg/l indole-3-butyric acid (IBA) with an average root numbers 4.0 ± 1.2. The histochemical analysis of nodules authenticates the distinctive anatomy exhibiting the vascular elements bounded with the cortex and epidermal layering. In the present study, morphogenetic features of papaya regeneration through nodular cultures reveal distinct characteristics from organogenesis and somatic embryogenesis.

1. Introduction

Papaya (*Carica papaya* L.) is an essential all season fruit crop widely grown in the tropics and sub-tropics around the world. In India, it is cultivated on an area around 1.33 lakh ha with an annual production of 5.69 million tons (FAOSTAT, 2016). Papaya fruits contain high levels of vitamins A and C. It is consumed as fresh fruit, whereas unripe fruits synthesize significant quantity of the proteolytic enzyme 'papain', utilized in chill proofing of beer, meat tenderization, and several pharmaceutical products (Manshardt, 1992; Bhattacharya and Khuspe, 2001). It is also a rich source of pectin and alkaloids like carpaine, which have a great potential for export.

Cross-pollinated papaya shows greater genetic variability through seed propagation and therefore vegetative propagation from selected clones has turned out to be very alluring (Bhattacharya and Khuspe, 2001; Fernando et al., 2001). However, its cultivation is constrained because of the inherent heterozygosity, creation of non-true-to-types and also to papaya ring spot viral infection (Chen et al., 1987; Fitch, 1993; Yang et al., 1996; Bhattacharya and Khuspe, 2001; Clarindo et al., 2008). Thus, the advancement of a proficient regeneration protocol is fundamental to *in vitro* mass proliferation and the genetic engineering of papaya (Anandan et al., 2012).

Till date, *in vitro* regeneration of papaya was accomplished through the adaptation of conventional regeneration methods based on organogenesis (Litz et al., 1983; Bhattacharya and Khuspe, 2001; Anandan et al., 2011; Teixeira da Silva, 2016; Kanth et al., 2017), embryogenesis (Jordan et al., 1982; Fitch and Manshardt, 1990; Ascencio-Cabral et al., 2008; Posada-Pérez et al., 2017) and suspension cultures (Litz and Conover, 1983; Anandan et al., 2012).

Tissue demonstrating nodule like structures that emerge straightforwardly from explants or callus and from cell suspension cultures has been proposed as reasonable for *in vitro* plant proliferation. McCown et al. (1998) detailed the nodules are made of meristematic cell clusters containing independent, dense, spherical cells, which plays an important role in a steady internal cell and tissue differentiation. Earlier research on *in vitro* regeneration of popular tissues uncovered the nodules are comprised of three cell types (meristematic cells, plastid-thick parenchyma cells, and vascular components) and two cell layers (epidermal and inward cortex/vascular) as suggested by McCown et al. (1998).

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Nodules can multiply to vast quantities of little minimal daughter nodules and have a high regenerative potential by means of organogenesis. In this manner, this morphogenetic pathway, having distinctive characteristics from organogenesis and somatic embryogenesis, was delegated as a third pathway of *in vitro* plant morphogenesis (George, 1993). There have been a few reports concerning the regeneration of woody and herbaceous species from nodular, for example, *Pinus radiata* (Aitken-Christie and Singh, 1988), *Eucalyptus grandis* (Warrag et al., 1991), *Lilium longiflorum* (Godo et al., 1998), *Humulus lupulus* (Batista et al., 2000), *Acacia mangium* (Xie and Hong, 2001) and *Ananas comosus* (Teng, 1997; Scherer et al., 2015).

Albeit a few reports on conventional regenerative systems have been published; a compelling *in vitro* plant regeneration system through nodular culture in papaya has not been accounted till date. Here, we report a proficient and speedy *in vitro* regeneration system through callus derived nodular culture in papaya cultivar CO7.

2. Materials and methods

2.1. Explants preparation

Seeds of Indian papaya cultivar CO7 (80, 100, 120, 140 and 150-days old) were collected from the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, India. Collected fruits were washed thoroughly in running tap water and then immersed in 1.25% sodium hypochlorite solution containing two drops of Tween 20 for 30 min. The disinfected fruits were rinsed with sterile distilled water for three times. After cleansing, the fruits were cut opened aseptically and the seeds were gathered in 100 mm × 15 mm sterile Petri dishes (Borosil, India). Using a stereo-microscope (Leica, Switzerland), the seeds were dissected and embryos were excised aseptically in a laminar air flow chamber (Clean Air systems, Chennai, India).

2.2. Callus initiation and nodulation

The culture media for callus induction and resulting nodulation comprised of Murashige and Skoog (1962) (MS) medium containing 3% sucrose, 4% (m/v) phytagel and supplemented with either abscisic acid (ABA; 0.5, 2.0, 4.0 and 6.0 mg/l) or indole-3-acetic acid (IAA; 0.5, 2.0, 4.0 and 6.0 mg/l) or 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T; 0.5, 1.0, 2.0, 4.0 and 6.0 mg/l). The pH of the medium was changed in accordance with 5.8 using 1 N HCl or 1 N NaOH prior to autoclaving at 121 °C and 1.06 kg cm^{-2} pressure for 20 min. After sterilization, around 25 ml of medium was dispensed into culture plates of size 100 × 15 mm (Borosil, India) and cultures were incubated at 25 °C in complete darkness. The cultures were maintained up to 6–8 weeks with frequent sub-culturing for every 15 days to fresh medium with similar plant growth regulators (PGRs) supplements for further proliferation of callus as well as to induce nodule formation. The nodules developed on ABA and 2, 4, 5-T and the calli developed on IAA were considered as starting tissues for regeneration experiments. Data on the frequency of callus induction and nodule formation of callus were recorded after 6 weeks of culture. Callus frequency was determined as follows: Callus induction frequency (%) = (number of explants with callus/total number of explants cultured) ×100.

2.3. Plantlets regeneration from nodules

The nodules obtained from ABA, 2, 4, 5-T and IAA containing media were transferred onto 250 ml Magenta vessel (Himedia, India) containing 50 ml of MS medium supplemented with casein hydrolysate (100 mg/l), malt extract (100 mg/l), sucrose (30 g/l) phytagel (4 g/l) and varying concentrations of 6-benzylaminopurine (BAP; 0.2, 0.4, 0.6, 0.8, 1.0 mg/l) or kinetin (0.2, 0.4, 0.6, 0.8, 1.0 mg/l) in combination with α-naphthaleneacetic acid (NAA; 0.02, 0.04, 0.06, 0.08, 0.1 mg/l) for adventitious shoot organogenesis. All the cultures were maintained in a plant growth chamber (25 ± 2 °C, 80–85% relative humidity, 16/8-h photoperiod, and 80 μmol $m^{-2} s^{-1}$ illumination). The MS basal medium devoid of PGRs was served as control. These cultures were sub-cultured on the same media once in every 15 days and maintained up to 6 weeks. Observations on the percentage of shoot organogenesis from nodules, mean shoot length and average number of leafs were recorded after 6 weeks of culture. The regeneration response was calculated using the following formula: Regeneration response (%) = (number of nodules showing shoot initiation/total number of nodules cultured) ×100.

2.4. Root induction and acclimatization

Regenerated healthy shoots (about 2 to 4 cm in length) with 2–3 trilobed leaves obtained from shoot organogenesis medium were cultured onto rooting media containing half-strength MS medium supplemented with varied levels of either indole-3-butyric acid (IBA; 0.5, 1.0, 1.5 and 1.0 mg/l) or NAA (0.5, 1.0, 2.0 and 4.0 mg/l) each for root induction. These cultures were maintained up to 4 weeks at 16 h photoperiod in a plant growth chamber. After a period of 4 weeks, data regarding the frequency of root induction, average number of roots per shoot and root lengths were recorded. Plantlets with a proper rooting system measuring a height of 4–6 cm were uprooted gently from magenta vessel and washed gently in tap water to remove the adhered media before transferring to a plastic pot containing sand, soil and vermiculite (1:1:1). The plastic pots were secured with polyethylene bags, and openings were expanded continuously to encourage trade of gases. These potted plants were kept in greenhouse with 50% sunlight reduction, 25 ± 2 °C temperatures, and intermittent mist. The plastic spreads were evacuated once the plants were settled. Hoagland solution was showered over the potted plants at an interval of 5 days. The plant survival rate in the greenhouse was recorded following two months. For each treatment, data on percentage of rooting response, average number of roots per shoot and mean root length were recorded after 4 weeks of culture.

2.5. Histochemical analysis of nodules

For histological examination, callus tissues bearing the nodules were fixed in FAA (95% ethanol + glacial acetic acid + formalin + distilled water in the proportion of 10:1:2:7) for 24 h and then stored in 70% (v/v) ethanol. Then the samples were dried using absolute ethanol and embedded in paraffin wax. Fine segments (3–5 μm thick) were sectioned using a microtome, mounted on glass slides and stained with Safranin (0.5%) and Fast green (0.1%) for microscopic examination using an Olympus BX51 microscope.

2.6. Statistical analysis

All experiments were conducted in a completely randomized design (CRD). The collected data was analyzed using IRRISTAT version 3/93 developed by Biometrics unit, International Rice Research Institute, Manila, The Philippines. The data were presented as a mean ± standard error (SE) of three replications. Significant differences were assessed by using Duncan's Multiple Range test (DMRT) at the 5% probability level (Duncan, 1955).

3. Results

3.1. Explant preparations

The zygotic embryos excised from 80, 100 and 140–150 days old fruits were failed to produce callus at all levels of hormones tested (data not shown). Whereas profuse callusing was observed among the zygotic embryos excised from white seeds of 120 day-old fruits. Hence, the