



Plantlet regeneration via direct and indirect somatic embryogenesis from inflorescence explants of arecanut palms (*Areca catechu* L.)

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Abstract

Plantlet regeneration from inflorescence explants of yellow leaf disease (YLD) resistant arecanut cultivar south kanara local was achieved by both direct and indirect somatic embryogenesis. This is the first report of direct somatic embryogenesis from inflorescence explants of arecanut. A total of 10 inflorescences were extracted from healthy palms of YLD - hot spot areas from Sullia in Dakshina Kannada during 2005 and 2006. Out of these, direct somatic embryogenesis was obtained from six inflorescences, which is ideal because it allows the production of plants without a callus phase leading to somaclonal variation and hence useful for efficient genetic transformation. The vigour of directly differentiated somatic embryos was much higher than indirect somatic embryo development. This was achieved in Y3 basal medium supplemented with two levels of picloram (100 μ M, 200 μ M). Induction of embryogenic potency and formation of somatic embryos were noticed when the cultures were passed from higher to lower concentration of picloram and then to a hormone free medium. Somatic embryos proliferated rapidly in subsequent cultures. Maturation of somatic embryos and germination occurred in 1/2 MS medium supplemented with 1mg/l BA and subsequent plantlet development was achieved by transferring them to same basal medium supplemented with 5 mg/l BA and 0.5mg/l IBA, followed by 10mg/l BA, 1mg/l IBA and 1mg/l NAA. Anatomical studies were conducted at various developmental stages of both direct and indirect somatic embryogenesis. Histological studies revealed that somatic embryos arose indirectly from a single callus cell whereas the origin of direct somatic embryos was multicellular, without a callus phase.

Key words : Arecanut, Yellow Leaf Disease, somatic embryogenesis, in vitro multiplication, picloram
Abbreviations : Yellow Leaf Disease., BA- 6-Benzyladenine; Picloram- 4-amino 3,5 6 trichloro picolinic acid; NAA- 1-naphthalene, acetic acid; IBA- Indole 3-butyric acid; MS- Murashige and Skoog (1962) medium

Introduction

Arecanut (*Areca catechu*. L) is an important commercial plantation crop in India. The economic produce is the fruit called betel nut and is used mainly for masticatory purpose. The major problem of arecanut cultivation is the Yellow Leaf Disease (YLD), a serious malady caused by *Phytoplasma* affecting areca palms in Kerala and Karnataka States. The yield loss is as high as 50% over a period of three years (Nambiar, 1949). Screening trials of all the released varieties and hybrids revealed all of them to be susceptible to YLD (Ravindran *et al.*, 2000). However, breeders have identified some

field tolerant palms in hot spot areas and they are being utilized for breeding to evolve resistant/tolerant lines. Large-scale multiplication of YLD- resistant arecanut palms through tissue culture would facilitate supply of disease free quality seedlings to the farmers.

Application of tissue culture technology for clonal multiplication of coconut (Blake and Eeuwens, 1983; Raju *et al.*, 1984.,Raju,2006), and oil palm (Raju *et al.*, 1989 Anitha Karun and Sajini, 1996; Rajesh *et al.*, 2003) has been well demonstrated. *In vitro* multiplication of arecanut has already been successfully achieved at CPCRI (Anitha Karun *et al.*, 2004). The protocol developed earlier was slightly modified for mass

multiplication of YLD- resistant arecanut palms. This paper reports direct somatic embryogenesis and plantlet regeneration from immature inflorescence explants of YLD resistant arecanut palms.

Material and Methods

A total of 44 palms were identified through serology and biochemical diagnosis as disease free elite palms in endemic hot spot areas of Sullia Taluk of Dakshina Kannada (Anon; 2003). For the present study, inflorescence explants from ten field resistant arecanut palms of 25-30 years age from Sullia Taluk were collected through non-destructive sampling during January (three Palms) and November (seven Palms), 2005. Excised spadices (2nd and 3rd leaf axil) of 20-40 cm length (Fig. A) were swabbed with alcohol in the field and stored in clean polythene bags, placed in an ice box and brought to the laboratory. In the laboratory, the spadices were again swabbed with alcohol to sterilize them and aseptically inoculated into culture media after chopping the rachillae into smaller pieces of 1mm. The middle and basal portion of the rachillae were utilized for regeneration study. From each inflorescence a total of 240-480 cultures (6-12 replications) could be raised initially, depending upon the size of the inflorescence excised (Table 1).

Table 1. Details of the number of explants inoculated from each of the ten inflorescence (25 to 30years old)

Palm no.	1	2	3	4	5	6	7	8	9	10
Infl. size(cm)	21	18.5	28	30	28	24	38	25	23	32
No. of Cultures initiated (Pic 100 and 200 μM)	240	240	480	240	480	480	240	240	240	240

Culture medium

CPCRI arecanut regeneration protocol (Anitha Karun *et al.*, 2004) already standardized was applied with slight modifications. Initially, all the cultures were inoculated into Eeuwens' Y3 medium (Eeuwens, 1978) supplemented with two levels of picloram (100 and 200 μM). Medium also contained 3% sucrose, 0.1% activated charcoal and 0.6% agar. The pH was adjusted to 5.7 using 1N NaOH/ 1N HCl before autoclaving for 20 minutes at 1.06 kg cm⁻².

Culture conditions

Initially, cultures were incubated in dark where the temperature and RH were maintained at 27 \pm 1 $^{\circ}\text{C}$ and 80% respectively. Once calli were initiated from the explants, the cultures (after 8 months) were transferred to illuminated room (40 $\mu\text{E}^{-2} \text{ s}^{-1}$) provided with yellow fluorescent tubes (Philips) with photoperiod of 16 hrs light and 8 hours darkness.

Somatic embryos, Callus Induction and maintenance

After one month *in vitro* the explants were sub-cultured to media containing lower levels of picloram 50, 25, 10, 5 μM (for cultures in 100 μM) and 100, 50, 25, 10, 5 μM (for cultures in 200 μM), and finally to a hormone-free medium. After 13 weeks of inoculation, swelling of the rachis and rachillae and callus induction was observed. Proliferation and germination of arecanut somatic embryos were obtained on 1/2 strength MS medium (Murashige and Skoog, 1962) supplemented with 1 mg/l BA. Later the embryos were transferred to media containing 5 mg/l BA and 0.5 mg/l IBA, followed by 10 mg/l BA, 1 mg/l IBA and 0.5 mg/l NAA for plantlet development.

Ex vitro establishment: Plantlets with balanced root and shoot system were removed individually from the culture tubes and washed with sterile water. Before transferring to pots, the plantlets were treated with 1% Bavistin. The potting mixture used was sterilized sand and soil in 5:1 ratio. Initially, the plantlets were covered with polythene bag (Fig.K). Gradually providing the perforated polythene bags regulated the relative humidity and later the bags were removed during the night. After 4 weeks, the bags were removed completely (Fig.L). The regenerated plantlets were successfully transferred to

glass house as described by (Anita Karun *et al.*, 2004)

Histology studies

Cultures of various stages of development of calli induction, direct somatic embryogenesis, secondary somatic embryogenesis and meristemoid formation were fixed in standard fixative [Carnoy's 'B' fluid (chloroform-30 ml; absolute alcohol-60 ml; glacial acetic acid-10 ml)] for 24 hours. Fixed materials were dehydrated serially with alcohol and butanol. The materials were embedded in bee's wax and paraffin wax in the ratio of 9:1. Uniformly thin sections of 10 μm were cut using a Leica rotary microtome. The fixed sections in micro slides were dehydrated and stained by using three histochemical stains, toluidine blue, periodic acid Schiff's reagent and mercuric bromophenol blue. The sections were subjected to histological staining for localization of different cellular metabolites like nucleic acid, polysaccharides and proteins. The photographs were taken with the help

of a Leica image analyzer.

Results and Discussions

Intense browning of the explants was observed initially in both the picloram levels (Fig. B). Browning of explants is a major problem in palm tissue culture. However, the browning induced by picloram had no harmful effect on induction of callus. Swelling of the rachis and rachillae was apparent within 13 weeks of subculture. These swellings lead to the subsequent production of direct somatic embryos. Upon transfer to these explants to low picloram medium, direct somatic embryogenesis was seen to develop from the peripheral regions of vascular bundle. Serial transfer of cultures from higher to lower levels of picloram and then transferring the explants into a hormone free medium resulted in the formation of direct somatic embryos without an intervening callus phase (Fig. E) In 10% of the cultures, embryogenic calli were formed after 25 weeks of culture. The direct somatic embryos were larger and they exhibited vigorous germination and growth, and also shoot development (Fig. I).

Histological studies revealed that depending upon the size of rachis, a number of direct somatic embryos of different stage of development were noticed. The number of somatic embryos obtained ranged from 0-40 in cultures containing picloram 200 μ M and 0-10 in cultures having picloram 100 μ M (Table 2). The direct somatic embryos were observed to originate from the peripheral vascular bundle of the rachilla explants (Fig. D) and it is of multicellular origin.

cultures, calli arose from the basal region. These calli, in subsequent transfer to the picloram media, resulted in secondary somatic embryos.

Direct somatic embryogenesis in culture proceeds from cells, which were already determined for embryogenic development prior to explanting i.e they are pre-embryogenic determined cells requiring on growth regulators or favourable conditions to allow release in to cell division at expression of embryogenesis (Williams and Maheswaran, 1986). Direct somatic embryogenesis was initiated from multicellular origin from the peripheral vascular bundle by the action of growth regulator (Picloram). There are fundamental homologies between direct or indirect somatic embryogenesis and between single cell and multiple cell initiation (William and Maheswaran, 1986.). However, the histological studies carried out revealed that the direct somatic embryos of arecanut had a multicellular origin. The embryoids were formed inside the parental tissue and later emerged out from the parental tissue. Direct somatic embryos formation was also reported in immature embryos of *Trifolium repens*, *Trifolium praetense* and *Medicago sativa* and rapid clonal propagation of *Trifolium repens*. (Maheswaran and Williams, 1984). A maximum of 26.25% of direct somatic embryos as obtained from inflorescence explants of palm No.3 after 30 weeks of culture.

Indirect somatic embryogenesis: Indirect somatic embryos developed through intermediary callus stage. Callus induction was noticed after 12 weeks of

Table 2. Details of callus induction (%) and formation of direct somatic embryos (numbers) after 7th month of subculture

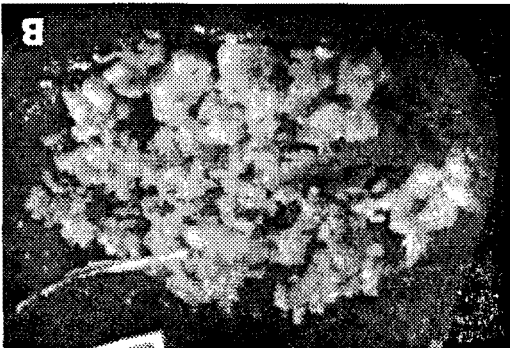
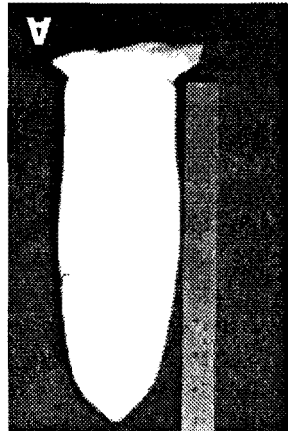
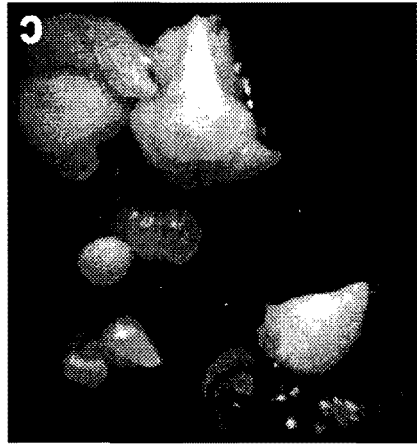
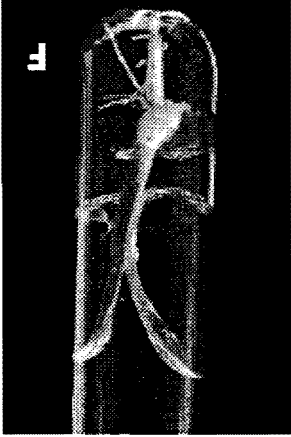
Palm no. →	1		2		3		4		5		6		7		8		9		10	
	C	SE	C	SE	C	SE	C	SE	C	SE	C	SE	C	SE	C	SE	C	SE	C	SE
Pic 100 μ M	33	0	42	10	33	10	23	0	52	0	25	3	25	0	9	0	40	0	30	0
Pic 200 μ M	42	0	50	13	63	40	53	8	33	3	77	12	50	3	5	0	25	0	25	0

C - Callus, SE - Direct somatic embryo

In arecanut, the presence of charcoal (2 gm/l) was found to be necessary for the callus induction and somatic embryogenesis. However, Tisserat (1982) reported that in date palm, calli could be sub-cultured and proliferated on medium containing auxin but devoid of charcoal. Proliferation of arecanut somatic embryos was obtained on ½ strength MS medium supplemented with 1mg/l BA and somatic embryo maturation and germination was observed in the same medium (Fig. H). Subsequent subcultures were done in same basal medium with 5 mg/l BA and 0.5 mg/l IBA followed by 10 mg/l BA, 1 mg/l IBA, 0.5 mg/l NAA for plantlet development. After germination of direct somatic embryos, in 1% of the

inoculation. Histological studies revealed that calli originated from vascular bundles of inflorescence rachis/rachillae. After complete development, it got detached from the parent tissue and developed into a complete plantlet. Culture medium, in vitro and ex vitro establishment of plantlets for both direct and indirect somatic embryogenesis are similar. A maximum of 67.5% of indirect somatic embryos were obtained from inflorescence of palm No. 3 after 52 weeks of culture.

Indirect somatic embryos had a unicellular origin as the embryoids were attached by a narrower suspensor to the parent tissue. In oil palm, the embryogenic calli originated from a group of cells (multicellular) present



towards the periphery of the calli (Schwendiman *et al.*, 1988). In peanut (Baker and Wetzstein 1992; Chengalrayan *et al.*, 1994) and in chickpea (Suhasini *et al.*, 1994; Barna and Wakhlu, 1995) somatic embryos have been reported to occur in two steps, one involving embryo induction in the presence of low levels of NAA along with high levels of BA and the second involving embryo development in the presence of IAA and BA. However in our studies direct somatic embryogenesis occurred in high auxin medium favouring embryo induction and the gradual reduction and omission of auxin resulted in embryo development. Goh *et al.* (1999) reported the superiority of picloram over other auxins like 2,4-D and also suggested an effective uptake and mobilization of this growth regulator coupled with rapid metabolization at target sites. Positive response of picloram recorded in the present study is in agreement with that reported in other palms.

Picloram has been used successfully in tissue culture of various plants with no adverse effect on the callus or subsequently regenerated plants (Conger *et al.*, 1983; Groll *et al.*, 2001). Stimulation of direct somatic embryo induction from non-embryogenic tissue by cytokinin supplementation has also been well documented in leguminous crops like Phaseolus and winged bean (Malik and Saxena, 1992; Ahamed *et al.*, 1996). However, in arecanut the induction of direct somatic embryo was stimulated by an auxin alone. Auxins primarily induce callus and promote development of somatic embryos (Groll *et al.*, 2001; Beyl and Sharma, 1983). Secondary somatic embryogenesis has been described in nearly one hundred species (Groll *et al.*, 2001). However, secondary embryogenesis frequently shows low conversion rate in to plants.

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