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Regeneration of Plantlets Through Somatic Embryogenesis from Nucellus Tissue of *Citrus macroptera* Mont. var. *anammensis* ('Sat Kara')

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Abstract

Somatic embryogenesis and plantlet regeneration were achieved in callus cultures of nucellus derived from undeveloped ovule of immature fruits of *Citrus macroptera*. Four types of media were used but only modified MS medium supplemented with malt responded well. Calli produced in malt supplemented MS medium were embryogenic in nature. After transfer of this embryogenic callus in hormone free MS medium, somatic embryos were developed. Independent plantlets were developed from these somatic embryos by further subculture in the same medium. After five weeks of culture the plantlets were transferred to pot soil mixed with cow-dung derived biogas slurry and survived well.

Introduction

Citrus macroptera Mont. var. *anammensis* is commonly known as Sat Kara, the most popular and expensive citrus fruits grown in greater Sylhet areas of Bangladesh. It is a semi wild species and used as medicine by local tribes of Assam, India (Ghosh, 1990). In Bangladesh, both green and mature fruits are used for cooking and pickle preparation. The sweet flavor of fruits due to essential oil of flavedo parts may be used in flavoring purposes in perfume industries. Every year, Bangladesh earns handsome amount of foreign currency by exporting these fruits especially to UK, USA and Middle East countries. Like other citrus species Sat Kara is also propagated conventionally by means of seeds, grafting and budding methods. Due to present demand of this fruit in both local and foreign markets it is necessary to develop a suitable protocol for mass propagation from existing elite cultivars.

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The process of somatic embryogenesis is a suitable method of micro-propagation and has the potential of mass propagation commercially at low cost per unit. Conventional breeding methods with woody perennial crops have hampered because of a large generation gap of 6 - 8 years before they can be assessed together with the difficulty associated with working on tissue of mature origin. Although there is no available information from *Citrus macroptera* yet, successful somatic embryogenesis from nucellus tissues of different citrus species was achieved by several researchers (Rangan and Murashige 1969; Bitters et al. 1972; Kochba et al. 1972; Starrantino and Russo 1980; Pasqual et al. 1984; Chen et al. 1990 and Gill et al. 1994. Moreover, the nucellus is a non-vascularized tissue of maternal origin and can be regarded as a pocket of juvenile tissue in an otherwise adult plant (Button and Kochba 1977).

The present investigation was undertaken to develop the *in vitro* suitable protocols for micropropagation of Sat Kara plants from nucellus through somatic embryogenesis.

Materials and Methods

Four to five weeks old immature fruits were collected from Citrus Research Station, BARI, Jaintapur, Sylhet. Fruits were washed thoroughly under running tap water to reduce dust and surface contaminants. Then they were dipped in 90 % alcohol for one minute followed by 4 % sodium hypochlorite solution for 10 min and finally rinsed four times with sterile distilled water under laminar airflow cabinet. The fruits were then placed on an autoclaved ceramic tile and cut open by sharp sterilized knife and then undeveloped ovules/immature seeds were separated. For somatic embryogenic callus induction the seeds were cut by scalpel and nucellus halves were separated and cultured on a semi-solid modified MS medium supplemented with 500 mg/l malt extract called it somatic embryogenic medium1 (SEM1) after Tisseret and Murashige (1977). At the same time nucellus halves were cultured into MS + 0.1 mg/l 2,4-D + 0.05 mg/l Kn (SEM2), MS + 0.5 mg/l 2,4-D + 0.1 mg/l Kn (SEM3) and MS + 1 mg/l 2,4-D + 0.5 mg/l Kn (SEM4) media. The nucellus halves were taken in conical flasks and containing five samples with three replicates for each medium.The responded calli were further subcultured on same media and simultaneously into hormone free MS medium.

The pH of all media was adjusted to 5.7 before addition of agar and sterilized by autoclaving for 20 minutes at 1.05 kg/cm^2 (15 psi) pressure at 121_C. An amount of 10 gm/l agar (BDH) was used for SEM1 and 0.8 gm/l for other three media. The flasks containing explants were incubated on culture

racks. The cultures were maintained at 25_C ± 2 under the cool white fluorescent lights for 16 hr photoperiod.

Results and Discussion

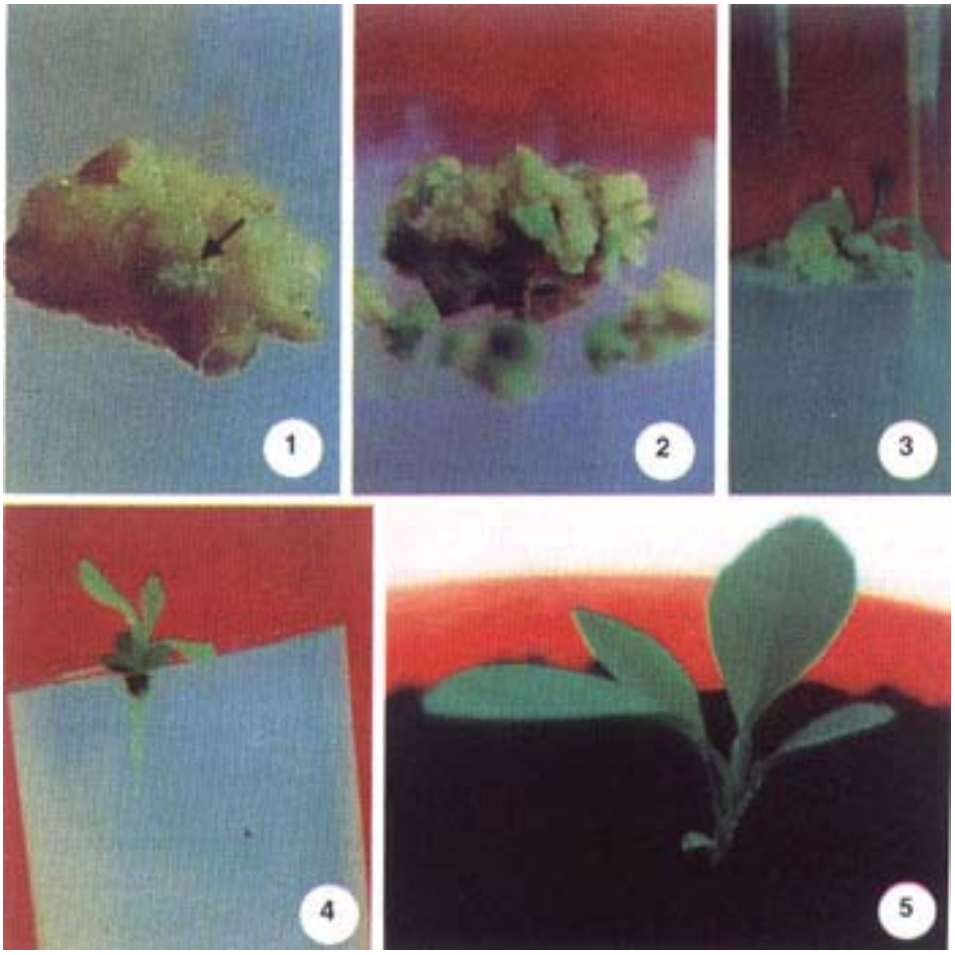
Four types of somatic embryogenic media were used to observe the embryogenic callus induction and plant regeneration from nucellus explants. Time for callus initiation was between 8 and 15 days in all media. The results obtained from this experiment are presented in Table 1. Among all the media tested, calli developed in SEM1 were only embryogenic in nature. These calli were loose light green and having good growth (Fig. 1). Calli obtained in other media were compact and initially green but with the lapse of time they turned brown. Embryogenic calli when transferred to hormone free MS medium somatic embryos were developed (Fig. 2). Complete plantlets were developed from these somatic embryos by further subculture in the same hormone free MS medium (Fig. 3).

Table 1. Effects of different somatic embryogenic media on somatic embryogenesis of *C. macroptera*.

Name of media	% of ncellus responded	Days to callus initiation	Nature of response	Response for somatic embryos
SEM1 MS + 500 mg/l malt extract	73.33	7 - 12	Loose, light green callus having good growth	Somatic embryos developed
SEM2 MS + 0.1 mg/I 2,4-D + 0.05 mg/l Kn	50.00	9 - 15	Compact callus, initially green but brown later. No further growth	Nil
SEM3 MS + 0.5 mg/I 2,4-D + 0.1 mg/l Kn	41.66	8 - 13	Compact callus, initially green but brown later. No further growth	Nil
SEM4 MS + 1 mg/I 2,4-D + 0.5 mg/l Kn	41.66	8 - 13	Compact, whitish swelling only. No further growth	Nil

Regeneration of plants via somatic embryogenesis has been preferred as a method for multiplication of viable germplasm in many woody plants (Bonga 1987). The somatic embryogenic callus and plantlets were obtained from nucellus of many citrus species on malt supplemented SEM1 medium. Tisserat and Murashige (1977) obtained that this medium was suitable to develop a protocol for somatic embryogenesis from nucellus of citrus species. However, some of the researchers used MT (Murashige and Tucker 1969) medium

supplemented with malt extract for somatic embryogenesis from nucellus of sweet orange (Pasqual et al. 1984), and some selected species of citrus (Pimental and Villegas 1993).



Figs. 1 - 5 : Regeneration of plantlets through somatic embryogenesis in *Citrus macroptera*. 1. Compact embryogenic callus from nucellus after four weeks of culture in malt supplemented modified MS. 2. Development of somatic embryos in callus after four weeks of subculture in basal MS. 3. Development of plantlets from somatic embryos after second subculture in basal MS. 4. Complete plantlet with shoot and root developed from somatic embryos. 5. Single established plant in pot soil.

A limited number of treatments were used in this experiment to induce somatic embryogenesis in *C. macroptera* from nucellus. Successful embryogenic calli were developed only from SEM1 medium. In case of auxin-cytokinin supplemented media initially calli were induced but these were not

embryogenic. But, Pasqual and Ando (1988) used 2,4-D and Kn for somatic embryogenesis from nucellus of sweet orange cv. *valecnia* and obtained some somatic embryogenic callus.

Single primary root growth (Fig. 4) was observed in each plantlet and the mean height of plantlets was 13 mm. Rajdan (1993) mentioned that embryos might germinate on agar medium without any growth regulators. However, Chen et al. (1990) observed GA_3 was effective for embryoid to plantlet regeneration. The plantlets thus obtained through somatic embryogenesis were transferred into soil mixed with biogas slurry 1 : 1 ratio (Fig. 5) and the rate of survival was 100%.

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