In vitro adventitive embryony in *Citrus*: A technique for *Citrus* germplasm exchange

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Seeds of *Citrus* spp. are moderately recalcitrant and lose viability within a short time. Furthermore, movement of bud wood is restricted for germplasm exchange because it is the potent source of invasive microorganisms, which have already destroyed several citrus-growing regions of the world. In this communication we report the protocol for storage and regeneration of plantlets of *Citrus reticulata* Blanco and *Citrus jambhiri* Lush from the cotyledonary segments. Murashige and Skoog basal medium fortified with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} kinetin + 1.0 mg l^{-1} NAA, gave the highest frequency of adventitive embryony. The regenerated plantlets were hardened and tested for clonal fidelity or variation using (RAPD) markers. The results showed that plantlets regenerated through this pathway are found to be true-to-the-type. It is proposed that the technique developed may be effectively used for the citrus germplasm exchange.

MORE stringent restrictions on the import of biotic materials could curb the rate of biotic invasions, and reduce their rates of spread[1]. Invasive species threaten native biodiversity, agricultural and other production systems[2–5]. Germplasm diversity for any crop must be protected from loss to ensure its availability for future plant improvement[6]. Germplasm is valuable because it contains the diversity of genotypes needed to develop new and improved lines. Among the fruit crops, the genus *Citrus* and its relatives are horticulturally important. In *Citrus*, most of the collections are conserved in field gene banks in different citrus-growing countries. Such collections are vulnerable to biotic and abiotic hazards[7]. For germplasm exchange, bud-wood and seeds are the most preferred tissues[8]. However, infected bud-wood used for germplasm exchange has unknowingly led to the spread of fungal, bacterial and viral diseases from one region to another within the country or even among continents, which threaten the species and regional biodiversity[9]. The real problem is detected only when the disease takes an epidemic form. Hence, many countries have restrictions on the import of bud-wood because of the hazards of introducing new and virulent diseases; and it is restricted only through seeds[8]. However, when the seed is used as a genetic material for germplasm exchange, desiccation and germinability loss during storage and transfer are the main problems[10]. Viral diseases are rarely seed-transmitted[11]. Furthermore, there is no evidence of the vertical transmission of fungal community through seeds, as seen in grasses[12]. Keeping in mind the problems associated with germplasm exchange and the threats of invasion of microorganisms to the plant species and its regional biodiversity, we propose a protocol for the regeneration of citrus from the cotyledonary explant excised from mature-stored seeds. This protocol may be effectively used for germplasm exchange.

Seeds were extracted from mature fruits of *Citrus reticulata* Blanco and *Citrus jambhiri* Lush and washed in running tap water. The seeds were then agitated in 0.75% sodium hypochlorite (v/v) for 15 min. They were then washed in tap water followed by air-drying up to 12–15 h under shade. Before storage, the seeds were treated with carbendazim (@1.0 g/kg) and packed in polythene bag (200 gauge) and stored at 4°C for six months.

After six months, the seeds were washed in running tap water for 30 min. Thereafter, the outer and inner seed coats were removed and surface sterilized using 0.75% sodium hypochlorite solution (v/v) with 0.1% Tween 20 for 10 min followed by rinsing five times with sterile distilled water. Zygotic and nucellar embryos were removed aseptically and cotyledons separated out with minimum damage. The cotyledon explants were then cultured on Murashige and Skoog (MS)[13] basal medium fortified with different growth regulator combinations such as: (i) 1.0 mg l^{-1} 6-benzylaminopurine (BAP) + 0.5 mg l^{-1} 6-furfuryl-aminopurine (kinetin) + 0.5 mg l^{-1} α-naphthalene acetic acid (NAA); (ii) 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} kinetin + 0.5 mg l^{-1} NAA; (iii) 1.0 mg l^{-1} BAP + 1.0 mg l^{-1} kinetin + 0.5 mg l^{-1} NAA; (iv) 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} kinetin + 1.0 mg l^{-1} NAA; (v) 2.0 mg l^{-1} BAP + 1.0 mg l^{-1} kinetin + 1.0 mg l^{-1} NAA, supplemented with 3% sucrose and solidified with 0.8% agar-agar. Before autoclaving (121°C for 15 min), the pH of the medium was adjusted to 5.7 and 25 ml aliquot was distributed in 25 × 150 mm culture tubes. Two cotyledons per tube were inoculated. All the cultures were maintained in culture room (26 ± 2°C) under complete darkness up to initiation of organogenesis on the explant; thereafter they were transferred to 16/8 h light/dark regime (45 µmol m^{-2} s^{-1}). Enlarging adventitious embryoids (1–2 cm length) were transferred to the rooting medium containing MS salt fortified with 2.0 mg l^{-1} IBA and 100 mg l^{-1}AC. *In vitro* hardening method was used as described by Singh *et al.*[14].

DNA was extracted from randomly selected plants using the method described by Cenis[15]. Amplification of DNA was performed in Biometra® thermocycler, programmed for initial denaturation at 94°C for 4 min. In each cycle, denaturation for 1 min at 94°C, annealing for 2 min at 32°C and extension for 2 min at 72°C was performed with final after 40 cycles for 7 min. The RAPD mixture contained 25–30 ng template DNA in 25 µl reaction volume with 2.5 µl of reaction buffer having 15 mM $MgCl_2$, 250 µM dNTPs, 1 unit of *Taq* DNA polymerase and 30 ng primer. Amplified DNA fragments were separated on 1.2% agarose gel and stained with ethidium bromide.

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We noted *in vitro* adventitive embryony on the cotyledonary explant of six-month-old stored seeds of citrus, viz. Nagpur mandarin (*C. reticulata* Blanco) and Rough lemon (*C. jambhiri* Lush.). When cotyledon segments were cultured on the regeneration medium containing MS basal medium fortified with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} kinetin and 1.0 mg l^{-1} NAA, the frequency of adventitive embryony was recorded higher in Rough lemon (28.7 embryos/explant) than the Nagpur mandarin (24.3 embryos/explant). Adventitive embryony was observed when the cultures were maintained initially under complete darkness; however, cultures kept in light or in different light/dark regimes did not show any response. Days taken for adventitive embryo induction ranged from 40.5 to 50.8 (Figure 1). Each adventitive embryo (1–2 cm length) was excised from the cotyledon explant and transferred onto MS basal medium fortified with 2.0 mg l^{-1} indole-3-butyric acid (IBA) and 100 mg l^{-1} AC. However, when initial cultures were kept for longer periods (up to 60–80 days) on the same regeneration medium, adventitive embryos grew as elongated shoots. After rooting, the plantlets were *in vitro* hardened on sterile peat : soilrite (1 : 1) filled in a glass jar fitted with polypropylene cap, prior to their *ex vitro* transfer[14]. Two-week-old hardened plantlets were transferred to the greenhouse for further acclimatization. Genetic fidelity was estimated in the regenerants by using five decamer RAPD primers. Result indicates the plantlets regenerated through this pathway are true-to-the-type. Figure 2 shows the RAPD profile amplification with 5′-GAAACGGGTG-3′ primer in Nagpur mandarin. The different stages of adventitive embryony from cotyledon explant of stored seed of Nagpur mandarin are shown in Figure 3 *a–c*.

Earlier, different regeneration systems were reported in *Citrus* using various explants[16–19]. However, regeneration of *C. jambhiri* Lush. and *C. reticulata* Blanco from cotyledons of stored seeds holds promise as a large number of plantlets can be produced, since the seeds of *Citrus* species are moderately recalcitrant[20] and cannot be stored conventionally in the cold room[7]. Furthermore, large numbers of

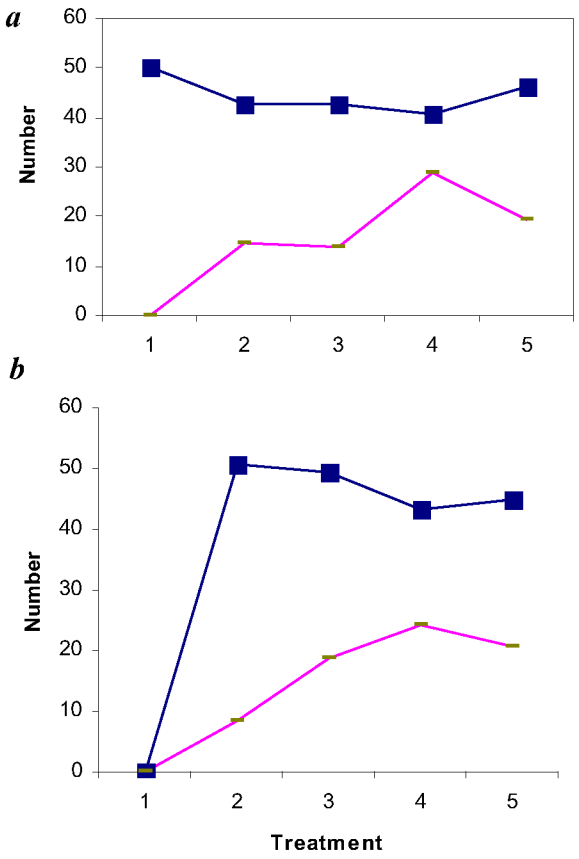

Figure 1. Effect of growth regulators on *in vitro* adventitive embryony in *Citrus*, (***a***) Rough lemon and (***b***) Nagpur mandarin. (■) Days to embryo induction; (–) No. of adventitive embryos/explant.

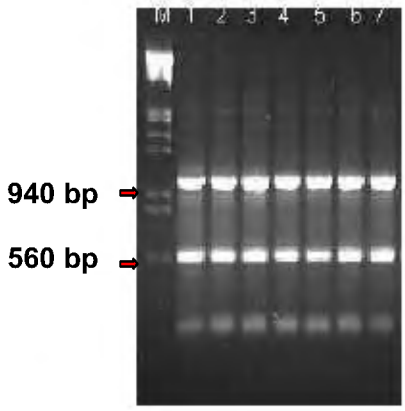

Figure 2. RAPD profile in Nagpur mandarin. Lane M, Lambda DNA digest with *Eco*RI/*Hin*dIII, lane 1, Mother plant, lanes 2–7, Plants developed through *in vitro* adventitive embryony from cotyledon segments of stored seeds.

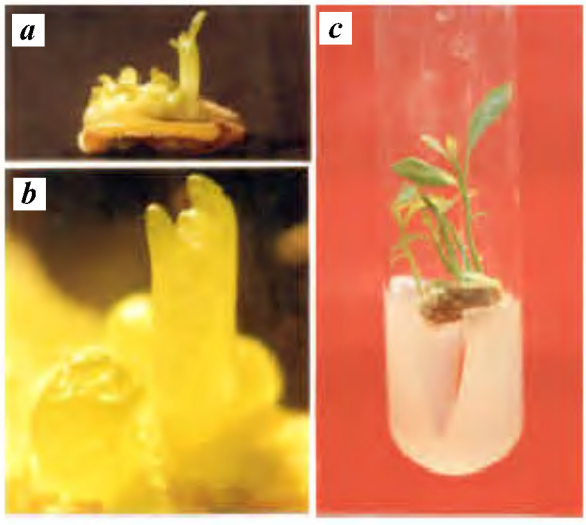

Figure 3. Different stages of adventitive embryony from cotyledon explant of stored seed in Nagpur mandarin. ***a***, Adventitive embryogenesis on cotyledonary segment; ***b***, Magnified view of ***a***, and ***c***, Shoot development.

virus(es) are not found to be transmitted through seeds[11]; rather almost all the citrus diseases are transmitted through bud-wood. Bud-wood exchange is restricted by many countries due to the hazards of invasion of new and old devastating diseases. This practice ruins the citrus industry in different regions of the world.

The technique developed is novel and can be effectively used for germplasm exchange, especially in those species which are moderately recalcitrant in nature and lose viability in a short time. Furthermore, this protocol with further experiments should be useful in (i) *Agrobacterium*-mediated genetic transformation; (ii) *in vitro* germplasm conservation; (iii) study on the nature of adventitive polyembryony in *Citrus* sp. and; (iv) role of growth regulators and other addenda on adventitive embryony.

The results described here have so far been reproduced three times with the two genotypes.

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Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm.

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Somatic embryogenesis and plant regeneration were obtained from cultured mature zygotic embryos of *Eucalyptus tereticornis* Sm. through callus. MS and B5 basal media containing different concentrations of NAA and 2,4-D were evaluated for callus induction and different BAP concentrations for somatic embryogenesis. The effect of different light conditions on somatic embryogenesis was also studied. Callus induction and somatic embryogenesis was found to be highest on MS medium compared to B5 medium. Highest frequency of friable callus was obtained on MS medium supplemented with 10.74 μM NAA. When the callus was transferred separately to the respective media on which callus induction occurred, containing various concentrations of BAP, somatic embryos developed after 1–2 weeks with highest frequency (54%) in MS medium containing 2.22 μM BAP. The embryos were germinated on MS basal medium. The rooted plants were successfully transferred to polybags after hardening.

EUCALYPTUS is an economically important multipurpose tree species belonging to the family Myrtaceae. It is widely used for establishing plantations in tropical and subtropical regions of the world[1]. The area under *Eucalyptus* plantations

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