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Influence of basal vitamins, growth regulators, and explants on *in vitro* organogenesis from synthetic seeds of *Citrus jambhiri* Lush.

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Abstract

An efficient shoot and root organogenesis protocol has been developed from synthetic seeds (synseeds) derived from various explants of *Citrus jambhiri* Lush. Optimum synseeds were developed using sodium alginate (0.5-0.75%) in 1.0% $CaCl_2$ solution. Shoot organogenesis was examined under various basal vitamin medium (MS Nitsch and MSB5) supplemented with various concentrations of adenine sulfate (ADS) and 6-benzyleaminopurine (BAP) from different explants such as cotyledonary junction, shoot tip, and nodal explants. The synseed regeneration response ranged between 60-100% among the vitamins, cytokinins and explants used. Number of shoots per synseeds was higher (13.4) in MSB5-BAP (1.5 mg L^{-1}), followed by 12.8 in MSN-ADS (2.0 mg L^{-1}), 11.2 in MSN-ADS (1.0 mg L^{-1}), and 10.8 in MSB5-ADS (1.0 mg L^{-1}) from the synseeds developed using 0.75% sodium alginate. The mean number of roots per explant was higher (4.2) in ½MSN+IAA (1.0 mg L^{-1}). Similarly, mean root length was higher (5.2 cm) in ½MSN+IAA (0.5 mg L^{-1}) followed by 4.2 cm in ½MSN+IAA (1.0 mg L^{-1}). Regenerants derived from synseeds have shown no somaclonal variations, confirming that the plantlets are true-to-type to their parental progenies. The encapsulated plantlets showed >90% survivability while transferred at Kachai village, Manipur, India. The results of the present study encourage the use of various vitamin medium and explants for large scale propagation of *C. jambhiri* through synseeds

Key words: Nitsch vitamin, MSB5, synseeds, organogenesis, genetic fidelity, Kachai lemon

Introduction

Citrus jambhiri Lush. is commonly known as rough lemon or Kachai lemon indigenous to Kachai village of Ukhrul, Manipur-a hilly province in the north-eastern hill region of India. It is a commercially important species among the citrus realm used as the most proficient rootstock for citrus propagation in southeast Asia (Savita *et al.*, 2012). Sexual incompatibility, heterozygosity, and nucellar polyembryony seeds are the major constraints in maintaining the true-to-type vigor (Savita *et al.*, 2015), which resulted in genetic erosion of this important citrus species. Genetic conservation using biotechnological tools needs urgent attention for the genetic improvement of citrus.

In vitro mass propagation techniques have been progressed in recent years for large-scale production of elite planting materials, genetic conservation, and improvement of citrus. Significant research has been carried out on direct and indirect organogenesis in *C. jambhiri* from shoot tip, nodal explants, epicotyl segment, cotyledons, and root explants. Low regeneration rate, clonal fidelity, and disease complexity from different explants are still major concerns in citrus micropropagation and conservation (Rattanpal *et al.,* 2011). In addition, collection of seeds and explants directly from the mother plants warrants virus infestation in *Citrus* spp. (Devi *et al.,* 2021). *In vitro* and *in vivo* storage, conservation, and transportation of seeds and plant propagules have certain limitations (Jang *et al.,* 2020). Polyembryony seed is another challenge in successful regeneration and maintenance of the genetic vigour of *citrus* plant (Wang *et al.,* 2017). Alternatively, micro encapsulation technology from somatic tissues would provide an avenue to resolve the difficulties in handling polyembryony zygotes, germplasm regeneration and conservation, and storage and transportation (Javed *et al.,* 2017).

Synthetic seed technology (synseed) is an emerging tool in biotechnology often used for commercial multiplication of uniform plants and large-scale conservation (Rihan *et al.,* 2017). Microencapsulation of *in vitro* explants using sodium alginate facilitates nutrients, vitamins, and growth regulator components for short- and long-term storage and maintenance of tissues by reducing transpiration (Gholami and Kaviani, 2018). Synthetic seed coat protects the synseeds from physical, mechanical, and environmental damage. However, *in vitro* basal media with vitamins and growth regulators accelerates the synseed germination and proliferation. Synseed production using direct meristematic tissues is proven to be efficient, cost-effective, and time-saving with low somaclonal variations among the regenerants (Savita *et al.*, 2012) over microencapsulation of somatic embryos. Thus, it is imperative to standardize an efficient protocol for synseed development and rapid regeneration for sustainable mass conservation and mass propagation *of C. Jambhiri*.

Basal vitamin medium and plant growth regulators (PGRs) play an important role in initiating dormancy and shoot/root proliferation (Nongmaithem *et al.*, 2020). MS medium

(Murashige and Skoog, 1962) is one of the most preferred media used in citrus micropropagation. However, the use of Gamborg's B5 (Gamborg *et al.*, 1968) and Nitsch vitamins (Nitsch and Nitsch, 1969) in citrus regeneration is limited (Devi *et al.*, 2021). Gamborg's B5 vitamins contain inositol (100 mg L^{-1}), nicotinic acid (1 mg L^{-1}), pyridoxine HCl (1 mg L^{-1}), thiamine HCl (10 mg L^{-1}), kinetin (0.1 mg L^{-1}), and 2,4-D (1 mg L^{-1}). However, Nitsch vitamins are comprised of folic acid (25 mg L^{-1}) and d-Biotin (2 mg L^{-1}) along with nicotinic acid (500 mg L^{-1}), pyridoxine HCl (25 mg L^{-1}), thiamine HCl (25 mg L^{-1}), and glycine (100 mg L^{-1}) in addition to the MS basal vitamins, which help in rapid plant organogenesis in combination with various cytokinins and auxins. The biodegradable gelling agents for synseed development, optimized basal vitamin medium, and plant growth regulators at lower concentration for regeneration and conservation often proven cost-effective, less toxic, and give healthy plant growth and development (Ghosh and Haque, 2019).

Hence, the present study focused on optimization of gel agent (sodium alginate) for encapsulation, vitamin medium (MSN and MSB5) for regeneration, and selection of *in vitro* explants (cotyledonary junction, shoot tip, and nodal explants) for synseed production and regeneration. Cryptic somaclonal variations among the regenerants were tested using reliable RAPD, SSR, and ISSR markers. The result of this study would encourage large scale mass propagation and commercialization of citrus through the inexpensive emerging synseed technology.

Materials and method

Experimental site, collection of experiments materials, and explant preparation: The present study was conducted in the Plant Tissue Culture Laboratory, ICAR Research Complex for North Eastern Hill Region, Manipur Centre, Imphal, India, located at a latitude of 24º50' N, the longitude of 93º55' E and an altitude of 860 m above mean sea level.

Healthy and mature fruits of *Citrus jambhiri* Lush. were collected from Kachai village of Ukhrul, Manipur, India. Fresh seeds were collected from ripened fruits and surface sterilized in 2% sodium hypochlorite (NaOCl) solution diluted from 4% (w/v) NaOCl solution, with 2-3 drops of 2% tween-20 by vigorous shaking for 5 min. inside the laminar air flow (Labtop Instruments Pvt. Ltd, India). The seeds were further treated with 70% ethanol for 30s, rinsed with distilled water thrice and allowed to be air-dried inside the laminar chamber. The surface-sterilized seeds were inoculated in MS basal medium with all growth regulators (1 lit sachet, HiMedia, India) for germination and seedling development. The cultures were maintained in 25x100 mL test tubes (Borosil, India) containing 25 mL of the culture medium. Six-week-old *in vitro* seedlings were selected as explant sources for the preparation of the synseeds.

Synthetic seed preparation: Shoot tips, nodal segments, and cotyledonary junctions (5 mm each) were excised from 6 weeks old *in vitro* raised seedlings for synthetic seed preparation (Fig. 1A-G). A drop of sodium alginate (HiMedia, India) at different concentrations of 0.5 and 0.75% were used to encapsulate explants in 1% $CaCl_2$, (HiMedia, India) aqueous solution. The synseeds were harvested from the $CaCl_2$ solution, washed in sterile water, and stored in a refrigerator at 4ºC.

Shoot organogenesis from synthetic seeds: Synthetic seeds were inoculated in MSN and MSB5 vitamins fortified with different concentrations of ADS and BAP (0.5, 1.0, 1.5, and 2.0 mg L^{-1}). The flasks containing the medium were steam sterilized in an autoclave (Equitron, India) for 15-20 min at 121ºC under 1.16 kg cm^{-2} pressure. Filter sterilized ADS and BAP was incorporated into the medium inside the laminar hood. About 25 mL media was poured into 25x100 mL test tubes (Borosil, India) and stored at room temperature for 72 h inside the culture room. Synseeds were inoculated aseptically into the medium, and the cultures were maintained in a culture room at 24±2°C with a 16/8 h light/dark cycle and 45 µmol m^{-2} s^{-1} white fluorescence irradiance level (Phillips, India) with 55-60% relative humidity (RH). Observations on days to synthetic seed germination, percent synthetic seed response, number of shoot formation, length of shoot, number roots, and root length were recorded.

Genetic fidelity studies: To assess the genetic homogeneity of *in vitro* regenerants derived from synseeds, gDNA was isolated from leaf tissues using DNeasy plant mini kit (QIAGEN, India) following the manufacturer's protocol in an automated DNA extractor (QIAcube, QIAGEN, Germany). Isolated gDNA were quantified in QIAexpert (QIAGEN, Germany) and visualized through 0.8% agarose gel electrophoresis (Tarson, India) in the E-Box gel imaging unit (Vilber, Germany). A concentration of 50 ng μL^{-1} of gDNA was used for PCR amplification of randomly amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and inter simple sequence repeat (ISSR) primers (Bioserve Biotechnologies, India) following the methodology described by Devi *et al.* (2021).

Acclimatization and hardening: *In vitro* grown plantlets at 4-6 leaves stage with 2-3 roots were acclimatized in sterile soil, sand, and vermicompost (1:1:1). The plantlets were maintained in a plant growth chamber (Thermofisher, USA) at 24ºC and 85% relative humidity for two weeks and subsequently transferred to a well-aerated mist house 26±2ºC at 95% relative humidity. The acclimatized plants were transplanted at its origin place at Kachai village, Manipur, located at a latitude of 25º14'29"N, a longitude of 94º16'22"E, and an altitude of 1381 m above mean sea level.

Statistical analysis: The experiment was conducted in a completely randomized design (CRD) twice with five replications under each treatment with duplicate determinations. Data were analyzed using analysis of variance (ANOVA) after square root transformation, differences among the mean values were compared using Tukey's test (Tukey, 1949), and graphs were designed in GraphPad Prism 9.2.0.

Results and discussion

Shoot organogenesis from synseeds

Days to bud break: Early bud break was observed in the synseeds developed from shoot tip compared to the cotyledonary junction and nodal explants (Fig. 2A-D). The bud break was earlier in the synseeds developed using 0.5% sodium alginate compared to 0.75%. MSB5 supplemented with ADS (0.5-1.5%) induced early bud break (8.8-10.4 days) in the synseeds developed from shoot tip cultures using 0.5% sodium alginate (Fig. 2A). The synseed regeneration response ranged between 60-100% among the vitamins, cytokinins and explants used (Fig. 2B). Previous reports showed the use of higher doses of sodium alginate (2.5-3.0% w/v) in encapsulation of *C. jambhiri* and *C. aurantifolia*

(Sharma and Roy, 2020). The appropriate concentration of sodium alginate and $CaCl_2$ is a critical factor for synseed production and capsule quality (Gholami and Kaviani, 2018). In the present study, we have demonstrated successful encapsulation at lower doses of sodium alginate (0.5-0.75%) with 1.0% $CaCl_2$ solution. Early bud break was obtained from shoot tip explants in MSB5 vitamins medium supplemented with ADS.

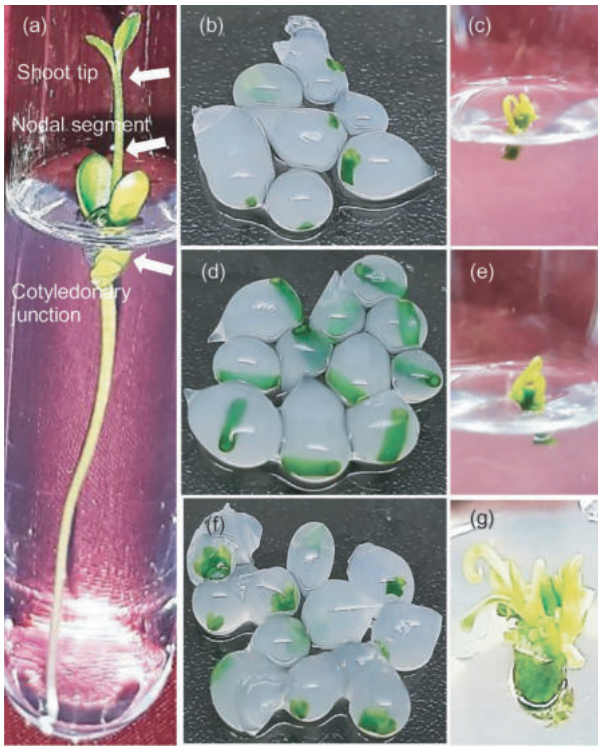

Fig. 1. Source of *in vitro* explant (A), synthetic seed preparation and regeneration from shoot tip (B-C), nodal segment (D-E) and cotyledonary junction (F-G)

Number of shoot formation and shoot length: Shoot organogenesis was better in the synseeds obtained from cotyledonary junction, followed by shoot tip culture and nodal explants. The number of shoot formations was recorded to be higher in the higher dose of sodium alginate (0.75%) than 0.5% sodium alginate. The number of shoots per synseeds was observed to be higher (13.4) in MSB5-BAP (1.5 mg L^{-1}), followed by 12.8 in MSN-ADS (2.0 mg L^{-1}), 11.2 in MSN-ADS (1.0 mg L^{-1}), and 10.8 in MSB5-ADS (1.0 mg L^{-1}) from the synseeds developed using 0.75% sodium alginate (Fig. 2C). Although early bud break occurred in a lower dose of sodium alginate (0.5%), further shoot proliferation was better in the synseeds developed from 0.75% sodium alginate. Shoot proliferation relies on the quality of vitamins and plant growth supplements and explants (Devi *et al.*, 2021). Use of MS basal medium and BAP in germination and shoot proliferation of synseeds was reported in several species (Micheli and Standardi, 2016). However, this study report demonstrated successful synseed proliferation in MSN and MSB5 vitamins. Better shoot organogenesis in MSN might be due to the additional contains of folic acid (25 mg L^{-1}) and d-biotin (2 mg L^{-1}), and glycine (100 mg L^{-1}) over MSB5 and MS medium.

Shoot length was significantly higher in the synseeds developed from cotyledonary junction using 0.75% sodium alginate compared to shoot tip and nodal explants. The mean shoot length was higher (8.2 cm) in MSN-ADS (1.0 mg L^{-1}) [Fig. 2D] followed by 8.0 cm in MSB5-ADS (1.0 mg L^{-1}), 6.2 cm each in MSN-ADS (2.0 mg L^{-1}) and MSB5-BAP (1.0 mg L^{-1}) [Fig. 2D] from the synseeds developed from cotyledonary junction using 0.75% sodium alginate. Several reports suggested the use of shoot tip as the most suitable explant for encapsulation followed by the nodal segment in MS medium fortified with BAP (1.0-2.0 mg L^{-1}) [Sharma and Roy (2021)]. However, this study reports the first attempt of synseed development from the cotyledonary junction.

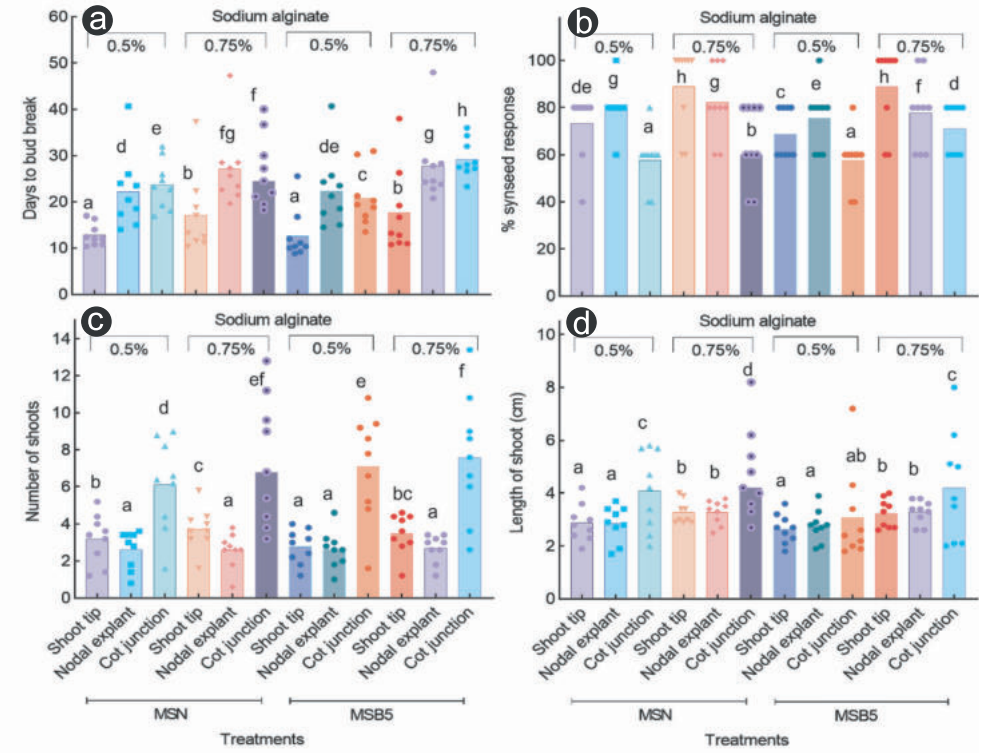

Fig. 2A-D. Effect of sodium alginate, vitamins, cytokinins supplements, and explants on synseed production and regeneration of *Citrus jambhiri* Lush. Data represent mean of two experiments with five replications under each treatment. Different letters indicated significant difference according to *Tukey's* test ($P \leq 0.05$).

Days to root initiation, number of root formation, and root length: In the present study, days to root initiation was 14.4 to 44.4 days in ½MSN vitamin medium and 20.0-42.4 days in ½MSB5 with NAA and IAA supplements. ½MSN+IAA (1.0 mg L^{-1}) registered early root initiation (14.4 days) [Fig. 3A]. The mean number of roots per explant was higher (4.2) in ½MSN+IAA (1.0 mg L^{-1}) followed by 3.6 in ½MSN+IAA (1.5 mg L^{-1}) and 3.2 in ½MSN+IAA (0.5 mg L^{-1}) [Fig. 3B]. Similarly, mean root length was higher (5.2 cm) in ½MSN+IAA (0.5 mg L^{-1}) followed by 4.2 cm in ½MSN+IAA (1.0 mg L^{-1}) [Fig. 3C]. Apical shoot tips and axillary shoot buds contain no root meristems (Rihan *et al.*, 2017). Thus, the encapsulated explants need a rooting medium for root induction after

shoot proliferation. Auxin concentration level controls the root induction and proliferation in different species (Sahoo *et al.,* 2018; Devi *et al.,* 2021). In our study, ½MSN+IAA (0.5-1.0 mg L^{-1}) was optimized as the best medium for root induction and proliferation.

Assessment of genetic fidelity of the regenerants from synseeds: The genetic homogeneity of *in vitro* regenerants derived from synseeds were showed a monomorphic profile while assessed using RAPD, SSR, and ISSR markers (Fig. 4A-C). The representative profiles showed five monomorphic bands (size: 400-2000bp) for OPC-12 (5' TGTCATCCCC 3'), three monomorphic bands (size: 150-200bp) for CMS-47 (SSR: 5' GGATCCTCCACCATCTCGTA 3' 5' TTCTTCTTCCATGCCGACTT 3'), and eight monomorphic bands (size: 600-1200bp) for UBC 834 [ISSR: (AG)8YT], which indicated the genetic uniformity among the regenerants. The main aim of *in vitro* micropropagation is to obtain true-to-type plantlets and avoid somaclonal variations (Jogam *et al.*, 2020), which could be verified using PCR-based RAPD, SSR, and ISSR markers (Elayaraja *et al.*, 2019). Genetic homogeneity has been routinely performed using reliable RAPD (Savita *et al.*, 2012; Savita *et al.*, 2015), SSR (Guzman *et al.,* 2017), and ISSR (Devi *et al.*, 2021) marker in *Citrus* spp.

Acclimatization and hardening: After genetic fidelity assessment, the *in vitro* plantlets derived from synseeds were transferred to a well-aerated polyhouse at the 3-4 leave stage. The results revealed that plantlets grown on MSN+ADS 1.0 mg L^{-1} and rooted in ½MSN+IAA 1.0 mg L^{-1} give rise to over 90% survivability (Fig. 5). About 100 *in vitro* grown plantlets were planted in its place of origin at Kachai, Ukhrul, Manipur. An efficient and successful *in vitro* regeneration protocol solely depends on the good establishment of true-to-type *in vitro* induced plantlets in soil (Sahoo *et al.,* 2018). Kour and Singh (2012) and Sharma and Roy (2020) reported 80-85% survivability *in vitro* regenerated plantlets of *Citrus jambhiri*.

In conclusion, synthetic seeds obtained from shoot tip cultures possessed early bud break. However, cotyledonary junction resulted in better shoot organogenesis. Sodium alginate (0.75%) was found to be suitable for the development of synseeds as compared to 0.5% sodium alginate. Among the vitamins, MSN induced better syn. seed response over MSB5. Cytokinins (ADS and BAP) at 1.0-1.5 mg L^{-1} were optimum to induce shoot organogenesis in synseeds. Regenerants derived from synseeds showed no genetic variations, which was validated using RAPD, SSR, and ISSR markers. The optimized protocol not only be helpful in rapid regeneration, but also be useful for genetic

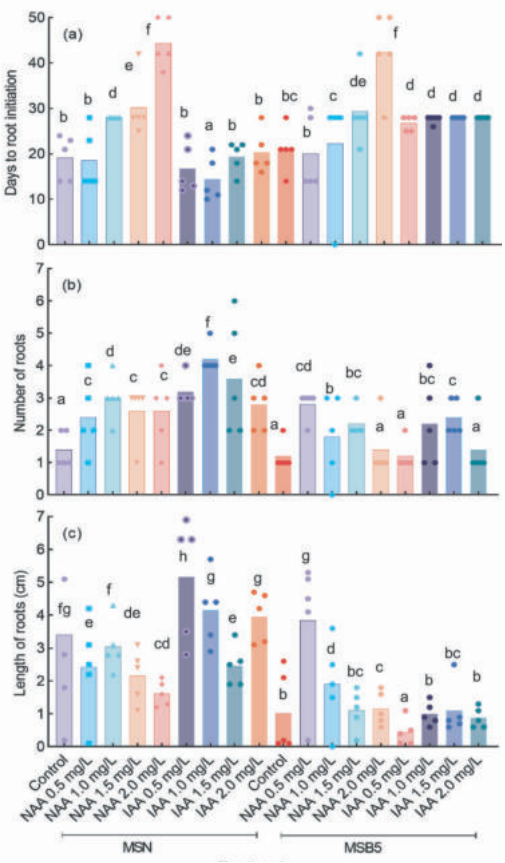

Fig. 3A-C. Effect of vitamins and auxin supplements on root induction in the plantlets derived from synseeds of *Citrus jambhiri* Lush. Data represent mean of two experiments with five replications under each treatment. Different letters indicated significant difference according to *Tukey's* test ($P \leq 0.05$).

Fig. 5. Acclimatization and hardening of *in vitro* plantlets derived from synseeds of *Citrus jambhiri* Lush.

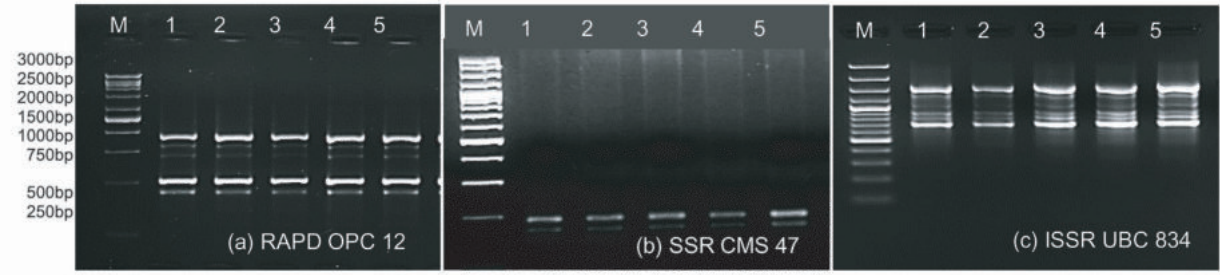

Fig. 4A-C. Genetic fidelity of synseed regenerants derived from various explants of *Citrus jambhiri* Lush. employing (A. RAPD OPC 12; B. SSR CMS 47; C. ISSR UBC 834)

conservation, mass propagation, storage and transportation of *Citrus* spp. sustainably.

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