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# Root-to-shoot organogenesis in *Citrus jambhiri* Lush.

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MANAS RANJAN SAHOO

## ABSTRACT

A protocol for high-frequency direct organogenesis from root explants of *Citrus jambhiri* Lush. was developed. Full-length roots (~3cm) were isolated from the *in vitro* grown seedlings and cultured on Murashige and Skoog basal medium supplemented with Nitsch vitamin (MSN) with different concentrations of cytokinin [6-benzylaminopurine, (BAP)] and gibberellic acid (GA<sub>3</sub>). The frequency of multiple shoot proliferation was very high, with an average of 34.3 shoots per root explant when inoculated on the MSN medium supplemented with BAP (1.0 mg L<sup>-1</sup>) and GA<sub>3</sub> (1.0 mg L<sup>-1</sup>). Optimal rooting was induced in the plantlets under half strength MSN medium supplemented with *indole-3-acetic acid* (IAA, 0.5-1.0 mg L<sup>-1</sup>). IAA induced better root structure than 1-naphthaleneacetic acid (NAA), which was evident from the result of scanning electron microscopy (SEM). The expressions of growth-regulating factor genes (*GRF1* and *GRF5*) and GA<sub>3</sub> signaling genes (*GA2OX1* and *KO1*) were high in the regenerants obtained using MSN+BAP (1.0 mg/L)+GA<sub>3</sub> (1.0 mg/L). The expressions of auxin regulating genes were high in roots obtained in ½ MSN+IAA1.0 mg L<sup>-1</sup>. Furthermore, the virus indexing of the regenerants confirmed that there were no virus amplicons detected for Huanglongbing and *Citrus tristeza*virus. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers detected no polymorphic bands amongst the regenerated plants. The high-frequency direct regeneration protocol in the present study provides an enormous significance in *Citrus* organogenesis, it's commercial cultivation and genetic conservation.

## ATTACHMENTS

[Figure 1.jpg](#)

## DOI

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## PROTOCOL CITATION

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## KEYWORDS

*Citrus jambhiri*, Root explants, organogenesis, Gene expression, Genetic fidelity, Virus indexing

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Figure1.jpg

MATERIALS TEXT

#### Plant materials and explant preparation

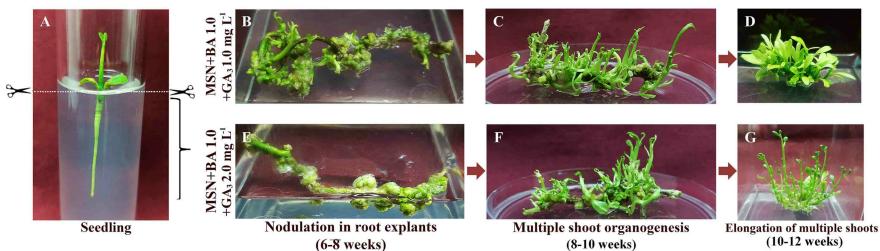
The mature and healthy fruits of *Citrus jambhiri* Lush. were collected from the Kachai village of Ukhru District, Manipur, India. Seeds were removed from the pulp, allowed to dry at room temperature for 2-3 days (d). Such dry seeds were soaked in distilled water for 20-30 min, and testa was removed by hand without any bruises on the thin inner layer of tegmen. After peeled off the hard outer seed coat testa, seeds were surface sterilized in 2% sodium hypochlorite solution (made from a 4% (w/v) NaClO solution; HiMedia®, Mumbai, India) with 2-3 drops of 2% Tween20 by vigorous shaking for 5 min. The seeds were then rinsed with sterile distilled water three times followed by washing with 70% ethanol for 30 s. The surface-sterilized seeds were allowed to dry completely inside the laminar airflow (LABTOP, Labtop Instruments Pvt. Ltd, India) and after that soaked in sterile distilled water overnight. The tegmen was removed carefully by using sterile forceps and scalpel. The white seeds portions, stripped of both the seed coats, were inoculated in 25x100 mL test tubes (Borosil®, Mumbai, India) containing 25 mL of culture medium.

#### Culture media and conditions

For germination of seeds, half-strength MS (Murashige and Skoog 1962) basal medium (1-L powder sachet from HiMedia®), supplemented with 3% sucrose, was used. The pH of the culture medium was adjusted to 5.8 with 1 N NaOH, and 0.3% (w/v) of clarigel™ (HiMedia®, India) was added as a gelling agent. The medium was sterilized in an autoclave (Equitron Medica Pvt. Ltd, Mumbai, India) at 121 °C, under 15 psi for 20 min, allowed to cool up to 55 °C. Before pouring the media, filter-sterilized PGRs were added, poured either in test tubes and in planton boxes (7.5x7.5x10 cm, Tarsons, Kolkata, India) and allowed to solidify. The seeds were inoculated vertically half-submerged into the medium. All the cultures were maintained at 24±2 °C, with a photoperiod of 16 h, under fluorescent light (Philips, New Delhi, India) with a light intensity of 40  $\mu\text{ mol m}^{-2}\text{ s}^{-1}$ .

#### Direct multiple shoot regeneration from *in vitro* raised roots

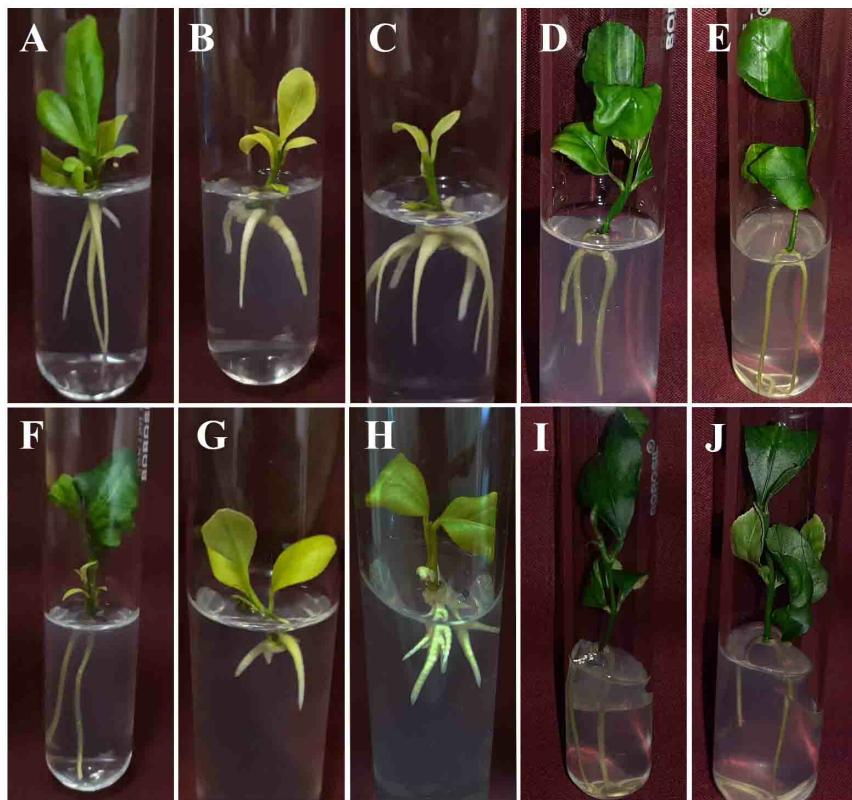
Full-grown roots, excised from 4-5 weeks (wks) old *in vitro* grown seedlings, were employed for direct regeneration of multiple shoots. MS medium with Nitsch vitamin (MSN, Nitsch and Nitsch, 1969) fortified with 1.0 mg L<sup>-1</sup> each of BAP, kinetin (KIN), adenine sulphate (ADS) and zeatin (ZEA) [HiMedia®, India] was used either alone or in combination with 1.0 or 2.0 mg L<sup>-1</sup> of GA<sub>3</sub> (HiMedia®, India). Data on the percent of explant response, days to shoot initiation, number of shoots per explant, shoot length (cm), and number of leaves per explant was recorded at an interval of 2 weeks (wks) after shoot initiation.



**Figure 1A-G** Direct multiple shoot organogenesis from *in vitro* root explants of *Citrus jambhiri* Lush. [A: Seedling; B, C, D: Nodulation in root explants, multiple shoot organogenesis, multiple shoot elongation at MSN+BA 1.0+GA<sub>3</sub> 1.0 mg L<sup>-1</sup>, respectively; E, F, G: Nodulation in root explants, multiple shoot organogenesis, multiple shoot elongation at MSN+BA 1.0+GA<sub>3</sub> 2.0 mg L<sup>-1</sup>, respectively]

### Induction of roots

To induce roots, the regenerants were cultured in various concentrations (0.5, 1.0, 1.5 and 2.0 mg L<sup>-1</sup>) of auxins such as NAA and IAA [HiMedia, India] were incorporated in both half ( $\frac{1}{2}$ MSN) and full-strength MS medium supplemented with Nitsch vitamins (MSN). Observations on days to root initiation, number, and length of roots (cm) per explant and the percent of rooting response was recorded after every 2 wks of culture initiation till 8 wks.

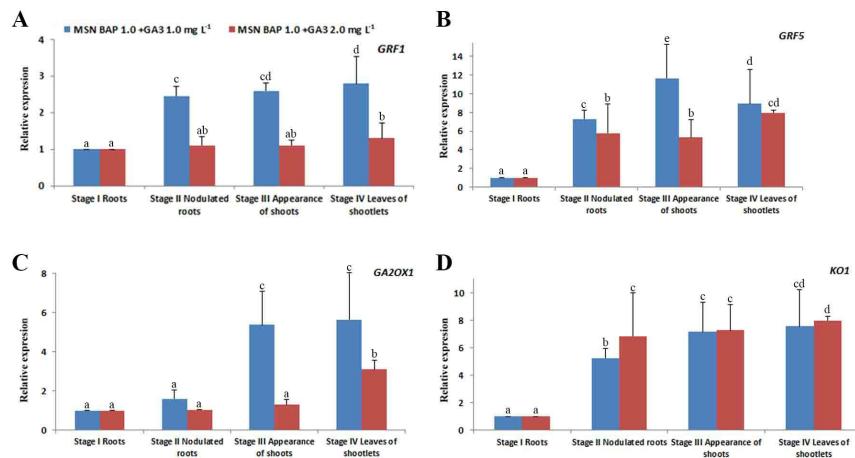


**Figure 2A–J** Root induction in *in vitro* plantlets of *Citrus jambhiri* Lush. [A:  $\frac{1}{2}$ MSN; B–C:  $\frac{1}{2}$ MSN+NAA 1.0 and 2.0 mg L<sup>-1</sup>; D–E:  $\frac{1}{2}$ MSN+IAA 1.0 and 2.0 mg L<sup>-1</sup>; F: MSN; G–H: MSN+NAA 1.0 and 2.0 mg L<sup>-1</sup>; I–J: MSN+IAA 1.0 and 2.0 mg L<sup>-1</sup>]

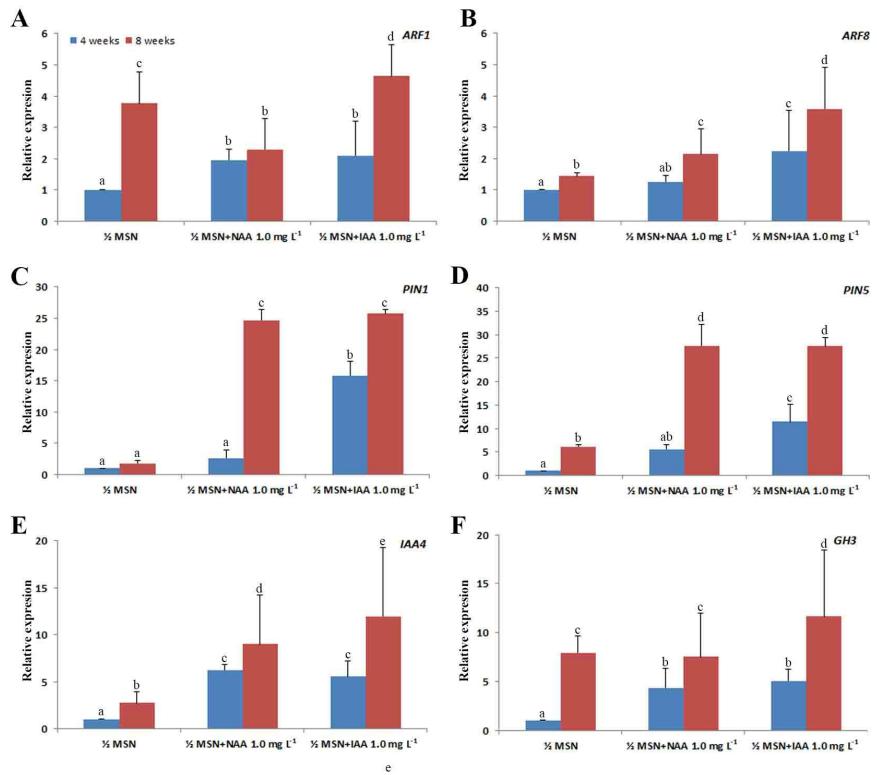
### Gene expression analyses

To understand the role of various growth-regulating factors (GRF) and GA<sub>3</sub> pathway genes during different stages of direct multiple shoot organogenesis from intact *in vitro* roots, quantitative real-time PCR (qRT-PCR) was performed. Total RNA was isolated from *in vitro* roots and leaves under different treatments and time points using RNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) following the manufacturer's protocol. The sampling was performed at four different growth stages (Phase I to Phase IV). The phase I represented roots without any response, phase II was comprised of nodulated roots, followed by phase III, where shootlets appeared. The phase IV consisted of fully grown plantlets in which young leaves have been sampled for qRT-PCR. The quality of the isolated RNA was quantified by QIAxpert (QIAGEN Hilden, Germany) and agarose gel electrophoresis before used for single-stranded cDNA synthesis. One microgram of the isolated RNA was converted into cDNA by dual step RT PCR kit (GCC Biotech (I) Pvt. Ltd., Kolkata, India) and proceeded for targeted gene expression analysis by using Rotor-Gene Q (QIAGEN Hilden, Germany). The expression of two growth-regulating factors, *viz.*, *GRF1* and *GRF5*, and two GA<sub>3</sub> pathway genes (*GA2OX1* and *KO1*), were studied (Supplementary Table 1). In a similar way, the expression of auxin regulating genes, *viz.*, *ARF1*, *ARF8*, *PIN1*, *PIN5*, *GH3* and *IAA4* (Supplementary Table 1), were analysed from *in vitro* roots obtained in various auxin supplemented media. The qRT-PCR

was performed in a 20  $\mu\text{L}$  reaction volume using 10  $\mu\text{L}$  of QuantiNova $\circ$  SYBR $\circ$ green PCR master mix (QIAGEN, India), 1  $\mu\text{L}$  each of forward and reverse primers, 1  $\mu\text{L}$  of cDNA and 7  $\mu\text{L}$  of RNase free water. The reaction was started by an incubation of 30 s at 60 °C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and 65-99°C for 30 s for melting curve as described by Liu et al. 2017. Each reaction was carried out in duplicate, and relative gene expression was calculated using  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen 2001) with *Citrus actin* gene (Mafra et al. 2012) as an endogenous control.



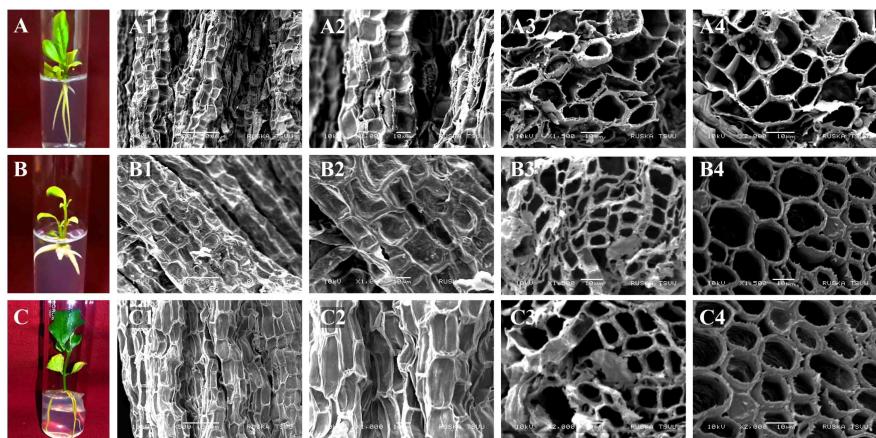
**Figure 3A-D** Relative expression of growth regulating factors (A. *GRF1* and B. *GRF5*) and GA<sub>3</sub> pathway genes (C. *GA2OX1* and D. *K01*) at different stages of organogenesis from *in vitro* root explants of *Citrus jambhiri* Lush.



**Figure 4A-F** Relative expression of auxin responsive factors (A. *ARF1* and B. *ARF8*), auxin efflux carrier protein genes (C. *PIN1* and D. *PIN5*), auxin/IAA family protein gene (E. *IAA4*) and GH3 family protein gene (F. *GH3*) at different stages of rooting of *Citrus jambhiri* Lush.

#### Scanning electron microscopy (SEM)

The microstructures of 8 wks old roots were studied using SEM imaging. Roots, from the control along with various auxin supplemented media, were viewed and photographed on SEM (Model: JOEL-JSM 5600) using an automated sputter coater (Model: JEOL, JFC-1600) for 3 min at required magnifications from 500x to 2000x as per the standard procedures (Bozzola and Russell 1998) at RUSKA Lab, College of Veterinary Science, P.V. Narasimha Rao Telangana Veterinary University (PVNR TVU), Rajendranagar, Hyderabad, India.

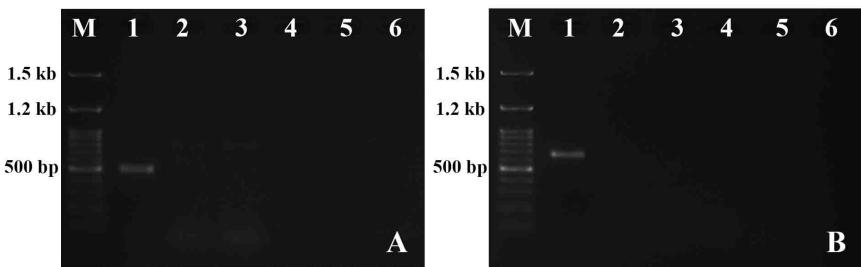


**Figure 5A-C** Scanning electron micrograph of the roots induced in *Citrus jambhiri* Lush. plantlets using various concentrations of auxins [A.  $\frac{1}{2}$ MSN (A1-A2: External at 500 and 1000x; A3-A4: Internal at 1500 and 2000x), B.  $\frac{1}{2}$ MSN+NAA  $1.0 \text{ mg L}^{-1}$  (B1-B2: External at 500 and 1000x; B3-B4: Internal at 1500 and 2000x), C.  $\frac{1}{2}$ MSN+IAA  $1.0 \text{ mg L}^{-1}$  (C1-C2: External at 500 and 1000x; C3-C4: Internal at 1500 and 2000x)]

#### Virus indexing

Screening of two major *Citrus* infecting viruses, namely, Huanglongbing (HLB) and *Citrus tristeza* virus (CTV), was carried out by reverse transcriptase PCR (RT-PCR) among the regenerants. For HLB detection, the genomic DNA (gDNA) was isolated from the midrib of the leaf tissues using the DNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) by following the manufacturer's protocol. The primers, *Las F* (5' GGAGAGGGTGAGTGGAATTCCGA 3') and *LasR* (5'ACCCAACATCTAGGTAAAAACC 3'), were designed from the hypervariable effector protein of the virus (Fujikawa and Iwanami, 2012). The PCR was carried out using Go Taq® G2 green master mix (Promega Corporation, US) under the conditions of 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min in SimpliAmp thermal cycler (Applied Biosystems, Life Technologies®).

For detection of CTV, total RNA was isolated from the leaf midrib of *in vitro* raised plantlets using RNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) by following the manufacturer's protocol and converted into cDNA by Verso cDNA synthesis kit (Thermo Scientific™, USA). The primers, namely, *K607F*(5'ACTCRCGTTGACTCTGTTAAA3') and *K608R* (5'GTACCGAACATATAACTCCAGT3'), were designed based on the viral coat protein according to Sharma et al. (unpublished data) and were procured from GCC Biotech (I) Pvt. Ltd., Kolkata, India. The PCR was carried out under the conditions of initial denaturation at 94°C 2 min, 35 cycles of 94°C 30 s, 59°C 45 s followed by 72°C 45 s, and 72°C 10 min of final extension. For the detection of amplicons, PCR products were run on 1.8% agarose gel, and pictures were taken on the E-Box gel documentation imaging system (Vilber Lourmat, France).



**Figure 6A-B** Virus indexing employing *Las* F and R (500 bp) to detect Citrus greening (Huanglongbing) para retrovirus (DNA) [A] and K607 F and R (648 bp) to detect *Citrus tristeza* retrovirus (RNA) [B] in *Citrus jambhiri* Lush. regenerants

[Lane M: 100 bp ladder (GCC biotech); Lane 1: Positive control (*C. greening* or CTV infected leaf midrib); Lane 2: leaf midrib from the mother plant; Lane 3: *In vitro* leaf midrib from the plantlets ( $\frac{1}{2}$ MSN); Lane 4: *In vitro* leaf midrib from the plantlets ( $\frac{1}{2}$ MSN+NAA 1.0 mg L $^{-1}$ ); Lane 6: *In vitro* leaf midrib from the plantlets ( $\frac{1}{2}$ MSN+IAA 1.0 mg L $^{-1}$ ); Lane 7: primercontrol (negative control)]

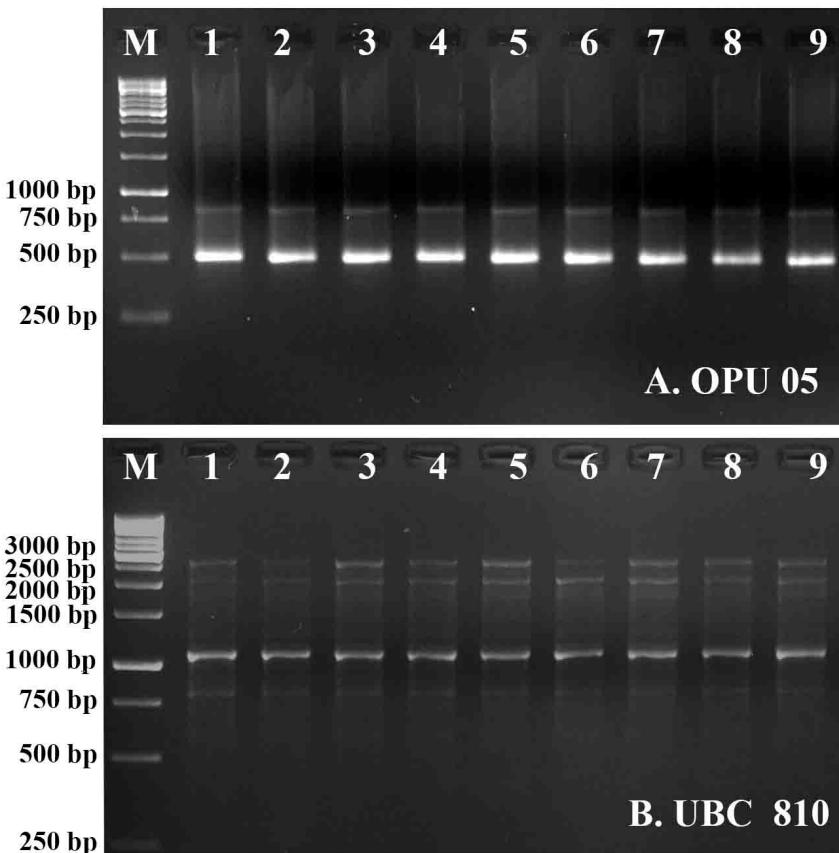
#### Assessment of genetic fidelity

To assess the genetic stability of *in vitro* raised plantlets, clonal fidelity test was carried out by using PCR based randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). Total gDNA were isolated from leaf tissues of the *in vitro* raised plantlets under different PGR treatments using DNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) using QIAcube by following manufacturer's protocol. The isolated gDNA were quantified using a QIAxpert (QIAGEN Hilden, Germany), checked on 0.8% agarose gel electrophoresis, and finally adjusted to a concentration of 50 ng  $\mu$ L $^{-1}$  for enabling PCR.

**RAPD:** Eight RAPD primers (Supplementary Table 2) were used to screen the genetic fidelity of the *in vitro* raised plants. PCR was performed in a total volume of 25  $\mu$ L comprising of 50 ng template DNA, 10 pM primer, 12.5  $\mu$ L of 2X Taq PCR master mix (QIAGEN, India (P) Ltd.). PCR amplification was carried out at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 32–34°C (varies with primer) for 2 min, extension at 72°C for 2 min and final extension of 7 min at 72°C in a thermocycler (Rohini et al. 2015).

**ISSR:** To assess the clonal fidelity of the *in vitro* regenerants, ten ISSR primers (Supplementary Table 2) were used. PCR amplification was carried out using 50 ng of gDNA, 1  $\mu$ L of 10 pM primer, 12.5  $\mu$ L of 2 X Taq PCR master mixes for a total reaction volume of 25  $\mu$ L in a thermocycler with reaction conditions by following Rohini et al. (2015). The annealing temperature was optimized at 42–54 °C by gradient PCR.

All the primers (Supplementary Tables 1 and 2) were procured from Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India. Genetic integrity, as revealed by the banding patterns of the PCR products, was checked on 1.8% agarose gel electrophoresis, and pictures were taken on E-Box gel documentation imaging system (Vilber Lourmat, France).

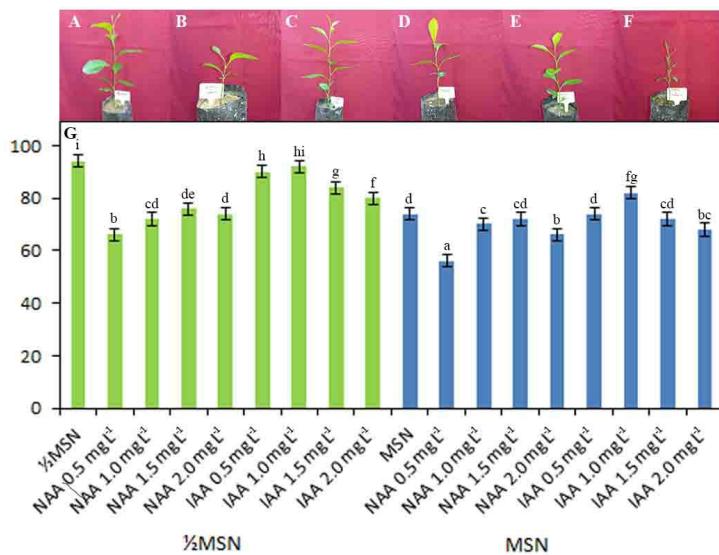


**Figure 7A-B** Genetic fidelity of *Citrus jambhiri* Lush. regenerants employing RAPD (**A.** OPU 05) and ISSR (**B.** UBC 810) markers

[Lane M: 1 kb DNA ladder for RAPD and ISSR; 100 bp DNA ladder for SSR; Lane 1: Mother plant from Kachai village, Ukhrul, Manipur; Lane 2: *In vitro* seedlings; Lane 3: Seedlings planted at Langol farm of ICAR, Manipur; Lane 4: Seedlings planted at polyhouse of ICAR, Manipur; Lane 5: Regenerants obtained from MSN+BAP 1.0+GA<sub>3</sub> 1.0 mg L<sup>-1</sup>; Lane 6: Regenerants obtained from MSN+BAP 1.0+GA<sub>3</sub> 2.0 mg L<sup>-1</sup>; Lane 7: Plantlets obtained from ½MSN; Lane 8: Plantlets obtained from ½MSN+NAA 1.0 mg L<sup>-1</sup>; Lane 9: Plantlets obtained from ½MSN+IAA 1.0 mg L<sup>-1</sup>]

#### Acclimatization and hardening

*In vitro* plantlets with well-developed roots were washed thoroughly with sterile distilled water and acclimatized in the mixture of sterile garden soil, sand, and vermicompost in 1:1:1 ratio. The plantlets were irrigated with Hoagland's solution (HiMedia®) [Hoagland and Arnon, 1938] twice a daily for the next 2 wks. For hardening, the plantlets were maintained at a well-aerated mist house with a temperature of 26±2°C. Well established, virus-free and true-to-type micro propagated plants of *C. jambhiri* were then maintained in the polyhouse.



**Figure 8A-G** Survivability of *in vitro* plantlets from different concentrations of auxins [A. ½MSN, B. ½MSN+NAA (1.5 mg L<sup>-1</sup>), C. ½MSN+IAA (1.0 mg L<sup>-1</sup>), D. MSN, E. MSN+NAA (1.5 mg L<sup>-1</sup>), F. MSN+IAA (1.0 mg L<sup>-1</sup>), G. %Survivability of the *in vitro* plantlets derived from MSN+BA 1.0+GA<sub>3</sub> 1.0 mg L<sup>-1</sup> and different concentrations of auxins]

## ABSTRACT

A protocol for high-frequency direct organogenesis from root explants of *Citrus jambhiri*Lush. was developed. Full-length roots (~3cm) were isolated from the *in vitro* grown seedlings and cultured on Murashige and Skoog basal medium supplemented with Nitsch vitamin (MSN) with different concentrations of cytokinin [6-benzylaminopurine, (BAP)] and gibberellic acid (GA<sub>3</sub>). The frequency of multiple shoot proliferation was very high, with an average of 34.3 shoots per root explant when inoculated on the MSN medium supplemented with BAP (1.0 mg L<sup>-1</sup>) and GA<sub>3</sub> (1.0 mg L<sup>-1</sup>). Optimal rooting was induced in the plantlets under half strength MSN medium supplemented with *indole-3-acetic acid* (IAA, 0.5-1.0 mg L<sup>-1</sup>). IAA induced better root structure than 1-naphthaleneacetic acid (NAA), which was evident from the result of scanning electron microscopy (SEM). The expressions of growth-regulating factor genes (*GRF1* and *GRF5*) and GA<sub>3</sub> signaling genes (*GA2OX1* and *KO1*) were high in the regenerants obtained using MSN+BAP (1.0 mg/L)+GA<sub>3</sub> (1.0 mg/L). The expressions of auxin regulating genes were high in roots obtained in ½ MSN+IAA1.0 mg L<sup>-1</sup>. Furthermore, the virus indexing of the regenerants confirmed that there were no virus amplicons detected for Huanglongbing and *Citrus tristeza*virus. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers detected no polymorphic bands amongst the regenerated plants. The high-frequency direct regeneration protocol in the

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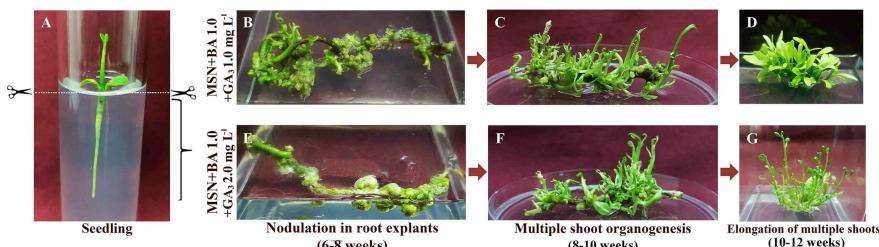
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## 3 Direct multiple shoot regeneration from *in vitro* raised roots

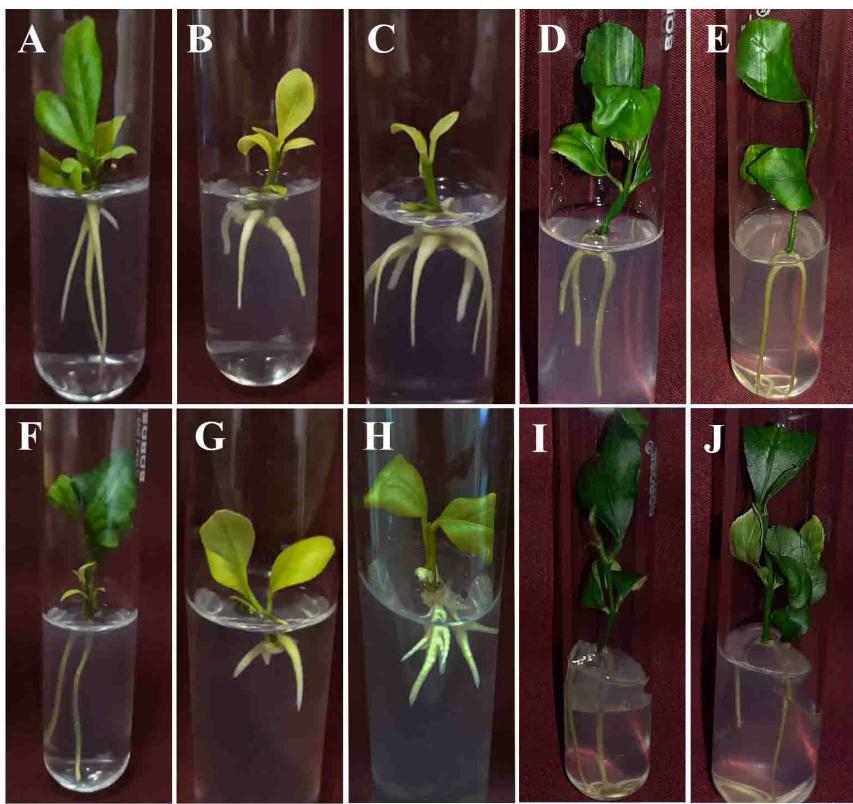
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## 4 Induction of roots

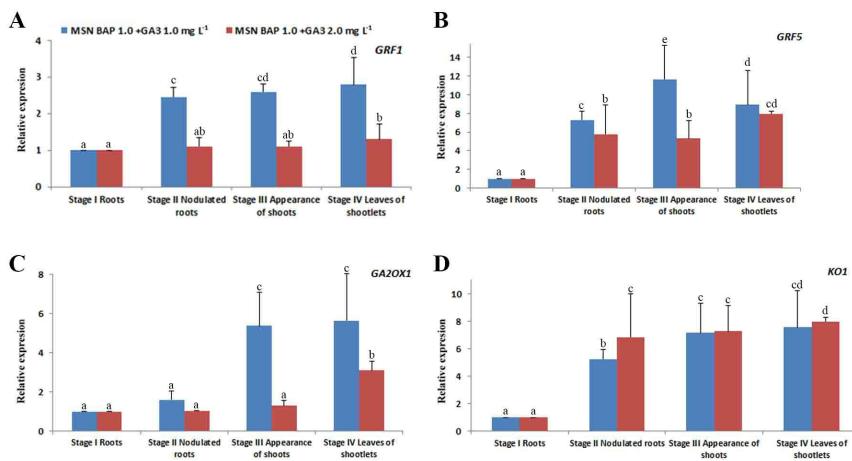
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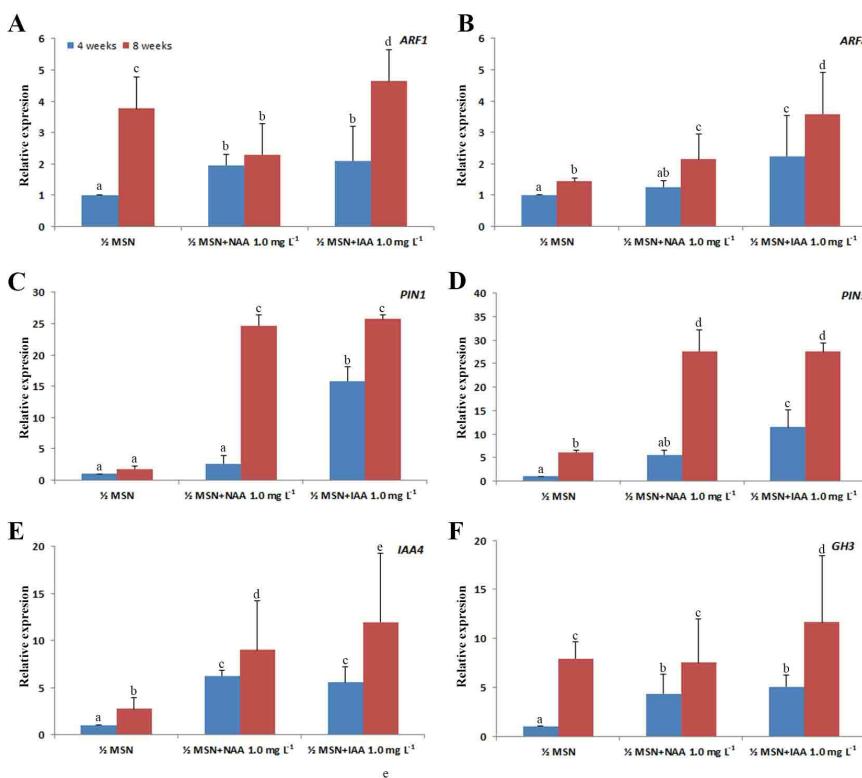
**Figure 2A-J** Root induction in *in vitro* plantlets of *Citrus jambhiri* Lush. [A: ½MSN; B-C: ½MSN+NAA 1.0 and 2.0 mg L<sup>-1</sup>; D-E: ½MSN+IAA 1.0 and 2.0 mg L<sup>-1</sup>; F: MSN; G-H: MSN+NAA 1.0 and 2.0 mg L<sup>-1</sup>; I-J: MSN+IAA 1.0 and 2.0 mg L<sup>-1</sup>]

## 5 Gene expression analyses

To understand the role of various growth-regulating factors (GRF) and GA<sub>3</sub> pathway genes during different stages of direct multiple shoot organogenesis from intact *in vitro* roots, quantitative real-time PCR (qRT-PCR) was performed. Total RNA was isolated from *in vitro* roots and leaves under different treatments and time points using RNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) following the manufacturer's protocol. The sampling was performed at four different growth stages (Phase I to Phase IV). The phase I represented roots without any response, phase II was comprised of nodulated roots, followed by phase III, where shootlets appeared. The phase IV consisted of fully grown plantlets in which young leaves have been sampled for qRT-PCR. The quality of the isolated RNA was quantified by QIAxpert (QIAGEN Hilden, Germany) and agarose gel electrophoresis before used for single-stranded cDNA synthesis. One microgram of the isolated RNA was converted into cDNA by dual step RT PCR kit (GCC Biotech (I) Pvt. Ltd., Kolkata, India) and proceeded for targeted gene expression analysis by using Rotor-Gene Q (QIAGEN Hilden, Germany). The expression of two growth-regulating factors, *viz.*, *GRF1* and *GRF5*, and two GA<sub>3</sub> pathway genes (*GA2OX1* and *KO1*), were studied (Supplementary Table 1). In a similar way, the expression of auxin regulating genes, *viz.*, *ARF1*, *ARF8*, *PIN1*, *PIN5*, *GH3* and *IAA4* (Supplementary Table 1), were analysed from *in vitro* roots obtained in various auxin supplemented media. The qRT-PCR was performed in a 20 µL reaction volume using 10 µL of QuantiNova® SYBR®Green PCR master mix (QIAGEN, India), 1 µL each of forward and reverse primers, 1 µL of cDNA and 7 µL of RNase free water. The reaction was started by an incubation of 30 s at 60 °C, followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and 65-99°C for 30 s for melting curve as described by Liu et al. 2017. Each reaction was carried out in duplicate, and relative gene expression was calculated using 2<sup>-ΔΔCt</sup> method (Livak and Schmittgen 2001) with *Citrus actin* gene (Mafra et al. 2012) as an endogenous control.



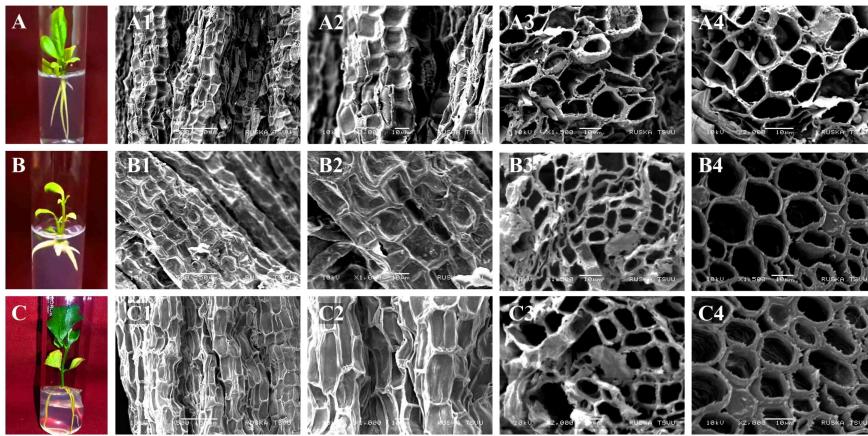
**Figure 3A-D** Relative expression of growth regulating factors (**A.** *GRF1* and **B.** *GRF5*) and GA3 pathway genes (**C.** *GA2OX1* and **D.** *K01*) at different stages of organogenesis from *in vitro* root explants of *Citrus jambhiri* Lush.



**Figure 4A-F** Relative expression of auxin responsive factors (**A.** *ARF1* and **B.** *ARF8*), auxin efflux carrier protein genes (**C.** *PIN1* and **D.** *PIN5*), auxin/IAA family gene (**E.** *IAA4*) and GH3 family protein gene (**F.** *GH3*) at different stages of rooting of *Citrus jambhiri* Lush.

## 6 Scanning electron microscopy (SEM)

The microstructures of 8 wks old roots were studied using SEM imaging. Roots, from the control along with various auxin supplemented media, were viewed and photographed on SEM (Model: JOEL-JSM 5600) using an automated sputter coater (Model: JEOL, JFC-1600) for 3 min at required magnifications from 500x to 2000x as per the standard procedures (Bozzola and Russell 1998) at RUSKA Lab, College of Veterinary Science, P.V. Narasimha Rao Telangana Veterinary University (PVNR TVU), Rajendranagar, Hyderabad, India.

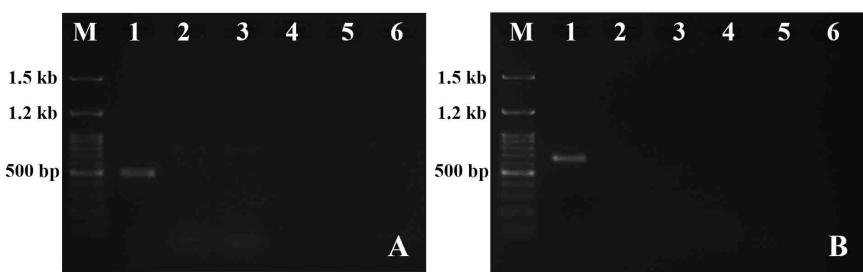


**Figure 5A-C** Scanning electron micrograph of the roots induced in *Citrus jambhiri* Lush. plantlets using various concentrations of auxins [A. ½MSN (A1-A2: External at 500 and 1000x; A3-A4: Internal at 1500 and 2000x), B. ½MSN+NAA 1.0 mg L<sup>-1</sup> (B1-B2: External at 500 and 1000x; B3-B4: Internal at 1500 and 2000x ), C. ½MSN+IAA 1.0 mg L<sup>-1</sup> (C1-C2: External at 500 and 1000x; C3-C4: Internal at 1500 and 2000x)]

## 7 Virus indexing

Screening of two major *Citrus* infecting viruses, namely, Huanglongbing (HLB) and *Citrus tristeza* virus (CTV), was carried out by reverse transcriptase PCR (RT-PCR) among the regenerants. For HLB detection, the genomic DNA (gDNA) was isolated from the midrib of the leaf tissues using the DNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) by following the manufacturer's protocol. The primers, *LasF* (5' GGAGAGGGTGAGTGGAATTCCGA 3') and *LasR* (5'ACCCAACATCTAGGTAAAAACC 3'), were designed from the hypervariable effector protein of the virus (Fujikawa and Iwanami, 2012). The PCR was carried out using Go Taq® G2 green master mix (Promega Corporation, US) under the conditions of 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min in SimpliAmp thermal cycler (Applied Biosystems, Life Technologies®).

For detection of CTV, total RNA was isolated from the leaf midrib of *in vitro* raised plantlets using RNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) by following the manufacturer's protocol and converted into cDNA by Verso cDNA synthesis kit (Thermo Scientific™, USA). The primers, namely, *K607F*(5'ACTCRCCGTTGACTCTGTTAAA3') and *K608R* (5'GTACCGAACATATACTCCAGT3'), were designed based on the viral coat protein according to Sharma et al. (unpublished data) and were procured from GCC Biotech (I) Pvt. Ltd., Kolkata, India. The PCR was carried out under the conditions of initial denaturation at 94°C 2 min, 35 cycles of 94°C 30 s, 59°C 45 s followed by 72°C 45 s, and 72°C 10 min of final extension. For the detection of amplicons, PCR products were run on 1.8% agarose gel, and pictures were taken on the E-Box gel documentation imaging system (Vilber Lourmat, France).



**Figure 6A-B** Virus indexing employing *Las F* and *R* (500 bp) to detect Citrus greening (Huanglongbing) para retrovirus (DNA) [A] and *K607 F* and *R* (648 bp) to detect *Citrus tristeza* retrovirus (RNA) [B] in *Citrus jambhiri* Lush. regenerants

[Lane M: 100 bp ladder (GCC biotech); Lane 1: Positive control (*C. greening* or CTV infected leaf midrib); Lane 2: leaf midrib from the mother plant; Lane 3: *In vitro* leaf midrib from the plantlets (½MSN); Lane 4: *In vitro* leaf midrib from the plantlets (½MSN+NAA 1.0 mg L<sup>-1</sup>); Lane 6: *In vitro* leaf midrib from the plantlets (½MSN+IAA 1.0 mg L<sup>-1</sup>); Lane 7: primercontrol (negative control)]

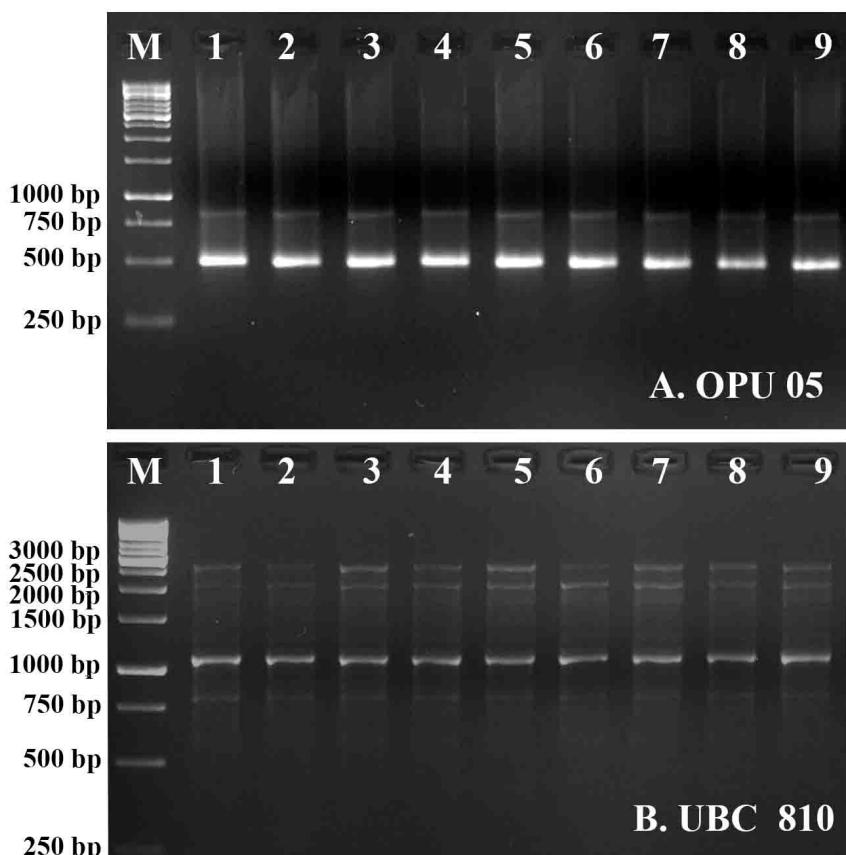
## 8 Assessment of genetic fidelity

To assess the genetic stability of *in vitro* raised plantlets, clonal fidelity test was carried out by using PCR based randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). Total gDNA were isolated from leaf tissues of the *in vitro* raised plantlets under different PGR treatments using DNeasy Plant Mini Kit (QIAGEN, India (P) Ltd.) using QIAcube by following manufacturer's protocol. The isolated gDNA were quantified using a QIAexpert (QIAGEN Hilden, Germany), checked on 0.8% agarose gel electrophoresis, and finally adjusted to a concentration of 50 ng  $\mu\text{L}^{-1}$  for enabling PCR.

**RAPD:** Eight RAPD primers (Supplementary Table 2) were used to screen the genetic fidelity of the *in vitro* raised plants. PCR was performed in a total volume of 25  $\mu\text{L}$  comprising of 50 ng template DNA, 10 pM primer, 12.5  $\mu\text{L}$  of 2X Taq PCR master mix (QIAGEN, India (P) Ltd.). PCR amplification was carried out at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 32-34°C (varies with primer) for 2 min, extension at 72°C for 2 min and final extension of 7 min at 72°C in a thermocycler (Rohini et al. 2015).

**ISSR:** To assess the clonal fidelity of the *in vitro* regenerants, ten ISSR primers (Supplementary Table 2) were used. PCR amplification was carried out using 50 ng of gDNA, 1  $\mu\text{L}$  of 10 pM primer, 12.5  $\mu\text{L}$  of 2 X Taq PCR master mixes for a total reaction volume of 25  $\mu\text{L}$  in a thermocycler with reaction conditions by following Rohini et al. (2015). The annealing temperature was optimized at 42-54 °C by gradient PCR.

All the primers (Supplementary Tables 1 and 2) were procured from Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, India. Genetic integrity, as revealed by the banding patterns of the PCR products, was checked on 1.8% agarose gel electrophoresis, and pictures were taken on E-Box gel documentation imaging system (Vilber Lourmat, France).

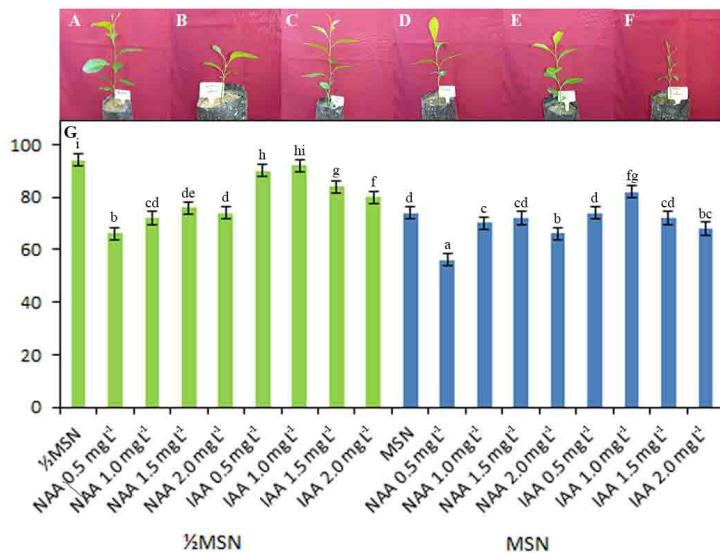


**Figure 7A-B** Genetic fidelity of *Citrus jambhiri* Lush. regenerants employing RAPD (A. OPU 05) and ISSR (B. UBC 810) markers

[Lane M: 1 kb DNA ladder for RAPD and ISSR; 100 bp DNA ladder for SSR; Lane 1: Mother plant from Kachai village, Ukhrul, Manipur; Lane 2: *In vitro* seedlings; Lane 3: Seedlings planted at Langol farm of ICAR, Manipur; Lane 4: Seedlings planted at polyhouse of ICAR, Manipur; Lane 5: Regenerants obtained from MSN+BAP 1.0+GA<sub>3</sub> 1.0 mg  $\text{L}^{-1}$ ; Lane 6: Regenerants obtained from MSN+BAP 1.0+GA<sub>3</sub> 2.0 mg  $\text{L}^{-1}$ ; Lane 7: Plantlets obtained from  $\frac{1}{2}$ MSN; Lane 8: Plantlets obtained from  $\frac{1}{2}$ MSN+NAA 1.0 mg  $\text{L}^{-1}$ ; Lane 9: Plantlets obtained from  $\frac{1}{2}$ MSN+IAA 1.0 mg  $\text{L}^{-1}$ ]

## 9 Acclimatization and hardening

*In vitro* plantlets with well-developed roots were washed thoroughly with sterile distilled water and acclimatized in the mixture of sterile garden soil, sand, and vermicompost in 1:1:1 ratio. The plantlets were irrigated with Hoagland's solution (HiMedia<sup>®</sup>) [Hoagland and Arnon, 1938] twice a daily for the next 2 wks. For hardening, the plantlets were maintained at a well-aerated mist house with temperature 26±2°C. Well established, virus-free and true-to-type micro propagated plants of *C. jambhiri* were then maintained in the polyhouse.



**Figure 8A-G** Survivability of *in vitro* plantlets from different concentrations of auxins [A.  $\frac{1}{2}$ MSN, B.  $\frac{1}{2}$ MSN+NAA ( $1.5 \text{ mg L}^{-1}$ ), C.  $\frac{1}{2}$ MSN+IAA ( $1.0 \text{ mg L}^{-1}$ ), D. MSN, E. MSN+NAA ( $1.5 \text{ mg L}^{-1}$ ), F. MSN+IAA ( $1.0 \text{ mg L}^{-1}$ ), G. %Survivability of the *in vitro* plantlets derived from MSN+BA 1.0+GA<sub>3</sub>  $1.0 \text{ mg L}^{-1}$  and different concentrations of auxins]