



Multiple plant regeneration from matricular substance released from explants of *Citrus jambhiri*

POOJA MANCHANDA¹, ANU KALIA², GURUPKAR S SIDHU³, H S RATTANPAL⁴, KULJOT KAUR⁵ and SIMRANJEET KAUR

Punjab Agricultural University, Ludhiana 141 001, India

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ABSTRACT

Comparative performance of epicotyl-, hypocotyl-, and cotyledonary (Cot) explants for direct organogenesis was evaluated with best response on BAP (3.5 mg/l) + NAA (0.5 mg/l) supplemented MS medium for Cot explants. This differential behaviour can be attributed to diversity and concentration of phytohormone(s), signal phenolics and other compounds. This study identifies the spatio-specific variabilities of these compounds leading to emergence of multiple shoots from explant's injured regions indicated by formation of foamy-white exudate, 'matrix'. Topographically, matrix appeared amorphous containing granulation. Further, FT-IR spectra indicated possible presence of wound-induced sugars and phytohormones. Likewise, LC-MS study revealed presence of diverse sugars, phytohormone (PH)/PH-like and signal compounds. This study illustrates possible interactive roles played by spatial co-localization of these compounds at wounded cut ends of explants for multiple *in vitro* shoot organogenesis. This study put-forth a novel concept of localized occurrence of multiple primary and secondary metabolites at cut/wounded ends of citrus explants which led to emergence of multiple shoots. These results are promising and could serve the basis for further investigations on various other citrus species for diversity and quantity of various metabolites during *in vitro* cultivation.

Key words: Cotyledons, Epicotyl, FT-IR Spectroscopy, LC-MS, SEM

Plant growth and development is controlled through a complex interactive action of diverse compounds comprising of both primary as well as secondary metabolites (Schafer *et al.* 2016). The role of these compounds in development of callus and direct organogenesis during micropropagation of explants derived from different organs of the plant or germinated seedling is not yet completely resolved (Hu *et al.* 2017). As micropropagation in tree plants can be beneficial for development of improved genotypes for climate resilience, tolerance to one or multiple abiotic stresses and for enhanced fruit quality, the possible mechanistic role of the individual compounds as well as their interactions during *in vitro* organogenesis have to be delineated (Hu *et al.* 2017). Further, the spatio-temporal localization of *in vitro* organogenesis inducing substances is critical particularly in citrus explants (Ng *et al.* 2016).

Micropropagation of rough lemon (*Citrus jambhiri* Lush.; 2n=18), generally involves the use of cotyledonary explants through regeneration from callus derived from stem explants on Murashige and Skoog's medium (MS) supplemented with 2,4-D (1.5 mg/l) (Ali and Mirza 2006) and on MS+2,4-D (2 mg/l) (Savita *et al.* 2011). Due to possibilities of somaclonal variations, direct organogenesis, i.e. formation of organs adventitiously and directly from the explant without an intervening callus phase will be most desirable (Stfaan *et al.* 1994). However, during direct organogenesis, the occurrence and concentration of various growth regulators, pH of the medium and culture environment have profound effect on the growth of *in vitro* raised plantlets.

Hence, the present investigation was carried out for direct organogenesis (single-step regeneration) in *Citrus jambhiri* (rough lemon) without an intervening callus phase using different explants of *in vitro* raised seedlings, i.e. hypocotyl, epicotyl, cotyledon and leaf. This work also explores the role of localized presence of certain specific metabolites at the damaged or cut ends of the explant for enhanced shoot regeneration possibility.

MATERIALS AND METHODS

Plant material and surface sterilization of seeds: Mature fruits from rough lemon growing in Citrus orchards of Punjab

¹Assistant Professor (poojamanchanda5@pau.edu), ³Assistant Horticulturist (gurupkar-soab@pau.edu), ^{5,6}Research Scholars (kuljotkaur006@gmail.com, simikaur.mehrok@gmail.com), School of Agricultural Biotechnology; ²Assistant Professor (kaliaanu@pau.edu), Department of Soil Science; ⁴Senior Horticulturist (hsrattanpal@pau.edu), Department of Fruit Science, Punjab Agricultural University, Ludhiana, India.

Agricultural University, Ludhiana were collected. Seeds were extracted from ripened fruits (Fig 1A) and washed with 'Teepol' detergent followed by washings under tap water. Outer seed coat (whitish in colour) was removed and the inner seed coat was kept intact. These seeds were further washed with 'Bavistin' to remove fungal contamination and subsequently washed with tap water. Under laminar air flow cabinet, the seeds were treated with mercuric chloride for 10 min followed by three washings with autoclaved distilled water.

In vitro raising of seedlings: Surface-sterilized seeds were inoculated onto MS medium (Murashige and Skoog

1962) supplemented with 100 mg/l myo-inositol and 30 g/l sucrose and subsequently; incubated at $24 \pm 2^\circ\text{C}$ in 16:8 hours light: dark conditions. Post-germination on 24th day of culturing, seedlings with established epicotyl and hypocotyl segment (Fig 1B) were formed in culture jars containing 50 ml of MS medium. The direct organogenesis was carried out in the commercial tissue culture laboratory of PAU, Ludhiana during 2018–19.

Direct organogenesis from different explants: After 3 weeks, *in vitro* raised seedlings were excised to obtain different explants like cotyledon, epicotyl, hypocotyl, leaves and inoculated on MS medium supplemented with different

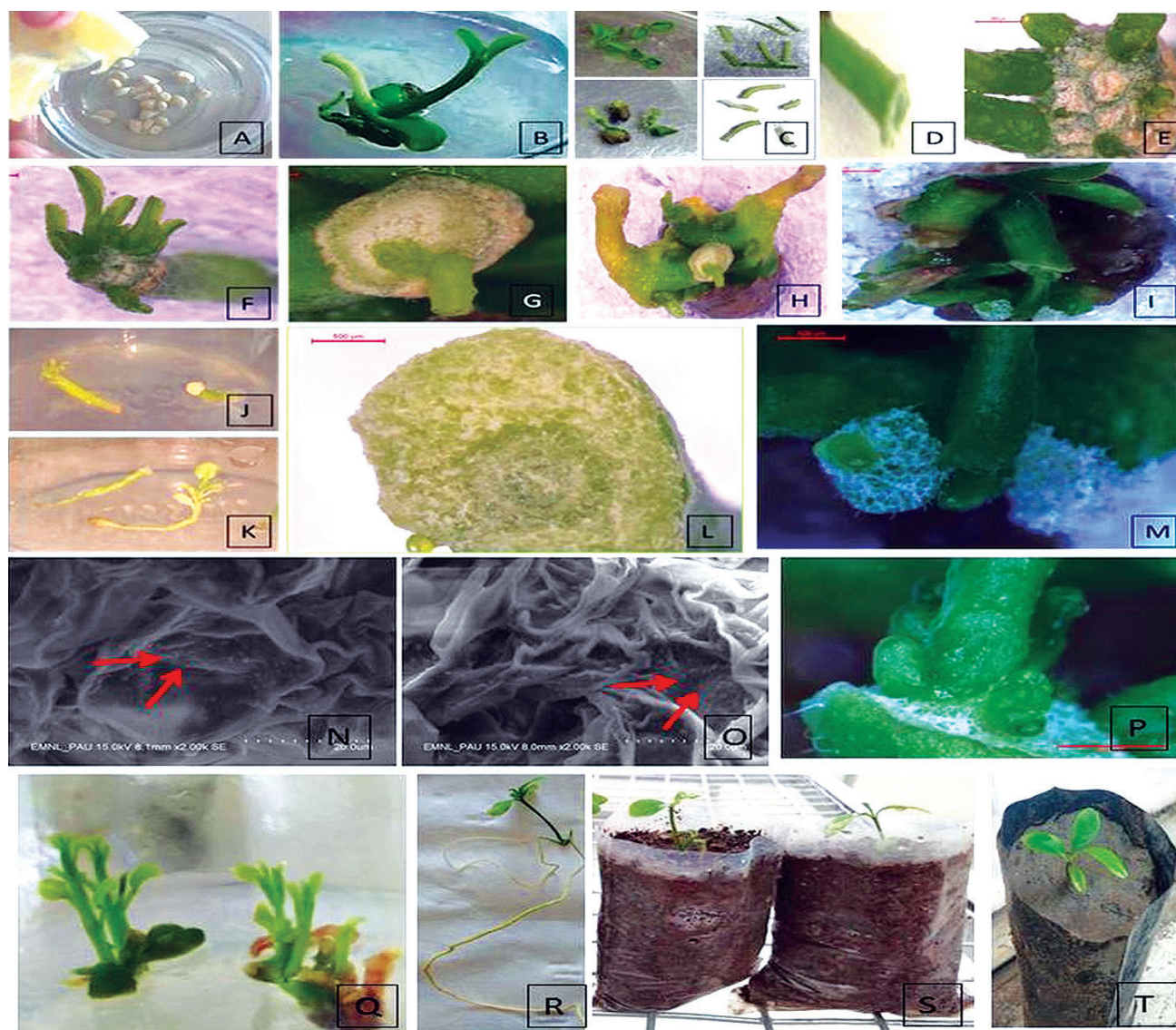


Fig 1 Direct adventitious organogenesis from different explants of *Citrus jambhiri* (A) Seeds obtained from ripened fruits of rough lemon; (B) Plantlet with established epicotyl and hypocotyl segment after 24 days of placement onto basal MS medium; (C) Different explants (leaves, hypocotyl, cotyledon and epicotyl) tested for direct organogenesis; (D, E, F, G) Plantlets started regenerating from cut ends of explants; (H, I) Multiple plantlets arising from top and middle portions of cotyledon; (J, K) Plantlet regenerating end of epicotyl showing phototropism; (L, M) Conspicuous white matrix formed at the cut ends of epicotyl and cotyledonary segment, respectively; (N, O) SEM study of white matrix revealed the occurrence of granulated nature of the matrix material (marked by red arrows); (P) Appearance of multiple shoot-bud like structures emerging from the cut end of the explant covered with white matrix; (Q) Elongation of shoots on MS + BAP (2 mg l⁻¹) + GA₃ (1 mg l⁻¹); (R) Plantlet with well-developed root and shoot system; (S) Plantlets transferred to glass-house after hardening; (T) One-month old plantlet growing in glass-house.

concentrations and combinations of auxins (Naphthalene acetic acid, NAA) and cytokinins (Benzyl amino-purine, BAP). MS basal + BAP (2 mg/l) served as the control (Rattanpal *et al.* 2011).

IR-spectroscopy: The white matrix from the cut wounded ends was collected and freeze-dried. The dried white powder analysed in Fourier Transform IR spectroscope (model Thermo 6700 NXT, USA) for mid IR region (4000–400/cm). The FT-IR spectra for the possible reference organic compounds at the cut end of the explant were also generated by using IR spectroscopy grade pure compounds/chemicals.

Liquid Chromatography-Mass Spectrometry analysis: The freeze-dried white matrix exudate sample was dissolved in HPLC grade methanol, and filtered. About 20 µl sample was injected into LC column (Water X bridge C18, USA) having separation module (Water Alliance 2795, USA) coupled with a hybrid high definition Accurate-Mass Quadrupole Time of Flight Mass Spectrometer (Q-TOF-MS) (Waters, Micromass Q-TOF micro, USA). The samples were run for 10 min in positive ion mode and the retention time was noted. The m/z ratio peaks were matched in Mass Bank using match spectra for obtaining the possible organic compounds.

Scanning Electron microscopy analysis: The cut ends of different explants were lyophilized, stubbed and made conductive by sputtering gold (10–20 nm gold layer) in Hitachi E-1010 Ion sputter coater before imaging in Hitachi s-3400 N SEM @ 15 kV acceleration voltage in secondary electron imaging mode.

Subculturing, elongation and rooting of shoots: The shoots raised from different explants were sub-cultured onto the same medium after 24 days for replenishment of the nutrients to the growing mass of plantlets which were further elongated on MS+BAP (2 mg/l)+Gibberellic acid; GA₃ (1 mg/l). The basal mass of elongated shoots was excised to obtain 2–3 plantlets which were transferred to root induction medium. The rooting of *in vitro* grown shoots is significant step for their establishment in soil. Therefore, rooting was induced on MS medium, supplemented with IBA (1.0 mg/l) + NAA (1 mg/l) (Saini *et al.* 2010).

In vitro hardening of plantlets and acclimatization to soil: The plantlets with well-developed root and shoot

system were removed from the rooting medium and washed thoroughly under running tap water. The hardening of plantlets was carried out on moist cotton for 6 days. After hardening, the plantlets were transferred to glasshouse maintained under controlled conditions containing a mixture of coco-peat, farmyard manure and garden soil. Half-MS liquid media without sucrose was added for the proper growth of plantlets grown under *in vitro* conditions for four days. Cultures were observed on daily basis and analysis under stereomicroscope was carried out on weekly basis.

Data analysis: The experiment was conducted with a minimum of three replications per treatment. The SPSS statistical software (version 16.0) was used for the analysis of variance (ANOVA) of the data according to completely randomized design analysis (Griffith 2007). The percentage data was transformed via arcsine before analysis. Means with significant difference were separated using Duncan least significant difference (LSD) at P<0.05 probability level of significance and used for comparing means of treatments for making interpretations.

RESULTS AND DISCUSSION

Raising of seedlings under in vitro conditions: During the present investigation, different explants from *in vitro* raised seedlings were placed on MS medium [fortified

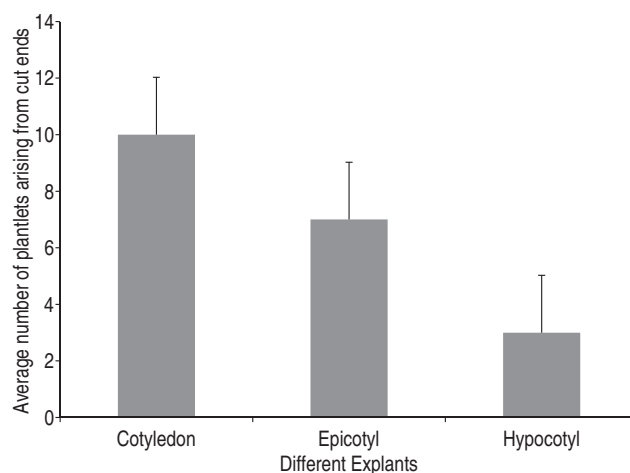


Fig 2 Average number of shoots arising from the cut ends of different explants.

Table 1 Mean per cent response of different explants for induction of direct formation of shoots in *Citrus jambhiri* (Rough lemon)

Medium	Mean % explants showing response towards direct organogenesis		
	Epicotyl	Cotyledon	Hypocotyl
MS + BAP (2 mg/l) [Control]	27.67	36.0	12.0
MS + BAP (2.5 mg/l)	31.33	36.67	12.67
MS + BAP (3.0 mg/l)	33.0	37.33	12.33
MS + BAP (3.5 mg/l)	36.33	39.33	14.33
MS + BAP (3.5 mg/l) + NAA (0.5 mg/l)	39.33	41.67	20.67
MS + BAP (3.5 mg/l) + NAA (1.0 mg/l)	36.33	38.0	17.0

Critical difference (CD) for treatment means for different explants at 5% level of significance was 3.04 for epicotyl, 2.41 for cotyledons and 3.27 for hypocotyl.

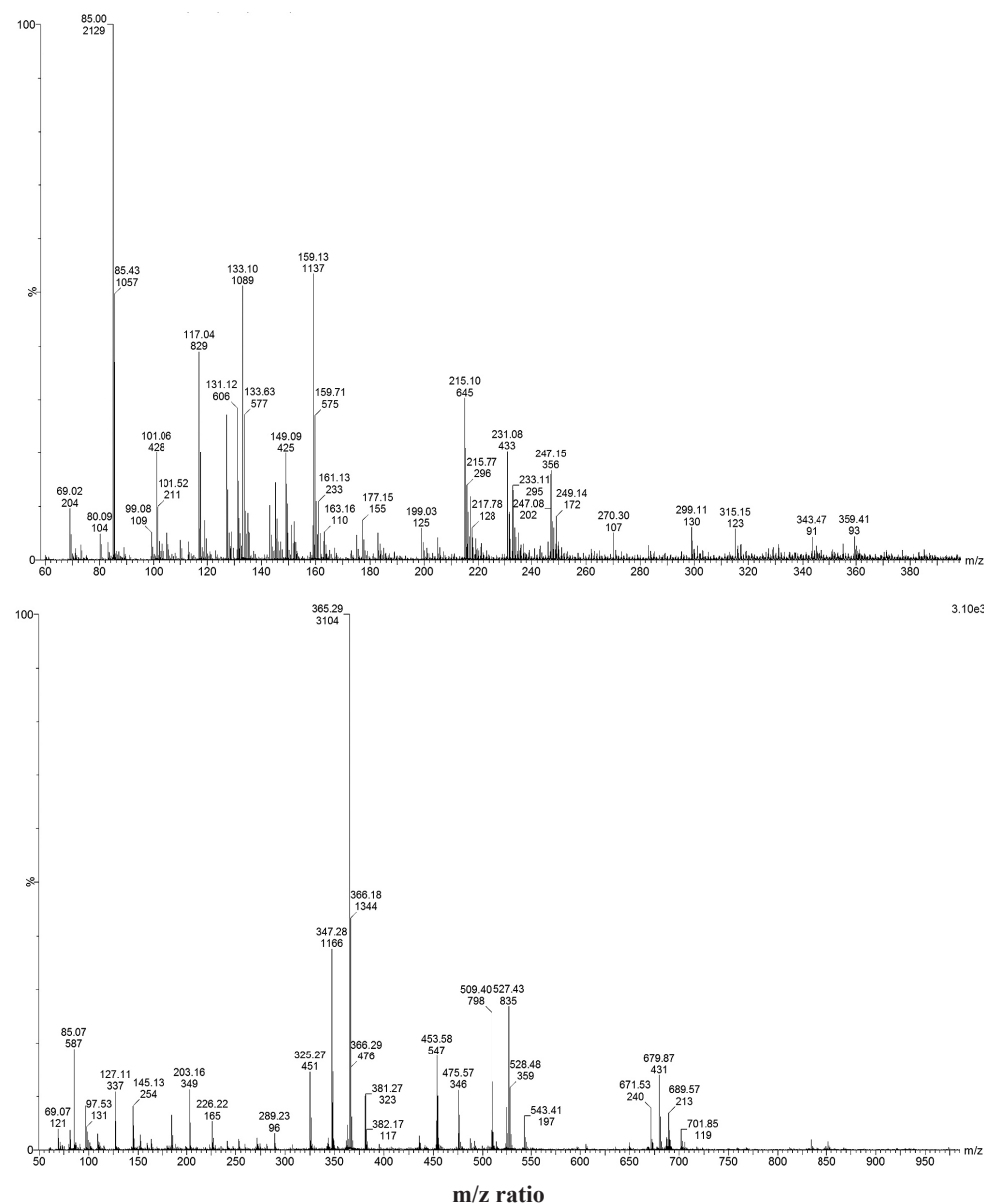
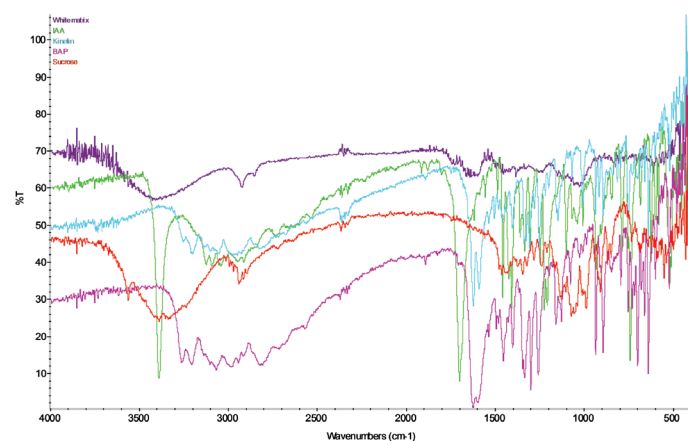


Fig 3a FT-IR spectra depicting the possible occurrence of specific organic compounds in the white matrix formed at the cut ends of explants of *Citrus jambhiri*. b. LC-MS spectra (1 and 2) of the white matrix substance depicting the occurrence of diverse organic compounds with predominance of sugars (ManglucNAc2 and sucrose), flavonoids (Fenfluramine, Rutin, Orientin) and phytohormones (N-6-isopentenyladenine-7-glucoside, benzyl amino purine, indole acetic acid)

with cytokinin (BAP) and auxin (NAA)] for direct organogenesis. For raising the seedlings, around 622 seeds were placed on the medium and out of that, 523 exhibited germination. Sixty two seeds were discarded due to contamination and 37 seeds did not germinate, probably due to dormancy phenomena.

Direct organogenesis from different explants: The different explants, i.e. cotyledons, hypocotyl, epicotyl and leaves derived from seedlings (Fig 1C) were tested for their potential of direct organogenesis on six different media. It was observed that from cut ends of different explants (Fig 1D); plantlets started regenerating (Fig 1E, F, G). Different explants exhibited significant variations for per cent response for

direct organogenesis on six different media. When MS medium supplemented with BAP (2 mg/l) was used, per cent response was 27.67, 36.0 and 12.0% in the epicotyl, cotyledon and hypocotyl segments respectively. Upon supplementing the medium with increased concentration of BAP and NAA (0.5 mg/l), i.e. MS + BAP (3.5 mg/l) + NAA (0.5 mg/l), significant increase in response for the epi-, cotyledon and hypocotyl segments were 39.33, 41.67 and 20.67%, respectively.

Cotyledonary explants exhibited best response followed by epicotyl and hypocotyl segments as mean per cent formation of shoot primordia was maximum and formed after 9 days in cotyledons (Table 1). Multiple direct bud-like structures were formed on the top and middle portions of the cotyledon (Fig 1 H, I). The number of plantlets formed were maximum in case of cotyledons (Fig 2) followed by epi- and hypocotyl segments.

Although, epicotyl segments had two cut ends for emergence of new shoots but invariably, only the proximal end formed shoots while the distal end does not (Fig 1 J, K). Further, it was observed that the wounded

ends of different explants, exhibited accumulation of tissue exudates which was recorded as white matrix under stereomicroscope (Fig 1 L, M). The scanning electron microscopy of the white matrix exuded from cut and wounded region showed the presence of polymeric matrix material impregnated with grainy and flaky crystals or granular structures which, further indicated the possibility of presence of primary or secondary metabolites as granules (Fig 1 N, O). Shoots started forming from the white exuded matrix (Fig 1P) and were elongated on MS medium supplemented with BAP (2 mg/l) and GA₃ (1 mg/l) (Fig. 1Q).

Rooting of shoots and acclimatization: Individual shoots were separated out from the basal mass and cultured on MS medium+IBA (1.0 mg/l)+NAA (1.0 mg/l) for rooting. Roots started forming after 17 days of culturing and after 28–30 days, plantlets with well-developed root and shoot system were formed (Fig 1R), which were hardened on moist cotton and were transferred to glass-house (Fig 1S, T). Rooting on Kinnow was achieved on MS + NAA (3 mg/l) (Rani *et al.* 2004) and on MS + NAA (10 mg/l) (Usman *et al.* 2005). Ali and Mirza (2006) also achieved rooting of regenerated shoots on medium supplemented with NAA (0.5 mg/l).

Fourier Transform-Infra Red spectroscopy characterization: The FT-IR analysis of the white exudate substance indicated the occurrence of diverse functional groups. The major peaks indicating the presence of sucrose include 3500–3000/cm (-OH stretching), 2940–2910/cm (-CH group stretching) and C=O group vibrations (1728–1700/cm) (Fig 3a). Likewise, the possibility of phytohormone such as Indole acetic acid can be presumed from 1700–500/cm peak and adenine specific cytokinins (kinetin and BAP) indicated from 1251.3 and 1116.7/cm peaks.

Damaged tissue is anticipated to exhibit the presence of cell-wall related and the phloem transportable sugars (Vatasescu-Balcan *et al.* 2008). The vibrational bands ranging from 1728–1536/cm and 1500–500/cm (Hart *et al.* 2016) may possibly indicate the presence of the phytohormones; most likely kinetin and IAA (Tamas and Davies 2016). Besides Hu *et al.* (2017) have reported the role of the endogenous IAA levels in stimulation/inhibition of *in vitro* shoot organogenesis. The conversion of various starch reserves present in the cotyledon into sucrose might be the reason behind the observance of sucrose spectra on the wounded regions (Tamas and Davies 2016). FT-IR analysis of these adenine-based cytokinins exhibited spectra characterized by the occurrence of most intense band at 1610/cm representing the N-H vibrations of secondary amines on adenine (Hart *et al.* 2016). The peak shifts from 1248 and 1133/cm to 1252 and 1148/cm respectively, indicated the possible occurrence of adenine (Hart *et al.* 2016). Similar peaks at 1251.3 and 1116.7/cm appeared in the IR spectra of the white matrix sample also.

Liquid Chromatography-Mass Spectrometry analysis: The acquired LC-ESI-Q-TOF MS spectra (Fig 3b) was compared in the MassBank database (Horai *et al.* 2010). Based on the measured mass to charge ratio of the protonated

molecules of white matrix substance (m/z 85.0, m/z 365.27) and the molecular masses were calculated, corresponding to the molecular formula of known compounds, i.e. piperidin, and N-6-isopentenyladenine-7/9-glucoside respectively besides signal flavonoids such as rutin, fenfluramine, and orientin (Brito *et al.* 2014, Sharma *et al.* 2015). Further, the localized occurrence of probable phytohormones was predominated by m/z peaks that matched to N-6-isopentenyladenine-7/9-glucoside, benzyl amino purine, and indole acetic acid.

In the present investigation, the direct organogenesis protocol was established from different explants resulting in the development of large number of plantlets. The results suggest that cotyledonary and epicotyl segments are best for organogenesis. The cut ends of the explants showed multiple shoot emergences possibly due to localized occurrence of signal compounds, phytohormones and carbon source (Ikeuchi *et al.* 2017). Plant regeneration from callus phase is common in rough lemon (Savita *et al.* 2011) but it leads to somaclonal variations and hence, interrogates the clonal fidelity of plants (Manchanda and Gosal 2012). Regeneration directly from explants can be a preferred mode for micropropagation, genetic transformation and genome editing processes. This study put-forth a novel concept of localized occurrence of multiple primary and secondary metabolites at cut/wounded ends of citrus explants which led to emergence of multiple shoots.

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