Wisdom Journal of Science and Technology 1(1), 44-56, June 2025
https://doi.org/10.64848/WJST.1.1.2025.44-56

WISDOM JOURNAL of Science & Technology

RESEARCH ARTICLE

Efficient clonal propagation of Rangpur lime (*Citrus limonia*) using mature nodal explants: An evaluation of lab to land production of disease-free plants

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Abstract: Rangpur lime is one of the most promising Citrus rootstocks for Indian conditions. An efficient micropropagation protocol from mature nodal explants of Rangpur lime was developed using the enhanced axillary branching method. In the present study, multiple shoots were obtained by stimulating axillary shoots from single nodes derived from field-grown trees on Murashige and Skoog's (MS) medium containing BA (1.11 µM), Kinetin (1.16 µM), and 3% sucrose and 100% rooting was obtained in half-strength MS medium. The rooted plants were then transferred to a potting mix containing soil and agropeat. Once again, 100% survival was observed during acclimatisation. Inter simple sequence repeats (ISSR) analysis of the in vitro derived progeny was used to determine their clonal fidelity of the regenerants vis-à-vis the mother plant. The results of this study have enormous commercial applications for the propagation of clean and healthy disease-free citrus rootstocks.

Keywords: *Micropropagation, Axillary branching, Rangpur lime, Rootstock, Clonal fidelity, ISSR*

Introduction

Rangpur lime (Citrus limonia) is a medium-sized tree with a spreading habit, round top and slender twigs with short thorns [1]. It is considered to be a natural hybrid between mandarin and acid limes/lemons [2]. This species is native to India and is a prolific bearer of flavoured acidic fruits [3]. Common names used for the fruit are Rangpur in India, Canton lemon in South China, Hime lemon in Japan, Cravo lemon in Brazil, Japanche citroen in Java, and Rangpur lime or mandarin-lime in the United States [4]–[6]. Other Indian names include Sylhet lime, Surkh nimboo, Sharbati and marmalade lime [7]. Its horticultural importance lies primarily in its use as a rootstock [3]. Rangpur lime is hardier than true limes, thereby identified as an ideal rootstock for Indian conditions, which makes a good union with sweet orange, mandarin, grapefruit, and pummelo and helps increase their productivity, quality, and survival [4], [8]. It is high yielding, vigorous, salt and drought-tolerant, precocious with early fruit maturity, has high resistance to citrus tristeza virus (which causes citrus decline), with good adaptability to a wide range of soil types, particularly heavy soils [2]–[4]. It has been found to have higher nutrient absorption efficiency than common rootstocks such as trifoliate orange. The greater salt resistance is associated with its capacity to limit the accumulation of chloride ions in the leaves. It is the most promising rootstock for Nagpur mandarin, Kinnow, Coorg mandarin, Mosambi, grapefruit, pummelos and Sathgudi sweet orange spread over north, central and south India [3], [5]. This is also the most widely used rootstock in Brazil and

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Received: 28/04/25, Accepted: 28/05/25, Published Online: 04/06/25

Argentina [2], [3], [5]. It has assumed commercial importance in the processing industry (juice and pickles) [7].

Citrus rootstocks such as Rangpur lime are mostly propagated by various methods, including seeds and vegetative approaches [3], [9]. However, it requires the skilful collection of seeds from healthy, virus-free old trees, which have a good pedigree performance and thereafter, efficient storage methods [5]. On average, one of five seeds of Rangpur lime gives rise to a healthy seedling [7]. Moreover, seeds of citrus species are moderately recalcitrant and conservation gets restricted due to a loss in germination frequency within a short span of time. Further, the germination is uneven and the plants grown from the seeds also exhibit extended juvenility [2]. Seed germination may be challenging due to the presence of multiple germination inhibitors in their seed coats [5]. Thus, it can be concluded that seeds are not the right option for Citrus propagation. The conventional vegetative methods of Citrus propagation are restricted to particular seasons and due to the larger size of the cutting of a particular thickness, the plant material usually becomes a constraint.

Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plant species [13], including Citrus [3], [11], [13]. However, to the best of our knowledge, there is only a single study aimed at developing a micropropagation protocol using axillary buds [14] for Rangpur lime and in that study, there was a complete failure in obtaining any shoot multiplication. In another micropropagation study, epicotyl and shoot tips were used from newly germinated seeds [11]. Other studies on the direct organogenesis of Rangpur lime by Almeida et al. [15] and Costa et al. [16] also showed a significantly low explant response, along with a low (0.6-1.6) shoot multiplication fold. Somatic embryos from callus cultures of nucellar origin were obtained by Tomaz et al. [17]. However, hardening was a complete failure in this case and no plants were obtained. All these studies reveal a big lacuna in the propagation of Rangpur lime. The study was thus undertaken to develop an efficient micropropagation protocol for the production of clonally uniform plants of this important rootstock.

Methodology

Plant material, growth conditions and establishment of explants in aseptic conditions

Authentic virus-free, certified germplasm of Rangpur lime from mature trees (5-7 years old) was collected from North India (Figure 1a). Nodal segments were used as the explants in this study. The leaves were trimmed and explants measuring approximately 1.5–2.0 cm in length were washed in running tap water for 10 min. After giving a quick dip in 70% alcohol, the excised shoots were air-dried on a blotting sheet and thoroughly washed for 30 min under running tap water again. Thereafter, the explants were rinsed with 4% Teepol (Reckitt Benckiser Ltd, India) solution for 10 min. The detergent was removed by constant shaking and then thorough washing under running tap water for another 30 min. The explants were surface sterilised with a 0.1% solution of mercuric chloride (Qualigens, India) and 2 drops of Tween 20 (Central Drug House, India) inside the laminar flow cabinet for 10-12 min. After three washings in sterile distilled water for 5 min each, the cut ends of the segments were trimmed and inoculated individually on basal Murashige and Skoog's (1962) medium having 3% sucrose and 0.8% agar in glass test tubes.

The pH of the media was adjusted to 5.8 before autoclaving at 1.05 kg cm-2 for 15 or 25 min for test tubes and glass jars, respectively. The cultures were incubated at 26 ± 1°C, under approximately 40 μmol m-2s-1 provided by cool, white fluorescent tubes (40W, Phillips, India) for a 16 h photoperiod.

Shoot multiplication

In a preliminary experiment, different cytokinins, namely, 2-isopentenyladenine (2ip), 6-benzylaminopurine (BA), and kinetin (Kn) at 2.5-10 μM, along with the basal MS medium as a control as well as Murashige and Tucker (MT) medium, were studied for their effect on the proliferation of shoots. In another experiment, the above-mentioned cytokinins were used in combinations with each other to study their interaction. In addition, thiadiazuron (TDZ; 0.1 μM) was also tried out in combination with BA and Kn.

Experiments were also conducted to study the effect of different carbohydrates namely sucrose (0-6%) and glucose (3%); and, various gelling agents such as agar (0.8%), agargel (0.4%), gelrite (0.2%) and a combination of 0.1% gelrite and 0.4% agar on the shoot multiplication of Rangpur lime. Further, to enhance the shoot proliferation rate and shoot length, various auxins such as Indole-3-acetic acid (IAA; 1.14–2.85 μM), Indole-3-butyric acid (IBA; 1.22–2.45 μM) and Naphthalene acetic acid (NAA; 1.34 –2.68 μM) were also tested along with BA as well as in combination with Kn. Growth adjuvants such as adenine sulphate (ADS; 40 mg l-1), casein hydrolysate (CH; 250–500 mg l-1), malt extract (ME; 250-500 mg l-1), coumarin (CO; 90-150 μM), glutamine (250-500 mg l-1) phloroglucinol (PG; 317.2 μM) and gibberellic acid (GA3; 0.28 μM) were supplemented to further improve the proliferation of shoots. Activated charcoal (AC; 250 mg l-1) was also added to the optimum treatment to study its effect on shoot growth and multiplication.

For the sub-culture of shoots, each cluster was divided into smaller clusters, shoot nodes and shoot tips. For every multiplication experiment, the total number of shoots formed in each shoot cluster, individual shoot length and multiplication rate were recorded at the end of the culture passage. Multiplication rates were calculated based on the number of propagules derived from a single cluster at the completion of each passage. During each subculture, all the dead tissue, callused tissue and adventitious buds were carefully removed from the proliferating clusters before transfer to fresh medium.

Rooting of shoots

Approximately 1cm long individual shoots were transferred to various rooting media containing MS medium, MS ½ (major salts reduced to half strength), MS 1/3 (major salts reduced to one third) and MS ¼ (major salts reduced to quarter strength). The media was further supplemented with different auxins at varying concentrations, such as IAA (0.57–5.7 μM), IBA (0.49– 4.9 μM) and NAA (0.53– 5.37 μM). The effect of varying sugar concentration (1%, 2% and 3%) was also observed on the root induction. The effects of liquid media supplemented with growth regulators on rooting in comparison to semi-solid medium were also studied. All the rooting experiments were initially performed in the test tubes and later in 400 ml glass jars. The parameters considered while standardising the rooting media were the extent of root induction, root quality, number of roots per plant and root length.

Hardening and acclimatisation

The plantlets obtained after 4 weeks of culturing in rooting medium were washed thoroughly in running tap water to remove all the traces of rooting media attached to roots. They were then transferred to polythene bags (13.5 cm × 8 cm) containing soil and agropeat (Varsha Enterprises, India) in different ratios (1:0, 1:1, 2:1, 3:1, 4:1, 0:1; v/v). Initially, the plants were placed closer to the cooling pads (RH: 80– 85%) and thereafter gradually shifted towards the exhaust fans over a period of 7-10 days (RH: 65%) in the greenhouse. Thereafter, the plants were shifted during the summer season to an open nursery at TERI's Micropropagation Technology Park (MTP), Gurugram, India.

Statistical analysis

All the multiplication and rooting experiments were repeated thrice for each treatment, with 24 replicates. The hardening experiments were repeated thrice with 50 plantlets per experiment. The effect of different treatments was quantified and the level of significance was determined by Analysis of Variance using the Costat statistical package. Significant differences between the means were assessed by Duncan's Multiple Range Test (DMRT) at $p=0.05$.

Clonal fidelity studies

Leaves from randomly selected shoots were collected at different intervals (5^{th}, 10^{th}, 15^{th}, 20^{th} and 25^{th} passages and hardened plantlets at nursery level) for comparison with the mother tissue. Along with that, one to two varieties were also chosen as outliers. They were properly labelled and lyophilised in the 'Virtis freezemobile G' lyophiliser for 48 hours at -70ºC. Total DNA was extracted following a modified 'Cetyl

Trimethyl Ammonium Bromide' (CTAB) DNA extraction procedure based on the Doyle and Doyle [18] protocol. Qualitative and quantitative assessment of total genomic DNA was performed by agarose (United States Biochemicals, USA) gel electrophoresis. Each DNA sample was diluted to a concentration of 25 ng µl-1 using sterile deionised water and stored at 4 °C. Seven inter simple sequence repeat (ISSR) primers (UBC, Canada) were used to evaluate clonal fidelity among the regenerated plantlets. Polymerase chain reaction (PCR) was performed in a volume of 20 µl containing 25 ng DNA, 10X Buffer (Biotools, USA), 50 mM of Magnesium chloride ($MgCl_2$; Biotools, USA), 10 mM deoxynucleotide triphosphate (dNTPs; Promega, USA), 10 mM primer and 1 unit of Taq (*Thermus aquaticus*) DNA polymerase (Biotools, USA). The optimised PCR conditions used for amplification consisted of an initial denaturation step at 94^0C for 5 min; continued denaturation for 35 cycles of 30s at 94^0C, annealing for 30 s at 42^0C, extension for 1 min at 72^0C; and one final extension at 72^0C for 5 min with a soak temperature of 4^0C performed in a 'Bio-Rad DNA Engine' (Peltier Thermal Cycler, USA). Amplified DNA was separated on a 2% agarose gel and electrophoresis was carried out at a voltage of 80 mV for 3–4 h. Lambda 1 kb ladder (Gibco BRL, USA) was used as the marker to interpret the PCR amplification products. The gel was visualised and photographed using a gel documentation system (Alphalmager® EC, Alpha Innotech Corporation, CA, USA). All the PCR reactions were repeated at least twice to check reproducibility. The ISSR amplification products were then scored based on presence (1) and absence (0) of bands across the tissue culture raised progenies, 1 parent (P) and outliers (O1: Alemow macrophylla, O2: C 35 citrange, O3: Swingle citrumelo) to determine clonal fidelity vis-à-vis the mother plant.

Results and discussion

Surface sterilisation and establishment of explants in aseptic conditions

Previous reports on Citrus micropropagation using nodal explants have mostly been successful using juvenile explants [12], [19]–[22]. This is one of the critical stages as it has been observed that explants from mature trees may not be able to withstand the harsh sterilisation procedure that is required to obtain aseptic cultures and even the surviving cultures may not show bud break as readily as tissues from juvenile sources [19], [23], [24]. However, in the present study, shoot cultures obtained from mature nodal explants of Rangpur lime were successfully established following the disinfection procedures as described under the Materials and Methods section. More than 90% of the primary explants were observed to be contamination-free. Axillary buds showed 100% viability along with the emergence of 2-3 shoots per node within four weeks of initiation (Figure 1b).

Shoot multiplication

On basal media, in some explants, bud break was observed, but the growth of the shoots remained restricted (Table 1). For this study, MS medium was found to be better as the base medium over MT, which was in contrast to the findings reported by Bashi et al. [21]. This may be due to the genotypic differences between Rangpur lime and C35 citrange. The application of plant growth regulators in a base medium is essential to achieve the best rates of shoot multiplication [10]. Of the various concentrations of the cytokinins tested in the preliminary experiment, BA at 2.5 µM was observed to be the best in terms of multiplication (2.82-fold multiplication every four weeks). Shoot elongation (of 1.5 cm average) was observed to be better on medium containing Kn (1.25-5 µM) and BA at 5 µM. Maximum numbers of shoots (3.64 and 3.48) were induced in media with BA (2.5 and 5 µM, respectively). 2ip at the tested concentrations (1.25-10 µM) did not show any favourable responses. BA has been shown to induce more shoot multiplication in various rootstocks, which contrasts with the results observed in this study [12]. Among all the media combinations attempted (Table 1), BA (1.11 µM) in synergy with Kn (1.16 µM) was observed to be the best media for shoot proliferation by axillary branching (Figure 1c, d). On this medium, a multiplication fold of 4.31 was obtained every 4 weeks and cultures were maintained for over 25 passages without the loss of morphogenic potential. The shoots were healthy and devoid of any callus. The synergistic effect of two or more cytokinins, especially BA and Kn, on shoot multiplication has been well documented in tissue culture of

many fruit species such as apple [25] and strawberry [26]. Similar reports that support the use of BA and Kn on axillary proliferation in some citrus species, such as Binhazir lime [27], Troyer citrange [20], Swingle citrumelo [19], *C. macroptera* [28] and Finger Lime [29] have been observed. This combination has been found to be successful in the propagation of citrus rootstocks in vitro using germinated seedlings [11]. An increase in the concentration of cytokinins beyond a certain threshold resulted in a decline in the multiplication rate for each of the cytokinins tested. Thus, higher combined concentrations of BA and Kn (6 μM or more) caused severe depression in shoot growth. The use of BA in conjunction with 2ip, even at low concentrations (0.6-1.8 μM), had an inhibitory effect on the proliferation rate, shoots per cluster as well as shoot length. TDZ also had an inhibitory effect on shoot multiplication as also observed in micropropagation of rootstocks, Troyer citrange [20], Swingle citrumelo [19] *and C. macroptera* [28] . The shoots showed necrosis and finally callused. The shoot inhibition might have occurred due to the increased level of endogenous cytokinins, which inhibit the action of cytokinin oxidase, the enzyme responsible for cytokinin degradation [30].

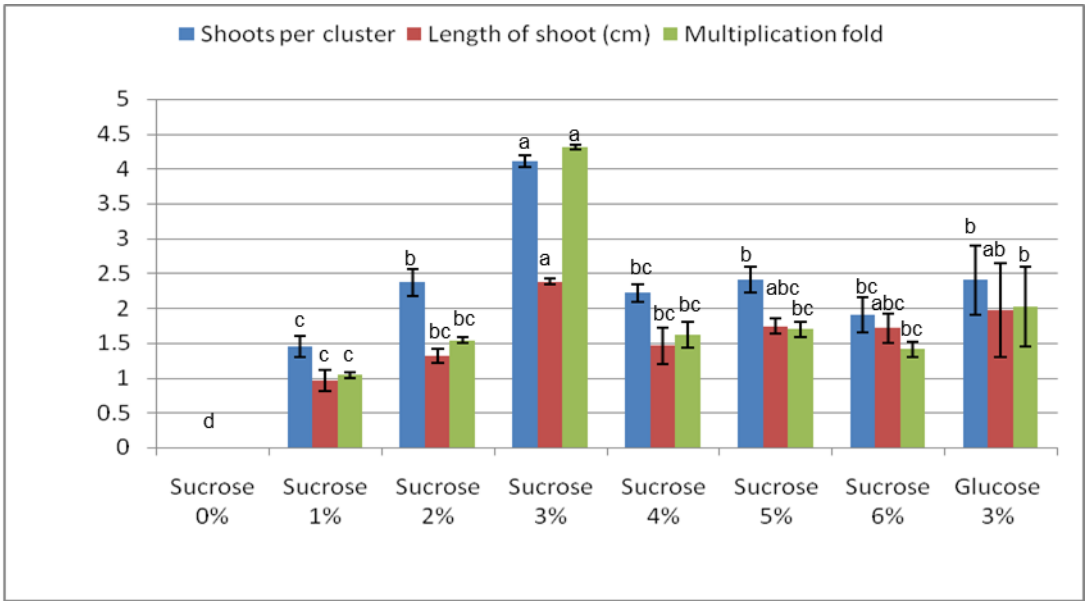
Table 1 Effect of cytokinin combinations on shoot multiplication in Rangpur lime

Cytokinin (μM)	Shoots per cluster*	Length of shoot (cm)*	Multiplication fold*
0 (Basal)	1.87 ± 0.12^{g}	1.19 ± 0.07^{def}	1.25 ± 0.2^{de}
BA (0.55) + Kn (0.58)	2.59 ± 0.26^{cde}	1.62 ± 0.22^{bc}	2.02 ± 0.46^{bcd}
BA (1.11) + Kn (0.58)	2.69 ± 0.33^{bc}	1.64 ± 0.17^{bc}	2.19 ± 0.41^{bc}
BA (1.11) + Kn (1.16)	4.11 ± 0.08^{a}	2.38 ± 0.04^{a}	4.31 ± 0.03^{a}
BA (1.66) + Kn (1.16)	3.15 ± 0.17^{b}	1.99 ± 0.16^{b}	2.75 ± 0.46^{b}
BA (0.55) + Kn (1.74)	2.94 ± 0.09^{bc}	1.60 ± 0.08^{bc}	2.38 ± 0.31^{b}
BA (1.66) + Kn (1.74)	2.74 ± 0.29^{bcd}	1.56 ± 0.19^{c}	1.97 ± 0.39^{bcd}
BA (2.22) + Kn (2.32)	2.71 ± 0.19^{bcd}	1.41 ± 0.13^{cd}	2.17 ± 0.31^{bc}
BA (3.33) + Kn (1.16)	2.15 ± 0.09^{efg}	1.08 ± 0.11^{def}	1.29 ± 0.12^{de}
BA (4.44) + Kn (2.32)	2.28 ± 0.19^{defg}	0.98 ± 0.03^{ef}	1.36 ± 0.16^{cde}
BA (1.11) + Kn (3.48)	2.49 ± 0.23^{cdef}	1.13 ± 0.19^{def}	1.46 ± 0.14^{cde}
BA (3.33) + Kn (3.48)	2.04 ± 0.06^{fg}	0.89 ± 0.03^{ef}	1.20 ± 0.1^{de}
BA (4.44) + Kn (4.65)	2.12 ± 0.22^{efg}	0.82 ± 0.04^{f}	1.29 ± 0.25^{de}
BA (1.11) + 2ip (0.61)	2.51 ± 0.18^{cdef}	1.60 ± 0.22^{bc}	1.52 ± 0.13^{cde}
BA (1.11) + 2ip (1.16)	2.31 ± 0.12^{defg}	1.31 ± 0.16^{cde}	1.44 ± 0.2^{cde}
BA (1.11) + 2ip (1.845)	2.01 ± 0.01^{fg}	1.11 ± 0.02^{def}	1.02 ± 0.04^{e}
BA (1.11) + Kn (1.16) + TDZ (0.1)	0.00 ± 0.00^{h}	0.00 ± 0.00^{g}	0.00 ± 0.00^{f}
P value	0.000*	0.000*	0.000*
LSD 0.05	0.481	0.483	0.758

*Values are means ± standard error
Values followed by the same letters within the column are not significantly different at 5% level
(Duncan's multiple range test)

Sugars at various levels displayed a significant effect on all the parameters for shoot multiplication (Figure 2). Among different carbon sources tested, sucrose at 3% proved to be the best source for shoot growth. A further increase in the sucrose concentration adversely affected the proliferation rate.

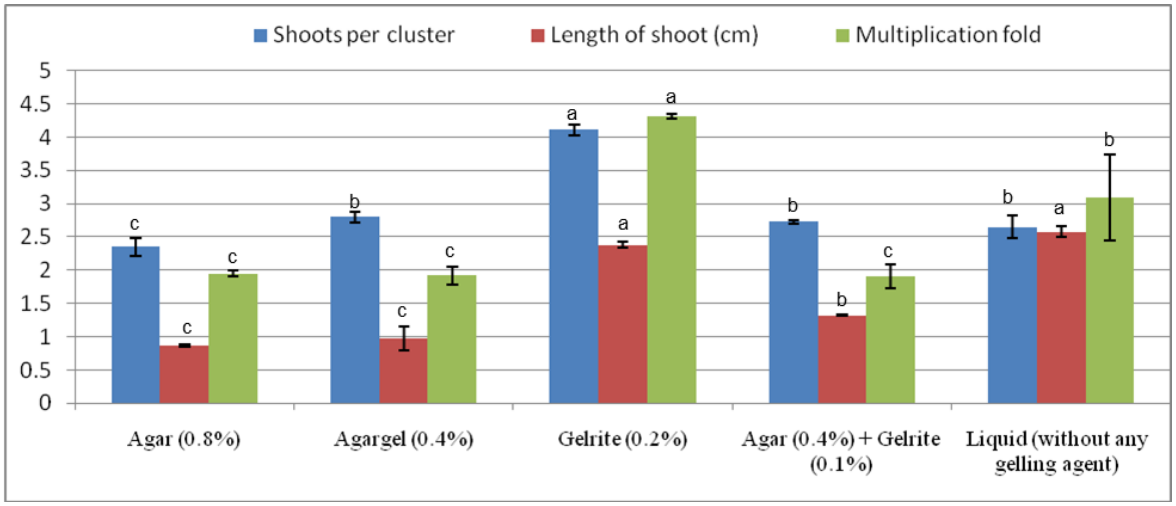
Figure 1 Micropropagation of Rangpur lime: (a) Mother tree, (b) initiation, (c) shoot multiplication, (d) One multiplied cluster (e) rooting, (f) bare root, (g) in polybag, (h) in nursery or (i) in field (prior to budding)



Values are means ± standard error, Means followed by the same letter within the column are not significantly different (Duncan's multiple range test, $p < 0.05$)

Figure 2 Effect of carbon source on shoot multiplication of Rangpur lime

Gelling agents like agargel, gelrite and agar were used to optimise shoot production (Figure 3). Gelrite at 0.2% concentration displayed the best shoot multiplication (4.31-fold every four weeks) compared to 1.95 and 1.92-fold achieved with agar and agargel, respectively. Gelrite is a clarified grade of gellan gum, an exocellular heteropolysaccharide obtained from cultures of the bacterium *Pseudomonas elodea* [31]. The superiority of gelrite as a gelling agent in micropropagation has been demonstrated for many plants due to its high purity, consistent quality, and its ability to solidify at lower concentrations compared to agar [32]. Also, the extra clarity enables easy detection of microbial contaminants, which is an added advantage [33]. Among Citrus, there are only two reports of the use of gelrite for shoot multiplication in pummelo [34], calamondin, grapefruit and sweet orange [35]. Gelrite is known to bind cations, particularly Ca^{2+}, Mg^{2+}, K^{+} and Fe^{3+} and also has high ash content relative to tissue-culture grade agar [36], which helps in shoot proliferation. Consequently, inorganic impurities may get introduced into the growth medium through agar, which can be toxic and cause vitrification in some cases [37]. A mixture of agar and Gelrite was also used to improve shoot development and to reduce the cost of production. However, this media was inferior as compared to gelrite alone. In the case of liquid media, it favoured shoot growth; however, the multiplication rate was lower than gelrite as vitrification caused suppression in the number of emerging shoots. This may be due to continuous contact of the liquid with explants, osmotic shock and low gas exchange [38]. Thus, all multiplication experiments were performed using 0.2% Gelrite only.



Values are means ± standard error, Means followed by the same letter within the column are not significantly different (Duncan's multiple range test, $p < 0.05$)

Figure 3 Effect of gelling agents on shoot multiplication of Rangpur lime

In the present study, a negative effect on overall shoot development was observed with auxins and a range of growth adjuvants that were tested. However, a combination of BA and NAA was found to be optimal for rough lemon [39] and for *C. limon* [22], which was also not observed in this study. Activated charcoal was observed to cause apical necrosis. Detrimental effects have also been reported when AC was included in the growth media of Rangpur lime [40] and this may be due to adsorption of growth-promoting culture substances [10].

Rooting

Individual shoots of Rangpur lime measuring about 1 cm obtained from the multiplication media were transferred to various rooting media. Rooting occurred readily in all media (Table 2), within 28-30 days after transfer. ½ MS was distinctly better than other media in terms of the frequency of rooting as well as shoot quality and length (Table 2; Figure 1e, f). On basal MS medium, negligible shoot growth was observed. However, there was no significant difference among the treatments with respect to the number of roots and root length. To further improve shoot and root quality, auxins were added to MS media with major salt strength reduced to half (Figure 4). A 100% rooting success was achieved on all the media tested. However, the root growth was restricted on IAA-supplemented medium coupled with basal callusing. Therefore, ½ MS (basal medium) was selected as ideal for the rooting of shoots. This is in contrast with the reports by El-Boray et al. (2015), where a combination of IBA and NAA were reported to be successful.

Table 2 Effects of MS and modified MS on in vitro rooting in Rangpur lime

Medium	Rooting %	Length of shoot (cm)*	No. of roots/plant*	Root length (cm) / root*
MS	33.33%	1.11 ± 0.04^{b}	2.00 ± 0.11^{a}	1.93 ± 0.45^{a}
MS ½	100.00%	2.89 ± 0.03^{a}	2.16 ± 0.06^{a}	2.13 ± 0.01^{a}
MS 1/3	66.66%	2.37 ± 0.07^{a}	1.63 ± 0.14^{a}	2.34 ± 0.02^{a}
MS ¼	33.33%	2.35 ± 0.27^{a}	1.50 ± 0.50^{a}	2.47 ± 0.65^{a}
P value		0.0009*	0.4672ns	0.5167ns
LSD 0.05		0.6076	1.050	1.429

*Values are means ± standard error
Values followed by the same letters within the column are not significantly different at 5% level (Duncan's multiple range test)

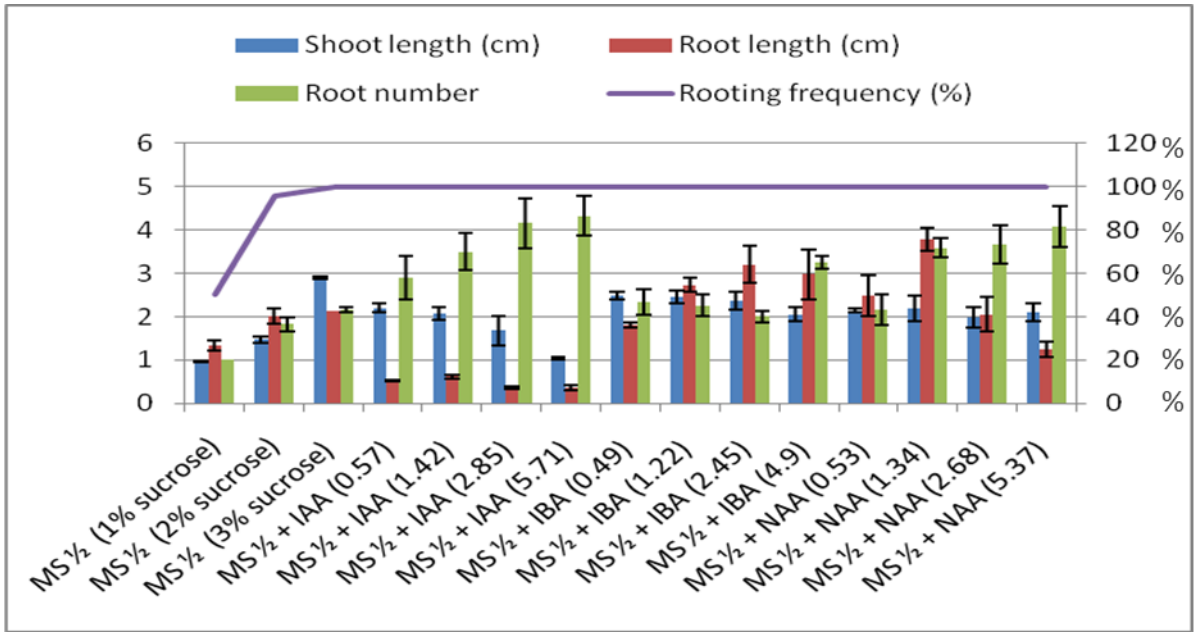


Figure 4 Effect of various auxins (µM) on rooting of Rangpur lime

Sucrose has a direct impact on rooting percentage and shoot growth (Figure 4). It was observed that rooting occurred spontaneously in 100% of the explants with 3% sucrose. Even though shoots augmented with 2% sucrose also displayed a very high rooting frequency (95%), the shoots failed to grow and thus were much shorter than on 3% sucrose-containing medium. Therefore, 3% sucrose was found to be optimum for rooting in Rangpur lime.

On liquid medium root number was almost double that of the gelrite solidified medium. However, the plantlets rooted in liquid medium had a lower hardening survival (75%) compared to those on the gelrite medium (100%). Since conventionally propagated Citrus plants have tap roots, a high number of roots obtained on liquid media can be inhibitory for plant growth. Subsequently, 0.2% gelrite was used as the gelling agent for all rooting experiments.

Hardening and acclimatisation

The survival rate varied between 70% and 100% depending upon the nature of the potting mixture used, and among all the tested combinations of substrates attempted, 1:1 soil: agropeat (v: v) favoured maximum shoot growth and leaf expansion and gave up to 100% hardening survival (Figure 1g). It is important to note that none of the prior studies on tissue culture of Rangpur lime have any mention of hardening methods or survival percentages [11] while Barlass and Skene [14] reported on the transplantation of rooted Rangpur lime plantlets on Jiffy's peat block. A high level of transplantation success was observed in this study. As part of our study to date, more than 10,000 plants of Rangpur lime have been produced, acclimatised and transferred to the open nursery (Figure 1h).

Clonal fidelity studies

In case of perennials, including horticultural species, micropropagation technology cannot be rewarding unless complete genetic fidelity is maintained [41]. This ensures that the advantages of the elite genotype, such as high yield and other unique traits, are inherited by the progeny. Moreover, retention of genetic uniformity for long durations in tissue culture has great commercial importance in species such as Citrus, where aseptic cultures have been difficult to establish. Thus, micropropagation using explants with preformed meristems (e.g. axillary buds, shoot tips) was chosen in the present study as it is well known that these shoots have much less variation than those arising from adventitious budding systems through direct organogenesis, callusing phase or through embryogenesis. However, there are some reports which document the occurrence of somaclonal variations in micropropagated plants raised through axillary

proliferation, such as teak [42] and apple [43]. The occurrence of somaclones is largely because the tissue culture environment induces enormous stress, which enhances the frequency of DNA methylation, somatic crossing over and further sister chromatid exchange [44]. In addition, plant cells in tissue culture may be especially susceptible to dNTP pool imbalances [45]. It has been observed that recurrent subculturing for an indefinite period and also longer passages hinder the maintenance of genetic fidelity since the tissue culture process appears to disrupt the cell cycle as the cells are dividing too fast and cause chromosome fragmentation [46]. Thus, it is important to determine the exact period of subculturing passage for each species. This was quite evident in a study on micropropagation of teak, where variation was observed at the 25th sub-culture passage [42].

To detect any clonal instability in the present study, a total of seven ISSR primers (UBC 812, 814, 818, 840, 842, 843 and 848) were initially screened, and six primers were found to be suitable for Rangpur lime (Table 3). DNA from the parent, its tissue-culture raised progenies and three outliers (Alemow macrophylla, C 35 citrange and Swingle citrumelo) were subjected to ISSR-PCR assay. These primers amplified a total of 85 bands in the size range from less than 506 bp to 2 Kb (Table 3). The number of bands generated by each primer varied from four to nine and the bands were scored for their presence or absence across the plantlets analysed. The highest number of bands was observed in the 506 bp to 1 Kb region. Each primer gave an average of over 6 bands in all the progenies and the mother plant. ISSR was also used for evaluating the genetic differences of micropropagated sweet oranges using microshoots [47], somatic embryos of Meyer Lemon [48] and somaclonal variants of Persian Lime [49]. In this study, aAll the micropropagated plantlets showed identical number and position of the bands as their donor plant in all the primers tested, confirming that the genetic stability is maintained in plants derived from *in vitro* axillary cultures even up to the 25th passage *in vitro* (Figure 5).

Table 3 ISSR primers: GC content, number and size range of bands produced by the mother plant, tissue culture raised plants of Rangpur lime and outliers to detect clonal stability

UBC ISSR Primer	Primer sequence[a]	GC content (%)	Total number of bands				Molecular size range (bp)	% polymorphism w.r.t. outliers
			Mother + TC raised progenies		TC including outliers			
			Total	Polymorphic	Total	Polymorphic		
812	$(GA)_8A$	47	9	0	15	14	<506 to 1600	93.33%
814	$(CT)_8A$	47	4	0	7	7	506 to 2000	100.00%
818	$(CA)_8G$	53	8	0	8	6	<506 to 1000	75.00%
840	$(GA)_8YT$	44-50	6	0	7	4	<506 to 1600	57.14%
842	$(GA)_8YG$	50-55	6	0	8	4	<506 to 1000	50.00%
848	$(GA)_8RG$	50-55	8	0	15	12	<506 to 1000	80.00%

[a]Key to base compositions R = A, G Y = C, T

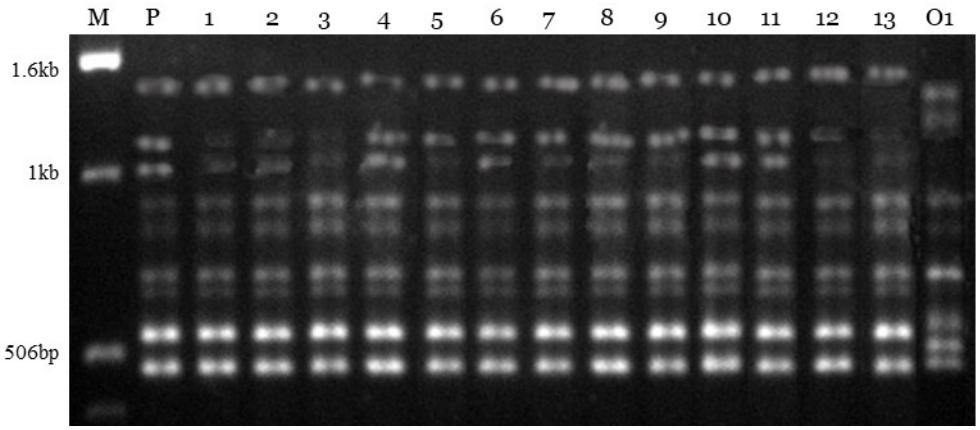


Figure 5 DNA amplification obtained with primer UBC 812; 1 Kb DNA ladder (lane M); mother plant (lane P); micropropagated plants at 5th (lane 1 and 2), 10th (lane 3 and 4), 15th (lane 5 and 6), 20th (lane 7 and 8) and 25th passages (lane 9 and 10); plant at nursery stage (lane 11, 12 and 13); outlier Alemow macrophylla (O1)

Also in the present study, the plantlets successfully transferred to the nursery were morphologically similar to the mother plants. Furthermore, the polymorphic bands scored for the outlier proved that the primers employed were competent enough to distinguish the plantlets based on genetic variations. The highest polymorphism with respect to outliers was observed in UBC primer 814 and the lowest in UBC Primer 842.

Conclusions

The present study establishes an efficient protocol for large-scale true-to-type production of plantlets of Rangpur lime using axillary multiplication. An optimised multiplication fold of 4.31 shoots every four weeks, with 100% rooting and hardening survival of plants, with healthy shoot growth and well-developed leaves, was achieved. Using this protocol, more than 100,000 plants can be produced starting from a single node within a year. Thus, this study has immense commercial potential for the mass-scale propagation of Rangpur lime.

Acknowledgements

The authors are thankful to TERI for the infrastructure support. Thanks are due to Dr S. B. Tripathi and Mr M. S. Negi for help in conducting the clonal fidelity experiments. The Senior Research Fellowship from the Council of Scientific and Industrial Research to Sangeeta Sen is gratefully acknowledged.

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