

Full Length Research Paper

Efficient micropropagation of *Citrus sinensis* (L.) Osbeck from cotyledonary explants suitable for the development of commercial variety

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In vitro regeneration of sweet orange (*Citrus sinensis* (L.) Osbeck Family: Rutaceae) has been performed via direct and indirect organogenesis. For indirect organogenesis, callus was induced and proliferated from leaf explants derived from *in vitro* grown seedlings on Murashige and Skoog (MS) media containing 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with benzyl adenine (BA) and α -naphthalene acetic acid (NAA). For direct organogenesis, explants were placed on MS media containing BA alone or in combination of NAA and gibberellic acid (GA_3). Well-developed microshoots were treated with different concentrations of NAA for rhizogenesis (a two-step procedure). Different responses to these treatments were recorded depending upon the procedure used. It appears that 4.53 μ M 2,4-D in combination with 5.37 μ M NAA induced 93.33% callus and proliferate 86.67% of callus into 6.93 shoots per explant. Exogenous addition of 4.44 μ M BA in combination with 1.54 μ M GA_3 enhanced shoot multiplication rate significantly (17.73 ± 1.69 shoots/explant) in comparison to control (0.00 ± 0.00 shoots/explant). Microshoots were rooted best ($75.00 \pm 14.43\%$) under the treatment 100 μ M NAA for 48 hrs. and rooted plantlets were transferred to soil, following acclimatization were taken to maturity in the polyhouse.

Key words: Malta, Himalaya, benzyl adenine (BA), callus.

INTRODUCTION

Citrus fruits are one of the most important commercial fruit crops of the world, cultivated in more than 100 countries (Barlass and Skene, 1999). *Citrus* (> 108 million

tons), ranks second among the most produced fruit crops of the world after banana (FAO, 2006). According to UN 2007 data, India is the largest producer of lemons

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and limes in the world and ranks sixth in the production of *Citrus* fruits.

Citrus sinensis is a member of family Rutaceae and commonly known as navel orange, sweet orange and 'Malta' in Uttarakhand, India (Christman, 2003). *C. sinensis* is a small evergreen tree having 7.5 m height and sometimes reached up to 15 m. Many biologically active, non-nutrient compounds found in *Citrus* fruits such as antioxidants (Tripoli et al., 2007), soluble and insoluble dietary fibres (Ejaz et al., 2006) known to be helpful in reducing the risk for cancers (Elangovan et al., 1994); many chronic diseases like arthritis; obesity (Walton et al., 1945) and coronary heart disease (Crowell, 1999). A single orange is said to have about 170 phytonutrients and over 60 flavonoids with anti-tumor, anti-inflammatory, blood clot inhibiting and antioxidant properties (Etebu and Nwauzoma, 2014). Due to high 'vitamin C' content and antioxidant potential of *C. sinensis*, it is consumed worldwide, therefore, considered as one of the major commercial fruit crops of the world (Kiong et al., 2008).

However, the cultivation of *C. sinensis* is difficult due to slow growth, short storage life (Mukhtar et al., 2005) and susceptibility to a large number of disease viz. Pierce's disease (Redak et al., 2004); *Citrus* chlorosis (Rossetti et al., 1977); *Citrus* greening (Bove, 2006); *Citrus* canker (Rossetti, 1977); Ring spot (Fawcett, 1933); Sweet orange scab (Kunta et al., 2013); *Citrus* black spot (Kotze, 1981); Powdery mildew (Sastra-Hidayat, 1992). Further, *Citrus* stands among difficult to root crops (Usman et al., 2005) and their seeds have a very short life because they are injured by drying during storage and thus, lose their viability (Johnston, 1968; Ali and Mirza, 2006).

Citrus species are propagated by both sexual and asexual methods; rootstocks are generally propagated sexually through seeds, while most of the commercial varieties are propagated by asexual means (Chaudhary, 1994). Micro propagation is an important asexual method that can be used for the production of disease-free rootstock plants in limited space and time under controlled conditions throughout the year (Usman et al., 2005; Hikada and Omura, 1989; Roistacher et al., 1976).

Techniques like *in vitro* culture made it easy to improve *Citrus* against different abiotic stresses, low yield and conserve important *Citrus* genotypes through exploiting somaclonal variations, transformation of high yielding cultivars (Deng et al., 2000, Koltunow et al., 2002). Regeneration of different species of *Citrus* has already been investigated, *C. paradisi* (Macf) (Costa et al., 2002); *C. reticulata* (Te-Chato and Nudoung, 1998), *C. sinensis*, Pena et al., 1995). Although small work has been done in *C. sinensis* but no work has been reported for Indian cultivars.

The study aimed to develop an *in vitro* regeneration system which can be used for effective propagation of selected rootstocks and regeneration of ideal genetically modified grafts which can be used to develop commercial

variety.

MATERIALS AND METHODS

Plant material and explant preparation

Mature fruits of *C. sinensis* (Malta) were collected from young elite plant (Plate 1 a) growing at Bhowali (1687 m asl; 29° 23' 5.39"N, 79° 31' 8.48"E), district Nainital, Uttarakhand India. Fruits were peeled and the pulp was removed to extract seeds. To remove all pulp adhering to the seed surface, seeds were washed with running tap water. Seeds were then subjected to floating test as described by Pandey and Tamta (2013), healthy and viable seeds were selected and then washed with a few drops of tween 20, a laboratory detergent, for 1 h; followed by five times rinsing in distilled water. Thereafter, seeds were subjected to fungicide treatment (bavistin, 1% w/v, 30 min) and shifted to laminar air flow bench for further steps. After five rinse with autoclaved distilled water, the seeds were dipped in mercuric chloride solution (HgCl₂, 0.1% w/v, 10 min), this treatment was followed by five times rinsing in autoclaved distilled water. Disinfected seeds, prior to inoculation, were scorched for 10 s to remove sticky cover present in the outer surface of the seed and cultured.

Culture medium

The nutrient medium consisted of Murashige and Skoog (MS; Murashige and Skoog, 1962) basal medium fortified with 3% sucrose and vitamins, having pH adjusted to 5.8 with 1 N NaOH and solidified with 0.8% agar. All chemicals were procured from Himedia, Mumbai, India. The prepared medium was autoclaved (in 1.05 Kg cm⁻², 121°C for 20 min) for sterilization. Cultures were maintained inside growth chamber at 25±2°C, 60% relative humidity and 16 h photoperiod, provided by cool white fluorescent light, under 42 µMm⁻² s⁻¹ illumination and subculturing was done every 45 days.

Callus induction and proliferation

For callus induction, leaf explants were excised from 45 days old *in vitro* grown seedlings and small pieces (0.5 cm²) were inoculated on MS medium fortified with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), either alone or in combination with BA or NAA (Table 1). Observations were taken visually in every five days and influence of different treatments was quantified in terms of percent callus induction, percent shoot proliferation.

Shoot regeneration

The cotyledonary node explants (1.5 cm) were excised from 45 day old seedlings growing in MS medium supplemented with 1 mg/l 6-benzylaminopurine (BA) (Pandey et al., 2011) and cultured on MS medium containing different concentrations-of BA (1-15 µM) alone or in combination with α-naphthaleneacetic acid (NAA) or gibberellic acid (GA₃) (Table 2). Explants in MS basal medium served as control. The shoot proliferation responses, in terms of number of shoots per explant and average length of shoots (cm) per explant, were evaluated 45 days after the inoculation in shoot multiplication medium.

Rhizogenesis

Actively growing shoots (2.5-3 cm. height) were used for *in vitro*

Table 1. Effect of different plant growth regulators on callus induction and proliferation of *C. sinensis*.

Treatment (μM)			Callusing (%)	Proliferation (%)	Average no. of shoots /callus
2,4-D	BA	NAA			
1			53.33	6.67	0.13
4.53			80.00	0.00	0.00
9.06			66.67	0.00	0.00
1	4.44		66.67	26.67	0.80
4.53	4.44		86.67	66.67	2.07
9.06	4.44		73.33	46.67	1.27
1		5.37	80.00	60.00	3.27
4.53		5.37	93.33	86.67	6.93
9.06		5.37	86.67	73.33	3.07
Control			0.00	0.00	0.00

Fifteen (15) explants were used per treatment and data of callus induction and proliferation was scored after 45 days of culture in MS medium supplemented with different concentrations of 2,4-D; BA and NAA, while average number of shoots per callus were recorded after 60 days of culture.

Table 2. Effect of plant growth regulators on shoot multiplication from *in vitro* derived cotyledonary node explants of *C. sinensis*.

Treatment (μM)			Number of shoots/ explant	Average shoot length/ explant (cm.)
BA	NAA	GA ₃		
2.22			2.27±0.44 ^{ab}	1.02±0.10 ^b
4.44			6.53±1.94 ^c	1.90±0.35 ^{cd}
8.88			4.33±0.47 ^{bc}	1.27±0.07 ^{bc}
2.22	2.68		4.67±0.59 ^{bc}	1.23±0.32 ^{bc}
4.44	2.68		6.13±0.18 ^c	1.23±0.32 ^{bc}
8.88	2.68		16.47±2.14 ^e	1.77±0.15 ^{cd}
2.22		1.45	10.40±1.22 ^d	2.30±0.25 ^{de}
4.44		1.45	17.73±1.69 ^e	2.63±0.09 ^e
8.88		1.45	11.50±0.70 ^d	1.97±0.09 ^d
Control			0.00±0.00 ^a	0.00±0.00 ^a

Values represent mean±SE, values within each column followed by the same letters are not significantly different by the Duncan test at 0.05% probability level. Fifteen explants were used per treatment (three replicates having five individuals in each) and data scored after 45 days of culture in MS medium supplemented with different concentrations of BA, NAA and GA₃.

rhizogenesis. The *in vitro* rhizogenesis was done by following two-step rooting procedure described by Pandey et al. (2013); Pandey and Tamta (2014) with some modifications. In this rooting procedure, microshoots were initially cultured in NAA (50 or 100 μM) supplemented MS medium for 24 or 48 h and cultures were kept in the dark during this step. In the second step, these NAA-treated microshoots were transferred to plant growth regulator-free half strength MS medium and exposed to normal light conditions (16 h photoperiod; under 42 $\mu\text{Mm}^{-2} \text{s}^{-1}$ illumination). Microshoots placed in MS basal medium (without NAA treatment in first step) served as control. Data for rooting percent, average number of roots per explant and length of roots per explant were recorded after 45 days of transfer to plant growth regulator-free half strength MS medium (Table 3).

Acclimatization

After successful rhizogenesis, the plantlets were taken out from

culture flasks and washed in distilled water to remove the traces of rooting media. Rooted plantlets were transferred to thermocol cups containing sterile sand: soil: farmyard manure, (1:1:1, v/v/v) and covered with transparent polybags and kept in culture room for 45 days, for first stage of hardening. They were later transferred to polythene bags containing a mixture of garden soil, farmyard manure and sand (1:1:1) and kept in polyhouse having relative humidity (60±4%) for second stage of hardening. After 6 months of growth in the polyhouse, the plants were transferred to the field.

Statistical analysis

All the experiments were set up in a completely randomized design. The data was subjected to analysis of variance (ANOVA) to detect significant difference between means. Means differing significantly were compared using Duncan's multiple range test at $p=0.05$. All the statistical analysis was done by using SPSS Ver 20 (SPSS Inc., Chicago, USA) Statistical software package.

Table 3. Effect of different concentrations of NAA on *in vitro* rhizogenesis of *C. sinensis*.

Treatment		Rooting (%)	Number of roots/ micro shoot	Average length of roots (cm.)
NAA (µM)	Time (h)			
50	24	25.00±14.43 ^a	1.17±0.17 ^b	2.27±0.18 ^b
100	24	41.67±08.33 ^{ab}	1.25±0.25 ^b	3.73±0.35 ^c
50	48	41.67±16.67 ^{ab}	1.17±0.17 ^b	2.47±0.18 ^b
100	48	75.00±14.43 ^b	1.67±0.17 ^b	2.83±0.50 ^{bc}
Control		0.00±0.00 ^a	0.00±0.00 ^a	0.00±0.00 ^a

Twelve (12) explants (three replicates having four individuals in each) were cultured on ½ MS medium. Data was evaluated after 45 days of culture in plant growth regulators free ½ MS media. Values represent mean±SE, values within each column followed by the same letters are not significantly different by the Duncan test at 0.05% probability level.

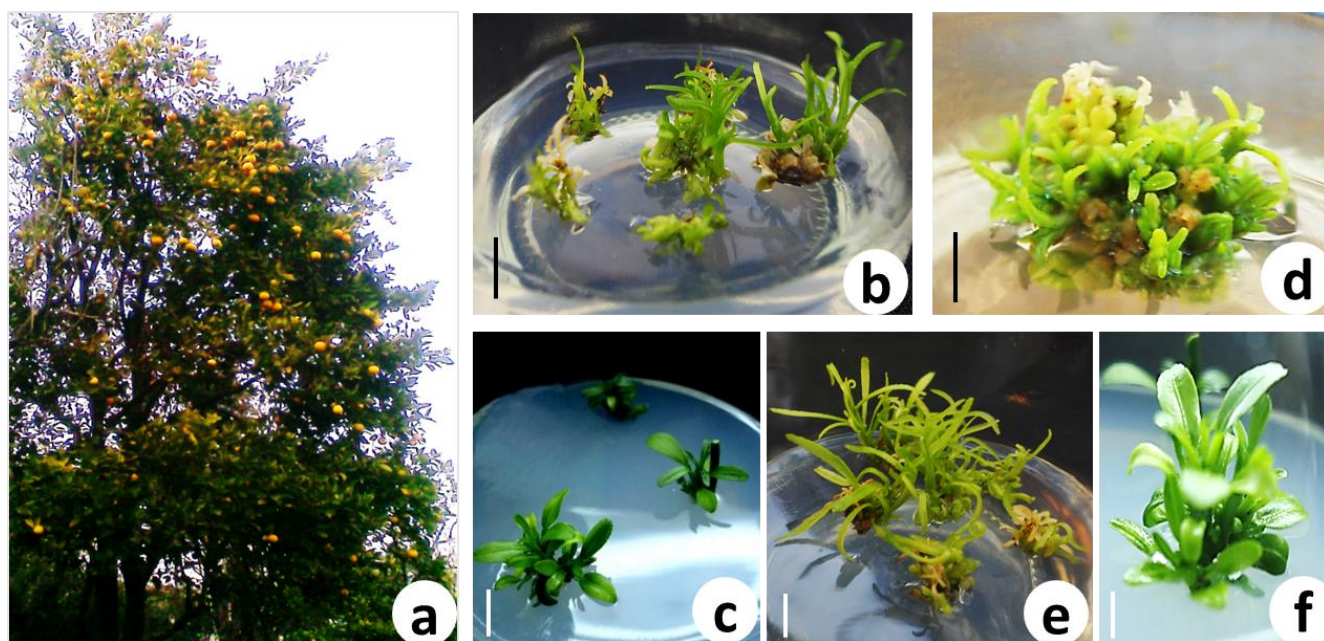


Plate 1. Micropropagation of *C. sinensis* through callus and nodal segments. **a.** Mature tree with fruits; **b.** shoot proliferation from leaf derived callus in 2,4-D+ NAA (4.53+5.37 µM) supplemented medium (30 days); **c.** bud proliferation from nodal segments derived from *in vitro* grown seedlings in BA+GA₃ (4.44+1.45 µM) supplemented medium (30 days); **d.** multiplication of shoots after 60 days of culture in BA+GA₃ (4.44+1.45 µM) supplemented medium; **e.** shoot proliferation from callus 2,4-D+NAA (4.53+5.37 µM) supplemented medium (45 days); **f.** well-developed shoots prior to rooting. Bar showing 1 cm scale.

RESULTS

Callus induction

To determine the best callus induction and proliferation response of leaf explants, derived from 6-week-old *in vitro* grown seedling of *C. sinensis*, different combinations of plant growth regulators were tested (Table 1). The optimal callusing response (93.33%) was observed in MS medium supplemented with 2,4-D+NAA (4.53+5.37 µM) followed by 2,4-D+NAA (9.06+5.37 µM) (86.67%). The lowest callus induction response (53.33%) was observed in MS medium supplemented with 2,4-D (1 µM) (Plate

1b). MS medium devoid of plant growth regulator (control) failed to induce callus.

Shoot proliferation and multiplication

For shoot proliferation and multiplication, the indirect and direct regeneration methods were performed. For indirect regeneration, green friable calli was proliferated and highest (86.62%) proliferation was observed in MS medium supplemented with 2,4-D+NAA (4.53+5.37 µM), the average number of proliferated shoots were also recorded maximum (6.93) for this treatment (Table 1 and

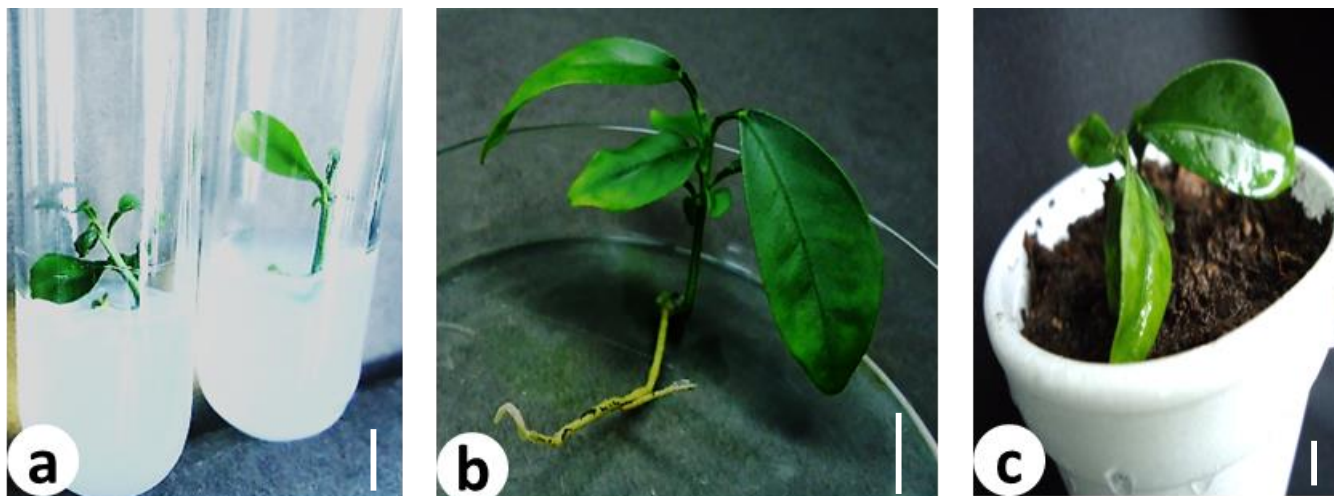


Plate 2. Rhizogenesis and acclimatization of plantlets of *C. sinensis*. **a.** Microshoot treated with 100µM NAA for 48 h in the first step; **b.** well-rooted plantlet after 45 days of transfer in half strength MS medium; **c.** well-developed *in vitro* grown plant of *C. sinensis* in polyhouse condition after (60 days). Bar showing 1 cm scale.

Plate 1b and e). The minimum (6.67%) proliferation response and average number of shoots (0.13) was observed in MS medium supplemented with 2,4-D (1 µM). The control treatment along with 2,4-D (4.53 µM, 9.06 µM) supplemented media were not able to proliferate.

For direct shoot multiplication, the responses under various concentrations and combinations of plant growth regulators were observed (Table 2). The number of shoots per explant (17.73 ± 1.69) and average shoot length (2.63 ± 0.09 cm) were recorded maximum in MS medium supplemented with BA+GA₃ ($4.44 + 1.45$ µM) (Plate 1c to d), followed by BA+NAA ($8.88 + 2.68$ µM) having 16.47 ± 2.14 shoots per explant and 1.77 ± 0.15 cm average shoot length, respectively. The lowest number of shoots (2.27 ± 0.44) per explant was observed in MS medium supplemented with 2.22 µM BA. Explants in 'control' failed to survive for 45 days and died within 25 days of culture.

Rhizogenesis

For *in vitro* rhizogenesis, the well-developed shoots (2.5-3 cm long) were cut off and cultured in rooting medium. The rooting responses were varied according to the treatment they received during first step. Furthermore, the treated microshoots showed significantly different responses to control (Table 3). $75.00 \pm 14.43\%$ rhizogenesis with 1.67 ± 0.17 roots per microshoot was observed in treated (100 µM NAA for 48 h) microshoots (Table 3 and Plate 2b). The average length of roots was observed maximum (3.73 ± 0.35 cm.) in microshoots treated with 100 µM NAA for 24 h. The lowest rhizogenesis ($25.00 \pm 14.43\%$), having 1.17 ± 0.17 roots per

microshoot and 2.27 ± 0.18 cm average root length, was recorded in microshoots treated with 50 µM NAA for 24 h. MS medium lacking auxin in first rooting step was not able to induce roots.

Acclimatization

Plantlets with well-developed roots (Plate 2b) were taken out carefully, and on acclimatization, plantlets exhibited normal growth under polyhouse (Plate 2c); and on transfer in soil, 50% survival of plantlets were recorded.

DISCUSSION

C. sinensis is among the most consumed fruits worldwide; therefore it has great economic importance, but like other fruits, *Citrus* is attacked by several pathogens that affect fruit quality (Bekele, 2007). Furthermore, it stands among difficult to root crops (Usman et al., 2005) which limit its cultivation by traditional means. Micropropagation offers rapid propagation of such difficult to root crops throughout the year under controlled conditions (Honda et al., 2001). This eliminates diseases (Grosser and Chandler, 2000) and also provides scope for the development of new cultivars. Micropropagation has been successfully used for the production of microbe free plants at commercial level (Parmessur et al., 2002). Further, it made easy to conserve important *Citrus* genotypes, improve *Citrus* against low yield and different abiotic stresses through exploiting possibilities in somaclonal variations and somatic cell hybridization (Deng et al., 2000; Koltunow et al., 2002). Indirect micropropagation is essential for

Agrobacterium-mediated genetic transformation (Khawale and Singh, 2005). Through transformation, disease free plants of high yielding cultivars can be produced e.g. nematode resistance *Citrus* rootstock etc.

MS medium was used as a nutrient source during the study, as it was found effective in seed germination of *C. sinensis* and also, successfully used for various citrus species e.g. *C. sinensis*; *Carrizo citrange* (Pandey et al., 2011; Germana et al., 2011). Response of explants to plant growth regulators was vigorous. The best callus induction (93.33%) and proliferation responses (86.67%) were observed in MS medium containing 2,4-D and NAA. Similarly, callus development in *C. sinensis* on MS medium supplemented with 2,4-D+NAA was reported by Das et al. (2000) which supports the present study, while in *C. reticulata* (83.0%), somatic embryogenesis was observed on MS medium supplemented with NAA + Kinetin ($10+1 \text{ mgL}^{-1}$) from callus (Gill et al., 1995).

In *Citrus* species, BA is reported as the best cytokinin for inducing organogenesis (Carimi and Pasquale, 2003; Germana et al., 2011), $22 \mu\text{M}$ BA in *Citrus reshni* (Moore, 1986); 2 mgL^{-1} BA in *C. limon*; *C. paradisi* (Kotsias and Roussos, 2001, Costa et al., 2004), etc. Kobayashi et al. (2003) was able to produce 3.1 shoots per explant with a response of 54% in *C. sinensis* on BA+GA₃ ($1.8+0.7 \mu\text{M}$) supplemented MS medium. In present study, comparatively better shoot multiplication (17.73 ± 1.69 shoots/explant) responses were observed in MS medium supplemented with BA+GA₃ ($4.44+1.45 \mu\text{M}$). It may be due to the increased concentrations of growth regulators and explant source. Therefore, present study reveals that the application of BA is effective in shoot multiplication but, BA in combination with GA₃ appeared more potent in shoot multiplication.

Many different *Citrus* species were subjected to rhizogenesis under different auxins (NAA or IBA) supplemented mediums (Carimi and Pasquale, 2003) and NAA was found to be the best rooting auxin for *Citrus* species (Kaneyoshi et al., 1994; Paudyal and Haq, 2000). Further, the highest (87%) rooting percentage in *C. reticulata* was also obtained on NAA (2 mgL^{-1}) supplemented media (Mukhtar et al., 2005). However, rooting response of *C. sinensis* was not satisfactory under continuous exposure of NAA and only 3.2%, rooting was observed after three months of transfer of shoots to rooting medium (Pena et al., 1995). Therefore, well-established two-step rooting procedure was adopted for *in vitro* rhizogenesis. Two-step rooting procedure was found to be the best for several species viz. *Berberis chitria* (Pandey et al., 2013), *Quercus serrata* (Pandey and Tamta, 2014), *Quercus leucotrichophora* (Pandey and Tamta, 2012) etc. In present study, moderate rooting (25 to 75%) responses were observed with 1.17 to 1.67 average number of roots-per-microshoot. The low rooting efficiency has also been reported as major problem for *in vitro* production of *Citrus* plants (Usman et al., 2005; Duran-Vila et al., 1989). The low survival of *C. sinensis*

plantlets may be due to less root length, that is, 2.27 ± 0.18 to 3.73 ± 0.35 cm.

Conclusion

The present study was done to develop an efficient micropropagation procedure for Malta (*Citrus sinensis*) through direct and indirect organogenesis. The direct organogenesis was achieved best in MS medium fortified with BA and GA₃ which may be useful for mass multiplication of selected elite cultivar. Similarly the best responses for indirect organogenesis were recorded in explants cultured in MS medium supplemented with 2,4-D and NAA, which might be useful in genetic transformation system and suitable for regeneration of new 'Malta Variety' in future. The NAA containing MS medium was able to induce $75.00\pm 14.43\%$ rhizogenesis in *in vitro* derived microshoots, but the further trials are required to achieve 100% rooting and better survival of these *in vitro* raised plants in field conditions.

Conflict of interests

The authors have not declared any conflict of interest.

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Abbreviations

MS, Murashige and Skoog media; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **BA**, benzyl adenine; **NAA**, α -naphthalene acetic acid.

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