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Effect of different growth regulators on *in vitro* regeneration of cotyledon explants of rough lemon (*Citrus jambhiri* Lush.) *via* callus induction

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Abstract
In order to establish *in vitro* protocols for raising aseptic cultures of cotyledon segments excised from five-week-old *in vitro* raised seedlings were used as explant material. Among surface sterilants, mercuric chloride 0.1% for three minutes provided least rate of contamination (24%) and the maximum explant survival (76%). The highest per cent culture establishment of 97.79% was obtained in cotyledon explants on Murashige and Skoog (MS) containing 2,4-D (3.0 mg/l) and maximum callus induction (95.41%) was observed on MS medium supplemented with 2,4-D (2.0 mg/l). Among different plant growth regulator treatments, maximum callus regeneration of 82.19% was observed on MS medium supplemented with BAP (3.0 mg/l) whereas, minimum callus regeneration (4.33%) was found in MS medium supplemented with BAP (3.0 mg/l) in combination with IAA (0.75 mg/l) and malt extract (500 mg/l). Maximum rooting response (96.00%) was recorded on MS (half strength) medium with NAA (2.0 mg/l) and was at par with rooting percentage of 92.00% on MS (half strength) medium supplemented with NAA (1.5 mg/l). The plantlets were successfully acclimatized in different potting mixtures and highest survival rate (91.35%) was achieved in potting mixture containing garden soil with sand and farmyard manure (1:1:1).

Keywords: Rough lemon, callus, regeneration, cotyledon, acclimatization, tissue culture

Introduction
Citrus (*Citrus* sp.) is collective generic term comprising a number of species and varieties of fruits known to the world for their characteristic flavour, attractive range of colours and uses (Liu *et al*., 2012) [13]. The well-known examples of group citrus are oranges, lemons, grapefruits and limes, they are long-lived perennial crops grown in more than 100 countries across the world (Saunt, 1990) [24]. Citrus is considered as the number one fruit of the world due to its high nutritional value, great production potential and preparation of large number of fruit products from them (Kour and Singh, 2012) [10].
Rough lemon (*Citrus jambhiri*) is the most commonly used rootstock for various scion cultivars of citrus in all over the world. It is native to North-eastern India, probably a natural hybrid because of its high degree of polyembryony compared to other lemon species. Rough lemon rootstock is suited to deep well drained sandy soil which are subject by the marked fluctuations in soil moisture (Kaur, 2018) [9]. It forms a normal union with all scion varieties, develops a deep root system, produces heavy yields and gives a long life to tree when planted on a suitable soil. It imparts resistance to tristeza and exocortis, viroid, tolerant to salt and drought. It ensures high yield with large size fruits in most of the scion cultivars and at the same time is resistant to most of the viruses (Altaf *et al*., 2008) [3]. It is an important rootstock for a number of citrus fruit crops including oranges, grape fruits, Kinnows, lemons, and mandarins all over the world (Kaur, 2018) [9].
In vitro culture is a method can produce crops on a comparatively large scale in comparison with conventional plant breeding and also results in superior soma clonal variants (Kasprzyk-Pawelec *et al*., 2015) [7]. The development of the *in vitro* regeneration protocol is essential to be used routinely as a research tool for the improvement of this plant species. Therefore, the present investigation was undertaken to standardize protocols for aseptic cultures of cotyledon segments of *Citrus jambhiri*.

Materials and Methods

The present investigation was carried out at Plant Tissue Culture Laboratory, Division of Fruit Science and School of Biotechnology, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu during 2016 to 2018.

Source of explants: Explants used for callus induction were cotyledon segments each of 0.5-1.0 cm size, excised from 5 weeks old *in vitro* grown seedlings. Seeds were extracted from ripened fruits of *Citrus jambhiri* and germinated in earthen pots containing sand: soil (1:1) mixture.

Surface sterilization: The explant segments were treated with Tween 20 (2-3 drops/100 ml of water) for 5 minutes and washed thoroughly with distilled water to remove surface contaminants. After that, they were treated with (0.2%) carbandazim for 5 minutes and washed thoroughly in running tap water for 10 minutes. Finally, sterilization procedures were carried under aseptic conditions in a laminar air flow chamber to standardize the most effective surface sterilization for isolate contamination free *Citrus jambhiri* explants.

Culture Establishment: The surface sterilized explants were inoculated on the establishment medium. The cotyledon segment was cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators like 2,4-dichlorophenoxyacetic acid; Naphthalene acetic acid and 6-benzylaminopurine. For each explant, 24 culture tubes were inoculated for individual treatment and the experiment was repeated three times.

Callus Induction: Cotyledon segments each of 0.5-1.0 cm size excised from 3-4 weeks old *in vitro* grown seedlings and callus was initiated in 25x150 mm culture tubes containing 40 ml of MS medium supplemented with different concentrations and combinations of plant growth regulators like 2,4-dichlorophenoxyacetic acid; Naphthalene acetic acid and 6-benzylaminopurine. For each explant, 24 culture tubes were inoculated for individual treatment and the experiment was repeated three times.

Shoot regeneration: For shoot regeneration, green healthy friable calli induced from cotyledon segments were divided into small pieces and cultured on MS medium supplemented with different concentrations and combinations of plant growth regulators like Kinetin; Indole-3-acetic acid; Naphthalene acetic acid; 6-benzylaminopurine and malt extract.

Rooting of regenerated shoots: For rooting, the regenerated shoots were separated out and cultured on half strength MS medium supplemented with different concentrations of growth regulators like Indole-3-acetic acid; Indole-3-butyric acid and Naphthalene acetic acid. Root formation from well-developed sub cultured shoots started after 8-10 days of inoculation.

Hardening and acclimatization: Regenerated plantlets were washed with water in order to remove any adhering medium and then transferred to autoclaved plastic pots containing autoclaved coco peat, vermiculite and perlite in the ratio of 1:1:1. Hardening of potted plantlets was accomplished in culture room set at 25±2 ºC and 16-hour day length by covering them with polyethylene bags to maintain high humidity. After 12-15 days, polyethylene bags were removed initially for a short duration (15-30 min) daily for about one week. Gradually, the daily exposure time was increased by 30 min for each day. Polyethylene bags were completely removed after 20 days. Subsequently, the plantlets were transferred to earthen pots containing only garden soil and kept in the greenhouse for one month for acclimatization, and then transferred to screen house.

Statistical analysis: The data were analysed according to Completely Randomized Block design (CRD) as described by Panse and Sukhatme (2000) [18]. Data scored on percentage wherever necessary were subjected to the arc sine transformation for the analysis of variance (ANOVA).

Results and Discussion

Surface sterilization

The explants collected from field grown seedlings harbour many microbial pathogens in addition to adhered soil particles. A critical stage in multiplication of plants through tissue culture is to obtain contamination and disease-free cultures and thus its necessities a thorough and effective surface sterilization of explants before culturing. Pre-sterilization of excised explants with Benomyl (0.2%) can improve a cleanness and aliveness of all types of explants, especially when followed through surface sterilization done by mercury chloride ($HgCl_2$) (Nurul, *et al*., 2012) [17]. In the present investigation (Table 1), mercuric chloride 0.1% for three minutes (T_2) gave best sterilization of epicotyl explants of *Citrus jambhiri* than all other treatments, which resulted in the least rate of contamination (24%) and the maximum epicotyl explant survival (76%). Kaur *et al*. (2005) [8], who worked on *in vitro* propagation of strawberry also found treatment of explants with 0.1% $HgCl_2$ for 3 minutes as most effective surface sterilization procedure registering maximum survival and minimum contamination of cultures. Our results are also supported by Rajiv *et al*. (2015) who used different concentrations of $HgCl_2$ and sodium hypochlorite (NaOCl) for surface disinfection of citrus seeds.

Table 1: Standardization of surface sterilization chemicals for *Citrus jambhiri* Lush. Cotyledon explants on MS medium

	Treatment	Contamination (%)	Survival (%)
T_1:	Mercuric chloride 0.1% (2 minutes)	67 (55.08) *	33 (34.88) *
T_2:	Mercuric chloride 0.1% (3 minutes)	24 (29.26)	76 (60.98)
T_3:	Mercuric chloride 0.1% (2 minutes) +Ethanol 70% (30 seconds)	42 (40.32)	58 (49.69)
T_4:	Mercuric chloride 0.1% (3 minutes) +Ethanol 70% (30 seconds)	33 (34.88)	67 (55.08)
T_5:	Sodium hypochlorite 5% (5 minutes)	91 (73.14)	09 (17.16)
T_6:	Sodium hypochlorite 5% (10 minutes)	82 (64.96)	18 (24.31)
T_7:	Sodium hypochlorite 5% (5 minutes) +Ethanol 70% (30 seconds) + Mercuric chloride 0.1% (2 minutes)	48 (43.82)	52 (46.14)
T_8:	Sodium hypochlorite 5% (5 minutes) +Ethanol 70% (30 seconds) + Mercuric chloride 0.1% (3 minutes)	44 (41.53)	56 (49.07)
T_9:	Sodium hypochlorite 5% (10 minutes) +Ethanol 70% (30 seconds) + Mercuric chloride 0.1% (2 minutes)	47 (43.24)	53 (46.73)
T_{10}:	Sodium hypochlorite 5% (10 minutes) +Ethanol 70% (30 seconds) + Mercuric chloride 0.1% (3 minutes)	42 (40.32)	58 (49.65)
	CD (0.05)	8.26	9.70

* Values in parenthesis are Arc sine transformed values

Culture establishment

It is obvious from the data (Table 2, Fig. 1A) that MS medium supplemented with 2,4-D (3.0 mg/l) recorded maximum culture establishment (97.79%) and was significantly higher as compared to all other treatments whereas, minimum culture establishment of (1.44%) was recorded on MS medium supplemented with 2,4-D (2.0 mg/l) in combination with BAP (0.25 mg/l) and NAA (1.0 mg/l). The results obtained are in line with the results of Mohammed *et al*. (2015) [14] wherein, maximum culture establishment had been reported from leaf and stem segments of *Citrus reticulata* on MS medium incorporated with 2,4 D (1.0 mg/l) either alone or in combination with NAA (0.25 mg/l). Savita *et al*. (2011b) [25] found greenish healthy callus from cotyledon explants of rough lemon on MS medium containing 2,4 D (2.0 mg/l). The results obtained in the present investigation regarding culture establishment are supported by the findings of Kaur (2018) [9] who worked on micropropagation of rough lemon and found MS medium incorporated with 2,4-D (1.0 mg/l) and BAP (1.0 mg/l) to be best for culture establishment.

Callus induction

In the present investigation, cotyledon segments excised from *in vitro* grown seedlings were inoculated on the MS medium supplemented with various concentrations and combinations of 2,4 D, NAA and BAP are presented in Table 3 and Fig 1B. Maximum callus induction (95.41%) was observed on MS medium supplemented with 2,4-D (3.0 mg/l. The results obtained in the present investigation regarding callus induction are supported by the findings of Mukhtar *et al*. (2005) [15] in *Citrus aurantifolia* cv. Kagzi lime and *Citrus sinensis* cv. Musambi; Ali and Mirza (2006) [1] in rough lemon; Mohammed *et al*. (2015) [14] in *Citrus reticulata* and Kaur (2018) [9] in rough lemon. Kaur (2018) [9] reported efficient protocol for *in vitro* embryogenic callus induction of rough lemon with 2, 4-D (1.0 mg/litre) in combination with BAP (1.0 mg/litre) produced early and highest percentage of callus with formation of somatic embryos.

Callus regeneration

For shoot regeneration, green healthy friable calli induced were divided into small pieces of 1.0-1.5 cm and cultured on MS medium supplemented with different concentrations of plant growth regulators. The results on per cent callus regeneration are presented in Table 4 and Fig 1C. The perusal of the data revealed that among different treatments tried, maximum callus regeneration (82.19%) was obtained on MS medium supplemented with BAP (3.0 mg/l) followed by callus regeneration of 68.14% in MS medium with BAP (2.0 mg/l) alone. Minimum callus regeneration (04.33%) was recorded in MS medium supplemented with BAP (3.0 mg/l) and in combination with IAA (0.75 mg/l) and ME (500 mg/l) and was at par with callus regeneration of 07.33% recorded on MS medium supplemented with BAP (3.0 mg/l) in combination with IAA (0.50 mg/l) and ME (500 mg/l). The results obtained in the present investigation are supported by the findings of different researchers in various *citrus* species (Ali and Mirza, 2006; Sharma *et al*., 2009; Saini *et al*., 2010; Savita *et al*., 2011a; Savita *et al*., 2011b; Sarker *et al*., 2015; Kanwar *et al*., 2016 and Kour, 2018) [1, 27, 22, 26, 25, 23, 6, 9].

***In vitro* rooting**

The results on the influence of combination and concentration of different auxins (NAA, IBA and IAA) on *in vitro* rooting are presented in Table 5 and Fig. 1D. The maximum rooting response was recorded on MS (half strength) medium with NAA (2.0 mg/l) with maximum number of roots per shoot on MS (half strength) medium containing NAA (0.5 mg/l) and maximum root length was found on MS (half strength) medium supplemented with NAA (0.5 mg/l). Our results corroborate with some of earlier studies in *Citrus* species where NAA was used for rooting of regenerated shoots (Wang *et al*., 2002; Ali and Mirza, 2006 and Kour, 2018) [28, 1, 9]. *In vitro* good quality of root induction is a known phenomenon due to plant growth regulators. The auxins (IAA, IBA and NAA) have been popularly considered as rooting hormones in plant tissue culture. Roderick *et al*. (1993) [21] studied the rooting in *in vitro* derived micro-shoots on half strength and full-strength MS medium supplemented with IBA, IAA and NAA. Similarly, Islam *et al*. (2005) [5] found rapid growth and multiplication of root on MS medium augmented with IBA, IAA and NAA. Khatun *et al*. (2010) [11] induced *in vitro* rooting on MS medium containing different combination and concentration of IBA, IAA, NAA and BAP. Leonid *et al*. (2011) [12] recorded variability within the auxins for growth and development of *in vitro* root in higher plants.

Hardening and acclimatization

The plantlets were removed from test tubes and transferred into different potting mixtures to the field. The data pertaining to Influence of different potting mixtures on survival of the plantlets of *Citrus jambhiri* is presented in Table 5 and depicted in Fig. 1E. The perusal of the data revealed that different kinds of potting mixture were found to influence significantly on survival percentage of rough lemon plantlets. Among different treatments of potting mixtures, maximum (91.35%) survival of plantlets were recorded in the potting mixture containing garden soil, sand and FYM (1:1:1), followed by plantlet survival percentage of 87.22 in the potting mixture of garden soil, sand and FYM (2:1:1). The potting mixture containing garden soil alone was significantly inferior to other potting mixtures with the plantlet survival of 39.92%. Normah *et al*. (1997) [16] reported 83.33% survival of regenerated plantlets of *Citrus halimii* under ex-vitro conditions. Al-Khayari and Al-Baharany (2001) [1] reported 90% survival of regenerated plantlets of *Citrus aurantifolia*. Filho *et al*. (2001) [4] reported 100% survival in Sweet Orange. Rani *et al*. (2004) [20] reported 67% survival rate of rooted plantlets of Kinnow. Altaf *et al*. (2008) [3] reported 76% survival of regenerated plantlets of *Citrus jambhiri*.

Table 2: Effect of growth regulators on the establishment of cotyledon culture of *Citrus jambhiri* Lush. On MS medium

Treatment	Culture establishment (%)
MS medium + 2,4-D 1.0 mg/l	92.02 (73.92) *
MS medium + 2,4-D 2.0 mg/l	93.98 (76.84)
MS medium + 2,4-D 3.0 mg/l	97.79 (82.29)
MS medium + NAA 1.0 mg/l	25.72 (30.36)
MS medium + NAA 2.0 mg/l	35.21 (36.34)
MS medium + NAA 3.0 mg/l	58.33 (49.79)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l	77.29 (61.53)

MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l	45.13 (42.18)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l	33.64 (35.42)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l	69.67 (56.59)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l	40.39 (39.42)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l	28.07 (31.96)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l	46.31 (40.94)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l	55.11 (47.92)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l	60.02 (50.78)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	13.30 (21.29)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	08.08 (16.91)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	02.43 (08.83)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	07.71 (16.09)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	03.22 (09.96)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	12.21 (20.40)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	05.61 (13.35)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	01.44 (06.56)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	06.19 (14.11)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	50.16 (45.07)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	41.19 (39.89)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	30.02 (33.16)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	34.11 (35.69)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	28.55 (32.27)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	12.63 (20.78)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	21.19 (27.39)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	14.18 (21.98)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	11.09 (19.43)
MS basal medium	00.00 (00.00)
CD $_{(0.05)}$	4.37

∗ Values in parenthesis are Arc sine transformed values

Abbreviations: MS-Murashige and Skoog; 2,4-D-2,4-dichlorophenoxyacetic acid; NAA-Naphthalene acetic acid; BAP-6-benzylaminopurine

Table 3: Effect of growth regulators on the callus induction from cotyledon of *Citrus jambhiri* Lush. on MS medium

Treatment	**Callus induction (%)**
MS medium + 2,4-D 1.0 mg/l	72.79 (58.56) *
MS medium + 2,4-D 2.0 mg/l	80.55 (63.85)
MS medium + 2,4-D 3.0 mg/l	95.41(78.69)
MS medium + NAA 1.0 mg/l	16.57 (23.91)
MS medium + NAA 2.0 mg/l	34.89 (36.17)
MS medium + NAA 3.0 mg/l	56.28 (48.59)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l	15.55 (23.15)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l	17.23 (24.49)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l	22.13 (28.04)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l	30.46 (33.44)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l	34.62 (36.02)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l	39.38 (38.85)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l	43.44 (41.19)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l	50.13 (45.06)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l	57.12 (49.08)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 1.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	00.00 (00.00)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	25.12 (29.99)

MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	15.16 (22.84)
MS medium + 2,4-D 2.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	10.32 (18.61)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	16.09 (23.58)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	4.42 (11.92)
MS medium + 2,4-D 2.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	2.20 (08.43)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	12.12 (20.32)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	3.99 (11.26)
MS medium + 2,4-D 2.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	2.02 (07.99)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 1.0 mg/l	62.21 (52.07)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 2.0 mg/l	57.82 (49.48)
MS medium + 2,4-D 3.0 mg/l + BAP 0.25 mg/l + NAA 3.0 mg/l	52.31 (46.31)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 1.0 mg/l	44.62 (41.89)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 2.0 mg/l	39.42 (38.86)
MS medium + 2,4-D 3.0 mg/l + BAP 0.50 mg/l + NAA 3.0 mg/l	33.22 (35.17)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 1.0 mg/l	28.63 (32.32)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 2.0 mg/l	21.34 (27.44)
MS medium + 2,4-D 3.0 mg/l + BAP 0.75 mg/l + NAA 3.0 mg/l	14.62 (22.40)
MS basal medium	00.00 (00.00)
CD $_{(0.05)}$	3.61

* Values in parenthesis are Arc sine transformed values

Abbreviations: MS-Murashige and Skoog; 2,4-D-2,4-dichlorophenoxyacetic acid; NAA-Naphthalene acetic acid; BAP-6-benzylaminopurine

Table 4: Effect of growth regulators on the regeneration of shoots from callus of cotyledon segments of *Citrus jambhiri* Lush. on MS medium

Treatment	Callus regeneration (%)
MS medium + BAP 1.0 mg/l	32.10 (32.40) *
MS medium + BAP 2.0 mg/l	68.14 (55.63)
MS medium + BAP 3.0 mg/l	82.19 (65.03)
MS medium + KN 1.0 mg/l	23.44 (28.92)
MS medium + KN 2.0 mg/l	48.90 (44.35)
MS medium + KN 3.0 mg/l	33.16 (35.72)
MS medium + BAP 1.0 mg/l + NAA 0.25 mg/l + ME 500 mg/l	40.21 (39.72)
MS medium + BAP 2.0 mg/l + NAA 0.25 mg/l + ME 500 mg/l	36.19 (36.95)
MS medium + BAP 3.0 mg/l + NAA 0.25 mg/l + ME 500 mg/l	60.12 (50.84)
MS medium + BAP 1.0 mg/l + NAA 0.50 mg/l + ME 500 mg/l	20.36 (26.70)
MS medium + BAP 2.0 mg/l + NAA 0.50 mg/l + ME 500 mg/l	25.11 (29.98)
MS medium + BAP 3.0 mg/l + NAA 0.50 mg/l + ME 500 mg/l	55.10 (47.92)
MS medium + BAP 1.0 mg/l + NAA 0.75 mg/l + ME 500 mg/l	20.43 (26.84)
MS medium + BAP 2.0 mg/l + NAA 0.75 mg/l + ME 500 mg/l	20.21 (26.67)
MS medium + BAP 3.0 mg/l + NAA 0.75 mg/l + ME 500 mg/l	16.60 (23.93)
MS medium + BAP 1.0 mg/l + IAA 0.25 mg/l + ME 500 mg/l	22.30 (28.15)
MS medium + BAP 2.0 mg/l + IAA 0.25 mg/l + ME 500 mg/l	35.51 (36.53)
MS medium + BAP 3.0 mg/l + IAA 0.25 mg/l + ME 500 mg/l	11.20 (19.44)
MS medium + BAP 1.0 mg/l + IAA 0.50 mg/l + ME 500 mg/l	16.00 (23.46)
MS medium + BAP 2.0 mg/l + IAA 0.50 mg/l + ME 500 mg/l	29.91(33.11)
MS medium + BAP 3.0 mg/l + IAA 0.50 mg/l + ME 500 mg/l	7.33 (15.61)
MS medium + BAP 1.0 mg/l + IAA 0.75 mg/l + ME 500 mg/l	12.61 (20.63)
MS medium + BAP 2.0 mg/l + IAA 0.75 mg/l + ME 500 mg/l	20.22 (26.67)
MS medium + BAP 3.0 mg/l + IAA 0.75 mg/l + ME 500 mg/l	4.33 (11.78)
MS basal medium	00.00 (00.00)
CD $_{(0.05)}$	4.19

* Values in parenthesis are Arc sine transformed values

Abbreviations: MS- Murashige and Skoog; KN-Kinetin; IAA-Indole-3-acetic acid; NAA-Naphthalene acetic acid; BAP-6-benzylaminopurine; ME-Malt extract

Table 5: Effect of growth regulators on rooting from the shoots regenerated from cotyledon segment callus of *Citrus jambhiri* Lush. on half strength MS medium

Treatment	Rooting (%)	No. of roots/shoot	Root length (cm)
½ MS medium + NAA 0.5 mg/l	82.52 (65.31) *	5.44	5.45
½ MS medium + NAA 1.0 mg/l	76.13 (60.76)	5.08	5.22
½ MS medium + NAA 1.5 mg/l	92.00 (72.67)	4.33	4.63
½ MS medium + NAA 2.0 mg/l	96.00 (79.09)	3.95	3.27
½ MS medium + IBA 0.5 mg/l	76.23 (60.83)	1.25	3.71
½ MS medium + IBA 1.0 mg/l	70.10 (56.88)	2.03	3.97
½ MS medium + IBA 1.5 mg/l	88.42 (70.18)	2.29	4.25
½ MS medium + IBA 2.0 mg/l	91.00 (72.67)	2.66	5.73
½ MS medium + IAA 0.5 mg/l	45.37 (42.32)	1.77	2.69
½ MS medium + IAA 1.0 mg/l	60.77 (51.21)	2.00	2.77

½ MS medium + IAA 1.5 mg/l	65.35 (53.93)	2.66	4.05
½ MS medium + IAA 2.0 mg/l	72.85 (58.61)	2.53	4.79
½ MS medium basal medium	00.00 (00.00)	-	-
CD (0.05)	4.46	1.45	0.35

* Values in parenthesis are Arc sine transformed values

Abbreviations: MS- Murashige and Skoog; IAA-Indole-3-acetic acid; IBA-Indole-3-butyric acid; NAA-Naphthalene acetic acid

Table 6: Influence of different potting mixtures on survival of the plantlets of *Citrus jambhiri* Lush.

Treatment	Survival percentage
Garden Soil	39.92 (38.58) *
Garden Soil + Sand (1:1)	73.27 (58.32)
Garden Soil + Sand (1:2)	71.11 (56.89)
Garden Soil + Sand (2:1)	65.47 (53.41)
Garden Soil + FYM (1:1)	78.09 (61.47)
Garden Soil + FYM (1:2)	69.28 (55.72)
Garden Soil + FYM (2:1)	71.26 (56.98)
Garden Soil + Sand + FYM (1:1:1)	91.35 (72.28)
Garden Soil + Sand + FYM (1:2:1)	80.64 (63.22)
Garden Soil + Sand + FYM (2:1:1)	87.22 (68.51)
Garden Soil + Sand + FYM (1:1:2)	73.58 (58.52)
CD (0.05)	3.44

* Values in parenthesis are Arc sine transformed values

FYM: Farm Yard Manure

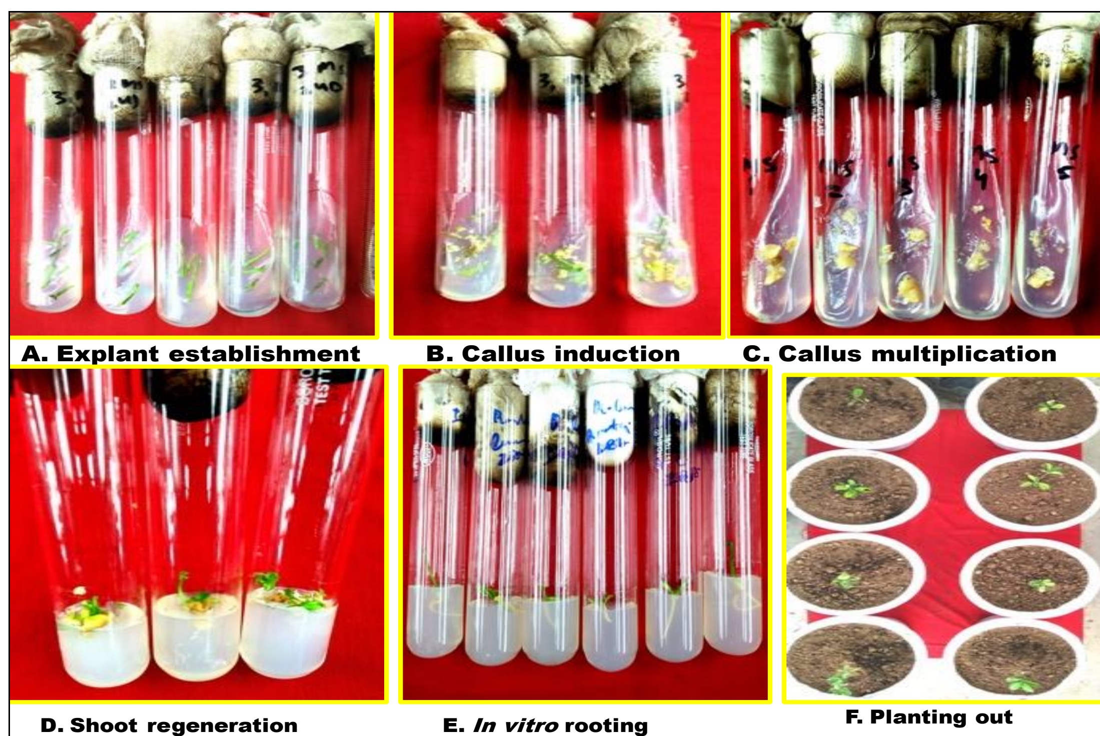

Fig 1: The perusal of the data revealed that different kinds of potting mixture were found to influence significantly on survival percentage of rough lemon plantlets

A- Culture establishment of cotyledon explants on MS medium containing 2,4-D (3.0 mg/l);
B- Callus induction on MS medium supplemented with 2,4-D (1.0 mg/l);
C- Callus multiplication on MS medium augmented with 2,4-D (1.0 mg/l);
D-Shoot regeneration on MS medium augmented with BAP (3.0 mg/l);
E- Rooting of regenerated shoots on MS medium (half strength) containing NAA (2.0 mg/l);
F- Transplanting of plants in plastic pots.

Conclusion

From the present investigation it can be concluded that *Citrus jambhiri* Lush. can be successfully raised under *in vitro* aseptic conditions employing cotyledon explants with good multiplication rate. The maximum callus induction, shoot regeneration and rooting was found on MS medium supplemented with 2,4-D (3.0 mg/l), BAP (3.0 mg/l) and NAA (2.0 mg/l), respectively. The present *in vitro* regeneration protocol will prove beneficial for the genetic improvement of rough lemon to biotic and abiotic stresses through somaclonal variations, genetic transformation and *in vitro* mutagenesis.

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