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In vitro somatic embryogenesis and regeneration from epicotyl segments of Rough lemon (*Citrus jambhiri* Lush.)

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Abstract
Rough lemon (*Citrus jambhiri* Lush.) is an important rootstock for a number of citrus fruit crops including lemons, oranges, grape fruits, kinnows and mandarins. The objective of this study was to develop an efficient protocol for *in vitro* embryogenic callus induction and regeneration of rough lemon (*Citrus jambhiri* Lush.). Epicotyl segments excised from *in vitro* raised seedlings were used as explants. Different growth regulators media were tried to produce somatic embryoes from epicotyls segments of rough lemon. Only MS medium supplemented with 2,4-D1.0mg/litre + BAP1.0 mg/litre produces early and highest percentage of callus with formation of somatic embryoes. Among different somatic callus regeneration media, MS medium fortified with NAA0.5mg/litre, BAP3.0 mg/litre and kinetin1.0mg/litre having good regeneration potential with minimum number of days taken for regeneration, highest number of shoots and shoot length. Rooting of regenerated shoots was highest in MS supplemented with NAA1.0mg/litre and IBA1.0 mg/litre^{-} with minimum number of days taken to rooting, highest roots and root length. The plantlets were successfully acclimatized in different potting mixtures and highest survival rate (96.45%) was achieved in potting mixture containing garden soil+ sand+ vermiculite (1:1:1).

Keywords: *Citrus jambhiri* Lush, epicotyl segments, indirect regeneration, somatic Embryogenesis

Introduction
Rough lemon (*Citrus jambhiri* Lush.) 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 to marked fluctuations in soil moisture. 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. Rough lemon (*Citrus jambhiri* Lush.) is the commercial citrus rootstock in India. This is a deep-rooted rootstock and is well adapted to the diverse agro-climatic conditions. 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) [5]. However, rough lemon is highly susceptible to Phytophthora (Naqvi, 2000) [52] and soil salinity (Ferguson, 2002) [20].
Rough lemon seedlings are not fast growing however budded trees on this rootstock are very vigorous, precocious and bear heavily as young trees but they have lesser longevity period than other stocks. Trees on all rootstocks were smaller except on the rough lemon (*Citrus jambhiri* Lush.). The potential of conventional methods of improvement of citrus rootstocks is limited by biological factors that inhibit breeding and selection, such as heterozygosity and inbreeding depression, pollen and ovule sterlity, sexual incompatability, apomixes, nucellar polyembryony and juvenility (Guo and Deng,2001;Grosser and Gmitter, 2005) [27, 26].
Under such circumstances, *in vitro* culture techniques hold potential and could offer solution to these problems. Besides improving the existing cultivars, micropropagation techniques have the potential for mass scale generation of novel plants in a comparatively short time span when compared to conventional breeding. Plant tissue culture has emerged as a powerful tool for propagation and improvement of many woody plant species including *Citrus*. *In vitro* culture has the potential to eliminate diseases and provides scope for development of new cultivars through somaclonal variations (Hammschlag *et al*., 1995) [28]. Production of callus and its subsequent regeneration are the prime steps in crop plants to be manipulated by

biotechnological means and to exploit somaclonal variations (Islam *et al*., 2005) [33]. The composition of culture medium and culture conditions have been shown to be crucial for the growth of *in vitro* cultures (Duran-Vila *et al*., 1992) [18].

Biotechnological tools such as genetic transformation and tissue culture techniques like somaclonal variation and *in vitro* mutagenesis are ideal alternatives of conventional breeding to expedite the genetic improvement of citrus genotypes(Kayim and Koe, 2006) [35]. As a pre-requisite to genetically improve rough lemon for phytophthora and salinity tolerance through these methods, an efficient *in vitro* regeneration protocol is must. *In vitro* regeneration system in rough lemon based on callus could prove useful both for the production of transgenics (young callus) and for induction of variants through somaclonal variation (old callus). Only few reports indicate regeneration from callus in rough lemon (Singh, 2000) [65] but with low regeneration frequency. Gill *et al*. (1994) [22] demonstrated the involvement of growth regulators in callus induction and regeneration in citrus. Like growth regulators, carbohydrates also influence the callusing behaviour in citrus (Oliveira *et al.*, 2001) [53]. This implies that the carbohydrate composition of callus induction medium could also influence the regeneration. Callus age is another factor, which modifies the *in vitro* response (Hao and Deng, 2002) [29].

Tissue culture and micropropagation protocols have been described for a number of *Citrus* species using a wide range of explant sources (Al-Khayri and Al-Bahrany,2001; Khawale and Singh, 2005; Ali and Mirza, 2006; Altaf *et al.*, 2008; Altaf *et al.*, 2009; Khan *et al*.,2009; Laskar *et al*., 2009; Sharma *et al*., 2009; Perez-Tornero *et al*., 2010; Singh and Rajam, 2009, 2010) [3, 40, 1, 5, 7, 38, 43, 64, 55, 66, 67]. However, a little work has been carried out on the tissue culture of *Citrus jambhiri* (Raman *et al*.,1992; Altaf and Ahmad, 1997; Khawale and Singh, 2005; Ali and Mirza, 2006; Altaf *et al*., 2008; Sharma *et al*., 2009; Saini *et al*.,2010; Kumar *et al*., 2011; Savita *et al*., 2010,2011) [57, 4, 40, 1, 5, 64, 61, 42, 63, 62]. Different protocols of callus induction and plant regeneration using various techniques and explants, including somatic embryogenesis and organogenesis have been reported for various citrus species (Al-Taha, 2009; Jajoo, 2010; Lombardo *et al*., 2011) [8, 34, 45]. Establishment of an efficient callus induction protocol is an essential prerequisite in harnessing the advantage of cell and tissue culture for genetic improvement. For the successful application of the tissue culture technique in crop breeding, callus growth and plant regeneration potential of each crop must be determined (Khaleda and Forkan, 2006; Altaf *et al*., 2009) [37, 7]. Citrus embryo explants were most responsive to callus induction and proliferation (Alka, 2010) [2].

Although all plant cells are derived from the fertilized egg cell and contain identical information, callus derived from somatic cells varies in competence to express totipotency (i.e. their genetic ability to produce plants). Mostly, explants that contain immature, meristematic cells develop callus, which are competent to express totpotency.In citrus, callus cultures have been established in species such as *Citrus grandis*(L) Osb, *Citrus aurantifolia* (Christm.) Swingle, *Citrus medica* L., *Citrus sinensis* (L) Osb, *Citrus madurensis* L., *Citrus paradise* Macf., *Citrus reticulata* Blanco and *Citrus limon* (Sabharwal, 1963; Murashige and Tucker,1969; Grinblat, 1972; Chaturvedi and Mitra, 1975; Moore, 1985; Duran-Vila *et al*., 1989; Gill, 1992; Gill *et al*., 1994) [60, 48, 25, 15, 47, 19, 24, 22]. Responses to different culture media are often genotype-specific. Genetic transformation through callus is quite easy and high frequency transgenic plants can be obtained from callus since in the case of direct regeneration, frequency of untransformed plants is quite high. Furthermore genetic transformation by protoplast isolation and fusion, agro bacterium mediated genetic transformation, induced mutation through gamma rays or chemical mutagenesis can also become efficient through callus formation and regeneration. Nucellar embryos or nucellar tissues and epicotyl segments can give an efficient callus induction and regeneration systems.

The development of tissue culture protocol is essential to be used routinely as a research tool for improvement of Rough lemon. Keeping this in mind, the present study was designed to develop an efficient and reproducible protocol for callus induction and somatic embyrogenesis from epicotyl segments of *Citrus jambhiri* and regeneration of calli into plantlets.

Materials and Methods

Explant collection and sterilization

The experimental material consisted of seeds extracted from ripened fruits of citrus rootstock rough lemon (*Citrus jambhiri* Lush.) collected from the New Orchard of Department of Fruit Science, Punjab Agricultural University, Ludhiana during 2009-10 and 2010-11. Fresh fruit taken from field grown trees were washed with water containing 1-2 drops of teepol and then washed thoroughly with running tap water. The seeds extracted from fruits were removed of testa (outer covering) and then surface sterilized under aseptic conditions with 0.1% mercuric chloride for 4-5 minutes. The seeds were thoroughly washed with sterilized distilled water thrice before inoculation to remove the toxic effects of mercuric chloride. After sterilization, seeds were sown in Murashige and Skoog (MS) (1962) [49] basal medium in culture jars. The cultured seeds were incubated at 25±2°C temperature in dark for two weeks for etiolation. After one week of sowing, seed germination started. After 4-5 weeks, these culture were shifted to light for 16 hours continuous fluorescent white light (2000lux) followed by a dark period of 8 hours (Fig1).

Callus induction

Epicotyl segments (1-2cm) from one month old seedlings (Fig1)were cultured in petri plates containing 40ml of callus induction MS media supplemented with different concentrations and combinations of plant growth regulators like Naphthalene acetic acid(NAA),3-Benzyl aminopurine (BAP), 2, 4-D(2, 4-Dichlorophenoxyacetic acid) and Kinetin (Fig2). For each treatment, 24 culture petri plates were inoculated for individual treatment and experiment was repeated thrice. These cultures were incubated at 25±2°C temperature for 16 hours continuous florescent white light (2000lux) followed by a dark period of 8 hours.

Shoot regeneration from callus

For regeneration, about 50 days old green healthy friable calli were divided into small pieces and cultured on different callus regeneration media containing MS medium supplemented with different concentrations and combinations of plant growth regulators like Naphthalene acetic acid(NAA),3-Benzyl aminopurine(BAP)and Kinetin. Further, to determine the regeneration capacity of long term callus cultures, calli of different age groups were transferred to optimized shoot regeneration medium. For each treatment, 24 culture tubes were inoculated for individual treatment and experiment was repeated thrice. Cultures were incubated at 25±2°C

temperature for 16 hours continuous florescent white light (2000lux) followed by a dark period of 8 hours.

Rooting of regenerated shoots

For rooting, the regenerated shoots (about 2-3 cm in length) which are induced on best shoot regeneration medium were separated out and cultured on MS medium supplemented with different concentrations of naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA). For each treatment, 24 culture tubes were inoculated for individual treatment and experiment was repeated thrice. Cultures were incubated at 25±2°C temperature for 16 hours continuous florescent white light (2000lux) followed by a dark period of 8 hours.

Visual observations were taken at every three days and data was recorded on effect of different treatments on callus induction, shoot regeneration and root induction. The experimental data was analyzed with Completely Randomized Block Design as described by Singh *et al*. (1998) [68].

Results and Discussion

Callus induction

The frequency of callusing varied with the composition of growth regulators in the medium. Effect of different concentrations and combinations of MS media with 2, 4-D, NAA, BAP and kinetins on callus induction and somatic embryogenesis from epicotyl segments of *Citrus jambhiri* Lush. have been tried (Table 1). Among the different media combinations, the MS medium supplemented with 2, 4-D 1.0 mg/litre and BAP 1.0 mg/litre produced soft, loose, nodular, friable and green colour of callus (Fig 3a & b). This type of callus produced somatic embryos (Table1) (Fig4 a&b) (Fig 5&b). Composition of growth regulators had great influence on the colour and texture of callus. Only green colour callus has morphogenetic potential (Tao *et al*., 2002) [70]. Essentially effects of auxins on citrus callus induction already have been reported in *Citrus grandis* (Huang *et al*., 2002) [31] and *Citrus jambhiri* (Savita *et al*., 2010) [31]. A similar result on *Citrus acida* (Chakravarty and Goswami, 1999) [13] shows that 2, 4-D in combination with BAP is one of the best combinations for induction and development of callus.

Among different combinations evaluated for their effects on callogenesis, MT medium supplemented with 1 mg/litre 2,4-D in combination with BAP at 0.5 mg/litre supported highest rate of callus induction (100%) with better growth and vigour (Ramdan *et al*., 2014) [58]. Gill *et al*. (1994) [22] demonstrated that the type of auxin used for establishment of callus not only affects the colour and texture of callus but also its regeneration potential. They reported that use of 2,4-D in the medium produced friable and non-embryonic callus while NAA induced compact nodular calli of embryonic nature.

Callus induction response in cotyledons of *Citrus jambhiri* was increased to 83.33% when 2,4-D 2mg/litre was used in combination with BA at 0.75 mg/litre (Savita *et al.*, 2011) [62].Maximum callus induction(98.66%) was observed from leaf segments on MS medium supplemented with 2,4-D 4mg/litre in *Citrus jambhiri* (Savita *et al.*, 2011)[62]. Kumar *et al*. (2011) [42] also observed that maximum callusing in epicotyl segments of *Citrus jambhiri* was observed on MS medium supplemented with NAA 10mg/litre+BA 1.0 mg/litre+kinetin 0.5 mg/litre+sucrose 6%+galactose.

MS medium alone with BAP 1.0 mg/litre and 1.0 mg/litre 2, 4-D induce highest percentage of callus (Waghmare and Pandhure, 2015) [72]. This shows that cell proliferation requires the presence of 2, 4-D as auxin essential for callogenesis. This acts as an inductive auxin signal to trigger the proliferative activity of explants.A similar result on *Citrus acida* (Chakraverty and Goswami, 1999) [13] shows that 2, 4-D in combination with BAP is one of the best combinations for induction and development of callus.

2,4-D in combination with BAP was also tested and it was found that 5 mg/litre 2,4-D and 1 mg/litre BAP was most effective in callus induction. At this concentration the response for primary callus induction was 88%. (Hussain *et al*., 2016) [32]. Al-Taha *et al*. (2012) [9] who also obtained similar findings in an attempt for primary callus induction in *Citrus sinensis.*

Hussain *et al*. (2016) [32] also reported that 2,4-D and BAP combination was more appropriate for embryogenic potential. These findings regarding to embryogenic callus induction for *Citrus reticulata* are in strongly agreement with Khan *et al*. (2006) [39], who also reported similar results. It was observed that MS medium containing 2.5 mg/litre 2,4-D and 0.5mg/litre BAP supplemented with 0.5gm/litre malt extract was suitable for embryogenic callus induction from both epicotyl segments and nucellar embryonic tissues (Kazmi *et al*., 2015) [36]. There are various previous reports available for direct and indirect micropropagation of citrus varieties including micro propagation of rough lemon through callus formation and regeneration in the presence of 2,4-D and BAP was performed by (Ali & Mirza, 2006) [1].

Kazmi *et al*. (2015) [36] reported that callus produced from epicotyl segments was soft, friable and embryogenic in the media with 2.5mg/litre 2,4-D and 0.5mg/litre BAP. These findings are in disagreement with (Nafees *et al*., 2009) [50] since they used BAP and 2,4-D combinations from 0 to 0.4mg/litre and also with Chakraverty & Goswami (1999) [13] as they found 1mg/litre 2,4-D and 0.5mg/litre BAP suitable for callus induction(90.2%) as well as 1mg/litre BAP for somatic embryogenesis(75.7%). These findings are in contrast with Gill *et al*., (1995) [23] since they found epicotyl parts more embryogenic as compared to other plant parts.

Chakravarty and Goswami, (1999) [13] reported highest callusing in 2,4-D and BAP combination at concentrations of 1 and 0.5 mg/litre respectively, although the presence of 2,4-D alone was sufficient to initiate callusing. BAP and 2,4-D gave better callusing response than NAA and Kinetins. NAA and Kinetins could induce callusing from the explants in *Citrus reticulata* (Gill *et al*., 1995) [23]. Altaf *et al.* (2009) [6] reported that the seeds formed callus in MS medium supplemented with BA + 2,4-D each at 1mg/litre. Callus induction occurred on half strength MS medium supplemented with BA at 1.0 mg/litre and 2,4-D at 5.0 mg/1itre were obtained by Miah *et al*.(2002) [46] for *Citrus macroptera*. Chakraborty and Goswami (1999) [13] reported best callus induction response on MS medium supplemented with 2,4-D (1 mg/litre) and BA (0.5 mg/litre).

Embryo germination and maturation occurred on half strength MS medium supplemented with BA at 1.0 mg/litre and 2,4-D at 5.0 mg/litre (Al Taha *et al*., 2012) [9]. Similar results were obtained by Miah *et al*. (2002) [46] for *Citrus macroptera* and Bhargara *et al.* (2003) [12] for date palm, who showed the importance of benzyl adenine and auxins, for embryo germination and maturation.

Shoot regeneration

For regeneration, 50 days old green healthy friable calli which produced somatic embryoes were divided into small pieces which were cultured on MS medium supplemented with different concentrations and combinations of NAA, BAP and Kinetin (Table 2).

Callus regeneration is initiated by the appearance of shoot buds on callus. The callus cultured on regeneration medium started some morphological changes. The callus started turning dark green, followed by its loosening. Most of the shoots regenerated either from the outer peripheral cells or from the middle cells of the callus. The histological analysis of callus cultures of *Citrus madurensis* by Grinblat (1972) [25] indicated that the outer peripheral and middle cells are of parenchymatous and meristematic origin and participate in cell division and differentiation. Shoot regeneration in callus started after 22 days of culturing (Fig 6a & b) and continued up to 54 days (Table 2).

Significantly maximum(80.52%) and earlier (22.00) callus regeneration was observed in calli cultured on MS medium fortified with NAA0.5mg/litre, BAP3.0mg/litre and Kinetin1.0mg/litre with maximum number of shoots (7.50), shoot length (5.50cm) and number of leaves (5.0) followed by MS medium supplemented with NAA0.5mg/litre BAP2.5mg/litre and Kinetin0.5mg/litre. Kumar *et al*. (2011) [42] reported that maximum shoot regeneration (76.09%) and number of shoots(8.15) per callus was observed on full MS medium with NAA 0.5mg/litre + kinetins 0.5mg/litre + BA 3.0mg/litre. But average length of regenerated shoots was highest (4.32cm) in the calli cultured on full MS medium supplemented with NAA 0.5 mg/litre, kinetin 0.5mg/litre and BA 1.0mg/litre.

Savita *et al*. (2010) [63] reported that nodal segments of *Citrus jambhiri* showed better regeneration (71.89%) with NAA 0.5mg/litre and BA 3mg/litre. Similarly, Savita *et al*. (2011) [62] maximum shoot regeneration (87.50%) was observed from callus induced from cotyledon segments of *Citrus jambhiri* on the MS medium with BA 3 mg/litre.

Cytokinins either alone or in combination with other growth regulators has been proved beneficial in the differentiation of callus cultures into shoots in different citrus species.In undifferentiated callus cultures of *Citrus grandis*(Li and Xu, 1992; Begum *et al*., 2003) [44, 10], *Citrus sinensis* (Rashad *et al*., 2005) [59], shoot differentiation was achieved with the supplementation of BAP alone in the MS medium. In *Citrus jambhiri*, Ali and Mirza, (2006) [1] also reported regeneration in stem segments derived callus on MS medium containing BAP 3.0 mg/litre. Chaturvedi and Sharma(1988)[14]reported that supplementation of BAP along with other auxins like NAA to the MS medium has been helpful in redifferentiation to form shoots in callus cultures of *Citrus grandis, Citrus sinensis* and *Citrus aurantifolia*. In Citrus, role of cytokinins in shoot regeneration were also noted by Bhansali and Arya, (1978b) [11]; Gill, (1992) [24]; Raman *et al*. (1992) [57]. The cytokinins are generally added to induce shoot formation and to inhibit root formation. Cytokinins have also been shown to activate RNA synthesis and to stimulate protein and enzymes activity in certain tissue (Torres, 1988) [71]. Rashad *et al*. (2005) [59] noted the maximum shoot induction in *Citrus sinensis* cultivar Musambi, when calli were cultured on MS media supplemented with BAP 2 mg/litre. Dhatt and Grewal (1997) [17] noted that shoots were regenerated from callus culture of citrus cultivars Mosambi, Baramasi lemon and Kinnow upon transfer to half strength MS medium supplemented with BAP 5 mg/litre. Singh (2000) [65] reported low regeneration frequency of 27% in hypocotyl and epicotyls derived callus of *Citrus jambhiri* on MS medium supplemented with NAA (0.5 mg/litre), BA (3.0mg/litre) and malt extract (0.5g/litre). In the present study, the relatively high regeneration from callus in this citrus species can be attributed to the difference in the composition of callus induction medium.

Root regeneration

For rooting, the regenerated shoots were separated out and cultured on MS medium supplemented with different concentrations of NAA and IBA.

In vitro regenerated shoots of about 2-3 cm height were separated out from the shoot clumps and transferred to the rooting media. The response of plantlets regenerated from callus is presented in Table 3. Among the six media tested for rooting response, the percent rooting (88.52) days to root initiation (12.0), number of roots (16.75) and root length (10.55cm) were significantly higher in MS medium supplemented with NAA1.0mg/litre and IBA1.0 mg/litre (Fig7) followed by MS medium fortified with NAA 1.0 mg/litre. The MS medium supplemented with NAA0.5 mg/litre and IBA 0.5 mg/litre induces minimum percent rooting (23.50), number of roots (3.17) with more number of days (22.50) taken for rooting. Auxin induces the rhizogenesis which include the division of meristematic cells, their elongation and differentiation into root primordial (Nanda, 1979) [51].

The number of roots produced per shoot, root length and thickness of roots varied with the concentration and combination of auxin used in the medium. Parkash *et al.* (2005) [56] observed that the earliest and maximum rooting in *C. jambhiri* on MS medium supplemented with NAA 1.0 mg/litre and IBA 1.0 mg/litre. Rooting response was maximum in *Citrus sinensis* cultivar Mosambi when shoots were cultured on MS medium supplemented with NAA1.5mg/litre (Rashad *et al*., 2005) [59] and MS medium fortified with NAA 0.75 mg/litre and IBA 2 mg/litre (Das *et al*., 2000) [16]. Gill and Gosal (2002) [21] noted that average number of roots per shoot was maximum in MS medium supplemented with NAA 2.0 mg/litre in pectinifera rootstock. Root length and root number were found 80% by 5.5 µM IBA + 2.5 µM NAA (Hasan *et al*., 2016) [30]. Kour and Singh, (2012) [41] reported that in *Citrus jambhiri* Lush., half strength MS medium fortified with 1.0 mg/litre NAA and IBA each was earlier (16.51 days) to show the root induction with highest per cent rooting (83.33) than IBA and NAA 1.0 mg/litre alone. Maximum number of roots per shoot (2.47) and length of longest root (3.57 cm) was observed in the medium supplemented with NAA and IBA 1.0 mg/litre each. Maximum rooting response (91.67%) was observed on half strength MS medium supplemented with NAA (0.5 mg/litre) (Savita *et al*., 2011) [62]. Kumar *et al.*, (2011) [42] reported the rooting from regenerated shoots of *Citus jambhiri* on MS medium supplemented with IBA 1.0mg/litre.

Auxins play an important role in rhizogenesis, which include division of meristematic cells, their elongation and differentiation into root primordial (Omura and Hidaka, 1992) [52]. The number of roots produced per shoot, root length and thickness of roots varied with the concentration and combination of auxins used in the medium.

Effect of increasing callus age on regeneration potential

The 50 days old calli had the maximum shoot regeneration potential. The increase in callus age showed a progressive and significant decline in shoot regeneration from 88.50% (50-days-old calli) to 15.68 % (130 days-old-calli) (Table4). Effect of increasing age of callus was studied which showed that callus retained regeneration capacity (58.33%) even after 420 days of culture (Savita *et al.*, 2011) [62]. It was observed

that with increase in age, the callus became more and more compact and therefore, it took more time to loosen prior to differentiation. With increase age of callus, the average number of regenerating shoots/ callus and their growth was also reduced significantly (Table 4) Similar results were noted by Kumar *et al.* (2011) [42] and Savita *et al*. (2011) [62]. With increase callus age, the time interval for shoot regeneration was also prolonged. The minimum time (24.25 days) for shoot regeneration with maximum number of shoots (7.25) and shoot length (5.35cm) were recorded in 50 days old callus, while 130 days old callus took maximum time (55 days) to regenerate with minimum number of shoots (2.65) and shoot length (2.5cm). Hao and Deng (2002) [29] linked decrease in regeneration from ageing callus to karyotypic changes. They observed numerical chromosomal changes in long-term callus cultures of *Citrus sinensis* cv 'Anliucheng' and found that 2.3 and 3.9% of the total examined cells were aneuploid and tetraploids, respectively. Although cytological examination of different age group callus has not been performed so far in citrus, but the study of Swedlund and Vasil (1985) [69] in *Pennisetum americanum* has shown that the relative frequency of the aberrant cells increases with the duration of culture. They reported that after 1 and 6 months of culture, the relative frequency of diploid cells was 92 and 76%, respectively. The aberrated cells, though in low frequency, but participate in the regeneration process via organogenesis along with normal diploid cells (Swedlund and Vasil, 1985) [69]. This implies that the old callus cultures have great potential in production of somaclonal variants. These somaclonal variants can later be screened to various biotic and abiotic stresses of interest.

Acclimatization

In vitro propagated plantlets of *Citrus jambhiri* Lush. having good shoot and root system are ready for acclimatization. The plantlets were removed from the culture vessels and washed with sterilized water to clean the root system from the remains of the growth medium. The plantlets were then placed in glass tubes containing half strength MS medium, ensuring the submergence of the root system. The glass tubes were then closed with aluminum foil and placed in a growth room under controlled conditions (temperature 27±2°C,16/8 hour photoperiod and light intensity 1500 Lux for 24 hrs. Then, the plantlets were planted in earthen pots containing an autoclaved potting mixtures containing different combinations of garden soil:sand:vermiculite and kept in polyhouse. Plantlets showed maximum survival (96.45%) was observed in garden soil + sand+vermiculite (1:1:1) potting mixture (Table 5). Similarly the Saini *et al*. (2010) [61] and Kumar *et al*. (2011) [42] reported the the maximum plantlet suvival in vermiculite and garden soil as potting mixture for in *Citrus jambhiri* Lush (Fig8). Rooted plantlets grown in all the vermiculite potting mixtures exhibited low mortality of the plantlets because vermiculite had high water holding capacity. After this, these plantlets were shifted to greenhouse. The plantlets exhibited normal growth with 3-4 leaves per plant. After 2-3 months in the greenhouse the plants were transplanted under the field conditions.

Table 1: Effect of different culture media on callusing and somatic embryogenesis in rough lemon (*Citrus jambhiri* Lush.)

Media	Days for callus initiation	Percent callus induction	Type of Callus	Colour of callus	Degree of callus growth	Response for somatic embryoes
MS+NAA1mg/litre +BAP1.0 mg/litre	13.82	75.55	Compact	White	Good	Nil
MS+NAA0.5mg/litre + BAP0.5 mg/litre	20.56	62.25	Rough and less compact	Creamish to yellow	Good	Nil
MS+2,4-D1.0mg/litre +kinetin 0.5 mg/litre	16.52	70.46	Compact	White	Good	Nil
MS+NAA1.0mg/litre +kinetin0.5 mg/litre	28.00	30.25	Rough and less compact	Creamish to yellow	Fair	Nil
MS+2,4-D1.0mg/litre+ BAP1.0 mg/litre	9.20	86.55	Soft, loose, Nodular and friable	Green	Very good	Somatic embryoes developed
MS+2,4D0.5mg/litre +BAP 0.5mg/litre	24.62	45.25	Compact	Creamish	Good	Nil
MS+2,4-D1.0 mg/litre + NAA 0.5 mg/litre	30.54	25.50	Compact	Creamish	Poor	Nil
CD (5%)	2.84	3.12	-	-	-	-

Table 2: Effect of different culture media on percent shoot regeneration, days to regeneration,average number of shoots, shoot length and number of leaves in callus culture of rough lemon (*Citrus jambhiri* Lush.)

Media	Percent shoot regeneration	Days to regeneration	Average number of shoots	Shoot length (cm)	Number of leaves
MS+NAA0.5mg/litre+BAP0.5mg/litre +Kinetin0.5mg/litre	20.00	51.63	2.80	2.50	3.00
MS+NAA0.5mg/litre +BAP3.0mg/litre +Kinetin1.0mg/litre	80.52	22.00	7.50	5.50	5.00
MS+NAA0.5mg/litre +BAP2.0mg/litre +Kinetin0.5mg/litre	60.00	34.50	5.00	2.95	3.20
MS+NAA0.5mg/litre +BAP1.0mg/litre +Kinetin0.5mg/litre	25.00	43.94	4.00	2.80	3.60
MS+NAA0.5mg/litre+BAP2.5mg/litre +Kinetin0.5mg/litre	65.25	30.42	6.25	3.33	4.00
MS+BAP0.5mg/litre+Kinetin0.5mg/litre	18.52	54.00	3.54	2.78	3.00
MS+BAP1.0mg/litre +Kinetin1.0mg/litre	60.52	32.25	4.56	3.00	2.50
CD (5%)	2.30	2.74	1.70	0.96	NS

Table 3: Effect of different media on rooting from callus regenerated shoots of rough lemon (*Citrus jambhiri* Lush.)

Media	Percent rooting	Days taken to root initiation	Number of roots	Root length(cm)
MS+NAA0.5mg/litre+IBA0.5 mg/litre	23.50	22.5	3.17	7.79
MS+NAA1.0mg/litre +IBA1.0 mg/litre	88.52	12.00	16.75	10.55
MS+NAA1.0 mg/litre +IBA0.5 mg/litre	50.55	18.50	6.00	8.00
MS+NAA0.5 mg/litre +IBA1.0mg/litre	60.25	17.00	8.52	8.25
MS+IBA1.0 mg/litre	75.40	16.00	10.35	8.45
MS+NAA1.0mg/litre	80.50	15.00	12.50	8.62
CD(5%)	3.31	3.02	3.01	NS

Table 4: Effect of callus age on days taken to regeneration, percent callus shoot regeneration, number of shoots and shoot length in callus culture of rough lemon (*Citrus jambhiri* Lush.)

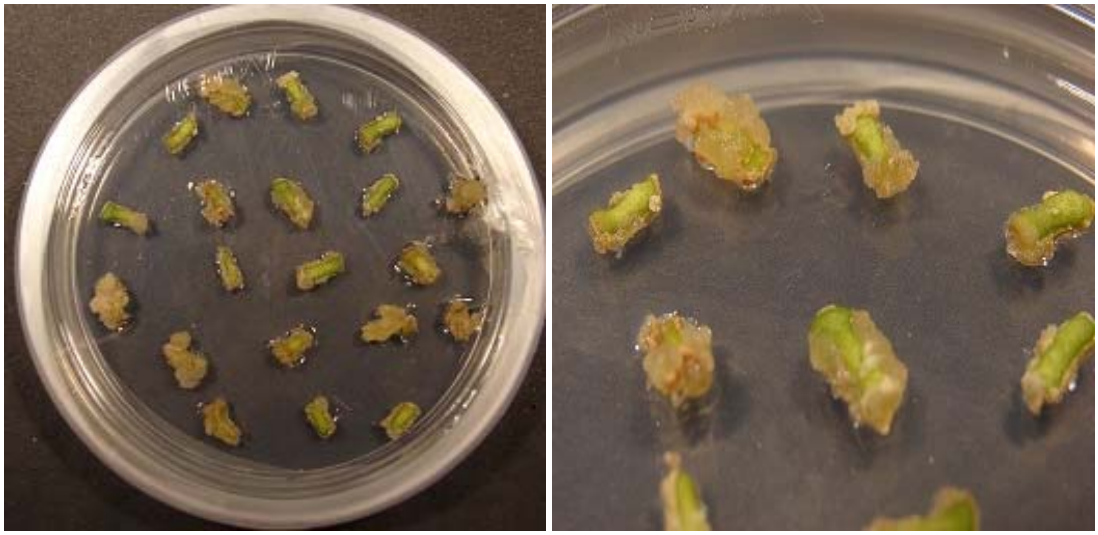
Age of callus(days)	Days taken to regeneration	Percent callus shoot regeneration	Number of shoots	Shoot length(cm)
50	24.25	88.50	7.25	5.35
70	35.55	70.34	4.55	3.75
100	46.35	52.50	3.50	3.20
130	55.00	15.68	2.65	2.50
CD(5%)	2.21	3.64	1.68	1.43

Table 5: Effect of different potting mixtures on the percent survival of the plantlets induced from callus of rough lemon (*Citrus jambhiri* Lush.)

Potting mixtures	Percent survival
Garden soil	44.50
Garden soil+sand(1:1)	74.53
Garden soil+sand(1:2)	70.20
Garden soil+sand(2:1)	66.72
Garden soil+Vermiculite(1:1)	80.00
Garden soil+Vermiculite(1:2)	70.25
Garden soil+Vermiculite(2:1)	72.32
Garden soil+sand+vermiculite(1:1:1)	96.45
Garden soil+sand+vermiculite(1:2:1)	82.16
Garden soil+sand+vermiculite(2:1:1)	88.15
Garden soil+sand+vermiculite(1:1:2)	75.00
CD (5%)	3.09

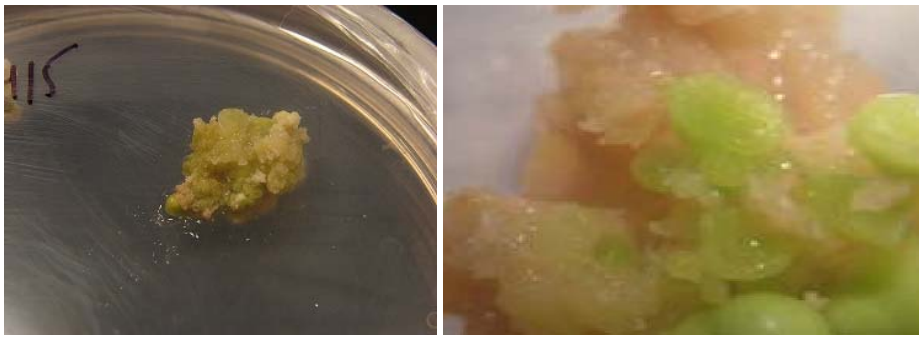
Fig1. Seed germination of *Citrus jambhiri* Lush. on MS medium

Fig2. Culturing of epicotyl segments on callus induction medium



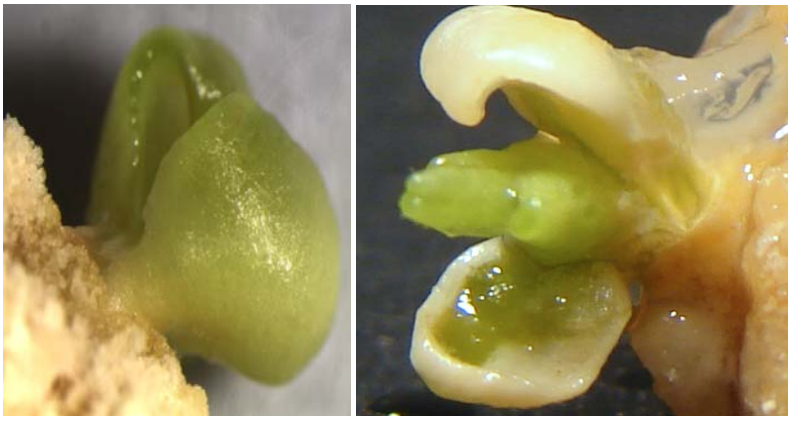
(a) (b)

Fig 3(a-b): Initiation of callus on callus induction medium



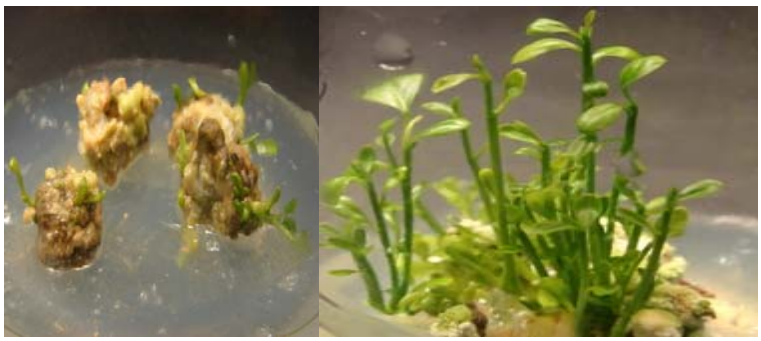
(a) (b)

Fig 4: Induction of somatic embryoes on MS medium with 2,4-D1.0mg/litre + BAP1.0 mg/litre



(a) (b)

Fig 5(a-b): Emergence of somatic embryoes



(a) (b)

Fig 6(a-b): Shoot proliferation from somatic embryoes on MS medium fortified with NAA0.5mg/litre, BAP3.0 mg/litre and Kinetin1.0mg/litre

Fig 7: *In vitro* rooting on MS medium+ NAA1.0 mg/litre and IBA1.0 mg/litre

Fig 8: Established full grown plant in potting mixture of garden soil +sand+vermiculite(1:1:1)

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