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Original Research Paper

Optimization of explants, media, plant growth regulators and carbohydrates on callus induction and plant regeneration in *Citrus jambhiri* Lush.

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ABSTRACT

Callus induction was attempted from the four explants *viz*. root, cotyledon, epicotyl, and leaf segments excised from *in vitro* raised seedlings of *C. jambhiri.* Among various MS media supplementations with growth regulators and carbohydrates, the maximum (95.50%) and the earliest (8.30 days) callogenesis was obtained in epicotyl segments, when cultured on MS medium supplemented with NAA (10.0 mgl^{-1}) + BAP (1.0 mgl^{-1}) + sucrose (8%). The modified MS (macro and micro-nutrients reduced to half) fortified with BAP (5.0 mgl^{-1}) + GA_3 (3.0 mgl^{-1}) recorded maximum shoot regeneration (43.10%) from callus, with an average of 5.30 shoots per callus after 35.50 days of culturing. However, prolonged exposure to GA_3 resulted in thin elongated shoots and leaves. The age of the callus substantially influenced the plant regeneration frequency. The potency of the callus to regenerate decreased significantly with an increase in the age of the callus. Shoot regeneration was recorded maximum (43.43%) in 60 days old calli, followed by 90 days old (30.48%) calli, whereas it was minimum (10.46%) in 150 days old calli. The maximum (79.50%) shoot proliferation was recorded in MS medium supplemented with BAP (1.0 mgl^{-1}) + Kin (0.5 mgl^{-1}) with an average of 5.06 shoots per culture. The MS medium fortified with NAA (1.0 mgl^{-1}) + IBA (1.0 mgl^{-1}) induced maximum (77.33%) rooting, with an average of 3.19 roots per shoot after 13.4 days of culturing. Rooted plants were hardened and survived the best (83.6%) on the potting mixture consisting of cocopeat + vermiculite + perlite (2:1:1).

Keywords : Callus, carbohydrates, *Citrus jambhiri*, explants, redifferentiation

INTRODUCTION

Rough lemon (*Citrus jambhiri* Lush.) is a commonly used citrus rootstock that is highly polyembryony and native to India (Wu *et al*., 2018). High vigour, profuse and deep root system, well adaptation to different agroclimatic zones, and resistance to viruses makes it a suitable rootstock for grafting scion of mandarins, sweet oranges, lime, lemons, and grapefruits in many countries (Russo *et al*., 2021). However, *Citrus jambhiri* is highly susceptible to *Phytophothora* fungus and yields moderate fruit quality. The genetic improvement through conventional plant breeding approaches is a difficult task because of its long juvenility, perennial habit, nucellar polyembryony, heterozygosity, etc (Salonia *et al*., 2020). Biotechnological tools such as genetic engineering, tissue culture-induced variations from somatic cells, and *in vitro* mutagenesis are alternate promising approaches for the genetic improvement of citrus genotypes (Kayim and Koc, 2006). *In vitro* manipulations through these techniques require an efficient and reproducible protocol for the production of callus and subsequent regeneration of plants through somatic embryogenesis (Moniruzzaman *et al*., 2021). The young and old embryogenic callus can be used for genetic transformation and induction of somaclonal variations, respectively. Earlier, embryogenic cultures were developed from reproductive organs *i.e.* isolated nucellar embryos or fertilized ovules (Maheshwari and Rangaswamy, 1958; Litz *et al*., 1985), abortive (Bitters *et al*., 1970), unfertilized (Button and Bornman, 1971), and undeveloped ovules (Carimi *et al*., 1998). But the availability of this reproductive tissue as explant at the right stage is only for a limited period. So to continue the tissue culture and transformation experiments throughout the year callus induction and shoot regeneration protocol from some alternate explant should be developed (Moniruzzaman *et al*., 2021). Gill *et al.* (1995) reported the embryogenic

potential of leaf, epicotyl, cotyledon, and root segments of *in vitro* grown seedlings of *C. reticulata*. Shoot regeneration from callus is very low in rough lemon (Raman *et al*., 1992). Although several *in vitro* propagation protocols are reported in citrus rootstocks (Hiregoudar *et al*., 2005; Singh and Rajam, 2009), few are available for rough lemon (Chaturvedi *et al*., 2001; Kumar *et al.,* 2011; Kaur 2018). A somatic embryo is a bipolar structure of single cell origin from which the whole organism develops and contains similar genetic information in all the somatic cells. Callus derived from different plant parts varies in their response to regenerate plants and its response is genotype-dependent. The culture media components and cultural conditions play an important role in the establishment of *in vitro* cultures (Rattanpal *et al*., 2011).

There are two pathways of shoot regeneration in plants *i.e.* direct and indirect regeneration. Direct regeneration produces more number of shoots per explant and induces fewer somaclonal variations among regenerants which is required for genetic transformation studies. Indirect regeneration (through the callus phase) is required for *in vitro* mutagenesis studies and induction of somaclonal variations. This study was conducted to optimize the tissue culture protocol to be used routinely by researchers for rough lemon (*C. jambhiri* Lush.). It aims to identify the best explant from *in vitro* raised seedling and plant growth hormones their concentration and combination for callus induction and callus maintenance, shoot regeneration from callus and rooting, and subsequent hardening of regenerated shoots.

MATERIALS AND METHODS

Preparation of explants

Explants, *viz.,* epicotyl, leaf, cotyledon, and root segments were collected from 2-3 week old *in vitro* grown seedlings of rough lemon. For growing seedlings, the seeds from mature fruits were excised aseptically using forceps and a scalpel under a laminar airflow cabinet.

Callus induction

Explants were cultured on Murashige and Skoog (MS) medium fortified with different concentrations and combinations of plant growth hormones including naphthalene acetic acid (NAA), indole butyric acid (IBA), benzyl amino purine (BAP), kinetin (Kin.), and carbohydrates (sucrose, maltose, and glycerol).

Culture conditions

The pH of the medium was adjusted to 5.8 and agar was added 0.8% w/v. Culture tubes/flasks after inoculation were incubated in the growth chamber by maintaining a 25 ± 2° C temperature with 16/8 hours day/night regime with 2500-3000 lux light intensity supplied through white fluorescent tubes.

Regeneration of calli

The calli induced on different media were put onto regeneration media consisting of MS salts with different combinations of benzyl amino purine (BAP) and gibberellic acid (GA_3).

Rooting and hardening of plantlets

The regenerated shoots were then transferred to a rooting medium. The rooted hardened plantlets were transplanted in the root trainer trays in the glasshouse The data were analyzed according to a completely randomized block design (CRD).

RESULTS AND DISCUSSION

Nature of explants

Response of various explants *viz*., root, cotyledon, leaf, and epicotyl segments to supplements added to medium are presented in Table 1. The explants exhibited swellings within 3-4 days of culturing and a little callusing was evident within 10 days of culturing on the callus induction media. Among the four explants studied, the maximum mean callus induction was recorded in epicotyls segments (69.19) which were significantly higher as compared to cotyledon (52.88), leaf (40.38) and root (13.70) segments, irrespective of the culture media used. M_4 medium induced the mean maximum (55.84%) callus irrespective of the explant used, whereas, M_1 media induced the minimum (34.36) per cent of callus. A maximum of 79.43 per cent of callus was induced in epicotyl segments on M_4 media while a minimum of 7.89 per cent was recorded in root segments on M_1. The explants cultured on M_1 and M_2 initiate callogenesis in 14-16 days, but after 20 days, they develop profuse rooting on their surface but it was not so in M_3 and M_4 media.

Researchers have reported successful induction of callus from various explants *viz*., from unopened

Table 1 : Effect of different media supplements on callus induction (%) in *C. Jambhiri* raised *in vitro* with different explants

Treatment	Media composition	Root Segments	Cotyledon	Leaf segments	Epicotyl segments	Mean
M_1	MS+NAA (7.5 mgl^{-1}) + Kin. (0.2 mgl^{-1})	7.89* (16.22)	43.56 (41.27)	29.20 (32.70)	56.79 (48.89)	34.36 (34.77)
M_2	MS+NAA (10.0 mgl^{-1}) + Kin. (0.2 mgl^{-1})	16.05 (23.50)	65.60 (54.08)	41.10 (39.86)	75.40 (60.25)	49.70 (44.42)
M_3	MS+NAA (7.5 mgl^{-1}) + BAP (1.0 mgl^{-1})	9.50 (17.80)	30.14 (33.26)	40.90 (39.74)	65.16 (53.80)	36.40 (36.15)
M_4	MS+NAA (10.0 mgl^{-1}) + BAP (1.0 mgl^{-1})	21.37 (27.46)	72.22 (58.19)	50.34 (45.17)	79.43 (62.92)	55.84 (48.44)
	Mean	13.70 (21.24)	52.88 (46.7)	40.38 (39.36)	69.19 (56.47)	-

*Values in parenthesis are *Arc sine* transformed values. CD at 5% : Medium (A): 1.75; Explant (B) : 1.75; A x B : 3.50

flower buds (stigmas and ovaries), *in vitro* seedlings (leaf, nodal and root segments), seeds (cotyledon and nucellar tissues) (Ali and Mirza, 2006; Savita *et al*., 2010, 2014) and epicotyl segments (Kumar *et al*., 2011; Kaur, 2018). Raman *et al*. (1992) observed callus initiation on MS with NAA (10 mgl^{-1}) and Kin (0.2 mgl^{-1}) in 7-10 days in *C. limon* and *C. jambhiri.* Callus induction frequency was higher in cultures derived from stem segments as compared to leaf and root segments. Similar behavior of different explants for callus formation was observed in *C. madurensis* (Grinblat, 1972) and *C. limon* cv. Pant lemon (Singh and Rana, 1997). Stem segments *viz*. shoot tip, epicotyl and hypocotyl were highly responsive for induction and proliferation of callus. Dhatt and Grewal (1997) reported the highest percent callusing from the stem segments in Mosambi and rough lemon, but from cotyledons in Baramasi lemon and Kinnow. Various reasons attributed to such differences in callus formation are endogenous hormone balance (Snijman *et al.,* 1977), medium, and genotype (Yeoman and Forche, 1980).

Explant excised from mature seedlings induced less callusing as compared to 2-3 weeks old seedling explants. Similar observations have also been reported in *C. aurantifolia* by Raman (1990). Furthermore, succulent nodal segments derived from fast-growing shoots induced more calli as compared to hard and slow-growing shoots. During culturing, mature tree explants derived calli, proliferated less as compared to seedling derived calli, which may be due to the maturity of tissue or secondary growth (Mukhopadhyay and Bhojwani, 1978). The root segments excised from the hypocotylar ends induced comparatively more callus than from the distal ends towards the root tip. This response may be due to optimum endogenous levels of auxins and cytokinins in this region.

Effect of carbohydrates on callus induction

The dedifferential response of epicotyl segments to varying concentrations of carbohydrates *i.e*. sucrose, maltose, and glycerol is depicted in Table 2. Out of various combinations of growth regulators studied, M_4 [MS + NAA (10 mgl^{-1}) + BAP (1.0 mgl^{-1})] emerged best (Table 1) and was supplemented with various concentrations of carbohydrates, to record their effect on the enhancement of callus induction (Table 2).

An increase in supplementation of sucrose from 3.0 to 10.0 per cent enhanced the callus initiation. Sucrose concentrations of 8.0 and 10.0 per cent were at par and were significantly better than all other treatments. In treatments from C_5 to C_8 the sucrose in the MS medium was completely replaced with the maltose at a varying concentration of 3, 6, 8, and 10 per cent. It could be perceived that the addition of 8 per cent of maltose only gave 10.0 percent callus initiation, whereas, other concentrations of maltose don't induce callogensis. Supplementation with glycerol at 2.0-3.0 percent in addition to 3 per cent sucrose doesn't induce callus in any of the explants. In the combination of sucrose and maltose, C_{10} [sucrose (3%) + maltose (5%)] induced 82.50 per cent callus in epicotyl segments. While C_9 and C_{11} induced only 65.15 and

Table 2 : Effect of various levels of carbohydrates on callus induction in *Citrus jambhiri* using epicotyl segments as explants

Treatment	Sucrose (%) (w/v)	Maltose (%) (w/v)	Glycerol (%) (v/v)	Callus induction (%)	Days for callus initiation
C_1 (Control)	3	-	-	79.43 (63.19)*	10.5
C_2	6	-	-	83.40 (66.21)	9.5
C_3	8	-	-	95.50 (78.89)	8.3
C_4	10	-	-	93.33 (76.60)	8.4
C_5	-	3	-	0.00 (0.00)	-
C_6	-	6	-	0.00 (0.00)	-
C_7	-	8	-	10.00 (18.39)	12.0
C_8	-	10	-	0.00 (0.00)	-
C_9	3	3	-	65.15 (53.83)	12.0
C_{10}	3	5	-	82.50 (65.63)	10.0
C_{11}	3	7	-	60.30 (50.94)	10.0
C_{12}	3	-	2	0.00 (0.00)	-
C_{13}	3	-	3	0.00 (0.00)	-
CD at 5%				6.88	2.9

*Values in parenthesis are *Arc sine* transformed values. MS + NAA (10mgl^{-1}) + BAP (1 mgl^{-1}) and agar @ 0.75 % (w/v) were added in all treatments

60.30 per cent callogensis, respectively. The addition of sucrose took 8.3-9.5 days to initiate callus, with a minimum of 8.3 days in C_3 followed by 8.4 days in C_4. All other treatments took 10 to 12 days for callus initiation.

The callogensis in sucrose supplements alone was the earliest and with maximum proliferation from 10 to 20 days in the first cycles. It had more bulgings on the surface and was less green than with maltose addition. With supplementation of maltose alone, less growth occurred and it was steady. A combination of maltose and sucrose (C_{10}) induced more green colour, whereas, sucrose and glycerol (2-3%) gave only swellings in the explants at cut ends. Carbohydrates play an important role in controlling the browning phenomenon in tissue culture. They control the activity of polyphenol oxidase and peroxidase enzymes which convert the phenolic substances (produced during browning) to pestilent compounds (Khosroushahi *et al*., 2011). In our study, higher concentration of sucrose results in less browning of cultures. In contrast to other plant species reduction in sucrose results in an increase in embryogenesis (Kochba *et al*., 1978). We find the optimum concentration of sucrose *i.e.* 8.0 per cent is best suitable for callus proliferation. The role of glycerol in embryogenesis is cultivar-dependent in citrus (Kayim and Koc, 2006). The addition of sucrose (0.15 M) to the basal medium promotes better growth and organization of *C. sinensis* callus followed by glucose, fructose, lactose, galactose, maltose, and sorbose (Button, 1978). Sucrose 3.0 per cent was more effective than 5.0 per cent sucrose or 2.0 per cent glycerol to induce callus in Satsuma mandarin on MT medium (Yun *et al.* 2006). Kayim and Koc (2006) reported that the best result of embryo formation from the callus of Clementine mandarin was obtained with 4.0 and 5.0 per cent glycerol concentrations.

Effect of various MT media modifications on callus induction

The effect of various MT media (Murashige and Tucker, 1969) modification on callus induction is presented in Table 3 and C_1/M_4 [MS + NAA (10.0 mgl^{-1}) + BAP (1.0 mgl^{-1})] was considered as control. The MT media was supplemented with the best combination of growth regulators and sucrose discussed in Tables 1 and 2. Moreover, in the best combination, MS was replaced with MT. It is apparent from Table 3 that MT_3 induced maximum (75.33%) callus initiation followed by MT_4 (70.00%) and MT_2 (65.00%) among different MT media modifications. MT_3 was at par with C_1 (control) in the callus

Table 3 : Effect of various MT media modifications on callus induction in *Citrus jambhiri* using epicotyl segments as explant

Treatment	Media composition	Callus Induction* (%)	Days for callus initiation	Degree of growth **	Remarks
C_1	MS+NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose (3%)	79.43 (63.20)*	10.50	+++	light green, less compact and globular surface
MT_1	MT+NAA (10 mgl^{-1}) + Kin. (0.1 mgl^{-1}) + Sucrose (8%)	40.00 (39.18)	20.20	++	small, creamish & rooting appeared
MT_2	MT+NAA (10 mgl^{-1}) + Kin. (0.2 mgl^{-1}) + Sucrose (8%)	65.00 (53.78)	14.20	++	profuse rooting after 20 days
MT_3	MT+NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose (8%)	75.33 (60.28)	12.00	+++	small and creamish
MT_4	MT+NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose (3%) + Maltose (5%)	70.00 (56.86)	11.50	+++	light greenish and compact
MT_5	MT+NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose (3%) + Glycerol (1%)	0.00 (0.00)	-	-	-
MT_6	MT+NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose (3%) + Glycerol (2%)	0.00 (0.00)	-	-	-
CD at 5%		6.52	4.17	-	-

*Values in parenthesis are *Arc sine* transformed values

**Degree of growth; + = poor; ++ = fair; +++ = good; ++++ = excellent

induction. MT supplementation with glycerol (1-2%) did not induce callus but just the swellings on the cut end of the explant. Considering the days for callus initiation, MT_2 (14.20), MT_3 (12.00), and MT_4 (11.50) were at par with control. The callus induction was delayed (20.20 days) in the MT_1 media, MT_4 took the least number of days (11.50) followed by MT_3 (12.00) and MT_2 (14.20) to induce callus in various combinations of MT with growth regulators and carbohydrates. It was noted that profuse rooting appears in MT_2 and MT_1 after 25 days of culturing.

MT medium may enhance callus induction due to more availability of vitamins in its composition. Hagagy *et al*. (2001) reported the highest callus induction in *C. jambhiri* when nucellar embryos were cultured on MT medium fortified with 2, 4-D (4.0 mgl^{-1}). Callus line maintained on MT medium supplemented with sucrose (5%) when transferred to MT + Maltose (150 µM) gave rise to globular embryos in *C. sinensis* and *C. limonia* (Tomaz *et al*. 2001). Handaji *et al*. (2005) reported maximum callus induction in *C. reticulata* ovules cultured on MT+Kin (1.0 mgl^{-1}).

Regeneration from callus cultures

Shoot regeneration on different media

The effect of various MS media modifications on shoot regeneration in 60 days old callus culture is presented in Table 4. Calli was induced on MS+NAA(10 mgl^{-1}) + BAP (1 mgl^{-1}) + sucrose (8.0%) and afterward transferred to regeneration media. Significantly higher (43.10%) regeneration was observed in calli cultured on Modified MS supplemented with BAP (5.0 mgl^{-1}) +GA_3 (3.0 mgl^{-1}) as compared to all other treatments. Modified MS fortified with BAP (5.0 mgl^{-1}) showed 26.80 percent regeneration, whereas the addition of GA_3 (5.0 mgl^{-1}) to the same induced 30.50 per cent regeneration.

Modified MS supplemented with sucrose (6.0%) + Gelrite (0.35%) induces 5.12 per cent shoot regeneration in 60-day old calli. Half strength MS

Table 4 : Effect of various MS media modifications on shoot regeneration in 60 days -old callus culture of *Citrus jambhiri*

Treatment	Media composition	Shoot regeneration* (%)	Shoots/callus (Average)	Days to regeneration
MR_1	½ MS basal**	0.00 (0.00)	-	-
MR_2	½ MS basal + BAP (5.0 mgl^{-1})	0.00 (0.00)	-	-
MR_3	Modified MS*** + BAP (5.0 mgl^{-1})	26.80 (31.10)	4.80	38.40
MR_4	Modified MS + BAP (5.0 mgl^{-1}) + GA_3 (3.0 mgl^{-1})	43.10 (40.99)	5.30	35.50
MR_5	Modified MS + BAP (5.0 mgl^{-1}) + GA_3 (5.0 mgl^{-1})	30.50 (33.49)	3.90	40.60
MR_6	Modified MS + BAP (5.0 mgl^{-1}) + GA_3 (7.0 mgl^{-1})	0.00 (0.00)	-	-
MR_7	Modified MS + Sucrose (6%) + Gelrite (0.35%)	5.12 (12.90)	1.00	34.00
MR_8	½ MS + BAP (5 mgl^{-1}) + Agar (0.9%)	0.00 (0.00)	-	-
MR_9	½ MS + BAP (5 mgl^{-1}) + Agar (1.0%)	0.00 (0.00)	-	-
CD at 5%		3.53	0.71	5.58

*Values in parenthesis are Arc sine transformed values, ** All five stocks reduced to half, *** Macro and micronutrients (first stock) reduce to half.

basal with supplementation of different concentrations of BAP did not give any regeneration response. Shoot induced on MR_3 was of normal morphology, whereas, the addition of various concentrations of GA_3 to MR_3 medium gave rise to the thinner shoots with elongated leaves. It is evident from Table 4 that a higher (5.30) number of shoots per callus were obtained on MR_4 medium which was at par with MR_3 (4.80) and both were significantly better than all other treatments. MR_5 gave rise to 3.9 shoots per callus, whereas, it was only 1.0 in MR_7.

Calli cultured on MR_7 was the earliest (34.00 days) to re-differentiate but did not differ significantly from MR_3 (38.40 days) and MR_4 (35.50 days). The Callus on MR_5 medium was late to regenerate, as it took 40.60 days to induce shoots in 60 days old calli.

The constitution of the medium used plays a vital role in the process of redifferentiation (Vasil and Hildebrandt, 1965; Reinert and Bocks, 1968). The presence of cytokinins was found essential for shoot regeneration, which pertains to the reason that more synthesis of nucleic acids and proteins is required in the process of organogenesis. The role of cytokinins in shoot regeneration in *Citrus* has been well documented (Bhansali and Arya, 1978, Gill, 1992 and Raman *et al*., 1992). Shoots were regenerated from the callus culture of citrus cultivars Mosambi, Baramasi lemon, and Kinnow upon transfer to half-strength MS medium supplemented with BAP (5.0 mgl^{-1}) (Dhatt and Grewal, 1997). Rashad *et al*. (2005) reported maximum shoot induction in *C. sinensis* cv. Mosambi, when calli were cultured on MS media supplemented with BAP (2.0 mgl^{-1}). Regeneration was best on MS + GA_3 (2.0 mgl^{-1}) + Adenine sulphateS (2.5 mgl^{-1}) (Khan and Nafees, 2005) and MS + NAA (10.74 µM) in Kinnow (Singh *et al*., 2006). Maximum indirect shoot regeneration response (70%) was observed on MS medium supplemented with BA (3.0 mgl^{-1}) in rough lemon (Ali and Mirza, 2006).

The capacity for *in vitro* regeneration varies considerably among different explants taken from the same plant. There are natural differences in the plant's ability to synthesize endogenous cytokinins and respond to external levels of cytokinins which play a significant role in the capacity for *in vitro* regeneration. It is irrelevant to increase the cytokinin

levels if the tissue is not responsive to external phytohormones. The calli derived from BAP-containing media turned brown and necrotic in appearance. This change could be due to enzymatic activity rather than hormonal toxicity as the callus was still regenerating. Similar results were also reported by Hill and Schaller (2013).

Effect of callus age on shoot regeneration

The effect of callus age on shoot regeneration is presented in Fig. 1. It is evident from the data that regeneration decreased with the increase in the callus age from 60 to 150 days. Redifferentiation was significantly higher (43.43) in 60 days old callus, whereas it was only 10.46 per cent in 150 days old callus. In 60 days old callus, the days to regenerate were significantly lower (35.42) than in 90 (52.31), 120 (87.58), and 150 (119.53) day-old calli. The effect of callus age on shoot regeneration and days to regenerate was significant. GA_3 initiated and enhanced regeneration in old callus as it may become dormant in the long-term maintenance by culturing.

It can be concluded that, besides, the media composition, the plant regeneration frequency also varied with the age of sub-cultured calli. The decrease in morphogenetic potential with the increase in callus age has earlier been reported, which may be due to karyotypic changes and altered hormonal balance within cells or tissue or sensitivity of the cells to growth substances (Yeoman and Forche, 1980). Raman (1990) reported no-shoot regeneration in sub-cultured calli of acid lime after 120 days. In contrast to this, Gill (1992) reported that the young calli (50-75 days old) exhibited significantly lower plant regeneration as compared to the older calli (125-150 days old). Hao et al. (2004) maintained the callus of Red Marsh grapefruit *in vitro* by slow growth culture method for one year and it survived with a significant weight increment over that period with fair regeneration.

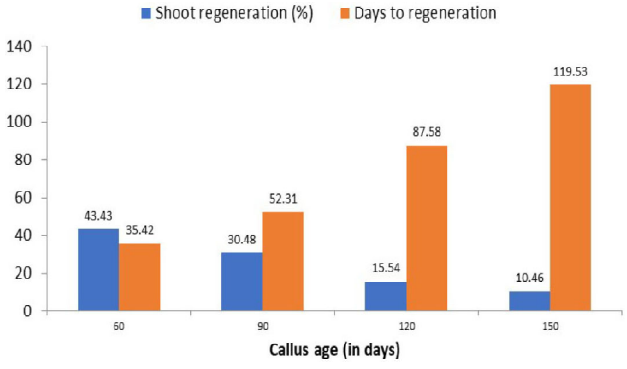

Fig.1 : Effect of callus age on shoot regeneration in *Citrus jambhiri* cultured on Modified MS + BAP (5 mgl^{-1}) + GA_3 (3 mgl^{-1})

Multiplication of shoot cultures

Data pertaining to the effect of various modifications of MS salts on shoot proliferation is presented in Fig. 2. Significantly higher percentage of shoot

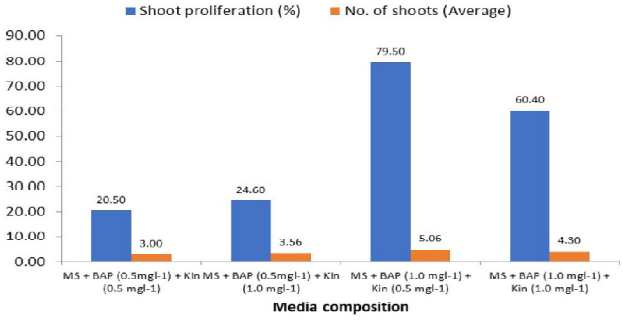

Fig. 2 : Effect of different media on shoot proliferation (Callus derived) in *Citrus jambhiri*

proliferation (79.50) was observed in cultures on MS + BAP (1.0 mgl^{-1}) + Kinetin (0.5 mgl^{-1}). MS supplemented with BAP (0.5 mgl^{-1}) and Kinetin (0.5 mgl^{-1}) resulted in the minimum (20.50%) shoot proliferation and mean number of shoots (3.00). Shoot proliferation was 60.4 per cent when it was cultured on MS + BAP (1.0 mgl^{-1}) + Kinetin (1.0 mgl^{-1}). The Maximum number of shoots (5.06) were noted in MS fortified with BAP (1.0 mgl^{-1}) and Kinetin (0.5 mgl^{-1}). The effect of medium composition on the number of shoots was not significant. In acid lime, six shoots per explant were achieved on MS+ BAP (5.0 mgl^{-1}) by Ramsunder *et al.* (1998). Similarly, El-Saway *et al.* (2006) reported maximum shoot proliferation and the maximum number of shoots and shoot length on MS+BAP (0.5 mgl^{-1}) + NAA (0.5 mgl^{-1}) in three *C. sinensis* cultivars and *C. jambhiri.*

Induction of rooting *in vitro*

In vitro regenerated shoots of more than 1.5 cm height were culled out from shoot clump and culture onto the rooting media. The response of plantlets regenerated from calli to rooting on different media is presented in Fig. 3. Among the four media studied for rooting response, the per cent rooting was maximum (77.33) on MS + NAA (1.0 mgl^{-1}) + IBA (1.0 mgl^{-1}), whereas, it was minimum (26.30) on MS medium supplemented with NAA (0.5 mgl^{-1}) and IBA (0.5 mgl^{-1}).

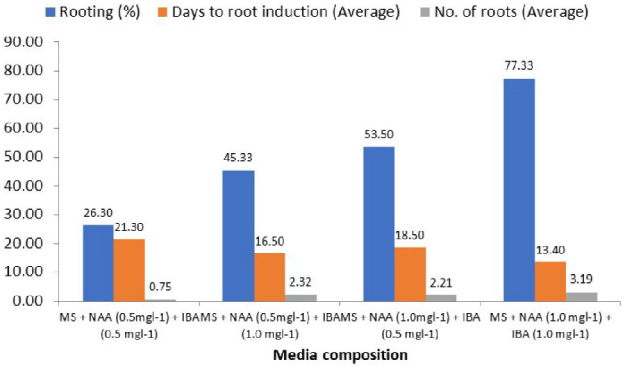

Fig. 3 : Effect of different media on rooting of callus derived shoots of Citrus jambhiri

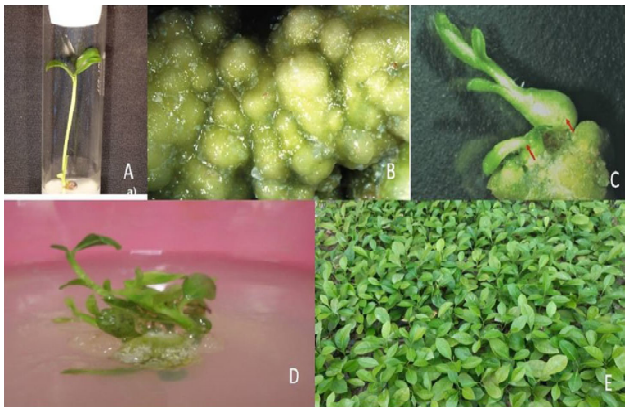

Fig. 4 : In vitro raised seedlings of rough lemon

A) On basal MS medium, B) Surface topography of embryogenic callus of rough lemon on C_3 [MS + NAA (10 mgl^{-1}) + BAP (1 mgl^{-1}) + Sucrose 8%(w/v)], C) Embryoids formation and germination on callus surface on modified MS + BAP (5.0 mgl^{-1}) + GA_3 (3.0 mgl^{-1}), D) Redifferentiation in 60 days old rough lemon callus on modified MS + BAP (5.0 mgl^{-1}) + GA_3 (3.0 mgl^{-1}), E) Plants of Citrus jambhiri in soil after hardening

The root initiation from the *in vitro* derived shoots of rough lemon started within 13-21 days on various modifications of MS medium. Rhizogensis was significantly earlier (13.4 days) on MS + NAA (1.0 mgl^{-1}) + IBA (1.0 mgl^{-1}), whereas, it was delayed on all other media, with maximum duration of 21.3 days on MS+NAA (0.5 mgl^{-1}) + IBA (0.5 mgl^{-1}). From the perusal of data, it is clear that the media studied had a significant influence on days to root induction.

Maximum (3.19) number of roots per shoot was observed on MS medium fortified with NAA (1.0 mgl^{-1}) and IBA (1.0 mgl^{-1}), whereas, minimum (0.75) was noted in MS + NAA (0.5 mgl^{-1}) + IBA (0.5 mgl^{-1}). However, the number of roots per shoot on the remaining two media was at par with each other. Auxins have a major role in rhizogenesis, which reflects from the multiplication of meristematic cells, their elongation, and differentiation into root primordia. The root length and thickness and the number of roots produced per shoot depend upon the concentration and combination of auxins used in the rooting medium. The rooting response was maximum in *C. sinensis* cv. Mosambi when shoots were cultured on MS + NAA (1.5 mgl^{-1}) (Rashad *et al.* 2005) and on MS + NAA (0.75 mgl^{-1}) + IBA (2.0 mgl^{-1}) (Das *et al.* 2000). Gill and Gosal (2002) found that the average number of roots per shoot was maximum on MS + NAA (2.0 mgl^{-1}) in *Pectinifera* rootstock. Similarly, Parkash (2003) observed the earliest and maximum (62.00%) rooting in *C. jambhiri* on MS + NAA (1.0 mgl^{-1}) + IBA (1.0 mgl^{-1}).

Hardening

Rooted plantlets were taken out of culture jars, medium adhering the roots was washed under running tap water, and plantlets were placed in a plastic stray lined with the wet cotton layer of about 0.5 cm Plantlets were placed in the tray in such a way that roots touched the wet cotton layer. The tray was covered with the transparent poly sheet to retain the moisture and kept at room temperature in the hardening room. The hardened plants had 83.6% survival in polyhouse when transplanted in root trainer trays with the potting mixture consisting of cocopeat + vermiculite + perlite (2:1:1).

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