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An efficient regeneration protocol from callus culture in rough lemon (*Citrus jambhiri*)

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ABSTRACT

A laboratory experiment was carried out during 2007–09 to develop an efficient and reliable regeneration protocol from callus cultures of rough lemon (*Citrus jambhiri* Lush.), a commercial citrus rootstock of India. Epicotyl segments and leaves excised from 3–4 weeks old *in vitro*-grown seedlings were used as explants for callus induction. Epicotyl segment was the most responsive explant and maximum callusing was induced on MS medium supplemented with naphthalene acetic acid [NAA (10.0 mg/litre)] + benzyl adenine [BA (1.0 mg/litre)] + kinetin [KIN (0.5 mg/litre)] + sucrose (6%) + galactose (3%). The percentage of regenerating calli and number of shoots per callus increased with increase in the concentration of BA from 1.0 to 3.0 mg/litre in the medium. Maximum shoot regeneration (76.09%) was achieved on MS medium supplemented with NAA (0.5 mg/litre) + BA (3.0 mg/litre) + KIN (0.5 mg/litre). Regeneration from callus was influenced by the carbohydrate composition of callus induction medium and its age. The regeneration potential was highest in the calli induced on medium supplemented with sucrose (6%) + maltose (2%) and it decreased progressively with increase in the age of callus from 40 to 120 days.

Key words: Callus age, Callus induction, Carbohydrates, *Citrus jambhiri*, Regeneration, Somaclonal variation

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 agroclimatic 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). However, rough lemon is highly susceptible to *Phytophthora* (Naqvi 2000) and soil salinity (Ferguson 2002). 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 Koc 2006). 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. The *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) but with low regeneration frequency. Gill *et al.* (1994) 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). 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). Therefore, the present investigation was undertaken to develop an efficient and reliable regeneration protocol from callus in rough lemon by studying the various factors associated with it.

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MATERIALS AND METHODS

The study was carried out in the Tissue Culture Laboratory of the Department of Horticulture, Punjab Agricultural University, Ludhiana during 2007–09.

The epicotyl segments and leaves (full and half leaves with petioles) excised from 3–4 weeks old *in vitro*-grown rough lemon seedlings were used as explants for studying the role of growth regulators [naphthalene acetic acid (NAA), 2,4–dichloro phenoxy acetic acid (2,4-D) and benzyl adenine (BA)] and carbohydrates (sucrose, galactose and maltose in

various combinations) in callus induction. The previously reported callus induction medium by Singh (2007) in rough lemon was used as control. Sucrose (8%) was used as a carbohydrate source for studying the influence of growth regulators. To study the influence of carbohydrates on callusing, the growth regulator composition of best callusing medium from growth regulators experiment was modified slightly by adding kinetin [KIN (0.5 mg/litre)] (Table 2). Observations on per cent explants producing callus, colour and type of callus were recorded after 40 days of culturing.

The 40 days old epicotyl segments derived calli, induced on control medium were transferred to full or half strength MS (Murashige and Skoog 1962) medium containing fixed levels of NAA and KIN (0.5 mg/litre each) and varying concentrations of BA (1.0–5.0 mg/litre) to standardize the regeneration medium. To study the influence of carbohydrates on regeneration, the epicotylar calli induced on the medium supplemented with various carbohydrates were regenerated on the standardized medium. Shoot regeneration potential of the calli of different age groups (40, 60, 90 and 120 days) was investigated. The already regenerated calli were also cultured in second cycle to test their morphogenic competence for regeneration. To distinguish new shoots from the existing ones on the regenerated callus, existing shoots were excised and retained as shoot stumps. Observations on per cent shoot regeneration, number of shoots/callus and shoot length, were recorded after 60 days of culturing.

The regenerated shoots of 3–4 cm length were transferred to auxin-free half strength MS medium and full strength MS medium supplemented with various auxins, viz indole butyric acid (IBA), indole acetic acid (IAA) and NAA at concentrations ranging from 0.25 to 1.0 mg/litre for *in vitro* rooting.

The media used in the study were solidified with agar (0.75%). The *p*H of the media was adjusted to 5.8 prior to autoclaving at 121.5°C temperature, 15 lbs/inch^2 for 30 min. The cultures were incubated under light/dark cycle of 16/8 hr with white light of 2000–3000 lux intensity, at a constant temperature of 25±2°C.

Twentyone explants or calli were used for each treatment and the experiments were repeated thrice. Experiments were analyzed according to completely randomized block design (CRD) and the analysis was carried out using CPCS 1 software (Punjab Agricultural University, Ludhiana).

RESULTS AND DISCUSSION

Callus induction

The results on the effect of various concentration and combinations of growth regulators, viz BA, NAA, 2,4-D and carbohydrates, viz sucrose, galactose and maltose on per cent callus induction in different explants are presented in Tables 1 and 2, respectively. The callus in the epicotyl segments and full leaves initiated from the cut ends and petioles, respectively. In half cut leaves, it generally originated from the petioles but at few occasions, it also appeared at transverse

Table 1 Effect of culture media on per cent callusing in epicotyl segments and leaves of *Citrus jambhiri*

Culture media	Callus induction (%)				Callus characters	
	Epicotyl segments	Full leaves	Half cut leaves	Means	Colour	Texture
MS + [b]BA (1 mg/litre) + 2,4-D (1.0 mg/litre) + NAA (2.5 mg/litre)	66.67 (54.71) [c]	22.22 (28.01)	11.11 (19.44)	33.33 (34.06)	Yellow to creamish-wheat straw coloured	Fibrous and friable, shiny, whole explant turned into callus
MS + BA (1 mg/litre) + 2,4-D (1.0 mg/litre) + NAA (5.0 mg/litre)	80.55 (63.86)	55.56 (48.18)	27.78 (31.78)	54.63 (47.94)		
MS + BA (1 mg/litre) + 2,4-D (1.0 mg/litre) + NAA (7.5 mg/litre)	83.33 (65.90)	0.00 (0.00)	0.00 (0.00)	27.77 (21.96)	Light-yellow	Soft, fibrous to compact
MS + BA (1 mg/litre) + 2,4-D (1.0 mg/litre) + NAA (10.0 mg/litre)	83.33 (65.87)	0.00 (0.00)	0.00 (0.00)	27.77 (21.96)		
MS + BA (1 mg/litre) + 2,4-D (2.0 mg/litre) + NAA (5.0 mg/litre)	26.39 (30.84)	0.00 (0.00)	5.56 (13.59)	10.65 (14.81)	Yellow to creamish-wheat straw coloured	Fibrous and friable, shiny, whole explant turned into callus
MS + BA (1 mg/litre) + 2,4-D (3.0 mg/litre) + NAA (5.0 mg/litre)	8.33 (16.76)	0.00 (0.00)	5.56 (13.62)	4.63 (10.13)		
MS + BA (1 mg/litre) + NAA (10.0 mg/litre): Control	92.00 (73.69)	55.00 (47.85)	71.38 (57.71)	72.79 (59.75)	Creamish-green	Compact and nodular
Mean	62.94 (53.09)	18.96 (17.72)	17.34 (19.45)			
CD (*P*=0.05)	A (culture medium): (1.72), B (explant): (1.12), A×B (interaction):(2.98)					

[a] Media with sucrose (8%) and agar (0.75%). [b] BA (benzyl adenine), 2,4-D (2,4-dichloro phenoxy acetic acid), NAA (naphthalene acetic acid). [c] Figures in parentheses are arc sine transformed values

Table 2 Effect of carbohydrate composition on per cent callus induction from epicotyl segments in *Citrus jambhiri*

Culture media[a]	Callus induction (%)
M1: MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + [b]KIN (0.5 mg/litre) + sucrose (6%) + galactose (2%)	64.28 (53.34)[c]
M2: MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + galactose (3%)	100.00 (89.96)
M3: MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + maltose (2%)	77.78 (62.62)
M4: MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + maltose (3%)	63.89 (53.22)
M5: MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + sucrose (8%)	86.79 (68.70)
CD (P=0.05)	(10.92)

[a]agar (0.75%) was added. [b]KIN (kinetin)
[c] Figures in parentheses are arc sine transformed values

cut portions. The callus initiation started after 8–10 days of culturing and attained culturable size (300–400 mg weight) within 20–25 days of its first appearance (Fig 1a).

Growth regulators and callusing

The frequency of callusing varied with the explant and the composition of growth regulators in the medium. Among the different explants, epicotyl segment was the most responsive explant (Table 1). The high amenability of epicotyl segments relative to the leaf and other explants to callusing has also been reported earlier in *Citrus aurantifolia* (Kamble *et al*. 2002) and *C. reticulata* (Khan *et al*. 2006). The MS medium containing BA (1.0 mg/litre) and NAA (10.0 mg/litre) was the most suitable medium for callus induction in this explant. For full leaves, MS medium supplemented with BA (1.0 mg/litre) + 2,4-D (1.0 mg/litre) + NAA (5.0 mg/litre) was the best callus induction medium, whereas in half leaves, the MS medium containing BA (1.0 mg/litre) and NAA (10.0 mg/litre) was the most suitable medium.

The composition of growth regulators had great influence on the colour and texture of callus. The MS medium containing BA (1.0 mg/litre) and NAA (10.0 mg/litre) induced creamish green callus of compact and nodular nature, while the MS medium containing BA (1.0 mg/litre) supplemented with 2,4-D and NAA initiated fibrous callus of light yellow to creamish brown colour (Table 1). Gill *et al*. (1994) 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. In the present study, it was shown that the friable callus was obtained, when NAA was supplemented

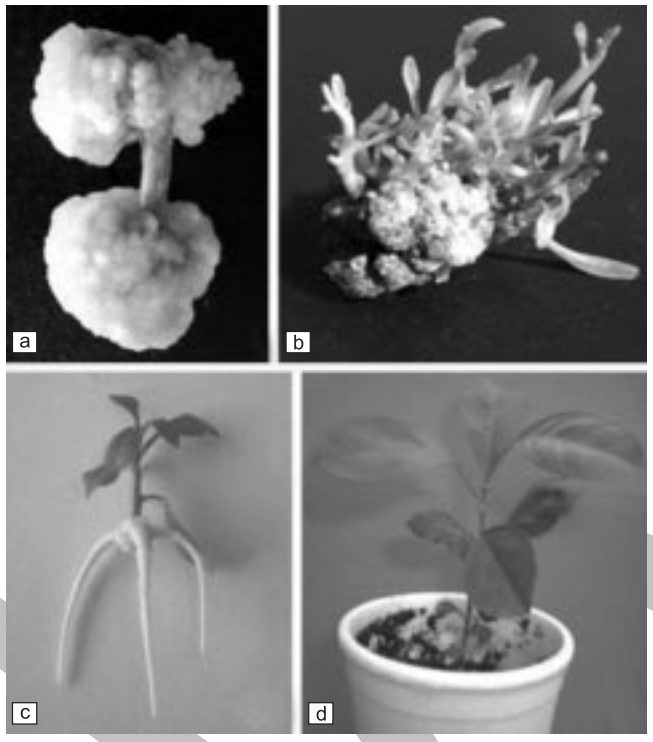

Fig 1 Plantlet regeneration from callus in *Citrus jambhiri*. (*a*) Callus induction from epicotyl segments after 35 days on MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + maltose (2%); (*b*) Shoot regeneration from 40 days old callus on MS + BA (3.0 mg/litre) + KIN (0.5 mg/litre) + NAA (0.5 mg/litre) after 60 days; (*c*) *In vitro* rooting on MS + IBA (1.0 mg/litre); (*d*) Established plant in pot

with 2,4-D in the medium. It could be interpreted that the presence of 2,4-D in the medium mask the effect of NAA. Thus, in order to establish callus of high regeneration potential in rough lemon, NAA should be used singly rather than in combination with 2,4-D.

Carbohydrates and callusing

The modified control medium (by addition of 0.5 mg/litre KIN to its growth regulator composition), when supplemented with different carbohydrates in various concentrations and combinations, influenced callusing (Table 2). The M2 medium, ie MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + galactose (3%) induced callus in all of the cultured epicotyls. It was significantly better over all other carbohydrate combinations and control. Previously, Oliveira *et al*. (2001) related the effect of individual carbohydrates on callus induction but did not report the possible influence of carbohydrate in combinations. However, they reported the promotory role of sucrose in callus growth.

The carbohydrate composition did not influence the type of the callus. Compact and nodular callus was obtained on all the carbohydrates evaluated. In general, irrespective of

Table 3 Effect of benzyl adenine (BA) on per cent shoot regeneration, average number of shoots/callus and average shoot length in callus cultures of *Citrus jambhiri*

Culture media	Shoot regeneration (%)	Average number of shoots/callus	Average shoot length (cm)
MS + BA (1.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	44.45 (41.74)[a]	2.11	4.32
MS + BA (2.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	64.70 (53.53)	4.26	3.05
MS + BA (3.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	76.09 (61.23)	8.15	2.80
MS + BA (5.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	50.00 (44.98)	4.28	2.77
½ MS + BA (3.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	71.43 (58.17)	5.00	1.91
½ MS + BA (5.0 mg/litre) + NAA (0.5 mg/litre) + KIN (0.5 mg/litre)	44.44 (41.79)	2.85	2.45
CD (*P*=0.05)	(12.19)	1.24	0.38

[a] Figures in parentheses are arc sine transformed values

the type and combination of carbohydrates, light/creamish green callus was induced on all the media except in the combination of sucrose (6%) + galactose (2%), where creamish yellow callus was obtained.

Shoot differentiation

The process of regeneration is signaled by the appearance of shoot buds on callus. The callus cultured on regeneration medium underwent 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 *C. madurensis* by Grinblat (1972) 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 27 days of culturing and continued up to 70 days. Maximum shoot regeneration (76.09%) and number of shoots (8.15)/callus was achieved on full MS medium + NAA (0.5 mg/litre) + KIN (0.5 mg/litre) + BA (3.0 mg/litre) (Table 3). Regeneration on this medium was significantly better over full strength MS medium + NAA (0.5 mg/litre) + KIN (0.5 mg/litre) + BA (1.0 mg/litre) and half strength MS medium + NAA (0.5 mg/litre) + KIN (0.5 mg/litre) + BA (5.0 mg/litre). But the number of shoots/callus in this medium was significantly higher over all other media. The number of shoots ranged from 4 to 20 (Fig 1b).

Average length of the regenerated shoots was highest (4.32 cm) in the calli cultured on full MS medium supplemented with NAA (0.5 mg/litre), KIN (0.5 mg/litre) and BA (1.0 mg/litre). The shoot length decreased with increase in the concentration of BA from 1.0 to 5.0 mg/litre in the full MS medium. Conversely, the relative increase in concentration of BA from 3.0 mg/litre to 5.0 mg/litre in the ½ MS medium containing NAA (0.5 mg/litre) and KIN (0.5 mg/litre) promoted shoot growth.

The cytokinins, either alone or in combination with other growth regulators, have proved beneficial in the redifferentiation of callus cultures into shoots in different *Citrus* species. The presence of BAP in the medium induced shoots in undifferentiated callus cultures of *C. grandis* (Begum *et al.* 2003), *C. sinensis* (Rashad *et al.* 2005) and *C. jambhiri* (Ali and Mirza 2006). The supplementation of BAP along with other auxins, like NAA in the MS medium proved helpful in shoot regeneration from callus cultures of *C. grandis*, *C. sinensis* and *C. aurantifolia* (Chaturvedi and Sharma 1983). Singh (2000) reported low regeneration frequency of 27% in hypocotyl and epicotyls derived callus of *C. jambhiri* on MS medium supplemented with NAA (0.5 mg/litre), BA (3.0 mg/litre) and malt extract (0.5 g/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.

Influence of carbohydrate composition of callus induction media on regeneration

The history of callus induction media in terms of carbohydrate composition significantly influenced the shoot regeneration potential of calli. Maximum shoot regeneration (80.00%) was achieved in the calli induced on M3 medium, ie MS + NAA (10.0 mg/litre) + BA (1.0 mg/litre) + KIN (0.5 mg/litre) + sucrose (6%) + maltose (2%) (Fig 2). It was significantly higher than all other media. With increase in the concentration of either galactose or maltose from 2 to 3% in the media, callus regeneration was reduced while the callus induced on the best callusing medium, ie M2 medium did not differentiate on regeneration medium. There is dearth of literature on the possible impact of carbohydrates on shoot regeneration from callus cultures in citrus. From the present study, it is clear that the callus induced on the best performing callusing media might not always have the maximum regeneration potential. Thus, for future investigations, it is advisable to check the compatibility of callus induction and regeneration media before establishing the callus regeneration protocol in a given *Citrus* species.

Callus age and regeneration

The data in Table 4 relate the effect of increasing callus

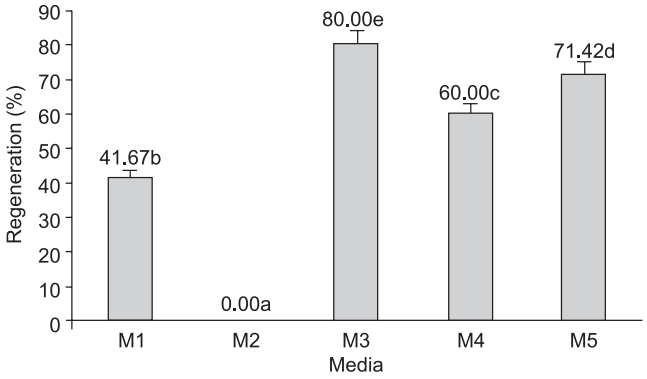

Fig 2 Influence of composition of carbohydrates on per cent shoot regeneration in 40 days old calli of *Citrus jambhiri.* The calli originated on M1, M2, M3, M4 and M5 media were cultured on standardized regeneration medium. Description of media is given in Table 2. Means with different letters are significantly different (P = 0.05). Vertical bars represent standard error

age on regeneration potential. The 40 days old calli had the maximum shoot regeneration potential. The increase in callus age showed a progressive and significant decline in shoot regeneration from 71.59 (40-days-old calli) to 11.11% (120 days-old-calli). However, the differences for shoot regeneration were more prominent amongst 60, 90 and 120 days old callus. 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.

Likewise, the average number of regenerating shoots/callus and their growth was also reduced significantly with increase in the age of callus (Table 4). With increase in age of callus, the time interval for shoot regeneration was also prolonged. The minimum time (31.22 days) for shoot regeneration was recorded in 40 days old callus, while 120 days old callus took maximum time (37 days) to regenerate.

Table 4 Effect of age of callus on per cent shoot regeneration, average number of shoots/callus, average shoot length and days to regeneration in callus cultures of *Citrus jambhiri*

Callus age (days)	Shoot regeneration (%)	Average shoots/ callus	Average shoot length (cm)	Days to regeneration
40	71.59 (57.76)[a]	5.07	2.89	31.22
60	66.33 (54.50)	3.16	1.04	32.42
90	43.11 (41.01)	3.04	1.07	35.52
120	11.11 (19.46)	2.13	0.75	37.00
CD (P=0.05)	(1.32)	0.27	0.14	0.41

[a]Figures in parenthesis are arc sine transformed values.

Hao and Deng (2002) 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) 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). 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.

Cycling of callus regeneration

The regenerated callus retained their competence for regeneration in the second cycle. The regeneration in second cycle started after 40–45 days of culturing. Though most of the shoots arised from the shoot stumps, but few shoots also regenerated from the new cells in the callus (data not shown). With advancement in the callus age, the frequency of appearance of variants also increases (Swedlund and Vasil 1985). In that context, the newly regenerated shoots obtained in the second cycle may be considered a possible carrier of somaclonal variation.

In vitro rooting of the excised shoots and plantlet establishment

Rooting in the *in vitro* shoots occurred within 8–17 days of culture and rooting was not significantly influenced by the composition of the rooting hormone (data not shown). The excised shoots were rooted on MS medium supplemented with IBA (1.0 mg/litre). The *in vitro* plantlets after hardening in jars with vermiculite were successfully transferred to the pots containing garden soil (Fig 1d).

In the present investigation, we studied various factors governing regeneration in callus cultures of rough lemon. Based on the findings of the investigation, we propose a scheme of micropropagation and somaclonal variation in callus cultures of this elite rootstock (Fig 3). We feel that the present regeneration protocol will prove beneficial for the genetic improvement of rough lemon for biotic stresses, like *Phytophthora* and abiotic stresses like soil salinity through somaclonal variation, *in vitro* mutagenesis and genetic transformation.

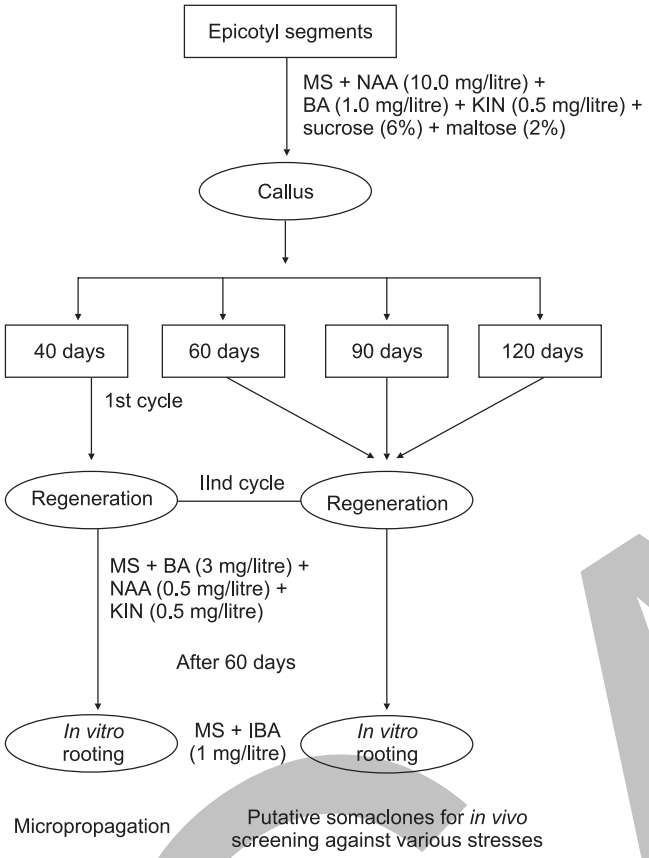

Fig 3 Schematic flow chart for micropropagation and somaclonal variation in rough lemon

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