RESEARCH PAPER | Botany | Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Effect of Explant Type and Different Plant Growth Regulators on Callus Induction and Plantlet Regeneration in Grapefruit, *Citrus decumana var. paradisi* (Macfad.) H. H. A. Nicholls

KEYWORDS: *In vitro*, indirect organogenesis, malt extract, BAP (6-Benzyl amino purine), NAA (Naphthalene acetic acid), IBA (Indole butyric acid),

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ABSTRACT *In vitro propagation of grapefruit using various explants results in rapid and efficient propagation, pathogen free plants, increased fruit yield and enhanced metabolic production useful to industry.The present investigation was carried out to evaluate the effect of types of explant, growth medium and growth regulators on callus induction and subsequent regeneration of grapefruit plant using direct and indirect organogenesis method. The nodal explants obtained from in vitro grown seedling cultured on Murashige and Tucker (MT) medium supplemented with Malt extract (0.5 gm/L) + kinetin (0.5 mg/L) + NAA (5mg/L) showed 88.70 percent of callus induction. The epicotyl segments cultured on MT medium containing 3 mg/l BAP, 0.5 mg/l NAA and malt extract @ 0.5 mg/l proved to be best explants for shoot bud induction with an average of 7.8 buds per explant. On the other hand for indirect organogenesis the callus derived from nodal tissue cultured on MT medium containing 1mg/l BAP, 0.5 mg/l NAA and malt extract @ 0.5 mg/l performed well for shoot bud induction. Regenerated shoots were rooted on half strength MT medium containing IBA (1 mg/l).*

Introduction

Grapefruit, *Citrus decumana* var. *paradisi* (Macfad.) H. H. A. Nicholls is an excellent source of many nutrients and phytochemicals those contribute to a healthy diet (Donatus E. O., 2008). Besides being a rich source of vitamin C, fiber and pectin and calcium, it is rich in nutrients like potassium, folate, thiamine, vitamin A, vitamin B_6 etc. 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. The *in vitro* regeneration system in grapefruit based on callus could prove useful both for production of transgenic (young callus) and for induction of variants through somaclonal variation (old callus). There are some reports of callus induction in related citrus cultivars, eg. Callus induction in Citrus jambhiri by Savita, Vijay and G.S. Virk, 2010; Regeneration from callus culture in *Citrus jambhiri* by Kumar, Kaur, Gill, Rattanpal and Kanika 2011; Indirect organogenesis in pommelo by Ibrahim M.A., 2012. The present investigation was undertaken to develop an efficient, reliable and easy to follow protocol for callus induction in leaf and nodal explants and micropropagation of grapefruit.

Materials and methods

In vitro seed germination

Source of seeds and sterilization methods

The mature fruits of the plant growing in the college campus were used as source of seeds in the month of January/February. The fruits were washed thoroughly with tap water followed by dipping in 95% ethanol. Fruits were cut to get the seeds. The seed coats were removed manually with the help of sterile pointed forceps. Seed sterilization involved treatment with 0.1% Bavistin, a fungicide, for 10 min. followed by 3 or 4 times rinsing with distilled water. Seeds were transferred to laminar air flow and dipped in 70% alcohol for 30 seconds and then in 2% sodium hypochlorite containing few drops of Tween 20, as surfactant for 10 to 15 min. followed by 3 to 4 times washing with autoclaved distilled water.

Culture medium and culture conditions

Seeds were cultured in Murashige and Skoog (MS) medium (Murashige T. & Skoog F., 1962) with 3% sucrose, solidified with 0.8-0.9% agar and adjusted to pH 5.8. MS medium was used with and without growth regulators to evaluate the effect of growth regulators on seed germination. Inoculated culture tubes were incubated in complete darkness at 27°C for 10- 12 days. After that seedlings were maintained at 25±2°C with16 h photoperiod of light intensity of 2500 lux which was provided by white fluorescent lamps. For each treatment 10 replicates were kept and each treatment was repeated thrice. Data was subjected to ANOVA and results were expressed as mean±SE.

Callus induction

Source of tissue and preparation of explants

Explants were collected both from in vitro grown seedlings and field growing grapefruit plant. 15-20 days old seedlings were cut using sterilized scissor and forceps to obtain leaf and nodal explants, while young shoots of about 2-3 cm long collected from field growing plant were washed thoroughly under running tap water for 10-15 minutes, followed by treatment with 0.1% Bavistin, for 10 minutes to eliminate any kind of fungal infection. Before sterilization shoots were treated with 0.01% ascorbic acid to prevent browning of explants during culturing, Shoots were surface disinfected with 70% alcohol for 30 seconds followed by sterilization with 0.1% mercuric chloride for 10 minutes and rinsed 3-4 times with autoclaved distilled water in laminar flow hood. Shoots were cut into 0.5-1 cm long nodal and leaf explants using sterilized scissor and forceps.

Culture medium

Culture medium consisted of MS and MT media prepared separately with various combinations of growth hormones (2, 4-D., BAP and NAA), solidified with 0.8 % agar (Hi media, India). Callus was initiated in borosil glass culture tubes (containing 20 ml of sterilized medium). Nodal explants, 1cm long were cultured upright with basal end of the node inserted a few millimeters into the semi- solid medium and leaf explants 0.5-1 cm long were placed on

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the surface of semi-solid medium with their dorsal surface in contact with medium.

Regeneration of shoots
From 20-days old seedlings, epicotyl and root segments were excised under aseptic conditions and cut into 1.0 cm pieces, while leaves and petioles were cut to a size of 0.5- 1.0 cm^2. The leaf segments were cut perpendicular to midrib. For indirect organogenesis the callus obtained as above was used as explant.

Culture medium and culture conditions
Culture medium used was MT medium containing BAP, NAA and kinetin supplemented with 50% sucrose with and without malt extract. Cultures were maintained at 25±2°C with 16 h photoperiod of light intensity of 2500 lux which was provided by white fluorescent lamps.

Explants with proliferated shoot buds formed under optimal condition were transferred to MT medium with BAP (1mg/l) + GA_3 (1 mg/l) supplemented with 30% sucrose and 500 mg/l malt extract for shoot elongation.

In vitro rooting of microshoots
Actively growing shoots with 4-5 nodes were used for rooting. Composition of rooting medium consisted of half strength MT medium fortified with IBA at different concentrations of 0.5, 1.0, 1.5 and 2.0 mg/l solely or combined with BAP (0.25 mg/l). Medium sterilization and culture conditions were same as described above.

Experimental design and statistical analysis
All experiments were conducted in a completely randomized block design with 18 explants per treatment repeated thrice. Effect of different treatments was quantified on the basis of the percentage of explants showing callus induction. Data on percentage of explants regenerating shoots and number of shoots per explant was recorded after 4 weeks interval and rooting experiments were evaluated after 3 weeks interval. The rooting percentage, number of roots and length of roots per rooted shoot were calculated. Data were subjected to one- way analysis of variance (ANOVA) with 5% significance level to determine variation within the treatments. The LSD test was used to study differences between treatments.

Results and discussion
Effect of growth regulators on seed germination
MS medium supplemented with GA_3 alone showed elongation of internodal regions only with average 1.3 shoots per seedling although the percent seed germination was improved (Fig. 1). MS medium supplemented with BAP+GA_3 (1 mg/L each) turned out to be the best possible medium with seed germination recorded 98% and 6.8 shoots per seedling (Fig 2, 3). Slow growth of seedling and reduced rate of seed germination was seen with treatment of BAP alone and as well as in the complete absence of growth regulators (Table 1).

Callus induction
Callus initiation started after 8-10 days of culturing (Fig. 4) and attained culturable size within 25-30 days of its first appearance. Maximum callus induction (88.70%) was observed in nodal explants cultured on MT medium Murashige T. & Tucker D.P.H., 1969) supplemented with Malt extract (0.5 gm/L) + NAA (5mg/L) + kinetin (0.5mg/L). Malt extract @ 0.5 mg/L improved the percent of callus induction in both nodal and leaf explants (Table 2). A decrease rate of callus induction was observed when NAA was used along with 2, 4-D. It could be interpreted that presence of 2, 4-D in the medium mask the effect of NAA. Composition of growth hormones had great influence on the color and texture of callus. NAA (5mg/L) + kinetin (0.5 mg/L) induced callus was compact and nodular and color varied from light to creamish green while 2,4-D (1 mg/L) + kinetin (0.5 mg/L) induced fibrous and friable callus with yellow to creamish colour. Callus induced from nodal explants was green and friable and whole explant changed to callus (Fig.5). Leaf explants were less responsive to all kinds of treatments (Fig. 6). High amenability of epicotyl segments relative to leaf and other explants to callusing has also been reported in *Citrus reticulata* (Khan, Jaskani, Abbas, Haider & Khan, M. M., 2006) and *Citrus jambhiri* (Kumar et al., 2011). Among different plant growth regulators tested maximum callus induction was observed on MS medium supplemented with 2,4-D (2 mg/l) in combination with malt extract (500 mg/l) in *Citrus jambhiri* (Nagpal, Vijay, Singh & Virk, 2011)

Regeneration of shoots
Of the four explants used for direct organogenesis the epicotyl segments gave the best response with 82.45 % bud induction (Fig. 7, 8, 9). The MT medium containing BAP (3 mg/l) + NAA (0.5 mg/l) supplemented with 50% sucrose and 500 mg/l malt extract led to maximum mean bud induction (7.8 buds per explant) irrespective of the explant used. It was noticed that higher concentration of BAP i.e. above 3.0 mg/l reduced the number of buds in all types of explant. For indirect organogenesis the nodal tissue derived callus performed well (75.20% bud induction) as compared to leaf callus (Table3,4). For shoot regeneration similar results were obtained in *Citrus jambhiri* (Kumar et al., 2011) with use of BAP and NAA. BAP and NAA were also used for shoot induction in one of the closely related genus pomelo (Ibrahim M.A., 2012).

Root induction
The best rooting medium proved was half strength MT medium fortified with IBA (1 mg/l) + BAP (0.25 mg/l) giving 79.89% root induction and mean number of roots recorded per explant was 4.3 (Fig. 10). IBA used solely not only delayed the initiation of root primordia but also reduced the number of roots per microshoot.

Conclusion
In this study the appropriate concentration of growth hormones BAP and NAA along with malt extract have given remarkable results for callus and shoot induction. The protocol has a great potential for *In vitro* propagation of grapefruit to overcome various barriers met by researches related with field culture of grapefruit.

Table 1 Effect of growth regulators on in vitro seed germination and multiple shoot induction

Growth regulators (mg/l)	% seed germination	Number of shoots per seedling Mean±SD
BAP (0.5)	65	1.9±0.16
GA_3 (0.5)	89	1.3±0.09
BAP+GA_3 (0.5)	95	3.2±0.52
BAP+GA_3 (1.0)	98	6.8±0.40
BAP+GA_3 (1.5)	80	3.4±0.24

Values represent mean±SD of three independent experiments (n=10).

Volume : 5 | Issue : 7 | July 2015 | ISSN - 2249-555X

Table 2 Effect of culture media on percent callusing in nodal and leaf explants of Citrus paradisi

Culture media	Percent callus induction		Rate of callusing	Texture	Colour
	Nodal explants	Leaf explants			
MS + 2,4-D (1 mg/l) + kinetin (0.5 mg/l)	63.30±2.21[a]	39.30±2.58[a]	Slow callusing	Soft, fibrous to compact	Yellow to creamish
MS + NAA (5 mg/l) + kinetin (0.5 mg/l)	68.80±2.10[c]	50.20±3.65[c]	Normal callusing	Compact and nodular	Creamish green
MS + 2, 4-D (1 mg/l) + kinetin (0.5 mg/l) + NAA (5mg/l).	58.80±1..89[c]	30.60±1.84[c]	Slow callusing	Fibrous and friable	Yellow to creamish
MT + Malt extract (0.5 gm/l) + 2,4-D (1 mg/l) + kinetin (0.5 mg/l)	73.40±2.67[d]	57.80±3.82[d]	Normal callusing	Nodular and friable	Creamish green
MT + Malt extract + NAA (5mg/l) + kinetin (0.5 mg/l)	88.70±2.67[e]	61.70±4.06[e]	Best callusing	Compact and nodular, shiny and whole explant turned into callus	Light green

Means within the same column not sharing a letter in common differ significantly according to LSD test at 5% probability level.

Table 3 Effect of type of exlpant, BAP concentration on regeneration of shoots by direct and indirect organogenesis of Citrus paradisi.

Culture medium	Shoot bud induction frequency (%)				Number of shoots per explant(Mean)
	Epicotyl	Root	Leaf	Callus	
MT + BAP (1 mg/l) + NAA (0.5mg/l)	49.40±2.83[a]	23.80±3.80[a]	29.50±3.20[a]	47.20±3.65[b]	2.34
MT + BAP (2 mg/l) + NAA (0.5mg/l)	57.30±4.49[b]	29.70±2.21[b]	38.30±4.10[b]	51.90±4.09[b]	4.67
MT + BAP (3 mg/l) + NAA (0.5 mg/l)	78.10±5.46[c]	48.90±5.48[c]	43.20±5.34[b]	75.20±3.67[b]	7.80
MT + BAP (4 mg/l) + NAA (0.5 mg/l)	39.20±3.58[a]	18.10±3.47[a]	18.50±2.24[a]	38.34±6.73[a]	2.89

Means within the same column not sharing a letter in common differ significantly according to LSD test at 5% probability level.

Table 4 Effect of malt extract on regeneration of shoots by direct and indirect organogenesis of Citrus paradisi.

Culture medium	Shoot bud induction frequency (%)				Number of shoots per explant(Mean)
	Epicotyl	Root	Leaf	Callus	
MT + BAP (1 mg/l) + NAA (0.5 mg/l) + malt extract (300 mg/l)	48.70±6.69[a]	30.30±6.70[a]	30.20±3.30[a]	46.30±3.20[a]	2.36
MT + BAP (2 mg/l) + NAA (0.5 mg/l) malt extract (300 mg/l)	60.30±4.43[b]	30.20±2.67[a]	40.50±4.45[b]	53.40±1.89[b]	4.70
MT + BAP (3 mg/l) + NAA (0.5 mg/l) + malt extract (300 mg/l)	82.40±7.64[c]	53.10±4.90[b]	42.10±5.52[b]	77.20±4.42[c]	7.82

Means within the same column not sharing a letter in common differ significantly according to LSD test at 5% probability level.

Plate 1 Effect of growth regulators on seed germination

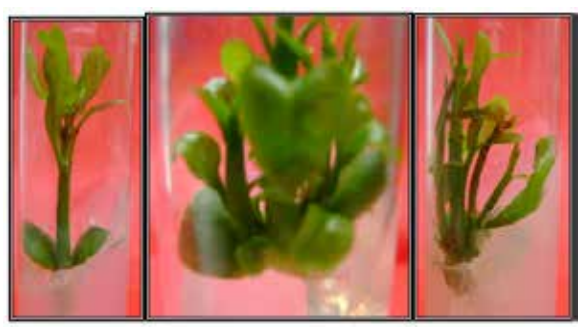

Fig. 1 Fig. 2 Fig. 3

Fig. 1 Effect of MS medium supplemented with GA_3 (0.5 mg/l). Fig. 2, 3 Effect of MS medium supplemented with both BAP and GA_3 (1.0 mg/l each).

Plate 2 Callus induction

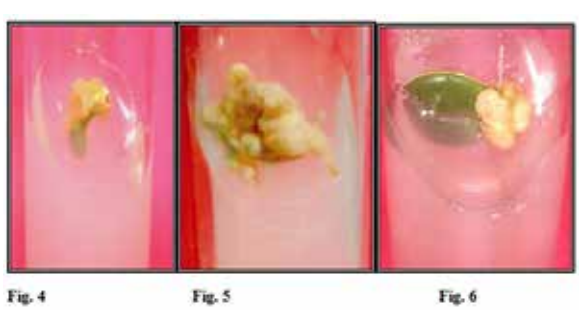

Fig. 4 Fig. 5 Fig. 6

Fig. 4 15-days old callus Fig. 5 Entire nodal explant transformed to callus. Fig. 6 Callus from leaf explant

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Plate 3 Regeneration of shoots

Fig. 7Proliferation of shoot buds

Fig. 8Elongation of microshoots

Fig. 9 Fully developed microshoots

Fig. 10 Rooting of microshoots

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