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Research Article

Effect of Phytohormones on *In Vitro* Morphogenesis of Citrus Cultivars Using Shoot Tip Explant

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Abstract

An attempt was made to examine the effect of varied concentrations and combinations of phytohormones on morphogenic response of four citrus cultivars viz., Rough lemon (C. Jambhiri), Kinnow (C. reticulata), Feutrell's early (C. reticulate × C. sinensis) and Musambi (C. sinensis) from shoot tip explants. To achieve in vitro shoot regeneration, in vitro maintained healthy shoot tips of four cultivars were excised and positioned on modified MS (Murashige and Skoog, 1969) medium fortifying the following benzylaminopurine (BAP)/Indole acetic acid (IAA) concentrations: 0.5/0.4, 1.0/0.4, 1.5/0.4, 2.0/0.4 and 2.5 mg L-1/0.4 mg L-1. Synergetic effect of BAP with IAA was best found on MS medium containing 1.0/0.4 mg L-1 concentration of BAP to IAA for shoot number/plant (2.25), shoot length (2.52cm) and leaf number/plant (10.58). The shoot multiplication rate was decreased in all four cultivars when BAP concentration was increased from 1.0-2.5 mg L-1. In order to assess the impact of different concentrations of auxins, well proliferated shoots were shifted to the rooting medium ((MS micro & macro elements, 100 mg L-1 myo-inositol, MS vitamins, 2 mg L-1 glycine, 30 g L-1 sucrose and 7.0 g L-1 agar) augmented with varied naphthalene-1-acetic acid (NAA)/ indole-3-butyric acid (IBA) concentrations i.e. 0.5/0.3, 1.0/0.3, 1.5/0.3, 2.0/0.3 and 2.5/0.3 mg L-1. Regenerated shoots started to rooting within 22.25 days, had more number of roots/plant (4.25) and root length (3.35cm) on medium containing 1.0 mg L-1 NAA and 0.3 mg L-1 IBA. Sub or supra optimal concentrations of phytohormones resulted in low plant regeneration in all the four cultivars assessed. It was also found that the morphogenic response was genotype dependent in citrus cultivars.

Keywords: *Phytohormones, Citrus, Feutrell's early, Shoot regeneration, Root induction*

Introduction

Genetic manipulation is among the key perspectives for citrus improvement that is being exploited to overcome biotic and abiotic stresses from many decades. Different biotechnological tools including embryo rescue, genetic transformation, in vitro grafting, and protoplast fusion have been found to utilized for successful citrus genotype improvement, circumventing the traditional breeding limits (Navarro *et al.*, 2004). For this, In vitro propagation protocols act as a pre-requisite for genetic manipulation and conservation of citrus species (Tao *et al.*, 2002) and a beneficial tool to overcome field related difficulties of such species (Mukhtar *et al.*, 2005). Leaf explants from In vitro grown

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plantlets of different citrus species are employed for protoplast isolation (Takayanagi *et al*., 1992; Grosser *et al*., 1996; Guo and Deng, 1998; Scarano *et al*., 2002; Khan and Grosser, 2004; Ananthakrishnan *et al*., 2006), In vitro shoot regeneration (khan *et al*., 2009; Kasprzyk-Pawelec *et al*.,2015) and callogenesis (Francisco and Mourao, 1992; Tao *et al*., 2002; Kamruzzaman *et al*., 2015; Mumtaz *et al*., 2015).

Development of an efficient micropropagation protocol involves the assessment of most effective phytohormones that could help in cell division and cell elongation (Tefera and Wannakrairoj, 2006). A balance between auxins and cytokinins in the culture medium is one of the critical factors for plant regeneration in various citrus species (Almeida *et al*., 2003; Silva *et al*., 2006). Singh *et al*. (1994) reported maximum shoot proliferation in *C. reticulate* and *C. limon* when MS medium was supplemented with BAP, kinetin and NAA. The synergetic effect of BAP with NAA in culture medium for shoot induction of Pera, Valencia, and Bahia (*Citrus sinensis*(L.) Osbeck) and Cravo (*Citrus limonia* Osbeck) has also been studied (Oliveira *et al*., 2010). Similarly, the combination of cytokinin with auxin in MS medium found to be most effective for shoot regeneration in *Citrus megaloxycarpa* (Haripyaree *et al*., 2011) and *Cassia angustifolia* (Siddique *et al*., 2015).

Role of plant growth regulators in *In vitro* organogenesis of different citrus species is well reported by various workers (Ali and Mirza, 2006; Altaf *et al*., 2009; Laskar *et al*., 2009; Sharma *et al*., 2009; Zeng *et al*., 2009; Jajoo *et al*., 2010; Savita *et al*., 2010; Haripyaree *et al*., 2011; Kumar *et al*., 2011; Tallon *et al*., 2012) but the effect of various phytohormones used in culture medium is genotype dependent (Bordon *et al*., 2000; Schinor *et al*., 2006). Hence, this research endeavor aimed to investigate the impact of varied concentration and combinations of phytohormones on morphogenic response of four citrus cultivars viz, Rough lemon, Kinnow, Feutrell's early and Musambi using shoot tip explants.

Materials and Methods

In vitro shoot proliferation

Stock cultures of citrus cultivars *viz*, Rough lemon (*C. Jambhiri*), Kinnow (*C. reticulata*), Feutrell's early (*C. reticulate* × *C. sinensis*) and Musambi (*C. sinensis*) maintained on MS (Murashige and Skoog, 1962) basal medium, were used as explant source. The aseptic plants comprising axillary buds from stock cultures were isolated and cut into small pieces (approx. 1cm) under aseptic conditions. Following excision, plants were inoculated into culture test tubes (25×150mm) containing modified MS (micro & macro elements, 100 mg L^{-1} myo-inositol, MS vitamins, 2 mg L^{-1} glycine, 30 g L^{-1} sucrose and 7.0 g L^{-1} agar) medium fortified with varied concentrations of BAP (0.5, 1.0,1.5,2.0 and 2.5mg L^{-1}) and 0.4 mg L^{-1} and IAA. pH was balanced at 5.8 and the medium was allowed to autoclave for 8 min at 121°C. The cultured tubes were kept in growth chamber under photoperiod of 2,000 lux for 16/8 h (25±1°C).

In Vitro Rooting

Actively growing shoots of four cultivars were selected for root initiation. Proliferated shoots (2cm long) with minimum 2 leaves were isolated under aseptic condition and were shifted to root induction medium (MS micro & macro elements, 100 mg L^{-1} myo-inositol, MS vitamins, 2 mg L^{-1} glycine, 30 g L^{-1}sucrose and 7.0 g L^{-1} agar) formulated with different composition of NAA (0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}) and 0.3 mg L^{-1}IBA after 60 days. The cultured tubes were kept in growth chamber under photoperiod of 2,000 lux for 16/8 h (25±1°C).

Data recording and statistical analysis

Data was visually observed every week and recorded after eight weeks for shoot number/plant, shoot length (cm), leaf number/plant, days to rooting, root number/plant and root length (cm). Individual test tube was considered an experimental unit for shoot regeneration and root induction. Each treatment consists of

three replications and ten explants were accounted for each replicate. Analysis of variance (ANOVA) method was used as a statistical mean and differences among treatment means were analyzed using Least Significance Difference (LSD) Test (p=1%) level (Steel *et al*., 1997).

Results

Efficacy of varied compositions of BAP and IAA on morphogenic response of Rough lemon, Kinnow, Feutrell's early and Musambi for shoot proliferation

Different compositions of phytohormones (BAP and IAA), cultivars and their interactions had notable effect on shoot length, shoot number and leaf number/plant at $p<0.01$(Table1). Mean shoot length (2.52cm), shoot number (4) and leaf number/plant (10.58) was best achieved on medium enriched with 1.0/0.4mg L^{-1} combination of BAP to IAA (Fig. 1a & b). Significant interaction between BAP/IAA concentrations and Feutrell's early was also observed on 0.5/0.4 and 2.5/0.4mg L^{-1} for mean shoot number and leaf number $plant^{-1}$ with 5.33±0.5 and 19.66±0.57 respectively. Mean shoot length (2.8±0.17 cm) by Rough lemon was relatively higher on 1.0/0.4mg L^{-1} comparative to other combinations screened. With increase in concentration of BAP from 1.0-2.5mg L^{-1} in culture media, explants exhibited poor shoot proliferation regarding mean shoot length, shoot number and leaf number/plant i.e., 1.32cm, 2.75 and 6.5 subsequently (Fig. 1c). None of the regenerates showed any callusing. Among four cultivars evaluated, shoots of Feutrell's early showed better morphogenic response towards mean shoot and leaf number/plant with 3.86 and 12.6 respectively (Fig 1d) while all four cultivars were at par with each other for shoot length.

Table 1: Morphogenic response of Rough lemon, Kinnow, Feutrell's early and Musambi shoot tip explants on modified MS medium fortifying different concentrations and combinations of BAP and IAA

Mean shoot number/plant±SE						Mean leaf number/plant±SE					Mean shoot length (cm)±SE				
Treatment BAP/IAA (mg L^{-1})	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean
0.5/0.4	3.33±0.57^{cdef}	3.0±0.5^{cdef}	5.33±0.5^{a}	2.33±0.5^{efg}	3.58b	3.66±0.57^{gh}	7.0±1^{f}	14±1^{c}	3.33±0.57^{a}	7.0 d	1.8±0.2^{defg}	2.23±0.05^{abcd}	2.63±0.49^{ab}	1.3±1.0^{h}	1.99 c
1.0/0.4	5±1.0^{ab}	3.66±0.5^{cd}	3.66±0.5^{cd}	3.66±0.5^{cd}	4a	13±1^{c}	11.66±0.57^{d}	10.66±0.57^{d}	7.0±1^{f}	10.58 a	2.8±0.17^{a}	2.56±0.25^{abc}	2±0.2^{cdef}	2.73±0.25^{a}	2.52 a
1.5/0.4	4±1.0^{b}	2.66±0.5^{defg}	3±1.0^{cdef}	3.33±0.5^{cde}	3.2 bc	13±1^{c}	4.66±0.57^{g}	10.66±0.57^{d}	4.33±0.57^{gh}	8.16 c	2.76±0.85^{a}	1.53±0.25^{gh}	1.66±0.30^{efgh}	2.1b±0.20^{cde}	2.01 b
2.0/0.4	4±1.0^{b}	1.2±0.1^{h}	3.33±0.5^{cde}	1.66±0.76^{g}	2.5 d	16±1.0^{b}	1.13±0.23^{j}	8.33±0.57^{e}	1.66±0.57^{i}	6.78d	2.33±0.15^{abcd}	0.83±0.15^{i}	1.4±0.26^{h}	1.56^{f}±0.5^{gh}	1.53d
2.5/0.4	0.83±0.15^{h}	5±1.32^{ab}	4±1.0^{bc}	2±0.5^{fg}	2.95c	1±0.2^{j}	13.66±0.57^{c}	19.66±0.57^{a}	2±1^{i}	9.08b	0.5±0.2^{i}	2.13±0.3^{bcd}	2.1±0.17^{cdef}	1.6±0.7^{fgh}	1.58d
Mean	3.43ab	3.17 b	3.86a	2.6 c		9.33 b	7.62c	12.66a	3.66 d		2.04 a	1.86b	1.96 ab	1.86b	
LSD$_{0.01}$	Treatment=0.59 Cultivar=0.53 Treatment*cultivar= 1.19					Treatment=0.58 Cultivar=0.52 Treatment*cultivar=1.17					Treatment=0.28 Cultivar=0.25 Treatment*cultivar=0.57				
Any two means showing a common letter do not differ significantly when separated by LSD Test at $P < 0.01$.															

Efficacy of varied compositions of NAA and IBA on morphogenic response of Rough lemon, Kinnow, Feutrell's early and Musambi for *In vitro* rooting

Number of days to rooting, root length and root number/plant were significantly influenced by different media compositions of NAA/IBA, cultivars and their interactions at p<0.01(Table 2). Micro shoots cultured on medium fortified with 1.0/0.3mg L^{-1} and 0.5/0.3mg L^{-1} concentration of NAA to IBA showed 6.08 mean root number/plant and 3.35cm root length respectively (Fig 1e & f). However, these two compositions i.e., 1.0/0.3mg L^{-1} and 0.5/0.3mg L^{-1} of NAA/IBA were at par with each other for number of days to rooting (22.25 and 22.83 d). Best interaction for days to rooting and root number/plant was achieved by Kinnow exhibiting 21.66±1.15d and 6.33±0.57 individually on 1.5/0.3mg L^{-1} and 1.0/0.3mg L^{-1} compositions of NAA/IBA. Maximum root length (6.16±0.15 cm) was attained by Rough lemon on medium containing 0.5/0.3mg L^{-1} (NAA to IBA conc.). Considering the genotype effect, Rough lemon had 5.06 root number/plant having 3.61 cm lengths whereas insignificant results were noticed for days to rooting among all cultivars assessed. Increase in NAA concentration from 1.0 to 2.5 mg L^{-1} negatively affected the morphogenic response in all citrus cultivars resulting in poor root growth (Fig. 1g)

Table 2: Efficacy of different concentrations and combinations of NAA and IBA supplemented to modified MS medium, on morphogenic response of Rough lemon, Kinnow, Feutrell's early and Musambi

Mean days to rooting±SE						Mean root number/plant±SE					Mean root length (cm)±SE				
Treatment NAA/IBA (mg L^{-1})	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean	Rough lemon	Kinnow	Feutrell's early	Musambi	Mean
0.5/0.3	21.33±0.57^{j}	22.33±0.57^{ghij}	23±1^{fghi}	24.66±0.57^{e}	22.83 cd	4.83±0.76^{cde}	5.33±0.57^{bcd}	5.16±0.28^{cd}	5±1^{cd}	5.08 b	6.16±0.15^{a}	1.96±0.15^{fg}	4.1±0.15^{gh}	1.7±0.1^{j}	3.35 a
1.0/0.3	21±1^{j}	22±1^{hij}	22.33±2.3^{ghij}	23.66±0.57^{efg}	22.25 d	7±1^{a}	6.33±0.57^{ab}	5.33±0.57^{bcd}	5.66±0.57^{bc}	6.08 a	3.06±0.11^{e}	1.96±0.15^{i}	4.1±0.1^{b}	2.6±0.1^{h}	2.93 b
1.5/0.3	24±1^{ef}	21.66±1.15^{ij}	23.33±0.57^{efgh}	24±1^{ef}	23.25 c	3.7±0.8^{efg}	5.33±0.6^{bcd}	3±1^{gh}	5±0.1^{cd}	4.27 c	1.46±0.05^{k}	2.5±0.15^{h}	1.06±0.15^{l}	2.66±0.05^{gh}	1.94 d
2.0/0.3	27±1^{cd}	26.66±0.57^{d}	26.33±0.57^{d}	27.33±0.57^{cd}	26.83 b	4.5±0.5^{def}	3.33±0.76^{gh}	2.3±0.57^{h}	3.66±0.15^{fg}	3.45 d	3.56±0.11^{d}	1.8±0.2^{ij}	3±2.9^{ef}	1.4±0.1^{k}	2.44 c
2.5/0.3	28.33±0.57^{c}	30.33±0.57^{b}	30.66±0.15^{b}	32.33±1.52^{a}	30.41 a	5.16±0.76^{cd}	3.06±0.5^{gh}	3±1^{gh}	2.33±0.5^{h}	3.39 d	3.8±0.1^{c}	0.56±0.15^{m}	0.4±0.1^{m}	0.5±0.1^{m}	1.31 e
	24.33 c	24.6 bc	25.13 b	26.4 a		5.05a	4.68a b	3.76c	4.33b		3.61 a	1.94 c	2.26 b	1.77 d	
LSD 0.01	Treatment=0.81 Cultivar=0.73 Treatment*cultivar= 1.63					Treatment=0.56 Cultivar=0.50 Treatment*cultivar= 1.13					Treatment=0.10 Cultivar=0.09 Treatment*cultivar= 0.20				
Any two means showing a common letter do not differ significantly when separated by LSD Testat $P < 0.01$.															

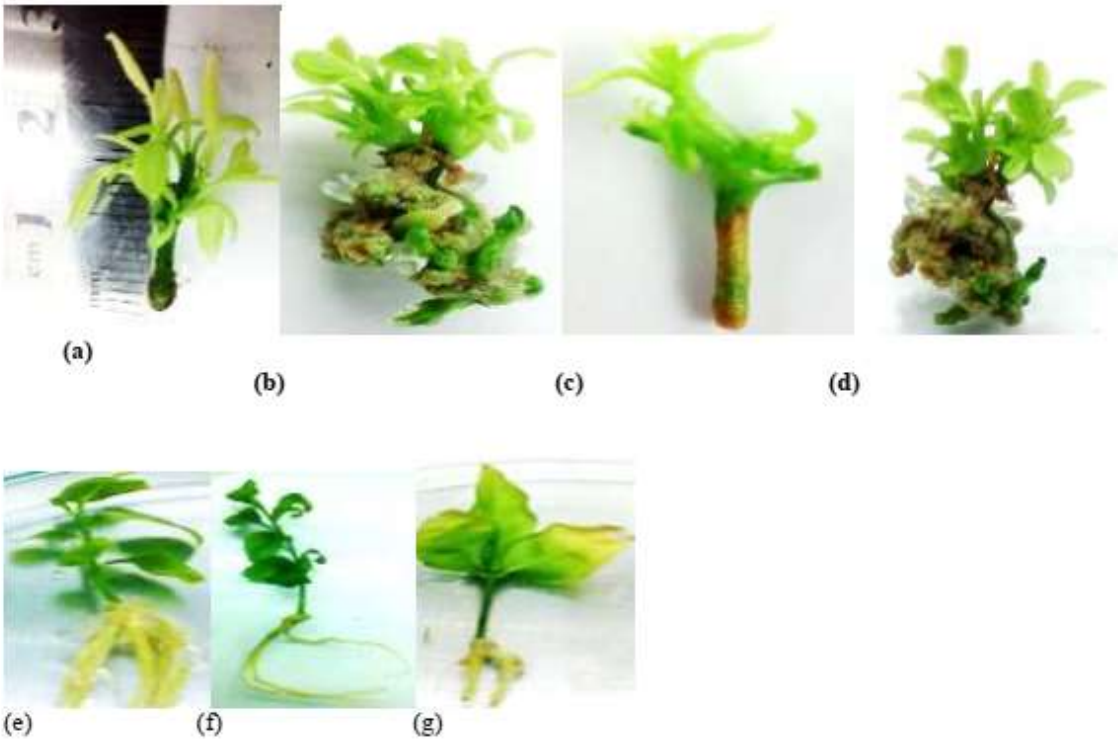

Fig. 1 Morphogenic response of Rough lemon, Kinnow, Feutrell's early and Musambi towards varying compositions of phytohormones (a) eight weeks old culture exhibiting shoot elongation and (b) multiple shoots having well developed leaves on modified MS medium enriched with1.0 mg L^{-1} BAP and 0.4 mg L^{-1} IAA (c) Stunted plant growth on medium containing higher concentrations of BAP(d) Feutrell's early showing mean shoot (3.86) and leaf number/plant (12.6) (e) well developed roots with 6.08 mean root number/plant and (f) 3.35cm root length on medium amended with 1.0 mg L^{-1} NAA/0.3 mg L^{-1} IBA and 0.5 mg L^{-1} NAA/0.3 mg L^{-1} IBA respectively(g) Micro shoots showing poorly developed roots.

Discussion

Determination of the suitable type and concentration of phytohormones as medium constituents is one of the most crucial aspects of *in vitro* propagation, among other factors studied (Daffalla *et al*., 2011). The synergetic effect of cytokinin with auxin is well documented for *in vitro* plant regeneration of numerous citrus species (Paudyal and Haq 2000; Rodriguez *et al*., 2008). The present study reveal that optimum concentrations of BAP and IAA have notable effect on shoot length, shoot and leaf number/plant in Rough lemon, Kinnow, Feutrell's early and Musambi whereas, supra-optimal concentrations of BAP demonstrated toxic effects on proliferated shoots of all four cultivars assessed. These results find support from Kim *et al*. (2002) and Vestri *et al*. (2003) who testified that the optimum concentration of different growth regulators has positive impact on shooting frequency of *C. Junos* and *C. jambheri* respectively.

George *et al*. (2008) investigated that the cytokinin concentrations at higher levels are responsible for senescence in plant tissues giving a smaller number of shoot and shoot length. Waseem *et al*. (2009) also reported that the higher dosage of growth regulator in chrysanthemum failed to make sure their impact positively and could be responsible for negative effect at higher meditations, while the ineffectiveness of the lower dose showed insufficient level of growth regulator

ensuing poor results. The simultaneous use of BAP with IAA at the concentration of 1.0 mg L^{-1} and 0.4 mg L^{-1} respectively in culture medium, gave best results in four cultivars regarding shoot length, shoot and leaf number/plant. A combined effect of BAP with IAA was more proficient in shoot proliferation of 'Garden Rue' as number of shoots per nodal segment was notably highest at MS medium containing 0.25 IAA mg L^{-1} and 1 mg L^{-1} BAP (Bohidar *et al*., 2008). A high concentration of cytokinin in combination with low level of auxin promotes shoot growth in *Withania somnifera* (Fatima and Anis, 2012) and in *Mentha arvensis* (Shasany *et al*., 1998).

Micropropagation techniques are routinely used for citrus improvement and their effects are genotype dependent (Brinstrubiene *et al*., 2004; Gitonga *et al*., 2010). In spite of same concentrations of phytohormones used, a discrepancy among Rough lemon, Kinnow, Feutrell's early and Musambi for shoot length, shoot and leaf number/plant is perceived. Disparity in response of Rough lemon, Pectinifera, Cleopatra mandarin and Troyer citrange towards varying concentration of PGRs assured that differences in organogenesis might be due to genetic makeup (Sharma *et al*., 2009). The outcomes achieved by Almeida *et al*. (2002) revealed differences in response to number of shoots/plant when Valencia, Natal, Hamlin (*Citrus sinensis*) and Rangpur lime (*C. limonia*) were compared. Bordon *et al*. (2000) testified that inconsistency of *C. reshni, C. aurantium, C. sinensis* and *C. macrophylla* towards varied frequency of phytohormones elucidates the genotypic effect. Moreover, morphogenic response affected by genotype in sour orange, grapefruit, alemow (*C. macrophylla*) (Ghorbel *et al*., 1998), sweet orange, rangpur lime (Oliveira *et al*., 2010) and in other citrus species (Carimi and Pasquale, 2003) is also reported.

In this study, it was found that certain level of NAA and IBA is quite necessary for promotion of rooting ability in Rough lemon, Kinnow, Feutrell's early and Musambi. Auxin application brought variations in RNA production and protein synthesis, hence exciting the cell division processes for enhancing root number (Iqbal *et al*., 2003; Husen and Pal, 2007). Rout, (2006) found that NAA endorses root number by promoting cell division in root primordia, similarly IBA produced better results of root number because of its effectiveness in increasing the endogenous auxin contents (George *et al*., 2008). Normah *et al*., (1997) also obtained *in vitro* rooting from aseptic shoots of *C. halimii* on MS basal medium enriched with NAA. NAA concentration more than optimal (i.e., 1.0 mg L^{-1} NAA) resulted in the decrease in root number and root length/plant in four citrus cultivars evaluated. The root elongation phase is much sensitive to auxin concentration and is suppressed by high levels of auxin in the culture medium of peanut (Baker and Wetzstein, 1994). These results find support from Ozel *et al*. (2006) who described that maximum level of auxin in culture medium inhibit the root development in *Centaurea tchihatcheffii* as per auxin in the root primordial is moved from the shoot apex. Daffalla *et al*. (2011) also obtained minimum root growth when a woody plant "*Boscia senegalensis*" was subjected to higher concentrations of IBA (1.0 mg L^{-1}).

Genetic potential could be the major factor affecting the root growth (Baig *et al*., 2011). All citrus cultivars i.e., Rough lemon, Kinnow, Feutrell's early and Musambi behave differently for days to rooting, root number and root length/plant regardless of the same concentrations of auxins used. Usman *et al*. (2005) stated that *in vitro* root formation in citrus cultivars is genotype dependent. Similarly, Costa *et al*. (2004) also confirmed the effect of various phytohormones used in culture medium is genotype dependent.

Conclusion

In present study, the impact of different phytohormones on morphogenic response of four citrus cultivars was assessed. It was found that, the combined use of BAP with IAA resulted in a significant synergistic effect on *in vitro* shoot induction and proliferation of different citrus cultivars. Additionally, it was also noticed that the synergistic effect is beneficial up to certain concentrations of phytohormones, for plant regeneration in different citrus cultivars. Considerable improvements in root induction were also observed when 1.0 mg L^{-1} NAA was used in combination with 0.3 mg L^{-1} IBA. However, substantial differences were noticed on the degree of effects exerted by the different combinations of phytohormones evaluated. Therefore, the prescribed protocol could be applied to other citrus species for mass scale clonal propagation and for conservation of important species.

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