

CALLOGENESIS AND PLANT REGENERATION FROM LEAF EXPLANTS OF CITRUS CULTIVARS

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We have established an efficient protocol for *in vitro* callogenesis from leaf explants of Troyer citrange (*Poncirus trifoliata* × *Citrus sinensis*) and Feutrell's early (*C. reticulata* × *C. sinensis*) using modified MS medium enriched with varied concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D), Naphthalene acetic acid (NAA) and Benzylaminopurine (BAP). Indirect shoot organogenesis was also achieved from leaf derived calli on medium comprising varied concentrations of BAP, Kinetin (KN) and NAA. Best interaction for callus induction was observed by Troyer citrange with modified MS medium containing 0.5 mg L⁻¹ 2,4-D, 0.5 mg L⁻¹ NAA and 0.5 mg L⁻¹ BAP, which resulted in 99.66% of leaf segments producing calli within 16.33 days. Morphological differences were also noticed for calli texture and color with different concentrations of 2,4-D, NAA and BAP used. Compact and hard calli were noticed on MS medium with 0.1 mg L⁻¹ NAA and 0.5 mg L⁻¹ BAP however, medium including 1.0 mg L⁻¹ 2,4-D, 0.9 mg L⁻¹ NAA and 0.5 mg L⁻¹ BAP resulted in friable and smooth calli by both cultivars. Exuberant shoot regeneration response (65.33%) with an average of 6.2 shoots/calli was recorded on MS medium with 2 mg L⁻¹ BAP and 0.5 mg L⁻¹ NAA by Feutrell's early. Our findings are the pre-requisite for various biotechnological tools like mutation breeding, somaclonal variations, genetic transformation and somatic hybridization which may be helpful in citrus varietal improvement.

Keywords: Callus induction, shoot regeneration, Troyer citrange, Feutrell's early, 2,4-D, BAP.

INTRODUCTION

Citrus, having a wide collection of rootstocks and edible species is considered as the commercially important fruit crop in Pakistan (Khan *et al.*, 1996; Jaskani and Khan, 2000; Perez-Tornero *et al.*, 2010). Pakistan is among top fourteen citrus producing countries with 2.15 million tones production per year occupying 194.0 thousand hectares area (GOP, 2014). Owing to its nutritional value and specific flavor, it is consumed fresh as well as processed worldwide (Dugo and Giacomo, 2002). Being well known fruit crop, it is susceptible to various stresses comprising biotic *viz.*, nematodes, viroids, citrus leaf minor, *Phytophthora*, citrus tristeza virus (CTV), citrus variegation virus (CVV), citrus greening as well as abiotic like salinity and drought which are accountable for its poor yield and low quality (Khan and Jaskani, 1992; Legaspi and French, 2003; Arif *et al.*, 2005). Other difficulties include alternate bearing, slow growth rate, more number of seeds/ fruit, long juvenility in seedlings and pre and post-harvest losses (Khan *et al.*, 1999; Mukhtar *et al.*, 2005; Jaskani *et al.*, 2007). Conventional breeding techniques are handicapped to circumvent the above mentioned threats because of sterility, heterozygosity, sexual incompatibility, nucellar embryony/ polyembryony (Ollitrault *et al.*, 2000; Louzada *et al.*, 2002; Naqvi *et al.*, 2011), apomixes and long juvenility (Khan and Grosser,

2004). Biotechnological applications open up the avenues for citrus genotype improvement by solving the problems limited by conventional breeding approaches through embryo rescue, somaclonal variations, somatic hybridization, genetic transformation and *in vitro* mutagenesis (Jaskani and Khan, 1993; Kayim and Koc, 2006; Kumar *et al.*, 2011). Hence, provision of a reliable indirect regeneration protocol via callus production is a pre-requisite for genetic improvement in citrus species (Tao *et al.*, 2002).

Callus serve as a basic material for many biotechnological tools like somaclonal variations, genetic transformation and protoplast manipulation (Grosser and Gmitter, 2011; Aftab, 2012). The success of callus induction and regeneration is evidently influenced by explant source, genotype, plant growth regulators, incubation conditions and organic and inorganic nutrient components of culture media (Singh and Rajam, 2010; Panathula *et al.*, 2014). Different plant growth regulators (PGRs) in the culture medium are also responsible for determining the type and quality of callus (Tamilselvan and Rajeswari, 2014). Medium enriched with NAA produced compact and brown calli whereas 2,4-D resulted into friable and creamy phenotype from immature cotyledon of walnut (Cai *et al.*, 2013). Similarly, non-regenerative hard calli was obtained on medium comprising NAA and TDZ from leaf explant of *Citrus indica* (Laskar *et al.*, 2009). In addition highest transformation frequency was achieved

from yellow phenotype calli which depicts the role of callus quality on transformation efficiency of *Coffea Arabica* (Ribas *et al.*, 2011). Herein, callus induction from leaf explants of Troyer citrange and Feutrell's early was investigated in response to modified MS (Murashige and Skoog, 1962) medium fortified by varied combinations of 2,4-D, NAA and BAP. Efforts were also made towards establishing a reproducible protocol for indirect shoot regeneration from Feutrell's early and Troyer citrange calli using BAP, KN and NAA in different combinations.

MATERIALS AND METHODS

Callus induction: Leaf segments of size 1 cm² from stock cultures of Troyer citrange and Feutrell's early were incubated in 25×190 mm test tubes with adaxial surface of leaf up. Modified MS (MS macro & micro elements, MS vitamins, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, 2 mg L⁻¹ glycine, 7.0 g L⁻¹ agar and 5.8 pH) medium supplemented with varied combinations of NAA (0.1, 0.3, 0.5, 0.7, 0.9 mg L⁻¹), 2,4-D (0, 0.25, 0.5, 0.75, 1.0 mg L⁻¹) and BAP (0.5 mg L⁻¹) was used for callus induction (Table 1). Cultured tubes were maintained at 25±1°C and 70±10% relative humidity provided with dark condition for 30-40 days.

Shoot regeneration: After three weeks of callus proliferation, well profused portion (5×5×5 mm) of calli was cultured on shoot regeneration medium (MS macro & micro elements, MS vitamins, 100 mg L⁻¹ myo-inositol, 30 g L⁻¹ sucrose, 2 mg L⁻¹ glycine, 7.0 g L⁻¹ agar and 5.8 pH) containing varied combinations of BAP (0, 0.5, 1.0, 2.0 mg L⁻¹), KN (0, 1.0, 2.0 mg L⁻¹) and NAA (0.5 mg L⁻¹) (Table 4). Cultures were incubated in growth chamber under photoperiod of 2,000 lux for 16/8 h (25±1°C).

Data recording and statistical analysis: Based on visual observation, data regarding days to callus induction, callus proliferation percentage (Number of leaf explants produced calli/ Total number of leaf explants cultured X 100), callus texture, callus color, shoot regeneration percentage (Number of calli produced shoots/ Total number of calli cultured X 100) and number of shoots/calli was recorded. Each test tube

was assumed an experimental unit for callus formation and shoot regeneration. Three replications were accounted for each treatment and each replicate consists of ten explants. Explants responded to callus formation were observed after 3 to 4 weeks of inoculation and 6 to 8 weeks were considered for shoot induction. Two-way analysis of variance (ANOVA) was used for statistical analysis. Data regarding treatment means was subjected to Least Significance Difference (LSD) test at 5% *p* level (Steel *et al.*, 1997).

RESULTS

Impact of varied concentrations of 2,4-D, NAA and BAP on callus induction and proliferation from leaf explants of Troyer citrange and Feutrell's early:

Callus induction in leaf explants of Troyer citrange and Feutrell's early varied among different PGRs tested at *p*<0.05 (Table 1 & 2). Significant interaction for days to callus induction was observed with Troyer citrange (16.33 d) and Feutrell's early (17 d) on medium fortifying 2,4-D (0.5 mg L⁻¹), NAA (0.5 mg L⁻¹) and BAP (0.5 mg L⁻¹). Same treatment exhibited better results for callus proliferation with 99.66% and 92% by Troyer citrange and Feutrell's early respectively. From the assessment of two cultivars for five different combinations of 2,4-D, NAA and BAP, efficient performance was presented by Troyer citrange showing 56.73% of calli proliferation with 27.4 days to calli initiation comparative to Feutrell's early which exhibits calli proliferation (52.13%) in 29.46 days (Fig. 1a & b). No single leaf explant of both cultivars showed necrotic symptom on different compositions of 2,4-D, NAA and BAP.

Based on careful observation, data elucidates that different types of auxins have potential for inducing varied color and texture in calli of both cultivars. Medium amended with NAA (0.1 mg L⁻¹) and BAP (0.5 mg L⁻¹) showed compact and nodular calli whereas, friable and smooth calli was produced by medium with 2,4-D (1.0 mg L⁻¹), NAA (0.9 mg L⁻¹) and BAP (0.5 mg L⁻¹) by both cultivars (Fig. 1c & d). From the two cultivars compared, Troyer citrange exhibited

Table 1. Influence of varied combinations of NAA, 2,4-D and BAP on days to callus induction from leaf explants of Troyer citrange and Feutrell's early.

NAA	PGRs (mg L ⁻¹)		Days to callus induction		
	2,4-D	BAP	Troyer citrange	Feutrell's early	Mean
0.1	0	0.5	38.00±1.00 de	41.00±0.89 f	39.50 e
0.3	0.25	0.5	31.33±1.52 c	35.33±0.57 d	33.33 d
0.5	0.5	0.5	16.33±1.52 a	17.00±0.90 a	16.66 a
0.7	0.75	0.5	24.00±0.90 b	24.00±0.89 b	24.00 b
0.9	1.0	0.5	27.33±0.57 bc	30.00±0.90 cd	28.67 c
Mean			27.40 a	29.46 b	

LSD (0.05) Cultivars = 0.81, Interaction (T×C) = 3.07, Treatments = 1.83

Data presented in column elucidates different treatment means ± SE followed by various alphabet (s) indicating substantial differences according to LSD test at *P* < 0.05.

Table 2. Influence of varied combinations of NAA, 2,4-D and BAP on callus proliferation percentage from leaf explants of Troyer citrange and Feutrell's early.

PGRs (mg L ⁻¹)			Callus proliferation percentage		
NAA	2,4-D	BAP	Troyer citrange	Feutrell's early	Mean
0.1	0	0.5	20.33±1.52 f	16.33±1.52 f	18.33 e
0.3	0.25	0.5	64.66±2.08 d	60.00±1.52 d	62.33 c
0.5	0.5	0.5	99.66±0.57 a	92.00±1.52 a	95.83 a
0.7	0.75	0.5	71.66±2.00 c	63.66±2.0 d	67.67 b
0.9	1.0	0.5	27.33±1.52 e	28.67±1.15 e	28.00 d
Mean			56.73 a	52.13 b	

LSD (0.05) Cultivars = 1.78, Interaction (T×C) = 6.75, Treatments = 4.03

Data presented in column elucidates different treatment means ± SE followed by various alphabet (s) indicating substantial differences according to LSD test at *P* < 0.05.

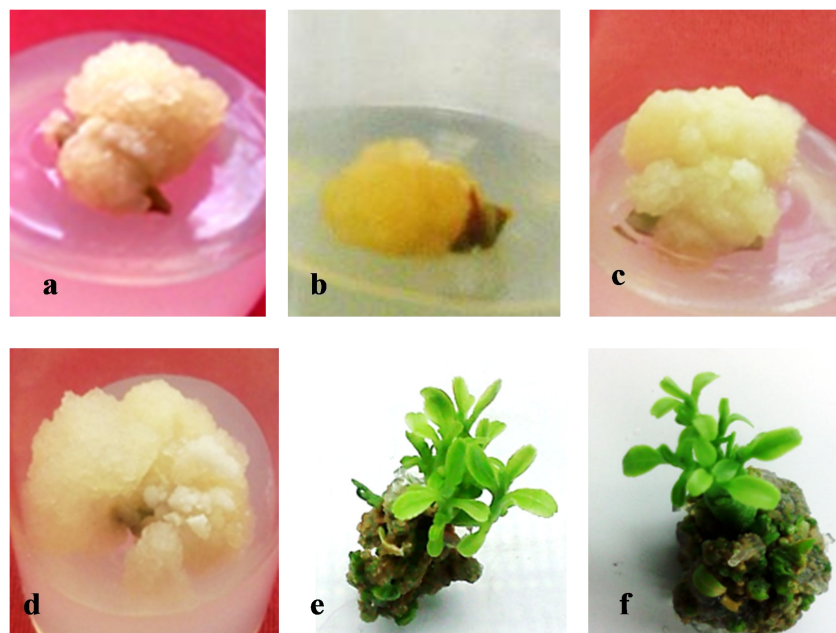


Figure 1. *In vitro* callogenesis and shoot regeneration in Feutrell's early and Troyer citrange: (a) Exuberant calli proliferation from leaf explants of Troyer citrange and (b) Feutrell's early; (c) compact and nodular calli produced with 0.1 mg L⁻¹ NAA and 0.25 mg L⁻¹ BAP by Troyer citrange; (d) medium with 1 mg L⁻¹ 2,4-D, 0.9 mg L⁻¹ NAA and 0.5 mg L⁻¹ BAP resulted friable and smooth calli by Feutrell's early; (e) vigorous adventitious shoot growth in Feutrell's early and (f) Troyer citrange.

compact and nodular calli with yellowish white color on medium with 2,4-D (0.5 mg L⁻¹), NAA (0.5 mg L⁻¹) and BAP (0.5 mg L⁻¹) conversely, Feutrell's early showed positive response towards friable and smooth calli with creamish white color on similar concentrations of NAA, BAP and 2,4-D (Table 3).

Impact of varied concentrations of BAP, KN and NAA on indirect shoot regeneration from leaf derived calli of Troyer citrange and Feutrell's early: Shoot regeneration response from *in vitro* derived calli of Troyer citrange and Feutrell's early was first observed by variation in calli color which turned into green after 45 days of incubation, following the development of leaf primordia. Strong

interaction was found between different PGRs concentration (BAP, KN and NAA) and citrus cultivars for shoot regeneration response (Table 4 & 5). Exuberant response in shoot regeneration (65.33%) with an average of 6.2 shoots/calli was observed on medium containing BAP (2 mg L⁻¹) and NAA (0.5 mg L⁻¹) by Feutrell's early, while 61.33% caullogenic response with 5.1 shoot/calli was displayed by Troyer citrange on same treatment. Of the two cultivars compared, average shoot regeneration percentage (44.66%) with 4.46 shoots/calli of Feutrell's early is better than Troyer citrange exhibiting 41.06% shooting response with 3.66 shoots/calli (Fig. 1e,f). None of the responded shoots turned brown or vitrified.

Table 3. Influence of varied concentrations of NAA, 2,4-D and BAP on callus color and texture of Troyer citrange and Feutrell's early.

PGRs (mg L ⁻¹)			Troyer citrange		Feutrell's early	
NAA	2,4-D	BAP	Callus texture	Callus color	Callus texture	Callus color
0.1	0	0.5	CN	CW	CN	C
0.3	0.25	0.5	CN	CW	CN	CW
0.5	0.5	0.5	CN	YW	FS	CW
0.7	0.75	0.5	FS	YW	FS	YW
0.9	1.0	0.5	FS	YG	FS	YG

CN: Compact and nodular; FS: Friable and smooth; C: Creamy; CW: Creamish white; YW: Yellowish white; YG: Yellowish green.

Table 4. Influence of varied combinations of BAP, KN and NAA on indirect shoot regeneration of Troyer citrange and Feutrell's early.

PGRs (mg L ⁻¹)			Shoot regeneration percentage		
BAP	KN	NAA	Troyer citrange	Feutrell's early	Mean
0.5	0	0.0	40.66±1.0d e	44.0±3.21 cd	42.33 c
1	0	0.5	50.33±1.52 bc	52.33±1.52 b	51.33 b
2	0	0.5	61.33±1.52 a	65.33±2.51 a	63.33 a
0	1	0.5	31.0±1.51 fg	35.0±3.21 ef	33.00 d
0	2	0.5	22.0±2.59 h	26.66±0.57 gh	24.33 e
			41.06 b	44.66 a	

LSD (0.05) Cultivars = 2.14, Interaction (T×C) = 8.12, Treatments = 4.85

Data presented in column elucidates different treatment means ± SE followed by various alphabet (s) indicating substantial differences by LSD test at $P < 0.05$.

Table 5. Influence of varied combinations of BAP, KN and NAA on number of shoots/calli of Troyer citrange and Feutrell's early.

PGRs (mg L ⁻¹)			Number of shoots/calli		
BAP	KN	NAA	Troyer citrange	Feutrell's early	Mean
0.5	0	0	3.3±0.57 cd	4.3±0.56 c	3.83 c
1	0	0.5	4.3±1.0 ab	5.0±0.56 bc	4.66 b
2	0	0.5	5.1±1.0 b	6.2±1.0 a	5.5 a
0	1	0.5	3.6±0.57 d	3.3±0.57 c	3.5 d
0	2	0.5	2.0±0.57 e	3.66±1.0 cd	2.8 e
Mean			3.66 b	4.46 a	

LSD (0.05) Cultivars = 0.59, Interaction (T×C) = 2.2, Treatments = 1.33

Data presented in column elucidates different treatment means ± SE followed by various alphabet (s) indicating substantial differences according to LSD test at $P < 0.05$.

DISCUSSION

A good quality callus is used as initial tissue for biotechnological study of many woody species and it could be regenerated into plant by using different PGRs. Here, in this report we have established a reproducible protocol for callus induction and indirect shoot regeneration of Troyer citrange and Feutrell's early via leaf explants with different PGRs used. Type and concentration of auxins are well known to affect the different parameters of *in vitro* derived calli in numerous species. The first noticeable response in

callogenesis was the initiation of undifferentiated tissues from wounded edges of leaf which gradually covered all the surface area of leaf in both cultivars. In fact cut ends are responsible for more absorption of growth regulators and nutrients from the culture medium (Sarwar and Skirvin, 1997). It is evident from the results that upto a certain concentration, PGRs positively affected days to callus induction and callus proliferation while above or below optimal doses of PGRs, calli initiation period and proliferation negatively affected. Increase in 2,4-D concentration prevents the callus development in leaf segments and it affects like a herbicide (Wernicke and

Mikovits, 1984). In previous reports it was found that higher or lower auxin level (2,4-D or NAA) may results in delaying of callus induction and proliferation in wheat grass (*Agropyron cristatum*) and Rauwolfia serpentine (Can *et al.*, 2008; Salma *et al.*, 2008).

Auxin/cytokinin ratio shows the best synergistic interaction for regulating the cell division in undifferentiated cells. The synergistic impact of auxin with cytokinin was also approved by the investigations of Tamilselvan and Rajeswari (2014) who obtained maximum callus induction (100%) from leaf explant of Chinease violet (*Asystasia gangetica*) on medium with NAA, BAP and KIN. Vujovic *et al.* (2014) illustrated that MS medium comprising 2,4-D with NAA resulted in 100% callus formation in *prunus* species. In spite of similar type and concentration of PGRs used, a marked disparity was observed between cultivars for days to callus induction and callus proliferation. Troyer citrange showed best response towards callogenesis with respect to days required to callus induction and callus proliferation as compared to Feutrell's early. Sharma *et al.* (2009) stated that variability in callus formation of different citrus species may also attribute to the genotype.

NAA and 2,4-D performed differently for callus texture and color of both the cultivars. A gradual change can be viewed in callus texture of both cultivars from compact to friable with deviation in 2,4-D and NAA combinations. Medium enriched with maximum amount of 2,4-D (0.9 mg L⁻¹) resulted into friable and smooth calli while compact calli was initiated on medium containing only NAA (0.1 mg L⁻¹) or combined with little amount of 2,4-D (0.25 mg L⁻¹). Michel *et al.* (2008) achieved loosely arranged mass of cells when cotyledons of *Gossypium hirsutum* was exposed to medium containing 2,4-D. However, Vujovic *et al.* (2014) highlighted hard calli from leaf explants of two *prunus* genotypes i.e. Gisela 5 (*P. cerasus* x *P. canescens*) and Cacanski Rubin' (*P. cerasus* L.) when cultured on medium containing NAA, BA and TDZ. Variation in calli color was also observed with alteration in PGRs concentration. Creamish calli was found on medium augmented with 2,4-D (0.25 mg L⁻¹) and yellowish green calli was accompanied by medium with more supplements of NAA (0.9 mg L⁻¹) by both cultivars. These results revealed that with deviation in combination of PGRs, calli color also changes. Gopi and Vastala (2006) got yellowish green calli from leaf explant of *Gymnema Sylvestre* when subjected to medium fortified with NAA. Panathula *et al.* (2014) explained that variability in callus texture and color quality of *Centella asiatica* might be due to different PGRs inclusion into the culture medium. Genotype has great effect on callus texture and color as well. Despite similar concentrations of PGRs, both cultivars behave differently for callus texture and color. The discrepancy in response of citrus cultivars regarding calli texture and color elucidates the genotypic effect as demonstrated by Bordon *et al.* (2000).

It is noteworthy to optimize auxin/cytokinin balance for efficient indirect shoot organogenesis in individual specie. Only compact and firm calli produced adventitious shoots, and not the friable ones in both cultivars assessed. In another report it was stated that no shoot regeneration was observed from friable callus induced from leaf explants of wild cherry (*Prunus avium* L.) (Grant and Hammat, 2000). Supplement of BAP and NAA proved to be the most favorable PGRs combination for shoot development of leaf derived calli in sweet orange (*Citrus sinensis*) (Khalil *et al.*, 2011) and in *S. involucrate* (Guo *et al.*, 2007). In previous investigations on *in vitro* organogenesis of citrus species, it was testified that optimum concentration of PGRs are responsible for the successful shoot formation in *Citrus junos* (Kim *et al.*, 2002), *C. jambheri* (Vestri *et al.*, 2003) and *Citrus reticulata* (Mukhtar *et al.*, 2005). Savita *et al.* (2011) obtained maximum shoot regeneration response from *in vitro* induced calli of *C. jambheri* on MS media with NAA and BAP.

With increasing meditation of BAP by 1 mg L⁻¹ to 2 mg L⁻¹, the shoot regeneration percentage becomes higher. Comparatively, varying concentrations of KN doesn't exhibit similar relationship with shoot regeneration percentage as shown by BAP. It can be clearly seen that BAP is the most essential cytokinin required for shoot induction. Efficiency of BAP over other cytokinin for *in vitro* shoot stimulation and proliferation is well documented in rangpur lime (Moura *et al.*, 2001), *citrus grandis* (Paudyal and Haq, 2000), sweet orange (Almeida *et al.*, 2002) and rough lemon (Rattanpal *et al.*, 2011). Caullogenetic response was best achieved by Feutrell's early in terms of shoot regeneration percentage and number of shoots/calli whereas, Troyer citrange showed less efficiency towards shooting frequency. Sharma *et al.* (2009) suggested that differences in regeneration capacity of citrus cultivars towards different PGRs concentrations might be due to genotype-specific behavior.

Conclusion: In this research endeavor, we have developed a proficient and reliable protocol for callus induction and indirect shoot regeneration of Troyer citrange and Feutrell's early via leaf explants. This investigation evidenced high synergistic effect of PGRs towards callogenic and shoot regeneration response of citrus cultivars. Additionally, it was observed that the synergistic effect was useful up to certain level of PGRs, for callogenic and shoot regeneration studies of both cultivars. PGR-dependent response was also noticed for calli texture and color. Compact calli was achieved with medium containing NAA however, 2,4-D enriched medium produced friable calli. Hence, the outcomes of this effort could be applied for genetic improvement in commercial cultivars via genetic transformation, protoplast fusion and somatic embryogenesis.

Acknowledgements: This research was supported by financial assistance from Plant Tissue Culture Laboratory, Department of Horticulture, PMAS-Arid Agriculture University Rawalpindi, Pakistan.

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