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Development of an efficient *in vitro* regeneration protocol for fig (*Ficus carica* L.)

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Abstract

The present investigation was undertaken to develop an efficient *in vitro* regeneration protocol in four fig cultivars *viz.,* Poona Fig, Brown Turkey, Conadria and Deanna. Highest shoot tip establishment was observed in Deanna (100 %), followed by Conadria (79.2 %) and Brown Turkey (76.7 %) on MS medium supplemented with 2.5 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L gibberellic acid (GA_3). Establishment of shoot tips was very poor in cultivar Poona Fig (11.7-13.3 %). Further inoculation of shoots on MS medium supplemented with 1.0 mg/L indole-3-butyric acid (IBA) resulted in both multiple shooting as well as rooting. Significant number of newly formed shoots were observed in Conadria (4.7) and Deanna (3.8) as against in Brown Turkey (1) and Poona Fig (0.6). Highest root induction was observed in Conadria (73.3 %), followed by Deanna (52.2 %), Brown Turkey (26.7 %) and Poona Fig (24.4 %). These results confirmed that the shoot bud establishment and multiple shoot induction in fig is highly genotype specific. As the response of popular cultivar Poona Fig to shoot tip culture was very poor, tender leaf explants were further used for regeneration study. Optimum regeneration was observed using MS medium supplemented with 4.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) for callusing; 7 mg/L thidiazuron (TDZ) and 0.25 mg/L α-naphthalene acetic acid (NAA) for shooting and 1.0 mg/L IBA for rooting.

Key words: Fig, regeneration, genotype-specific, shoot tip culture, multiple shooting, rooting

Introduction

The common fig (*Ficus carica* L.) is well known for its nutritive value and are consumed both as fresh and process dried. Figs are rich source of crude fibre, minerals, vitamin K and antioxidants compounds (Vallejo *et al.,* 2012). It is native to Southwest Asia and the Mediterranean region (from Afghanistan to Portugal). Worldwide fig is widely cultivated, with an area of 3.8 lakh ha and production of 10.93 lakh tonnes harvested in 2012. However despite of huge demand both cultivation area (5500 ha) and production (19,000 tonnes) is very low in India (FAOSTAT, 2014). Exotic cultivars do not find favor with Indian farmers as they are more prone to nematode. The seeds of fig are non-viable; hence the figs are propagated through vegetative approaches. However, these propagation approaches are slow and limited. Development of clonal propagation methods have numerous potential applications *e.g.*, plant transformation, germplasm conservation, synthetic seeds and mutation breeding (Ji *et al*., 2011). Micropropagation has been successfully employed for rapid multiplication of genetically identical and superior quality planting material in many fruit crops. *In vitro* propagation in fig serves the purpose of mass-scale production of high quality planting material (Rout *et al*., 2006). *In vitro* regeneration in fig using various explants such as shoot tips (Murithi *et al*., 1982; Haelterman and Docampo, 1994; Gella *et al*., 1998; Hepaksoy and Aksoy, 2006), nodal explants (Fraguas *et al.,* 2004), leaves (Kim *et al.,* 2007; Dhage *et al.,* 2012; Soliman *et al.,* 2010) and apical buds (Kumar *et al*., 1998; Gella *et al*., 1998) has been reported.

Development of an efficient regeneration protocol is essential for successful *in vitro* propagation and transformation in fig. Fig is recalcitrant in its production of adventitious shoots (Kim *et al.,* 2007) and factors affecting shoot proliferation have not been optimized (Fraguas *et al.,* 2004). Thus, the objective of our study was to optimize *in vitro* regeneration protocol in fig using shoot tips and leaves.

Materials and methods

Preparation of explants: Axillary shoot tips of four fig cultivars *viz*., Poona Fig, Brown Turkey, Conadria and Deanna as well as tender leaves of Poona Fig were collected in morning hours from healthy mature plants from experimental orchard of the All India Coordinated Research Project Arid Zone Fruits, Mahatma Phule Krishi Vidyapeeth, Rahuri. These explants were sterilized with 0.1, 0.2, 0.3 % (w/v) mercuric chloride (**$HgCl_2$**) or 4 % (w/v) sodium hypochlorite (NaClO) solution for 3, 5, 7, 9 min. They were further rinsed five times with sterile distilled water.

Shoot tip culture establishment: Initially shoot tip culture was attempted in cv. Poona Fig. However, very poor bud breaking was observed. In order to check whether initial poor results in cv. Poona Fig was due to genotype specific response, three other fig cultivars *i.e.* Brown Turkey, Conadria and Deanna were further included in the present study. Sterilized shoot tips were inoculated on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 2.5 or 3.5 mg/L 6-benzylaminopurine (BAP), 0.5 mg/L gibberellic acid (GA_3), 100 mg/L ascorbic acid and 150 mg/L citric acid. They were initially kept in dark for a week and then incubated at 25 °C temperature with 16 h light period and 8 h dark period.

Multiple shoot induction and rooting: Individual shoots from shoot tip induction medium were further transferred to MS medium with either of 1/2/3/4 mg/L of indole-3-butyric acid (IBA), 100 mg/L ascorbic acid, 150 mg/L citric acid and 0.2 % activated charcoal for further induction of shoots and roots.

Table 1. Effect of growth regulators on *in vitro* shoot tip establishment

Genotype	Treatment	Establishment (%)	Days to bud break/ sprouting	Days to leaf emergence	Shoots with more than one leaf (%)	Multiple shoot (%)
Poona Fig	EM1	13.3 ±0.70 [a]	6.7 ±0.02 [a]	7.6±0.03	18.3 ±0.61 [a]	0.00
	EM2	11.7 ±0.65 [a]	7.0±0.02	7.9±0.05	20.8 ±0.59 [a]	0.00
Brown Turkey	EM1	76.7±1.15	6.5±0.02	7. 5±0.03	90.8±0.85	0.00
	EM2	53.3±0.48	6.7 ±0.03 [a]	7.3 ±0.04 [a]	52.5±0.83	0.00
Conadria	EM1	79.2±0.59	6.1 ±0.01 [b]	7.1±0.04	95.0±1.96	52.50
	EM2	87.5±1.26	6.2 ±0.03 [bc]	7.2 ±0.02 [a]	60.8±0.49	39.17
Deanna	EM1	100	6.2±0.03 [c]	7.3 ±0.02 [a]	94.2±0.99	41.67
	EM2	62.5±0.50	6.4±0.03	7.3 ±0.03 [a]	54.2±0.48	60.00
CD at 5%		2.08	0.07	0.103	2.75	

EM1: MS + 2.5 mg/L BAP+ 0.5 mg/L GA_3 + 100 mg/L ascorbic acid +150 mg/L citric acid

EM2**:** MS + 3.5 mg/L BAP+ 0.5 mg/L GA_3 + 100 mg/L ascorbic acid +150 mg/L citric acid

*All values are means ±SE. Mean values in each column/row followed by the same lower-case letter(s) are not significantly different ($P < 0.05$) by the FCRD test.acid

EM2**:** MS + 3.5 mg/L BAP+ 0.5 mg/L GA_3 + 100 mg/L ascorbic acid +150 mg/L citric acid

*All values are means ±SE. Mean values in each column/row followed by the same lower-case letter(s) are not significantly different ($P < 0.05$) by the FCRD test.

In vitro **regeneration using leaf explants in cv. Poona Fig:** Tender leaves were cut across the midrib and placed with adaxial surface up for callusing on MS medium supplemented with six different hormonal combinations (Table 3) along with 100 mg/L ascorbic acid and 150 mg/L citric acid. They were initially kept in dark for a week and then incubated at 25 °C with 16 h light period and 8 h dark period. All treatments of regeneration experiments had three replicates with 25 explants in each replication. Nine weeks after culture, the calli were transferred to shooting medium *i.e.* MS medium with ten different hormonal combinations (Table 4). Subculturing of cultured material was done after every 4 weeks. Shoots were transferred to MS medium with 1.0 mg/L IBA for rooting. Plantlets thus produced were transferred to pots containing coco peat and farmyard manure (2:1), and irrigated with water at regular intervals. They were initially covered with plastic bags for a week and then kept in polycarbonated polyhouse.

Results and discussion

Preparation of explant: Contamination of axillary shoot tip and leaf explants was major problem in fig, during establishment of *in vitro* culture. In all the genotypes tested 0.2 % $HgCl_2$ for 7 min was found to be optimum for shoot tip sterilization. Though 0.2 % $HgCl_2$ for 9 min gave highest sterilization but percent explant establishment was low. Decrease in concentration of disinfectant and duration of treatment resulted in high percentage of contamination while increase in concentration leads to browning of shoot tip. Sterilization with 0.1 % $HgCl_2$ for 7 min was found to be optimum for leaf explants of Poona Fig. Higher $HgCl_2$

Table 2. Response of fig genotypes to multiple shoots and root induction

Genotype	Treatment	Rooting (%)	Shoot length (mm)	Number of leaves/ shoot	Newly formed shoots (#)
Poona Fig	RM1	24.4±0.75[a]	23.0±0.04[a]	1.7±0.02[a]	0.6±0.04[b]
	RM2	0[c]	14.0±0.04	1.0±0.02[c]	0
	RM3	0[c]	23.0±0.03[a]	2.2±0.02	0
	RM4	0[c]	19.1±0.04	1.8 ±0.02[a]	0.5±0.02[b]
Brown Turkey	RM1	26.7±1.26[b]	15.1±0.04	1.8 ±0.03[a]	0.8±0.04
	RM2	0[c]	10.1±0.09	1.0 ±0.03[c]	0
	RM3	0[c]	22.0±0.02	2.3±0.03	1.0±0.04
	RM4	0[c]	18.0±0.04	1.8 ±0.02[a]	0
Conadria	RM1	73.3±1.23	47.5±0.04	8.1±0.09	4.7±0.07
	RM2	30±1.19	37.6±0.04	6.5±0.04	3.3±0.02
	RM3	25.6±0.75[ab]	37.8±0.03	5.7±0.04[b]	3.5±0.03
	RM4	0[c]	50.2 ±0.06	5.7 ±0.04[b]	2.7±0.04
Deanna	RM1	52.2±0.63	30.0±0.05	6.3±0.04	2.5±0.04
	RM2	0[c]	42.7±0.06	9.0±0.04	3.2±0.02 [c]
	RM3	0[c]	35.3±0.03	7.5±0.04	3.8±0.03
	RM4	0[c]	25.0±0.02	4.7±0.04	3.3±0.02 [c]
CD at 5%		1.82	0.13	0.10	0.09

RM1: MS + 1 mg/L IBA, RM2: MS + 2 mg/L IBA, RM3: MS + 3 mg/L IBA, RM4: MS + 4 mg/L IBA

*All values are means ±SE. Mean values in each column/row followed by the same lower-case letter(s) are not significantly different ($P < 0.05$) by the FCRD test.

concentrations (0.2-0.4 %) and 4 % NaClO proved to be more toxic leading to browning and death of leaf explants.

Shoot tip culture establishment: Browning due to oxidation of phenolic compounds, released from the cut ends of the explants is a major problem during *in vitro* cultures of woody plant species. In preliminary experiments it was observed that browning of explants hindered explant establishment in cultivar Poona Fig. The shoot tips that survived showed poor growth in terms of shoot elongation and leaf emergence. There was high-browning in both shoot buds and leaf tissues. Therefore in the present study antioxidants such as ascorbic acid and citric acid were used in all medium to reduce browning. Soliman *et al*. (2010) used different antioxidants such as polyvinyl-pyrrolidone, citric acid and ascorbic acids to control browning.

During shoot tip culture, it was observed that establishment of explants was significantly influenced by genotype and growth regulator used (Table 1). Highest shoot tip establishment was observed on MS medium supplemented with 2.5 mg/L BAP and 0.5 mg/L GA_3. However in Conadria optimum shoot tip establishment was observed on MS medium supplemented with 3.5 mg/L BAP and 0.5 mg/L GA_3. Establishment was higher in three genotypes *i.e.* Deanna (62.5-100 %), Conadria (79.2-87.5 %) and Brown Turkey (53.3-76.7 %). However, response to shoot tip establishment was very poor in Poona Fig (11.7-13.3 %). BAP have been reported to be useful in fig shoot establishment and proliferation (Kumar *et al.,* 1998; Kim *et al*., 2007). Mustafa and Taha (2012) reported enhanced shoot multiplication and callus formation from shoot tip explants of different fig cultivars using 2.5 mg/L BAP. Hepaksoy and Aksoy (2006) used combination of BAP, GA_3 and IBA for *in vitro* shoot tip culture as well as in multiplication medium in fig. Genotype specific response during *in vitro* culture in fig has also been reported earlier (Hepaksoy and Aksoy, 2006; Kim *et al*., 2007; Dhage *et al*., 2012).

Number of leaves per shoot also varied with genotype and culture medium used (Table 1). Cultivar Poona Fig showed poor leaf bearing with only 17.7 % shoots having multiple leaves as compared to other three genotypes (52.5-95 %). There was not much effect of genotype and growth regulator on days to bud sprouting (6.1-7.0 days) and leaf emergence (7.1-7.9 days). Earliest bud sprouting (6.1 days) and leaf emergence (7.1 days) was observed in Conadria genotype. Callus formation at the base was observed in Brown Turkey and Poona Fig after 12-16 days.

Multiple shoot formation was observed only in Deanna (41.7 and 60.0 %) and Conadria (52.5 and 39.17%) at BAP concentrations of 2.5 mg/L and 3.5 mg/L, respectively with GA_3 (0.5 mg/L). However, no multiple shooting was observed in genotype Poona Fig and Brown Turkey in either treatment. Shoot tip culture studies clearly indicated that Poona Fig was very poor in response to initial *in vitro* morphogenesis as compared to other genotypes.

Multiple shoot induction and rooting: Individual shoots obtained from shoot tip culture were transferred to rooting medium containing four different IBA combinations (1-4 mg/L). However, multiples shoots were observed in addition to rooting. Response to shoot multiplication was highly genotype specific (Table 2). Higher new shoot induction was observed in Conadria (2.7-4.7 additional shoots/explant) and Deanna (2.5-3.8 additional shoots/explant). Negligible new shoot induction was observed in Poona Fig (0-0.6 additional shoots/explant) and Brown Turkey (0-1.0 additional shoots/explant). Multiple shoots may be likely due to carry over effect of previous medium.

Genotype specific response to IBA treatment was observed in terms of shoot length after 30 days of culture. Higher shoot length was observed in Conadria (37.6-50.2 mm) and Deanna (25.0-42.7 mm), as compared to Poona Fig (14.0-23.0 mm) and Brown Turkey (10.1-22.0 mm). Average number of leaves / shoots after 30 days of culture, was highest in Deanna (9.0) at 2 mg/L IBA followed by Conadria (8.1) at 1 mg/L IBA concentration. Even in other IBA treatments, high leaf bearing was observed in Conadria (5.7-6.5) and Deanna (4.7-7.5). However leaf bearing was very poor in Poona Fig (1.0-2.2) and Brown Turkey (1.0-2.3), with best treatment being 3 mg/L IBA in both cases.

Highest rooting percentage was observed in MS medium supplemented with 1 mg/L IBA in all genotypes *i.e.* Conadria (73.3 %), followed by Deanna (52.2 %), Brown Turkey (26.7 %) and Poona Fig (24.4 %) (Table 3). However, in Brown Turkey shoots exhibited only aerial rooting. Cultivar Conadria also showed significant rooting at 2 mg/L and 3 mg/L IBA concentrations. However, there was almost no rooting in rest of genotype-IBA treatment combinations. Earlier, there are reports of rooting in fig at varying levels of IBA *i.e.* 0.5 mg/L IBA and NAA (Danial *et al*., 2014); 1 mg/L IBA (Yakushiji *et al.*, 2003; Soliman *et al*., 2010); 2 mg/L IBA (Kumar *et al.,* 1998) and hormone free medium (Yakushiji *et al.*, 2003; Kim *et al.,* 2007).

In vitro **regeneration using leaf explants in cv. Poona Fig:** Poona Fig is very popular local cultivar in western India due to its good flavor, easily removable skin and soft seeds. However this variety showed very poor response to shoot tip culture therefore leaf explants from field were used for regeneration study. Most of the reports published are based on use of leaf explants derived from shoot tip culture in fig (Yakushiji *et al.*, 2003; Soliman *et al*., 2010; Dhage *et al.,* 2012).

Browning was observed in all media, of which least browning (18.7-28.0 %) was observed on medium containing only 2,4-dichlorophenoxy acetic acid (2,4-D), while highest incidence of browning (58.7-68.0 %) was observed on both combinations of BAP and α-naphthalene acetic acid (NAA). Days required for callus treatments also varied with treatments, ranging from 35.3-45.7 days. Late callus formation (48.3-49.0 days) was observed on medium containing combinations of kinetin and 2,4-D.

Among all treatments, calli induction frequency varied from 21.3 % to 89.3 % (Table 3). Highest (89.3 %) and earliest callus

Table 3. Callus formation in cv. Poona Fig after 60 days of inoculation

Hormonal concentration (mg/L)		Callusing (%)	Browning (%)	Days to callus initiation
BAP	NAA			
8.0	8.0	21.3±0.92	68.0±1.4	45.7±0.88
10 .0	10.0	60.0±1.64	58.7±0.77	40.3±0.88 [a]
2,4-D	Kinetin			
2.0	0	66.7 ±1.32	28.0±1.5	41.7 ±0.33 [a]
2.0	0.2	48.0±1.64	40.0±1.4	49.0±0.58 [b]
4.0	0	89.3±2.38	18.7±0.99	35.3±0.33
4.0	0.4	41.3±0.77	41.3±0.77	48.3 ±0.33 [b]
LSD (P=0.05)		4.75	3.60	1.87

*All values are means ±SE. Mean values in each column/row followed by the same lower-case letter(s) are not significantly different ($P < 0.05$) by the CRD test.

induction was observed when leaf explants were cultured on MS medium supplemented with 4.0 mg/L 2,4-D. Next highest calli formation (66.7 %) was observed on medium containing 2.0 mg/L 2,4-D. This is as expected since it is well known that 2,4-D serves as a callusing agent at higher concentrations. Addition of kinetin resulted in significant reduction of callusing and increase in browning at both 2,4-D concentrations. This result contradicts earlier reports of Soliman *et al.* (2010) of higher callus formation (86%) obtained on MS medium supplemented with 2 mg/L 2,4-D and 0.2 mg/L kinetin.

Sixty per cent callusing was observed on MS medium supplemented with 10.0 mg/L BAP and 10.0 mg/L NAA. Lowest callus formation (21.33 %) was observed on medium supplemented with 8.0 mg/L BAP and 8.0 mg/L NAA. Previously, Soliman *et al.* (2010) reported 73 and 68% callusing, respectively on MS medium with 10.0 mg/L BAP and NAA each and 8.0 mg/L BAP and NAA each.

For shoot induction ten different medium were used, out of which shoot induction was observed only in five of them, three of which were supplemented with thidiazuron (TDZ) and NAA (Table 4). These three NAA and TDZ combinations showed earliest shooting (26.0-35.7 days); longest shoot length (19.2-22.3 mm); most shoots/ callus (2.4-2.8). Highest shoot induction (82.7 %) was observed in MS medium supplemented with 7.0 mg/L TDZ and 0.25 mg/L NAA. TDZ has been reported to be efficient in stimulating adventitious shoot production in several recalcitrant woody plants including fig (Huetteman and Preece, 1993). Soliman *et al.* (2010) reported optimum indirect shooting on medium comprising 7 mg/L TDZ in combination with 0.25 mg/L NAA.

Shooting was also observed in MS medium supplemented with 8.0 mg/L N6-[2-Isopentyl] adenine (2iP) + TDZ 2.0 mg/L and 8.0 mg/L BAP + 2.0 mg/L kinetin. Later treatment showed delayed shooting (44 days), poor shoot growth (12.2 mm) and lowest multiples (1.3). High browning was observed in the treatments that failed to induce shoot. After shooting for 4-5 weeks regenerated shoots were rooted on full-strength MS medium with 1 mg/L IBA and acclimatized into pots containing cocopeat and manure in polycarbonate polyhouse.

In summary, highly efficient shoot multiplication and regeneration protocol was developed. Shoot bud breaking at 2.5-3.5 mg/L BAP + 0.5 mg/L GA_3 and further inoculation at 1.0 mg/L IBA resulted in multiple shooting as well as rooting. Best regeneration was observed after callusing from leaf explants on 4.0 mg/L 2, 4 D followed by subsequent shooting on 7 mg/L TDZ and 0.25 mg/L NAA. The present investigation may be helpful for commercial micropropagation of different varieties of fig.

Table 4. Details of shoot induction from calli of cv. Poona Fig

Hormonal concentration (mg/L)		Percent shoot induction	Days to shoot induction	Shoot length (mm)	Number of shoots/ explant
NAA	TDZ				
0.25	7.0	82.7±0.99	30.7±0.88	20.2±0.09	2.8±0.15
0.50	7.0	64.0±1.38	26.0±0.58	22.3±0.09	2.5±0.09[a]
0.25	8.0	25.3±2.31[a]	35.7±0.88	19.2±0.09	2.4±0.06[a]
2iP	TDZ				
8.0	2.0	45.3±2.03	41.0±0.58	21.6±0.09	2.0±0.06
10.0	2.0	0.0	0.0	0.0	0.0
20.0	2.0	0.0	0.0	0.0	0.0
2iP					
30.0		0.0	0.0	0.0	0.0
BAP	Kinetin				
8.0	2.0	22.7±0.92[a]	44.0±0.58	12.2±0.12	1.3±0.06
8.0	4.0	0.0	0.0	0.0	0.0
10.0	2.0	0.0	0.0	0.0	0.0
LSD (P=0.05)		3.39	2.25	0.30	0.28

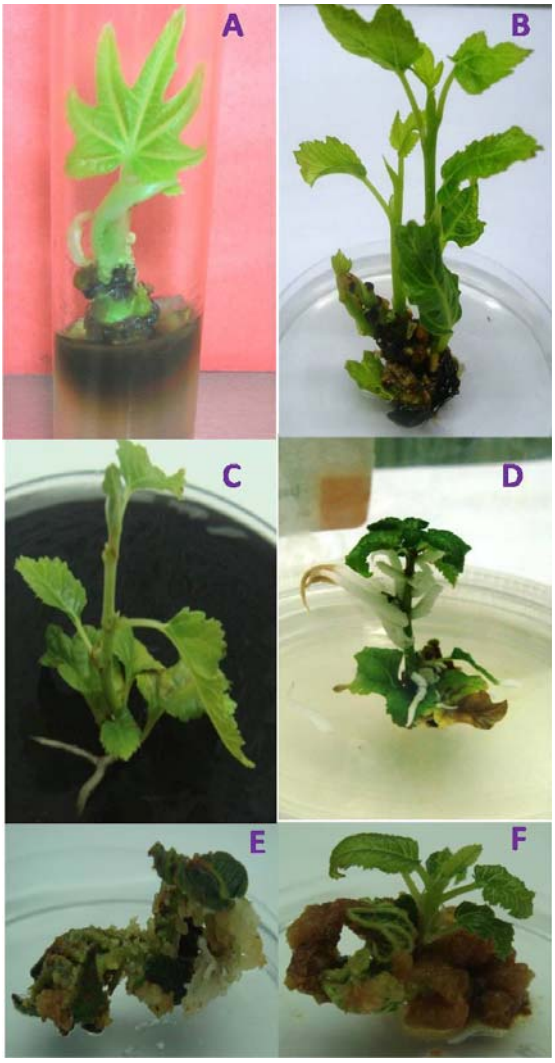

Fig. 1. *In vitro* regeneration in fig. (A) Shoot tip establishment on MS+2 mg/L BAP + 0.5 mg/L GA_3 in Conadria; (B) Multiple shoot induction MS + 1 mg/L IBA in Conadria; (C) Rooting; (D) Aerial rooting in cv. Brown Turkey:; (E): Callus initiation from cv. Poona Fig leaf explant on 4 mg/L 2,4-D; (F): Shoot induction from the callus on MS + 0.25 mg/L NAA + 7 mg/L TDZ in cv. Poona Fig.

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