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Direct *In Vitro* Regeneration of Fig (*Ficus carica* L. CV. Benaty) from Apical and Lateral Buds

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Abstract

Benaty fig plant was successfully propagated by tissue culture technique and a reproducible and reliable micropropagation protocol was developed through this investigation. At initiation stage, healthy aseptic cultures from lateral and terminal bud explants of about 85% were achieved when the explants were treated with 0.15% $HgCl_2$ for 7 minutes. A combined solution of ascorbic acid and citric acid (100 and 150 mgl^{-1}) was successfully used to overcome the problem of phenolic compounds exudation from the explants into the culture media. At shoot multiplication stage, the addition of 2.0 mgl^{-1} from both BA and kinetin gave the highest number of shoots per explant estimated at 2.66 and 3.33 shoots/ explant respectively. As well as, activated charcoal promoted shoots multiplication in all multiplication parameters. The highest number of shoots per explants (5 shoots/ explant) was obtained on MS medium containing 4.0 gl^{-1} AC. At root formation stage, the interaction treatment of full MS salt strength with the use of 0.25 mgl^{-1} NAA gave the highest rooting percentage reaching 87.5%. The highest number of roots (18.5 roots/ explant) was recorded as well from the combined treatment of full MS salt strength with the use of 0.25 mgl^{-1}NAA. While the longest roots (6.66 cm) were recorded from the interaction treatment of half salt strength and 0.50 mgl^{-1}IBA. The addition of activated charcoal to the rooting culture medium decreased the rooting percentage from 87.5% at control treatment to 50% and 62.5% when 2.0 and 4.0 gl^{-1}AC were respectively added. The *in vitro* propagated fig plantlets were gradually moved in a successful way from lab to field conditions with 95% survival rate.

Introduction

Fig (*Ficus carica* L.) belongs to the family *Moracea* also known as common fig. it is considered as one of the most traditional Mediterranean crops.Fig is one of the important trees cultivated in Iraq but not widely as other fruits like apple, apricot and nut fruits. It is cultivated in Kurdistan more than other parts of Iraq and has a great success in Duhok Governorate. It is not cultivated in large areas as big orchards but present in small orchards and is cultivated with other fruits. Fig trees are sometimes cultivated as ornamentals in some parts of Kurdistan Region of Iraq. Fig trees are low water required crops (Mustafa and Taha 2013).Fig trees are usually propagated through several vegetative ways. Sexual seed propagation of fig by using of seeds is not the preferred one; this is because their seeds are nonviable. So it is propagating through vegetative ways including cuttings, grafting and layering, are the only traditional ways to propagate fig trees. In propagation by cuttings only 20-30% of the cuttings are usually survive due to poor rooting (Mustafa and Taha, 2012).

Nowadays, micropropagation is considered as the most important technique for propagation of most woody plants.

In the case of large scale propagation, a high number of cuttings will be required for mass production to increase cultivated area of fig and that will not be available, thus micropropagation may be a better success method to overcome such problems. Generally, *in vitro* rejuvenation has been known as a base in changing vegetative characters such as leaf shape and shoots vigor and increase in rooting ability. The use of tissue culture techniques offers new prospects for fast multiplication of many plant species. Recently, studies on *in vitro* propagation of woody plants have found that these techniques may be suitable for rapid propagation of selected trees (Bonga and Durzan, 1987; Ahuja, 1991).

By plant tissue culture of fig plant, it is possible to produce a pathogen-free plant which is one of the greatest objectives for a successful commercial fig orchard. Also plant tissue culture technique has advantages of mass production, giving plantlets whenever needed (Pasqual & Ferreira, 2007). The usage of this technique is advantageous in production of qualified disease free plants, true to type plants production independent on seasonal and other environment conditions in a smaller space (Debergh and Zimmerman, 1991).

The current investigation aimed to develop a reliable and successful *in vitro* culture protocol for fig plant to overcome the obstacles that usually face the conventional propagation of such kinds of trees and developing a procedure for mass production for the micropropagated plantlets after testing various growth regulators at different concentrations.

Materials and methods

This study was done in the laboratory of Plant Tissue Culture at the Department of Horticulture, Faculty of Agriculture- University of Duhok, Iraqi Kurdistan Region. The explants including lateral and terminal buds were surface sterilized by immersion in 0.15% mercuric chloride ($HgCl_2$) for 7 and 10 minutes. Working within the confines of a Laminar-air-flow hood, lateral and terminal buds with about 1cm length were removed and then cultured on a premixed modified MS medium supplemented with 0.2 mgl^{-1} NAA, 1 mgl^{-1} GA_3 and 1 mgl^{-1} BA. The medium contained as well 30 gl^{-1} sucrose, 7 gl^{-1} agar and 0.4 mgl^{-1} Thiamine-HCl. The pH of the medium was adjusted to 5.7 prior to autoclaving for 20 minutes at 121ºC and 1.05 kg/ m^2 pressure. The explants were put into Petri dishes by using forceps and fine tip scalpels. Three explants were cultured on each culture vessel and their cut bases embedded in the culture medium and each treatment was replicated five times. The cultures were kept at constant temperature (25± 2°C) and exposed to 16 hours daily to 1000 lux illumination. The responded explants from the establishment stage were transferred to the multiplication stage by testing BA at 0, 1.0, 2.0, 3.0 and 4.0 mgl^{-1} and kinetin at 0, 2.0, 4.0, 6.0 and 8.0 mgl^{-1}. Activated charcoal was added at this stage with the best cytokinin concentration by testing its concentrations at 0, 2 and 4 gl^{-1}. At rooting stage, IBA and NAA were used at 0, 0.25, 0.50, 0.75 and 1.00 mgl^{-1} with the use of different MS salts concentrations including ¼, ½ and full strengths. Activated charcoal was added at this stage with the best auxin concentration and MS salt strength 0, 2 and 4 gl^{-1} activated charcoals. All the experiments were designed according to a completely randomized design (CRD). Duncan's multiple range test ($P \leq 0.05$) was used for the comparison between means by using a computerized program of SAS (SAS, 2001).

RESULTS AND DISCUSSION

When both kinds of explants were cultured on the nutrient media, a common phenomenon was observed regarding the exudation of phenolic substances from the explants (Figure 1, A). These oxidized phenols accumulated in the culture medium as brown-black dye. The uptake of the phenols and polyphenols by the explants caused their intoxification, turned black in color and eventually died. This problem has been

reported by several investigators regarding woody trees cultures (George *et al.*, 2008). To overcome this, anti-oxidant agents including ascorbic and citric acids were used to prevent browning.

A combined solution of ascorbic acid and citric acid (100 and 150 mgl^{-1}) was prepared and then the explants were soaked on for 30 minutes prior sterilant disinfestation. This step enables us to get rid of phenolic compounds at this stage (Figure 1, B)**.**

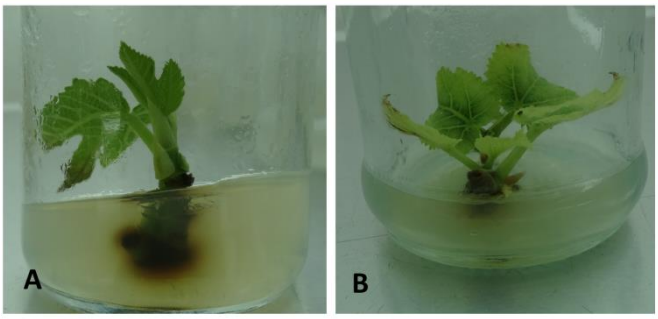

Figure (1): A. Phenolic compounds exudation from fig explants after culture on MS medium.
B. Healthy cultures without any phenolic compounds exudation after treating with ascorbic acid and citric acid (100 and 150 mgl^{-1}).

In general, terminal buds performed better than lateral buds at initiation stage. So, the microshoots produced from terminal buds were transferred to the next stage for shoot multiplication.

At shoot multiplication stage, the problem of phenolic compounds exudation appeared again, but it was overcome by reculture after 20 days for one time. In addition of that, the cultures were covered by an aluminum foil at first three days from culture (Pasqual & Ferreira, 2007 and Komlekcioglu et al., 2007).

Table (1) shows the effects of BA and Kinetin on shoot multiplication stage of Benaty fig trees. It can be noticed that the addition of 2.0 mgl-1 from both cytokinins gave the highest number of shoots per explant estimated at 2.66 and 3.33 shoots/ explant for both BA and Kinetin respectively. The addition of high BA and kinetin concentrations (4.0 mgl-1 BA and 8.0 mgl-1 kinetin) reduced the number of shoots per explant even when compared with the control by giving only 1.5 and 1.66 shoots per explant respectively. The addition of 1.0 mgl-1 BA gave the longest shoots (3.50 cm). Whereas, the highest number of leaves (11.00 leaves/ explant) was achieved by the addition of 2.0 mgl-1 kinetin which was the superior treatment significantly except when compared to 4.0 mgl-1 which gave 10.00 leaves per explant (Figure 2).

Table (1): The effect of different BA and Kinetin concentrations on the shoot multiplication of Benaty figs six weeks after culture

Cytokinins (mgl^{-1})	Number of Shoots/ Explant	Mean Length of Shoots (cm)	Number of Leaves/ Explant
Control (0.0)	1.33ef	2.73a-c	5.66cd

BA (1.0)	2.66a-c	3.50a	9.33ab
BA (2.0)	2.66a-c	2.50bc	7.00cd
BA (3.0)	2.33a-e	2.66a-c	7.33b-d
BA (4.0)	1.50d-f	2.75a-c	6.50cd
Kinetin (2.0)	3.33a	3.23ab	11.00a
Kinetin (4.0)	2.66a-c	3.33ab	10.00a
Kinetin (6.0)	2.50a-d	3.08a-c	7.50bc
Kinetin (8.0)	1.66c-f	2.66a-c	6.00cd

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

The reasons behind the positive role of cytokinins on the multiplication stage might be due to their profound role in releasing lateral buds from the dominance of terminal buds without the need to remove the apical bud by promoting formation of xylem tissues of buds which facilitate the transformation of water and nutrient leading to lateral bud growth (Mohammed and Al-Younis, 1991). Nevertheless, the important role of cytokinins in the increasing the synthesis of RNA, protein and enzymes inside the cell which enhance bud growth as well (Al-Rifae´e and Al-Shobaki, 2002).

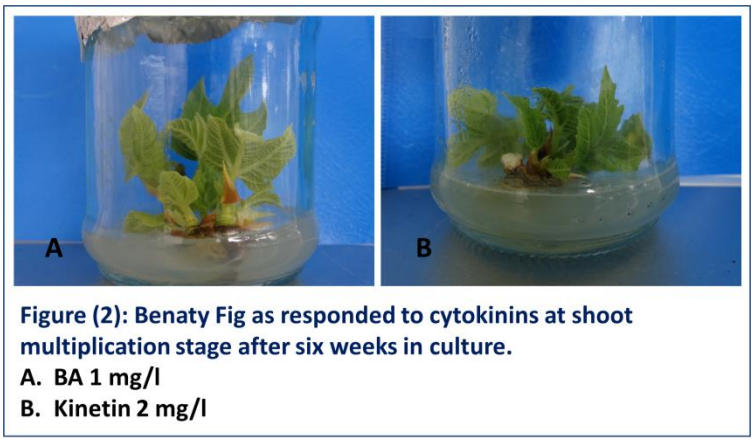

Figure (2): Benaty Fig as responded to cytokinins at shoot multiplication stage after six weeks in culture.
A. BA 1 mg/l
B. Kinetin 2 mg/l

Activated charcoal was added at this stage with the best cytokinin concentration (2 mgl^{-1} kinetin) with Benaty cultivar at 0, 2.0 and 4.0 gl^{-1} activated charcoal to test its role in multiplication of fig. The data in Table (2) show that activated charcoal promoted shoots multiplication in all multiplication parameters. The highest number of shoots per explants (5 shoots/ explant) was obtained on MS medium containing 4.0 gl^{-1} AC, which was significantly different from activated charcoal-free medium. The longest mean length of shoots (4.50 cm) was obtained with 2.0 gl^{-1} AC in which was significantly different from control medium. On the other hand, the highest number of leaves (16 leaves/ explant) was recorded on the medium containing 4.0 gl^{-1} AC and significantly differed from both media containing 0.0 and 2.0 gl^{-1} AC which gave 11.00 and 11.33 leaves per explants respectively.

Table (2): Effect of activated charcoal on shoot multiplication stage of Benaty fig plants after six weeks in culture on MS medium supplemented with 2.0 mgl^{-1} kinetin

Activated Charcoal (gl^{-1})	Number of Shoots/ Explant	Mean Length of Shoots (cm)	Number of leaves/ Explant
0.0	3.33 b	3.23 b	11.00 b
2.0	3.66 ab	4.50 a	11.33 b
4.0	5.00 a	4.33 ab	16.00 a

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

The enhanced role of AC may be due to the adsorption of inhibitory and undesirable substances existing in the culture medium, regardless to its role in inhibiting growth due to adsorbing of growth regulators and other growth promoting substances and releasing of some helpful natural substances that promote growth (Pan and van Staden, 1998 and Fridborg*et al.*, 1978 and Horner *et al*.,1977).

At rooting stage, two auxins including IBA and NAA were tested at 0, 0.25, 0.50, 0.75 and 1.00 mgl^{-1} with the use of different MS salts strengths quarter, half and full Benaty cultivar. Table (7) shows that the addition of NAA at 0.75 mgl^{-1} gave the highest rooting percentage (45.83%), whereas the addition of 0.5 mgl^{-1} NAA gave the highest number of roots (11.44 roots/ explant). On the other hand, the longest roots (4.00 cm) were obtained when 0.50 mgl^{-1} IBA was added to the culture medium. Concerning the effect of MS salts strength, the highest rooting percentage (52.77%); the highest number of roots (9.72 roots/ explant) and the longest roots (3.26 cm) were recorded with the use of full strength MS salts. These parameters were significantly different from the two other MS salt strengths except the number of roots which did not differ from the number recorded with half salt strength (8.27 roots/ explant). The interaction treatment of full MS salt strength with the use of 0.25 mgl^{-1} NAA gave the highest rooting percentage (87.5%) which was significantly different from the rest of treatments except the treatment of full strength salts with 0.75 mgl^{-1} IBA which gave 75.00%. The highest number of roots (18.5 roots/ explant) was recorded as well from the combined treatment of full MS salt strength with the use of 0.25 mgl^{-1} NAA (Figure 3). The longest roots were recorded from the interaction treatment of half salt strength and 0.50 mgl^{-1} IBA reached to 6.66 cm which was significantly different from the rest combined treatments except the treatments of full salts strength with both 0.25 mgl^{-1} NAA and 0.50 mgl^{-1} IBA.

Table (3): Rooting Stage of Benaty fig tree after six weeks in culture on different MS salts strengths and various concentrations of NAA and IBA

MS Salts Strength	Auxins Concentration (mgl^{-1})	Rooting Percentage (%)	Mean of MS Salts for Rooting Percentage	Number of Roots/ Explant	Mean of MS Salts for Number of Roots/ Explant	Mean Length of Roots (cm)	Mean of MS Salts Strength for Length of Roots
¼ MS Salts	0.0	0 f	2.77 c	0 e	0.59 b	0 g	0.16 c
	NAA 0.25	0 f		0 e		0 g	
	NAA 0.50	25 e		5.33 c-e		1.5 fg	
	NAA 0.75	0 f		0 e		0 g	
	NAA 1.0	0 f		0 e		0 g	
	IBA 0.25	0 f		0 e		0 g	
	IBA 0.50	0 f		0 e		0 g	
	IBA 0.75	0 f		0 e		0 g	
	IBA 1.0	0 f		0 e		0 g	
½ MS Salts	0.0	0 f	38.88 b	0 e	8.27 a	0 g	2.25 b
	NAA 0.25	37.5 d		7.66 cd		2.5 ef	
	NAA 0.50	50 cd		15.33 ab		1.5 fg	
	NAA 0.75	37.5 d		6 cd		1.5 fg	
	NAA 1.0	50 cd		13.33 ab		2 ef	
	IBA 0.25	0 f		0 e		0 g	
	IBA 0.50	62.5 bc		15.5 ab		6.66 a	
	IBA 0.75	62.5 bc		3 de		2 ef	
	IBA 1.0	50 cd		13.66 ab		4.33 bc	
	0.0	0 f	52.77	0 e	9.72	0 g	3.26

1/1 MS Salts	NAA 0.25	87.5 a	a	18.5 a	a	5.5 ab	a
	NAA 0.50	50 cd		13.66 ab		2.83 d-f	
	NAA 0.75	50 cd		16.66 a		2.5 ef	
	NAA 1.0	50 cd		10.66 bc		3.26 c-e	
	IBA 0.25	37.5 d		7 cd		1.5 fg	
	IBA 0.50	62.5 bc		10 bc		5.5 ab	
	IBA 0.75	75 ab		7.16 cd		4.43 bc	
	IBA 1.0	62.5 bc		4 de		3.96 cd	

Mean of Auxins			
Auxins Concentration (mgl^{-1})	Rooting Percentage(%)	Number of Roots/ Explant	Mean Length of Roots (cm)
0.0	0 e	0 e	0 f
NAA 0.25	41.66 ab	8.72 ab	2.66 bc
NAA 0.50	41.66 ab	11.44 a	1.94 c-e
NAA 0.75	29.16 c	7.53 b	1.30 e
NAA 1.0	33.33 bc	7.93 b	1.74 de
IBA 0.25	12.50 d	2.33 de	0.50 f
IBA 0.50	41.66 ab	8.50 ab	4.00 a
IBA 0.75	45.83 a	3.38 cd	2.14 b-d
IBA 1.0	37.5 a-c	5.88 bc	2.76 b

These results proved that auxins have a role in rooting process since they promote adventitious roots initiation in the bases of cultured shoots (Abdul, 1987 and Saleh 1990). The differences in the potency of IBA and NAA in promoting rooting might be attributed to the structure of the auxins under study, the endogenous hormone level, as well as the genetic makeup of species under consideration (George *et al*., 2008).The superior effect of full salts strength upon the lower strengths might be due to the high level of endogenous auxin levels in the plant material used in culture which might compensate the need of a higher C/N ratio.

Figure (3): Rooting Stage of Benaty fig tree after six weeks in culture on full MS salts strength enriched with 0.25 mg/l NAA.

Table (4) shows that the addition of activated charcoal to the culture medium decreased the rooting percentage from 87.5% at control treatment to 50% and 62.5% when 2.0 and 4.0 gl^{-1} AC were respectively added. On the other hand, the addition of AC was beneficial in increasing the mean length of roots from 5.5 cm to 7.0 cm and 9.3 cm respectively (Figure 4).

Table (4): Effect of activated charcoal on root formation stage of Benaty fig plants after six weeks in culture on MS medium supplemented with 0.25 mgl^{-1} NAA

Activated Charcoal (gl^{-1})	Rooting Percentage (%)	Number of Roots/ Explant	Mean Length of Roots (cm)
0.0	87.5 a	18.5 a	5.5 a
2.0	50.0 b	11.0 b	7.0 a
4.0	62.5 ab	12.5 b	9.3 a

Different letters within each comparison represent significant differences according to Duncan´s multiple range test at 5% level.

The ideal effect of activated charcoal on rooting is for enhancement but the decreased effect at this study might be due to its act in auxin adsorption especially when lower auxin concentrations are added to the medium (Sharma *et al.*, 2007).

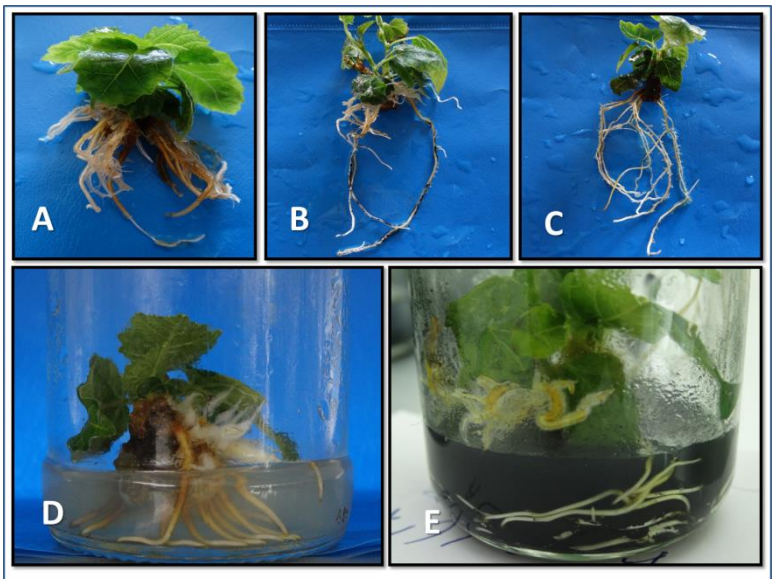

Figure (4): Effect of Activated Charcoal on Benaty Fig Rooting Stage after six weeks in culture on MS medium supplemented with 0.25 mg/l NAA.
A. and D. AC free medium, B. and E 2.0 g/l AC, C. 4.0 g/l AC.

After developing good shoot and root systems, the produced fig plantlets were ready for transplanting to soil. They were removed from culture flasks and their roots were washed with distilled water and immersed in benomyl fungicide (concentration 0.1% for 10 min.) to avoid fungus infections. They were transferred to pots containing a steam sterilized soil mix (peatmoss+ Styrofoam 1: 0.5, v:v) suitable to grow fig transplants. Pots were enclosed in transparent plastic containers, which were closed and placed in a shaded area of a temperature-controlled greenhouse set at 23-25ºC. The plants were irrigated with a nutrient solution containing ¼ strength of MS salts. After 8 to 10 days, the containers were opened and after further 8 to 10 days, the plantlets were removed from the containers and grown under regular greenhouse conditions. This was a critical period since the plantlets were shifted from heterotrophic phase into an auotrophic phase to face the real world. Under *in vitro* conditions, the plantlets had a carefully controlled supply of nutrient, humidity, temperature and photoperiod. During this hardening period, the plants developed normal culticular system with good rooting. Furthermore, the stomata system started regulating water loss by evapotranspiration processes (Maheswaran*et al*., 2000). The above mentioned steps which make fig plantlets to grow under natural conditions are collectively called hardening and this process enhances the plant survival after transplanting. A good performance was found with fig plantlets transferred to soil. A survival rate of about 95% was achieved from the gradually moved plantlets. The most of the plantlets began to grow well and did not show any morphological abnormalities (Figure 5).

Figure (5): Acclimatization stage.

A. Plantlets kept in closed transparent plastic containers.
B. Plantlets kept in opened transparent plastic containers.
C. Plantlets after three weeks from transplanting under greenhouse conditions.
D. After six weeks.
E. After eight weeks.
F. After ten weeks in bigger sized pots.

In conclusion, this investigation provides an optimized and reliable micropropagation protocol for *in vitro* mass production of *Ficus carica* L. Benaty cultivar and can be used for reproduction and conservation of such important fruit crop. It is highly recommended to test more effective factors on fig micropropagation like the use of 2iP, TDZ or other growth regulators at different concentrations and combinations.

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