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REGENERATION AND *Agrobacterium* - MEDIATED TRANSFORMATION STUDIES IN FIG; *Ficus carica* L.

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ABSTRACT

A system for regeneration and *Agrobacterium*- mediated transformation of the common fig*Ficus carica* L. of the cultivar Sultani was developed. Different combinations of BA and NAA or 2,4-D and kinetin of callus formation from leaf segment explants, and the best % formation of callus was found to be 2.0 mg/L 2,4-D and 0.2 mg/L kinetin. Callus produced somatic embryogenesis which differentiated to plantlets on media containing TDZ in combination with NAA or 2ip.The best concentrations which produce somatic embryogenesis and differentiated to plantlets on media were containing30 mg/l 2ip and 7 mg/L TDZ with 0.25 mg/L NAA, which gave the regeneration efficiencies 83 and 79 of respectively.The callus failed to induce organogenesis on media containing BA and kinetin. The best concentrations of growth regulators to produce shoots formation by direct somatic embryogenesis with leaf explants were on media containing 2 mg/L TDZ and 4 mg/L 2ip, which gave the regeneration efficiencies (89%).The best results of shoots forming roots was reached the highest value 95% by using MS medium supplemented with 1.0 mg/ L IBA. Leaf explants of *in vitro* propagated plants were co-cultivation with *Agrobacterium* cell culture containing the binary plasmid pISV2678 which harbor the *gus*-intron and *bar* genes, the results showed that the highest transformation efficiency was obtained when explants incubated for 30 min. with bacterial suspension (17.5%). The transgenic nature of regenerated plants was confirmed by histochemical GUS, leaf painting assay and PCR analysis.

Keywords: Fig (*Ficus carica*); Transformation; Callus; Regeneration; GUS; *bar*; *Agrobacterium tumefaciens*

INTRODUCTION

Fig trees are one of the earliest fruit-bearing trees cultivated. *Ficus carica* (Moraceae), the well-known fig of commerce, is indigenous to wide areas including, Turkey, England, China, India, Hungary, Australia and several countries in the Mediterranean region (Küden,1996). Fig fruit is well known for its nutritive value, and is consumed fresh or dry worldwide. Fig fruits are also known for their mild laxative activity and high alkalinity, and substances derived from them are used in various drug preparations. Other parts of fig trees have also been shown to have a commercial value (Kirtikar and Basu, 1986).Figs are woody plants with highly varied forms: bushes, shrubs, small trees, and very tall and large trees. Roots are very extended, and leaves are usually simple, alternate, entire, or lobate. The plants are usually monoecious, seldom dioecious, with unisexual, small flowers without petals and nectarines, gathered in inflorescences in a closed receptacle. After fertilization, the ovary develops into a small achene, while the whole inflorescence becomes a false-fruits called the syconium (Bajaj, 1991).The application of genetic engineering techniques to stably incorporate

homologous and/or heterologous genetic material into woody species, including fruit trees, offers the potential of obtaining improved planting stocks for agricultural use in a short period of time compared to traditional breeding techniques. In addition, efficient transformation methods can be used for the production of heterologous polypeptides having nutritional and/or pharmaceutical value. The overall efficiency of techniques for genetically modifying plants depends upon the efficiency of the transformation technique(s) used to stably incorporate the desired genetic material into plant cells or tissues, and the regeneration technique(s) used to produce viable plants from transformed cells. Plant formation from callus could be difficult in certain plant species. Studies on differentiation and growth of *Ficus* spp. have been carried out by some workers using *Ficus benjamina*, *Ficus lurate*, and *ficus nitida* (Ibrahim, and Nasr EL-Din, 1990; Arafa, *et.al.*, 1993). Within *ficus* sp. adventitious shoot regeneration has been reported only for F. lyrata from young leaves isolated from plants maintained under greenhouse conditions (Debergh, 1977). *In vitro* studies using *F.carica* were restricted to the development of protocols for micropropagation and production of fig mosaic virus-free plants by single shoot tip culture (Muriithi *et al.*, 1982; Pontikis and Melas, 1986; Demiralay *et al.*, 1998; Günver and Ertan, 1998; Gella *et al.*, 1998) as well as to biochemical assessment of active compounds in fig calli grown *in vitro* (Nassar and Newbury, 1987; Cormier *et al.*, 1989).Within the genus *Ficus*, several reports described regeneration and organogenesis from calli and other explants. *F. religiosa* plants were regenerated from calli of stem segments (Jaiswal and Narayan, 1985) and from axillary buds of mature trees (Deshpande *et al.*, 1998).Regeneration of *F. carica* plants from the apical buds of a mature tree was also reported by (Kumar *et al.*, 1998). However, in all mentioned *in vitro* studies, plant regeneration has been restricted to the use of single shoot tips and apical buds. Recently, (Yakushiji *et al.*, 2003) reported a method for the induction of organogenesis from leaf explants of *F. carica* using phloroglucinol (PG). However, by this method the frequency of adventitious bud differentiation from leaf fragments was relatively low, and no adventitious buds were observed without PG.

The overall objective of the current research was to develop an optimized protocol for *in vitro* regeneration of fig that allows for successful application of *Agrobacterium*-mediated transformation in fig (*F. carica*) commercial cultivar Sultani Egypt (fresh consumption).

MATERIAL AND METHODS

1. Material:

1.1.Explant source:

Shoot tip explants were collected from 7 to 8 years-old mature infected trees of *Ficus carica* L cv. Sultani in Marsa Matrouh and Siwa Oasis of the Northern West Coast. Explants were washed under running tap water for 3 hours.

1.2.Bacterial strain and plasmid vector:

The plasmid pISV2678 (unpublished data) harboring *gus*–intron and *bar* genes (kindly provided by Institut des Sciences Vegetales (ISV), Center

National de la Recherche Scientifique (CNRS), Gif- Sur-Yvette, France) and *Agrobacterium tumefaciens* strain LBA 4404 (Horsch *et al.*, 1985) were used in establishing Fig transformation methods.

2. Methods

2.1. Callus induction of *Ficus carica* L.

The leaves of *F. carica* were taken from *in vitro* plants produced from shoot apexes cultured and cut into 0.5-1.0 cm segments and Explants were placed on Murashige and Skoog (1962) salts and vitamins supplemented with 100 mg/L myo-inositol ,30 g/L sucrose , 2.5 g/L phytagel and contained ((2-10 mg/L BAP combined with 0-10 mg/L NAA)) ,and (1- 5 mg/L 2,4-D combined with 0.0- 0.5 mg/L kinetin) in the present 100 mg/l Ascorbic acid +150 mg/l Citric acid.

2.2. *Ficus carica* regeneration:

2.2.1. Effect of growth regulators on indirect somatic embryogenesis.

Regeneration experiments were carried out initially with *F. carica* cv. Sultani. To examine the effect of culture media on callus differentiated to plantlets, the basal media MS, containing different concentration of (0.0-10 mg/L) BAP combination with (0.0-10 mg/L) Kinetin, or (1.0-10 mg/L) TDZ combination with (0.0-1.25 mg/L) NAA, or (0.0-10 mg/L) TDZ combination with (0.0-30 mg/L) 2ip, or (0.0-5 mg/L) BAP combination with (0.0-5 mg/L) 2ip. Calli were continuously sub cultured on this medium every 3 weeks.

2.2.2. Effect growth regulators on direct somatic embryogenesis.

The leaves of *Ficus carica* were used as explants from *in vitro* (Rooting stage) were taken leaves and preexisting buds were removed. Explants were cut into two pieces longitudinally and cultured on MS media, including different concentration of cytokinins; (0.0-10 mg/L) BAP in combination with (0.0-10 mg/L) Kinetin, or (0.0-10) TDZ in combination with (0.0-10 mg/L) 2ip, or (0.0-10 mg/L) TDZ in combination with (0.0-1.0 mg/L) NAA in order to determine the best combined concentration of BAP and Kinetin or TDZ and 2ip or TDZ and NAA on direct somatic embryogenesis. The regeneration media contained MS basal salt mixture, supplemented wit 100 mg/L myo-inositol, vitamins, 2-4% sucrose (w/v) and 0.3% phytagel at pH 5.7, with 100 mg/L ascorbic acid and 150 mg/L citric acid. The cultures were kept for 7 days in low light intensity at 25°C, in a 16/8 h photoperiod. Explants were examined after 35 days and the percentage of explant producing shoots (regeneration capacity) and the mean of shoots formed per regeneration explant were recorded. All experiments were repeated at least three times..

2.3. Rooting and acclimatization:

Shoots derived from regeneration stage (about 3 cm in length) were transferred to Murashige and Skoog salts and vitamins in addition to 100 mg/L myo-inositol, 30 g/L sucrose and different treatments of 3-Indole Butyric Acid (IBA) and α-Naphthalene Acetic Acid (NAA). Cultures were incubated under the same environmental conditions used for shoot proliferation.

Plantlets produced from rooting stage were transferred from the test tubes under tap water to minimize injury and to free the roots from phytagel. The plantlets were transferred to pots containing a mixture media of peatmoss and sand (1:1), plastic pots enveloped in polyethylene bags were incubated under 3000 Lux light intensity derived from cool white flurescent lamps for 16 hours photo period at 25 ±1°C in growth cabinets. After 4 weeks polyethylene bags were completely opened and after 4 weeks more polyethylene bags were removed and plantlets were maintained under greenhouse conditions.

2.4. *Ficus carica* L transformation:

2.4.1. *Agrobacterium*-mediated transformation

A. tumefaciens strain LBA4404 containing the binary vector pISV2678 with the *bar* and gus-intron reporter genes was used for adapting fig transformation system. The leaf segment explants were incubated for different times (ranged between 5 up to 60 minutes) with *Agrobacterium* culture, blotted on sterile filter paper and the explants were cultured on co-cultivation medium (without antibiotics).After two days at 28±2°C in the dark. The explants were washed in a sterile solution of strength MS medium with 250 mg/L cefatoxime and Leaf explants were transferred to the regeneration medium containing (3.0 mg/L bialaphos and 250 mg/L cefotaxime). Bialaphos was added to select the transformed fig cells whereas the other antibiotic was added to inhibit *Agrobacterium* growth. The cultures were kept in the growth chamber at 26±2°C under 16 hours photoperiod of 3000 Lux supplied with cool white fluorescent lamps. Transformation efficiency was determined by the number of explants expressing GUS.

2.5. Histochemical GUS assay:

Histochemical GUS assay was carried out on transformed explants and regenerated shoots to detect the GUS activity. Tissues were immersed in GUS buffer containing 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-B-D-glucuronoide) and incubated overnight at 37°C as described by Jefferson (1987).

2.6. Assay of BASTA resistance:

Transformants were tested in the greenhouse for the expression of the *bar* gene by painting the leaflets of the transgenic plants with BASTA (a commercial formulation of PPT containing 200 g/L ammonium glufosinate) dilution at a concentration of 1 g/L ammonium glufosinate. The young plants (around 6-8 weeks after acclimatization) were painted by 1 g/L of BASTA solution. Resistances of leaflets were scored after 3-7 days.

2.7. PCR analysis:

Plant genomic DNA was isolated from the youngest three leaves excised from bailaphos-resistant shoots according to Dellaporta *et al.* (1983).

The oligonucleotide primers used for PCR amplification of a 2.07 kbp fragment of the gus-intron gene were: direct GUS-F primer 5´- CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C -3´; GUS-R primer ´5- CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC-3´.Amplification was

performed in aliquots of 25 µl using a thermal cycler. The PCR conditions for amplification of gus-intron gene fragment were 95°C for 5 min, followed by 35 cycles at 94°C for 1 min, 57°C for 1 min, 72°C for 2 min and a final extension at 72°C for 7 min. Applicants were visualized by electrophorsis on 1% agarose gel stained with ethidium bromide.

2.8. Statistical analysis:

Variance analysis of data was done using Anova program for statistical analysis. The differences among means for all treatments were tested for significance at 5% level by using Duncan (1955) new multiple range test as described by Snedecor and Cochran (1967). Means followed by the same letter are not significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

Callus induction of *Ficus carica* L:

Leaves of *F. carica* were taken from virus free *in vitro* plants and cut into 0.5-1.0 cm segments and cultured on solidified medium containing BA and NAA or 2,4-D and kinetin. Six only from all media had ability to form callus on the media used and under that conditions compared to the other treatments which gave negative results for callus formation.

Table 1: The best concentrations of different growth regulators on callus formation of *Ficus carica* from leaf segments explants after five weeks.

concentration (mg/L)	Survival		Formation of Callus	
	No.	%	No.	%
8 mg/L NAA+8 mg/L BA	25 b	83	17 b	68
10 mg/LNAA+10 mg/LBA	30 a	100	22 a	73
2 mg/L 2,4-D	22 c	73	17 b	78
0.2 mg/L Ki+2 mg/L2,4-D	28 ab	93	24 a	86
4 mg/L 2,4-D	20 c	66	16 b	80
0.4 mg/LKi+4 mg/L2,4-D	26 b	87	21 a	81

The best results obtained from these treatments are shown in table (1).The date indicated that the highest survival percentage was 100% on MS medium supplemented 10.0 mg/l of each of BA and NAA, whereas the lowest one was found with 2 and 4 mg/L 2, 4-D. The highest callus formation percentage was 86% on MS medium supplemented with 0.2 mg/L kinetin and 2.0 mg/L 2,4-D compared to the other treatments. Shown in figure (1). Callus was subcultured and maintained on the same medium every month. These results agree with Nasser and Newbury, 1987 obtained callus from stem explants of *F. carica* cv. Brown Turkey on MS medium containing 10 mg/L BAP and 10mg/L NAA, also Narayan and Jaiswal (1986) who obtained callus from leaf explant of *F. religiosa* on MS medium containing 0.5 mg/L 2,4-D. And similar results were observed of peach by Gentile *et al.*, 2002 when

culturing leaf from micropropagated peach shoots on MS medium supplemented with BA and NAA.

***F. carica* regeneration:**

Two methods were evaluated for establishing suitable regeneration methods; indirect somatic embryogenesis and direct somatic embryogenesis.

Effect of growth regulators on direct somatic embryogenesis:

The calli and formed embryo-like structure produced from 10 mg/l of each of NAA and BA were transferred on medium containing different concentrations of NAA and TDZ in order to establish the best concentration for producing somatic embryogenesis. Calli on the concentrations of 7.0 and 8.0 mg/l of TDZ with 0.25 and 0.5 mg/l NAA only were able to develop embryos compared to the other concentrations which gave negative results for somatic embryogenesis. While, the effects of different concentrations of BAP and kinetin on shoot formation from callus were evaluated. It was found that embryos were only performed on 8.0 or 10.0 mg/l of BAP in combination with 2..0 or 4.0 mg/l kinetin, while low concentration of BA up to 6 mg/l had no effect on embryos and the embryos were growing in size and in some rare cases developed into globular and heart stage. Also, different concentrations of 2ip and TDZ were evaluated for the development of somatic embryos from calli. Only 2.0 mg/l of TDZ in combination with 10 and 20 mg/l of 2ip and 30 mg 2ip alone to produce somatic embryogenesis and differentiated to plantlets, whereas the other treatments had no ability to form somatic embryos from callus.

Table:The best concentration of growth regulators of to produce shoots from indirect somatic embryogenesis of *F.carica* cv. Sultani.

Concentration (mg/L)		% of explant forming shoots	Mean number of shoots/explant	Mean length of shoots (cm)
NAA	**TDZ**			
0.25	7.0	79	5.25 **b**	1.75 **c**
0.50	7.0	60	4.20 **c**	1.50 **c**
0.25	8.0	67	3.60 **d**	1.80 **c**
2ip	**TDZ**			
8.0	2.0	43	2.20 **f**	2.40 **b**
10.0	2.0	50	2.40 **f**	2.40 **b**
20.0	2.0	67	3.00 **e**	2.50 **b**
30.0	0.0	83	6.50 **a**	2.75 **a**
BAP	**Kin**			
8.0	2.0	The embryos developed into globular, heart and torpedo stages		
8.0	4.0			
10.0	2.0			

The best concentrations of growth regulators of to produce shoots formation from callus was experiments are shown in Table 2 and figure 2. Showed the best results of the percentage of explant producing shoots were produced at a concentration of 30 mg/l 2ip compared to the other treatments;

was reached (83%). Also, the best results of mean number of shoots perexplant were produced on concentration of 30 mg/l 2ip and 7 mg/L TDZ and 0.25 mg/L NAA ; was reached (6.5 and 5.25,respectively) while, length of axillary shoots formed on the explant were produced on concentration of 30 mg/l 2ip compared to the other treatments; was reached (2.75 cm).These results agree with (Arafa *et al.*, 1993), they found that callus induction and organ differentiation from leaves of *Ficus lyrata* on MS medium containing high concentration of 2ip (30 mg/L). While, (Lai *et al.*, 1988) they found that globular, heart and torpedo shaped somatic embryoids were formed on the surface of the calli at 4-5 weeks of *Ficus religiosa* L. after transfer on MS medium containing 0.1 mg/L BAP and 0.05 mg/L NAA. Also, (Gamage and Nakanishi, 2000) they found that an efficient in vitro system to regenerate shoots from the leaf tissue of apple on MS medium in presence of 20µM TDZ and 0.1 µM NAA in dark.

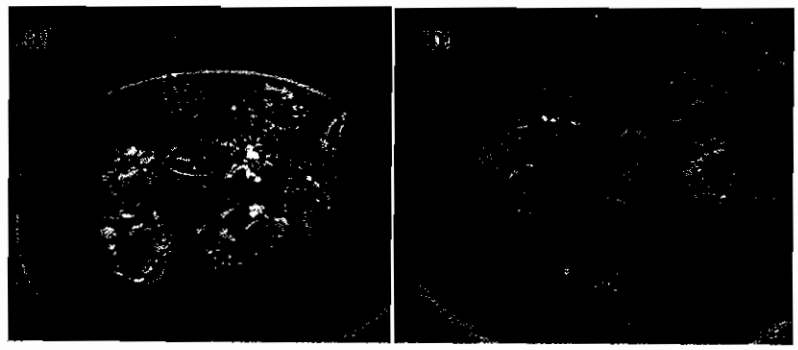

Figure1: Different developmental stages of callus formation on MS medium containing 0.2 mg/L kinetin and 2.0 mg/L 2,4-D. (a) after three weeks, (b) after five weeks.

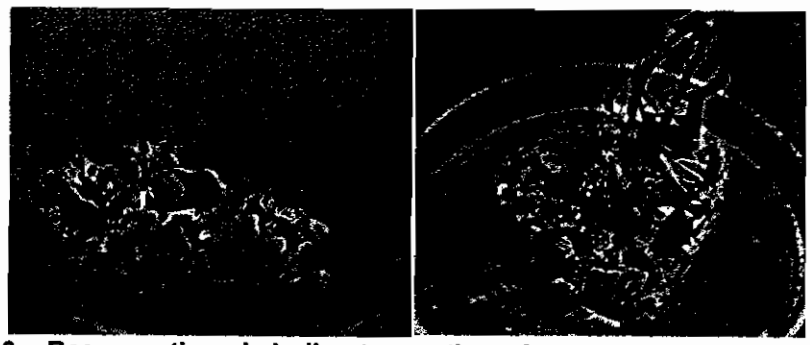

Figure 2: Regeneration via indirect somatic embryo- genesis

Effect growth regulators on direct somatic embryogenesis:

Leaves of *F. carica* were used as explants and preexisting buds were removed. Explants were cultured on MS medium containing different concentrations combinations of BA and kinetin or TDZ and 2ip or TDZ and NAA to establish the best condition for forming direct embryogenesis.

Table 3: The best concentration of growth regulators of to produce shoots from direct somatic embryogenesis of *F.carica* cv. Sultani using leaf segment explants.

Concentration (mg/L)		% of explant forming shoots	Mean number of shoots/explant	Mean length of shoots (cm)
NAA	**TDZ**			
0.2	2.0	25	1.2 **d**	0.5 **d**
0.4	4.0	28	1.4 **d**	0.6 **d**
2ip	**TDZ**			
2.0	2.0	78	2.0 **c**	1.6 **c**
4.0	2.0	89	2.6 **a**	2.1 **b**
6.0	2.0	79	2.2 **bc**	2.8 **a**
BAP	**Kin**			
4.0	4.0	70	2.4 **ab**	1.4 **c**
6 0	4.0	64	2.0 **c**	1.5 **c**

The best concentrations of growth regulators of to produce shoots formation from direct somatic embryogenesis with leaf explant are presented in table 3. The best results of the percentage of explant producing shoots and mean number of shoots perexplant were produced at a concentration of 2mg/l TDZ in combination with 4 mg/l 2ip compared to the other treatments; was reached (89%) and (2.6). While, the best results of mean length of shoots formed perexplant were produced on concentration of 2 mg/l TDZ and 6 mg/l 2ip; was reached 2.8 cm compared to the other treatments which gave the regeneration efficiencies (25-79%), mean number of shoots perexplant (1.2-2.4 shoots/explant) and mean length of shoots formed perexplant (0.5-2.1 cm.) were obtained when leaf explants were grown in a medium containing different concentration of growth regulators. Shown in figure 3. In general the best results for the percentage of explant producing shoots were produced at the concentration of 2mg/l TDZ and 4 mg/l 2ip which gave 83%, while longer formed shoots were produced on the concentration of 2 mg/l TDZ and 6 mg/l 2ip compared to the other treatments. The results agree with (Yakushiji *et al.*, 2003) reported a method for the induction of organogenesis from leaf explants of *F. carica* on MS medium supplemented with different combinations of 2,4-D, TDZ and phloroglucinol (PG) but, this method the frequency of adventitious bud differentiation from leaf fragments was relatively low only (22%), and no adventitious buds were observed without PG. (Lakshmi *et al.*, 1994) similar results were observed of direct somatic embryogenesis and plant regeneration from leaf discs of *Nicotiana tabacum* L. when culturing on MS medium supplemented with 0.1mg/L NAA and 1.0 mg/L BAP.(Griga, 1998) obtained direct somatic embryogenesis from shoot apical meristems of pea on MS medium supplemented with 10 µM (TDZ). TDZ was also able to induce shoot bud regeneration on embryoids without a differentiated shoot apex. TDZ has been reported to be very efficient in stimulating adventitious shoot production in several recalcitrant woody plants (Leblay *et al.*, 1991; Perez *et al.*, 2000). Additional evidence for the influence of TDZ in combination with different auxins on the morphogenic potential of apples was recently provided (Yancheva *et al.*, 2003). The results confirmed the stimulatory effect of TDZ in combination with different auxins or cytokinins on apple adventitious shoot regeneration.

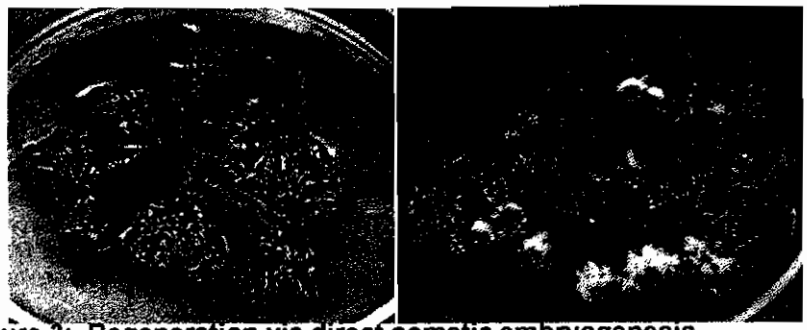

Figure 3: Regeneration via direct somatic embryogenesis

Rooting and acclimatization:

In the present study, *in vitro* elongated shoots for at least 3 cm and shoots of *Ficus Carica* L cv. Sultani were cultured on Murashige and Skoog basal nutrient medium containing 1 mg/L 3-Indole Butyric Acid (IBA) and 0.5 mg/L IBA in combination with 0.5 mg/L α-Naphthalene Acetic Acid (NAA) for 4 - weeks culture peroid. The results in table (4) indicated that the percentage of shoots forming roots was reached the highest value 95% by using MS phytagel gelled nutrient medium supplemented with 1.0 mg/ L IBA under 28 days of light and 92%.with MS medium containing 0.5 mg / L IBA + 0.5mg / L NAA. These results are agreed with those obtained by pontikis and Melas (1986) who found that higher rooting responses (80%) for *Ficus Carica* L. were obtained after 28 - days in light on MS medium containing 1 mg/ L IBA However, Muriithi *et al.* (1982) reported that shoots of *Ficus Carica* L. were rooted on MS medium with 0.5 mg/L NAA + 0.5 mg/ L IBA in darkness for 7 days and transfer to in the presence of light for 3 weeks.Plants produced from rooting stage which were containing 3- 5cm, then rinsed once with water and then transfer into pots containing equal parts of peat and sand, then incubated under transparent plastic bags on 16h photo period at 25C for 4 weeks before transfer to a green house, after 8 weeks from transfer into green house, they were repotted into sterile soil consists equal parts of peat and sand (v/v). Figure (4).These results are agreed with those obtained by (Muriithi *et al.* 1982 and Pontikis and Melas (1986), who reported that for acclimatization of *Ficus Carica* L. the plantlets were transplanted into pots containing equal parts of peat and vermiculite (v/v) before transfer to a greenhouse after 4 weeks. After 8 weeks they were repotted into sterile soil containing equal parts of peat and vermiculite (v/v).

Table 4: The efficiency of shoots forming roots for *Ficus carica* L cv. Sultani after growing on MS medium supplemented with different auxin concentration.

Auxin conc.		% of shoot forming roots	Mean number of roots/shoot	Mean length of roots (cm)
IBA	NAA			
0.0	0.0	7	2.3 c	1.33 d
1.0	0.0	95	3.0 b	3.53 b
0.5	0.5	89	4.87 a	4.07 a

Figure 4: Rooting and acclimatization of Fig plants from tissue culture.

Transformation of *F. carica* L.

Effect of incubation time:

The leaf segment explants were incubated with the *Agrobacterium* cell culture containing the binary plasmid pISV2678 with the *bar* and *gus-intron* reporter genes for 5, 10, 20, 30, 40, 50 and 60 min. shows the results in table (5) which indicated that at 5 and 10 min. only 4.0 and 7.0 explants were transformed out of 80. The rate keeps increasing to reach the highest rate 17.5% for leaf segment explants at 30 min. Increasing the incubation time might have increased the rate of transformation but this could not be shown because the explants were totally destroyed at 40 min.

Table 5: Effect of the time of incubation of the explants with *Agrobacterium* suspension on the transformation efficiency of leaf segment explants.

Time (min.)	Total No. of explant	No. of expressing GUS	% of transformation
5	80	4	5.0
10	80	7	8.7
20	80	9	11.2
30	80	14	17.5
40	80	explants died	explants died
50	80	explants died	explants died
60	80	explants died	explants died

Regeneration of transgenic shoots:

Agrobacterium cell culture containing the binary plasmid pISV2678 which harbor the *gus*-intron and bar genes was cocultivated with divided leaf tissues. The transformed explants were transferred into selective medium supplemented with 3mg/L bialaphos (selective herbicide) and 250mg/L cefatoxime to inhabit *Agrobacterium* growth on the medium. Explants were exposed for 7 days to low intensity light and then transferred to normal light. Table 6, showed the survival percentage of Sultani fig cultivar before and after treatment with *Agrobacterium* on the regeneration medium. It was obvious that the highest callus formation percentage was 83% on the control

medium compared to medium supplemented with bialaphos as a selectable agent (26.5 %), it was also clear that the co cultivation with *Agrobacterium* considerably decrease the regeneration percentage (24.5) compared to control regeneration percentage (71). While, the transformation efficiency was 17.5%.

Table 6: Regeneration and transformation percentages of *Ficus carica* L cv. Sultani.

treatment	% formation of callus	% of forming shoots	Transformation efficiency %
control	83	71	–
Agrobacterium	26.5	24.5	17.5

Detection and screening of transformed plants:

Histochemical colorimetric analysis was according to the method of Jefferson (1987). GUS enzyme activity could be detected in the transformed plants that were resistant to bialaphos contained the bar gene, by histochemical gus assay. The results indicated that GUS activity in the transformed callus and plantlets which co-cultivated with *Agrobacterium* could be detected histochemical for GUS expression after 4 weeks of the callus as the first evidence of transformation figure 5. Plantlets were incubated overnight at 37°C in GUS buffer. The transformation efficiency was determined by calculating the numbers of explants expressing the GUS gene.The results are in agreement with other different plant such as apple, peach, and radish reported by (Martin *et al.* 1990), they reported that, the GUS marker system is useful for early selection.

Figure 5: Histochemical GUS assay for *Agrobacterium*- mediated transformed fig plants.

Leaf painting assay:

The plants were analyzed by testing the expression of the bar gene in the greenhouse by applying the leaf painting assay the young plants with 1 g/L of the herbicide. Within 3 days necrotic spots appeared on the untransformed leaves. Four days after BASTA application, the treated

transgenic leaflets showed complete tolerance, in contrast to non-transformed plants showed leaflets, which were completely necrotic (Figure 6).

Figure 6: Herbicide leaf painting test showing the resistance of transgenic leaf to BASTA application (left) and the control non transgenic leaflet necrotic after painting (right).

PCR analysis of transgenic plants:

PCR analysis of the putative transgenic shoots confirmed the stable incorporation of the transgenic into the *F. carica* genome. All plants selected after transformation with plasmid pISV2678 showed the predicted bands: the 2.07 kbp for the gus-intron. No fragment was amplified in the untransformed plant (Fig. 7).

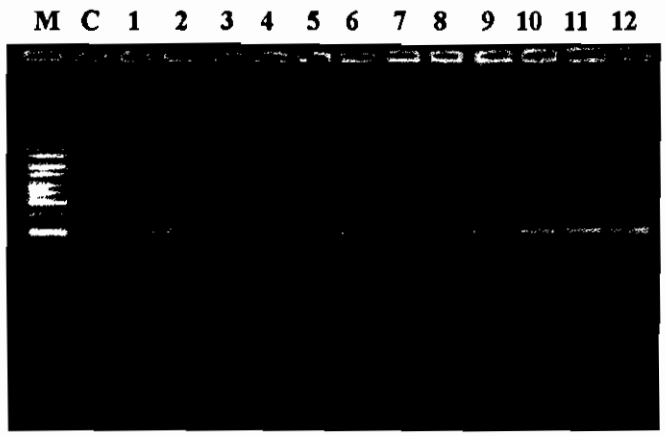

Figure 7: PCR analysis of the putative transgenic plants showed the predicted bands for gus-intron gene in *F. carica* cv. Sultani; M 1Kbp ladder plus; C, untransformed plants; lanes1-12 transgenic plants.

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دراسة أعادة التكشف و التحول الوراثى بأستخدام ألأجروباكتيريم لنبات التين

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أستهدفت الدراسة تأسيس نظام لأعادة التكشف Regeneration بأستخدام أسلوب التكشف الجنينى المباشر و الغير مباشر و التحول الوراثى Transformation لصنف التين سلطانى. تم أستخدام تركيزات مختلفة من BA و NAA أو 2,4-D و Kinetin لتكوين كالس و كانت أفضل تركيز لتكوين الكالس ٢ملليجرام 2,4-D مع ٢ و ملليجرام Kinetin و الكالس قادر على تكوين أجنة جسمية و التكشف الى نباتات مع البيئة المحتوية على TDZمع NAAأو 2ip بينما أفضل التركيزات القادرة على تكوين أجنة جسمية والتكشف الى نباتات من الكالس كانت ٣٠ ملليجرام 2ip و ٧ ملليجرام TDZ مع ٢٥و ملليجرام NAA والتى أعطت نسبة تكشف ٨٣و٧٩ % على التوالى بينما الكالس فشل فى تكوين نباتات مع البيئة المحتوية على BA و Kinetin و أفضل التركيزات من منظمات النمو لأنتاج أفرع بواسطة التكشف الجينيى المباشر كانت مع البيئة المحتوية على ٢ملليجرام TDZ مع ٤ملليجرام 2ip والتى أعطت نسبة تكشف ٨٩% و أحسن النتائج لتكوين الجزور على الأفرع والتى أعطت ٩٥ % كانت مع البيئة MS المحتوية على ١ملليجرام IBA. بلغت نسبة أعادة التكشف للأنسجة المحورة وراثيا ٥و ١٧% وذلك بعد العدوى ببكتيريا الأجروباكتيريم الحاملة للجينات المستهدفة (*bar, gus* genes) وتم الكشف المبدئى عن حدوث عملية التحول الوراثى من خلال أختبار ال GUS activity و leaf painting وكذلك التأكد من وجود GUS gene بأستخدام تفاعل البلمرة المتسلسل (PCR).