



An efficient protocol for *in vitro* propagation of Fig (*Ficus carica* sp) and evaluation of genetic fidelity using RAPD and ISSR markers

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ARTICLE INFO

Article history:

Received on: 30/03/2016

Revised on: 18/05/2016

Accepted on: 04/06/2016

Available online: 26/08/2016

Key words:

Micro-propagation, *Ficus carica* sp, Genetic homogeneity, RAPD, ISSR.

ABSTRACT

Fig had been proliferated *in vitro* using tissue culture techniques. However, factors affecting shoot proliferation have not been optimized. In the present study, sufficient protocol for *in vitro* propagation of fig trees (*Ficus carica* sp) using shoot tips and nodal explants were reported. High percentage of shoots proliferation (95%) was obtained by culture of nodal explants containing two buds that were excised from greenhouse plants on MS medium supplemented with 3.0 mg/l BAP and 0.1mg/l Kin. Multiple shoots (12 multiple shoots per explant) were obtained after subculture of formed shoots on MS medium supplemented with 2.0 mg/l BAP, 0.1 mg/l Kin and 0.1 mg/l Gibberellic acid (GA3). Subsequently, high frequency of rooting (100%) were obtained after transferring the plantlets to half strength MS basal medium supplemented with 2.0 mg/l indole-3-Acetic acid, 0.1mg/l Indole butyric acid and 2g/l activated charcoal. Rooted plantlets were planted in pots containing a sterile soil and kept in the green house; the acclimatization was performed successfully with 100% survival rate. One year old plants were exhibited normal morphological characters comparing with the mother plant. Genetic homogeneity of the micro-propagated plants was evaluated by molecular analysis using nine randomly selected 1-year-old fig plants along with the mother plant. A total of six RAPD primers and 5 inter-simple sequence repeat (ISSR) primers produced a total of 109 resolvable, reproducible and scorable bands ranging from 250 to 1550 bp in size. Among these bands, 103 bands were monomorphic (94.5%) and 6 bands were polymorphic (5.5%). This low polymorphism ration between mother plants and micro-propagated plants indicates the little effect of somaclonal variations, the high genetic similarity between mother plants and micro-propagated plants and demonstrates the reliability of our *in vitro* propagation system for fig trees.

1. INTRODUCTION

Fig (*Ficus carica* L.) is a deciduous tree belonging to the Moraceae family [1] which grows in many different environments and soil types [2]. It is recognized to essay the adeptness to linger predominant shortfall and blend salinity highlight [3] making it a suitable species for cultivation in semi-arid environments such as the Mediterranean and middle-East. Fig tree plantations cover 426.244 hectare area worldwide with an overall yield of 1.070.676 million tones [4]. Turkey, Egypt, Iran are the largest fig producing countries that using the

vegetative propagated plants, especially by the rooting of cuttings. However, the propagation by conventional method of cuttings, grafting and layering is slow and limited and results in poor rooting and only 20–30% of the cuttings survive [5]. In addition, these methods contributed significantly to the dissemination of pathogens which affect the yield potential of the crop. Figs have been restricted to producing Fig-mosaic-virus-free plants by single-shoot-tip elongation [6]. Hence, *in vitro* micro-propagation of *Ficus* species has been widely studied as an alternate method for mass-scale production of high quality planting material being a faster multiplication technique in comparison with meristem culture [7-10]. Only a limited number of reproducible regeneration methods have been reported and most of the successful results were obtained from using shoots tip and apical buds [5, 11].

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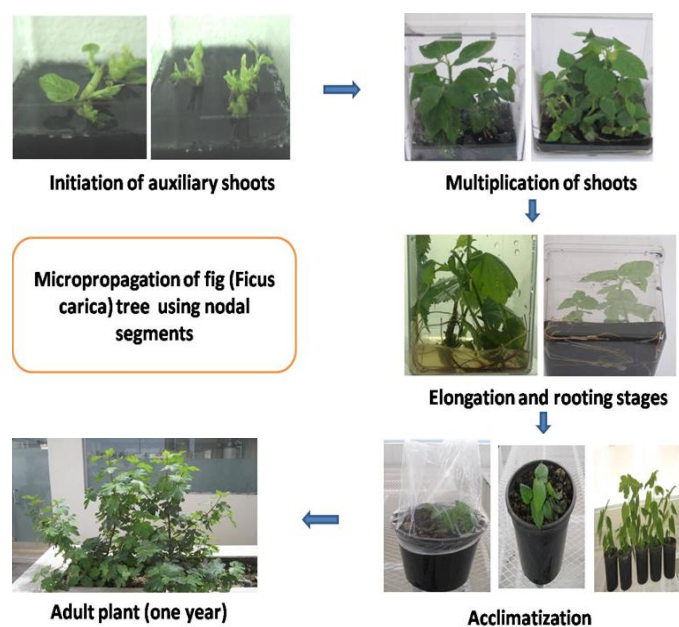


Fig 1: *In vitro* propagation of fig tree (*Ficus carica* sp).

Effect of different factors like growth regulators on the *in vitro* shoot proliferation has also been evaluated. A feasible protocol for shoot proliferation of *F. carica* 'Gular' by growing on MS medium supplemented with 2.0 mg/l benzyladenine (BA) and 0.2 mg/l naphthaleneacetic acid (NAA) has been reported [5,12]. A similar method was also tested for *F. carica* 'Roxo de Valinhos' with higher levels of BA and lower levels of NAA [10], but results were less promising. Although the propagation fig using tissue culture techniques has several potential advantages over conventional propagation method like high multiplication rate in short time, season-independent production of plants, production of disease-free plant and germplasm conservation [13], the maintenance of true-to-type nature of the *in vitro* propagated plants is an important requisite for upholding certain agronomic and horticultural traits when using elite genotype. It has been reported that tissue culture environment and influence of culture conditions like culture media, type of explant, successive transfer of culture, temperature, pH etc. cause alterations in cellular controls, resulted genomic changes in the *in vitro* raised plantlets [14,15]. While, plantlets regenerated through enhanced axillary branching or direct somatic embryogenesis has been reported to be genetically uniform [14], the possibility of somaclonal variations cannot be ruled out completely [16]. Thus, the genetic stability of *in vitro* propagated plants should be assessed as early as possible especially in long living woody trees, so that the deleterious effects that might be expressed later in growth do not result in having a severe economic impact [17]. The genetic fidelity of *in vitro* raised plants has been assessed using several strategies [18,19]. Previously, some workers have been used isozyme electrophoresis for evaluation of genetic integrity of *in vitro* raised plants, but the effects of environmental and developmental variation on it is one of the major drawbacks in its widespread use [20]. Recently, DNA-based molecular markers have served as an

important tool to assess the true to type nature and genetic homogeneity of the micro-propagated plants. Restriction fragment length polymorphism (RFLP) and Amplified fragment length polymorphism (AFLP) are shown to be reliable for screening of genetic fidelity in tissue cultured plants, but the risk of using radioactive labeling, extensive care and using the high cost make it unsuitable for genetic fidelity testing purposes [21]. Randomly Amplified Polymorphic DNA (RAPD) markers have been used for assessment of genetic fidelity of *in vitro* propagated plants in many reports [21-24]. In recent years, ISSR markers have been proved to be more efficient than dominant RAPD markers for the detection of somaclonal variations in tissue culture plants [16]. Many reports were documented the assessment of genetic fidelity of micro-propagated plants using microsatellite markers [16, 17, 25-28] and ISSR markers [21,23,29-33]. In tree species, assessment of genetic stability of tissue culture raised plants through molecular markers at early stage is desirable as morphological variations in these plants can be detected usually only at maturity and fruiting stage [34]. Therefore, the aims of the present study were to establish an *in vitro* shoot proliferation protocol for the important KSA variety of *F. carica*, assess the applicability of RAPD and ISSR markers for determining genetic stability of *in vitro* regenerated plants and the reliability of developed micro-propagation protocol for true to type cloning. The results may lead to the development of a protocol for rapid shoot proliferation, which can supplement the conventional method of cuttings.

2. MATERIALS AND METHODS

2.1. Plant material

Healthy shoot tips and nodal explants were collected from greenhouse of *Ficus carica* L. Explants were washed first under running tap water for 30 min, to remove the superficial dust followed by immersion in 70% ethanol for 1min, then incubated in 15 % Clorox (5.25% of sodium hypochlorite) for 12 min and rinsed for three times with sterile distilled water and rinsed with sterile anti-oxidant solution (0.01% L-ascorbic acid).

2.2. Culture conditions

The sterilized nodal cuttings containing axillary buds were transferred to shoot initiation media containing full strength MS medium (4.4 g/l+30 g/l sucrose) and supplemented with different concentrations and combinations of auxins and cytokinins. all media was adjusted to 5.7 pH using 1.0 N hydrochloric acid (HCl) or 1.0 N potassium hydroxide (KOH), before adding 8g /l plant agar. Media were autoclaved for 20 min. at 121°C and 1.5 k/ cm² pressure. All *in vitro* cultures were placed on a 16/8 hour light/dark cycle in a culture room maintained at 26 ± 2°C.

2.3. Micro-propagation stages

For shoot initiation, nodal cuttings containing axillary buds explants were cultured on shoot initiation media containing full strength MS medium with various concentrations of BAP and

Kin. for four weeks. Shoot multiplication and elongation stages were performed by sub-culturing of proliferated shoots on MS media sublimated with different combined concentrations of BAP, Kin and Gibberellic acid (GA3). For rooting stage, shoots (5-6 cm length) were transferred in half strength Murashige and Skoog (MS) medium supplemented with different combinations and concentrations of IAA and IBA for four weeks and containing 2 g/l activated charcoal. Rooted explants were planted in pots containing a sterile soil and kept in the green house for acclimatization.

2.4. Statistical analysis

Statistical analysis of data, analysis of variance (ANOVA) and mean separation were carried out using Duncan's multiple range test and significance was determined at the ($p < 0.01$) level. Data analysis was performed using ASSISTAT Version 7.7 beta (2015).

2.5. Isolation of genomic DNA for molecular markers analysis

Fresh young healthy leaves were collected and grounded to powder with liquid N₂ using a mortar and pestle. Genomic DNA was isolated from leaf samples using the procedure described by the plant isolation kit (Jena Bioscience, Germany).

2.5.1. RAPD analysis

PCR reaction and condition: Sequence of the six RAPD random primers that used to detect the polymorphism and produce a clear scorable and reproducible banding pattern is shown in (Table 1). The amplification reactions were performed in a 25 μ l volume containing about 30 ng genomic DNA and 300 pg random primer (Operon Technologies Inc.) using 12.5 μ l of 2x green master mix (Promega) in Bio-Rad C1000 thermal cycler (Germany). The PCR temperature profile was adjusted as 5 min at 94°C for initial denaturation followed by 40 cycles of 60 s at 94°C, an annealing step of 1 min at 37°C and an elongation step of 1 min at 72°C; and finally a 7 min extension at 72°C was applied. Electrophoresis in a 1, 5% agarose gel containing ethidium bromide (0.5 μ g/ml) in 1X TBE buffer at 95 volts was used for the separation of the amplification products. Subsequently, the separated PCR products were visualized on UV light and photographed using a gel documentation system (Bio-Rad® Gel Doc-2000).

Table 1: Effect of different concentrations and combinations of BAP and Kin on shoot formation of Fig Tree (*Ficus carica* sp.).

Supplement (mg/l)		No. of explants	Shoot proliferation (%)
BAP	Kin		
0.0	0.0	40	9 d
1.0	0.3	40	58 c
2.0	0.2	40	70 b
3.0	0.1	40	95 a

Each treatment consisted of 3 replicate in which 40 explants were used. The averages followed by the same letter do not differ statistically between themselves at ($p < .01$).

2.5.2. ISSR analysis

Ten ISSR primers were tested using a specific and optimal annealing temperature for each one (Table 2). PCR reactions were performed in a total volume of 20 μ l using the Bio-Rad C1000 thermal cycler (Germany).

Table 2: Effect of different concentrations and combinations of BAP, Kin and GA3 on multiplication and elongation of Fig Tree (*Ficus carica* sp.).

Supplement (mg/l)			No. of shoots / explants	Elongated shoots (%)
BAP	GA3	Kin		
0.0	0.0	0.0	2	30c
0.5	0.1	0.1	5	50bc
1.0	0.1	0.1	8	70ab
2.0	0.1	0.1	12	90a

Each treatment consisted of 3 replicate in which 30 explants were used. The averages followed by the same letter do not differ statistically between themselves at ($p < .01$).

The reaction mixture contained 60 pg of each primer, 100 μ M of each deoxynucleotide, 0.5 units of GoTaq polymerase (Promega), 10xTaq buffer containing 2.5 mM MgCl₂ and 10 ng of template DNA. Amplification reaction condition was 94°C/5 min, followed by 30 cycles of 94°C/1 min, 4-60°C (specific for each primer)/1 min and 72°C/2 min and ending with an extension step of 7°C / 7 min. PCR products were analyzed using agarose (2% w/v) electrophoresis gels stained with ethidium bromide and only bands with high intensity and well separated were selected.

2.5.3. Data scoring and statistical analysis

To ensure the absence of artifacts, bands were carefully selected from replicated amplifications (three times). Amplified bands designated by their primer code and their size in base pairs. Data recorded as discrete variables: 1 for the presence and 0 for the absence of a similar band. Only intense and reproducible bands appearing on the gel were scored. Band scoring was analyzed using Gene Tools-gel analysis software of SPSS ver. 16.

3. RESULTS

3.1. Optimization of Figs micro-propagation protocol

The shoot tips and nodal explants were incubated on MS medium without Plant Growth Factors or fortified with different concentrations of BAP (0.0, 1.0, 2.0 and 3.0 mg/l) with (0.0, 0.1, 0.2 or 0.3 mg/l) Kin. The results are summarized in (Table 1). The axillary bud initiation was observed in all medium used after two to three weeks. Increase of BAP and decrease in kin concentrations resulted in a gradual increase in the shoot formation frequency. However, the best frequency of break of axillary buds was achieved in MS medium supplemented with 3 mg/l BAP and 0.1 mg/l Kin. At this concentration, the best percentage of explants (95%) showed shoot initiation and more significance comparing with other concentrations used at the ($p < 0.01$) (Table 1). Multiple shoots were emerged directly from axillary nodes of the cultured explants after four weeks in medium containing different concentrations of BAP with combination with Kin and GA3 (Table 2).

The medium supplemented with a combination of 2, 0.1 and 0.1 mg/l of BAP, Kin and GA3, respectively, was found to be the ideal concentration to induce maximum shoots (12) per nodal explant (Table 2). In addition, subculture in the same medium resulted in the best shoots elongation frequency (90%), however, shoots elongation was recorded in all the medium-used. For root formation, the *in vitro* regenerated shoots were excised aseptically and implanted on half strength MS medium with growth regulators IAA and IBA. Rooting was recorded only when half strength MS medium was used in combination with different concentrations of IBA and IAA (Table 3).

Table 3: Effect of different concentrations and combinations of IAA and IBA on root formation of Fig Tree (*Ficus carica* sp).

Supplement (mg/l)		No. of shoots	Rooted shoots (%)
IAA	IBA		
0.0	0.0	30	00 e
0.5	2.0	30	40 d
1.0	1.0	30	80 bc
1.5	0.5	30	90 ab
2.0	0.1	30	100 a

Each treatment consisted of 3 replicate in which 30 explants were used. The averages followed by the same letter do not differ statistically between themselves at ($p < .01$).

Induction of roots was noticed after 20 days of implantation in all medium. Rooting frequency of 40, 80 and 90 % was obtained on the concentrations of 0.5 and 2, 1 and 1, 1.5 and 0.5 mg/l IAA and IBA respectively ($p < 0.01$; Table 3). However, the concentration of 2 mg/l IAA and 0.1 IBA was found optimal with best rooting formation (100%). For acclimatization, rooted plantlets were planted in pots containing a sterile soil (peat moss and sand) and kept in the green house. The adaptation was performed successfully with 100% survival rate.

3.2. Genetic fidelity of micro-propagated plants

Out of the 16 screened primers of RAPD and ISSR that used to assess the genetic stability in nine randomly selected one-year old micro-propagated plants along with the mother plant, 14 produced resolvable, reproducible and scorable bands (Table 4 and Table 5).

Table 4: List of random primers that have been used for RAPD analysis, numbers of amplicons and % polymorphism.

PN	PS	NA	NPB	P%
OPA-01	5'-CAGGCCCTTC-3'	10	1	10.00
OPA-03	5'-AGTCAGCCAC-3'	7	0	00.00
OPA-07	5'-GAAACGGGTG-3'	5	0	00.00
OPA-10	5'-GTGATCGCAG-3'	4	0	00.00
OPB-03	5'-CATCCCCCTG-3'	12	1	08.30
OPH-05	5'-AGTCGTCCCC-3'	7	1	14.30
Total		45	3	06.60

PN: Primer Name, PS: Primer sequence, NA: No. of amplicons, NPB: No. of polymorphic bands and P%: Polymorphism% .

Table 5: List of ISSR primers that have been used for ISSR analysis, numbers of amplicons and % polymorphism.

PN	PS	AT	NA	NPB	P%
ISSR-06	(AC)8YT	49.0	06	0	0.00
ISSR-07	HBH(AG)7	50.0	12	1	8.30
ISSR-08	BHB(GA)7	52.0	10	1	10.00

ISSR-10	BDB(AC)7	50.0	12	1	8.30
ISSR-11	(AG)8T	50.0	13	0	0.00
ISSR-12	(AG)8C	48.5	03	0	0.00
ISSR-13	(AG)8G8	48.5	04	0	0.00
ISSR-15	(GA)8C	48.5	04	0	0.00
Total			64	3	4.70

PN: Primer Name, PS: Primer sequence, AT: Annealing Temperature, NA: No. of amplicons, NPB: No. of polymorphic bands and P: Polymorphism% .

Total of 109 bands were obtained from the two markers in which only 6 bands were polymorphic representing 5.5% polymorphism. In RAPD analysis, six primers produced 45 bands with 6.6% polymorphism (3 bands). Number of bands produced by the six primers was ranged between 4 and 12 with average of 7.5 bands per primers. The three polymorphic bands were produced by the 3 primers OPA-01, OPB-03 and OPH-05 as one band for Each that represent 10%, 8.3% and 14% of the total bands produced by them, respectively (Table 4). The other three primers (50%) showed 100% monomorphic pattern. The banding pattern produced by the primers OPA-01, OPH-05 and OPA-03 are shown in (Fig. 2).

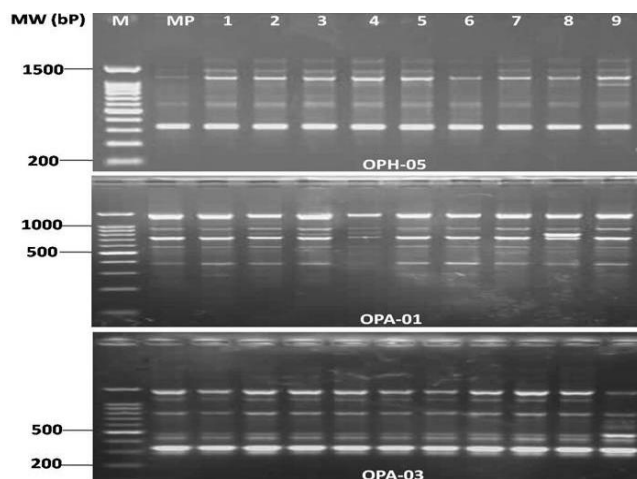


Fig. 2: RAPD profile of mother plant and *in vitro* raised plants of Fig Tree (*Ficus carica* sp) using three random primers.

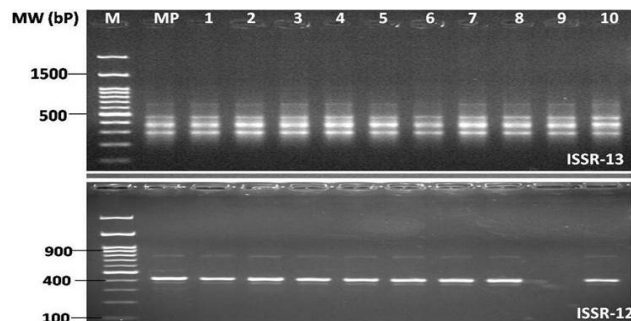


Fig 3: ISSR profile of mother plant and *in vitro* raised plants of Fig Tree (*Ficus carica* sp) using primers ISSR-12 and ISSR-13.

In ISSR analysis, eight primers produced 64 bands with 4.7% polymorphism (3 bands). Number of bands produced by the eight primers was ranged between 3 and 13 with average of 8

bands per primers (Table 5). Each of the three primers ISSR-7, ISSR-8 and ISSR-10 produced one polymorphic band among the total bands produce by each of them (Table 5).

While, all the bands produced by the other five primers ISSR-6 and ISSR-11, ISSR-12, ISSR-13 and ISSR-15 was monomorphic (Table 5). ISSR amplification pattern (Fig. 3) obtained with the two primers ISSR-12 and ISSR-13 (with 100% monomorphic pattern).

4. DISCUSSION

Development of efficient and reproducible protocol for micro-propagation of tree species has many advantages, however, morphological variations in these tissue culture raised plants can be detected usually only at maturity and fruiting stages, therefore assessment of genetic reliability in these plants through molecular markers at early stage is desirable before their exploitation for routine propagation. In the present study, an efficient protocol for mass production of the fig trees using the nodal explants was developed (Fig. 1). Different combinations of the two cytokinins BAP and Kin were used for break of axillary buds and shoots proliferation. In the presence of 0.5 mg BA, the shoots of some fig cultivars such as 'Bermuda' and 'Lampa' proliferated well [35]. However, the presence of Kin alone in the medium did not support good callus proliferation of fig [36]. At lower concentrations of cytokinin, lateral buds from the base of the leaves grew into individual shoots (Fig. 1); however, increasing BAP concentration to 3 mg/l and 0.1 mg/l Kin resulted in the best frequency of break of axillary buds (95%) and adventitious shoot proliferation (Table 3). Further increases in cytokinin concentration to 6.0 mg /l, resulted in a decreased proliferation rate. Similar results were observed in sprouting of apical buds on MS medium with the same combination of BAP and Kin. Fráguas *et al.* [37] showed that, the regulator strength of (Woody Plant Medium) WPM in combination with 0.5 mg/l Kin was the best condition for shoot proliferation of fig "Roxo de Valinhors" plants. It was indicated that, BA stimulated shoot proliferation and multiplication of *Morus alba* cv. Kokoso and Moritiana [38].

Increasing the number of shoots regenerated from the explant with increases in BAP concentration in the medium combined with stable concentration of GA3 and Kin with BAP at 5.0 mg/l, the number of shoots regenerated from the single explants decreased, which indicated that BAP had an adverse effect when used at a higher than optimal concentration. Proliferation behavior of BAP in banana was observed [39]. The positive role of BAP on micro-propagation stage might be due to cytokinins great role in releasing lateral buds from the dominance of terminal buds without need to remove the apical bud by promoting the formation of xylem tissues of buds which will facilitate the transformation of water and nutrients leading to lateral bud growth [40] as well as, the important role of cytokinins in increasing the synthesis of RNA, proteins and enzymes inside the cell which enhance bud growth as well [41,42]. It has been reported that improvement in the shoot quality of fig can be

achieved by culturing explants on medium containing 3.0 mg activated charcoal [43]. Percentage of a survival rate was (100%) when rooted plantlets were transfer to pots containing a sterile soil and kept in the green house. Hemaïd *et al.* [44] reported that, the percentage of acclimatized fig plants was high (95 - 97%) and was obtained in two months. A similar method has been reported by Pontis and Melas [45], for acclimatization of *Ficus carica* L. The one-year old micro-propagated trees were found to be phenotypically normal and essentially identical with their mother plant which partly suggests the minimal or absence of somaclonal variations. The two molecular markers (RAPD and ISSR) were used to test these results at the molecular level. The usages of two markers, which amplify different regions of the genome, allow better chances for the identification of genetic variations in the clones [46]. Palombi and Damiano [47] suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation on micro-propagated plants of kiwi fruit.

The random nature of RAPD and the fact that a simple sequence repeat targets the fast evolving hyper variable sequences makes these two markers suitable to detect variations among micro-propagated plants [25, 48]. The high ratio of monomorphic banding pattern in micro-propagated and mother plant and the low frequency of polymorphism in RAPD and ISSR profile of micro-propagated plantlets (6.6 and 4.7, respectively) suggested the occurrence of little effects of somaclonal variations.

There are many reports in literature suggested that, plants regenerated through organized tissues like meristems or direct somatic embryogenesis maintain genetic integrity of the plantlets with a least risk of genetic variation [14,46,48,49]. This finding further supports the fact that the axillary multiplication is the safest mode of micro-propagation to produce true to type progeny. In addition, these results confirm the high genetic homogeneity of the *in vitro* raised plants and also indicated that the developed micro-propagation protocol is efficient enough to maintain genetic stability. Similar results were reported by Srivastava *et al.* [50] who evaluated < 6% frequency polymorphism of *in vitro* raised sugarcane varieties produced from shoot-tip culture through RAPD markers.

5. CONCLUSION

In conclusion, the obtained results representing an efficient protocol for the mass production of figs tree and indicated good genetic fidelity obtained in the *in vitro* raised plantlets when examined by the two markers system. Hence we concluded that, the developed protocol could be effectively used for rapid micro-propagation and commercial utilization of figs without much risk of genetic instability.

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How to cite this article:

El-Dessoky S. Dessoky, Attia O. Attia, El-Awady A. M. Mohamed., An efficient protocol for in vitro propagation of Fig (*Ficus carica* sp) and evaluation of genetic fidelity using RAPD and ISSR markers. J App Biol Biotech. 2016; 4 (04): 057-063. DOI: 10.7324/JABB.2016.40406