Journal of Plant Biochemistry and Biotechnology (April–June 2021) 30(2):304–316
https://doi.org/10.1007/s13562-020-00590-9

ORIGINAL ARTICLE

Factors affecting in vitro regeneration of *Ficus carica* L. and genetic fidelity studies using molecular marker

M. Moniruzzaman[1,2] · Zahira Yaakob[1] · Nurina Anuar[1]

Received: 17 May 2020 / Accepted: 19 August 2020 / Published online: 28 August 2020

Abstract

A facile method for regeneration of fig (*Ficus carica* L.) is in demand given the inability of the varieties having persistent type fruiting habit to produce viable seeds for germination, whereas asexual propagation features certain limitations. This article reports factor affecting in vitro regeneration of three fig cultivars, Masui Dauphine, Orphan, and A134. Ammonium nitrate, calcium chloride, sugar concentration in Murashige and Skoog (MS) medium, explants genotype, culture system (liquid or solid media), and light intensity of culture room affect regeneration. Half-calcium-modified MS (HCMS) liquid medium with 1 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l naphthaleneacetic acid (NAA), and 0.02 mg/l gibberellic acid (GA_3) responded well for shoot induction and proliferation. In average, 18 harvestable shoots were observed per-explant of Orphan cultivar. For elongation, HCMS liquid medium with 0.6 mg/l BAP, 0.1 mg/l NAA, and 0.1 mg/l GA_3 performed well among the studied media combinations. Hormone-free regular MS liquid medium produced the highest percentage of rooted explants for all cultivars. Root induction reached 85% for Orphan. In vitro rooted plantlets were successfully acclimatized on soil. Inter simple sequence repeat marker based study for somaclonal variation detection showed genetic uniformity of regenerated and donor plants. This regeneration method may be useful for large-scale production of identical plantlets and genetic transformation studies to further improve fig.

Keywords Common fig · Genetic stability · Liquid media · Media optimization · Micro-propagation · Plant biotechnology

Abbreviations

BAP	6-Benzylaminopurine
bp	Base pair
DNA	Deoxyribonucleic acid
GA3	Gibberellic acid
HCMS	Half-calcium-modified Murashige and Skoog
IBA	α-Indole-3-butyric acid
ISSR	Inter simple sequence repeat marker
ME	Media for shoot elongation
MI	Media for shoot induction
MR	Media for root induction
MS	Murashige and Skoog
mg/l	Milligram/liter
mm	Millimeter
NAA	Naphthaleneacetic acid
PCR	Polymerase chain reaction
PGR	Plant growth regulator
SPSS	Statistical product and service solutions
UV	Ultra violet
%	Percentage

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13562-020-00590-9) contains supplementary material, which is available to authorized users.

✉ M. Moniruzzaman
monirbge@gmail.com

[1] Research Center for Sustainable Process Technology (CESPRO), Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 Bangi, Malaysia

[2] Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou 510640, China

Introduction

The fig (*Ficus carica* L.) is an economically important fruit crop. Fruits bear enormous food and medicinal value (Barolo et al. 2014). Considering its importance in nutrition, food, medicine, and religion, fig is spread and cultivated in most parts of the world (Dueñas et al. 2008). There is a huge demand of quality plantlets of elite cultivars. Our

previous work demonstrated that Masui Dauphine, Orphan, and A134 cultivars performed well in tropical climatic conditions (Moniruzzaman et al. 2017a, b).

Fig varieties belonging to common type generally produce no viable seeds for germination. Vegetative propagations, including cuttings, air layering, or grafting, are being practiced for their propagation. These methods are season dependent, insure uniformity, require considerable labor and large area; furthermore, relatively low multiplication rates are achieved as such materials can be obtained only from upright branches, resulting in poor rooting (Kumar et al. 1998). The plant also experiences difficulties in controlling nematodes (Melidogyne spp.), mites (Eriophyes spp.), and mosaic virus (*Heterodera fici*) (Campos 1997; Comlekcioglu et al. 2007).The mysterious floral biology and dependency of Smyrna type figs on fig wasp (*Blastophaga psenes*) (Ferrara et al. 2016; Rosianski et al. 2016; Weiblen 2002) results in fig breeding, which is focused on achieving quality traits, that is highly complex and time consuming (Kim et al. 2007). Genetic engineering of plants relies on a tissue culture systems to regenerate transformations (Kumar et al. 2010). Classical breeding methods aided by genetic engineering can accelerate the development of new cultivars with improved traits (Lemaux 2009; Sharma et al. 2005).

Micropropagation protocols have been successfully used for rapid and large-scale production of a number of fruit trees (Bajaj 1988). There are some reports on *F. carica* regeneration (Al-Zahrani et al. 2018; Fráguas et al. 2004; Kumar et al. 1998; Ling et al. 2018; Moniruzzaman et al. 2017b; Sharma et al. 2015). None of them dealt with the effect of different factors on regeneration and result was not reproductive enough. Nevertheless, a facile and efficient in vitro regeneration method was demanding. Factor affecting regeneration study can optimize media and culture system for efficient regeneration (Sharma et al. 2011; Zhang et al. 2013). This article represents the factors affecting regeneration of *F. carica* L. for the development and improvement of an efficient regeneration method. This method could be useful for bulk production of fig plantlets and genetic transformation studies for further improvement.

Materials and methods

Explants and surface sterilization

Tiny shoots of three cultivars (Masui Dauphine, Orphan, and A134) were collected from poly-house fig garden of living lab, UKM at Kuala Pilah. Explants were collected separately early in the morning (7.00 AM) using a sharp knife, which was frequently sterilized using alcohol. Leaves were removed quickly and carefully and placed in a beaker (500 ml) containing water with 2–3 drops of mild liquid soap (Deepol, Bendosen-Malaysia). Afterwards the beaker was shaken for 30–40 s to produce bubbles. Then, the explants were washed under running tap for 45 min to remove dirt and latex (young fig tissue contains high amounts of latex-phenolic substances) that cause cell oxidation and eventual death of explants. Then, the shoots were put into 300 ml autoclaved distilled water containing 350 µl Myzim SC (Mycrop Sdn, Bhd.) and 3 drops of Tween 20 for 15 min. After washing the explants carefully, it was transferred to laminar air-flow hood. Here, the explants were washed with 0.1% $HgCl_2$ (w/v) for 1 min and 5% bleach (KAO Bleach, Japan) with 2–3 drops of Tween 20 for 4 min. Finally, the explants were rinsed 3–4 times using autoclaved distilled water and placed on media.

Shoot induction and proliferation

Eight different MS liquid media (MI 1 to MI 8; Table 1) in combination with three hormones, BAP, NAA, and GA_3, were prepared and subjected to shoot induction and proliferation. Hormone dose and combination were determined based on the works of Bayoudh et al. (2015) and Sharma et al. (2015) with certain modifications.

Three chemicals, ammonium nitrate, calcium chloride, and sugar, and light intensity, as another parameter were tested to study the factors influencing in vitro regeneration. Media MI 6, which performed the best in shoot induction and proliferation experiments was used for further media modifications while studying the factors affecting regeneration. Media modifications were achieved as half ammonium nitrate (that is, NH_4NO_3 was added into media at 825 mg/l instead of 1650 mg/l) half calcium chloride (that is, $CaCl_2{\cdot}2H_2O$ was added into media at 220 mg/l instead of 440 mg/l), and sugar, which was added at 15 gm/l instead of 30 gm/l. The half-calcium modified MS medium (HCMS) liquid and solid media with 1 mg/l BAP, 0.1 mg/l NAA, and 0.02 mg/l GA_3 were examined for culture system and genotype effect study as these modifications yielded the best responses. For preparation of solid media, 8 gm/l agar was added and boiled to melt before autoclaving.

Shoot elongation

Individual shoots of around 7–8 mm were isolated from clusters and placed in hormone-free HCMS liquid medium for the first five days to achieve homogeneity among all shoots from different media. Then, the shoots were transferred on elongation media. The HCMS liquid media with four different concentrations and combinations of BAP,

Table 1 Effect of different concentration and combination of hormones with MS media on shoot induction and proliferation of different cultivars (mean with same letter in each column are not significantly different according to DMRT at $\alpha = 0.05$)

Hormone combination (mg/l)				Masui Dauphine			Orphan			A134		
Media code	BAP	NAA	GA_3	Shoot induction %	No of Shoots per explant	Shoot length (mm)	Shoot Induction %	No of Shoots per explant	Shoot length (mm)	Shoot induction %	No of Shoots per explant	Shoot length (mm)
MI 1	0.5	0.1	–	55.0 ± 2.8b	1.0 ± 0.1b	4.7 ± 0.1ab	65.0 ± 2.8c	2.0 ± 0.1b	5.1 ± 0.1b	52.5 ± 4.7b	1.0 ± 0.1b	4.1 ± 0.1bc
MI 2	1	0.1	–	57.5 ± 2.5b	6.0 ± 0.3c	7.2 ± 0.4bc	67.5 ± 4.7c	8.0 ± 0.8c	7.9 ± 0.3d	55.0 ± 2.8b	5.0 ± 0.3c	6.1 ± 0.3d
MI 3	1.5	0.1	–	15.0 ± 2.8a	2.0 ± 0.1b	5.9 ± 1.8abc	24.5 ± 4.7b	2.0 ± 0.1b	4.9 ± 0.1b	12.5 ± 2.5a	1.0 ± 0.1b	4.3 ± 0.2c
MI 4	2	0.1	–	07.5 ± 2.5a	1.0 ± 0.1ab	3.9 ± 0.2ab	12.5 ± 2.5a	2.0 ± 0.1b	3.9 ± 0.2a	05.0 ± 2.8a	1.0 ± 0.1b	3.3 ± 0.2b
MI 5	0.5	0.1	0.02	55.0 ± 2.8b	2.0 ± 0.1b	8.5 ± 2.3c	70.0 ± 4.0c	2.0 ± 0.2b	6.8 ± 0.1c	55.0 ± 2.8b	1.0 ± 0.1b	6.4 ± 0.1d
MI 6	1	0.1	0.02	57.5 ± 4.7b	10.0 ± 0.9d	11.9 ± .5d	75.0 ± 2.8c	17.0 ± 6.0d	10.2 ± 0.4e	47.5 ± 2.5b	9.0 ± 0.9d	7.9 ± 0.3e
MI 7	1.5	0.1	0.02	12.5 ± 2.5a	1.0 ± 0.1b	3.7 ± 0.2ab	23.5 ± 6.2b	1.0 ± 0.1ab	4.5 ± 0.2ab	12.5 ± 2.5a	1.0 ± 0.1b	3.4 ± 0.2b
MI 8	2	0.1	0.02	07.5 ± 2.5a	1.0 ± 0.1a	3.5 ± 0.2a	15.0 ± 2.8ab	1.0 ± 0.1a	3.7 ± 0.4a	07.5 ± 2.5a	1.0 ± 0.1a	2.3 ± 0.4a

NAA, and GA_3 were used to determine the best medium for elongation (Table 3).

In vitro rooting and acclimatization

Green and healthy elongated shoots were placed in hormone-free HCMS liquid medium for the first five days. Then, the shoots were cultured on half- and full-strength MS liquid media with different hormone combinations for root induction (Table 4). To determine the culture system's effect on rooting, both liquid and solid media of hormone-free MS were tested as these media yielded the best rooting effect.

The rooted shoots were prepared for planting on soil. First, the bases of plantlets were soaked with extremely low concentration (0.05 mg/ml) of fungicide, Bavistin (Carbendazim Powder, BASF India Ltd.) mixed water for 30 s. Then, the bases were planted separately in small disposable paper cups containing a mixture of peat moss (40%), garden soil (20%), compost (15%), fired soil (15%), and ash (10%). These materials were mixed and placed in a disposable paper cup and autoclaved the previous day. After planting the plantlets in soil mixture, autoclaved distilled water was poured to wet the soil (80%). Then, individual pots were covered by plastic bag and tightened by a rubber band (Fig. 4b). The plantlets were then placed into a culture room. Starting from the next day, each pot was given approximately 10 ml autoclaved distilled water and covered again. After 15 days, the plastic cover was removed. After 30 days, when the plantlets established on soil and thrived well, they were transferred to a large pot containing the same soil medium composition used previously and placed under ambient room condition for hardening.

Culture condition, data recording, and analysis

Uniform culture conditions were applied in all experiments. Cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with light intensity of 40 µmol m^{-2} s^{-1} (cool-white fluorescent tubes). For the study of light effect on regeneration, the explants were also cultured under 20 µmol m^{-2} s^{-1} intensity of light.

For the data analysis (ANOVA) of culture system effect, all explants on liquid media were considered one group irrespective of genotypes; for example, explants of all three cultivars on liquid media were considered as one group. Same thing happened in case of solid media: for example, explants of all three cultivars on solid media were considered as another group. For the analysis (ANOVA) of genetic effect, all explants of the same genotype on both solid and liquid media were considered as one group; for example, explants of "Orphan" cultivar on both solid and liquid media were considered as one group. Same thing happened in case of other two cultivars. In other cases of analysis explants were grouped according to the represented tables. Data from individual groups was recorded and used as separate group during ANOVA analysis. Data on shoot induction percentage, the number of shoots per explants, and average shoot length were recorded after 6 weeks of incubation. Shoot induction percentage was calculated based on the number of explants cultured and the number of those induced to shooting. The number of shoots that were easily distinguishable from each explant was counted. Average shoot length was calculated by considering the total length of all shoots and total number of shoots of individual explants.

Data were recorded from three replications for each of three cultivars. Statistical difference among the means was analyzed by Duncan's multiple-range test using SPSS (version 23), and results were expressed as mean ± standard error of three independent experiments.

Genetic stability determination by inter simple sequence repeat (ISSR Marker)

DNA extraction from plant materials

Fresh leaves were treated with liquid nitrogen, and leaf samples were ground to fine powder by mortar and pestle. The fine-powder leaf samples were utilized in DNA extraction. DNA was extracted using innuPREP Plant DNA Kit (AJ Innuscreen GmbH, Germany) according to manufacturer's instruction. Quality and quantity of DNA were assessed on 0.8% agarose gel and using a spectrophotometer (Eppendorf, Germany).

DNA amplification with ISSR marker

Thirteen ISSR primers (Supplement S6) from two sets (IMA and UBC) were amplified with DNA from donor and progeny plants for primer screening. Amplified DNA from a mother plant and one plant from each of the three replicas using each primer were run in gel electrophoresis. Finally, two primers from the two sets with distinct and easily scorable bands were selected. Selected primer amplification with DNA from mother and six progeny plants of each replica was run in gel electrophoresis. UV trans-illumination images were captured.

PCR conditions were optimized using different concentrations of template DNA and primers and varied annealing temperatures and time. Based on optimization experiments, PCR was carried out in 50 µl final volume as follows: template DNA 1 µl (200 ng DNA/µl), primer (20 µM) 1 µl, My Taq Mix (2X) 25 µl (Bioline, UK), and 23 µl double-distilled water. PCR reactions were performed in Eppendorf master cycler (Germany)

programmed for an initial denaturation step at 95 °C for 4 min, followed by 45 cycles at 94 °C for 15 s, 49 °C for 15 s, and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. Amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels (Biobasic, Canada) in Tris-acetate-ethylenediaminetetraacetic acid, visualized by GelRed™ Nucleic Acid Gel Stain (Biotium USA) staining, and photographed under UV light with a Gel Doc system (UVP, Bio Doc, Upland, USA). Each sample was amplified at least twice to verify reproducibility.

Results

Best responding shooting media

The responses of explants to media varied significantly based on different hormone combinations with MS media (Fig. 1). Shoot induction percentage ranged from 05.0 ± 2.8 to 75.0 ± 2.8 among the studied cultivars and media composition. Orphan showed the highest percentage (75.0 ± 2.8) of shoot induction on MI 6 (MS with 1 mg/l BAP, 0.1 mg/l NAA, and 0.02 mg/l GA_3). For Masui Dauphine, the highest percentage of shoot induction was 57.5 ± 4.7 on media MI 6, and for A134, the value was55.0 ± 2.8 on media MI 2 and MI 5 (Table 1). A significant difference was observed in the number of shoots per explants among the studied cultivars and medium composition. The number of shoots per explants was 17, 10, and 9 on media MI 6 for Orphan, Masui Dauphine, and A134, respectively. The number of shoots per explants ranged from 0 to 10 for Masui Dauphine, 0 to 17 for Orphan, and 0 to 9 for A134 among the different media used. Addition of GA_3 to media significantly influenced the number of shoots per explants and shoots length. The longest shoots (11.9 ± 0.5 mm) were observed on media MI 6 for Masui Dauphine. The shoot length was 10.2 ± 0.4 and 7.9 ± 0.3 mm for Orphan and A134, respectively, after 6 weeks of culture.

Effect of Ca^+, NH_4^+, sugar and light on shoot induction and proliferation

Reduced Calcium positively influence shoot induction. HCMS exhibited the best performance among the studied media with modifications (Supplement S1 and Fig. 2). The

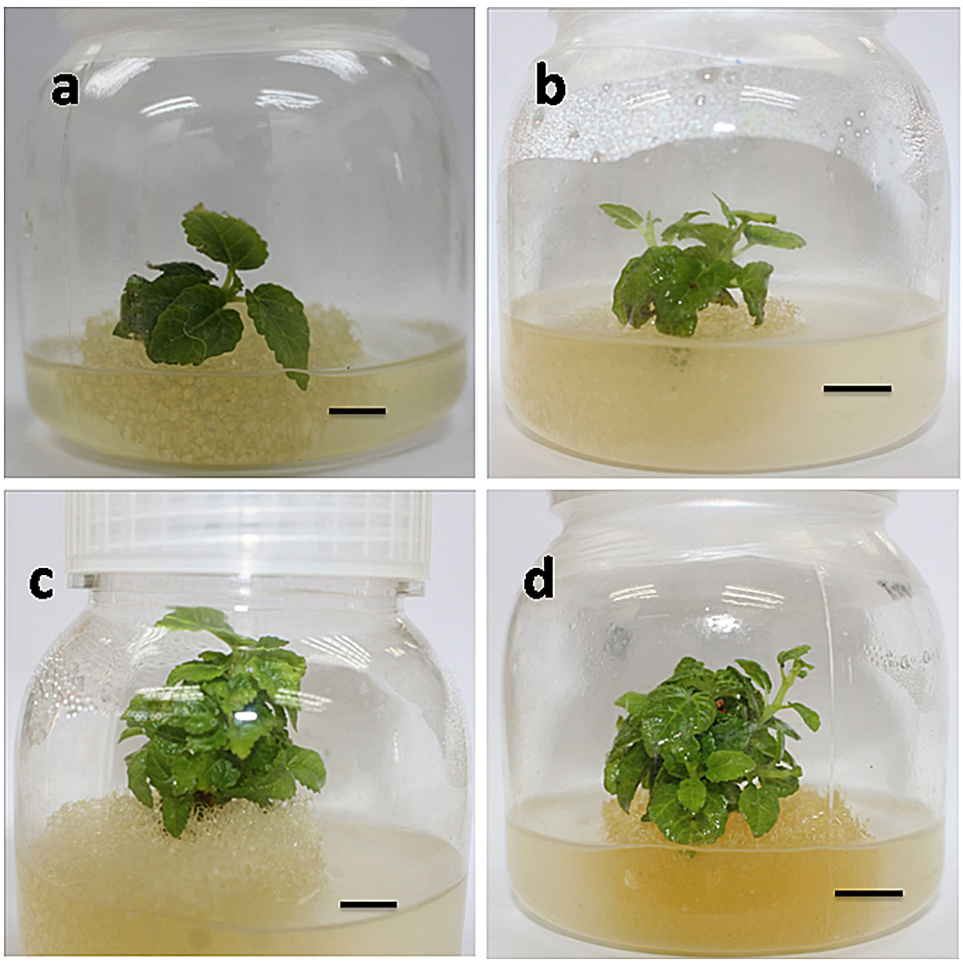

Fig. 1 Shoot induction responses on different media. **a**, **b** Explants on media MI 1, MI 3, MI 4 or MI 7. **c** Explant on media MI 2. **d** Explant on media MI 6. Pictures were taken 6 weeks after culture. The scale represents 10 mm; cultivar: Orphan

HCMS media induced the highest percentage of shooting (65%). Increased numbers of shoots (13) and shoot length (10.5 mm) were observed in HCMS media. On the other hand, half sugar (15 gm/l) and lower light intensity (20 µmol m^{-2} s^{-1}) negatively affected shoot induction. Shoot induction percentage amounted to 52.5% on half-sugar medium and 60% in regular MS media (Table 2). Half sugar and less intense light reduced the number of shoots per explants and shoot length. The average numbers of shoot per explants and shoot length were 8.0 and 8.8 mm, respectively, for half-sugar modification; these values reached 10 and 9 mm under less intense light. No significant difference was observed in the shoot number and shoot length between regular MS and half-NH_4^+ modification. However, a downward trend of shoot growth (shoot length) was observed for half NH_4^+ media.

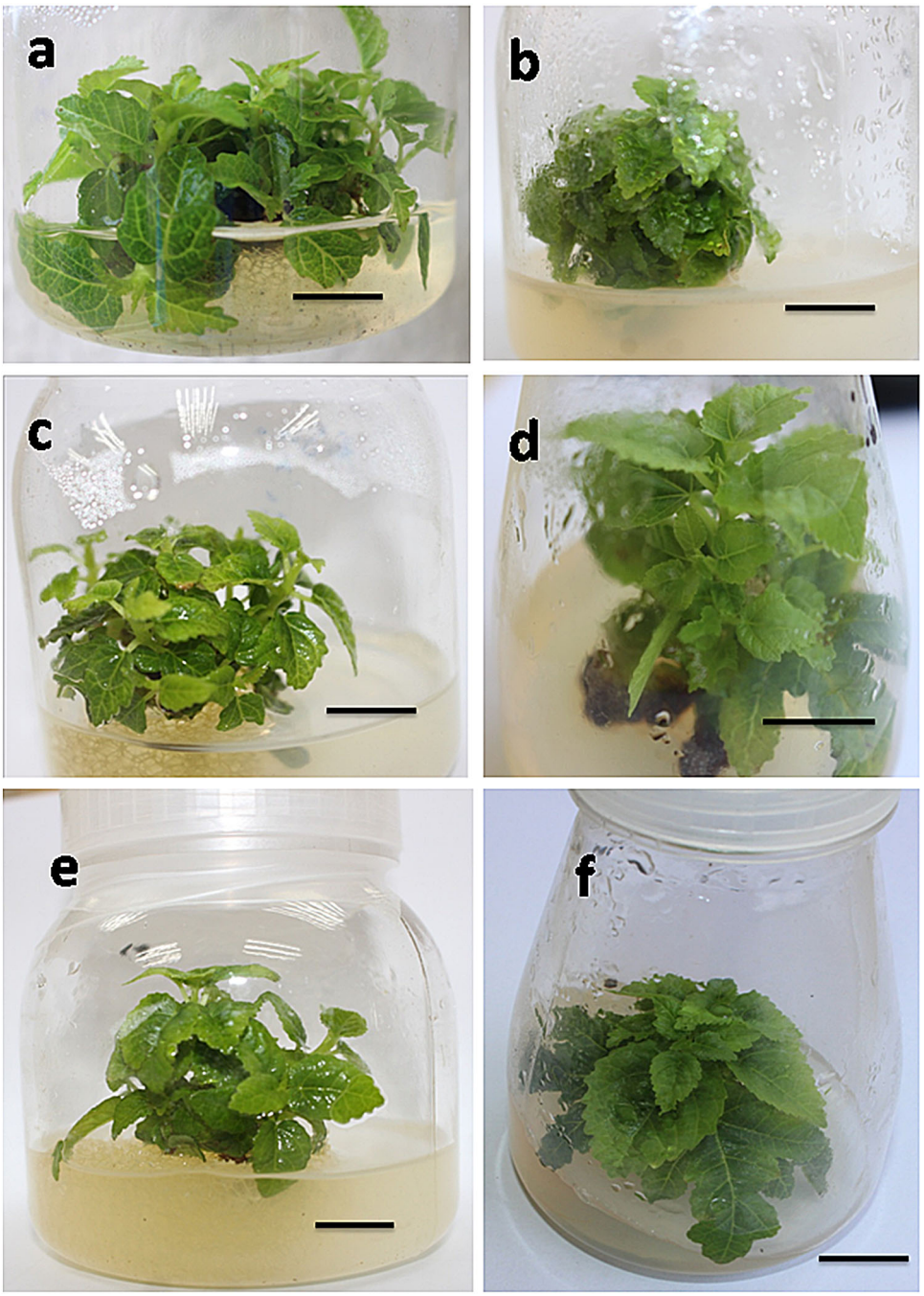

Fig. 2 Culture system and genotype effect on HCMS media with 1 mg/l BAP, 0.1 mg/l NAA and 0.02 mg/l GA_3. **a** Orphan explants on liquid media, **b** Orphan explants on solid media, **c** A134 explants on liquid media, **d** A134 explants on solid media, **e** Masui Dauphine explants on liquid media, **f** Masui Dauphine explants on solid media. Pictures were taken 6 weeks after culture. The scale represents 10 mm

Table 2 Effect of different factors on regeneration of explants (mean with same letter in each column are not significantly different according to DMRT at $\alpha = 0.05$)

Media	Shoot induction %	No of shoot	Shoot length
MS	60.0 ± 3.8ab	10.0 ± 0.6b	10.0 ± 0.3bc
1/2 NH_4	61.6 ± 3.6ab	10.0 ± 0.5b	9.3 ± 0.3ab
1/2 Ca	65.0 ± 3.1b	13.0 ± 0.5c	10.5 ± 0.3c
1/2 Sugar	52.5 ± 3.2a	9.0 ± 0.3a	8.8 ± 0.2a
Low light[a]	62.5 ± 3.2b	10.0 ± 0.2b	9.7 ± 0.3a

[a]20 μmol m^{-2} s^{-1}

Effect of culture system (liquid and solid media) on shoot induction and proliferation on HCMS media

Differences between liquid and solid culture system effects were not statistically significant. However, in general liquid culture system stimulated shoot induction, number of shoots per explants, and shoot length (Fig. 2). Shoot induction percentage increased from 65% (solid media) to 69.1% (liquid media). For individual cultivars, Masui Dauphine exhibited higher shoot induction on liquid culture media. However, no stimulation of the liquid culture system was observed for shoot induction in case of Orphan (Supplement S2). The highest stimulation in case of the number of shoots per explants was observed for Orphan. A total of 18 and 12 shoots were noted on average per explant on liquid and solid media, respectively. Considering all cultivars, the average shoot number increased from 12 in solid media to 15 on liquid. Long shoots were observed on liquid culture system for Orphan. Around 2.0 mm extra long shoots of Orphan were detected on liquid media in compared with solid media.

Genotype effect on shoot induction and proliferation

Genotype affect significantly on regeneration (Fig. 2). Shoot induction reached 77.5%, 65% and 58.7% for Orphan, Masui Dauphine and A134, respectively. The number of shoots per explant ranged from 12 to 16 among the studied cultivars. The highest number of shoot (16) was observed for Orphan. The highest shoot length (13.5 mm) was observed for Masui Dauphine, whereas a short shoot length (10.5 mm) was noted for A134 (Supplement S3).

Shoot elongation

The best elongation medium was ME 3 (HCMS media with 0.6 mg/l BAP, 0.1 mg/l NAA, and 0.1 mg/l GA_3), which produced the longest shoots among the studied media (Fig. 3). Concentration of GA_3 with elongation media significantly affected shoot length. The longest shoots measured 43.5 ± 1.2 mm for Orphan cultivar. Shoot length ranged from 20.2 ± 1.2 mm to 37.2 ± 2.0 mm and from 19.3 ± 1.3 mm to 29.8 ± 1.4 mm for Masui Dauphine and A134, respectively (Table 3). The longer shoots produced more leaves. The highest numbers of leaves per shoot were 6, 7 and 8 for A134, Masui Dauphine, and Orphan, respectively, on ME 3 media.

Rooting and acclimatization

Figure 4 shows rooted plants on medium and on soil. The plant growth regulator (PGR)-free MS medium (MR_0), half-strength MS medium with 0.5 mg/l NAA and half-strength MS medium with 1 mg/l NAA can induce roots. Percentage of root induction, the number of roots per explants, and root length significantly differed among the tested media. MR_0 was the best rooting medium for all tested genotypes. The highest (85%) percentage of rooted plants was recorded for Orphan on MR_0 media. In case of Masui Dauphine and A134, the root induction percentage was 82.50% and 62.50% respectively on the same media. The number of root induction varied from 2.9 ± 0.3 to 6.4 ± 0.3 among the studied cultivars and tested media. The highest number of roots was 6 for both Masui Dauphine and Orphan and 5 for A134 on half-strength MS medium with 1 mg/l NAA (Table 4). However, fibrous and thin roots were observed on this medium. The average number of roots per explants on MR_0 medium was significantly lower than that of roots per explants in half-strength MS with 1 mg/l NAA medium. The MR_0 medium produced long roots. The average root length on this medium measured 13.0 ± 1.8, 11.9 ± 0.4 and 7.8 ± 0.4 mm for Masui Dauphine, Orphan and A134, respectively. Rooted shoots adapted well on soil (mixture of peat moss: compost: black soil: fired soil) without showing any abnormalities.

Culture system effect on rooting

Figure 5 shows the rooted explants on MR_0 medium (PGR-free MS media) of liquid and solid culture systems. Significant differences in shoot induction percentage, the number of shoots, and shoot length were observed between liquid and solid culture systems. In liquid medium, 76% of total incubated shoots were rooted, but this value was around 35% on solid media. The average number of roots per explants was above 4 on liquid media and around 3 on solid media. On average, 3 mm-long shoots were observed on liquid media compared with solid media (Supplement S4).

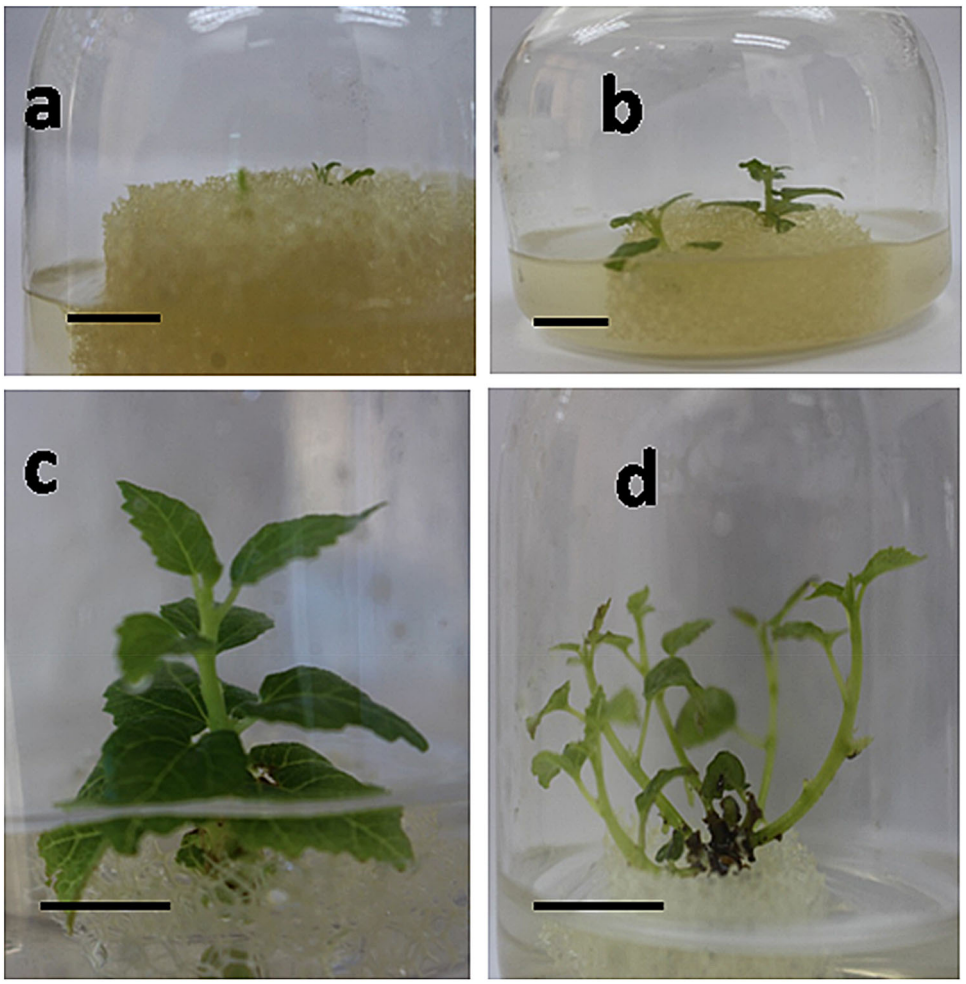

Fig. 3 Shoot elongation on ME 3 media. **a** Shoot incubation on media, **b** growing shoot on media, **c** elongated shoot on media after 4 weeks of incubation, **d** elongated shoot cluster on media after 4 weeks of incubation. The scale represents 10 mm; cultivar: Orphan

Table 3 Effect of different concentration of BAP and GA_3 with HCMS media on shoot elongation and leaf number of different cultivars (mean with same letter in each column are not significantly different according to DMRT at $\alpha = 0.05$)

Hormone combination (mg/l)				Masui Dauphine		Orphan		A134	
Media code	BAP	NAA	GA_3	Shoot length (mm)	No of leaves	Shoot length (mm)	No of leaves	Shoot length (mm)	No of leaves
ME 1	0.6	0.1	0.05	25.0 ± 1.2a	5.0 ± 0.2ab	26.7 ± 1.1a	5.0 ± 0.2ab	22.7 ± 1.1a	5.0 ± 0.2ab
ME 2	1	0.1	0.05	23.1 ± 1.3a	6.0 ± 0.5b	25.3 ± 1.2a	6.0 ± 0.3b	21.4 ± 1.4a	6.0 ± 0.4bc
ME 3	0.6	0.1	0.1	37.2 ± 2.0b	7.0 ± 0.3c	43.5 ± 1.2b	8.0 ± 0.2c	29.8 ± 1.4b	6.0 ± 0.2c
ME 4	1	0.1	0.1	20.2 ± 1.2a	4.0 ± 0.2a	22.9 ± 1.3a	5.0 ± 0.2a	19.3 ± 1.3a	4.0 ± 0.2a

Genotype effect on shoot elongation and rooting

Genotype significantly affected the overall number of leaves, root induction percentage, the number of roots and root length. Orphan produced the highest average number of leaves (6) on elongation media. Approximately 60% of shoots of Masui Dauphine cultivar produced roots, following that of Orphan (68%). Masui Dauphine produced the highest (5) number of roots and longest (8.45 mm) roots (Supplement S5).

Molecular marker selection for finger printing and genetic stability determination

Among 13 primers, 12 primers produced reproducible bands. A total of 47 distinct and scoreable loci ranging

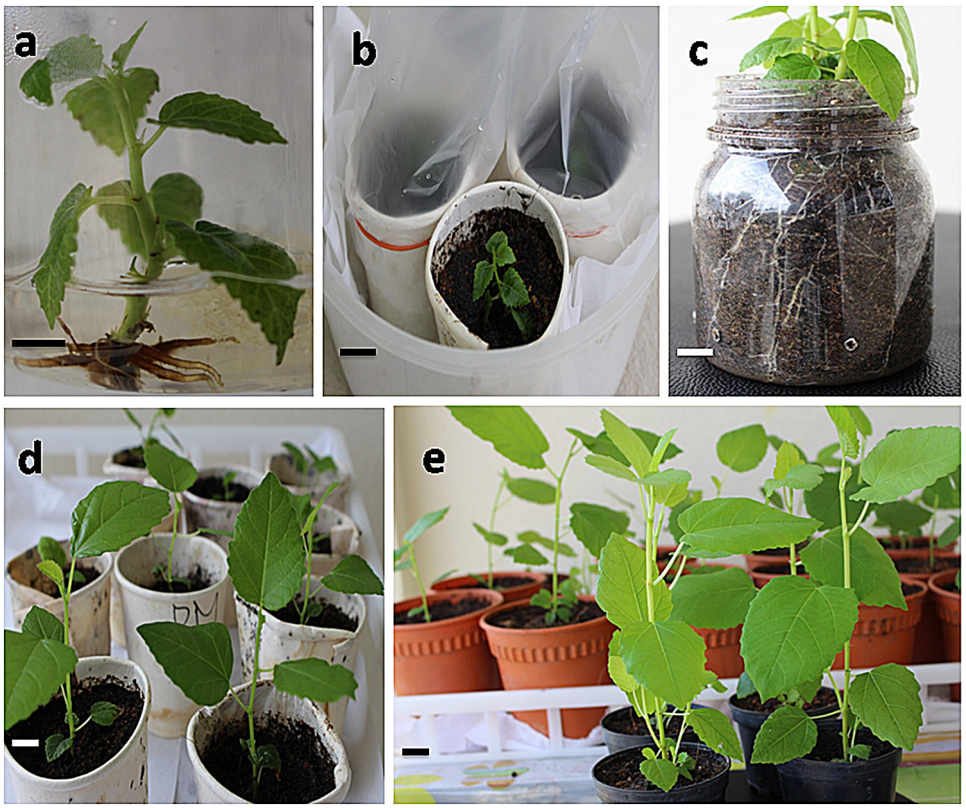

Fig. 4 In vitro rooting and plant established on soil. **a** Rooted shoot on MR_0 media after 4 weeks, **b** rooted shoot on soil media at culture room condition, **c** root elongation and proliferated in soil media after 8 weeks, **d** successfully adaptation of in-vitro regenerated plants on soil media at room condition, **e** Plantlets established on soil media at outdoor condition without showing any phenotypic abnormalities. The scale represents 10 mm; cultivar: Orphan

from 300 to 1500 bp were recorded. Primer IMA 5 produced three bands between 600 and 1400 bp; IMA 8, six bands between 550 and 1500 bp; IMA 9, five bands between 550 and 1500 bp; IMA 12, two bands between 600 and 1000 bp; IMA 303, six bands between 550 and 1500 bp; IMA 834, two bands between 600 and 900 bp; UBC 807, five bands between 500 and 1500 bp; UBC 811, five bands between 300 and 1000 bp; UBC 818, four bands between 500 and 1500 bp; UBC 827, two bands between 600 and 1000 bp; UBC 834, four bands between 300 and 1200 bp; UBC 841, three bands between 600 and 1100 bp. Primer UBC 891 produced no distinct band. A total of 853 bands (number of samples analyzed × number of scorable loci with all 12 primers) were generated during ISSR analysis, and all bands were monomorphic (Supplement S6 and S7).

UV trans-illumination image of gel showed similar banding patterns and positions for all primers. Primer UBC 841and IMA 9 were selected to cross check all 18 randomly selected plants form three replicas as they produce highly visible band loci. Supplement S8 and S9 are showing homogenous band patterns for primers UBC 841 and IMA 9, respectively.

Discussion

Concentration of PGRs and culture system (liquid or solid) affect shoot induction

The optimized medium was HCMS liquid medium with 1 mg/l BAP, 0.1 mg/l NAA, and 0.02 mg/l GA_3. On this media, around 77% of shoots from Orphan cultivar were induced to shooting, and more than 18 shoots were observed per explants. Sharma and co-workers reported the use of BAP for multiple shoot induction of fig shoot bud and short stem segments. They reported that the medium containing 7.5 µM/l (∼ 1.7 mg/l) BAP with 0.5 µM/l (0.1 mg/l) NAA is the best for shoot induction (Sharma et al. 2015). Another study reported 2 mg/l BAP with small amount of NAA (0.2 mg/l) as the best medium for shoot induction and multiplication (Kumar et al. 1998). Moreover, MS medium containing 1 mg/lα-indole-3-butyric acid (IBA), 1 mg/lGA_3, and 5 mg/l6-benzyladenine was also reported as the best multiplication medium for fig shoot tip culture (Hepaksoy and Aksoy 2006). On the other hand, low concentration of (0.5 mg/l) BAP with NAA (Bayoudh et al. 2015) or without NAA (Nobre and Romano 1997) are also reported for shoot induction and

Table 4 Root induction and growth on different strength MS liquid media with different concentration of NAA for different cultivars (mean with same letter in each column are not significantly different according to DMRT at $\alpha = 0.05$)

Hormone combination (mg/l)			Masui Dauphine			Orphan			A134		
Media code	Media strength	NAA	Root induction %	No of roots per explant	Root length (mm)	Root induction %	No of roots per explant	Root length (mm)	Root induction %	No of roots per explant	Root length (mm)
MR 0	Full MS	0	82.5 ± 3.2c	5.0 ± 0.2b	13.0 ± 1.8c	85.0 ± 2.0c	5.0 ± 0.2b	11.9 ± 0.4c	62.5 ± 3.2b	4.0 ± 0.2b	7.8 ± 0.4c
MR 1	1/2MS	0.5	37.5 ± 3.2a	3.0 ± 0.2a	3.9 ± 0.9b	43.7 ± 3.1a	4.0 ± 0.3a	4.5 ± 0.2a	27.5 ± 3.2a	3.0 ± 0.3a	4.0 ± 0.2a
MR 2	1/2MS	1	62.5 ± 4.3b	6.0 ± 0.3c	8.0 ± 1.9a	76.2 ± 1.2b	6.0 ± 0.3c	7.5 ± 0.4b	52.5 ± 3.2b	5.0 ± 0.2c	5.2 ± 0.2b

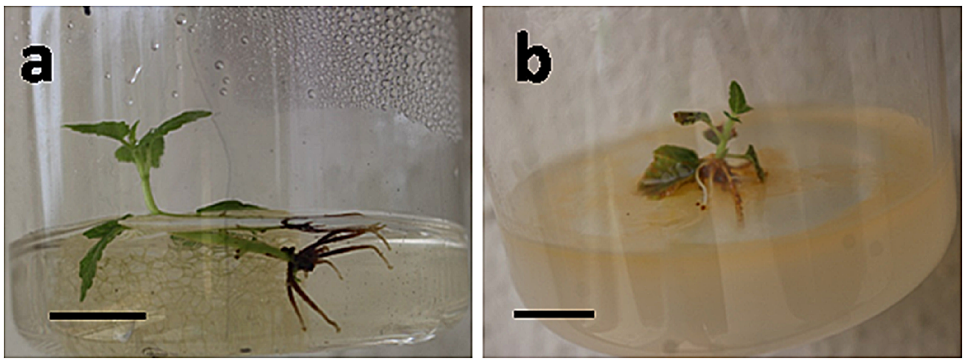

Fig. 5 Effect of culture system on MR_0 media for in vitro rooting. **a** Rooted plantlets on liquid media, **b** rooted plantlets on solid media. The scale represents 10 mm; cultivar: Orphan

multiplication. The results could be explained as the sourcing of explants from different age-old plants of different endogenous hormones activity and use of different media (solid or liquid). The young and vigorously growing shoots from one-year-old plants were biologically more active.

Half Ca^{2+} modified MS liquid media performed well for shoot regeneration

Half amount of Ca^{2+} in liquid media positively influenced shoot induction rate, the number of shoots, and shoot length in comparison with regular MS media. Reduced concentration of ammonium nitrate (825 mg/l) and calcium chloride (220 mg/l) produced more shoot buds per explants of date palm (Mazri et al. 2016). Sharma and co-researcher reported that half Ca^{2+} concentrations (220 mg/l) in the germination medium increased the number of responding explants for *Jatropa curcas* in vitro regeneration (Sharma et al. 2011). These findings support our results. Half-ammonium-nitrate-modified MS liquid medium also influenced shoot induction rate and the number of shoots per explants, though it was not statistically significant. However, later on, this medium negatively affected shoot growth (data not shown). This happened may be due to reduced phenolic oxidation of explants by low concentration of ammonium nitrate. Though, shoot growth was hampered possibly due to poor supply of nitrogen. Study reported that low concentrations of NH_4^+ and K^+ in media reduced phenolic oxidation of *Adhatoda vasica* (Nath and Buragohain 2005). For *Syringa vulgaris* L. full concentration of ammonium nitrate and potassium nitrate in MS media provide better response in compared with lower concentrations (Gabryszewska 2011). These studies support our findings. Reduced sucrose (15 mg/l) in MS media negatively affects shoot regeneration. Similar result was reported in date palm regeneration (Mazri et al. 2016).

Light intensity affects regeneration

High percentages of shoot induction but small shoots were observed from the explants cultured under reduced intensity of light. This occurred may be because reduced phenolic oxidative stress may enhance shoot induction. However, shoot growth was hampered possibly owing to low rate of photosynthesis because of low light intensity. Similar observation was reported for regeneration of cotyledonary nodes of cotton (Gupta et al. 2000).

GA_3 plays an important role on shoot elongation

The HCMS liquid medium with 0.6 mg/l BAP, 0.1 mg/l NAA, and 0.1 mg/l GA_3 was selected as the best elongation medium, producing 43.5 mm-long shoot with eight leaves. Similar observations were reported in case of GA_3 in combination with cytokine and auxin for shoot proliferation and elongation of *F. carica* (Hepaksoy and Aksoy 2006; Yakushiji et al. 2003). Significant influence of GA_3on shoot proliferation and elongation is reported. Bayoudh and others reported MS medium supplemented with BAP, NAA, and 0.1 mg/l GA_3 as the best medium for shoot tip culture (Bayoudh et al. 2015).

PGR-free MS liquid medium (MR_0) is good for rooting

More than 85% of Orphan shoots were rooted on (MR_0) media. Similar observations were reported in case of other *F. carica* rooting trials (Kim et al. 2007; Yakushiji et al. 2003). However, low percentage of rooting was reported on PGR-free MS medium and medium with low concentration of auxin was recommended (Hepaksoy and Aksoy 2006).

Genotypes affect regeneration

Genotype affects in vitro plant regeneration (Feyissa et al. 2005). In this study, significant differences were noted

among three genotypes for elongation of regenerated shoot buds, percentage of in vitro rooting, and root elongation. Other studies reported that regeneration of *F. carica* is genotype dependent (Bayoudh et al. 2015; Hepaksoy and Aksoy 2006). Similar findings were reported for *Morus alba* (Chitra and Padmaja 2005), *Hagenia abyssinica* (Feyissa et al. 2005), and *Jatropha curcas* (Kumar et al. 2010). This result could be due to different concentration of endogenous growth regulators, particularly cytokinin levels, during the induction period (Pellegrineschi 1997; Schween and Schwenkel 2003).

Regeneration retains genetic uniformity

The banding patterns and positions showed that the in vitro regenerated protocol retained the genetic stability of donor plants. The possible reason may be due to multiple shoot bud differentiation without intervening callus phase and the use of small shoots as explants. This finding supports that meristem-based micropropagation systems are considerably more genetically stable than those in which regeneration occurs via the callus phase (Dong et al. 2018; Golombek and Lüdders 1993; Polat and Caliskan 2017). The absence of somaclonal variation was reported for tree species, such as *Pithecellobium dulce* (Roxb.) Benth (Mlinarić et al. 2016), and woody species, including *Guadua angustifolia* Kunth (Vangelisti et al. 2019). Similar results were obtained by Caruso and his group during clonal fidelity analysis of in vitro propagated plantlets by random amplified polymorphic DNAand ISSR markers (Caruso et al. 2017).

Conclusion

This article reports factors affecting in vitro regeneration of *F. carica* cvs Masui Dauphine, Orphan, and A134 for the development and optimization of an efficient propagation system. The HCMS liquid medium with BAP 1 mg/l, NAA 0.1 mg/l and GA_3 0.02 mg/l was the best medium for shoot induction and proliferation. Shoot induction reached up to 77.50%, with the shoot number amounting to 18 per explants. For elongation, the HCMS liquid medium with BAP 0.6 mg/l, NAA 0.1 mg/l and GA_3 0.1 mg/l exhibited the best performance. Root induction was recorded 85% as the maximum, and the number of roots per explant as 7 depending on cultivar, medium modification, and culture system. PGR-free full-strength regular MS liquid medium was the best for in vitro rooting. In vitro rooted plantlets were successfully acclimatized on soil at outdoor conditions, showing no abnormalities. This multiplication system is faster and efficient. Molecular marker (ISSR) studies of regenerated plantlets and donor plant also confirmed that the process retains genetic stability. This optimized multiplication system may be useful in rapid clonal propagation and genetic transformation studies of fig for further research and improvement.

Acknowledgements The authors thanked University Kebangsaan, Malaysia (UKM) for providing research facilities and fund. This study would not have been possible without the cooperation of the staff and practical students of Living lives and future crop centre, UKM. This project is financed under grant FRGS/1/2019/TK02/UKM/01/2 and the Dean's Foundation of Guangdong Academy of Agricultural Sciences, Guangzhou, China (BZ201902).

Author contributions MM performed the experiments and drafted the manuscript. MM, NA and ZY reviewed and revised the manuscript. All authors read and agreed on the final manuscript.

Compliance with ethical standards

Conflict of interest Authors do not have any conflict of interest.

References

Al-Zahrani HS, Almaghrabi OA, Fuller MP, Soliman HI, Farooq M, Metwali EM (2018) Micropropagation of virus-free plants of Saudi fig (*Ficus carica* L.) and their identification through enzyme-linked immunosorbent assay methods. In Vitro Cell Dev Biol Plant 54:626–636

Bajaj Y (1988) Biotechnology in agriculture and forestry 6. Crops II. Springer, Berlin

Barolo MI, Mostacero NR, López SN (2014) *Ficus carica* L. (Moraceae): An ancient source of food and health. Food Chem 164:119–127

Bayoudh C, Labidi R, Majdoub A, Mars M (2015) *In vitro* propagation of caprifig and female fig varieties (*Ficus carica* L.) from shoot-tips. J. Agric. Sci. Technol. 17:1597–1608

Campos V (1997) Nematóides na cultura da figueira Informe Agropecuário Agropecuário. Belo horizonte 18:36–38

Caruso G, Gennai C, Ugolini F, Marchini F, Quartacci MF, Gucci R (2017) Tolerance and physiological response of young *Ficus carica* L. plants irrigated with saline water. Acta Hortic 1173:137–142

Chitra DV, Padmaja G (2005) Shoot regeneration via direct organogenesis from *in vitro* derived leaves of mulberry using thidiazuron and 6-benzylaminopurine. Sci Hortic 106:593–602

Comlekcioglu S, Kuden AB, Kacar YA, Kamberoglu MA (2007) Meristem culture of two fig cultivars in Turkey. Fruits 62:125–131

Dong J-l, Li X, Nazim G, Duan Z-Q (2018) Interactive effects of elevated carbon dioxide and nitrogen availability on fruit quality of cucumber (*Cucumis sativus* L.). J Integr Agric 17:2438–2446

Dueñas M, Pérez-Alonso JJ, Santos-Buelga C, Escribano-Bailón T (2008) Anthocyanin composition in fig (*Ficus carica* L.). J Food Compost Anal 21:107–115

Ferrara G et al (2016) Characterization of edible fig germplasm from Puglia, southeastern Italy: is the distinction of three fig types (Smyrna, San Pedro and Common) still valid? Sci Hortic 205:52–58

Feyissa T, Welander M, Negash L (2005) *In vitro* regeneration of *Hagenia abyssinica* (Bruce) JF Gmel. (Rosaceae) from leaf explants. Plant Cell Rep 24:392–400

Fráguas CB, Pasqual M, Dutra LF, Cazetta JO (2004) Micropropagation of fig (*Ficus carica* L.) 'Roxo de Valinhos' plants. Vitro Cell Dev Biol Plant 40:471–474

Gabryszewska E (2011) Effect of various levels of sucrose, nitrogen salts and temperature on the growth and development of *Syringa vulgaris* L. shoots in vitro. J Fruit Ornam Plant Res 19:133–148

Golombek SD, Lüdders P (1993) Effects of short-term salinity on leaf gas exchange of the fig (*Ficus carica* L.). Plant Soil 148:21–27

Gupta SK, Singh PK, Sawant SV, Chaturvedi R, Tuli R (2000) Effect of light intensity on in vitro multiple shoot induction and regeneration of cotton (*Gossypium hirsutum* L. cv Khandawa-2). Indian J Exp Biol 38:399–401

Hepaksoy S, Aksoy U (2006) Propagation of *Ficus carica* L. clones by *in vitro* culture. Biol Plant 50:433–436

Kim K-M, Kim MY, Yun PY, Chandrasekhar T, Lee H-Y, Song P-S (2007) Production of multiple shoots and plant regeneration from leaf segments of fig tree (*Ficus carica* L.). J Plant Biol 50:440–446

Kumar N, Anand KV, Reddy MP (2010) Shoot regeneration from cotyledonary leaf explants of *Jatropha curcas*: a biodiesel plant. Acta Physiol Plant 32:917–924

Kumar V, Radha A, Chitta SK (1998) *In vitro* plant regeneration of fig (*Ficus carica* L. cv. Gular) using apical buds from mature trees. Plant Cell Rep 17:717–720

Lemaux PG (2009) Genetically engineered plants and foods: a scientist's analysis of the issues (part II). Ann Rev Plant Biol 60:511–559

Ling WT, Liew FC, Lim WY, Subramaniam S, Chew BL (2018) Shoot induction from axillary shoot tip explants of fig (*Ficus carica*) cv. Japanese BTM 6. Trop Life Sci Res 29:165

Mazri MA, Meziani R, El Fadile J, Ezzinbi A-E (2016) Optimization of medium composition for in vitro shoot proliferation and growth of date palm cv. Mejhoul. 3 Biotech 6:111

Mlinarić S, Antunović Dunić J, Štolfa I, Cesar V, Lepeduš H (2016) High irradiation and increased temperature induce different strategies for competent photosynthesis in young and mature fig leaves. S Afr J Bot 103:25–31

Moniruzzaman M, Yaakob Z, Khatun R, Awang N (2017a) Mealybug (Pseudococcidae) infestation and organic control in fig (*Ficus carica*) orchards of Malaysia Biology and Environment. Proc R Ir Acad 117B:25–32

Moniruzzaman M, Yaakob Z, Taha R (2017b) *In vitro* production of fig (*Ficus carica* L.) plantlets. Acta Hortic 1173:231–236

Nath S, Buragohain AK (2005) Micropropagation of *Adhatoda vasica* Nees—a woody medicinal plant by shoot tip culture. Indian J Biotechnol 4:396–399

Nobre J, Romano A (1997) *In vitro* cloning of Ficus carica L. adult trees. Acta Hortic 480:161–164

Pellegrineschi A (1997) *In vitro* plant regeneration via organogenesis of cowpea [*Vigna unguiculata* (L.) Walp.]. Plant Cell Rep 17:89–95

Polat AA, Caliskan O (2017) Effect of different environments on fruit characteristics of table fig (*Ficus carica* L.) cultivars. Mod Agric Sci Technol 3(1–2):11–14. https://doi.org/10.15341/mast(2375-9402)/01.03.2017/002

Rosianski Y, Freiman ZE, Cochavi SM, Yablovitz Z, Kerem Z, Flaishman MA (2016) Advanced analysis of developmental and ripening characteristics of pollinated common-type fig (*Ficus carica* L.). Sci Hortic 198:98–106

Schween G, Schwenkel H-G (2003) Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of Primula ssp. Plant Cell Tissue Organ Cult 72:53–61

Sharma KK, Bhatnagar-Mathur P, Thorpe TA (2005) Genetic transformation technology: status and problems. Vitro Cell Devl BiolPlant 41:102–112

Sharma S, Kumar N, Reddy MP (2011) Regeneration in *Jatropha curcas*: factors affecting the efficiency of *in vitro* regeneration. Ind Crop Prod 34:943–951

Sharma S, Shahzad A, Mahmood S, Saeed T (2015) High-frequency clonal propagation, encapsulation of nodal segments for short-term storage and germplasm exchange of *Ficus carica* L. Trees 29:345–353

Vangelisti A et al (2019) How an ancient, salt-tolerant fruit crop, *Ficus carica* L., copes with salinity: a transcriptome analysis. Sci Rep 9:2561

Weiblen GD (2002) How to be a fig wasp. Annu Rev Entomol 47:299–330

Yakushiji H, Mase N, Sato Y (2003) Adventitious bud formation and plantlet regeneration from leaves of fig (*Ficus carica* L.). J Hortic 78:874–878

Zhang C, Fu S, Tang G, Hu X, Guo J (2013) Factors influencing direct shoot regeneration from mature leaves of Jatropha curcas, an important biofuel plant. Vitro Cell Dev BiolPlant 49:529–540