In Vitro Adventitious Shoot Regeneration through Direct and Indirect Organogenesis from Seedling-derived Hypocotyl Segments of *Ficus religiosa* L.: An Important Medicinal Plant

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Abstract. *Ficus religiosa* is an important industrial, medicinal, and ornamental plant, so in vitro regeneration is of high paramount in this valuable germplasm. Two efficient protocols were developed for indirect and direct shoot organogenesis through hypocotyl explants. In the first experiment, different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and indole butyric acid (IBA) (0.5, 1.0, and 1.5 mg·L⁻¹) in combination with 6-benzyl amino purine (BAP) (ratio 10:1, respectively) were used for callus formation. Two types of callus were obtained from different concentrations of plant growth regulators (PGRs). Also, 2,4-D produced yellow-brownish and friable callus (Type I), whereas the green and compact callus (Type II) was achieved in IBA. The highest callus fresh weight (2.43 g) was observed in Murashige and Skoog (MS) medium containing 0.5 mg·L⁻¹ 2,4-D plus 0.05 mg·L⁻¹ BAP. In the later experiments, various concentrations of thidiazuron (TDZ), 6-furfuryl amino purine (KN), and BAP (0.25, 0.5, 1.0, and 1.5 mg·L⁻¹) in combination with IBA (ratio 10:1, respectively) were applied for shoot regeneration (direct and indirect organogenesis). In shoot regeneration from callus, the highest regeneration frequency (86.66%) and shoot number per callus (4.13) were achieved in MS medium supplemented with 1.5 mg·L⁻¹ BAP plus 0.15 mg·L⁻¹ IBA from type I calli. However, no regeneration was observed in type II calli. In direct shoot regeneration, the highest regeneration frequency (96.66%) and shoot number (6.26) were obtained in the medium mentioned previously. In root induction experiment, different concentrations of naphthalene acetic acid (NAA) and IBA alone or in combination were applied, and MS medium containing 2.0 mg·L⁻¹ IBA along with 0.1 mg·L⁻¹ NAA was the best hormonal balance for root induction. The rooted plantlets' survival rate was more than 95% in the acclimatization stage. These results demonstrated that the direct regeneration method provides more shoot regeneration frequency and take a less time for shoot organogenesis than the indirect regeneration method. Based on our knowledge, this study is the first report of direct and indirect shoot organogenesis of *F. religiosa* via hypocotyl from in vitro-grown seedling.

Ficus is the genus of 1000 species in the family Moraceae, mainly distributed throughout tropical and subtropical regions. Many of these species have ornamental values and are also known as medicinal plants. *Ficus religiosa* L. is a long-lived, large, fuel wood, medicinal, ornamental, and evergreen perennial tree with glossy green foliage, native to India

and mainly found in Pakistan, Bangladesh, Ceylon, China, Burma, Thailand, and Iran (Singh et al., 2011). It is also known as a roadside tree that is found most frequently near temples. Different parts of *F. religiosa* are extensively used in indigenous medicine especially for their antibacterial (Pawar and Nabar, 2010), anticonvulsive (Patil et al., 2011), antidiabetic (Kirana et al., 2009), antinephropathic (Ballabh et al., 2008), wound healing (Ghosh et al., 2016), anti-inflammatory and analgesic (Singh et al., 2011), antimicrobial and antiviral (Cagno et al., 2015), antihyperlipidemic (Keshari et al., 2016), antioxidant

(Pandit et al., 2010), immunostimulant (Mallurwar and Pathak, 2008), antiasthmatic (Vinutha et al., 2007), and anticancer activities (Sankar et al., 2014), parasympathetic modulatory (Dwivedi et al., 2014), as well as nootropic effects (Bhangale et al., 2016). Mostly, the characterization and isolation of metabolites in this plant are carried out from naturally occurring sources. Such sources are time- and labor consuming and are influenced by environmental conditions and high religious values (Siwach and Gill, 2014). Hence, commercial processing demands substitute sources (Singh et al., 2011). *Ficus religiosa* generally propagates by seed or stem cutting. However, these two methods have a slow growth pattern and just rely on collecting or cutting mother plants (Salmi and Hesami, 2016). Singh et al. (2011) and Siwach and Gill (2011) reported that the rate of seed germination in this species is limited to 50%, and seeds cannot be stored for a long time. However, these methods are not sufficient under different climatic conditions (Salmi and Hesami, 2016). In addition, these traditional methods of propagation cannot describe the rising demand of pharmaceutical and ornamental purposes as well as cosmetic industries. Thus, it is necessary to establish an efficient in vitro micropropagation and plant organogenesis protocol to enhance the conservation, development, and utilization of this plant resource. There are only four studies on direct organogenesis of *F. religiosa* (Deshpande et al., 1998; Hassan et al., 2009; Siwach and Gill, 2011, 2014) and only a single tissue culture and plant organogenesis protocol via callus phase (Jaiswal and Narayan, 1985) exist, but all of those studies were based on mature explants. Plant tissue culture is known as a very applicable technique for mass production and genetic engineering of plant germplasm. In the Moraceae family, a callus-mediated organogenesis protocol could be useful in some programs that related to genetic improvement of genus *Ficus* via transgenic engineering (Sharma et al., 2015). According to the study that is about shoot regeneration via indirect organogenesis through callus phase conducted by Jaiswal and Narayan (1985), the maximum 50% regeneration frequency was achieved from a stem callus on Murashige and Skoog (MS) medium 1 mg·L⁻¹ benzyl amino purine (BAP). However, the mentioned study was related to indirect shoot organogenesis from mature stem segments via the callus phase and does not discuss about the effect of color and texture of callus in shoot regeneration. Direct shoot organogenesis in *F. religiosa* L. has been indicated by Deshpande et al. (1998) and according to their study, the maximum 68% regeneration frequency was achieved from a mature nodal explant in MS medium supplemented with 1.5 mg·L⁻¹ benzyl adenine (BA) and 1.5 mg·L⁻¹ adenine sulfate. Hassan et al. (2009) reported that the maximum shoot proliferation (78%) via apical and axillary buds was achieved in MS medium supplemented with 0.5 mg·L⁻¹ BAP plus 0.1 mg·L⁻¹ indole acetic acid (IAA). Siwach and Gill (2011) indicated that

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the highest budbreak frequency (100%) via mature nodal segments was obtained on Woody Plant Medium (WPM) supplemented with $1.0 \text{ mg}\cdot\text{L}^{-1}$ BAP along with $0.5 \text{ mg}\cdot\text{L}^{-1}$ IAA. Also, Siwach and Gill (2014) reported that the highest regeneration frequency (100%) via mature leaf segments was reached in MS medium supplemented with $5.0 \text{ mg}\cdot\text{L}^{-1}$ BAP. According to the mentioned studies, it became clear that the response of mature explants and the contamination frequency could be influenced by the season of explants collection, restricting the culture initiation experiment to a particular time period of the year (Siwach et al., 2011). Siril and Dhar (1997) and Siwach et al. (2011) reported that the seasonal factor has a massive impact on in vitro shoot organogenesis of perennial trees because of their periodic development that was known as an important limited factor in commercialization of micro-propagation of woody plants in case that it cannot be overcome by environmental or nutritional manipulations. Thus, there is a dire need of introducing an efficient and applicable protocol for *F. religiosa* to overcome this major difficulty. On the other hand, explants obtained from in vitro-grown seedlings do not depend on seasonal constraint, and also their regeneration and callus formation potentials are more than mature explants (Bhojwani and Dantu, 2013). However, there is no report for plant regeneration from immature hypocotyls of *F. religiosa*. Studies about in vitro regeneration of the Moraceae family (Bayoudh et al., 2015; Sharma et al., 2015) have mainly focused on the formation of shoots. According to this study, we introduce efficient protocols for high-frequency regeneration by two pathways, direct and indirect shoot regeneration, from *F. religiosa* hypocotyl explants. To conserve some plants that have a great value and low reproductive ability, it is essential to have alternative pathways that can lead to mass production and rapid multiplication. Although indirect organogenesis is not only a useful method for plant propagation, it is also known as a powerful tool for plant genetic improvement, germplasm preservation, and production of useful secondary metabolites. We also investigated the effects of different types of plant growth regulators (PGRs) and their concentrations on the formation of each of these pathways to establish prolific and rapid in vitro shoot organogenesis.

Materials and Methods

Seed germination and explant preparation.

The fruits were collected from 45 to 50-year-old *F. religiosa* mother plants grown in the campus of Ramin Agriculture and Natural Resources University, Khuzestan, Iran. The fruits were washed with tap water for 30 min and then washed with a liquid soap solution followed by washing with tap water as well. Further surface sterilization treatment was applied in a laminar air flow cabinet. The seeds were surface sterilized with 70% aqueous ethanol for 10 s, dipped for 5 min in 10% (v/v) NaOCl solution, and then washed three

times in sterilized distilled water. The sterilized seeds were inoculated on one-tenth strength MS medium. After 8–10 d, the seeds were germinated and the hypocotyl from in vitro germinated plants was used as a source of explant for the latter experiment.

Media and culture condition. MS medium that fortified with $30 \text{ g}\cdot\text{L}^{-1}$ sucrose (Duchefa biochemie, Haarlem, Netherlands) and gelled with 0.6% agar (Duchefa biochemie) was used as the basic culture medium. Also, the pH of the medium was adjusted to 5.7 ± 0.2 with 0.1 N KOH or 0.1 N HCl after adding the different concentrations of growth regulators into the medium. The medium was dispensed into a flask and autoclaved at 121°C for 30 min and also, all the cultures were maintained in a sterilized culture room at $26 \pm 2^\circ\text{C}$, under 16 h photoperiods provided by cool white fluorescent light ($65 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and with 55% to 60% relative humidity.

Callus induction. The hypocotyls of the 3-week-old single seedling were used as the explants for callus formation. The hypocotyl was cut into sections of 5 mm long, and the explants were placed horizontally on MS medium containing auxin—2,4-dichlorophenoxyacetic acid (2,4-D) or indole butyric acid (IBA)—in combination with cytokinin, BAP, in the absence of light. The auxins were used at three concentrations (0.5 , 1.0 , and $1.5 \text{ mg}\cdot\text{L}^{-1}$). The ratio of auxin and cytokinin in the media was 10:1. Data of callus formation frequency (%) and its fresh weight (g) were recorded after 4 weeks of culture.

Morphogenesis from different callus types. Type I (yellow-brown and friable) and II (green and compact) calli were excised from cultured hypocotyl segments and cut into 8- to 9-mm-diameter callus bulk that were inoculated on MS medium containing cytokinin—BAP, furfuryl amino purine (KN), or TDZ—in combination with auxin, IBA, in the presence of light. The cytokinins were used at four concentrations (0.25 , 0.5 , 1.0 , and $1.5 \text{ mg}\cdot\text{L}^{-1}$). The ratio of cytokinin and auxin in the media was 1:10. Calli that generated from hypocotyl explant were sub-cultured every 3 weeks on the same composition of fresh MS medium. The percentage of shoot regeneration from callus, average number of shoots per inoculum, and shoot length (cm) were recorded on the 60th d after transferring the callus on shoot organogenesis media.

Direct shoot organogenesis. The hypocotyl segment (about 5 mm long) separated from the same 3-week-old seedling of *F. religiosa*, which was used to begin the callus culture. The explants were placed horizontally on MS medium containing BAP, KN, or TDZ in combination with auxin, IBA, in the presence of light. The cytokinins were used in the following concentrations: 0.25 , 0.5 , 1.0 , and $1.5 \text{ mg}\cdot\text{L}^{-1}$. The ratio of cytokinin to auxin in the media was 10:1. The cultures were sub-cultured on the fresh medium after 3 weeks. The percentage of explants forming adventitious shoots (organogenesis frequency), the average number of shoots per explant, and shoot length (cm) were recorded after 45 d.

Shoot elongation and rooting. Individual shoots of 2–3 cm length were excised from the multiple shoots and inoculated on elongation MS medium containing $0.5 \text{ mg}\cdot\text{L}^{-1}$ BAP and $0.5 \text{ mg}\cdot\text{L}^{-1}$ gibberellic acid (GA_3). After 4 weeks, elongated shoots were transferred to rooting medium containing different concentrations and combinations of IBA and naphthalene acetic acid (NAA) or no PGRs (Table 4). The percentage of root induction, number of roots per shoot, and the root length were recorded after 5 weeks.

Acclimatization and transplantation. The plantlets were removed from the culture tubes within 4 weeks after transferring to MS medium and washed several times with sterilized water to remove some traces of media on root surfaces. Afterward, the plants were transferred to perlite and cocopeat with the ratio of 1:1. The pots were placed in plastic covers ($50 \text{ cm} \times 30 \text{ cm} \times 3 \text{ cm}$) at $26 \pm 2^\circ\text{C}$ under a 16-h photoperiod for 4 weeks. To reduce the relative humidity inside the covers, after the first week, the covers were gradually opened and 4 weeks after transplanting those plants, the covers were completely removed. Also, plant survival (%) was recorded after this period.

Statistical analysis. The experiments were set up in completely randomized design, and there were 10 replications per treatment and each treatment was repeated in three sets. The data were analyzed by analysis of variance followed by Duncan's multiple range test ($P < 0.05$). Data analysis was carried out by using SAS version 9.3 and SPSS version 21.

Results and Discussion

The effect of different PGRs on callus induction. In vitro plant regeneration from different parts of in vitro-grown seedlings has received notable attention, and many researchers have used various parts of in vitro-grown seedling as explants (Jafari et al., 2017; Mali and Chavan, 2016; Niazi et al., 2017; Phulwaria et al., 2013; Singh et al., 2016) to improve the micropropagation method for many plant species. This study demonstrated the candidature of hypocotyl segments, which are obtained from axenic seedling, as a source of explant for in vitro regeneration of *F. religiosa* for the first time (Fig. 1A). By investigating hypocotyl explants cultured on MS media, the effect of different concentrations of PGRs on callus formation of *F. religiosa* was determined (Table 1; Fig. 1B and C). According to our result, the explants failed to respond on PGR-free MS medium. The phenolic exudation caused blackening of the cut ends, and finally of the whole explant. Thus, phenolic exudation leads explants to death within 2 weeks of culture. The hypocotyl segments induced to form callus when the medium contained different concentrations of auxins. However, the frequency of callus formation was low in the case of $0.5 \text{ mg}\cdot\text{L}^{-1}$ IBA with $0.05 \text{ mg}\cdot\text{L}^{-1}$ BAP and also, the explant exhibited the signs of stress, such as becoming yellowish, wrinkled surface, and finally led to necrosis,

Fig. 1. In vitro shoot regeneration through direct and indirect organogenesis from seedling-derived hypocotyl segments of *Ficus religiosa* L. (A) Seedling from in vitro seed germination. (B) Yellow-brown and friable callus induction on MS + 0.5 mg·L⁻¹ 2,4-D + 0.05 mg·L⁻¹ BAP. (C) Green and compact callus induction on MS + 1.5 mg·L⁻¹ IBA + 0.15 mg·L⁻¹ BAP. (D) Shoot regeneration from callus on MS + 1.5 mg·L⁻¹ BAP + 0.15 mg·L⁻¹ IBA. (E) Shoot regeneration from hypocotyl segment on MS + 1.5 mg·L⁻¹ BAP + 0.15 mg·L⁻¹ IBA. (F) In vitro root formation on MS + 2.0 mg·L⁻¹ IBA + 0.1 mg·L⁻¹ NAA. (G) Acclimatized regenerated plants after 8 weeks. BAP = benzyl amino purine; 2,4-D = 2,4-dichlorophenoxyacetic acid; IBA = indole butyric acid; MS = Murashige and Skoog; NAA = naphthalene acetic acid.

Table 1. Effect of 2,4-D or IBA in combination with BAP in Murashige and Skoog medium on callus induction of *Ficus religiosa* from hypocotyl explant.

Plant growth regulator (mg·L ⁻¹)			Callus formation frequency (%)	Fresh wt of callus (g)
2,4-D	IBA	BAP		
—	—	—	0.000 d	0.000 e
0.5	—	0.05	100.00 a	2.433 a
1.0	—	0.1	100.00 a	2.233 b
1.5	—	0.15	93.333 a	2.033 c
—	0.5	0.05	66.667 c	1.567 d
—	1.0	0.1	83.333 b	1.867 c
—	1.5	0.15	96.667 a	2.367 ab

Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$.

2,4-D = 2,4-dichlorophenoxyacetic acid; BAP = benzyl amino purine; IBA = indole butyric acid.

whereas 2,4-D was indicated as an appropriate auxin for inducing callus. PGRs play an important role in the organogenic response of any plant tissue or organ under in vitro

conditions (Bhojwani and Dantu, 2013). Synthetic auxins such as 2,4-D are critical PGRs which are mainly used in most of the embryogenic cell and tissue culture systems

and also involved in callus formation and the establishment of cell suspension culture (Ge et al., 2016). Some studies have confirmed the positive effect of 2,4-D on callus formation during the physiological and molecular process in many cases, and those studies demonstrated that 2,4-D regulates the endogenous IAA metabolism, promotes specific proteins, and controls DNA methylation (Pan et al., 2010). After 1–2 weeks of culture, the callus formation began from the cut edges of the explants. Different frequencies of callus induction were reached on MS medium supplemented with different concentrations of PGRs. The highest callus fresh weight achieved in two treatments (0.5 mg·L⁻¹ 2,4-D plus 0.05 mg·L⁻¹ BAP and 1.0 mg·L⁻¹ 2,4-D plus 0.1 mg·L⁻¹ BAP) is completely in agreement with Siwach et al. (2011) in *F. religiosa* through nodal, internode, and shoot apices explants. In another study, Jaiswal and Narayan (1985) obtained the callus formation via mature internode explant of *F. religiosa* based on the MS basal medium with 0.5 mg·L⁻¹ NAA plus 1.0 mg·L⁻¹ BAP. Bhojwani and Dantu (2013) reported that 2,4-D may possess herbicidal property at high concentrations which might prevent callus induction. An early response to callus formation was obtained on 0.5 mg·L⁻¹ 2,4-D plus 0.05 mg·L⁻¹ BAP, whereas on increasing the concentration of 2,4-D, the rate of callus formation decreased exponentially. However, Parasharami et al. (2014) reported that the best callus induction from fruit segments of *F. religiosa* was achieved in MS medium containing 2.4 mg·L⁻¹ 2,4-D along with 1.0 mg·L⁻¹ BAP. The particular concentration of PGRs has a massive impact on callus formation in the culture medium. On the other hand, Bhojwani and Dantu (2013) indicated that concentrations of the PGRs can completely depend on plant species and rely on the source and age of the explant.

According to growth regulators on basal medium, two different callus types [type I (yellow-brown and friable) and II (green and compact)] were obtained. The hypocotyl explants that cultured on MS medium containing 2,4-D produced yellow-brown and friable calli (Fig. 1B). However, green and compact calli (Fig. 1C) were achieved in MS medium supplemented with IBA. The calli obtained from the same explant may show considerable variation in terms of their color, the amount of water content, texture, and morphogenic potential. Also, the calli might have a compact or friable texture and light or dark color. These features may also change by time in cultures because of genetic or epigenetic changes or some amendments in culture medium (Bhojwani and Dantu, 2013). Mohajer et al. (2012) reported that the synthesis of phenolic substances on the cells of callus can lead to changes in the color of the callus. Changes in the color of the callus are a sign for decreasing growth of callus. According to Jiménez and Bangerth (2000), the cells of callus were still active and easily defended when the color of callus changed from white to yellow, and brown color

indicates symptoms of aging of cells. Moreover, Hu et al. (2015) suggested that changes in the color of the callus can lead to changes in the growth step and regeneration of cells. Also, the color of the callus explains the visual appearance of callus that is a sign for the activity level of the cells of the callus. Bhojwani and Dantu (2013) indicated that cells in friable callus have poor bonds and they easily separate from each other, but it would be vice versa in compact callus. Also, Chen et al. (2016) reported that friable callus has a great quality than the compact one because it can easily separate into a single cell. Despite that, there is no report on callus formation via different types of callus of *F. religiosa*; the effect of color and texture of callus on callus formation of other plants has been investigated in many studies. Garcia et al. (2011) reported that different PGRs produced different callus types (compact, friable, and mucilaginous) which were obtained from the leaf segments of *Passiflora suberosa*. Likewise, Karami et al. (2009) obtained various callus types from *Elaeagnus angustifolia* because of different growth regulators. In contrast, Lavanya et al. (2014) indicated that callus morphology was similar among the different growth regulators in *Hildegardia populifolia*.

The effect of different PGRs on morphogenesis from different callus types. All treatments except control (no PGR) induced shoot regeneration after 1–2 weeks in type I calli (Fig. 1B), whereas there was no shoot generation observed in type II calli. Different concentrations of BAP, TDZ, or KN in combination with IBA had a significant difference in terms of regeneration frequency and number of shoots per explant in type I calli (Table 1). The highest regeneration frequency (86.66%) and the maximum shoot number (4.13) were obtained from type I calli in media containing 1.5 mg·L⁻¹ BA in combination with 0.15 mg·L⁻¹ IBA (Table 2; Fig. 1D). Also, BAP produced higher number of shoots than other cytokinins. According to other studies, Karami et al. (2009) and Murthy et al. (2010) reported that BAP exerts a powerful influence on shoot multiplication in several cases. Jaiswal and Narayan (1985) reported that shoots regenerated via stem segments of adult plants of *F. religiosa* callus cultured on the MS medium with 1.0 mg·L⁻¹ BA. However, they did not indicate the type of callus that they used in their study. In our experiments, the high frequency of regeneration was obtained from type I calli in MS medium containing 1.5 mg·L⁻¹ BA in combination with 0.15 mg·L⁻¹ IBA. Preliminary experiments indicated that the texture of the callus is an important factor in organogenesis response (Chen et al., 2016; Karami et al., 2009). In accordance with our results, Karami et al. (2009) demonstrated that different regeneration responses through various callus types from cotyledon segments of *E. angustifolia* were obtained, according to the growth regulators. Chen et al. (2016) investigated the different growth regulators and obtained various regeneration frequencies via

different callus types from leaf explants of *Chirita swinglei*.

The effect of different PGRs on direct organogenesis. Regeneration potential from hypocotyl segments was investigated in MS medium containing various PGRs (Table 3). Apart from the types and concentrations of PGRs in MS medium, shoots regenerated from the hypocotyl explants within 2 weeks of incubation. However, control MS medium did not support the shoot induction. The shoot regeneration frequency was varied with the types and concentrations of growth regulators. Media containing 1.5 mg·L⁻¹ BAP in combination with 0.15 mg·L⁻¹ IBA proved to be most efficient in shoot regeneration, shoot number, and shoot length with 96.66%, 6.26, and 1.83 cm, respectively (Fig. 1E). The frequency of shoot regeneration was comparatively low and there were fewer shoots per explant in the MS medium containing KN or TDZ instead of BAP (Table 3). The shoot regeneration frequency increased with increasing concentrations of BAP up to 1.5 mg·L⁻¹. In contrast, shoot regeneration suppressed gradually when TDZ concentration increased beyond 0.25 mg·L⁻¹. Among all concentrations of TDZ, a maximum number of multiple shoots (4.33) was observed in MS medium

supplemented with 0.25 mg·L⁻¹ TDZ. TDZ is known as a potential substituted for phenyl urea (N-phenyl-1,2,3-thiadiazol-5-yl urea) that has vast potentials as a cytokinin in shoot multiplication in several plant systems, especially in the woody species (Ahmad and Anis, 2007; Mansouri and Preece, 2009; Siwach and Gill, 2011). On the other hand, many studies also confirmed that TDZ has a negative impact on shoot organogenesis. Thus, when it is used above the threshold level, the shoots regenerated as deformed ones showing different levels of hyper-hydration (Bosela and Michler, 2008; Feng et al., 2010). Hyper-hydration is known as a physiological disorder that occurs during plant tissue culture which exerts a negative influence on the ability of growth and regeneration of cultures which leads to explants that is not able to maintain or propagate (Bhojwani and Dantu, 2013). It is recommended that hyper-hydration of cultures mainly occurs because of the higher concentration of PGRs as well as water potential of the culture medium (Siwach and Gill, 2011). Therefore, it would be important in the time that the cultures are established, growth regulators and medium should be optimized. Regarding our study, after 8 weeks of culture period, the negative

Table 2. Effects of TDZ, BAP, or KN in combination with IBA in Murashige and Skoog medium on shoot regeneration from callus of *Ficus religiosa*.

Plant growth regulator (mg·L ⁻¹)				Regeneration frequency (%)	Number of shoots per explant	Length of the shoots (cm)
TDZ	BAP	KN	IBA			
—	—	—	—	0.000 g	0.000 i	0.000 f
0.25	—	—	0.025	73.333 bc	2.367 e	1.033 e
0.5	—	—	0.05	73.333 bc	1.533 f	1.023 e
1.0	—	—	0.1	70.000 c	1.267 g	0.967 e
1.5	—	—	0.15	53.333 e	1.067 h	0.933 e
—	0.25	—	0.025	56.667 de	2.567 d	1.633 b
—	0.5	—	0.05	76.667 abc	2.700 d	1.833 a
—	1.0	—	0.1	83.333 ab	3.567 c	1.700 b
—	1.5	—	0.15	86.667 a	4.133 a	1.667 b
—	—	0.25	0.025	33.333 f	1.167 gh	1.333 d
—	—	0.5	0.05	46.667 e	1.433 f	1.267 d
—	—	1.0	0.1	66.667 cd	2.333 e	1.367 cd
—	—	1.5	0.15	76.667 abc	3.867 b	1.467 c

Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$.

TDZ = Thidiazuron; KN = furfuryl amino purine; IBA = indole butyric acid; BAP = benzyl amino purine.

Table 3. Effect of TDZ, BAP, or KN in combination with IBA in Murashige and Skoog medium on regeneration response of *Ficus religiosa* from hypocotyl explant.

Plant growth regulator (mg·L ⁻¹)				Regeneration frequency (%)	Number of shoots per explant	Length of the shoots (cm)
TDZ	BAP	KN	IBA			
—	—	—	—	0.000 g	0.000 i	0.000 e
0.25	—	—	0.025	93.333 ab	4.333 c	1.267 c
0.5	—	—	0.05	86.667 abc	3.767 e	0.967 d
1.0	—	—	0.1	56.667 d	2.533 f	0.833 d
1.5	—	—	0.15	46.667 de	1.067 h	0.967 d
—	0.25	—	0.025	16.667 f	2.533 f	1.867 a
—	0.5	—	0.05	56.667 d	4.033 d	1.967 a
—	1.0	—	0.1	86.667 abc	5.033 b	1.933 a
—	1.5	—	0.15	96.667 a	6.267 a	1.833 a
—	—	0.25	0.025	36.667 e	1.233 g	1.367 c
—	—	0.5	0.05	46.667 de	1.267 g	1.567 b
—	—	1.0	0.1	76.667 c	3.667 e	1.567 b
—	—	1.5	0.15	83.333 bc	4.133 d	1.633 b

Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$.

TDZ = Thidiazuron; KN = furfuryl amino purine; IBA = indole butyric acid; BAP = benzyl amino purine.

effect of TDZ was observed and was probably due to extending the time of explant exposure to TDZ or using above the threshold level of this growth regulator. Although the exact mechanism of TDZ is not understandable enough, it is supposed to be involved in the regulation of endogenous levels of different growth regulators. Based on our results, the BAP had better performance in shoot regeneration than other PGRs. There was much evidence which elucidated that determination of a suitable concentration and type of PGRs had a massive impact on the rate of successful regeneration of *F. religiosa*. Many studies proved the positive influence of cytokinin on cell division and shoot regeneration (Arab et al., 2014; Jafari et al., 2017; Siwach and Gill, 2011, 2014). Hesami et al. (2017a) and Siwach and Gill (2014) reported that TDZ is more effective than BAP on the number of shoot regeneration, whereas various studies take a radical point of view and alleged that BAP is more effective than TDZ (Deshpande et al., 1998; Hassan et al., 2009; Siwach and Gill, 2011). Therefore, these conflicting results might be related to the use of various explants and effects of various genotypes. Deshpande et al. (1998), in proliferation of *F. religiosa* via mature nodal segments, showed that BAP is the most effective cytokinin. Also, Hassan et al. (2009) reported that shoot organogenesis was obtained from apical and axillary buds of *F. religiosa* in MS medium containing 0.5 mg·L⁻¹ BAP + 0.1 mg·L⁻¹ IAA. In another study, Siwach and Gill (2011) reported that the maximum number of multiple shoots from nodal segments of *F. religiosa* was achieved on WPM containing 1.0 mg·L⁻¹ BAP along with 0.5 mg·L⁻¹ IAA, and these findings confirmed our results. The influence of cytokinins on micropropagation can be varied based on the kind of culture medium, the variety of plants, and the age of explants (Bhojwani and Dantu, 2013; Hesami et al., 2017b; Siwach and Gill, 2014).

Root formation and acclimatization. After third subculturing on MS medium, the shoots were inoculated on MS medium containing a combination of 0.5 mg·L⁻¹ BAP and 0.5 mg·L⁻¹ GA₃ (elongation medium). After shifting to the elongation medium, the shoots showed a significant increase in the length (2–3 cm) after 3–4 weeks of culture. The long shoot length was reached in the MS medium containing with different concentrations of BAP (Fig. 1D). Siwach and Gill (2011) reported that the conducive effect of BAP on shoot length was also reached in *F. religiosa*. Gibberellins exert a positive influence on cell division and shoot elongation (Xiao et al., 2016). Other studies proved that Gibberellin in the medium promotes the elongation of in vitro shoots (Inthima et al., 2017; Siwach and Gill, 2011). Other studies, on the other hand, take a radical point of view and allege that incubating in the culture medium containing low PGR (lower to the threshold level on which shoot regeneration obtained) or PGR-free medium are known as a profitable way for shoot elongation (Sivanesan et al., 2011). For

this study, we applied both of the methods mentioned previously (adding 0.5 mg·L⁻¹ GA₃ plus 0.5 mg·L⁻¹ BAP) and the successfully elongated the shoot (2–3 cm). To induce the root, the elongated shoots were shifted to MS medium containing different concentrations of NAA and IBA. There was no root induction observed in control MS medium, whereas root induction with various frequencies was observed in MS medium containing different concentrations of NAA, IBA, or both (Table 4). By increasing the concentration of IBA, the root frequency was increased exponentially up to 2 mg·L⁻¹ IBA. Also, this result was obtained in high concentrations of NAA. Generally, MS medium containing 2.0 mg·L⁻¹ IBA with 0.1 mg·L⁻¹ NAA had the highest frequencies of root induction (96.66%) and number of roots per shoot (5.56) as well as root length (4.9 cm) (Fig. 1F). IBA and NAA that belong to auxins are most frequently involved in the medium for inducing root (Siwach and Gill, 2011). Jaiswal and Narayan (1985) investigated the various concentrations of NAA for root induction, and they found that the most suitable NAA concentration for inducing root in *F. religiosa* is 1.0 mg·L⁻¹. Also, Deshpande et al. (1998) found that 2 mg·L⁻¹ IBA in

combination with 0.1 mg·L⁻¹ NAA is the most effective PGR balance for root induction of in vitro shoots of *F. religiosa*, whereas Hassan et al. (2009) reported that 2.0 mg·L⁻¹ IBA along with 0.1 mg·L⁻¹ NAA is the most suitable one. The plantlets showed more than 90% survival in the greenhouse after acclimatization (Fig. 1G). Our results are in agreement with Deshpande et al. (1998), Hassan et al. (2009), and Siwach and Gill (2014).

Conclusions

In some plants that have an immense ornamental or medicinal benefit, a single tissue culture and plant organogenesis method via adventitious shoot regeneration are not sufficient for conservation purposes. To conserve these ornamental and medicinal plants, it is necessary to establish multiple plant organogenesis pathways. In addition, indirect organogenesis is known as a powerful tool for plant genetic engineering when it is used to accompany with conventional agricultural techniques. It also plays an important role in understanding plant growth pattern and mechanisms of cell differentiation. Our study was developed based on the previous studies

Table 4. Effect of auxins (IBA or NAA) in Murashige and Skoog medium on in vitro root induction in regenerated shoots of *Ficus religiosa*.

Plant growth regulator	Rooting frequency (%)	Number of roots per explant	Root length (cm)
Control (free of plant growth regulators)	0.000 h	0.000 h	0.000 h
1.0 mg·L ⁻¹ IBA	46.667 g	2.333 g	2.900 d
1.5 mg·L ⁻¹ IBA	66.667 de	2.567 f	2.600 e
2.0 mg·L ⁻¹ IBA	76.667 bc	4.033 c	2.867 de
1.0 mg·L ⁻¹ NAA	43.333 g	2.267 g	1.367 g
1.5 mg·L ⁻¹ NAA	63.333 def	2.767 f	2.033 f
2.0 mg·L ⁻¹ NAA	70.000 cd	3.667 d	2.167 f
1.0 mg·L ⁻¹ IBA + 0.1 mg·L ⁻¹ NAA	60.000 ef	2.633 f	2.633 de
1.5 mg·L ⁻¹ IBA + 0.1 mg·L ⁻¹ NAA	76.667 bc	3.633 d	3.400 c
2.0 mg·L ⁻¹ IBA + 0.1 mg·L ⁻¹ NAA	96.667 a	5.567 a	4.900 a
0.1 mg·L ⁻¹ IBA + 1.0 mg·L ⁻¹ NAA	56.667 f	2.733 f	1.367 g
0.1 mg·L ⁻¹ IBA + 1.5 mg·L ⁻¹ NAA	76.667 bc	3.033 e	2.700 de
0.1 mg·L ⁻¹ IBA + 2.0 mg·L ⁻¹ NAA	83.333 b	4.433 b	4.333 b

Means in each column followed by the same letters are not significantly different according to Duncan's multiple range test at $P < 0.05$.

IBA = indole butyric acid; NAA = naphthalene acetic acid.

Fig. 2. Simplified diagram of two shoot organogenesis pathways (direct and indirect) from *Ficus religiosa* hypocotyl explants.

on shoot organogenesis system of *F. religiosa*, which was limited to the simple shoot regeneration. This is the first report of efficient in vitro shoot regeneration for *F. religiosa* from young derived hypocotyl explants by two distinct pathways, after indirect shoot regeneration and direct shoot organogenesis (Fig. 2). According to indirect organogenesis pathway, two types of callus were obtained and just one type of callus (yellow brownish and friable callus achieved in MS medium containing 2,4-D) regenerated. This protocol provides a basic knowledge of future transgenic and other biotechnological applications for this plant. The highest shoot multiplication was obtained in MS medium supplemented with 1.5 mg·L⁻¹ BAP and 0.15 mg·L⁻¹ IBA in both direct and indirect organogenesis pathways. The present study presents a cost-effective, prolific, and efficient direct shoot organogenesis system of *F. religiosa* using in vitro-grown seedling-derived hypocotyl explants. In addition, this study introduced an efficient protocol for *F. religiosa* during acclimatization stage. Also, it has a significant potential for supplying mass propagation in short duration that proved the economic value of this protocol. Based on our results, the direct regeneration pathway provides more shoot regeneration frequency and takes a less time for shoot organogenesis than another pathway. Also, this study serves as a practical and powerful technique for mass propagation methods for this medicinal and ornamental plant.

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