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Micropropagation of three endemic and endangered fig (*Ficus carica* L.) genotypes

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Key words: explant, rooting, single node, shoot proliferation.

Abstract: In this research, *in vitro* propagation of three endangered and endemic fig genotypes named 'Bargchenari', 'Dehdez' and 'Runu' were investigated. For shoots proliferation, nodal explants on Murashige and Skoog (MS) medium were treated with different concentrations of growth regulators. Rooting of shoots was initiated by the use of MS and MS/2 media and various concentrations of Indole-3-butyric acid (IBA) (0, 0.5, 1 or 1.5 mg l^{-1}). The experiments were conducted in a completely randomized design. Results showed that shoots proliferation improved as subcultures increased. The highest shoot numbers per explant 13.67 and 8.8 were achieved eight weeks after second subculture in 'Runu' and 'Bargchenari' genotypes respectively, when MS medium supplemented with 0.5 mg l^{-1} benzyl adenine (BA) and 0.2 mg l^{-1} N^6-(Δ^2-isopentenyl)adenine (2ip). In 'Dehdez' genotype, the number of shoots was 4.4 per explant, when culture media were supplemented with 6 mg l^{-1} Kinetin (Kn) and 0.2 mg l^{-1} 6-α-naphthaleneacetic acid (NAA). The highest root numbers (2.23) were obtained in 'Bargchenari' genotype on MS/2 medium containing 1.5 mg l^{-1} IBA.

1. Introduction

Figs are one of the earliest cultivated fruit-bearing trees. Iran is one of the major producing countries of fig (*Ficus carica* L.) and it stands fifth after Turkey, Egypt, Algeria and Morocco (FAO, 2012). In Iran, some of the genotypes, including 'Bargchenari', 'Runu' (Faghih and Sabet-Sarvestani, 2001) and 'Dehdez' (Gholami, 2012) are endangered. Compatible old native varieties and their wild relatives as genetic resources are the basis of the breeding programs. In addition, the continuing the search for high yielding varieties with resistance to pests and pathogens ensures the availability and maintenance of a large genetic resources that guarantee accessibility of a useful genetic material at any time (Chawla, 2009).

The number of endangered plant species in the world has increased in recent years due to anthropogenic and environmental pressures. Numerous species are described as endangered, and integrated programs are required to protect and preserve current biodiversity (Sarasan *et al.,* 2006). Special attention must be given to endemic flora, which is a critical target in conservation strategies because it is restricted to small areas (Mallon *et al*., 2008). Genetic conservation of plant can be performed in situ or *in vitro*. Field collections (*in situ*), loose genetic biodiversity because of pests, diseases and adverse weather conditions and their maintenance is labor-intensive and expensive. Recently, the use of *in vitro* tools is an important way of safeguarding the germplasm of endangered plants and become increasingly popular for conservation purposes (Sarasan *et al.,* 2006; Bunn *et al.,* 2007; Mallon *et al.*, 2008; Piovan *et al.,* 2010). *In vitro* techniques can be used for not only the production of large number of plantlets in short period of time, but also for conservation of rare and endangered plant species, including genetic resources of recalcitrant seed and vegetatively propagated of elite genotypes (Engelmann, 2011). Several reports described shoot regeneration and organogenesis from various explants by the use of different culture media, types and concentrations of plant growth regulators in *F. carica* L. (Brum *et al.,* 2001; Fráguas *et al.,* 2004). Somatic embryogenesis from leaf segment of fig (Sultani genotype) was reported on MS medium supplemented with 30 mg l^{-1}

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N6-(Δ2-isopentenyl)adenine (2ip) and 7 mg l^{-1} Thidiazuron (TDZ) and 0.25 mg l^{-1} 6-α-naphthaleneacetic acid (NAA) (Soliman *et al.,* 2010). Dhage *et al.* (2012) obtained shoot regeneration from leaf callus initiated of four fig genotypes on MS medium supplemented with 0.5 mg l^{-1} NAA and 7 mg l^{-1} TDZ. For successful micropropagation axillary buds or shoot tip cultures are preferred as preexisting meristem easily develop into shoots while maintaining clonal fidelity and reduce the risk of somaclonal variability (Anis *et al.,* 2003; Ning *et al.,* 2007).

The present study was undertaken with the aim to evaluate the regeneration potential of three endangered genotypes and to establish an efficient *in vitro* method for their rapid propagation by culture of nodal explants using various plant growth regulators.

2. Materials and Methods

Preparation and decontamination of explants

Shoots with a length of 5-20 cm were collected from mother plants of three different genotypes ('Bargchenari', 'Dehdez' and 'Runu') grown in a greenhouse with 40% relative humidity, day and night average temperatures of 30±2°C and 24±2°C respectively. The shoots were kept for one hour under the running tap water then dipped for 20 min in a solution of 4 g l^{-1} benomyl. Explants were surface sterilized with 70% ethanol for 5 min, then were disinfected with 15% Clorox (commercial bleach, 5.25% sodium hypochlorite) for 15 min and they were rinsed 3-4 times with sterile distilled water. Then, the single-node segments were isolated and inoculated on a proliferation medium (MS) containing 100 mg l^{-1} ceftriaxone (Darusazy Exir, Iran) to control bacterial contamination. For control of phenolic substances 2 g l^{-1} activated charcoal was added in the culture medium.

Shoot proliferation

Based on availability of explants different experiments were conducted as follow:

In the first experiment the effects of different concentrations of Kinetin (kn) (2, 4, 6 and 8 mg l^{-1}) supplemented with 0.2 mg l^{-1} NAA and control (without plant growth regulator) were separately studied for two genotypes 'Bargchenari' and 'Dehdez'. Data were collected after 4 weeks of culture.

The second experiment was carried out on three genotypes of 'Bargchenari', 'Dehdez' and 'Runu'. The explants were sub cultured two times with the interval of 4 weeks and a little change in growth regulators in culture medium as follows:

Culture. The single-node explants cultured on MS (Murashige and Skooge, 1962) medium containing 0.5 mg l^{-1} benzyl adenine (BA).

First subculture. After 4 weeks of culture and data collection, the explants were subcultured into the same medium.

Second subculture. After another 4 weeks and data collection, the explants were subcultured on a new medium with the same growth regulator composition plus 0.2 mg l^{-1} 2ip. In this step, after 4 weeks, the proliferated shoots were compact and short, thus, they were transferred into new medium containing 1 mg l^{-1} gibberellic acid (GA), and data were recorded after another 4 weeks. The experiments were conducted in completely randomized design with 4 replications of at least three explants in each.

Rooting

The produced shoots (above 1 cm length) were used for rooting. In this experiment, the effects of different concentrations of indole-3-butyric acid (IBA; 0, 0.5, and 1.5 mg l^{-1}) and different media (MS and MS/2) on shoot rooting performance were investigated. A factorial test was conducted in a completely randomized design with 8 replications. After 4 weeks, the rooted shoots were transferred to pots containing soil mixture (field soil, leaf mold and sand; 1V:1V:1V).

Data were subjected to analysis of variance by SAS software, version 9.1 (SAS Institude, Cary, NC, USA) and means comparison were done by Duncan Multiple Range test at probability of 5%.

3. Results and Discussion

Shoot proliferation

First experiment. In 'Dehdez' genotype, the results showed that the highest shoots number per nodal segment (4.4 shoots/explant) was obtained at concentration of 6 mg l^{-1} Kn which was significantly higher than the other treatments (Table 1, Fig. 1). However, by increasing of Kn from 6 to 8 mg l^{-1} significantly reduced the number of shoots. The positive effect of Kn in suitable concentration on shoot proliferation have also been reported previously in fig (Fráguas *et al.,* 2004) and strawberry (Balakrishnan *et al.,* 2009).

Table 1 - Effects of different treatments of Kn and NAA on shoot number, shoot length and leaf number per explant in 'Dehdez' genotype

Treatments (mg l^{-1})	Shoot number	Shoot length (mm)	Leaf number
Control	1.67 c	27.67 a	12.67 c
Kn 2 + NAA 0.2	1.67 c	9.78 c	5.33 e
Kn 4 + NAA 0.2	3.00 b	9.96 c	17.86 b
Kn 6 + NAA 0.2	4.40 a	11.59 b	26.40a
Kn 8 + NAA 0.2	2.22 c	7.56 d	9.89 d
Significant	**	**	**

Mean values followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.

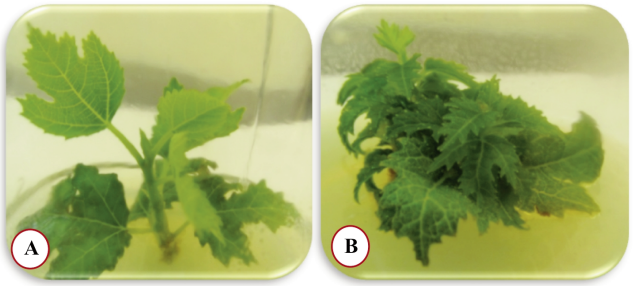

Fig. 1 - Effect of NAA and Kn on nodal explant proliferation in 'Dehdez', A= control, B= 0.2 mg l^{-1} NAA and 6 mg l^{-1} Kn, 8 weeks after culture.

With increasing Kn in culture medium, the shoot length significantly decreased so that the highest shoot length was observed in control (27.67 mm) and the lowest one in 8 mg l^{-1} Kn (7.56 mm). Our results was in agreement with the findings of Fráguas *et al*. (2004), they reported that although the Kn is required for the induction of shoot proliferation, in fig 'Roxo de Valinhos', the supra optimal concentrations can be toxic, and reduce the number and length of shoots. Nevertheless, it was in disagreement with the results reported by Mustafa and Taha (2012) in which 2.5 mg l^{-1} Kn encouraged shoot length. The highest number of leaves (26.4) was gained at the concentration of 6 mg l^{-1} Kn and lowest one (5.33) at 2 mg l^{-1}.

In 'Bargchenari' genotype, the results showed different concentration of Kn had no significant effect on shoots number (Fig. 2 A), although with adding Kn in culture media, shoot number increased. With increasing Kn in medium culture the shoots length decreased (Fig. 2 B). The M2 medium produced the highest numbers of leaf/explant that was significantly higher than control (Fig. 2 C). Shoot length reduction in M1 and M2 media and increment of number of leaves in the same medium is indicative of internodes length reduction (Fig. 2).

Second experiment. The results showed that after 4 weeks of nodal segments culture, there were not significant differences between three genotypes in relation to shoot number, shoot length and leaf number. In the first subculture, the number of shoot (1.78 per explant) and shoot length (18.11 mm) in genotype 'Bargchenari' were significantly higher than 'Dehdez' and 'Runu' (Table 2). In second subculture the maximum shoot numbers was obtained in 'Runu' (13.67 shoots/explant) (Fig. 3, A3) which was not significantly different from 'Bargchenari' (8.08 shoots/explant), and 'Dehdez' (2.8 shoots/explant) produced the lowest number of shoots (Fig. 3, B3). It seems that in the second subculture, the adding of 0.2 mg l^{-1} 2ip in addition to 0.5 mg l^{-1} BA into the medium improved shoot proliferation. Previous reportes, on *Ficus* shoot proliferation with implication of BA and 2ip in culture media, showed BA achieved better than 2ip on shoot proliferation on *Ficus benjamina* (Rzepka-Plevnes and Kurek, 2000) and *Ficus anastasia* (Al Malki and Elmeer, 2010).

It has been also reported that BA (a synthetic

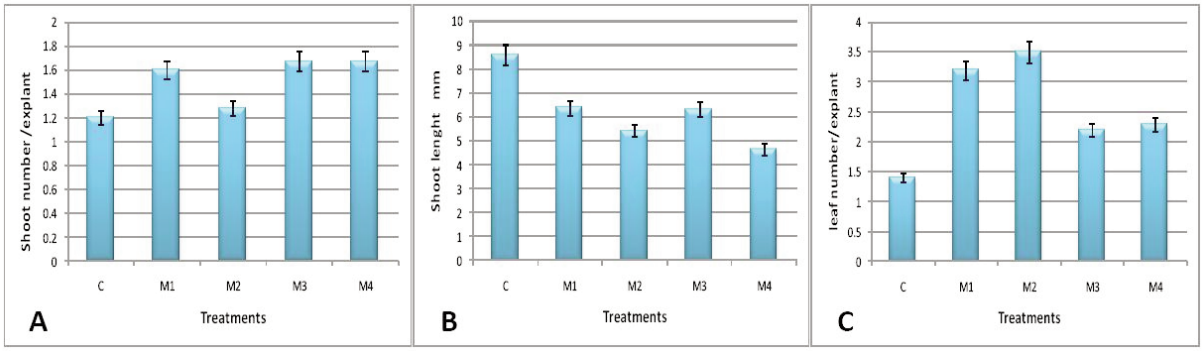

Fig. 2 - Effects of different treatments of Kn and NAA on shoot number (A), shoot length (B) and leaf number (C) per explant in 'Bargchenari' genotype. C= Control (no growth regulator); M1= Kn 2 mg l^{-1}+NAA 0.2 mg l^{-1}; M2= Kn 4 mg l^{-1}+NAA 0.2 mg l^{-1} ; M3= Kn 6 mg l^{-1}+NAA 0.2 mg l^{-1} ; M4= Kn 8 mg l^{-1}+NAA 0.2 mg l^{-1}.

Table 2 - Effects of genotype and subculture on shoot numbers, shoot length and leaf numbers per explants on MS medium

Genotype	Subculture	Shoot number	Shoot length (mm)	Leaf number
'Dehdez'	Culture	1.00 c	9.90 bc	2.50 c
	First subculture	1.00 c	10.30 bc	3.25 c
	Second subculture	2.80 b	6.45 c	10.80 c
'Bargchenari'	Culture	1.00 c	14.89 ab	5.44 c
	First subculture	1.78 b	18.11 a	7.67 c
	Second subculture	8.08 a	17.18 a	38.50 b
'Runu'	Culture	1.00 c	14.20 ab	3.60 c
	First subculture	1.12 c	8.62 c	3.87 c
	Second subculture	13.67 a	13.52 ab	66.67 a

Mean values followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.

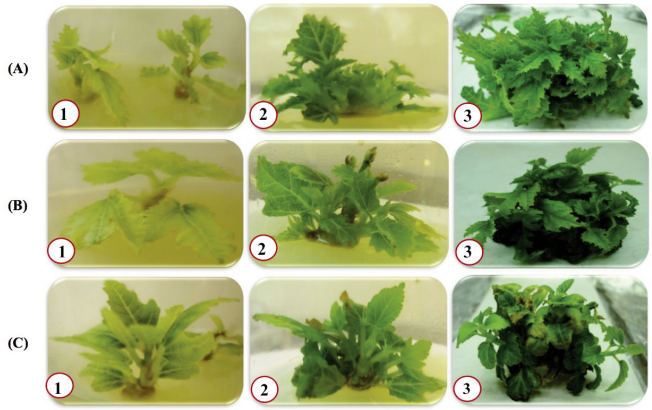

Fig. 3 - Compared growth of nodal explants in 'Runu' (A), 'Dehdez' (B) and 'Bargchenari'(C) genotypes in different cultures, 1= culture, 2= 4 weeks later, first subculture, 3= 8 weeks later, second subculture.

cytokinin) effect is stronger than the other cytokinins on shoot regeneration (Torres, 2013). However, in this study, simultaneous application of 2ip and BA in culture media highly improved shoot proliferation of the three fig genotypes. This indicates that two kinds of cytokinins had synergistic effect and enhanced shoot proliferation. The positive effect of transferring explants to a new medium on explant growth have also been reported by other researchers on different species of *Ficus,* including *carica* (Mustafa and Taha, 2012) and *religiosa* (Hassan *et al.,* 2009; Siwach *et al.,* 2011; Siwach and Gill, 2014).

The results showed in 'Dehdez' and 'Bargchenari', there were no significant differences in shoot length with increasing subcultures. However, the shoot length of 'Bargchenari' was significantly higher than 'Dehdez'. In 'Runu', the shoot length decreased in first subculture and increased in second subculture. As described in Materials and Methods, 4 weeks after the second subculture explants were transferred to a new medium containing GA. The results showed in spite of high shoot proliferation in second subculture, positive effect of GA on stem elongation. It has been reported GA induces internode elongation, meristem and buds growth in *in vitro* culture (Torres, 2013). Fráguas *et al.* (2004) also reported a positive effect of GA on shoot length elongation on fig 'Roxo de Valinhos'.

Eight weeks after second subculture the highest number of leaves (66.67) was observed in 'Runu' and the lowest number (10.8) in 'Dehdez' genotype. The results showed that all measured parameters, especially shoot proliferation are genotype dependent. In other species such as *Prunu mume* (Ning *et al.*, 2007), pomegranate (Al-Wasel, 1999; Naik *et al*., 1999) the effect of genotype on shoot proliferation rate has been reported.

Rooting

The highest rooting percentage (84.61), root number (2.23) and root length (1.51 cm) per explant were observed in MS/2 medium containing of 1.5 mg l^{-1} IBA which were significantly higher than their controls (Table 3). On MS/2 medium supplemented with 0.5 mg l^{-1} IBA did not produce any roots (Fig. 4).

Table 3 - Effects of different concentrations of IBA and media (MS and MS/2) on rooting characteristics in 'Bargchenari' genotype

Medium	IBA (mg l^{-1})	Rooting (%)	Root number	Root length (cm)
MS	0	18.18 c	0.45 bc	0.11 b
	0.5	71.43 ab	1.14 ab	0.30 b
	1	16.67 c	0.33 bc	0.08 b
	1.5	28.57 bc	0.71 bc	0.09 b
MS 1/2	0	30.77 bc	0.92 bc	0.10 b
	0.5	0.00 c	0.00 c	0.00 b
	1	28.57 bc	0.28 bc	0.43 b
	1.5	84.61 a	2.23 a	1.51 a
Significant		*	**	**

Mean values followed by the same letters are not significantly different according to Duncan's multiple range test (DMRT) at $p<0.05$.

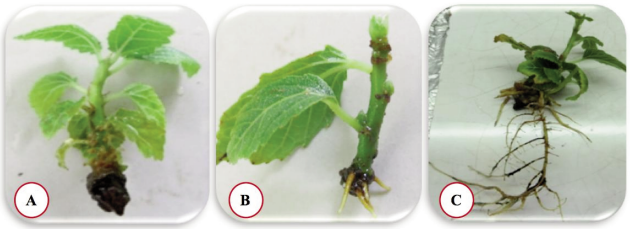

Fig. 4 - Effects of IBA on rooting of shoots in 'Bargchenari' genotype in *in vitro* condition, A= control, B= 0.5 mg l^{-1} IBA on MS medium, C= 1.5 mg l^{-1} IBA on MS 1/2 medium.

The results showed a high interaction between rooting media and different concentrations of IBA on rooting percentage and root number, such that in MS/2, the best results was obtained with 1.5 mg l^{-1} IBA, where in MS medium it was 0.5 mg l^{-1} IBA that produced the best result. Hepaksoy and Aksoy (2006) also reported that IBA is necessary for *in vitro* rooting of fig, Sarilop cultivar, that is in consistent with our results. The obtained results are in disagreement with those reported by Brum *et al*. (2001) and Fráguas *et al.* (2004) who found IBA are not essential for *in vitro* rooting of fig cv. Roxo de Valinhos. In this experiment, the best rooting occurred on MS/2 medium. Dhage *et al.* (2012) also introduced MS half strength medium for *in vitro* fig rooting, while Yakushiji *et al.* (2003) and Kim *et al.* (2007) reported MS full strength was the best medium for *in vitro* rooting of fig cultivars.

Adventitious root formation on proliferated shoots in a commercial propagation is very important. The ability of plant tissue to form adventitious roots depends on the interaction of many different endogenous and exogenous factors. The role of auxins in root development was reviewed, and it is a well-established fact that auxins are the main factors involved in the root formation (Németh, 1986).

4. Conclusions

Simultaneous application of 2ip and BA in culture media highly improved shoot proliferation of the three fig genotypes. This indicates that two kinds of cytokinins had synergistic effect and enhanced shoots proliferation. The highest shoot numbers per explant 13.67 was achieved eight weeks after 2nd subculture in 'Runu' genotype. The highest root number (2.23) was obtained in 'Bargchenari' genotype on MS/2 medium containing 1.5 mg l^{-1} IBA.

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References

AL MALKI A.A.H., ELMEER K.M.S., 2010 - *Influence of auxin and cytokinine on* in vitro *multiplication of* Ficus Anastasia. - Afr. J. Biotech., 9(5): 635-639.

AL-WASEL A.S., 1999 - In vitro *colonel propagation of "Al-Belehi" Pomegranate (Punica* granatum L.) - Journal of king Saud University, Agri. Sci., 11: 3-14.

ANIS M., FAISAL M., SINGH S., 2003 - *Micropropagation of mulberry (*Morus alba *L.) through* in vitro *culture of shoot tip and nodal explants.* - Plant Tissue Cult., 13: 47-51.

BALAKRISHNAN V., LATHA M.R., RAVINDRAN K., ROBINSON J.P., 2009 - *Clonal propagation of* Morus alba *L. through nodal and axillary bud explants*. - Bot. Res. Inter., 2: 42-49.

BRUM G., PASQUAL M., SILVA A., CHALFUN N., 2001 - *Sucrose, culture media, and their interactions during in vitro proliferation of 'Roxo de Valinhos' (*Ficus carica *L.).* - Acta Horticulturae, 605: 131-135.

BUNN E., TURNER S., PANAIA M., DIXON K.W., 2007 - *The contribution of* in vitro *technology and cryogenic storage to conservation of indigenous plants* - Aust. J. Bot., 55: 345-355.

CHAWLA H.S., 2009 - *Introduction to plant biotechnology.* - CRC Press, Taylor and Francis Group, Abingdon, UK, pp. 730.

DHAGE S., PAWAR B., CHIMOTE V., JADHAV A., KALE A., 2012 - In vitro *callus induction and plantlet regeneration in fig* (Ficus carica *L.).* - J. Cell Tiss. Res., 12: 3395-3400.

ENGELMANN F., 2011 - *Use of biotechnologies for the conservation of plant biodiversity*. - *In Vitro* Cell. Develop. Biol. Plant., 47: 5-16.

FAGHIH H., SABET-SARVESTANI J., 2001 - *Fig, plant and harvesting*. - 1st ed - Rahgosha, Shiraz, Iran, pp. 292. (In Persian).

FAO, 2012 - *FAOSTAT agricultural statistics database.* - FAO, http://www.fao.org.

FRÁGUAS C.B., PASQUAL M., DUTRA L.F., CAZETTA J.O., 2004 - *Micropropagation of fig (*Ficus carica *L.) 'Roxo de Valinhos' plants. - In Vitro* Cell. Develop. Biol. Plant., 40: 471-474.

GHOLAMI M., 2012 - *Evaluation of drought tolerant genetic resources in the fig* (Ficus carica L.*) using physiological and proteomics analysis indexes*. - Ph.D. Thesis, Shiraz University, Shiraz, Iran, pp. 120 (In Persian).

HASSAN A.S., AFROZ F., JAHAN M.A.A., KHATUN R., 2009 - In vitro *regeneration through apical and axillary shoot proliferation of* Ficus religiosa *L.- A multi-purpose woody medicinal plant.* - Plant Tiss. Cult. Biotech., 19: 71-78.

HEPAKSOY S., AKSOY U., 2006 - *Propagation of* Ficus carica *L. clones by* in vitro culture. - Biol. Plantarum, 50: 433-436.

KIM K.M., KIM M.Y., YUN P.Y., 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.).* - Plant Biol., 50: 440-446.

MALLON R., BUNN E., TURNER S.R., GONZALEZ M.L., 2008 - *Cryopreservation of* Centaurea ultreiae *(Compositae)*

a critically endangered species from Galicia (Spain). - Cryo Letters, 29: 363-370.

MURASHIGE T., SKOOG F., 1962 - *A revised medium for rapid growth and bioassays with tobacco tissue cultures*. - Physiologia Plantarum, 15: 473-497.

MUSTAFA N., TAHA R.A., 2012 - *Influence of plant growth regulators and subculturing on* in vitro *multiplication of some fig (*Ficus carica*) cultivars.* - Appl. Sci. Res., 8: 4038-4044.

NAIK S.K., PATTNAIK S., CHAND P.K., 1999 - In vitro *propagation of pomegranate (*Punica granatum *L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree*. - Sci. Hort., 79: 175-183.

NÉMETH G., 1986 - *Induction of rooting,* pp. 49-64. - In: BAJAJ Y.P.S. (ed.) *Biotechnology in Agricultural Forestry*. Springer Verlag, Berlin, Germany, pp. 515.

NING G.G., FAN X.L., HUANG W.J., BAO M.Z., ZHANG J.B., 2007 - *Micropropagation of six* Prunus mume *cultivars through axillary shoot proliferation, and ISSR analysis of cloned plants.* - Acta Biologica Cracoviensia Series Botanica, 49: 25-31.

PIOVAN A., CANIATO R., CAPPELLETTI E.M., FILIPPINI R., 2010 - *Organogenesis from shoot segments and via callus of endangered* Kosteletzkya pentacarpos *(L.) Ledeb.* - Plant Cell, Tiss. and Org. Cult., 100: 309-315.

RZEPKA-PLEVNES D., KUREK J., 2000 - *The influence of media composition on the proliferation and morphology of* Ficus benjamina *plantlets.* - Acta Horticulturae, 560: 473-476.

SARASAN V., CRIPPS R., RAMSAY M.M., ATHERTON C., McMICHEN M., PRENDERGAST G., ROWNTREE J.K., 2006 - *Conservation* in vitro *of threatened plants-progress in the past decade.* - *In Vitro* Cell. Dev. Biol. - Plant., 42: 206-214.

SIWACH P., GILL A.R., 2014 - *Micropropagation of* Ficus religiosa *L. via leaf explants and comparative evaluation of acetylcholine esterase inhibitory activity in the micropropagated and conventionally grown plants.* - 3 Biotech., 4(5): 477-491.

SIWACH P., GILL A.R., KUMARI K., 2011 - *Effect of season, explants, growth regulators and sugar level on induction and long term maintenance of callus cultures of* Ficus religiosa *L.* - Afr. J. Biotech., 10: 4879-4886.

SOLIMAN H.I., GABR M., ABDALLAH N.A., 2010 - *Efficient transformation and regeneration of fig (*Ficus carica *L.) via somatic embryogenesis.* - GM crops, 1: 40-51.

TORRES C.K., 2013 - *Tissue culture techniques for horticultural crops.* - Van Nostrand Reinhold, New York, USA, pp. 283.

YAKUSHIJI H., MASE N., SATO Y., 2003 - *Adventitious bud formation and plantlet regeneration from leaves of* fig (Ficus carica *L.)*. - Hort. Sci. Biotech., 78: 874-878.