An improved procedure for *in vivo* and *in vitro* propagation of *Buxus hyrcana*, an ornamental shrub critically at risk of extinction

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Abstract

The ornamental shrub, *Buxus hyrcana* is a threatened species due to the attacks of some pathogens. Rooting of this valuable shrub using propagation techniques such as cuttings is very slow. The present study was carried out to evaluate the effect of different concentrations of indole-3-butyric acid (IBA) and kinetin (KIN) on rooting of semi-hardwood cuttings and *in vitro* propagation of *B. hyrcana*. In propagation experiment, the basal region of the cuttings was treated with IBA and KIN solutions (0.00, 500, 1000 and 2000 mg/l of each). The cuttings were then transferred to rooting beds filled with perlite. After a rooting period of 60 days, some morphological parameters were measured. The 500 mg/l KIN along with 1000 mg/l IBA induced the largest number of roots (16.80 per plantlet). A maximum of 8.16 cm root length per plantlet was obtained with 1000 mg/l IBA together with 1000 mg/l KIN. The largest number of leaves (105.00) was observed in rooted stem cuttings treated with 2000 mg/l IBA along with 1000 mg/l KIN. In tissue culture experiment, the medium enriched with 2.00 mg/l KIN together with 0.50 mg/l IBA was found to be suitable for obtaining the maximum leaf number (11.43 per explant). A higher rooting frequency was also observed with the highest rooting of 6.50 roots per cutting achieved in plantlets grown in a medium supplemented with KIN and IBA (1.00 mg/l each). *In vitro* rooted plantlets were cultivated in plastic pots containing peat and perlite (in ratio of 1 : 1). The plantlets were successfully acclimatized in an adaptation greenhouse with a survival rate of 95% exhibiting normal developmental patterns.

Key words: endangered plants, ornamentals, plant growth regulators, rooting, stem cutting, tissue culture

Introduction

Buxus from the family Buxaceae is a genus containing about 70 species of evergreen shrubs and trees found in habitats ranging from rocky hills to woodlands in Europe, Asia, Africa and Central America. Common boxwood (*Buxus hyrcana* or *Buxus sempervirens*) is a bushy, rounded shrub or small tree with ovate to oblong, glossy and dark green leaves. Boxwoods are grown mainly for their foliage, which may be variegated (Brickell and Zuk, 1996). This species is in danger of extinction due to the invasion with some pathogens.

Propagation of boxwood is done by seeds and cuttings. Seeds propagation is a slow process; it is time consuming and results in heterogeneous plants with segregation of traits. Vegetative propagation is an alternative method for conservation of endangered plants threatened with extinction; it also helps to maintain certain desired characteristics in the plants (Hartmann et al., 2002). Roots produced in the cultures *in vitro* through the use of plant growth regulators (PGRs) are stronger and emerge faster and more in number than those produced without application of PGRs (Sağlam et al., 2014). The formation of root primordium cells depends on the presence of endogenous auxins in the cutting area or on application of exogenous auxins. Adventitious root formation in cuttings is an important process for vegetative propagation of many plant species, especially hard- and semi-hard wood cuttings of trees and shrubs (Husen et al., 2015; Kaviani and Negahdar, 2017).

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The success of vegetative reproduction depends on the interaction of hereditary factors and some biological and external factors particularly PGRs (de Oliveira et al., 2003; Davidović et al., 2015). The application of exogenous PGRs, especially the auxins group, can increase the success of adventitious rooting in the stem; IBA is the most commonly used auxin (Hartmann et al., 1997; Brondani et al., 2012; Davidović et al., 2015; Kaviani and Negahdar, 2017). Rooting of tree species through stem cuttings is affected by several factors particularly cultivation beds and PGRs (Hartmann et al., 2002). Application of auxins, especially IBA and α-naphthaleneacetic (NAA) is recommended for production of adventitious roots in stem-cutting propagation of many shrubs and trees (Hartmann et al., 2002; Hunt et al., 2011; Husen et al., 2015; Kaviani and Negahdar, 2017). Tsipouridis et al. (2003) demonstrated that IBA at 2000 mg/l concentration stimulated rooting of hardwood and semi-hardwood cuttings of peach trees (*Prunus persica* (L.) Batch). Hartmann et al. (1997) revealed that the application of IBA at 2500 to 4000 mg/l concentration had the best results in rooting of the semi-hardwood cuttings of apple, plum, and olive. The positive effect of 3000–4000 mg/l auxin in increasing rooting the cuttings of woody ornamental shrubs such as roses and Chinese hollyhock has been demonstrated (Al-Sagri and Alderson, 1996). Usually, a concentration of 2000–4000 mg/l will result in a proper rooting for most shrubs and evergreens (Poston, 2007).

The application of biotechnology and tissue culture in particular, is an appropriate method for reproduction of rare and threatened plants that are difficult to propagate. This method is becoming increasingly important for propagation and preservation of rare and endangered plant species (Kaviani and Negahdar, 2017). Plant tissue culture and micropropagation techniques also play an important role in preservation programs and management of botanical collections (Mahendran and Bai, 2009). Kaviani and Negahdar (2017) reported that the maximum *in vitro* multiple shoot formation in *B. hyrcana* was obtained through the use of 1.00 mg/l 6–benzylaminopurine (BAP). The optimum PGR combination for the largest number of roots was found to be 0.50 mg/l BAP and 1.00 mg/l NAA. There are very few reports regarding propagation of *B. hyrcana* through the rooting of semi-hardwood stem cuttings. Furthermore, the only reference found regarding the *in vitro* propagation of this species was the previous study by the present group on *B. hyrcana* (Kaviani and Negahdar, 2017). Therefore, the objectives of this study were to 1) develop an effective protocol for root induction from hard-wood stem cuttings; and 2) to establish a reliable procedure for *in vitro* propagation of *B. hyrcana*, a threatened ornamental shrub.

Materials and methods

Boxwood (*Buxus hyrcana* Pojark.), an ornamental shrub species, was obtained from a commercial landscape and garden company in Mazandaran province, Iran. The acquired plants were used as mother plants. The mother plants were transported to the plant tissue culture laboratory located in the Hyrcan Agricultural Science and Biotechnology Research Institute, Amol, Iran.

Experiment 1. *In vivo* propagation

Plant materials and treatments

Stems collected from two-years-old plants grown in a greenhouse were used as the source of cuttings. Stem cuttings of 20 cm length with 16 leaves (Fig. 1A) were disinfected by immersion in silver Nano-particles at a concentration of 250 mg/l for 10 s. To induce adventitious rooting, the basal region of the stem cuttings was dipped in solutions containing 0 (control, no IBA and KIN), 500, 1000 and 2000 mg/l of both IBA and KIN for 10 s.

Cultivation bed and growth conditions

After the treatments, the cuttings were placed in pots filled with perlite. They were maintained in a greenhouse to induce rhizogenesis. Fertilization was not performed. Plastic pots (70 × 38 × 4 cm^3) were used as the cultivation container (Fig. 1F). The temperature of greenhouse was adjusted to 24 °C and the air relative humidity (RH) so that it exceeded 80%.

Measured parameters

Evaluations were performed after a rooting period of 60 days. Plantlets height, stem diameter, shoot number, leaf number, node number, root length and root number were measured.

Experimental design and statistical analysis

A full factorial experiment in a completely randomized design was employed. Four replicates of five cut-

tings were randomly assigned per treatment; thus, a total of 320 cuttings were used. All the statistical analyses were done by using Microsoft Excel 2013 and Statistical Package for Social Sciences (SPSS) v 16.0. The analysis of variance (ANOVA) procedure for factorial experiments was used to test for the significant effect of treatments, followed by least significant difference (LSD) test for comparisons of different means of different treatments.

Experiment 2. *In vitro* micropropagation

Explant source and sterilization

Shoot tips obtained from the mother plants was used as explants for the establishment of cultures. Shoots (4–5 cm long) were washed under running tap water for 60 min. with a few drops of washing liquid. Shoots were transported to aseptic conditions in a laminar air hood. They were then surface sterilized with 20% sodium hypochlorite (NaOCl) for 10 min and rinsed with autoclaved water 3–5 times. Then, explants were disinfected with 0.5% (w/v) $HgCl_2$ for 15 min and rinsed with sterilized distilled water for 5 min. Finally, they were placed in the 5% ethanol for 5 min and then fully rinsed with sterilized distilled water. Under aseptic conditions in a laminar air hood, shoot tips (8–10 mm) were excised from the shoots and used as explant (Fig. 2A).

Culture medium, treatments, and measured parameters

The surface-sterilized explants were cultured in MS (Murashige and Skoog, 1962) medium fortified with 0.50, 1.00 and 2.00 mg/l of KIN and IBA or no PGRs as control. Sucrose (3%) was used as carbon source and media were solidified with Agar–agar (0.8%). The pH of the medium was adjusted to 5.7 ± 0.10 and autoclaved at 15 psi, 121 °C for 30 min. The cultures were maintained in a growth chamber at 24 ± 1 °C with a 16-h photoperiod and a photosynthetic photon density flux of 50 µmol $m^{-2} s^{-1}$ provided by cool white fluorescent lamps and 75–80% RH. After 2 months of culture, the parameters including leaf length, leaf diameter, leaf number, root length, root number and viability percentage of plantlets were calculated.

Plantlets acclimatization

In vitro rooted plantlets were washed with sterile water to remove the nutrient agar adhered to the roots and were cultivated in plastic pots containing peat and perlite (in a ratio of 1 : 1). Plantlets were covered by thin plastic bags, transferred to the greenhouse and kept at 25 ± 1 °C and 70–80% RH. They were then watered regularly and were exposed gradually to external environment by removing the plastic bags around the pots.

Experimental design

A factorial experiment in a completely randomized design was conducted. The experiment consisted of four replicates with one plant per replicate. Data were recorded from 200 explants after 8 weeks of culture. All the statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) v 16.0. The ANOVA procedure for factorial experiments was used to test whether the treatments had significant effects; this was followed by least significant difference (LSD) test for comparisons of different means of different treatments.

Results

Effect of PGRs on in vivo propagation of stem cuttings

Stem cuttings treated with different concentrations of KIN and IBA showed varied responses (Tables 1–3, Fig. 1). Adventitious roots were emerged 4–5 weeks after imposing of treatments. KIN and IBA at 500, 1000 and 2000 mg/l were tested for their influence on the number of roots produced per shoot and the root length in *B. hyrcana* (Fig. 1E). It was observed that 100% of the shoots tested produced roots following the different KIN and IBA treatments (including the control). A significantly higher number of roots were generated on shoots in the presence of 500 mg/l KIN and 1000 mg/l IBA (16.80) compared to the control (6.10). Shoots subjected to KIN and IBA treatments at 1000 and 2000 mg/l, respectively, also produced a high number of roots (14.23) (Table 3). Treatments without the presence of IBA yielded the least number of roots (with an average of 7.15) per shoot. Tables 1 and 2 show that among all treatments containing KIN and IBA used alone, higher number of roots were generated on shoots in the presence of 500 and 1000 mg/l IBA (11.78 and 10.45, respectively). The concentration of 1000 mg/l KIN in combination with 1000 mg/l IBA resulted in the best values of the length of the root (8.16 cm per cutting) (Fig. 1E). The treatments of 500 and 2000 mg/l IBA without the presence of KIN also showed promising effects on the root length (more than 7.00 cm) (Ta-
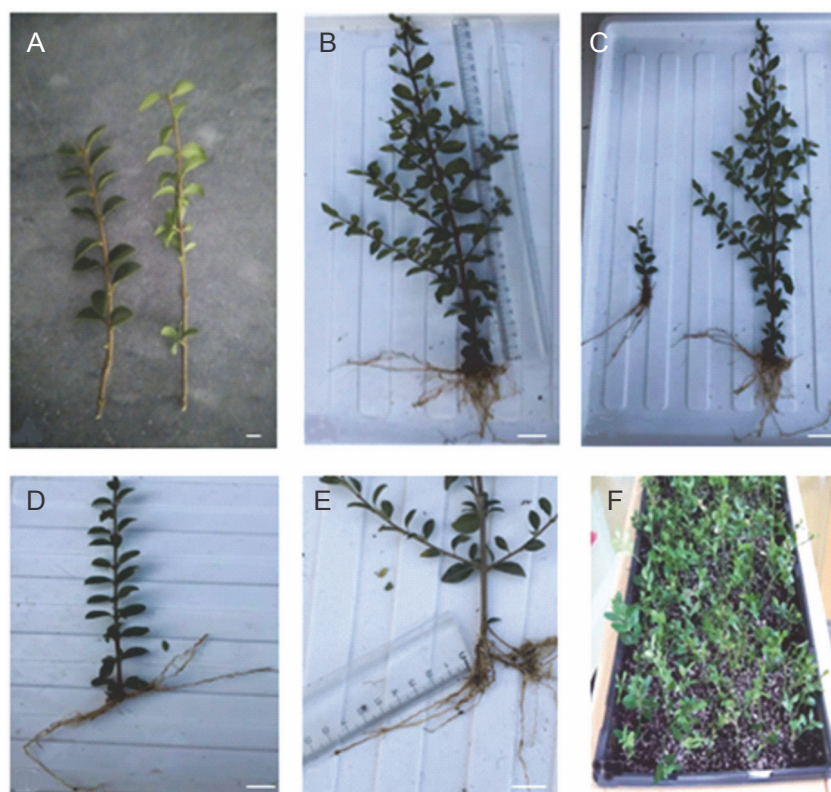
Fig. 1. Root and shoot induction on stem cuttings of *Buxus hyrcana* induced by different concentrations of KIN and IBA. A) standardized stem cuttings; B) plantlets height (treated with 1000 mg/l IBA); C) shoot number (treated with 500 mg/l KIN plus 1000 mg/l IBA – right and control – left); D) node number (treated with 500 mg/l IBA); E) root length (treated with 1000 mg/l KIN plus 1000 mg/l IBA); F) acclimatized plants in tray (scale bar = 1 cm)

Table 1. Mean comparison of the effect of different concentrations of KIN on measured parameters of *Buxus hyrcana* (*in vivo* conditions)

KIN [mg/ l]	Node number	Plantlet height [cm]	Shoot number	Root length [cm]	Root number	Length of the longest root	Stem diameter [cm]	Leaf number
0	10.67 [a]	23.57 [a]	6.40 [b]	6.75 [a]	6.68 [c]	6.19 [a]	0.36 [a]	80.00 [a]
500	6.34 [d]	15.20 [c]	7.67 [a]	6.62 [a]	7.85 [b]	6.95 [a]	0.38 [a]	84.30 [a]
1000	8.58 [c]	15.91 [c]	6.98 [ab]	6.56 [a]	8.32 [a]	7.29 [a]	0.36 [a]	82.50 [a]
2000	9.61 [b]	18.22 [b]	7.05 [ab]	6.84 [a]	6.94 [c]	6.77 [a]	0.40 [a]	84.30 [a]

Means with different letters on the same column are significantly different ($P < 0.05$) based on LSD test

ble 3). Differences between root length in cuttings treated with each one of KIN and IBA singly were not significant (data not shown). The longest root (11.40 cm) was obtained with the treatment of 1000 mg/l KIN (Table 3). The longest root obtained under control (without application of KIN and IBA) was 4.06.

Shoots in the control (without KIN and IBA) produced significantly more node compared to those in the presence of all KIN and IBA treatments, alone or in combination (Table 3). However, there were many nodes on shoots treated with 500 mg/l (Fig. 1D) and 2000 mg/l KIN plus 500 mg/l IBA (Table 3). Treatments

Table 2. Mean comparison of the effect of different concentrations of IBA on measured parameters of *Buxus hyrcana* (*in vivo* conditions)

IBA [mg/l]	Node number	Plantlet height [cm]	Shoot number	Root length [cm]	Root number	Length of the longest root	Stem diameter [cm]	Leaf number
0	10.45 [a]	16.84 [b]	5.48 [c]	6.87 [a]	6.89 [d]	6.31 [ab]	0.31 [b]	74.80 [c]
500	9.32 [b]	18.35 [a]	6.44 [b]	6.80 [a]	11.78 [a]	5.08 [b]	0.45 [a]	69.00 [d]
1000	6.25 [c]	19.24 [a]	8.05 [a]	6.53 [a]	10.45 [b]	7.58 [a]	0.43 [a]	90.10 [b]
2000	9.18 [b]	18.49 [a]	8.14 [a]	6.56 [a]	9.21 [c]	8.23 [a]	0.30 [b]	97.30 [a]

Means with different letters on the same column are significantly different ($P < 0.05$) based on LSD test

with 1000 mg/l IBA and control induced faster growth of shoots in relation to the other treatments ($P < 0.01$), with 26.70 and 24.30 cm length at 65 days, respectively (Table 3). The lowest value of the length of the shoots was obtained with the treatment of 500 mg/l KIN; this treatment resulted in shoots 9.20 cm long. Needless to say, too high length of plantlets is not an advantage for ornamental species from aesthetic point of view; the ornamentals with more compact shoots are more beautiful.

The treatment without KIN and IBA (control) produced only 3.60 shoots ($P < 0.01$) and 42.30 leaves ($P < 0.01$ for KIN together with IBA and IBA alone, not for KIN alone) per cutting at 65 days (data not shown). The concentrations of 1000 mg/l KIN and 2000 mg/l IBA induced the maximum shoot number (9.26) (Fig. 1C) and leaf number (105.00) per cutting (Table 3). These amounts are about three fold higher than those of produced in control cuttings. Table 3 shows the production of more than 8 shoots per cutting when 500 or 2000 mg/l KIN in combination with 500, 1000 or 2000 mg/l IBA were applied. This result suggests the importance of applying both KIN and IBA for achieving greater number of shoots and leaves. There was no positive relationship between increasing KIN and IBA concentrations and increase in the number of shoots and leaves (Table 3). The concentration of 1000 mg/l KIN along with 500 mg/l IBA and 2000 mg/l KIN along with 1000 mg/l IBA resulted in better values for the stem diameter (both with 0.53 cm per cutting) (Table 3). The treatment without KIN and the treatment containing the highest concentrations of KIN and IBA showed least effects on stem diameters.

Effect of PGRs on *in vitro* propagation of shoot tips

The number, length and width of leaves formed per shoot explants were strongly dependent on the composition of media. Successful leaf length (2.26 and 2.70 cm per explant) was obtained in media fortified with 2.00 mg/l KIN together with 1.00 mg/l IBA, and 1.00 mg/l KIN together with 1.00 mg/l IBA, respectively (Table 6, Fig. 2D). Minimum leaf length (0.96 cm) was calculated in a medium without KIN and IBA (control). Medium with 0.50 mg/l KIN and 0.50 mg/l IBA formed leaves with a low frequency (8.10 per explant) – Table 6. A combination of 2.00 mg/l KIN and 0.50 mg/l IBA was found to be the most suitable PGRs regime, achieving the largest number of leaves with 11.43 leaves per explant (Table 6, Fig. 2B). IBA at a concentration of 1.00 mg/l was found to be more effective for leaf width (Fig. 2E). The combination of 2.00 mg/l KIN and 0.50 mg/l IBA also did not improve the leaf width. Plantlets produced in media containing 2.00 mg/l IBA, 2.00 mg/l KIN, and 0.50 mg/l KIN together with 1.00 mg/l IBA showed 90% viability percentage (Table 6).

Table 6 shows that different concentrations of PGRs combinations may induce different rates of root length and root number. We found that the application of 2.00 mg/l KIN alone resulted in the highest length of root (9.60) and the largest number of roots (6.10) per explant (Fig. 2G and Fig. 2H). The explants treated with 1.00 mg/l KIN in combination with 1.00 mg/l IBA produced the same root number (6.50) as those on 2.00 mg/l KIN containing medium (Table 6). The poorest rooting (6.00 cm in length and 3.50 in number) was noticed on shoot tips cultured on media without PGRs. KIN at 2.00 mg/l was better than the other KIN concentrations

Table 3. Mean comparison of the effect of different concentrations of KIN and IBA on measured parameters of *Buxus hyrcana* (*in vivo* conditions)

KIN × IBA [mg/l]	Node number	Plantlet height [cm]	Shoot number	Root length [cm]	Root number	Length of the longest root	Stem diameter [cm]	Leaf number
0 × 0	14.50 ^{a} ± 0.50	24.30 ^{a} ± 3.29	3.60 ^{e} ± 0.00	6.50 ^{ab} ± 0.50	6.10 ^{h} ± 0.50	4.06 ^{f} ± 0.56	0.33 ^{bc} ± 0.05	42.3 ^{g} ± 0.57
0 × 500	12.50 ^{b} ± 1.32	21.00 ^{bc} ± 0.50	7.83 ^{ab} ± 0.15	7.36 ^{c} ± 0.30	12.12 ^{c} ± 1.20	4.80 ^{ef} ± 1.21	0.36 ^{b} ± 0.05	86.3 $^{c-e}$ ± 2.88
0 × 1000	6.50 ^{fg} ± 0.50	26.70 ^{a} ± 0.72	6.96 ^{bc} ± 0.63	5.70 ^{b} ± 0.40	10.44 ^{cd} ± 1.21	8.80 ^{abc} ± 3.27	0.47 ^{a} ± 0.00	90.0 ^{cd} ± 9.00
0 × 2000	9.20 ^{cd} ± 0.26	20.30 ^{cd} ± 0.20	6.23 ^{c} ± 1.95	7.43 ^{a} ± 0.40	8.56 ^{cde} ± 0.43	5.80 $^{b-f}$ ± 1.94	0.26 ^{c} ± 0.05	101.6 ^{ab} ± 4.50
500 × 0	6.83 $^{e-g}$ ± 0.35	9.20 ^{j} ± 0.17	4.56 ^{d} ± 2.03	6.86 ^{a} ± 1.06	7.54 ^{de} ± 0.32	4.80 ^{ef} ± 1.21	0.33 ^{bc} ± 0.05	81.3 $^{d-f}$ ± 6.65
500 × 500	5.50 ^{gh} ± 0.86	22.70 ^{b} ± 0.17	8.76 ^{a} ± 1.20	6.50 ^{ab} ± 0.50	13.78 ^{ab} ± 1.02	4.36 ^{f} ± 0.56	0.46 ^{a} ± 0.05	76.0 ^{ef} ± 3.00
500 × 1000	7.86 $^{d-f}$ ± 0.28	14.93 ^{gh} ± 0.40	8.93 ^{a} ± 0.73	6.56 ^{ab} ± 0.66	16.80 ^{a} ± 0.60	9.86 ^{ab} ± 1.64	0.36 ^{b} ± 0.05	88.6 ^{cd} ± 3.21
500 × 2000	8.86 ^{cd} ± 0.32	14.00 ^{hi} ± 1.30	8.73 ^{a} ± 0.41	6.56 ^{ab} ± 0.57	11.86 ^{cd} ± 0.75	8.80 $^{a-e}$ ± 4.20	0.36 ^{b} ± 0.02	91.3 $^{b-d}$ ± 3.05
1000 × 0	10.20 ^{c} ± 1.21	12.83 ^{i} ± 0.45	6.53 ^{bc} ± 0.77	6.70 ^{a} ± 0.43	8.10 ^{cde} ± 1.04	11.40 ^{a} ± 2.90	0.26 ^{c} ± 0.05	94.3 $^{a-c}$ ± 8.51
1000 × 500	6.46 ^{fg} ± 2.66	17.23 ^{ef} ± 1.47	4.60 ^{d} ± 0.20	6.50 ^{ab} ± 0.50	9.67 ^{d} ± 0.47	4.26 ^{f} ± 0.15	0.53 ^{a} ± 0.05	45.0 ^{g} ± 2.65
1000 × 1000	7.50 $^{d-f}$ ± 1.32	16.43 ^{fg} ± 1.00	7.83 ^{ab} ± 0.85	8.16 ^{a} ± 0.30	12.42 ^{c} ± 1.28	5.53 $^{c-f}$ ± 2.10	0.33 ^{bc} ± 0.05	90.0 ^{cd} ± 6.00
1000 × 2000	10.16 ^{c} ± 0.75	17.16 ^{ef} ± 1.80	9.26 ^{a} ± 0.47	5.70 ^{b} ± 0.40	14.23 ^{ab} ± 0.87	9.16 $^{a-d}$ ± 4.35	0.33 ^{bc} ± 0.05	105.0 ^{a} ± 2.64
2000 × 0	10.26 ^{c} ± 0.80	19.03 $^{c-e}$ ± 1.15	6.23 ^{c} ± 0.15	6.83 ^{a} ± 0.40	6.89 $^{f-g}$ ± 0.48	5.70 $^{b-f}$ ± 2.11	0.33 ^{bc} ± 0.05	81.3 $^{d-f}$ ± 6.65
2000 × 500	12.83 ^{ab} ± 1.25	12.46 ^{i} ± 0.97	4.56 ^{d} ± 0.28	6.56 ^{ab} ± 1.06	10.54 ^{cd} ± 0.86	7.10 $^{a-f}$ ± 4.63	0.46 ^{a} ± 0.05	72.6 ^{f} ± 7.51
2000 × 1000	6.84 $^{e-g}$ ± 0.43	18.90 ^{de} ± 1.24	8.76 ^{a} ± 0.37	6.50 ^{ab} ± 0.50	10.76 ^{cd} ± 0.90	5.13 $^{d-f}$ ± 1.88	0.53 ^{a} ± 0.05	92.0 $^{b-d}$ ± 5.64
2000 × 2000	8.50 ^{cde} ± 1.80	22.50 ^{b} ± 0.50	8.63 ^{a} ± 0.55	6.56 ^{ab} ± 0.66	6.60 $^{f-g}$ ± 0.88	9.16 $^{a-d}$ ± 1.68	0.26 ^{c} ± 0.05	91.3 $^{b-d}$ ± 6.05

Means with different letters on the same column are significantly different ($P < 0.05$) based on LSD test; mean ± std. dev.

Table 4. Mean comparison of the effect of different concentrations of KIN on measured parameters
of *Buxus hyrcana* (*in vitro* conditions)

KIN [mg/l]	Root length [cm]	Leaf length [cm]	Leaf diameter [cm]	Leaf number	Root number	Viability percentage
0.00	6.67 c	1.50 c	0.79 a	9.22 b	4.60 b	78.30 ab
0.50	7.14 bc	1.60 bc	0.76 ab	9.13 b	5.22 a	81.80 a
1.00	7.53 b	1.84 a	0.66 bc	9.60 ab	5.25 a	75.80 b
2.00	8.47 a	1.66 b	0.62 c	10.18 a	5.21 a	81.60 a

Means with different letters on the same column are significantly different (*P* < 0.05) based on LSD test

Table 5. Mean comparison of the effect of different concentrations of IBA on measured parameters
of *Buxus hyrcana* (*in vitro* conditions)

IBA [mg/l]	Root length [cm]	Leaf length [cm]	Leaf diameter [cm]	Leaf number	Root number	Viability percentage
0.00	7.41 a	1.38 d	0.71 a	9.66 a	5.17 a	75.16 b
0.50	7.15 a	1.50 c	0.65 a	9.81 a	4.62 b	83.33 a
1.00	7.43 a	1.94 a	0.74 a	9.44 a	5.36 a	75.00 b
2.00	7.82 a	1.79 b	0.74 a	9.22 a	5.13 a	84.16 a

Means with different letters on the same column are significantly different (*P* < 0.05) based on LSD test

as determined by measured root length. There was no significant difference in the length of roots per explants treated with different concentrations of IBA. Other treatments showed significant differences in root length and root number (data not shown).

Plantlets were acclimatized in the plant growth chamber benefited from the high humidity and other incubation conditions during the 2 weeks and showed 95% survival (Fig. 2I and Fig. 2J). Plantlets were then taken out of the growth chamber and transferred to an adaptation greenhouse for another 2 weeks after repotting in pots containing a mixture of peat and perlite (in ratio of 1 : 1). All the acclimatized plantlets were successfully established and formed new leaflets (Fig. 2H). Acclimatized plants had normal leaf development and lacked detectable morphological variation and showed apparently uniform growth and true-to-type morphology.

Discussion

In vivo propagation

B. hyrcana is a species critically at the risk of extinction mainly due to the invasion of pathogens (Kaviani

and Negahdar, 2017). The main pathogen is a type of witch moth, *Cydalima perspectalis*. The population of *B. hyrcana* in northern part of Iran has been reduced to 20–30%. This plant has great commercial potential. Boxwoods are evergreen shrubs and trees; they are popular in gardens and landscapes (Kaviani and Negahdar, 2017). Thus, they should be protected. Propagation by rooting of semi-hard wood cuttings and *in vitro* propagation are the effective methods for conservation and rapid multiplication of the species in danger of extinction. Effective production of shoots and adventitious roots in plant tissue culture laboratory and greenhouse ensures a constant production of clones throughout the year. Just a single work was reported (by the authors of this article) about propagation and micropropagation of *B. hycana* Pojark (Kaviani and Negadar, 2017).

In the current study, *B. hyrcana* responded positively to rooting in concentrations of 500 and 1000 mg/l IBA. Authors in their previous study revealed that 1000 mg/l IBA along with 1000 mg/l NAA induced the largest number of roots per cutting (Kaviani and Negahdar, 2017). Similar findings were reported on other trees and
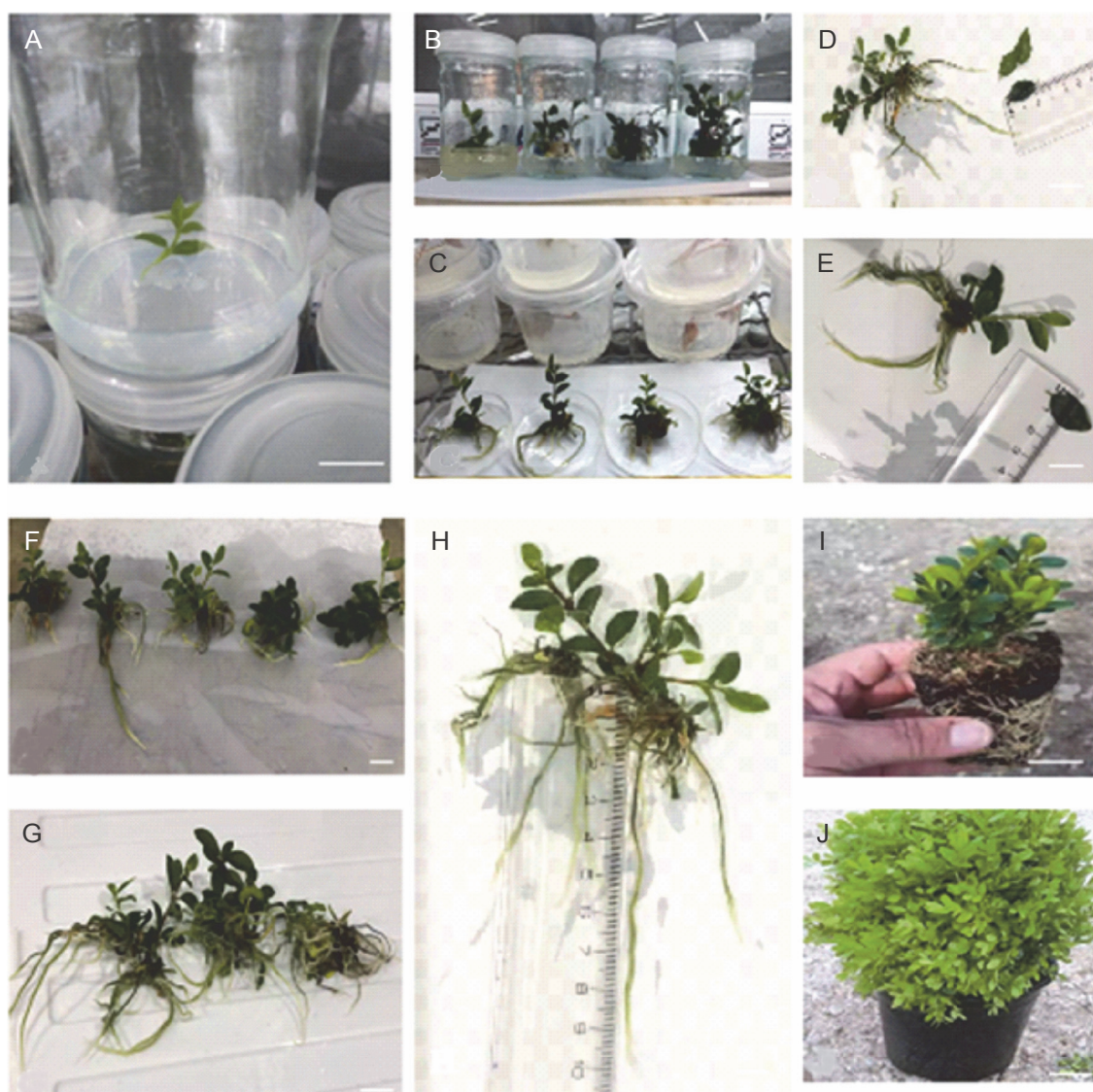
Fig. 2. *In vitro* development of *Buxus hyrcana*. A) early stage of *in vitro* culture (the explants were shoot apex (scale bar = 1 cm)); B) shoot multiplication from explants cultured on Murashige and Skoog (MS) medium containing different concentrations of KIN and IBA; C) fully developed plantlets out of culture media; D) measurement of leaf length; E) measurement of leaf width; F) plantlets containing shoots and roots ready for acclimatization; G) regenerated plantlets from media containing different concentrations of KIN and IBA with fully developed roots; H) measurement of root length; I) acclimatization phase; J) viable *ex vitro* plants growing under standard greenhouse conditions (scale bar = 2 cm except for A)

shrubs species such as *Pausinystalia johimbe* and *Pongamia pinnata* (Hartmann et al., 2002; Tchoundjeu et al., 2004; Kesari et al., 2009). Auxins, natural or artificially applied, are known to play a significant role in stimulating root initiation in stem cuttings of woody species (Hartmann et al., 2011). The application of auxins in the basal region of cuttings and mini-cuttings is prevalent worldwide to promote the adventitious rooting of woody species (Hartmann et al., 2011; Brondani et al., 2012). A great variation was observed among the types,

concentrations, formulations and forms of application (Wendling et al., 2010; Brondani et al., 2012). It is well known that IBA is the most widely used auxin for the induction of adventitious roots in vegetative propagules, but the concentrations should be adjusted to obtain better rooting rates (Corrêa and Fett-Neto, 2004; Kaviani and Negahdar, 2017). Adventitious rooting appears to be particularly controlled by endogenous levels of auxins (Zhu et al., 2010). Exogenous application of auxins in appropriate concentration enhances the rooting induction

Table 6. Mean comparison of the effect of different concentrations of KIN and IBA on measured parameters of *Buxus hyrcana* (*in vitro* conditions)

KIN × IBA [mg/l]	Root length [cm]	Leaf length [cm]	Leaf width [cm]	Leaf number	Root number	Viability percentage
0.00 × 0.00	6.00 [e] ± 0.60	0.96 [g] ± 0.20	0.60 [de] ± 0.10	9.00 [c–f] ± 1.15	3.50 [g] ± 0.52	70.00 [ef] ± 0.00
0.00 × 0.50	6.10 [e] ± 0.40	1.06 [g] ± 0.20	0.80 [a–d] ± 0.10	10.10 [bc] ± 0.90	3.93 [g] ± 0.15	86.60 [ab] ± 5.77
0.00 × 1.00	6.53 [de] ± 0.49	1.90 [bc] ± 0.10	0.90 [a] ± 0.10	9.26 [c–f] ± 1.40	5.10 [c–e] ± 0.45	66.60 [f] ± 5.77
0.00 × 2.00	7.73 [bcd] ± 1.01	1.95 [bc] ± 0.10	0.86 [ab] ± 0.05	8.53 [ef] ± 0.35	4.90 [d–f] ± 0.10	90.00 [a] ± 5.00
0.50 × 0.00	6.30 [e] ± 0.45	1.63 [def] ± 0.15	0.83 [a–c] ± 0.05	9.16 [c–f] ± 1.10	4.33 [fg] ± 0.15	70.60 [ef] ± 4.50
0.50 × 0.50	7.20 [b–e] ± 0.36	1.63 [def] ± 0.15	0.70 [a–d] ± 0.00	8.10 [f] ± 0.70	5.36 [a–d] ± 0.20	86.60 [ab] ± 5.77
0.50 × 1.00	6.93 [cde] ± 0.96	1.40 [f] ± 0.10	0.76 [a–d] ± 0.20	9.53 [b–e] ± 0.40	5.33 [b–d] ± 0.25	90.00 [a] ± 0.00
0.50 × 2.00	8.13 [bc] ± 1.28	1.73 [cde] ± 0.11	0.76 [a–d] ± 0.05	9.43 [b–f] ± 0.80	5.86 [ab] ± 0.65	80.00 [b–d] ± 0.00
1.00 × 0.00	7.43 [b–e] ± 0.41	1.40 [f] ± 0.10	0.63 [cd] ± 0.11	10.43 [ab] ± 1.36	5.76 [abc] ± 0.50	70.00 [ef] ± 2.64
1.00 × 0.50	7.20 [b–e] ± 0.34	1.83 [cd] ± 0.20	0.70 [a–d] ± 0.20	9.33 [b–f] ± 1.09	4.73 [d–f] ± 0.90	76.60 [c–e] ± 5.77
1.00 × 1.00	8.30 [ab] ± 1.21	2.70 [a] ± 0.10	0.63 [cd] ± 0.20	8.90 [def] ± 0.88	6.50 [a] ± 0.20	70.00 [ef] ± 5.00
1.00 × 2.00	7.20 [b–e] ± 0.10	1.93 [bc] ± 0.20	0.70 [a–d] ± 0.10	9.76 [b–d] ± 0.66	4.40 [e–g] ± 0.52	86.60 [ab] ± 7.02
2.00 × 0.00	9.60 [a] ± 1.22	1.53 [ef] ± 0.05	0.80 [a–d] ± 0.20	10.06 [bc] ± 0.85	6.10 [a] ± 0.55	90.00 [a] ± 5.00
2.00 × 0.50	8.10 [bc] ± 0.80	1.46 [f] ± 0.05	0.40 [e] ± 0.10	11.43 [a] ± 0.41	4.46 [e–g] ± 0.37	83.30 [a–c] ± 5.77
2.00 × 1.00	7.96 [bc] ± 0.70	2.26 [ab] ± 0.11	0.66 [bcd] ± 0.15	10.06 [bc] ± 0.45	4.93 [d–f] ± 0.11	73.30 [d–f] ± 3.51
2.00 × 2.00	8.23 [bc] ± 1.23	1.40 [f] ± 0.10	0.63 [cd] ± 0.05	9.16 [c–f] ± 1.25	5.36 [a]–d ± 0.41	80.00 [b–d] ± 0.00

Means with different letters on the same column are significantly different ($P < 0.05$) based on LSD test; mean ± std. dev.

in the base of stem cuttings. IBA increases the concentration of endogenous auxins; the accumulation of IBA in the basal region of stem cuttings acts as a metabolizing agent and signal to produce roots (Husen, 2008). PGRs, especially IBA, change the metabolism of enzymes, carbohydrates, RNA, DNA and proteins (Dai et al., 2004; Husen and Pal, 2007; Brondani et al., 2012). IBA is the best auxin for general use because it is nontoxic to plants over a wide concentration range than other auxins; it is also effective in promoting the root formation of a large number of plant species (Hartmann et al., 2002).

In this study, various combinations of different concentrations of an auxin (IBA) and a cytokinin (KIN) were tested on rooting of semi-hard-wood cutting of *B. hyrcana*. The authors succeeded in inducing roots twice as much as possible (16.80 per cutting) by treating the cutting with 500 mg/l KIN in combination with 1000 mg/l IBA, compared to the control. Moreover, the longest roots were observed in stem cuttings treated with 1000 mg/l KIN in combination with 1000 mg/l IBA. Contrary to author's finding, successful rooting without the

application of auxin has been reported in a number of tropical tree species like *Allanblackia floribunda* (Atangana et al., 2006). Such contrasting results may be due to the variation in endogenous auxin contents in a particular species.

Auxin regulates various aspects of plant growth and development by affecting numerous processes including cell growth, development and differentiation (Husen et al., 2015). The different concentrations of IBA, NAA and cytokinins applied lead to a rooting response varied from species to species. In the present study, IBA in combination with KIN increased the rooting capacity of stem cuttings. Cuttings treated with median level of PGRs (1000 mg/l IBA in combination with 1000 mg/l KIN) produced more roots than other levels of IBA and KIN and untreated cuttings. The stimulatory effects of IBA in root induction of stem cuttings of several other woody plant species such as *Dalbergia melanoxylon*, *Eucalyptus benthamii*, *Morus alba*, and *Macleania rupestris* have been demonstrated by other researchers (Amri et al., 2010; Brondani et al., 2012; Veloza et al., 2014; Husen et al., 2015). The success of IBA may be due to

its low activity and slow degradation by auxin degradation enzymes (Singh et al., 2014). However, high concentrations of auxin can cause damage to the base of the cutting. Auxin can be effective to rooting cuttings at a certain concentration, depending on the species and cultivar; on the other hand, it will have an inhibitive effect at higher concentrations (Galavi et al., 2013). Contrary to the authors' findings, Husen et al. (2015) and Singh et al. (2014) showed that a high concentration of IBA (3000 and 2000 mg/l, respectively) was most effective for root production in terms of number and length in *Morus alba* L. Induction of more roots with higher IBA concentration has been reported in various plants such as *Dalbergia melanoxylon, Vitis vinifera, Thuja compaecta* and *Macleania rupestris* (Amri et al., 2010; Galavi et al., 2013; Singh et al., 2013; Veloza et al., 2014). Alizadeh and Grigorian (2002) demonstrated that the increase in NAA concentration from 1000 to 2000 mg/l increased rooting in semi-hardwood cuttings of almond–peach hybrid, whereas decrease in rooting was observed at concentration of 3000 mg/l. Investigation of Shirzad et al. (2012) on *Ficus benjamina* revealed that the maximum root number and root length was obtained with 4000-6000 mg/l IBA. Sağlam et al. (2014) showed superiority of IAA than NAA and IBA for root induction and growth in *Salvia fruticosa* Mill. These researchers showed that the maximum root number values (77.40) were obtained in stem cuttings treated with 400 mg/l of IAA, NAA and IBA, respectively. In IBA application, especially at concentrations of 300 and 400 mg/l, notable increases in root length were observed (Sağlam et al., 2014).

Present study showed the positive effect of KIN and IBA on shoot and leaf number, because the smallest number of shoots and leaves were produced in control plantlets. Maximum shoot and leaf number was obtained in cuttings treated with 1000 mg/l KIN in combination with 2000 mg/l IBA. The author's work showed the synergic effect of KIN and IBA in terms of stimulating the production of highest shoot and leaf number. The investigation of Singh et al. (2014) on the rooting of stem cuttings of *Morus alba* L. demonstrated that the number of leaves per cutting was maximum in 2000 mg/l of IBA, followed by 1500 mg/l of IBA. The minimum number of leaves per cutting was calculated in control. Increase in leaf number may be due to vigorous rooting induced by the PGRs enabling the cuttings to absorb more nutrients

and thereby produce more leaves (Stancato et al., 2003; Singh et al., 2014).

In vitro propagation

Management of tissue cultures of a particular species requires adequate knowledge concerning both its nutritional needs and the optimal combination of PGRs (Lucía et al., 2015). The choice of appropriate PGRs to induce shoot and leaf production is an essential process for *in vitro* propagation. The highest leaf induction was achieved using appropriate concentrations of both KIN and IBA. The explants treated with 1.00 and 2.00 mg/l KIN in combination with 1.00 mg/l IBA promoted the longest leaves (over than 2 cm). Moreover, 2.00 mg/l KIN in combination with 0.50 mg/l IBA induced the formation of maximum leaf number with an average of 11.43. Leaves were also formed in a KIN- and IBA-free medium, but in lower numbers. Previous study on *B. hyrcana* (Kaviani and Negahdar, 2017) indicated that BAP alone was able to induce the maximum shoot number, but plantlets growth, node number, leaf number, root length, and root number could be increased when it was used in combination with NAA or IBA. Maximum number of leaves was observed in a medium fortified with 1.00 mg/l BAP in combination with 1.50 mg/l IBA (Kaviani and Negahdar, 2017). Optimum concentration for highest shoot multiplication was different among different plant species. This might be due to the content of endogenous PGRs in particular species. Moreover, the response to exogenous PGRs varies with plant species and the percentage of response varies with PGRs treatments and types of explants (Kaviani and Negahdar, 2017). The combination of cytokinins and different auxins did not improve shoot proliferation in some trees and shrubs species such as *Vitis thunbergii, Prunus tomentosa* and *Prunus* spp. (Lu, 2005; Pruski et al., 2005; Kalinina et al., 2007). These results are in contrast to our findings, in which the highest shoot proliferation was found in treatments containing KIN plus IBA. Similar results were reported by some researchers (Ďurkovič, 2008; Noroozi Sharaf et al., 2011). A study by Ďurkovič (2008) on *Cornus mas*, a shrub species showed that 0.70 mg/l BAP in combination with 0.05 mg/l NAA was capable of stimulating continuous axillary and adventitious shoot proliferation. For *Philodendron*, the BA and KIN treatments showed a higher shoot formation percentage compared to the control (Chen et al., 2012).

The results of the present study showed 100% of rooting on all the media tested (including the control) a month after the treatment. Maximum root number was produced in medium containing 1.00 mg/l of both KIN and IBA. Similar observation was made with *B. hyrcana* where explants cultured on MS medium supplemented with 0.50 mg/l BAP in combination with 1.00 mg/l NAA produced the largest number of roots (Kaviani and Ne-gahdar, 2017). IBA is more effective for rooting as compared with other auxins as reported for many woody species (Onay, 2000; Kalinina et al., 2007). IBA and NAA promoted rooting of different trees and shrubs species (Takihira et al., 2007; Noroozi Sharaf et al., 2011; Chen et al., 2012). The current investigation revealed that explants cultured on medium fortified with 2.00 mg/l KIN without IBA induced high root number and maximum root length. It is possible that, the content of auxins in the explants of *B. hyrcana* is adequate for root growth. The requirement of PGRs for shoot and root regeneration is species-specific. In the present study, all the plantlets were successfully acclimatized with a survival rate of 95%.

Conclusions

B. hyrcana is in danger of extinction. Complementary *in vivo* and *in vitro* approaches represent important tools for *ex situ* conservation of plants germplasms particularly those threatened. The present study constitutes the second report on the propagation and micropropagation of *B. hyrcana* Pojark. Results in the current study demonstrated that the use of both KIN and IBA in appropriate concentrations induced higher rooting percentage and shoot proliferation in both propagation and micropropagation experiments. Following transfer to the adaptation greenhouse, 95% of the rooted shoots survived. The procedure presented in this study can be implemented for the mass production of *B. hyrcana* on a commercial scale.

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