RESEARCH ARTICLE

In vitro propagation of some old Persian cypress accessions (*Cupressus sempervirens* L.) by embryo culture

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Abstract In order to study in vitro propagation of some old Persian cypress genotypes (*Cupressus sempervirens* L.), embryos of twenty old cypress accessions were cultured on MS and SH media containing 100 and 200 mg L⁻¹ myo-inositol. Germination percentage and growth parameters of produced plantlets and their hardening off were evaluated. Results showed that the highest germination percentage and germination rate was obtained with MS medium containing 100 mg L⁻¹ myo-inositol, although no significant difference was observed with MS containing 200 mg L⁻¹ myo-inositol and SH containing 100 mg L⁻¹ myo-inositol. Furthermore, based on the results, the highest root length was gained with SH medium containing 200 mg L⁻¹ myo-inositol. Germination percentage of isolated embryos and shoot length of produced plantlets were affected by genotype, so that the highest germination percentage and shoot length was obtained with KB and KT genotypes, respectively. The oldest genotype which was 4000 years old (Abarkuh cypress) showed no significant difference with other genotypes in terms of shoot and root length. In hardening-off step, first all genotypes were initially established but after 2 weeks, KJ2, KK, KSHN, KD1, KB genotypes died.

Keywords Cypress · Hardening · Embryo culture

Introduction

Cupressaceae with ubiquitous distribution is found in all continents excluding Antarctica (Manescu et al. 2011; Shi et al. 2011; Yang et al. 2012). *Cupressus* L. includes as many as 25 species and is widespread in the northern hemisphere (Afzal-Rafi and Dodd 1994; Giovanelli and De Carlo 2007). *Cupressus sempervirens* L. is native to Persia (Iran), and some adjacent countries such as Syria, Turkey, Cyprus and several Greek islands and was introduced into all the Mediterranean countries during the era of the Roman Empire (Giovanelli and De Carlo 2007).

Common cypress as a multipurpose species plays historical, ecological, economical and ornamental roles in many parts of the world. The species is drought tolerant and capable of growing in adverse conditions such as calcareous, clayish or rocky soils. Thus, common cypress has been and is valuable from various horticultural perspectives (Teissier du Cros 1999). The use of cypress as an evergreen landscape tree has increased in the recent decades in public and private landscape and gardens for unique characteristics reasons (Karimi et al. 2013; Farahmand 2015; Farahmand et al. 2015; Farahmand and Karimi 2016).

Cypress is traditionally reproduced by seeds in Iran, although it can also be propagated by wood cuttings and grafting (Hartman et al. 2011; Giovanelli and De Carlo 2007; Sparapano and Bruno 2004; Stankova and Panetsos 1997; Capuana and Lambardi 1995). Seed germination in common cypress is commonly lower than 50% due to high seed emptiness (Giovanelli and De Carlo 2007; Giannini et al. 1999). This emptiness is also a common characteristic found in some old Persian cypress accessions. Low germination percentage in cypress might be related to unfavorable environmental condition during seed development

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stage. Commonly, drought stress accompanied with extreme temperature, and imposed water stress on trees may result in aborted seeds during developmental stages and decreased seed viability (Maher et al. 2010). Thus, seed germination is largely influenced by ecological factors and genetic background as reported for *Cupressus sempervirens* var. *horizontalis* (Darikvand and Zolfaghari 2014). In Iran, there are genotypes of cypress 100–4000 year old, possessing seeds unable to germinate (Karimi et al. 2013; Farahmand and Karimi 2016). Lower cone formation and the inability of seeds to germinate, are two main factors affecting seed germination and subsequent seedling growth particularly in old Persian cypress accessions. These old cypress accessions are also difficult and slow-to-root based on our preliminary studies (data not presented). Consequently, optimization of the efficient tissue culture systems can be used for propagation of old genotypes and rejuvenation of old accessions. Surprisingly, little attention has been directed towards the old accessions and researches are mainly available on in vitro regeneration with juvenile materials (Fossi et al. 1981; Lambardi et al. 1995, 1997; Spanos et al. 1997; Lambardi 2000), but rarely from adult plants (Capuana and Gannini 1997).

Embryo culture or in some cases embryo rescue is an efficient method of in vitro propagation used for different purposes including breeding, multiplication and conservation approaches (Bridgen 1994; Chee 1994). Embryo culture is a promising method of in vitro propagation particularly for endangered, rare and old plant species (Ghorpade et al. 2010; San and Yidirim 2009; Song et al. 2014). Embryo culture is also commercially used for propagation of orchids species (Paudel et al. 2012; Kauth 2005; Kauth et al. 2006; Shadang et al. 2007). In woody plants, micropropagation is also used for shortening generation cycle when moist chilling for 2–3 months is required for successful germination (Kaur et al. 2006). Embryo culture is a suitable technique for overcoming seed dormancy, reducing breeding cycle, rescue embryos and produce new plants through crosses between seedless cultivars (Razi et al. 2013). In vitro seed and embryo culture and the subsequent seedling development of some *Taxus* species, depend on seed developmental stage and growth media (Chee 1994; Song et al. 2014; Zhiri et al. 1994). In *T. baccata* L. cv. *Stricta*, 100% germination occurred when embryos were cultured on MS medium or Heller (H) medium (Zhiri et al. 1994). Additionally, successful germination and development occurred when embryos were cultured on modified H (H⁺) or modified MS (MS⁺) media while desirable results were not obtained when embryos were cultured on MS, H, Gamborg basal medium (B₅), and White and Risser basal (WR) medium. In the latter media, embryos germinated but full development was not gained (Zhiri et al. 1994).

Common cypress or Italian cypress is locally known as Persian cypress in Iran and is a highly sacred and praised tree among Iranians. Iran is a rich germplasm for Persian cypress in the world. There are many old accessions of cypress in Iran distributed across the country particularly in central, southern and southeastern regions. Some of these old accessions are invaluable gene pool and deserve conservation projects including optimizing propagation protocols (Farahmand and Karimi 2016). Consequently, the objective of this research was to study in vitro germination of old cypress genotypes' embryos and evaluation of produced plantlets.

Materials and methods

Seed collection

Female mature cone of 20 old and two young genotypes of *Cupressus sempervirens*, mostly grown in Kerman and Yazd provinces (Table 1) were picked from trees for seed collection (Fig. 1a). The weight and dimension of seeds were measured after separation from cones (Table 2).

Embryo separation from seed and disinfection

In first step, isolated seeds were soaked in distilled water for 48 h and were decontaminated for 2 min with 70% ethanol, 2 min in sodium hypochlorite and 2 min in 0.2% HgCl₂, followed by three times rinses with sterile distilled water (Fig. 1b). The embryos were then dissected from the seeds under laminar-flow hood (Fig. 1c).

Culture media

SH (Schenk and Hildebrandt 1972), and MS (Murashige and Skoog 1962) culture media were used for embryo culture of genotypes according to the formulations reported in Table 3.

Embryo culture

After excision of the embryos from seeds with a scalpel, embryos were used for culture initiation in media containing 5.0 mg per liter cefotaxime for 10 days (Fig. 1d). The explants were cultured on hormone-free MS and SH mediums, with 100 and 200 mg L⁻¹ myo-inositol. Germination was defined as the emergence of the radicle (about 1 mm long) accompanied by opening and greening of cotyledons (Fig. 1e). Meanwhile, percentage of seedlings produced from isolated embryos was calculated 38 and 45 days after culture. The germination rate of embryos was calculated using the following equations.

Table 1 Geo-location of genotypes used for embryo culture

No	Genotype	Location	Latitude (UTM)	Longitude (UTM)	Sea level (m)	Approximate age	Seed source
1	YA	Yazd	39R0717386	3445429	1534	4000	CS
2	YD	Yazd	40R0268409	3552000	2064	2000	CS
3	YT ₁	Yazd	40S0514990	3721261	1383	2000	CS
4	KS	Kerman	40R0553926	3328293	1692	1200	CS
5	KT	Kerman	40R0452061	3473344	2369	1000	CS
6	YCH	Yazd	40R0240206	3520327	1426	1000	CS
7	KG	Kerman	40R0570969	3221598	1783	900	CS
8	YZ	Yazd	40R0240066	3522137	1395	900	CS
9	YKH	Yazd	40R0237352	3518016	1483	800	CS
10	YT ₂	Yazd	40S0514940	3721274	1382	800	CS
11	KJ ₂	Kerman	40R0591214	3191760	2163	700	CS
12	KB	Kerman	40R0628778	3340720	1109	700	CS
13	KP ₂	Kerman	40R0380278	3305643	2369	500	CS
14	KP ₁	Kerman	40R0380299	3305629	2369	500	CS
15	KSHZ ₁	Kerman	40R0527127	3321440	2043	400	CS
16	KSHZ ₂	Kerman	40R0527220	3321441	2014	400	CS
17	KSHN	Kerman	40R0527061	3325500	1919	400	CS
18	KK	Kerman	40R0529647	3373387	1881	100	CS
19	KD ₁	Kerman	40R0607110	3347046	1712	50	CS
20	KD ₂	Kerman	40R0510023	3346956	1758	50	CS

CS Collect seed by researcher

GR (number germinated embryo per day) = N/T

where, GR is germination rate; N is the number of germinated embryos, and T is number days of culture.

Culture conditions

The planted embryos were incubated at 25 ± 1 °C at 16:8 h light/dark photoperiod. After 4 days, germination percentage and germination rate were calculated. Also, percentage of plantlets produced from isolated embryos was calculated, 38 and 45 days after culture (Fig. 1f, g). Furthermore, fresh weight of plantlets, shoot length, root length and number of lateral shoots was measured at this time.

Plantlet hardening

After 45 days of culture, plantlets were transferred to pots filled with a medium composed of coco peat and perlite (1:1 v:v) (Fig. 1h). To retain moisture, pots were covered with a glass. The pots were incubated at 20 °C in 24 h photoperiod at an irradiance of 4000 Lux for 2 weeks. Then plantlets were transplanted to pots containing a sandy loam soil and placed in greenhouse. After 60 days,

plantlets were evaluated for shoot length and number of lateral shoots.

Experimental design and statistical analysis

The characteristics of seeds were compared in a complementary design by Duncan test at $P \leq 0.05$. The experiments performed for in vitro culture and hardening-off was as nonparametric factorial in the framework of a completely randomized design with four replications each containing 3–5 embryos. Analysis of variance (ANOVA) was performed using SAS software. If ANOVA determined that the effects of the treatments were significant ($P \leq 0.05$), then the means were compared by LSD test.

Results

Characteristics of seeds

Significant differences were found among accessions in terms of seed characteristics such as seed weight and seed length, so that the highest seed weight and seed length were observed in KB accession and the lowest was obtained with old accessions. YA accession, oldest cypress in the world (Ca. 4000 years) (Fig. 1i), had seeds with 2.55 mm length

Fig. 1 Stages of embryo culture of *Cupressus sempervirens*; **a** cypress fruits, **b** pre-culture treatment, **c** separating the embryos, **d** culture initiation, **e** elongation, **f** rooting, **g** a plantlet 45 days after embryo culture, **h** a plantlet transferred to the soil, **i** mother plant (“Abarkouh”)

Table 2 Comparison means of seeds weight and seed length of studied cypress genotypes

Characteristics	Genotype									
	KSHZ ₁	KSHZ ₂	KP ₁	KG	KD ₂	KJ2	KK	KSHN	KD ₁	KS
100 seeds weight (g)	0.37 l	0.92 d	0.68 fg	0.86 de	1.16 b	0.44 kl	0.49 jk	1.01 c	0.57 hi	0.53 ij
Seed length (mm)	2.79 ghi	3.24 efgh	2.75 ghi	3.60 def	4.65 ab	2.90 ghi	2.73 ghi	4.76 c	2.88 ghi	3.15 e-i
	KT	KP ₂	KB	YA	YD	YCH	YZ	YKH	YT ₁	YT ₂
100 seeds weight (g)	1.00 c	0.61 gh	1.38 a	0.67 fg	0.70 f	0.72 f	0.58 hi	0.87 de	0.80 e	0.80 e
Seed length (mm)	4.06 bcd	4.49 ab	4.87 a	2.55 hi	2.63 ghi	2.46 i	2.89 ghi	3.61 def	4.28 abc	3.79 cde

Different letters within a row indicate significant differences by LSD test

and 100 seeds weight 0.67 gr. In general, comparison of

100 seeds weight showed that older accessions had lower seed weight (Table 2).

Table 3 Contents and media culture used in embryo culture of old cypress

Contents	Medium			
	MS + 100 mg L ⁻¹	MS + 100 mg L ⁻¹	SH + 100 mg L ⁻¹	SH + 200 mg L ⁻¹
Macro-elements	MS	MS	SH	SH
Micro-elements	MS	MS	SH	SH
Myo-inositol	100	200	100	200
Nicotinic acid	MS	MS	SH	SH
Pyridoxine	MS	MS	SH	SH
Thiamine	MS	MS	SH	SH
Glycine	MS	MS	SH	SH
Sucrose	MS	MS	SH	SH
pH	5.8	5.8	5.8	5.8

Effect of medium culture on germination

Means comparison revealed that germination percentage, germination rate and root length of plantlets were affected by culture medium whereas other parameters were unaffected by medium. The highest germination percentage (Table 4) and germination rate was observed with MS medium containing 100 mg L⁻¹ myo-inositol, although significant difference was not observed with MS containing 200 mg L⁻¹ myo-inositol and SH containing 100 mg L⁻¹ myo-inositol. The lowest germination percentage and germination rate was observed with SH medium containing 200 mg L⁻¹ myo-inositol. Mean comparison indicated that the highest root length was obtained with SH medium containing 200 mg L⁻¹ myo-inositol and the lowest with the same medium with 100 mg L⁻¹ myo-inositol. Root length was not influenced by myo-inositol concentration in MS medium (Table 4).

Effect of genotype on germination

There were significant differences between accessions considering germination percentage and rate in which accessions had germination percentage of 52.27%–100%. The highest germination percentage and rate were

measured in KB (100%), KSHN (100%), KD2 (94.47%) and KT (92.68%) accessions. The lowest germination percentage was observed in YD (52.27%) and KP1 (56.25%) accessions. The oldest accessions (YA), had germination percentage of 80% with a rate of 21 embryo per day (Table 5).

Effect of genotype on percentage of plantlets produced from embryo

After 38 days of culture, most accessions produced plantlets from embryo, although varying regeneration was found among accessions. Accessions such as KJ2, KG, KS, YZ and YT1 that had high germination percentage were unable to produce plantlet. Also, the highest percentage of plantlet production was obtained with KT accession and the lowest figure was obtained with KK and KP1 accession. In YA accession, 60% of isolated embryos were able to produce plantlet. After 48 days of culture, accessions which were not already responsive produced plantlets, although the percentages of produced plantlets were less than 50%. The rest of accessions, showed no significant increase in the number of plantlets (Table 5).

Table 4 Effect of culture media on germination parameters, produced plantlets percentage and their growth parameters

Culture media	Germination percentage	Germination rate	Produced plantlets after 38 days from culture (%)	Produced plantlets after 45 days from culture (%)	Root length (cm)	Shoot length (cm)	Plantlet weight (g)	Number of lateral shoot
MS100	90.49 a	24.0 a	44.18 a	44.18 a	4.06 bc	1.05 a	0.043 a	0.79 a
MS 200	89.06 a	23.0 a	40.62 a	42.62 a	3.34 c	0.73 a	0.380 a	0.69 a
SH100	67.16 b	17.0 b	51.49 a	51.49 a	7.16 a	1.04 a	0.048 a	0.82 a
SH200	92.06 a	25.0 a	50.09 a	50.0 a	5.80 ab	0.82 a	0.032 a	0.63 a

Different letters within a column indicate significant differences by LSD Test

Table 5 Effect of genotype on germination parameters and produced plantlets percentage

Parameters	Genotype									
	KSHZ ₁	KSHZ ₂	KP ₁	KG	KD ₂	KJ ₂	KK	KSHN	KD ₁	KS
Germination percentage	77.78 abc	90.0 a	56.25 cd	88.0 a	94.47 a	100 a	60.87 bcd	100 a	100 a	87.5 a
Germination rate	21.0 ab	23.0 ab	14.0 c	23.0 a	24.0 a	26.0 a	16.0 bc	26.0 a	26.0 a	23.0 a
PSP38	66.67 ab	40.0 a-e	12.50 de	*	66.67 ab	*	8.70 e	33.33 b-e	28.57 cde	*
PSP45	66.67 ab	40.0 a-e	12.50 de	48.0 abc	66.67 ab	66.67 ab	8.70 e	33.33 cde	28.57 cde	43.75 a-d
	KT	KP ₂	KB	YA	YD	YCH	YZ	YKH	YT ₁	YT ₂
Germination percentage	92.68 a	96.67 a	100 a	80 ab	52.27 d	85.29 a	87.5 a	90.32 a	89.19 a	77.78 abc
Germination rate	24.0 a	25.0 a	26.0 a	21.0 ab	13.0 c	22.0 a	23.0 a	23.0 a	23.0 a	20.0 a
PSP38	75.0 a	43.33 abcd	57.14 abc	60.0 abc	34.09 b-e	55.88 abc	*	54.84 abc	*	29.63 cde
PSP45	75.0 a	43.37 abcd	57.14 abc	60.0 ab	43.09 b-e	55.88 abc	43.75 a-d	58.60 abc	62.16 abc	29.63 cde

Different letters within a row indicate significant differences by LSD Test; PSP38 and PSP45 show produced plantlets percentage after 38 and 45 days from culture respectively

*Genotypes unable to produce plantlets

Effect of genotype on growth parameters of produced plantlets

Root length varied in accessions and ranged from 0.37 to 1.12 cm. The highest and lowest root length was obtained with KT, YKH and KP1, respectively. The highest shoot height was recorded in KT and KSHZ2 accessions with a mean of 2.51 and 1.24 cm, respectively. The lowest shoot length was observed in KP1 accession. No significant difference was observed between YA accession (the oldest one), and other accessions in the case of shoot and root length. There was no significant difference among most accessions in terms of plantlet weight, although the highest plantlet weight was observed for KT and YA. The number of lateral shoot was different among accessions and ranged from 0.12 to 1.78 shoot per plantlet. The highest shoot per plantlet, was observed in KT and KSHN accessions and the lowest was obtained in KP1 accession (Table 6).

Evaluation of shoot length and number of lateral shoot after hardening

Although, all accessions were established in pots containing cocopeat and perlite, accessions KJ2, KK, KSHN, KD1, KB died after transfer to pots containing soil (Fig. 1). Results obtained from evaluating growth parameters of plantlet, showed that accessions KSHZ1, KSH2, KG, KT, YA, YCH and YKH grew successfully, in which, the highest shoot length and number of lateral shoots were observed in KSHZ1 and YCH, respectively. YA, the oldest accessions, had 2.7 cm length with an average of more than 2 lateral shoots per plantlet although not significantly different compared to other accessions (Table 7).

Discussion

Many factors such as genetic background, age of the mother plant, nutritional status, irrigation regime, time of seed collection and embryo culture, developmental stage, the composition of the medium, and temperature and light affect the successful recovery of plants from embryos. Two main types of basal media are generally used for embryo rescue research viz. Murashige and Skoog medium (MS) and Gamborg's B-5 media (Bridgen 1994). In addition, the composition of the media is considerably different, considering the concentrations of media supplements required. The stage of embryo development is critically important affecting media composition so that for young embryos, a complex medium with high sucrose concentration is required, while mature embryos usually develop on a simple medium with low levels of sucrose. Temperature and light requirements are closely species-specific and two crucial factors for embryo culture. So, the range of temperature and light, should be adjusted considering the origin of the crop i.e. whether it is a cool-season or a warm-season crop. In the present study, results related to the effect of medium culture on germination indicated that the highest germination percentage was obtained with MS medium without significant difference between two applied myo-inositol concentrations. Germination parameters were decreased in SH medium with 200 mg L⁻¹ myo-inositol. These negative effects may be related to increased osmotic potential induced by myo-inositol.

Myo-inositol has been classified as a plant vitamin, although some researchers regarded it as a supplementary carbohydrate. In the present research, myo-inositol at higher level was unable to affect embryo development. It

Table 6 Effect of genotype on growth parameters of produced plantlets

Parameters	Genotype									
	KSHZ ₁	KSHZ ₂	KP ₁	KG	KD ₂	KJ ₂	KK	KSHN	KD ₁	KS
Root length (cm)	7.20 bcd	3.60 cde	0.37 e	5.09 bcde	5.51 bcde	4.81 bcde	0.94 e	5.5 bcde	2.0 de	3.04 cde
Shoot length (cm)	0.98 bc	1.24 b	0.17 c	0.90 bc	1.03 bc	0.93 bc	0.29 bc	0.75 bc	0.51 bc	0.73 bc
PSP38	66.67 ab	40.0 a-e	12.50 de	*	66.67 ab	*	8.70 e	33.33 b-e	28.57 cde	*
PSP45	66.67 ab	40.0 a-e	12.50 de	48.0 abc	66.67 ab	66.67 ab	8.70 e	33.33 cde	28.57 cde	43.75 abcd
Weight of plantlet (g)	0.046 bc	0.040 bc	0.006 e	0.039 bc	0.044 bc	0.038 bc	0.012 c	0.042 bc	0.014 c	0.019 c
	KT	KP ₂	KB	YA	YD	YCH	YZ	YKH	YT ₁	YT ₂
Root length (cm)	12.01 a	7.90 abc	3.12 cde	4.84 b-e	5.0 bcde	6.40 bcd	2.70 cde	9.55 a	4.89 b-e	2.77 cde
Shoot length (cm)	2.51 a	1.01 bc	1.08 bc	1.08 bc	0.56 bc	0.84 bc	0.54 bc	1.04 bc	0.90 bc	0.49 bc
PSP38	75.0 a	43.33 abcd	57.14 abc	60.0 abc	34.09 b-e	55.88 abc	*	54.84 abc	*	29.63 cde
PSP45	75.0 a	43.37 abcd	57.14 abc	60.0 ab	34.0 b-e	55.08 abc	43.75 a-d	58.60 abc	62.16 abc	21.63 cde
Weight of plantlet (g)	0.11 a	0.05 bc	0.027 c	0.095 ab	0.035 c	0.05 bc	0.025 c	0.047 bc	0.023 c	0.016 c

Different letters within a column indicate significant differences by LSD Test; PSP38 and PSP45 show produced plantlets percentage after 38 and 45 days from culture respectively

Table 7 Comparison means of genotypes in terms of number of lateral shoot and shoot length in hardening step

Parameters	Genotype									
	KSHZ ₁	KSHZ ₂	KP ₁	KG	KD ₂	KJ ₂	KK	KSHN	KD ₁	KS
Number of lateral shoot	5.0 a	4.0 abc	*	5.0 a	2.60 abc	*	*	*	*	1.66 c
Shoot length (cm)	3.2 abcd	3.3 abc	*	4.4 a	2.2 bcd	*	*	*	*	2.8 abcd
	KT	KP ₂	KB	YA	YD	YCH	YZ	YKH	YT ₁	YT ₂
Number of lateral shoot	3.12 abc	2.0 c	*	2.5 abc	2.14 bc	3.6 abc	2.0 c	4.8 ab	1.8 c	3.0 abc
Shoot length (cm)	3.2 abcd	1.5 d	*	2.7 abcd	2.08 bcd	4.3 a	2.4 bcd	3.8 ab	2.3 bcd	2.0 cd

Different letters within a column indicate significant differences by LSD Test; * genotypes died after translation to pots containing soil

may be related to the concentration of internal myo-inositol. Myo-inositol accumulates in plants under stress. Some of the oldest accessions of cypress are found in areas near desert lands in Iran with low rainfall and saline soil. Although all studied accessions are found in Kerman and Yazd provinces from central and southeastern parts of Iran with similar climatic conditions, some inevitable differences exist in these areas considering the microclimate and even edaphic conditions. It might be assumed that higher concentrations of internal myo-inositol, have already been accumulated under these stressful conditions in the accessions. The findings indicated that varying germination percentage and growth parameters among accessions could be associated with genetic differences, not age. For instance, no significant difference was found among YA, (the oldest accession) and other accessions considering germination percentage. It should also be noted that YA

accession is fortunately under strict governmental protection as a champion tree and may benefit from better irrigation regime and other maintenance management. So, this difference could be partially related to climatic, cultural and maintenance factors and not merely genetic ones. The highest germination percentage and rate were observed in KB (100%), KSHN (100%), KD2 (94.47%) and KT (92.68%) accessions which had the high seed weight. Accessions YD (52.27%) and KP1 (56.25) with low seed weight, had less germination. It may be pertained to genetic abnormalities in pollination, fertilization and seed development or unfavorable environmental conditions during seed growth. The cypress accessions studied in this research are from southeastern and central and parts of Iran. Arid and semi-arid conditions are the prevailing climatic conditions in these areas with sodic soils and ground water may also be considered a second source of salinity.

High temperature along with drought stress during seed development, have been reported to decrease seed quality and following germination in *C. sempervirens* and *C. arizonica* (Maher et al., 2010). Based on the present data, 38 days after culture, most accessions with high germination percentage (KT, KB, YCH, YKHIYM, IYCH and IKSI), had the high produced plantlets percentage. KG, KJ2, KS, YZ and YT1 accessions were unable to produce plantlet. Damage to embryos during excision from seeds and undeveloped or rudimentary embryos are also two other probable influencing factors in this regard. Seed comparison of accessions also showed that these genotypes had the lowest 100-seed-weight, most possibly possessing weaker embryos. Karimi et al. (2013) in a study evaluated the same accessions morphologically and reported that the least amount of seed weight were observed in KJ2, KS and YZ. Furthermore, some accessions such as KSHN, KD1 and YT2 that had high germination percentage were unable to produce enough plantlets 45 after culture. This may be due to damage caused during excision of embryos from seeds.

According to growth parameters data, the highest root length was obtained with SH medium containing 200 mg L^{-1} myo-inositol. Root growth is a high-energy-demanding process that can only occur at the expense of available metabolic substrates which are mainly carbohydrate. Root growth is affected by C/N ratio and SH medium have higher C/N ratio compared to MS, which can impact the quality of produced roots.

The results of this study confirm the conclusion of Romano et al. (1995) on cork oak that number of adventitious roots produced per shoot increased with increasing carbohydrate concentration. In hardening-off step, accessions KJ2, KK, KSHN, KD1 and KB were unable to grow in soil pots due to weak root system. Establishment of an effective in vitro root system is essential for subsequent success during acclimatization to autotrophic conditions in cork oak (Romano et al. 1995). Inoculation of plantlets with mycorrhizal fungi has been reported as an efficient method to reduce the decline and loss of plantlets during acclimatization process and hardening -off step. The application of mycorrhizal fungi is an option to improve in vitro produced plantlets (Gianinazzi and Gianinazzi-Pearson 1988) and shorten the acclimatization period required for micropropagated plants (Rai 2001). Thus, the in vitro incorporation of mycorrhiza fungi is suggested as an efficient method to improve the quality of plantlets and increase the chance of their survival during acclimatization period.

Old accessions of cypress in Iran are considered to be at severe environmental risk, as recently reviewed in detail for geophytes of Iran by Farahmand and Nazari (2015). Detailed information in the case of Persian cypress status in

Iran and the potential biotic and abiotic stresses have already been reported by some Iranian authors (Farahmand et al. 2015; Mohammadi et al. 2014). Additionally, national and international efficient cooperation for germplasm conservation and transfer, establishment of germplasm bank, cryopreservation of plant materials, development of propagation methods and especially tissue culture techniques for suitable multiplication and biotechnological means for gene transfer are some suggestions offered for cypress accessions in Iran. Human pressures have recently been mentioned as one of the main concerns responsible for endangered status of *Cupressus chengiana* (Hao et al. 2006). The genus *Seiridium* is a serious cause of cypress canker (Spanos et al. 1997; Xenopoulos and Tsopelas 2000; Smith 2010; Danti et al. 2013). Although cypress canker as a disastrous disease, has not been reported in Iran but the potential risk of pests and especially this devastating disease should be seriously monitored, as far as the prominent value of old cypress genotypes in Iran is concerned.

Conclusion

In conclusion, according to this study, seeds of old cypress accessions were unable to germinate in natural condition, most probably due to poor endosperm status or underdeveloped embryos. Furthermore, vegetative cutting propagation of these old accessions is difficult due to their lower regenerative capacity linked to age. Therefore, embryo culture is a suitable and promising technique for their propagation and conservation. Furthermore, seedlings produced from embryo culture, are juvenile and can be used as highly regenerative materials for various micropropagation and biotechnological purposes.

Based on the results of the present study, MS medium with 100 and 200 mg L^{-1} myo-inositol was suitable for embryo culture of old cypress accessions. Meanwhile, it was revealed that embryo growth of old cypress accessions was more affected by genotype and not age. The results of this preliminary study would pave the way for many horticultural and ornamental objectives including in vitro conservation and management of the genus *Cupressus*.

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