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Indirect organogenesis from bud explants of *Juniperus communis* L.: Effects of genotype, gender, sampling time and growth regulator combinations

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Abstract: Common juniper (*Juniperus communis* L.) is a valuable tree species in forestry and source of many natural products. However, natural regeneration of common juniper is difficult. To develop micropropagation procedures for this species callus induction and organogenesis experiments were carried out. It was found that genotype, gender, sampling time and different growth regulator-combinations had significant effects on callus induction in common juniper. Murashige and Skoog medium supplemented with Benzylaminopurine 0.1 mg l^{-1} and indole-3-butyric acid at concentrations ranging between 0.5–4.0 mg l^{-1} were the best among the treatments to induce callus formation when spring buds were used as explant sources. In organogenesis experiments, the combination of 2 mg l^{-1} Benzylaminopurine and 1 mg l^{-1} 2,4-Dichlorophenoxyacetic acid was found to be essential to bring about adventitious bud and shoot development from calli. Genotype, gender and sampling times had significant effects on promoting adventitious bud and shoot development. Although several experimental trials with 0.005, 0.03 and 0.05 mg l^{-1} indole-3-butyric acid and varying media compositions produced adventitious root like structures, none of these structures further developed into a true root system. However, the results of this pioneering study provide a foundation for further experiments concerning the in vitro regeneration of common juniper.

Additional key words: 2,4-D, BAP, IBA, indirect organogenesis

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Introduction

The natural regeneration process of common juniper (*Juniperus communis* L.) exhibits low efficiency. Major constraints for the natural regeneration of common juniper, especially on Mediterranean mountains, are seed abortion, low germination and high seedling mortality (Garcia 2001). Due to the problems in germination, seedling establishment of common juniper is also difficult in nature (Diotte and Bergeron 1989; Tirmenstein 1999).

In certain insular areas, common juniper populations have serious problems in regard to regression (Ward 1973; Clifton et al. 1997). Both remnant population dynamics and low resilience against disturbances reduce the distribution area of common juniper. Also consequent attempts for the restoration of common juniper populations have not been as successful as anticipated (Garcia et al. 1999). The common juniper species in Turkey are considered under risk due to their low regeneration capacity (Ekim et al. 2000). These problems necessitate the application

of biotechnological tools to propagate juniper plants in vitro. Through micropropagation, rare genotypes could be cloned; thereby, the risk of rare populations being lost due to demographic stochasticity could be eliminated.

To date, in vitro propagation of common juniper has not been reported other than the establishment of embryogenic cell lines from limited germinating zygotic embryos (Helmerson and von Arnold 2009). However, some tissue culture studies have been conducted in other species of the *Juniperus* genus such as morphogenetic capacity of mature *J. oxycedrus* L. leaves (Gomez and Segura 1994), micropropagation of *J. oxycedrus* L. (Gomez and Segura 1995b), plantlets from mature embryos of *Juniperus cedrus* Webb and Berth (Harry et al. 1995) and in vitro induction of multiple buds of *Juniperus excelsa* (Negussie 1997).

This study describes the first *in vitro* testing of vegetative propagation ability of common juniper (*Juniperus communis* L.) species. Our objectives were to determine the effects of genotype, gender, sampling time, along with different growth regulator-combinations on induction of callus and on indirect organogenesis from bud explants of common juniper trees.

Methods

Plant material and explant preparation

Shoots with buds were collected from four individuals of common juniper trees about three meters in height (two female and two male trees) located in the Middle East Technical University (METU) campus forest (Latitude: 39°53'18''N; Longitude: 32°46'29'' E; Elevation: 920 m). To provide plant materials for callus induction and following organogenesis experiments, explants were collected during three different periods, that is, November 2003 (sampling time 1, ST1), March 2004 (ST2), and September 2004 (ST3).

Being easily accessible and containing meristematic tissues, buds on newly emerged or fully developed shoots, depending on sampling times, were chosen as the explant material. They were collected and surface sterilized with 15% (v/v) of commercial bleach (a local brand containing 5.25% NaClO) including 1–2 drops of dishwashing detergent for 15 minutes. The 1–2 mm long bud explants were dissected and rinsed with sterile distilled water for four times.

Basal media and culture conditions

Murashige and Skoog (1962) basal medium (MS medium) including vitamins (Sigma M5519) with 3% sucrose and 0.7% agar, at pH 5.7 was used in all experiments (Torres 1989). Petri dishes containing cultures were maintained at 25 ± 2 °C with 16-h light / 8-h dark photoperiod (330 μmol m^{-2} sec^{-1} light) provided by cool-white fluorescent lamps. The plant material was sub-cultured onto fresh media at the end of every 30 days culture period.

Callus induction experiments

For callus initation, excised buds were cultured on MS medium, supplemented with 0.1 mg l^{-1} Benzylaminopurine (BAP: Sigma B3408), 3% sucrose (w/v) and 0.7% agar (w/v), at pH 5.7. To the medium, four different auxin and auxin-like growth regulators; Indole-3-acetic acid (IAA: Sigma I2886), indole-3-butyric acid (IBA: Sigma I5386), Naphthalene acetic acid (NAA: Sigma N0640) or 2,4-Dichlorophenoxyacetic acid (2,4-D: Sigma D8407) at 5 different concentrations (0.5, 1.0, 2.0, 4.0, 10.0 mg l^{-1}) were added separately to obtain 20 different auxin-type-concentration treatments. Buds (5 / Petri dish with three replications) were placed on each medium. As controls explants were incubated in hormone-free MS media.

Data concerning explant survival (ES), callus induction (CI), callus size (CaS) and spontaneous shoot formation (SSF) were collected according to the criteria defined and summarized in Table 1.

Followed by the two months incubation in callus induction media, the calli were transferred to 15 different MS basal media including vitamins supplemented with BAP at 5 different concentrations (0.1, 0.5, 1.0, 2.0 and 4.0 mg l^{-1}) and 2,4-D at 3 different concentrations (0, 0.5 and 1.0 mg l^{-1}) to induce adventitious bud and shoot formation with three replications. After a month of incubation in a growth room, the calli were transferred to fresh media excluding auxin, but including previously applied BAP concentrations. The calli were incubated in these media again for another month. Conditions of the growth room were the same as explained in the previous section. As controls calli were incubated in hormone-free MS media.

Before each transfer we recorded the survival (S), amount of green (alive) callus tissue (GC), meristemoid formation (MF), number of adventitious buds and shoots according to the criteria defined in Table 1.

Organogenesis: Adventitious rooting experiments

After the incubation period in the organogenesis media, newly emerged adventitious shoots from the calli were transferred to the rooting media. For root induction, we tried several different medium compositions. These were; 1) MS media supplemented with four different concentrations of IBA (0, 0.005, 0.03, 0.05 mg l^{-1}) and 1 g l^{-1} activated charcoal 2) Half strength McCown (MC : Sigma M6774) woody plant media (Kyte 1987) supplemented with 0.25 mg l^{-1}

Table 1. Description of traits and the assigned codes in callus induction and organogenesis experiments

Traits	Unit
Callus Induction:	
Explant Survival (ES)	Classes: 1=Alive 0= Dead
Callus Initiation (CI)	Count: 1=Initiation 0= None
Callus Size (CaS)	Millimeter (mm)
Spontaneous Shoot formation (SSF)	Count: 1=shoot induction 0=no induction
Organogenesis:	
Callus or SSF Survival (CSO)	Class: 1=alive 0=dead
Amount of Green Callus (GC)	Class: 0=none 1=low 2=medium 3=high
Meristemoid formation (MF)	Class: 0=none (no MF) 1=low (MF covers less than half of callus induced) 2=medium (MF covers half of the callus induced) 3=high (MF covers more than half to entire surface of callus induced)
Adventitious bud formation (ABF)	Buds counted
Adventitious shoot development (ASD)	0=none (closed),1=very little (closed but swollen) 2=little (barely open with small shoot) 3=good (open with a significant shoot) 4=very good (larger shoots) 5=perfect (shoots~1 cm with own needles)

IBA, 3) MC woody plant media supplemented with 0.05 mg l^{-1} IBA. As controls adventitious shoots were incubated in hormone-free MS media.

The buds and shoots from the organogenesis experiment were transferred into baby jars containing the media composition 1, 2 or 3 and incubated for two months. At the end of this period, survival of explants, adventitious shoot development, and presence or absence of adventitious roots were recorded according to the criteria described in Table 1.

Statistical analyses

In order to determine the effects of genotype and treatments on explant survival (ES), callus initiation (CI), callus size (CaS), spontaneous shoot formation (SSF), amount of green callus (GC), callus survival in indirect organogenesis (CSO), meristemoid formation (MF), adventitious bud development (ABF) and adventitious shoot development (ASD), an analysis of variance (ANOVA) was conducted. Since callus induction and organogenesis related data were collected as scores which are categorical variables and violate ANOVA assumptions, a nonparametric ANOVA was employed. The trait scores first ranked using PROC RANK and then analysis of variance (ANOVA) was conducted using PROC GLM of SAS statistical package (SAS Institute Inc. 2003) using ranks instead of actual scale scores.

Explant survival data were normalized by ArcSin *vP* transformation prior to analysis to meet the assumptions of ANOVA. For all traits, the GLM procedure of SAS (SAS Institute Inc. 2003) was used for analysis of variance by considering the linear model employed by Kaya et al. (1989).

The genotype, treatment, gender and sampling time means for callus induction, adventitious buds and shoots were calculated again using the mean procedures of SAS statistical package (SAS Institute Inc. 2003).

Results and discussion

Callus Induction

The analysis of variance indicated that genotypes and treatments had significant effects on explant survival, callus initiation, callus size and spontaneous shoot formation traits (Table 2). The magnitude of component of total variance due to genotypes ranged from 0.09% in SSF to 0.77% in callus initiation while the variance components due to treatments were between 0.31 in survival and 3.84 in callus size (Table 2). When the spontaneously formed shoots were examined, they are found to be originated from the original buds, therefore the calli that formed were separated and transferred to organogenesis media.

Since explants were collected in different seasons, we considered growing season as an important parameter regarding dormancy status for callus initiation and production of the common juniper explants. Sampling done during March, 2004 (ST-2) yielded significantly (χ^2=13.758, P<0.01) high explant survival (91.2%, Table 3A). When we examined the callus initiation capacity and resultant callus size with respect to sample collection times, again statistically significant results were obtained from the explants sampled in the spring of 2004 (ST-2). At the ST-2, callus initiation frequency and callus size were 90% (χ^2=13.397, P<0.01) and 7.24 mm respectively, whereas other sampling times were less successful for both callus initiation and size (Table 3A). This could be due to high physiological activities of plants prior to their natural flushing times.

The tested genotypes showed significant variation on callus initiation rate. Callus initiation rate ranged

Table 2. Analysis of Variances for the traits recorded in the callus initiation experiments. MS: mean squares; VC: component of total variance; df: degrees of freedom

	Replication (df=2)	Genotype df=3		Treatment (Genotypes) df=76		Error df=1772	
Traits	MS	MS	VC%	MS	VC%	MS	VC%
Survival (S)	13.09	4.06**	0.62	0.62^{ns}	2.73	0.31	96.65
Callus initiation (CI)	76.88	16.02**	0.77	4.26**	4.36	2.08	94.87
Callus size (CaS)	76.88	16.02**	0.24	4.26^{ns}	0.00	3.84	99.76
Spontaneous Shoot Formation (SSF)	75.76	16.01**	0.09	4.23**	5.85	1.72	93.24

*significant at $p< 0.05$; **significant at $p< 0.01$

from 60% in *Genotype 3 to* 74% in *Genotype 1*. When the mean values of callus-size were compared among genotypes, *Genotype 1* responded better than the others by forming larger calli (Table 3B). Furthermore, in the case of mean callus size produced by the explants of female trees was significantly higher (6.18 ± 0.17; n=992) than that by the explants of male trees (4.52 ± 0.14; n=862) (Table 3B).

Since the variation in responses of different genotypes within species can be enormous, results of micropropagation studies of a species often varies with genotype (Bonga and von Aderkas 1992). According to Confalonieri et al. (2003), besides explant type, genotype of the source plant is critical for callus induction in poplar trees. Yiqun and Wagner (1995) also establish the relationship between genotype and callus induction in Ponderosa pine. Our findings also indicated that genotype of the source tree was an important parameter for callus induction in common juniper. However, the relationship between the gender and the callus production is established for the first time in our study for common juniper. But, considering a low sample size (four genotypes) used in this study, interpretation of the results require caution and further studies are needed to have a firm conclusion on the matter.

We found significant differences among the treatments applied (Table 2, Fig. 1). Although, it was evident that Treatments 8, 9, 10 (where IBA at 2, 4, and 10 mg l^{-1} respectively), 16 and 17 (where 2,4-D at 0.5 and 1 mg l^{-1} respectively) gave better callus initiation responses, it was hard to clarify which auxin type at which concentration should be ideal for callus formation in common juniper explants; since all treatments produced acceptable callus size (ranging from 2.7 to 7.8 mm). Also the effects of treatments were signifi-

Table 3. A) Sampling-time means for survival, callus induction, and callus size. Sampling time–1: November 2003, Sampling time-2: March 2004, Sampling- time-3: September 2004. B) Genotype means for the traits of callus induction and organogenesis

A)

	Sampling time – 1	Sampling time – 2	Sampling time – 3
Traits	(ST–1) (N=432)	(ST-2) (N=658)	(ST-3) (N=764)
Survival (S)	0.71 ± 0.02	0.91 ± 0.01	0.83 ± 0.01
Callus initiation (CI)	0.81 ± 0.02	0.90 ± 0.01	0.69 ± 0.02
Callus size (CaS)	4.06 ± 0.19	7.24 ± 0.19	4.59 ± 0.17

B)

Callus Induction	Genotype1 (Female)	Genotype2 (Male)	Genotype3 (Male)	Genotype4 (Female)
Survival (S)	0.88a*	0.85a	0.75b	0.84a
Callus initiation (CI)	0.74a	0.67b	0.60c	0.73a
Callus size (CaS)	7.67a	4.80c	3.76d	5.54b
Spontaneous Shoot Formation (SSF)	0.06a	0.17b	0.10b	0.03a
Organogenesis				
Survival (S)	0.73a	0.79a	0.67b	0.52c
Amount of Green Callus (GC)	1.60b	2.03a	1.10c	1.18c
Meristemoid Formation (MF)	0.39b	0.93a	0.22c	0.37b
Adventitious Bud Formation (ABF)	0.78a	0.79a	0.68b	0.50c
Adv. Shoot Development (ASD)	0.39b	0.56a	0.18c	0.33b

*the means followed by the same letters are not significant at $p<0.05$

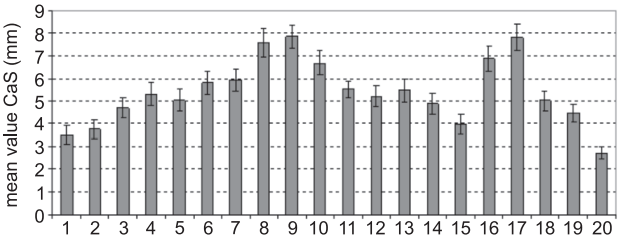

Fig. 1. Effect of treatments on callus formation. Each 5 treatments represents concentrations of growth regulators at 0,5, 1.0, 2.0, 4.0 and 10 mg $^{-1}$ sequentially. Vertical lines on the bar graphs indicated the standard error of estimations

cant on callus biomass. Especially, the types of auxins produced significantly different callus biomass, the mean callus biomass produced by auxin types ranged from 4.46 ± 0.206 (n=465) for IAA to 6.72 ± 0.24 (n=469) for IBA. For the others, the values were 4.99 ± 0.20 (N= 463) for NAA; and 5. 45 ± 0.22 (N=457) for 2,4 D.

Concerning the auxin types, it was clear that explants treated with IBA and 2,4-D showed the best response by forming the largest calli. The remaining growth regulators displayed non-significant differences among themselves.

Organogenesis

After deciding the parameters affecting the common juniper's callus induction capacity, the formed calli were transferred onto 15 different organogenesis medium-treatments. We investigated the effects of genotypes and treatments by recording the traits such as amount of green calli (GC), amount of meristemoid formation (MF), number of adventitious buds (ABF) and adventitious shoot development (ASD) (Fig. 2, panel a and b).

The statistical analysis indicated that there were significant differences among genotypes for organogenesis capacity of the common juniper (Tables 3B and 4). The genotype effects were significant for all traits and variance components due to genotypes ranged from 0.001 in survival to 4.5 in adventitious shoot formation. Genotype 2 yielded the highest green callus mass, meristemoid formation, number of adventitious buds and shoots while Genotypes 3 and 4 yielded the lowest values for these traits (Table 3B). A similar relationship between shoot formation and genotype was also established on *Pinus radiata* (Bergmann and Stomp 1994) as well as for adventitious bud formation and genotype in *Pinus pinaster* Ait (Tereso et al. 2006).

Treatment effects (Table 5) were significant for all traits except for GC and ABF. The component of total variance due to treatment effects ranged from 0.11% in survival to 8.4% in ASD (Table 4).

As can be seen in Table 5, the best results regarding MF, ABF, and ASD were obtained from *Treatment*

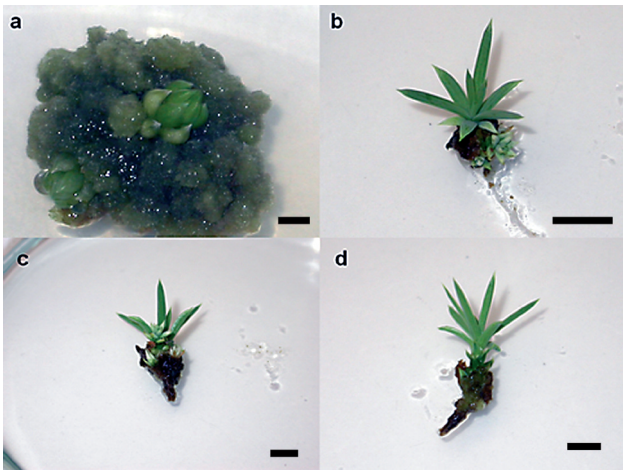

Fig. 2. Organogenesis a) Meristemoids emerging from green callus tissue, b) Adventitious shoot formation, c) and d) root like formations. Horizontal lines are scale bars 1mm in panel a and 1 cm in panels b, c and d.

14 (2 mg l^{-1} BAP + 1 mg l^{-1} 2,4-D). Within the MF values a seven fold difference between the lowest and the highest values were observed. However, around three-fold differences were observed within ABF and ASD values. Browning of the meristemoids and the subsequent necrosis in *Treatment 6* might be attributed to damage-associated phenolic compound accumulation which is common occurrence in tissue culture studies (Rout et al. 2000).

The best treatments supporting adventitious buds were *Treatments 14, 9* and *11* in which the frequencies were 47.5 (± 16.2%), 45.7 (± 14.6%) and 32.9% (± 10%), respectively. This was consistent with the results demonstrated in many studies that high cytokinin concentrations increase the formation of adventitious buds. Confalonieri et al. (2003) reported that when cytokinin concentration was lowered, it resulted in the reduction of the frequency of adventitious buds and increase in shoot elongation in poplar. In our trials, it was clear that adventitious bud formation increased when the auxin component was excluded from the nutrient media, but it was needed in the first stage of the adventitious shoot development in common juniper to increase the yield. Negussie (1997) reported that explants of the family Cupressaceae responded favorably to relatively low levels of cytokinins. Higher concentration of BAP, especially when combined with high levels of NAA, resulted in inhibition or reduction in the percentage of adventitious bud formation.

Limited number of studies were conducted on in vitro propagation of other juniper species. In a study on *J. oxycedrus*, Gomez & Segura (1995a) found that 1/3 strength MS media supplemented with different concentrations of BA was ideal for apical explants than nodal explants. They also concluded that nutrient medium and BA concentration affected the length of regenerated shoots.

Table 4. Analysis of Variances for the traits recorded in the organogenesis experiments; MS: mean squares; VC: component of total variance; df: degrees of freedom

	Replication (df=1)	Genotype df=3		Treatment (Genotypes) df=55		Error df=429	
	MS	MS	VC%	MS	VC%	MS	VC%
Traits							
Survival (S)	11.03	1.66^{ns}	0.01	1.31**	0.11	0.36	0.36
Amount of Green Callus (GC)	1.77	7.58**	2.90	1.04^{ns}	0.50	1.57	96.60
Meristemoid Formation (MF)	2.78	11.91**	3.60	1.639*	4.10	1.189	92.10
Adventitious Bud Formation (ABF)	2.78	11.91**	3.30	1.639^{ns}	2.90	1.427	93.64
Adventitious Shoot Development (ASD)	2.78	11.91**	4.50	1.639**	8.40	0.908	86.90

*significant at $p < 0.05$; **significant at $p < 0.01$

In our study, when 2 mg l^{-1} BAP and 1 mg l^{-1} 2,4-D were used, highest results are obtained for MF, ABF and ASF (Table 5). Therefore, we concluded that the presence of both growth regulators in the media is required to initiate tissue re-differentiation leading to adventitious shoot formation. A study on *Pinus pinaster* Ait (Tereso et al. 2006) also demonstrated the requirement of cytokinin and auxin in successful induction of adventitious buds.

We cultured the four months old shoots in different sizes in adventitious rooting media. In order to induce root formation in common juniper explants, adventitious shoots were incubated in three different sets of treatments.

Although adventitious root-like formations have been observed (Fig. 2 c, d), none of these further developed into true adventitious roots either in MS based medium or in McCown Woody Plant Medium.

Lambardi et al. (1995) stated that as adventitious shoots aged and transfer intervals were increased, rooting occurred spontaneously in *Cupressus sempervirens*. On the contrary, it was reported that rooting of adventitious shoots proliferated by in vitro techniques was difficult in *Juniperus oxycedrus* (Gomez & Segura 1995a). They tried to induce adventitious roots in different treatments supplemented with IAA, NAA or IBA, or in combinations of two of them at different concentrations, but were unsuccessful.

In the studies conducted with *Juniperus excelsa*, Negussie (1997) could not achieve rooting of the adventitious shoots by pre-treating them with IBA and NAA or with activated charcoal during the first eight weeks of culture. However, only four shoots were rooted in hormone-free full MS medium after four months of culture, but they subsequently died. Only when the shoots were transferred to non-sterile compost, 59% of the shoots survived and 31% of them rooted after four months.

Our experiments showed that *Juniperus communis*, as in other *Juniper* species, is a difficult species to induce adventitious roots under *in vitro* conditions.

In conclusion, the effects of genotype, gender and sampling time on tissue culture parameters were reported neither for common juniper nor any other member of the Cupressaceae family. This study clearly indicated the significant effects of the source

Table 5. Effect of BAP and 2,4-D growth regulators on indirect organogenesis.

Treatment #	BAP mg l^{-1}	2,4-D mg l^{-1}	Green Calli # (GC)	Meristemoid # (MF)	Adv. Bud # (ABF)	Adv. Shoot # (ASD)
1	0.1	0	1.89 ± 0.21	0.60 ± 0.14	0.23 ± 0.11	1.60 ± 0.40
2	0.5	0	1.77 ± 0.21	0.37 ± 0.13	0.18 ± 0.07	0.71 ± 0.36
3	1.0	0	0.93 ± 0.20	0.10 ± 0.06	0.31 ± 0.13	1.86 ± 0.14
4	2.0	0	1.00 ± 0.22	0.40 ± 0.16	0.18 ± 0.13	2.00 ± 0.58
5	4.0	0	0.77 ± 0.15	0.33 ± 0.13	0.32 ± 0.12	1.67 ± 0.21
6	0.1	0.5	2.15 ± 0.18	0.66 ± 0.14	0	0
7	0.5	0.5	1.97 ± 0.19	0.81 ± 0.16	0.28 ± 0.09	2.00 ± 0.26
8	1.0	0.5	1.97 ± 0.21	0.49 ± 0.14	0.29 ± 0.10	2.13 ± 0.30
9	2.0	0.5	1.97 ± 0.18	0.40 ± 0.15	0.46 ± 0.15	2.22 ± 0.36
10	4.0	0.5	1.45 ± 0.23	0.79 ± 0.20	0.20 ± 0.08	1.00 ± 0.37
11	0.1	1.0	1.68 ± 0.23	0.68 ± 0.15	0.33 ± 0.10	2.20 ± 0.33
12	0.5	1.0	1.66 ± 0.19	0.44 ± 0.12	0.27 ± 0.08	2.00 ± 0.41
13	1.0	1.0	1.49 ± 0.23	0.73 ± 0.16	0.28 ± 0.09	2.11 ± 0.61
14	2.0	1.0	1.64 ± 0.23	0.93 ± 0.20	0.48 ± 0.16	2.44 ± 0.44
15	4.0	1.0	0.79 ± 0.24	0.13 ± 0.09	0.21 ± 0.13	1.00 ± 0.58

plants on the examined traits. Also for the first time, we demonstrated the effect of gender on callus production. In addition, adventitious bud induction increased when the auxin was removed from the nutrient media after the first stage. The rate of adventitious shoot development improved considerably at high concentrations of BAP in the absence of auxins. Furthermore, we showed that the explant collection time was also an important parameter for in vitro propagation of common juniper as it was reported for some other tree species.

In conclusion, although we could not develop whole plantlets from callus tissues, we were able to produce considerable amount of buds and shoots. In future studies, alternatives on rooting should be exhausted in order to obtain whole common juniper plantlets.

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