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Effect of different plant growth regulators on callus induction of stem explants in *Pistacia atlantica* subsp. kurdica

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Abstract

Wild pistachio (*Pistacia atlantica* subsp. kurdica) is one of tree species that cover much of the forest in northwest of Iran and used in industries**,** medicine and a**s** food. The present study describes callus induction of the *P. atlantica* subsp. kurdica using seedling stem explants and the influence of different plant growth regulators including kinetin, benzyladenine (BA), thidiazuron (TDZ), 6-Benzylaminopurine (BAP) on the growth of calli. To determine the best concentration and composition of plant growth regulators to induce callus in wild pistachio, 13 treatments were compared in a completely randomized design with five replicates. Explants were excised from 7-10 days old *in vitro* grown seedlings and transferred to Woody Plant Medium (WPM) containing 3% sucrose and 0.7% agar supplemented different plant growth regulators. The data were collected for callus fresh weight, callus dry weight and callus induction percentage. GC-MS analysis of essential oil constituents of *P. atlantica* subsp. kurdica callus was performed. Results showed that concentrations and combination of various plant growth regulators had significant effect on callus induction and callus weight. The high efficient callus formation was observed in the medium containing different concentration of 6-BAP individually. The lowest and highest percentage of callus induction were in the treatment of BA 2 mg/l with NAA 1mg/1 (40%) and 6BAP 1mg/l (85%), respectively. GC-MS separated eight kinds of substances in callus. Main compositions of callus were Bornyl acetate (9.18%), Spathulenol (5.89%) and Ledol (5.37%).

Keywords: Auxin, callus, cytokinin, GC-MS, *Pistacia atlantica* subsp. Kurdica.
Abbreviations: MS_Murashige and Skoog's medium; BA_N6-benzyladenine; BAP_6-Benzylamino-purine, NAA_1-naphthylacetic acid; IAA_indole-3-acetic acid; TDZ_Thidiazuron; WPM_woody plant medium; PGR_plant growth regulators.

Introduction

Wild pistachio (*Pistacia*) is a genus of plants in tropical *Anacardiaceae* family. Three *Pistacia* species grow naturally in Iran, including *P. vera* Linnaeus, *P. khinjuk* and *P. atlantica* Desf. Large populations of *P. atlantica* subsp. kurdica trees growing in the wild acro**ss** Kurdistan province of Iran. *P. atlantica* subsp. kurdica, with the local name of Baneh is used for medicine, food and industrial purposes. Baneh nuts are used by the natives after grinding and mixing with other nuts. Its gum is also used in the production of chewing gum (Daneshrad and Aynehchi, 1980). The biological effects of gum compounds including anti-atherogenic, hypoglycemic, anti-inflammatory, antipyretic, antifungal, antimicrobial, anti-viral, anti-insecticide and anticancer activities for *Pistacia* species has been established (Rezaei et al., 2011). Plant cell and tissue culture has been used as a strategy to improve trees through somaclonal variation, germplasm conservation, and genetic transformation and also for the production of secondary metabolites. Most research has focused on the species *P. vera* L. and less emphasis has been placed on some of the other important species of this genus. *P. atlantica* subsp. kurdica is the major source of a gum in Kurdistan, Iran and has not been known well to the world. *P. atlantica kurdica* is a sub-species of *P.atlantica* because of the presence of leaf rachis wing that narrower than the type of *P. atlantic*a (Zohary, 1952). Most works on *P. atlantica* were focused on gum biological and antioxidant activities ((Rezaei et al., 2011). Regeneration of *P. vera* L. from seed or seedling tissues has been the most successful method to date (Yang and Ludders 1993, Parfitt and Almehdi 1994, Onay et al. 1995, 1996, Onay 2000). An efficient protocol for inducing direct shoot organogenesis from mature leaf explants of *P. vera* L. and the subsequent recovery of the pistachio plantlets has been reported (Tilkat and Onay 2009). There have also been several previous reports on plant regeneration from seedling explants of other wild pistachio species (Onay 2000, Tilkat et al. 2005). Onay (2000) produced embryogenic mass from kernels of mature fruits of *P. atlantica* cultured in liquid MS) media (Murashige and Skoog (1962), supplemented with 100 mg/l casein hydrolysate, 100 mg/l l-ascorbic acid, and 6BAP. Later matured somatic embryos germinated on the maturation medium without growth regulators and developed into plantlets.Plant tissue culture systems represent a potential renewable source of valuable medicinal compounds, flavors, fragrances, and colorants, which cannot be produced by microbial cells or chemical synthesis. Advances in biotechnology particularly methods for culturing plant cell cultures, should provide new means for the commercial processing of even rare plants and the chemicals they provide So far, there are no reports on the induction of callus in *P.*

Table 1. Treatment combinations and callus induction percentage of *P. atlantica* subsp. kurdica

Treatments	Cytokinins	Auxin	Callus induction percentage
A	Kin 2	-	75
B	BA 4	-	55
C	BA 1	NAA 0.1	55
D	BA 0.5	IBA 0.1	70
E	BA 1	IBA 0.1	50
F	BA 2	IBA 0.1	60
G	6BAP 0.5	-	60
H	6BAP 1	-	85
I	6BAP 2	-	75
J	6BAP 3	-	70
K	BA 1	NAA 1	75
L	BA 2	NAA 1	40
M	**BA 3**	**NAA 1**	**55**

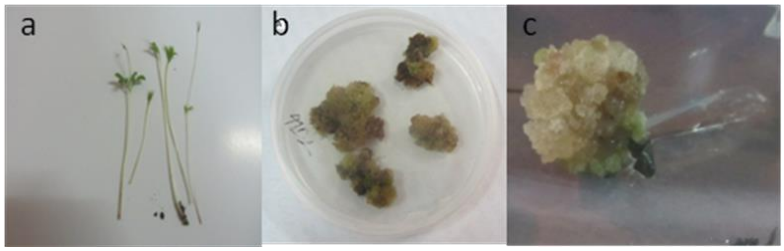

Fig 1. Callus induction from seedling stem. *P. atlantica* subsp. kurdica seedlings (a), callus (b &c).

atlantica subsp. kurdica. This study was undertaken to standardize the protocol for callus induction of the *P. atlantica* subsp. kurdica using seedling stem explants and to analyze essential oil of callus using GC-MS.

Result and discussion

Callus induction

The applications of different PGRs on callus induction of *P. atlantica* subsp. kurdica were effective. The PGRs combinations that were used in the culture establishment are presented in Table 1. Analysis of variance showed significant differences (α=0.01) between the different treatments (Table 3). Most of PGRs combinations tested for *in vitro* culture resulted in callus production after 2 weeks of culture. Initially, the shoot explants enlarged and developed globular structures on the surface of explants and latter callus mass were proliferated. The lowest and highest percentage of callus induction were in the treatment of BA 2 mg/l with NAA 1mg/1 (40%) and 6BAP 1mg/l (85%), respectively. Treatments containing BA in the medium produced the greatest amount of callus fresh weight and dry weight of callus (Fig 2, 3). The lowest fresh and dry weights of callus were in treatments with different concentrations 6BAP. The callus growth rate results were different from callus fresh and dry weights. Treatments BA 4 mg/l, BA + NAA and BA with low concentration of IBA had the highest rate of callus production. Also the lowest growth rate callus was related to treatments 6BAP 0.5, 1 and 2 mg / l is. To our knowledge, this is the first report for *P. atlantica* subsp. kurdica on callus induction from seedling stem explants to date. In the present study, a strong influence of BA on the callus induction was also observed. However, a combination of BA with auxins, IBA and NAA, was crucial for callus production. A similar result was reported by Tilkat and Onay (2009) on *P. vera*. Among different cytokinin- auxin combinations they used BA individually gave the highest callus proliferation frequency. In *Coffea arabica* leaf explants cultured on medium with 5 **µ**M BA as the sole plant growth regulator produced white friable calli that formed somatic embryos and calli have been subcultured on the same medium for more than 2 years and maintain the ability to produce somatic embryos (Yasuda et al. 1985). These results show that BA has an important role in callus induction of some plants.

including *P. atlantica* subsp. kurdica.. Results showed that treatments with NAA showed better callus weight and growth rate. Similarly, Yang et al. (2006) produced compact greenish nodule callus in *Acacia crassicarpa* on medium (MS) containing NAA and TDZ in combination after 40 days of . In addition, Arumugam et al. (2009) showed that under optimized culture conditions, the high rate of callus induction and proliferation in *Acacia confusa* Merr immature leaflet explants was obtained in 35days on MMS medium supplemented with 2,4-D (3 mg /l) + NAA (0.01 mg/ l) + Kin (0.05 mg/ l). The present study highlighted that BA alone or in combination with NAA could enhance callus growth, suggesting the involvement of BA in the modulation of endogenous growth regulators, especially auxins and cytokinins. The formation and growth of callus, requires more culture conditions including darkness, temperature, medium composition, especially with regard to PGRs. We kept explants on darkness condition for callus induction. Ying et al. (2000) showed that induction of *Phalaenopsis* callus, darkness play important role in callus induction and plantlet regeneration. In other work the effects of kinetin and 4PU-30 on the growth and content of polyphenols in tobacco callus, darkness at 26°C showed the best results (Yordanka et al. 2001).

Table 2. Components identified in the essential oil of the stem callus of *P. atlantica* subsp. kurdica

Compound No.	Compound Name	Retention Time (Rt)	Percentage (%)
1	Bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl	7.26	4.96
2	Beta Pinene	8.69	0.61
3	Bornyl acetate	20.49	9.18
4	Caryophyllene	25.40	1.44
5	Spathulenol	30.85	5.89
6	Globulol	31.08	4.99
7	Ledol	31.30	5.37
8	Oleic Acid	47.02	0.83

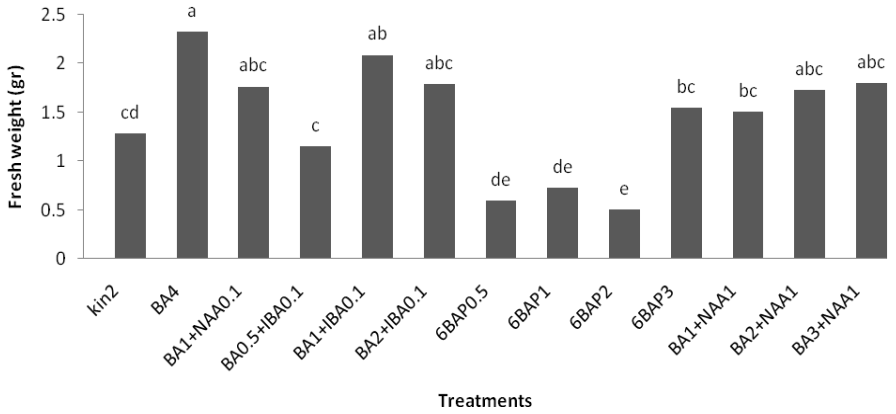

Fig 2. Effect of plant growth regulators on callus fresh weight of explants of *P. atlantica* subsp. Kurdica. Means were compared using LSD. Similar letters show non-significant differences ($p<0.05$).

GC-MS analysis of callus

The essential oil was extracted by the hydrodistillation from the dried callus of *P. atlantica* subsp. kurdica, and their constituents were analyzed by GC/MS. The identified combinations in essential oil, retention time index (Rt) and quantitative percentage of the compounds from leaves and fruits are presented (Tables 2). A total of eight compounds were identified in the essential oil with total percentage of 33.27%. The combinations of bornyl acetate (9.18%), Spathulenol (5.89%) Ledol (5.37%) Globulol (4.99%),

Bicyclo[2.2.1]heptan-2-ol,1,7,7-trimethyl-(4.96%), Caryophyllene (1.44%) Oleic Acid (0.83%) Beta Pinene (0.63%) were detected. ß-Pinene also has been reported in all analytical data from the literature from essential oils of this species (Shariffi and Hazell 2011 and Alma et al. 2004). Shariffi and Hazell (2011) analyzed the volatile oil from the crude gum of *P. atlantica subsp. Kurdica* and reported that the major compound were α-Thujene α-Pinene, Camphene, Sabinene,β-Pinene,Δ3-Carene and Limonene. These results showed that the callus oil compound was different from that of gum oil.

Materials and Methods

Plant materials

Seeds of *P. atlantica* subsp. kurdica were collected from Kamyaran city, south of Kurdistan province. Seeds were soaked in water and shaken for two days at 150 rpm. The surface layer was gently removed. Scarification of seeds was done by soaking them in concentrated sulfuric acid. Seeds are placed in a glass container and covered with sulfuric acid (30N). The seeds were gently stirred and allowed to soak for 10 minutes. When the seed coat was modified (thinned), the seeds were removed, washed with distillated water and sown. One month after the seedlings emerged from seed and was about 10 cm high; their stems were used as explants. Seedlings were washed with dish soap and running water for 30 minutes to wash the surface, and then were sterilized under laminar hood was as follows: ethanol 75% for 30 seconds, 8% (w/v) calcium hypochlorite (58% available chlorine) 15 minutes and finally washed with sterile distilled water three times 15, 10 and 5 minutes.

Callus induction

Explants were excised from 7-10 days old *in vitro* grown seedlings and transferred to Woody Plant Medium (WPM) containing 3% sucrose and 0.7% agar supplemented different plant growth regulators. Treatments are listed in Table 1. Stems were cut in 1-2 cm pieces and four samples were placed in each petri dish. After cultivation, explants were transferred to the growth chamber with 25° C and darkness. After two weeks of culture the callus appeared and continued taking notes until the end of sixth week. Traits measured in this study include fresh weight of callus mass, dry weight of callus mass, percentage of callus induction and callus growth rate relative. The percentage of callus induction and callus samples were counted and recorded. Callus fresh weight by scale with 0.0001 g was measured and then were transferred to the oven with aluminum foil and kept at 75° C for 48 hours for drying. After drying the callus dry weight was measured and the data were recorded for statistical analysis. Callus relative growth rate also was calculated using the formula provided by Galiba et al. (1993).

Table 3. ANOVA for callus traits.

		Mean square		
Source of variation	DF	Fresh weight(g)	Dry weight (g)	Relative Growth rate
Treatments	12	1.539**	0.0188**	128478.09**
Error	49	0.295	0.0045	28452.02

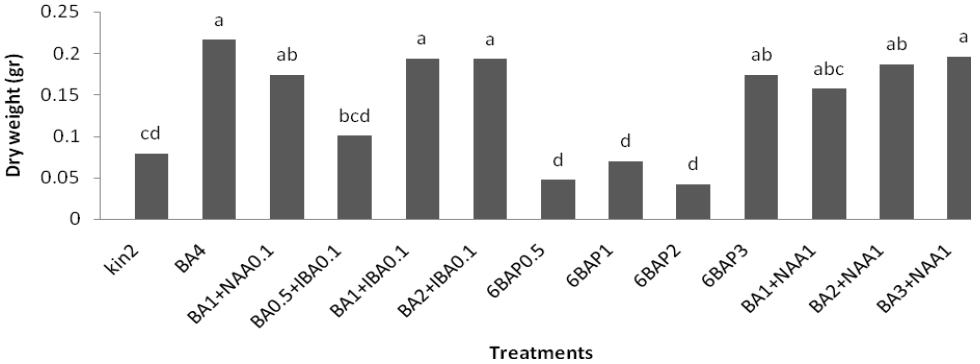

Fig 3. Effect of plant growth regulators on callus dry weight of explants of *P. atlantica* subsp. Kurdica. Means were compared using LSD. Similar letters show non-significant differences ($p<0.05$).

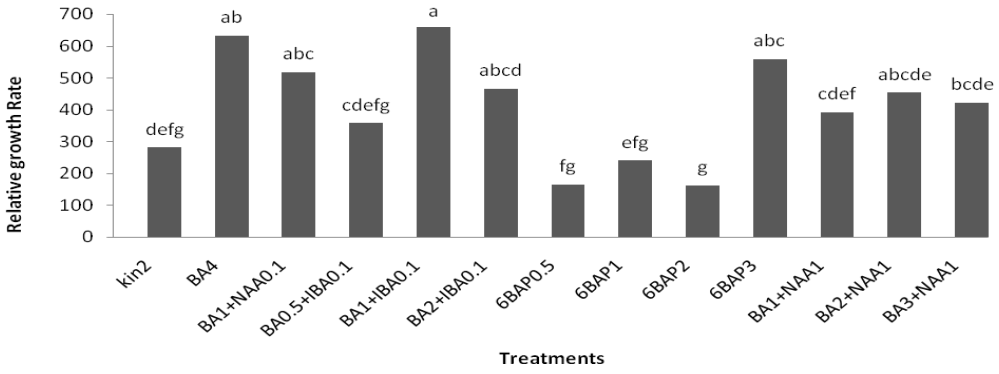

Fig 4. Effect of plant growth regulators on callus relative growth rate of explants of *P. atlantica* subsp. Kurdica. Means were compared using LSD. Similar letters show non-significant differences ($p<0.05$).

$$RGR = \left[\left(\ln\left(w_2\right) - \ln\left(w\right)\right)\left(t_2 - t_1\right)\right] \times 10^3$$

The base-10 logarithm lnw1 is callus fresh weight in 15 days after culture (t1) and base-10 logarithm lnw2 is callus fresh weight after 30 days of culture (t2).

Medium preparation and culture

The explants were inoculated into Woody Plant Medium (WPM) (Lloyd and Mc Cown 1980) with 0.7% agar with pH 5.8. Woody plant medium was supplemented with different combinations of growth regulators for callus induction.The media were autoclaved at 20 psi, at 121°C for 20 min. Cultures were maintained in 25 ± 1°C, under darkness for callus initiation. The effect on callus induction was studied with different concentrations of N 6-Benzyladenine (BA), 6-Benzylaminopurine (6 BAP), Kinetin, Naphthalene Acetic Acid (NAA), Indole-3-butyric acid (IBA) and their combination(s).

Preparation of plant materials and extraction for callus

The *in vitro* plant materials were removed from their respective culture medium and washed to remove any traces of culture medium before they were air dried at 25 ± 2 ˚C until constant weight was obtained. They were then macerated to powder form with a mortar and pestle. The determination of active compound was carried out according to the method described by Leng et al. (2011) with slight modification. One gram (1.0 g) of the macerated plant materials were then soaked with 20 ml methanol (MeOH) and allowing the homogenate to stand for 3 days. This extraction procedure was repeated three times. The combined supernatant was concentrated to 10 ml below 40°C and 300 mbar by using a rotary evaporator (Heidolph HEI-VAP Advantage Vacuum rotary Evaporator). The solution was transferred into separatory funnel and extracted three times with 20 ml of n-hexane. After the bottom layer of n-hexane extract was collected from the funnel, they were concentrated to dryness below 40˚C. Just before injection of samples into GC-MS column, the dried extracts were eluted with 1 n-hexane: 1 diethyl ether. Since, external standard was not employed here; all the chromatograms and mass spectra of

the samples were then matched with Mass Spectrometer (HP5975 MSD, EI made in USA) library for authenticity.

Statistical analysis

Data collected in the experiments were analyzed using SAS (version 9.00) analysis of variance procedures. The means and the differences within the treatments were compared using one way analysis of variance (ANOVA). Post hoc LSD test was also performed at $p \leq 0.05$.

Conclusion

Effect of BA, KIN, IBA, 6-BAP, NAA on callus initiation and maintenance as an efficient protocol for the callus induction of *P. atlantica* subsp. kurdica was developed from seedling stem explants. Further exploitation of this protocol for mass propagation, transformation, secondary metabolites clonal propagation and commercial production should assist in large scale. GC-MS analysis of essential oils of callus showed that it can be used as new source for secondary metabolites in *P. atlantica* subsp. kurdica.

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