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Effects Chemical Treatments and Stratification on Seedlings Emergence of Persian Parrotia (*Parrotia Persica* (DC.) and Assessment of Genetic Diversity in its Seedlings

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Abstract: *Persian parrotia (Parrotia persica C.A. Mey.) is native to northern Iran and Azerbaijan, along the Caspian Sea. Persian parrotia is an important tree species in Hyrcanian forests and also well known for its landscape values and the wood is also used by wood turners and for weaving shuttles, telephone poles. Very limited agronomic information exists regarding the cultivation of Iron tree as a industrial crop. In this study we investigated effects of cold stratification (25, 50 and 75 days) in combination with putrescine (5, 10 and 15 mM), BA (1, 2 and 3 mM), KNO_3 (25, 50 and 75 mM) and warm stratification (42 days) on seed germination and emergence of P. persica and assessment of genetic diversity in it seedlings using RAPD markers. The results of germination treatments showed that the highest emergence percentage was obtained with 42 days warm moist chilling followed by 50 days cold stratification. To assessment of genetic diversity, 18 RAPD primers were used. The results indicated that 6 out of 18 primers produced no band on the studied genotypes and the rest primers (12) produced 92 bands overall. The average polymorphism bands were 7.66%. The lowest number of bands was three bands with 66.6% polymorphism which obtained with OPB-10 primer and the highest number of polymorph bands was 12 bands with 100% polymorphism gained with OPD-05 primer. The highest resolving power (0.48 and 0.47) was found with OPA-10 and OPB-10 primers, respectively. The lowest resolving power (0.23) was related to OPE-06 and TIBMBC-13 primers. The molecular results showed that OPD-05 and OPA-10 and OPA09 primers can use for ongoing genetic variation studies in Persian parrotia.*

Keywords: *Parrotia persica, seed germination, treatment.*

1. INTRODUCTION

The family Hamamelidaceae comprises 30 genera and 144 species and has been the subject of a great attention due to its phylogenetic position and extensive fossil records (Zhou et al. 2001; Andrew 1997; Zhi-yun and An-ming 1995). Persian parrotia, Persian ironwood, Iron tree or Ironwood tree (*Parrotia persica* C.A. Mey.) was described by C.A. Meyer in 1831 and named in honor of F.W. Parrot, a German physician, naturalist and explorer (More and White 2003; Li and Tredici 2006; Andrews 2007). Persian parrotia is native to northern Iran and Azerbaijan, along the Caspian Sea, a phytogeographic region consists of northern Iran (Gilan, Mazandarn, Gorhgan, north-west Khorasan) and Talish in Azerbaijan (Sabeti 1994; Mozaffarian 2003; Mozaffarian 2005; Ahanjan 2007; Andrews 2007). The species with some its cultivars are now found in many famous botanic gardens around the world (Andrews 2007). Persian ironwood is a highly ornamental tree or large shrub that can grow to 20-25 m and has been in cultivation since 1840 (More and White 2003; Andrew 2007). It makes a round-year feature with red or

brown bark patches in winter, lustrous green leaves in summer following by quite outstanding color change in autumn which makes it ideal as specimen, accent or street tree (More and White 2003; Gilman 2014). It is also a tough species that tolerates drought, heat, wind and cold (Gilman 2014). The species is also one of the most lime tolerate ones in Hamamelidaceae (Andrew 2007).

This species has some local Persian names including "Anjeeli" and is praised for many characteristics including its fabulous autumn color change particularly in mixed woods and used in landscape and botanical gardens in Iran, as well. The wood is extremely hard and durable and this is why it is called iron tree. The charcoal made from it is highly valued. The wood is also used by wood turners and for weaving shuttles, telephone poles or other uses in Iran and Azerbaijan (Sabeti 1994; Andrew 1997). Many herbalists use Persian ironwood in the treatment of various fevers and respiratory infections in Iran due to its flavonoids compounds and is also used for food coloring and food flavoring (Ahanjan et al. 2007). There are various kinds of dormancy in which physiological dormancy may be decreased by cold or warm stratification or application of hormones (Takos 2001; Zhou et al. 2003; Garcia- Gusano et al. 2004; Rehman et al. 2000; Duan et al. 2004; Fang et al. 2006; El- Refaey 2014). Plant growth regulators such as GA_3 (Gibberellic acid) and BA (Benzyl adenine) and chemical compounds including KNO_3 (Potassium nitrate) and putrescine may improve seed germination and/or early seedling growth in plant species (Bryan and Seiler 1991; Matilla 1996; Strik et al. 2005; El-Tohamy et al. 2008; Tzortzakis 2009). A combination treatment of alternating temperatures (25/15 or 23/11ºC) for 6 months followed by 5 ºC for 3 months was successful in overcoming seed dormancy in *Taxus mairei* (Chien et al. 1998). Warm stratification followed by cold stratification has also been applied in some *Rosa* taxa (Alp et al. 2009) and *Styrax japonicas* (Alp et al. 2009) to overcome seed dormancy. Warm stratification has also been reported as a requirement for breaking dormancy in seeds with intermediate physiological dormancy such as *Empetrum hermaphroditum* (Baskin et al. 2002). Seed germination of Japanese stewartia (*Camelia japonica*) was promoted by GA_3 treatment and warm and cold stratification (Olesksak and Struve 1999). The application of GA_3 during and after stratification significantly increased the length, trunk diameter, internodes length, leaf area and fresh and dry weight of seedlings of two species of *Pistacia* (Rahemi and Baninasab 2000). According to Airi et al. (2009), treating the seeds of *Hippophae salicifolia* with GA_3 shortened mean germination time and increased germination percentage. GA_3 treatment followed by warm stratification for 3 months and 7 months of cold stratification has been reported as a practical procedure for seed germination in *Stewartia pseudocamellia* (Brian et al. 1999). In *Prunus avium*, treating the seeds with 7500 ppm KNO_3 after 120 days of stratification was very effective and resulted in 65% germination of seeds with coat (Cetnibas and Koyunncu 2006). The positive effect of potassium nitrate has reported for the genus *Rubus* (Wada and Reed 2011). Polyamines are implicated in seed germination and their concentrations altered by the process of stratification (Matilla 1996). Application of polyamins (Spd, Spm and Put) on pistachio, increased both seed germination percentage and mean germination rate and seedling growth (Sedaghat and Rahemi 2011). Chapurro et al. (1988) studied effects of BA on peach seeds germination and reported that the highest germination was obtained by 24 hr exposure to 50-100 mg L^{-1} of BA. Osman Sarihan et al. (2005) investigated the effects of GA_3 and KNO_3 on *Plantago lanceolata* seeds and reported the positive effects of these compounds on seed germination. Some members of Hamamelidaceae require warm and cold stratification treatments to germinate successfully. Seed germination of 17 species of this family was investigated at Batumi Botanical Garden using scarification and stratification treatments and a long period of dormancy reported for some species including *Parrotai persica* (Metreveli and Bregvadze 2007). *P. persica* is propagated by layering, grafting on *Hammamelis virginiana*, root off-shoots or root suckers, cutting under mist and micropropagation (Sabeti 1994; Andrew 1997; Hartman et al. 2011). It can also be raised by seed (Hartman et al. 2011). The natural regeneration of the species through seed is also very scanty. *Parrotia persica* is a slow- growing tree, preservation of the present populations and a genetic database information for future purposes are of vital importance. Consequently, optimizing protocols for genetic assessment and adoption the efficient means to pave the ground for breeding and biotechnological goals are very decisive. On the other hands, there are just few reports (Yosefzadeh et al. 21010, Sattarian et al. 2011) covering the phylogenetic relationship among Persian parrotia populations in Iran merely based on morphological markers.

Recent years have witnessed increasingly rapid development of molecular phylogenetics and systematics. This is due to the development of new diverse methods of analysis of molecular DNA markers (Grechko 2002). To investigate the phylogenetic relationship among the populations of each plant with DNA markers, the first step is the adoption of efficient primers. In the case of Persia parrotia, no report is available about using molecular markers for genetic variation assessment. There are a few reports on seed germination of iron wood tree and its genetic variation assessment . Thus, the objective of this study was to investigate effect of cold and warm stratification and some chemical compounds on germination of Persian parrotia and assessment of genetic diversity in its seedlings using RAPD marker as an important forest species and suitable for landscape.

2. MATERIAL AND METHODS

2.1. First Experiment (Seed Germination Treatments)

The mature seeds with dark brown colour were collected from Noor forest in the western

Hyrcanian forest of Iran. The geographical location of the seed collection was 52° E and 36° N with annual mean of temperature and precipitation 16.5° C and 850 mm respectively (Sattarian et al. 2011). The seeds were collected from 20 trees and were stored at 4 ° C to start testing. The teatments were done on the same year. The seeds were surface sterilized with sodium hypochlorite (1%) for 5 min and kept in distilled water for 24hr. For cold stratification treatment, the seeds were mixed with perlite (1:3 seed/perlite) as medium and put in refrigerator at 4±1 Cº for 25, 50 and 75 days (Baninasab and Rahemi 2008). At the end of stratification period, the seeds were taken out and treated with KNO_3 (25, 50 and 75 mM), putrescine (5, 10 and 15 mM) (Sedaghat and Rahemi 2001) and BA (1, 2 and 3 mM) (Khalil and Al-Eisawi 1998) and were sown in pots with perlite medium. For warm stratification, seeds were treated at 25±1º C for 42 days in moistened perlite (Bujarska-Borkowska 2002). After warm stratification, the seeds were sown in pots with perlite medium and kept in greenhouse at 24± 2 ºC. The present study was done in foure parts that the first part was putrescine treatment with cold stratification, the second and third parts were KNO_3 and BA tratments respectively with cold stratification and final part was warm stratification treatment with cold stratification. In all treatments, control was 25 days cold stratification. Each parts of study had 10 treatments except to final part that was contained 4 treatments. Each treatments had four pots as replication. In total 100 seeds were used in each treatment. Pots were irrigated during experiment period with Hogland solution. Seedling emergence percentage was recorded 30 days after sowing. All treatments of this experiments are presented in Tables 2, 3, 4 and 6.

Seedling emergence percentage and emergence rate were calculated 30 days after seed sowing using the following equations (Salim Azad et al. 2011; Bian et al. 2013). Meanwhile, some growth parameters such as leaf area, leaf number, stem length and shoot and root dry mass were measured.

Emergence rate$_{=}$ number of seedlings/days to first count $_{+}$ …….$_{+}$ number of seedlings/days to final count

Emergence (%) = a × 100/N

Where, E is emergence percentage; a is the number of produced seedlings after 30 days, and N is the total number of planted seeds. Leaf area of three youngest, fully expanded leaves from the terminal shoots were measured with a portable leaf area meter (LI-COR 3000, Lincoln, Neb.) in 90 days after seed sowing. At the end of the experiment; 90 days after seed sowing; plants were cut at the pot level and roots were washed and separately oven- dried at 70 ºC for measurement of root and shoot dry weigth. In part of experiment design was as completely randomized design (CRD) with four replications each contained 25 seeds per pot (100 seeds per each treatment). Analysis of variance (ANOVA) was performed using the SAS version 9.4 software's for each part of experement. If ANOVA determined that the effects of the treatments were significant ($P \leq 0.05$), then the means were compared by Tukey's multiple range test.

2.2. Second Experiment (Molecular Evaluation)

Eighteen genotypes (seedling) produced from the first study were used in this study, but 5 did not produce any bands by used primers or did not amplify clear products. In total, 13 genotypes which produced good and reproducible bands were used for further analysis. The leaf samples were washed three times in sterile distilled water, frozen in liquid nitrogen and kept at -20 ◦ C until used. Genomic DNA was extracted using the CTAB method of Doyle and Doyle (1987) with minor modifications (Karimi et al. 200; Karimi and Sadeghi Seresht 2015). One gram of needle was ground in liquid nitrogen and mixed with 6 mL of CTAB buffer (100 mM Tris- HCl, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 2% polyvinylpyrrolidone, 0.2% β-mercaptoethanol, 0.1% Na_2S20_5). The samples were then incubated at 65^o C for 1 h, following by extraction with an equal volume of chloroform- isoamylalcohol (24/1). The aqueous phase was recovered and mixed with an equal volume of cold isopropanol and kept at -24^o C for 24 hour. The precipitated nucleic acids were recovered by centrifugation at 1000 rpm for 2 min, washed with ammonium acetate in 76% ethanol, dried and resuspended with double distil water. DNA concentration was estimated spectrophotometrically and confirmed by electrophoresis in 8% agarose gels using known concentration of bacteriophage lamda DNA (CinnaGen, Tehran, Iran)

Table1. *List of used primers to assessment of genetic diversity in iron wood genotypes (seedlings)*

Row	Primer	Primer sequence
1	OPD-03	GTCGCCGTCA
2	OPD-05	TGAGCGGACA
3	OPD-O6	ACCTGAACGG
4	OPD-07	TTGGCACGGG
5	OPD-08	GTGTGCCCCA
6	OPD-20	ACCCGGTCAC
7	OPAD-02	CTGAACGCTG
8	OPA-09	GGGTAACGCC
9	OPA-08	GTGACGTAGG
10	OPA-10	GTGATCGCAG
11	OPB-10	CTGCTGGGAC
12	OPC-02	GTGAGGCGTC
13	OPE-06	AAGACCCCTC
14	OPG-02	GGCACTGAGG
15	OPZ-10	CCGACAAACC
16	OPAE-10	CTGAAGCGCA
17	TIBMBB-12	GTGTGCCCCA
18	TIBMBC-13	TCGGTGAGTC

At the beginning of the experiment, 72 Operon 10-mer primers (Operon Technologies, Alameda, CA, USA) and 100 TIB 10-mer primers (TIBMOLBIOL, Berlin, Germany), were tested on three genotypes and 18 primers was selected for the next step in this study (Table 1). Polymerase chain reactions (25 µ) each contained 10 ng template DNA, 1× PCR buffer (CinnaGen, Tehran, Iran), 0.875 mM $MgCl_2$, 200 µM each of dNTPs, 0.2 µM each decamer primer, and 1 unit of *Taq* DNA polymerase (CinnaGen, Tehran, Iran). Amplification reactions were performed in termocycler (iCycler, Bio Rad, Hercules, CA, USA) programmed as follow: 94ºC for 4 min, followed by 35 cycle of 92ºC for 1 min, 37ºC for 1 min, 72ºC for 2 min and a final extension at 72º C for 5 min. Amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels in Tris-borate-EDTA (TBE) buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA. Na_2, pH=8.0), visualized by ethidium bromide staining and photographed under UV light with a Gel Doc system (UVP: Bio Doc, Upland, CA, USA). The Simple Matching Cofficient similarity matrix was calculated using numerical taxonomy and multivariate analysis system NTSYSpc Ver 2.11 (Rohlf 2004) and the dendrogram produced using the UPGMA. For each primer, Polymorphism Information Content (PIC) was measured (Farahmand et al. 2015).

3. RESULTS

3.1. First Experiment (Seed Germination)

3.1.1. Effects of Cold Stratification and Putrescine on Emergence

Table2. *Effects of cold stratification and putrescine on seedling emergence and growth parameters of Iron tree.*

Treatments	Leaf area (cm^2)	No of leaf	Stem length (cm)	Shoot dry mass (gr)	Root dry mass (gr)	Emergence (%)	Emergence rate (seedling per day)
Control (25 days cold stratification)	0.38 c	3.60 a	4.0 a	0.13 h	0.06 d	20 e	1.2 d
Cold stratification (25 days) + KNO_3 (25 mM)	2.45 b	4.80 a	5.38a	0.21 c	0.15 a	40 b	1.95 b
Cold stratification (25 days) + KNO_3 (50 mM)	3.54 b	5.0 a	5.50 a	0.24 b	0.11 c	35 c	1.7 c
Cold stratification (25 days) + KNO_3 (75 mM)	4.30 b	4.60 a	5.80 a	0.17 e	0.12 b	25 d	1.5 cd
Cold stratification (50 days) + KNO_3 (25 mM)	2.40 b	4.20 a	4.30 a	0.18 d	0.12 b	45 a	2.7 a
Cold stratification (50 days) + KNO_3 (50 mM)	2.33 b	3.40 a	4.80 a	0.14 g	0.11 c	40 b	1.95 b
Cold stratification (50 days) + KNO_3 (75 mM)	4.26 b	3.60 a	4.0 a	0.15 f	0.06 d	35 c	1.7 c
Cold stratification (75 days) + KNO_3 (25 mM)	3.90 b	6.60 a	7.30 a	0.18 d	0.06 d	20 e	0.95 e
Cold stratification (75 days) + KNO_3 (50 mM)	4.60 b	6.0 a	7.75 a	0.25 a	0.05 e	15f	0.70 f
Cold stratification (75 days) + KNO_3 (75 mM)	12.91a	5.50 a	6.45 a	0.13h	0.02 f	10 g	0.45 g

In each column, means with the similar letter (s) are not significantly different at 5% level of probability using Tukey's multiple range test.

The application of putrescine up to 10 mM increased emergence percentage and rate when it was used after 50 days stratification, so that, the highest emergence rate was obtained in combined treatment of 50 day stratification and 5 mM putrescine in compared to control. Seedling emergence percentage and rate were decreased when the seeds receiving 75 days stratification, treated with 10 and 15 mM putrescine (Table 2).

3.1.2. Effects of Cold Stratification and Putrescine on Seedling Growth Parameters

All treatments increased leaf area compared to control significantly, but no significant difference was found among the treatments. The number of leaf was unaffected by the interaction of cold stratification and putrescine and there was no difference among the treatments. Stem length was increased in all treatments except 75 days stratification and 15 mM putrescine. Shoot and root dry weight was increased at all concentrations of putrescine after 25 and 50 days of stratifications but these parameters were significantly decreased compared to control treatment, when putrescine (15 mM) was applied after 75 days of stratification. The highest shoot and root dry mass were obtained with 5 mM putrescine after 75 and 25 days of stratification (Table 2).

3.1.3. Effects of Cold Stratification and KNO3 on Emergence

All concentrations of KNO_3 increased emergence percentage and rate in comparison to control after 25 and 5 days of stratification but emergence percentage and rate were decreased when KNO_3 applied after 75 days of stratification. The highest seedling emergence was obtained with 50 days stratification and 25 mM KNO_3 (Table 3).

3.1.4. Effects of Cold Stratification and KNO3 on Seedling Growth Parameters

Table3. *Effects of cold stratification and KNO_3 on seedling emergence and growth parameters of Iron tree.*

Treatments	Leaf area (cm^2)	No of leaf	Stem length (cm)	Shoot dry mass (gr)	Root dry mass (gr)	Emergence (%)	Emergence rate (seedling per day)
Control (25 days cold stratification)	0.38 c	3.60 a	4.0 a	0.13 h	0.06 d	20 e	1.2 d
Cold stratification (25 days) + KNO_3 (25 mM)	2.45 b	4.80 a	5.38a	0.21 c	0.15 a	40 b	1.95 b
Cold stratification (25 days) + KNO_3 (50 mM)	3.54 b	5.0 a	5.50 a	0.24 b	0.11 c	35 c	1.7 c
Cold stratification (25 days) + KNO_3 (75 mM)	4.30 b	4.60 a	5.80 a	0.17 e	0.12 b	25 d	1.5 cd
Cold stratification (50 days) + KNO_3 (25 mM)	2.40 b	4.20 a	4.30 a	0.18 d	0.12 b	45 a	2.7 a
Cold stratification (50 days) + KNO_3 (50 mM)	2.33 b	3.40 a	4.80 a	0.14 g	0.11 c	40 b	1.95 b
Cold stratification (50 days) + KNO_3 (75 mM)	4.26 b	3.60 a	4.0 a	0.15 f	0.06 d	35 c	1.7 c
Cold stratification (75 days) + KNO_3 (25 mM)	3.90 b	6.60 a	7.30 a	0.18 d	0.06 d	20 e	0.95 e
Cold stratification (75 days) + KNO_3 (50 mM)	4.60 b	6.0 a	7.75 a	0.25 a	0.05 e	15f	0.70 f
Cold stratification (75 days) + KNO_3 (75 mM)	12.91a	5.50 a	6.45 a	0.13h	0.02 f	10 g	0.45 g

Interaction of cold stratification and KNO_3 had no significant effects on leaf number and stem length and there was no significant difference between treatments and control. Leaf area was significantly increased in all treatments in compression to control. The highest leaf area was gained with 75 days stratification and 75 mM KNO_3. In all cold stratification treatments with the exception of 75 days stratification and 75 mM KNO_3, shoot dry mass was increased compared to control. Root dry mass was increased when KNO_3 was used after 25 and 50 days of stratification but it was reduced when KNO_3 (50and 75mM) applied after 75 days stratification (Table 3).

3.1.5. Effects of Cold Stratification and BA on Emergence

Emergence percentage and rate were increased compared to control when BA was used after 50 days of stratification treatment. The highest emergence was observed with 2 mM BA. Seedling emergence was decreased when BA was applied after 75 days of stratification (Table 4).

3.1.6. Effects of Cold Stratification and BA on Seedling Growth Parameters

Table4. *Effects of cold stratification and BA on seedling emergence and growth parameters of Iron tree.*

Treatments	Leaf area (cm^2)	No of leaf	Stem length (cm)	Shoot dry mass (gr)	Root dry mas (gr)	Emergence (%)	Emergence rate (seedling per day)
Control (25 days cold stratification)	0.38f	3.60 a	4.0 a	0.13de	0.06 de	20 e	1.2 d
Cold stratification (25 days) + BA (1mM)	1.25e	4.0 a	5.0 a	0.11 e	0.09 d	22 d	1.1 d
Cold stratification (25 days) + BA (2 mM)	1.25 e	4.90 a	5.60 a	0.22 b	0.11 c	19 e	0.96 e
Cold stratification (25 days) + BA (3 mM)	2.68 d	3.50 a	4.23 a	0.33 a	0.23 a	9 g	0.45 j
Cold stratification (50 days) + BA (1mM)	4.37 b	2.25 a	3.60 a	0.09 f	0.02 e	29 b	1.45 b
Cold stratification (50 days) + BA (2 mM)	3.56 c	5.0 a	3.40 a	0.15 d	0.02 e	34 a	1.7 a

Cold stratification (50 days) + BA (3 mM)	2.28 d	4.0 a	3.54 a	0.18 c	0.12 b	24 c	1.5 c
Cold stratification (75 days) + BA (1mM)	4.0 bc	6.0 a	6.60 a	0.11 e	0.01f	14 f	0.7 f
Cold stratification (75 days) + BA (2 mM)	4.05 bc	6.0 a	6.60 a	0.11 e	0.01f	14 f	0.45 g
Cold stratification (75 days) + BA (3 mM)	5.70 a	8.0 a	9.0 a	0.25 c	0.02 e	9 g	0.45 g

In each column, means with the similar letter (s) are not significantly different at 5% level of probability using Tukey's multiple range test.

Leaf area was increased in all treatments by BA. Leaf number and stem length were not affected by BA treatment, and there was no significant difference compared to control.

The highest leaf area was observed in 75 days stratification and 3 mM BA. Shoot dry mass was increased at 3 mM BA in all stratification levels. Root dry weight was increased at 3 mM BA after 25 and 50 days stratification, but was not affected after 75 days stratification (Table 4).

3.1.7. Effects of Putrescine, KNO3 and BA on Emergence

Mean comparison of the solely effects of chemical treatments on emergence percentage and rate indicated that seedling emergence and rate were affected by kind of chemical material, so that the highest emergence percentage and rate were observed with KNO_3 treatment. There were not significantly difference between BA and putrescine related to seedling emergence (Table 5).

3.1.8. Effects of Putrescine, KNO3 And BA On Seedling Growth Parameters

Table5. *Comparison of KNO3, BA and putrescine tratments seedling emergence and growth parameters of Iron tree.*

Treatments	Leaf area (cm^2)	No of leaf	Stem length (cm)	Shoot dry mass (gr)	Root dry mass (gr)	Emergence (%)	Emergence rate (seedling per day)
Putrescine	3.26 b	4.65 b	5.03 b	0.19 a	0.14 a	22.70 b	1.30 b
KNO_3	2.73 b	3. 59 c	3.90 c	0.13 b	0.07 b	34 a	1.70 a
BA	4.82.a	6.57 a	7.43 a	0.03 b	0.03 c	13 b	0.67 c

In each column, means with the similar letter(s) are not significantly different at 5% level of probability using Tukey's multiple range test.

Effects of chemical treatments on measured parameters showed that there was no significant difference between chemical treatments for leaf number, stem length and root dry mass whereas the highest shoot dry mass was obtained with putrescine (Table 5).

3.1.9. Effects of Cold and Warm Stratification on Emergence

Table6. *Effects of warm and cold stratification on seedling emergence and growth parameters of Iron tree.*

Treatments	Leaf area (cm^2)	No of leaf	Stem length (cm)	Shoot dry mass (gr)	Root dry mass (gr)	Emergence (%)	Emergence rate (seedling per day)
Control (25 days cold stratification)	0.38 d	3.60 b	4.0 c	0.13 c	0.06 a	20 d	1.2 c
Warm stratification (42 days) + without Cold stratification	6.01 c	6.0 a	8.32 b	0.11 d	0.03 b	29 c	1.45 d
Warm stratification (42 days) + cold stratification (25 days)	11.01 b	6.60 a	10.0 ab	0.31 b	0.05 b	34 b	1.7 b
Warm stratification (42 days) + cold stratification (50 days)	16.34 a	7.60 a	11.90 a	0.34 a	0.06 a	44 a	2.2 a

In each column, means with the similar letter(s) are not significantly different at 5% level of probability using Tukey's multiple range tests.

Warm stratification increased seedling emergence in comparison to control. A synergistic effect was found combining warm and cold stratification treatments. The highest emergence percentage in the entire experiment was observed when seeds received 50 days of cold stratification subjected to 42 days of warm stratification (Table 6).

3.2. Second Experiment (Molecular Evaluation)

3.2.1. Discriminative Ability and Efficiency of Primers in Polymorphism

Table7. *The number of bands, percentage of polymorphism bands produced by used primers in iron tree genotypes.*

Row	Primer	Number of produced bands	Percentage of polymorphism *	RP	PIC
1	OPA-08	7	71.42	1.31	0.44
2	OPB-10	3	66.6	0.87	0.47
3	OPD-03	4	75.0	0.76	0.41
4	OPD-05	12	100	0.93	0.41
5	OPE-06	5	80.0	0.27	0.23
6	OPG-02	10	100	0.49	0.33
7	OPZ-10	8	100	0.86	0.45
8	OPA-09	10	100	0.83	0.48
9	OPAE-10	9	88.8	0.42	0.26
10	OPA-10	11	100	0.37	0.25
11	TIBMBB-12	8	87.5	0.48	0.33
12	TIBMBC-13	5	100	0.27	0.23
Total		92	-	7.33	
Mean		7.66	89.11	0.61	

**(b/a)×100*

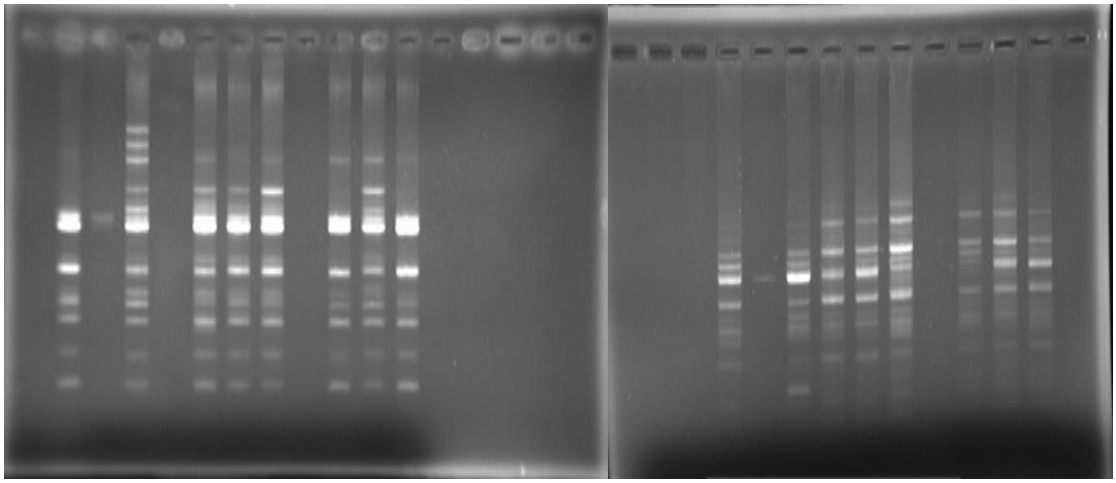

Figure1. *RAPD amplified with the arbitrary primer OPD-05 using DNAs of 18 iron tree genotypes.*

To investigate the amount of polymorphism among the genotypes of Persian parrotia, only the bands produced by 12 primers were included and the data of 6 primers were not used for analysis (Table 7). In general, 92 bands with average of 7.66 bands per primer were produced by the applied primers. Most of the primers (12 out of 18) exhibited polymorphism. The number of multiplied bands ranged from 3-12 among the 12 primers. The highest band number was produced by OPD-05 (12) and OPA-10 (11) primers (Fig 1). The overall resolving power of primers was 7.33 and 0.61 for each primer. The highest resolving power (0.93) among the used primers was related to OPD-05 primer. Based on the results, the highest polymorphic information content (PIC) 0.48, 0.47 and 0.45, were identified with OPA-09, OPB-10 and OPZ-10 primers, respectively. Thus, the polymorph bands produced in response to PCR were higher in these primers compared to others and these four primers indicated the genetic distance among Persian parrotia more efficiently.

3.2.2. The Amount of Similarity and Cluster Analysis Between the Studied Genotypes

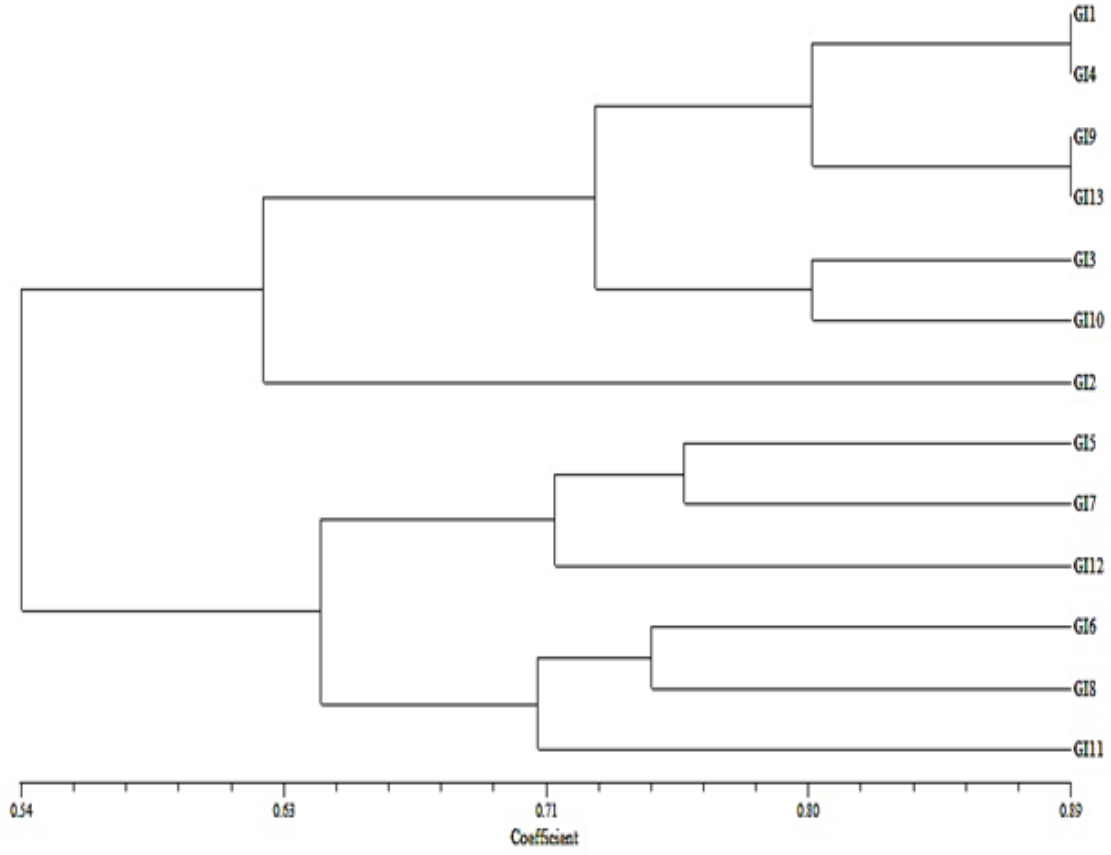

Figure2. *UPGMA dendrogram of 13 iron tree genotypes based on 12 random RAPD primers.*

Table8. *Similarity matrix between genotypes based on SMC similarity.*

Row	Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13
1	GI_1	1												
2	GI_2	0.65	1											
3	GI_3	0.76	0.47	1										
4	GI_4	0.89	0.71	0.65	1									
5	GI_5	0.57	0.51	0.70	0.68	1								
6	GI_6	0.33	0.31	0.53	0.40	0.62	1							
7	GI_7	0.48	0.42	0.64	0.55	0.76	0.73	1						
8	GI_8	0.34	0.30	0.52	0.39	0.59	0.75	0.72	1					
9	GI_9	0.89	0.63	0.78	0.80	0.66	0.44	0.53	0.39	1				
10	GI_{10}	0.78	0.56	0.80	0.69	0.64	0.53	0.64	0.54	0.67	1			
11	GI_{11}	0.48	0.35	0.68	0.48	0.62	0.71	0.67	0.70	0.53	0.68	1		
12	GI_{12}	0.64	0.51	0.72	0.68	0.73	0.63	0.69	0.51	0.75	0.59	0.56	1	
13	GI_{13}	0.80	0.63	0.73	0.71	0.57	0.53	0.51	0.41	0.89	0.71	0.64	0.64	1

Based on the results of similarity matrix, the highest similarity (0.98%) was found between GI4 with GI1 genotypes, GI9 and GI13 and between GI1 with GI9. The lowest similarity also was observed between GI8 and GI2 and GI6 and GI2 genotypes (Table 8). Cluster analysis gained from UPGMA divided the genotypes at 0.6 into two groups. The first group included GI1, GI4, GI9, GI13, GI3 and GI10 genotypes and the second group was formed with GI5, GI9, GI7, GI12, GH6, GI8 and GI11 genotypes. In the first group, GI1, GI4, GI9 and GI13 were not separated by the applied primers (Fig 2).

3.2.3. D-plot Analysis

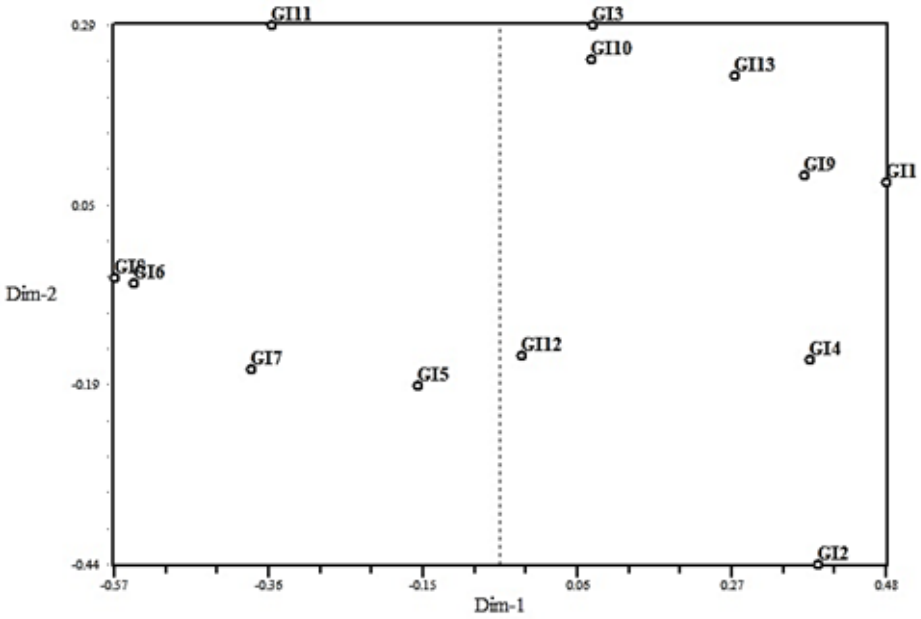

Figure3. *Two-dimensional model ofthe distribution of genotypes using the first two components factors of RAPD data.*

This way is used for exhibition of genotypes in bidimentional space based on the effective characters in the first and second factor. The accumulation of one point in plot indicates its genetic similarity. So, based on D- plot analysis, the genotypes which are close to each other in an rear have similar effective traits for factor 1 and factor 2 and are classified in one group. In this study, GI3, GI10, GI13 and GI9 genotypes have higher similarity considering the effective traits in factor 1 and 2 and formed one group. Meanwhile, considering the effective traits in factor 1 and 2, GI11 genotype was at the highest level (positive area) and GI7 genotype was the the lowest level and negative area (Fig 3).

4. DISCUSSION

Seed germination is a mechanism, in which morphological and physiological alterations lead to activation of the embryo. Seed dormancy, on the other hand, is a mechanism by which seeds can inhibit their germination to wait for more favorable conditions (Miransari and Smith 2014). Thus, dormancy and germination are complex phenomena that are controlled by a large number of genes, which are affected by both developmental and environmental factors (Kucera et al. 2005). There are various kinds of dormancy in which physiological dormancy may be decreased by cold or warm stratification or application of hormones (Takos 2001; Zhou et al. 2003; Garcia- Gusano et al. 2004; Rehman et al. 2000; Duan et al. 2004; Fang et al. 2006; El- Refaey, 2014). Based on the present results, combined cold and warm stratification is an efficient way to improve seedling emergence in *Persia parrotia*, as the highest EP was obtained after 42 days warm stratification followed by 50 days cold stratification. Cold and warm stratification had similar effects in species as *Taxus mairei* (Chien et al. 1998), *Stewaritia pseudocamellia* (Oleska and Struve 1999), *Rosa* taxa (Alp et al. 2009) and *Styrax japonicas* (Alp et al. 2009). Transverse cut of imbibed seeds of this species indicated that the seeds have a cartilage endosperm most probably having inhibitory compounds, delaying radicle emergence. During stratification, the cartilage endosperm which retards seed germination physically or chemically becomes softened enabling the roots to emerge easily. The existence of thick endosperm has previously reported by Baladan et al. (1995), Bevilaqua et al. (1998), Rascio et al. (1998), Farahmand (1999), and Farahmand and Khosh-Khui (2001) in *Cercis siliguastrum*. Our results showed that after 25 and 50 days of stratification, KNO_3 increased seed germination. KNO3 has been shown to promote the germination of species such as *Sorbus pohuashanesis*

(Bian et al. 2013) and *Plantago lanceolata* (Osman Sarihan et al. 2005). It is reported that polyamines are involved in the germination process (Matilla 1996). The findings of the present study indicated that putrescine at 5 and 10 mM with 25 and 50 days stratification, increased seedling emergence. This result agrees with Sedaghat and Rahemi (2011), considering the positive effect of putrescine on germination rate and seedling growth of pistachio (*Pistacia vera* L.). Polyamines were increased during seed germination in *Araucaria angustifolia* and *Ocotea odorofera* (Pierruzi et al. 2011). Thus, exogenous application of putrescine may mimic the same role in Persian ironwood.

The present study also indicated that warm stratification could be replaced by exogenously applied putrescine, an indication of possible polyamine increase during cold and warm stratification as previously reported by Matilla (1996). In this study, it was found that after 50 days stratification, emergence was increased by application of BA. The positive effect of BA in this rserch may directly be related to its mechanism of action or indirectly to it permissive effect on GA_3 (Kucera et al. 2005). Although this claim needs a thorough study tracing and measuring the concentrations of these compounds during stratification and germination. In interaction of chemical treatment with stratification, the highest stem length obtained with putrescine when applied after 75 days of stratification. Application of polyamins (Spd, Spm and Put) on pistachio increased both seedling emergence percentage and emergence rate and seedling growth (Sedaghat and Rahemi 2011). Foliar application of putrescine increased plant height, leaves, number of branches and fresh weight of eggplant (*Solanum melongena* L.) under sandy soil conditions (El-Tohamy et al. 2008). The interaction of effects chemical treatment and stratification showed that the highest shoot and root dry mass were obtained with 3 mM BA and 25 days of stratification. Chapurro et al. (1988) studied effects of BA on peach seeds germination and reported that the highest germination was obtained by 24 hr exposure to 50-100 mg L^{-1} of BA. Stratification at 5 °C for 20 days led to a marked increase in the cytokinins in *Acer saccharum* (Weed et al. 1973). BA increased plant weight of *Gladiolus* (Ram et al. 2002). BA is a cytokinin known to increase cell division and favor shoot formation. The improvement of shoot and root dry mass, may be the result of enhanced photosynthetic activity by BA. Cytokinens are plant regulators that stimulate cell division, photosynthesis and seed germination (Ram et al. 2002). Cytokinins are also important in the mobilization phenomena of plants and chlorophyll formation in plants (Hare and Van Staden 1997; Fletcher and Mc-Cullagh 1971). It has been proposed that the endosperm is a source of cytokinins needed for promotion of cell division in the embryo. After radicle protrusion, a cytokinin peak is associated with a- amylase accumulation (Kucera et al. 2005). Thus, it appears that BA may activate some genes and the resultant enzymes (such as a- amylase) would ultimately lead to accelerated seedling growth through the activation of related pathways. In general, it seems that morphological, structural and biochemical characters are combined to form a barrier against seed germination in endosperm and embryo of *P. persica*. As the transverse cut in the imbibed seed did not improve seed germination, it appears that the nature of dormancy in this species is affected by both seed reserves (endosperm) and embryo. In previous studies have been showed that the collection time of seeds related to humidity level of the seeds during maturation can be effect on seed dormancy (Hidayati et al. 2001). In regard to the high rainfall and relative humidity in the habitat of parrot tree in Iran, it seems that seed dormancy of parrot no affected humidity of seeds. *P. persica* occurs in the moist deciduous forest region south and south-west of the Caspian Sea. The species grows mainly on lying plains and mountain foothills. Its population decreases as the elevation increases. The optimal condition for its growth is in stations from 125-400m (Andrew 1997). In Northern parts of Iran, the annual precipitation is high (2000 mm in some years) in comparison with other parts of the country. So, it appears that this high rainfall which is combined with warm and cold days have an important role in breaking seed dormancy of this endemic species. So, the positive effect of warm and cold stratification treatments on seed germination and seedling growth is a kind of mimic naturally occurs in Persian parrotia habitat.

The assessment of genetic diversity within and between populations is routinely performed at the molecular level using various laboratory-base techniques such as allozyme or DNA analysis which measure level of variation directly (Mondini et al. 2009). In this research based on cluster analysis, the genotypes were classified into two groups. The researches on this species are scarce. Based on only two morphological studies by Yosefzade et al. (2010) and Sattarian et al. (2011), morphological variations

that were found in leaves of Persian parrotia genotypes, is attributed to elevation, temperature and average rainfall. It is previously concluded that the populations grown in lower latitudes with warmer climate, have lower leaf area. As previous reported (Yosefzadeh et al., 21010, Sattarian et al., 2011), the morphological characteristics of leaves are closely orrelated with climatic conditions. But it appears that the analysis of genetic diversity in *Parrotia persica* based merely on morphological markers is not persuasive. The present study indicated that RAPD markers are better means in this regard and can separate the genotypes of Persian ironwood without the influence of climatic conditions.

In the present research, RAPD markers could separate the genotypes of *Persia parrotia* which are in line with those reported on *Hammamelis* (Marquard et al. 1997). According to Marquard et al. (1997) RAPD markers reasonably discriminated the species with North American origin from the Asiatic species. Based on the present results, the highest resolwing power was found with OPD-05 primer and the highest polymorphism was obtained with OPD-05, OPA-10 and OPA-09 primers. Thus, these primers can possibly use for ongoing genetic variation studies in *Persian parrotia*. In this study, 92 bands with the average of 7.66 bands per primer was produced and most produced bands were polymorphic indicating high genetic variation in the studied genotypes. As *Persian parrotia* is a wind- pollinated species, variation is inevitably found in seed-derived genotypes. Meanwhile, this species is naturally grown in several habitats in north of Iran and it is possible that genetic balance has been occurred due to geographical factors. So, this variation needs a comprehensive program evaluating the variation between and among genotypes. Furthermore, *Parrotia persica* is clonally propagated by root suckers in nature. This species has reported as one of the key trees of Caspian-Hyrcanian forests of Iran (Heshmati, 2007). The Hyrcanian forests in Iran and Azerbaijan, together with the Colchic forests of Georgia, are the most important relics of the so-called Arcto- Tertiary forests in western Eurasia and an important biodiversity 'hot spot'. Many tree genera like *Pterocarya*, *Albizzia*, *Parrotia*, or *Gledetsia*, survived the last ice age only in this area (Scharnweber *et al*., 2007, Andrew, 2007). Because of the fact that most of the temperate deciduous forests in Europe and western Asia are converted into artificial plantations, secondary woodlands or agricultural and urban land, these remnants of primary forests have to be considered as of irreplaceable value (Scharnweber et al. 2007). On the other hand, *Parrotia persica* is a slow growing tree or small shrub and conservation of the present populations and gathering genetic database information for future purposes are of vital importance. Consequently, optimizing protocols for genetic assessment and seed germination to pave the ground for breeding and biotechnological goals, are very decisive.

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