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Effect of activated charcoal, growth supplements and storage on removing dormancy, germination indices and vigour of Ash (*Fraxinus excelsior* L.)

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ABSTRACT

In order to conquest of ash seed dormancy, we used seeds of this species that were collected from Haftkhal region, northern Iran, during 2005, 2007 and 2008 years by Forest Seed Center of Khazar (FSCK). Seeds were sterilized with mercuric colorid 1% for 20 min, and using 70% ethanol for 30 sec. Then half of seeds put in Gibberellic acid 500 mg/l density, the other half of seeds put in sterile distilled water for 24 hours. Then each group of seeds cultured on MS and enrich - MS medium with Ascorbic acid, Casein hydrolisat and yeast each 100 mg/l and 5 mg/l activated charcoal. In general for each year seeds 6 treatments, for each treatment 5 replication and in each replication 6 ash seeds have been cultured. Analysis of variance is showing that storage period had an influence on germination index in %5 confidence limit. Also for plantlet length, germination index and vigour after that period had a difference in %1 confidence limit. In this study the most desirable period for seed storage was 1 year, so after this period the germination index percentage (%14.815), plantlet length (2.766 mm) germination rate (2.601), germination index (0.756) and vigour (2.676) had increased. Also we observed meaningful difference on %1 confidence limit for effect of treatments for all those values, which in treatment number 6 with using Giberlic acid, growth supplement and activated charcoal showed an increasing in the germination percentage (%53.706), plantlet length (6.49 mm), germination rate (7.584), germination index (4.235) and vigour (8.351). In analyses variance any meaningful differences observed between seed storage and kind of treatments.

Keywords: Germination rate, MS medium, Gibberellic acid, Ascorbic acid.

INTRODUCTION

Common Ash as *Fraxinus excelsior* L. is one of 65 native species in moderate north hemisphere region and is an important species in Hyrecaian Forest and has economic and genetic importance. This species diffusion form plain to highest elevations and from west to east of Caspian seashore forests and tolerate semi-humid climate with moderate to very cold winter [31].

But it seems to due this climate and increasing in temperature, intensive logging and grazing in these forests make some difficulties for natural regeneration of Ash. there would be an overthrow for Ash, if this increasing in temperature continues [13]. Ash trees also are very sensitive to natural destruction of north of Iran forest ecosystems, because in other hand during last years in many regions, the seeds on the trees didn't reach to final evolution and/or direct grazing in this sites hamper the natural regeneration. In fact continuance this process probably led to overthrow of Ash in north of Iran [1]. Artificial seedling propagation is very important. However seeds of Ash have endodormacy with type of physiological dormancy (embryo dormancy) that finding a way to over come this dormancy is the first step to produce seedlings. Embryo dormancy involves some controls in the embryo that its growth requires 1-3 month chilling that in this period hydration and access air is possible [16]. Most woody species in moderate region have this type of dormancy due to germination doesn't occur in the warm weather condition in winter. The time of germination in spring depend on both chilling period and vigour [9].

Some researchers indicated the positive effect of plant hormones in removing dormancy and increasing in germination percentage. In this case researchers found that after initial ripen of dormant seeds of ash, they need a low temperature to germinate. First they found that Abscisic acid prevents the germination of non-dormant embryos that this state in admixture with GA_3 and kinetin can remove [29]. Some researchers studied the *Fraxinus excelsior* and dormancy in admixture with different medium with agar (MS medium with macronutrients, MS medium with micronutrients, Sucrose, and GA_3 for embryo culture). Differences in responses to different medium in initial phase represented more growth and in maturation phase showed low growth [35]. In this study the protocol for callus induction and regeneration in Boerhaavia diffusa has been developed in culture medium. Young apical leaves, nodal region and roots was used as explants for callus induction on MS medium containing 2-4- D and Kinetin. Callus initiation was first recorded in the lamina of leaf and nodal region. The optimum % of calli was obtained from leaf lamina. The calli in most of the cultures were yellowish white and friable in nature [14]. Some researchers also represented that seeds with 60 mg/l GA_3 likewise 80 mg/l 2, 4-D, had 80 percentages in germination [36]. Nodal explants excised from Eclipta alba L. plants maintained in vitro formed yellowish white, friable calluses after three weeks of culture on Murashige and Skoog (MS) medium supplemented with 10.75 µM α-napthaleneacetic acid (NAA) and 9.04 µM 2,4-dichlorophenoxyacetic acid (2,4-D). These calluses were subsequently transferred to MS basal medium where after an additional four weeks of culture, approximately 50% of the calluses could form somatic embryos [7].

Some researchers found that scarification with GA_3 had the most effect on seed germination [28]. A study with in-vitro breaking seed embryo dormancy and without growth regulator, principle, found that 6-BA and GA_3 had an effect not only in breaking seed dormancy, but also in increasing germination rate [5]. also pointed that Hydrogen cyanide and peroxide Hydrogen have appositive effect on removing embryo dormancy and germination [21].

Growth supplements used to removing dormancy and increasing embryo growth for in-vitro culture. In this case to remove yew embryo dormancy used enrich-MS medium with casein hydrolisat, ascorbic acid and yeast, each 100 mg/l and 5 mg/l activated charcoal and succeed to grow them [10, 19]. A study succeed in somatic embryo genesis using mature seed's core as explants in MS medium with 100 mg/l casein hydrolisat, 100 mg/l Ascorbic acid and 0.5 - 4 mg/l bansilaminaporin [20]. Activated charcoal is one of effective material in increasing germination. They material due to its porosity are available to imbibe growth inhibitor and toxic compounds. Activated charcoal often use in tissue culture for growth and cell propagation and have an vital role in micro propagation, seed germination, somatic embryogenesis, protoplast culture, increase

in stem length, rooting, etc. and gradual produces some of material inclusive nutrients and growth regulator that are necessary for plants. Some researchers found that activated charcoal has a positive effect on germination. Furthermore, using 2gr/l activated charcoal in culture medium produce larger plantlet (1.6 = 0.1 mm diameter) [8, 32].

Some studies, emphasis the seed storage period effect on percentage, and germination, and germination rate and seed vigour. A study on stored European larix (*larix decidua* Mill.) in 4^{o}c temperature and during 3 - 24 years showed that there is a negative correspond between storage period and germination percentage and a positive correspond between storage period and germination rate (the necessity days to reach 15% germination) [6]. In addition, obtained these results that fresh collected beech seeds with high vigour, represented the lowest germination, but in stored seeds there was slight differences between vigour and germination percentage [23]. In this case a study indicate that with increasing in storage period, seeds vigour decrease, as seeds germination percentage that were collected in 1990, was higher than seeds collected in 1995, 1998 and 2000 years [18]. Also a study represented that keeping seeds in silica gel for 12 month and in 25^{o}c temperature cause increase in germination and seed vigour [25]. The present study is an attempt to determine the effect of activated charcoal, growth supplements and storage on removing dormancy, germination indices and vigour of Ash (*Fraxinus excelsior* L.).

MATERIALS AND METHODS

Seeds and site characteristic

In this study we took seeds at Haftkhal site from Forest Seed Center of Khazar (FSCK). The used seeds inclusive fresh collected and 1 and 3 years storage seeds in this center store in 2 - 4˚ C temperature that their viability with tetrazolium test that determined in forest seed center was 81, 72 and 64 percent respectively. All seeds collected in late September month. Haftkhal site place between 530˚, 29', 8" and 530˚, 36', 36" longitude and 360˚, 19', 43" and 360˚, 22', 16" latitude. In this site the forest type is beech with Ilex, Hornbeam, and individual trees like Ash, Oak, Lim tree, Maple and individual Ash trees in this site have a goal quality and produce seeds with high vigour. The height from sea is 1800 m, the bedrock type is calcic and soil type is randzin, the site climate basis on Amberge climatograph is cold moderate [2].

Research Methods

To sterilize seeds first washed them with teepol and then after washing with distilled water placed them in %1 Mercuric chloride ($HgCl_2$) for 20 min. Then washed it with sterile distilled water and lied in %70 ethanol for 30 sec. and next washed with sterile distilled water [10]. After wards half of seeds placed in sterile distilled water and another half placed in sterile GA_3 solution with 500 mg/l density. In general 6 treatments for spot each collected year's seeds and to name the treatment easily, we assign a number for each treatment. We use this number instead of the treatments name. Before implement these treatments, 6 similar treatments as initial tests had used, with this different that we placed seeds without excision in their coat on medium that due to their failure, we don't represent them in results.

1. Soaking seeds in sterile distilled water for 24 hours then excision on seed coat in seeds border and culture on Ms-Medium.
2. Soaking seeds in GA_3 with 500 mg/l density for 24 hours then an excision on seed coat in seeds border and culture on Ms-Medium.
3. Soaking seeds in sterile distilled water for 24 hours then an excision on seeds coat and culture on Ms-Medium with casein hydrolisat, ascorbic acid and yeast each 100 mg/l.

4. Soaking seeds in 500 mg/l for 24 hours then an excision on seeds coat in seeds border and culture on Ms-Medium with casein hydrolisat, ascorbic acid and yeast each 100 mg/l.
5. soaking seeds in sterile distilled water then on excision on seeds coat in seeds border and culture on Ms-Medium with casein hydrolisat, ascorbic acid and yeast each 100 mg/l and 5 gr/l activated charcoal.
6. Soaking seeds in 500 mg/l GA_3 then an excision on seeds coat in seeds border and culture on Ms-Medium with casein hydrolisat, ascorbic acid and yeast each 100 mg/l and 5 mg/l activated charcoal.

To disconnect the relation between seeds and any kind of external pollution, the vessels cover with nylon, after 24 hours seeds under Laminar Floo Hood, emitted from the liquid and then with using sterile scalpel and pans we created an excision on seeds border then cultured on the medium. To sterilize medium, we put 30ml of medium in 250 ml vessels with cap, then this vessels sterilize in autoclave in 121˚ C for 20 min. All of culture use in 5 replication and 6 seeds in each replication.

Measuring seed germination's sample Methods

In all treatments and in each replication, number of growth seeds and their plantlets length measured at day 7 and 14 after culture. The measured index inclusive germination percentage, germination rate, germination index and seed vigour, that their values obtained from follow equations.

(1) Germination percentage: $GP = \frac{n_i}{N} \times 100$

Where GP is germination percentage; n_i number of germinated seeds, and N total number of seeds [22].

(2) Germination rate: $GS = \sum_{i=1}^{n} (\frac{n}{t})$

Where GS is germination rate; n percentage of the seeds that germinate in a specific time, and t is the time from culture [22].

(3) Germination index: $GI = \frac{(\sum T_i N_i)}{S}$

Where GI is germination index; Ti the days after culture; N number of seed germinate, in day i and s total number of planted seeds [3]. In the perusing the germination index must notice that the low value of this index represents the short time of germination.

(4) Seeds vigour index = [Average length of plantlet (mm) × Germination n percentage]/100 [3].

Statistical analysis

Normality of the variables was checked by Kolmogrov - Smirnov test and Levene test was used to examine the equality of the variances. Afterward to analyze data we used factorial T - test with 2 factors include storage period in store (in 3 levels) and used treatments (in 6 levels) in completely random from plan and for group comparison multi-range averages we used Duncan method. All of analyzes implemented in SPSS 17 software.

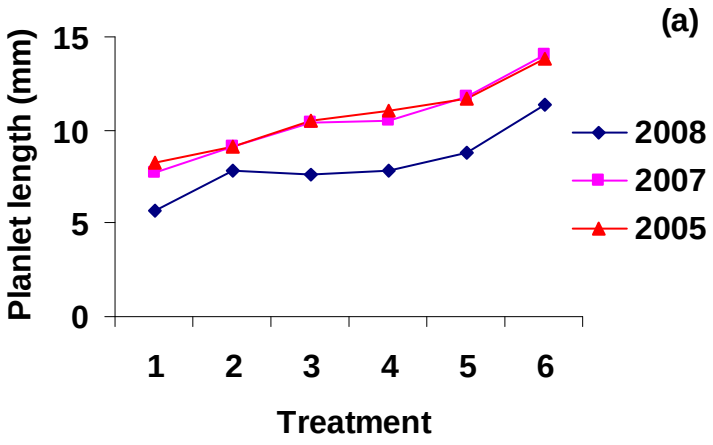
RESULTS

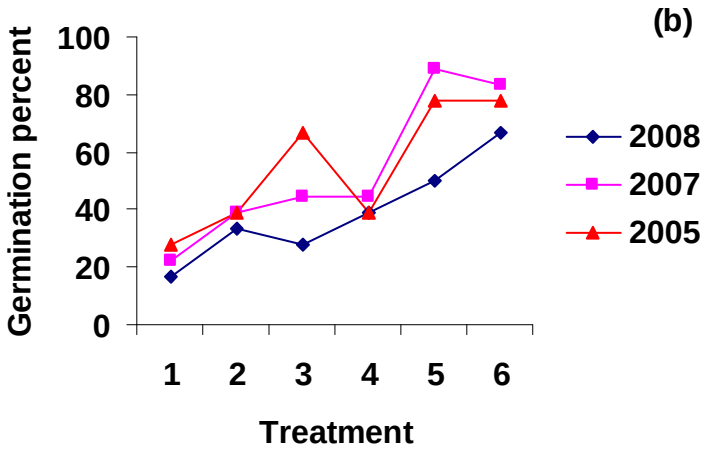
Results from variance analyze showed that there is meaning full difference in %99 confidence limit for seed storage period (2005, 2007, 2008) effect on plantlet length, germination rate, seed vigour index and germination index and for germination percentage in %95 confidence limit. As always highest values for plantlet length, germination percentage, germination index and seed vigour relevant to seeds of year 2005 (10.705, 54.627, 6.221, 6.155, respectively) and lowest values for plantlet length, germination percentage, germination index and seed vigour relevant to seeds of year 2008 (7.846, 38.885, 4.001, 4.034, 3.443, respectively). Just germination rate index represents the highest value (6.612) for seeds of year 2007 (Table 1 and Figure 1).

Table 1: Average (±standard error) effect of seed storage period in store on studied characters

Years	Plantlet's length	Germination percentage	Germination rate	Germination index	Seedy vigour
2008	7.846 ± 0.616b	38.885 ± 5.042b	4.011 ± 0.570c	4.039 ± 0.485b	3.443 ± 0.583b
2007	10.612 ± 0.497a	53.700 ± 7.200a	6.612 ± 0.885a	4.795 ± 0.491b	6.119 ± 1.007a
2005	10.705 ± 0.495a	54.627 ± 6.292a	5.355 ± 0.742b	6.221 ± 0.597a	6.155 ± 0.885a

The small words shows the group differences in germination percentage index in %5 probability level and other item in %1 probability level for different years of seed storage.





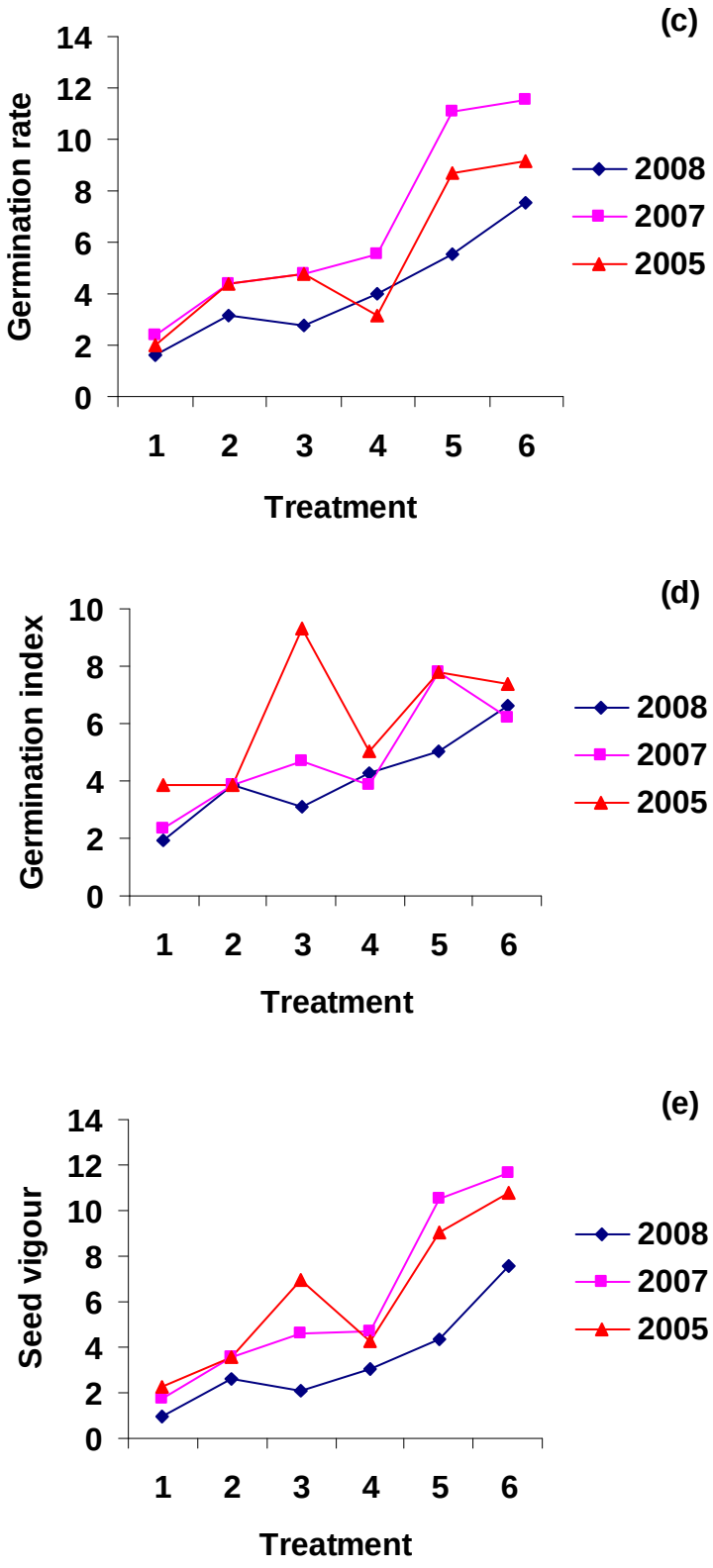


Figure 1: effect of seed storage period on plantlet length (a), germination percentage (b), germination rate (c), germination index (d) and seed vigour (e) in 14 days after culture.

With regard to the variance analyze, we observed that there is significance in %99 confidence limit for treatment effect (number 1 - 6) on plantlet length, Germination percentage, germination rate, germination index and seeds vigour, as the highest values obtained for treatment number six

(13.08, 75.923, 9.39, 6.74, 9.998, respectively) and lowest values for treatment number one (6.611, 22.217, 1.806, 2.505, 1.647, respectively) (Table 2 and Figure 1).

Table 2: average (± standard error) effect of treatment 1-6 on studied characters

Treatment number	Plantlet's length	Germination percentage	Germination rate	Germination index	Seedy vigour
1	6.611 ± 0.893 ^{e}	22.217 ± 3.928 ^{c}	1.806 ± 0.350 ^{c}	2.505 ± 0.486 ^{d}	1.647 ± 0.303 ^{c}
2	8.740 ± 0.272 ^{d}	37.034 ± 3.704 ^{bc}	3.967 ± 0.543 ^{b}	3.888 ± 0.341 ^{c}	3.239 ± 0.350 ^{bc}
3	9.255 ± 0.638 ^{cd}	46.292 ± 9.930 ^{b}	4.099 ± 0.460 ^{b}	5.703 ± 1.094 ^{ab}	4.573 ± 1.153 ^{b}
4	9.924 ± 0.581 ^{bc}	40.737 ± 4.900 ^{bc}	4.231 ± 0.593 ^{b}	4.406 ± 0.386 ^{bc}	3.998 ± 0.514 ^{b}
5	10.716 ± 0.510 ^{b}	72.220 ± 0.833 ^{a}	8.463 ± 0.968 ^{a}	6.869 ± 0.416 ^{a}	7.979 ± 1.169 ^{a}
6	13.080 ± 0.503 ^{a}	75.923 ± 6.280 ^{a}	9.390 ± 0.751 ^{a}	6.740 ± 0.656 ^{a}	9.998 ± 0.997 ^{a}

Small words represent the group differences in %5 probability level for treatments 1- 6.

Therefore GA_3, ascorbic acid, casein hydrolisat, yeast and activated charcoal with their specific density in treatment number 6 the highest effect on removing dormancy and increase in percentage and rate of germination and seed vigour. The results from polar effect of seed storage and type of treatment on plantlet length, germination percentage, germination rate, germination index and seed vigour, index represent that there is no significance for them. The plantlet form seed cultures in during years 2007 and 2008 presented in figures 2 and 3.

Figure 2: Plantlet from seed culture of year 2007 in treatment 6.

Figure 3: Plantlet from seed culture of year 2008 in treatment number 6.

DISCUSSION AND CONCLUSION

When a seed separates from a plant, has a little primary dormancy that not only prevents the quick seed germination, but adjusts time conditions and place of germination. Ash step is to remove its dormancy to propagate this worth species. Results of this study indicate that breaking ash seeds dormancy in - vitro is possible. One way to overcome this dormancy is using special

Hormones like GA_3 and medium supplements. Gibberellins inclusive a group of hormones that have the most usage of them in research and commerce is Gibberellic acid (GA_3) [16]. Many researchers aim that using GA_3 is suitable for breaking dormancy. In this case some researchers reported that GA_3 cause removing dormancy and increasing in germination value [17, 36, 28].

Results of this research represented that in treatment 1 - 6 which we made on excision on seed border, a direct relation between embryo and medium cause success in seed germination. Therefore presence of inhibitor compounds in seeds endosperm can be a reason for miscarriage in initial tests that seeds without an excision was cultured on Medium, and this means that Ash seeds hare chemical dormancy. Some researchers reported that mechanical stratification of endocarp cause breaking dormancy and increasing germination power [12, 27, 33]. Also removing seeds coat cause increasing germination percentage, germination rate and seed vigour [4, 24, 26].

Analysis of Variance showed the plantlet length and measured indexes value in culture treatments on MS medium that this values in presence of GA_3 is Slights more than those treatments without GA_3, Results from culture treatments in enrich – MS medium with supplements and activated charcoal, indicated the importance of this materials in increasing this values than GA_3. Also with comparison between results of supplemented and activated charcoal we can find that the positive effect of activated charcoal is higher than supplements. Investigation about positive effect of presence of activated charcoal in Medium represents the vital role of this material in increasing in seed germination and elongation of stem length. Basically positive effect of activated charcoal on morphogenesis may due to one side surface imbibe of inhibitor compounds in medium and in fact minimizing toxic metabolism and phenolic compounds exude [32].

Thus Amino acids and casein hydrolisat phosphate provide nutrients for embryo and increase embryo growth rate [15]. In other hand, the role of ascorbic acid in adjusting main peroxides of germinating embryos tip activity (ferrolit acid and giacoal) is significant as motivate cell, propagation in embryos tip [30]. In present study the positive effect of casein hydrolisat and ascorbic acid in plantlet length increase has been reported. Some researchers to overcome seed embryo dormancy used enrich – MS medium with casein hydrolisat, ascorbic acid and yeast each with 100 mg/l density and reported similar results [10, 19].

Results about seed storage period in this study represent the positive effect on plantlet growth and measured indexes values than fresh collected seeds. Also regard to results about stored seeds for 1 and 3 years had no significance differences, we can derive that the best period for storing seeds is 1 year. In this case some researchers found that storing seeds cause increasing in germination percentage and seeds vigour [6, 18, 23, 25, 34]. Finally it's necessary to expression that with regard to the high importance of ash in North Iran forests and some researcher's advice about threating of its progency in this forest [1, 13]. We should attend precision conservation of sites of destruction and grazing and also produce its seedling for planting in forests.

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