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Effect of Various Pretreatments on Germination of Turkish Endemic *Achillea gypsicola* Hub.-Mor. Species under *In Vivo* and *In Vitro* Conditions

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ABSTRACT: There has been an increasing interest to overcome seed dormancy in medicinal and aromatic plants using various pretreatments. This study was carried out to determine effect of several pretreatments on germination of Turkish endemic *Achillea gypsicola* Hub.-Mor. species under *in vivo* and *in vitro* conditions. The seeds were subjected to three pretreatments as various concentrations of GA_3 and KNO_3 and 91day cold stratification, with a seven-day interval. *In vivo* germination test produced no germination in the seeds pre-treated with cold stratification and various concentrations of GA_3, whereas KNO_3 treatments both *in vivo* and *in vitro* explicitly increased germination percentage. Cold stratification showed no inciting effect on germination both *in vitro* and *in vivo* conditions. *In vitro* combined application of KNO_3 and GA_3 produced higher germination percentages than single applications. The stimulating effect of KNO_3 and GA_3 on germination percentage increased as the duration of seed immersing in KNO_3 increased. The seeds hold in 200 µM KNO_3 for 48 hours and planted on MS medium supplemented with 2 mg L^{-1} GA_3 showed 100% germination. In conclusion, this study suggested that combined pre-treatment of KNO_3 with GA_3 had a great potential in breaking seed dormancy and increasing germination of Turkish endemic *Achillea gypsicola* Hub.-Mor. species.
Keywords: Cold stratification, dormancy, gibberellic acid, potassium nitrate, seed

In Vivo ve *In Vitro* Koşullar Altında Çeşitli Ön Uygulamaların Türkiye Endemiği *Achillea gypsicola Hub.-Mor.* Türünün Çimlenmesi Üzerine Etkisi

ÖZET: Tıbbi ve aromatik bitkilerde tohum dormansisinin üstesinden gelmek için çeşitli ön uygulamalara karşı artan bir ilgi vardır. Bu çalışma, çeşitli ön uygulamaların Türkiye endemiği *Achillea gypsicola* Hub.-Mor. türünün *in vivo* ve *in vitro* koşullarda çimlenmesi üzerine etkisini belirlemek amacıyla yapılmıştır. Tohumlar, GA_3 ve KNO_3'ün çeşitli konsantrasyonları ve yedi günlük aralıklarla 91 günlük soğukta katlama gibi üç ön muameleye tabi tutulmuştur. *In vivo* çimlenme testinde, soğukta katlama ve çeşitli GA_3 dozları ile ön muamele edilmiş tohumlarda çimlenme görülmezken, hem *in vivo* hem de *in vitro* koşullarda KNO_3 uygulamaları çimlenme yüzdesini arttırmıştır. Soğuk katlama, hem *in vitro* hem de *in vivo* koşullarda çimlenme üzerinde hiçbir teşvik edici etki göstermemiştir. KNO_3 ve GA_3'ün *in vitro* koşullarda kombine uygulanması, tek başlarına uygulamalarından daha yüksek çimlenme yüzdeleri üretmiştir. Tohumların KNO_3'te bekletme süresi arttıkça KNO_3 ve GA_3'ün çimlenme yüzdesi üzerindeki uyarıcı etkisi artmıştır. 48 saat boyunca 200 µM $KN0_3$ çözeltisinde bekletilen ve 2 mg L^{-1} GA_3 ile takviye edilmiş MS ortamına ekilen tohumlarda, %100 çimlenme görülmüştür. Sonuç olarak, bu çalışma KNO_3'ün GA_3 ile birleştirilmiş ön uygulamasının tohum dormansisini kırmada ve endemik *Achillea gypsicola* Hub.-Mor. türünün çimlenmesini artırmada büyük bir potansiyele sahip olduğunu göstermiştir.
Anahtar Kelimeler: Soğuk katlama, dormansi, giberellik asit, potasyum nitrat, tohum

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INTRODUCTION

Turkey, located at the convergence point of three floristic regions, is known as one of the most biologically diverse countries in the temperate zone. Its plant diversity is manifested by the presence of over 12 000 plant species, around 30% of which are endemic (Uludag et al., 2017). Medicinal and aromatic plants belonging to Lamiaceae, Apiaceae and Asteraceae, with high rate of endemism, represent an important component of this biodiversity endowment of the country. The potential benefits of medicinal and aromatic plants are largely accounted for naturally occurring bioactive compounds, called secondary metabolites. The genus of *Achillea* as known "civanperçemi" in Turkish, a member of Asteraceae family, is represented by 59 species in Turkey, 31 of which are endemic (Tabanca et al., 2016; Demirci et al., 2018). The genus of *Achillea* has been proven to have an important secondary metabolite; the terpene camphor which has long been used in medicine and cosmetics industry due to its antimicrobial and anti-carcinogenic properties (Abdel-Rahman et al., 2015; Almadiy et al., 2016). Some previous studies have revealed that camphor content showed a wide range of variation in the species of *Achillea* genus, changing between 0.6% and 32.7% (Sampietro et al., 2016; Ghasemi, 2017). The main source of camphor is the species of *Cinnamomum camphora* Sieb. known as camphor tree and growing wild in the Far East countries, contains around 68% camphor compound (Frizzo et al., 2000; Zuccarini, 2009). *Achillea gypsicola* Hub.-Mor., an endemic yarrow species of Turkey, has 40% camphor content, although it has an herbaceous growth habit (Baser, 2016). With its relatively short growing period, it is apparent from these results that, *Achillea gypsicola* is a very good source in terms of camphor in the plants kingdom including woody plants. Within their native environments, endemic plants have been exposed to several serious threat and, in consequence of this, the production of certain secondary metabolites with sufficient amount and quality form the plants grown in wild are inhibited (Paunescu, 2009). Furthermore, growing medicinal and aromatic plants with conventional techniques and producing secondary metabolites are of time consuming and rather costly. Plant cell cultures are of considerable importance and commonly used in production of these valuable phytochemicals of certain quality standards, without seasonal limitations (Bourgaud et al., 2001; Abdin et al., 2007). The first milestone in plant cell cultures providing rapid, easy and mass production of valuable secondary metabolites is to overcome seed dormancy and obtain a good level of germination *in vitro*. It has been a well-known fact that the seeds of certain medicinal and aromatic plant species have some degree of dormancy, easily germinating within their native environments, but failing to show good germination under laboratory conditions (Gupta, 2003; Zare et al., 2011). The need, thus, is apparent to develop some methods breaking dormancy in seeds and promoting germination, particularly *in vitro* condition. Foremost among these applications are such processes as subjecting seeds to cold stratification and keeping them in hormones and osmotic solutions (Bhardwaj et al., 2016). In the scientific literature, there has been no information concerning the potential seed dormancy problems and a study presenting the effect of seed pretreatments on germination of *Achillea gypsicola* species. Furthermore, a study we carried out with *Achillea gypsicola* indicated that the seeds exhibited some degree of dormancy and the present study will be the first attempt to overcome seed dormancy in this species (Acikgoz, 2017). In view of these points, this study was aimed to determine the effect of some pretreatments such as immersing seeds in gibberellic acid and potassium nitrate solutions and cold stratification on germination of Turkish endemic *Achillea gypsicola* Hub.-Mor. species both *in vivo* and *in vitro* conditions.

MATERIALS AND METHODS

The seeds of Turkish endemic *Achillea gypsicola* species collected from its natural habitat were used in the study. *Achillea gypsicola* plants were detected on the right-side hills at the 26th and 47th kilometers of Corum-Iskilip (latitude 40°73′N, longitude 34°47′E) road and the plant samples of both above- and below-ground organs were taken for species diagnosis on June 7, 2017. A herbarium of the plant samples was kept at normal room temperatures until species diagnosis. Species diagnosis was carried out by Assist. Prof. Dr. Sevda TÜRKİŞ, a member of the Science Education Department, Education Faculty of Ordu University. Voucher plant specimens were kept in the herbarium at Field Crops Department of Agricultural Faculty, Ordu University. The seeds were collected from the representing samples of the whole plants found at the region on August 13, 2017 and properly cleaned seeds were kept in jars with cork stoppers until they were subjected to germination tests.

Seed surface sterilization

The seeds were immersed at first in 70% alcohol for 10-15 sec for pre-sterilization and then rinsed with distilled water. Further, the seeds were surface sterilized by shaking in 25% sodium hypo-chloride (13% NaOCl) for 45 minutes and rinsed with distilled water.

***In vivo* germination tests**

In this part of germination test, the seeds were subjected to three pretreatments as;

a) Gibberellic acid (GA_3): The seeds were kept in solutions containing various GA_3 concentrations (25, 50, 75, 100, 200, 400 and 800 ppm) for 24 hours, along with the control (water).
b) Cold stratification: The seeds were exposed to 13 cold stratifications with seven days interval in a period of 91 days.
c) Potassium nitrate (KNO_3): The seeds were soaked in solutions containing 100 and 200 µM KNO_3 for several hours (12, 24, 36 and 48 hours), along with the control (water).

The germination tests were carried out with eight replications. Fifty seeds were placed in two layers of filter paper in petri dishes of 18 cm and the petri dishes were stored at 16/8 hours light/dark conditions at 25 ºC for 14 days.

***In vitro* germination tests**

The germination tests were repeated 6 times with petri dishes containing eight seeds each. The pH of the culture media of MS (Murashige and Skoog, 1962) was adjusted to 5.8. The petri dishes were stored at 16/8 hours light/dark conditions at 25 ºC. A number of *in vitro* germination tests were carried out using GA_3, KNO_3 and cold stratification as seed pretreatments:

a) GA_3 application to MS media; the seeds were planted in MS media including various concentrations (0, 0.1, 0.5, 1, 2, 3, 5, 7, 9, 11 and 15 mg L^{-1}) of GA_3.
b) Immersing seeds in GA_3; the seeds were planted in MS media with several concentrations (0, 0.5, 1 and 2 mg L^{-1}) of GA_3, after keeping them in solutions containing various levels (0, 25, 50, 75, 100, 200, 400 and 800 ppm) of GA_3 for 24 hours.
c) Cold stratifications; the seeds exposed to 13 cold stratifications at 2 ºC, in a period of 91 days with a seven-day interval, were planted in MS media having different concentrations (0, 0.5, 1 and 2 mg L^{-1}) of GA_3.
d) Potassium nitrate (KNO_3) and gibberellic acid (GA_3) applications; the seeds were planted in MS media with four concentrations (0, 0.5, 1 and 2 mg L^{-1}) of GA_3 after soaking in KNO_3 solutions (100 and 200 µM) and in water (the control) for 12, 24, 36 and 48 hours.

The seeds, both *in vivo* and *in vitro*, with 2 mm of radicle were considered as germinated. Germination percentage was calculated as the ratio of the number of daily germinated seeds to the total number of seeds tested. Germination percentages were arcsine transformed and then data were subjected to an ANOVA. The assumptions of data normality and homogeneity

of variance were tested with the Kolmogorov-Smirnov test and the Levene's test (Steel and Torrie, 1980). Means compared with Tukey's HSD test and all calculations were performed with Minitab 17 statistical software.

RESULTS AND DISCUSSION

In vivo germination tests

In vivo germination tests revealed that no germination occurred with gibberellic acid (GA_3) and cold stratification, but *in vivo* potassium nitrate applications stimulated seed germination explicitly (Figure 1). Increasing concentrations of KNO_3 resulted in a significant increase in germination percentage, with no germination in the control solution (water). The interaction effect of KNO_3 concentrations x immersing hours was found to be non-significant, that is, the stimulating effect of potassium nitrate concentrations on germination increased depending on immersing hours of the seeds in KNO_3 solution. The maximum germination percentages recorded in the seeds kept in 200 µM KNO_3 for 12, 24, 36 and 48 hours were found to be 10%, 12%, 27% and 33%, respectively. These results suggest that the stimulating effect of KNO_3 concentrations on germination percentage increases as the duration of seed immersing increases. The previous studies also indicated that KNO_3 applications played an effective role in breaking seed dormancy and increasing germination (Fariman et al., 2011; Gupta et al., 2011; Gashi et al., 2012).

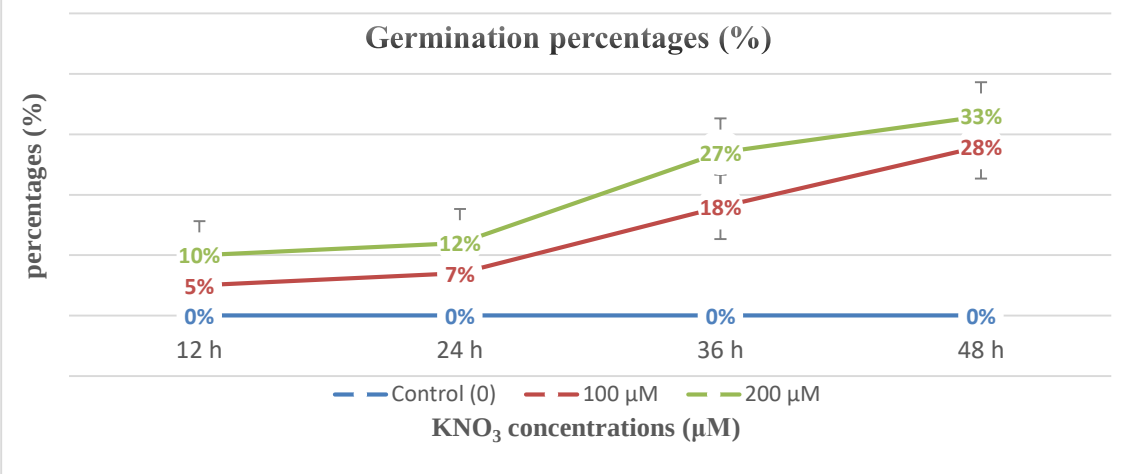

Figure 1. *In vivo* germination percentages (%) of the seeds kept in KNO_3 solutions of different concentrations for different hours

In vitro germination tests

In the first *in vitro* germination test, adding increasing concentrations of GA_3 to MS media resulted in a significant increase in germination percentage, as compared to the control. Germination percentages obtained from GA_3 treatments ranged from 14% to 19%, with no germination in the control treatment (Figure 2). With the first three GA_3 concentrations, germination percentage linearly increased from 17% to 19%, but decreased to 14% with additional concentrations.

The second *in vitro* germination test was consisted of two stages; at first, the seeds were kept in GA_3 solutions of several concentrations for 24 hours and then they were planted in MS media containing three levels of GA_3 solution, along with the control. GA_3 application of increasing levels to MS media produced a significant and regular increase in germination percentage, with the highest obtained in media containing the highest (2 mg/l) GA_3 level (Figure 3). Immersing seeds in increasing concentrations of GA_3 up to 200 ppm, before planting to MS

media, resulted in a constant increase in germination percentage up to 27%, with almost no changes in additional GA_3 concentrations. The germination percentage increased as GA_3 concentrations added to MS medium increased, irrespective of GA_3 concentration of immersing solution, suggesting that two-way interaction of GA_3 concentrations MS media and immersing solution.

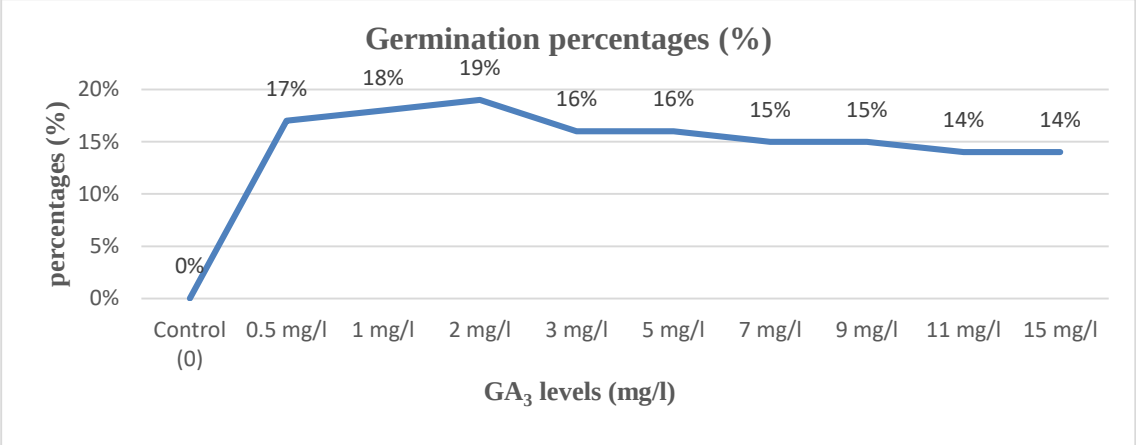

Figure 2. Germination percentages (%) of the seeds planted in MS media with various levels of GA_3

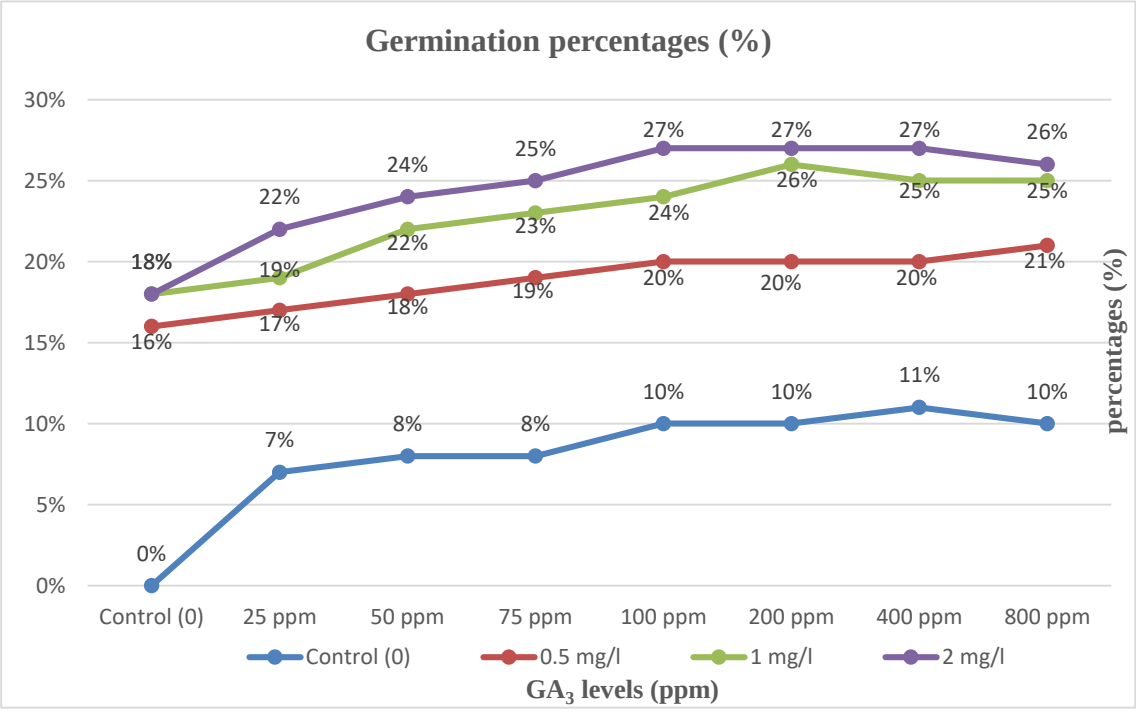

Figure 3. Germination percentages (%) of the seeds planted in MS media with several GA_3 levels, after keeping in GA_3 solutions of various concentrations for 24 hours

Several studies reported that certain *in vitro* pre-treatments such as soaking seeds in GA_3 solution for some times produced good results in breaking primer seed dormancy and reducing the time taken for germination (Warakagoda and Subasinghe, 2014; Kadi et al., 2015; Elhindi et al., 2016). It was recorded that, dipping the seeds of *C. fenestratum* in 2250 mg L^{-1} GA_3 solution for

24 h reduced the time taken for germination by removing inhibitory chemicals, facilitating embryo growth and reducing inherent ABA/GA_3 ratio (Warakagoda and Subasinghe, 2014). There was no germination in the seeds exposed to 13 cold stratifications at 2 ºC, in a period of 91 days with a seven-day interval, before planting to MS media including several concentrations (0, 0.5, 1 and 2 mg L^{-1}) of GA_3. On the other hand, combined application of potassium nitrate and gibberellic acid significantly enhanced seed germination, ranging from 2.08 to 100% (Table 1). In combined application, the seeds were dipped in KNO_3 solutions of 100 and 200 µM and in water (the control) for 12, 24, 36 and 48 hours before planting in MS media with 0.5, 1 and 2 mg L^{-1} concentrations of GA_3, and the control. The germination percentages of the seeds steadily increased in accordance with the concentration of KNO_3 solution, incubation time in KNO_3 solution and GA_3 concentrations added to the growth medium. Three-way interaction of KNO_3 and GA_3 concentrations and immersing hours was found to be significant, suggesting that the effect of addition of increasing concentrations of GA_3 to MS media varied depending on KNO_3 concentrations and immersing hours of the seeds in KNO_3 solutions, and/or vice versa.

Table 1. Germination percentages* (%) of the seeds, stored in KNO_3 solutions of different concentrations for different hours, planted in media including GA_3 of different concentrations.

KNO_3 µM	GA_3 mg L^{-1}	Immersing hours in KNO_3			
		12 h	24 h	36 h	48 h
0	0	0.00±0.00l	12.50±2.08ı	12.50±0.00ıj	29.17±2.64g
	0.5	0.00±0.00l	14.58±2.64ı	16.67±0.00ıj	52.08±4.17f
	1	2.08±2.08k	22.92±3.23h	25.00±3.84h	52.08±2.64c
	2	6.25±2.80j	31.25±2.08g	35.42±2.80g	54.17±3.23b
100	0	4.17±2.64jk	18.75±2.64h	20.83±2.80ı	81.25±3.84b
	0.5	4.17±2.64jk	20.83±4.17h	29.17±2.64g	89.58±2.08b
	1	8.33±2.64j	29.17±2.64g	33.33±2.64g	89.58±2.08c
	2	8.33±2.64j	35.42±3.84g	52.08±3.84f	89.58±2.08b
200	0	4.17±2.64jk	16.67±3.84h	22.92±2.64ı	85.42±4.27d
	0.5	10.42±2.08j	27.08±3.84gh	35.42±3.84g	85.42±2.08b
	1	18.75±2.80ı	56.25±2.80f	70.83±4.17de	89.58±2.08c
	2	25.00±3.23h	68.75±2.80e	81.25±2.80d	100.00±0.00a

Mean±Standard Error of Mean; Means with similar letter in % 5 level of Tukey test are not significant.

Among the three treatments, immersing hour appeared to be the most effective factor on seed germination, suggesting that effects of KNO_3 and GA_3 concentrations were predominantly determined by the duration of immersing hour. For example, the seeds pre-treated just in water for 48 hours showed 29.17% germination, higher than those of seeds treated with different concentrations of KNO_3 and GA_3 for 12 hours. The seeds exposed to pre-treatments of 100 and 200 µM KNO_3 solutions for 48 hours had much higher germination percentage than the other applications. The highest germination (100%) was obtained from the combined application of immersing seed in 200 µM KNO_3 solution for 48 hours and adding 2 mg L^{-1} GA_3 to MS medium. In order to break seed dormancy and increase germination, a number studies have been carried out using GA_3, KNO_3, cold stratification and acid scarification alone or together (Nadjafi et al., 2006; Cirak et al., 2007; Fariman et al., 2011; Elhindi et al., 2016). It is well documented that GA_3 enhances the biosynthesis of starch-digesting enzyme α amylase and this is assumed

to be an initially essential process of seed germination (McCrate et al., 1981; Kolumbina et al., 2006). Furthermore, it was concluded that using certain chemical stimulants as seed pre-treatments could result in a reduction in seed growth inhibitors such as abscisic acid, one of the reasons for the positive effects of potassium nitrate on breaking dormancy and promoting germination. The effect of KNO_3 was discovered when it was proven that Knop's Solution encouraged germination of some plant species. The beneficial effect of KNO_3 was explained by the nitrate reductase enzyme activity in the production of nitrite/nitric oxide, which acted to remove dormancy and promote faster germination (Delian and Lagunovschi-Luchian, 2015). Most of the studies in the literature showed that germination percentages were higher with combined applications of these pre-treatments, indicating a very good consistency with the results of the present study. When cold stratification and GA_3 was individually applied, the germination percentage was at most 68%, but when these two tested together, the germination percentage reached up to 91.66% (Zare et al., 2011). In a study with *Echinacea purpurea* L. seeds, using GA_3 and cold stratification alone gave 32% germination percentage as the highest, but with combined application the highest percentage was found to be 90.02% (Zahed et al., 2015). Gibberellic acid has been reported to induce an increase on the effect of certain germination promotors such as HNO_3, H_2SO_4, chilling and soaking with water in *Ferula gummosa* Boiss and *Teucrium polium* L. species, traditional medicinal plants in Iran (Nadjafi et al., 2006). The response to acid scarification and water soaking in both species was stronger when GA_3 was combined, indicating an apparent synergistic response. It was reported that highest germination percentage was 32% when KNO_3 and cold stratification used separately, but with combined application germination percentage significantly increased up to 76% (Raisi et al., 2013). In a study with *Ramonda serbica* Panc. and *Ramonda nathalie* Panc. endemic species of Balkan Peninsula, the highest germination percentage was obtained in the seeds treated 1000 ppm GA_3 + 0.3% KNO_3 (92.26%) and 500 ppm GA_3 + 0.2% KNO_3 (91.81%), compared to the seeds (9.26%) without pretreatment (Gashi et al., 2012). Delian and Lagunovschi-Luchian (2015) stated that potassium phosphate and ascorbic acid could be employed as alternative carrot seed priming treatments to provide rapid, highest germination percentage and early vigorous seedling growth under saline stress conditions. The effects of germination stimulants on breaking seed dormancy and increasing germination can be independent of each other, additive, and interactive (Chuanren et al., 2004).

CONCLUSION

In vivo germination test resulted in no germination in *Achillea gypsicola* seeds pre-treated with GA_3 and cold stratification, while KNO_3 applications both *in vivo* and *in vitro* highly promoted germination percentage. Cold stratification at 2 ºC had no inciting effect on seed germination both *in vitro* and *in vivo* conditions. *In vitro* combined applications of both KNO_3 and GA_3 produced higher germination percentages than separate applications. *In vitro* germination percentage of *Achillea gypsicola* seeds pre-treated with 200 µM KNO_3 for 48 hours and planted on MS medium supplemented with 2 mg L^{-1} GA_3 reached to 100%. In conclusion, the present study has established a highly effective strategy for breaking seed dormancy and increasing seed germination of *Achillea gypsicola* through combined application of KNO_3 and GA_3 solutions.

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