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EFFECTS OF TEMPERATURE, GA_3 AND CYTOKININS ON BREAKING SEED DORMANCY OF *FERULA ASSA-FOETIDA* L.*

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Abstract – *Ferula assa-foetida* L. (Apiaceae) is one of the most important endangered medicinal plants, which is rare in nature due to poor seed germination. In an effort to improve and promote the cultivation of this plant, the effects of two temperatures (23°C and 4°C), exogenous GA_3 and cytokinins (kinetin and BAP) were investigated on dormancy breaking and germination of *F. assa-foetida* L. seeds. Among the treatments, cold stratification (4°C) significantly stimulated seed breaking dormancy. The highest mean germination index (2.6 germinated seed per week) was obtained by treatment of seeds with 5 mg/L kinetin at 4°C. But, under no hormone treatment, the highest final percentage germination (73%) was obtained when the seeds were soaked in distilled water and then incubated at 4°C. Also, under this condition the germination index was achieved to 2.5 germinated seeds per week. Treatment of the seeds by GA_3 not only could not significantly enhance the germination index and percentage at 23°C, but also the existence of GA_3 caused a marked decrease in those values at 4°C. This result demonstrated that GA_3 was not effective to overcome dormancy for this species.

Keywords – *Ferula assa-foetida* L., dormancy breaking, seed germination, temperature, hormone treatment

1. INTRODUCTION

Ferula assa-foetida L. (Apiaceae) is a medicinal plant indigenous to Iran and Afghanistan. This plant is one of the most important among the thirty species of *Ferula* distributed in Iran. It is an herbaceous and perennial plant that grows up to 2 m high. One part used is an oleo-gum resin, called asa-foetida or anghouzeh in Persian, obtained by incision from the roots [1, 2]. It has been reported in Iranian folk medicine to be antispasmodic, aromatic, carminative, digestive, expectorant, laxative, sedative, nervine, analgesic, anthelmintic, aphrodisiac and antiseptic [2, 3].

The demand for medicinal plants has increased globally due to the resurgence of interest in and acceptance of herbal medicine. Most of the demand is being met through collection of large quantities of medicinal plants and plant parts from wild populations. The methods of extraction employed are almost invariably crude and unsystematic. As a consequence, the rates of exploitation may exceed those of local natural regeneration. Also, the natural habitats are quickly being depleted depleting. There is thus an urgent need to develop and implement conservation strategies for exploited medicinal plant species.

The medicinal plant is propagated through seeds. However, its natural populations are very limited in native habitats, which may be due to poor seed germination. Low seed germination in Apiaceae is known [4]. The seed of many medicinal plant species are dormant and do not germinate unless specific environmental signals or events occur [5]. One of the main problems preventing sustainable use of

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medicinal plants native to arid lands is that they can germinate within the native environment, but fail to show good germination under laboratory conditions or when cultivation is attempted [6]. Seed germination is an important event in the life of every sexually reproduced higher plant. Seed dormancy is a common phase of the plant life cycle, and several parts of the seed can contribute to dormancy [7]. The seeds of most angiosperms are dormant at maturity, and dormancy must be lost before germination can occur [8].

Plant hormones are found to play an important role in the germination process [9]. Gibberellic acid (GA_3) is one of the hormones proposed to control primary dormancy by inducing germination [6]. External application of gibberellins to seeds can break seed dormancy and aid seedling establishment [10, 6]. It has been demonstrated that other plant growth substances, particularly those with cytokinin activity, could be effective on seed germination or dormancy breaking in some plants [11, 12, 13]. However, the stimulating effects of cytokinins on seed germination have been widely tested in dicot plants [7, 14]. It has been stated that cold stratification also plays an important role in providing the stimulus required to overcome dormancy. Cold stratification has been reported to induce an increase in GA_3 concentration [15, 6].

Although there are some studies on chemical composition and medicinal effects of resin found in *F. assa-foetida* [1, 2, 3, 16], there is currently no information on the seed germination of this plant. Since the regeneration of this species is possible only through seeds, studies to understand germination behavior assume great importance. The present study focuses on improvement of dormancy breaking and germination of *F. assa-foetida* seeds using temperature, GA_3 and cytokinins treatments. Enhancing seed germination is crucial for this purpose.

2. MATERIALS AND METHODS

a) Seed collection

Mature seeds of *F. assa-foetida* were collected from hillsides in the Shir kuh mountains in the east of Tabas (Yazd province, Iran) during September 2006. Seeds were stored in small cotton bags at room temperature (about 23 ˚C). Germination studies were started in October 2006.

b) Seed germination procedure

The seeds were surface sterilized by pre-washing with tap water for 1 h. Then, in a laminar flow cabinet, the seeds were soaked in 70 % alcohol for three minutes, soaked in 1% of sodium hypochloride + 2 drops of Tween 20 per 100 mL for 30 minutes, and washed with sterile distilled water 3 times for 5 minutes.

To determine if plant hormones (kinetin, BAP and GA_3) can overcome dormancy in *F. assa-foetida*, four replicates of fifty seeds were incubated in Petri dishes on filter paper (Whatman No. 1) moistened with kinetin or benzylaminopurine (BAP) solution at concentrations of 5 and 10 mg/L or GA_3 solution at concentrations of 25, 50, 75 and 100 mg/L. One set of untreated seeds acted as control (distilled water). During the following weeks, filter papers were kept moist with distilled water. For germination, the Petri dishes were left in a growth chamber at 23±2 °C or in a refrigerator at 4 °C in continuous darkness. The experiments lasted for 12 weeks and germinated seeds were counted every week. Seed germination was recorded at periodic intervals, and the radicle emergence (2-4 mm) served as index of germination [6].

The germination index (GI) was calculated as described in the Association of Official Seed Analysts [17] by the following formula:

$$GI = X_1/W_1 + (X_2-X_1)/W_2 + \ldots + (X_n-X_{n-1})/W_n$$

Where X_n is the germination percentage on the n^{th} week and W_n is the number of the week from the first week of the experiment.

All tests were conducted following the recommendations of the International Seed Testing Association [18].

c) Statistical analysis

All the experiments were conducted by using a Randomized Complete Block Design (RCBD). Results obtained as percentages and indexes were arcsine-square root transformed to normalized data before statistical analysis. To detect significant differences among means, the data from the experiments were subjected to one-way ANOVA by Duncan's multiple range test (DMRT, $P < 0.05$) using the SPSS software version 9 [19]. A three-way ANOVA was used to test the effects and interactions of temperature, and the kind and concentration of cytokinins on germination of the seeds. Two-way ANOVA was used to test the effects and interaction of temperature and concentration of GA_3 on seed germination.

3. RESULTS

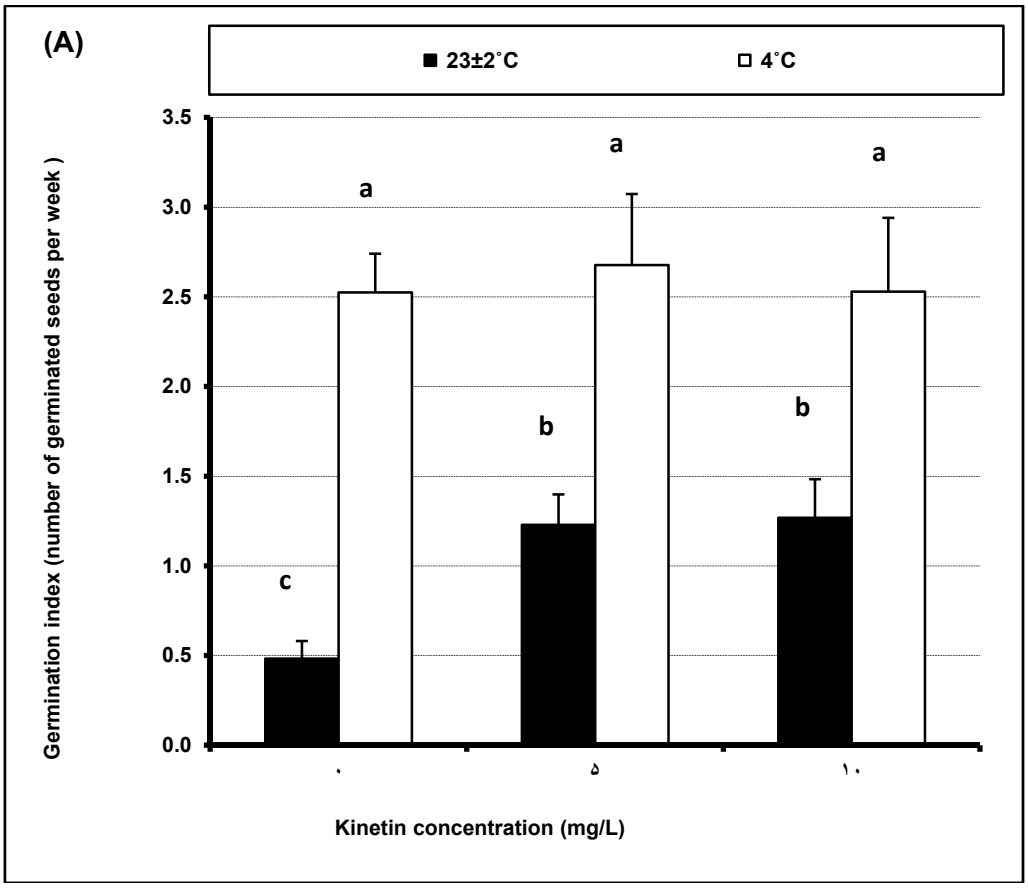
a) Effect of temperature and cytokinin treatment on seed germination

The effects of 0, 5 and 10 mg/L kinetin or BAP concentrations on germination of *F. assa-foetida* seeds were investigated at 23 ˚C and 4 ˚C. Seed germination varied significantly ($P<0.01$) according to temperature and cytokinin types (Table 1). Significant differences were also observed between different concentrations of the cytokinin ($P<0.05$) for germination percentage and index (Table 1). The double interactions were significant for temperature×cytokinin types ($P<0.05$), temperature×cytokinin concentration ($P<0.01$) and type×concentration of cytokinin ($P<0.01$) (Table 1). No significant interaction effect was observed between incubation temperature, type and concentration of cytokinin on germination of seeds (Table 1). The germination index of seeds was improved by cold stratification (4˚C) or with concomitant application of kinetin (Fig. 1a). Higher values for germination index (ranged 2.52-2.68 seeds germinated per week) were observed for seeds incubated at 4˚C with or without kinetin. The opposite was observed for seeds treated with 5 and 10 mg/L BAP at both temperatures compared by ANOVA (Figs.1a & 1b). A slight increase in the final germination percentage with increasing the cytokinin concentration was recorded in seeds incubated at 23˚C, therefore there was no significant difference ($P < 0.05$) between the two applied concentrations of 5 and 10 mg/L of cytokinins (Figs. 2a & 2c). Up to 65% germination was recorded in seeds incubated in 10 mg/L kinetin at 4 ˚C, while low germination percentage (%18) was obtained after incubating the seeds at 23˚C (Figs. 2b & 2d). Germination in seeds that were cold stratified at 4 ˚C alone had begun by the 2^{nd} week, and was up to 73 % on the 10^{th} week. The maximum final germination recorded for all treated seeds ranged from 8% in 5 mg/L BAP to 73% in control at 4 ˚C (Fig. 2). In our experiments, application of kinetin at 23˚C improved both germination percentage and index significantly. Although kinetin, even in combination with 4˚C treatment could not be as effective as the 4˚C alone on seed germination, it was mostly statistically superior to BAP.

Table 1. Results of three-way ANOVA showing the effects of temperature, kind and concentration of cytokinins in *F. assa-foetida* seeds

Source of variation	df	Germination index			Germination percentage		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
Temperature (A)	1	0.20	0.20**	24.31	0.27	0.27**	9.90
Type of Cytokinin (B)	1	0.47	0.47**	55.61	1.33	1.33**	48.22
Concentration of Cytokinin (C)	2	0.07	0.03*	4.10	0.26	0.13*	4.62
AB	1	0.08	0.08**	9.37	0.19	0.19*	6.77
AC	2	0.19	0.10**	11.54	1.19	0.60**	21.57
BC	2	0.23	0.12**	13.93	0.68	0.34**	12.36
ABC	2	0.04	0.02 ns	2.42	0.14	0.07ns	2.45
Error	36	0.30	0.01		0.99	0.03	

Abbreviations: d.f., degrees of freedom; * and ** significant at P<0.05 and P<0.01 respectively; ns, non-significant at P< 0.05.



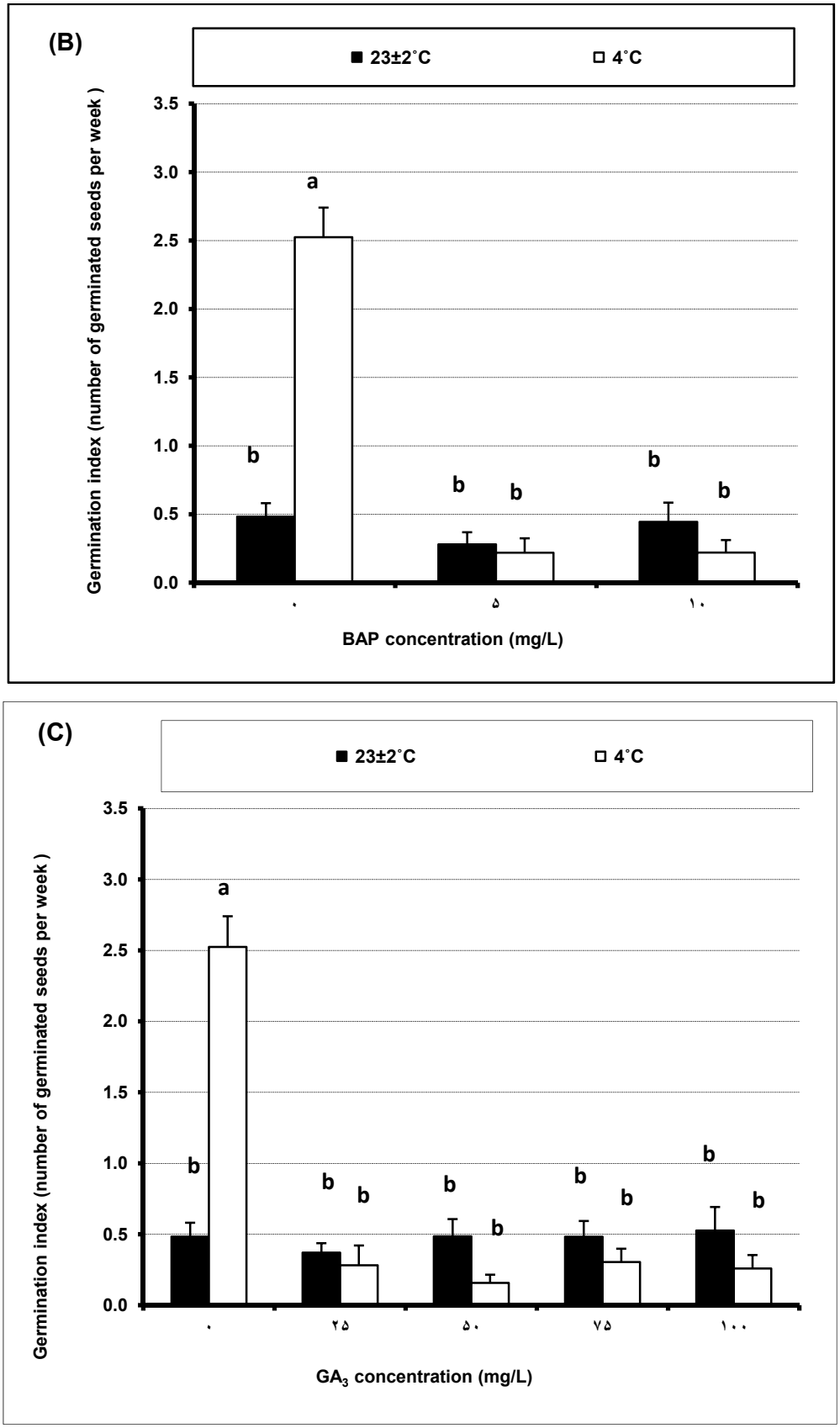
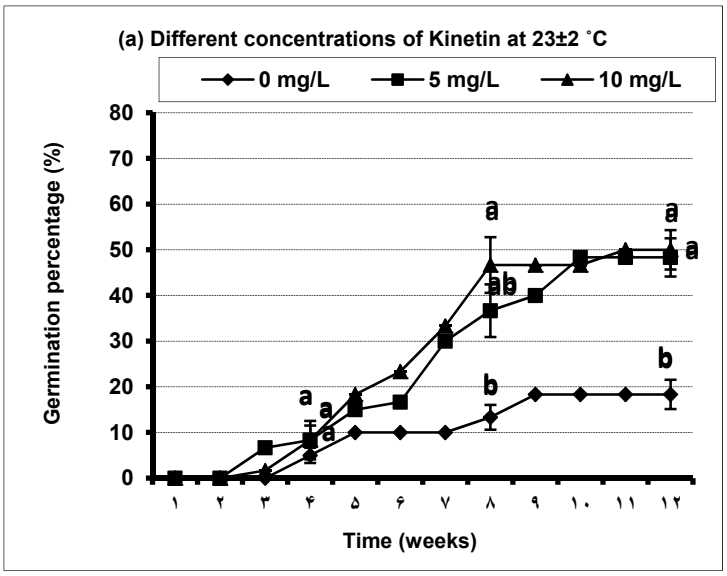
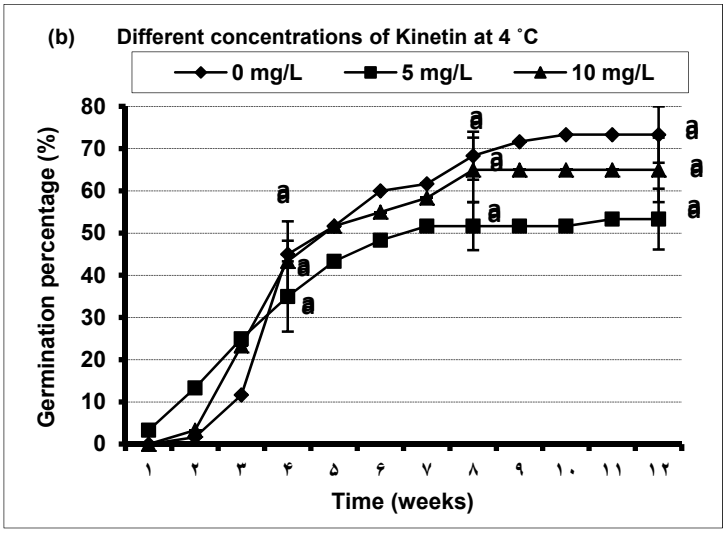
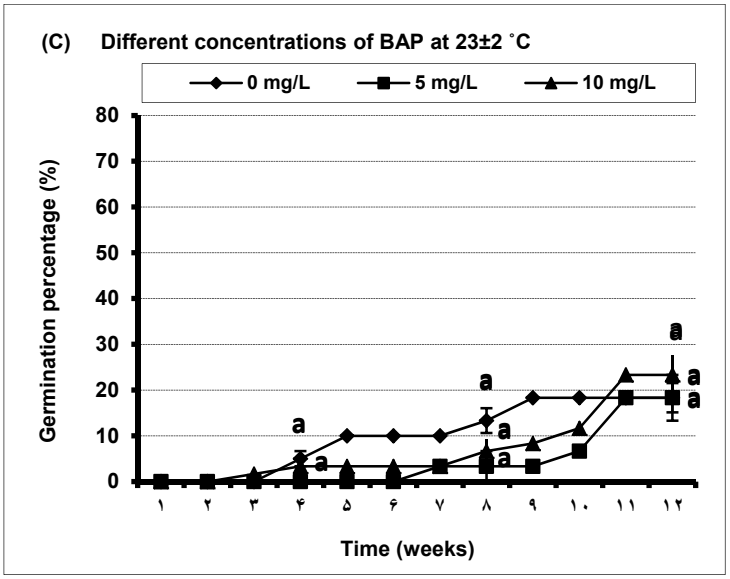


Fig. 1. Germination index of *F. assa-foetida* L. seeds in different concentrations of kinetin (a), BAP (b) and GA_3 (c) at +23°C and +4°C during 12 weeks. Error bars indicate standard error of means; the same letter above the columns indicates no significant differences ($P< 0.05$) among seed treatments at both temperatures


(a) Different concentrations of Kinetin at 23±2 ˚C
0 mg/L
5 mg/L
10 mg/L
Germination percentage (%)
Time (weeks)


(b) Different concentrations of Kinetin at 4 ˚C
0 mg/L
5 mg/L
10 mg/L
Germination percentage (%)
Time (weeks)


(C) Different concentrations of BAP at 23±2 ˚C
0 mg/L
5 mg/L
10 mg/L
Germination percentage (%)
Time (weeks)

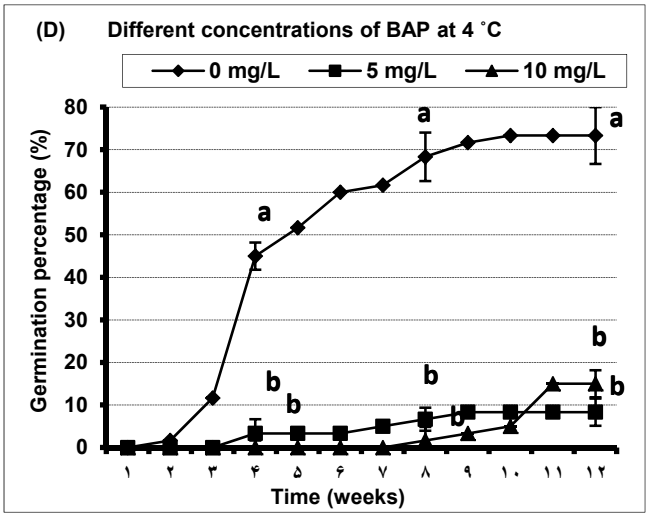
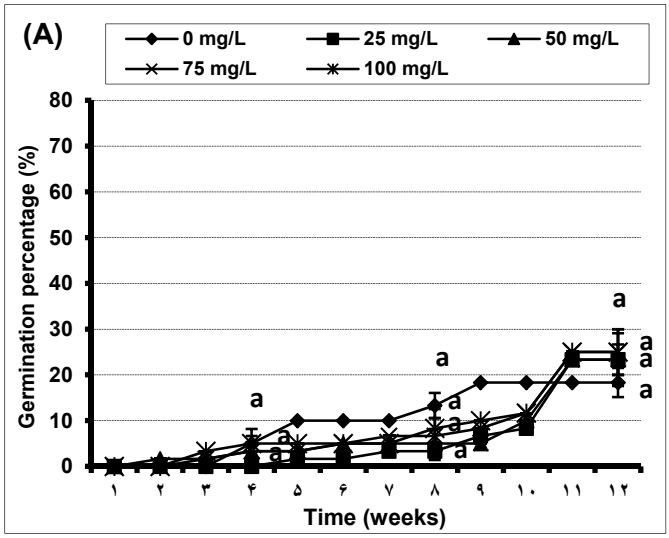


Fig. 2. Progress of germination (as percentage) in response to different concentrations of kinetin and BAP at +23°C (a and c) and at+4°C (b and d), respectively, during 12 weeks. Error bars indicate standard errors; different letters in 4th, 8th and 12th weeks show significant differences at P< 0.05 level

b) Effect of temperature and GA_3 treatment on seed germination

The concentration of GA_3 had a slight effect on the improvement of the germination indexes and percentages after 12 weeks of incubation in all temperature conditions (Figs. 1c & Fig. 3). Two-way ANOVA showed a significant effect ($P<0.01$) of GA_3 concentration and its interaction with temperature on the final germination percentage and germination index (Table 2). The effect of temperature was not significant at $P<0.05$ level (Table 2), meaning that germination was similar within different GA_3 concentrations at 23˚C (Fig. 3a), and the same results were obtained for cold stratified seeds treated with 25-100 mg/L GA_3 (Fig. 3b). Application of exogenous GA_3 in all concentrations at both temperatures could not enhance the germination percentage as compared to seeds incubated at 4˚C without GA_3 (8-25% against 73%) (Fig. 3). Seeds germinated to 73% after 10 weeks of incubation at 4˚C, with a peak of germination between the 8th and 12th week (Fig. 3). When the seeds were incubated at 4 or 23˚C with GA_3 concentrations, about 10-23% of the seeds had germinated after only 10 weeks, after which very little additional germination was recorded. In addition, it seems breaking dormancy occurred seven weeks earlier in seeds that were treated at 4˚C (Fig. 3b).



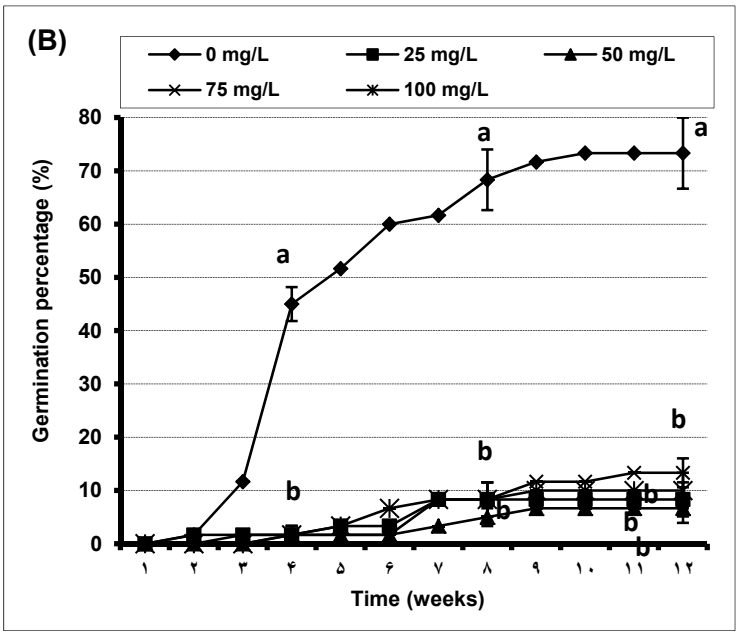


Fig. 3. The progress of seed germination (as percentage) in response to different concentrations of GA_3 at +23°C (a) and +4°C (b) during 12 weeks. Error bars indicate standard errors; different letters in 4th, 8th and 12th weeks show significant differences at P< 0.05 level

Table 2. Results of two-way ANOVA showing the effects of temperature and concentration of GA_3 in *F. assa-foetida* seeds

Source of variation	Df	Germination index			Germination percentage		
		Sum of squares	Mean square	F	Sum of squares	Mean square	F
Temperature (A)	1	0.001	0.001^{ns}	0.03	0.04	0.04 ^{ns}	2.20
Concentration of GA_3 (B)	4	0.25	0.06**	13.37	0.78	0.19**	11.04
AB	4	0.23	0.06**	12.41	1.10	0.28**	15.62
Error	30	0.14	0		0.53	0.02	

Abbreviations: d.f., degrees of freedom; * and ** significant at P<0.05 and P<0.01 respectively; ns, non-significant at P< 0.05.

4. DISCUSSION

Seeds which do not germinate within 30 days are considered to be dormant [20]. Deep complex morphophysiological dormancy (MPD) has previously been observed in a number of other Apiaceae species [21]. In this trial, very few seeds of *F. assa-foetida* (5%) germinated within 4 weeks at 23˚C, but cold stratification (4˚C) yielded 45% seed germinated at the same time. It has been reported by other researchers that cold stratification is successful in ending dormancy and accelerating the germination of dormant seeds in some Apiaceae species [6, 22, 23]. Similar to our observations, the effectiveness of low temperature in causing dormancy removal has also been reported in *Ferula gummosa* by Nadjafi *et al* [6]. Their results showed that washing and cold stratification of dormant seeds at 5˚C for a period of 14 days resulted in the highest germination index (0.45 seed per week) and percentage (26.1%) of *Ferula gummosa*. In accordance with the results obtained by Nadjafi *et al* [6], the present study showed that a long period of cold stratification could be effective increasing seed germination. Prolonged time of cold

stratification has been previously found to improve the seed germination of several plants [10]. Cold stratification is a standard procedure that plays an important role in providing the stimulus required to overcome dormancy [18]. The action of low temperatures in terminating dormancy may be: to promote a fall in the level of inhibitors, or to increase the seeds capacity for production of high levels of promotive hormones [24, 23].

Seed germination study on *F. assa-foetida* revealed significant ($P < 0.05$) improvement in germination under cytokinin treatment compared to control at 23˚C. However, consistent with Bhatt et al. [25], our result showed that the extent of improvement varied by the kind of applied cytokinins. This response was not dependent on the concentration of the applied cytokinins. It has been demonstrated that cytokinins alone break seed dormancy in many species, although the germination response of plants to different cytokinins may differ considerably [26]. It has been demonstrated that BAP is more active than any other cytokinin in germination and in breaking the dormancy of celery and lettuce seeds [13, 26]. On the contrary, based on our results kinetin was the most effective cytokinin on seed germination of *F.assa-foetida* at both temperature treatments. The different responses of plants to cytokinins and their different types may also change according to species, ecotype and even presumably the location of plants in taxonomy [13].

An interesting feature of our experiment was the ineffectiveness of GA_3 at both temperatures on seed germination enhancement of *F. assa-foetida*. Apparently, GA_3 treatment of the seeds along with cold stratification decreased the maximal germination by means of 65%. In confirmation of the same results on *Heracleum sphondylium* [22] and *Chaerophyllum temulum* (Apiaceae) [23], application of exogenous GA_3 did not replace cold stratification after incubation of *F. assa-foetida* seeds at 23˚C. Also, the seeds treatment with GA_3 at 4˚C caused at least a marked 6 to 12-fold decrease in seed germination as compared to control. Based on our current knowledge, similar to *Coffea arabica* [27], *F. assa-foetida* is another species that displays inhibition of germination by GAs at physiological concentrations. Essentially the events of dormancy and germination are controlled by a balance between hormones [24]. Previously, it has been emphasized that seeds of the same or different species may contain different levels of gibberellins, cytokinins and inhibitors leading to various depths of dormancy, from no apparent dormancy to absolute dormancy. Therefore, the seeds should not be expected to give the same response to application of a gibberellin or cytokinin [13]. Seed dormancy and germination are complex physiological processes that are controlled by a range of developmental and external cues [28].

Regardless of the applied hormone treatment to seeds, the germination index was a positively correlated germination percentage ($r= 0.95$, $P<0.05$) for both temperatures. Therefore, fast germination was associated with high germination percentage.

In conclusion, the results of this study imply that the seeds of *F. assa-foetida* are temperature dependent for germination. The application of the cold stratification ended the seed dormancy of this plant in the third month and is a good technique for the germination and conservation of this very important medicinal plant. In general, the present study recommends cold stratification as an economic and easily applicable procedure in seed germination over costly plant growth regulators and associated technicalities. Additional research on dormancy of *F. assa-foetida* could further elucidate protocols for fast germination and dormancy breaking of this species.

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