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Germination improvement and α-amylase and β-1,3-glucanase activity in dormant and non-dormant seeds of Oregano (*Origanum vulgare*)

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Abstract

Oregano plays a primary role among temperate culinary herbs in world trade. It is native of Southern Europe and is one of the most popular herbs in Mediterranean cooking. The germination response of this species to various germination improvement treatments including mechanical, physical and chemical scarification, were studied. Mechanical and chemical scarification of seed coat improved seed germination parameters and suggesting that *O. vulgare* seeds have an exogenous dormancy. Moreover, seed germination of oregano affected by other treatments such as moist chilling and chemicals, and proposing that oregano seeds also have endogenous dormancy. Standard germination test showed that germination percentage of untreated seeds is 25.8% while after chilling treatment at 4 ˚C for 7 days germination percentage reached to 39%. Soaking seeds in 100 ppm of GA_3 for 36 h resulted in 48% germination. The highest value of germination parameters detected by soaking seeds in -10 bar polyethylene glycol solution at 20 ˚C for 72 h. Total germination percentage reached to 60% in this treatment. Combination of chilling and PEG improved seed germination significantly, but was not as effective as PEG solely. Enzyme activity of α-amylase and β-1,3-glucanase in germinating seeds at 12 h after start of imbibition were assayed. Dormant seeds showed lower enzyme activity and enzyme activity in treated seeds increased significantly.

Keywords:; *Origanum vulgare*; Seed dormancy; α-amylase; β-1,3-glucanase.
Abbreviations: GA_3- Gibberellic acid; GI- Germination Index; Gn- Normal germination percentage; Gt- Total germination percentage; MGT- Mean Germination Time; PEG- Polyethylenglycol; VI- Vigor Index.

Introduction

The genus *Origanum* (Lamiaceae) includes 39 species widely distributed in the Mediterranean region (Kintzios, 2002). Oregano (*O. vulgare*) is a woody rhizomatous perennial and a Mediterranean endemic (Thanos, 1995). Oregano has been a valuable source of natural products for maintaining human health for a long period of time, especially in the last decades (Force et al., 2000). The volatile oil of oregano has been used traditionally for respiratory disorders, indigestion, dental caries, rheumatoid arthritis, and urinary tract disorders (Chaudhary et al., 2007). Today, oregano is the "largest selling" culinary herb (Kintzios, 2002). Americans consume 379,000 metric tons of oregano per year, the majority of which (63%) is imported. Increases in consumption in recent years may be due in part to popularity of low-fat, low-salt diets, which rely more on seasonings (Oliver, 1997). Origanum species can be grown from seed, cuttings, division or layering, and the best method will depend on the plant's growth habit and variability (Belsinger, 2004). Propagating by dividing established plants or from cuttings is the preferred method and growing oregano from seed is not always reliable due to seed dormancy. Seed dormancy is a temporary failure of a viable seed to germinate (Vleeshouwers et al., 1995) and is of agricultural and ecological importance. It can lengthen the longevity of seeds and has a large impact on the survival and emergence of plants in the wild habit. However, it also affects the uniformity of germination of crops during field establishment (Wang et al., 2007). The degree of dormancy may be investigate to show some variation related to climate of origin, and can vary considerably among different clones, from seed lot to seed lot and among seeds within one seed lot. The seed dormancy must be broken to induce germination. Various methods are used for this, depending on the plant species and type of dormancy (Koyuncu, 2005). Seeds with physical dormancy remain dormant until some factors render the covering layers permeable to water (Baskin et al., 2000). In morphophysiological dormancy, seeds must be exposed to cold, heat, gibberellic acid or chemical materials for dormancy breaking (Otroshy et al., 2009). Some seeds with combined dormancy characteristics require two types of treatment for good germination. One of the main problems that prevent sustainable use of medicinal plants is that they easily germinate within the native environment, but fail to show good germination under laboratory conditions (Gupta, 2003) due to dormancy. Also, seed germination studies on medicinal plants have proved to be useful in developing appropriate conservation strategies (Kandari et al., 2008). Some enzymes and hormones have critical role in removing dormancy and germination of seeds. The production of α-amylases in aleurone layers is believed to be essential for seed germination in cereal grains, which is tightly regulated by GAs synthesized in the embryonic. On the contrary, ABA blocks the production of α-amylases and suppresses seed germination (Xie et al., 2007; Ogawa et al.,

Table 1. Details of pretreatments to overcome seed dormancy of *O. vulgare*

Pretreatments	Concentration/Duration	Method	Remarks
Mechanical scarification	Until seeds were scarified	Using sandpaper	
Chemical scarification	1, 3 , 5 min	Using concentrated H_2SO_4(75% v/v)	Washed with DH_2O thoroughly
Moist Chilling	7, 14 , 21 days	Seeds placed between wet towel at 4 ˚C	
GA_3	100, 300, 600, 1000 ppm	Seeds soaked for 48h at room temp.	Washed with DH_2O thoroughly
PEG	-6, -10, -14 bar	Seeds soaked for 72h at 20 ˚C	Washed with DH_2O thoroughly
Scarification+ Chilling	Until seeds scarified+ 7 days	Used sandpaper and chilled at 4 ˚C	
Chilling+ GA3	7 days+ 100 ppm	Seeds chilled and soaked for 48h	Washed with DH_2O thoroughly
Chilling+ PEG	7 days+ -10 bar	Seeds chilled and soaked for 72h	Washed with DH_2O thoroughly

H_2SO_4- Sulphuric acid, GA_3- gibberellic acid, PEG- Polyethylenglycol (Merck), DH_2O-Distilled water

Table 2. Effects of treatments on *O. vulgare* seed germination parameters

Treatments	Gt	Gn	MGT	VI	GI
Control	25.87 f	20.85 ef	6.30 cd	4.90 hi	0.09 hij
Mechanical scarification	48.99 b	43.73 bc	9.75 a	12.5 c	0.26 bcd
Chemical scarification					
1 minute	37.95 cd	28.90 de	10.02 a	4.95 hi	0.15 ghi
3 minutes	41.97 bcd	34.93 cd	8.80 ab	10.86 cd	0.22 c-f
5 minutes	26.84 ef	16.69 fg	8.42 ab	2.77 ij	0.92 hij
Moist Chilling					
7 days	38.92 bcd	31.97 d	9.34 a	10.09 de	0.2 d-g
14 days	23.80 fg	4.37 h	4.17 efg	0.12 k	0.03 j
21 days	22.74 fg	10.50 g	7.55 bc	1.43 jk	0.08 ij
GA_3, 48 h					
100 ppm	47.92 bc	33.80 cd	5.75 de	7.81 fg	0.22 cde
300 ppm	31.94 def	16.86 fg	3.57 fg	5.59 h	0.14 ghi
600 ppm	41.95 bcd	21.54 ef	4.23 efg	4.61 hi	0.16 e-h
1000 ppm	35.98 de	20.65 ef	5.05 def	5.82 gh	0.15 f-i
PEG, 72h					
-6 bar	39 bcd	36.99 cd	2.37 g	8.44 ef	0.30 b
-10 bar	60.02 a	58.03 a	3.42 fg	24.18 a	0.39 a
-14 bar	60 a	53.00 ab	2.69 g	15.28 b	0.41 a
Scarification+ Chilling	15.80 g	12.80 fg	5.21 def	1.9 jk	0.12 hi
Chilling+ GA3	16.86 g	11.86 g	4.25 efg	2.41 j	0.10 hi
Chilling+ PEG	64.07 a	57.05 a	3.47 fg	12.08 cd	0.27 bc

Data that do not share the same letters differ significantly at $p < 0.05$

2003). Alpha amylase plays an important role in hydrolyzing the endosperm starch into sugars, which provide the energy for the growth of roots and shoots (Kaneko et al., 2002).It has been shown that β-1,3-glucanase develops prior to germination in the micropylar region of the endosperm of tobacco seeds (Leubner-Metzger et al., 1995) and it was suggested that the enzyme contributed to the hydrolysis of cell wall components resulting in endosperm weakening at the site of radicle protrusion from tobacco seeds (Wu and Bradford, 2003). It was also observed that β-1,3-glucanase activity developed specifically in the micropylar endosperm portion of tomato seeds, similarly to tobacco seeds (Morohashi and Matsushima, 2000). β-1,3-Glucanase is widely distributed in higher plants and have been found during germination of pea, barley, maize, and wheat. β-1,3-glucanase is well known as PR proteins, belonging to the PR-2 families (Wu et al., 2001). Seeds of oregano have a long dormancy and germinate poorly, therefore have restricted dispersal. For this reason, it is almost, a forgotten species in Iran. Therefore, cultivation of this species could be easier and also more production could be reached by increasing germination of its seeds under field conditions. Few information are available for how to break seed dormancy of oregano and which dormancy breaking treatment is the best in order to provide germination requirements of *O. vulgare* seeds. Hence, it is essential to investigate seed dormancy mechanisms and germination improvement methods in oregano. Most of genus in this family exhibit high viability in contrast to low germination percentage because of high seed dormancy. The objectives of this study were to devise an effective method for germination improvement of oregano. Then, activity of two important germination enzymes, α-amylase and β-1,3-glucanase, in germinating seeds were investigated to find the possible dormancy mechanism.

Materials and methods

Study site

The study was conducted in the Seed Lab, Department of Agronomy and Plant Breeding, University of Tehran, Iran in 2009.

Seed source

Mature seeds of *O. vulgare* were purchased in September, 2008 from Pakanbazr Co. Isfahan, Iran. Seeds were stored in room temperature till the start of experiment in November, 2008. Weight of 1000 seeds was 0.25 g and seed viability (using tetrazolium test) at the start of the experiment was 86%.

Pretreatments and experimental conditions

Details of various pretreatments used to improve germination of *O. vulgare* are presented in Table 1. The surface of treated and untreated seeds was sterilized by soaking in 3% sodium hypochlorite solution for 5 minutes and subsequently rinsed thoroughly with sterilized water. After sterilization, four replicates of 25 seeds were used for each treatment. Seeds were placed in 90 mm diameter disposable petri dishes on double layers of Whatman No. 1 filter paper that was moistened with 5 ml distilled water. Then, petri dishes were put in a germinator with alternating temperatures cycle (20/30˚C, 16h dark/8h light). Seeds with 2 mm radical emergence were considered germinated. The observations were recorded daily up to 21 days. Infected seeds were removed during counting.

Seed germination parameters calculation

Total germination percentage (Gt) was calculated as Gt= (n/N*100), where n= total number of germinated seeds (normal and abnormal) at the end of the experiment and N= total number of seeds used for germination test. Normal and Abnormal seedlings were determined base on ISTA, (1997). Normal germination percentage (Gn) was calculated the same as Gt. But in Gn only normal seedlings were counted and calculated. Mean germination time (MGT) was calculated based on the following equation (Manjkhola et al., 2003):

$$MGT = \frac{\Sigma(ni \times di)}{N}$$

Where n_i is the number of seeds germinated after each period of incubation in days (di) and N is the total number of seed germinated normally at the end of the experiment. The seed vigor index was calculated as following (Rahnama and Tavakkol-Afshari, 2007):

$$VI = \frac{Ls \times Pg}{100}$$

Where VI is vigor index, Ls is the mean of seedling length (mm) and Pg is germination percentage. For calculating germination index (GI), the following equation was used (Walker-Simmons and Sesing, 1990):

$$GI = \frac{\sum_{i}^{t} gi(t-j)}{N \times t}$$

Where g_i is germination in time interval i (i varying from 1 to t); t is total number of time intervals and j=i–1. N is the number of normal seedlings at the end of the experiment.

Alpha-amylase assay

Enzyme activity only assayed at the treatment resulted in the highest germination parameters. For assay of alpha-amylase activity, both treated and untreated seeds placed in petri dishes on moistened filter paper and were left under germination conditions for 12 h of imbibition. Then seeds were powdered thoroughly in liquid nitrogen and were grounded thoroughly with a pestle in a chilled mortar with 10 ml of cold phosphate buffer (0.1 M pH 7.2). The homogenate was centrifuged in a refrigerated centrifuge at 4˚C for 25 min at 10000 rpm. Clean supernatant was decanted and stored in -80˚C for enzyme assay (Biswas et al., 1978, with some modifications). Alpha-amylase activity of the seed extract was determined following the method of Baker (1991) and Bernfeld (1991) with some modification. 200 µl of 1% starch solution (Merck) in phosphate buffer was added to 50 µl of enzyme extract. The reaction mixture was incubated at 37˚C for 30 min. Then 100 µl dinitrosalicylate reagent was added and placed at boiling water for 10 min for the termination of the reaction. The mixture was then diluted with 350 µl distilled water, mixed well, and the absorbance was read immediately at 540 nm in a spectrophotometer.

β-1,3-glucanase assay

Enzyme activity only assayed at the treatment with the highest germination parameters which selected as the most effective treatment. For assay of β-1,3-glucanase activity, both treated and untreated seeds placed in petri dishes on moistened filter paper and were left under germination conditions for 12 h of imbibtion. Then seeds were powdered thoroughly in liquid nitrogen. To prepare enzyme extract, powdered seeds were homogenized in 10 ml of 15 mM sodium acetate buffer, pH 5.5, in a chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm in a refrigerated centrifuge at 4˚C for 5 min. The supernatant was decanted and stored in -80˚C for enzyme assay (Morohashi and Matsushima, 2000, with some modifications). Total β-1,3-glucanase activity was assayed with laminarin (Sigma) as substrate according to Celestino et al. (2006) with some modifications. The assay system consisted of 1% (wt/vol) laminarin dissolved in 100 mM sodium acetate buffer, pH 5.0, and 50 µl enzyme sample. The reaction was allowed to proceed for 30 min at 50 ˚C, and was then stopped by the addition of 300 µl dinitrosalicylate reagent and 5 min of boiling. The absorbance of the reaction mixture was determined at 550 nm using spectrophotometer.

Data analysis

All the experiments were conducted in a randomized completely blocks design (RCBD) with four replications. Analysis of variance was performed using the SAS statistical software package, version 6.12 (SAS Institute, 1998). Treatments means were compared using Duncan at 5% level of probability.

Results and discussion

Seed germination parameters increased when seeds imposed to different treatments (Table 2, Fig. 1-5). Oregano seed germination was affected by both mechanical and chemical

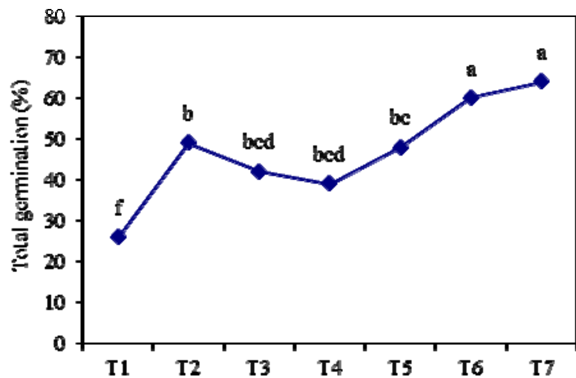


Fig 1. Effect of selected pretreatments on Gt; T1- Control, T2- Mechanical scarification, T3- Chemical scarification (3 minutes), T4- chilling (7 days), T5- GA_3 (100 PPM), T6- PEG solution (-10 bar), T7- Chilling (7 days)– PEG solution (-10 bar)

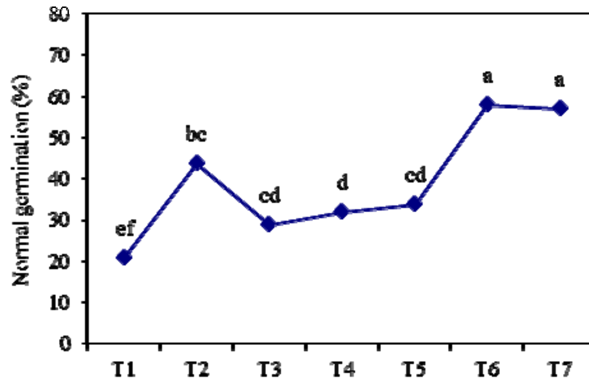


Fig 2. Effect of selected pretreatments on Gn; T1- Control, T2- Mechanical scarification, T3- Chemical scarification (3 minutes), T4- chilling (7 days), T5- GA_3(100 PPM), T6- PEG solution (-10 bar), T7- Chilling (7 days)– PEG solution (-10 bar)

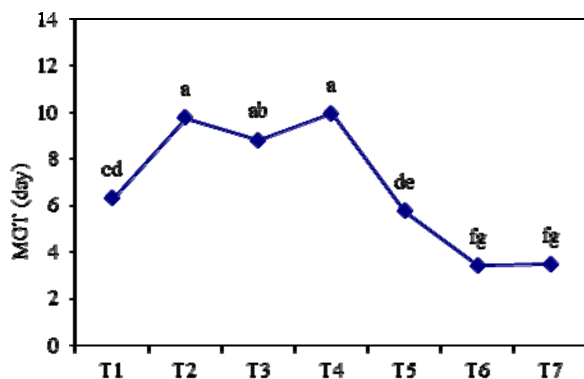


Fig 3. Effect of selected pretreatments on MGT; T1- Control, T2- Mechanical scarification, T3- Chemical scarification (3 minutes), T4- chilling (7 days), T5- GA3(100 PPM), T6- PEG solution (-10 bar), T7- Chilling (7 days)– PEG solution (-10 bar)

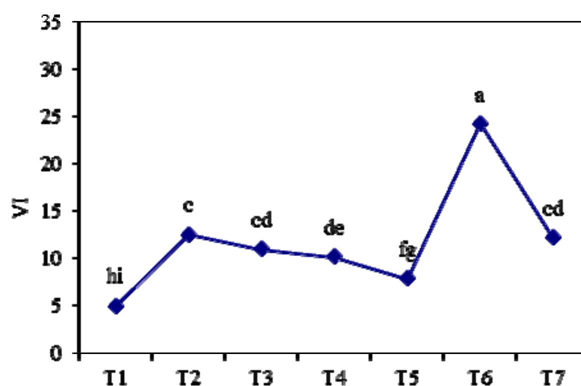


Fig 4. Effect of selected pretreatments on VI; T1- Control, T2- Mechanical scarification, T3- Chemical scarification (3 minutes), T4- chilling (7 days), T5- GA3(100 PPM), T6- PEG solution (-10 bar), T7- Chilling (7 days)– PEG solution (-10 bar)

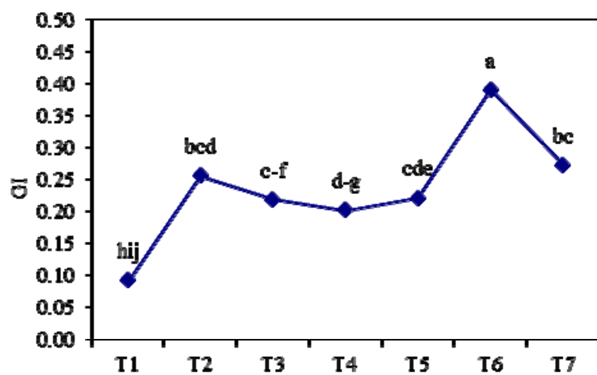
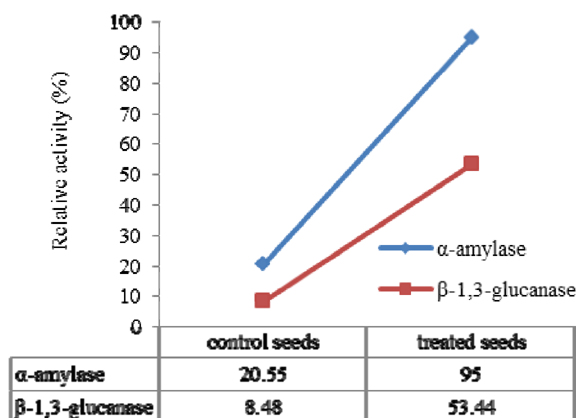


Fig 5. Effect of selected pretreatments on Gt; T1- Control, T2- Mechanical scarification, T3- Chemical scarification (3 minutes), T4- chilling (7 days), T5- GA3 (100 PPM), T6- PEG…



	control seeds	treated seeds
α-amylase	20.55	95
β-1,3-glucanase	8.48	53.44

Fig 6. Activity of α-amylase and β-1,3-glucanase in control and treated seeds

seed scarification (Table 2). All parameters except mean germination time were improved by mechanical scarification (Fig. 3). In chemical scarification, 3 minutes scarification showed better results but was less effective than mechanical scarification (Fig. 1-5). Chemical scarification more than 3 minutes had negative effects. Longer period of time for acid treatment resulted in damaged embryo and seed (Wang et al., 2007). In *ferula gummosa*, similar to our results, a reduction in seed vigor index, germination rate and MGT detected by increasing immersion time in H_2SO_4 (Rahnama and Tavakkol-Afshari, 2007). Moist chilling or cold stratification has been widely used as a pre-sowing treatment for breaking seed dormancy and enhancing the maximum rate and germination percentage of dormant seeds of many species (Baskin et al., 1992). Moist chilling of oregano seeds at 4 ˚C for 7 days enhanced seed germination indices (Fig. 1-5). Chilling treatment beyond 7 days, reduced germination indices as there was no significant difference with control (Table 2). In contrast to our results, seed germination of *Ferula gummosa* (Rahnama and Tavakkol-Afshari, 2007) and *Teucrium polium* (Nadjafi et al., 2006) significantly improved at longer periods of prechilling treatment. Chilling has been reported to induce an increase in GA_3 concentration and sensitivity (Nadjafi et al., 2006). Compared to control, different concentrations of GA_3 improved germination percentage to 48% at concentration of 100 ppm (Table 2). Higher GA_3 concentrations resulted in lower germination. Similar to our results, in a population of *Heracleum candicans,* application of 100 ppm GA_3 showed the highest germination percentage and the lowest MGT and increase in GA_3 concentration, decreased germination percentage (Joshi and Dhar, 2003). However, in European *Chaerophyllum temulum*, the final germination percentage increased with increasing concentration of GA_3 in all temperature conditions (Vandelook et al., 2007). Germination of *Ferula gummosa* and *Teucrium polium* seeds increased at higher concentrations of GA_3 (Rahnama and Tavakkol-Aafshari, 2007; Nadjafi et al., 2006). It seems that higher concentrations of GA_3 have poisonous effect on germination of oregano seeds. Polyethylenglycol (PEG) is a common chemical for osmopriming treatment. In this treatment, seeds were exposed to PEG to imbibe sufficient water to initiate the germination process, but radicle emergence is prevented by the osmotic potential of the solution (Bradford, 1986). In our experiment, germination parameters improved when seeds soaked in PEG solutions. Compared to all other treatments, soaking seeds in -10 bar PEG solution, showed the highest germination and was the most effective treatment (Fig. 1-5). . Also soaking seeds in -10 bar PEG solution, resulted in higher germination (Fig. 1), normal germination (Fig. 2), MGT (Fig. 3) and significantly higher vigor (Fig. 4, Fig. 5). Combination of scarification plus chilling and also chilling followed by 100 ppm GA_3 was not effective and germination decreased (Table 2). However, combination of chilling and PEG gave promising results and showed higher germination percentage. Manjkhola et al. (2003) concluded that prechilling plus GA_3 pretreatment at different concentrations produced variable germination responses in alpine and subalpine populations of *Arnebia benthamii*. Ruhi et al. (2010) reported that stratification for 7 weeks was more effective treatment on germination characteristics of waterlily tulip than 5 weeks. They suggested that cold stratification was a better treatment on breaking seed dormancy of waterlily seeds than GA_3 and KNO_3 treatments. Application of 500 ppm concentration of GA_3 and 0.1 of KNO_3 after stratification resulted in higher germination in waterlily dormant seeds. Enhancing seed germination due to seed coat scarification emphasizes that an impermeable covering layer restricts *O. vulgare* seed germination. Although scarification stimulates germination, however, seed coat cannot be the only factor in seed dormancy of *O. vulgare.* Also cold and chemicals such as GA_3 and PEG enhanced oregano seed germination. Insomuch chemicals alleviate endogenous dormancy (Baskin and Baskin, 2000; Alan Cohn, 1996), it seems oregano seeds possess both exogenous and endogenous dormancy (Combined dormancy). But it is interesting that combined treatments except combination of chilling and PEG didn't show promising results. Scarifying seeds following with chilling them did not show any increase in all of the traits compared to control. We guess exposing coat damaged seeds to low temperatures led to necrosis of the embryo, but needs more investigation. Combination of chilling and GA_3 was less effective than chilling and soaking seeds alone. While most of the studies show a synergistic response to GA_3 and prechilling, Gupta (2003) reported that dormant seeds which require chilling are often treated with GA_3 for breaking their dormancy. In *ferula gummosa* seeds the response to prechilling was stronger when it was combined with GA_3 (Rahnama and Tavakkol-Afshari, 2007). Study of α-amylase and β-1,3-glucanase activity in control and superior treatment showed that enzyme activity in dormant seeds was very low. However, enzyme activity was increased in germinating seeds of oregano dormancy after breaking treatment. Therefore, α-amylase and β-1,3-glucanase play important roles in oregano seed germination. Activity of α-amylase was more influenced by application of dormancy breakage treatment comparing to β-1,3-glucanase (Fig. 6). Biswas et al. (1978) reported that dormant seeds of large crabgrass contained very little or no activity of α-amylase, whereas non-dormant seeds showed appreciable activity. In this study, increase in α-amylase activity of dormant seeds at various periods of hydration was slow, but non-dormant seeds showed a rapid increase in activity and reached 90 units at 76h. β-1,3-glucanase could also plays an indirect role as signalling molecule to mediate loosening or breakage of the micropylar endosperm by releasing elicitor-active oligo-ß -1,3- glucans from plant wall components. These processes, like expression of β-1,3-glucanase and endosperm rupture, are promoted by GA and ethylene and could be inhibited by ABA. Therefore, it seems that β-1,3-glucanase appears as a key factor in regulating dormancy and germination in response to environmental and hormonal conditions (Leubner, 2003). In tomato seeds, the completion of germination and β-1,3-glucanase activity are causally related (Morohashi and Matsushima 2000). Wu et al. (2001) also reported that a doubling of β-1,3-glucanase activity occurred after 36 h of imbibition on water and activities continued to increase until at least 60 h. The increase in both activities occurred only in the micropylar region, not in the rest of the tomato seed.

Conclusion

This paper proposed that seed germination of *O. vulgare* can be significantly improved by subjecting seeds to different treatments. Soaking seeds in -10 bar PEG solution at 20 ˚C for 72 h resulted in the highest germination percentage and the lowest mean germination time and therefore, propose for improving germination of oregano seeds. It seems that a combined dormancy mechanism restricted oregano seed germination. Considering enzyme activities in germinating seeds, two-stage germination is possible germination mechanism and rupture of the testa and rupture of the endosperm are separate events. More investigations such as

electron microscopic studies are needed to corroborate this thought.

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