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Effect of polyethylene glycol and its interaction with ascorbate on seed germination index in *Pimpinella anisum* L.

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Abstract

Cell stability is impaired during environmental stresses such as drought stress and subsequent Reactive Oxygen Species (ROS) production. Seed antioxidant defense system controls a few levels of radicals. Ascorbate (ASC), an important antioxidant, has been known as a cellular redox system supporter. *Pimpinella anisum* L. is a very aromatic and medicinal plant that has great export value. This study was designed for the evaluation of the effect of polyethylene glycol (PEG) 6000 and its interaction with ascorbate on seed germination index in this plant. In a Petri dish study, control and drought induced treatments were introduced to 9 levels of osmotic potentials by PEG 6000 (ψ_s = 0.0 to -2.0 MPa) and one level of ASC (1.4 mM), with 5 replications in the presence and absence of ASC for different stress levels. The results of this study revealed that in lower water potentials, germination percentage was significantly reduced. Comparing the means of the stressed treatments showed that germination percentage increased by using exogenous ASC. This increase was significant in the moderate stress levels. ASC has protective effect against drought stress.

Key words: *Pimpinella anisum* L., osmotic potential, PEG, exogenous ascorbate, germination.

Introduction

Pimpinella anisum L. (Umbelliferae, Apiaceae) (anise, aniseed) is a herbaceous annual plant, native to Mediterranean region. Its oil is used commercially in perfumery, tobacco manufacture and pharmaceutical production [3]. P-anisaldehyde is a caricidal component of *Pimpinella anisum* seed oil used against the house dust mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* [15]. It is used in healing peptic ulcer [1]. It has antispasmodic effects [26], anticonvulsant effects [21] and antifungal activity [14].

Water availability of the soil is considered one of the principal causes of low germination in seeds [17]. Water stress acts by decreasing the percentage and rate of germination [9]. It requires a coordinated series of events during dehydration that are associated with preventing oxidative damage and maintaining the native structure of macromolecules and membranes [12].

Reactive oxygen species (ROS) generation in the embryonic axis of germinating seeds at the onset of germination suggests a risk of oxidative damage at that stage of development [22]. If the ROS are not rapidly removed, they would cause considerable structural and functional damages to the cells [27].

Ascorbic acid is the most abundant low molecular weight antioxidant in aqueous compartments of plants [20]. Ascorbate is a major primary antioxidant, reacting directly with hydrogen peroxide, hydroxyl radicals, superoxide and singlet oxygen [7]. The involvement of antioxidative mechanisms in the maturation of orthodox seeds is poorly documented, with studies performed only in *Vicia faba* [2], bean [4], sunflower [5] and *Triticum durum*[8]. However, it has been widely reported that orthodox seeds are completely devoid of ASC and ascorbate peroxidase (APX), two key factors in ROS detoxification directly involved in H_2O_2 removal. They contain only moderate amounts of dehydroascorbate (DHA) and have ascorbate free radical (AFR) reductase and DHA reductase, enzymes catalysing the reduction of the two products of ASC oxidation into ASC. Indeed, the enzymes and metabolites responsible for ROS scavenging have particular importance for the success of germination [27]. Therefore, control of oxidative stress is an important ability of plant to tolerate drought.

According to studies have been done so far, besides the risks of oxidative stress threaten the germination, environmental water restriction will also be added to this damage. Therefore, due to the importance of this plant in modern science and periodic and continuous drought in recent years, this study was designed for evaluating the effects of different drought levels and applying exogenous ASC as an important antioxidant in scavenging ROS produced by drought stress under different osmotic potentials on aniseed germination index. The ability and germination rate of the seeds and the effect of antioxidant on this process were also assessed in this study.

Materials and Methods

Since anisum is an annual plant, its fresh and physiologically active seeds were selected for this experiment. Seeds were purchased from Isfahan Pakan Bazar Seed Production Company in Iran. The seeds were labeled for complete properties, including purity, germination percentage, harvesting time and physiological features.

Determination of germination percentage: After being sterilized with 12% sodium hypochlorite for 3 min, the seeds were washed and germinated in Petri dishes containing filter papers, placed in autoclave for 15 min in high pressure and temperature of 120°C. The germination treatments in the Petri dishes included distilled water (0 MPa) and various polyethylene glycol (PEG 6000) solutions with different osmotic potentials (-0.25, -0.5, -0.75, -1, -1.25, -1.5, -1.75 and -2 MPa) to induce drought stress. PEG solutions were prepared using the non-linear model proposed by Money [18]. Two experiments were carried out to evaluate the effects of drought stress induced by PEG and exogenous application of ASC on aniseed germination. In order to prepare these solutions with different osmotic potentials ranging from -0.25 to -2 MPa, 2.4, 4.7, 6.5, 8.1, 9.4, 10.3, 11 and 11.4 g PEG were dissolved in 100 ml distilled water to produce -0.25, -0.5, -0.75, -1, -1.25, -1.5. -1.75 and -2 MPa osmotic potentials, respectively. Petri dishes were divided into two groups with 9 treatments in each group. In a completely randomized design experiment, each treatment was replicated 5 times.

The first group (called no ASC-treated group) with 9 treatments consisted of 8 different concentrations of PEG 6000 (as mentioned above) plus control (0 MPa). The second group (called ASC-treated group) also consisted of 9 treatments (8 PEG levels and control) plus ASC (1.4 mmol L^{-1}). After conducting several preliminary experiments with various concentrations of ASC (from 0.25 to 2 mmol L^{-1}), the 1.4 mmol L^{-1} concentration was the most efficient one for aniseed. Twenty seeds were placed in each Petri dish and sealed with parafilm, then incubated in a growth chamber (GROEC germinator) at 23°C. The seeds were irrigated daily with distilled water by moistening the filter papers to provide moisture for seed germination. The number of germinated seeds (radical appearance) was counted once a day during 10 days. Germination percentage was calculated by the following equation [28]: Germination percentage = 100 n/N, n = number of germinated seeds, N = total number of seeds.

Statistical analysis: The data were subjected to analysis of variance (ANOVA) using SPSS software package. Means were compared using Tukey multi-components test at $P<0.05$. The results are presented in the form of mean comparisons ± S.E.M at $P<0.05$.

Results

Germination: The variance analysis revealed that a significant difference among the treatments occurred with the highest germination percentage (%G) in the ψ_s of 0.0 MPa (control), in the absence and presence of ASC (94 and 97%, respectively) (Table 1). It was observed that the %G of the control treatment (ψ_s = 0.0 MPa) did not differ significantly from the -0.25 MPa stress level and this value in the absence and presence of ASC was 89 and 93%, respectively.

There was progressive decrease in %G of aniseed in this study with decrease in osmotic potentials starting with -0.5 MPa, varying from 75 to 51% in the absence of ASC and 79-66% in the presence of ASC at -0.5, -0.75 and -1 MPa stress levels. It was verified that the %G markedly decreased at the -1.25 MPa stress level compared to the control treatment with %G 44-58 in the absence and presence of ASC, respectively. The lowest %G was observed at -2 MPa stress level in this experiment (Table 1), with 15 and 21% in the absence and presence of ASC, respectively.

Table 1. Aniseed germination percentage under different osmotic potentials in the absence and presence of ASC.

Osmotic potential (MPa)	Mean germination (%) ± S.E.M	
	No ASC	ASC
0	94±1.87a	97±1.22a
-0.25	89±1a	93±1.22a
-0.5	75±1.58b	79±1.87b
-0.75	61±1c	76±1b
-1	51±2.44d	66±1.85c
-1.25	44±1.87de	58±2d
-1.5	37±1.87e	47±1.87e
-1.75	27±2f	30±2.23f
-2	15±1.58g	21±1.87g

Values are mean±S.E.M. of 5 replicates, in each column, means are compared between different osmotic potentials at P<0.05, Means in a column followed by the same letter are not significantly different (p>0.05), no ASc: absence of ASC, ASC: presence of ASC.

Comparison of %G between the absence and presence of ASC at each osmotic potential: Mean germination percentages in different potentials during 10 days in the absence and presence of ASC are presented in Table 2. Analysis of variance showed significant difference in some levels when comparing the absence and presence of ASC at each same osmotic potential.

At early stage of germination, there was significantly different %G in control and -0.25 MPa treatments with applying ASC compared to its absence between same days and osmotic potentials. Applying ASC caused obvious acceleration in germination process on first days (Table 2). In regards to -0.5 and -0.75 MPa stress levels, ASC significantly increased %G and decreased the time necessary for germination compared to its absence. ASC not only caused significant difference in %G at -0.75, -1, -1.25 and -1.5 MPa stress levels but also progressive increase in %G with ASC maintained persistently to Day 10. At -1.75 and -2 MPa stress levels, ASC partially increased %G compared to its absence, but the difference was not significant.

Comparing Figs 1 and 2 between the absence and presence of ASC showed germination delay with decrease in osmotic potential of environment. The minimal time needed for germination belonged to control and -0.25 MPa treatments during 10 days, starting from Day 4 and differing from other treatments. This time was also the same for -0.5 and -0.75 treatments with applying ASC. The latest time of germination was 6 days at -1.5, -1.75 and -2 MPa stress levels. However, germination started on Day 5 at -1 and -1.25 MPa stress levels.

Discussion

In the present study, the effects of various concentrations of PEG 6000 solutions with different osmotic potentials on aniseed germination percentage were evaluated. It was observed that seed germination percentage was reduced with increasing PEG concentrations and decreasing water potentials of the media. The effects of PEG on seed germination of *Pimpinella anisum* [25], *Pterogyne nitens* [19] and *Sorghum bicolor* [16] support our results.

Table 2. Comparison of mean of aniseed germination percentage between the absence and presence of ASC at the same osmotic potentials during 10 days.

Osmotic potential (MPa)	ASC and no ASC	Germination(%)															
		Day 3	sig	Day 4	sig	Day 5	sig	Day 6	sig	Day 7	sig	Day 8	Sig	Day 9	Sig	Day 10	sig
0	no ASc	0	-	2±1.22	**	18±1.22	**	48±2.5	*	76±1.87	-	81±1.87	-	91±1.87	-	94±1.87	-
	ASc	0		18±1.22		28±1.22		57±1.22		81±1.87		88±2.55		94±1.87		97±1.22	
-0.25	no ASc	0	-	1±1	**	12±1.22	-	32±1.22	*	71±1.87	*	79±2.91	-	87±2	-	89±1	-
	ASc	0		8±1.22		17±1.22		41±1.87		81±1.87		84±1.87		91±1		93±1.22	
-0.5	no ASc	0	-	0	-	7±1.87	**	18±2	*	37±2	*	60±1.58	-	73±2.55	-	75±1.58	-
	ASc	0		2±1.22		14±1.87		27±2		46±1.87		64±1.87		78±1.22		79±1.87	
-0.75	no ASc	0	-	0	-	6±1	-	12±1.22	*	30±1.58	*	46±1.87	*	56±1.87	*	61±1	*
	ASc	0		1±0		10±1.58		24±1.87		43±1.22		56±1.87		75±2.55		76±1	
-1	no ASc	0	-	0	-	6±1	-	7±1.22	*	13±1.22	*	25±1.58	*	44±1	-	51±2.44	*
	ASc	0		0		8±1.22		20±1.87		37±1.22		40±1.55		49±2.44		66±1.85	
-1.25	no ASc	0	-	0	-	4±1	-	6±1.87	*	9±2.44	*	17±1.22	**	36±1.87	-	44±1.87	*
	ASc	0		0		6±1.87		18±1.70		30±1.34		22±1.22		40±2.23		58±2	
-1.5	no ASc	0	-	0	-	1±1	-	4±1.87	-	7±2	-	15±1.58	*	33±2	-	37±1.87	*
	ASc	0		0		3±1.22		6±1		9±1.87		30±1.87		37±2		47±1.87	
-1.75	no ASc	0	-	0	-	0	-	2±1.22	-	5±1.58	-	10±1.58	-	24±1.87	-	27±2	-
	ASc	0		0		0		4±1.87		8±1.22		14±1		28±3.39		30±2.23	
-2	no ASc	0	-	0	-	0	-	2±1.22	-	3±1.22	-	7±2	-	15±1.58	-	15±1.58	-
	ASc	0		0		0		3±2		6±1		10±0		21±1.87		21±1.87	

Significant differences are compared between the absence and presence of ASC at the same osmotic potential and on the same day. Values are mean±S.E.M. of 5 replicates, * for $p<0.05$, ** for $p<0.01$, no ASc: absence of ASC, ASC: presence of ASC, sig: significant difference.

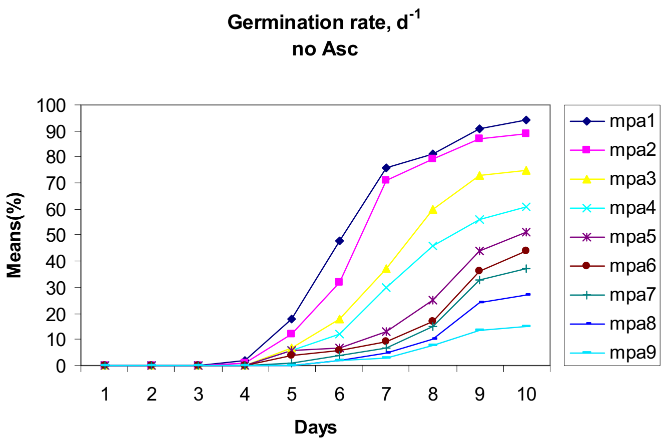

Figure 1. Comparison of mean of aniseed germination percentage in different PEG levels in the absence of ASC during 10 days.

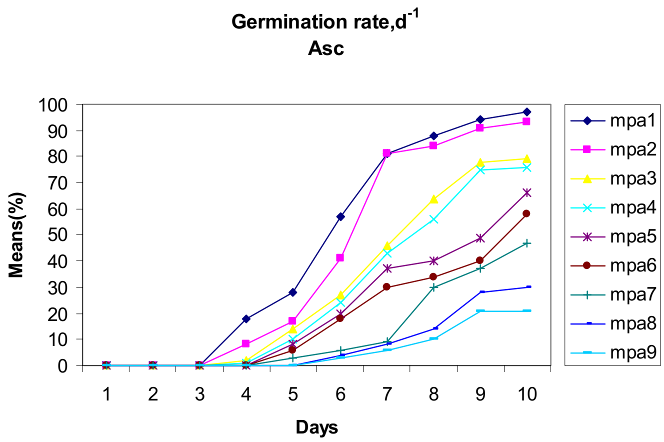

Figure 2. Comparison of mean of aniseed germination percentage in different PEG levels in the presence of ASC during 10 days.

The progressive fall in the germination percentage with decreasing water potential of the environment, observed in this experiment, probably was caused by the low hydraulic conductivity of the environment, where PEG 6000 makes water unavailable to seeds, affecting the imbibition process of the seed which is fundamental for germination [16].

In the present study, the highest germination percentage occurred at 0.0 and -0.25 MPa treatments (Table 1), and this value is higher than those achieved by Stefanello *et al.* [25] on aniseed at the same osmotic potentials at 20-25°C. The lowest %G was observed at -2.0 MPa stress level (Table 1). This larger reduction with PEG solution could be attributed to high viscosity, where solubility and diffusion of oxygen were reduced compared to water[9].

In this study, with applying ASC and evaluation of its interaction with different stress levels due to PEG, %G of *Pimpinella anisum* increased. It seems that in addition to increasing osmotic stress, higher PEG concentration caused more ROS production and its progressive increase during germination and oxidative damage. In our study, applying exogenous ASC as an important antioxidative along with coordinate induction of the antioxidative mechanisms could scavenge more ROS produced under stress conditions and improved germination process. So, the efficacy of ASC increased aniseed germination percentage (Table 2, Figs 1 and 2).

ROS generation in the embryonic axis of germinating seeds at the onset of germination suggests a risk of oxidative damage at that stage of development [22]. It requires a coordinated series of events during dehydration that are associated with preventing oxidative damage and maintaining the native structure of macromolecules and membranes [12].

ASC and APX are two key factors in ROS detoxification [27]. Because cell division and cell expansion require ASC [10] and these two processes are fundamental for seedling development, ASC could also be involved directly or by maintaining the opportune cellular redox balance in the process responsible for rendering the reserve substances available for the germinating embryos [27]. The early availability of ascorbic acid (AA) is necessary to meet the large demands for AA by the restored cell metabolism and because AA is, in some way, required to elicit AA peroxidase. It has been reported that decreases in AA peroxidase occurrence was correlated with the onset of a biochemical pathway leading to morphological anomalies of seedlings and to the loss of seed germination capacity [2]. Therefore, in the present study, it is possible to conclude that while oxidative stress increased due to rising PEG, applying exogenous ASC along with PEG solutions could have increased the redox capacity. This event has apparently supported germination and cell division and expansion in drought-stressed treatments and growth process in the present study.

In addition to ASC, tocopherols are also lipophilic antioxidants, and the highest tocopherol level was observed in seeds. ASC plays a role in reducing α-tocopherol radical to α-tocopherol [11]. In soybean, it was shown that the α-tocopherol content increased in embryonic axes upon imbibition and post-germination under oxidative stress [24]. Therefore, it could be concluded that using exogenous ASC would probably protect germination of aniseed against lipid peroxidation of membrane in storage period. Since ASC could be considered as the best supporter for seed tocopherol storage and germination process might be protected with high confidence against ROS oxidative damage, control of oxidative stress would be an important ability of plant to tolerate drought stress. In the present study, concurrent with increasing stress levels, germination process occurred with delay in the low water potential media compared to the control treatment (Table 1). Presence of ASC provoked acceleration in the germination process. In regards to the results of this study, it is believed that presence of exogenous ASC affected the biochemical processes of germination, because the enzymes involved have ranges of action that will retard or accelerate seed germination [6].

According to the studies performed on the *Senna occidentalis*[9], *Sorghum bicolor* [16] and *Pterogyne nitens* [19], germination rate decreased with increasing PEG concentrations. The primary action of osmotic inhibition is retardation of water uptake, which is crucial for germination [13]. Furthermore, the fact should be observed that under water restriction the velocity of water absorption is affected, where the absorption and consequently the hydrolysis of carbohydrates are slower [16]. The results demonstrated that the aniseeds are more tolerant to water stress than other species like *Senna occidentalis* [9], *Ateleia glazioviana* [23] and *Pterogyne nitens*[19], besides revealing that the germination behavior of seeds of this species under water restriction is to some extent similar to what was found by Lobato *et al.*[16] on *Sorghum bicolor*. Besides revealing the adaptability of aniseeds at low environmental water potentials, exogenous ASC provoked the increase in %G and reduction in the time necessary for germination, even in the treatments under strong water deficiency.

The di- and oligosaccharides, compatible solutes and specific proteins, such as the late embryogenesis abundant proteins (LEAs) and heat shock proteins (HSPs), confer protection to cells during dehydration [12]. Indeed, the enzymes and metabolites responsible for ROS scavenging have particular importance for the success of germination [27], since control of oxidative stress is an important ability of plant to tolerate drought.

Conclusions

Results of this study revealed that severe drought stress can negatively affect germination of *Pimpinella anisum*. This effect probably interferes with oxidative system and ROS production. ASC has protective effect against drought stress with several mechanisms and positive effect on germination rate and percentage of this plant. Indeed, the enzymes and metabolites responsible for ROS scavenging have particular importance for the success of germination [27], since control of oxidative stress is an important ability of plant to tolerate drought. Further studies are needed to evaluate the exact protective effect of exogenous ASC on interaction with PEG inducing drought stress. Applying exogenous ASC can be an effective method in increasing growth of this plant in greenhouses and fields.

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