

THE IMPORTANCE OF BRACTEOLES IN ENSURING *Atriplex halimus* GERMINATION UNDER OPTIMAL CONDITIONS

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ABSTRACT

Atriplex halimus L. is a native halo-nitrophilous scrub that grows in salt-marsh environments and saline soils of the Mediterranean basin.

The presence of bracteoles did not change the germination rate of seeds and fruits (seeds with bracteoles) in water, although bracteoles did slow down the process, from 2.5 to 5.7 days to reach the 50% of the final germination in seeds and fruits, respectively.

Bracteoles prevented germination NaCl solutions greater than 0.3M. The seeds without bracteoles germinated at concentrations higher than 0.3M NaCl, but had smaller cotyledons than seeds germinated in water (3.1 and 5.6 mm, respectively), and all the roots had necrotic zones. The results showed that bracteoles play an important role in preventing seeds from germinating in NaCl concentrations higher than 0.3M, ensuring that they remained dormant and only germinated when freshwater was available. The recovery experiments showed that the seeds from the fruits that did not germinate in salt were not unviable, but remained dormant, awaiting the proper conditions to germinate. In the presence of water, the final germination was higher than 95%, and there was little necrosis found in the roots. The results showed how the presence of bracteoles prevented germination under unfavourable conditions for plant development.

KEYWORDS:

Chenopodiaceae; marshes; salt; sodium chloride

1 INTRODUCTION

Atriplex halimus (Chenopodiaceae) is a monoecious perennial bush, native to the shores of the Mediterranean

basin. It is associated with emerged soil in salt marshes [1], and exhibits a great tolerance to drought and salinity [2], which has meant that this species has colonized arid zones across North Africa and the eastern Mediterranean. It is sometimes known as *A. halimus* var. *schweinfurthii* Boiss and has become an important component in the rehabilitation of soils affected by excessive salinity and low moisture levels [3, 4]. Salt negatively affects the germination and early seedling development of *A. halimus* [3, 5], as it does in other *Atriplex* [6, 7] and halophyte species [8]. *A. halimus* seeds have the ability to be dormant for extended periods of time, avoiding hypersaline conditions while they are dispersed, and germinating when the salt levels decrease [9, 10].

A. halimus dispersal units consist of one-seed with a membranous pericarp protected by two bracteoles, which play an important role in seed germination of *Atriplex* species. The inhibitory or retarding effect of bracteoles in germination has been described for a number of *Atriplex* species, e.g. *A. canescens* (Pursh) Nutt., *A. dimorphostegia* Kar. & Kir., *A. griffithii* Moq., *A. inflata* F. Muell., *A. nummularia* Lindl., *A. portulacoides* L., *A. semibaccata* R. Br., *A. spongiosa* F. Muell., *A. undulata* (Moq.) D. Dietr. and *A. vesicaria* Heward ex Benth. [6, 7, 11-15]. The retarding and inhibitory effects have been attributed to different causes. Among these, the reasons highlighted by different authors are: (1) the high concentration of salt accumulated in bracteoles, whose effect is evident by the stimulation of germination after washing, as has been shown to be the case in *A. canescens* [12], *A. dimorphostegia* [13], *A. hortensis* L. [16], *A. nummularia*, *A. vesicaria*, *A. semibaccata*, *A. inflata* and *A. spongiosa* [6], or by the inhibitory effect of bracteole leachate in *A. centralasiatica* Iljin [17]; (2) the physical barrier imposed on the seed [17-19]; (3) the presence of growth regulating substances (e.g. ABA) or allelopathic compounds (e.g. saponin) in bracteoles that delay germination [17, 18]; (4) a reduction in oxygen diffusion, or (5) the effects caused by bracteoles on the quality of light falling on the seed [13, 16, 17, 20]. In

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addition to the reduction in germination rate, bracteoles have a retarding effect on germination, as demonstrated in *Atriplex portulacoides* [7] and *A. inflata*, *A. nummularia*, *A. semibaccata*, *A. spongiosa* and *A. vesicaria* [6].

The bracteoles could play an important ecological role in the establishment of *A. halimus* populations, and the present study investigated the germination of *A. halimus* fruits and seeds under different salt conditions and their recovery potential when transferred to fresh water. The aims of this study were to identify the effects of bracteoles on germination rate and timing, seedling characteristics, and to investigate whether germination inhibitory processes had roles other than avoiding seed germination during dispersal in sea water.

2 MATERIALS AND METHODS

Fruits of *A. halimus* were collected from the Parque Natural Marismas del Odiel (SW Spain), a coastal salt marsh, in September 2010. The fruits were taken from 10 bushes, and mixed and stored in paper bags at room temperature for future use. The germination study was undertaken using two different propagules: fruits (seed with bracteoles) and seeds (fruits without bracteoles).

Germination tests were carried out in Petri dishes (9 cm diameter) on two layers of autoclaved filter paper watered with 5 ml of the different treatment solutions and sealed with adhesive tape (Parafilm™) to avoid desiccation. There were three replicates of 25 fruits or seeds per treatment, and germination was considered to be complete following radicle emergence. The experiments were carried out under controlled-environmental conditions with 12/12-h of day/night at 24/20 °C, respectively. The light was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$. The experiment lasted 30 days, during which germination was recorded every 2 or 3 days. Germination percentage rates were then calculated for each dish.

Fruits and seeds were surface-sterilized before use in 5% (v/v) sodium hypochlorite for 10 min and rinsed three times with sterile water. To check the effect of the surface sterilization, three dishes had seeds and fruits that had not undergone surface sterilization. No significant differences ($p < 0.001$) were found in the germination rates between the sterile and non-sterile treatments but the sterilization treatment was found to stop seed infections. Following this result, all seeds used in the experiments were surface-sterilized.

2.1 Effects of salt on seed germination

The seeds were separated from the bracteoles by hand. The resulting seeds were surface-sterilized, then placed in Petri dishes and treated with 0, 0.1, 0.2, 0.3, 0.6 and 0.9 M NaCl for 30 days. In order to study recovery after the salt treatments, the seeds that did not germinate after this time were rinsed in deionized water, and then transferred to

new dishes, containing deionized water, for 30 days and the germination recorded.

2.2 Effects of salt on fruit germination

Fruits were surface-sterilized, placed in Petri dishes and treated with 0, 0.1, 0.2, 0.3, 0.6 and 0.9 M of NaCl for 30 days. In order to study recovery after the salt treatments, the fruits that did not germinate were divided into two groups. The first group of fruits was rinsed in deionized water, transferred to new plates for 30 days, and the germination rate recorded. In the second group, the fruits had their bracteoles removed by hand, and then the seeds were rinsed in deionized water. Following this, the seeds were transferred to new dishes, containing deionized water, for 30 days and the germination rate was recorded.

For each experiment, 25 seeds or fruits were sown in triplicate. However, in the case of fruits, the final number of seeds included in the calculations was lower. The fruits were sown completely and, in all cases, apparently intact fruits were selected. At the end of the treatments (salt exposure and recovery), the fruits that did not germinate were opened and those without seeds were recorded. This data was taken into account in all the calculations, as not only fruits with seeds were included in the final results.

The germination dynamics were analyzed using the final germination percentage after 30 days. T_{50} (number of days necessary to reach 50% of the final germination percentage) was used as the measure of germination, as described in previous germination assays [21]. The length of the cotyledons and the number of roots with necrotic zones were recorded 15 days after germination for 10 seedlings per dish (or all the germinated seedlings when there were less than 10 seeds germinated per dish).

Statistical analyses of the data were performed using SPSS. The normality of the data was tested, and Kruskal-Wallis and Mann-Whitney U test were used due to the non-normality of the data. The significance levels used are indicated in each table.

3 RESULTS AND DISCUSSION

Increasing the salt concentration increased the germination time of the *A. halimus* seeds that managed to germinate (Fig. 1A). A significant decline ($p < 0.05$) in the final germination percentage was also observed as NaCl concentration increased (Table 1). The results showed that there was a 50% reduction in germination between the 0 (control) and 0.1 M NaCl treatments. However, as indicated by T_{50} , only at 0.6 M NaCl was there a significant delay ($p < 0.05$) in germination time, which confirmed results from previous studies using *A. halimus* [3, 22] where the reduction was attributed to the osmotic effects of the salt [23]. They also agree with studies on other *Atriplex* [5-7] and halophyte species [8-10, 24, 25].

There was no significant difference in cotyledon size ($p < 0.05$) between the 0 and 0.1 M NaCl treatments, but

cotyledons got significantly smaller as NaCl concentration rose. Not only the size of the cotyledons was affected, but also the percentage of exerted cotyledons dropped when salt concentration rose, and the number of roots with necrotic zones increased when NaCl concentration rose above 0.1 M (Table 1).

Seed recovery results (Fig. 1B) showed that the seeds that did not germinate in the presence of salt were still viable, and there were no significant differences in the final germination percentage between seeds from different pre-treatments, or between seeds initially germinated in deionized water (control) ($p < 0.05$) (Table 1), but the germination rate was significantly ($p < 0.05$) accelerated in seeds previously exposed to NaCl concentrations over 0.2 M, as indicated by the T_{50} results, results that agree with find-

ings from previous studies that had investigated *Atriplex* species found in Africa [3, 23]. No significant differences were found ($p < 0.05$) in the size of the cotyledons. All the cotyledons were exerted, and there was almost no root damage (Table 1).

In order to analyze the role bracteoles play in the germination process, the germination of the complete fruit, with bracteoles, was tested. The germination percentage of fruits placed in deionized water (Fig. 1C, Table 2) showed no significant differences when compared to the germination rate of seeds in deionized water (Tables 1 and 2), but the presence of bracteoles significantly delayed the germination process ($p < 0.05$). Germination T_{50} for seeds was reached on day 2.5, while for fruits, this was not reached until day 5.7. The seedlings had significantly smaller coty-

TABLE 1 - Germination dynamics and morphological parameters of *A. halimus* seeds exposed to different concentrations of NaCl and their subsequent recovery when exposed to deionized water (The data shows the mean \pm SD of three different replicates; in each column, different letters indicate significant differences ($p < 0.05$) (lower-case for the initial salt treatments and capital letters for the recovery treatments)).

	Germination dynamics			Morphological parameters			
	Total number of seeds	Final germination (%)	T_{50}	Number of seedlings analysed	Cotyledon size (mm)	Exerted cotyledons (%)	Necrotic roots (%)
Seeds							
0.0 M NaCl (Control)	75	98.7 \pm 2.3 a A	2.5 \pm 0 a A	30	5.6 \pm 0.8 a ABC	100	13.3
0.1 M NaCl	75	58.7 \pm 14.0 b	5.8 \pm 5.1 ab	22	5.2 \pm 0.9 a	73.3	60
0.2 M NaCl	75	28 \pm 6.9 c	3 \pm 1.5 ab	17	3.9 \pm 0.9 b	53.3	59.1
0.3 M NaCl	75	26.7 \pm 2.3 c	4.3 \pm 2.8 ab	14	3.1 \pm 1 c	46.7	100
0.6 M NaCl	75	13.3 \pm 4.4 d	5.3 \pm 1.4 b	3	2.7 \pm 1.6 bc	10	100
0.9 M NaCl	75	1.3 \pm 2.3 e	7	0	-	-	-
Recovery treatments in deionized water							
0.1 M NaCl	6	100 \pm 0 A	2.5 \pm 0.9 AC	6	6 \pm 0.5 A	100	0
0.2 M NaCl	17	100 \pm 0 A	1.5 \pm 0 C	16	5.6 \pm 0.7 ABC	100	5.9
0.3 M NaCl	16	100 \pm 0 A	1.5 \pm 0 C	16	5.3 \pm 0.4 B	100	0
0.6 M NaCl	11	100 \pm 0 A	1.5 \pm 0 C	11	5.4 \pm 0.5 BC	100	0
0.9 M NaCl	13	85 \pm 13.2 A	1.5 \pm 0 C	11	5.8 \pm 0.6 AC	100	0

TABLE 2 - Germination dynamics and morphological parameters of *A. halimus* fruits (seeds with bracteoles) exposed to different concentrations of NaCl and the recovery of fruits and seeds in deionized water (The data shows the mean \pm SD of three different replicates; in each column, different letters indicate significant differences ($p < 0.05$) (lower-case for the initial salt treatments and capital letters for the recovery treatments)).

	Germination dynamics			Morphological parameters			
	Total number of seeds	Final germination (%)	T_{50}	Number of seedlings analyzed	Cotyledon size (mm)	Exerted cotyledons (%)	Necrotic roots (%)
Fruits							
0 M NaCl (Control)	58	100 \pm 0 a A	5.7 \pm 2 a A	30	6.4 \pm 1.1 a B	100	0
0.1 M NaCl	49	98 \pm 3.6 a	16 \pm 1 b	30	7.1 \pm 1 b	100	6.7
0.2 M NaCl	42	13 \pm 13.3 b	20.5 \pm 3.5 b	6	7.1 \pm 1.4 ab	100	0
0.3 M NaCl	47	0 \pm 0	-	-	-	-	-
0.6 M NaCl	45	0 \pm 0	-	-	-	-	-
0.9 M NaCl	58	0 \pm 0	-	-	-	-	-
Recovery treatments in deionized water of fruits							
0.2 M NaCl	17	100 \pm 0 A	1.8 \pm 1.4 BC	17	6 \pm 0.8 AB	100	0
0.3 M NaCl	21	100 \pm 0 A	3 \pm 0.9 BD	20	6.4 \pm 0.8 B	100	0
0.6 M NaCl	22	95.2 \pm 8.2 A	3.5 \pm 0 B	21	6.1 \pm 0.8 BC	100	9.5
0.9 M NaCl	27	100 \pm 0 A	6.3 \pm 3 AB	27	5.6 \pm 1 AC	100	3.8
Recovery treatments in deionized water of seeds							
0.2 M NaCl	17	96.3 \pm 6.4 A	1 \pm 0 C	16	5.6 \pm 0.9 AC	100	6.2
0.3 M NaCl	24	96.7 \pm 5.8 A	1 \pm 0 C	24	5.7 \pm 1.2 AC	100	8.7
0.6 M NaCl	21	95.8 \pm 5.2 A	2.7 \pm 1.4 BC	20	5.7 \pm 1.2 AC	100	5
0.9 M NaCl	28	96.3 \pm 3.4 A	3.5 \pm 0 B	25	5.3 \pm 1.1 A	100	15.4

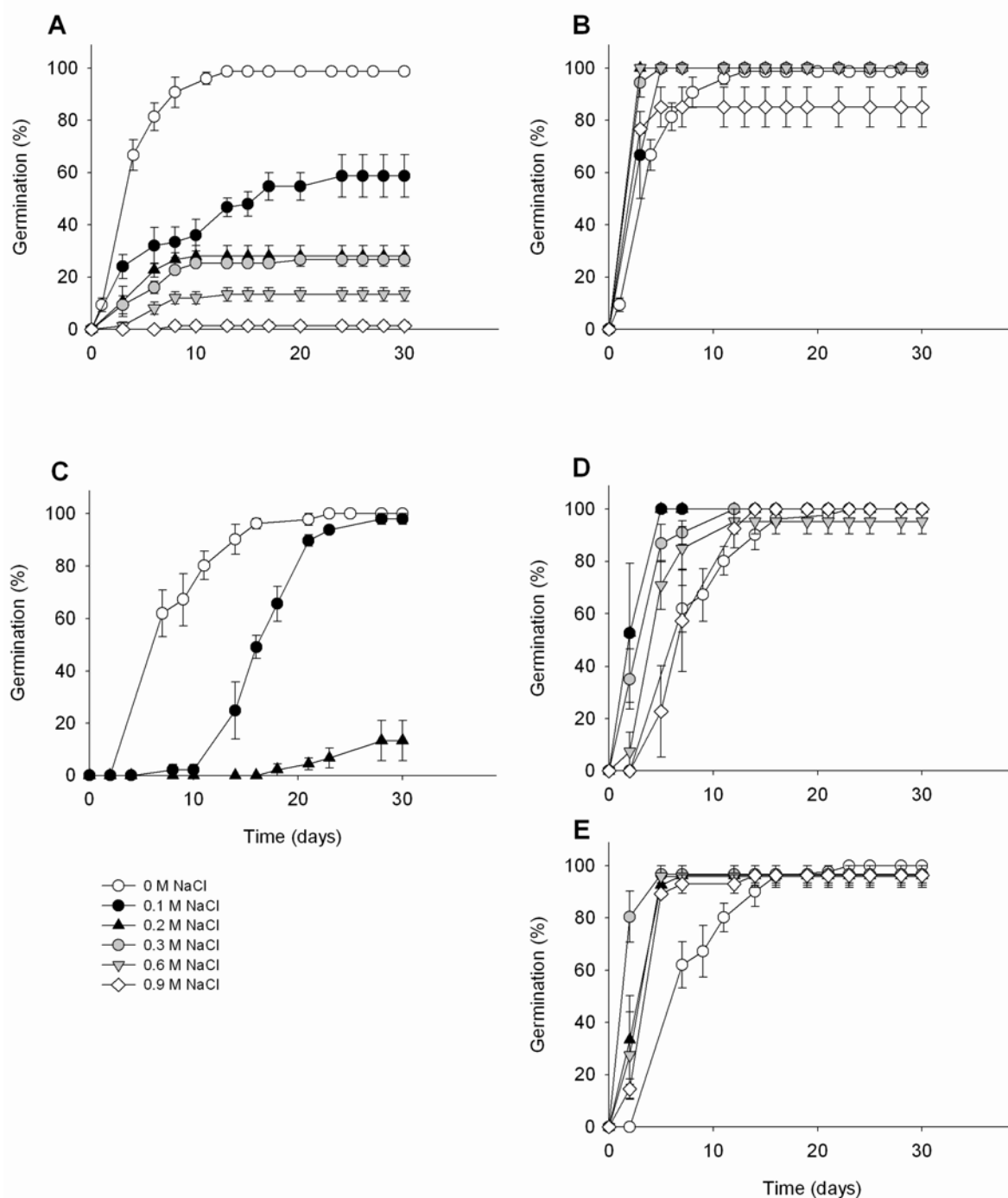


FIGURE 1 - Cumulative germination percentage over 30 days: A) *A. halimus* seeds exposed to different NaCl treatments; B) *A. halimus* seeds that did not germinate when exposed to NaCl and being transferred to deionized water; C) Cumulative germination percentage of *A. halimus* fruits exposed to different concentrations of NaCl; D) *A. halimus* fruits that did not germinate when exposed to NaCl and being transferred to deionized water; E) *A. halimus* fruits that did not germinate when exposed to NaCl. Their bracteoles were removed and the seeds transferred to deionized water (Data shows the mean \pm SD of three independent Petri dishes with 25 seeds in each dish)

ledons ($p < 0.05$) when the bracteoles were removed (Tables 1 and 2); data that were opposite to the study by [14], who found germination rates of 98% in seeds and only 35% when the fruits with bracteoles were tested. It could be that the sterilization of the fruits removed soluble compounds

from the bracteoles, although other researchers have found that there was no significant inhibition of germination when the bracteole leachate was tested. This suggests that the sterilization had no effect on germination. Germination inhibition by bracteoles has been shown in other *Atri-*

plex species [6, 11, 13, 15, 16], but no connection has been established between salt levels and seedling development.

In the presence of salt, the fruits showed a significant reduction in germination rate when exposed to 0.2 M NaCl, compared to 0 and 0.1 M NaCl. No germination occurred at concentrations over 0.3 M NaCl. Germination was also significantly delayed ($p < 0.05$), with a T_{50} of 5.7 days in the control rising to 16 and 20 days in concentrations of 0.1 and 0.2 M NaCl, respectively. However the seedlings produced significantly larger cotyledons as NaCl level rose to 0.2 M (Table 2). Above 0.2 M there was no germination at all.

When the fruits that did not germinate in the presence of salt were placed in deionized water for recovery (Fig. 1D, Table 2), no significant changes in germination percentage ($p < 0.05$) were found when compared to the control fruit that had been placed in water from the beginning but germination was accelerated compared to the fruit that had not been exposed to NaCl ($p < 0.05$). The exception to this was the fruits exposed to the 0.9 M NaCl solution. The cotyledons on the fruits recovered from the 0.9 M solution were significantly smaller than the control. A small percentage of seedlings had necrotic roots at 0.6 and 0.9 M NaCl.

When the bracteoles were removed so that seed recovery in deionized water (Fig. 1E, Table 2) could be investigated, no significant differences ($p < 0.05$) were found in the final germination percentage but germination time accelerated. The seedlings all had significantly smaller cotyledons ($p < 0.05$) and a small number of necrotic roots were found (in all cases less than 15%).

The higher sensitivity of fruits to salt compared to seeds, in terms of germination percentage and time, has been previously reported in *Atriplex portulacoides* [7], and described as an adaptive advantage as it prevents germination of fruits if they are dispersed into sea water, or during unfavorable periods such as drought [19]. This hypothesis has been supported by the fact that fruits of *A. portulacoides*, removed from highly saline solutions after 1 month, displayed germination rates similar to those that germinated in deionized water [7]. *A. halimus* seeds were able to germinate in concentrations up to 0.9 M but the germinated seedlings had much smaller cotyledons (Table 1) and damage in roots. The salt affected not only seedling size, since all cotyledons produced in concentrations over 0.2 M were smaller than the ones exposed to deionized water, but also the health of the seedlings as necrotic roots were detected when the seeds germinated in the presence of salt. This is clearly indicative of unhealthy plants. The fruits did not germinate in the presence of high levels of salt, which suggested that the presence of bracteoles prevented germination under unfavorable conditions and kept the seeds dormant until fresh water conditions prevailed in the environment. When this occurred, the germination time was lower (T_{50}) and the seedlings produced larger cotyle-

ons and healthy roots, as shown by the recovery experiments carried out with fruits.

ACKNOWLEDGEMENTS

The authors are grateful to the institution and all the staff of the Odiel Marshes Natural Park.

The authors declare no conflict of interest.

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Received: March 19, 2012

Accepted: May 21, 2012

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