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BRIEF COMMUNICATION

Effects of salt stress on the reproductive biology of the halophyte *Plantago crassifolia*

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Abstract

Floral phenology, pollen quality and seed set of *Plantago crassifolia* plants, grown in the presence of increasing NaCl concentrations, were studied to test how this Mediterranean halophyte responded to salt stress during the reproductive phase of its life cycle. "Reproductive success" was maximal in plants grown in non-saline conditions, or in the presence of 100 mM NaCl, but it was negatively affected by higher salinities, due to a progressive reduction of pollen fertility, seed set, and seed viability.

Additional key words: flower development, pollen viability, pollen germination, seed set, seed germination.

Halophytes, plants naturally adapted to live in saline environments, have been the subject of intensive research for a long time (Flowers *et al.* 1986). Many studies on halophytes deal with the effect of salinity on seed germination (*e.g.* Dell'Aquila 2000, Gulzar and Khan 2002) or vegetative plant growth (*e.g.* Misra *et al.* 1996, Gadallah and Ramadan 1997), but only a few papers refer to flowering and fruiting phenology in conditions of high salinity. *Plantago crassifolia* is a perennial halophyte spread in salt marshes throughout the Mediterranean region. In a recent paper (Vicente *et al.* 2004), we reported on the general responses to salt stress during vegetative development of *P. crassifolia*. Seed germination and seedling development were very sensitive to salt stress, but salt tolerance increased with the age of the plants. Adult plants grew optimally in the absence of salt, and a concentration-dependent inhibition of plant growth was observed in the presence of NaCl. Nevertheless, the plants were even able to tolerate external concentrations of up to 500 mM NaCl. The present study was carried out to investigate the effects of salinity on the reproductive biology of this plant. There are some reports on different reproductive aspects in other species of this genus, regarding male sterility, protogyny or gynodioecy (Dinnétz 1997, Dinnétz and Jerling 1997), or the mechanisms of pollen release (Young and Schmitt 1995), but, to our knowledge, none in relation to responses to salinity.

Salt treatments (100, 200, 300, 400 and 500 mM NaCl), plus a control treatment without salt, were applied to three month-old *Plantago crassifolia* plants, obtained and grown as previously described (Vicente *et al*. 2004), for five months (from April to August 2002). For each treatment, fifteen plants in individual pots were placed in a plastic tray, to which the corresponding salt solution (or water, for the control) was added periodically, ensuring that the pots were maintained wet throughout the experiment. The number of spikes produced by each plant was counted every ten days. After anthesis, all spikes were separately enclosed in paper bags, to avoid the loss of seeds, which were collected after ripening. The length of scapes and spikes was measured prior to sampling.

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To check the effect of salinity on pollen development and fertility, cytological studies were performed on pollen grains isolated from fresh anthers, immediately after dehiscence. Pollen viability was determined as described in Alexander (1980), a method which enables the distinction between aborted and non-aborted pollen grains. Pollen germination tests were carried out in small Petri dishes containing GK medium (Wilson *et al.* 1997); after incubation at 25 ºC for 2 h in a germination chamber, pollen grains were examined under a microscope. Pollen was considered as having germinated when the presence of a pollen tube of at least double the length of the grain's diameter was observed. At least 1500 pollen grains, from three randomly selected plants per treatment, were counted.

To calculate the seed set, seeds were collected from more than 50 % of the fertile spikes obtained for each group of salt-treated plants and the controls (15 to 35 spikes, depending on the treatment), separated from floral debris, counted and weighed on an analytical balance. The mean number of seeds per spike, and the mean seed mass were multiplied by the number of fertile spikes per plant (not taking into account the aborted ones) obtained after the different salt treatments, to estimate seed production, in terms of total number of seeds and their total mass. Finally, germination tests were carried out by placing 100 seeds obtained after each treatment in Petri dishes, on filter paper moistened with distilled water (Table 1).

Plantago crassifolia showed a complex pattern of flower formation in the presence of increasing external NaCl concentrations (Fig. 1). The highest salt concentrations tested completely inhibited flower development: only two spikes were produced including all plants subjected to the 400 mM NaCl treatment, and not a single one in the presence of 500 mM NaCl; therefore, these plants were not analysed further. In the control and 100 mM NaCl treatments, flowering continued throughout the five months of the assay, whereas in 200 and 300 mM NaCl-treated plants it stopped after the first three months. Regarding the total number of spikes formed, in the 100 and 200 mM NaCl treatments most plants flowered, producing up to seven spikes per plant; they also showed the lowest percentages of aborted spikes (spikes without any seed). Fewer plants flowered in the control sample, producing at most four inflorescences per plant, thus resulting in a lower total number of spikes as compared to the former treatments; in addition, a higher fraction of aborted spikes was observed. Flower development was more severely affected in the 300 mM NaCl-treated plants, which produced a lower total number of spikes, with a much higher percentage of aborted ones (Fig. 1, Table 1). These

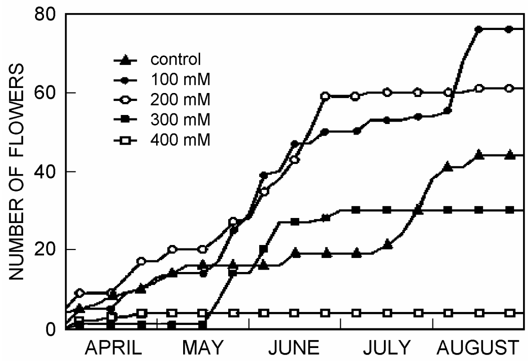

Fig. 1. Floral phenology of *Plantago crassifolia* plants in the presence of increasing NaCl concentrations. The accumulative number of flowers formed for each treatment was counted every 10 d, from April to August 2002.

Table 1. Effects of salt stress on the formation of reproductive structures, pollen development, seed set and seed viability in *Plantago crassifolia*. Absolute, percentage or mean values (± SD, where indicated) are shown for the different measurements.

NaCl [mM]	0 (control)	100	200	300
Spikes [total number]	44.00	76.00	61.00	30.00
Aborted spikes [%]	15.90	9.20	13.11	36.66
Fertile spikes [plant^{-1}]	2.47	4.60	3.53	1.27
Scape maximal length [cm]	45.00	34.50	25.00	17.00
Scape mean length [cm]	27.13 ± 1.70	19.35 ± 0.97	11.99 ± 0.98	6.06 ± 0.85
Spike maximal length [cm]	9.00	6.70	6.00	3.00
Spike mean length [cm]	4.37 ± 0.34	3.90 ± 0.20	2.73 ± 0.19	1.62 ± 0.14
Pollen viability [%]	95.13	87.47	85.08	79.93
Germinating pollen [%]	55.95	41.72	29.76	22.69
Mean seed number [spike^{-1}]	122.93 ± 8.28	81.85 ± 7.34	70.41 ± 5.70	29.83 ± 5.44
Mean seed mass [mg]	0.47 ± 0.06	0.40 ± 0.09	0.31 ± 0.08	0.23 ± 0.06
Mean seed mass [mg spike^{-1}]	58.26 ± 2.50	33.62 ± 3.30	22.72 ± 2.15	7.79 ± 1.63
Total seed number [plant^{-1}]	304.00	377.00	249.00	38.00
Total seed mass [mg plant^{-1}]	143.90	154.65	80.20	9.89
Seed germination [%]	97.00	97.00	97.00	56.00

data indicate that formation of fertile spikes is optimal at a moderate NaCl concentration of 100 mM, and still higher than in the control at 200 mM NaCl. However, measurements of other parameters, such as the length of floral scapes and spikes, showed that growth of reproductive structures is maximal in the absence of salt, being progressively inhibited with increasing NaCl concentrations (Table 1).

Pollen viability was also maximal, and very high (over 95 %), in control plants grown in the absence of NaCl, decreasing, albeit only slightly, with increasing salinity (Table 1). Pollen fertility, estimated by the percentage of germinating grains in the total pollen population, was strongly affected by salt, decreasing from 56 % in the non-treated plants, to less than 23 % for the 300 mM NaCl treatment (Table 1). It should be mentioned, that the *in vitro* germination medium developed for tobacco pollen (Wilson *et al.* 1997), was not optimised for germination of *P. crassifolia* pollen.

All parameters regarding seed production and quality (number of seeds per spike, mean seed mass, mean mass of seeds per spike) were maximum for plants grown under non-saline conditions, decreasing progressively with increasing NaCl concentrations. Only in the case of the 100 mM NaCl treatment (but not for the plants treated with 200 mM NaCl), the higher number of fertile spikes, as compared to the control, compensated the lower number and mass of seeds per spike, resulting in a higher total number, and also in a slightly higher total mass, of the seeds per plant. Despite differences in size, almost all seeds produced by control plants and by those grown in the presence of 100 or 200 mM NaCl were viable and germinated in water. Plants were still able to complete their life cycle in the presence of 300 mM NaCl, but this treatment was very detrimental to seed production: these plants not only produced fewer seeds and of a smaller size, as compared to the previous treatments; in addition, a significant fraction of them were not viable, and did not germinate in the absence of salt (Table 1).

The data presented here and those previous reported (Vicente *et al*. 2004), taken together, indicate that the reproductive phase of the life cycle of *Plantago crassifolia* is less sensitive to salinity than seed germination. "Reproductive success", estimated by the number and viability of the seeds obtained after the different treatments, was maximal in plants grown in the absence of salt, or in the presence of 100 mM NaCl, a concentration which strongly inhibited germination of seeds. On the other hand, vegetative growth appears to be more tolerant to salt than reproductive development, as it could be maintained, albeit at a reduced rate, in conditions of high salinity (400 - 500 mM NaCl) which completely inhibited seed production. However, these high salt concentrations can not be considered as physiological: in nature, *P. crassifolia* grows at the edges of salt marshes, where soil salinity is never so extreme. The behaviour of *P. crassifolia* plants observed in our experiments appears to be adapted to the conditions under which the plant grows in its natural habitats, as it correlates well with the ecology of the species. *P. crassifolia* blooms by the end of spring and in summer, just when the soil is dry and salt concentration is at its highest level, whereas seed germination occurs in autumn, after strong rainfalls have leached the salts from the soil.

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