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Research Article

Effects of salinity on pollen germination in wild and cultivated leguminous species

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Abstract

Plant sexual reproduction plays a crucial role in species persistence, land colonisation, and the enhancement of genetic diversity. Sexual reproduction in plants encompasses several key processes, including gametogenesis, fertilisation, zygote development, and fruit production. Environmental stresses can affect each of these processes, with male gametogenesis, pollen germination and tube elongation showing special vulnerability. We investigated the effects of salinity on pollen fitness across several species of Fabaceae, a large family which includes economically important crops. We first assessed the *in vitro* germination capacity of pollen from 14 different species and identified four distinct response patterns to salinity stress. A more detailed analysis was conducted on pollen germination and tube elongation in *Medicago marina* (dune habitats), *Spartium junceum* (Mediterranean scrub), and *Cicer arietinum* (cultivated). For *C. arietinum*, a positive correlation was observed between pollen germination and tube length, whereas no such correlation was found in the wild species. Furthermore, we examined the fitness of pollen produced by plants of *Medicago marina*, *Lotus creticus*, and *Ononis ramosissima* subjected to salt stress under greenhouse conditions. These species exhibited varying degrees of salt sensitivity, although the three are characteristic of dune habitats. In conclusion, our study reveals that pollen from different species within the Fabaceae family exhibits distinct responses to salinity stress, with variations in salt sensitivity amongst species. These findings contribute to a deeper understanding of how environmental stressors, particularly salinity, affect pollen fitness in plants adapted to different environments.

Keywords: Fabaceae; pollen germination; pollen tube length; reproductive fitness; salinity

Introduction

The Fabaceae family, with about 800 genera and 20,000 species, is the third largest of the angiosperms after Asteraceae and Orchidaceae but the second most economically important plant family after Poaceae (Hasanuzzaman *et al.*, 2020). Many of its species are of great economic importance as human and animal food due to their high protein and carbohydrate content, as well as raw materials for different industries.

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Leguminous species are important components of different plant communities in forests, shrublands and grasslands. They have a high ecological value in natural and agricultural ecosystems due to their unique ability to improve soil fertility by fixing atmospheric nitrogen through a symbiotic association with bacteria of the genus *Rhizobium*, in which the plant provides the medium for bacterial fixation in its roots, generating nodules and providing carbon as an energy source. Representatives of this cosmopolitan family occupy an enormous variety of habitats, including coastal dunes. Regardless of the geographical or climatic zone where dune systems develop, they have a set of environmental characteristics derived from their connection with the sea, which create an inhospitable living environment for most plants. Limiting environmental factors for coastal dune plants include wind, sand accumulation and erosion, substrate mobility, salty water spray and saline soils, water stress, highly permeable coarse-grained substrates with low field capacity, and nutrient poverty (Maun, 2009; Du and Hesp, 2020). The dune vegetation is dominated by a few species that have evolved a range of adaptations and responses to cope with the unique and restrictive conditions of this habitat, resulting in a specific coastal vegetation succession (Ruocco *et al*., 2014). Dune plant communities show a high functional diversity and complexity of adaptive strategies to withstand the harsh environmental conditions in this habitat type (Ciccarelli, 2015). Although taxonomically diverse, there are only a few families with exceptional relevance in the composition of dune plant communities, such as Poaceae and Fabaceae, the former with a role in dune fixation and the latter due to its ability to enrich the soil in nitrogen content. Consequently, introducing leguminous species has become a common practice in dune restoration efforts (Douglas *et al*., 2004).

In the littoral areas near Valencia (E Spain), three Fabaceae species are frequent and contribute to the structure of specific plant communities. *Medicago marina* L. is essential in the constitution of the association *Medicago marinae-Ammophiletum arundinaceae* Br.-Bl. (1931) 1933, which is a community of large perennial grasses contributing to the stabilisation of mobile dunes (Costa and Mansanet, 1981). In the Valencian territory, this community is also characterised by the high frequency of *Lotus creticus* L., a species characteristic of a different plant association, *Crucianelletum maritimae* Br.-Bl. (1931) 1933, where, together with another legume, *Ononis ramosissima* Desf., and other species such as *Helichrysum stoechas* (L.) Moench., *Teucrium dunense* Sennen, and *Crucianella maritima* L. constitute the primary biomass of this community (Costa and Mansanet, 1981).

Numerous studies have documented the effects of soil salinity on plants, including tissue necrosis, leaf abscission, reduced photosynthetic activity, inhibited growth, and altered biochemical responses (Farieri *et al*., 2016; van Puijenbroek *et al*., 2017; Toscano *et al*., 2022). However, only scarce information on its impact on floral development and reproductive organs is available (Berkheimer *et al*., 2006; Griffiths *et al*., 2006; Sánchez-Vilas and Retuerto, 2012). Pollen is the male gametophyte, which is transferred to the female gametophyte in the pollination process, followed by pollen tube elongation, double fertilisation, embryogenesis and seed production. Therefore, the impact of environmental stress, such as salinity, on pollen biology is of considerable interest as it can reduce or even prevent the sexual reproduction of plants. Indeed, early studies in *Petunia hybrida* (Reddy and Goss, 1971) indicated the susceptibility of pollen grains to the presence of salts in the germination medium. *In vitro* pollen germination assays in the presence of different concentrations of NaCl were proposed as a screening method for testing more tolerant genotypes (Tyagi and Rangaswamy, 1992; Soleimani *et al*., 2010; Khaleghi *et al*., 2019).

This study aimed to examine the impact of NaCl supplementation on *in vitro* pollen germination in wild leguminous species inhabiting coastal dunes and neighbouring habitats, compared to cultivated species of the same family. The central hypothesis posits that wild plants, which grow in harsher environmental conditions—including elevated salinity due to salt spray that can directly influence floral development—produce pollen with greater tolerance to salinity. Consequently, these wild plants are expected to exhibit higher *in vitro* pollen germination rates in the presence of NaCl relative to cultivated species. Additionally, this study

investigates whether closely related species have similar patterns of pollen germination and explores the potential effects of salt treatment during the reproductive stage on the germination capacity of pollen.

Materials and Methods

Plant material

Pollen was collected from six wild species of Fabaceae located in the littoral dunes and neighbouring areas in the Albufera Natural Park (Table 1 and Figure 1). Pollen grains were isolated from ten individual flowers per species collected from April to May 2024.

Table 1. Species sampled in the wild and their habitat type and distribution

Species	Habitat and distribution
Anthyllis cytisoides L.	On different soil types in different habitat types, including littoral dunes in W Mediterranean area (NW Africa, Iberian Peninsula, Balearic Isles, and France)
Coronilla juncea L.	Roadsides and stony soils, preferably calcareous, cleared scrubland, preferably on calcareous, clayey or loamy substrate in S Europe and NW Africa
Lotus creticus L.	Maritime sandy soils, sporadically inland, in S Europe, NW Africa, SW Asia, Macaronesia
Medicago marina L.	On maritime sands and dunes, coastal habitats throughout the Mediterranean
Ononis ramosissima Desf.	Coastal dunes and coastal scrub in W Mediterranean and the Canary Islands
Spartium junceum L.	Stabilised dunes, substitution scrub on basic soils, naturalised and cultivated in ditches and road dividers in the Circum-Mediterranean and Macaronesian area

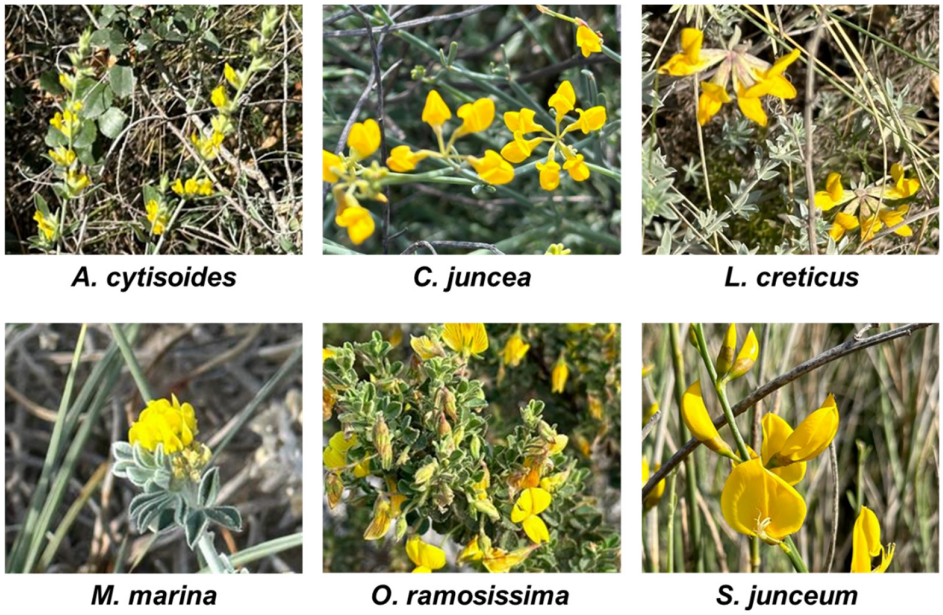

Figure 1. Photographs of flowers belonging to wild species collected in the Natural Park of Albufera, Valencia

In addition, pollen grains were collected from eight cultivated species from the agronomic experimental plot at the Universitat Politècnica de València, Spain (Table 2 and Figure 2).

Table 2. Cultivated species and their uses, and native distribution range

Species	Uses	Geographic area
Cicer arietinum L.	Human consumption	The second most important pulse in the world, native from Turkey and Middle East
Coronilla viminalis Salisb.	Forage and ornamental	Wild in Morocco and the Canary Islands
Lathyrus sativus L.	Forage	Cultivated in C and E Europe, SW Asia, India, N Africa, S America, and Australia, native from the Balkan Peninsula
Medicago arborea L.	Ornamental and soil stabilisation	S Europe, introduced in Africa, Asia, Australia and N America
Medicago sativa L.	Forage	Native of Crimea and Anatolia, cultivated and naturalised throughout the world
Ornithopus sativus Brot.	Forage	Mediterranean native and introduced to Africa, Australia and S America
Vicia incana Gouan	Edible and cover crop	Turkey and Central and S Europe
Vicia villosa Roth.	Forage and cover crop	Europe and Western Asia

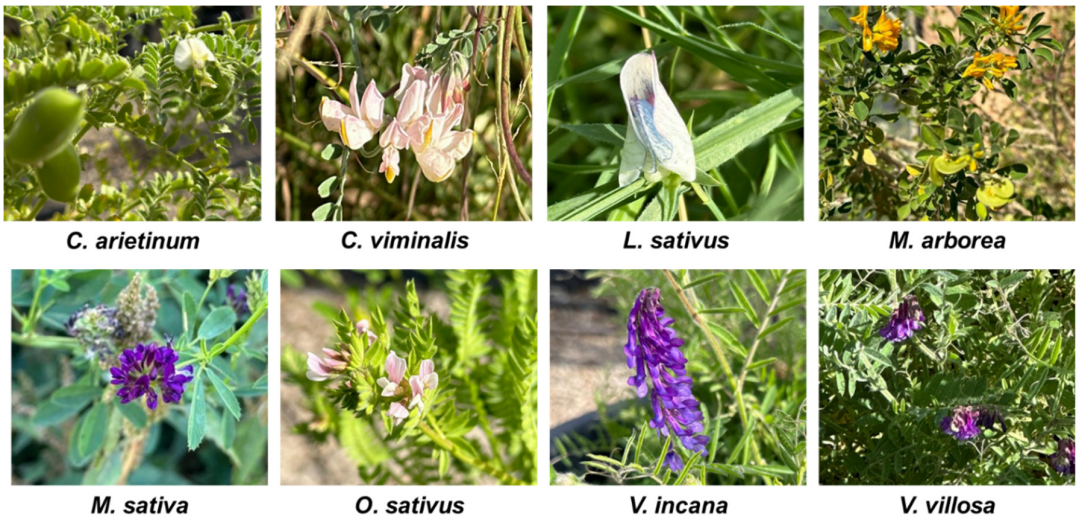

Figure 2. Photographs of flowers belonging to cultivated species grown on the experimental plot UPV

Greenhouse experiment

Three target species growing in dune habitats, namely *Lotus creticus*, *Medicago marina* and *Ononis ramossisima,* were subjected to salt stress under greenhouse-controlled conditions. Plants were obtained from seeds and grown in individual pots. Upon flowering, a watering solution containing 0 mM (control), 150 mM, or 300 mM NaCl was applied every three days. Pollen grains were collected from open flowers after one week of salt treatment. A total of five plants per treatment were used for each species.

In vitro germination assay

Pollen grains were collected from 10 flowers of each species to ensure a representative sampling. The pollen from these flowers was pooled to create a homogeneous sample for each species. Three technical replicates were prepared for each treatment from the pooled pollen. Pollen germination was assessed *in vitro* using a growth medium supplemented with different concentrations of NaCl to simulate varying salinity conditions. The germination rates were recorded and analysed to evaluate the effect of NaCl concentration on pollen viability and germination. Pollen grains were carefully isolated and placed into 2 mL of freshly prepared GK germination medium (Brewbaker and Kwack, 1963), with some modifications, containing 1.3 mM

$Ca(NO_3)_2$, 1 mM KNO_3, 3.2 mM H_3BO_3, 0.8 mM $MgSO_4$, and 0.29 M sucrose, and adjusted to a pH of 6. This pollen suspension was softly homogenised to ensure an even distribution of pollen grains.

The pollen suspension was then aliquoted into multi-well culture plates, with each well receiving 250 µL of the pollen suspension. Three wells were assigned to one of the four treatments: a control with no additional NaCl and experimental treatments with NaCl concentrations of 20 mM, 50 mM, and 100 mM. The NaCl was added to the GK medium before pollen introduction to ensure even exposure. The plates were incubated at room temperature in darkness.

On average, 500 pollen grains were counted to determine the germination frequency. Germination was defined as the emergence of a pollen tube at least as long as the diameter of the pollen grain. Data were analysed using ANOVA to determine the effects of NaCl concentration on germination rates and pollen tube length.

Pollen monitoring and measurements

Pollen germination and tube growth were determined with a Zeiss AXIOVERT 40 CFL Inverted Microscope. It took about 10 min to prepare the pollen suspension and proceed to the first microscopic observation of each sample (time 0), which was repeated after three, six and 24 hours of incubation. Image analysis was performed using Digimizer v.4.6.1 software (Med-Calc Software, Ostend, Belgium, 2005–2016).

Statistical analysis

Statgraphics Centurion XVI (Statgraphics Technologies, The Plains, VA, USA) and IBM SPSS Statistics - statistical software version 16 were used to conduct the statistical data analysis.

For assessing the effect of stress treatments on pollen germination and pollen tube length, a one-way analysis of variance (ANOVA) was employed. If the null hypothesis was rejected, the Tukey test was performed as a post hoc test with a p-value of 0.05 ($p < 0.05$) to examine the statistical significance of differences.

Results

Pollen germination

Germination of pollen from 14 Fabaceae was performed in the presence of increasing NaCl concentrations to determine to what extent salinity affects pollen germination rates, which are critical for plant reproductive success. The results are presented in a comparative analysis of pollen germination percentages at different NaCl concentrations (0, 20 mM, 50 mM and 100 mM) during three-time intervals (three, six and 24 hours).

Pollen germination rates in control treatments gradually increased over time, peaking at 24 hours in most species, except in *Medicago marina*, for which the highest germination rate was found at six hours. For most taxa studied, maximum germination rates exceeded 70%, except for *Medicago arborea*, *Ononis ramosissima*, and *Lathyrus sativus*, which recorded pollen germination rates at 24 hours of 16.2%, 26.9%, and 62.2%, respectively (Table 3). After three hours of incubation, pollen germination increased considerably in all studied species, reaching the highest values for *Vicia incana* (90.9%, Table 3), *Cicer arietinum* (75.1%, Table 3), and *Medicago sativa* (100%, Table 3). After six and 24 hours, the percentages of pollen germination generally increased but did not vary significantly with respect to those registered at three hours, except for *Lotus creticus,* where values at 24 hours more than doubled those from three hours.

In the absence of NaCl, a large variation in pollen germination percentages was observed, even for plants belonging to the same genus. For instance, a 100% germination rate was registered upon six hours of incubation of pollen grains isolated from *Medicago sativa*, whereas germination rates of 11.9 and 16.2 upon six and 24 h, respectively, were observed for *Medicago arborea* pollen. Moreover, pollen collected from *Vicia incana* rapidly activated germination (85.5% at time 0, Table 3); that was not the case for *Vicia villosa* pollen (no germination

observed at time 0, Table 3). Large differences between germination percentages at time 0 were also observed in other two congeners, *Coronilla viminalis* (69%, Table 3) and *C. juncea* (0%, Table 3). These observations indicate a different behaviour of pollen during *in vitro* germination, not only within species belonging to the same family but also to the same genus.

Table 3. Pollen germination percentages of the studied species at each NaCl tested concentration

Species	Time (h)	NaCl concentration			
		0 mM	20 mM	50 mM	100 mM
Anthyllis cytisoides (W)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	53.1 ± 10.4 bcde	69.6 ± 9.8 de	15.3 ± 2.9 ab	56 ± 9.9 cde
	6	54.5 ± 10.4 cde	50 ± 9.1 bcde	15.7 ± 1.7 ab	45.4 ± 12.5 bcde
	24	76.4 ± 0.6 e	77.1 ± 0.8 e	36.8 ± 3.9 bcd	28.9 ± 7.2 abc
Cicer arietinum (C)	0	58.2 ± 5.6 cde	63.8 ± 2.8 cde	37 ± 0.7 ab	21.2 ± 1.1 a
	3	89.2 ± 8.6 e	65.9 ± 4.6 cde	48.4 ± 1.8 bcd	46.7 ± 7.7 bcd
	6	87.4 ± 2.6 e	68.6 ± 4.7 de	65.5 ± 2.7 cde	47 ± 5.9 abc
	24	75.1 ± 8.4 e	67.9 ± 2.3 cde	65.5 ± 2.7 cde	44.4 ± 5.7 bcd
Coronilla juncea (W)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	69.3 ± 6.7 e	29.5 ± 2.5 cd	16.8 ± 4.7 bc	1.6 ± 0.2 a
	6	76.7 ± 4 e	39 ± 7.3 d	19.9 ± 0.9 c	1.6 ± 0.7 ab
	24	77.9 ± 0.8 e	37.9 ± 0.6 d	17.7 ± 0.4 c	1.1 ± 0.1 a
Coronilla viminalis (C)	0	69 ± 2.5 bcd	58 ± 3.7 b	51.5 ± 0.7 b	7.9 ± 1.1 a
	3	85.5 ± 4.5 cde	88 ± 3.8 e	63.6 ± 9.9 bc	9.8 ± 4.6 a
	6	92.7 ± 1.7 e	89.2 ± 2.6 e	61.7 ± 3.6 b	4.6 ± 0.8 a
	24	92 ± 2.4 e	85.8 ± 3 de	65.9 ± 3.9 bc	6.2 ± 1.3 a
Lathyrus sativus (C)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	50.7 ± 5.9 bc	35.8 ± 5.2 b	47.4 ± 1.7 bc	6.8 ± 1.2 a
	6	54 ± 6.5 bc	50 ± 3.6 bc	48.1 ± 7 bc	10.6 ± 0.1 a
	24	62.2 ± 0.6 c	53.7 ± 1.3 bc	47.3 ± 9.4 bc	11.7 ± 0.5 a
Lotus creticus (W)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	37.8 ± 6.8 bc	32.6 ± 6.6 bc	28.5 ± 3 b	0 ± 0 a
	6	71.6 ± 6.9 e	48.6 ± 6.9 cd	50.7 ± 8.3 bcd	0 ± 0 a
	24	86 ± 2.6 e	80.7 ± 1.3 e	73.5 ± 3.3 de	0 ± 0 a
Medicago arborea (C)	0	5.4 ± 0.1 abc	4.8 ± 0.6 abc	4.5 ± 0.8 ab	2.1 ± 0.7 a
	3	13.7 ± 1.6 abc	12.6 ± 0.2 abc	11 ± 0.8 abc	3.9 ± 0.5 ab
	6	11.9 ± 4.3 abc	21.3 ± 11.6 c	18.3 ± 0.6 abc	4.2 ± 0.9 ab
	24	16.2 ± 8.2 abc	23.8 ± 10.3 bc	21.7 ± 0.2 abc	4.6 ± 1.2 ab
Medicago marina (W)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	69 ± 6 cde	53.5 ± 0.9 bcde	58 ± 3.4 bcde	29.5 ± 6.2 ab
	6	80.7 ± 6.2 e	86.6 ± 5.4 e	89.3 ± 10.4 e	40.9 ± 12.1 bcd
	24	73.3 ± 3.5 de	76.3 ± 3.2 de	77.9 ± 6.1 de	35 ± 10.1 abc
Medicago sativa (C)	0	14 ± 0.8 ab	52.4 ± 10.2 bcdef	23.5 ± 4.3 ab	3.4 ± 0.5 a
	3	89.6 ± 7.8 g	78.8 ± 2.9 efg	59 ± 13.2 cdefg	39.2 ± 10 bcde
	6	100 ± 0 g	75.4 ± 8 defg	71 ± 9 defg	34.8 ± 2.7 abc
	24	100 ± 0 g	80 ± 9.6 fg	73.6 ± 10.5 efg	38.9 ± 6.8 abcd
Ononis ramosissima (W)	0	3.5 ± 1.5 ab	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	37.5 ± 8.4 cde	42.4 ± 3.8 de	44.4 ± 7.9 de	9 ± 1.6 abc
	6	50.3 ± 10.5 e	38.6 ± 5.4 de	34.6 ± 3.4 bcde	14.8 ± 1.3 abcd
	24	26.9 ± 13.9 cde	47.4 ± 8.9 de	27.8 ± 2 abcde	21.1 ± 1.8 abcd
Ornithopus sativus (C)	0	3.6 ± 0.9 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	82.6 ± 5.4 cd	43.4 ± 6.1 b	62.1 ± 3.4 bc	53.2 ± 3.7 b
	6	90.5 ± 4.5 d	56.9 ± 9.6 b	56 ± 3.9 b	52.2 ± 3.7 b
	24	91 ± 2.9 d	52.7 ± 6.7 b	53 ± 2.9 b	53.7 ± 7.1 b
Spartium junceum (W)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	69.2 ± 4.9 ef	73 ± 5.3 ef	50.1 ± 5.6 bcd	31.4 ± 6.8 bc
	6	85.7 ± 4.9 f	77 ± 6.6 ef	70.2 ± 2.5 def	33.4 ± 3.9 b

	24	86.6 ± 2.2 f	80.4 ± 6.3 f	80.9 ± 3.5 ef	60.2 ± 4.5 cde
Vicia incana (C)	0	85.5 ± 3 cd	75.2 ± 7.3 cd	75.1 ± 5.3 cd	13.1 ± 3.2 a
	3	90.1 ± 3.1 cd	87.3 ± 5.5 cd	72.2 ± 0.6 c	24 ± 4.2 ab
	6	87.3 ± 3.5 cd	89.5 ± 4 cd	83.5 ± 3.5 cd	34.6 ± 5.5 b
	24	90.9 ± 3.7 d	88.8 ± 2.2 cd	85.5 ± 1.6 cd	35.6 ± 3.7 b
Vicia villosa (C)	0	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
	3	79.4 ± 3.9 e	56 ± 7.6 c	55.3 ± 6.5 cd	24.5 ± 3.1 b
	6	81.2 ± 1.7 e	75 ± 7.2 de	82.7 ± 0.8 e	41.5 ± 2.3 bc
	24	81.6 ± 0.5 e	76.9 ± 2.9 de	82.9 ± 0.8 e	44.5 ± 2.4 c

Values shown are means per plate ± SE; n = 3. Different lowercase letters indicate significant differences between treatments for each species, according to the Tukey test ($p < 0.05$). Abbreviations: (C) cultivated, (W) wild.

The presence of salt in the germination medium generally resulted in a dose-dependent reduction of pollen germination rates at 24 hours of incubation for all studied species. The lowest salt concentration (i.e., 20 mM) had no or mild negative effect on the germination rate of pollen of most species, except for *Coronilla juncea* and *Ornithopus sativus,* for which a reduction of 42.1 and 51.4%, respectively, was registered when the germination medium was supplemented with 20 mM NaCl. However, the intermediate salt concentration (50 mM NaCl) had a clear inhibitory effect on the germination of pollen belonging to all species tested, although these differences were statistically significant only in *Anthyllis cytisoides*, *Cicer arietinum*, *Coronilla juncea*, *C. viminalis* and *Ornithopus sativus*. Finally, the addition of 100 mM NaCl to the germination medium resulted in a dramatic reduction of pollen germination in *Lotus creticus, Coronilla juncea*, *C. viminalis*, *Lathyrus sativus* and *Medicago marina*, with germination rates lower than 10%. In contrast, some species maintained high pollen germination rates under high saline conditions, such as *Ornithopus sativu*s, with more than 50% at all incubation times, followed by *Cicer arietinum*, *Anthyllis cytisoide*s at short incubation times (three and six hours), and *Spartium junceum*, with more than 60% of germinated pollen upon 24 hours of incubation (Table 3).

For a better understanding of the response to the presence of NaCl in the germination medium, ratios between pollen germination in the salt treatments relative to the control were calculated for each species and incubation period. As the germination percentages after three and six hours of incubation were very similar, ratios were only calculated for times three and 24 hours. These ratios were graphically represented as trend graphs, each containing information about several species presenting similar patterns at three (Figure 3) and 24 hours (Figure 4) incubation times.

After three hours of incubation, four patterns of response were observed. First, a dose-dependent reduction of pollen germination was observed for *Cicer arietinum*, *Coronilla juncea, Lotus creticus, Medicago arborea,* and *M. sativa*, although the severity was different in each species (Figure 3A). Pollen harvested from *C. juncea* was the most sensitive to salinity since low (i.e., 20 mM NaCl) salt concentration resulted in a 60% decrease in the germination rate and complete inhibition of germination when the medium was supplemented with 100 mM NaCl (Figure 3A). The reduction in germination of pollen belonging to the other species was gradual with increasing salt concentration, with *Lotus creticus* pollen being severely affected under high salt conditions (100 mM NaCl) (Figure 3A). For pollen isolated from *Cicer arietinum*, *Medicago arborea,* and *M. sativa*, the lowest germination ratios were detected at 100 mM NaCl: 0.5, 0.3 and 0.4, respectively (Figure 3A).

A second pattern observed was the absence of an inhibitory effect on pollen germination in media supplemented with 20 mM NaCl and a further decline in germination at 50 and 100 mM NaCl. This pattern was observed for *Coronilla viminalis* and *Vicia incana*, for which the lowest germination ratio was measured as 0.1 and 0.3, respectively, at 100 mM NaCl (Figure 3B). Pollen of both species behaved similarly in their response to the presence of NaCl in the germination media. Furthermore, the data obtained for *Lathyrus sativus*, *Medicago maritima, Ornithopus sativus* and *Vicia villosa* pollen showed a stronger inhibitory effect on their germination by low salinity conditions (20 mM NaCl) than by higher salinity treatments (i.e., 50 mM

NaCl). For all four species, the inhibitory effect of the highest applied salinity (100 mM NaCl) was variable, with the lowest observed for *Ornithopus sativus* (0.6; Figure 3C) and the strongest for *L. sativa* (0.1; Figure 3C). Finally, an unexpected increase in germination was observed for pollen incubated in a medium supplemented with 20 mM NaCl in *Anthyllis cytisoides*, *Ononis ramosissima* and *Spartium junceum* (Figure 3D). Reduced germination ratios at the highest NaCl concentration tested (100 mM) were observed for *Spartium junceum* and *Ononis ramosissma*, being 0.5 and 0.2, respectively, whereas the calculated pollen germination ratio for *Anthyllis cytisoides* was 1.1 (Figure 3D), indicating an enhancement of pollen germination by high salinity in this species.

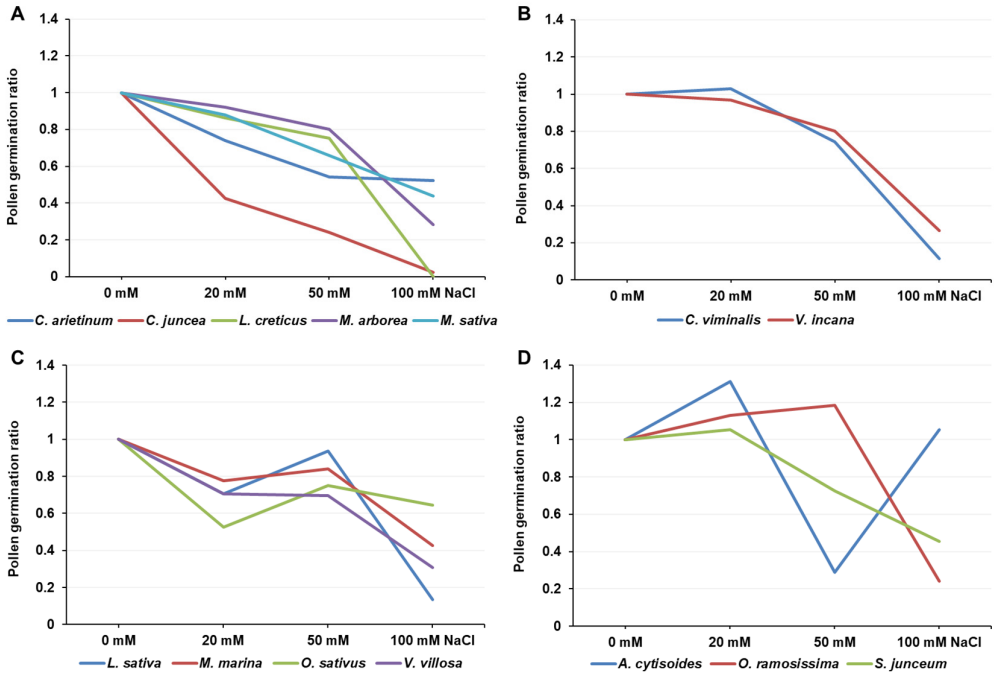

Figure 3. Trends in pollen germination after 3 hours of incubation on media with different NaCl concentrations

Values represent ratios between percentages in media with NaCl relative to the control. Each panel includes ratio values registered for several species with similar trends

In addition, ratios were calculated from the data recorded after 24 hours of pollen incubation in increasing salt concentrations relative to control media, and four trends were observed (Figure 4). The ratios calculated for *Coronilla juncea*, *C. viminalis*, *Medicago sativa* and *Ornithopus sativus* indicate a dose-dependent inhibitory effect of salt on the germination of pollen collected from these species (Figure 4A), although the effect at different concentrations was species-dependent. Among these, *Coronilla juncea* pollen was the most salt-sensitive, with complete inhibition of germination at 100 mM NaCl, and the least sensitive pollen was that harvested from *Ornithopus sativus* (Figure 4A).

A second detected trend was pollen insensitivity to low salt concentrations (20 mM NaCl) and reduced germination at higher salinity. This trend was observed for pollen harvested from *Anthyllis cytisoides* and *Vicia incana* (Figure 4B). Pollen from both species showed a 60% reduction of pollen germination at the highest salt concentration tested, as a ratio of 0.4 was estimated (Figure 4B). A similar trend was observed for *Cicer arietinum*, *Lotus creticus*, *Medicago marina*, *Spartium junceum* and *Vicia villosa*. However, for pollen collected from these species, germination was not affected at both low (20 mM) and medium (50 mM) NaCl concentrations (Figure 4C). The highest concentration tested (100 mM) resulted in a decrease in germination

compared to germination on the control medium; *Lotus creticus* pollen was the most affected, with complete inhibition of germination under such conditions (Figure 4C). The ratios calculated for the other four species ranged from 0.5 to 0.7 (Figure 4C). Finally, as observed for the data obtained at 3 hours of incubation, pollen germination of *Medicago arborea* and *Ononis ramosissim*a was favoured by low salt concentrations (20 mM NaCl), with calculated ratios of 1.5 and 1.8, respectively (Figure 4D). High salt concentrations resulted in reduced pollen germination of both species, with calculated ratios of 0.3 and 0.8, respectively.

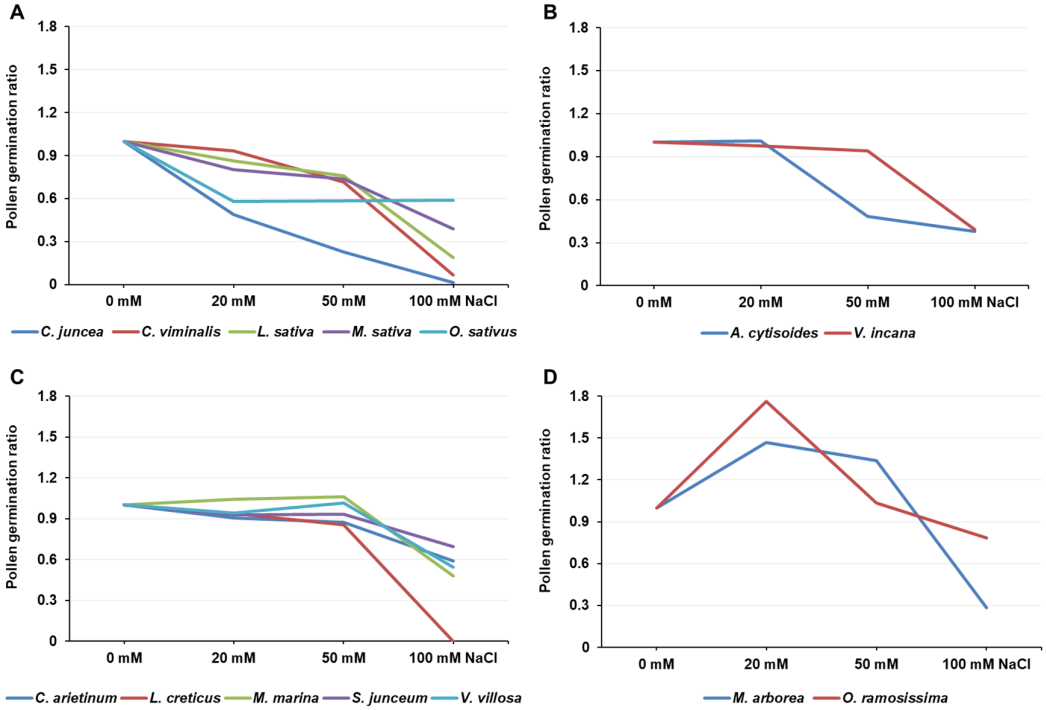

Figure 4. Trends in pollen germination after 24 hours of incubation on media with different NaCl concentrations

Values represent ratios between percentages in media with NaCl relative to control. Each panel includes ratio values registered for several species with similar trends

Pollen tube length after three hours of incubation

To further analyse the inhibitory effect of salt on pollen behaviour, pollen tube length was monitored. The effect of salinity on pollen tube length under such conditions may result from delayed germination, reduced pollen tube growth, or both. The exact effect of saline media during pollen germination is difficult to assess, but it gives an idea of the salt sensitivity of pollen belonging to different species.

Pollen tubes were very long and difficult to measure individually in most cases at 24 hours of incubation; therefore, tube lengths were recorded at three hours of incubation, as indicated in the Materials and Methods section. Furthermore, in most cases, pollen germinated at a reasonable rate at this time point at all salt concentrations, except in the case of *L. creticus* incubated in the presence of 100 mM NaCl, which allowed accurate and consistent measurement of pollen tube lengths. In addition, the three-hour window minimises the risk of contamination, which can become a significant problem at longer incubation times. At this stage, pollen tubes are less likely to suffer from nutrient depletion or osmotic stress, factors that could affect their growth and skew the results. Measurements were made under control conditions and in three NaCl treatments (20 mM, 50 mM and 100 mM) to determine the extent to which increased salinity inhibits pollen tube elongation. Measurements of pollen tube length in the 14 Fabaceae species after three hours of incubation under different NaCl concentrations are shown in Table 4. Increasing NaCl concentrations generally result in

significant reductions in pollen tube length in all Fabaceae species studied, with some showing a more pronounced sensitivity to salinity than others. Pollen tube length varied considerably amongst species in response to NaCl treatments. Some species showed a dose-dependent reduction in pollen tube length. For example, *Cicer arietinum* showed significant sensitivity to NaCl, with pollen tube length halved under 20 mM NaCl (126.5 µm versus 228.5 µm) and decreased to 48.1 and 42.1 µm at 50 and 100 mM NaCl, respectively. *Medicago sativa* and *Coronilla viminalis* showed similar trends (Table 4). Under control conditions, *Anthyllis cytisoides* exhibited the longest pollen tube (612.1 µm), slightly increasing at 20 mM NaCl (620.7 µm). However, a significant decrease in pollen tube length was observed at higher salt concentrations, with significant reductions to 116.6 µm at 50 mM and 96.1 µm at 100 mM NaCl. Similarly, pollen from *Coronilla juncea, Medicago arborea, M. marina, Ononis ramosissima* and *Ornithopus sativus* showed a significant decline in its tube length at medium and high salt concentrations (i.e., 50 and 100 mM NaCl, respectively), whereas it was not significantly affected under low salinity (20 mM NaCl; Table 4).

Finally, pollen tube length was slightly affected at low and medium salt concentrations in *Lathyrus sativus*, *Lotus creticus*, *Spartium junceum Vicia incana* and *V. villosa* (Table 4). However, when incubated in a medium with high (100 mM NaCl) salt concentration, the pollen grains belonging to these species showed a substantial reduction in tube length (Table 4). Interestingly, pollen sampled from *Spartium junceum* showed longer tube lengths under low and mild saline conditions (216.8 and 194.6 µm, respectively, *versus* 176.7 µm under control conditions), and highly saline conditions (100 mM NaCl) only reduced pollen tube length by 30% with respect to the control (125.3 µm; Table 4).

Table 4. Pollen tube length (µm) of the studied species after 3 hours of incubation

Species	Control	NaCl concentration		
		20 mM	50 mM	100 mM
Anthyllis cytisoides (W)	612.1 ± 6.6 b	620.7 ± 9.8 b	116.6 ± 10.9 a	96.1 ± 6.9 a
Cicer arietinum (C)	228.5 ± 8.3 c	126.5 ± 10.4 b	48.1 ± 0.8 a	42.1 ± 0.8 a
Coronilla juncea (W)	184.5 ± 10.3 c	199.1 ± 8.8c	85.2 ± 8.6 b	24.2 ± 0.9 a
Coronilla viminalis (C)	380.1 ± 8.2 d	260.3 ± 6.1 c	228 ± 6 b	57.7 ± 1.5 a
Lathyrus sativus (C)	142.7 ± 3.6 c	117.5 ± 4.3 b	114.6 ± 1.9 b	48.2 ± 8.5 a
Lotus criticus (W)	178.8 ± 11 b	183.8 ± 13.5 b	165.5 ± 12.9 b	0 ± 0 a
Medicago arborea (C)	331.7 ± 3.7 c	353 ± 9.7 c	192.7 ± 6.7 b	100.6 ± 5.8 a
Medicago marina (W)	256.3 ± 3.4 c	268.4 ± 6.5 c	147.3 ± 5.8 b	47.7 ± 5 a
Medicago sativa (C)	539.3 ± 10.6 d	447.6 ± 8.6 c	271.2 ± 8.4 b	40.5 ± 6.6 a
Ononis ramosissima (W)	150.5 ± 6 c	132.9 ± 2.3 c	96.9 ± 6.1 b	32.6 ± 5.2 a
Ornithopus sativus (C)	310.6 ± 10.5 c	294.4 ± 3.2 c	213.8 ± 5.1 b	83.8 ± 2.1 a
Spartium junceum (W)	176.7 ± 10.9 b	216.8 ± 4.1 c	194.6 ± 6.1 bc	125.3 ± 8.1 a
Vicia incana (C)	126.4 ± 10.4 bc	97.3 ± 3.3 b	128.5 ± 7.3 c	47.7 ± 0.7 a
Vicia villosa (C)	88.8 ± 4.6 c	65.4 ± 6 b	69.8 ± 3.4 bc	33.5 ± 3.1 a

Values shown are means per plate ± SE; n = 3. Different lowercase letters indicate significant differences between treatments for each species, according to the Tukey test ($p < 0.05$). Abbreviations: (C) cultivated, (W) wild.

Comparative analysis of three species from different habitats

To assess the possible relationship between species adapted to different habitats and responses to salinity during their *in vitro* pollen germination, three species were compared: *Medicago marina* (dune habitat), *Spartium junceum* (Mediterranean scrub) and *Cicer arietinum* (cultivated). *In vitro* pollen germination and pollen tube length were compared at three hours of incubation in media with increasing salt concentrations and the control without NaCl (Figures 5-7).

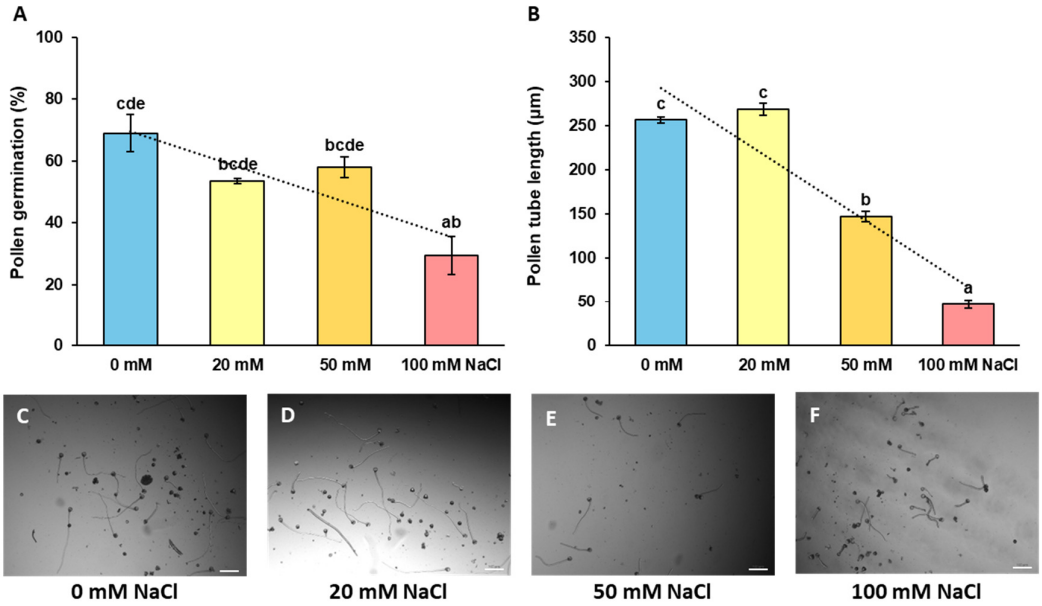

Figure 5. Pollen behaviour of *Medicago marina* after three hours of incubation in control medium and media supplemented with increasing salt concentrations. A. Graphical representation of pollen germination rates in control and media supplemented with increasing salt concentrations, as indicated. B. Graphical representation of pollen tube length in control and media supplemented with increasing salt concentrations, as indicated. C-F. Representative microscopy images of pollen incubated in different media used to collect the data shown in panels A and B

A-B: values shown are means per image ± SE; n = 3. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$). C-F: scale bar 100 µm

Medicago marina showed a significant decrease in pollen germination percentages from 69% under control conditions to 29.5% in the presence of 100 mM NaCl (Figures 5 A, C, F), whereas low (i.e., 20 mM NaCl) and mild (i.e., 50 mM NaCl) salinities resulted in a slight reduction of the germination rate to 53.5 and 58%, respectively (Figures 5 A, D, E). Accordingly, treatment with 100 mM NaCl resulted in a severe decrease in pollen tube length compared to the control (47.5 µm vs. 256.3 µm; Figures 5B, C, F), whereas no significant changes were observed when pollen was incubated in the medium supplemented with 20 mM NaCl (Figures 5 B, D). Interestingly, a mild salt concentration of 50 mM NaCl, although it did not affect the germination rate as mentioned above, had a significant effect on pollen tube length, with a reduction of pollen tubes down to 147.3 µm (Figures 5 B, E). This observation points to a different effect of salinity on both processes, *in vitro* pollen germination and pollen tube growth during *in vitro* incubation, which could result from an uncoupled regulation of both responses. Thus, pollen collected from *M. marina* could withstand low salt concentrations during i*n vitro* culture, but high concentrations caused the arrest of both, germination and tube growth.

On the other hand, pollen from *Spartium junceum* was unresponsive to low salinity during germination, as germination rates under 20 mM NaCl (73%) were comparable to those observed under control (69.2%) conditions (Figure 6 A, C, D). In contrast, higher salt concentrations caused a dose-dependent reduction in germination, with rates declining to 50.1% and 31.4% under 50 and 100 mM NaCl, respectively (Figure 6 A, E, F). However, low and moderate NaCl concentrations did not affect pollen tube growth substantially. Notably, the lowest concentration (20 mM NaCl) even resulted in a significant increase in tube length compared to the control (216.8 µm vs. 176.7 µm; Figure 6 B, C, D), and concentrations up to 50 mM NaCl did not negatively impact tube length (194.6 µm; Figures 6 B, E) after three hours of incubation. The highest concentration tested (100 mM NaCl) caused a significant reduction in pollen tube growth, which decreased to

125.3 µm (Figures 6 B, F). These results indicate that pollen from *S. junceum*, adapted to Mediterranean scrublands, is tolerant to low and moderate salinity, particularly concerning pollen tube growth. Although NaCl concentrations up to 100 mM strongly inhibit pollen germination, their impact on pollen tube growth is less pronounced. Interestingly, low salt concentrations appear to stimulate pollen tube growth (Figure 6 B).

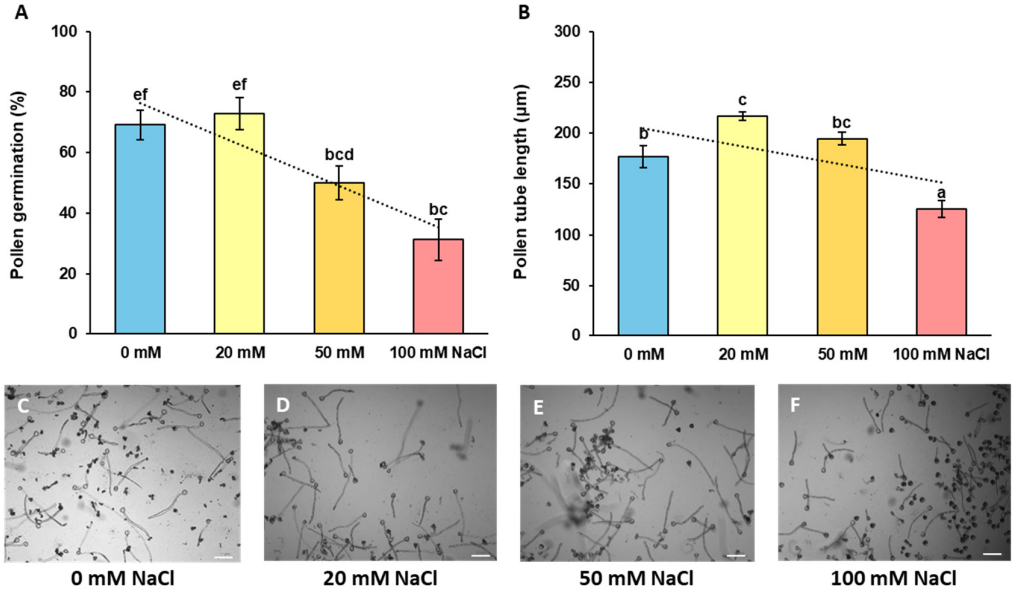

Figure 6. Pollen behaviour of *Spartium junceum* after three hours of incubation in control medium and media supplemented with increasing salt concentrations. A. Graphical representation of pollen germination rates in control and media supplemented with increasing salt concentrations, as indicated. B. Graphical representation of pollen tube length in control and media supplemented with increasing salt concentrations, as indicated. C-F. Representative microscopy images of pollen incubated in different media used to collect the data shown in panels A and B

A-B: values shown are means per image ± SE; n = 3. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$). C-F: scale bar 100 µm

Finally, data collected for pollen harvested from the cultivated species *Cicer arietinum* was used for this analysis (Figure 7). Pollen from *C. arietinum* showed the highest *in vitro* germination rate of all three species tested under control conditions (89.2%, Figures 7 A, C). Both pollen germination rate and tube length were severely affected by salinity. Salt presence in the germination media caused a reduction in the germination rate already at 20 mM NaCl (65.9%, Figures 7A, D), which reached minimum values at 50 mM NaCl (48.4%, Figure 7 A, E). Higher salt concentrations up to 100 mM NaCl did not cause a further significant reduction of the pollen germination rate (46.7%; Figures 7 A, F). Similarly, low salt concentration almost halved pollen tube length with respect to control (126.5 µm *versus* 228.5 µm; Figures 7 B, C, D), and minimum values for pollen tube length were obtained in germination media supplemented with 50 (48.1 µm) and 100 mM NaCl (42.5 µm; Figures 7 B, E, F). These results indicate that the cultivated chickpea is the most susceptible to saline stress among the three studied species, with both pollen germination and pollen tube length significantly reduced by low salt concentrations in the germination medium.

Altogether, these results suggest that plants of the Fabaceae family, adapted to wild environments, such as Mediterranean scrubland and dune habitats, exhibit greater resilience in terms of reproductive fitness, as indicated by pollen behaviour, compared to cultivated plants of the same family.

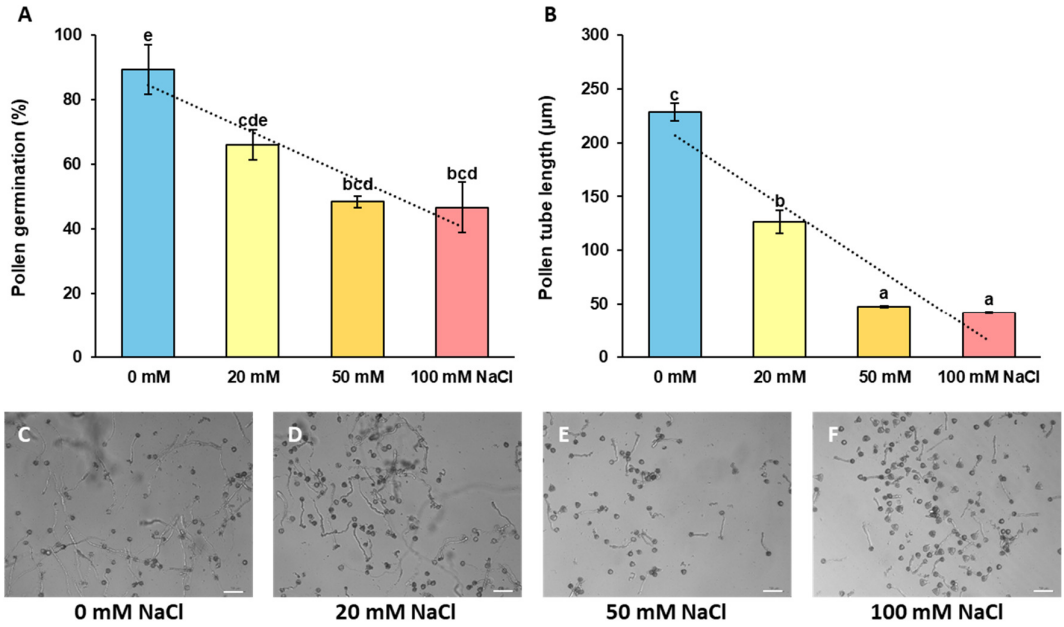

Figure 7. Pollen behaviour of *Cicer arietinum* after three hours of incubation in control medium and media supplemented with increasing salt concentrations. A. Graphical representation of pollen germination rates in control and media supplemented with increasing salt concentrations, as indicated. B. Graphical representation of pollen tube length in control and media supplemented with increasing salt concentrations, as indicated. C-F. Representative microscopy images of pollen incubated in different media used to collect the data shown in panels A and B

A-B: values shown are means per image ± SE; n = 3. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$). C-F: scale bar 100 µm

Viability and germination of pollen harvested from salt-stressed plants

The *in vitro* pollen behaviour presented in this study reveals distinct differences in the salinity responses of the gametophyte haploid phase across several *Fabaceae* species adapted to different environments. While salinity is a factor that can influence both pollen germination and tube elongation during pollination, particularly in coastal landscapes, the primary limitation to the reproductive fitness of plants is the presence of salt in soils or irrigation water. To further investigate the plants' responses to salt stress at the reproductive phase, pollen viability and germination rates were assessed in grains harvested from plants irrigated with solutions of increasing salt concentration. Three species—*Medicago marina*, *Lotus criticus*, and *Ononis ramosissima*—were selected for this analysis due to their relevance in littoral dune ecosystems and their importance for ecological restoration. Five plants of each species, grown individually in pots in a greenhouse, were watered with tap water supplemented with 0, 150, or 300 mM NaCl, beginning when the first flower buds appeared. After one week, pollen was collected from these plants and germinated in a modified GK medium without the addition of salt.

The viability of pollen belonging to *Medicago marina* and *Lotus creticus*, was unaffected by salinity. Pollen viability observed for *M. marina* was slightly higher when plants were watered with 300 mM NaCl (84.8%) than when harvested from control plants (73. 8%, Figure 8A). Pollen from *L. creticus* presented the highest viability under all conditions tested, with values of 100, 99.2 and 96.1% when plants were watered with control, 150 NaCl and 300 mM NaCl solutions, respectively (Figure 8A). For pollen harvested from *Ononis ramosissima*, however, a decrease in viability was observed when plants were watered with tap water supplemented with 300 mM NaCl (50%), compared to control plants (96,4%, Figure 8A). Thus, pollen

viability was differently affected by one-week of plant growth under saline conditions, in the three species studied.

Pollen harvested from *Medicago marina* exhibited rapid germination, with images taken at time 0 showing already germinated pollen under both control and 150 mM NaCl conditions. However, higher salt concentrations (300 mM NaCl) inhibited rapid germination (Figure 8B). Pollen from control plants reached a maximum germination rate of 68.7% after three hours of incubation, which was comparable to the germination rates observed at six hours (70.3%) and 24 hours (72.4%) (Figure 8B). In contrast, saline conditions applied to *M. marina* plants, with 150 mM and 300 mM NaCl, resulted in a reduction in germination rates to 39.3% and 37.5%, respectively, after three hours of incubation, and these reductions were maintained over longer incubation periods (e.g., 24 hours) (Figure 8B). These results suggest that salinity negatively affects pollen fitness in *M. marina*, potentially reducing its reproductive potential. The most pronounced decrease in pollen fitness occurred at the lower salt concentration (150 mM NaCl) and was sustained even at the highest concentration tested (300 mM NaCl) (Figure 8B).

Pollen harvested from *Lotus creticus* plants watered with tap water showed the highest germination rate at 24 hours (96.9%, Figure 8C), and 150 mM NaCl had no significant effect on its germination potential (96.2%, Figure 8C). However, higher salt concentrations resulted in a reduction in pollen germination compared to control plants, with germination rates of 59.4% versus 77.4% at three hours, 76.3% versus 63.7% at six hours, and 82.5% versus 96.7% at 24 hours (Figure 8C). Thus, pollen germination from *L. creticus* plants watered with 300 mM NaCl was reduced, but no such effect was observed for pollen from plants watered with 150 mM NaCl compared to control plants.

Finally, pollen germination rates for *Ononis ramosissima* plants watered with tap water were nearly 100% after three hours of incubation (98.5%), and these rates were sustained at six hours (97.5%) and 24 hours (96.7%) (Figure 8D). Irrigation with a 150 mM NaCl solution had a mild negative impact on pollen germination, with rates of 93.6%, 89.3%, and 86.7% at three, six, and 24 hours of incubation, respectively (Figure 8D). At higher salinity (300 mM NaCl), the reduction in pollen germination was more pronounced, with rates of 75.9%, 86.2%, and 86.2% at 3, 6, and 24 hours, respectively (Figure 8D).

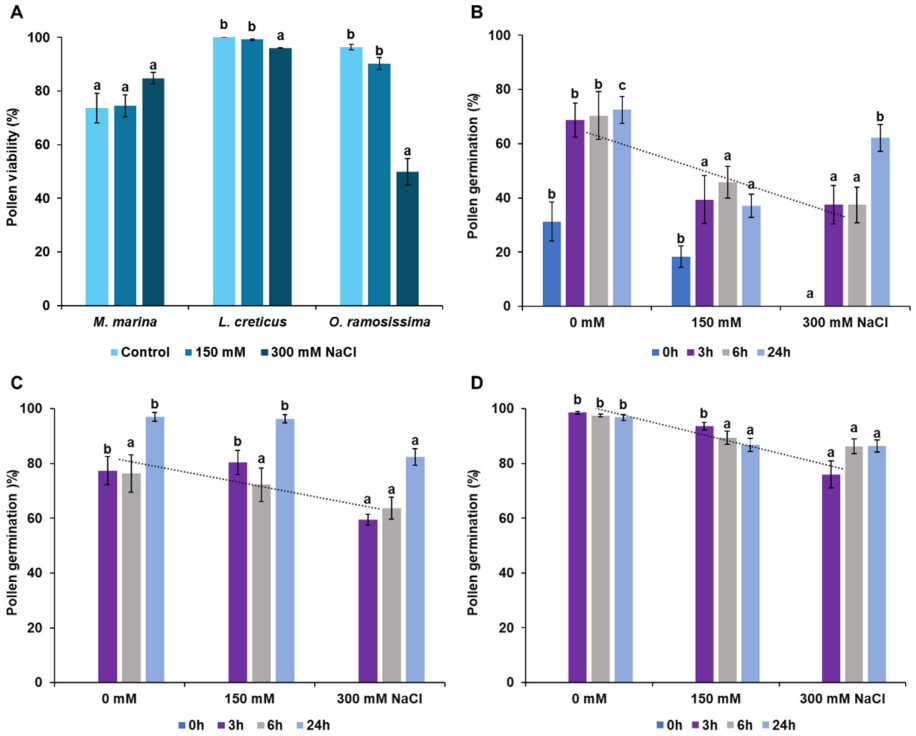

Figure 8. Effect of salt stress on pollen viability and germination of pollen harvested from the dune species *Medicago marina, Lotus creticus* and *Ononis ramosissima*. A. Pollen viability of different species, as indicated, measured with FDA-staining. B-D. Germination percentages of pollen harvested from *M. marina* (B), *L. creticus* (C), and *O. ramosissima* (D) at different incubation times (0, 3, 6 and 24 hours) and salt concentration (0, 150 and 300 mM NaCl) in irrigation solution, as indicated

Values shown are means per plate ± SE; n = 5. Different lowercase letters indicate significant differences between treatments for each determined variable, according to the Tukey test ($p < 0.05$)

Effect on pollen tube growth harvested from salt-stressed plants

To further investigate the impact of salinity during irrigation on pollen fitness, the effect of salt treatment on *in vitro* pollen tube length was examined. Pollen tube length was measured after 3 hours of incubation for pollen grains harvested from plants watered with either control solutions or salt-treated irrigation water. The longest pollen tubes were observed in *Medicago marina*, with an average length of 198.3 µm when harvested from control plants (Figures 9 A, B). Pollen harvested from salt-treated *M. marina* plants, however, exhibited a drastic reduction in tube length, measuring only 44.5 µm and 49.5 µm for plants irrigated with 150 mM and 300 mM NaCl, respectively (Figures 9 A, C, D). These results indicate that salinity during flowering significantly impaired pollen tube length in *M. marina*.

On the other hand, pollen harvested from *Lotus creticus* plants watered with 150 mM NaCl exhibited a longer pollen tube length (81.2 µm, Figures 9 A, F) than those from control-treated plants (64.3 µm; Figures 9 A, E). At higher salt concentrations, irrigation with 300 mM NaCl resulted in a reduction of pollen tube length to 54.7 µm (Figure 9 A, G).

Finally, a dose-dependent reduction in pollen tube length was observed in *Ononis ramosissima*, with an average tube length of 112.7 µm for pollen harvested from control-treated plants (Figures 9 A, H), which decreased to 71.5 µm and 66.6 µm when the plants were watered with 150 mM and 300 mM NaCl, respectively (Figures 9A, I, J).

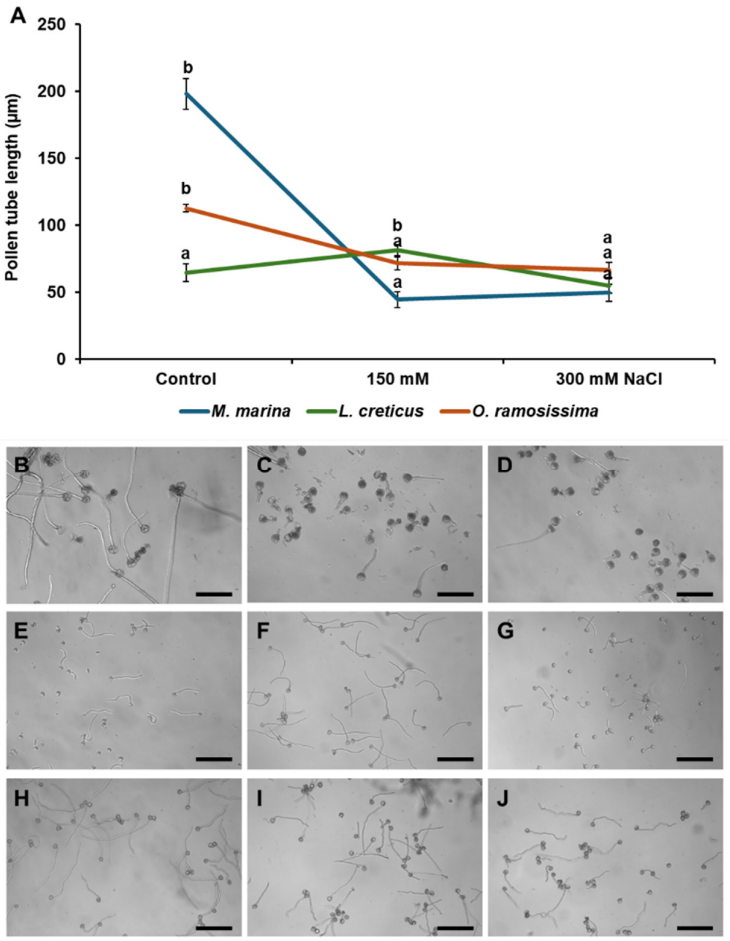

Figure 9. Effect of salt stress on pollen tube length upon three hours incubation of pollen harvested from the dune species *Medicago marina, Lotus creticus* and *Ononis ramosissima*. A. Graphical representation of data collected from microscopy images. Salt concentration used in irrigation solution is indicated
Values shown are means per plate ± SE; n = 5. B-D. Representative images of pollen harvested from *M. marina* upon 3 hours of incubation on germination media supplemented with 0 (B), 150 (C) and 300 (D) mM NaCl. E-G. Representative images of pollen harvested from *L. creticus* upon 3 hours of incubation on germination media supplemented with 0 (E), 150 (F) and 300 (G) mM NaCl. H-J. Representative images of pollen harvested from *O. ramosissima* upon 3 hours of incubation on germination media supplemented with 0 (H), 150 (I) and 300 (J) mM NaCl. Scale bar: 100 µm

Discussion

After developing inside the anther, mature pollen grains are released in the environment once the anther reaches maturity, in a state of dehydration and metabolic dormancy. Cross-pollinated species require pollinating agents to transport pollen to the compatible and receptive stigma within a short period of time until the pollen grain maintain a sufficient level of viability. Since the *in vivo* pollen germination process on the stigmatic surface is complicated, and challenging technologically, *in vitro* pollen germination could be used to assess pollen qualities (Heslop-Harrison, 1979). The ability to identify the physiological and biochemical parameters necessary for successful pollen germination and pollen tube development underscore the practical significance of *in vitro* pollen germination techniques. Furthermore, *in vitro* pollen germination and pollen

viability have been shown to be closely related (Schori *et al*., 1992; Shivanna *et al*., 1991), with both factors often demonstrating a strong correlation with fruit and seed set in many species (Kumar *et al*., 2016).

Soil salinity poses a significant threat to soil fertility, stability, and biodiversity, making it an increasingly critical concern in the context of global warming (Hassani *et al*., 2021), and represents one of the most harmful environmental stress factors affecting plant growth and survival, and reducing crop yields worldwide (Mahajan and Tuteja, 2005). Several studies have reported similar responses to abiotic stress during the gametophytic and sporophytic stages of the life cycle in plants (Vasiliy, 2000; Ravikumar *et al*., 2003). *In vitro* pollen selection is regarded as a useful screening method, as it allows for the direct application of stress factors to large male gametophytic populations (Agr *et al*., 2010). Comparative studies between the male gametophyte (pollen) and sporophyte (seeds) indicated similar patterns of response, though pollen exhibits a higher susceptibility to salt stress (Martínez-Pallé *et al*., 1995).

Of the 14 species analysed in this study, the three target species are specific to littoral dunes (*Lotus creticus*, *Medicago marina* and *Ononis ramosissima*), three grow in neighbouring areas, and the remaining eight are cultivated. Species inhabiting dune ecosystems are considered as halophytes, as they naturally thrive in environments affected by salinity. On the contrast, the large majority of cultivated plants are glycophytes, susceptible to salt stress. Several studies have investigated the salt tolerance of dune species during the vegetative stage, but there is a lack of information regarding the effects of salinity on their male gametophytes. A study on two Tunisian populations of *Lotus creticus* indicated that both were relatively salt-tolerant at growth stage to the tested concentrations, ranging from 0 to 400 mM NaCl (Rejili *et al*., 2007). Salt stress to up 140 mM NaCl even enhanced dry mass accumulation in this species (Morales *et al*., 2000). A comparative study of different *Lotus* species revealed that *L. creticus* exhibited superior survival after exposure to long-term, lethal salinity and was more effective at excluding Cl^- from the shoots compared to the glycophytes (Sanchez *et al*., 2011). Although *Medicago marina* is a halophyte, it was found that its optimal growth under greenhouse conditions occurred on peat or garden substrate. Plants grown on sand and saline soil sampled in natural environments achieved smaller biomass in the same experimental conditions (Grigore *et al*., 2012). Although there are no specific studies on the salt tolerance of *Ononis ramosissima*, the third species of dune habitats here investigated, several reports identify it as a halophyte, characteristic of plant communities developed on saline soils (Laguna *et al*., 2021).

Of the 14 investigated species, only *Lotus creticus* pollen did not germinate at all at 100 mM NaCl, although the species is considered extremophilic (Sanchez *et al*., 2011). However, lower NaCl concentrations did not affect germination and pollen tube growth. In *Medicago marina* and *Ononis ramosissima* only 100 mM NaCl concentration significantly reduced pollen germination, while pollen tube growth was inhibited already by 50 mM NaCl. When comparing species from three different habitats, *Medicago marina* showed a smaller reduction in germination rates and pollen tube length compared to *Spartium junceum* and *Cicer arietinum*, with the latter showing the most significant reduction in both parameters. Despite this strong reduction, chickpea (*Cicer arietinum*) maintained relatively high germination percentages at 100 mM NaCl. A previous study on the salt tolerance of this widely cultivated crop found that germination was unaffected by 40 mM NaCl (Turner *et al*., 2013); however, in the present investigation, 50 mM NaCl led to a significant reduction in pollen germination compared to the control.

Of all the species analysed here, the highest germination percentages at 100 mM NaCl were observed in the cultivated species, *Ornithopus sativus* (French or pink seradella) a Mediterranean forage crop. A relatively high tolerance of the male gametophyte was also found in other crops, such as *Vicia villosa*, *Vicia incan*a and *Medicago sativa*. Several differences were found between the congeneric species included in the present study, especially regarding germination speed and pollen tube length. *Coronilla juncea* had a slower germination speed and was more affected by salt than *C. viminalis*, even when pollen was collected from wild plants located near

the littoral zone. Of the three *Medicago* species tested, *M. marina* was the most tolerant to salinity, while *M. sativa* showed the fastest germination and pollen tube growth. In contrast, *M. arborea* had very low germination under all conditions. *Vicia incana* and *V. villosa* were both relatively tolerant to salinity; however, the former exhibited immediate germination at time 0 and produced longer pollen tubes after three hours. Notably, germination percentages were higher under 100 mM NaCl in *V. villosa,* and this trait is considered a more reliable indicator of salinity tolerance than pollen tube length.

The results obtained from plants irrigated with saline solutions clearly indicate a negative effect of salinity on pollen quality. In all three species analysed (*Lotus creticus*, *Medicago marina* and *Ononis ramosissima*), pollen produced by plants irrigated with 300 mM NaCl solutions had reduced germination capacity. Although the three species share the same habitat type and seeds used for the generation of the plants used in the experiment were collected from the same area in the Natural Park of Albufera, they responded in a different manner to stress, as *Medicago marina* was the most susceptible to salinity, both in terms of pollen germination percentage and pollen tube length. It is well known that environmental conditions of the parental plant influence offspring performance, including pollen quality (Delph *et al*., 1997; Boscaiu *et al*., 2005; Mircea *et al*., 2024). Salinity stress has been shown to reduce the mobilisation of soluble carbohydrates to the inflorescence, leading to a significant decline in pollen viability (Ghanem *et al*., 2009). Furthermore, it was found that Na^+ accumulation did not occur in the tapetum cells or anthers, suggesting that the reduction in pollen viability was not due to the toxic effects of ions, but rather to a lack of photosynthate mobilisation to the inflorescence (Razzaq *et al*., 2019).

Conclusions

In summary, this study shows that pollen fitness within Fabaceae family varies greatly in response to salinity stress, with different species exhibiting different patterns of response. The 14 species examined showed a high variability, as indicated by the large differences in percentages of germination of their pollen grains and pollen tube elongation in response to salt stress. Notably, while a positive correlation was found between pollen germination and tube length in the cultivated species *Cicer arietinum*, such a relationship was absent in the wild species examined. The variability of responses of male gametophyte to salinity, even among species that share the same environment, were highlighted by the differences in fitness of pollen produced by plants of *Medicago marina*, *Lotus creticus*, and *Ononis ramosissima* grown under controlled greenhouse conditions and irrigated with saline solutions. However, when comparing species of Mediterranean scrubland (*Spartium junceum*) and dune habitats (*Medicago marina*) with a cultivated one, the first exhibit greater resilience in terms of reproductive fitness. Overall, these findings improve our knowledge on the effect of the salt stress on the pollen viability and germination capacity, which has consequences for the management and conservation of both cultivated and wild members of the Fabaceae family in saline environments.

Authors' Contributions

Conceptualisation: MB, RM and OV; Data curation: DMM; Formal analysis: MB; Funding acquisition: OV; Investigation: DMM and PB; Methodology: DMM, PB and RM; Project administration: MB, OV and RM; Resources: MB, JP and RM; Software: DMM and RM; Supervision: MB and RM; Validation: MB, OV, JP and RM; Visualisation: DMM; Writing - original draft: DMM, RM and MB; Writing - review and editing: OV and JP. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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