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ECOLOGÍA Y FITOQUÍMICA DE *ERYNGIUM MARITIMUM* L. EN LAS DUNAS DE MALLORCA

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Programa de Doctorado en Biología de las Plantas

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Doctor por la Universitat de les Illes Balears

A Paco y Marianela,

los cuales me han dado las oportunidades que ellos nunca tuvieron

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Declaración de la tesis doctoral

La presente tesis doctoral se ha elaborado a partir de un compendio de artículos publicados o en vías de publicación. La estructura consta de Introducción, cuatro Capítulos principales en los cuales se incluyen cinco manuscritos, Discusión, Conclusiones y Anexos. El lenguaje usado en la estructura de la tesis doctoral es el castellano, a excepción de los cuatro capítulos principales cuyo idioma principal es el Inglés.

Los artículos publicados o en vías de publicación cuyo contenido se presenta en la tesis doctoral son los siguientes:

Cortés-Fernández, I., Cerrato, M. D., Ribas-Serra, A., & Gil Vives, L. (2022). Floral traits and reproductive success variation among inflorescence orders in *Eryngium maritimum*. *Plant Biology*. *24(2),* 249-258. https://doi.org/10.1111/plb.13354 (Q1, IF 2021 = 3.877)

Cortés-Fernández, I., Cerrato, M.D., Ribas-Serra, A., Cardona, C., González, C., & Gil, L. (2021). Evidence of interpopulation variation in the germination of *Eryngium maritimum* L. (Apiaceae). *Plant Ecology 222*: 1101–1112. https://doi.org/10.1007/s11258-021-01164-y (Q2, IF 2021 = 1.99)

Cortés-Fernández, I., Cerrato, M. D., Ribas-Serra, A., & Gil, L. (2022). Salinity effects on the germination and reproduction of *Eryngium maritimum* L. (Apiaceae). *Flora*, *291*, 152062. https://doi.org/10.1016/j.flora.2022.152062 (Q2, IF 2021 = 2.22)

Cortés-Fernández, I., Cerrato, M. D., Ribas-Serra, A., Ferrà, X. C., & Gil-Vives, L. (2022). The role of *E. maritimum* (L.) in the dune pollination network of the Balearic Islands. *Ecology and Evolution*, *12*, e9164. https://doi. org/10.1002/ece3.9164 (Q2, IF 2021 = 3.167)

Cortés-Fernández, I., Sureda, A., Adrover, Caprioli, G., Maggi, F., Gil-Vives, L., Capó, X. (2022). Antioxidant and anti-inflammatory potential of rhizome aqueous extract of sea holly (*Eryngium maritimum* L.) on Jurkat cells. *Molecules*. *In review.* (Q2, IF 2021 = 4.927)

RESUMEN

Los hábitats costeros presentan una elevada biodiversidad y se encuentran entre los más amenazados del planeta. Entre ellos, los hábitats dunares son especialmente susceptibles a los efectos de la antropización. En Baleares, una comunidad autónoma particularmente dependiente del turismo, estas zonas han sufrido durante décadas los efectos de la explotación de nuestras costas. Las plantas, durante miles de años de procesos de selección, han desarrollado mecanismos para hacer frente a las condiciones climáticas y edáficas de las dunas. Sin embargo, las cambiantes condiciones actuales, entre las que destacan el aumento en el nivel del mar y el aumento de la temperatura global, escapan a los tiempos necesarios para que las especies desarrollen mecanismos adaptativos. Entre las especies que podemos encontrar en las dunas de Baleares se encuentra *Eryngium maritimum* L. (Apiaceae), un geófito rizomatoso con un característico tono azulado y una marcada espinescencia, utilizado en algunos lugares del mundo en medicina tradicional y jardinería. En el Norte de Europa es considerada como una especie gravemente amenazada, mientras que en el Mediterráneo sus poblaciones exhiben un buen estado de conservación. Debido a este hecho, existe una falta de estudios que analicen las características clave de la especie, tales como su biología reproductiva, germinación y polinización, en esta zona geográfica. Asimismo, aunque la especie presenta muchos usos tradicionales, se desconoce cuál es su composición química y las propiedades medicinales que presenta. Por otro lado, relativamente pocos estudios han evaluado sus capacidades antinflamatorias y antioxidantes in-vivo, las cuales son, a priori, las que presentan un mayor potencial. El presente proyecto de tesis pretende arrojar luz a nivel de la ecología de la especie y a nivel fitoquímico, estableciendo los siguientes objetivos:

1- Analizar la estructura floral y el éxito reproductivo de la especie.
2- Comprender las necesidades específicas de germinación de la especie.
3- Analizar la tolerancia a la sal de la especie.
4- Conocer sus polinizadores y evaluar cual es el papel que juega la especie dentro de la red de polinización dunar.
5- Evaluar la composición química de la especie y los efectos de su administración en líneas celulares.

El análisis reproductivo muestra que la especie presenta inflorescencias con diversos verticilos, los cuales son diferentes a nivel morfológico y reproductivo, siendo los primeros verticilos los responsables del éxito reproductivo femenino de la especie. Por otro lado, los análisis morfométricos sugieren que la translocación de recursos ocurre de los verticilos interiores a los exteriores, actuando los rizomas como almacén de recursos en época desfavorable. Se han obtenido los porcentajes de germinación más altos que se conocen para la especie, superiores al 90%. Se ha constatado que la especie requiere de un periodo de estratificación en frío para germinar, el cual es variable entre poblaciones, presentando las de latitudes más altas periodos de estratificación más largos. La especie resulta poco halotolerante, tanto a nivel germinativo como a nivel reproductivo. Su distribución en la duna parece responder parcialmente a la concentración de sales en el medio, viéndose inhibida su presencia en las zonas más cercanas a la costa, aunque otros factores como la compactación del suelo, el spray salino o la competencia interespecífica podrían ser claves en su distribución a lo largo de la duna. La especie presenta una fuerte dependencia hacia sus polinizadores, presentando capacidades de autofecundación casi nulas. Es una especie ampliamente generalista, y ejerce un papel importante dentro de la red de polinización dunar. Presenta una situación fenológica muy particular, siendo la última especie generalista en flor de la duna, potencialmente debido a fenómenos de competencia con *Teucrium dunense* y *Helichrysum stoechas*. Los extractos acuosos de los rizomas de la especie muestran una alta capacidad antioxidante y antinflamatoria, superior a otras especies de apiáceas ampliamente utilizadas (como el eneldo o el comino). Su composición apunta hacia una acción

sinérgica de diversos compuestos antioxidantes y antiinflamatorios, que evitarían la peroxidación lipídica en los tejidos y conducirían a una mejora general en los parámetros fisiológicos. Los ensayos de citotoxicidad muestran que la administración de *E. maritimum* no es tóxica para líneas celulares, lo cual resulta clave para avanzar en los ensayos clínicos de las propiedades medicinales evidenciadas.

La presente tesis describe la biología reproductiva de la especie, establece su protocolo de germinación, evidencia su baja tolerancia a la sal y describe su papel dentro de la red de polinización dunar. Además, muestra que es una especie con una alta capacidad antioxidante y antiinflamatoria, evidenciando las necesidades específicas de la especie de cara a su producción y, a la vez, a su conservación.

RESUM

Els hàbitats costaners presenten una elevada biodiversitat i es troben entre els més amenaçats del planeta. Entre aquests, els hàbitats dunars són especialment susceptibles als efectes de l'antropització. A les Balears, una comunitat autònoma particularment dependent del turisme, aquestes zones han patit durant dècades els efectes de lexplotació de les nostres costes. Les plantes durant milers d'anys de processos de selecció han desenvolupat mecanismes per fer front a les condicions climàtiques i edàfiques de les dunes. Tot i això, les canviants condicions actuals, entre les quals destaquen l'augment en el nivell del mar i l'augment de la temperatura global, escapen als temps necessaris perquè les espècies desenvolupin mecanismes adaptatius. Entre les espècies que podem trobar a les dunes de Balears hi ha *Eryngium maritimum* L. (Apiaceae), un geòfit rizomatós amb un característic to blavós i una marcada espinescència, utilitzat en alguns llocs del món en medicina tradicional i jardineria. Al Nord d'Europa és considerada una espècie greument amenaçada, mentre que a la Mediterrània les seves poblacions exhibeixen un bon estat de conservació. A causa d'aquest fet, hi ha una manca d'estudis que analitzin les característiques clau de l'espècie, com ara la seva biologia reproductiva, germinació i pol·linització, en aquesta zona geogràfica. Així mateix, encara que l'espècie presenta molts usos tradicionals, se'n desconeix quina és la composició química i les propietats medicinals que presenta. D'altra banda, relativament pocs estudis han avaluat les seves capacitats antiinflamatòries i antioxidants in-viu, les quals són, a priori, les que presenten més potencial. Aquest projecte de tesi pretén donar llum a nivell de l'ecologia de l'espècie i fitoquímic, establint els objectius següents:

1- Analitzar l'estrucura floral i l'èxit reproductiu de l'espècie.
2- Comprendre les necessitats específiques de germinació de l'espècie.
3- Analitzar la tolerància a la sal de l'espècie.
4- Conèixer els seus pol·linitzadors i avaluar quin és el paper que juga l'espècie dins la xarxa de pol·linització dunar.
5- Avaluar la composició química de l'espècie i els efectes de la seva administració en línies cel·lulars.

L'anàlisi reproductiva mostra que l'espècie presenta inflorescències amb diversos verticils, els quals són diferents a nivell morfològic i reproductiu, i els primers verticils són els responsables de l'èxit reproductiu femení de l'espècie. D'altra banda, les anàlisis morfomètriques suggereixen que la translocació de recursos passa dels verticils interiors als exteriors, actuant els rizomes com a magatzem de recursos en època desfavorable. S'han obtingut els percentatges de germinació més alts que es coneixen per a l'espècie superiors al 90%. S'ha constatat que l'espècie requereix un període d'estratificació en fred per germinar, que és variable entre poblacions, presentant les de latituds més altes períodes d'estratificació més extensos. L'espècie resulta poc halotolerant, tant a nivell germinatiu com a nivell reproductiu. La seva distribució a la duna sembla respondre parcialment a la concentració de sals al medi, veient-se inhibida la seva presència a les zones més properes a la costa, encara que altres factors com la compactació del sòl, l'esprai salí o la competència interespecífica podrien ser claus en distribució al llarg de la duna. L'espècie és fortament dependent dels pol·linitzadors, presentant capacitats d'autofecundació gairebé nul·les. És una espècie àmpliament generalista, i exerceix un paper important dins de la xarxa de pol·linització dunar. Presenta una situació fenològica molt particular, sent l'última espècie generalista a flor de la duna, potencialment a causa de fenòmens de competència amb *Teucrium dunense* i *Helichrysum stoechas*. Els extractes aquosos dels rizomes de l'espècie mostren una alta capacitat antioxidant i antiinflamatòria, superior a altres espècies d'apiàcies àmpliament utilitzades com a anet o comí. La seva composició apunta cap a una acció sinèrgica de diversos compostos antioxidants i antiinflamatoris, que evitarien la peroxidació lipídica

als teixits i conduirien a una millora general en els paràmetres fisiològics. Els assaigs de citotoxicitat mostren que l'administració d' *E. maritimum* no és tòxica per a línies cel·lulars, cosa que resulta clau per avançar en els assaigs clínics de les propietats medicinals evidenciades.

Aquesta tesi descriu la biologia reproductiva de l'espècie, n'estableix el protocol de germinació, n'evidencia la baixa tolerància a la sal i en descriu el paper dins la xarxa de pol·linització dunar. A més, mostra que és una espècie amb una alta capacitat antioxidant i antiinflamatòria, evidenciant les necessitats específiques de l'espècie de cara a la producció i, alhora, a la conservació.

ABSTRACT

Coastal habitats are highly biodiverse and among the most threatened on the planet. Among them, dune habitats are particularly susceptible to the effects of anthropization. In the Balearic Islands, an autonomous community particularly dependent on tourism, these areas have suffered for decades from the effects of the exploitation of our coasts. Over thousands of years of selection processes, plants have developed mechanisms to cope with the climatic and soil conditions of the dunes. However, today's changing conditions, including rising sea levels and increasing global temperatures, are beyond the time needed for species to develop adaptive mechanisms. Among the species found in the Balearic dunes is *Eryngium maritimum* L. (Apiaceae), a rhizomatous geophyte with a characteristic bluish hue and marked spinescence, used in some parts of the world in traditional medicine and gardening. In Northern Europe it is considered a seriously endangered species, while in the Mediterranean its populations are in a good state of conservation. Due to this fact, there is a lack of studies analysing key characteristics of the species, such as its reproductive biology, germination and pollination, in this geographical area. Furthermore, the species has many traditional uses, but its chemical composition and medicinal properties are unknown. Moreover, relatively few studies have evaluated its anti-inflammatory and antioxidant capacities *in-vivo*, which are, a priori, those with the greatest potential. This thesis project aims to shed light on the ecology of the species and its phytochemistry, with the following objectives:

1- To analyse its floral structure and reproductive success of the species.

2- To understand the specific germination requirements of the species.

3- To analyse the salt tolerance of the species, a key fact considering that it is a coastal habitat plant.

4- To find out about its pollinators and assess the role played by the species in the dune pollination network.

5- To evaluate the chemical composition of the species and the effects of its administration on cell lines.

The reproductive analysis shows that the species has inflorescences with different whorls, which are morphologically and reproductively different, with the first whorls being responsible for the female reproductive success of the species. On the other hand, morphometric analyses suggest that resource translocation occurs from the inner to the outer whorls, with the rhizomes acting as a resource store in unfavourable seasons. The highest germination percentages known for the species were obtained, over 90%. It has been found that the species requires a cold stratification period to germinate, which varies between populations, with those at higher latitudes having longer stratification periods. The species is not very halotolerant, both at germination and reproductive levels. Its distribution on the dune seems to respond partially to the concentration of salts in the environment, its presence being inhibited in the areas closest to the coast, although other factors such as soil compaction, salt spray or interspecific competition could be key to its distribution along the dune. The species is strongly dependent on pollinators, with almost no self-fertilisation capacity. It is a broadly generalist species and plays an important role in the dune pollination network. It has a very particular phenological situation, being the last generalist species in flower on the dune, potentially due to competition with *Teucrium dunense* and *Helichrysum stoechas*. The aqueous extracts of the rhizomes of the species show a high antioxidant and anti-inflammatory capacity, superior to other widely used apiaceae species such as dill or cumin. Their composition points to a synergistic action of various antioxidant

and anti-inflammatory compounds, which would prevent lipid peroxidation in tissues and lead to an overall improvement in physiological parameters. Cytotoxicity assays show that administration of *E. maritimum* is non-toxic to cell lines, which is key to advancing clinical trials of the medicinal properties demonstrated.

This thesis describes the reproductive biology of the species, establishes its germination protocol, demonstrates its low salt tolerance and describes its role in the dune pollination network.

INTRODUCCIÓN

“Aunque estemos ciegos a este hecho, la realidad es que las plantas son la base de todo, directa o indirectamente. Las plantas nos proporcionan el aire que respiramos; nos visten, nos curan y nos protegen; las plantas nos procuran cobijo y casi toda nuestra comida y bebida diarias. Pensemos en las medicinas, los materiales de construcción, el papel, el caucho, los anticonceptivos, el algodón para los vaqueros y el lino para los vestidos; en el pan, las judías, el té, el zumo de naranja, la cerveza, el vino y la Coca-Cola, y pensemos también que las vacas comen hierba, pienso o forraje y que obtenemos de ellas carne y leche; que las gallinas comen trigo y otras semillas y nos dan huevos, carne y sopas; que las ovejas comen hierba y nos dan lana, etcétera. Así que las plantas son nuestros mejores y más humildes sirvientes; se ocupan de nosotros cada día, en cada aspecto. Sin ellas, no podríamos sobrevivir. Es tan simple como eso.”

Carlos Magdalena, El Mesías de las Plantas (2018)

Los hábitats dunares

Los hábitats costeros presentan una elevada biodiversidad y se encuentran entre los más amenazados del mundo (Culbertson et al., 2009; Prisco et al., 2013). En la costa confluyen una serie de factores externos, como son: la influencia marina, la diversidad de sustratos, los aportes de materia orgánica y de agua dulce, que crean una amplia diversidad de entornos y ecotonos que son particularmente propicios para albergar una elevada cantidad de especies (Maun, 2009). Los hábitats costeros en general, y los dunares en particular, han sufrido especialmente los efectos de la antropización, en forma de urbanización de la costa (Marignani et al., 2017), uso recreativo de las playas (Pagán et al., 2016), introducción de especies invasoras (Basnou et al., 2015) y destrucción de los hábitats marinos, vitales para la regeneración del sustrato dunar (Dugan & Hubbard, 2010). Ello comporta que actualmente, según la Lista Roja de Hábitats Europeos (Janssen et al., 2016), el 45% de los hábitats costeros están catalogados como amenazados. Además, a los factores de amenaza anteriormente citados hay que sumar la creciente preocupación por los efectos del cambio climático, los cuales son particularmente alarmantes en el Mediterráneo (IPCC, 2021). Mientras que en gran parte del planeta se espera un aumento de la precipitación, en el Mediterráneo, una zona particularmente estresada hídricamente, se esperan reducciones de hasta el 40% de las precipitaciones invernales, lo que implicará problemas en la producción de alimento y ocasionará un fuerte impacto en la biodiversidad (Tuel & Eltahir, 2020). A su vez, el incremento esperado de la temperatura global se espera que sea mucho más acusado en el Mediterráneo (4-5 ºC, Giorgi & Lionello, 2008) que en el resto del planeta (1.7-4.4 ºC; Escenario A1B IPCC; Bernstein et al., 2008). Por otro lado, se estima que se produzcan aumentos muy significativos en el nivel del mar, entre 342 ± 211 mm (RCP 2.6) y 570 ± 255 mm (RCP 8.5) para el año 2100 (Vecchio et al., 2019).

Las plantas, por el hecho de carecer de movilidad (o ser muy reducida), han tenido que desarrollar una serie de mecanismos para afrontar las condiciones bioclimáticas y geomorfológicas que presentan los hábitats costeros (Hesp, 1991). Así pues, a modo de ejemplo, la influencia del viento condiciona la morfología de las especies que en ella habitan, que se desarrollan con portes distintos a los que pueden presentar las mismas especies en zonas de interior. Estos condicionantes no son solo físicos, sino también químicos, siendo la salinidad y el estrés hídrico factores clave para el establecimiento de especies eminentemente costeras, las cuales desarrollan mecanismos para hacer frente a dichas situaciones de estrés (Lum & Barton, 2020). Entre estos mecanismos, destacan la exudación de sales, aumento de la concentración y variabilidad de compuestos antioxidantes y la compartimentalización de osmolitos (Parida & Das, 2005). Sin embargo, dichas adaptaciones son fruto de millones de años de presión evolutiva, y en ningún caso la capacidad de respuesta es lo suficientemente rápida para afrontar las presiones fruto de la antropización y el cambio climático, y sus efectos en las poblaciones de plantas costeras son inciertos (Anderson & Song, 2020).

Entre los ecosistemas costeros, los hábitats dunares son los más amenazados debido a los efectos de la antropización (EEA, 2008; Drius et al., 2013) y el cambio climático (Bruelheide, 2003; Jackson et al., 2019). Entre los efectos ecológicos de estos factores destaca un aumento en la duración de la etapa vegetativa, lo cual facilita el establecimiento de las especies termófilas (Provoost et al., 2011), incrementos locales de la superficie de masa vegetal (Jones et al., 2013) y la reducción de la distribución de especies nativas (Mendoza-González et al., 2013). En las dunas, la movilidad del sustrato juega un papel clave dentro de la dinámica de las poblaciones (Hesp & Martínez, 2007). Entre los diferentes hábitats dunares, las dunas móviles (Hábitat 2120, Directiva 92/43) y las fijas (Hábitat 2210) son aquellas que presentan un mayor grado de amenaza, mientras que los hábitats próximos al mar podrían incluso incrementar su área de distribución, al ser primocolonizadoras y estar adaptadas a condiciones climáticas muy desfavorables (Prisco et al., 2013). Por la proximidad a la línea de costa, la salinidad juega un papel clave en el establecimiento de las especies dunares, debido a los efectos del spray marino y al nivel freático (Maun., 2009). En las dunas se considera que, generalmente, la salinidad es baja, debido a la percolación de sales en el sustrato debido a las lluvias, aunque su concentración es muy variable y depende fuertemente de la pluviometría (Bolhuis et al., 2013). En cuanto a las especies dunares, su tolerancia a la sal es heterogénea, siendo las especies de los primeros frentes dunares las más resistentes (Cardona, 2019), lo cual es especialmente relevante a fin de establecer medidas de recuperación, conservación y manejo.

Caracterización fitosociológica de las dunas de Baleares

La estructura del sustrato, el viento, los aportes de materia orgánica y la salinidad crean un gradiente que se ve reflejado en una amplia variedad de comunidades vegetales que se distribuyen, generalmente, de forma paralela a la línea de costa en forma de catenas de vegetación (**Figura 1**). En las Baleares orientales, en ocasiones estas dunas pueden presentarse de forma perpendicular a la línea de costa. Ello puede ser consecuencia de acciones antrópicas (por ruptura del primer frente de dunas) o bien por causas naturales. Éste es el caso de algunas calas y playas expuestas a los vientos del N, NE y NW que inciden de forma oblicua, y muy intensamente, sobre la línea de costa. A menudo, estas calas o playas están situadas en desembocaduras de torrentes (Llorens & Gil, 2017).

Según Llorens et al. (2022), el esquema sintaxonómico de las dunas de Mallorca es:

- Clase ***Cakiletea maritimae*** Tüxen & Preising ex Br.-Bl. & Tüxen 1952
    - Orden ***Cakiletalia integrifoliae*** Tüxen ex Oberdorfer 1949 corr. Rivas-Martínez, Costa & Loidi 1992
        - Alianza ***Cakilion maritimae*** Pignatti 1953
            - Asociación ***Salsolo kali - Cakiletum maritimae*** Costa & Mansanet 1981
            - Asociación ***Hypochoerido radicatae - Glaucietum flavi*** Rivas Goday & Rivas-Martínez 1958
- Clase ***Ammophiletea*** Br.Bl. & Tüxen *ex* Westhoff, Dijk & Passchier 1946
    - *Orden* ***Ammophiletalia*** Br.-Bl. 1933
        - Alianza ***Agropyro - Mynuartion peploidis*** Tüxen in Br.-Bl. & Tüxen 1952
            - Asociación ***Cypero mucronati - Agropyretum juncei*** Kühnholtz ex Br.-Bl. 1933
        - Alianza ***Ammophilion australis*** Br.-Bl. 1921 corr. Rivas-Martínez, Costa & Izco in Rivas-Martínez, Lousã, T.E. Díaz, Fernández-González & J.C. Costa 1990
            - Asociación ***Eryngio - Pancratietum maritimi*** *Llorens & Gil prov.*
            - Asociación ***Medicago marinae - Ammophiletum arundinaceae*** *Br.-Bl. 1921 corr. F. Prieto & T.E. Díaz 1991*
        - Alianza ***Sporobolion arenarii*** Géhu & Géhu-Franck ex Géhu & Biondi 1994

            - Asociación ***Eryngio maritimi - Sporoboletum arenarii*** Arènes ex Géhu & Biondi 1994 Rivas Mart. & Cantó 2002.
    - Orden ***Crucianellietalia maritimae*** Sissingh 1974
        - Alianza **Crucianellion maritimae** Rivas Goday & Rivas-Martínez 1963
            - Asociación ***Loto cretici - Crucianelletum maritimae*** Alcaraz, T.E. Díaz, Rivas-Martínez & P. Sánchez 1989
            - Asociación ***Fumano laevis - Scrophularietum ramossisimae*** Llorens & Gil prov.
            - Asociación ***Ononido crispae - Scrophularietum minoricensis*** O. Bolòs, Molinier & P. Montserrat 1970

Así pues, en la zona más próxima al mar, correspondiente a la clase fitosociológica *Cakiletea maritimae*, encontramos especies capaces de hacer frente a las salpicaduras del agua marina, fuertemente ligadas a los aportes de materia orgánica procedentes de corrientes y mareas y capaces de habitar en sustratos altamente compactados puntualmente. Por su localización, es el hábitat más afectado por la antropización y los usos recreativos de la costa, siendo difícil encontrar en Baleares zonas donde se encuentre bien representada (Llorens et al., 2022). Dependiendo de si el sustrato es arenoso o rocoso, encontraremos la asociación *Salsolo kali - Cakiletum maritimae*, de arenas finas dominadas por terófitos, o *Hypochoerido radicatae–Glaucietum flavi*, de arenas gruesas y rocas con dominancia de criptófitos.

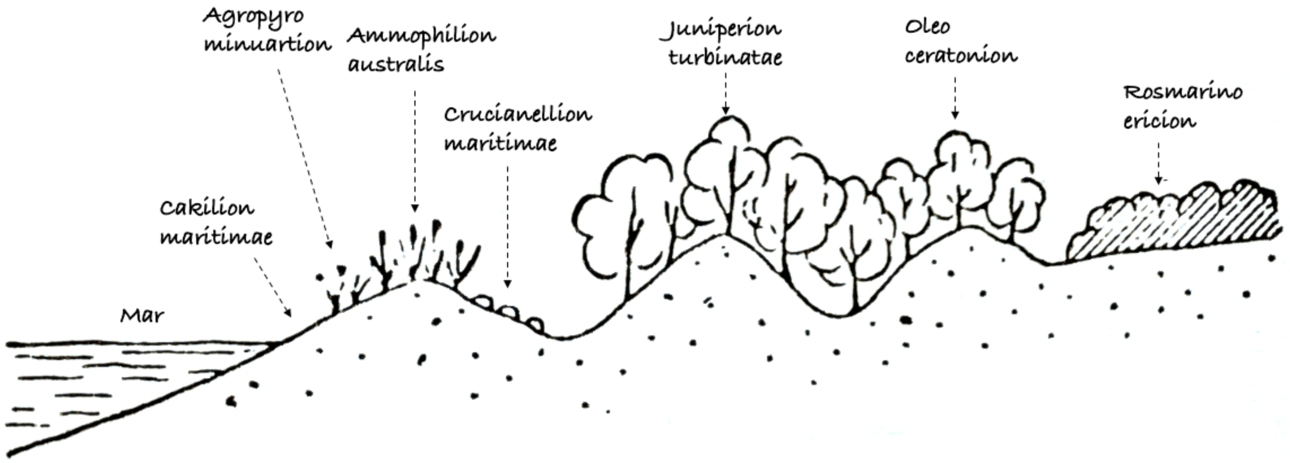

Figura 1. Esquema simplificado de la catena de vegetación de las dunas de Mallorca, en el que se indican las alianzas principales. Modificado de Bolòs & Moliner (1984).

Continuando hacia el interior, encontramos la zona propiamente dunar, la cual se caracteriza por una influencia marina más leve y en la cual la arena tiene la posibilidad de comenzar a acumularse, presentando una alta movilidad. En esta zona habitan comunidades vegetales de la clase *Ammophiletea*, dominadas por gramíneas perennes y geófitos. A diferencia de otras zonas costeras, como los roquedos litorales o saladares, la salinidad no ejerce un papel tan determinante en la distribución de las comunidades que aquí habitan, debido a que las sales, aunque son capaces de acumularse en el sustrato, sufren procesos de lavado tras las precipitaciones que disminuyen su concentración en el medio (Cardona, 2019). Por ello, otros factores como la movilidad del sustrato o la influencia marina ejercen una mayor presión selectiva sobre las especies que habitan en dichas zonas (Fenu et al., 2013). En la duna móvil, orden *Ammophiletalia*, podemos diferenciar dos tipos de vegetación principal. En primer lugar, hallamos las dunas embrionarias, caracterizadas por una fuerte influencia del viento, una salinidad edáfica muy variable (elevada en algunos momentos), una alta movilidad de la arena y donde las gramíneas inician el proceso de fijación del sustrato. Son dunas de escasa elevación incluidas en la asociación *Cypero mucronati-Agropyretum juncei*, caracterizada por una diversidad específica muy escasa. En las zonas donde se producen mayores aportes de materia orgánica (normalmente en forma de restos de *Posidonia oceánica* (L.) Delile) y, como consecuencia, la movilidad del sustrato decrece y la salinidad edáfica aumenta, se desarrolla la alianza *Sporobolion arenarii*, más escasa y representada en Baleares casi exclusivamente por la asociación *Eryngio maritimi-Sporoboletum arenarii*, caracterizada fundamentalmente por poblaciones prácticamente monoespecíficas de *Sporobolus pungens* L. (Llorens et al., 2022). Seguidamente, encontramos la vegetación de dunas blancas, caracterizadas por habitar en zonas con un mayor volumen de arena, representada en Baleares por la alianza *Ammophilion australis*, teniendo como asociaciones principales *Medicago marinae–Ammophiletum arundinaeae* y *Eryngio-Pancratietum maritimi*. En la primera de ellas, la presencia de *Ammophila arenaria* (L.) Link es mayoritaria, posibilitando su sistema radicular una mayor acumulación de arena y llegando a alcanzar las dunas en las que habita más de 2 metros de altura (Llorens et al., 2022). Cuando el volumen de arena es ligeramente inferior y *Ammophila arenaria* encuentra dificultades para establecerse, sobre restos de *Posidonia oceanica* se desarrolla el *Eryngio-Pancratietum*, caracterizado por la abundancia de los geófitos *Pancratium maritimum* L. y *Eryngium maritimum* L., y la ausencia prácticamente total de gramíneas fijadoras. Generalmente esta asociación se localiza en zonas donde la presencia de algún islote frente a la costa provoca una disminución del movimiento de la arena. Son dunas más ricas en materia orgánica y, por tanto, con una mayor concentración de salinidad edáfica que las dunas blancas de *Ammophila arenaria*.

Finalmente, tras las dunas móviles el sustrato adquiere una cierta protección frente a la influencia marina, sobre todo, al viento, posibilitando la aparición de las dunas semimóviles, orden *Crucianelletalia*, dominadas por caméfitos y representadas en Baleares por comunidades pertenecientes a la alianza *Crucianellion maritimae*. Tras ellas, la arena prácticamente se ha fijado, la salinidad ha disminuido de forma importante y la influencia eólica es menor. Ello permite el asentamiento de la vegetación fanerofítica del *Juniperion turbinatae* Rivas-Martínez 1975 *corr.* 1987 y *Oleo-Ceratonion siliquae* Br.-Bl. ex Guinochet & Drouineau 1944 em. Rivas-Martínez 1975.

En Baleares, podemos encontrar hábitats dunares distribuidos en todas sus islas, si bien en Formentera Mallorca y Menorca es donde se hallan las formaciones más desarrolladas.

Taxonomía y filogenia del género *Eryngium*

El género *Eryngium*, formado por aproximadamente 250 especies, es el género más diverso y taxonómicamente complejo de la familia Apiaceae. Alrededor de dos tercios de las especies del género se encuentran en el continente americano, con otros dos núcleos de diversidad localizados en el Mediterráneo Occidental y en el suroeste de Asia (**Figura 2**; Cerceau-Larrival, 1971; Calvino et al., 2008). La mayoría de los autores coinciden en que el género se originó en algún punto del sudeste asiático, conformando el clado de los *Eryngium* subgénero *Eryngium* (conocidos como del Viejo Mundo). Sin embargo, en la actualidad encontramos que gran parte de las especies pertenecen al subgénero *Monocotyloidea*, o del Nuevo Mundo, cuyo origen se cree que tuvo lugar en el Mediterráneo Occidental (Calvino et al., 2008). Desde allí se produjo una rápida especiación potencialmente debida a eventos de hibridación y presión selectiva durante la colonización del continente europeo (Seehausen, 2004). Posteriormente, eventos múltiples de dispersión transatlántica, relacionados con la viabilidad y flotabilidad de las semillas en el océano, dieron lugar a las especies del continente americano (Calvino et al., 2008). Aunque el género es extremadamente variable a nivel morfológico (**Figura 3**), se distingue de otras umbelíferas por presentar inflorescencias capituliformes, con una sola bráctea por flor. Aunque la mayoría de las especies son herbáceas perennes, también hay algunas especies anuales y otras leñosas (Calvino et al., 2008).

Descripción y distribución de *Eryngium maritimum*

El cardo corredor (*Eryngium maritimum* L., subfamilia Saniculoidae) es un geófito rizomatoso que habita comúnmente en las dunas móviles (Isermann & Rooney, 2014). Se la conoce con el nombre de "cardo corredor" debido a que el escapo en su conjunto, al completar la maduración de los frutos y secarse, se vuelve frágil y por factores externos tiende a romperse, dispersándose de forma estepicursora. *Eryngium maritimum* presenta una elevada espinescencia en márgenes foliares, brácteas y frutos, así como hojas trilobadas, basales, coriáceas, crasas, glabras y glaucas, a veces persistentes, con nerviación palmati-reticulada, densamente espinescentes en sus márgenes (Nieto-Feliner, 2003; Isermann & Rooney, 2014). Presenta una raíz pivotante muy desarrollada, así como un extenso sistema rizomatoso. Su inflorescencia es capituliforme y de gran tamaño (10-30 x 10–25 mm), formada por un escapo de 0.15-0.60 cm que surge de la roseta basal foliar, de la que surgen a su vez diversos verticilos de capítulos (Nieto-Feliner, 2003). Estos son ovoides o globulares, con 5-7 brácteas lanceoladas similares a las hojas. Sus bractéolas son tricuspidadas y espinosas de 2.5 a 4 mm de longitud, de color azul violáceo, más largas que las flores. Presenta flores hermafroditas, protandras, provistas de nectarios en su base, con 5 sépalos espinosos, más largos que los pétalos, y 5 pétalos blanquecinos, oblongos y emarginados. Presenta 5 estambres prominentes, azulados o blanquecinos, con anteras azuladas o amarillas, así como dos estilos blancos, más cortos que los estambres. Su fruto es un esquizocarpo, consistente en 2 mericarpos con una semilla cada uno, que a menudo se dispersan de forma separada al completar su maduración. La planta en su conjunto adquiere una vistosa tonalidad azulada, muy característica, durante el comienzo de la floración, la cual es estival y se extiende alrededor de 1 mes (Gil, 1994). En el territorio español se diferencia del resto de miembros del género por presentar hojas basales palmatisectas, con segmentos laterales no decurrentes en el peciolo y limbo cordiforme (Nieto-Feliner, 2003).

La especie presenta una estructura de la inflorescencia muy definida. Cada individuo produce un solo escapo floral, el cual termina con una inflorescencia capituliforme terminal (**Figura 4**). Por debajo de esta surgen de forma dicasial nuevos pedúnculos con sus respectivas inflorescencias terminales, generando inflorescencias con un numero variable de capítulos. De este modo, *E. maritimum* presenta una estructura floral en la cual los capítulos se distribuyen en pisos (en adelante verticilos) de diferente número, tamaño y, atendiendo a observaciones personales y a estudios previos, con diferente éxito reproductivo. Ello podría dar lugar a una diversidad morfométrica y funcional a nivel intraindividual que puede ser vital para entender la funcionalidad de la inflorescencia en su conjunto.

La especie presenta una distribución mediterráneo-atlántica (**Figura 2**). Se puede observar como el género se encuentra distribuido por Europa, América y Australia, con una presencia menos destacada en Asia e incluso África. *E. maritimum*, por el contrario, se encuentra distribuido de forma casi exclusiva en el continente Europeo y en la costa Norte de África de forma puntual. Algunos individuos utilizados en jardinería han llegado al contiene americano y australiano. En el Mediterráneo occidental es una especie característica de las comunidades vegetales asociadas a dunas blancas (Marcenò & Jiménez-Alfaro, 2017). En Baleares, utilizando los inventarios realizados en literatura previa de referencia (Bolòs et al., 1970; Bolòs & Molinier, 1984; Rivas-Martínez et al., 1992) y realizando un análisis de componentes principales se puede observar que *E. maritimum* se encuentra como especie característica de Ammophiletea, a caballo entre las dunas más embrionarias (*Cypero-Agropyretum*) y las crestas dunares (*Medicago-Ammophiletum;* **Figura 5**).

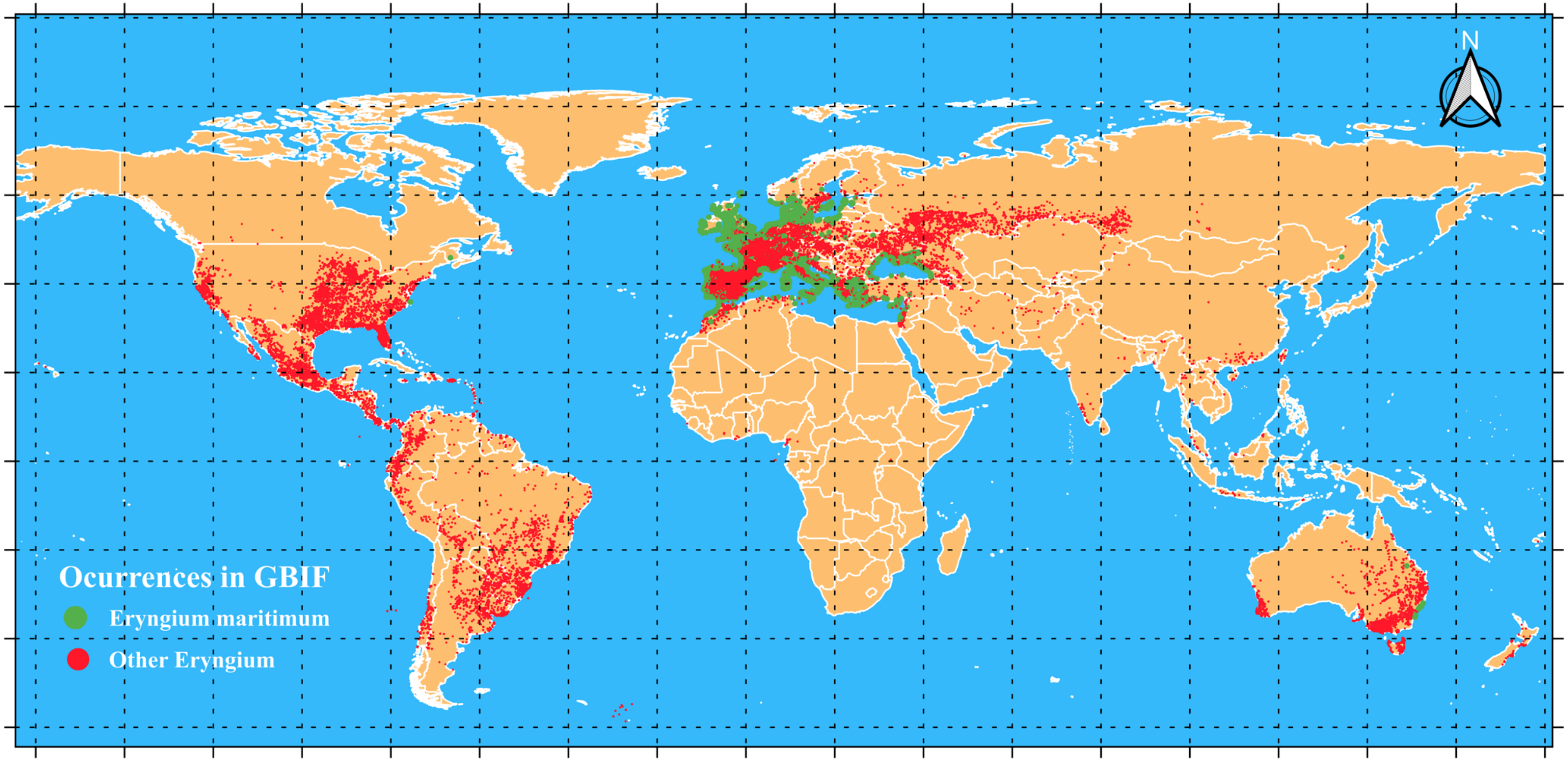

Figura 2. Mapa de ocurrencias del género *Eryngium*. Con puntos verdes se indican las ocurrencias que corresponden específicamente a *Eryngium maritimum* L. Los datos han sido extraídos de la *Global Biodiversity Information Facility* (GBIF), utilizando todos los datos con coordenadas latitud-longitud que corresponden a observaciones humanas

Figura 3. *Eryngium campestre* (A, Mallorca, Islas Baleares), *E. alpinum* (B, Attinghausen, Suiza), *E. bourgatii* (C, fuente: Flickr) y *E. yuccifolium* (D,fuente: Alamy).

Figura 4. Individuo de *E. maritimum* visitado por *Pryonix viduatus*. Se puede observar l*a* inflorescencia dicasial capituliforme, formada por verticilos de morfología y tamaño variable.

Figura 5. El análisis de componentes principales de los inventarios realizados en literatura previa de referencia (Bolòs et al., 1971; Bolòs & Molinier, 1984; Rivas-Martínez et al., 1992).

La ecología de *Eryngium maritimum*

El cardo corredor es una especie gravemente amenazada en el Norte de Europa, donde se encuentra listada en los Libros Rojos de Lituania (Balevičius, 1992), Estonia (Paal, 1998), Noruega (Fremstad & Moen, 2001) y Rusia (Seregin & Suslova, 2007). La pérdida de hábitat y la fragmentación de sus poblaciones debido a la antropización han sido propuestas como sus principales causas de amenaza (Curle et al., 2004). Sin embargo, las poblaciones del Mediterráneo Occidental, área geográfica que también sufre un intenso uso de sus costas, no están amenazadas. Las poblaciones mediterráneas parecen presentar un mayor número de plántulas e individuos juveniles (Stasiak 1986), mientras que en las poblaciones del Norte de Europa los individuos presentan un fitness reducido con una baja producción de semillas (Bengtsson et al., 2009), asegurando la continuidad de las poblaciones por reproducción vegetativa. Aunque la especie presenta, a priori, altos porcentajes de germinación en su hábitat, los estudios previos que analizan las condiciones óptimas de germinación de la especie bajo condiciones controladas han obtenido, en la mayoría de los casos, porcentajes de germinación muy bajos (Walmsley & Davy, 1997; Curle et al., 2004, Fernández-Pascual et al., 2017). Ello podría deberse a que las semillas de la especie presentan una dormición morfofisiológica, en la cual el embrión no está completamente formado en el momento de la dispersión (Necajeva & Ievinsh, 2013). Sin embargo, existe una falta de estudios que analicen la germinación de la especie en el Mediterráneo Occidental, siendo la aproximación más cercana los ensayos de germinación realizados en el Sur de Mallorca (Gil, 1994) y en Cerdeña (Porceddu et al., 2019).

Estudios previos sugieren que *E. maritimum* parece ser particularmente dependiente de la polinización cruzada, aunque la autoincompatibilidad no es completa, dando lugar a autogamia y geitonogamia parcial (Gil, 1994; Klotz et al., 2002). Sin embargo, se desconocen cuáles son sus polinizadores y qué papel juega dentro de la red de polinización dunar y, en consecuencia, si existen fenómenos de competencia y/o facilitación entre *E. maritimum* y las especies con las que habita. En este sentido, la mayoría de estudios que analizan las redes de polinización dunar en Baleares se han realizado en la duna fija de Son Bosc (Castro-Urgal et al., 2012 Tur et al., 2013; Castro-Urgal & Traveset, 2014; Traveset et al., 2017; Lázaro et al., 2020), siendo la aproximación más cercana al hábitat de *E. maritimum* un estudio realizado por Castro-Urgal & Traveset (2014) en la duna de Cala Mesquida, y en el cual no se hace mención al papel concreto de la especie. Por otro lado, la base de datos Pollinib contiene la información de mayor calidad en lo relativo a visitantes florales de la especie, lo cual, aunque de gran utilidad, no es suficiente para entender cuáles son los condicionantes de su polinización y qué papel juega *E. maritimum* en la red de polinización dunar.

Fitoquímica, citotoxicidad y etnobotánica de la especie

El metabolismo secundario de las plantas ha creado el almacén químico más extenso a nivel global, tanto en cantidad como en diversidad de estructuras. Durante 500 millones de años su evolución ha conllevado la producción de moléculas destinadas a un conjunto heterogéneo de finalidades, tan dispares como: i) la defensa ante depredadores; ii) evitar la competencia intra e interespecífica; iii) o establecer mecanismos de comunicación entre ellas (Li & Weng, 2017).

El estudio de las propiedades medicinales en plantas usualmente comienza a través de textos clásicos (Dioscorides, 1829; Bigelow, 1934; Mattioli, 1971; Fuchs, 1990), que establecen efectos observados tras la administración de material vegetal a pacientes enfermos, documentando los efectos observados tras su aplicación. El estudio preliminar realizado por C. Amengual (*com.pers.* 2019) basado en la administración de rizoma pulverizado de *E. maritimum* como complemento alimenticio en pacientes con disfunción renal, mostró que los pacientes obtuvieron una mejora notable en los indicadores de dicha enfermedad después de la administración. Dicha planta fue seleccionada y administrada ya que ensayos realizados por Touchard (1925) mostraron efectos satisfactorios en el tratamiento de la nefritis crónica albuminúrica o hematúrica. Además, la especie ha sido consumida como alimento en algunos países como Inglaterra y Turquía durante generaciones (Grieve, 1994), lo que es indicador de su palatabilidad y su falta de toxicidad relativa. Finalmente, diversos textos clásicos, tales como los textos de Dioscórides (1829), y estudios recientes en otras especies del género, como es el caso de *Eryngium caeruleum* M. Bieb., con elevado potencial anti-diabético (Rehman et al., 2017); *Eryngium creticum* Lam., con capacidad para suprimir la viabilidad de células tumorales (Barakat et al., 2017), o *Eryngium duriaei* Gay ex. Boiss. subsp. *juresianum* (M. Laínz) M. Laínz, con gran potencial antiinflamatorio, avalan que se trata de un género con un amplio potencial medicinal (Rufino et al., 2015).

Las infusiones de partes aéreas y raíces de *E. maritimum* han sido utilizadas por la medicina popular como tratamiento antitusivo, diurético y afrodisiaco (Baytop, 1999) y como inhibidor de la formación de piedras en el riñón y para eliminar obstrucciones en hígado, vesícula y riñones (Andrew, 2001). La aplicación de los extractos de rizoma y semillas de *E. maritimum* en ratas ha constatado su potencial antinflamatorio (Lisciani et al., 1984; Suciu & Pârvu, 2012) y como atenuante de la toxicidad inducida por otras moléculas (Mejri et al., 2017). Sin embargo, y a pesar de todo ello, todavía no ha sido descrito si este efecto se reproduce en humanos, ni cuáles son las moléculas (principios activos) responsables de los mismos, aunque actualmente se comercializa como nutracéutico por diversas empresas (*Homeomart, Bjain Pharmaceuticals, Schwabe*). Si bien ha sido ya descrita parte de la composición de sus extractos (Darriet et. al, 2013; Darriet et. al, 2014; Palá Paúl, 2004, Lajnef et al., 2017), la gran diversidad de metodologías existentes para la extracción y cuantificación de los compuestos hace que el perfil fitoquímico de la especie se encuentre difuso en la literatura, lo que contribuye a que la gran diversidad de propiedades que presentan los extractos no haya sido atribuida a ningún compuesto o familia de compuestos concreta, por lo que su mecanismo de acción es hasta el momento desconocido. La mejor aproximación sobre el conocimiento de las propiedades de los extractos acuosos de la especie la encontramos en Kholkhal et al. (2012), los cuales demostraron *in-vitro* su alto potencial antioxidante, antifúngico y antibacteriano. Sin embargo, el potencial antioxidante de los extractos acuosos de los rizomas de la especie sobre modelos *in-vivo* es desconocido, pudiendo ser una de las vías principales de actuación en enfermedades renales (Shah & Iqbal, 2010) y vital para las futuras pruebas en modelos animales.

OBJETIVOS

Este trabajo pretende profundizar en el conocimiento de la especie desde un punto de vista ecológico, a fin de comprender los mecanismos que regulan la biología reproductiva, la germinación y la tolerancia salina y la polinización de la especie. Por otro lado, pretende profundizar en la composición química de los extractos acuosos del rizoma evaluando su potencial antioxidante, antiinflamatorio y citotoxicidad.

Por todo ello, el objetivo principal de la tesis doctoral se constituye como:

Analizar la ecología de la especie *E. maritimum*, a fin de comprender los mecanismos que rigen los principales puntos críticos de desarrollo de la especie, tales como: 1- la germinación; 2- el crecimiento; 3- la tolerancia a la salinidad; 4- la formación de flores y frutos; 5- sus mecanismos de polinización. Asimismo, se pretende evaluar la composición química y las propiedades medicinales de la especie, profundizando en su potencial antioxidante y la citotoxicidad.

Este objetivo general se desglosa en los siguientes objetivos específicos:

Ecología de la especie

1. Profundizar en la biología reproductiva de la especie, a fin de comprender la estructura de las inflorescencias, así como sus diferencias morfométricas y funcionales entre verticilos.

2. Comprender los mecanismos que regulan la germinación de la especie, a fin de desarrollar un protocolo óptimo de germinación.

3. Analizar la tolerancia a la salinidad de la especie durante la germinación, el crecimiento y la fase reproductiva.

4. Evaluar la dependencia de polinizadores de la especie, evaluando qué papel desempeña la especie dentro de la red de polinización dunar.

Fitoquímica y citotoxicidad de la especie

5. Establecer la composición de los extractos acuosos de la especie en sus distintos órganos.

6. Evaluar la citotoxicidad de la especie en líneas celulares.

7. Establecer el potencial antioxidante y antiinflamatorio de los extractos de rizoma de la especie.

Los objetivos se resolverán mediante la elaboración de cinco análisis, de los cuales se propone obtener la información para conseguir resultados útiles, así como se resumen en la **Figura 6**.

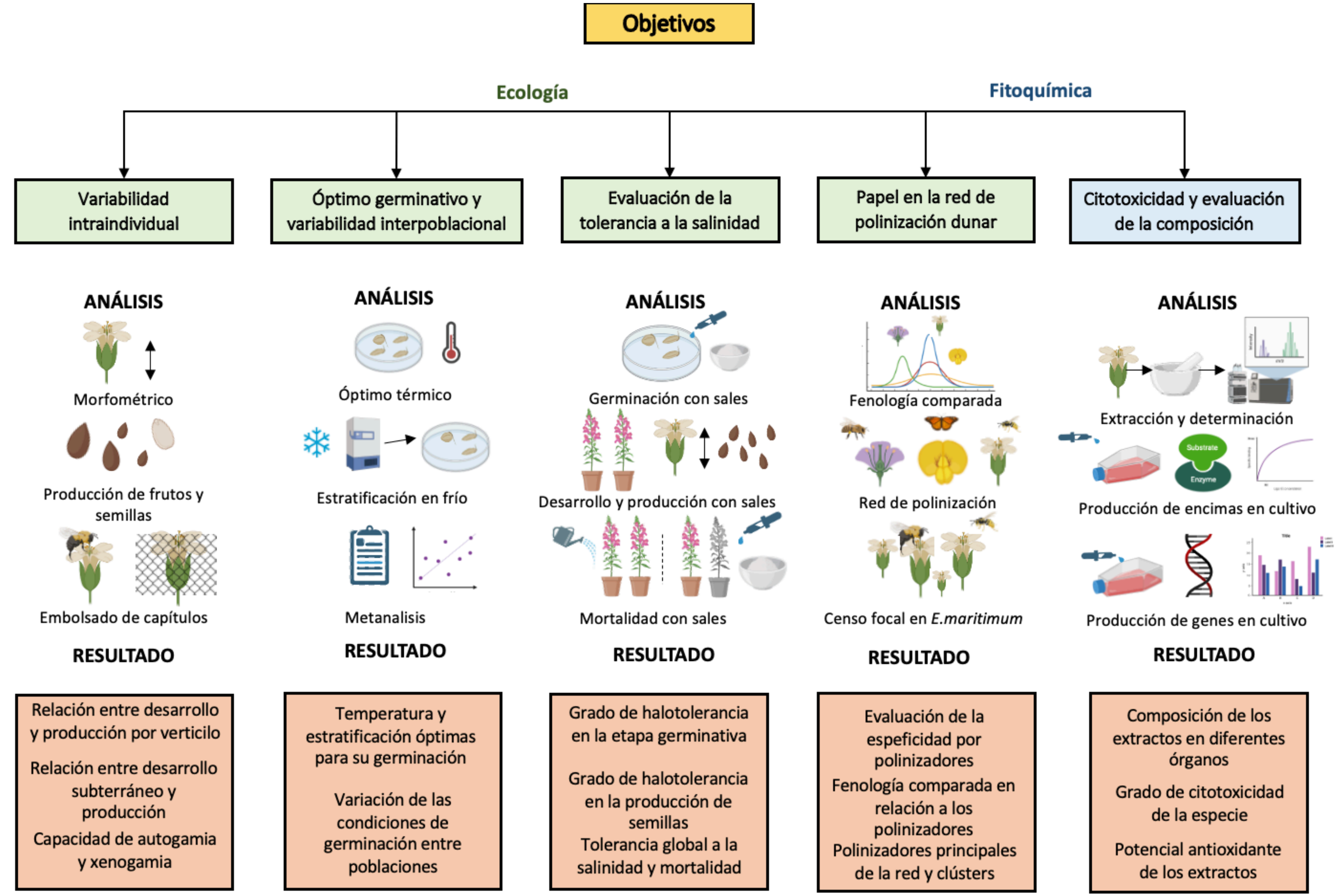

Figura 6. Síntesis de los objetivos de la tesis doctoral, los análisis que se proponen para resolverlos y la información que se espera obtener.

Capítulo 1.

Biología reproductiva y germinación de *Eryngium maritimum* L.

Manuscript I
Cortés-Fernández, I., Cerrato, M. D., Ribas-Serra, A., & Gil Vives, L. (2022). Floral traits and reproductive success variation among inflorescence orders in *Eryngium maritimum*. *Plant Biology*. *24(2),* 249-258. https://doi.org/10.1111/plb.13354

Manuscript II
Cortés-Fernández, I., Cerrato, M.D., Ribas-Serra, A., Cardona, C., González, C., & Gil, L. (2021). Evidence of interpopulation variation in the germination of *Eryngium maritimum* L. (Apiaceae). *Plant Ecology 222*: 1101–1112. https://doi.org/10.1007/s11258-021-01164-y

Manuscript I. Floral traits and reproductive success variation among inflorescence orders in *Eryngium maritimum*

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RESEARCH PAPER

Floral traits and reproductive success variation among inflorescence orders in Eryngium maritimum

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Keywords
Eryngium maritimum; coastal dune communities; capitulum; inflorescence orders; reproductive traits; floral visitors.

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ABSTRACT

- *Eryngium maritimum* L. is considered a key species in dunes of the western Mediterranean. Although Northern European populations are endangered, the Mediterranean populations show a good conservation status, despite a lack of studies of the reproductive requirements and strategies of this species. This study aims to analyse the reproductive biology of a population of *E. maritimum* in the western Mediterranean (Balearic Islands, Spain), specifically focusing on differences in morphometric and reproductive traits among inflorescence orders.
- Capitulum size, number of flowers, fruit set and seed set were analysed to evaluate the morphological and reproductive differences among inflorescence orders. The association between subterranean development and investment in reproductive structures was also studied along the dune location. Breeding system was analysed, evaluating self-pollination capacity at capitulum level and the pollen/ovary ratio. Finally, floral visitors were assessed.
- Morphometric and reproductive traits showed a decline in size and production from early to subsequent inflorescence orders. A correlation between width of stem base (caudex) and number of reproductive structures was observed, whereas seashore distance did not affect the reproductive development. Flowering occurred during summer, from May until late July, while fructification took place from the middle of August until the last days of September. Pollinator exclusion treatments induced a fruit and seed set decline. Twenty-one species of floral visitor were found.
- First and second inflorescence orders contributed to the generation of seeds, displaying high fruit and seed production, while other inflorescence orders seemed to attract pollinators. Reproductive and non-reproductive development could depend on the capacity to reallocate resources from underground to aerial structures, which seemed to be superior in samples with highly developed caudices. In the studied population, inner areas closest to the seashore showed a delay in anthesis, which could be related to microclimate differences along the coast-to-inland gradient. Pollination is strongly entomophilous and showed low levels of self-compatibility at capitulum level. Considering these results, for habitat conservation the reported microclimate diversity should be taken into consideration, with a focus on conservation of the pollinator populations

INTRODUCTION

Eryngium maritimum L. is considered a key species in mobile dunes (Walmsley & Davy, 1997) because of its contribution to soil stabilisation of sand dunes (Hibilik *et al.* 2016). However, in many coastal regions, *E. maritimum* is one of the rarest and most threatened plant species and is listed in Red Data Books (*e.g.* in Latvia, Estonia, Norway, Israel, along the Russian Black Sea coast; Sapir *et al.* 2003; Seregin & Suslova 2007; Isermann & Rooney, 2014). This species is widely distributed across European and Mediterranean coasts, reaching northern and southern distribution limits in Sweden (Tyler 1969) and Morocco (Hanifi *et al.* 2007; Isermann & Rooney, 2014), respectively. The study of reproductive biology of the species is essential in order to understand mechanisms underlying reproduction, in order to assure the appropriate development of habitat and species conservation strategies (Evans *et al.* 2003; Moza & Bhatnagar, 2007; Cogoni *et al.* 2015). These reproductive strategies have been documented in northern *E. maritimum* populations, which due to their endangered status have been extensively studied. Nonetheless, less attention has been given to the southern populations.

The *E. maritimum* inflorescence is structured as a flowering stalk with a central capitulum, under which new capitula emerge in a dichasial cyme. The quantity and density of flowers in this type of inflorescence means that pollen can be easily transferred to the stigma of the same flower (autogamy) or to the stigma of other flowers (geitonogamy; Harder *et al.* 2004). The available data suggest that, as in other taxa of the Apiaceae family (Lindsey, 1982), its reproductive biology is characterized by a marked protandrous dichogamy (Bell, 1971; Reuther & Claßen-Bockhoff, 2010), with two clearly defined phases. In the first phase, pollen is delivered to the pollinators and the stigma remains inactive, while in the second phase the stigma becomes active and is receptive to pollen for fecundation. Hence, dichogamy is considered a mechanism to avoid self-pollination. However, in *E. maritimum*, synchrony among capitula may not occur as described in other taxa of the same family (Reuther & Claßen-Bockhoff, 2010), so geitonogamy between flowers from different inflorescence orders is not potentially avoided by dichogamy. In this respect, references that mention variability in the flower, fruit and seed production among the specific levels of the inflorescence stalk (inflorescence orders) in species of *Eryngium* are scant (Gaudeul & Till-Bottraud 2004; Bujan & Real, 2014). In addition, size differences among inflorescence orders have also been indicated in other Apiaceae species (Gutterman, 2000), this variation being critical to understand mechanisms regulating reproduction in this species. Although *E. maritimum* is considered an entomophilous species mainly pollinated by general insects (Fitter & Peat, 1994; Isermann & Rooney, 2014), there is a lack of pollinator data in the western Mediterranean populations, and the available information mainly comes from the Balearic Islands Pollinator Database (Pollinib). In many of the northern European populations, these data provide essential information as this species has been suggested to be affected by limited reproduction, due to low seed production and low germination (Curie *et al*., 2004; Aviziene *et al*., 2008).

Eryngium maritimum is considered to be a rhizome geophyte (Raunkiaer, 1934), as the aerial part is lost in each reproductive cycle and new shoots emerge from the subterranean rhizome system (Isermann & Rooney, 2014) that stores energy for the next season (Howard & Cellinese, 2020). Although this species is restricted to sandy dunes, where the sand volume is highly mobile (Isermann & Rooney, 2014), plants can also be found hundreds of meters inland (Gil, 1994), displaying a gradient from the coast to inland areas (Sorce *et al.* 2019). This means that species along the dune are exposed to different environmental and microclimate conditions (Sorce *et al.* 2019), which could influence reproductive behaviour of the plants, involving morphometric or phenological changes. However, the specific effect of location on the investment in reproductive structures has not been sufficiently analysed in the western Mediterranean, which could be critical to understanding differences between the inner and outer coastal subpopulations.

The present study aims to describe in detail the forms and mechanisms of the reproductive biology of the species *E. maritimum* in a population of the western Mediterranean, near to the southern distribution range. Moreover, the variation in size, flowering and fruiting investment and timing among inflorescence orders, as well as the potential associations between phenology, location, subterranean development and the investment in reproductive structures, were evaluated.

Our main hypothesis is that the rank order in inflorescence production is related to fitness in first and second orders of inflorescences and to attraction–pollen donation in the others, with a strong association between the production of reproductive structures and subterranean development. Furthermore, taking into account our previous observations, we expected to find differences in the flowering and fruiting timing between samples located inland and those located closer to the seashore, together with a strong decrease in fruiting due to pollinator exclusion.

MATERIAL AND METHODS

Plant species and study site

Eryngium maritimum L. (Apiaceae) is a rhizomatous psammophilous geophyte, characteristic of dune habitats (Isermann & Rooney, 2014; Pignatti *et al.* 2017). It is a perennial plant with a woody stem and large, coriaceous, blue-green leaves with thorny margins, arranged in a basal rosette. Every stem produces only one flowering stalk with a central capituliform inflorescence from which new capituliform inflorescences in a dichasial cyme originate (Fig. 1), although some second-order whorls can occasionally be found under the central capituliform cyme, which develop equally to a dichasial cyme. Fruits are formed by two mericarps that remain joined until completely ripe, which change colour from green to brown at maturity. Individual plants are difficult to differentiate due to the complex root and developed rhizome system. In the Balearic Islands, *E. maritimum* can be found in most dune systems, although it is more abundant in the eastern islands (Mallorca and Menorca) than in the western ones (Ibiza and Formentera; Llorens & Gil, 2017).

The study was carried out in the dunes of Son Serra de Marina, Mallorca (Spain; X: 520245 Y: 4397909; ETRS89 EPSG:25831) in 2019 because of the *E. maritimum* population size and the well-conserved status of these dunes. The dominant vegetation in this area can be defined as the phytosociological association *Medicagini marinae-Ammophiletum arundinaceae* 1921 corr. F. Prieto & T.E. Díaz 1991 (Class Ammophiletea Br.-Bl. & Tüxen ex Westhoff, Dijk & Passchier 1946; Order Ammophiletalia Br.-Bl. 1933), where *E. maritimum* is mainly accompanied by *Pancratium maritimum* L., *Ammophila arenaria* subsp. *arundinacea* H. Lindb, *Lotus cytisoides* L., *Calystegia soldanella* (L.) R. Br. and *Cynanchum acutum* L.

Reproductive traits

The reproductive traits of *E. maritimum* were quantified from the development of an inflorescence in the form of an umbel of flower heads (compacted umbels, hereafter capitulum). In order to establish the reproductive traits, plants were selected randomly (n = 30) and capitulum morphology (height, diameter and weight without bracts) and flower, seed and fruit production were evaluated among inflorescence orders. Capitulum

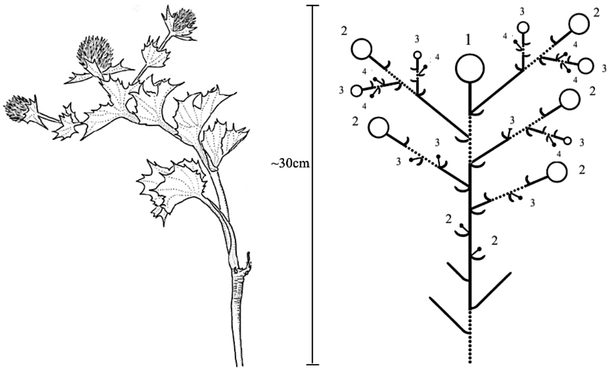

Fig. 1. Morphometric schema and drawing of *E. maritimum* inflorescences (modified from Burmester, 2008).

size was measured by measuring capitulum length and diameter with and without bracts, plus the length and width of flowers and fruits (Fig. 2). Flowers with receptive stigmas were weighed before and after drying for 24 h at 80 °C. All measurements were done in capitula and flowers of six different inflorescence orders.

Reproductive success was studied using samples (n = 30) selected randomly along the sampling area, with at least 2 m between samples. The capitula of each flowering stalk were individually collected and stored, considering the inflorescence order of origin for subsequent fruit set and seed set calculation. The proportion of viable seeds per capitulum were assessed using the cut test method (Leadem, 1984).

Biometric analysis

A biometric analysis was carried out in order to explore the relationship between the inversion on reproductive structures and the size of reserve structures. This analysis was based on the detailed morphometric study of plants (n = 100) that were randomly selected and labelled in June 2019 during the flowering period. For each member, the number of capitula was counted and correlated to the caudex width in order to examine whether the inversion on reproductive structures is related to the size of reserve structures. A coast-to-inland gradient was considered to include different environmental conditions, and therefore the coordinates of each plant were obtained with a GPS system (Leika GS07) to evaluate the effect of seashore distance on vegetative development (Figure S1). Geographic data were processed using QGIS 3.0 software (QGIS Development Team, 2016). Stem width was measured 10 cm over the ground using precision calipers, while base stem width (hereafter caudex) was measured 5 cm below the start of the rhizome tissue, as identified by a colour change from intense brown to yellow.

Phenology

A detailed phenological survey was carried to analyse flowering and fruiting timing of the species. Flower development was assessed by daily observation of the flowering stage of flowers (n = 10) from individual plants until the anthers and stigma became inactive. During each phase, all changes, as well as the time span, were recorded. Criteria for male phase began with anther dehiscence, and the female phase was considered initiated with pistil elongation and stigma receptivity. Flowering and fruiting timing was evaluated at both plant and population scale. Different plants (n = 30) were randomly selected and used for phenological study by recording open capitula per plant each week from May to September. Population flowering was evaluated by recording the first, last and maximum peak of flower production (day with maximum number of flowered capitula) in the first and second whorls. In addition to the biometric analysis, the coast-to-inland gradient was considered in the analysis, and three distinct areas were selected (ten plants per area) that were located at different distances (100, 150 and 200 m) from the seashore in order to evaluate the effect of sea proximity and represented the most proximal and most distal plant aggregations of the analysed population.

Breeding system

First-order capitula from plants (n = 30) were assigned to three treatments by bagging with paper bags, net bags (2-mm opening), with unmanipulated capitula as controls. Each treatment was conducted to evaluate wind and pollinator exclusion, or only pollinator exclusion and natural pollination, respectively. The number of flowers, fruits and seeds was subsequently counted, and fruit and seed set calculated considering the number of seminal primordia.

Pollen production from first inflorescence orders was studied by counting the number of pollen grains per flower using ten flowers sampled from ten different plants, in order to evaluate the pollen/ovule ratio to predict the theoretical mating system, according to Cruden (1977). Although hierarchical differences among inflorescence orders in pollen production have already been analysed by van der Pluym & Hydeux (1997), they did not address the purposes of the present study. Two stamens were collected from each of the ten flowers before dehiscence of the anthers and preserved in acetic acid (70%) for later analysis in the laboratory (Cursach & Rita, 2012). Due to the high number of pollen grains, each sample was diluted in 2 ml distilled water and mixed with 2 drops dishwashing soap for homogenization (Fairy Liquid; Cursach & Rita, 2012) and then five aliquots per sample were counted under the microscope using a haematocytometer (Fuchs-Rosenthal bright-line superior, Germany). Two grids (each 4 × 4 × 0.2 mm divided into 256 cells of 0.0625 mm^2) were counted per aliquot (ten measurements per sample) and the number of grains per flower calculated accordingly.

Floral visitors

Phenology was accompanied by floral visitor observations between 08:00 and 17:00 h. Insects were followed, photographed and captured when appropriate identification in the field was not possible. These surveys were carried out in parallel

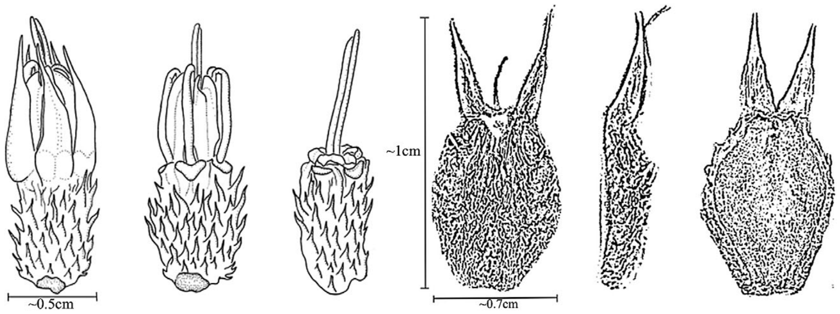

Fig. 2. Morphometric schema of flowers of *E. maritimum* (left) at different flowering phases (from left to right: flowering, petal fall and anther dehiscence) and fruits (right) in different planes (from left to right: outer frontal, sagittal and inner frontal plane). Modified from Burmester, 2008).

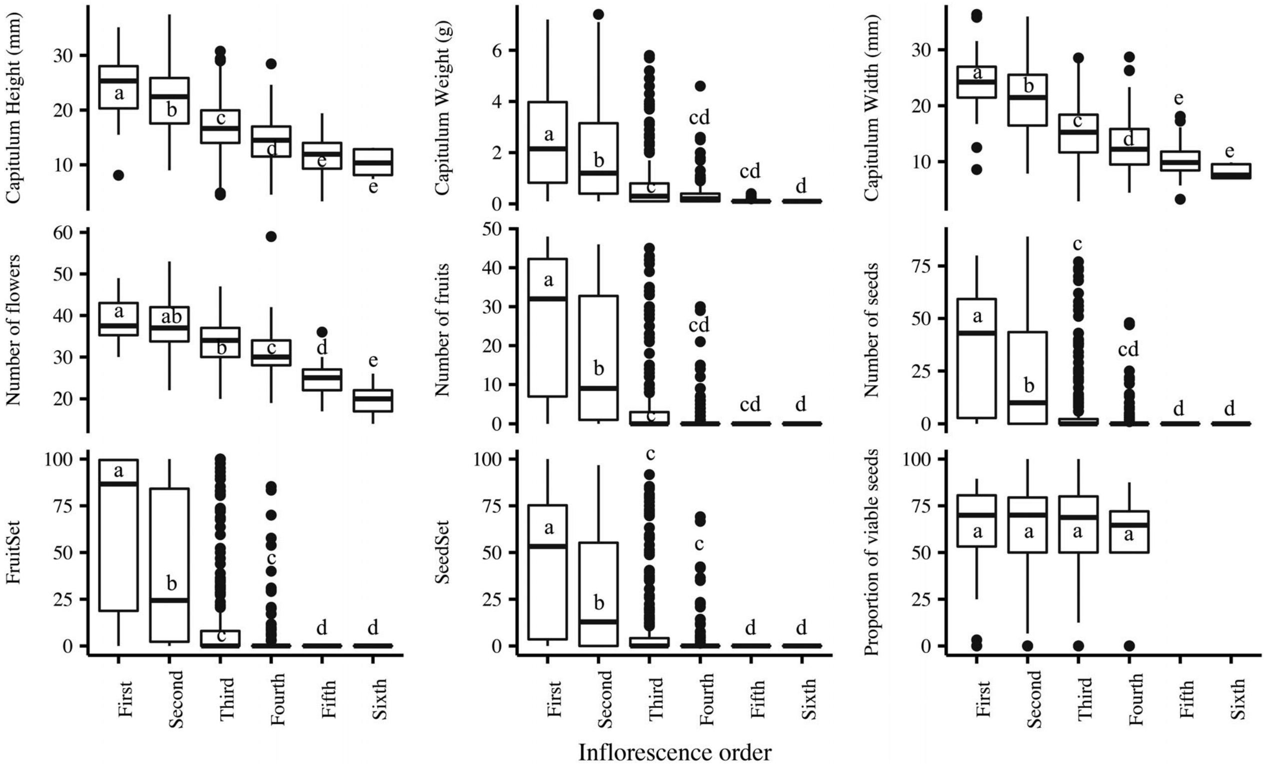

Fig. 3. Developmental (Capitulum height – A; Capitulum weight – B; Capitulum width – C) and reproductive traits (Number of flowers – D; Number of fruits – E; Number of seeds – F; Fruit set – G; Seed set – H; Proportion of viable seeds – I) by inflorescence order in *E. maritimum*. Different letters indicate significant differences among orders with the Tukey test ($P < 0.05$).

with other methodologies (pollinator exclusion, biometric analysis or phenological observation). For this reason, no detailed pollination data (time spent in visits, frequency, behaviour description) were gathered.

Statistical analysis

Linear and generalized mixed-effect models and ANOVA were used to evaluate the hypothesis that inflorescence order had no effect on biometric and reproductive traits, considering inflorescences belonging to the same plant as a random effect. Generalized mixed-effect models were applied for count variables (number of fruits, seeds and capitula) using the Poisson family with log link (Bolker *et al.* 2009). Moreover, linear mixed-effect models were used for continuous response variables (capitulum weight and height, and scape height). Model selection was carried out using the Akaike Information Criterion (AIC; Sakamoto *et al.* 1986). Specific differences among inflorescence orders were established using Tukey test (Abdi & Williams, 2010). Correlations between seashore distance, number of inflorescences and caudex width were assessed using adjusted Pearson correlation coefficient for normal data and non-normal data (package *rsq*; Zhang, 2018). Generalized mixed-effect models using Poisson family (log link) and ANOVA were used to evaluate the hypothesis that exclusion treatments have an impact on fruit and seed production. Statistical analysis was carried by using the statistical software R (R Core Team 2018), and descriptive data using the specific R packages *ggplot2* (Wickham, 2011) and *tidyverse* (Wickham *et al.* 2019).

RESULTS

Reproductive traits

Each plant developed up to six successive inflorescence orders. Occasionally, accessory capitula emerged under the first inflorescence orders from the leaf axil, with morphological and phenological similar characteristics to the second inflorescence orders of the capitula (Fig. 1).

Variability within capitula was observed in the capitulum size (Table S1) . Primary inflorescence orders displayed higher, wider and heavier capitula than the other inflorescence orders

Table 1. Results of variance and deviance analysis evaluating the effect of inflorescence order of origin on morphometric and productive response variables per capitulum. Plant was considered as a random effect. For continuous variables (Weight, Width, Height) Linear Mixed-Effect Models were carried out, while for count data (Number of flowers, Fruits, Seeds) and proportion data (Fruit Set and Seed Set) Generalized Linear Mixed-Effect models where used.

Response variable	*SS*	*df*	*MS*	*F*	*P*
Capitulum weight	355.03	5	71.005	63.886	<0.05
Capitulum width	12122	5	2424.5	166.9	<0.05
Capitulum height	10798	5	2159.6	171.79	<0.05
Number of flowers	256.02	5	51.203	51.203	<0.05
Number of fruits	342.83	5	68.566	68.566	<0.05
Number of seeds	299.57	5	59.913	59.913	<0.05
Fruit set	3035.7	5	607.13	607.13	<0.05
Seed set	4198.3	5	839.66	839.66	<0.05

(Fig. 3, Table 1). However, the number of capitula was maximum in the third inflorescence orders (9.50 ± 0.19 inflorescences plant^{-1}) and only two plants presented capitula from the sixth inflorescence orders. The number of flowers and fruits per inflorescence decreased from the first inflorescence orders to the sixth (Fig. 3), and flower size displayed an equal tendency until the fourth inflorescence order, after which flower measurements were too small for accuracy (Fig. 3).

Fruit and seed production were larger in the first inflorescence order than in other levels (Fig. 3, Table S2). Mean fruit set was 63.3 ± 8.2%; the first inflorescence order had a fruit production of 57.5%, which was three times higher than the second and third inflorescence orders. Following this trend, a mean seed set of 45.6 ± 6.9% was observed in the first inflorescence order, which was 88.5% and 490% higher than in the second and third inflorescence orders, respectively. However, third and fourth inflorescence orders showed high variability among specimens (Fig. 3). Negligible values were found under the fourth inflorescence order.

Biometric analysis

The results of biometrical analysis showed that the number of capitula per inflorescence was correlated with the width of the caudex (Fig. 4A). However, there was no significant difference in the caudex width of specimens regarding seashore distance (Fig. 4B).

Phenology

Anthesis began with petal exertion and anther opening. The duration of the male phase was short (1.54 ± 0.78 days), while the female phase was initiated 1.03 ± 0.54 days after the end of the male phase, with falling petals, style exertion and stigma opening; remaining in this condition for 9.34 ± 0.89 days. Flower opening started from the base to the apical axis of the inflorescence. Flowers were pale violet to violet in colour (18A3; Komerup & Wanscher, 1978). At the plant scale, mean flowering lasted 35.0 ± 1.0 days. At the scale of the entire population, the flowering peak was on 24 June 2019, while the first flowerings occurred on 30 May 2019. Plants inhabiting more inland areas displayed a delay of 14.82 ± 3.21 days compared with those located in areas closer to the seashore (Fig. 5). Inflorescences from primary orders flowered 6.87 ± 2.12 days earlier than inflorescences from secondary orders, these differences being more notable in inland areas. Flowering ended in all three study areas in mid-July.

Fruiting began with fruit ripening in the first days of August and extended for 25 days until 26 August, while the fructification peak was on 12 August. During this process, the stem base became fragile to external agents such as wind, collisions and water, which facilitated its breaking, occurring between September and November and overlapping with the first autumn squalls. The broken portion was rolled over the sand by wind action, releasing the mericarps, similar to tumbleweed species. This type of dispersal was favoured by the presence of thorny structures on the outer case of the fruit and also by the floral bracts.

Breeding system

The complete exclusion of pollinators (bagged, self-pollination + wind) led to low to null fruit set (0.8 ± 1.0%) and seed set (0.4 ± 0.8%), whereas when the exclusion was partial (net bag), fruit and seed production was more than 50% less than the untreated (control) group (Fig. 6). Exclusion treatments had a similar percentage of fruits with one and two seeds, while 25% of fruits in the control treatment generated only one seed. The results were consistent in both the first and second inflorescence orders (Fig. 6).

In addition, mean pollen content per flower was 34.56 ± 1.96 grains (n = 10). The mean P/O ratio was 17.28 ± 0.98 (n = 10).

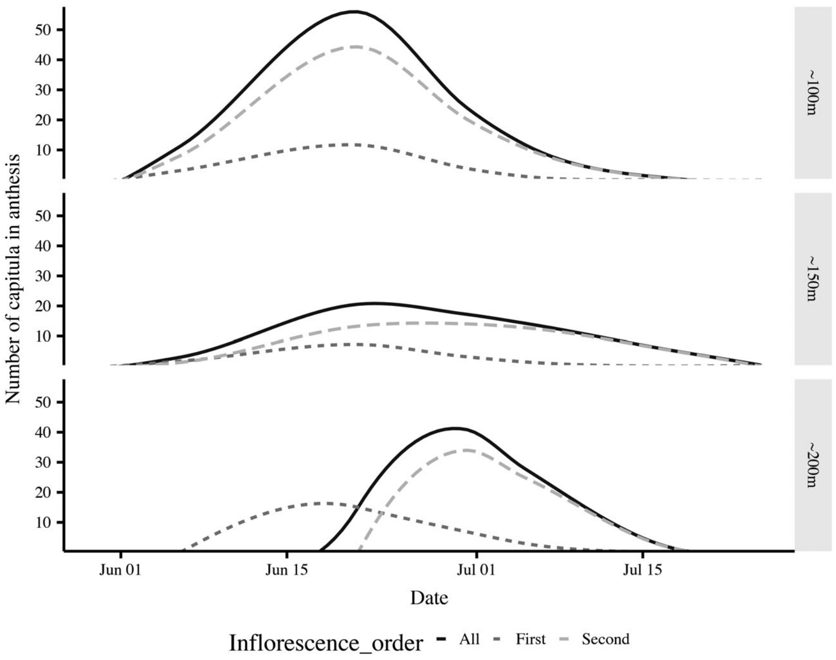

Fig. 4. Correlations between caudex width and number of flower heads (A). Seashore distance displayed no correlation with number of flower heads (B).

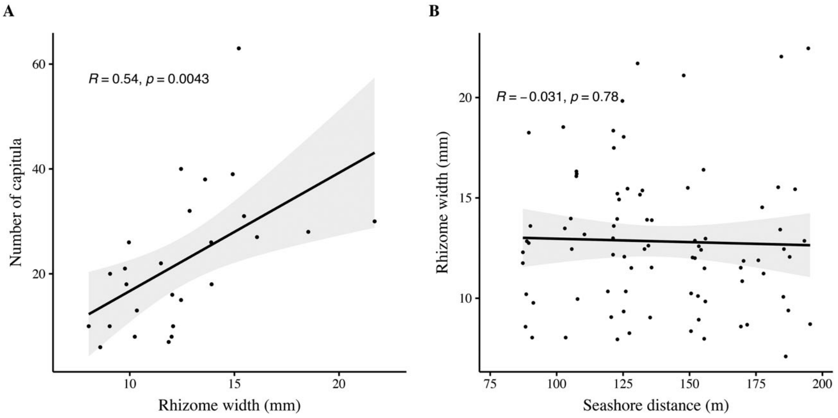

Fig. 5. Phenological curve for anthesis of *E. maritimum* in the three sampled areas, considering the total number of capitula (dark solid line) and the primary (dark-grey dashed line) and secondary (light-grey dashed line) inflorescence orders. Mean distance to the seashore was measured for each area.

Floral visitors

Twenty-one species of floral visitor were observed and successfully identified as belonging to 18 different families of four orders (Table 2), with most insects belonging to the Hymenoptera order. Some of the observed species carrying out potential pollination include *Apis mellifera* (Linnaeus, 1758*)*, *Bombus terrestris* (Linnaeus, 1758), *Megascolia maculata flavifrons* (Fabricius, 1775) and *Vanessa cardui* (Linnaeus, 1758) (Fig. 7).

DISCUSSION

The results of the present study have shown marked differences in reproductive and biometric traits among inflorescence orders in *E. maritimum*. First and second orders were larger with higher fruit and seed production than in subsequent orders. Flowering phenology is long, about 35 days, beginning in June and ending in the last days of July. However, specimens displayed variations in phenology along the inland gradient. Complete and partial exclusion of pollinators had a significant effect on fruit and seed production. Finally, 21 floral visitors from 18 different families were detected.

Reproductive traits

Reproductive traits within *E. maritimum* showed that flowers in the first inflorescence orders were larger with higher probability of producing viable fruits than those in other inflorescence orders. This variability is consistent with previous studies on northern populations of *E. maritimum* (Germany; Burmester, 2008). Similar results have also been described in *Eryngium alpinum L.*, where removal of terminal capitula led to increases in seed set of the remaining reproductive structures (Gaudeul & Till-Bottraud 2004). Inter-whorl morphometric variability is not uncommon among sequential flowering species and is considered to be a result of higher resource allocation to inflorescences of basal position (Wyatt, 1982; Gutterman, 2000; Harder & Prusinkiewicz, 2012; Baskin & Baskin, 2014) or early temporal development (Hendrix, 1984; Medrano *et al.* 2000).

Fruit and seed production per capitulum observed in the present study was similar to that in previous studies (Burmester, 2008; Isermann & Rooney, 2014). However, other studies have suggested reduced fitness in northern regions (Stasiak, 1986; Ievina *et al.* 2019), where populations seem to maintain themselves through vegetative reproduction (Andersone *et al.* 2011). Furthermore, Burmester (2008) reported that half of the

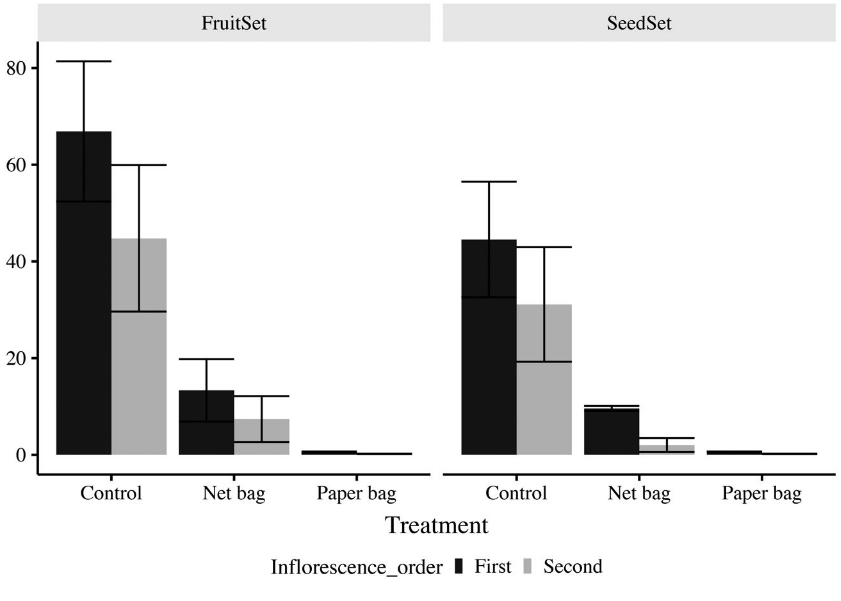

Fig. 6. Mean ±SE (error bars) of fruit and seed set of *E. maritimum* after the complete exclusion (paper bag) and the partial exclusion (net bag) treatments in the two main productive inflorescence orders (first and second). Different letters indicate significant differences among treatments after the Tukey test ($P < 0.05$).

Table 2. Floral visitors observed in the present study.

Order	Family	Species
Coleoptera	Buprestidae	*Anthaxia umbellatarum* (Fabricius, 1787)
Coleoptera	Cerambycidae	*Stenopterus rufus* (Linnaeus, 1767)
Coleoptera	Mordellidae	*Mordellistena* sp.
Diptera	Syrphidae	*Syritta flaviventris* (Macquart, 1842)
Diptera	Tephritidae	*Euaresta* sp.
Hymenoptera	Andrenidae	*Andrena* sp.
Hymenoptera	Apiidae	*Bombus terrestris* (Linneus, 1758)
Hymenoptera	Apiidae	*Apis mellifera* (Linnaeus, 1758)
Hymenoptera	Cabronidae	*Bembix oculata* Panzer, 1801
Hymenoptera	Colletidae	*Hylaeus variegatus* (Fabricius, 1798)
Hymenoptera	Crabronidae	*Philanthus triangulum* (Fabricius,1775)
Hymenoptera	Halictidae	*Halictus scabiosae* (Rossi, 1790)
Hymenoptera	Halictidae	*Lasioglossum* sp.
Hymenoptera	Scoliidae	*Scolia sexmaculata* (Müller 1766)
Hymenoptera	Sphecidae	*Podalonia tydei* (Le Guillou, 1841)
Hymenoptera	Sphecidae	*Prionyx viduatus* (Christ, 1791)
Hymenoptera	Tiphiidae	*Meria tripunctata* (Rossi 1790)
Hymenoptera	Xylocopidae	*Xylocopa violacea* (Linnaeus, 1758)
Hymenoptera	Scoliidae	*Megascolia maculata flavifrons* (Fabricius 1775)
Lepidoptera	Lycaenidae	*Celastrina argiolus* (Linnaeus, 1758)
Lepidoptera	Nymphalidae	*Vanessa cardui* (Linnaeus, 1758)

plants of the studied population (in northwest Germany) produced only one inflorescence per plant, while only 15% produced more than five inflorescences. They also described up to three inflorescence orders. In contrast, in the present study, 90% of the analysed plants produced more than five inflorescences and displayed up to six orders. In this context, differences in fitness could be related to higher fruit production per plant because of higher resource investment in reproductive structures and/or higher seed quality (*e.g.* seed germination).

Biometric analysis

The biometric analysis revealed that plants with larger caudex width had higher numbers of capitula per flowering stalk, which is consistent with previous studies performed with several geophyte species (Howard & Cellinese, 2020). Moreover, the lack of association between caudex width and seashore distance suggests that the environmental conditions, which strongly vary along the dune (Ciccarelli, 2015), did not have a direct effect on the investment in reproductive structures. These results agree with a previous study of Andersone *et al.* (2011), which suggested that seasons with high investment in reproductive structures are followed by seasons with mostly vegetative shoots and *vice versa*, as flowering is strongly dependent on rhizome carbohydrate storage. However, specific analysis of the C:N ratio in rhizomes before and after flowering are needed to assess this hypothesis. In addition, Burmester (2008) suggested that the number of reproductive structures was correlated with the plant age, while *ex-situ* observations seem to support rhizome size being more likely a combination of resource availability (*e.g.* soil nutrients or light) and age. Previous studies in dune systems have showed that biomass can be extremely variable, depending on water availability and temperature (Luo *et al.* 2017). Considering dune microsite variability, higher reproductive potential cannot be directly linked to older plants, but to better environmental conditions for development. Nevertheless, more specific data on plant density are needed in order to focus on this topic.

Phenology

The flowering timing observed in the studied area was 45 days, which is consistent with other *E. maritimum* populations of the western Mediterranean (Gratani *et al.* 1986; Burmester, 2008). However, in northern populations, flowering timing is even shorter, almost 1 month, and occurs during mid-August (Isermann & Rooney, 2014). This suggests that this species was able to adapt flowering to specific climate conditions of the areas which it inhabits. This capacity could have contributed to its wide distribution, among other factors, as it has already been proposed for other species widely distributed across the Mediterranean (Burghardt *et al.* 2015; Berger *et al.* 2017; Marcer *et al.* 2018).

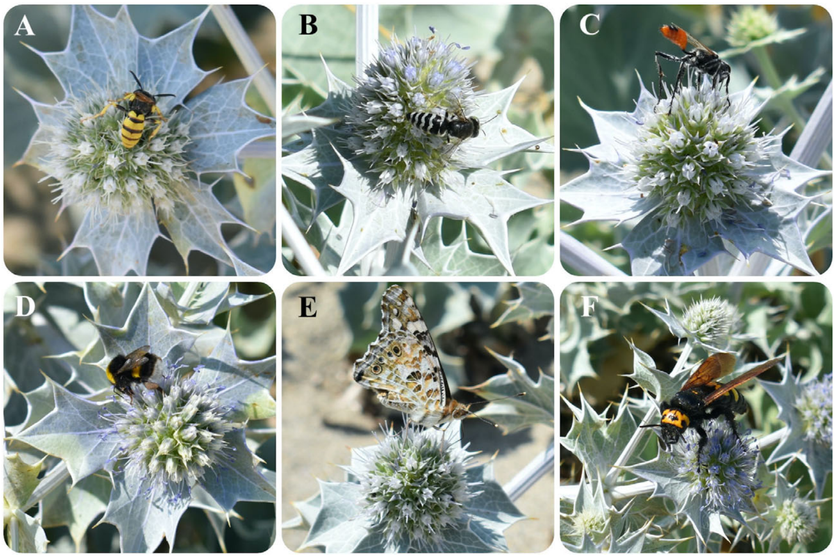

Fig. 7. Floral visitors observed in the study: *Philanthus triangulum* (A), *Bembix oculata* (B), *Prionyx viduatus* (C), *Bombus terrestris* (D), *Vanessa cardui* (E) and *Megascolia maculata flavifrons* (F).

In the studied population, slight differences in flowering were observed among patches located at different distances from the seashore. Plants far from the sea flowered almost 15 days after than those located closer to the seashore. These differences could be due to the specific ecological requirements of the species to start flowering, such as water uptake and the influence of soil conditions, which differ substantially since patches located inland are exposed to less soil mobility. Contrary findings have been observed in other species in dunes of the western Mediterranean (Gil, 1994), suggesting that patches located closer to the seashore are exposed to a buffered climate, thus delaying flowering. Although differences among species are possible, the slight differences observed among patches were not sufficiently significant to establish a pattern. For this reason, future studies should focus on this topic in order to demonstrate the presence of patterns at a community level.

Breeding system

Reproductive success in plants is an aggregate process that depends on the reproductive outcomes of all flowers (Harder *et al.* 2004; Danderson & Molano-Flores 2010). The current observations after the exclusion of pollinators and floral visitors suggest that the *E. maritimum* is mainly entomophilous, as net bagged capitula presented low fruit and seed set, which has been suggested as a mechanism to avoid self-pollination and promote allogamy (Jersakova & Johnson, 2007). A large proportion of the inflorescences of the specimens, mainly in the most external inflorescence orders, presented low fruit and seed production, suggesting that they act as attractors for pollinators and pollen donors (Harder *et al.* 2004). This large floral display has been proposed to result in a higher plant visitation rate (Makino *et al.* 2007) and, at the same time, decreases flower visitation rate (Grindeland *et al.* 2005). Considering these facts, a large floral display should be accompanied by mechanisms to limit geitonogamy (Harder *et al.* 2004), which will increase with display size (Crawford, 1984; Karron *et al.* 2004) and entails lower genetic exchange in the population (Muñoz & Devesa, 1987). Self-fertilization at capitulum level could be partially avoided by remarkable protandry, which could be a mechanism to avoid pollen exchange among flowers of the same capitulum, as in other *Eryngium* species (Molano-Flores, 2001; Gaudeul & Till-Bottraud 2004). However, in *E. maritimum* and other species of the genus (Gaudeul & Till-Bottraud 2004), flowering is not synchronous among inflorescence orders. For this reason, flower- and capitulum-level protandry will potentially avoid self-pollination, although male and female phases at the plant level are not uncoupled. Thus, self-pollination among capitula of the same plant is indeed possible. Nonetheless, previous studies have described that, at a plant level, some species of the Apiaceae show a multicycle dichogamy (Reuther & Claßen-Bockhoff, 2010), promoting outcrossing (Harder & Barrett, 1995). Studies in other *Eryngium* species have suggested that even though fruit set in self-fecundated specimens is possible, it is low when compared with outcross pollination (Molano-Flores, 2001; Gaudeul & Till-Bottraud 2004). Taking this into account, it can be hypothesized that the geitonogamy potential for reproduction is probably very low compared to xenogamy in *E. maritimum*. Furthermore, the P/O ratio is consistent with values proposed by Cruden (1977) for species with a xenogamous reproductive system. The results of the exclusion of floral visitors also suggest that wind and undetermined micro-pollinators could play a limited role in reproductive success, because they were observed inside net bags interacting with stamens, promoting movement or transporting pollen over short distances. Hence, future studies should focus on the specific role of micro-pollinators in the reproductive potential of this species.

Floral visitors

The results of the preliminary pollinator survey suggest that *E. maritimum* reproduction seemed to depend on a wide range of pollinator species. Orders and families were congruent with observations in the Balearic Islands Pollinator Database (Pollinib). However, pollen studies are needed to prove the interaction with floral visitors. The current results are also consistent with previous studies carried out in other populations (Hegi, 1975; De Rond, 2009; Isermann & Rooney, 2014) and with other species in the genus *Eryngium* (Gaudeul & Till-Bottraud 2004). More common potential pollinators were hymenopterous of medium to large size. Moreover, potential micro-pollinators were also observed inside the net bags interacting with stamens. This could have implications on the observed results that should be considered in future studies.

The results of the present work suggest that, at least in the studied population, the first and second inflorescence orders of *E. maritimum* produce most of the viable seed, while other inflorescence orders might be mainly used to attract pollinators. Reproductive structure development of this species is correlated with caudex development, while distance to the seashore did not seem to influence reproductive and non-reproductive inversion. However, inner areas furthest to the seashore showed a delay in anthesis, which suggests that the microclimatic and environmental differences from coast to inland areas could determine the reproductive output of specimens. The flowering of *E. maritimum* began in the last week of May and lasted until the end of July. On the other hand, fructification began in the middle of August and ended in late September. Finally, pollination seemed to be entomophilous and generalist.

Conclusions

Some implications can be extracted from these findings. Although the studied *E. maritimum* population presents a high investment in reproductive structures and high fruit and seed set, the species seemed to be mainly xenogamous. However, considering that exclusion of pollinators proved that anemophily did not play an important role in pollination, it is possible that the species is mainly entomophilous, so its reproductive success may depend on the conservation status of pollinator populations. This suggests that appropriate management of the species should necessarily be associated with correct management of the dune system. This is even more necessary when taking into account the microclimatic and environmental differences along the coast-to-inland gradient that affect the reproductive output of plants directly. These conditions are particularly important in the Balearic Islands, where a high degree of coastal anthropization is observed (Burjachs *et al.* 2017) that can also directly affect populations of pollinator

(Traveset *et al.* 2018), and consequently the reproductive success of *E. maritimum*. Future studies should consider the role of germination on the reproductive success of the species, which is essential for a better understanding of its reproductive biology and development.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Distribution map of the areas and plants used in the biometric study.

Table S1. Morphometric characters of the capitulum by inflorescence orders (X ± SE).

Table S2. Flower, fruit and seed production of capitulum by inflorescence orders (X ± SE).

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Evidence of interpopulation variation in the germination of *Eryngium maritimum* L. (Apiaceae)

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Abstract Germination is considered as one of the most crucial steps in a plant's lifetime. The germination of *Eryngium maritimum*, a dune species located across all European coasts, has been extensively analysed in Northern populations, where it is considered a locally endangered species. However, less attention has been given to southern populations, where the knowledge about the germination of the species is very limited. The main objective of the present study was to analyse the effect of cold stratification in one Mediterranean and one Atlantic population of the species, as well as to compare the potential variation of seed dormancy among a latitudinal gradient in European populations based on current literature. Seeds collected from Mallorca (Mediterranean, Spain) and Asturias (Atlantic, Spain) were germinated at different temperatures and cold stratification periods ranging from 4 to 20 weeks. These results were merged with the results of previous studies to test the potential effect of latitude and climatic variables in germination. Although the optimal incubation temperature was 10 °C, the highest final germination percentages (up to 96%) was obtained in both populations combining specific cold stratification periods (5 °C) and constant incubation temperatures (15–20 °C). Atlantic seeds needed longer cold stratification times to reach high germination percentages compared with Mediterranean seeds. Apart from the stratification response, significant differences in viability and in germination were observed between the Atlantic and the Mediterranean

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populations. Considering data from previous studies in the analysis, the results suggest that there is a potential effect of latitude in the ecological germination requirements, by which the higher the latitude, the longer the cold stratification period required to germinate, with a correlation with the mean annual temperature. These results suggests that two factors, modulation of dormancy relating to adaptative or maternal effects and viability, govern the germination of the species.

Keywords Dormancy · *Eryngium maritimum* · Germination · Seeds · Cold Stratification

Introduction

Coastal dune systems are considered one of the most endangered habitats, threatened by several natural and anthropogenic factors, such as sea-level rise, flooding, human trampling and infrastructures (Ciccarelli 2014; Pinna et al. 2015; Gigante et al. 2018). In this context, specific studies that focus on the species that inhabit these areas are extremely important to ensure habitat health (Vallés and Cambrollé 2013).

Eryngium maritimum (L.) is a species from the Apiaceae family that inhabits coastal areas of the Atlantic shores of Europe, including the British islands, the west and east Mediterranean coasts, and more sparingly the Black and Azov Sea coasts (Isermann and Rooney 2014). *E. maritimum* grows on sandy soils, being present in dune systems where it occurs in several communities mainly in white dunes (shifting coastal dunes), even though it can also be found in grey dunes and occasionally in other coastal or near-dune systems communities (Isermann and Rooney 2014). In the Mediterranean dunes, it develops in the Ammophiletalia phytosociological class, being mainly established in the embryonic and white dunes (Embryonic shifting dunes, Habitat 2110, EUNIS). Clausing et al. (2000) identified two different genetic clusters, the Atlantic and the Mediterranean, because of the Quaternary Würm glacial. As a result of the distribution of ice cover, permafrost and sea surface temperatures in that period, the distribution area of the species must have been dramatically reduced in West, Central and North Europe, while in the Western Mediterranean, temperatures might have been appropriate for the populations survivorship. The conservation status of E. maritimum has been extensively discussed and reviewed for northern European populations, where it is considered a locally endangered species due to habitat loss and potential pollination issues (Maarel and Maarel-Versluys 1996; Aviziene et al. 2008). However, Mediterranean stands have been given less specific attention.

Germination is considered as one of the most crucial phases in a plant's lifetime and also as the bottleneck for plant species' successful establishment (Baskin and Baskin 2014; Del Vecchio et al. 2020). To ensure that it is carried out in the optimal season, many species develop specific germination strategies entailing different types of dormancy. Seed dormancy is considered one of the main factors determining the adaptive value of germination, thereby ensuring initiation of the next generation (Fenner and Thompson 2005; Donohue et al. 2010; Willis et al. 2014). Apart from genetic origin, the level of primary dormancy in seeds may be determined by other factors, such as maternal environment during maturation, age of the mother plant during maturation and position of the seeds on the plant (Andersson and Milberg 1998; Fenner and Thompson 2005). Seed dormancy and germination are complex traits of spermatophytes that are influenced by many genes and environmental factors (Finch-Savage and Leubner-Metzger 2006; Donohue et al. 2010) both in the long term (through ecotypes or clines) and in the short term (through the influence of the seed maturation environment) (Fernández-Pascual et al. 2013).

Many species in the Apiaceae are reported to exhibit morphological dormancy (MD) or morphophysiological dormancy (MPD) (Finch-Savage and Leubner-Metzger 2006; Baskin and Baskin 2014), including the Genus *Eryngium* (Wolkis et al. 2020). In MPD, the seed coat is water permeable, embryos are undeveloped and also have a physiological inhibiting mechanism that requires an ecological signal to elicit germination (i.e. physiological dormancy; PD; Baskin and Baskin 2014). In the case of *E. maritimum*, Necajeva & Ievinsh (2013) reported a morphophysiological dormancy, in which the embryo is not fully formed when the seed is dispersed, obtaining higher germination percentages with cold stratification treatments of 5 °C. This study, carried out with populations of the North of Europe, has shown that cold stratification treatments increase germination up to 90% after

16 weeks of cold. However, it has also been reported that cold stratification periods longer than 14 weeks can produce negative effects (Walmsley and Davy 1997). Moreover, Necajeva & Ievinsh also found that the cold stratification treatment could be substituted by addition of gibberellic acid. Optimal temperatures for germination have been reported with alternating temperatures ranging from 10 °C to 20 °C (Walmsley and Davy 1997), increasing embryo development at higher incubation temperatures (Necajeva & Ievinsh, 2013). However, there is a lack of studies analysing the germination of *E. maritimum* populations of different latitudes, which could be critical to understand the potential capacity of modulation of MPD of the species.

The main aim of this study is to analyse the germination of *E. maritimum* in one Atlantic and one Mediterranean population, in order to evaluate the potential differences between populations. The main hypothesis of the present study is that the temperature differences between one Mediterranean and one Atlantic Spanish population of *E. maritimum* will have a strong impact on germination, the required length of cold stratification increasing at higher latitudes. We also aim to analyse if, considering previous studies carried out with the species, there is a relationship between latitude and the length of the cold stratification to break PD in *E. maritimum*.

Material and methods

Seed collection

Fruits were collected in Son Serra de Marina (3.237619 / 39.731218, WGS84, EPSG: 4326), a dune system located in the northern area of Mallorca (hereafter Mediterranean population), and in Playa de Xagó (– 5.918455 / 43.604510, WGS84, EPSG: 4326), in Gozón, Asturias (hereafter Atlantic population), both representing two different climatic profiles occurring in Spain coasts (Fig. 1). Only seeds from the central capitulum of the *E. maritimum* inflorescence were collected, in order to avoid potential inter-whorl variability. Two thousand seeds were collected from approximately fifty random individuals during September 2018 (Mediterranean population) for the incubation experiment and September 2019 (Atlantic and Mediterranean populations) for the cold stratification experiment. Controls were compared between both years to avoid the possible effect of interannual variation. Seeds were maintained at 23 °C and 60% of relative humidity until pre-germination treatments for 60 days when subsequent germination tests were carried out.

General germination procedure

The experiments were carried out in the University of the Balearic Islands, Spain. In all trials, seeds were sown in Petri dishes, to avoid desiccation, with paper filters moistened in distilled water, and then incubated at constant temperatures in dark conditions, as Carta et al. (2017) proved that photoinhibition in *E. maritimum* is negligible. Each trial was conducted using 4 replicates of 25 seeds (total of 100 per trial), which were assessed every two days till a maximum of 90 days since sowing per trial. Seeds were considered germinated when radicle protrusion was visible, and a cut test was assessed on the ungerminated seeds (Baskin and Baskin 2014), considering non-germinated seeds with an embryo as viable seeds and without it as unviable seeds.

In all trials, several germination parameters were calculated as proposed by Aravind et al. (2019). Germination Speed (percentage of germinated seeds per day), final germination percentage, days to reach first and last germination, synchrony of germination and germination uncertainty were calculated as indicated by Dastanpoor et al. (2013).

Optimal incubation temperature

As a pre-step before collecting Atlantic seeds, a first trial was conducted to establish the optimal incubation temperature of Mediterranean seeds in 2018. So, seeds were incubated at 5, 10, 14, 18, 20 and 23 °C in dark conditions for 90 days, as established in the *general germination procedure*.

Effect of cold stratification

A second trial was conducted with seeds from both populations collected in 2019, in which seeds were incubated at 6, 8, 10, 12 and 14 weeks of cold stratification at 5 °C in both populations. Seeds were germinated at constant 15 and 20 °C in the dark. While carrying out the experiment, considering the low

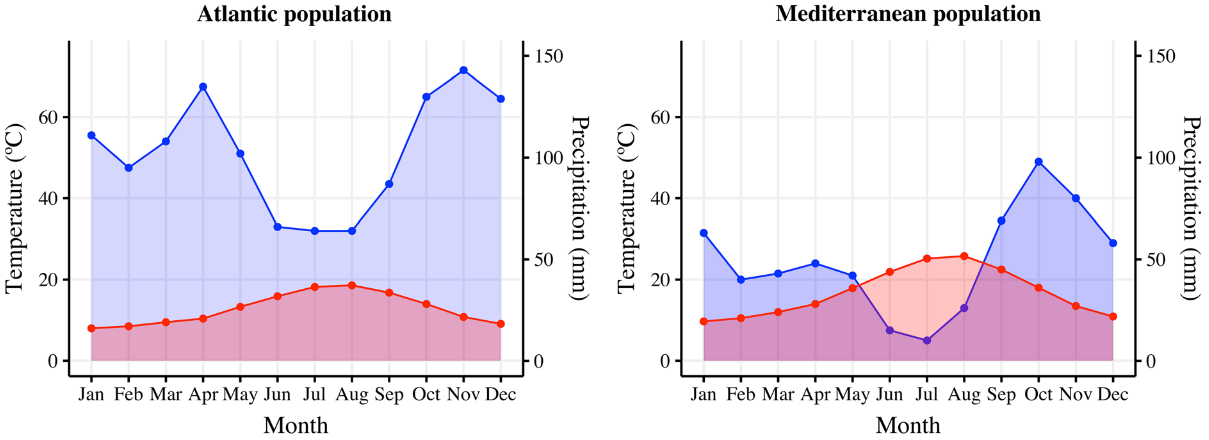

Fig. 1 Ombrothermic diagram of the study sites. Mean precipitation is plotted in blue, while the mean temperature is plotted in red. Data: AEMET 2019

germination percentages of Atlantic seeds (see Results), a surplus of Atlantic seeds (not available in the Mediterranean population) was incubated after 16, 18 and 20 weeks of cold stratification at 5 °C.

Latitudinal variation

The existing literature regarding germination of *E. maritimum* was gathered to make further analysis, considering the temperature of incubation, latitude, longitude, stratification time and final germination percentage of each trial. Environmental data of each population were extracted using Worldclim (Fick and Hijmans 2017). To make it possible to compare among studies with different stratification times, final germination was weighed with the number of weeks under cold stratification of each treatment, as follows:

$$\text{Weighted Germination} = \frac{\text{Final Germination Percentage}}{\text{Cold stratification length (weeks)}}$$

Statistical analysis

Germination parameters were calculated using the package *Germinationmetrics* (Aravind et al. 2019) in R software (R. C 2013), using the user interface RStudio (RStudio Team 2020). Descriptive statistics and plots were carried out using the *Tidyverse* library (Wickham 2019).

In the stratification experiment, cumulative germination in the different treatments was modelled considering the germination temperature and the stratification period as the experimental variables, considered in all analyses as numeric variables. For count response variables (day of the first germination), generalised linear models using the Poisson family (link *log*) and zero-inflated models were used. On the other hand, for percentage data (Final Germination Percentage), logistic Generalised Linear Models with a binomial error distribution were used. In all cases, model selection was carried out according to the Corrected Akaike selection Criterion using AICc (Bozdogan 1987; Parmoon et al. 2015). Model accuracy was evaluated using Q-Q plots and McFadden Pseudo-R2 when possible (Veall and Zimmermann 1996). Significant effects of the experimental variables were evaluated using ANOVA (Rutherford 2011).

Data of the present study were gathered with the final germination percentage results of the existing literature to analyse the potential effects of latitudinal variation on the germination. Final germination was modelled using Beta regression models (Zeileis et al. 2016) considering Latitude, Longitude and environmental data as explanatory variables. Final models were achieved via backwards elimination (log-likelihood ratio test, $P < 0.05$) using the lmertest package (Kuznetsova et al. 2015). The effect of explanatory variables on final germination was evaluated using ANOVA (Rutherford 2011).

Results

Optimal incubation temperature

No germination was observed in the Mediterranean population seeds at 18, 20 and 23 °C. Germination increased from 5 °C (Mean (M) = 6.67%, Standard Deviation (SD) = 3.34, Number of samples (N) = 4) to 10 °C ($M = 30.04\%$, SD = 6.00, $N = 4$) and decreased again before 18 °C ($M = 4.55\%$, SD = 2.21, $N = 4$). The low germination observed prevented other variables from being taken into consideration.

Effect of cold stratification

The percentage of viability among lots was significantly higher ($p < 0.001$) in the Mediterranean ($M = 29.5$, SD = 2.02, $N = 52$) than in the Atlantic population ($M = 15.1$, SD = 1.37, $N = 43$) but showed no difference between temperatures ($p = 0.177$, Table 1). A final germination percentage of 88.4 ± 0.21% was obtained in Mediterranean seeds under 12 weeks of cold stratification at 5 °C, followed by constant incubation at 20 °C, while in Atlantic seeds, the maximum mean germination of 70.22 ± 0.44% was observed at an incubation temperature of 15 °C after 20 weeks of cold stratification. The cold stratification treatment had a significant ($p < 0.001$) effect on final germination in both populations, as well as temperature ($p < 0.001$) and their interaction (p value < 0.001, Table 2). Mediterranean seeds germinated significantly more than Atlantic seeds for the same stratification time ($p < 0.001$, Fig. 2). In contrast to Mediterranean seeds, Atlantic seeds germinated significantly better at 15 °C than at 20 °C ($p < 0.01$).

First and last days of germination, as well as the germination time range, were not explained by the analysed variables (Table 2), although Atlantic seeds germinated a few days later than Mediterranean seeds at the same stratification time (Fig. 3), with no significative differences among treatments. In general, seeds germinated quicker at longer cold stratification times. On the other hand, Mediterranean seeds germinated quicker than Atlantic at the same stratification time. Finally, differences in germination synchrony were observed between regions and temperatures, being higher in Mediterranean than in Atlantic seeds and at 15 °C than at 20 °C.

Latitudinal variation

Previous literature data were gathered and merged with our results to analyse potential trends at latitudinal level. Seeds reached higher germination percentages at the same stratification time in southern than in northern populations, suggesting a negative correlation between latitude and germination (Fig. 4). The combination of latitude, longitude and the cold stratification period strongly explained final germination ($p < 0.001$, R2 = 0.743), achieving higher germination percentages as latitude decreases ($p < 0.001$). On the other hand, the coldest temperature of the coldest month and the Annual Mean Temperature explained part of the variability observed on the final germination per week of stratification ($p < 0.001$, $F = 39.09$, R2 = 0.768).

Discussion

Optimal incubation temperature

The results of the pre-study with the Mediterranean population suggest that the optimal incubation temperature is 10 °C without a cold stratification treatment. However, when applying cold stratification temperatures, this same population reached higher germination percentages at 20 °C than at 15 °C. This supports the hypothesis that *E. maritimum* seeds are physiological (PD) or morphophysiological dormant (MPD), and so they need a trigger, in this case, a cold stratification period to begin germination. Considering that MPD was reported by Necajeva and Ievinsh (2013), it can be suggested that embryos are incompletely developed as morphophysiological dormant seeds at the end of the fruiting period (Wolkis et al. 2020), and so they have to develop during autumn and winter, while they need some high temperatures at spring to begin the germination process (Vandelook et al. 2012). Moreover, seeds from the Atlantic population germinated better at 15 °C, which could be linked with the same hypothesis, and so suggests that the species has been able to adapt its ecological germination requirements to the specific

Table 1 Results of the ANOVA test of the temperature and stratification experiment

Factor		Number of Rotten seeds	Germination Percentage by day	Final Germination Percentage	First Germination Day	Last Germination Day	Days from first to last germination	Germination Speed	Germination Uncertainty
Stratification	*df*	1	**1**	**1**	**1**	**1**	1	**1**	**1**
	F	0.566	**6792.34**	**133.962**	**12.473**	**33.611**	2.383	**14.844**	**43.090**
	p value	0.453	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.001*****	0.126	**< 0.001*****	**< 0.001*****
Temperature	*df*	1	**1**	**1**	**1**	**1**	**1**	1	**1**
	F	0.1845	**146.02**	**18.135**	**6.280**	**105.716**	**36.942**	0.646	**28.844**
	p value	0.177	**< 0.001*****	**< 0.001*****	**0.014***	**< 0.001*****	**< 0.001*****	0.423	**< 0.001*****
Region	*df*	**1**	**1**	**1**	**1**	**1**	**1**	**1**	**1**
	F	**88.932**	**3312.52**	**47.494**	**49.487**	**49.487**	**15.032**	**303.729**	**94.045**
	p value	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.004****	**< 0.001*****	**< 0.001*****	**< 0.001*****
Day	*df*		**1**						
	F		**4817.76**						
	P value		**< 0.001*****						
Stratification: Temperature	*df*	**1**			1	1	1	1	
	F	**9.906**			0.137	2.106	2.253	0.176	
	p value	**0.002****			0.712	0.149	0.136	0.675	
Stratification:Region	*df*	**1**			**1**	**1**	1	**1**	
	F	**27.911**			**5.107**	**10.900**	0.433	**52.360**	
	P value	**< 0.001*****			**0.026***	**0.001***	0.512	**< 0.001*****	
Temperature:Region	*df*	1	**1**	**1**	1	1	1	**1**	1
	F	1.628	**88.97**	**2.962**	1.940	1.286	0.104	**8.413**	0.024
	p value	0.205	**< 0.001*****	**0.023**	0.167	0.259	0.748	**0.004****	0.878
Stratification: Temperature: Region	*df*	**1**			1	**1**	**1**	1	
	F	**10.024**			0.292	**4.899**	**5.147**	2.990	
	P value	**0.002****			0.589	**0.029***	**0.026***	0.087	
Residuals		109	10,254	109	99	99	99	99	109
	R2	0.20	**0.692**	**0.713**	0.12	0.09	0.03	**0.66**	**0.624**
	Model p value	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.001*****	**0.002***	**< 0.001*****	**< 0.001*****
	Model family	GLM—Poisson	GAM	GLM—Poisson	GLM—Negative Binomial Poisson	GLM—Negative Binomial Poisson	GLM—Negative Binomial Poisson	GLM—Poisson	GAM

A single model was carried out with each response variable (first row) with the explanatory variables (first column). Interactions among variables are indicated with ":" Model coefficients for each variable are indicated, as well as model general accuracy (bottom portion). *df* degrees of freedom, *F* Fisher coefficient. Asterisks are used to highlight *p* values below the 0.05 significant threshold

Bold text is used to highlight significant variables environmental conditions, as occurs in other species (Finch-Savage and Leubner-Metzger 2006).

Effect of cold stratification

Considering the effect of cold stratification on the germination, the results of the present study show that *E. maritimum* seeds reach higher germination percentages in the Mediterranean population than in the Atlantic population at shorter stratification periods. So, it can be considered that seeds from the Mediterranean population are less dormant than Atlantic. Compared to Northern European populations, Mediterranean seeds from the present study have reached the highest germination percentages observed in this species (up to 96%, Fig. 2) (Jankeviciene 1978; Walmsley and Davy 1997; Klavina et al. 2006; Curle et al. 2007; Necajeva & Ievinsh, 2013) which could be linked to environmental differences among populations (Fig. 1), overall considering climatic interannual variations at the time of seed maturation. However, more populations are required to understand if these differences are related to a latitudinal trend (see *Latitudinal variation*).

Comparison with previous studies could be biased by the fact that some studies have not made any viability test (Necajeva et al. 2003), which can strongly decrease the maximum germination percentages due to inviable seeds (i.e. empty seeds) (Necajeva et al. 2003). Empty seeds can reach 30% of the total seed lot (Walmsley and Davy 1997), and sometimes up to 60% (Aviziene et al. 2008).

The cold stratification treatment proved to have a substantial effect on germination, improving the final germination percentage of both populations. These results are contrary to those obtained in a previous study carried out by Walmsley and Davy (1997) with *E. maritimum* seeds from Sizewell, United Kingdom, which stated that more than 6 weeks of cold stratification did not increase germination. Similarly, Fernández-Pascual et al. (2017), who analysed the effect of cold stratification requirements of several dune species in the Atlantic seaboard of Northern Spain, proposed that cold stratification did not improved the germination of *E. maritimum*. However, the optimal stratification period has been suggested to be longer in higher latitudes, like in Asturias or Northern European populations (Necajeva et al. 2003; Klavina et al. 2006).

Latitudinal variation

The results of the present study combined with the results of the previous studies suggest that there is a trend in the germination of this species, in which the higher the latitude and the lower the mean annual temperature, the more weeks of cold stratification are needed to germinate (Fig. 4A, B). Our results suggest that *E. maritimum* has been able to couple germination dormancy to the specific climatic conditions of each population, preventing seeds in regions with a clear winter season from germinating in summer or autumn, which is a mechanism to decrease the risk of frost damage during the vulnerable seedling stage in winter (Baskin and Baskin 2014; Nikolaeva 2004; Vandelook et al. 2012). This is consistent with the findings of Vandelook et al. (2012), who obtained in a wide analysis of species belonging to the Apiaceae family that germination was mainly related to temperature conditions and altitude. Carta and collaborators (2016) obtained similar results for *Hypericum elodes* (L.), finding that the effect of cold stratification was weaker in southern populations with no relationship to population genetic differentiation, highlighting that physiological dormancy can be modulated by local climate.

Because of a lack of studies carried out in northern populations applying long cold stratification periods (> 14 weeks), in *E. maritimum,* it is difficult to establish if seeds from northern populations are more dormant or germinate poorly due to other factors such as genetic inbreeding (Isermann and Rooney 2014). In our results with Spanish populations, we have observed a significant interaction between the stratification length and the region, supporting the hypothesis that dormancy increases at higher latitudes. Differences among populations at a latitudinal gradient could also be attributed to genetical variations, as suggested by Clausing and collaborators (2000). Considering these results, populations at the same latitude but influenced by different seas could germinate at different rates, which could explain some observed irregularities in the latitudinal trend. This hypothesis is consistent with our findings, considering the significant effect of the region when leaving aside the effect of the stratification length, and also the differences in the seed viability of both populations. So, it can be suggested that both factors, differences in dormancy due to maternal or genetic effects and differences in seed viability, govern the observed

Table 2 Results of the regression models of the temperature and stratification experiment

	Cumulative germination	Final germination percentage	First germination day	Last germination day	Time spread germination	Germination speed	Germination uncertainty
Predictors	*Estimates*	*Estimates*	*Log-Mean*	*Log-Mean*	*Log-Mean*	*Log-Mean*	*Estimates*
(Intercept)	**36.10*****	**88.35*****	**5.57*****	**5.31*****	3.37	**1.65*****	**3.28*****
	(32.79–39.42)	**(57.90–118.79)**	**(2.49–8.66)**	**(3.34–7.29)**	(− 3.49–10.35)	**(− 2.06– − 1.25)**	**(2.03–4.53)**
Temperature	**− 1.41*****	**− 3.78*****	− 0.09	− 0.07	− 0.05	**− 0.08*****	**− 0.14*****
	(− 1.60– − 1.22)	**(− 5.50– − 2.06)**	(− 0.27–0.09)	(− 0.18–0.05)	(− 0.45–0.35)	**(− 0.10– − 0.06)**	**(− 0.21– − 0.07)**
Region [Mediterranean population]	**18.23*****	9.17	− 1.49	1.63	6.25	0.18	1.75
	(13.43–23.03)	(− 34.97–53.30)	(− 5.06–2.08)	(− 0.67–3.93)	(− 2.04–14.49)	(− 0.27–0.62)	(− 0.06–3.55)
Temperature* Region [Mediterranean population]	**1.57*****	2.44	0.08	− 0.08	− 0.33	**0.09*****	0.01
	(1.29–1.84)	(− 0.06–4.94)	(− 0.13–0.28)	(− 0.22–0.05)	(− 0.80– 0.15)	**(0.06–0.11)**	(− 0.09–0.11)
s (Day)	**2.00*****						
s (Stratification)	**2.00*****	**1.96*****					**1.91*****
						1	
Stratification			− 0.06	0.04	0.16	**0.07*****	
			(− 0.28–0.15)	(− 0.10–0.17)	(− 0.31–0.63)	**(0.05–0.10)**	
Stratification*Temperature			0	0	− 0.01	0	
			(− 0.01–0.01)	(− 0.01–0.01)	(− 0.03–0.02)	(0–0)	
Stratification*Region [Mediterranean population]			− 0.03	− 0.20	− 0.61	0	
			(− 0.34–0.28)	(− 0.40–− 0.01)	(− 1.32–0.10)	(− 0.03–0.03)	
Stratification*Temperature*Region [Mediterranean population]			0	0.01	0.04	0	
			(− 0.02–0.01)	(0–0.02)	(− 0.01–0.08)	(0–0)	
Observations	10,260	114	107	107	107	114	115
R2	**0.692**	**0.713**	0.12	0.09	0.03	**0.66**	**0.624**
Model p value	**< 0.001*****	**< 0.001*****	**< 0.001*****	**< 0.001*****	**0.002***	**< 0.001*****	**< 0.001*****
Model family	GAM	GAM	GLM—Negative Binomial Poisson	GLM—Negative Binomial Poisson	GLM—Negative Binomial Poisson	GLM—Poisson	GAM

A single model was carried out with each response variable (first row) with the explanatory variables (first column). Interactions among variables are indicated with ":". When GAM models were applied, smooth variables are indicated with "s". Coefficients are indicated for each combination of variable, indicating positive coefficient positive effects of the explanatory variable on the response variable. 95% confidence intervals are indicated between brackets

Bold text is used to highlight significant variables

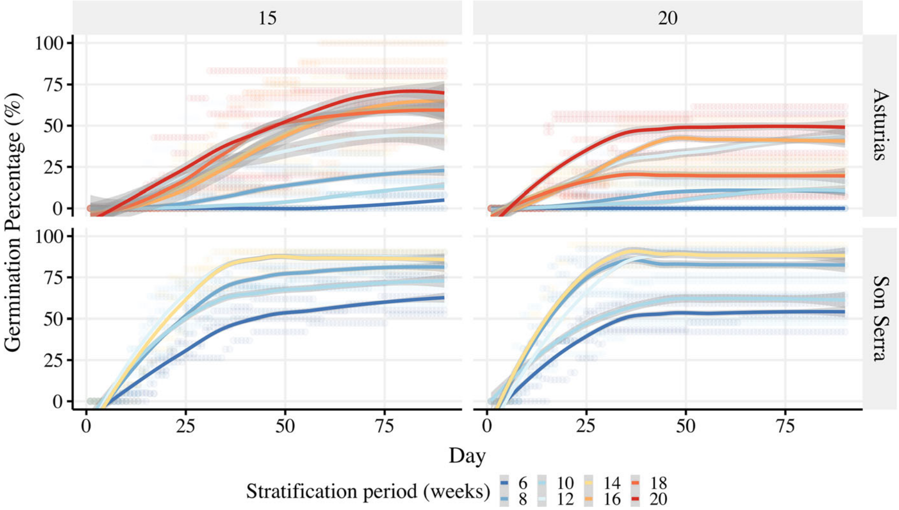

Fig. 2 Germination Percentage (left axis) by day (base axis) at two different germination temperatures (15 °C and 20 °C) and 8 different stratification times (6–20 weeks, blue to red gradient) in the two studied populations

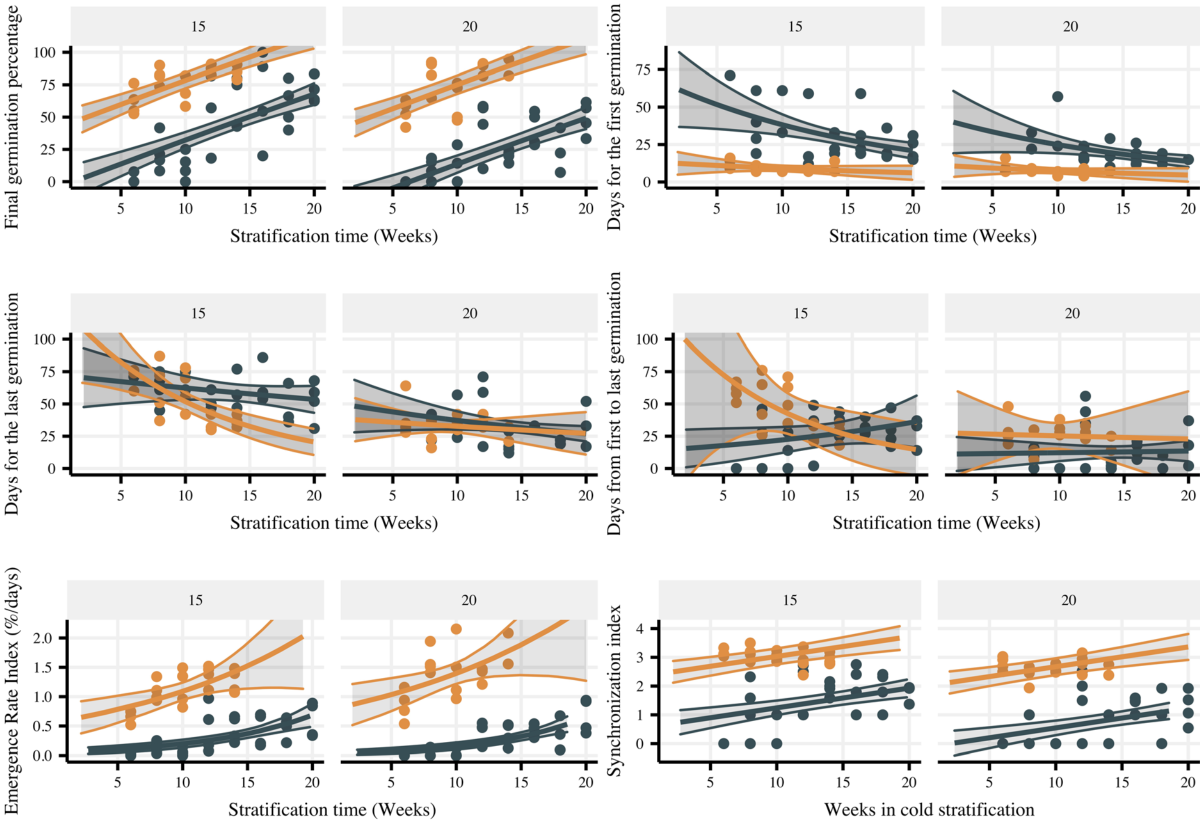

Fig. 3 Observed and predicted data of the germination variables in two different incubation temperatures (15 and 20 °C) in the two studied populations (Atlantic—Dark blue; Mediterranean—Orange)

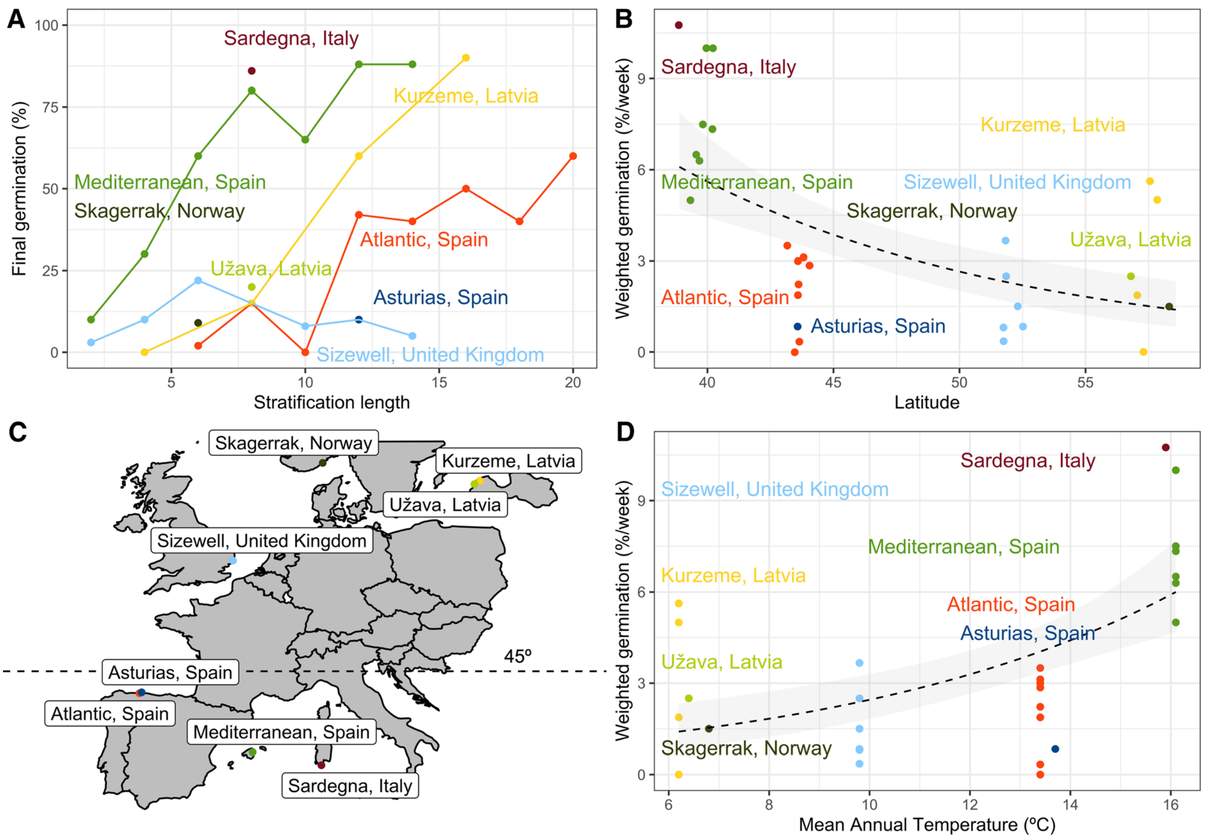

Fig. 4 Meta-analysis considering the potential latitudinal variation of the germination of *E. maritimum* (More information in Supplementary material—Table S1). In (**a**), the relation between final germination percentage and the cold stratification length (weeks) is shown. In (**b**), the weighted germination (final germination percentage/stratification length) is plotted against latitude. **c** represents the analysed populations, emphasising the analysis below and above the 45 °C latitude limit. **d** analyses the relationship between weighted germination and the annual mean temperature of the populations

dynamics of *E. maritimum* germination along a latitudinal trend across European populations. However, an extensive study considering both factors should be carried out avoiding interannual and methodological variation to prove this trend.

Conclusions

Germination in *E. maritimum* is variable depending on the temperature and the stratification length. Cold stratification at 5 °C increased germination in both populations. Seeds from the Mediterranean population, which are located near the meridional border of its distribution range, need a shorter period of cold temperatures to germinate than those located in Northern populations with colder climates. On the other hand, apart from the effect of stratification, the Atlantic population has lower viability rates and lower final germination percentages than the Mediterranean population. It can be suggested that two factors, differences in dormancy due to maternal or genetic effects and differences in seed viability, govern the observed dynamics of *E. maritimum* germination along a latitudinal gradient across European populations.

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Declarations

Conflicts of interest The authors of the present study declare that no conflicts of interest have been present while carrying out this work.

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Capítulo 2.

Evaluación de la halotolerancia de *Eryngium maritimum* L.

Manuscript III

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Salinity effects on the germination and reproduction of *Eryngium maritimum* L. (Apiaceae)

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ABSTRACT

Coastal ecosystems are globally threatened. As sea level rises, flooding, salt-water intrusion and coastal regression are dramatically changing their environmental conditions. *Eryngium maritimum* L. (Apiaceae) mainly inhabits white dunes of the Mediterranean and Atlantic coasts. The present study aimed to analyse the effect of salinity on the germination and reproduction of the species. Seeds from Mallorca (Spain) were germinated with four different salts (NaCl, Na_2SO_4, $MgCl_2$, $MgSO_4$) at concentrations ranging from 0 to 0.4 M. On the other hand, seedlings were grown and watered with different seawater (SW) concentrations ranging from 6.25% SW to full SW. Reproductive investment was assessed considering the number of inflorescences, fruit set and seed set per plant per whorl in the different treatments. Complementarily, field electrical conductivity measurements were carried out in a natural population to assess its variability along the dune. Germination was inhibited by salts, with nearly null germination at concentrations higher than 0.1 M, with significant differences among salts. Specifically, Na_2SO_4 and $MgCl_2$ strongly affected germination rather than NaCl and $MgSO_4$. Plant dieback increased in concentrations higher than 50% SW. Fruit and seed production was inhibited by the presence of salts even at concentrations of 12.5% SW. Embryonic dune was the most saline area, with decreasing electrical conductivity in white and semi-fixed dunes. These results suggest that *E. maritimum* can deal with high salt concentrations along its life cycle. However, to germinate and reproduce, it tolerates only low salt concentrations. So, it should be considered as a halotolerant species. Considering its distribution along the dune, the salinity of the strandline could be, along with other factors, responsible for its decrease in fitness in front dunes. As a result of sea-level rise and coastal salinization, it can be hypothesized that the optimal distribution of *E. maritimum* along the dune will change, shifting from actual foredunes to dune-crests.

1. Introduction

Although coastal ecosystems are globally threatened, they are largely understudied (Lum and Barton, 2020). As sea level rises, flooding, salt-water intrusion, and coastline recession will drastically impact coastal areas across all the globe (Culbertson et al., 2009), especially in the Mediterranean Sea (Enríquez et al., 2017, 2019). As belowground sea levels in coastal regions rise, some coastal plant communities will be exposed to intense salinity levels. These projected changes will result in a loss of habitats. Although it seems likely that coastal plants have adapted to tolerate saline conditions, high salinity was observed to limit recruitment, shift phenology, and alter plant species distributions in a variety of coastal ecosystems (Lum and Barton, 2020), including sand dunes (Feagin et al., 2005; Greaver and Sternberg, 2007).

Eryngium maritimum L. (Apiaceae) mainly inhabits dunes of the Mediterranean and Atlantic coasts, although very rarely it can be found growing on rocks (Isermann and Rooney, 2014). In the North coast of Europe, it is considered as a strongly endangered species, while in the Mediterranean it exhibits a better conservation status (Isermann and Rooney, 2014). Its populations are subjected to different environmental stresses such as salt spray, episodic over-wash, highly permeable and mobile substrates, low field capacity, high temperatures, drought and strong winds (García-Mora et al., 1999; Isermann and Rooney, 2014). In the Mediterranean, *E. maritimum* is a diagnostic species of the phytosociological alliance *Ammophilion australis Br.-Bl. 1921 corr. Rivas-Martínez, Costa & Izco & Rivas-Martínez, Lousã, T.E. Díaz, Fernández-González & J.C. Costa 1990* (class Ammophiletea Br.-Bl. et Tx. ex Westhoff et al. 1946, shifting dunes or white dunes; Marcenò and Jiménez-Alfaro,

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2017), being related to soils with high percentages of sand, with less than 2.5% of clays and silts (Özcan et al., 2010). Low nutrient concentrations were reported in the soils where *E. maritimum* grows (Fitter and Peat, 1994), with low concentrations of nitrogen and potassium (Isermann and Rooney, 2014) and variable concentrations of phosphorus (Andersone et al., 2011; Aviziene et al., 2008).

Salinity is one of the main environmental factors that modulate coastal species distribution. Halophyte species are able to deal with salinity, deploying several biochemical and metabolic strategies (Kataria and Verma, 2018). Seawater can be considered as a source of nutrients, but at the same time high Na^+ concentration inhibits uptake of K^+ ions which is an essential element for growth and development that results in lower productivity and may even lead to death (James et al., 2011; Eisa et al., 2012). On the other hand, alterations in stomatal conductance, reduced photosystem II efficiency, decreased photosynthetic capacity, reduced biomass and poor yield were associated with the ionic phase (Isla et al., 1998; Tester and Davenport, 2003; Netondo et al., 2004; Tavakkoli et al., 2011). Although *E. maritimum* has been systematically considered as a halophyte in previous literature (Meot-Duros et al., 2008, Öztürk et al., 2010; Ivanova et al., 2015; Zitouni et al., 2016; Pereira et al., 2019), to our knowledge, the effects of salinity on the germination and reproduction of the species have never been assessed before. Germination can be inhibited by salts in mainly two ways: by osmotic effect, in which uptake of water is inhibited as a result of high concentration of solutes in the soil; and by ionic toxicity, in which the accumulation of ions inside of seeds is responsible for embryo death (Ungar, 1982; Cardona, 2017). Due to this last effect, different salts must be evaluated when analysing the potential effect of salinity on germination, as previous studies have suggested that the recovery rate is not equal among ions (Pujol et al., 2000; Cardona, 2017). Understanding the effect of salinity on germination of *E. maritimum* is particularly important as new populations establish largely along coasts (Clausing et al., 2000), after dispersal of seeds by sea (Kadereit et al., 2005). However, Curle et al. (2007) observed that floatability is limited, with 50% of mericarps sinking after 3 days. Moreover, the distribution of the species along the dune system, ranging from strandline to grey dunes (Isermann and Rooney, 2014), indicates that the species is somehow capable of dealing with salinity. In this sense, it is vital for its conservation to understand how salinity modulates the distribution of the species as a result of the implications that salinity has on the reproduction of the species.

The aim of the present study was to test how different salts at different concentration levels affected the germination and to evaluate the effects of constant salinity on the reproductive success of the species. Our approach complements experiments applying pulses of salinity stress to simulate extreme climatic events, which have documented considerable vulnerability of coastal plants to salinity (White et al., 2014; Hanley et al., 2020; Lum and Barton, 2020).

2. Material and methods

2.1. Seed collection

Seeds were collected in Son Serra de Marina (3.237619 E / 39.731218 N; WGS84, EPSG: 4326), a dune system located in the northern area of Mallorca. Seeds ($N = 2000$) were collected from random individuals ($N \approx 80$) during September 2019. Seeds were also collected haphazardly in each plant without considering its whorl of origin, as this factor has been previously tested indicating an absence of effect on germination (Necajeva and Ievinsh, 2013). Seeds were maintained at room temperature (≈21 °C, 40% relative humidity) until pre-germination treatments and subsequent germination tests were carried out.

2.2. General germination procedure

The experiments were carried out in the University of the Balearic Islands, Spain. In all trials, seeds were sown in Petri dishes to avoid desiccation, with paper filters moistened in distilled water. Before germination, seed viability was assessed applying a slight pressure with a laboratory scalpel to the seeds, with fruits lacking seeds being discarded previously to germination procedures. Each trial was conducted using 4 replicates of 25 seeds (total of 100 per trial), which were assessed every two days until a maximum of 90 days of germination per trial. As the seeds of *E. maritimum* are morphophysiologically dormant (Necajeva and Ievinsh, 2013), the optimal germination conditions previously established for the population were used (Cortés-Fernández et al., 2021). So, seeds were pretreated with 10 weeks of cold stratification, in which seeds were stored in wet perlite at a constant temperature of 5 °C (Cortés-Fernández, 2021), and then put to germinate at 20 °C under different salt treatments. Seeds were germinated in the dark, as the species does not benefit from photoperiod variations (Carta et al., 2017). Salt effect on germination was tested by germinating seeds with 4 different salts (NaCl, $MgCl_2$, $MgSO_4$, Na_2SO_4), the most abundant in saline soils (Vicente et al., 2009), at 4 different concentrations (0.1, 0.2, 0.3 and 0.4 M), corresponding 0.4 M with the maximum concentration of NaCl found in Western Mediterranean seawater (See Table S1 for correspondences between molarity and concentration in g/L; Margalef, 1991). Different salts were used as it has been indicated to produce different stress in germination (Vicente et al., 2007; Cardona, 2017). In parallel, a control treatment was established using distilled water. The ungerminated seeds of the different saline treatments were rinsed with distilled water and resown on distilled water for an additional 30 days to study germination recovery (Cardona, 2017). Seeds were considered germinated when radicle protrusion was visible, and a cut test was assessed on the final ungerminated seeds (Baskin and Baskin, 2014). Unsound fruits, which were not identified in the first phase, were discarded from further calculations.

In all trials, germination speed (percentage of germinated seeds per day), final germination percentage (number of germinated seeds out of total number of seeds), days to reach first and last germination and the germination uncertainty (or synchrony of germination, the number of germinated seeds per time interval) were calculated as proposed by Aravind et al. (2019). Theoretical osmotic potential of each salt was considered for analysis as suggested by Zehra et al. (2013).

2.3. Growing conditions

Plants were cultivated in 5 L pots with culture substrate composed of 61.5% coconut fibre, 33.0% blond peat moss and 5.5% of expanded perlite, fertilised with 4.4 mg/l of Osmocote NPK 19-10-19, a slow-release fertilizer which provides plants optimum mineral nutrient levels, including N, P, K, Mg, Fe, Cu, Zn and Mo. Plants were cultivated in November from germinated seeds and were grown for one and a half year until sea-water treatments started in mid-May. Because *E. maritimum* is a xenogamous species strongly dependent on pollinators, with a nearly null autocompatibility (Cortés-Fernández et al., 2022), plants were maintained through all the growing and experimentation periods outdoors under a green shade cloth (50% light exclusion) at the University of the Balearic Islands (Mallorca, Spain). Seawater treatments were established by combining the corresponding proportion of seawater (collected in Sa Ràpita locality, Mallorca) with tap water from the experimental field facility. Sixty individuals were then randomly allocated to six seawater treatments (10 plants/treatment) (EC in mS/cm): control–tap water (1.05), 6.25% SW (5.07), 12.5% SW (9.30), 25% SW (16.34), 50% SW (30.30) and full-SW (55.69). Each treatment was applied during a 15-day period by progressively watering with increasing seawater concentration starting with 6.25% SW level until full treatment concentration was achieved in each treatment (intervals of 5 days between watering), to avoid osmotic shock (Amor et al., 2005).

In this way, a pre-treatment period was established which lasted for 15 days, followed by a complete treatment of two months before flowering period started in mid-May, and continued until fruiting ended in September. Watering was performed with tap water until field capacity with a frequency that varied from weekly to 3 times per week. EC was measured weekly (XS Instruments Cond 51+) to ensure that soil salinity was maintained at the corresponding treatment EC value.

2.4. Reproductive measurements

Reproductive traits were measured in September in the second year of plant growth, when fruiting period ended and before fruit dispersal occurred. Each inflorescence was cut, and all measurements were conducted both at the inflorescence and capitulum level. At inflorescence level, scapus height and diameter, as well as the number of capitula per whorl, were recorded. At capitulum level, diameter, length, number of flowers, number of aborted flowers, seed viability based on the cut test, and fruit set and seed set were considered for analysis. Finally, mericarp length and width were also measured among treatments.

2.5. Field electrical conductivity measurements

Complementarily, electrical conductivity (EC) was measured along a year (2020) using Sa Rapita dune system to analyze the potential correlation between the distribution of *E. maritimum* along the dune system and EC (Fig. S1). A straight transect was established along the dune selecting 4 sampling points, one for each vegetation community, considering the following syntaxonomical alliances as proposed in Rivas-Martínez et al. (2001): vegetation of embryonic dunes (*Agropyro-Minuartion peploidis* Tüxen in Br.-Bl. & Tüxen 1952), with *Elymus farctus* as the main indicator species, white dunes (*Ammophilion australis*), with *Ammophila arenaria* (L.) Link, semi-fixed dunes (*Crucianellion maritimae* Rivas Goday & Rivas-Martínez 1958), with a predominance of *Crucianella maritima* L., and dunes with sclerophyllous vegetation (*Halimienion halimifolii* Rivas-Martínez & Costa in Rivas--Martínez, Costa, P. Soriano, Pérez- Badia, Llorens & Roselló 1992; cf. Crespo & Serra, 2021), with *Teucrium dunense* Sennen. The density of *E. maritimum* is maximum in the *Ammophilion australis* community, while it develops eventually in the other alliances (See syntaxon description for further details). Soil samples were collected haphazardly at 15 cm depth around these 4 points two times per month and EC was measured by diluting soil samples in distilled water in a 1:5 ratio with a magnetic shaker during a two-hour period. Samples where then filtered, and EC was measured (XS Instruments Cond 51+).

2.6. Statistical analysis

Germination parameters were calculated using the package *Germinationmetrics* (Aravind et al., 2019) in R software (R Core Team, 2013). Descriptive statistics and plots were carried out using the *Tidyverse* library (Wickham et al., 2019) and the *ggplot2* package (Wickham, 2016).

Cumulative germination was analysed considering the type of salt, the concentration, and the osmotic potential as the experimental variables. Statistical models considering salt and concentration were compared with models considering only the osmotic potential, to establish if there is a specific ion toxicity of salts. For count response variables (day of the first germination, number of germinated seeds) and rate response variables (germination rate, final germination percentage) generalized linear models (GLM) using the Poisson family (link *log*) and zero-inflated models were used. To model the effect of salts and concentrations over time generalized additive models (GAM) were fitted considering the variables and their interaction.

Reproductive measurements were analysed considering the concentration of salt and the whorl of origin, when possible, as the explanatory variable. The potential effect of interindividual variation was evaluated using linear and generalized mixed models (Kumle et al., 2021). In all cases model selection was carried out according to the Akaike selection Criterion using AICc (Bozdogan, 1987; Parmoon et al., 2015). Model accuracy was evaluated using Q-Q plots, R^2 and McFadden Pseudo-R2 when possible (Veall and Zimmermann, 1996). Significant effects of the experimental variables were evaluated using ANOVA (Rutherford, 2011), while differences among treatments were analysed using the Tukey Honest Significance Test (Abdi and Williams, 2010). Finally, EC of sampling sites was analysed using linear models, while the effect of sampling site on EC was evaluated using ANOVA (Rutherford, 2011).

3. Results

3.1. Effect of salinity on germination

Salt concentration had a significant decreasing effect on final germination percentage (Table 1, Fig. 1). The control treatment germinated at 70 ± 4.67%, while in salt treatments the maximum observed was 32%. Significant differences were observed among salts (Table 1), *E. maritimum* being more tolerant to $MgSO_4$ and NaCl, and less to Na_2SO_4 and $MgCl_2$, with almost no seed germinating at concentrations higher than 0.1 M (Fig. 2A). The recovery test slightly improved germination, but not at significant levels [F (1,127) = 1.66, $P = 0.20$]. First and last day of germination, as well as the germination time range, did not seem to be explained by the treatment factors (Table 1), although salt concentration seemed to provoke a delay in germination (Fig. 2B). However, the low number of germinated seeds in high salt concentration treatments did not allow us to analyse these germination metrics deeper. Germination speed (considered as the % of germinated seeds per day, or Emergence Rate Index) was negatively affected by the concentration of salts (Fig. 2E). Na_2SO_4 and $MgCl_2$ strongly affected germination rather than NaCl and $MgSO_4$ (Table S2). Finally, the different salts and concentration exerted a significant decreasing effect on germination uncertainty (Table S2). Salts and concentration explained better final germination percentage than the osmotic potential of the salts alone ($P < 0.05$, $\Delta AIC = 76.31$, Table 2), although the observed effect of salinity on the germination metrics was similar (Figure S2). No relation was observed between treatments and the percentage of rotten seeds (Table 1). A relatively high percentage of rotten seeds was observed in all treatments (33.01 ± 0.70%, $N = 264$).

3.2. Effect of salinity on development, phenology, and reproduction

All individuals died when exposed to full SW, and one died in the 50% SW treatment. However not all individuals died without producing inflorescences. In the full SW treatment 50% of the individuals produced at least one capitulum, decreasing the number of flowered individuals at salt concentrations higher than 25% SW [F (1,66) = 8.05; $P < 0.005$, $R^2 = 0.61$; Fig. S3]. Among flowered individuals, production of reproductive structures was strongly affected by salinity. The number of capitula varied among whorls, with capitula belonging to secondary, tertiary and quaternary whorls being more numerous and decreasing with the addition of salts, with a significant interaction between variables, by which the effect of salinity was stronger in outer whorls (Table 3; Fig. 3A). Flowering and fruiting were inhibited by the presence of salts, overall in external whorls even at concentrations below 25% SW (Fig. 3C). Phenology was strongly affected by salt addition (Fig. 3B, C). Plants exhibited a delay in flowering and fruiting even at low concentrations of salt (below 50% SW) [F (1,136) = 21.67, $P < 0.005$; Table 3], while plants exposed to seawater presented early anthesis and fruiting. Exceptionally, fourth order capitula exposed to 6.25% SW presented early anthesis.

Salinity exerted a significant effect on inflorescence morphology (Fig. 4A, B), decreasing diameter [F (1,40) = 32.24, $P < 0.005$] and length [F (1,40) = 16.31, $P < 0.005$]. Capitulum diameter also behaved in a similar pattern, with inner whorls being bigger than outer whorls and decreasing in size when treated with high salt concentrations

Table 1
Results of the ANOVA test of the salinity tolerance experiment considering salts and concentrations as the explanatory variables.

Factor		Number of Rotten seeds	Germination Percentage by day	Final Germination Percentage	First Germination Day	Last Germination Day	Days from first to last germination	Germination Speed	Germination Uncertainty
Salt	*df*	4	**4**	**4**	4	4	4	**4**	**4**
	F	1.958	**1628**	**134.311**	2.818	0.429	2.301	**157.201**	**13.24**
	P	0.112	**<0.001**	**<0.001**	0.105	0.522	0.082	**<0.001**	**<0.001**
Concentration	*df*	1	**1**	**1**	1	1	1	**1**	**1**
	F	0.054	**1237**	**8.363**	1.171	0.621	0.961	**26.152**	**53.13**
	P	0.817	**<0.001**	**<0.001**	0.346	0.654	0.335	**<0.001**	**<0.001**
Day	*df*		**1**						
	F		**1847**						
	P		**<0.001**						
Residuals	*df*	62	62	62	26	15	30	62	62
	R2	0.022	**0.656**	**0.591**	**0.121**	0.061	0.02	**0.762**	**0.60**
	Model *P*-value	0.521	**<0.001**	**<0.001**	**<0.001**	**0.045**	0.326	**<0.001**	**<0.001**
	Model family	GLM - Poisson	GAM	GLM - Poisson	GLM - Poisson	GLM - Poisson	GLM - Negative Binomial Poisson	GLM - Poisson	LM

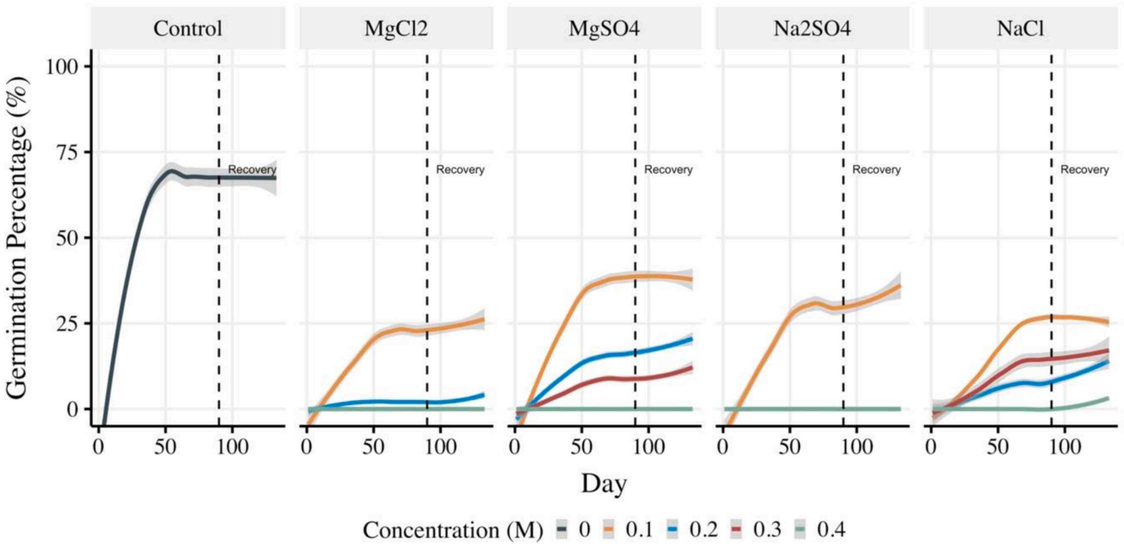

Fig. 1. Germination percentage by day of *Eryngium maritimum* seeds exposed to four different salts at concentrations ranging from 0 to 0.4 M. Dashed line represents the moment in which recovery was carried out. Shaded area represents the 25–75% confidence interval using GAM models. Statistics related to this data an found in Tables 1 and S2.

(Tables 4, S7). While first and second whorls only aborted at the seawater treatment, third and fourth whorls experienced high rates of abortion even in the 1/8 SW treatment. On the other hand, the number of flowers slightly decreased because of salinity with inner whorls having more flowers than outer (Tables 4, S7).

Plants exposed to concentrations higher than 25% SW were not able to develop fruits nor seeds. Fruit set was lower in outer whorls and decreased with the addition of salts (Table 4), reaching decreases up to a 70% in the 50% SW treatment and being completely blocked in full SW (Fig. 4C). Seed set experienced similar patterns (Fig. 4D), being lower in outer whorls and decreasing because of salts concentrations (Tables 4, S7). Fruit size was also altered in presence of high concentrations of salts, decreasing in width and length (Tables 4; S8) as seawater concentration increased.

3.3. *Field electrical conductivity measurements*

EC varied strongly among vegetation communities [F (3,91) = 47.070, $P < 0.005$; Fig. 5] and along the year [F (1, 91) = 4.412, $P < 0.05$]. The alliance *Agropyro-Minuartion peploidis*, located in embryonic dunes and represented in this area by *Elymus farctus*, presented the highest EC values. However, EC declined rapidly inland, with the other alliances (*Ammophilion australis, Crucianellion maritimae* and *Halimienion halimifolii)* presenting similar EC values.

4. Discussion

Germination, phenology and reproduction of *E. maritimum* were strongly altered in the presence of salts. Germination was severely inhibited at concentrations higher than 0.1 M, with slight differences depending on the salt, while recovery did not allow seeds to germinate. A high mortality was observed in adult plants exposed to salt concentrations higher than 25% SW. Plants exposed to salinity presented reduced and less numerous capitula, low fruit and seed production, and modifications in flowering and fruiting timing. However, the effect of salinity on reproduction and phenology was different depending on the whorl of the capitulum, with salt exerting a slighter effect in inner than in outer whorls.

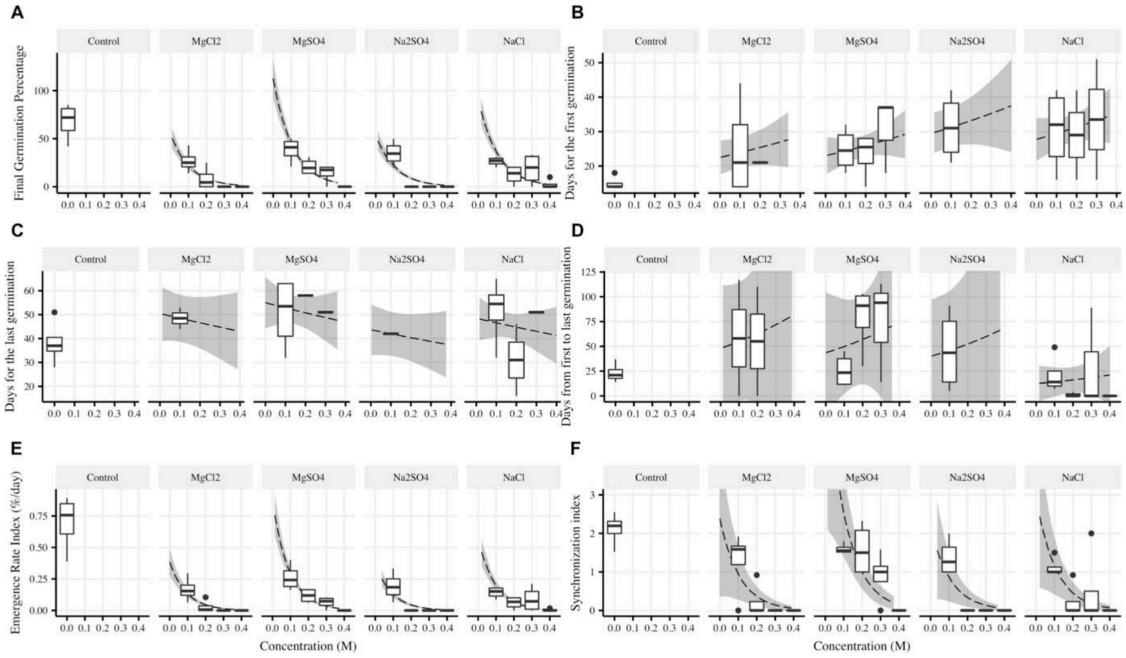

Fig. 2. Observed and predicted data of the germination metrics of *Eryngium maritimum* seeds exposed to four different salts ($MgCl_2$, $MgSO_4$, Na_2SO_4 and NaCl) at concentrations ranging from 0 to 0.4 M. Models of each variable and supplementary statistics can be found in Tables 1 and S2. Shaded area represents the 25–75% confidence interval. Boxes indicate median ± 2nd and 3rd quartiles, while whiskers indicate 1st and 4th quartiles. Outliers are indicated with dots.

Table 2
Results of the ANOVA test of the salinity tolerance experiment considering the osmotic potential as the explanatory variable.

Factor		Number of Rotten seeds	Germination Percentage by day	Final Germination Percentage	First Germination Day	Last Germination Day	Days from first to last germination	Germination Speed	Germination Uncertainty
Osmotic Potential	*df*	1	**1**	**4**	1	1	1	**1**	**1**
	F	0.507	**2951**	**65.04**	3.12	0.077	0.252	**43.3**	**60.31**
	P	0.479	**<0.001**	**<0.001**	0.0875	0.789	0.619	**<0.001**	**<0.001**
Day	*df*		**1**						
	F		**772.1**						
	p		**<0.001**						
Residuals	*df*	66	62	66	26	19	34	62	66
	R2	0.001	**0.656**	**0.55**	0.093	0.004	0.02	**0.762**	**0.60**
	Model P-value	0.479	**<0.001**	**<0.001**	0.0875	0.784	0.326	**<0.001**	**<0.001**
	Model family	LM	GAM	GLM - Poisson	LM	LM	LM	GLM - Poisson	GLM - Poisson

4.1. *Effect of salinity on germination*

The physical process of water uptake leads to the activation of metabolic processes as the dormancy of the seed is broken following hydration (Katembe et al., 1998). Elevated salinity slows down water uptake by seeds, thereby inhibiting their germination and root elongation (Uhvits, 1946; Simon, 1984; Werner and Finkelstein, 1995). Considering the results of the present study, *E. maritimum* seeds are not able to tolerate high percentages of salinity. This fact could contribute to the survival of *E. maritimum* preventing germination in adverse conditions where nude seeds could germinate but young plants could die (Conesa et al., 2008). Our results are consistent with the findings of Walmsley and Davy (1997a) and Woodell (1985), who obtained null germination of *E. maritimum* seeds at about 300 mM of NaCl. However, Praeger (1915) and Ridley (1930) obtained germination rates of 50% experimentally floating fruits in seawater for 40 days, which is considerably different from our findings.

Although final germination proved to be strongly related to the osmotic potential, the decrease in germination was different among salts. This suggests that there is a slight effect of specific ion toxicity, which is different among salts (Redmann, 1974). Specifically, Na_2SO_4 and $MgCl_2$ strongly affected germination rather than NaCl and $MgSO_4$ (Table S2). These results are consistent with the findings of Tobe et al. (2003) and Duan et al. (2004), who obtained that $MgCl_2$ and Na_2SO_4 exerted the most aggressive effect on germination in *Artemisia ordosica* Krasch., *Aristida adscensionis* L. and *Bassia dasyphylla* (Fisch. & C.A.Mey.) Kuntze; and *Chenopodium glaucum* L., respectively. The recovery test did not allow seeds to germinate, which could indicate that seeds died by specific ion toxicity (Woodell, 1985; Walmsley and Davy, 1997b). However, osmotic stress could have exerted the most aggressive effect, as suggested in previous studies carried out in other Apiaceae species (Solima and El-Shaieny, 2014). In this context, certain dispersal patterns described for *E. maritimum* such as hydrochory of fruits seem to be implausible, at least in long voyages of weeks, although fruits have a considerably good floatability up to 14 days, with 50% of floatability after 4 days (Curle et al., 2007). In this same study, Curle et al. (2007) evidenced that the cold stratification treatment decreases the floatability of seeds. Attending to these observations, the strong coat of fruits could also be involved in the salinity tolerance of the species, and cold stratification could exert a significant effect increasing the sensitivity of

Table 3
Statistical significance of the effect of seawater concentration on flowering timing and number of capitula per whorl.

		Number of capitula	First day of flowering	First day of fruiting
Seawater concentration				
	Df	1	1	1
	Df Residuals	243	133	136
	F	**50.13**	**21.34**	**3.98**
	P	**<0.001**	**<0.001**	**<0.05**
Whorl				
	Df	3	3	3
	Df Residuals	243	133	136
	F	**24.96**	**37.40**	**41.23**
	P	**<0.001**	**<0.001**	**<0.001**
Seawater concentration: Whorl				
	Df	3	3	1
	Df Residuals	243	133	136
	F	**8.30**	0.30	0.013
	P	**<0.001**	0.826	0.998
Model				
	P	<0.001	<0.001	<0.001
	R2	0.94	0.89	0.72
	Type	GLM - Poisson	GLM - Poisson	GLM - Poisson

seeds to salinity, by increasing the moisture of the thick walls of the fruit which naturally protect seeds. Considering this fact, fruits could be able to be dispersed by the sea a few days, protected by a dry coat, and be buried in the sand protected from salts until germination starts when the embryo is fully grown, soil electrical conductivity is low as a result of winter rainfalls and fruit walls have been softened by continuous moisture. However, specific analysis of salinity tolerance of seeds with and without cold pre-treatment will be compulsory to test this hypothesis. On the other hand, previous studies analysing the genetic relationship among populations stated that sea dispersal is the major means of long-distance dispersal (Weising and Freitag, 2007), although it is probably unusual (Curle et al., 2007). In this sense, an extensive study carried out by Kadereit et al. (2005) suggested that *E. maritimum* genetic distribution is highly connected with sea-dispersal, with three main clusters in the Atlantic, Western Mediterranean and Black/Aegean Sea which correspond with different sea currents. It is also possible that other organs, such as rhizomes, would be partly responsible for the sea dispersal of the species, as *E. maritimum* is known to have a considerable regrowth capability (Curle et al., 2007), which could explain the observed distribution. In this sense, Walmsley and Davy (1997b) suggested that seedling establishment may be of secondary importance in Northern European populations of *E. maritimum* compared with establishment from vegetative fragments, as a result of the observation of poor germination rates *in-situ*. However, Mediterranean populations could behave differently, as it can be concluded from seed stratification requirements (Cortés-Fernández et al., 2021). On the other hand, interpopulation variation in salinity tolerance has been suggested in the model species *Arabidopsis thaliana* (DeRose-Wilson and Gaut, 2011), so more research is needed to contrast these hypotheses.

The slight salinity tolerance observed in the seeds of the present study suggests that *E. maritimum* is not able to establish new populations in high salinity substrates, which is consistent with the observations of Walmsley and Davy (1997a). However, their study was carried out with seeds coming from United Kingdom, and they obtained maximum germination rates of 16% in the control treatment due to infertile seeds, which is notably lower compared to our observations. Considering the low salinity tolerance of the seeds of the species, it is rational to consider that substrate composition can strongly influence the germination capacity of *E. maritimum* populations, determining the ratio of salts remaining in the substrate after raining events. Greiß et al. (1995) and Andersone et al. (2011) reported relatively low salt concentrations in the

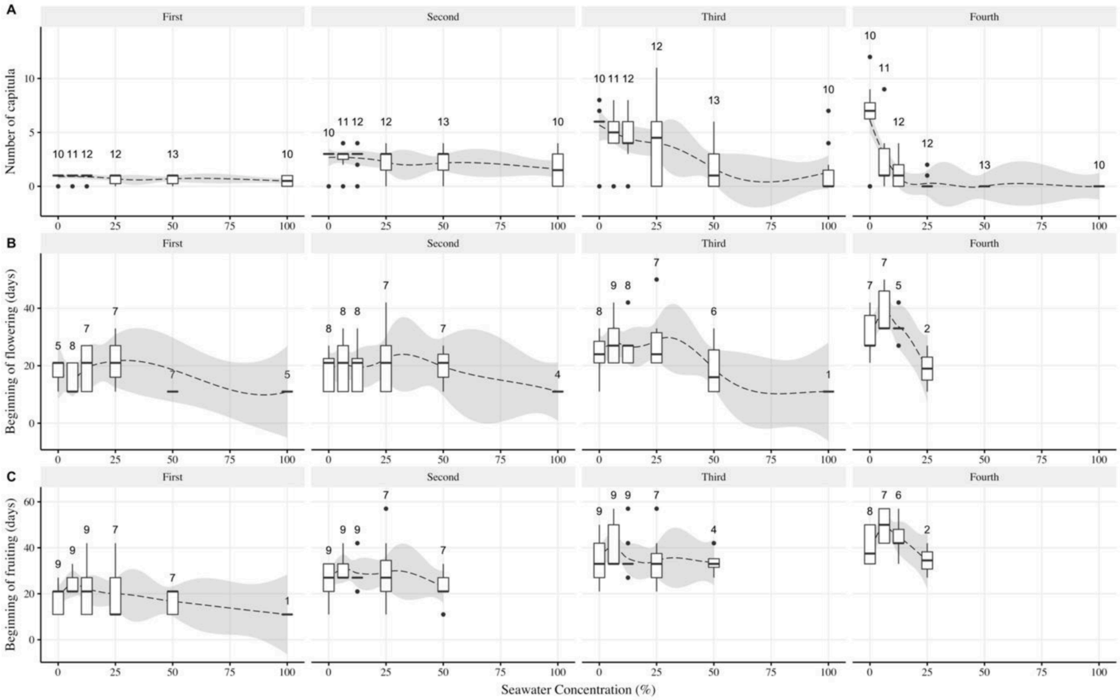

Fig. 3. Effect of salt concentration (ranging from 0 to 100% Seawater) as boxplots of the number of capitula (A), first day of flowering (B) and fruiting (C) per whorl (first, second, third and fourth from inner to outer) of *Eryngium maritimum*. Number of replicates are indicated on the top of each group. Dashed line represents the GAM model fitted for each variable, while shaded area represents the 25–75% confidence interval for each model. Boxes indicate median ± 2nd and 3rd quartiles, while whiskers indicate 1st and 4th quartiles. Outliers are indicated with dots.

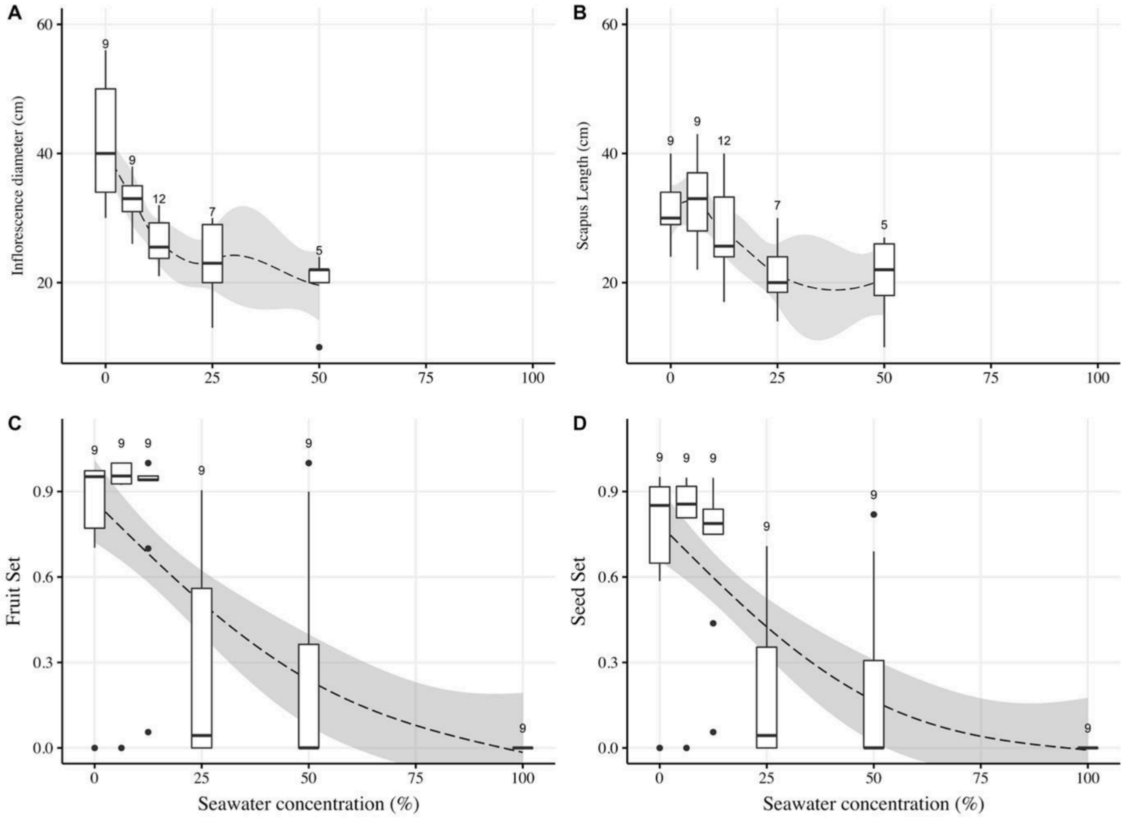

Fig. 4. Effect of salts as boxplots of the morphometric and reproductive characters of *Eryngium maritimum*. Number of replicates are noted on the top of each group. Dashed line represents the model fitted for each variable (GAM in inflorescence diameter and Scapus length and GLM – quasipoisson for Fruit and Seed Set), while shaded area represents the 25–75% confidence interval for each model. Boxes indicate median ± 2nd and 3rd quartiles, while whiskers indicate 1st and 4th quartiles. Outliers are indicated with dots.

Table 4
Statistical significance and estimates of the effect of seawater concentration on the reproductive outputs per whorl.

		Number of flowers	Capitulum diameter	Fruit set	Seed set	Fruit width	Fruit length
Seawater concentration							
	Estimate	–6.27	–37.06	–-1.30	–1.37	–2.65	–5.82
	Standard error	0.85	3.15	0.33	0.30	1.33	1.93
	Df	2	2	2	2	2	2
	χ^2	**66.22**	**81.47**	**19.82**	**23.18**	**18.10**	**14.41**
	P	**<0.001**	**<0.001**	**<0.01**	**<0.001**	**<0.001**	**<0.001**
Whorl							
	Estimate	–0.18	–6.17	–0.33	–0.30	–0.09	–0.31
	Standard error	0.01	0.22	0.01	0.01	0.23	.25
	Df	2	2	2	2	2	2
	χ^2	**486.47**	**607.76**	**462.81**	**444.39**	**15.50**	**11.24**
	P	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.01**
Seawater concentration: Whorl							
	Estimate	0.10	4.67	0.17	0.23	–3.45	–1.78
	Standard error	0.076	1.63	0.12	0.11	1.63	1.85
	Df	1	1	1	1	1	1
	χ^2	1.49	**8.13**	2.16	**4.48**	**4.35**	0.94
	P	0.223	**<0.001**	0.142	**<0.01**	**<0.01**	0.33
Model							
	P	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Marginal R2	0.36	0.40	0.42	0.40	0.22	0.22
	Conditional R2	0.99	0.80	0.52	0.57	0.30	0.49
	Type	GLMER - Poisson	LMER	GLMER - Binomial	GLMER - Binomial	LMER	LMER

soils where *E. maritimum* is growing. However, this hypothesis is contrary to the results obtained by Hitchmough et al. (2001), who obtained similar germination rates varying the substrate.

4.2. Effect of salinity on development, reproduction and phenology

Eryngium maritimum adults were able to tolerate moderate salinity concentrations up to 50% SW without dying. In the light of this specific result, it can be considered as a halophyte species, as it can survive at

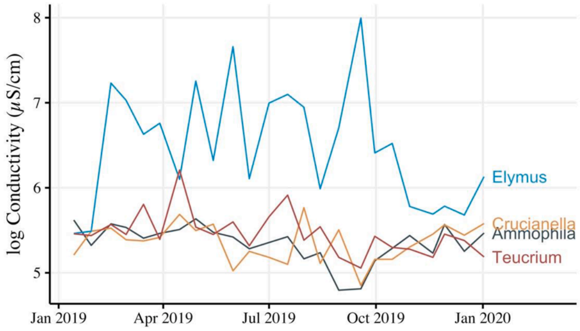

Fig. 5. Electrical conductivity measurements per syntaxon alliance from sea-line to inwards: Elymus = *Agropyro–Minuartion peploidis*; Ammophila = *Ammophilion arenarii*; Crucianella = *Crucianellion maritimae*; Teucrium = *Halimienion halimifolii*. Distribution of sampling areas can be found in Figure S1.

concentrations above 200 mM of NaCl. However, more than survivorship must be considered in order to evaluate this affirmation (Flowers and Colmer, 2008; Flowers et al., 2015; Yuan et al., 2019). Although the species was able to survive at moderate concentrations of salts, fruit set and seed set of the remaining capitula were strongly reduced by the presence of salt concentrations above 12.5% SW, while plants exposed to concentrations higher than 25% SW were not able to develop fruits. However, low concentrations of salts provoked a slight increase in fruit set, which could be linked to nutrient relocation, as resources invested for external whorl production already start to be depleted at early stages in favor of inner whorls, as suggested by Cortés-Fernández et al. (2022) as a potential explanation for the observed differences in reproductive production between whorls. This contrasts with the observations of Debez et al. (2004) in *Cakile maritima*, a neighbor species inhabiting slightly near to the coastline in the *Salsolo-cakiletum maritimae* phytosociological association (Sykora et al., 2003), who obtained clear increasing patterns in all reproductive traits under study, both at low and medium salinity values of 50–100 mM (≈12.5–25% SW) and subsequent drastic reduction only at higher concentrations.

The presence of salts strongly altered *E. maritimum* reproductive traits. Individuals exposed to high percentages of salts developed short and narrow inflorescences compared with plants exposed to low percentages of salts, which could have implications for male and reproductive success and thus the capacity to complete the reproductive cycle. As suggested by previous studies (Cortés-Fernández et al., 2022), in *E. maritimum* only first and second whorls can be considered effective reproductive structures, being bigger, less numerous and displaying higher fruit and seed set than outer whorls. On the other hand, outer whorls seem to function as pollen donors or as attractive structures for pollinators. Considering these facts, it was suggested that allocation of nutrients is carried out preferably in inner whorls (Cortés-Fernández et al., 2022). This could be linked with the observed response to salinity, by which stressed individuals displayed less external capitula or even aborted them, possibly to reallocate nutrients to inner whorls and ensure reproduction.

Previous studies carried out in other coastal species (Franks et al., 2007; Lum and Barton, 2020) observed that salinity provoked a significant delay in flowering. However, some other factors, such as the specific concentration of salts and the whorl of origin, must be taken into account. In this study, salt concentrations above 25% SW tended to accelerate anthesis and fruiting, while concentrations below that range tended to provoke a delay. On the other hand, differences among whorls were observed considering phenology of flowering and fruiting, being more stable in inner whorls even at moderate concentration of salts, while in outer whorls flowering and fruiting were completely inhibited and accelerated even at low concentrations.

4.3. Field electrical conductivity measurements

EC was variable along the dune series decreasing inland. The vegetation of embryonic dunes has to deal with the highest EC, with notable differences as sand increases generating higher white dunes, that protect against wind-blown salt more inland (Du and Hesp, 2020). So, considering that *E. maritimum* can be found in embryonic dunes but rarely in strandlines, it can be suggested that salinity, along with flooding and sand dynamics, could be a limitation for the settlement of the species in the front line. However, more factors have to be implicated in the distribution of *E. maritimum* along the dune, as soils from vegetation of inner sites (*Crucianellion maritimae* and *Halimienion halimifolii)* display similar EC values but *E. maritimum* density strongly decreases. Soil compaction could be responsible for this reduced fitness in inner sites, as it increases soil strength, decreases air permeability, hydraulic conductivity and prevents the elongation of roots (Whalley et al., 1995). In this sense, Walmsley and Davy (1997b) proposed that seeds of *E. maritimum* germinate better in sandy seaward plots compared to landward plots, which is consistent with our hypothesis. On the other hand, EC was not constant along the year, experiencing intense fluctuations, which considering our observations could be related with precipitations (Ungar, 1982; Gil, 1994). In this sense, germination of *E. maritimum*, mainly occurring on February in our study area, concurs with a period when EC is similar along all the dune.

In the light of these results, *E. maritimum* can be considered as a salinity tolerant species, but not a halophyte, because salt concentration can strongly influence its germination, growth and reproduction. As a result of sea-level rise and inland coastal salination, the optimal distribution of *E. maritimum* along the dune could change, shifting from actual foredunes to dune-crests to avoid hypersaline sites, as it has been documented in other coastal species (Mendoza-González et al., 2013), while in inner sites its distribution will continue to be limited as a result of other factors, like soil compaction and interspecific competition.

5. Conclusion

Germination of *E. maritimum* is strongly inhibited by the presence of relatively low salt concentrations. At osmotic potentials lower than -2 MPa, germination is almost null. In the adult stage, reproduction of the species is strongly inhibited at concentrations higher than 0.125 SW. Electrical conductivity strongly varies along the dune, with high conductivity in the front line were *E. maritimum* is found rarely. This process can inhibit the germination and seed production of the species stopping recruiting, reducing its distribution range along the dune. Finally, this study has shown that more than plant dieback should be considered in order to define a species as a halophyte. In the case of *E. maritimum*, although it can withstand low to moderate concentrations of salts, germination and reproduction is strongly inhibited in presence of salts, which is particularly important considering that this species inhabits coastal areas that will suffer from increased sea-levels and coastal salination.

CRediT authorship contribution statement

I. Cortés-Fernández: Software, Formal analysis, Investigation, Writing – original draft, Project administration. **M.D. Cerrato:** Conceptualization, Investigation. **A. Ribas-Serra:** Resources, Visualization, Data curation. **L. Gil:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2022.152062.

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Capítulo 3.

Papel de *Eryngium maritimum* L. en la red de polinización dunar

Manuscript IV

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The role of *E. maritimum* (L.) in the dune pollination network of the Balearic Islands

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Abstract

Eryngium maritimum L. (Apiaceae) is a geophyte that inhabits in the dunes of the Mediterranean and Atlantic. Although it is a highly entomophilous species, there is little literature on its pollinator assemblage. The aim of this study is to analyze the role played by *E. maritimum* in the dune pollination network of the Balearic Islands, where there is an intense anthropogenic impact in its habitat. For this purpose, two populations located in the North and South of Mallorca were chosen, in which diurnal transects were carried out to observe and capture pollinators on 15 plant species during the anthesis period of *E. maritimum*. The flowering period of 10 plant species flowering at the same period than *E. maritimum* was analyzed to identify periods of competition. A total of 71 pollinator species were found, belonging to 30 different families. *Eryngium maritimum* is a strongly generalist species, with a total of 45 pollinator species. Two new species, *Odice blandula* and *Leucospis gigas*, were found for the first time in Mallorca. In terms of pollinators, *Teucrium dunense* and *Helichrysum stoechas* are the most similar species to *E. maritimum*. However, analysis of phenology suggests that these three species have been able to decouple their blooms to avoid competition. The present study shows that *E. maritimum* plays an important role in the dune pollination network, being its anthesis located at the end of the dune flowering season, when there are no functionally similar species in flower.

KEYWORDS
connectance, dune, *Eryngium maritimum*, pollination network, resilience

TAXONOMY CLASSIFICATION
Behavioural ecology; Botany; Community ecology; Entomology; Functional ecology

1 | INTRODUCTION

Pollination is considered as one of the most crucial plant–animal interactions, influencing on dynamics and diversity of plant communities (Fantinato, Del Vecchio, Giovanetti, et al., 2018). Widespread declines in pollinators had led to a concern about a global pollination crisis (Burkle et al., 2013; Tylianakis, 2013). By reducing pollinator availability and nesting sites due to habitat modifications, cross-pollination levels can be modified influencing plant fruit and seed production (Traveset et al., 2018; Vanbergen et al., 2014). At the same time, anthropization is jeopardizing the conservation of ecosystems and the ability to resist future environmental changes (MacDougall et al., 2013).

Among ecosystems, coastal habitats, such as seashores and dune ridges, are considered some of the most threatened habitats (Gigante et al., 2018), due to habitat loss (Coverdale et al., 2013), global warming (Culbertson et al., 2009) and coastal salination due to an increased sea-level (Chu-Agor et al., 2011). Concretely, coastal dune ecosystems are a hotspot for specialized pollinator species (Cane, 1991), displaying highly specialized species and interactions higher risks of extinction (Aizen et al., 2012; Burkle et al., 2013). Understanding plant–pollinator interactions is vital to give light to coevolutionary processes in highly diverse communities (Bascompte & Jordano, 2007) and to evaluate the maintenance of ecosystem's resilience over time (Fantinato et al., 2019). So, pollinators are used as a bioindicator species as the decline of their populations are strongly associated with anthropogenic influence (Biesmeijer et al., 2006). At the same time, some attributes of the pollination network (selectiveness, nestedness, connectance) have an ecological meaning in the assessment of habitat resilience to various forms of disturbances (Fantinato et al., 2019; Lázaro et al., 2016; Traveset et al., 2018).

Ecological indicators enable the analysis of complex systems processed in a reliable way (Dale & Beyeler, 2001). Selectiveness or specialization is defined as the number of partners, or links, of a species (Blüthgen et al., 2006). Specialist species are usually the first to go extinct from a network (Henle et al., 2004), Connectance (or link density) is the most common way to characterize specialization and is calculated as the proportion of the observed interactions to all possible interactions (Olesen & Jordano, 2002). It is considered as a useful metric to analyze functional redundancy of interactions, which is related with resilience, due to its ease of calculation (Tylianakis et al., 2010). On the other hand, the interactions in a network are said to be nested when the species interacting with specialists are a proper subset of the species interacting with generalists (Tylianakis et al., 2010). The ecological implication of nestedness is that, if an species goes extinct and the network is nested, the remaining species will have others with which to interact, providing a buffer to secondary extinctions (Fortuna & Bascompte, 2006; Tylianakis et al., 2010). Compartmentalization is interpreted as a subset of an interaction network which tend to interact frequently with another, but little with the species outside of the compartment (Tylianakis et al., 2010). Compartmentalization may be caused by coevolution, and it is considered that increases stability of networks (Krause et al., 2003). Highly connected species within a compartment are considered as "module hubs", while species interacting with various compartments are considered as connectors (Olesen & Jordano, 2002). In terms of conservation, the extinction of module hubs and connectors is related with cascading extinctions across compartments (Olesen et al., 2007; Tylianakis et al., 2010).

Eryngium maritimum L. is a geophyte from the Apiaceae family that inhabits in sand dunes of the Atlantic and Mediterranean coasts (Isermann & Rooney, 2014), being a diagnostic species of mobile dunes (Marcenò & Jiménez-Alfaro, 2017). Each individual produces one flowering stalk, from which multiple capituliform inflorescences (5–40) emerge in a dichasial disposition (Cortés-Fernández, Cerrato, Ribas-Serra, & Gil Vives, 2022). Flowers per capitula are numerous (25–50), hermaphrodite, with nectaries at the base, while stamens are prominent, purplish to bluish (Isermann & Rooney, 2014). Its role in the coastal pollination network has never been assessed, and its pollinators are unstudied, with only a few studies that give light to some of its pollinators (Gil, 1994) and most of them carried out in Northern European populations (Fitter & Peat, 1994; Hegi, 1935; Westrich, 2001; Zanella et al., 2009), where it is considered as a highly-threatened species (Aviziene et al., 2008; van der Maarel & van der Maarel-Versluys, 1996). In Northern populations, it displays low fruit and seed set production, and conversely, Balearic populations exhibit high fruit and seed set, with low levels of incompatibility (Cortés-Fernández et al., 2021). These differences could be related to pollinators but, to validate this hypothesis, firstly it is compulsory to understand how the species behaves in Mediterranean populations, where its populations exhibit a good conservation status.

In the Balearic Islands, *E. maritimum* develops optimally in embryonic and white dunes, where perennial grasses are not dominant (Llorens et al., 2021). The dune systems of the Balearic Islands are exposed to intense levels of anthropization, mainly due to the touristic pressure (García & Servera, 2003), but also to invasive species introduction (Hulme et al., 2008; Moragues & Traveset, 2005), and will be specially affected by coastal retreat (Enríquez et al., 2017). In Mallorca, it coinhabits with a great variety of plant species which are strongly pollinator-dependent, including members of the Lamiaceae (*Teucrium dunense* Sennen), Leguminosae (*Lotus cytisoides* L.), Asteraceae (*Helichrysum stoechas* (L.) Moench) and Papaveraceae (*Glaucium flavum* Crantz). The best approaches to give light to the dune pollination networks of the Balearic Islands have been carried out in two locations: Son Bosc and Cala Mesquida. Son Bosc is a fixed dune of the North of Mallorca (Castro-Urgal & Traveset, 2014; Lázaro et al., 2020; Traveset et al., 2017; Tur et al., 2013), which displays a substantially differential floral diversity than where *E. maritimum* optimally inhabits (Marcenò & Jiménez-Alfaro, 2017). On the other hand, Cala Mesquida is the studied location more representative of the optimal habitat of *E. maritimum* but in the only study available in this area (Castro-Urgal & Traveset, 2014), the specific role of *E. maritimum* is not analyzed.

The main objective of the present study is to understand which are the pollinators of *E. maritimum* and what is its specific role in the dune pollination network of the Balearic Islands. The main hypothesis is that *E. maritimum* is visited by a great number of pollinators, as attending to previous studies the capacity of the species to self-fertilize is low, as well as its anemophily, which suggests that the species is strongly entomophilous (Cortés-Fernández, Cerrato, Ribas-Serra, & Gil Vives, 2022). This study will give light to the ecology of the species in the Balearic Islands, continuing a series of studies which analyzed its reproductive biology, germination and salinity tolerance in this area (Cortés-Fernández et al., 2021; Cortés-Fernández, Cerrato, Ribas-Serra, & Gil, 2022; Cortés-Fernández, Cerrato, Ribas-Serra, & Gil Vives, 2022).

2 | MATERIAL AND METHODS

2.1 | Study area

Pollinator surveys were carried out in two different coastal dunes of Mallorca (Balearic Islands, Spain), one located in the North and one in the South of the island (Figure 1). First sampling area was located in Son Serra de Marina (SS, 39.7309 N, 3.2382 E), in the North of the island. Although the area is relatively well-conserved compared with other areas of the island, it suffers from severe anthropogenic impact mainly due to tourist pressure, overall in the drift line zone. On the other hand, the other sampling area was located in Es Trenc (ET, 39.3382 N, 2.9903 E), in the south of the island, which is protected. We carried out three 50 m linear transects along the seashore. Transects were randomly located at a minimum of 100 m from each other in order to enhance the chances of a fair sampling of most of the flora. In both areas, vegetation, and so transects, followed a clear sequence from the seashore inland. The sequence starts from therophytes in the drift line zone (Aliance *Cakilion maritimae*), very altered by the presence of tourism, followed by embryonic dune (*Agropyro-Minuartion peploidis*) and white dune communities (*Ammophilion australis*), which lead to semi-fixed dunes (*Crucianellion maritimae*) landwards (Llorens et al., 2021).

2.2 | Pollinator surveys

Areas were sampled for 10 weeks, from the beginning of *E. maritimum* flowering in the first week of June until the second week of July, plus 2 weeks extra (one after and one before) in order two observe pollinator diversity variation. Surveys took place between 08:00 and 18:00 h under favorable weather conditions.

A pollinator survey involved an observer slowly walking (40 min) along a transect and recording only those insects that contacted the plant's reproductive structures while actively searching for pollen and/or nectar. As the focus was put on gathering the highest diversity of pollinators, we opted to not gather information about abundance, building qualitative (binary) networks. So, in each interaction, pollinator and plant species was noted, and photographs were taken to ensure proper identification. Search was limited to those insects belonging to the insect orders most associated with pollination (Coleoptera, Diptera, Hymenoptera and Lepidoptera). Due to the great quantity of pollinators in both areas, only first interaction per day per transect was recorded, in other to construct a presence-absence interaction matrix per session. When floral visitors were not possible to identify they were captured and placed into individually labeled vials. To minimize our impact on local insect populations, only subsets of individuals from each non-identified species were netted. Insects were frozen and transferred to the laboratory where they were stored until identification. Insects were identified, if possible, to species level. The observed species were compared with reference studies and with international, national and local databases (GBIF, Biodibal, BioAtles, Pollinib) to evaluate the presence of new cites and species with reduced distribution.

2.3 | Network analysis

Sampling coverage was evaluated as an indicator of sampling completeness, using the statistical software R (R Core Team, 2013)

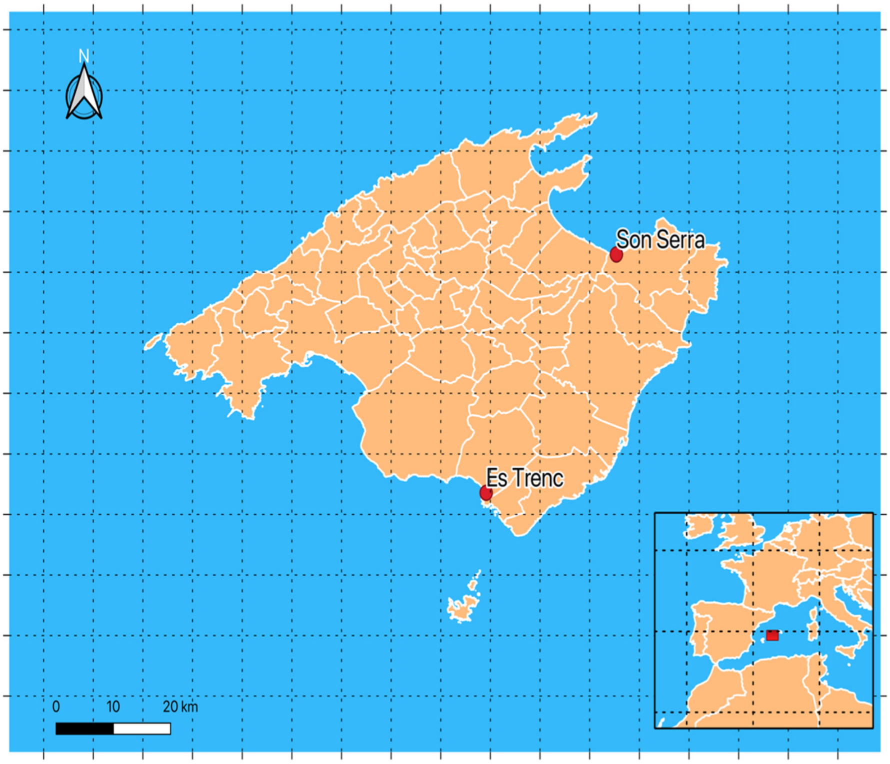

FIGURE 1 Map of the studied areas. Red points indicated the sampling areas, each one divided in 3 transects of 50 m separated by 100 m.

and the package iNEXT (Hsieh et al., 2016). Three qualitative plant–pollination networks (presence and absence of interactions between taxons), were carried out, one for each population and one for the whole observations. Descriptors for structure and resilience of pollinator interactions were calculated as described by Fantinato et al. (2019) and Traveset et al. (2017) using the *bipartite* R-based package (Dormann et al., 2008). At the network level, Connectance (*C*; Dunne et al., 2002), Nestedness (*N*; Almeida-Neto et al., 2008), Shannon diversity Index (*S*; Shannon, 1948), Links per species or Connectivity (*LP*) and number of compartments (*NC*) were calculated. Connectance is a proportion of the observed links divided by the number of total of possible links (Dunne et al., 2002). Nestedness is measure of departure from systematic arrangement of species by niche width (Dormann et al., 2009), and is considered as the ecological tendency of specialist species to interact with a subset of species that interact with more generalist species (Almeida-Neto et al., 2008; James et al., 2012). The Shannon diversity Index measures species diversity on the basis of species richness and evenness in abundance (Santini et al., 2017). Links per species indicates the number of different species a taxon interacts with. Finally, the number of compartments reflects the degree of clustering of the network. At the species level, Specificity (*Spec*) was considered for analysis (Poisot et al., 2012), which is considered as the coefficient of variation of interactions, and ranges from 0 (low specificity) to 1 (high specificity). Specialization level is, similarly, the level of selectiveness of a species. Using this metric, the degree of selectivity of pollinators and plant species was established (highly selective, $Spec > 0.75$; selective, $0.75 > Spec > 0.5$; opportunistic, $0.5 > Spec > 0.25$ and highly opportunistic, $Spec < 0.25$), as suggested by Castro-Urgal and Traveset (2014).

For each of the three networks, total number of plant species with interactions (NP) and total number of pollinators (NS) were calculated. Same analyses were carried out after removing singletons (pollinators that visited only one species and detected once in the whole experiment), to evaluate the potential increase of specialization as a result of rare species (Blüthgen et al., 2008; Dormann et al., 2008). In order to confirm that our results described patterns that are different from random, the observed interaction network was compared with a null model based on a number of random networks (Dormann et al., 2008). To do so, 1000 null versions (null model) of each community matrix were generated using the *mgen* algorithm implemented in the *bipartite*, which returns a list of randomized matrices without keeping any variable constant (Dormann et al., 2008).

Parallelly, the diversity of pollinators per plant species per session was analyzed, in order to describe potential temporal shifts of pollinators between species.

2.4 | Phenology

In each sampling area, plant surveys were carried out to assess the phenological distribution of plants coinhabiting with *E. maritimum*. Each sampling day a phenological survey was carried out in 10 key species of the habitat, in order to analyze the relationship between flowering and pollinator surveys. To do so, for each species individuals, inflorescences or flowers were followed and flowered units were counted as proposed in Gil (1994). For each species, a flowering peak and a standard deviation of flowering was calculated to estimate phenological curves, and then it was plotted using the ggplot2 (Wickham, 2011) package and the statistical software R.

2.5 | Pollinators behavior

Specific observations of *E. maritimum* pollinators were carried out to assess pollinators behavior and diversity. In each transect random individuals of *E. maritimum* were selected and observations were made for 20 min each transect, with a total observation time of 20 (minutes per transects)·8 (transects per day)·4 (sampling sessions per area) = 640 min in each area. For each interaction, the number of visited capitula, its whorl and the total time spend in an individual was recorded. The number of visited capitula was evaluated across families using generalized linear models assuming a Poisson distribution, while the time spend in visits was modeled using linear models, evaluating the potential effect of species and whorls (see Cortés-Fernández, Cerrato, Ribas-Serra, & Gil Vives, 2022 for detailed description about whorls in the species). The effect of the above mentioned factors in response variables was evaluated using Analysis of Variance and Deviance in R (Faraway, 2002), respectively.

3 | RESULTS

3.1 | Pollinator surveys

We recorded 353 contacts, involving 71 pollinator species (Table S2) and 15 plant species. The sampling coverage showed that species richness per session was still growing, but decelerating, at the end of the sampling sessions (Figure 2). Although observed species diversity was higher in SS than in ET, nearly similar richness would be obtained in both areas after 8 sampling sessions. Diversity of pollinators and Shannon diversity Index indicated that SS was richer in pollinators than ET (Table 1), recording interactions in 15 different plant species in SS, while only 7 in ET. However, only in both populations and in SS the Shannon Index was statistically different from null models. Connectance was higher in ET than in SS, while Links per species were lower. Nestedness was higher in ET than in SS, but only significant in the matrix of both populations. The analysis of the number compartments showed that two different compartments were identified considering both populations and ET individually, while SS displayed only one compartment (but not statistically significant from null models, Table 1).

Network evaluation indicated that SS web was more complex than ET, displaying more nodes and links (Table 1). In both webs, *E. maritimum*, *T. dunense* and *H. stoechas* displayed the higher diversity of pollinators and interactions (Figure 3). On the other hand, in other

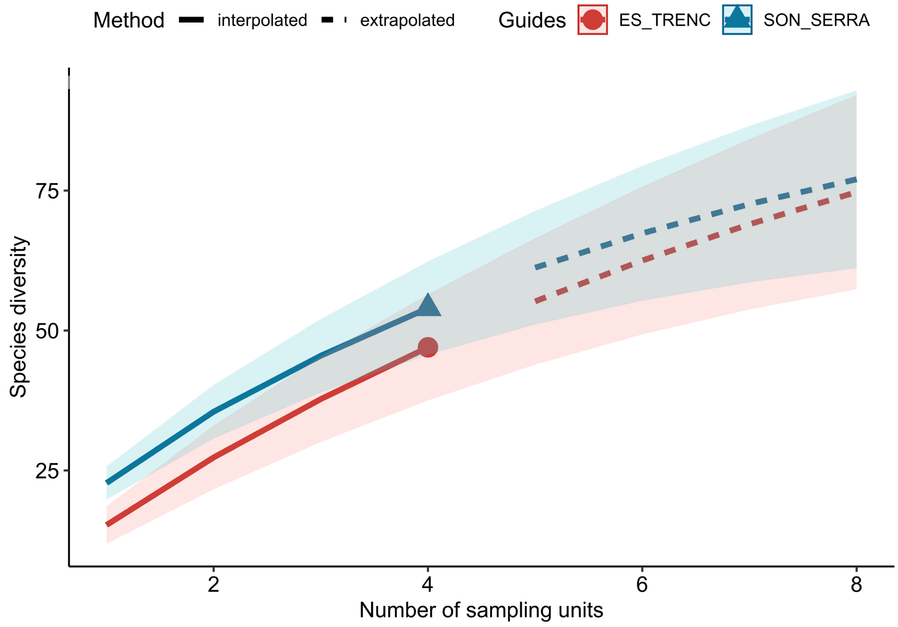

FIGURE 2 Sampling coverage per studied area. Solid lines indicate the model for the effective sampling units (sampling sessions), while dashed line indicates the prediction for future sessions.

TABLE 1 Network metrics of the different populations (Son Serra—SS, Es Trenc—ET) and both (All) in the full network (up) and with omitted singletons (down). NS = number of pollinator species found interacting with plants, NP = number of plant species visited by potential pollinators, *C* = connectance, *S* = Shannon Diversity Index, *N* = nestedness, *LS* = links per species, *NC* = number of compartments. The significance of observed results was tested by constructing 1000 randomized networks with identical margin totals as the empirical networks and comparing the observed and random values using the null model 'r2d' (*$p < .05$).

Population	*NS*	*NP*	*C*	*S*	*N*	*LS*	*NC*
All	70	15	0.12*	4.88*	8.40*	1.54*	2
Es Trenc	39	7	0.19*	3.95	27.13	1.13*	2
Son Serra	48	14	0.14*	4.57*	11.83	1.56*	1
All	41	12	0.12*	4.08*	17.66	1.11*	5*
Es Trenc	19	6	0.17	2.94	29.09	0.76	6
Son Serra	25	11	0.15	3.69	26.19	1.11	4

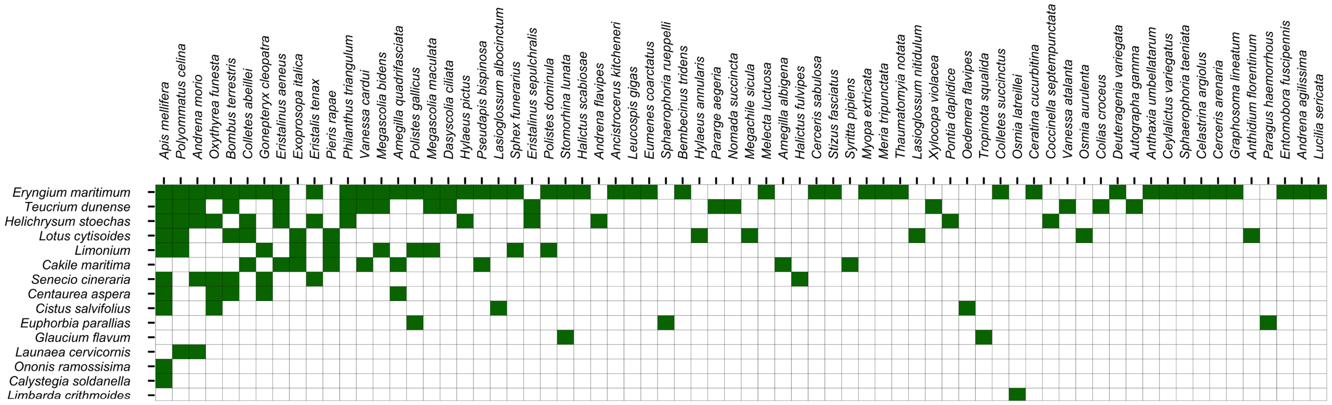

FIGURE 3 Matrix of pollinators species per plant species. Pollinators are ordered by diversity of interactions (grade of selectiveness) from left to right. In the genus matrix, color gradient indicates de diversity of taxons per interaction.

plant species like *Ononis ramosissima* Desf., *Calystegia soldanella* (L.) R. Br. and *Limbarda crithmoides* (L.) Dumort only one pollinator species was found (Figure 3). Although no data about pollinator abundancy were gathered, less pollinators were observed in ET than in SS. On the other hand, the specificity indicated that in SS pollinators were more opportunistic than in ET (Figure 4). However, it must be considered that network from ET was not significantly different from null models, and so no clear patterns can be obtained, contrary to SS and to the full network of both areas (Table 1). Twenty-nine species were characterized as singletons (pollinator species only observed once visiting one plant species). When omitted for the analysis, nestedness increases in SS and the number of compartments in the three networks. In this case, only with the combination of both populations variables are significantly different from null models, with the exception of Nestedness (Table 1).

Apis mellifera L. was the most polylectic species of pollinator, visiting 10 of the 15 plant species, while 45 pollinator species were found only visiting a single plant species (Figure 4). Of the 30 families found, *Apidae*, *Syrphidae* and *Colletidae* were the more diverse families in the network. On the other hand, two families (*Satyridae*, *Bruchinidae*) were represented by only one species. Hymenoptera was the most diverse family in the network, followed by Diptera and Lepidoptera. Three plant species presented very low levels of selectiveness, *H. stoechas*, *T. dunense* and *E. maritimum*, while *C. soldanella*, *O. ramossisima* and *L. crithmoides* presented only one interaction and were considered as highly selective (Table 2). The vast majority of pollinator species

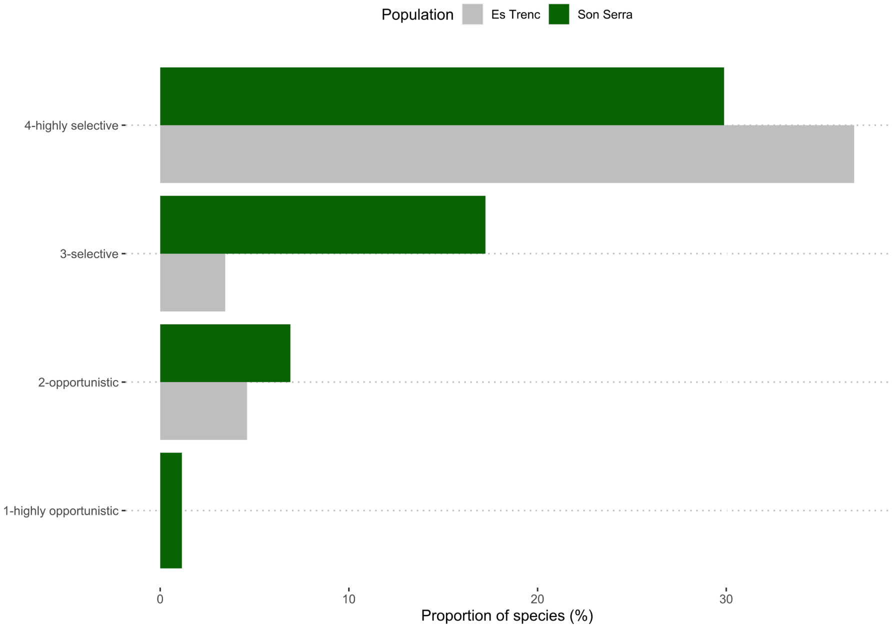

FIGURE 4 Proportions of pollinator species in the different categories of selectivity at each of the study sites. The degree of selectivity is calculated based on the *Specificity index* (highly selective, *Spec* > 0.75; selective, 0.75 > *Spec* > 0.5; opportunistic, 0.5 > *Spec* > 0.25; highly opportunistic, *Spec* < 0.25).

TABLE 2 Plant species metrics of the different populations (Son Serra, Es Trenc) And both (All). *D* = number of different pollinators species, *Spec* = Specificity. Selectiveness is calculated based on the *Specificity index* (highly selective, *Spec* > 0.75; selective, 0.75 > *Spec* >0.5; opportunistic, 0.5 > *Spec* > 0.25; highly opportunistic, *Spec* < 0.25).

	Es Trenc		Son Serra		All		
Species	***D***	***Spec***	***D***	***Spec***	***D***	***Spec***	***Specificity***
Cakile maritima	5	0.42	4	0.48	9	0.31	Opportunistic
Calystegia soldanella			1	1	1	1	Highly selective
Centaurea aspera			5	0.43	5	0.43	Opportunistic
Cistus salvifolius			4	0.48	4	0.49	Opportunistic
Eryngium maritimum	22	0.14	28	0.12	45	0.09	Highly opportunistic
Euphorbia parallias			3	0.56	3	0.57	Selective
Glaucium flavum			2	0.7	2	0.7	Selective
Helichrysum stoechas	6	0.38	11	0.27	13	0.25	Highly opportunistic
Launaea cervicornis			2	0.7	2	0.7	Selective
Limbarda crithmoides	1	1			1	1	Highly selective
Limonium sp.	6	0.38	5	0.43	10	0.29	Opportunistic
Lotus cytisoides	4	0.48	8	0.33	11	0.28	Opportunistic
Ononis ramossisima			1	1	1	1	Highly selective
Senecio cineraria			7	0.35	7	0.36	Opportunistic
Teucrium dunense	8	0.32	16	0.21	17	0.21	Highly opportunistic

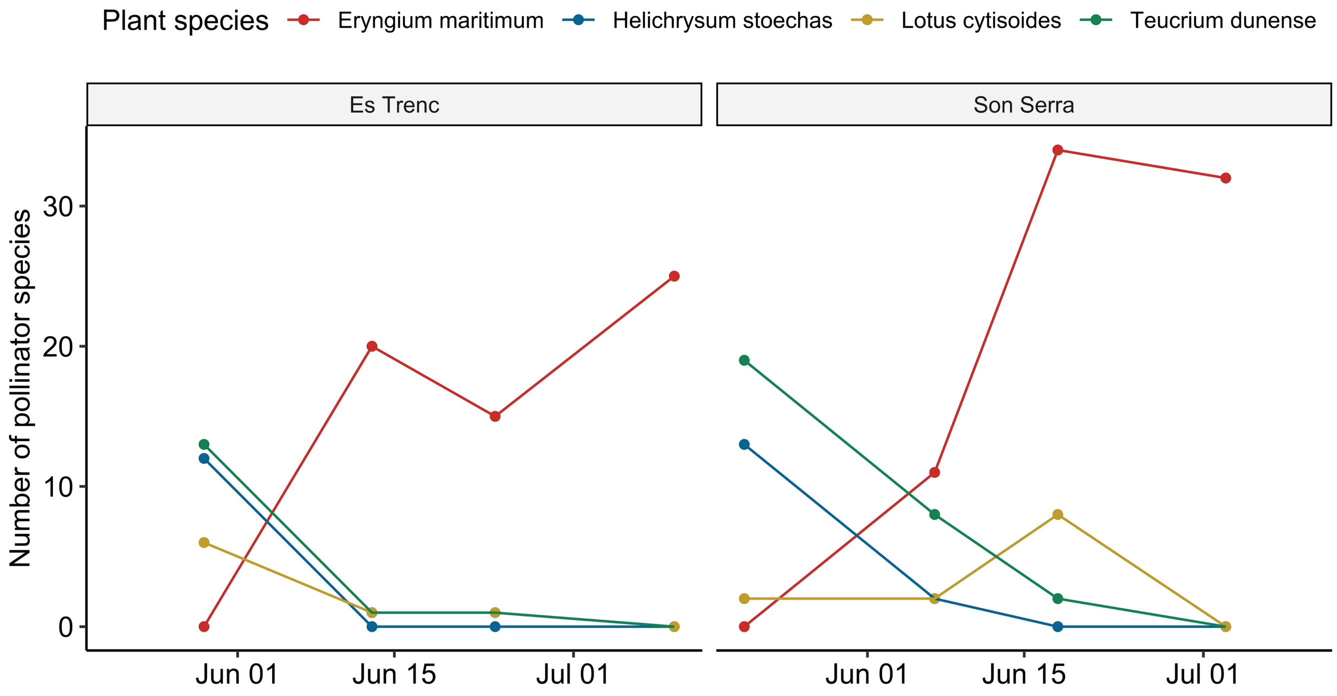

FIGURE 5 Changes in pollinator diversity of the more generalist plant species in the two studied populations. Only plant species visited by more than 10 pollinators species are indicated.

in both populations were highly selective, while high-opportunistic species were proportionally negligible, representing <2% of species (Figure 4; Table S3). In ET highly selective and selective species were more representative than in SS, where selective and opportunistic species were more represented. Attending at pollinator families, most of them were classified as highly selective, being the only opportunistic families *Andrenidae*, *Apidae*, *Lycaenidae*, *Pieridae*, *Scarabeidae* and *Syrphidae* (Table S4; Figure S1). Considering pollinator orders, Diptera, Hymenoptera and Lepidoptera can be considered as highly opportunistic while Coleoptera can be considered as opportunistic (Table S5).

Pollination networks strongly varied among sessions (Figure 5). During first sessions, *E. maritimum* was outside of its flowering period and *T. dunense* and *H. stoechas* gathered the vast majority of interactions. Then, *T. dunense* and especially *H. stoechas* experimented a decrease while *E. maritimum* began to attract more interactions. In the third session, *E. maritimum* was already the stronger species in terms of pollinator diversity. A slight decrease in pollinator diversity was observed comparing first and last sampling sessions in each area.

3.2 | Phenology

Seven of the ten plant species displayed Gauss-like phenological curves, with a defined flowering peak, while *L. cytisoides*, *G. flavum* and *E. pithyusa* presented a diffuse flowering period (Figure 6). Considering the most abundant species in the habitat, the flowering period of *E. maritimum* is located after *T. dunense* and *H. stoechas*, being the last species before *P. maritimum*. Observations of the flowering periods between SS an ET during pollinators samples, although not quantitatively evaluated, suggested that plant populations at ET flowered with a delay of, at least, 1 week respect to SS.

3.3 | Pollination behavior

A relation between time spent in visits and the whorl of the visited capitulum was observed, decreasing the time spent in visits in outer whorls (df = 4, $F = 2.21$, $p = .04$). Time spent in visits and number of visited capitula was variable among families (Figure 7). *Apidae* pollinators visited more capitula per plant than any other family, while *Lycaenidae* pollinators spent more time per visits (Table S6). Data about some families were insufficient to analyze behavior deeper.

4 | DISCUSSION

4.1 | Dune pollination networks

Most plant species are generalist rather than specialists (Gómez & Zamora, 2006; Herrera, 1996), and similarly the great majority of pollinators visit a great variety of plant species (Bosch et al., 2009). The evaluation of the level of specialization is particularly important as more specialized networks are more prone to collapse (Thébault & Fontaine, 2010). Most of the species observed in the present study could be considered as specialists, as their interactions were found in one or a few plant species. However, Petanidou et al. (2008) observed than 90% of species labeled as specialist are indeed generalist

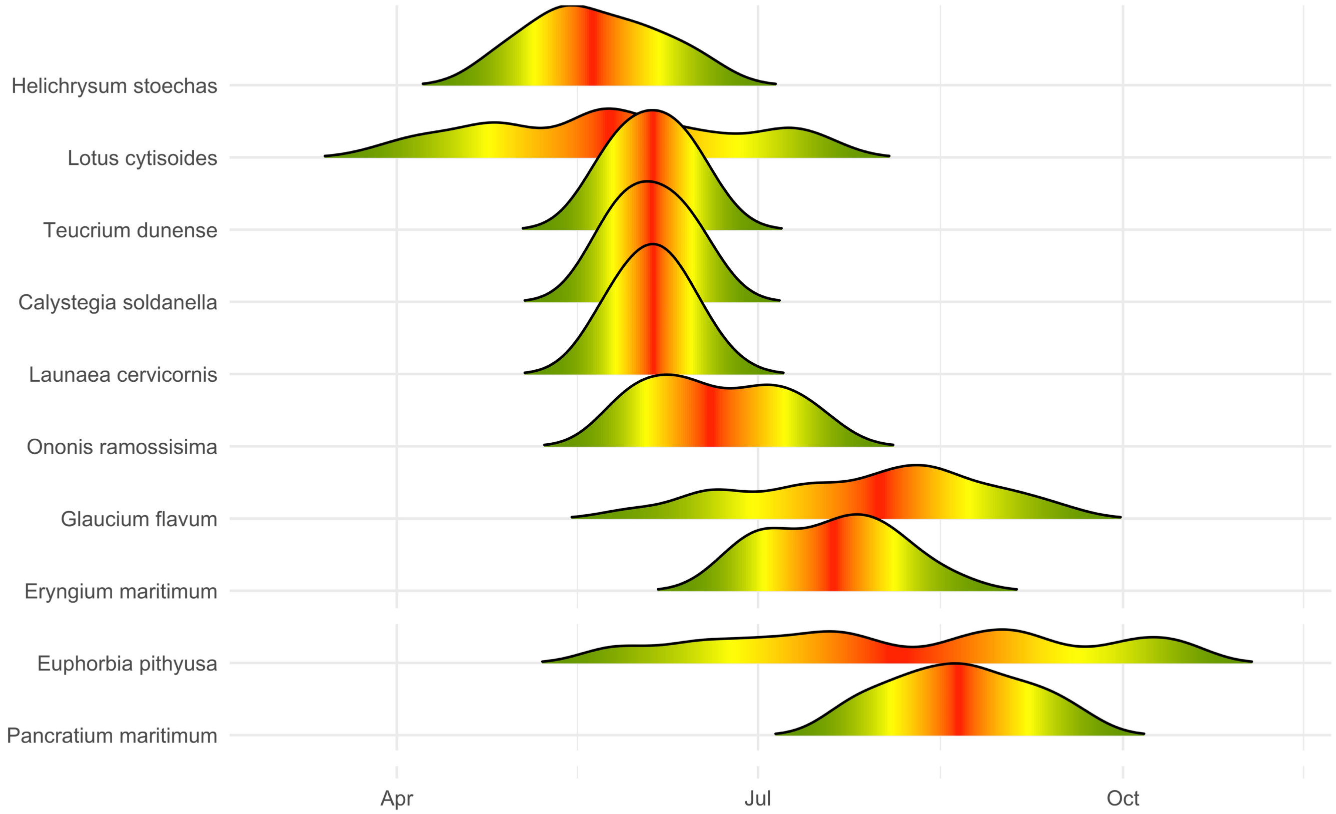

FIGURE 6 Flowering schedules of the analyzed dune species. Color gradient is used to indicate the flowering peaks. Curves indicate the number of opened flowers or flowering units in a certain time (see Gil, 1994, for specific methodological details).

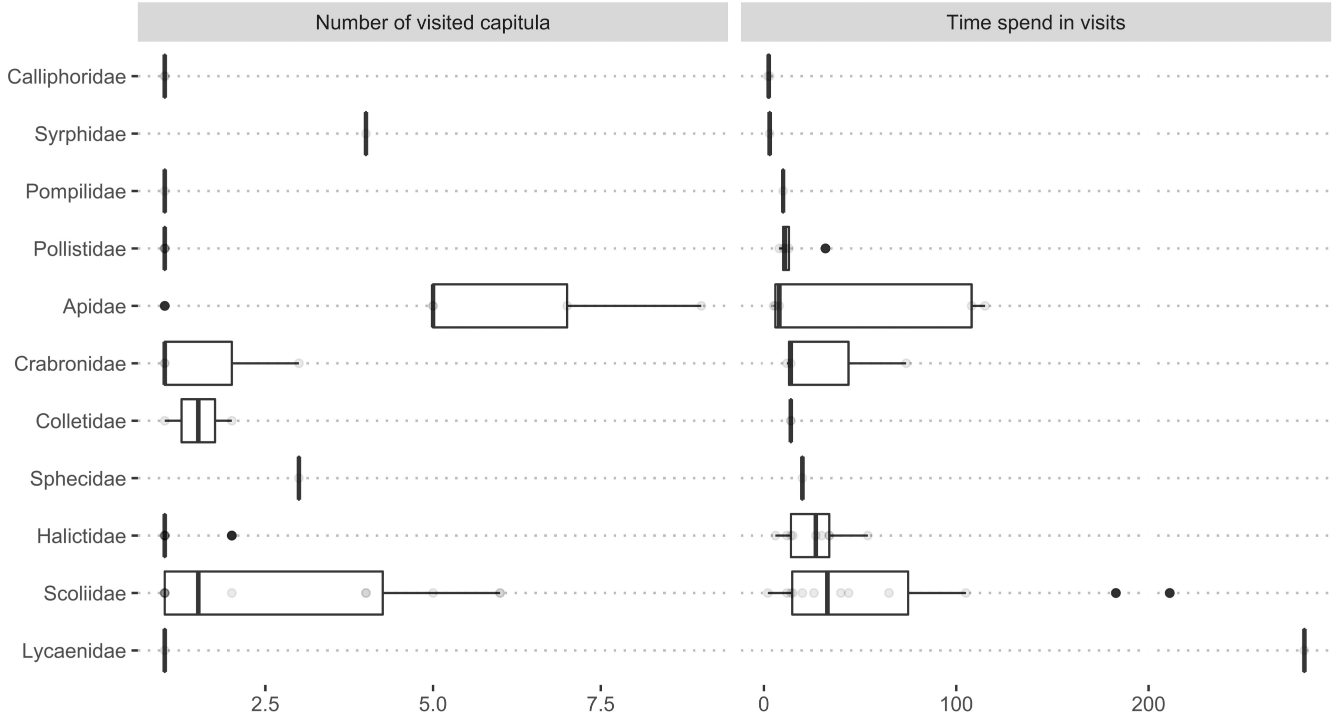

FIGURE 7 Results of the focal observation of pollinators behavior on *E. maritimum* individuals. For comparison, species have been grouped in families.

when multiple-year data are added, not being specialist but rare (less abundant). The high proportion of singletons observed is reasonable considering that the study was focused on finding the highest diversity of pollinator species possible. On the other hand, as suggested by Armbruster (2006), generalist pollinators can behave as specialist species in local scale, as a consequence of ecological specialization. Including the interactions found by pollen analysis is known to reduce the proportion of extreme specialist and increase connectance, as pollen remains in the body of pollinators for long period providing a record of visitation history rather than a single sample (Bosch et al., 2009; Courtney et al., 1982).

Two main areas, ET and SS were analyzed in order to gather the maximum diversity of pollinators and evaluate the differences between the two populations, being the first a protected area, in which users are not allowed to walk through the dune vegetation system. As a consequence of this protection character, it was expected that ET would display a more diverse network than SS, which would be more impacted by touristic pressure. However, SS presented a higher pollinator richness than ET, which could be related to differences in plant species abundance between populations, that although were not objective of the present work, were observed in the field. In this sense, floral abundance and plant diversity was higher in SS, factors that have been associated with a high density and abundance of interactions (Blüthgen et al., 2007; Hagen & Kraemer, 2010). On the other hand, a high pollinator richness can be considered as an indicator of moderate disturbance levels. As proposed by Connell (1978) in the intermediate disturbance hypothesis (IDH), moderate disturbances levels maintain the highest levels of species richness, although it should be taken into consideration with caution as IDH is considered as an oversimplification of nature (Fox, 2013). In sandy dune ecosystems, it has been documented that human disturbance increases micro-site diversification (Slaviero et al., 2016) but, at the same time, it increases competition between pollinators and local exclusion of weaker species, as a result of a reduction of floral resources availability (Wojcik et al., 2018).

Differences in connectance between populations could be considered as an indicator of differences in populations resilience (Heleno et al., 2012), by which the protected area, ET, would be more resilient than SS. However, it is known that species richness strongly influences connectance (Fantinato et al., 2019; Olesen & Jordano, 2002). In this sense, rarefaction curves indicated that more sampling effort would have conducted similar species richness in both populations. Moreover, low values of nestedness, as occurs in SS, are associated with low levels resistance and resilience (Bastolla et al., 2009) sometimes as a result of intense disturbances (Revilla et al., 2015; Welti & Joern, 2018), although not always (Spiesman & Inouye, 2013). However, when singletons are omitted, SS displays similar, but slightly lower, nestedness values than ET, but cannot be further discussed as they are not statistically significant from null models. Considering these findings, no clear effect of protection measures on pollination networks can be extracted from our results, and so they should be evaluated in future studies.

Hymenoptera was the most diverse order of pollinators in the dunes, which is consistent with the observations of previous studies (Castro-Urgal & Traveset, 2014; Fantinato, Del Vecchio, Silan, & Buffa, 2018). Other orders, such as Diptera and Lepidoptera, also played an important role in dune pollination (Gil, 1994). In our study areas, Apidae were the most diverse family of pollinators and the most opportunistic. This is rational considering than bees are generally polylectic, usually alternating visits between a pollen-rich source and a nectar-rich source (Bosch et al., 2009) and exploiting pollen resources from various species (Minckley & Roulston, 2006), although even oligolectic bees visit various species for nectar.

An invasive plant species, *Senecio cineraria* DC., was found in our sampling sessions in SS. Attending to the observed interactions, it behaves as a generalist species, although one pollinator species, *Halictus fulvipes*, was only found exploiting its floral resources. Previous studies carried out in a very close area suggested that alien species (in this case *Carpobrotus edulis* (L.) L. Bolus) influence the quantitative component of pollination, influencing negatively (competition) or positively (facilitation; Moragues & Traveset, 2005). So, the present results describe the effect of another alien species, quite abundant in the Balearic Island coasts due to its commercialization as garden plant, producing pollinator species displacements from autochthonous to allochthonous species, altering the structure of coastal networks.

4.2 | Phenology

A strong phenological variation was present among sampling sessions. For most pollinator species, flowering phenology is the main driver for pollinator distribution rather than flower traits (Bosch et al., 1997), usually presenting short activity periods of pollination (Farré-Armengol et al., 2015). However, a few species presented irregular and long phenological periods (*L. cytisoides*, *G. flavum* and *E. pithysusa*). In this sense, bivoltine insects have been proposed to be especially dependent upon plants with long or late flowering periods, as in *E. maritimum* (Howe et al., 2010). Although *L. cytisoides* and *E. pithyusa* are strongly entomogamous, *G. flavum* presents higher levels of autocompatibility (Gil, 1994), which could be related with their irregular phenology and the low pollinator diversity observed in the species, which is particularly visited by coleoptera.

The phenological distribution of species with very low selectiveness (*H. stoechas*, *T. dunense* and *E. maritimum*) suggests that there is a selective pressure to decouple flowering between species as to avoid interspecific competition for pollinators. At community level, competition is thought to be the primary selective force molding flowering schedules (Rathcke, 2014; Waser, 1978). However, when pollinator abundance is optimal, also facilitation among species can occur (Rathcke, 1983), as a sequential mutualism, in which early-flowering species support pollinators of late-flowering species (Waser & Real, 1979), or as a result of synchronous blooms that attract more pollinators that single species alone (Rathcke, 1983). Differences in phenological timing between populations could be related with temperature, as southern coastal populations due to sea currents are colder (Guijarro, 1986; Table S7), which is known to produce a delay in flowering (Gil, 1994; Llorens et al., 2021). On the other hand, the effect of wind is known to have a great impact in pollinators, which enhances the importance of multiannual data to reduce the potential impact of this variable. On the other hand, more focus should be put on pollinator species abundance in both areas, because attending to our observations strongly varied among sampling sessions.

4.3 | Pollinator behavior

Remarkable differences were found between families attending at pollination behavior. Apidae, the most represented family in both populations, visited more capitula per foraging bout, spending few time per visit, which is consistent with the results of previous studies (Brunet, 2009). The high variability observed in the behavior of Apidae could be attributed to the presence of different functional groups within the family (i.e., bumble-bees, solitary bees and social bees) with usually present different pollination behaviors, spending bumble-bees less time per visit but interacting with more flowers per visit (Brunet, 2009). The high values of time per visit observed in Lepidoptera, overall in *Polyommatus celina*, could be attributed to behavioral thermoregulation (Kevan & Shorthouse, 1970), and not really to extensive periods of foraging. In this sense, it must be considered that not all insect visitors may actually be pollinators, and also that pollination is not equally probable among pollinator species, due to differences in carrying capacity, morphology, foraging behavior and the degree of fidelity (Lindsey, 1984). For example, Coleoptera is known to visit a great variety of plant species, but at the same time is generally considered to have a low effective pollination rate (Sayers et al., 2019; Thayer et al., 2003). However, the evaluation of all these parameters in a single study is prohibitive, and so simplifications, as we did, should be carried out.

4.4 | The pollinators of *E. maritimum*

As suggested by our previous observations, *E. maritimum* is strongly entomophilous (Table 3; Cortés-Fernández, Cerrato, Ribas-Serra, & Gil Vives, 2022), being visited by a wide variety of pollinators. Forty-five species of 29 families can be considered as potential pollinators of the species, being Diptera and Hymenoptera the most diverse orders. Although some of the detected species have only a few cites in GBIF (Figure S2), all the detected plant and animal species in the study were previously cited Mallorca, except for *Leucospis gigas* and *Odice blandula* (Table S2; Figure S3). *Leucospis gigas* is an hymenoptera of the family Leucospidae widely distributed in the warmer parts of the Paleartic Region, as proposed by Madl and Schwarz (2014). In this same study, they propose that *Leucospis gigas* is found in the Balearic Islands, but no exact location or island is provided. So, to our knowledge, it is the first cite of the species in Mallorca, which was found only once in SS pollinating *E. maritimum*. On the other hand, *Odice blandula*, is a Lepidoptera of the family Erebidae which was detected previously in Ibiza and Formentera (Férriz et al., 2006), but similarly, no references about its presence in Mallorca are available, although its distribution in the island is known within the local experts (Truyols, pers. commun.)

Eryngium maritimum can be considered as an extreme opportunistic species, which is consistent with previous observations carried out in northern populations (Fitter & Peat, 1994; Hegi, 1935; Westrich, 2001; Zanella et al., 2009). Apiaceae species are known to be visited by a large quantity of insects (Davila, 2006; Zych et al., 2019) as a result of not presenting floral restrictions accessing to pollen and nectar (Lindsey, 1984). While myophile pollination is known to be usually focused on species with little odorless flowers, psychophyle pollination is carried out preferentially in species with more intense aromas and big tubular flowers (Aguado Martín et al., 2015). In our study, both orders exploited floral resources of a high diversity of plant species, including *E. maritimum*, but attending to flower morphology the species would not be optimal for lepidoptera. In previous quantitative studies carried out in other *Eryngium* and Apiaceae species, flies, bees and beetles made up the majority of insect visitors while butterflies and moths were rarely observed (Danderson & Molano-Flores, 2010; Zych, 2007), which is consistent with our results and field observations, but should be proved in future studies considering specifical pollinator abundance.

The species presented the greater pollinator richness in both populations. This is logical considering that the focus of the study was put on the species, and as a consequence of oversampling, the diversity of *E. maritimum* pollinators in relation with the other species could be overestimated, as the study comprises its whole flowering period. However, a central role of the species can be defined considering the diversity of pollinators that exploit the floral resources of the species, in a period in which *E. maritimum* is the last generalist species in flower previously to the arrival of cold temperatures. In this sense, *P. maritimum*, which flowers after *E. maritimum*, is not functionally similar, as it is known to be strongly related to evening and nocturnal rather than diurnal pollinators (Eisikowitch & Galil, 1971). So, it can be concluded that conservation of *E. maritimum* is important in terms of dune pollinators conservation, as a vast variety of pollinators depend on the species at the end of the flowering season, previously to the decrease in activity due to temperature changes (Mellanby, 1939; Taylor, 1963). However, the specific functionality of the species in the dune pollination network should be evaluated incorporating abundance data, as our study have already focused on diversity. Finally, our results suggest that it is improbable that the decrease in fruit and seed set in northern European populations could be attributed to specific pollinator extinctions, as the species behaves as an extremely opportunistic in terms of pollination. However, as suggested by Armbruster (2006), some species can behave as a specialist locally, so replicas of this study in northern populations would be critical to evaluate a potential cause of its decrease in fitness.

TABLE 3 Pollinator species detected in *Eryngium maritimum*. Reference indicates if the pollinator species was previously detected in the literature as a potential pollinator. Hegi (1935) [1], Gil (1994) [2], Cortés-Fernández, Cerrato, Ribas-Serra, and Gil Vives (2022) [3] and Polinib Database [4]

Order	Family	Genus	Species	ET	SS	References
Coleoptera	*Buprestidae*	*Anthaxia*	*Anthaxia umbellatarum*	1	0	[3]
	Pentatomidae	*Graphosoma*	*Graphosoma lineatum*	1	0	
	Scarabaeidae	*Oxythyrea*	*Oxythyrea funesta*	0	1	
Diptera	*Calliphoridae*	*Lucilia*	*Lucilia sericata*	1	0	
		Stomorhina	*Stomorhina lunata*	0	1	
	Cloropidae	*Thaumatomyia*	*Thaumatomyia notata*	1	0	
		Myopa	*Myopa extricata*	0	1	
	Pompilidae	*Deuteragenia*	*Deuteragenia variegata*	0	1	
	Syrphidae	*Eristalinus*	*Eristalinus aeneus*	0	1	
		Eristalis	*Eristalis tenax*	0	1	
		Sphaerophoria	*Sphaerophoria taeniata*	1	0	
Hymenoptera	*Andrenidae*	*Andrena*	*Andrena agilissima*	0	1	
			Andrena morio	1	1	
	Apidae	*Amegilla*	*Amegilla quadrifasciata*	0	1	
		Apis	*Apis mellifera*	0	1	[3–4]
		Bombus	*Bombus terrestris*	0	1	[3–4]
		Ceratina	*Ceratina cucurbitina*	1	0	
		Melecta	*Melecta luctuosa*	0	1	
	Colletidae	*Colletes*	*Colletes abeillei*	0	1	[2]
			Colletes succinctus	1	0	
		Hylaeus	*Hylaeus pictus*	1	0	
	Crabronidae	*Cerceris*	*Cerceris arenaria*	1	0	[1]
			Cerceris sabulosa	1	0	
		Philanthus	*Philanthus triangulum*	0	1	[2–3-4]
		Stizus	*Stizus fasciatus*	0	1	
	Halictidae	*Ceylalictus*	*Ceylalictus variegatus*	1	0	[4]
		Halictus	*Halictus scabiosae*	1	1	[3]
		Lasioglossum	*Lasioglossum albocinctum*	0	1	
		Pseudapis	*Pseudapis bispinosa*	1	0	[4]
	Leucospidae	*Leucospis*	*Leucospis gigas*	0	1	
	Polistidae	*Bembecinus*	*Bembecinus tridens*	0	1	
		Polistes	*Polistes dominula*	1	1	[4]
			Polistes gallicus	1	0	[2–4]
	Pompilidae	*Entomobora*	*Entomobora fuscipennis*	1	0	
	Scoliidae	*Dasyscolia*	*Dasyscolia ciliata*	0	1	
		Megascolia	*Megascolia bidens*	1	1	
			Megascolia maculata	1	1	[3–4]
	Sphecidae	*Sphex*	*Sphex funerarius*	0	1	
	Typhiidae	*Meria*	*Meria tripunctata*	1	0	[3]
	Vespidae	*Ancistrocerus*	*Ancistrocerus kitcheneri*	0	1	
	Vespidae	*Eumenes*	*Eumenes coarctatus*	1	0	
Lepidoptera	*Lycaenidae*	*Celastrina*	*Celastrina argiolus*	0	1	[3]
		Polyommatus	*Polyommatus celina*	1	0	[4]
	Nymphalidae	*Vanessa*	*Vanessa cardui*	0	1	[3]
	Pieridae	*Gonepteryx*	*Gonepteryx cleopatra*	0	1	
	Lycaenidae	***Polyommatus***	***Polyommatus celina***	**281**	**1**	
	Nymphalidae	*Vanessa*	*Vanessa cardui*	79		

5 | CONCLUSIONS

1. *Eryngium maritimum* is a widely generalist species, mainly pollinated by Diptera and Hymenoptera.
2. It flowers after the other two main generalist species, *H. stoechas* and *T. dunense*.
3. The pollination network of the protected area (ET) did not present clear differences with the non-protected area (SS), displaying a lower diversity but higher connectance values.
4. Hymenoptera are the main pollinators of dunes, although Diptera and Lepidoptera also play a major role.
5. *Eryngium maritimum* plays an important role as it is the last generalist species in flower before the end of the dune flowering season.

AUTHOR CONTRIBUTIONS

Arnau Ribas-Serra: Conceptualization (equal); investigation (equal); supervision (equal); validation (equal); writing – review and editing (equal). **Xavier Canyelles Ferrà:** Conceptualization (equal); investigation (equal); methodology (equal); validation (lead). **Iván Cortés-Fernández:** Conceptualization (equal); data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); project administration (equal). **Lorenzo Gil:** Conceptualization (equal); funding acquisition (lead); project administration (equal); supervision (lead); writing – review and editing (lead). **Marcello Dante Cerrato:** Conceptualization (equal); investigation (equal); methodology (equal); resources (equal); validation (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository https://doi.org/10.5061/dryad.p8cz8w9s8.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Capítulo 4.

Fitoquímica y citotoxicidad de *Eryngium maritimum*

Manuscript V

Cortés-Fernández, I., Sureda, A., Adrover, Caprioli, G., Maggi, F., Gil-Vives, L., Capó, X. (2022). Antioxidant and anti-inflammatory potential of rhizome aqueous extract of sea holly (*Eryngium maritimum* L.) on Jurkat cells. *Molecules*. *In review.*

Manuscript V. Antioxidant and anti-inflammatory potential of rhizome aqueous extract of sea holly (Eryngium maritimum L.) on Jurkat cells.

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Article

Antioxidant and anti-inflammatory potential of rhizome aqueous extract of sea holly (*Eryngium maritimum* L.) on Jurkat cells

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Abstract: The genus *Eryngium* is known for presenting a wide range of compounds with proved medicinal properties. In last years. Research has focused on *E. maritimum*, with previous studies reporting anticancer, antimicrobial, antioxidant, and anti-inflammatory activities. Being rhizomes one of the most bioactive organs, the effects of its extracts on the antioxidant and inflammatory response of immune cells have not been determined, being this the main aim of this study. Rhizome extracts were obtained from individuals located in Mallorca (Balearic Islands), and its composition was determined using HPLC-DAD, highlighting simple phenolic compounds such as *trans*-ferulic acid, catechin, chlorogenic acid, epicatechin and rosmarinic acid as the major constituents. Total antioxidant capacity was determined using the FRAP assay. Jurkat cells were cultured to analyse cytotoxicity by the cell viability assay. Parallelly, cells were stimulated with phytohemagglutinin and treated with different extract concentrations. Gene and protein expression, as well as nitrite and cytokine levels, were evaluated as indicators of metabolic responses. The species presented a high diversity of pharmacologically bioactive compounds with potential therapeutic uses. The extract presented null cytotoxicity and exerted antioxidant and anti-inflammatory effects by inducing an antioxidant response and reducing cytokine and nitric oxide release and the expression of pro-inflammatory genes. The results suggest that *E. maritimum* is a promising phytotherapeutic species because of its strong antioxidant and anti-inflammatory potential.

Keywords: *Eryngium maritimum, rhizome extracts, phytochemical screening, antioxidant, anti-inflammatory*

1. Introduction

The Apiaceae family, texto also called Umbelliferae, is known to present an extraordinary and diverse secondary metabolism, with a wide range of biological activities. Extracts obtained from members of this family possess antimicrobial [1,2], antioxidant [3], anti-inflammatory [4,5], antidiabetic [6,7] and anticancer activities [8]. There are approximately 3,780 species in the Apiaceae family, including caraway, carrot, celery, chervil, coriander, cumin, dill, fennel, hemlock, parsley and parsnip, which are important in the production and consumption in food industry [9].

The genus *Eryngium* is the most taxonomically diverse in the Apiaceae family, and is widely distributed around the world, mostly in Eurasia, North of Africa and South of

America [10]. This genus is drawing attention of the research community due to its potential medicinal applications, which include treating diabetes, renal and liver disfunctions and cancer [11]. The mechanisms that lead to these beneficial effects are not always known and strongly vary among species. For example, the administration of *E. carlinae* F.Delaroche has been proved to reduce hyperlipidaemia related to cardiovascular risk in diabetes mellitus, probably by improving renal function with reduction of acid uric levels [12]. This effect, among others observed in taxonomically closed species, could be related to the high antioxidant potential of the species of this genus, related to the presence a variety of compounds such as carotenes, tannins, saponins, alkaloids, acetylene, flavonoids, coumarin and terpenes (mainly sesquiterpenes, monoterpenes and triterpenes) [13-16] .

Eryngium maritimum L., known as sea holly or seaside eryngo, is a geophyte inhabiting white dunes of the European and Mediterranean coasts [17,18]. It is a long-lived perennial herb with a high-developed taproot and an extensive rhizomatous system extending laterally from buried stems [19]. In recent years, *E. maritimum* has become one of the most promising species as a source of bioactive compounds due to their proved medicinal potential. Many works carried out *in-vitro* reported the antioxidant potential of *E. maritimum* using diverse plant extracts [20-23]. Additionally, various studies have shown the beneficial effects of *E. maritimum* extracts in animal models associated with their antioxidant capacity. Thus, it has evidenced the capability of *E. maritimum* to reduce the toxicity associated with chemotherapeutic agents such as cisplatin as well as the potential to improve renal dysfunction [24]. Moreover, the application of extracts of rhizome and aerial parts in rat models of carrageenan-induced paw oedema and 12-O-tretradecanoylphorbol-13-acetate (TPA)-induced ear oedema has also been proven to exert anti-inflammatory effects [25-27].

However, in these studies, the potential effects of the extracts on the antioxidant and inflammatory response of immune cells have not been determined. With all this, the main objective of the present study was to analyse the phytochemical profile of rhizomes and to evaluate the *in-vivo* antioxidant and anti-inflammatory potential of the rhizome extracts of *E. maritimum* in JurKat cells.

2. Results

2.1 Phytochemical composition

The composition of the rhizome extract of *E. maritimum* was analysed and presented in Table S1. The concentration of bioactive compounds, mainly polyphenols in the rhizome extracts, was 3514.1±40.5 mg/Kg. A total of 25 different compounds in quantifiable amounts were found in the extract. The most abundant compound was *trans*-ferulic acid, with a mean concentration of 718 ± 9 mg/Kg of extract, followed by catechin (603 ± 26 mg/Kg) and chlorogenic acid (513 ± 6 mg/Kg) (Figure 1). Among the non-phenolic compounds, the triterpenes oleanolic acid (5292 ± 10 mg/Kg) and ursolic acid (230 ± 7 mg/Kg) can be highlighted.

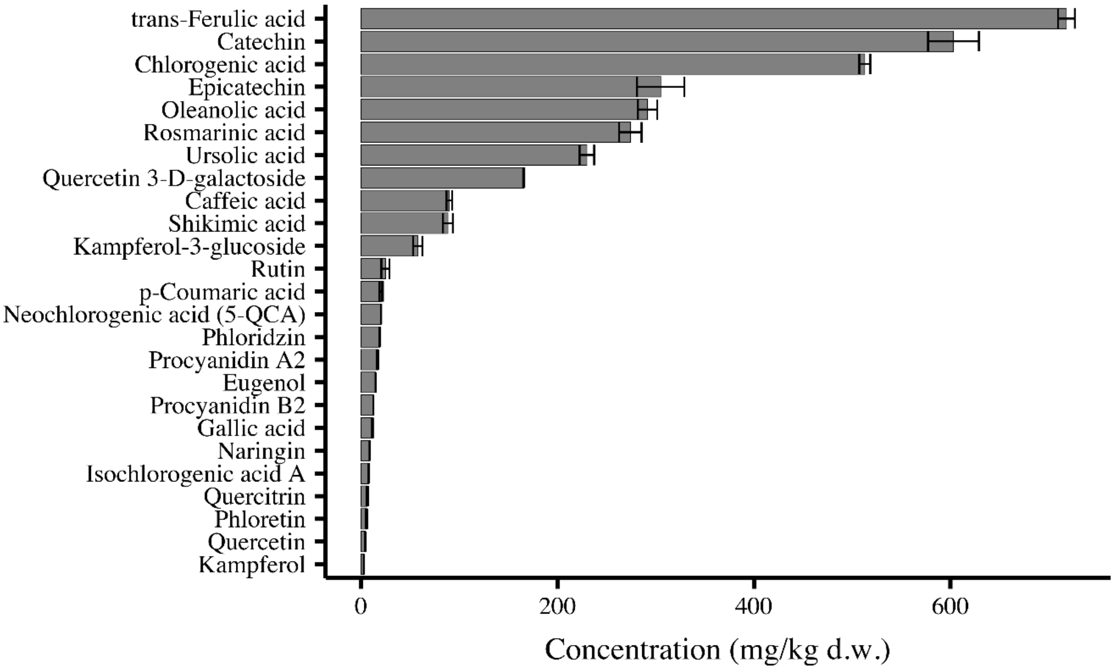

Figure 1. Composition of *E. maritimum* rhizome extracts expressed as the mean concentration of each compound (mg/kg d.w.).

2.2 Total antioxidant capacity (FRAP)

The total antioxidant capacity of the rhizome aqueous extract was determined using the FRAP assay. The results showed that antioxidant capacity of rhizome extracts was 0.10 ± 0.03 mM Trolox Equivalents (2 μmol Trolox equivalents/g rhizome).

2.3 Cell viability

Cell viability evaluated with the MTT assay evidenced that the rhizome extract at the doses used was not toxic for the JurKat cells up to 500 μg/mL.

2.4 Antioxidant and anti-inflammatory activity

TNF-α and IL-2 production by stimulated JurKat cells were measured after 4 and 24 h of treatment in the culture medium (Figure 2). Treatment with PHA induced an increase in TNF-α production both at 4 and 24 h of treatment. The treatment with *E. maritinum* extract induced a decrease in TNF-α production both at the concentration of 100 and 500 μg/ml of extract. The decrease in TNF-α production was significantly higher in the treatment at 500 μg/ml of extract in both studied periods. IL-2 showed a response pattern similar to that observed for TNF-α (Figure 2B). PHA activation increased IL-2 production both at 4 and 24 h, and this increase was significantly higher at 24 h stimulation. The treatment with extract at 100 and 500 μg/ml significantly reduced IL-2 production both at 4 and 24 h.

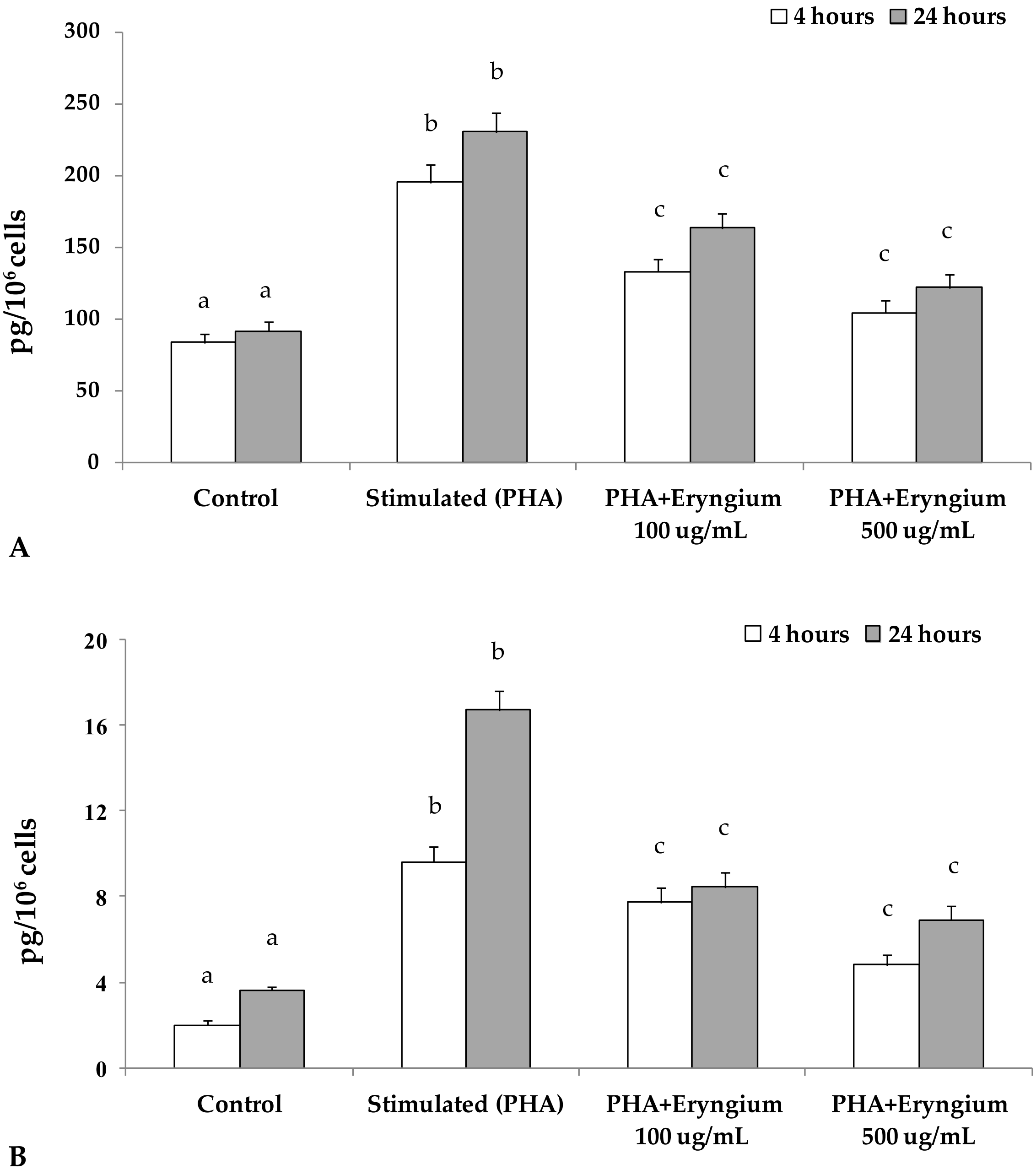

Figure 2. Effects of *E. maritimum* rhizome extracts on the concentration of TNF-α (A) and IL-2 (B) in the culture medium (pg/10^6 cells) measured at 4 and 24 h. Different letters are used to indicate significant differences among treatments. Results are expressed as mean ± S.E.M.

Nitrite levels, as an indicator of NO production, were presented in Figure 3. Similar to cytokine production, PHA caused an increase in nitrite production both at 4 and 24 h. The treatment at 500 µg extract/mL significantly reduced nitrite production to values similar to the control, while the decrease observed at the 100 µg/mL treatment was not statistically significant.

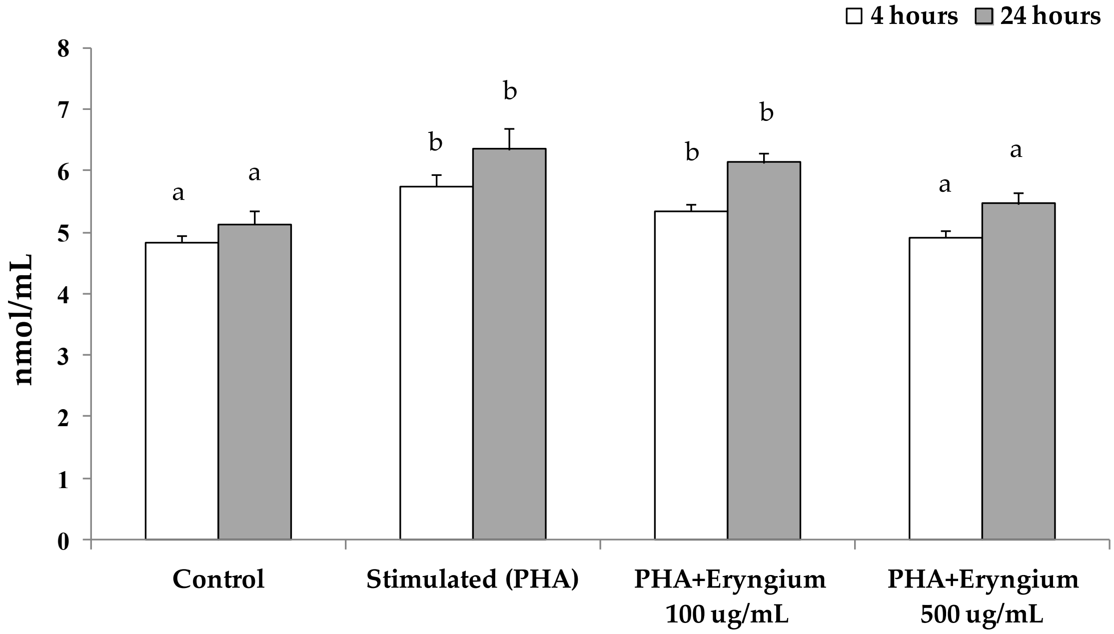

Figure 3. Effects of *E. maritimum* rhizome extracts on the production of nitrite (nmol/mL) in Jurkat cells at 4 and 24 h. Different letters are used to indicate significant differences among treatments. Results are expressed as mean ± S.E.M.

Table 1 shows the effects of PHA and extract at 4 h of treatment on JurKat cells gene expression. PHA induced an increase in CAT, MPO, TNF-α and NFκB expression, but this increase was not significant in the case of CAT. The treatment with extract at 100 µg extract/mL significantly reduced MPO, TNF-α and NFκB gene expression, while no effects on CAT gene expression were observed. The treatment with 500 µg/ml of extract reduced even more MPO, TNF-α and NFκB gene expression. On the other hand, CAT gene expression significantly increased as a consequence of the treatment with extract at 500 µg/mL. In the case of iNOS, although there is a tendency to increase after stimulation with PHA and to decrease with the different extracts, the differences were not statistically significant.

Table 1. Gene expression per treatment. Effects of *E. maritimum* rhizome extracts on the gene expression of antioxidant and anti-inflammatory genes in Jurkat cells at 4 hours. Different letters are used to indicate significant differences among treatments. Results are expressed as mean ± S.E.M.

Treatment	CAT	NF-κB	TNF-α	COX-2	iNOS
Control	1.00 ± 0.21a	1.00±0.17a	1.00 ± 0.14a	1.00 ± 0.11a	1.00 ± 0.21
PHA-Activated	1.26 ± 0.15ab	1.65±0.08b	1.95 ± 0.31b	1.65 ± 0.06b	1.53 ± 0.23
Eryngium 100 µg/mL	1.27 ± 0.11ab	1.51±0.27b	1.75 ± 0.25b	1.56 ± 0.13b	1.51 ±0.22
Eryngium 500 µg/mL	1.50 ± 0.13b	1.22 ± 0.11ab	1.42 ± 0.17ab	1.34 ± 0.15ab	1.23 ± 0.15

CAT and MPO protein levels measured by western blot were measured in samples after 24 h of treatment (Figure 4). PHA induced an increase in MPO protein levels, and this increase was avoided by the treatment with the extract, although the reduction was only statistically significant at 500 µg/ml. In the case of CAT protein levels PHA treatment did not induce any significant change in CAT protein levels. However, the treatment with extract induced a progressive increase in the expression of CAT, which was statistically significant at 500 μg/ml.

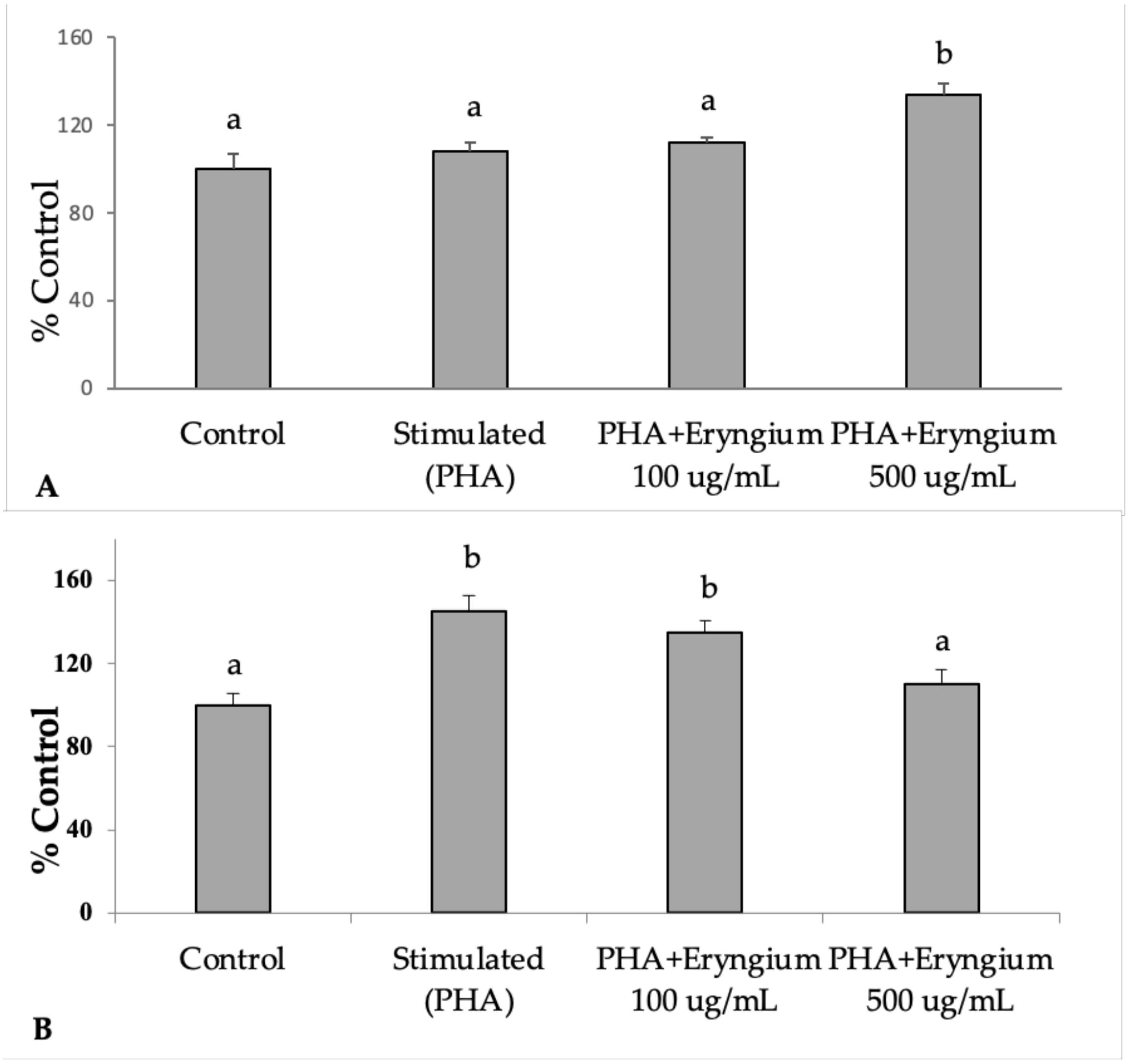

Figure 4. Effects of *E. maritimum* rhizome extracts on the protein levels of CAT and MPO in Jurkat cells at 24 h, expressed as % respect to the control group. Different letters are used to indicate significant differences among treatments. Results are expressed as mean ± S.E.M.

3. Discussion

Numerous studies have shown a wide variety of biological activities of different extracts of members from the *Eryngium* genus as well as of the vast majority of its components in isolation. The main finding of the present study was to evidence the antioxidant and anti-inflammatory capacity of aqueous extracts of *E. maritimum* rhizomes using immortalized Jurkat lymphocytes as a cell model. In addition, the phytochemical screening has allowed quantifying a total of 25 bioactive compounds in the extracts. Within the main determined compounds, those found in greater quantity are simple phenolic compounds such as *trans*-ferulic acid, catechin, chlorogenic acid, epicatechin and rosmarinic acid. Among the non-phenolic compounds, the oleanolic and ursolic acid triterpenes stand out. Although the composition of bioactive compounds is similar to those reported in other studies, there are differences in the concentrations obtained between the different studies [20,23,28]. These differences may derive from different factors such as the geographical area and collection season or the extraction and quantification method.

The extract of *E. maritimum* has an important antioxidant capacity, evidenced *in vitro* from the FRAP assay, derived from the presence of numerous bioactive compounds. In this sense, various studies have evaluated the antioxidant capacity of the rhizome of *E. maritimum*, although their comparison is complex since the extracts were obtained from different geographical areas but also because of the different methodologies [22,29]. The antioxidant capacity of the rhizome extracts of *E. maritimum* was higher than that observed for other widely used Apiaceae species, such as coriander (*Coriandrum sativum*), dill (*Anethum sowa*), cumin (*Cuminum cyminum*) and fennel (*Foeniculum vulgare*) [30]. The antioxidant ability of the extract mainly comes from the chemical nature of the molecules that contained. Mostly of them were polyphenols that are able to neutralize free radicals such as hydroxyl, superoxide, or peroxyl radicals [31,32]. In addition, polyphenols are also able to chelate metal ions, thus preventing their participation in free radical formation processes [31,33]. Some of the major constituents found on *E. maritimum* extracts present, attending to literature, strong antioxidant properties, including trans-ferulic acid [34,35], catechins [36], chlorogenic acid [37,38], rosmarinic acid [39], ursolic acid [40] and oleanolic acid [41,42]. Among the main antioxidant effects of these compounds has been proposed inhibition of lipid peroxidation [39], intracellular and extracellular radical scavenging [36], DNA protection [40], and up-regulation or inhibition of key enzymes [35]. Thus, the composition of the extracts could derive in a synergistic, and even stronger, antioxidant capacity, as it has been documented for specific combinations of phenolic compounds [43].

In addition to the direct antioxidant capacity of the extract, treatment with the same extract induces an increase in the expression and levels of catalase in Jurkat cells, without altering cell viability. Catalase is the main antioxidant enzyme since is responsible for decomposing hydrogen peroxide in water and oxygen [44], and together with SOD and GPX is considered as part of the first line defence antioxidants, preventing the formation of reactive species in cells [45]. The antioxidant capacity of the extract is also evident when evaluating the levels of the pro-oxidant enzyme MPO [46]. The results showed how the treatment with PHA induced an increase in the expression of the enzyme, which was reduced by the presence of the extract in a dose-effect relationship. These results are in line with previous studies proving that polyphenols induce an antioxidant response, reflected in an increase in the cellular antioxidant capabilities and a decrease in the oxidative damage markers [47,48]. Polyphenols can induce the up-regulation of antioxidant enzyme expression via activation of specific nuclear signalling pathways including nuclear factor-erythroid 2-related factor 2 (Nrf2) and forkhead box 0 (FOX0) and proliferator-activated receptor gamma coactivator 1α (PGC-1 α) [49-51]. Accordingly with our results, with the present results, Mejri and collaborators [24] evidenced increases in catalase activity as a result of the administration of *E. maritimum* extracts in rats protecting against protect against CCl_4 and cisplatin induced. Other taxonomically close species like *E. carlinae* have also shown to exert catalase restoring capabilities in streptozotocin-induced diabetic rats [52], which could be attributed to the capacity of some antioxidants to enhance endogenous antioxidant enzymes activities [53]. Moreover, the topical application of *E. foetidum* L. was found to induce a significant decrease in MPO activity in auricular oedema mouse model induced by 12-0-tetradecanoylphorbol acetate (TPA) [54].

In addition to the antioxidant activity, the treatment of PHA-activated Jurkat cells with *E. maritimum* extract induced an anti-inflammatory response that resulted in the inhibition of the expression of NF-κB, a transcriptional regulator of inducible inflammatory genes including COX-2 and iNOS. In addition, a reduction in the release of TNF-α, IL-2 and nitrite (as marker of NO production) were also observed. The anti-inflammatory effects of *E. maritimum* became also evidenced from its potential to protect against inflammation by reducing the number of total leukocytes and neutrophil percentage in the acute phase of inflammation induced by turpentine oil in rats [55]. An increase in ROS production has been evidenced to stimulate NF-κB activation, while polyphenols are capable of suppressing NF-κB pathways, ameliorating the oxidative and inflammatory responses [56,57]. Accordingly with our data, *E carlinae* inhibited the activation of NF-κB downregulating the production of TNF-α and IL-1β and stimulating the production of the anti-inflammatory IL-10 [58,59]. NO produced by the inducible isoform of NO (iNOS) is implicated in many physiological and pathological processes, including host defence, vasodilation, and acute and chronic inflammation [60]. However, when produced at high levels, NO alters cell cycle, promotes the formation of nitrogen reactive species (RNS) favouring inflammatory responses and induces apoptosis [61-63]. The reduction in the production of NO could avoid the excessive production of RNS and potentially reduce the inflammatory damage. In a previous study, Taskin and collaborators (2020) obtained similar NO inhibitory effects using *E. kotschyi* Boiss. and *E. campestre* L. ethanolic extracts, in LPS stimulated Raw 264.7 cells [64]. Also, similar results were obtained in murine endothelial cells treated with *E. campestre* and *E. planum* evidencing an almost completely inhibition of nitrite accumulation but, at the same time, without altering cell viability [65]. COX-2 is an inducible key enzyme in inflammatory response acting as the most important source of prostaglandins and, consequently, its inhibition is a main target of anti-inflammatory drugs [66,67]. A previous research, evidenced a significant reduction in COX-2 but also in iNOS gene expression in RAW264.7 cells stimulated with LPS [11]. The process was mediated by the inhibition of LPS-induced phosphorylation of IkB and reducing the activation of the NF-κB pathway. Moreover, the levels of TNF-α, as the most important cytokine in the inflammation cascade, and IL-2, as the most representative inflammatory cytokine secreted by Jurkat cells in the culture medium were reduced by *E. maritimum*. The anti-inflammatory effects of some other species of the *Eringium* genus evidenced by the inhibition of cytokine release were also reported. In this sense, the administration of *E. bungei* Boiss. and *E. foetidum* extracts inhibited the release L-6 and TNF-α in a dose-dependent manner in RAW264.7 cells and rats [11]. Similar results were obtained with the decoction of *E. carlinae* F.Delaroche, which decreased the accumulation of IL-1α, IL-2, IL-4, PDGF, prolactin, and thymus chemokine-1 in obsess rats [52]. Like the antioxidant properties, some of the major constituents of the extract have also been associated with anti-inflammatory processes [38,41,68]. Different anti-inflammatory effects have been documented for these compounds, including the downregulation of NF-kB [68], the inhibition of TNF-α, NO and IL-1ß production [69,70] and the inhibition of the expression of COX-2 and iNOS [71]. Moreover, some of them have been proposed to present synergistic effects, such as quercetin and catechin [72].

In conclusion *E. maritimum* presents a high diversity of pharmacologically bioactive compounds, with broad therapeutic potential, especially derived from its antioxidant activities. The hydrophilic extract of *E. maritimum* rhizomes exerted antioxidant and anti-inflammatory effects on Jurkat cells by inducing an antioxidant response and reducing cytokine and nitric oxide release and the expression of pro-inflammatory genes. The results of the present study suggest that *E. maritimum* is a promising species as a source of bioactive compounds because of its strong antioxidant and anti-inflammatory potential.

4. Materials and Methods

4.1 Plant material

Rhizomes of *E. maritimum* were collected during February 2019 in Son Serra de Marina (31S; 52050N-4397928E), a dune system located in the northern coast of Mallorca (Balearic Islands, Spain). Six samples of each organ were collected from 15 individuals, which were haphazardly selected along the dune. The freshly cut plants were dried in the drying room at ambient temperature with active ventilation for two weeks [73]. Samples were milled with a sample mill (Taurus 9085) and conserved at -20ºC.

4.2 Extraction

The extraction was carried out shaking flasks with 10g of sample and 200ml of solvent (miliQ water) in a magnetic heated stirred (JoanLab SH-3) at 80ºC for 30 minutes [73]. Extracts were then centrifuged at 4500 rpm and 4ºC for 30 minutes and then the supernatant was filtered with a Whatman No. 2 filter paper and conserved at -20ºC for further analysis. The *E. maritimum* rhizome extracts were dissolved in methanol and the solutions were filtered through a 0.45 µm pore size nylon membrane filter (Phenex, Phenomenex, Torrance, CA, USA) before HPLC-DAD analysis. Each sample was analysed in triplicate.

4.3 HPLC analysis of extracts

In order to evaluate the phytochemical profile of the rhizomes of *E. maritimum*, HPLC-DAD studies were performed with fruits, flowers, leaves and rhizomes extracts using a Hewlett-Packard HP-1090 Series II (Palo Alto, CA, USA), equipped with a vacuum degasser, a binary pump, an autosampler and a model 1046A HP photodiode array detector (DAD). The HPLC-DAD studies were performed following two different chromatographic procedures developed in our laboratory [74]. Briefly, the chromatographic separation was accomplished on a Synergi Polar-RP C18 (4.6 mm x 250 mm, 4 µm) analytical column from Phenomenex (Chesire, UK). The column was preceded by a security cartridge. The column temperature was set at 30°C and the injection volume was 10 µl. UV spectra were recorded in the range 210-400 nm allowing to detect 25 compounds. 210 nm was used for ursolic acid and oleanolic acids. 230 nm for shikimic acid, procyanidin A2 and procyanidin B2. 256 nm for quercitrin. 265 nm for rutin, quercetin-3-D-galactoside and kaempferol-3-glucoside. 272 nm for gallic acid. 280 nm for (+)-catechin hydrate, (-)-epicatechin, cinnamic acid, eugenol, trans-cinnamaldhehyde, carnosic acid, carnosol, naringin, phloretin, phlorizin. 325 nm for 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,5-di-caffeoylquinic acid, rosmarinic acid, caffeic acid, p-coumaric acid and trans-ferulic acid. 365 nm for kaempferol and quercetin, and 520 nm for cyanidin-3-glucoside.

4.4 Total Antioxidant Capacity (FRAP)

The total antioxidant capacity of rhizome extracts was measured using the ferric reducing antioxidant power (FRAP) assay, following the method described by Benzie and Strain [75]. Briefly, 300 µL of rhizome extract was incubated for 30 min with a solution of ferric chloride (2 mM) and 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in acetate buffer pH 3.6. After incubation, the absorbance at 593 nm was measured. The results are expressed as µM of Trolox/mg of prot.

4.5 Cell culture

JurKat cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), 100 units/ml penicillin, 0.1 ng/ml streptomycin and 2mM L-glutamine, in a humidified atmosphere containing 5% CO2 at 37°C.

4.6 Cell viability assay

Cell viability was measured using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay. The cells were cultured in 96-well plates in standard conditions at 6 x 10^5 cells/ml density, and after 24 hours of incubation, the medium of culture was changed with medium containing various concentrations of plant extract (100, 250, and 500 µg/mL). The plates were incubated for 24 hours and untreated cells were used as control. After the 24 hours, the cells were washed with PBS (to remove the remaining of *E. maritimum* rhizome extract) and the MTT reagent was added to each well (0.5 mg/ml) and cells were incubated for 4 h at 37ºC. The plates were then centrifuged, and the supernatant discarded. Tetrazolium crystals were resuspended in DMSO and the absorbance was measured at 570/620 nm [76,77].

4.7 Cell treatments and experimental design

All the procedures were performed in 6-well plates containing 6 x 10^5 cells/ml. Cells stimulated with phytohemagglutinin (PHA) 10µg/mL were treated with the extract using two different non-toxic concentrations (100 and 500 µg extract/ml). A negative control without stimulation and a positive control with only PHA were also used. Treatments were maintained for 4 hours and 24 hours. At the end of treatments, the content of each plate was centrifuged at 1200 rpm for 5 minutes to pellet cells and media were saved for the determination of TNFα, IL-2 proteins and nitrite levels. All treatments were carried out in triplicate.

4.8 Gene expression

mRNA expressions were determined by real time RT-PCR in cells collected after 4h treatment. For this purpose, mRNA was isolated by extraction with Tripure Isolation Reagent (Roche Diagnostics). cDNA was synthesized from 1 µg total RNA using reverse transcriptase with oligo-dT primers. Quantitative PCR was performed using the LightCycler instrument (Roche Diagnostics) with DNA-master SYBR Green I. Amplification was performed at 55ºC and 45 cycles. The relative quantification was achieved by standard calculations considering $2^{(\Delta\Delta Ct)}$. Gene expression was normalized using 18S rRNA as housekeeping. mRNA levels at the beginning of the study were arbitrarily referred to as 1. The primers and conditions used for the amplification are listed in Table 2.

Table 2. Primers and conditions used in the real-time PCRs.

Gene	Sequence	Conditions
18S	Fw: 5′-ATG TGA AGT CAC TGT GCC AG-3′ Rev: 5′-GTG TAA TCC GTC TCC ACA GA-3′	95 °C 10 Seg 60 °C 10 Seg 72 °C 15 Seg
CAT	Fw: 5′ -TTT GGC TAC TTT GAG GTC AC-3′ Rv: 5′ -TCC CCA TTT GCA TTA ACC AG-3′	95 °C 10 Seg 60 °C 10 Seg 72 °C 15 Seg
NF-kB	Fw:5′ -AAACACTGTGAGGATGGGATCTG-3′ Rv:5′ -CGAAGCCGACCACCATGT-3′	95 °C 10 Seg 60 °C 10 Seg 72 °C 15 Seg
TNFα	Fw: 5′-CCCAGGCAGTCAGATCATCTTCTCGGAA-3′ Rev: 5′-CTGGTTATCTCTCAGCTCCACGCCATT-3′	95 °C 10 Seg 63 °C 10 Seg 72 °C 15 Seg
COX-2	Fw: 5′-TTGCCTGGCAGGGTTGCTGGTGGTA-3′ Rev: 5′-CATCTGCCTGCTCTGGTCAATGGAA-3′	95 °C 10 Seg 63 °C 10 Seg 72 °C 15 Seg
iNOS	Fw: 5′–TCTGCAGACACGTGCGTTACT-3′ Rv: 5′ -ATGCACAGCTGAGCATTCCA-3′	95 °C 10 Seg 62 °C 10 Seg 72 °C 15 Seg

4.9 Western blot analysis

CAT and MPO protein levels were determined by Western blot after 24h of treatment. Cells were lysed with RIPA buffer (250 mM Tris/HCl, pH 8.0, 4.4% NaCl, 5% IGEPAL®, 2.5% deoxycholic acid, 0.5% sodium dodecyl sulphate (SDS)). Proteins of total cell extract were separated by size using 12% SDS polyacrylamide electrophoresis and transferred onto a nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were incubated overnight at 4ºC with primary monoclonal antibodies: anti-CAT (1:1000, rabbit) purchased from Calbiochem (Merck KGaA, Darmstadt, Germany) and anti-MPO (1:1000, mouse) purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Blots were incubated with a secondary peroxidase-conjugated antibody (1:5000) against the specific primary antibodies. Development of immunoblots was carried out using an enhanced chemiluminescence kit (Immun-Star® Western C® Kit reagent, Bio-Rad Laboratories, Hercules, CA, USA). Protein bans were visualised and quantified using the image analysis program Quantity One (Bio-Rad Laboratories).

4.10 Cytokine assay Kits

The levels of TNFα and IL-2 were measured in the culture media obtained after 4 and 24 h of treatment. TNFα levels were determined in culture media using an individual ELISA kit (Diaclone, Besancon Cedex, France) following the manufacturer's instructions for use. The intra- and inter-assay coefficients of variation were 3.2% and 10.9%, respectively. IL-2 levels TNFα levels were measured using an ELISA kit (Cusabio, Houston, TX, USA) following the manufacturer's instructions for use. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively. Detection wavelength for both assays was at 450 nm in a microplate reader (BioTek®, PowerWaveXS).

4.11 Nitrite levels

Nitrite levels were determined in culture media by the acidic Griess reaction using a spectrophotometric method. Samples were deproteinized with acetone and kept overnight at −20 ◦C. Then samples were centrifuged for 10 min at 15,000× g at 4 ºC, and supernatants were recovered. A 96-well plate was loaded with the samples or nitrite standard solutions (100 µL) in duplicate. A total 50 µL of sulphanilamide (2% w/v) in 5% HCl was added to each well, and 50 µL of N-(1-naphthyl)-ethylenediamine (0.1% w/v) in water was later added. After an incubation of 30 min, the absorbance was measured at 540 nm.

4.12 Statistical analysis

Statistical analysis was carried out using R (R Core Team, 2022). Results are expressed as mean ± SEM. A Kolmogorov–Smirnov test was applied to assess the normal distribution of the data. The statistical significance of the data was assessed by one-way analysis of variance (ANOVA). Differences among treatments were evaluated using the Tukey Test. Levels of significance were set at $p \leq 0.05$.

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DISCUSIÓN

Biología reproductiva

La variabilidad interindividual e interpoblacional suele ser el principal objeto de análisis de la mayoría de los estudios que hacen referencia a la biología reproductiva de las especies (Sawyer & Anderson, 1998; Cursach & Rita, 2012; de Lima Nadia & Machado, 2014; Hayafune et al., 2019). Sin embargo, la presente tesis pone de manifiesto la necesidad de profundizar en la variabilidad intraindividual, a fin de entender en su conjunto los mecanismos que rigen los requerimientos reproductivos y las estrategias de una especie. En el caso de *E. maritimum,* se ha mostrado que los capítulos primarios son la pieza central a nivel de éxito reproductivo femenino de la especie, siendo los principales responsables de la generación de flores y frutos. Diferencialmente, los capítulos exteriores serían los responsables del éxito reproductivo masculino de la especie, siendo más numerosos y encargándose de la generación de néctar y dispersión de polen. Asimismo, se ha evidenciado una prioridad en la translocación de recursos a los verticilos primarios, lo cual ya había sido propuesto por estudios de remoción de capítulos en *Eryngium alpinum* L. (Gaudeul & Till-Bottraud, 2004).

La especie ha resultado tener una casi nula capacidad de autofecundación, que puede entenderse como un mecanismo para favorecer el intercambio de material genético entre individuos. A este mecanismo se suma la marcada protandria de las flores, que disminuye la probabilidad de que la autofecundación tenga lugar (Forrest, 2014). Por otro lado, la elevada inversión de la especie en estructuras reproductivas, con varios verticilos de decenas de capítulos con diferente sincronía floral, eleva la probabilidad de que se produzcan eventos de geitonogamia. Sin embargo, y atendiendo a la gran variabilidad y cantidad de polinizadores que presenta, muchos de los cuales visitan de forma continuada varios verticilos de la misma planta, es lógico considerar que, aun existiendo intercambio de polen entre flores de diferentes verticilos, la capacidad de autofecundación es muy reducida, siendo una probable causa de la drástica disminución en el cuajado de frutos y semillas de los verticilos exteriores.

El estudio de los rizomas de la especie ha puesto de manifiesto que existe una relación entre sus dimensiones y el desarrollo de estructuras reproductivas, lo cual podría deberse a una mayor capacidad para reservar nutrientes durante la fase vegetativa (finales de otoño-primavera) como ocurre en otras especies de geófitos (Werger & Huber, 2006; Puntieri et al., 2014; Salachna et al., 2015). Asimismo, no se ha encontrado ninguna relación entre la distancia a la línea de costa y la anchura del rizoma, lo que podría indicar que otros factores como la edad de los individuos o la compactación del sustrato tienen una mayor influencia en el desarrollo de estructuras subterráneas. Estos resultados deben complementarse con una evaluación interpoblacional de los parámetros reproductivos, en especial entre las poblaciones amenazadas y las Mediterráneas, a fin de establecer cuál es la causa de los problemas reproductivos de las poblaciones del Norte de Europa y elaborar en consecuencia acciones de conservación.

Germinación

Los mecanismos de dormición en semillas resultan ser vitales para la supervivencia de las futuras plántulas (Crocker, 1916; Taylorson & Hendricks, 1977; Cardona, 2019). Las semillas procedentes de diferentes poblaciones de *E. maritimum* han manifestado mecanismos de adaptación que se han puesto de manifiesto en el estudio comparativo de su respuesta a la estratificación bajo diferentes regímenes de temperatura. En *E. maritimum*, la dormición morfofisiológica permite a la semilla pasar el invierno y germinar en el momento justo en el que las condiciones son viables para su desarrollo. Ello implica que existe una fuerte dependencia entre el régimen de temperaturas y la capacidad de germinación de la especie, que podría verse alterada por los efectos del cambio climático (Mondoni et al., 2012; Zogas et al., 2020). Un aumento en la temperatura global podría hacer que las semillas de las poblaciones vean atrasada su germinación, ya que, aunque se alcance la temperatura óptima de germinación en el ambiente, el desarrollo del embrión no será completo por no haber transcurrido el tiempo de estratificación necesario. Ello podría comportar un menor fitness y una mayor competencia con especies más eurioicas frente a dichas condiciones de temperatura. Sin embargo, queda pendiente dilucidar como la especie establece sus estrategias germinativas a largo plazo, es decir, que papel juega el banco de semillas persistente dentro del reclutamiento de la especie, así como la variación interanual en la germinación, lo cual ha sido reportado como un factor clave en especies que habitan en sistemas áridos y semiáridos para asegurar la germinación en años posteriores (Zogas et al., 2020).

Los resultados obtenidos en los análisis de halotolerancia muestran que *E. maritimum* es una especie halotolerante, pero no halófita como se le atribuye en la bibliografía (Meot-duros et al., 2008; Zitouni et al., 2016; Lajnef et al., 2017; Pereira et al., 2019). Ello radica parcialmente en la consideración imprecisa de que las especies dunares, por el hecho de ser costeras, tienen una elevada tolerancia a la salinidad. Sin embargo, las diferentes especies despliegan valores de halotolerancia muy diferentes entre sí, como se ha puesto de manifiesto en el análisis de la conductividad a lo largo de la duna realizado en el estudio de salinidad. Se ha de tener en cuenta que las dunas blancas, por su composición, presentan una acumulación de sales que en ningún caso es continua, sino puntual, ya que las lluvias permiten que se realicen lavados en los cuales las sales percolan hasta estratos más profundos, disminuyendo la concentración de sales en el medio (Etherington, 1967; Sloet van Oldruitenborgh & Heeres, 1969). Por ello es razonable que las especies del *Ammophilion*, entre las que se encuentra *E. maritimum*, desplieguen adaptativamente mecanismos para hacer frente a incrementos puntuales, moderados e incluso altos, de sales, tales como la suculencia moderada, la formación de vacuolas o la producción de exudados (Deinlein et al., 2014; Liang et al, 2018). Sin embargo, en ningún caso es plausible que cuenten con estrategias dirigidas a hacer frente a concentraciones elevadas continuas de dichas sales, tal y como presentan las plantas halófitas que habitan en zonas salinas e hipersalinas (Flowers & Colmer, 2015). Ello explicaría el bajo éxito reproductivo observado en los individuos adultos de *E. maritimum* sometidos a condiciones de stress salino, que aunque toleran porcentajes de agua marina superiores al 25% sin morir presentan una gran disminución en la producción de frutos y semillas. A su vez, las semillas también han presentado una reducida capacidad de germinación en presencia de sales, muriendo por toxicidad iónica. La heterogeneidad en la tolerancia a la salinidad de las semillas de especies dunares ya sido evidenciada por Cardona (2019), estableciendo que la salinidad por sí sola no es capaz de explicar la catena de vegetación dunar que se observa en el medio natural y, por ello, que las especies dunares no presentan todas ellas altos grados de halotolerancia. En Baleares, la concentración máxima de sales en el sustrato dunar tiene lugar en el mes de agosto debido a las bajas precipitaciones fruto de la sequía estival, mes en el que tiene lugar el pico máximo de fructificación de *E. maritimum* (Gil, 1994). Sin embargo, no es hasta el mes de septiembre, coincidiendo con las fuertes lluvias, cuando se produce la dispersión de semillas (Gil, 1994). En este momento, se produce el lavado de sales de la duna, que continuará durante todo el Otoño e Invierno, dando paso a las nuevas plántulas de la siguiente generación, hecho que condiciona las concentraciones de sales a la que se ve expuesta la semilla hasta su germinación. Por otro lado, aunque la bibliografía sugiere que la especie presenta una elevada capacidad de talasocoria (dispersión por el mar), y que ha sido básica para la distribución de la especie por las costas Mediterráneas, según los resultados de la presente tesis dicha dispersión parece ser poco plausible. Sin embargo, en futuros estudios se deberá evaluar qué papel juega la estratificación de la semilla en relación con la flotabilidad y la tolerancia a las sales de la especie, ya que es posible que la exposición de la semilla a condiciones de humedad continua a bajas temperaturas altere las propiedades fisicoquímicas de la cubierta de la semilla, haciéndola más sensible a la presencia de sales de lo que sería en su medio natural.

En cuanto al nicho ecológico de la especie y su distribución a lo largo de la duna, atendiendo a los análisis de conductividad realizados, se puede hipotetizar que la salinidad puede ejercer un factor de selección que evita que *E. maritimum* se desarrolle de manera óptima en las comunidades halonitrófilas del primer frente (*Cakilion maritimae*). Sin embargo, a medida que se avanza hacia el interior la conductividad decrece, al igual que la densidad de *E. maritimum*, lo que sugiere que otros factores más allá de la salinidad son claves en el establecimiento de la especie. En este sentido, parece ser que la compactación del suelo pueda ejercer una presión selectiva significativa sobre la especie, impidiendo la formación del sistema radicular y rizomatoso (Whalley et al., 1995), lo cual es clave para esta especie geófita. Este factor podría actuar también en primera línea, lo cual explicaría su tendencia a habitar en dunas móviles de manera óptima (Isermann & Rooney, 2014). Además, otros factores como la abrasión, la sequía y la pobreza en nutrientes, así como la competencia interespecífica, han sido propuestos como factores clave en la limitación del crecimiento de las plantas en frentes dunares (Rozema et al., 1985), cuyos efectos sobre el establecimiento de *E. maritimum* son inciertos y deberán evaluarse en futuros estudios.

Polinización

Eryngium maritimum es una especie generalista con una gran diversidad de polinizadores y que realiza una producción floral considerable, presentando escapos de gran tamaño y con una gran cantidad de capítulos por planta (20.07±12.69). Teniendo en cuenta los resultados obtenidos en los experimentos de exclusión de vectores de polinización (embolsados), se puede corroborar que, al menos en las poblaciones estudiadas, la producción de frutos, y por ello el reclutamiento de la especie, es particularmente dependiente del estado de conservación de las poblaciones de polinizadores. Esto es especialmente relevante teniendo en cuenta que en las poblaciones donde la especie está amenazada, en el Norte de Europa, se ha propuesto que las poblaciones se mantienen por reproducción vegetativa (Stasiak, 1986), lo que podría estar ligado tanto a un menor éxito reproductivo como a un problema de polinización.

Considerando los periodos de floración de las especies dunares, se han puesto de manifiesto potenciales mutualismos entre *E. maritimum*, *H. stoechas* y *T. dunense*. Todas ellas son especies oportunistas, de gran abundancia y con picos de floración muy establecidos, que podrían haber desacoplado sus floraciones, sobre todo en el caso de *E. maritimum*, para evitar la competencia interespecífica por los polinizadores, mientras que en el caso de *H. stoechas* y *T. dunense*, más acopladas, podrían intervenir mecanismos de facilitación entre ellas para captar un mayor número de polinizadores. Por otro lado, se ha evidenciado el papel central de *E. maritimum* en la red de polinización dunar de las poblaciones estudiadas, ya que de sus recursos florales se nutren una gran variedad de especies de polinizadores al final de la época de floración de las especies diurnas de la duna. En estudios futuros queda pendiente evaluar, de todos los polinizadores observados, cuales juegan un papel más importante dentro de la red, llevando a cabo muestreos cuantitativos en lugar de cualitativos, lo que permitirá destacar, entre todos los polinizadores observados, aquellos que juegan un papel más trascendental como piezas centrales de los módulos que conforman la red y aquellos que son conectores entre módulos, lo cual es fundamental para la correcta conservación de las comunidades (Olesen et al., 2007).

Composición fitoquímica

La especie parece carecer de compuestos exclusivos, siendo la gran mayoría de sus compuestos ampliamente conocidos por sus propiedades medicinales. Sin embargo, presenta un amplio espectro de compuestos que podrían conducir hacia acciones sinérgicas que darían lugar a las propiedades medicinales observadas. Atendiendo a la bibliografía existente, la composición de sus extractos presenta fuertes variaciones según el órgano, el método de extracción y la población estudiada (Darriet et al., 2014; Traversier, 2020). Sin embargo, se puede afirmar que sus rizomas, de forma general, presentan altas concentraciones de catequinas, sesquiterpenos (Germacreno D, 15-Hydroxy-α-muuroleno), fenoles (ácido 3,4-Dihydroxyphenylacetico, Epicatequina, ácido rosmarínico), triterpenos (ácido oleanólico, ácido ursólico), flavonoides (Rutina, Quercetin 3-D-galactósido) y ácidos carboxílicos (ácido shikimico).

Potencial medicinal

El análisis bibliográfico y los resultados obtenidos en los análisis de citotoxicidad y de efectos sobre líneas celulares evidencian que nos encontramos ante una especie con un elevado potencial antioxidante y cuya administración no genera efectos nocivos en modelos *in-vivo*. Este resultado es crucial para llevar a cabo futuros ensayos que evalúen los efectos específicos en humanos, considerando que en ratas el efecto nefroprotector de la especie ya ha sido constatado (Lisciani et al., 1984; Mejri et al., 2017). Sin embargo, no es posible establecer el compuesto o compuestos responsables de su mecanismo de acción, ya que es necesario un estudio exhaustivo que valore el efecto preciso de cada uno de ellos y que profundice en aquellos compuestos minoritarios que no se han podido identificar. Por otro lado, solo se han evaluado los extractos acuosos de rizomas, debiéndose estudiar con más detalle otros métodos de extracción y órganos para dilucidar si hay otros compuestos con bajos coeficientes de reparto en agua que puedan contribuir a los beneficios observados. Asimismo, es de vital importancia relacionar los requerimientos ecológicos de la especie, tales como la salinidad, con la producción de metabolitos, a fin de evaluar si su producción es dependiente de las condiciones de desarrollo, tal y como sucede en otras especies ampliamente utilizadas para la producción de compuestos bioactivos (Banerjee & Roychoudhury, 2017).

Una potencial vía de actuación de *E. maritimum* podría ser su elevado potencial antioxidante. Diversas patologías, como la obesidad o la diabetes, promueven que los ácidos grasos libres se acumulen en los glomérulos generando lipogénesis renal (Kamijo et al., 2002) y a su vez un fuerte estrés oxidativo (Gröne & Gröne, 2008), que da lugar a un estado inflamatorio (Pérez-Ramírez et al., 2016). La acumulación de citoquinas proinflamatorias conduce a una excesiva proliferación (Zhang et al., 2005), a fibrosis (producción excesiva de matriz extracelular) y a la apoptosis celular (Bashkaran et al., 2003). Por ello, su administración podría prevenir dicha cascada de respuestas pro-oxidantes, desencadenando una mejora sustancial en los biomarcadores indicados en las analíticas de los pacientes. Por otro lado, estudios previos han evidenciado otros efectos, como su potencial anticancerígeno (Yurdakök and Baydan, 2013; Beeby et al., 2020), antimicrobiano (Kholkhal et al., 2012, Traversier et al., 2020) y antidiabético (Pereira et al., 2019), que parecen estar ligados a otras vías metabólicas, tales como la inhibición de la alfa-amilasa en los ensayos con modelos diabéticos (Pereira et al., 2019) o promoviendo la acumulación de compuestos quimioterapéuticos en las células cancerígenas, inhibiendo su transporte hacia el exterior. Dilucidar los mecanismos causantes de las propiedades medicinales demostradas es vital para su uso futuro como nutracéutico o fuente de fórmulas farmacológicas específicas.

Implicaciones para la conservación

Eryngium maritimum es una especie clave de las dunas de Baleares. Contribuye, con sus rizomas, a la estabilización y acumulación del sustrato dunar y juega un papel clave en la red de polinización, otorgando conectividad al hábitat y nutriendo a una gran cantidad de polinizadores en una época del año en la que no se encuentran otras especies similares en flor. Se ha constatado que se trata de una especie que no tolera altas concentraciones de sal de forma continuada, generando graves problemas de reclutamiento. Por ello, es plausible considerar que un incremento en el nivel del mar provoque un drástico descenso en el éxito reproductivo de la especie, lo que provocará un estrechamiento de su área de distribución dentro de la propia duna, limitada hacia el interior por la presencia de especies mejor adaptadas a las condiciones de la duna semimóvil. Además, el aumento de la temperatura global podría comportar un descenso en la capacidad de germinación de la especie, al reducirse la duración de la época de bajas temperaturas, necesarias para el crecimiento del embrión debido a la dormición morfofisiológica de las semillas. De poder extrapolar los resultados de la presente tesis a las poblaciones del Norte de Europa, donde la especie se encuentra fuertemente amenazada y ya se han evidenciado cambios en su distribución (Stasiak, 1986; 1988), implicaría graves problemas para la conservación futura de la especie.

De requerirse una explotación farmacológica de la especie, la presente tesis establece los protocolos para su desarrollo *ex situ*. La recolección de semillas debe realizarse focalizándose en los primeros verticilos al inicio de la época de fructificación, los cuales presentan un mayor cuajado de frutos. Asimismo, sometiendo a las semillas a las condiciones establecidas de temperatura y estratificación, será posible obtener gran cantidad de plántulas que puedan ser cultivadas bajo condiciones controladas. A raíz de los resultados de los ensayos de halotolerancia, se puede establecer que la concentración de sal en el sustrato y en el agua de riego debe ser controlada, ya que limita la producción de biomasa, si bien queda pendiente la evaluación de la influencia de la salinidad sobre la composición cualitativa y cuantitativa de los componentes químicos en la especie. Asimismo, la compactación del suelo, aunque debe evaluarse su efecto específicamente, parece ser un factor clave en el desarrollo de la especie, hecho que deberá tenerse en cuenta para el diseño de los sustratos de cultivo. Para el cuajado de los frutos y la producción ex situ de semillas, el papel de los polinizadores es clave, aunque la especie es ampliamente generalista y es previsible que sea capaz de generar frutos en una gran diversidad de localizaciones. El análisis de la composición y de la actividad antioxidante y anti-inflamatoria de los rizomas de la especie juntamente con la bibliografía previa, ha puesto de manifiesto su elevado potencial medicinal y su ausencia de toxicidad. Sin embargo, previamente a su uso, es vital un ensayo clínico de sus efectos específicos para dilucidar el potencial real de la especie.

CONCLUSIONES

La presente tesis doctoral ha analizado la biología reproductiva y la fitoquímica de una especie dunar, *Eryngium maritimum*, en las costas de Mallorca. Concretamente, se han descrito mecanismos de variabilidad intraindividual en cuanto a la funcionalidad de las inflorescencias, se han establecido los protocolos óptimos de germinación de la especie, se han evidenciado procesos de adaptación de la germinación a nivel interpoblacional, se ha analizado su tolerancia a la salinidad en diferentes etapas de su ciclo vital, su papel dentro de la red de polinización dunar, su composición, potencial antioxidante y citotoxicidad.

Por ello, las conclusiones de la presente tesis doctoral son:

Biología reproductiva y germinación

1. La especie presenta una diversidad morfométrica y funcional entre verticilos, de forma que los verticilos internos son más grandes y presentan un mayor porcentaje de fructificación y de cuajado de semillas, mientras que los externos son más pequeños y no contribuyen a la generación de frutos.
2. La capacidad de autogamia de la especie es prácticamente nula, jugando la xenogamia un papel fundamental en el éxito reproductivo de la especie.
3. La relación masa aérea – masa subterránea parece ser clave para el óptimo desarrollo de los escapos florales, presentando los individuos con mayores rizomas (y por tanto mayor capacidad de reserva energética) un mayor desarrollo floral.
4. Las semillas de *Eryngium maritimum* de las poblaciones de Mallorca presentan porcentajes de germinación prácticamente del 100% a 20ºC con un periodo de estratificación en frío de 8 semanas.
5. Existe una variabilidad interpoblacional en el periodo de estratificación en frío necesario para la ruptura de la dormición de las semillas, por la cual las poblaciones localizadas en zonas más frías requieren de un periodo de exposición al frío más largo, lo que evidencia procesos de adaptación a las condiciones ambientales para asegurar la supervivencia de las plántulas.

Evaluación de la tolerancia a sal

6. *Eryngium maritimum* es una especie halotolerante, y no halófita, ya que, aunque es capaz de tolerar concentraciones moderadas de sal sin morir, su éxito reproductivo se ve gravemente afectado por la presencia de sales, impidiendo la producción de frutos y con ello el reclutamiento.
7. La presencia de sales en el medio impide la germinación de la especie por toxicidad iónica. Por otro lado, no todas las sales ejercen el mismo efecto, siendo Na_2SO_4 y $MgCl_2$ las que ejercen una toxicidad más alta.
8. La salinidad juega un papel clave en la distribución de la especie a lo largo de la duna. Sin embargo, otros factores, como la compactación del sustrato, podrían ejercer un efecto significativo en su distribución, que es previsible que disminuya como consecuencia del cambio climático y la antropización

Papel de *Eryngium maritimum* L. en la red de polinización dunar

9. *Eryngium maritimum* es una especie altamente oportunista que juega un papel clave dentro de la red de polinización dunar, presentando una alta diversidad de polinizadores.
10. *Eryngium maritimum* florece más tarde que el resto de las especies funcionalmente similares de su clúster de polinización (*Helichrysum stoechas* y *Teucrium dunense*), lo que puede ser ocasionado por un desplazamiento fenológico para evitar la competencia, evidenciando fenómenos de mutualismo en la red de polinización dunar.

11. La distribución fenológica de *Eryngium maritimum* comporta que sea la última especie de alta densidad y altamente oportunista en florecer del sistema dunar. Por ello, muchas especies de polinizadores dependen de su presencia para nutrirse previamente a la finalización de la época de floración diurna.

Fitoquímica y citotoxicidad

12. Los extractos acuosos de los diferentes órganos muestran una concentración muy elevada de compuestos antioxidantes, entre los que destacan el ácido rosmarínico, las catequinas, el ácido trans-ferúlico y el cafeico.
13. Los extractos acuosos presentan una considerable actividad antioxidante y antiinflamatoria, superior a otras especies de apiáceas ampliamente utilizadas como el eneldo o el comino.
14. Su composición y actividad antioxidante ponen de manifiesto que se trata de una especie con un elevado potencial medicinal.

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MATERIAL SUPLEMENTARIO

Manuscript I. Floral traits and reproductive success variation among inflorescence orders in *Eryngium maritimum*

Figure S1. Distribution map of the areas and plants used in the biometric study.

Table 3. Morphometric characters of the capitulum by inflorescence orders ($\bar{X}$ ± SE)

Inflorescence orders	N	Capitulum diameter with bractea (cm)	Capitulum diameter without bractea (cm)	Capitulum length (cm)	Flower width (cm)	Flower length (cm)
First	10	7.8±0.72	2.93±0.04	2.82±0.10	0.72±0.02	1.20±0.03
Second	24	6.7±0.18	2.52±0.07	2.47±0.08	0.66±0.02	1.09±0.01
Third	34	4.7±0.16	2.12±0.05	1.89±0.05	0.53±0.02	0.87±0.03
Fourth	12	3.2±0.29	1.63±0.10	1.45±0.08	0.47±0.01	0.54±0.01
Fifth	2	3.73±0.19	1.56±0.01	1.36±0.02	-	-

Table 4. Flower, fruit and seed production of capitulum by inflorescence orders ($\bar{X}$ ± SE).

Inflorescence order	N	Number of flowers	Number of fruits	Number of seeds	FruitSet	SeedSet
First	26	39.12±1.0	25.73±3.5	36.96±5.7	63.3±8.2	45.6±6.86
Second	136	37.23±0.4	16.12±1.4	23.02±2.2	40.5±3.4	28.8±2.7
Third	220	33.88±0.4	4.79±0.7	6.99±1.1	13.0±1.8	9.5±1.4
Fourth	141	30.45±0.4	1.45±0.4	1.95±0.6	4.6±1.2	3.1±0.9
Fifth	37	25.22±0.6	0	0	0	0
Sixth	5	19.80±2.1	0	0	0	0

Manuscript II. Evidence of interpopulation variation in the germination of *Eryngium maritimum* L. (Apiaceae).

Table S1. Previous literature and trials considered in the meta-analysis. In Incubation Temperature "/" indicates alternate temperatures, while "-" indicates the range of temperatures considered in the trials.

Population	Reference	Incubation Temperature	Photoperiod (light-dark)	Viability test	Stratification length (weeks)	Final germination (%)
SPAIN						
Asturias	Fernandez-Pascual et al., 2017	22/12	12-12		12	10
Atlantic	Cortes et al., 2020	20-15	0-24	Cut-test	6-20	31.12 ± 22.5
Mediterranean	Cortes et al., 2020	20-15	0-24	Cut-test	2-14	60.14 ± 29.98
LATVIA						
Kurzeme	Necajeva & levinsh,2013	25/10	12-12		4-16	41.25 ± 41.31
Uzava	Klavina et al., 2006	22-25	16-8		8	20
ITALY						
Sardegna	Porceddu et al., 2019	15	12-12		8	86
UNITED KINGDOM						
Sizewell	Walsmley & Davy, 1997	25/15	12-12	Tetrazolium test	2-14	9.67 ± 6.65
NORWAY						
Skagerrak	Curie et al., 2004	20/10	12-12		6	9

Manuscript III. Salinity effects on the germination and reproduction of Eryngium maritimum L. (Apiaceae)

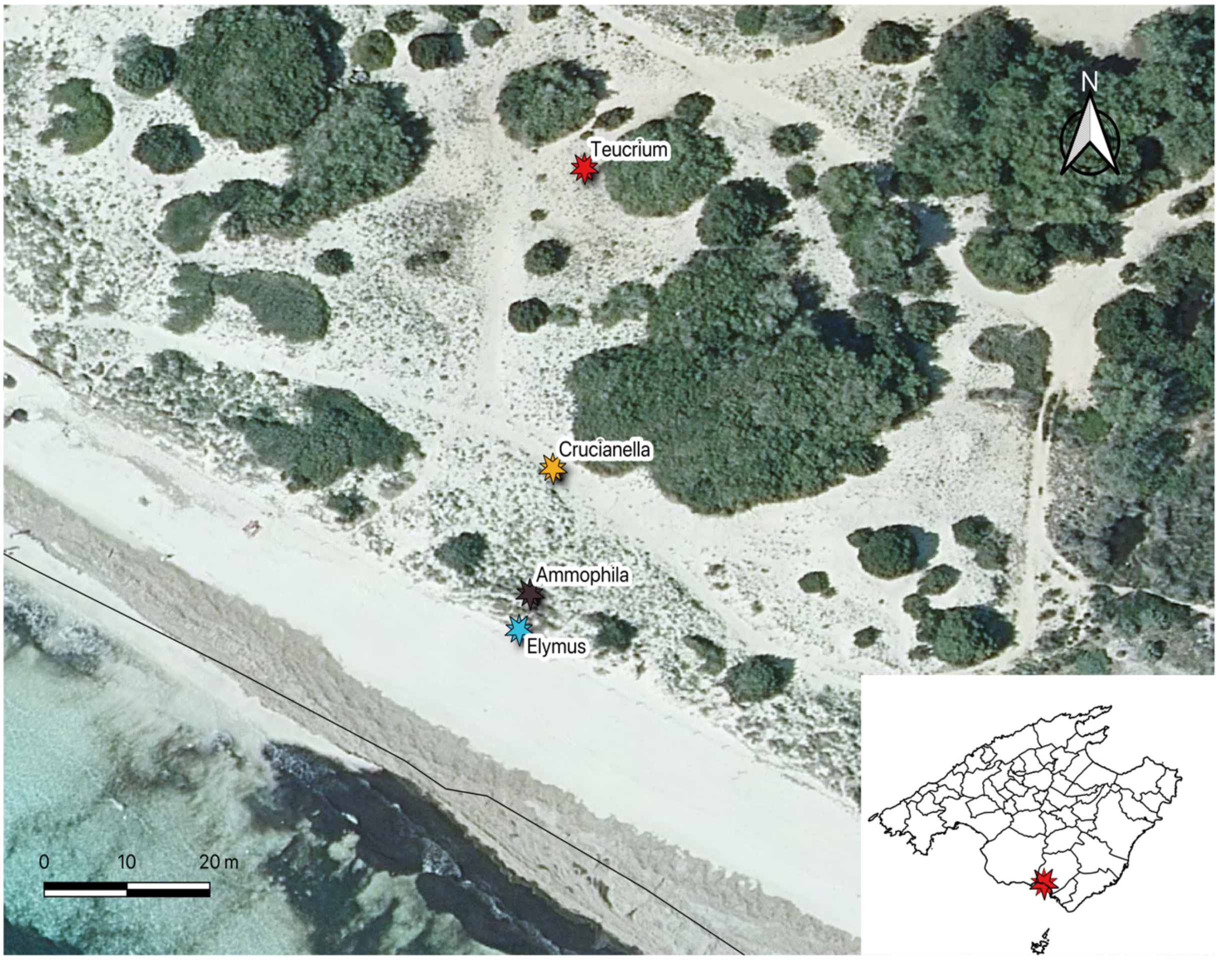

Figure S1. Distribution of the sampling areas where field electrical conductivity measures were carried out. Syntaxonomic alliances are indicated from sea-line to inwards: Elymus = *Agropyro–Minuartion peploidis*; Ammophila = *Ammophilion arenarii*; Crucianella = *Crucianellion maritimae*; Teucrium = *Halimionenion halimifolii*

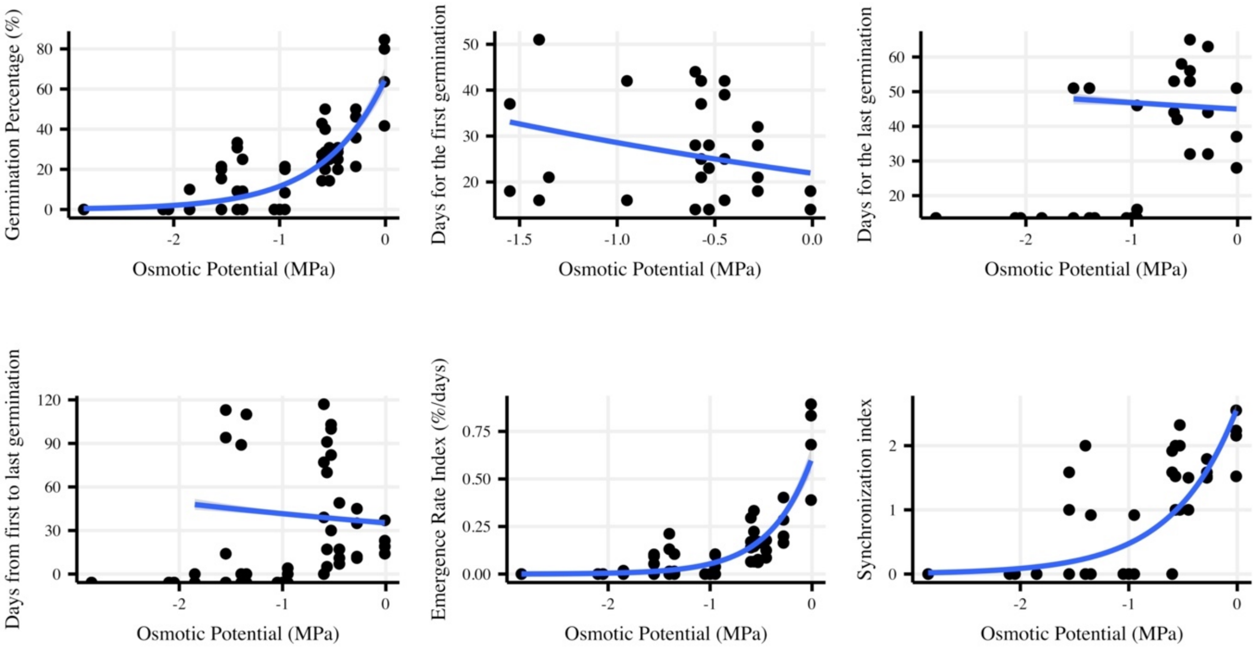

Figure S2. Observed and predicted data of the germination metrics of *Eryngium maritimum* seeds exposed to different salt concentrations ($MgCl_2$, $MgSO_4$, Na_2SO_4 and NaCl at concentrations ranging from 0 to 0.4 M) expressed as the osmotic potential (MPa) of each treatment. Blue lines represent the predicted curve. Models used for each variable and supplementary statistics can be found in Table 2 and Table S3.

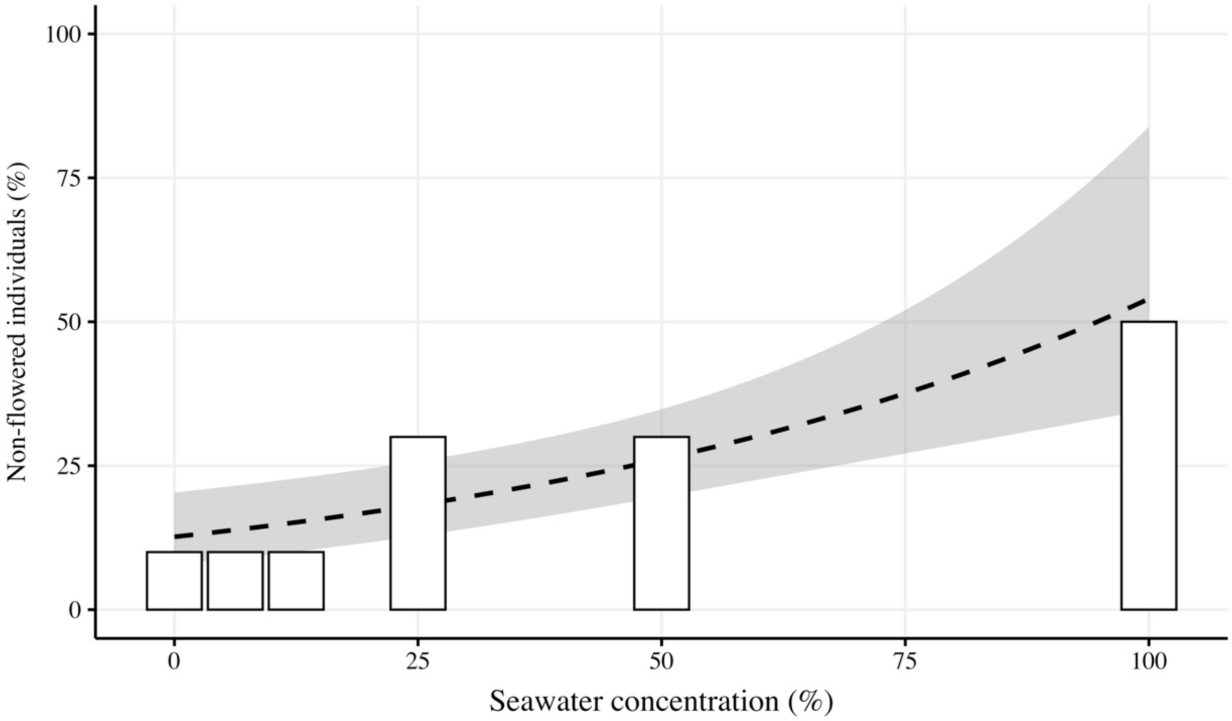

Figure S3. Percentage of non-flowered individuals per treatment are indicated with bars. Dashed line indicates the estimate of the GLM model, while shaded area represents the 25–75% confidence interval for each model.

Table S1. Correspondence between salt concentrations and g/L. Cl^- is the predominant ion in the Mediterranean seawater (22.1 g/L), followed by Na^+ (12.50 g/L), SO_4^{2-} (3.41 g/L), Mg^{2+} (1.45 g/L), Ca^{2+} (0.45 g/L) and K^+ (0.45 g/L) (Fritzmann et al., 2007).

Concentration (M)	**NaCl**	**$MgCl_2$**	**$MgSO_4$**	**Na_2SO_4**
0.1	5.84	9.52	12.04	14.20
0.2	11.69	19.04	24.07	28.41
0.3	17.53	28.56	36.11	42.61
0.4	23.38	38.08	48.15	56.82

Table S2. Results of the regression models of the salinity tolerance experiment considering salts and concentrations as the explanatory variables. Range of estimates are indicated in parentheses.

	Cumulative Germination	Final Germination Percentage	First Germination Day	Last Germination Day	Time Spread Germination	Germination Speed	Germination Uncertainty
Predictors	*Estimates*	*Estimates*	*Log-Mean*	*Log-Mean*	*Log-Mean*	*Log-Mean*	*Estimates*
(Intercept)	**25.07***	**-0.39*****	**2.71*****	**3.64*****	**3.15*****	**-0.36*****	**2.12*****
	(4.07-46.08)	**(-0.51 - -0.27)**	**(2.44 - 2.95)**	**(3.48 - 3.80)**	**(2.07 - 4.77)**	**(-0.40 - -0.32)**	**(1.60 - 2.63)**
MgCl2	-19.33	**-0.22**	**0.41***	**0.28***	0.72	**-0.60*****	**-0.69***
	(-41.65 - 2.99)	**(-0.47 - 0.02)**	**(0.08 - 0.74)**	**(0 - 0.55)**	(-1.27 - 2.57)	**(-0.70 - -0.50)**	**(-1.34 - 0.05)**
MgSO4	-13.49	**0.51*****	**0.43***	**0.36****	0.63	**-0.21*****	-0.05
	(-35.83 - 8.83)	**(0.30 - 0.72)**	**(0.09 - 0.77)**	**(0.11 - 0.61)**	(-1.50 - 2.61)	**(-0.13 - -0.29)**	(-0.69 - 0.59)
Na2SO4	-18.72	-0.24	**0.67*****	0.13	0.54	**-0.63*****	**-0.72***
	(-41.05 - 3.60)	(-0.48 - 0)	**(0.35 - 1.00)**	(-0.16 - 0.42)	(-1.49 - 2.57)	**(-0.73 - -0.53)**	**(-1.37 - -0.08)**
NaCl	-16.83	**0.30****	**0.61*****	0.24	-0.63	**-0.20*****	-0.60
	(-39.15 - 5.49)	**(0.08 - 0.51)**	**(0.28 - 0.96)**	(-0.02 - 0.49)	(-2.68 - 1.30)	**(-0.28 -- - 0.11)**	(-1.25 - 0.04)
s (Day)	**5.78*****						
s (Concentration)	**2.99*****						
Concentration		**-9.16*****	0.60	-0.40	1.34	**-10.71*****	**-4.19*****
		(-10.01 - - 8.34)	(-0.42 - 1.61)	(-1.48 - 0.65)	(-4.93 - 7.76)	**(-11.10 - - 10.32)**	**(-5.34 - -3.04)**
Observations	6052	68	32	21	36	68	68
R2	**0.66**	**0.59**	0.12	0.06	0.02	**0.76**	**0.60**
Model p-value	**<0.001*****	**<0.001*****	**<0.001*****	**0.045***	0.326	**<0.001*****	**<0.001*****
Model family	GAM	GLM - Poisson	GLM - Poisson	GLM - Poisson	GLM - Negative Binomial Poisson	GLM - Poisson	LM

***, p<0.0001; **, p<0.001; *, p<0.01

Table S3. Results of the regression models of the salinity tolerance experiment considering the osmotic potential as the explanatory variable. Range of estimates are indicated in parentheses.

	Number of Rotten seeds	Cumulative germination	Final Germination Percentage	First Germination Day	Last Germination Day	Time Spread Germination	Germination Speed	Germination Uncertainty
Predictors	*Estimates*	*Estimates*	*Log-Mean*	*Estimates*	*Log-Mean*	*Estimates*	*Log-Mean*	*Estimates*
(Intercept)	**8.64**** **(7.39-9.88)**	**8.98** **(8.85- 9.11)**	**4.18***** **(4.09 - 4.27)**	**21.51***** **(14.90 - 28.11)**	**44.93***** **(35.53 - 54.94)**	**34.97***** **(10.89 - 50.06)**	**-0.49***** **(-0.53 - - 0.48)**	**0.95***** **(0.94 - 0.97)**
Osmotic potential	0.32 (-0.58 - 1.21)		**-1.74***** **(-1.87 - - 1.62)**	7.27 (-1.14 - 15.69)	1.91 (-12.50 - 6.32)	6.77 (-20.62 - 34.15)	**-2.43***** **(-2.49 - - 2.37)**	**-1.70***** **(-1.72 - 1.68)**
Smooth term Day		**5.65***						
Smooth term Osmotic potential		**4.00**						
Observations	68	6052	68	68	21	36	68	68
R2	0.01	**0.64**	**0.55**	0.06	0.01	0.01	**0.72**	**0.55**
Model p-value	0.479	**<0.001*****	**<0.001*****	0.087	0.784	0.326	**<0.001*****	**<0.001*****
Model family	LM	GAM	GLM - Poisson	LM	LM	LM	GLM - Poisson	GLM - Poisson

Table S4. Number of capitula per salt concentration and per whorl

Seawater concentration (%)	*First*	*Second*	*Third*	*Fourth*
0	0.90 ± 0.10	2.70 ± 0.30	5.70 ± 0.67	6.30 ± 1.17
6.25	0.91 ± 0.09	2.64 ± 0.31	4.91 ± 0.61	2.45 ± 0.79
12.5	0.91 ± 0.09	2.73 ± 0.30	4.64 ± 0.64	1.18 ± 0.38
25	0.75 ± 0.13	2.25 ± 0.43	4.00 ± 1.02	0.25 ± 0.18
50	0.77 ± 0.12	2.23 ± 0.38	1.62 ± 0.56	0 ± 0
100	0.50 ± 0.17	1.60 ± 0.54	1.30 ± 0.76	0 ± 0

Table S5. First day of flowering, fruiting and death per salt treatment and whorl

First_day	*Seawater concentration (%)*	*First*	*Second*	*Third*	*Fourth*	*Fifth*
Dead	50	22.00 ± 0				
Dead	100	16.75 ± 1.15	16.75 ± 1.15	16.75 ± 1.15	16.75 ± 1.15	16.75 ± 1.15
Flowered	0	11.60 ± 4.7	13.80 ± 2.6	15.77 ± 1.85	11.70 ± 2.71	17.00 ± 5
Flowered	6.25	13.80 ± 2.6	14.00 ± 2.38	16.00 ± 1.98	15.44 ± 2.95	12.00 ± 0
Flowered	12.5	16.50 ± 4.11	14.50 ± 3.6	13.90 ± 3.4	19.75 ± 2.43	
Flowered	25	11.75 ± 4.57	11.82 ± 4.15	8.22 ± 4.05		
Flowered	50	3.44 ± 1.62	3.36 ± 1.25	2.10 ± 1.1		

Flowered	100	2.38 ± 1.37				
Fruited	0	18.07 ± 0.86	17.83 ± 0.95	17.14 ± 1.07	16.48 ± 1.16	22.00 ± 0
Fruited	6.25	24.82 ± 0.56	24.70 ± 0.61	24.14 ± 0.7	23.29 ± 0.93	22.00 ± 0
Fruited	12.5	20.65 ± 1.27	21.05 ± 1.41	20.26 ± 1.44	19.12 ± 1.81	
Fruited	25	17.88 ± 1.56	16.45 ± 1.46	16.89 ± 1.68	12.00 ± 0	
Fruited	50	18.15 ± 0.79	17.14 ± 0.83	19.5 ± 1.12		
Fruited	100	12.00 ± 0				

Table S6. Measurement of inflorescences per salt treatment (expressed as % of seawater)

Variable	*0%*	*6.25%*	*12.5%*	*25%*	*50%*
Full Length (cm)	48.64 ± 1.66	47.33 ± 2.51	39.54 ± 2.17	32.86 ± 3.7	27.40 ± 3.08
Inflorescence diameter (cm)	40.56 ± 3.15	33.00 ± 1.27	26.24 ± 1.01	23.43 ± 2.39	19.60 ± 2.48
Number of 2nd branches	6.11 ± 0.31	5.67 ± 0.65	5.25 ± 0.46	5.86 ± 0.46	3.80 ± 0.58
Number of 3rd branches	11.11 ± 0.7	8.44 ± 0.96	6.67 ± 0.88	7.43 ± 1.99	2.40 ± 1.36
Number of 4th branches	9.22 ± 1.66	4.11 ± 1.41	1.42 ± 0.38	0.43 ± 0.43	0 ± 0
Number of 5th branches	1.33 ± 0.87	0.11 ± 0.11	0 ± 0	0 ± 0	0 ± 0
P length (cm)	38.57 ± 1.51	38.67 ± 2.42	32.80 ± 2.04	26.29 ± 2.9	24.60 ± 3.44
Scapus Length (cm)	31.00 ± 1.55	32.44 ± 2.3	27.76 ± 1.92	21.29 ± 2.15	20.60 ± 3.09

Table S7. Measurement of capitula per salt treatment and whorl (expressed as % of seawater)

Variable	*Seawater concentration (%)*	*First*	*Second*	*Third*	*Fourth*	*Fifth*
Capitulum diameter without bracteas (mm)	0.00	29.06 ± 1.52	25.87 ± 0.6	19.18 ± 0.54	12.80 ± 0.36	10.08 ± 0.74
Capitulum diameter without bracteas (mm)	6.25	27.57 ± 0.71	23.92 ± 0.6	16.55 ± 0.61	11.27 ± 0.28	6.84 ± 0
Capitulum diameter without bracteas (mm)	12.50	26.59 ± 1.28	22.48 ± 0.55	15.28 ± 0.51	10.77 ± 0.47	
Capitulum diameter without bracteas (mm)	25.00	16.22 ± 3.72	18.40 ± 0.69	14.11 ± 0.6	13.45 ± 1.06	
Capitulum diameter without bracteas (mm)	50.00	10.48 ± 3.52	16.45 ± 0.56	12.41 ± 0.25		
Capitulum diameter without bracteas (mm)	100.00	0 ± 0				
Capitulum length (mm)	0.00	32.11 ± 2.05	28.29 ± 0.87	21.24 ± 0.48	15.06 ± 0.37	10.77 ± 0.6
Capitulum length (mm)	6.25	31.60 ± 0.7	26.70 ± 0.75	19.07 ± 0.39	13.99 ± 0.47	6.84 ± 0
Capitulum length (mm)	12.50	28.03 ± 1.33	24.48 ± 0.5	17.82 ± 0.41	12.37 ± 0.66	
Capitulum length (mm)	25.00	17.45 ± 3.94	21.50 ± 0.61	15.77 ± 0.62	13.37 ± 1.05	
Capitulum length (mm)	50.00	11.38 ± 3.85	18.14 ± 0.86	14.01 ± 0.37		
Capitulum length (mm)	100.00	0 ± 0				
Diameter-Length Relation	0.00	0.92 ± 0.05	0.95 ± 0.03	0.91 ± 0.02	0.86 ± 0.01	0.94 ± 0.05
Diameter-Length Relation	6.25	0.87 ± 0.02	0.92 ± 0.03	0.86 ± 0.02	0.82 ± 0.02	1
Diameter-Length Relation	12.50	0.95 ± 0.03	0.92 ± 0.01	0.86 ± 0.02	0.89 ± 0.04	
Diameter-Length Relation	25.00	0.94 ± 0.07	0.86 ± 0.02	0.92 ± 0.03	1.03 ± 0.17	
Diameter-Length Relation	50.00	0.93 ± 0.06	0.94 ± 0.06	0.89 ± 0.02		

Diameter-Length Relation	100.00					
Number of flowers	0.00	41.56 ± 1.55	41.35 ± 0.91	35.35 ± 0.55	28.68 ± 0.79	22.33 ± 1.29
Number of flowers	6.25	45.88 ± 2.09	42.36 ± 1.26	35.21 ± 0.7	28.30 ± 1.35	6
Number of flowers	12.50	39.42 ± 1.73	38.32 ± 0.82	31.66 ± 0.75	24.59 ± 1.84	
Number of flowers	25.00	29.30 ± 6.78	41.26 ± 1.37	32.56 ± 1.18	30.67 ± 2.91	
Number of flowers	50.00	19.00 ± 6.62	38.89 ± 1.95	32.92 ± 1.26		
Number of flowers	100.00	0				
Number of aborted flowers	0.00	8.89 ± 5.16	8.83 ± 1.74	24.15 ± 1.33	27.64 ± 0.86	22.33 ± 1.29
Number of aborted flowers	6.25	1.62 ± 0.5	9.28 ± 1.54	28.24 ± 1.3	28.30 ± 1.35	6
Number of aborted flowers	12.50	4.92 ± 2.8	12.60 ± 1.6	24.90 ± 1.05	24.41 ± 1.85	
Number of aborted flowers	25.00	18.00 ± 6.04	31.33 ± 1.97	26.35 ± 1.7	30.67 ± 2.91	
Number of aborted flowers	50.00	6.60 ± 3.44	24.79 ± 3.02	33.75 ± 1.41		
Number of aborted flowers	100.00	0				
FruitSet	0.00	0.80 ± 0.11	0.77 ± 0.04	0.30 ± 0.04	0.03 ± 0.02	0
FruitSet	6.25	0.96 ± 0.01	0.75 ± 0.04	0.19 ± 0.04	0	0
FruitSet	12.50	0.87 ± 0.08	0.65 ± 0.05	0.19 ± 0.03	0.01 ± 0.01	
FruitSet	25.00	0.42 ± 0.14	0.23 ± 0.04	0.16 ± 0.05	0	
FruitSet	50.00	0.62 ± 0.17	0.31 ± 0.08	0 ± 0		
FruitSet	100.00	0				
SeedSet	0.00	0.73 ± 0.1	0.71 ± 0.04	0.27 ± 0.03	0.03 ± 0.01	0
SeedSet	6.25	0.88 ± 0.02	0.67 ± 0.04	0.17 ± 0.03	0	0
SeedSet	12.50	0.75 ± 0.07	0.59 ± 0.04	0.17 ± 0.03	0.01 ± 0.01	
SeedSet	25.00	0.32 ± 0.11	0.19 ± 0.03	0.15 ± 0.04	0	
SeedSet	50.00	0.51 ± 0.14	0.26 ± 0.07	0		
SeedSet	100.00	0				

Table S8. Fruit measurements per whorl and salt treatment (0–25% seawater)

Variable	*Whorl*	*0%*	*6.25%*	*12.5%*	*25%*
Length	First	8.81 ± 0.26	8.46 ± 0.27	7.24 ± 0.2	7.60 ± 0.35
Length	Second	8.35 ± 0.26	7.76 ± 0.25	6.93 ± 0.18	6.54 ± 0.31
Width	First	6.12 ± 0.2	5.93 ± 0.25	5.34 ± 0.14	5.53 ± 0.26
Width	Second	5.88 ± 0.19	5.60 ± 0.21	4.91 ± 0.15	4.38 ± 0.33

Manuscript IV. The role of *E. maritimum* (L.) in the dune pollination network of the Balearic Islands

Table S1. Climatic data of SS and ET populations. Data collection has been restricted until the end of the fruiting season in final August of 2021. This data complements the analysis of Guijarro (1986), which suggested that southern dunes are colder than northern Mallorcan dunes. Data source: Wunderground (Code: ISANTA224) and Balearsmeteo (Club Nautico del Sa Rapita de Campos), respectively.

ES TRENC (ET)						
	Mean T (ºC)	Absolute min. T (ºC)	Mean min. T (ºC)	Absolute max. T (ºC)	Mean max. T (ºC)	Precipitation (mm)
January	10,8	-1,4	6,3	19,4	14,5	29
February	13,1	2,9	8,1	21,5	16,8	2
March	12,7	3,6	8	21,5	16,8	47,2
April	14,5	1,9	10,3	21,9	18,1	8,6
May	18,1	10,6	13,9	27,1	21,4	65,2
June	23,4	14,7	18,2	35	27,5	35
July	25,3	17,3	20,6	33,1	29	0
August	26,3	18,8	21,9	37,8	30,6	10

SON SERRA DE MARINA (SS)						
	Mean T (ºC)	Absolute min. T (ºC)	Mean min. T (ºC)	Absolute max. T (ºC)	Mean max. T (ºC)	Precipitation (mm)
January	10,4	-0,1	5,8	25,3	15,6	66,97
February	12,8	2,9	7,8	25,1	18,2	3,6
March	12,8	4,5	7,5	23,8	17,5	40,6
April	13,8	2	9,6	23,5	19	8,9
May	17,2	15	13,6	27,9	22,8	51,6
June	23,8	15	17,3	34	28,1	46,7
July	26	16,6	20,4	38,1	32,1	8,7
August	26,1	18	21,6	40,8	31,5	60,9

Table S2. List of the pollinator taxons observed per population. Ones and zeroes denote presence and absence of interactions. Endemic species are indicated with an asterisk *. In references, previous cites of species on GBIF [https://www.gbif.org , 1], Baldock et. al (2020) [2], Vives (1994) [3], Tolrà (2002)[4], Cobos [5], Gomila (1999) [6], Ebejer (2006) [7], Polinib Database [http://polinib.info/?lang=en, 8], Biodibal [https://biodibal.uib.cat/ca/, 9] and Bioatles [http://bioatles.caib.es, 10] are indicated.

Order	*Family*	*Genus*	*Species*	*ET*	*SS*	*References*
Coleoptera				1	1	
	Bruchidae			1	0	
		Bruchidius		1	0	
	Buprestidae			1	0	
		Acmaeodera		1	0	
			Acmaeodera cylindrica (Fabricius, 1775)	1	0	[1-5-8-9-10]
		Anthaxia		1	0	
			Anthaxia umbellatarum (Fabrizius, 1787)	1	0	[5]
	Coccinellidae			1	1	
		Coccinella		1	1	
			Coccinella septempunctata L. (1758)	1	1	[1-8-9-10]
		Exochomus		1	0	
	Mordellidae			1	0	
		Mordellistena		1	0	
	Oedemerinae			0	1	
		Oedemera		0	1	
			Oedemera flavipes (Fabricius, 1758)	0	1	[1-8-10]
	Pentatomidae			1	0	
		Graphosoma		1	0	
			Graphosoma lineatum (Müller, 1766)	1	0	[1-8-10]

	Scarabaeidae			0	1	
		Oxythyrea		0	1	
			Oxythyrea funesta (Poda, 1761)	0	1	[1-8-9-10]
		Tropinota		0	1	
			Tropinota squalida (Scopoli, 1783)	0	1	[1-8-9-10]
	Scraptiidae			1	0	
		Anaspis		1	0	
	Tenebrionidae			0	1	
		Pimelia		0	1	
			*Pimelia cribra** (Solier, 1836)	0	1	[1-6-9-10]
Diptera				1	1	
	Asilidae			1	0	
		Machimus		1	0	
	Bombyllidae			1	1	
		Exoprosopa		1	1	
			Exoprosopa italica (Rossi, 1794)	1	1	[1-4]
		Petrorossia		1	0	
			Petrorossia hespera (Rossi, 1790)	1	0	[1-4-8]
	Calliphoridae			1	1	
		Lucilia		1	0	
			Lucilia sericata (Meigen, 1826)	1	0	[1-4-8]
			Lucilia silvarum (Meigen, 1826)	1	0	[1-8-10]
		Stomorhina		1	1	

		Stomorhina lunata (Fabricius, 1805)	1	1	[1-4-8]
Cloropidae			1	0	
	Thaumatomyia		1	0	
		Thaumatomyia notata (Meigen, 1830)	1	0	[7-10]
Conopidae			0	1	
	Myopa		0	1	
		Myopa extricata Collin, 1960	0	1	4
Ephydriidae			1	0	
Muscidae			0	1	
Phoridae			1	0	
	Megaselia		1	0	
Pompilidae			1	1	
	Deuteragenia		0	1	
		Deuteragenia variegata (Linnaeus, 1758)	0	1	[1]
Sarcophagidae			1	1	
	Sarcophaga		1	1	
Syrphidae			1	1	
	Eristalinus		1	1	
		Eristalinus aeneus (Scopoli, 1763)	1	1	[1-4-8-10]
		Eristalinus sepulchralis (Linnaeus, 1758)	0	1	[1-4-8-10]
		Eristalinus taeniops (Wiedemann, 1818)	0	1	[4-8-10]
	Eristalis		1	1	
		Eristalis tenax (Linnaeus, 1758)	0	1	[1-4-8-10]

		Eumerus		0	1	
		Paragus		0	1	
			Paragus haemorrhous Meigen, 1822	0	1	[4-10]
		Sphaerophoria		1	1	
			Sphaerophoria rueppelli (Wiedemann, 1830)	0	1	[1-4-8-10]
			Sphaerophoria taeniata (Linnaeus, 1758)	1	0	[1-4]
		Syritta		1	0	
			Syritta pipiens (Linnaeus, 1758)	1	0	[1-4-8-10]
Hymenoptera				1	1	
	Andrenidae			1	1	
		Andrena		1	1	
			Andrena agilissima (Scopoli, 1770)	0	1	[1-2-8-10]
			Andrena flavipes Panzer, 1799	1	0	[1-2-8-10]
			Andrena morio Brullé, 1832	1	1	[1-2-8-10]
	Apidae			1	1	
		Amegilla		1	1	
			Amegilla albigena (Lepeletier, 1841)	1	0	[1-10]
			Amegilla quadrifasciata (de Villers, 1789)	0	1	[1-2-8-10]
		Anthophora		0	1	
		Apis		1	1	
			Apis mellifera Linnaeus, 1758	1	1	[1-2-8-9-10]

	Bombus		0	1	
		Bombus terrestris Linnaeus, 1758	0	1	[1-2-8-9-10]
	Ceratina		1	0	
		Ceratina cucurbitina (Rossi, 1792)	1	0	[1-2-8-10]
	Megachile		0	1	
	Melecta		0	1	
		Melecta luctuosa (Scopoli, 1770)	0	1	[2-9-10]
	Nomada		1	0	
		Nomada succincta Panzer, 1798	1	0	[1-2-8]
	Thyreus		0	1	
	Xylocopa		0	1	
		Xylocopa violacea (Linnaeus, 1758)	0	1	[1-2-8-9-10]
Crabronidae			1	1	
	Cerceris		1	0	
		Cerceris arenaria (Linnaeus, 1758)	1	0	[1-2-8-10]
		Cerceris sabulosa (Panzer, 1799)	1	0	[1-2-8-10]
	Oxybelus		1	0	
	Philanthus		1	1	
		Philanthus triangulum (Fabricius, 1775)	1	1	[1-2-8-10]
	Stizus		0	1	
		Stizus fasciatus	0	1	[1-2-10]

		(Fabricius, 1781)			
	Tachysphex		1	1	
Colletidae			1	1	
	Colletes		1	1	
		Colletes abeillei (Pérez, 1903)	1	1	[1-2-8-10]
		Colletes succinctus (Linnaeus, 1758)	1	0	[1-2-8-10]
	Hylaeus		1	1	
		Hylaeus annularis (Kirby, 1802)	0	1	[1-2-8-10]
		Hylaeus pictus (Smith, 1853)	1	1	[1-2-8-10]
		Hylaeus punctatus (Brullé, 1832)	1	0	[1-2-8-10]
		Hylaeus taeniolatus (Förster, 1871)	1	0	[1-2-8-10]
Halictidae			1	1	
	Ceylalictus		1	0	
		Ceylalictus variegatus (Olivier, 1789)	1	0	[1-2-8-10]
	Halictus		1	1	
		Halictus Fulvipes (Klug, 1817)	0	1	[1-2-8-10]
		Halictus scabiosae (Rossi, 1790)	1	1	[1-2-8-10]
	Lasioglossum		1	1	
		Lasioglossum albocinctum (Lucas, 1849)	0	1	[1-2-8-10]
		Lasioglossum nitidulum (Fabricius, 1804)	1	0	[1-2-8-10]
	Pseudapis		1	0	

		Pseudapis bispinosa (Brullé, 1832)	1	0	[2-8-10]
	Sphecodes		0	1	
		Sphecodes sf. ruficrus	0	1	
Leucospidae			0	1	
	Leucospis		0	1	
		Leucospis gigas (Fabricius, 1793)	0	1	-
Megachilidae			1	1	
	Anthidium		0	1	
		Anthidium florentinum (Fabricius, 1775)	0	1	[1-2-10]
	Heriades		1	0	
	Megachile		0	1	
		Megachile sicula (Rossi, 1792)	0	1	[1-2-8-10]
	Osmia		1	0	
		Osmia aurulenta (Panzer, 1799)	1	0	[1-2-8-10]
		Osmia latreillei (Spinola, 1806)	1	0	[1-2-10]
	Rhodanthidium		1	1	
		Rhodanthidium septemdentatum (Latreille, 1809)	1	1	[1-2-8-10]
		Rhodanthidium sticticum (Fabricius, 1787)	0	1	[1-2-8-10]
Pollistidae			1	1	

	Bembecinus		0	1	
		Bembecinus tridens (Fabricius, 1781)	0	1	[1-2-10]
	Polistes		1	1	
		Polistes dominula (Christ, 1791)	1	1	[1-2-8-9-10]
		Polistes gallicus (Linnaeus, 1767)	1	1	[1-2-8-9-10]
Pompilidae			1	1	
	Entomobora		1	1	
		Entomobora fuscipennis (Vander Linden, 1827)	1	0	[2-10]
Scoliidae			1	1	
	Dasyscolia		0	1	
		Dasyscolia ciliata (Fabricius, 1787)	0	1	[1-2-8-10]
	Megascolia		1	1	
		Megascolia bidens (Linnaeus, 1767)	1	1	[1-2-8-9-10]
		Megascolia maculata (Drury, 1773)	1	1	[1-2-8-9-10]
Sphecidae			0	1	
	Sphex		0	1	
		Sphex funerarius (Gussakovskij, 1934)	0	1	[1-2-8-10]
Syrphidae			1	1	
	Sphaerophoria		1	1	
Typhiidae			1	0	
	Meria		1	0	
		Meria tripunctata (Rossi, 1790)	1	0	[1-2-8-10]

	Vespidae			1	1	
		Ancistrocerus		0	1	
			Ancistrocerus kitcheneri (Dusmet, 1917)	0	1	[1-2-8-10]
		Eumenes		1	0	
			Eumenes coarctatus (Linnaeus, 1758)	1	0	[1-2-8-10]
Lepidoptera				1	1	
	Lycaenidae			1	1	
		Celastrina		0	1	
			Celastrina argiolus (Linnaeus, 1758)	0	1	[1-8-9-10]
		Polyommatus		1	1	
			Polyommatus celina (Austaut, 1879)	1	1	[1-8-9-10]
	Noctuidae			0	1	
		Autographa		0	1	
			Autographa gamma (Linnaeus, 1758)	0	1	[1-8-9-10]
		Odice		0	1	
			Odice blandula (Rambur, 1858)	0	1	10
	Nymphalidae			1	1	
		Vanessa		1	1	
			Vanessa atalanta (Linnaeus, 1758)	0	1	[1-8-9-10]
			Vanessa cardui (Linnaeus, 1758)	1	1	[1-8-9-10]
	Pieridae			1	1	
		Colias		1	1	
			Colias	1	1	[1-8-9-10]

		croceus (Fourcroy, 1785)			
	Gonepteryx		0	1	
		Gonepteryx cleopatra (Linnaeus, 1767)	0	1	[1-8-9-10]
	Pieris		1	1	
		Pieris rapae (Linnaeus, 1758)	1	1	[1-8-9-10]
	Pontia		1	0	
		Pontia daplidice (Linaneus, 1758)	1	0	[1-8-9-10]
Satyridae			0	1	
	Pararge		0	1	
		Pararge aegeria (Linnaeus, 1758)	0	1	[1-8-9-10]

Table S3. Pollinator species metrics of the different populations (Son Serra, Es Trenc) And both (All). *D*= Number of different plant species visited, *Spec*= Specificity. Selectiveness is calculated based on the *Specificity index* (highly selective, *Spec* > 0.75; selective, 0.75 > *Spec* > 0.5; opportunistic, 0.5 > *Spec* > 0.25; highly opportunistic, *Spec* < 0.25).

	Es Trenc		Son Serra		All		
Species	*D*	*Spec*	*D*	*Spec*	*D*	*Spec*	*Specificity*
Amegilla albigena	1	1			1	1	Highly selective
Amegilla quadrifasciata			3	0,53	3	0,53	Selective
Ancistrocerus kitcheneri			1	1	1	1	Highly selective
Andrena agilissima			1	1	1	1	Highly selective
Andrena flavipes	1	1			1	1	Highly selective
Andrena morio	1	1	5	0,37	5	0,38	Opportunistic
Anthaxia umbellatarum	1	1			1	1	Highly selective
Anthidium florentinum			1	1	1	1	Highly selective
Apis mellifera	3	0,47	10	0,18	10	0,19	Highly opportunistic
Autographa gamma			1	1	1	1	Highly selective
Bembecinus tridens			1	1	1	1	Highly selective

Bombus terrestris			5	0,37	5	0,38	Opportunistic
Celastrina argiolus			1	1	1	1	Highly selective
Ceratina cucurbitina	1	1			1	1	Highly selective
Cerceris arenaria	1	1			1	1	Highly selective
Cerceris sabulosa	1	1			1	1	Highly selective
Ceylalictus variegatus	1	1			1	1	Highly selective
Megachile sicula			1	1	1	1	Highly selective
Coccinella septempunctata	1	1	1	1	1	1	Highly selective
Colias croceus	1	1	1	1	1	1	Highly selective
Colletes abeillei	3	0,47	2	0,68	4	0,44	Opportunistic
Colletes succinctus	1	1			1	1	Highly selective
Dasyscolia ciliata			2	0,68	2	0,68	Selective
Dipogon variegatus			1	1	1	1	Highly selective
Entomobora fuscipennis	1	1			1	1	Highly selective
Eristalinus aeneus	1	1	4	0,44	4	0,44	Opportunistic
Eristalinus sepulchralis			2	0,68	2	0,68	Selective
Eristalis tenax			3	0,53	3	0,53	Selective
Eumenes coarctatus	1	1			1	1	Highly selective
Exoprosopa italica	1	1	2	0,68	3	0,53	Selective
Gonepteryx cleopatra			4	0,44	4	0,44	Opportunistic
Graphosoma lineatum	1	1			1	1	Highly selective
Halictus fulvipes			1	1	1	1	Highly selective
Halictus scabiosae	1	1	1	1	1	1	Highly selective
Hylaeus annularis			1	1	1	1	Highly selective
Hylaeus pictus	1	1	1	1	2	0,68	Selective
Lasioglossum albocinctum			2	0,68	2	0,68	Selective
Lasioglossum nitidulum	1	1			1	1	Highly selective
Leucospis gigas			1	1	1	1	Highly selective
Lucilia sericata	1	1			1	1	Highly selective
Megascolia bidens	3	0,47	2	0,68	3	0,53	Selective
Megascolia maculata	2	0,65	2	0,68	3	0,53	Selective
Melecta luctuosa			1	1	1	1	Highly selective
Meria tripunctata	1	1			1	1	Highly selective
Myopa extricata			1	1	1	1	Highly selective
Nomada succinta	1	1			1	1	Highly selective
Oedemera flavipes			1	1	1	1	Highly selective
Osmia aurulenta	1	1			1	1	Highly selective

Osmia latreillei	1	1			1	1	Highly selective
Oxythyrea funesta			5	0,37	5	0,38	Opportunistic
Paragus haemorrhous			1	1	1	1	Highly selective
Pararge aegeria			1	1	1	1	Highly selective
Philanthus triangulum	1	1	3	0,53	3	0,53	Selective
Pieris rapae	1	1	2	0,68	3	0,53	Selective
Pollistes dominula	1	1	2	0,68	2	0,68	Selective
Pollistes gallicus	1	1	2	0,72	3	0,57	Selective
Polyommatus celina	5	0,26	4	0,44	6	0,33	Opportunistic
Pontia daplidice	1	1			1	1	Highly selective
Pseudapis bispinosa	2	0,65			2	0,68	Selective
Sphaerophoria rueppellii			1	1	1	1	Highly selective
Sphaerophoria taeniata	1	1			1	1	Highly selective
Sphex funerarius			2	0,68	2	0,68	Selective
Stizus fasciatus			1	1	1	1	Highly selective
Stomorhina lunata			2	0,68	2	0,68	Selective
Syritta pipiens	1	1			1	1	Highly selective
Thaumatomyia notata	1	1			1	1	Highly selective
Tropinota squalida			1	1	1	1	Highly selective
Vanessa atalanta			1	1	1	1	Highly selective
Vanessa cardui	2	0,65	2	0,68	3	0,53	Selective
Xylocopa violacea			1	1	1	1	Highly selective

Table S4. Pollinator family metrics. *D*= Number of different plant species visited, *Spec*= Specificity. Selectiveness is calculated based on the *Specificity index* (highly selective, *Spec* > 0.75; selective, 0.75 > *Spec* > 0.5; opportunistic, 0.5 > *Spec* > 0.25; highly opportunistic, *Spec* < 0.25).

Family	*Number_species*	*Number_plants*	*Spec*	*Specificity*
Andrenidae	4	5	0.38	Opportunistic
Apidae	9	11	0.17	Highly opportunistic
Bombyllidae	2	3	0.54	Selective
Bruchidae	1	1	1.00	Highly selective
Buprestidae	2	1	1.00	Highly selective
Calliphoridae	3	2	0.68	Selective
Cloropidae	1	1	1.00	Highly selective

Coccinellidae	2	1	1.00	Highly selective
Colletidae	7	4	0.45	Opportunistic
Conopidae	2	3	0.54	Selective
Crabronidae	5	3	0.54	Selective
Ephydriidae	1	1	1.00	Highly selective
Halictidae	8	7	0.29	Opportunistic
Leucospidae	1	1	1.00	Highly selective
Lycaenidae	2	6	0.33	Opportunistic
Megachilidae	7	2	0.68	Selective
Mordellidae	1	1	1.00	Highly selective
Muscidae	1	1	1.00	Highly selective
Noctuidae	3	1	1.00	Highly selective
Nymphalidae	2	3	0.54	Selective
Oedemerinae	1	1	1.00	Highly selective
Pentatomidae	1	1	1.00	Highly selective
Phoridae	1	1	1.00	Highly selective
Pieridae	4	8	0.26	Opportunistic
Polistidae	4	3	0.54	Selective
Polistinae	3	1	1.00	Highly selective
Pollistidae	1	1	1.00	Highly selective
Pompilidae	3	1	1.00	Highly selective
Sarcophagidae	1	1	1.00	Highly selective
Satyridae	1	1	1.00	Highly selective
Scarabaeidae	2	6	0.33	Opportunistic
Scoliidae	4	3	0.54	Selective
Scraptiidae	1	1	1.00	Highly selective
Sphecidae	1	2	0.68	Selective

Syrphidae	9	6	0.33	Opportunistic
Typhiidae	1	1	1.00	Highly selective
Vespidae	3	1	1.00	Highly selective

Table S5. Pollinator order metrics of the different populations. *D*= Number of different plant species visited, *Spec*= Specificity. Selectiveness is calculated based on the *Specificity index* (highly selective, *Spec* > 0.75; selective, 0.75 > *Spec* > 0.5; opportunistic, 0.5 > *Spec* > 0.25; highly opportunistic, *Spec* < 0.25).

Orden	*Number of species*	*Number of plants*	*Spec*	*Specific*
Coleoptera	9	6	0.33	Opportunistic
Diptera	17	9	0.23	Highly opportunistic
Hymenoptera	47	15	0.07	Highly opportunistic
Lepidoptera	12	9	0.23	Highly opportunistic

Table S6. Pollinator behaviour results of the focal census on *E. maritimum* individuals. Time spent in visits and number of visited capitula are indicated. For some species only one observation is available.

Order	***Family***	***Genus***	***Species***	***Time (s)***	***Capitula***
Coleoptera					2
Diptera				67 ± 2.35	1
	Calliphoridae			2.5 ± 0.5	1
Diptera	*Syrphidae*			3 ± 0	4
Hymenoptera				81.45 ± 21.41	3 ± 0.48
	Apidae	*Amegilla*	*Amegilla quadrifasciata*	5.5 ± 0.5	5
		Bombus	*Bombus terrestris*	36.78 ± 14.62	5.67 ± 2.4
		Pseudapis	*Pseudapis bispinosa*	30	2
	Crabronidae			74	3
		Cerceris		14	1
			Cerceris arenaria	12	1

		Philanthus	*Philanthus triangulum*	117 ± 23	
	Colletidae			10 ± 4	1.5 ± 0.5
		Colletes	*Colletes succintus*	14	2
	Halictidae			14	1
		Ceratina	*Ceratina cucurbitina*	20 ± 14	1
		Halictus	*Halictus scabiosae*	28.4 ± 7.54	1.2 ± 0.2
	Polistidae			13	1
		Polistes	*Pollistes dominula*	16.67 ± 7.69	1
			Pollistes gallicus	11	1
Hymenoptera	*Pompilidae*	*Entomobora*	*Entomobora fuscipennis*	10	1
	Scoliidae			197 ± 14	6
		Dasyscolia	*Dasyscolia ciliata*	65	4
		Megascolia	*Megascolia bidens*	32.67 ± 9.4	2.33 ± 0.88
			Megascolia maculata	32.75 ± 9.7	1.67 ± 0.67
	Sphecidae	*Sphex*	*Sphex funerarius*	20	3
Lepidoptera					
	Lycaenidae	*Polyommatus*	*Polyommatus celina*	281	1
	Nymphalidae	*Vanessa*	*Vanessa cardui*	79	

SUPPLEMENTARY FIGURES

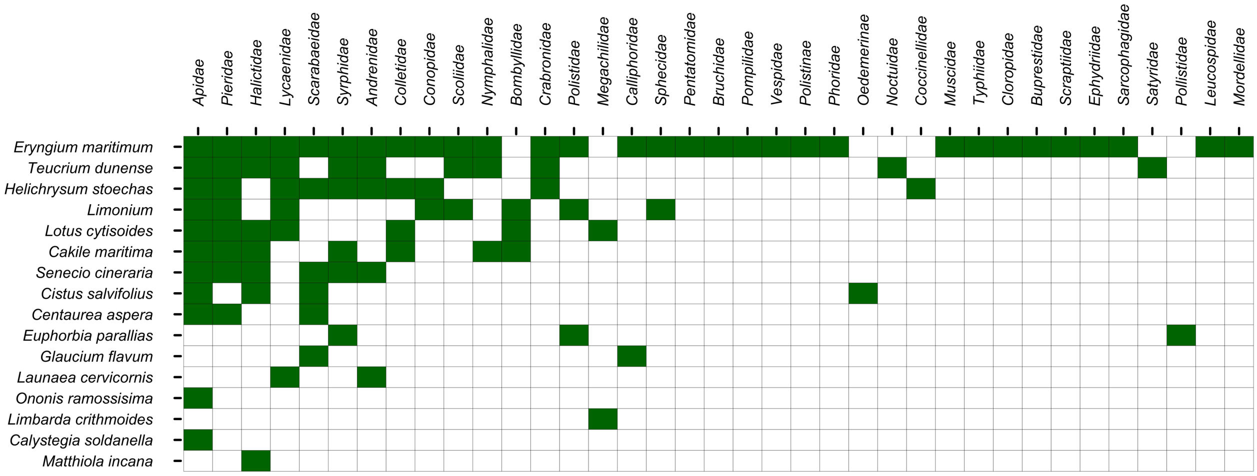

Figure S1. Matrix of pollinators families per plant species. Pollinators are ordered by diversity of interactions (grade of selectiveness) from left to right. The colour gradient indicates de diversity of taxons per interaction.

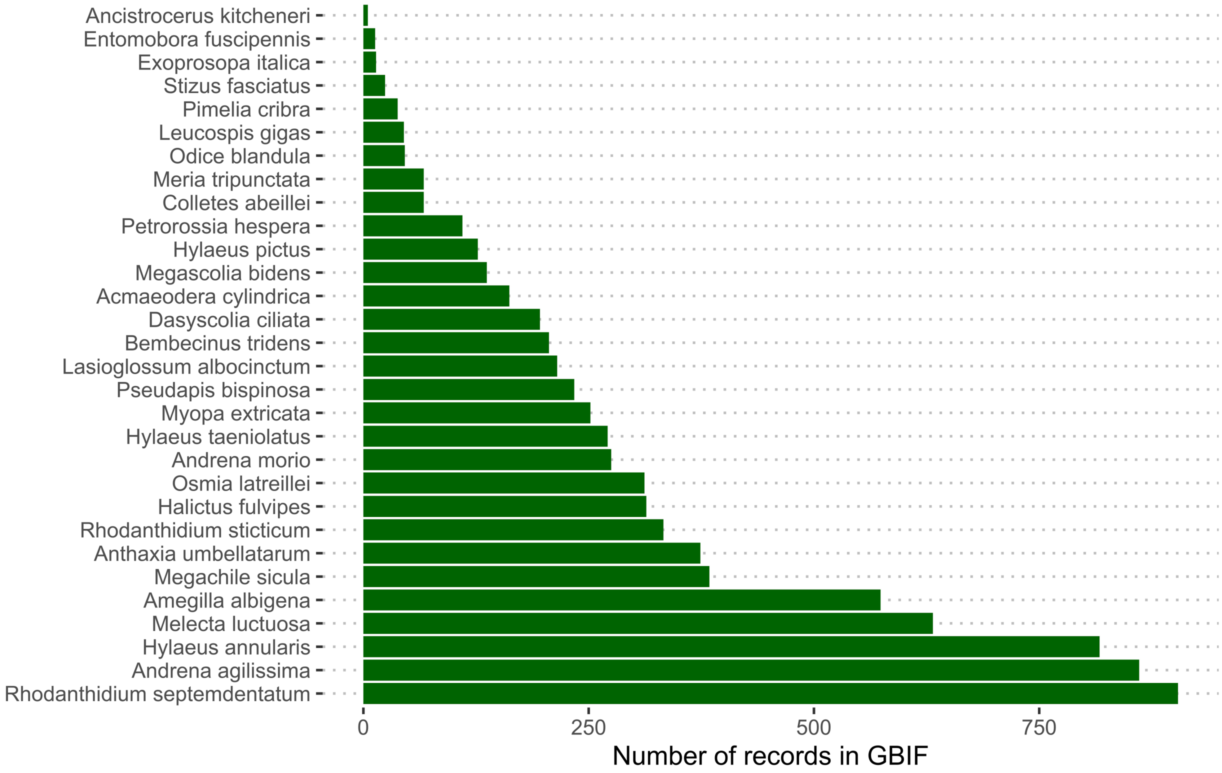

Figure S2. Pollinator species detected in the study with less occurrences in GBIF.

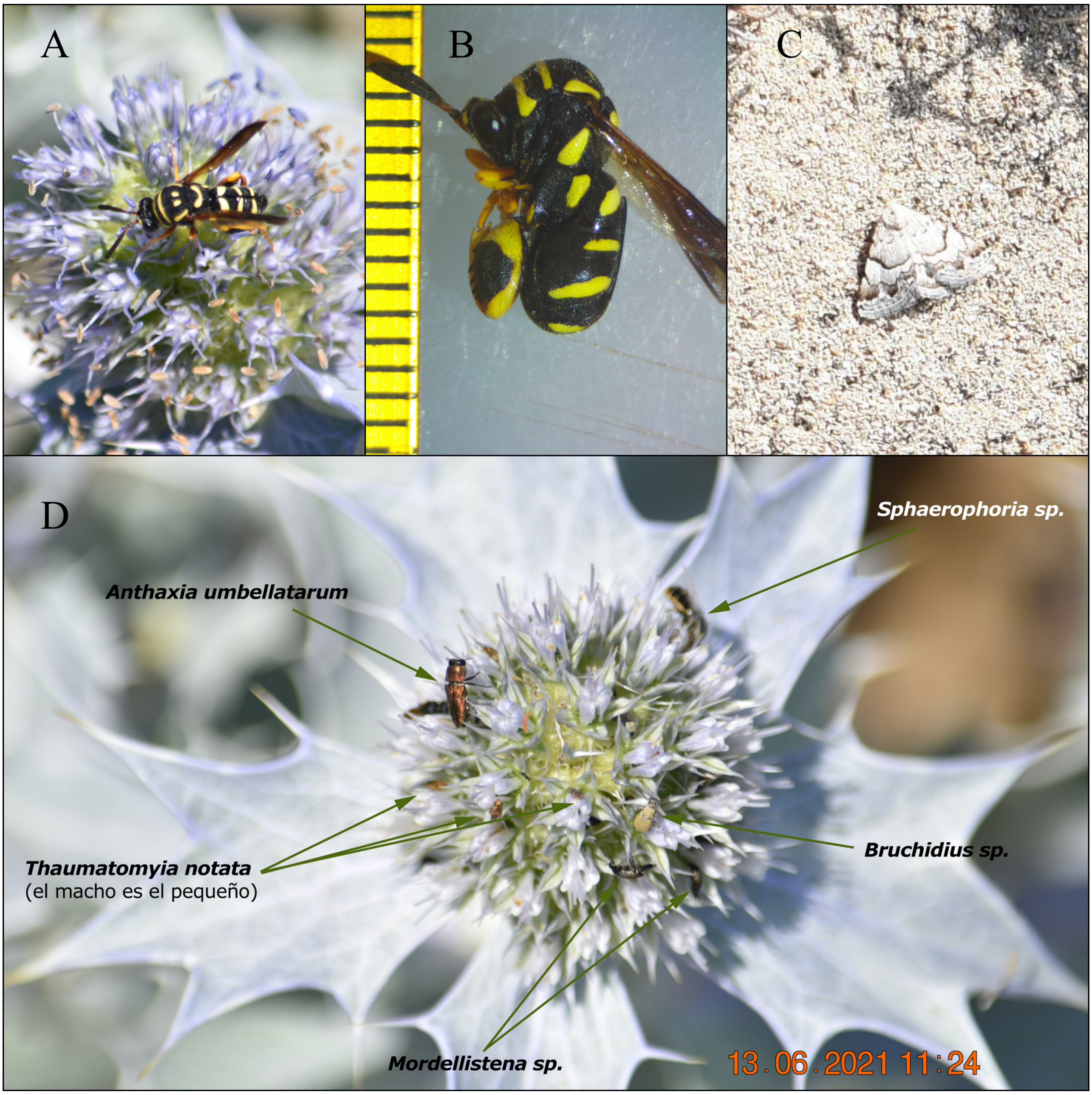

Figure S3. *Leucopsis gigas* pollinating *Eryngium maritimum* (A) and body detail (B). Individual of *Odice blandula* laying in the ground (C). Photography of multiple pollinator species on *E. maritimum*. Date, hour and species are indicated (D).

ANEXO FOTOGRÁFICO

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Capítulo 1. Biología reproductiva y germinación de *Eryngium maritimum* L.

Fotografía 1. Inflorescencia de *E. maritimum*. Se puede observar cómo está compuesta por un capítulo de mayor tamaño central bajo el cual surgen los diferentes verticilos.

Fotografía 2. Mericarpos de *Eryngium maritimum* utilizados para el conteo de frutos y semillas por verticilo.

Fotografía 3. Uso recreativo del espacio donde habita *Eryngium maritimum* en la población de Son Serra de Marina. Los paseos a caballo por las dunas son habituales en el Norte de la Isla de Mallorca.

Fotografía 4. Detalle de las hojas densamente espinescentes de *E. maritimum*.

ANEXO FOTOGRÁFICO

Capítulo 2. Tolerancia a la sal de *Eryngium maritimum* L.

Fotografía 9. Muestras cultivadas de *E. maritimum* para su desarrollo y evaluación de la producción.

Fotografía 10. Toma de muestras de hojas de *E. maritimum*.

Fotografía 11.. Área de distribución óptima de *E. maritimum* en Es Trenc, reducida al espacio restante entre la zona turística y el sabinar trasero, delimitado con barreras físicas de protección.

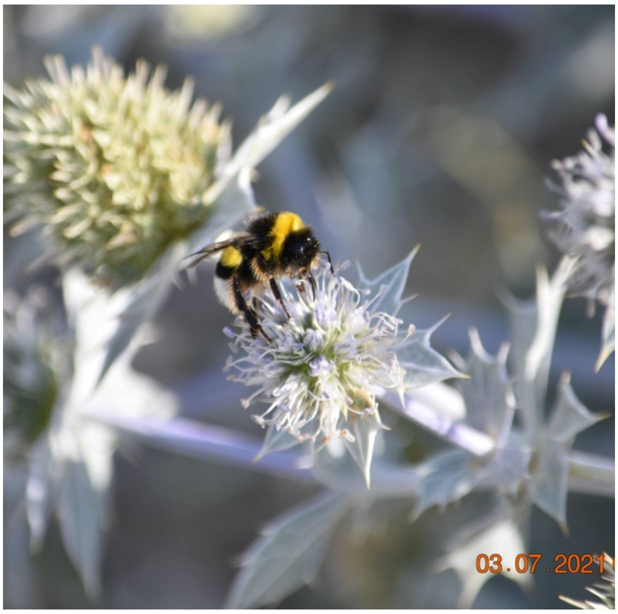
Fotografía 12. Individuo de *E. maritimum* con restos de basura de los usuarios de la playa. Toallitas, papeles, bolsas de basura y cigarrillos se encuentran muy frecuentemente en su zona de ocupación.

ANEXO FOTOGRÁFICO

Capítulo 3. Papel de Eryngium maritimum en la red de polinización dunar

Fotografía 17. Pareja de *Polyommatus celina* observada durante los muestreos.

Fotografía 18. Ejemplar de *Apis mellifera* capturado para el análisis del polen de los individuos.

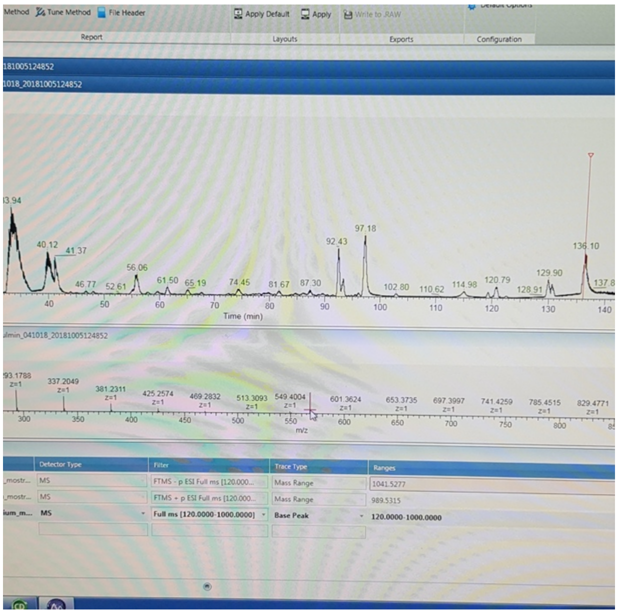


Fotografía 19. Ejemplar de *Bombus terrestris* sobre *Eryngium maritimum.*

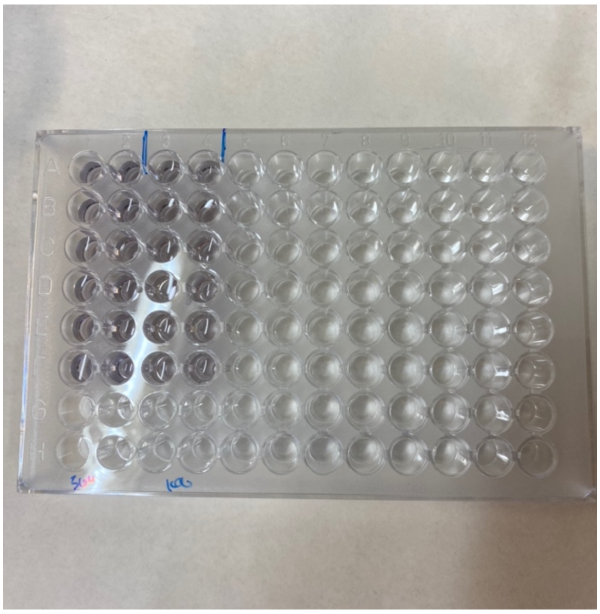
Fotografía 20. Muestreo preliminar de polinizadores nocturnos en la duna de Son Serra.

ANEXO FOTOGRÁFICO

Capítulo 4. Fitoquímica y citotoxicidad de *Eryngium maritimum* L.



Fotografía 13. Fotografía de los cromatogramas obtenidos mediante HPLC de los extractos de *E. maritimum.*



Fotografía 14. Placas multipocillo para la realización de la prueba de capacidad antioxidante mediente FRAP (Ferric Reducing Atnioxidant Power Assay.

Fotografía 15. HPLC empleado para la puesta a punto de los ensayos cromatográficos necesarios para la determinación de la composición de los extractos.

Fotografía 16. Extracción de los compuestos acuosos mediante agitación controlada en baño maría.