Universidad de Huelva

Departamento de Ciencias Integradas

Biología de la reproducción de las especies de *Spartina* presentes en las marismas del Golfo de Cádiz

Memoria para optar al grado de doctora
presentada por:

María Dolores Infante Izquierdo

Fecha de lectura: 21 de febrero de 2020

Bajo la dirección de los doctores:

Jesús Manuel Castillo Segura

Adolfo Francisco Muñoz Rodríguez

Francisco Javier Jiménez Nieva

Huelva, 2020

Universidad de Huelva

Biología de la reproducción de las especies de *Spartina* presentes en las marismas del Golfo de Cádiz

Reproductive biology of *Spartina* species present in saltmarshes of the Gulf of Cádiz

María Dolores Infante Izquierdo
2020

Memoria de tesis doctoral:

Biología de la reproducción de las especies de *Spartina* presentes en las marismas del Golfo de Cádiz

Presentada para optar al grado de Doctora por
María Dolores Infante Izquierdo

Directores:
Dr. Jesús Manuel Castillo Segura
Dr. Adolfo Francisco Muñoz Rodríguez
Dr. Francisco Javier Jiménez Nieva

2020

Programa de Doctorado
"Ciencia y Tecnología Industrial y Ambiental"
Departamento de Ciencias Integradas

Ph. D. Thesis:

Reproductive biology of *Spartina* species present in saltmarshes of the Gulf of Cádiz

Submitted to apply for the degree of PhD by:
María Dolores Infante Izquierdo

Supervisors:
Dr. Jesús Manuel Castillo Segura
Dr. Adolfo Francisco Muñoz Rodríguez
Dr. Francisco Javier Jiménez Nieva

2020

Doctoral Program
“Ciencia y Tecnología Industrial y Ambiental”
Department of “Ciencias Integradas”

A mis padres.
A Mariana Labrado Márquez.

AGRADECIMIENTOS

Agradecer en primer lugar a los directores de esta Tesis: Adolfo Muñoz, Jesús Castillo y Javier Jiménez, por su apoyo y su laborioso esfuerzo sin los cuales su realización no habría sido posible.

A las personas que han colaborado en la elaboración de los trabajos de la tesis. A Enrique Sánchez por llevarnos y ayudarnos en tantas ocasiones a realizar los muestreos y por enseñarnos cada rincón de las marismas, a Ruth Romero, por su gran aportación a uno de los capítulos de esta tesis, y a mis compañeros y amigos de laboratorio: Alejandro, Jesús, Israel, por su trabajo, por su ayuda, por su ánimo, por compartir tantos momentos de muestreos en las marismas y tantos experimentos de laboratorio, por ser un equipo y ayudarnos en todo.

Al Paraje Natural Marismas del Odiel y a todo su personal, por autorizarnos y facilitarnos trabajar allí.

A Malika Ainouche, que tan amablemente me acogió durante mi estancia en la Universidad de Rennes 1, y a todas las personas de las que aprendí cada día que estuve allí, Olivier, Virginie, Oscar, Abdelkader, Armel, Rosa, Julien, Delphine, que hicieron de mi estancia una experiencia muy enriquecedora.

A Brenda Grewell por su revisiones e interesantes aportaciones a los capítulos de esta tesis.

A Javi Macías y Lola Muñoz por su considerable ayuda proporcionándonos el apoyo técnico necesario para nuestra investigación.

A los alumnos que han pasado por el laboratorio y han colaborado de alguna manera con este trabajo.

Quiero agradecer por supuesto a toda mi familia, especialmente a mis padres, el apoyo incondicional y constante ánimo recibido durante estos cuatro años. A mi director y amigo Adolfo, por compartir tantos momentos de agobios, risas, consejos, por estar siempre disponible y por todo lo que he aprendido y sigo aprendiendo a su lado.

A mis amigos por su apoyo y su comprensión.

Y en general a todas aquellas personas que con su inestimable aportación han permitido el desarrollo y la finalización de esta tesis doctoral.

ÍNDICE

Abstract

Spartina is a genus of perennial grasses (cordgrasses) that mostly inhabit salt marshes around the world. Many *Spartina* species have successfully invaded areas beyond their native range, and frequently form hybrids between native and invasive species.

Tidal salt marshes are one of the most productive ecosystems in the world and they provide many ecosystem services. Salt marsh biodiversity is in risk due to the effects of biological invasions and the formation of new hybrids taxa. In this context, the main objective of present work was to study the reproductive biology of the *Spartina* taxa present in the salt marshes of the Gulf of Cádiz (Southwest Iberian Peninsula): native *Spartina maritima* (Curtis) Fernald., invasive *Spartina densiflora* Brongn., invasive *Spartina patens* (Aiton) Muhl., and the reciprocal transgressive hybrids between *S. densiflora* and S. maritima: *Spartina* × *onubensis* Sánchez Gullón, M.D. Infante & B. Gallego subsp. *onubensis* and *Spartina* × *onubensis* subsp. *tartessiana* Sánchez Gullón, B. Gallego & M.D. Infante.

- *S. maritima* is the only native cordgrass in European and African Atlantic Coasts and grows in low marsh areas forming tussocks that promote sedimentation and facilitate ecological succession development.

- *Spartina densiflora* is native from the Atlantic Coast of South America and it has invaded salt marshes in North America, Europe and Africa. It shows a high tolerance to contrasted environmental conditions and high phenotypic plasticity, which allows it to colonize different habitats along the intertidal gradient.

- *Spartina patens* (=*S. versicolor* Fabre) is native from the Atlantic Coast of North America, Gulf of Mexico, and Central American and Caribbean Coasts. It was introduced in Atlantic and Mediterranean European and African marshes, and it is distributed along the Mediterranean, Atlantic and Cantabrian Coasts in the Iberian Peninsula.

- In marshes of the Southwest Iberian Peninsula, invasive *S. densiflora* has reciprocally hybridized with native *S. maritima* forming the transgressive hybrids *S.* × *onubensis* subsp. *onubensis*, with *S. densiflora* as maternal species, and *S.* × *onubensis* subsp. *tartessiana*, with *S. maritima* as maternal species. Both F1 hybrids subspecies are sterile but, nevertheless, represent a potential threat to native biodiversity since their invasion could accelerate if they acquire the capacity to produce viable caryopsis through allopolyploidization.

Present PhD dissertation includes seven studies about these taxa in salt marshes along the Gulf of Cádiz:

Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae)

Interspecific hybridization is a major mechanism for generating evolutionary novelty in plants. Despite a large body of knowledge on the ecology and evolution of *Spartina* species, taxonomic resources for distinction of complex taxa in the genus are scarce. The general aim of this study was to characterize parental species *S. maritima* (native) and *S. densiflora* (invasive) and their reciprocal hybrids formed in the Gulf of Cadiz (Southwest Iberian Peninsula). Our approach was to quantify and compare morphological and anatomical characters of tufts, culms, leaves and inflorescences and the limits of their variation in different ecological settings. Our results document discriminating trait variables from populations of both hybrids that can be used to distinguish them from their parental species and each other. From these findings and the verification of type specimens, we describe two new nothotaxa, *Spartina* × *onubensis* subsp. *onubensis* and *Spartina* × *onubensis* subsp. *tartessiana*, and develop taxonomic keys for identification of the four studied taxa. Floral traits such as the length of the terminal spike, the spikelet and its glumes and lemma allow us to differentiate *S. densiflora* (with lower values) from the other three taxa. The presence of exerted stamens, shorter culms and shorter leaf blades separated *S. maritima* from both hybrids. Each hybrid can also be distinguished since *S.* × *onubensis* subsp. *onubensis* develops more spikes per inflorescence and consistently has longer leaves than *S.* × *onubensis* subsp. *tartessiana*.

Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula

Many aspects of *Spartina maritima* reproductive biology are unknown. Viable seed production has been described as potentially very low to non-existent. Our primary objective was to evaluate the seed viability of *S. maritima*. We hypothesized that *S. maritima* was capable of producing viable seeds since we observed many new patches of this cordgrass. We analyzed 400 inflorescences in fruiting state from 20 different tussocks in three estuaries in the Southwest Iberian Peninsula and carried out a germination experiment. *Spartina maritima* was able to set moderate numbers of highly viable caryopses. Mature caryopses with embryos were recorded in 12.5 ± 2.9% of total spikelets, with high variation ranging from 0 to 45% among tussocks. Caryopses showed a high level of viability (89.0 ± 4.9%) and final germination fraction (85.3 ± 2.9%). The percentage of spikelets with all their stamens exerted was 38.3 ± 4.5%, being positively correlated with the percentage of mature caryopses with embryo, indicating that tussocks in which inflorescences reached a more advanced state of flowering when fruit set started would ultimately produce more fruits.

Variation in sexual reproductive capacity among exotic *Spartina* taxa

Most plant species with both sexual and asexual reproductive modes reach a balance between them. Sexual reproduction can enhance the colonization and spread of invasive plants, including the successful multi-continent invasion of coastal marshes by cordgrasses from the *Spartina* genus. We studied a broad spectrum of sexual reproductive traits in exotic *Spartina* taxa from salt marshes by the Gulf of Cádiz. Density of inflorescences, spikelets per inflorescence, percentage of spikelets with caryopses, and density of caryopses were recorded for *S. patens*, *S. densiflora*, and reciprocal hybrids of invasive *S. densiflora* with native *S. maritima* (*S.* ×*onubensis* subsp. *onubensis* and *S.* ×*onubensis* subsp. *tartessiana*). For *S. patens* and hybrids we also assessed pollen viability, and the percentages of reabsorbed caryopses and spikelets with all stamens exerted. For fertile *S. densiflora*, we evaluated plant sexual traits and characteristics of the sedimentary environment, and conducted germination experiments from discrete habitats long the intertidal gradient (low, middle, high and terrestrialized marshes, and saltpans). We found interannual variation for reproductive traits of *S. densiflora* which also differed among habitats. Plants with highest inflorescence and caryopsis densities occupied low marshes and saltpans, lowest caryopsis production was in low marsh where abiotic stress from frequent inundation was high, while viability of caryopses was highest in middle and high marshes. Our results indicate neither hybrid produced any mature caryopses, due to male sterility. However, male fitness did not explain infecundity of *S. patens* at our study sites, where ca. 80 % of spikelets had all stamens exerted and pollen viability was ca. 60 %. To reduce invasive spread and impact, conservation efforts to protect biodiversity should prioritize rapid eradication of *Spartina* hybrids before they attain sexual reproductive capacity, and removal of *S. densiflora* within two years of establishment before it produces viable caryopses.

Is dichogamy responsible of differences in sexual reproduction success between native *S. maritima* and invasive *S. densiflora*?

Spartina maritima and *Spartina densiflora* share similar reproductive traits such as spikelet characteristics, wind pollination and protogyny at flower and inflorescence levels. However, the causes of their different reproductive success are unknown, in spite of the interest in conserving *S. maritima* and in controlling *S. densiflora* invasion. This study aims to address this knowledge gap by investigating the reproductive biology in natural populations of both *Spartina* species in tidal salt marshes of Southwest Iberian Peninsula: inflorescences development patterns and degree of overlap between sexual phases, production, viability, liberation, loss of germinability and dispersal of pollen, development and pollination of stigmas, and self-compatibility. In both species, stamens and stigmas were exerted from the spikelet early in the morning, and stigmas were receptive for the first two days. Their pollen liberation and dispersal rates were extremely low, and pollen lost its germinability is a few hours, which determined low rates of germinated pollen on stigmas. Both species presented protogyny. *S. maritima* showed only 28% of inflorescences overlapping sexual phases, while in *S. densiflora* this percentage was up to 87%. Both species were self-compatible. *S. maritima* fruit-set was associated positively with sexes overlap in its inflorescence. The lower rates of fruit set that presents *S. maritima* in comparison with *S. densiflora* are explained by some disadvantages in its sexual breeding system such as its low rates of pollen dispersal that reduced the chance of crossing, and its low degree of overlap between the two sexual phases in a inflorescence that reduced the chances of geitonogamy. Environmental conditions during flowering may also influence *S. maritima* low fruit set in relation to high temperature and humidity, and tidal immersion that would increase pollen germinability loss, incomplete opening of anthers, and a levels of spikelets with non-exerted stamens.

Seed viability, spikelet dispersal, seed banks, and seed storage requirements for native and invasive *Spartina* cordgrasses across salt marsh intertidal gradients

Seed banks play an important role in determining the spatial and temporal distribution of halophytes in salt marshes. We tested the ability of native *Spartina maritima* and invasive *S. densiflora* spikelets to disperse by flotation on water with different salinity concentrations, and the longevity of spikelets relative to environmental conditions including dry or wet storage at low or moderate temperatures, and aqueous salinity concentrations from fresh to sea water. We quantified pre-dispersal seed production and pre- and post-dispersal focal *Spartina* spp. presence in seed banks along intertidal gradients within salt marshes at the Gulf of Cádiz (Southwest Iberian Peninsula). *S. densiflora* spikelets, especially from middle and high elevation marshes, showed greater ability than *S. maritima* to remain afloat, which suggests this species could be dispersed over longer distances supporting possible colonization of new areas. Wet-stored *Spartina* seeds were able to maintain viability for months, while seeds under dry storage rapidly lost viability. This decline was most significant for *S. maritima*, and for seeds stored at moderate temperatures. Storage of spikelets under wet and cold conditions optimized spikelet viability. Native *S. maritima* did not establish transient or persistent seed banks, while invasive *S. densiflora* established transient seed banks mainly at higher marsh elevations. Our results on the dynamics of seed dispersal and seed banks and seedling recruitment provide fundamental knowledge that can be applied for conservation of native *S. maritima,* management of invasive *S. densiflor*a, and ecological restoration of tidal salt marshes.

Differential effects of increasing salinity on germination and seedling growth of native and exotic invasive cordgrasses

Soil salinity is a key environmental factor influencing germination and seedling establishment in salt marshes. Global warming and sea level rise are changing estuarine salinity, and may modify the colonization ability of halophytes. We evaluated the effects of increasing salinity on germination and seedling growth of native *Spartina maritima* and invasive *S. densiflora* from wetlands of the Odiel-Tinto Estuary. Responses were assessed following salinity exposure from fresh water to hypersaline conditions and germination recovery of non-germinated seeds when transferred to fresh water. The germination of both species was inhibited and delayed at high salinities, while pre-exposure to salinity accelerated the speed of germination in recovery assays compared to non-pre-exposed seeds. *S. densiflora* was more tolerant of salinity at germination than *S. maritima*. *S. densiflora* was able to germinate at hypersalinity and its germination percentage decreased at higher salinities compared to *S. maritima*. In contrast, *S. maritima* showed higher salinity tolerance in relation to seedling growth. Contrasting results were observed with differences in the tidal elevation of populations. Our results suggest *S. maritima* is a specialist species with respect to salinity, while *S. densiflora* is a generalist capable of germination of growth under suboptimal conditions. Invasive *S. densiflora* has greater capacity than native *S. maritima* to establish from seed with continued climate change and sea level rise.

Effects of metals on germination and early seedling growth in native *Spartina maritima* and invasive *S. densiflora*

Seed germination and seedling establishment are the most sensitive stages to abiotic stress in the plant cycle. Metal exposition of seeds can affect germination and seedling growth. We analyzed the effects of Cu, Zn and Ni on seed germination and early seedling growth of native *Spartina maritima* and invasive *S. densiflora* from tidal marshes in the Southwest Iberian Peninsula. In addition, we analyzed metals effects on *S. densiflora* seeds coming from polluted and non-polluted estuaries. Germination percentage of both *Spartina* species was not affected by any metal at any tested concentration (from 0 to 2000 μM). However, *S. maritima* seedlings were more tolerant to metals than *S. densiflora*, since the increase in Cu, Zn and Ni had negative effects on the early seedling growth of *S. densiflora*, mainly on the radicle length, while *S. maritima* seedlings were not affected. Comparing our results to metal concentrations in the field, we expect invasive *S. densiflora* seedling development may be impacted in most metal-polluted areas in Odiel and Tinto Estuaries. *S. densiflora* seedlings coming from seeds from Tinto Estuary showed higher tolerance to metals than those from Odiel and Piedras Estuaries.

Resumen

Spartina es un género de gramíneas perennes que habita principalmente las marismas de todo el mundo. Muchas especies de *Spartina* han invadido con éxito zonas alejadas de su distribución nativa, formando con frecuencia híbridos entre las especies nativas e invasoras.

Las marismas mareales son uno de los ecosistemas más productivos del mundo y proporcionan muchos servicios ecosistémicos. La biodiversidad de las marismas está en riesgo debido a los efectos de las invasiones biológicas y la formación de nuevos taxones híbridos. En este contexto, el objetivo principal de esta tesis es estudiar la biología reproductiva de los taxones de *Spartina* presentes en las marismas del Golfo de Cádiz (Suroeste de la Península Ibérica): la especie nativa *Spartina maritima* (Curtis) Fernald, la invasora *Spartina densiflora* Brongn, la invasora *Spartina patens* (Aiton) Muhl., y los híbridos recíprocos y transgresivos entre *S. densiflora* y *S. maritima*: *Spartina* × *onubensis* Sánchez Gullón, MD Infante y B. Gallego subsp. *onubensis* y *Spartina* × *onubensis* subsp. *tartessiana* Sánchez Gullón, B. Gallego y M.D. Infante.

- *S. maritima* es la única especie de *Spartina* nativa de las costas atlánticas europeas y africanas, y crece en zonas de marisma baja formando matas que favorecen la sedimentación y facilitan el desarrollo de la sucesión ecológica.

- *Spartina densiflora* es nativa de la costa atlántica de América del Sur y ha invadido marismas en América del Norte, Europa y África. Esta especie tiene una alta tolerancia a condiciones ambientales contrastadas y una gran plasticidad fenotípica, lo que le permite colonizar diferentes hábitats a lo largo del gradiente intermareal.

- *Spartina patens* (=*S. versicolor* Fabre) es nativa de la costa atlántica de América del Norte, el Golfo de México y las costas de América Central y El Caribe. Fue introducida en las marismas europeas y africanas del Atlántico y el Mediterráneo, y se distribuye a lo largo de las costas mediterránea, atlántica y cantábrica en la Península Ibérica.

- En las marismas del suroeste de la Península Ibérica, la especie invasora *S. densiflora* ha hibridado recíprocamente con la nativa *S. maritima* formando los híbridos transgresivos *S.* × *onubensis* subsp. *onubensis*, con *S. densiflora* como especie materna, y *S*. × *onubensis* subsp. *tartessiana*, con *S. maritima* como especie materna. Ambas subespecies de híbridos F1 son estériles, pero, sin embargo, representan una potencial amenaza para la biodiversidad nativa ya que su invasión podría acelerarse si adquieren la capacidad de producir cariopsis viables a través de la alopoliploidización.

La presente tesis incluye siete estudios sobre estos taxones en marismas del Golfo de Cádiz:

Evidencias morfológicas y anatómicas apoyan la diferenciación de nuevos híbridos interespecíficos entre la especie nativa *Spartina maritima* y la invasora *S. densiflora* (Poaceae, subfamilia Chloridoideae)

La hibridación interespecífica es un mecanismo principal que genera evolución en las plantas. A pesar del gran conocimiento sobre la ecología y la evolución de las especies de *Spartina*, los recursos taxonómicos para la distinción de taxones complejos en el género son escasos. El objetivo general de este estudio fue caracterizar las especies parentales *S. maritima* (nativa) y *S. densiflora* (invasora) y sus híbridos recíprocos formados en el Golfo de Cádiz (Suroeste de la Península Ibérica). Nuestro enfoque fue cuantificar y comparar caracteres morfológicos y anatómicos de macollas, tallos, hojas e inflorescencias y los límites de su variación en diferentes entornos ecológicos. Nuestros resultados muestran características discriminatorias de las poblaciones de ambos híbridos que se pueden utilizar para distinguirlos de sus especies parentales y entre sí. A partir de estos hallazgos y la verificación de especímenes tipo, describimos dos nuevos nothotaxa, *Spartina* × *onubensis* subsp. *onubensis* y *Spartina* × *onubensis* subsp. *tartessiana*, y desarrollar claves taxonómicas para la identificación de los cuatro taxones estudiados. Rasgos florales como la longitud de la espiga terminal, de la espiguilla y de sus glumas y lemas nos permiten diferenciar *S. densiflora* (con valores más bajos) de los otros tres taxones. La presencia de estambres exertos, tallos más cortos y láminas foliares más cortas separó a *S. maritima* de ambos híbridos. Cada híbrido también se puede distinguir ya que *S.* × *onubensis* subsp. *onubensis* desarrolla más espigas por inflorescencia y tiene hojas más largas que *S.* × *onubensis* subsp. *tartessiana*.

Fructificación, viabilidad y germinación de semillas de la especie nativa europea *Spartina maritima* en el suroeste de la Península Ibérica

Muchos aspectos de la biología reproductiva de *Spartina maritima* son desconocidos. La producción de semillas viables se ha descrito como potencialmente muy baja o inexistente. Nuestro objetivo principal fue evaluar la viabilidad de las semillas de *S. maritima*. Nuestra hipótesis es que *S. maritima* es capaz de producir semillas viables ya que hemos observado muchos rodales nuevos de esta especie. Analizamos 400 inflorescencias en estado de fructificación de 20 clones diferentes en tres estuarios en el suroeste de la Península Ibérica y realizamos un experimento de germinación. *Spartina maritima* fue capaz de producir un número moderado de cariopsis altamente viables. Las cariopsis maduras con embrión se registraron en 12.5 ± 2.9% del total de espiguillas, con una alta variación de entre 0 y 45% entre los clones. Las cariopsis mostraron un alto nivel de viabilidad (89.0 ± 4.9%) y porcentaje de germinación final (85.3 ± 2.9%). El porcentaje de espiguillas con todos los estambres exertos fue de 38.3 ± 4.5%, y se correlacionó positivamente con el porcentaje de cariopsis maduras con embrión, lo que indica que los clones en los que las inflorescencias alcanzaron un estado de floración más avanzado cuando comenzó la fructificación podrían producir más frutos.

Variación en la capacidad reproductiva sexual entre los taxones exóticos de *Spartina*

La mayoría de las especies de plantas con modos ambos reproductivos, sexuales y asexuales, alcanzan un equilibrio entre ellos. La reproducción sexual puede mejorar la colonización y la propagación de las plantas invasoras, mencionando la exitosa invasión de las marismas costeras de distintos continentes por especies del género *Spartina*. Estudiamos un amplio espectro de rasgos de reproducción sexual en taxones exóticos de *Spartina* de marismas mareales del Golfo de Cádiz. Se cuantificó la densidad de las inflorescencias, las espiguillas por inflorescencia, el porcentaje de espiguillas con cariopsis y la densidad de cariopsis en *S. patens*, *S. densiflora* y en los híbridos recíprocos entre la invasora *S. densiflora* y la nativa *S. maritima* (*S.* × *onubensis* subsp. *onubensis*) y *S.* × *onubensis* subsp. *tartessiana*). En *S. patens* y los híbridos también evaluamos la viabilidad del polen y los porcentajes de cariopsis reabsorbidas y de espiguillas con todos los estambres exertos. En la especie fértil *S. densiflora*, evaluamos rasgos sexuales de las plantas y las características de los sedimentos, y realizamos experimentos de germinación en distintos hábitats a lo largo del gradiente intermareal (marisma baja, media, alta y continentalizada, y cubetas hipersalinas). Encontramos variaciones interanuales en los rasgos reproductivos de *S. densiflora*, los cuales también se diferenciaron entre los hábitats. Las plantas con la mayor densidad de inflorescencias y de cariopsis ocuparon las zonas de marisma baja y las cubetas hipersalinas, la producción de cariopsis más baja fue en zonas de marisma baja, donde el estrés abiótico producido por la inundación frecuente fue alto, mientras que el mayor porcentaje de viabilidad de cariopsis fue en zonas de marisma media y alta. Nuestros resultados indican que ninguno de los híbridos produjo ninguna cariopsis madura, debido a la esterilidad masculina. Sin embargo, la aptitud masculina no explicó la infecundidad de *S. patens* en nuestros sitios de estudio, donde cerca del 80% de las espiguillas tuvieron todos los estambres exertos y la viabilidad del polen fue de aproximadamente el 60%. Para reducir la propagación y el impacto de las invasoras, los esfuerzos de conservación para proteger la biodiversidad deberían priorizar la rápida erradicación de los híbridos de *Spartina* antes de que alcancen la capacidad reproductiva sexual, y la eliminación de *S. densiflora* dentro de los dos años posteriores al establecimiento antes de que produzca cariopsis viables.

¿Es la dicogamia responsable de las diferencias en el éxito de la reproducción sexual entre la especie nativa *S. maritima* y la invasora *S. densiflora*?

Spartina maritima y *Spartina densiflora* comparten rasgos reproductivos, como las características de las espiguillas, la polinización por el viento y la protoginia a nivel de flor e inflorescencia. Sin embargo, se desconocen las causas de su diferente éxito reproductivo, a pesar del interés en conservar *S. maritima* y controlar la invasión de *S. densiflora*. Este estudio tiene como objetivo abordar este desconocimiento mediante la investigación de la biología reproductiva en poblaciones naturales de ambas especies de *Spartina* en marismas mareales del suroeste de la Península Ibérica: patrones de desarrollo de inflorescencias y grado de solapamiento entre fases sexuales, producción, viabilidad, liberación, pérdida de germinabilidad y dispersión del polen, desarrollo y polinización de estigmas y autocompatibilidad. En ambas especies, los estambres y los estigmas salieron de la espiguilla por la mañana temprano, y los estigmas estuvieron receptivos durante los primeros dos días. Las tasas de liberación y dispersión de polen fueron extremadamente bajas, y el polen perdió su germinabilidad en unas pocas horas, lo que determinó las bajas tasas de polen germinado en los estigmas. Ambas especies presentaron protoginia. Solo el 28% de las inflorescencias de *S. maritima* presentó solapamiento de las fases sexuales, mientras que en *S. densiflora* este porcentaje fue de hasta el 87%. Ambas especies son autocompatibles. La producción de frutos de *S. maritima* se asoció de forma positiva con el solapamiento de sexos en sus inflorescencias. Las bajas tasas de fructificación que presenta *S. maritima* en comparación con *S. densiflora* se explican por algunas desventajas en su sistema de reproducción sexual, como sus bajas tasas de dispersión de polen que reducen la posibilidad de cruzamiento, y su bajo grado de solapamiento de las dos fases sexuales en una inflorescencia lo cual reduce las posibilidades de geitonogamia. Las condiciones del ambiente durante la floración también podrían influir en la baja formación de frutos de *S. maritima* en relación con la alta temperatura y humedad, y la inmersión por las mareas que aumentaría la pérdida de germinabilidad del polen, la apertura incompleta de las anteras y el porcentaje de espiguillas con estambres no exertos.

Viabilidad de semillas, dispersión de espiguillas, banco de semillas y requerimientos de almacenamiento de las especies de *Spartina* nativa e invasora a lo largo de un gradiente intermareal

Los bancos de semillas juegan un papel importante en la determinación de la distribución espacial y temporal de halófitas en las marismas mareales. Se evaluó la capacidad de las espiguillas de la especie nativa *Spartina maritima* y la invasora *S. densiflora* para dispersarse por flotación en agua con diferentes concentraciones salinas, así como la longevidad de las espiguillas en relación con las condiciones ambientales, incluido el almacenamiento en seco o en húmedo a temperaturas bajas o moderadas y las concentraciones salinas desde agua dulce a agua de mar. Se cuantificó la producción de semillas antes de la dispersión y la presencia en el banco de semillas de las especies de *Spartina* antes y después de la dispersión a lo largo de gradientes intermareales en marismas del Golfo de Cádiz (Suroeste de la Península Ibérica). Las espiguillas de *S. densiflora*, especialmente en las zonas de marisma media y alta, mostraron una mayor capacidad que *S. maritima* para flotar, lo que sugiere que esta especie podría dispersarse a distancias más largas y hacer posible la colonización de nuevas zonas. Las semillas de *Spartina* almacenadas en condiciones de humedad mantuvieron la viabilidad durante meses, mientras que las semillas almacenadas en seco rápidamente perdieron viabilidad. Esta disminución fue más significativa para *S. maritima* y para las semillas almacenadas a temperaturas moderadas. El almacenamiento de espiguillas en condiciones húmedas y frías optimizó la viabilidad de las espiguillas. La especie nativa *S. maritima* no estableció bancos de semillas transitorios o persistentes, mientras que la invasora *S. densiflora* estableció bancos de semillas transitorios principalmente en zonas elevadas de la marisma. Nuestros resultados sobre la dinámica de dispersión de semillas y los bancos de semillas y el reclutamiento de plántulas proporcionan conocimientos fundamentales que pueden aplicarse para la conservación de *S. maritima*, el manejo de la invasora *S. densiflora* y la restauración ecológica de las marismas mareales.

Efectos diferenciales del aumento de la salinidad en la germinación y el crecimiento de plántulas de una especie nativa y una exótica invasora de *Spartina*

La salinidad del suelo es un factor ambiental clave que influye en la germinación y el establecimiento de plántulas en marismas. El calentamiento global y el aumento del nivel del mar están cambiando la salinidad de los estuarios, y podrían modificar la capacidad de colonización de las halófitas. Evaluamos los efectos del aumento de la salinidad en la germinación y el crecimiento de plántulas de la especie nativa *Spartina maritima* y la invasora *S. densiflora* de humedales del estuario Odiel-Tinto. Las respuestas se evaluaron mediante la exposición a la salinidad desde agua dulce a condiciones hipersalinas y la recuperación de las semillas no germinadas cuando se transfirieron a agua dulce. La germinación de ambas especies se inhibió y retrasó a altas salinidades, mientras que la preexposición a la salinidad aceleró la velocidad de germinación en los ensayos de recuperación en comparación con las semillas no preexpuestas. *S. densiflora* fue más tolerante a la salinidad en la germinación que *S. maritima*. *S. densiflora* fue capaz de germinar en condiciones de hipersalinidad y su porcentaje de germinación disminuyó a salinidades más altas en comparación con *S. maritima*. En cambio, *S. maritima* mostró una mayor tolerancia a la salinidad en relación con el crecimiento de las plántulas. Se observaron diferentes resultados en relación con la elevación de las poblaciones en la marisma. Nuestros resultados sugieren que *S. maritima* es una especie especialista con respecto a la salinidad, mientras que *S. densiflora* es una especie generalista capaz de germinar y crecer en condiciones subóptimas. La invasora *S. densiflora* tiene mayor capacidad que la nativa *S. maritima* para establecerse a partir de semillas con el cambio climático y el aumento del nivel del mar.

Efectos de los metales en la germinación y el crecimiento temprano de plántulas en la especie nativa *Spartina maritima* y la invasora *S. densiflora*

La germinación de semillas y el establecimiento de plántulas son las etapas más sensibles al estrés abiótico en el ciclo de la planta. La exposición de las semillas a metales puede afectar a la germinación y al crecimiento de las plántulas. Analizamos los efectos de Cu, Zn y Ni sobre la germinación de semillas y el crecimiento temprano de plántulas de *Spartina maritima* y *S. densiflora* de marismas mareales del suroeste de la Península Ibérica. Además, analizamos los efectos de los metales en las semillas de *S. densiflora* provenientes de estuarios contaminados y no contaminados. El porcentaje de germinación de ambas especies de *Spartina* no se vio afectado por ningún metal a ninguna de las concentraciones testadas (de 0 a 2000 μM). Sin embargo, las plántulas de *S. maritima* fueron más tolerantes a los metales que las de *S. densiflora*, ya que el aumento de las concentraciones de Cu, Zn y Ni tuvo efectos negativos en el crecimiento de las plántulas de *S. densiflora*, principalmente sobre la longitud de la radícula, mientras que las plántulas de *S. maritima* no se vieron afectadas. Comparando nuestros resultados con las concentraciones de metales en el campo, esperamos que el desarrollo de plántulas de la invasora *S. densiflora* pueda verse afectado en la mayoría de las áreas contaminadas con metales en los estuarios del Odiel y del Tinto. Las plántulas de *S. densiflora* provenientes de semillas del estuario del Tinto mostraron mayor tolerancia a los metales que las de los estuarios del Odiel y del Piedras.

1

Introduction

Tidal salt marshes are one of the most productive ecosystems in the world, providing many ecosystem services (Pennings & Bertness 2001). Marsh vegetation is dominated by species that tolerate high salinity (halophytes) and flooding levels, which are distributed according to zonation patterns that responds to different levels of tidal flooding, soil salinity and biological interactions (Contreras-Cruzado et al. 2017; Pennings & Callaway 1992). In this context, the spread of exotic species is an important threat to biodiversity salt marsh conservation (Adam 2002).

Spartina Schreb. is a monophyletic genus of perennial grass species (cordgrasses) belongs to the Poaceae family. Most of *Spartina* species inhabit salt marshes around the world, and many of them have successfully invaded areas beyond their native ranges. Cordgrasses usually are powerful ecological engineers with substantial influence on productivity, plant and animal communities and food webs (Ainouche & Gray 2016; Strong & Ayres 2013). Hybridization between *Spartina* species is very frequent and the formation of hybrids between invasive and native species is well documented (Ainouche et al. 2009; Strong & Ayres 2013).

Spartina species present in the salt marshes of the Gulf of Cádiz (Southwest Iberian Peninsula) are invasive *Spartina densiflora* Brongn., invasive *Spartina patens* (Aiton) Muhl., native *Spartina maritima* (Curtis) Fernald., and the reciprocal transgressive hybrids of *S. densiflora* and *S. maritima*: *Spartina* × *onubensis* Sánchez Gullón, M.D. Infante & B. Gallego subsp. *onubensis* and *Spartina* ×*onubensis* subsp. *tartessiana* Sánchez Gullón, B. Gallego & M.D. Infante.

Spartina densiflora (2n = 70) is a rhizomatous perennial species, densely caespitose, with a dense phalanx growth-form (sensu Lovett Doust & Lovett Doust 1982) with multiple culms forming tufts above the soil surface that together form discrete tussocks. This species is native from the Atlantic Coast of South America and it has invaded salt marshes in North America, Europe and Africa (Bortolus 2006). In Southwest Iberian Peninsula, it was introduced around the sixteenth century (Strong & Ayres 2013), where its invasion has become a serious ecological problem (Nieva et al. 2001a). *S. densiflora* shows a high tolerance to contrasted environmental conditions and high levels of phenotypic plasticity, which allows it to colonize different habitats along the intertidal gradient (Bortolus 2006; Di Bella et al. 2014; Nieva et al. 2001a). In addition, this species produces many viable caryopses that can increase its invasive potential (Kittelson & Boyd 1997; Nieva et al. 2001b).

Spartina patens (2n = 40) is a rhizomatous perennial grass native from tidal wetlands along the Atlantic Coast of North America, Gulf of Mexico, and Central American and Caribbean coasts, where it colonizes middle to high intertidal marshes, beaches and coastal dunes (Silander & Antonovics 1979). It was introduced in Atlantic and Mediterranean European and African marshes at the beginning of the nineteenth century (Baumel et al. 2016). In the Iberian

Peninsula, *S. patens* is distributed along the Mediterranean, Atlantic and Cantabrian Coasts (Sanz Elorza et al. 2004). Until recently, European populations of *S. patens* were considered as *Spartina versicolor* Fabre, but recent phylogenetic studies concluded that European and African populations of *S. versicolor* came from North American *S. patens* populations (Baumel et al. 2016; Prieto et al. 2011). *S. patens* seems to have a moderate invasive potential in European coastal marshes and dunes due to its low level of sexual reproduction that limits its dispersion (Castillo et al. 2017).

S. maritima (2n = 60) is a rhizomatous perennial, sparsely caespitose, that grows in low marsh areas with a guerilla growth-form (sensu Lovett Doust and Lovett Doust 1982) forming tussocks with sparse tillers that promote sedimentation and facilitate ecological succession development. Thus, *S. maritima* plays an important role as primary colonizer of salt marshes (Castellanos et al. 1994). This species is the only native cordgrass in European and African Atlantic Coasts (Mobberley 1956). *S. maritima* communities constitute a Habitat of Community Interest according to Directive 92/43/CEE (1320 Spartinion maritimae) in Europe. Due to its limited distribution and because its populations are decreasing in many coastal areas, *S. maritima* appears in the Red List in some European coastal regions (Cabezudo et al. 2005; Cooper 1993). The low genetic diversity recorded within and among *S. maritima* populations (Yannic et al. 2004) together with that this species occupies low elevations in tidal marshes (Castellanos et al. 1994), suggests this cordgrass is highly vulnerable to predicted future changes in the littoral environment. Until very recently, seed production in *S. maritima* had been described as very low or non-existent (Castellanos et al. 1994; Castillo et al. 2010; Marchant & Goodman 1969).

In Southwest Iberian Peninsula, invasive *S. densiflora* has reciprocally hybridized with native *S. maritima* forming the transgressive hybrids *S.* × *onubensis* subsp. *onubensis* (2n = ca. 64-66) and *S.* × *onubensis* subsp. *tartessiana* (2n = ca. 92-94) (Castillo et al. 2010; Infante-Izquierdo et al. 2019).

Spartina × *onubensis* subsp. *onubensis* is a rhizomatous perennials species, densely caespitose, with *S. densiflora* as maternal species (Castillo et al. 2010). It grows in middle marshes near *S. densiflora*, appearing sporadically in the estuaries of Tinto and Piedras rivers and being very abundant in a salt marsh location in the Guadiana Estuary (Castillo et al. 2010). *Spartina* × *onubensis* subsp. *tartessiana* (2n = ca. 92-94 chromosomes) is a rhizomatous perennials species, densely caespitose, with *S. maritima* as maternal species, that grows in low marshes nearby *S. maritima* with isolated tussocks in Odiel, Piedras and Guadiana Estuaries (Castillo et al. 2010). Both hybrids subspecies are sterile, which could limit their spread (Gallego-Tévar et al. 2018; Infante-Izquierdo et al. 2019). However, hybrids represent a potential threat to native biodiversity since their invasion could accelerate if they acquire the capacity to produce viable caryopsis through allopolyploidization, as occurred with *Spartina anglica* C.E. Hubb., an allopolyploid species resulting from the sterile hybrid *S.* × *townsendii* H. Groves & J. Groves (Baumel et al. 2001).

The incidence of invasive species and the new hybrids taxa put the conservation of biodiversity in salt marshes in the Gulf of Cadiz in serious risk. In addition, many aspects of the reproductive biology of the threatened native species *S. maritima*, the invasive species and the hybrids between native *S. maritima* and invasive *S. densiflora* are unknown.

In this context, the main goal of this PhD dissertation was to study the sexual reproductive biology of the five *Spartina* taxa present in the salt marshes of the Gulf of Cádiz (Southwest Iberian Peninsula). Improving our knowledge of the sexual reproductive biology of native *S. maritima* is important for the conservation of this European cordgrass. In addition, results may provide fundamental knowledge for restoration and management of tidal marshes invaded by exotic cordgrasses.

The main goal was divided in the following specific objectives:

1. To describe and differentiate the two reciprocal hybrids formed in the Gulf of Cádiz between native *S. maritima* and invasive *S. densiflora*, and to develop taxonomic keys to identify the phenotypes of the hybrids in relation to their parental species.

2. To evaluate the ability of *S. maritima* to produce viable seeds, to test caryopsis germination, and to identify environmental factors influencing its fruit set.

3. To analyze and compare a broad spectrum of sexual reproductive traits, as inflorescences and caryopsis density, in all exotic *Spartina* taxa in coastal wetlands of the Southwest Iberian Peninsula, and to evaluate differences in sexual reproductive traits from contrasting habitats along the intertidal gradient in *S. densiflora*.

4. To look for explanations of the low and variable seed production in *S. maritima*, using as comparison the behavior of invasive *S. densiflora* that shows high fruit set levels, and studying the following sexual reproductive traits: the efficiency of protogyny, production, viability and dispersion of pollen, receptivity and effective pollination of stigmas, and presence or absence of self-incompatibility.

5. To evaluate the ability of native *S. maritima* and invasive *S. densiflora* spikelets to disperse by flotation on water with different salinity concentrations, to evaluate the spikelet longevity relative to different environmental conditions, and to quantify the pre-dispersal seed production and the pre- and post-dispersal seed banks of both cordgrasses along the intertidal gradient.

6. To evaluate the effects of increasing salinity (from fresh water to hypersaline conditions) on germination and early seedling growth of

native *Spartina maritima* and invasive *S. densiflora* from wetlands of the Odiel-Tinto Estuary, and evaluate the recovery of non-germinated seeds when transferred to fresh water. To evaluate salinity responses of *S. densiflora* seeds that were produced in different habitats along the intertidal gradient.

7. To analyze and compare the effects of heavy metals on seed germination and early seedling growth of native *S. maritima* and invasive *S. densiflora*. In addition, analyze in *S. densiflora* the metals effects on seeds coming from polluted and non-polluted estuaries.

References

Adam, P. (2002). Saltmarshes in a time of change. Environmental conservation 29: 39-61. https://doi.org/10.1017/S0376892902000048

Ainouche, M.L., Fortune, P.M., Salmon, A., Parisod, C., Grandbastien, M.A., Fukunaga, K., Ricou, M., Misset, M.T. (2009). Hybridization, polyploidy and invasion: lessons from *Spartina* (Poaceae). Biological Invasions 11: 1159-1173. https://doi.org/10.1007/s10530-008-9383-2

Ainouche, M., Gray, A. (2016). Invasive *Spartina*: lessons and challenges. Biological Invasions 18: 2119-2122. https://doi.org/10.1007/s10530-016-1201-7

Baumel, A., Ainouche, M.L., Levasseur, J.E. (2001). Molecular investigations in populations of *Spartina anglica* C. E. Hubbard (Poaceae) invading coastal Brittany (France). Molecular Ecology 10: 1689-1701. https://doi.org/10.1046/j.1365-294X.2001.01299.x

Baumel, A., Rousseau-Gueutin, M., Sapienza-Bianchi, C., Gareil, A., Duong, N., Rousseau, H., Coriton, O., Amirouche, R., Sciandrello, S., Duarte, B., Caçador, I., Castillo, J.M., Ainouche, M. (2016). *Spartina versicolor* Fabre: Another case of *Spartina* trans-Atlantic introduction? Biological Invasions 18: 2123-2135. https://doi.org/10.1007/s10530-016-1128-z

Bortolus, A. (2006). The austral cordgrass *Spartina densiflora* Brong.: its taxonomy, biogeography and natural history. Journal of Biogeography 33: 158-168. https://doi.org/10.1111/j.1365-2699.2005.01380.x

Cabezudo, B., Talavera, S., Blanca, G., Salazar, C., Cueto, M., Valdés, B., Hernández-Bermejo, J.E., Herrera, C.M., Rodríguez-Hiraldo, C., Navas, D. (2005). Lista roja de la flora vascular de Andalucía. Consejería de Medio Ambiente. Junta de Andalucía, Sevilla.

Castellanos, E.M., Figueroa, M.E., Davy, A.J. (1994). Nucleation and facilitation in saltmarsh succession: interactions between *Spartina maritima* and *Arthrocnemum perenne*. Journal of Ecology 82: 239-248. DOI: 10.2307/2261292

Castillo, J.M., Ayres, D.R., Leira-Doce, P., Bailey, J., Blum, M., Strong, D.R., Luque, T., Figueroa, E. (2010). The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Diversity and Distributions 16: 547-558. https://doi.org/10.1111/j.1472-4642.2010.00673.x

Castillo, J.M., Leira-Doce, P., Figueroa, E. (2017). Biomass and clonal architecture of the cordgrass *Spartina patens* (Poaceae) as an invasive species in two contrasted coastal habitats on the Atlantic coast of the Iberian Peninsula. Plant Ecology and Evolution 150: 129-138. https://doi.org/10.5091/plecevo.2017.1290

Contreras-Cruzado, I., Infante-Izquierdo, M.D., Márquez-García, B., Hermoso-López, V., Polo, A., Nieva, F.J.J., Cartes-Barroso, J.B., Castillo, J.M., Muñoz-Rodríguez, A. (2017). Relationships between spatiotemporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma 305: 173-187. https://doi.org/10.1016/j.geoderma.2017.05.037

Cooper, M.A. (1993). The status of *Spartina maritima* in Suffolk. Suffolk Naturalists' Society Transactions 29: 48-54.

Di Bella, C.E., Striker, G.G., Escaray, F.J., Lattanzi, F.A., Rodríguez, A.M., Grimoldi, A.A. (2014). Saline tidal flooding effects on *Spartina densiflora* plants from different positions of the salt marsh. Diversities and similarities on growth, anatomical and physiological responses. Environmental and Experimental Botany 102: 27-36. https://doi.org/10.1016/j.envexpbot.2014.02.009.

Gallego-Tévar, B., Curado, G., Grewell, B.J., Figueroa, M.E., Castillo, J.M. (2018). Realized niche and spatial pattern of native and exotic halophyte hybrids. Oecologia 188: 849-862. https://doi.org/10.1007/s00442-018-4251-y

Infante-Izquierdo, M.D., Gallego-Tévar, B., Sánchez-Gullón, E., Nieva, F.J.J., Grewell, B.J., Castillo, J.M., Muñoz-Rodríguez, A.F. (2019). Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae). Plant Systematics and Evolution 305: 531-547. https://doi.org/10.1007/s00606-019-01591-5

Kittelson, P.M., Boyd, M.J. (1997). Mechanisms of expansion for an introduced species of cordgrass, *Spartina densiflora*, in Humboldt Bay, California. Estuaries 20: 770-778. https://doi.org/10.2307/1352250

Lovett Doust, L., Lovett Doust, J. (1982) The battle strategies of plants. New Scientist 91: 81-84.

Marchant, C.J., Goodman, P.J. (1969). *Spartina maritima* (Curtis) Fernald. Journal of Ecology 57: 287-291. DOI: 10.2307/2258222

Mobberley, D.G. (1956). Taxonomy and distribution of the genus *Spartina*. Iowa State College Journal of Science 30: 471-574.

Nieva, F.J.J., Díaz-Espejo, A., Castellanos, E.M., Figueroa, M.E. (2001a). Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel Marshes (SW Spain). Estuarine, Coastal and Shelf

Science 52: 515-527.
https://doi.org/10.1006/ecss.2000.0750

Nieva, F.J.J., Castellanos, E.M., Figueroa, M.E. (2001b). Effects of light and salinity on seed germination in the marsh invader *Spartina densiflora* Brong., 1829 (Gramineae) from Gulf of Cádiz, Spain. Boletín de la Real Sociedad Española de Historia Natural 96: 117-124.

Pennings, S.C., Bertness, M.D. (2001). Salt marsh communities. In: Bertness, M.D., Gaines, S.D., Hay, M.E. (eds). Marine community ecology. Sinauer Associates, Sunderland, pp 289-316.

Pennings, S.C., Callaway, R.M. (1992). Salt marsh plant zonation: the relative importance of competition and physical factors. Ecology 73: 681-690.
https://doi.org/10.2307/1940774

Prieto, J.A.F., Cires, E., Sánchez Corominas, T., Vázquez, V.M. (2011). Systematics and management of natural resources: the case of *Spartina* species on European shores. Biologia 66: 1011-1018.
https://doi.org/10.2478/s11756-011-0109-z

Sanz Elorza, M., Dana Sánchez, E.D., Sobrino Vesperinas, E. (2004). Atlas de las plantas alóctonas invasoras en España. Dirección General para la Biodiversidad, Madrid.

Silander, J.A., Antonovics, J. (1979). The genetic basis of the ecological amplitude of *Spartina patens*. I. Morphometric and physiological traits. Evolution 33: 1114-1127. DOI: 10.2307/2407471

Strong, D.R., Ayres, D.R. (2013). Ecological and evolutionary misadventures of *Spartina*. Annual Review of Ecology, Evolution, and Systematics 44: 389-410.
https://doi.org/10.1146/annur ev-ecols ys-110512-135803

Yannic, G., Baumel, A., Ainouche, M. (2004). Uniformity of the nuclear and chloroplast genomes of *Spartina maritima* (Poaceae), a salt-marsh species in decline along the Western European coast. Heredity 93: 182-188.
https://doi.org/10.1038/sj.hdy.6800491

2

Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native Spartina maritima and invasive S. densiflora (Poaceae, subfamily Chloridoideae)

Publicado en:
María D. Infante-Izquierdo[1], Blanca Gallego-Tévar[2], Enrique Sánchez-Gullón[3], F. Javier J. Nieva[1], Brenda J. Grewell[4], Jesús M. Castillo[2], Adolfo F. Muñoz-Rodríguez[1] (2019).
Plant Systematics and Evolution 305: 531-547. https://doi.org/10.1007/s00606-019-01591-5

[1] Dpto. de Ciencias Integradas, Universidad de Huelva, Huelva, Spain
[2] Dpto. Biología Vegetal y Ecología, Universidad de Sevilla, Seville, Spain
[3] Paraje Natural Marismas del Odiel, Ctra. del Dique Juan Carlos I, Apdo. 720, Huelva, Spain
[4] USDA-ARS Invasive Species and Pollinator Health Research Unit, Department of Plant Sciences MS-4, University of California, Davis, CA, USA

Factor de impacto (2018): 1,585. Plant Science: 106 de 228, Cuartil 2

Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae)

Abstract

Interspecific hybridization is a major mechanism for generating evolutionary novelty in plants. Halophytic *Spartina* species are often abundant in coastal salt marshes around the world, and they frequently hybridize. Despite a large body of knowledge on the ecology and evolution of *Spartina* species, taxonomic resources for distinction of complex taxa in the genus are scarce. The general aim of this study was to characterize parental species *S. maritima* (native) and *S. densiflora* (invasive) and their reciprocal hybrids formed in the Gulf of Cadiz (Southwest Iberian Peninsula). Our approach was to quantify and compare morphological and anatomical characters of tufts, culms, leaves and inflorescences and the limits of their variation in different ecological settings. Our results document discriminating trait variables from populations of both hybrids that can be used to distinguish them from their parental species and each other. From these findings and the verification of type specimens, we describe two new nothotaxa, *Spartina* × *onubensis* subsp. *onubensis* and *Spartina* × *onubensis* subsp. *tartessiana*, and develop taxonomic keys for identification of the four studied taxa. Floral traits such as the length of the terminal spike, the spikelet and its glumes and lemma allow us to differentiate *S. densiflora* (with lower values) from the other three taxa. The presence of exerted stamens, shorter culms and shorter leaf blades separated *S. maritima* from both hybrids. Each hybrid can also be distinguished since *S.* × *onubensis* subsp. *onubensis* develops more spikes per inflorescence and consistently has longer leaves than *S.* × *onubensis* subsp. *tartessiana*.

Keywords:

Cordgrass, Gulf of Cádiz, Hybridization, Invasive species, Phenotypic plasticity, Salt marsh

Introduction

Interspecific hybridization is a major mechanism for generating evolutionary novelty in plants (Ellstrand et al. 1996; Hegarty and Hiscock 2005; Mallet 2007). In this context, when exotic species are introduced to new locations by humans, hybridization between the exotic and sometimes invading species and a native species results in new hybrids that may evolve as new species (Abbott 1992; Mooney and Cleland 2001). Exotic species can evolve rapidly after hybridization (Vila et al. 2000) and can lead to the evolution of invasiveness (Rieseberg et al. 1999; Sloop et al. 2009).

Spartina Schreb. is a monophyletic genus of perennial grass species (cordgrasses) from the Poaceae, subfamily Chloridoideae Kunth ex Beilschm., tribe Zoyseae Benth., integrated by 12 non-hybrid species (Mobberley 1953, 1956) with a preference for saline habitats (Peterson et al. 2010). Thus, *Spartina* species are frequently abundant halophytes in coastal salt marshes around the world. All *Spartina* species are polyploids with basic number $x = 10$ chromosomes and natives of the New World, except hexaploid *Spartina maritima* (Curtis) Fernald, which is native to European and African salt marshes (Marchant 1968a, b; Ainouche et al. 2004). Many species of *Spartina* are powerful ecological engineers with substantial influence on productivity, communities and food webs (Strong and Ayres 2013; Ainouche and Gray 2016). Some hybrids between native species have been described such as *Spartina* × *caespitosa* A.A. Eaton that seems to have originated from repeated crosses involving *Spartina patens* (Aiton) Muhl. and *Spartina pectinata* Link forming a hybrid swarm that propagates vegetatively in Atlantic North America marshes (Mobberley 1953; Ainouche et al. 2004). Nevertheless, human-caused intra- and intercontinental exchanges of plant species coupled with human-induced habitat disturbances over the two last centuries have increased the dispersal, naturalization and invasion of plant species beyond their native range (Hulme et al. 2008).

Extensive biotic exchanges have led to the appearance of different hybrids between alien and native *Spartina* species (Ainouche et al. 2004, 2009; Strong and Ayres 2013; Ainouche and Gray 2016). For example, hexaploid *Spartina alterniflora* Loisel. native to the Atlantic and Gulf Coasts of North America has been introduced on the Pacific coasts of North America, New Zealand and China, and the Atlantic coasts of South America, Europe and the tip of South Africa (Partridge 1987; An et al. 2007; Saarela 2012; Bortolus et al. 2015; Ainouche and Gray 2016). Introduced populations of *S. alterniflora* have hybridized with native *Spartina* species when and wherever the two species co-occur. *Spartina longispica* Hauman & Parodi ex St.-Yves. is a hybrid between introduced *S. alterniflora* and native *S. densiflora* Brongn. in Rio de la Plata (Mobberley 1953; Ainouche et al. 2004; Bortolus 2006). In California, exotic populations of *S. alterniflora* and native hexaploid *Spartina foliosa* Trin. hybridized in both directions (Anttila et al. 2000) that together with recurrent backcrossing resulted in an invasive hybrid swarms (Daehler and Strong 1997; Ayres et al. 1999, 2008a; Ainouche et al. 2004). Data on distribution and spread of these swarms support the prediction that if no actions were taken to control the invasion of these hybrids, the extinction of both parental species as distinct genetic entities would occur within several decades to centuries (Hall et al. 2006; Ayres et al. 2004a, b; Strong and Ayres 2013). In Europe, hybridization of exotic *S. alterniflora* with native *S. maritima* resulted in two morphologically different homoploid sterile hybrids: *Spartina* × *neyrautii* Foucaud (in the South of France) and *Spartina* × *townsendii* H. Groves & J. Groves (in the British Islands) (Groves and Groves 1880). Chromosome doubling of *S.* × *townsendii* less than 150 years ago resulted in the fertile allododecaploid *Spartina anglica* C.E. Hubb. that displayed wider ecological amplitude than its parents and has expanded around the world (Raybould et al. 1991; Thompson 1991; Baumel et al. 2001; Ainouche et al. 2004). Marchant (1968b) reported a nonaploid plant of *S.* × *townsendii* $2n = 9x = 90$ composed of $2n$ *S. alterniflora* and $1n$ *S. maritima*. Renny-Byfield et al. (2010) reported *Spartina* plants $2n = 75$-76 that they presumed to be the progeny of the fertile nonaploid taxon described by Marchant (1968b). These observations led Strong and Ayres (2013) to suggest a triploid taxon (triploid bridge) may have played a role in the speciation of *S. anglica*. The formation of transgressive phenotypes observed in *S. alterniflora* hybrids is an example of how hybridization may promote extreme phenotypic characters that accelerate the evolution of invasive hybrids (Ellstrand and Schierenbeck 2000).

Spartina densiflora is a caespitose graminoid species with a dense phalanx growth-form (sensu Lovett Doust and Lovett Doust 1982) with multiple culms forming tufts above the soil surface that together form discrete tussocks in wetlands. This heptaploid species ($2n = 70$) is native from the Atlantic coast of South America and is considered to be the product of an ancient hybridization between tetraploid *Spartina arundinacea* (Thouars) Carmich. and hexaploid *S. alterniflora* or their ancestors (Fortune et al. 2008). In 1972, *S. densiflora* was mistaken for a native species and introduced to San Francisco Bay (California) in a wetland restoration project. Since that time, F1 hybrids with other *Spartina* species have been detected: reciprocal hybrids between *S. densiflora* and native *S. foliosa* (one of them a triploid hybrid containing $2n$ *S. foliosa* and $1n$ *S. densiflora*), and hybrids between *S. densiflora* and the hybrids of *S. alterniflora* and *S. foliosa* (Ayres et al. 2008b). In California, hybrids between *S. densiflora* and *S. foliosa* have exhibited greater salinity tolerance that both parental species (Strong and Ayres 2013; Lee et al. 2016). In the Gulf of Cádiz (Southwest Iberian Peninsula), invasive *S. densiflora* has repeatedly and reciprocally hybridized with native *S. maritima*. *S. maritima* grows mainly in low marsh areas with a guerilla growth-form (sensu Lovett Doust and Lovett Doust 1982) forming tussocks with sparse tillers that promote sedimentation and facilitate ecological succession development (Castellanos et al. 1994). This hybridization has produced two F1 hybrids. To date, distinct biological nomenclature has not been assigned to these hybrid taxa that are useful for organizing information about biological diversity. Since both hybrids share the same parental species, they cannot be published as two different nothospecies (Turland et al. 2018). However, as they have different chromosome numbers and different seed parents, we propose they should be assigned subspecies rank under the hybrid taxon *Spartina* × *onubensis*. One of these hybrid taxa that we herein describe as *Spartina* × *onubensis* subsp. *onubensis* has a 1*n* contribution by each species (homoploid; ca. 64-66 chromosomes) and has the chloroplast DNA (cpDNA) haplotype of *S. densiflora*, denoting its maternal origin (Castillo et al. 2010). This hybrid grows in middle marshes near *S. densiflora*, appearing sporadically in the estuaries of Tinto and Piedras rivers and being very abundant and invasive (hundreds of tussocks) in a salt marsh location in the Guadiana Estuary (Castillo et al. 2010). The other hybrid taxon that we herein name *Spartina* × *onubensis* subsp. *tartessiana* is a triploid hybrid (ca. 92-94 chromosomes) containing 2*n* from *S. maritima* and 1*n* from *S. densiflora* (Castillo et al. 2010). This hybrid has cpDNA haplotype of *S. maritima* and grows in low marshes nearby *S. maritima* with isolated tussocks in Odiel, Piedras and Guadiana estuaries (Castillo et al. 2010). Castillo et al. (2010) transplanted hybrids between *S. densiflora* and *S. maritima* along the whole intertidal gradient, showing that they grew taller, rapider and at more elevations than the parental species, and Gallego-Tévar et al. (2018a) reported that these hybrids show higher tolerance to salinity than both parental species. At present, both hybrid subspecies of *S.* × *onubensis* are sterile, which initially limits their invasive spread (Gallego-Tévar et al. 2018b). However, Castillo et al. (2010) suggest the hybrids need not be fertile to pose conservation threats and that the formation of a new fertile allopolyploid species would lead to rapid hybrid spread as happened with *S. anglica* (Raybould et al. 1991). Invasive *Spartina* taxa become dominant in salt marshes, diminish the species richness and diversity of biota, increase costs of managing wildlife and interfere with human uses of estuaries (An et al. 2007; Strong and Ayres 2013). The ability to clearly identify the exotic hybrids is key for environmental management and conservation (Abbott 1992). To date, a detailed characterization and taxonomic key to identify the phenotypes of the hybrids between *S. maritima* and *S. densiflora* in relation to their parental species is lacking.

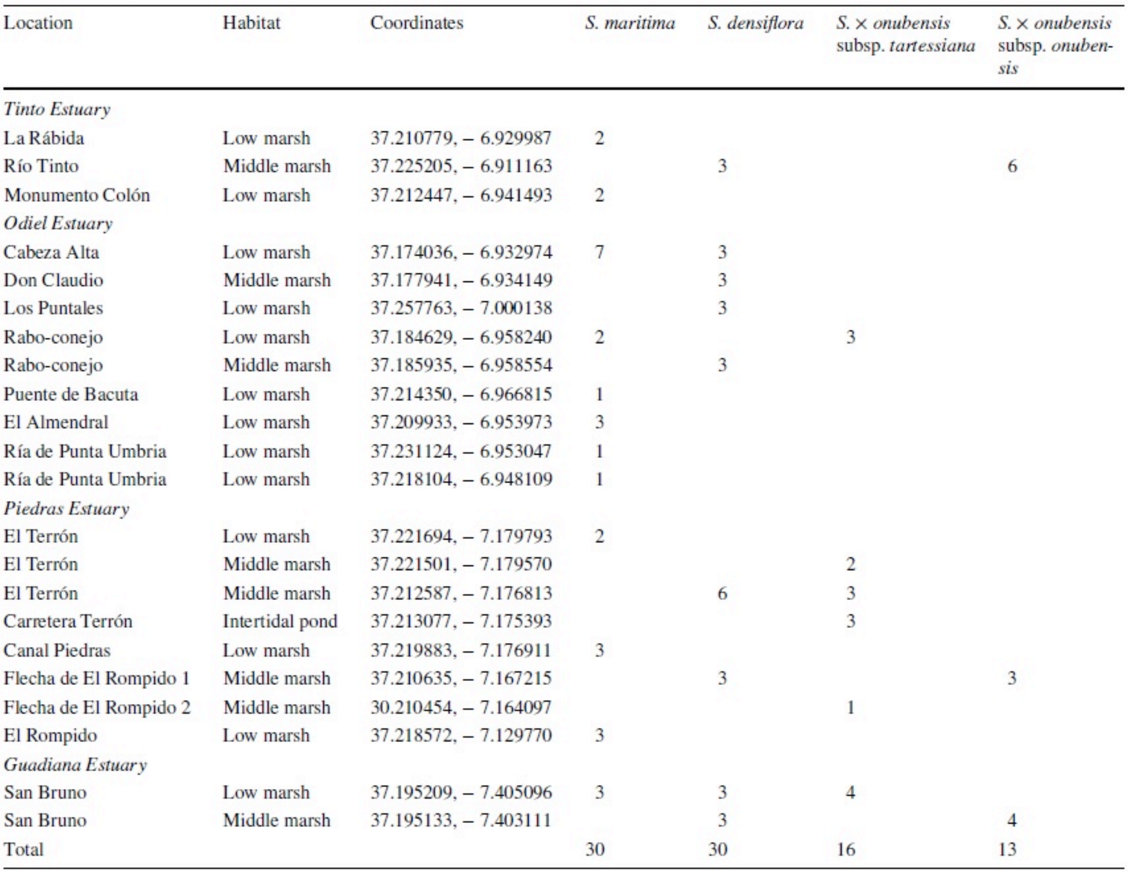
Despite a large and broad body of knowledge on the ecology and evolution of *Spartina* species (Strong and Ayres 2013), taxonomic resources for the genus are scarce (Saarela 2012). The general aim of this study was to characterize the parental species *S. maritima* and *S. densiflora* and of their reciprocal hybrids in which genetic determination of their maternal origin have been previously made (Castillo et al. 2010). Our approach was to quantify and compare morphological and anatomical characters of tufts, culms, leaves and inflorescences. Since *Spartina* species such as *S. densiflora* showed high phenotypic plasticity depending on environmental conditions (Castillo et al. 2014, 2018), the range of variation for each studied trait among plants from different ecological settings was also considered. From these findings and the verification of type specimens, our objectives were to describe and differentiate two new nothotaxa, and to develop taxonomic keys for identification of the four studied taxa.

Materials and methods

Plant specimen and sedimentary data collections

Table 1 shows location, habitat type and number of tufts of *Spartina maritima*, *S. densiflora* and their two reciprocal hybrids that were evaluated. Every hybrid population known at the time of this study was sampled. One flowering culm with caryopses and one adult unflowered culm were chosen randomly from every studied *Spartina* tuft, in September 2017 for *S. maritima* and in November-December 2017 for *S. densiflora* and both hybrids. At each location, three sediment samples were obtained using stainless steel cores of 50 mm diameter and 50 mm height. Sediment samples were kept in hermetically closed polyethylene bags and stored at -20 °C until analysis in the laboratory. Water content (%) was calculated by weight loss of 50 g samples dried in a forced air oven at 80 °C for 48 h. Organic matter content (%) was determined by the loss-on-ignition method from samples of dehydrated soil (5 g) using a muffle oven at 500 °C for 5 h. In a mix of 5 ml of wet sediments and the same volume of distilled water (1:1, v:v), electrical conductivity (mS cm^{-1}) was measured with a conductivity meter (Crison Instruments 50 64, Hach Lange, Barcelona, Spain) and sediment pH using pH meter (Crison pH meter 25, Hach Lange Spain, Barcelona, Spain) (Contreras-Cruzado et al. 2017).

Table 1 Location (local name), habitat, geographical coordinates (N, W) and number of studied tufts of native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids in three estuaries along the coast of the Gulf of Cadiz (Southwest Iberian Peninsula)



Location	Habitat	Coordinates	*S. maritima*	*S. densiflora*	*S.* × *onubensis* subsp. *tartessiana*	*S.* × *onubensis* subsp. *onubensis*
Tinto Estuary						
La Rábida	Low marsh	37.210779, – 6.929987	2			
Río Tinto	Middle marsh	37.225205, – 6.911163		3		6
Monumento Colón	Low marsh	37.212447, – 6.941493	2			
Odiel Estuary						
Cabeza Alta	Low marsh	37.174036, – 6.932974	7	3		
Don Claudio	Middle marsh	37.177941, – 6.934149		3		
Los Puntales	Low marsh	37.257763, – 7.000138		3		
Rabo-conejo	Low marsh	37.184629, – 6.958240	2		3	
Rabo-conejo	Middle marsh	37.185935, – 6.958554		3		
Puente de Bacuta	Low marsh	37.214350, – 6.966815	1			
El Almendral	Low marsh	37.209933, – 6.953973	3			
Ría de Punta Umbria	Low marsh	37.231124, – 6.953047	1			
Ría de Punta Umbria	Low marsh	37.218104, – 6.948109	1			
Piedras Estuary						
El Terrón	Low marsh	37.221694, – 7.179793	2			
El Terrón	Middle marsh	37.221501, – 7.179570			2	
El Terrón	Middle marsh	37.212587, – 7.176813		6	3	
Carretera Terrón	Intertidal pond	37.213077, – 7.175393			3	
Canal Piedras	Low marsh	37.219883, – 7.176911	3			
Flecha de El Rompido 1	Middle marsh	37.210635, – 7.167215		3		3
Flecha de El Rompido 2	Middle marsh	30.210454, – 7.164097			1	
El Rompido	Low marsh	37.218572, – 7.129770	3			
Guadiana Estuary						
San Bruno	Low marsh	37.195209, – 7.405096	3	3	4	
San Bruno	Middle marsh	37.195133, – 7.403111		3		4
Total			30	30	16	13

Plant trait characterization

Most of the traits evaluated were the same as those used by Mobberley (1953) in his revision of the genus *Spartina* and for the study of the possible hybrid origin of some species such as *Spartina longispica* and *Spartina* × *caespitosa*. Other measured traits, such as the number of culm nodes, were suggested by other authors to be of taxonomic interest (Marchant 1967). Following Kern et al. (2008), we included evaluation of inflorescences. Among the plant (P) characters studied, we included six culm traits: maximum height of flowering culms (cm) (PFH), maximum height of unflowered culms (cm) (PUH), tuft diameter (cm) (PMD), culm density (culms cm^{-2}) (PCD), number of culm nodes (PNN) and culm diameter at its base (mm) (PTD). We measured five morphological foliar traits using the longest leaf (L) of each studied culm: leaf position (numbered from bottom of the culm) (LLP), sheath length (cm) (LSL), blade length (cm) (LBL), blade width (mm) (LBW) and ligule length (mm) (LLL). Three inflorescence (I) traits were evaluated: inflorescence length (cm) (IIL), terminal spike length (cm) (ISL) and number of spikes per inflorescence (INS). Eight characters were recorded for the spikelets (S) from the base of the terminal spike: pedicel length (mm) (SPD), lower glume length (mm) (SLG), upper glume length (mm) (SUG), upper-to-lower glume length ratio (SGR), upper glume width (mm) (SGW), lemma length (mm) (SLM), palea length (mm) (SPL) and number of exerted stamens during anthesis (SNS).

Following Maricle et al. (2009), we evaluated anatomical (A) structures of the leaves. First, using a stereoscopic microscope, the adaxial ridges on the longest leaf were counted (ANR). Next, transversal cuts at the widest part of the longest leaf were made and evaluated under an optical compound microscope. Traits recorded included counts of aerenchyma channels and sclerenchyma strands in the leaf, and the ratio of the number of major ribs to that of the minor and medium ribs (ARR). We also recorded the ratio between the number of aerenchyma channels and the number of depressions between two ribs without aerenchyma (AAC), and the ratio between the number of abaxial sclerenchyma strands and the number of ribs (ASS). Then, we made three measurements on the midrib of each leaf: midrib length (µm) (ARH), maximum midrib width (µm) (ARW) and maximum width of the colorless aqueous parenchyma (µm) (ACP). For the study of epidermal characters, abaxial epidermis was manually peeled using a scalpel and mounted on a microscope slide for observation. The four studied taxa presented the long cells of the epidermis with sinuous edges, as in the rest of *Spartina* species (Maricle et al. 2009). The recorded epidermal characters were the length and width (µm) of these epidermal cells (AEL and AEW, respectively) (n = 5 cells per leaf), and the density of stomata per mm^{-2} recorded in visual fields of 0.95 mm^2 (ADS) (n = 3 fields per leaf).

Phenotypic inheritance

We assessed phenotypic inheritance of each hybrid taxa. Dominant inheritance (D) was attributed when the character for one hybrid was equal to one of the parents (D-Sm for *S. maritima*; D-Sd for *S. densiflora*). We concluded parental codominance (D-Sm, Sd) when a trait expressed by a hybrid was the same as both parents. Parental additivity (I) was attributed when traits were intermediate between both parents. Transgressive (T) inheritance was recorded when trait expression was different from both parental species. In this case, we defined “transgressive vigorous” as a positive change for the fitness of the hybrid in relation to its parents and “transgressive depressed” when the trait expression was a negative change for the hybrid. These inheritance types were quantified as percentages in relation to the 32 studied morphological and anatomical traits evaluated for each hybrid taxon.

Statistical analysis

Statistical analyses were performed using the software package Statistica 8.0 (StatSoft Inc.). Deviation of the mean was calculated as standard error (SE). A probability value (P) < 0.05 was applied for every significant analysis. Data sets were analyzed for normality and homogeneity of variance using Kolmogorov-Smirnov and Levene’s test, respectively. Then, we used one-way ANOVA and Tukey’s HSD post hoc test to compare means of the recorded plant traits among taxa. When the homogeneity of variance or normality was not reached, means were compared using a non-parametric Kruskal-Wallis test, followed by post hoc Mann-Whitney U-test. Principal component analysis (PCA) on plant traits was carried out to establish the relative importance of the specific characters that differentiated hybrids and parental species. We selected those plant traits that showed absolute factor loading values > 0.600 and analysed the correlation matrix with 50 maximum iterations for convergence to extract independent principal components factors with eigenvalues > 1. The data matrices were standardized prior to analyses. Relationships between plant traits and sedimentary factors were analyzed using Spearman’s correlation coefficient.

Results

Characterization of Spartina taxa

Significant differences between studied *Spartina* taxa were observed in 31 out of 32 studied morphological and anatomical traits (ANOVA or Kruskal-Wallis test, P < 0.05). Only culm diameter was not significantly different among taxa (Table 2).

Spartina maritima presented shorter flowered (– 38%) and unflowered (– 37%) culms than *S. densiflora*, and both hybrids had taller (> + 21%) culms than either parental species. Culm density was also lower (< – 77%) for *S. maritima* than for the other three taxa. In contrast, tuft diameter of *S. maritima* and *S.* × *onubensis* subsp. *onubensis* was larger (> + 44%) than for *S. densiflora* and *S.* × *onubensis* subsp. *tartessiana*. The number of culm nodes was minimal for *S. densiflora*, intermediate for *S.* × *onubensis* subsp. *onubensis* and *S. maritima*, and maximum for *S.* × *onubensis* subsp. *tartessiana* (Table 2).

Leaf traits showed a different pattern. The position of the longest leaf on the culm was higher (> + 64%) for *S. maritima* and *S*. × *onubensis* subsp. *tartessiana* than for *S. densiflora*, while longest leaves of *S*. × *onubensis* subsp. *onubensis* were at intermediate heights. The leaf sheath and the blade were shorter for *S. maritima* than for *S. densiflora* and *S*. × *onubensis* subsp.

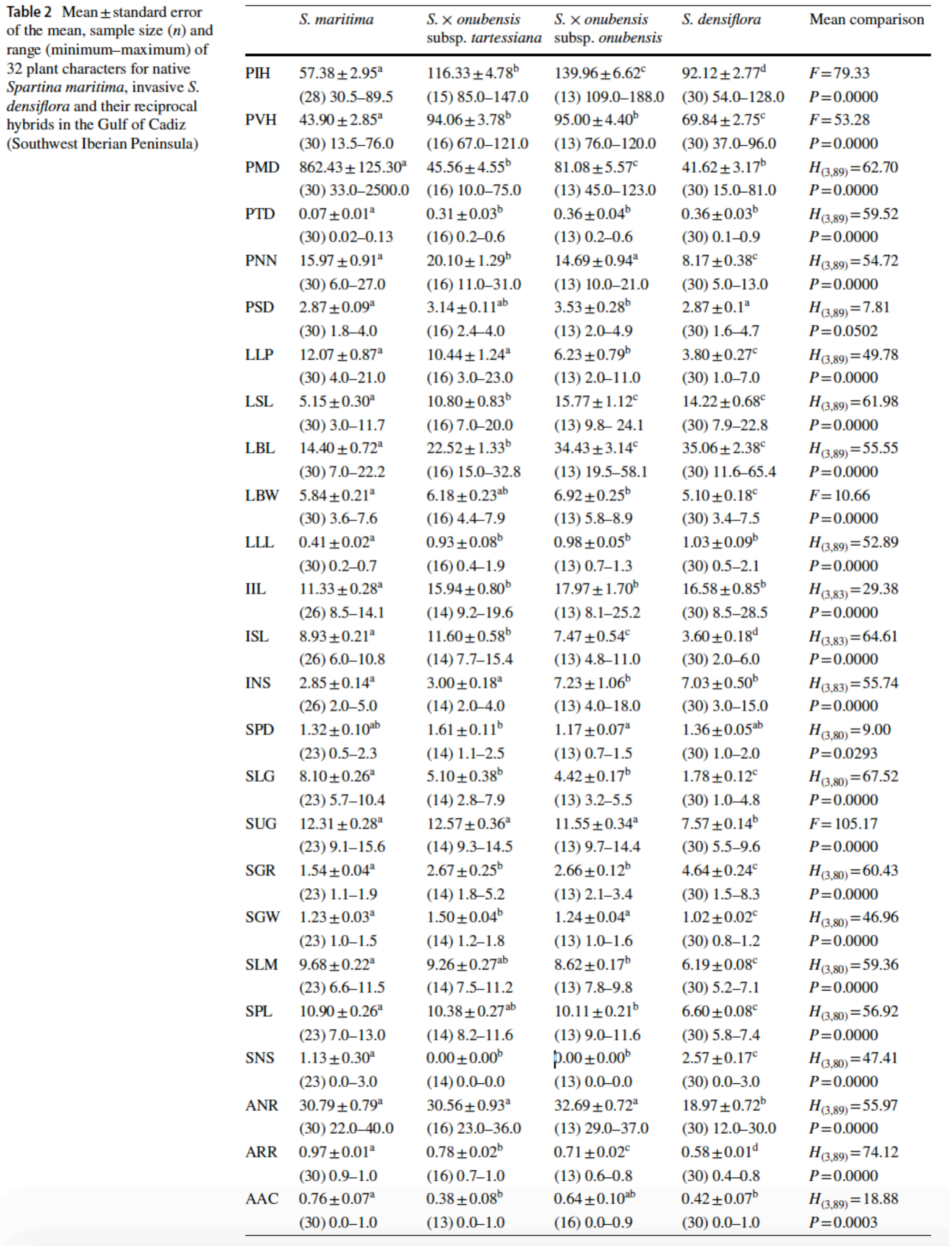


Table 2 Mean ± standard error of the mean, sample size (*n*) and range (minimum–maximum) of 32 plant characters for native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids in the Gulf of Cadiz (Southwest Iberian Peninsula)

	S. maritima	*S.* × *onubensis* subsp. *tartessiana*	*S.* × *onubensis* subsp. *onubensis*	*S. densiflora*	Mean comparison
PIH	57.38 ± 2.95^{a}	116.33 ± 4.78^{b}	139.96 ± 6.62^{c}	92.12 ± 2.77^{d}	$F = 79.33$
	(28) 30.5–89.5	(15) 85.0–147.0	(13) 109.0–188.0	(30) 54.0–128.0	$P = 0.0000$
PVH	43.90 ± 2.85^{a}	94.06 ± 3.78^{b}	95.00 ± 4.40^{b}	69.84 ± 2.75^{c}	$F = 53.28$
	(30) 13.5–76.0	(16) 67.0–121.0	(13) 76.0–120.0	(30) 37.0–96.0	$P = 0.0000$
PMD	862.43 ± 125.30^{a}	45.56 ± 4.55^{b}	81.08 ± 5.57^{c}	41.62 ± 3.17^{b}	$H_{(3,89)} = 62.70$
	(30) 33.0–2500.0	(16) 10.0–75.0	(13) 45.0–123.0	(30) 15.0–81.0	$P = 0.0000$
PTD	0.07 ± 0.01^{a}	0.31 ± 0.03^{b}	0.36 ± 0.04^{b}	0.36 ± 0.03^{b}	$H_{(3,89)} = 59.52$
	(30) 0.02–0.13	(16) 0.2–0.6	(13) 0.2–0.6	(30) 0.1–0.9	$P = 0.0000$
PNN	15.97 ± 0.91^{a}	20.10 ± 1.29^{b}	14.69 ± 0.94^{a}	8.17 ± 0.38^{c}	$H_{(3,89)} = 54.72$
	(30) 6.0–27.0	(16) 11.0–31.0	(13) 10.0–21.0	(30) 5.0–13.0	$P = 0.0000$
PSD	2.87 ± 0.09^{a}	3.14 ± 0.11^{ab}	3.53 ± 0.28^{b}	2.87 ± 0.1^{a}	$H_{(3,89)} = 7.81$
	(30) 1.8–4.0	(16) 2.4–4.0	(13) 2.0–4.9	(30) 1.6–4.7	$P = 0.0502$
LLP	12.07 ± 0.87^{a}	10.44 ± 1.24^{a}	6.23 ± 0.79^{b}	3.80 ± 0.27^{c}	$H_{(3,89)} = 49.78$
	(30) 4.0–21.0	(16) 3.0–23.0	(13) 2.0–11.0	(30) 1.0–7.0	$P = 0.0000$
LSL	5.15 ± 0.30^{a}	10.80 ± 0.83^{b}	15.77 ± 1.12^{c}	14.22 ± 0.68^{c}	$H_{(3,89)} = 61.98$
	(30) 3.0–11.7	(16) 7.0–20.0	(13) 9.8– 24.1	(30) 7.9–22.8	$P = 0.0000$
LBL	14.40 ± 0.72^{a}	22.52 ± 1.33^{b}	34.43 ± 3.14^{c}	35.06 ± 2.38^{c}	$H_{(3,89)} = 55.55$
	(30) 7.0–22.2	(16) 15.0–32.8	(13) 19.5–58.1	(30) 11.6–65.4	$P = 0.0000$
LBW	5.84 ± 0.21^{a}	6.18 ± 0.23^{ab}	6.92 ± 0.25^{b}	5.10 ± 0.18^{c}	$F = 10.66$
	(30) 3.6–7.6	(16) 4.4–7.9	(13) 5.8–8.9	(30) 3.4–7.5	$P = 0.0000$
LLL	0.41 ± 0.02^{a}	0.93 ± 0.08^{b}	0.98 ± 0.05^{b}	1.03 ± 0.09^{b}	$H_{(3,89)} = 52.89$
	(30) 0.2–0.7	(16) 0.4–1.9	(13) 0.7–1.3	(30) 0.5–2.1	$P = 0.0000$
IIL	11.33 ± 0.28^{a}	15.94 ± 0.80^{b}	17.97 ± 1.70^{b}	16.58 ± 0.85^{b}	$H_{(3,83)} = 29.38$
	(26) 8.5–14.1	(14) 9.2–19.6	(13) 8.1–25.2	(30) 8.5–28.5	$P = 0.0000$
ISL	8.93 ± 0.21^{a}	11.60 ± 0.58^{b}	7.47 ± 0.54^{c}	3.60 ± 0.18^{d}	$H_{(3,83)} = 64.61$
	(26) 6.0–10.8	(14) 7.7–15.4	(13) 4.8–11.0	(30) 2.0–6.0	$P = 0.0000$
INS	2.85 ± 0.14^{a}	3.00 ± 0.18^{a}	7.23 ± 1.06^{b}	7.03 ± 0.50^{b}	$H_{(3,83)} = 55.74$
	(26) 2.0–5.0	(14) 2.0–4.0	(13) 4.0–18.0	(30) 3.0–15.0	$P = 0.0000$
SPD	1.32 ± 0.10^{ab}	1.61 ± 0.11^{b}	1.17 ± 0.07^{a}	1.36 ± 0.05^{ab}	$H_{(3,80)} = 9.00$
	(23) 0.5–2.3	(14) 1.1–2.5	(13) 0.7–1.5	(30) 1.0–2.0	$P = 0.0293$
SLG	8.10 ± 0.26^{a}	5.10 ± 0.38^{b}	4.42 ± 0.17^{b}	1.78 ± 0.12^{c}	$H_{(3,80)} = 67.52$
	(23) 5.7–10.4	(14) 2.8–7.9	(13) 3.2–5.5	(30) 1.0–4.8	$P = 0.0000$
SUG	12.31 ± 0.28^{a}	12.57 ± 0.36^{a}	11.55 ± 0.34^{a}	7.57 ± 0.14^{b}	$F = 105.17$
	(23) 9.1–15.6	(14) 9.3–14.5	(13) 9.7–14.4	(30) 5.5–9.6	$P = 0.0000$
SGR	1.54 ± 0.04^{a}	2.67 ± 0.25^{b}	2.66 ± 0.12^{b}	4.64 ± 0.24^{c}	$H_{(3,80)} = 60.43$
	(23) 1.1–1.9	(14) 1.8–5.2	(13) 2.1–3.4	(30) 1.5–8.3	$P = 0.0000$
SGW	1.23 ± 0.03^{a}	1.50 ± 0.04^{b}	1.24 ± 0.04^{a}	1.02 ± 0.02^{c}	$H_{(3,80)} = 46.96$
	(23) 1.0–1.5	(14) 1.2–1.8	(13) 1.0–1.6	(30) 0.8–1.2	$P = 0.0000$
SLM	9.68 ± 0.22^{a}	9.26 ± 0.27^{ab}	8.62 ± 0.17^{b}	6.19 ± 0.08^{c}	$H_{(3,80)} = 59.36$
	(23) 6.6–11.5	(14) 7.5–11.2	(13) 7.8–9.8	(30) 5.2–7.1	$P = 0.0000$
SPL	10.90 ± 0.26^{a}	10.38 ± 0.27^{ab}	10.11 ± 0.21^{b}	6.60 ± 0.08^{c}	$H_{(3,80)} = 56.92$
	(23) 7.0–13.0	(14) 8.2–11.6	(13) 9.0–11.6	(30) 5.8–7.4	$P = 0.0000$
SNS	1.13 ± 0.30^{a}	0.00 ± 0.00^{b}	0.00 ± 0.00^{b}	2.57 ± 0.17^{c}	$H_{(3,80)} = 47.41$
	(23) 0.0–3.0	(14) 0.0–0.0	(13) 0.0–0.0	(30) 0.0–3.0	$P = 0.0000$
ANR	30.79 ± 0.79^{a}	30.56 ± 0.93^{a}	32.69 ± 0.72^{a}	18.97 ± 0.72^{b}	$H_{(3,89)} = 55.97$
	(30) 22.0–40.0	(16) 23.0–36.0	(13) 29.0–37.0	(30) 12.0–30.0	$P = 0.0000$
ARR	0.97 ± 0.01^{a}	0.78 ± 0.02^{b}	0.71 ± 0.02^{c}	0.58 ± 0.01^{d}	$H_{(3,89)} = 74.12$
	(30) 0.9–1.0	(16) 0.7–1.0	(13) 0.6–0.8	(30) 0.4–0.8	$P = 0.0000$
AAC	0.76 ± 0.07^{a}	0.38 ± 0.08^{b}	0.64 ± 0.10^{ab}	0.42 ± 0.07^{b}	$H_{(3,89)} = 18.88$
	(30) 0.0–1.0	(13) 0.0–1.0	(16) 0.0–0.9	(30) 0.0–1.0	$P = 0.0003$

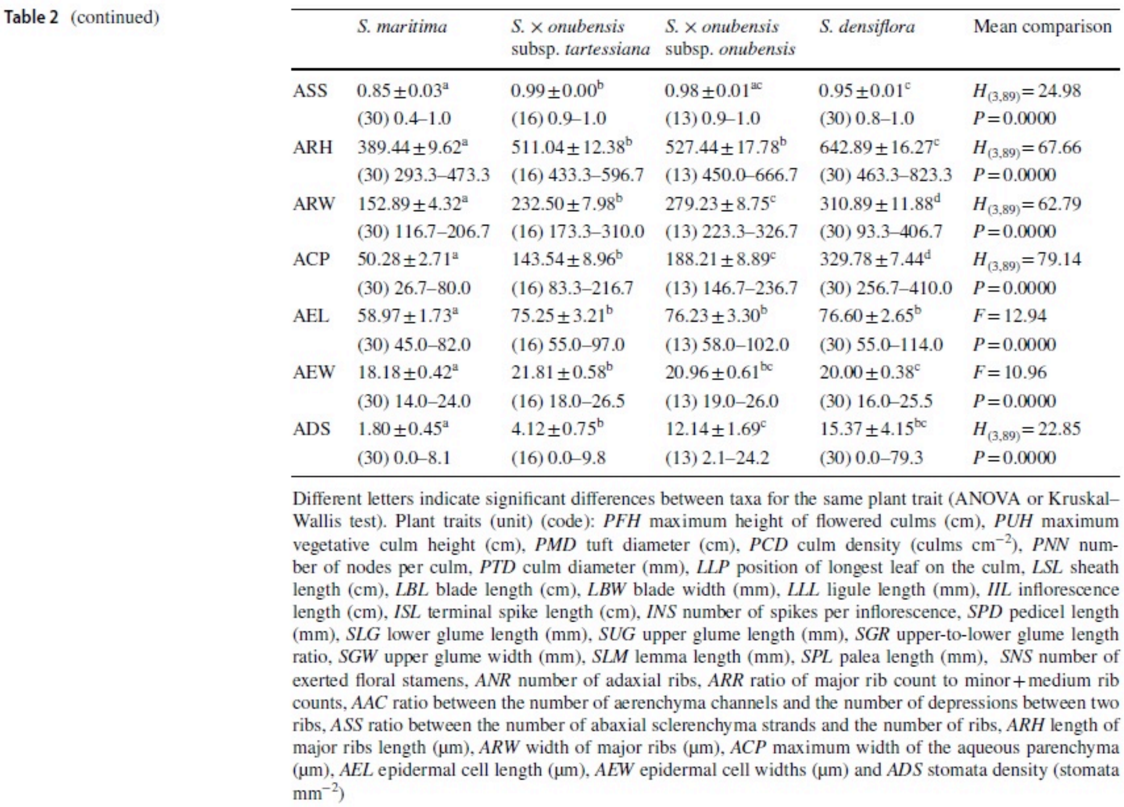


Table 2 (continued)

	S. maritima	*S.* × *onubensis* subsp. *tartessiana*	*S.* × *onubensis* subsp. *onubensis*	*S. densiflora*	Mean comparison
ASS	0.85 ± 0.03^{a}	0.99 ± 0.00^{b}	0.98 ± 0.01^{ac}	0.95 ± 0.01^{c}	$H_{(3,89)}=24.98$
	(30) 0.4–1.0	(16) 0.9–1.0	(13) 0.9–1.0	(30) 0.8–1.0	$P=0.0000$
ARH	389.44 ± 9.62^{a}	511.04 ± 12.38^{b}	527.44 ± 17.78^{b}	642.89 ± 16.27^{c}	$H_{(3,89)}=67.66$
	(30) 293.3–473.3	(16) 433.3–596.7	(13) 450.0–666.7	(30) 463.3–823.3	$P=0.0000$
ARW	152.89 ± 4.32^{a}	232.50 ± 7.98^{b}	279.23 ± 8.75^{c}	310.89 ± 11.88^{d}	$H_{(3,89)}=62.79$
	(30) 116.7–206.7	(16) 173.3–310.0	(13) 223.3–326.7	(30) 93.3–406.7	$P=0.0000$
ACP	50.28 ± 2.71^{a}	143.54 ± 8.96^{b}	188.21 ± 8.89^{c}	329.78 ± 7.44^{d}	$H_{(3,89)}=79.14$
	(30) 26.7–80.0	(16) 83.3–216.7	(13) 146.7–236.7	(30) 256.7–410.0	$P=0.0000$
AEL	58.97 ± 1.73^{a}	75.25 ± 3.21^{b}	76.23 ± 3.30^{b}	76.60 ± 2.65^{b}	$F=12.94$
	(30) 45.0–82.0	(16) 55.0–97.0	(13) 58.0–102.0	(30) 55.0–114.0	$P=0.0000$
AEW	18.18 ± 0.42^{a}	21.81 ± 0.58^{b}	20.96 ± 0.61^{bc}	20.00 ± 0.38^{c}	$F=10.96$
	(30) 14.0–24.0	(16) 18.0–26.5	(13) 19.0–26.0	(30) 16.0–25.5	$P=0.0000$
ADS	1.80 ± 0.45^{a}	4.12 ± 0.75^{b}	12.14 ± 1.69^{c}	15.37 ± 4.15^{bc}	$H_{(3,89)}=22.85$
	(30) 0.0–8.1	(16) 0.0–9.8	(13) 2.1–24.2	(30) 0.0–79.3	$P=0.0000$

Different letters indicate significant differences between taxa for the same plant trait (ANOVA or Kruskal–Wallis test). Plant traits (unit) (code): *PFH* maximum height of flowered culms (cm), *PUH* maximum vegetative culm height (cm), *PMD* tuft diameter (cm), *PCD* culm density (culms cm^{-2}), *PNN* number of nodes per culm, *PTD* culm diameter (mm), *LLP* position of longest leaf on the culm, *LSL* sheath length (cm), *LBL* blade length (cm), *LBW* blade width (mm), *LLL* ligule length (mm), *IIL* inflorescence length (cm), *ISL* terminal spike length (cm), *INS* number of spikes per inflorescence, *SPD* pedicel length (mm), *SLG* lower glume length (mm), *SUG* upper glume length (mm), *SGR* upper-to-lower glume length ratio, *SGW* upper glume width (mm), *SLM* lemma length (mm), *SPL* palea length (mm), *SNS* number of exerted floral stamens, *ANR* number of adaxial ribs, *ARR* ratio of major rib count to minor + medium rib counts, *AAC* ratio between the number of aerenchyma channels and the number of depressions between two ribs, *ASS* ratio between the number of abaxial sclerenchyma strands and the number of ribs, *ARH* length of major ribs length (µm), *ARW* width of major ribs (µm), *ACP* maximum width of the aqueous parenchyma (µm), *AEL* epidermal cell length (µm), *AEW* epidermal cell widths (µm) and *ADS* stomata density (stomata mm^{-2})

onubensis, while these traits were intermediate for *S.* × *onubensis* subsp. *tartessiana*. On the contrary, blade width was less (< – 13%) for *S. densiflora* than for *S. maritima* and *S.* × *onubensis* subsp. *tartessiana*, and *S.* × *onubensis* subsp. *onubensis* had the widest leaf blades. The ligula was shorter (< – 56%) for *S. maritima* than for the other three taxa (Table 2).

In relation to the inflorescences, their length was lower (< – 29%) for *S. maritima* than for the other three taxa, whereas the terminal spike length was the lowest for *S. densiflora* followed by *S.* × *onubensis* subsp. *onubensis* and *S. maritima*, with *S.* × *onubensis* subsp. *tartessiana* presenting the highest values (> + 22%). *S. densiflora* and *S.* × *onubensis* subsp. *onubensis* had more (> + 57%) spikes per inflorescence than *S. maritima* and *S.* × *onubensis* subsp. *tartessiana* (Table 2). *Spartina* × *onubensis* subsp. *tartessiana* had longer (+ 27%) spikelet pedicels than *S.* × *onubensis* subsp. *onubensis*. Every taxon had hairy glumes. The lower and the upper glumes were longer for *S. maritima* than for *S. densiflora*, with both hybrids showing intermediate values for the lower glume and similar values than *S. maritima* for the upper glume (Fig. 1). The ratio of upper to lower glume length was smaller (– 67%) for *S. maritima* than for *S. densiflora*, being intermediate between parents for both hybrids. The upper glume was wider (> + 18%) for *S. maritima* and *S.* × *onubensis* subsp. *onubensis* than for *S. densiflora*, with *S.* × *onubensis* subsp. *tartessiana* showing the widest upper glumes. In florets, the lemma and the palea were both wider for *S. maritima* and *S.* × *onubensis* subsp. *tartessiana* than for *S. densiflora*, while intermediate widths were recorded for *S.* × *onubensis* subsp. *onubensis*. With respect to the number of stamens exerted, both parents had most of their stamens exerted, while the hybrids showed none exerted stamens (Table 2).

Through anatomical evaluation of foliar traits, we observed that *S. maritima* and both hybrids had more (> + 42%) adaxial ribs than *S. densiflora*. The ratio of major ribs to minor and medium ribs counts was maximum for *S. maritima* and minimum for *S. densiflora*, but intermediate for both hybrids. *Spartina maritima* had more (> + 50%) aerenchyma channels than *S. densiflora* and both hybrids. In contrast, the ratio between abaxial sclerenchyma strands and adaxial rib counts was maximum for *S. densiflora* and both hybrids. The height and width of ribs and the width of the aqueous parenchyma were minimum for *S. maritima*, intermediate for both hybrids and maximum for *S. densiflora*. Epidermal cells were shorter (< – 23%) and thinner (< – 17%) for *S. maritima* than for *S. densiflora* and both hybrids. The density of stomata on the abaxial foliar side was the lowest for *S. maritima* followed by *S.* × *onubensis* subsp. *tartessiana*, *S.* × *onubensis* subsp. *onubensis* and *S. densiflora* (Table 2; Fig. 2).

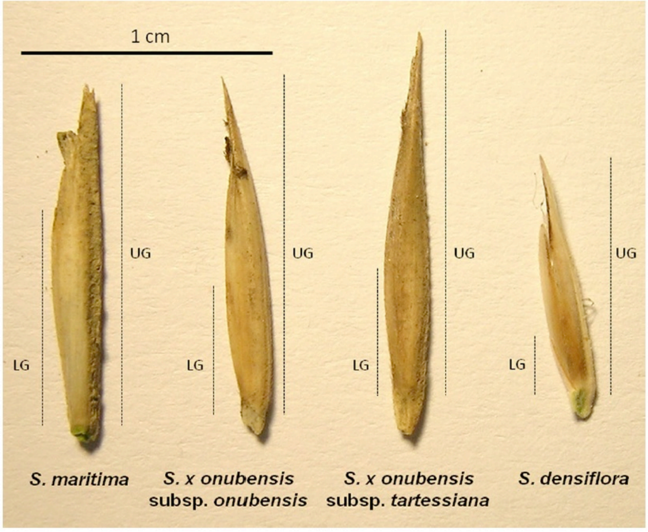


Fig. 1 Photographs of spikelets of native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids in the Gulf of Cadiz (Southwest Iberian Peninsula). Plant characters: Length of lower glume (LG) and upper glume (UG)

Considering all these morphological and anatomical results, the PCA clearly separated the parental species into discrete groups (Fig. 3). Hybrid taxa were also clearly differentiated from parental taxa with only minimal overlap between some hybrid individuals (Fig. 3). The first component (PCA 1) explained 41.6% of the variance, ordinating the studied taxa along Axis 1 (Fig. 3). This axis was derived from data of 19 plant characters with factor loadings > 0.600. Nine of these characters had positive factor loadings, with highest values for *S. densiflora* and its maternal hybrid *S.* × *onubensis* subsp. *onubensis*. The other ten plant characters had negative factor loadings with higher values for *S. maritima* and its maternal hybrid *S.* × *onubensis* subsp. *tartessiana* (Table 3). Thus, *S. densiflora* and *S.* × *onubensis* subsp. *onubensis* presented higher culm densities with longer leaves, more spikes per inflorescence and bigger leaf ribs with more aqueous parenchyma than *S. maritima* and *S.* × *onubensis* subsp. *tartessiana* that produced larger tufts, longer spikelets and more foliar

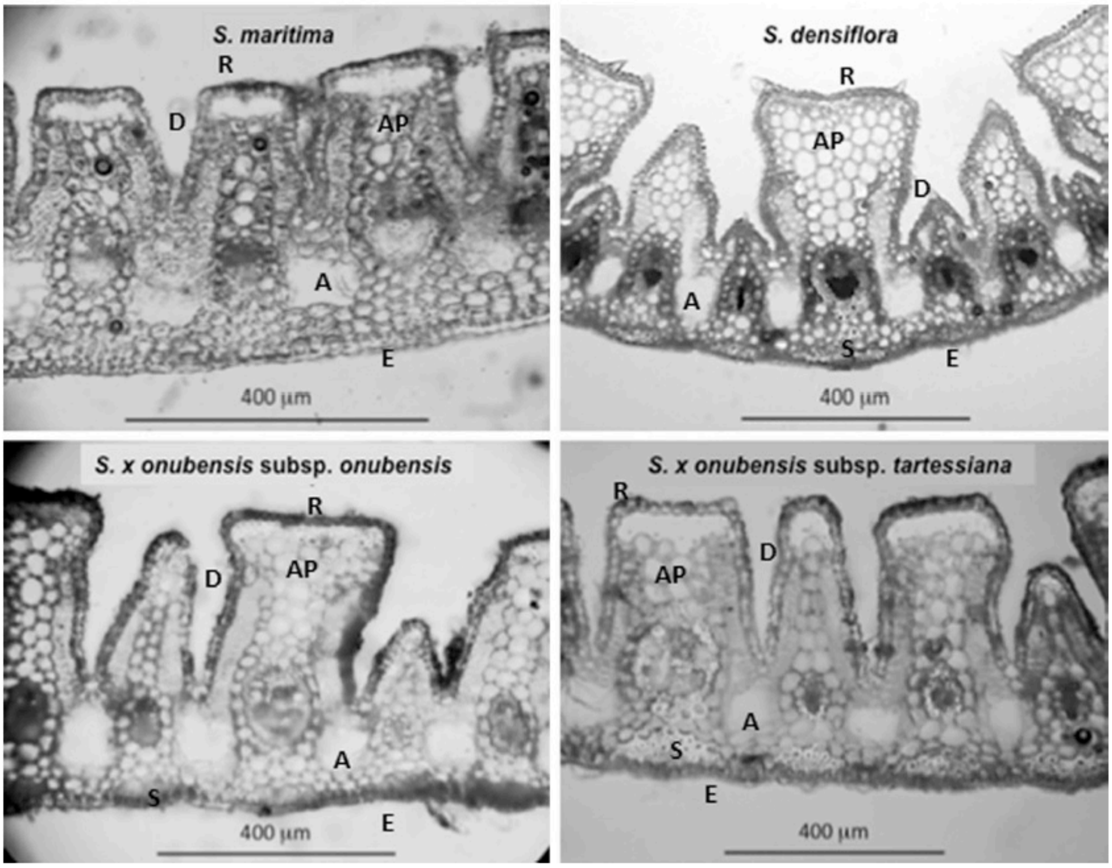


Fig. 2 Transversal cuts at the central area of the leaves of native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids. Recorded anatomical structures: leaf *A* aerenchyma channel; *AP* aqueous parenchyma; *D* depressions between two ribs; *E* epidermis; *R* adaxial rib; *S* abaxial sclerenchyma strand

ribs. The second component (PCA 2) explained 15.7% of the variance and separated both hybrids from their parents along Axis 2 (Fig. 3), which was positively correlated with the height of flowering and unflowered culms, being both hybrids taller than their parents. Axis 2 was negatively correlated with just one character, the number of exerted stamens, that was maximum for both parental species since neither hybrid had exerted stamens (Table 3).

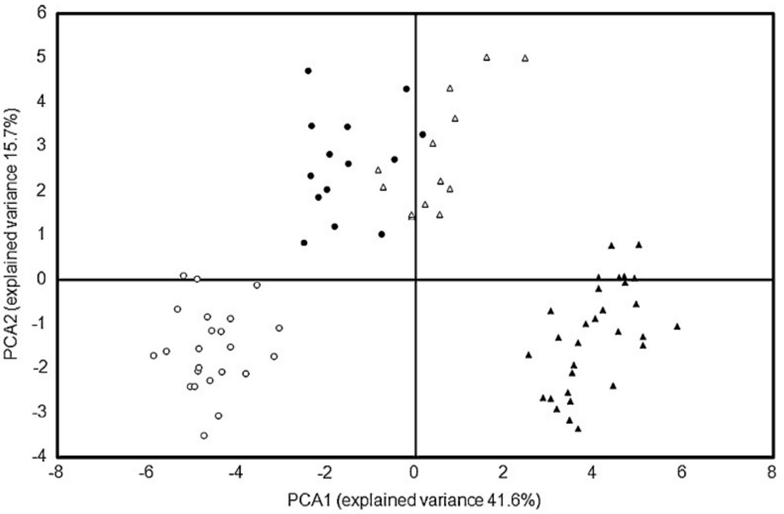


Fig. 3 Principal component analysis plots for native *Spartina maritima* (white circles), invasive *S. densiflora* (black triangles) and their reciprocal hybrids *S.* × *onubensis subsp. tartessiana* (black circles) and *S.* × *onubensis subsp. onubensis* (white triangles) in the Gulf of Cadiz (Southwest Iberian Peninsula) based on 32 morphological and anatomical plant characters

Table 3 Explained variance and factor loadings for the three first factors obtained by principal component analysis using 32 plant morphological and anatomical traits for native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids in the Gulf of Cadiz (Southwest Iberian Peninsula)

	PCA 1	PCA 2	PCA 3
% Total var.	41.60	15.72	7.22
PIH	+ 0.35	**+ 0.84**	+ 0.06
PVH	+ 0.31	**+ 0.82**	+ 0.25
PMD	**− 0.61**	− 0.36	− 0.26
PTD	**+ 0.61**	+ 0.17	+ 0.48
PNN	**− 0.68**	+ 0.36	+ 0.35
PSD	− 0.02	+ 0.46	− 0.51
LLP	**− 0.77**	+ 0.01	+ 0.17
LSL	**+ 0.76**	+ 0.44	− 0.08
LBL	**+ 0.72**	+ 0.32	− 0.19
LBW	− 0.32	+ 0.58	− 0.49
LLL	**+ 0.61**	+ 0.34	− 0.09
IIL	+ 0.46	+ 0.48	− 0.35
ISL	**− 0.73**	+ 0.48	+ 0.15
INS	+ 0.67	+ 0.21	− 0.58
SPD	− 0.07	+ 0.08	+ 0.36
SLG	**− 0.93**	+ 0.02	− 0.22
SUG	**− 0.84**	+ 0.38	+ 0.02
SGR	**+ 0.80**	− 0.18	+ 0.23
SGW	− 0.55	+ 0.47	+ 0.28
SLM	**− 0.88**	+ 0.28	− 0.03
SPL	**− 0.87**	+ 0.33	− 0.08
SNS	+ 0.43	**− 0.61**	− 0.07
ANR	**− 0.72**	+ 0.49	− 0.20
ARR	**− 0.91**	− 0.09	− 0.14
AAC	+ 0.49	+ 0.30	+ 0.15
ASS	+ 0.30	+ 0.59	+ 0.11
ARH	+ 0.40	+ 0.00	− 0.46
ARW	**+ 0.83**	+ 0.07	− 0.03
ACP	**+ 0.82**	+ 0.16	− 0.03
AEL	**+ 0.96**	− 0.04	+ 0.05
AEW	− 0.28	− 0.06	− 0.06
ADS	+ 0.26	+ 0.48	+ 0.34

Factor loadings > 0.600 are marked in bold. See Table 2 for plant characters codes

Phenotypic inheritance

In *Spartina* × *onubensis* subsp. *onubensis*, 34% of the evaluated plant characters had intermediate values between both parents. Ten percent of the characters presented transgressive vigorous inheritance, and 31% of the traits of the hybrids were dominated by *S. densiflora*. A smaller number of traits, 13%, were dominated by *S. maritima* and 9% were co-dominated by both parents (Table S1, Fig. 4). In *S.* × *onubensis* subsp. *tartessiana*, 25% of the measured characters exhibited values that were intermediate between both parents, and were significantly different from parental species. Transgressive vigorous inheritance was detected for 22% of the traits. Each parental species dominated the expression of 22% of traits and 6% of traits were co-dominated by both parents. Only the number of exerted stamens presented a transgressive depressed inheritance for both hybrids (Table S1, Fig. 4).

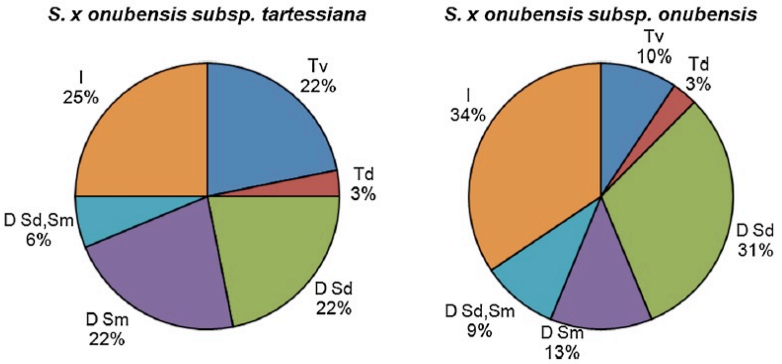


Fig. 4 Percentages of phenotypic inheritances for two reciprocal hybrids between native *Spartina maritima* and invasive *S. densiflora*. Phenotypic inheritance types: *Tv* transgressive vigorous; *Td* transgressive depressed; *D-Sd* dominated by *S. densiflora*; *D-Sm* dominated by *S. maritima* parent; *D-Sd, Sm* dominated by the both parents; *I* intermediate value between both parents

Plant trait expression with variation in sediment environment

Spartina maritima had more nodes per culm, shorter spike pedicels and less aerenchyma channels when growing in low marshes with higher sediment water content (ca. 60%). Culm density of *S. maritima* was lower and its leaf sheaths longer at higher sediment pH (ca. 7.8). The maximum width of the aqueous parenchyma and the ratio between the number of abaxial sclerenchyma strands and the number of ribs increased on plants growing in sediments with higher conductivity (ca. 16 mS cm^{-1}) (Tables 2, 4; Table S2). On the other hand, *S. densiflora* presented shorter inflorescences, lower stomatal density and more aerenchyma channels in middle marshes with higher sediment water content (80%) and more organic matter (28%). When growing on less acidic sediments (pH ca. 8.0), *S. densiflora* showed less aerenchyma channels, taller flowered culms, larger leaves with more adaxial ribs and higher stomatal densities, longer spikes and longer inflorescences with more spikes. Moreover, *S. densiflora* also showed shorter inflorescence with less spikes and lower stomatal densities in middle marshes with higher conductivity (ca. 16 mS cm^{-1}) (Tables 2, 4; Table S2).

Spartina × *onubensis* subsp. *tartessiana* showed wider leaves with more adaxial ribs, wider upper glumes and shorter lemma and palea growing in intertidal ponds and middle marshes with higher sediment water content (ca. 66%) than in the low marshes (ca. 37%). The number of culm nodes and of adaxial foliar ribs increased together with sediment organic matter (changing between 7 and 27%) and with conductivity (varying between 10.1 and 16.4 mS cm^{-1}). Moreover, plant specimens from middle marshes and intertidal ponds with higher conductivity also produced longer inflorescences (Tables 2, 4; Table S2). *Spartina* × *onubensis* subsp. *onubensis* from middle marshes had more nodes per culm, higher position of the longest leaf on the culm, and smaller leaves and epidermal cells on less basic sediments (varying between pH 7.2 and 8.1) in sediments with higher salinity (conductivity ca. 16 mS cm^{-1}) and in sediments with higher water and organic matter content (69% and 27%, respectively). Its flowering culms were shorter and thinner in more organic sediments with higher salinities, and its culm density increased and the number of spikes per inflorescence decreased at saltier sediments (Tables 2, 4; Table S2).

Discussion

Our results showed that populations of both reciprocal hybrids of native *Spartina maritima* and invasive *S. densiflora* have unique morphological and anatomical characteristics that can be used to distinguish them from their parental species and each other. Thus, floral traits such as the length of the terminal spike, the spikelet and its glumes and lemma allow us to differentiate *S. densiflora* (with lower values) from the other three taxa. The presence of exerted stamens, shorter culms and shorter leaf blades separated *S. maritima* from both hybrids, which were set apart among them since *S.* × *onubensis* subsp. *onubensis* showed more spikes per inflorescence and longer leaves than *S.* × *onubensis* subsp. *tartessiana*. On the other hand, plants from the same habitat for a given taxon were more alike than those from the same location and estuary, which pointed to relatively high phenotypic plasticity for some plant traits as reported previously for different *Spartina* species (Thompson 1990; Di Bella et al. 2014; Castillo et al. 2018). In this sense, a common garden experiment should be carried out to determine whether distinct phenotypes recorded at contrasted habitats in the field are due to genetic differentiation and/or phenotypic plasticity.

Eighteen out of the 32 assessed plant traits (56%) maintained the same phenotypic inheritance type for both *Spartina* hybrids. Two of them (6%) were transgressive vigorous: the height of flowered and unflowered culms, although *S.* × *onubensis* subsp. *onubensis* showed taller flowered culms than *S.* × *onubensis* subsp. *tartessiana*. Castillo et al. (2010) previously reported taller culms for these hybrids than for their parents in Guadiana Estuary, and Ayres et al. (2004a, b) also reported much taller culms for some hybrids between native *S. foliosa* and invasive *S. alterniflora* in San Francisco Bay. Only one character (3%), the number of exerted stamens, presented a transgressive depressed behavior for both hybrids, reflecting their infertility as for other F1 *Spartina* hybrids (Marchant 1968a, b; Ayres et al. 1999). These three characters with

Table 4 Spearman correlation coefficient for significant correlations ($P < 0.05$) between plant characters of native *Spartina maritima*, invasive *S. densiflora* and their reciprocal hybrids, and water content (*WC*), organic matter content (*OMC*), pH and conductivity (*CO*) of the sediments

Plant character	*S. maritima*				*S.* × *onubensis* subsp. *tartessiana*				*S.* × *onubensis* subsp. *onubensis*				*S. densiflora*			
	WC	OMC	pH	CO	WC	OMC	pH	CO	WC	OMC	pH	CO	WC	OMC	pH	CO
PIH	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.64**	**+ 0.57**	**− 0.73**	n.s.	n.s.	**+ 0.47**	n.s.
PTD	n.s.	n.s.	**− 0.48**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**+ 0.60**	n.s.	n.s.	n.s.	n.s.
PNN	**+ 0.43**	n.s.	n.s.	n.s.	n.s.	**+ 0.54**	n.s.	**+ 0.54**	**+ 0.75**	**+ 0.73**	**− 0.77**	**+ 0.71**	n.s.	n.s.	n.s.	n.s.
PSD	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.55**	n.s.	**− 0.57**	n.s.	n.s.	n.s.	n.s.
LLP	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**+ 0.80**	**+ 0.75**	**− 0.79**	**+ 0.69**	n.s.	n.s.	n.s.	n.s.
LSL	n.s.	n.s.	**+ 0.46**	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.62**	**− 0.62**	**+ 0.68**	**− 0.61**	n.s.	n.s.	**+ 0.49**	n.s.
LBL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.69**	**− 0.77**	**+ 0.77**	**− 0.82**	n.s.	n.s.	**+ 0.65**	n.s.
LBW	n.s.	n.s.	n.s.	n.s.	**+ 0.53**	n.s.	n.s.	**+ 0.58**	n.s.	n.s.	**+ 0.56**	n.s.	n.s.	n.s.	**+ 0.51**	n.s.
IIL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**+ 0.62**	n.s.	n.s.	n.s.	n.s.	**− 0.57**	**− 0.54**	**+ 0.45**	**− 0.51**
INS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.55**	n.s.	n.s.	**+ 0.50**	**− 0.47**
SPD	**− 0.50**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SGR	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.64**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SGW	n.s.	n.s.	n.s.	n.s.	**+ 0.56**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SLM	n.s.	n.s.	n.s.	n.s.	**− 0.60**	n.s.	**− 0.60**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
SPL	n.s.	n.s.	n.s.	n.s.	**− 0.57**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ANR	n.s.	n.s.	n.s.	n.s.	**+ 0.66**	**+ 0.62**	**+ 0.74**	**+ 0.62**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**+ 0.37**	n.s.
AAC	**− 0.53**	**− 0.42**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.40**	**+ 0.39**
ASS	n.s.	n.s.	n.s.	**+ 0.43**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ACP	n.s.	n.s.	n.s.	**+ 0.44**	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
AEL	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.61**	**− 0.56**	**+ 0.55**	n.s.	n.s.	n.s.	n.s.	n.s.
AEW	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.68**	**− 0.66**	**+ 0.67**	**− 0.64**	n.s.	n.s.	n.s.	n.s.
ADS	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	**− 0.56**	**− 0.51**	**+ 0.37**	**− 0.50**

See Table 2 for plant character abbreviations. *n.s.* not significant transgressive phenotypes for both hybrids allowed us to clearly distinguish the hybrids from the parental species across the measured environmental conditions. On the other hand, five characters were transgressive for just one hybrid (leaf blade width, culm nodes number, terminal spike length, upper glume width and epidermal cell width), with culm nodes number and leaf blade width changing depending on the sedimentary environment for three out of the four taxa. Terminal spike length was intermediate between both parents for *S.* × *onubensis* subsp. *onubensis*, but it was transgressive vigorous for *S.* × *onubensis* subsp. *tartessiana*, which made it useful to identify this hybrid in the field. This may be a result of the diploid maternal influence of *S. maritima* (Yao et al. 2013).

Only two plant characters (culm diameter and spikelet pedicel length) (6%) were co-dominated by both parents for both hybrids, so they were not useful to differentiate hybrids from parents regardless of environmental setting. On the other hand, six characters (19%), related to glumes morphology and foliar anatomy, presented intermediate and significantly different values from the parents, for both hybrids. These intermediate characters were useful to differentiate both hybrids from the parental species (e.g., the ratio between upper and lower glume lengths, lower glume and major ridge lengths) and some of them also separated the hybrids between each other (e.g., the ratio between the number of major ribs and minor plus medium ribs, midrib width and aqueous parenchyma width). Thus, *S.* × *onubensis* subsp. *onubensis* showed wider foliar ribs with more aqueous parenchyma than *S.* × *onubensis* subsp. *tartessiana*, reflecting the maternal effect of *S. densiflora*. A low ratio between the number of foliar major ribs and shorter ridges, large major ridges and low number of ribs, as recorded in *S. densiflora*, all facilitate the longitudinal rolling of the leaf on its adaxial surface as a way to avoid leaf desiccation. These results are in accordance with Maricle et al. (2009) reporting higher ratios and smaller ribs for *Spartina* species from low marshes. In this sense, *S. maritima* increased the presence of foliar sclerenchyma and aqueous parenchyma when growing at higher salinities, but showing always less foliar sclerenchyma than *S. densiflora*. *Spartina* species differ in the distribution of sclerenchyma in leaves being more developed in high-marsh species (Maricle et al. 2009).

The maternal effect of both parents was recorded for both hybrids in just one character (3%), the number of spikes per inflorescence that was dominated by *S. maritima* in *S.* × *onubensis* subsp. *tartessiana* and by *S. densiflora* in *S.* × *onubensis* subsp. *onubensis*. Thus, this trait was useful to separate the two hybrids. However, the number of spikes per inflorescence decreased on more acidic sediments with higher salinities for *S. densiflora* and on sediments with lower organic matter contents for its maternal hybrid, which pointed to nutrient availability as limiting factor for the flowering (Tilman and Wedin 1991). Inflorescence morphology is central to the delimitation of grass taxa at all levels (Clayton and Renvoize 1986; Kern et al. 2008). In contrast to our results, the inflorescence structures of the hybrid *S.* × *townsendii* and of the allopolyploid *S. anglica* were similar to the masculine parent *S. maritima* (Saarela 2012).

Twelve characters (38%) were dominated by the neophyte *S. densiflora* for any of the two hybrids, and five of them were shared by both hybrids: culm density, length of the ligulas, inflorescences and epidermal cells, and stomatal density. Culm density and ligula length were characters easily to measure in the field without needing the plants to be flowering, so they may be useful to differentiate *S. maritima* from the other three taxa; however, culm density of *S. maritima* and *S.* × *onubensis* subsp. *onubensis* changed depending on the sedimentary environment. On the other hand, stomatal density was higher for *S.* × *onubensis* subsp. *onubensis* than for *S.* × *onubensis* subsp. *tartessiana*, reflecting again the maternal effect of *S. densiflora*, which lowered its stomatal density on saltier sediments at higher elevation on the intertidal gradient probably to limit water loss through transpiration (Maricle et al. 2009).

When compared in the similar sedimentary environments, the maternal effect of *S. densiflora* was also recorded in the lengths of the sheath and the blade that were intermediate between both parents for *S.* × *onubensis* subsp. *tartessiana*, and longer and dominated by *S. densiflora* for *S.* × *onubensis* subsp. *onubensis*. Thus, these were interesting characters to differentiate between hybrids in their vegetative state, even when leaf morphology is often a highly plastic trait in response to environmental conditions such as light or soil nutrient availability (Castillo et al. 2014). Thus, *S.* × *onubensis* subsp. *tartessiana* presented wider leaves in waterlogged and saltier sediments, *S.* × *onubensis* subsp. *onubensis* showed larger leaves on sediments with higher organic matter contents, and *S. densiflora* produced larger leaves at less acidic sediments, which may be related to nutrients availability as reported for *S. alterniflora* (Darby and Turner 2008).

The dominance of native *S. maritima* appeared in nine characters (28%) in any of the hybrids, with two of them shared by both hybrids: upper glume length and number of adaxial foliar ribs. These two characters were useful to separated *S. densiflora* from the other three taxa. On the other hand, the maternal effect of *S. maritima* was reflected in the position of the longest leaf on the culm, and the length of the lemma and palea that were intermediate between both parents for *S.* × *onubensis* subsp. *onubensis* and dominated by *S. maritima* for *S.* × *onubensis* subsp. *tartessiana*.

The recorded differences between the two studied hybrids and comparison of our results with similar evaluations make us think that heritability of morphological and anatomical characters expressed by different species crosses do not follow easily predictable rules, since the mechanisms regulating gene expression in hybrids are not completely understood (Yoo et al. 2014; Bird et al. 2018). For example, Mobberley (1953) found a very distinct inheritance profile than ours studying twenty plant traits of *S.* × *caespitosa*, out of which ten presented parental additivity, whereas six of these traits were transgressive vigorous or dominated by parental species in our study. In this sense, hybrids swarms between *S. foliosa* and *S. alterniflora* in California were highly variable in vegetative, floral and reproductive aspects, as much as in phenological and ecological aptitudes, being some of them transgressive (Strong and Ayres 2013). Rieseberg et al. (1999) found that 97% of hybrids showed at least one transgressive character, and 58% of examined traits were transgressive from a survey of 171 studies that reported phenotypic variation in segregating hybrid populations, but transgressivity was between 13 and 25% in our study. Baumel et al. (2003) identified multiple factors that appear to contribute to the expression of transgressive traits in *S.* × *neyrautii* and *S.* × *townsendii*, both with *S. alterniflora* as the seed parent but proceeding from different genotypes of *S. alterniflora* and *S. maritima*. In this context, our finding suggests that different ploidy levels of the studied hybrids (*S.* × *onubensis* subsp. *onubensis* is a homoploid, while *S.* × *onubensis* subsp. *tartessiana* is a triploid), maternal and parental effects when there is parental sex reversal as in our reciprocal hybrids, and genotypic differences between parents may influence trait expression in their hybrids.

Taxonomic treatment

Both hybrids share the same parental species, yet they are morphologically and anatomically distinguishable and they have different chromosome numbers and different seed parents. Given these factors, they should not be published as two different nothospecies in accordance with article H4.1 of the International Code of Nomenclature (Turland et al. 2018). We therefore propose they should be assigned subspecies rank.

Key for species and new hybrids

1a. Glumes with longest hairs < 0.2 mm; terminal spike 2-6 cm; spikelet 5.5-10 mm long; lower glume 1-3.5(5) mm; upper glume 5.5-10 mm; lemma 5-7.5 mm ……………………………..…………………………………………….. ***S. densiflora***

1b. Glumes with longest hairs ≥ 0.2 mm long; terminal spike 4.8-15.4 cm; spikelet 9-16 mm; lower glume 2.5-10.5 mm; upper glume 9-16 mm; lemma 6.5-12 mm……………………………………………………………………………… 2

2a. Stamens usually exerted from the florets; Culm 30.5-89.5 cm; leaf blade 7-23 cm long; lower glume 5.5-10.5 mm……………………………..… ***S. maritima***

2b. Stamens never exerted from the florets; Culm 85-188 cm; leaf blade 15-58.5 cm long; lower glume 2.5-6.5(8) mm
……………………………..…………………………………………………… 3

3a. Glumes with longest hairs < 0.35 mm; inflorescence with 4-18 spikes, the upper glume 4.5-11 cm…………………….. ***S. × onubensis* subsp. *onubensis***

3b. Glumes with longest hairs > 0.40 mm; inflorescence with 2-4 spikes, the upper glume 7.5-15.5 cm………………….. ***S. × onubensis* subsp. *tartessiana***

Spartina maritima (Curtis) Fernald, Rhodora 18: 180. 1916.

Description: Rhizomatous perennials, sparsely caespitose with 1.5-12.5 culms dm^{-2}. Culm 30-90 cm × 1.8-4 mm, (6)10-27 nodes. Longest leaf of the culm with sheath 3-8(12) cm long; ligule 0.2-0.7 mm long; blade 7-23 × 3.5-8 cm. Inflorescence 8-15 cm long, a raceme with 2-5 spikes, the upper 6-11 cm long. Spikelet 9-16 mm long, with 1 floret. Pedicel 0.5-2.3 mm long. Glumes 2, unequal, with hairs up to 0.8 mm, the lower 5.5-10.5 mm long, the upper (9)10.5-16 mm × 1-1.5 mm. Lemma 6.5-12 mm long; palea 7-13 mm long. Floret with 3 stamens exerted during anthesis, dehiscent, with fertile pollen. Anatomy of the leaf blade: adaxial side with 22-40 ribs, regular in size, 293-474 µm height × 116-207 µm width, colorless (aqueous) parenchyma in the ribs 26-80 µm width, valleys between ribs frequently with air channels. Chromosome number: $2n = 60$.

Spartina densiflora Brongn., Voy. Monde 2(2): 14-15. 1829.

Description: Rhizomatous perennials, densely caespitose with 14-85 culms dm^{-2}. Culm (54)75-130 cm × 1.6-4.7 mm, 5-13 nodes. Longest leaf of the culm with sheath 7.5-23 cm long; ligule 0.5-2.1 mm long; blade (11.5)23-66 × 3.4-8 cm. Inflorescence 8-29 cm long, a raceme with 3-15 spikes, the upper 2-6 cm long. Spikelet 5.5-10 mm long, with 1 floret. Pedicel 1-2 mm long. Glumes 2, unequal, with hairs up to 0.2 mm, the lower 1-3.5(5) mm long, the upper 5.5-9.6 mm × 0.8-1.2 mm. Lemma 5-7.5 mm long; palea 5.5-7.5 mm long. Floret with 3 stamens exerted during anthesis, dehiscent, with fertile pollen. Anatomy of the leaf blade: adaxial side with 12-30 ribs, with different sizes, the bigger usually less than 2/3 in number of total and 463-824 µm height × 93-410 µm width, colorless (aqueous) parenchyma in the ribs 256-410 µm width, valleys between ribs with or without air channels. Chromosome number: 2n = 70.

Spartina* × *onubensis E.Sánchez Gullón, M.D.Infante & B.Gallego, **nothosp. nov.** [Spartina densiflora × S. maritima].—HOLOTYPE: Spain, Huelva, Palos de la Frontera, Marismas del Tinto, middle marsh, 37.225205–6.911163, 13 Nov 2017, J.M. Castillo, B. Gallego, M.D. Infante, A.F. Muñoz and E. Sánchez (SEV287441).—ISOTYPES: Spain, Huelva, Palos de la Frontera, Marismas del Tinto, middle marsh, 37.225205–6.911163, 13 Nov 2017, J.M. Castillo, B. Gallego, M.D. Infante, A.F. Muñoz and E. Sánchez (SEV287440).—PARATYPES: Spain, Huelva, Ayamonte, Estero de San Bruno, middle marsh, 37.195133–7.403111, 24 Nov 2017, B. Gallego, M.D. Infante and A.F. Muñoz (SEV287445); Spain, Huelva, Lepe, Marismas del Río Piedras, middle marsh, 37.210635–7.167215, 24 Nov 2017, J.M. Castillo, M.D. Infante and & A.F. Muñoz (SEV287446) (Fig. S1).

Description: Rhizomatous perennials, densely caespitose with 15-59 culms dm^{-2}. Culm 85-175 (190) cm × 2-5 mm, 10-31 nodes. Longest leaf of the culm with sheath 7.0-24.5 cm long; ligule 0.4-2.0 mm long; blade 15.0-58.5 × 4.4-9.0 cm. Inflorescence 8.0-25.5 cm long, a raceme with 2-18 spikes, the upper 4.5-15.5 cm long. Spikelet 9.0-14.5 mm long, with 1 floret. Pedicel 0.7-2.5 mm long.

Glumes 2, unequal, with hairs up to 0.6 mm, the lower 2.5-6.5(8) mm long, the upper 9-14.5 mm × 1-1.8 mm. Lemma 7.5-11.5 mm long; palea 8-12 mm long. Floret with 3 stamens not exerted during anthesis, not dehiscent, with sterile pollen. Anatomy of the leaf blade: adaxial side with 23-37 ribs, frequently with different sizes, the bigger usually more than 2/3 in number of total and 433-667 µm height × (173) 200-327 µm width, colorless (aqueous) parenchyma in the ribs 83-237 µm width, valleys between ribs with or without air channels.

Etymology: The nothospecific name onubensis refers to "Onuba," the Latin name of Huelva, the type locality.

Spartina* × *onubensis* subsp. *onubensis [□Spartina densiflora × □S. maritima]

Description: Rhizomatous perennials, densely caespitose with 15-59 culms dm^{-2}. Culm 109-175(190) cm height, with 10-21 nodes. Longest leaf of the culm with sheath 9.5-24.5 cm long; blade 19.5-58.5 × 5.5-9 cm. Inflorescence 8-25.5 cm long, a raceme with 4-18 spikes, the upper 4.5-11 cm long. Spikelets pedicel 0.7-1.5 mm long. Glumes with hairs up to 0.35 mm. Chromosome number: 2n = ca. 64, ca. 65, ca. 66.

Phenology: Flowering July-December (February).

Habitat: Middle marshes.

Distribution area: Southwest Iberian Peninsula.

Spartina* × *onubensis* subsp. *tartessiana E.Sánchez Gullón, B.Gallego & M.D.Infante, **nothosubsp. nov.** [□Spartina maritima × □S. densiflora].—HOLOTYPE: Spain, Huelva, Lepe, Marismas del Río Piedras, middle marsh, 37.221501–7.179570, 24 Nov 2017, J.M. Castillo, M.D. Infante and A.F. Muñoz (SEV287450).—PARATYPES: Spain, Huelva, Ayamonte, Estero de San Bruno, low marsh, 37.195209–7.405096, 24 Nov 2017, B. Gallego, M.D. Infante and A.F. Muñoz (SEV287449); Spain, Huelva, Huelva, Marismas del Odiel, low marsh, 37.184629–6.958240, 13 Nov 2017, M.D. Infante, A.F. Muñoz and E. Sánchez (SEV287448); Spain, Huelva, Lepe, Marismas del Río Piedras, middle marsh, 30.210454–7.164097, 24 Nov 2017, J.M. Castillo, M.D. Infante and A.F. Muñoz (SEV287451); Spain, Huelva, Lepe, Marismas del Río Piedras, middle marsh, 37.212587–7.176813, 5 Dec 2017, M.D. Infante and F.J.J. Nieva (SEV287452); Spain, Huelva, Lepe, Marismas del Río Piedras, intertidal pond, 37.213077–7.175393, 5 Dec 2017, M.D. Infante and F.J.J. Nieva (SEV287453) (Fig. S2).

Description: Rhizomatous perennials, densely caespitose with 17-55 culms dm^{-2}. Culm 85-150 cm height, with (11)18-31 nodes. Longest leaf of the culm with sheath 7-14.5 (20) cm long; blade 15-33 × 4.4-8.0 cm. Inflorescence 9-20 cm long, a raceme with 2-4 spikes, the upper 7.5-15.5 cm long. Spikelet pedicel

1.1-2.5 mm long. Glumes with hairs up to 0.6 mm. Chromosome number: 2n = ca. 92, ca. 94.

Etymology: The nothosubspecific name tartessiana refers to “Tartessos,” a semi-mythical kingdom on the south coast of the Iberian Peninsula associated with one river that could be Guadalquivir, Tinto or Guadiana.

Phenology: Flowering July-December (February).

Habitats: Low and middle marshes and intertidal ponds.

Distribution area: Southwest Iberian Peninsula.

Notes: Previous studies of infraspecific taxa for *S. maritima* and *S. densiflora*: Some infraspecific taxa have been described for *S. maritima* (*S. maritima* var. *alterniflora* (Loisel.) St.-Yves, *S. maritima* var. *brasiliensis* (Raddi) St.-Yves, *S. maritima* subvar. *fallax* St.-Yves, *S. maritima* subsp. *glabra* (Muhl. ex Elliott) St.-Yves, *S. maritima* var. *glabra* (Muhl. ex Elliott) St.-Yves, *S. maritima* f. *gracilis* St.-Yves, *S. maritima* subvar. *pilosa* (Merr.) St.-Yves and *S. maritima* subvar. *radii* St.-Yves), but in accordance with Mobberley (1953) all of them belong to *S. alterniflora* and they cannot be identified as the nothospecies described here. Some infraspecific taxa have also been described for *S. densiflora*. One of them, following Mobberley (1953), belongs to *S. foliosa* (*S. densiflora* f. *acuta* St.-Yves); some others belong to *S. spartinae* (Trin.) Merr. ex Hitchc. Sensu Mobberley (1953) (*S. densiflora* subvar. *argentinensis* (Parodi) St.-Yves, *S. densiflora* var. *junciformis* (Engelm. & A.Gray) St.-Yves and *S. densiflora* var. *obtusa* Hack.). Finally, *S. densiflora* subvar. *pauper* St.-Yves is a taxón reported for Patagonia (South Argentina), so it could never be any of the hybrids characterized in this study.

Acknowledgements

Blanca Gallego-Tévar thanks the University of Seville for a research contract (Plan Propio de Investigación). M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte, Spanish Government, for the FPU Grant (Ref. FPU14/06556). F. Muñoz-Rodríguez thanks Ministerio de Economía, Industria y Competitividad of Spanish Government for the research project CGL2017-85204-C3-3-P. The authors thank the Directorate of the Tinto, Odiel, Piedra and Guadiana Natural Parks for supporting field work conducted in this study. A cooperative agreement between the United State Department of Agriculture, Agricultural Research Service (USDAARS) Invasive Species and Pollinator Health Research Unit and the University of Seville facilitated this collaboration.

References

Abbott RJ (1992) Plant Invasions, interspecific hybridization and the evolution of new plant taxa. Trends Ecol Evol 7:401–405. https://doi.org/10.1016/0169-5347(92)90020-C

Ainouche M, Gray A (2016) Invasive *Spartina*: lessons and challenges. Biol Invasions 18: 2119–2122. https://doi.org/10.1007/s10530-016-1201-7

Ainouche ML, Baumel A, Salmon A, Yannic G (2004) Hybridization, polyploidy and speciation in *Spartina* (Poaceae). New Phytol 161:165–172. https://doi.org/10.1046/j.1469-8137.2003.00926.x

Ainouche ML, Fortune PM, Salmon A, Parisod C, Grandbastien MA, Fukunaga K, Ricou M, Misset MT (2009) Hybridization, polyploidy and invasion: lessons from *Spartina* (Poaceae). Biol Invasions 11:1159–1173. https://doi.org/10.1007/s1053 0-008-9383-2

An SQ, Gu BH, Zhou CF, Wang ZS, Deng ZF, Zhi YB, Li HL, Chen L, Yu DH, Liu YH (2007) *Spartina* invasion in China: implications for invasive species management and future research. Weed Res 47:183–191. https://doi.org/10.1111/j.1365-3180.2007.00559.x

Anttila CK, King AR, Ferris C, Ayres DR, Strong DR (2000) Reciprocal hybrid formation of *Spartina* in San Francisco Bay. Molec Ecol 9:765–771. https://doi.org/10.1046/j.1365-294x.2000.00935.x

Ayres DR, Garcia-Rossi D, Davis HG, Strong DR (1999) Extent and degree of hybridization between exotic (*Spartina alterniflora*) and native (*S. foliosa*) cordgrass (Poaceae) in California, USA determined by randomly amplified polymorphic DNA (RAPDs). Molec Ecol 8:1179–1186. https://doi.org/10.1046/j.1365-294x.1999.00679.x

Ayres DR, Smith DL, Zaremba K, Klohr S, Strong DR (2004a) Spread of exotic cordgrasses and hybrids (*Spartina* sp.) in the tidal marshes of San Francisco Bay, California, USA. Biol Invasions 6:221–231. https://doi.org/10.1023/B:BINV.00000 22140.07404.b7

Ayres DR, Zaremba K, Strong DR (2004b) Extinction of a common native species by hybridization with an invasive congener. Weed Technol 18:1288–1291. https://doi.org/10.1614/0890-037X(2004)018%5b1288:EOACNS%5d2.0.CO;2

Ayres DR, Zaremba K, Sloop CM, Strong DR (2008a) Sexual reproduction of cordgrass hybrids (*Spartina foliosa* × *alterniflora*) invading tidal marshes in San Francisco Bay. Diversity Distrib 14:187–195. https://doi.org/10.1111/j.1472-4642.2007.00414.x

Ayres DR, Grotkopp E, Zaremba K, Sloop CM, Blum MJ, Bailey JP, Anttila CK, Strong DR (2008b) Hybridization between invasive *Spartina densiflora* (Poaceae) and native *S. foliosa* in San Francisco Bay, California, USA. Amer J Bot 95:713–719. https://doi.org/10.3732/ajb.20073 58

Baumel A, Ainouche ML, Levasseur JE (2001) Molecular investigations in populations of *Spartina anglica* C.E. Hubbard (Poaceae) invading coastal Brittany (France). Molec Ecol 10:1689–1701. https://doi.org/10.1046/j.1365-294X.2001.01299.x

Baumel A, Ainouche ML, Misset MT, Gourret JP, Bayer RJ (2003) Genetic evidence for hybridization between the native *Spartina maritima* and the introduced *Spartina alterniflora* (Poaceae) in South-West France: *Spartina × neyrautii* re-examined. Pl Syst Evol 237:87–97. https://doi.org/10.1007/s0060 6-002-0251-8

Bird KA, VanBuren R, Puzey JR, Edger PP (2018) The causes and consequences of subgenome dominance in hybrids and recent polyploids. New Phytol 220:87–93

Bortolus A (2006) The austral cordgrass *Spartina densiflora* Brong.: its taxonomy, biogeography and natural history. Biogeography 33:158–168. https ://doi.org/10.1111/j.1365-2699.2005.01380.x

Bortolus A, Carlton JT, Schwindt E (2015) Reimaging South American coasts: unveiling the hidden invasion history of an iconic ecological engineer. Diversity Distrib 21:1267–1283. https ://doi.org/10.1111/ddi.12377

Castellanos EM, Figueroa ME, Davy AJ (1994) Nucleation and facilitation in saltmarsh succession: interactions between *Spartina maritima* and *Arthrocnemum perenne*. J Ecol 82:239–248. https://doi.org/10.2307/22612 92

Castillo JM, Ayres DR, Leira-Doce P, Baile J, Blum M, Strong DR, Luque T, Figueroa E (2010) The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Diversity Distrib 16:547–558. https://doi.org/10.1111/j.1472-4642.2010.00673.x

Castillo JM, Grewell BJ, Pickart A, Bortolus A, Peña C, Figueroa E, Sytsma M (2014) Phenotypic plasticity of invasive *Spartina densiflora* (Poaceae) along a broad latitudinal gradient on the pacific coast of North America. Amer J Bot 101:448–458. https://doi.org/10.3732/ajb.14000 14

Castillo JM, Gallego-Tévar B, Figueroa E, Grewell BJ, Vallet D, Rousseau H, Keller J, Lima O, Dréano S, Salmon A, Aïnouche M (2018) Low genetic diversity contrasts with high phenotypic variability in heptaploid *Spartina densiflora* populations invading the Pacific coast of North America. Ecol Evol 8:4992–5007. https://doi.org/10.1002/ece3.4063

Clayton WD, Renvoize SA (1986) Genera graminarum. Grasses of the world. Kew Bull 13:1–389

Contreras-Cruzado I, Infante-Izquierdo MD, Márquez-García B, Hermoso-López V, Polo A, Nieva FJJ, Cartes-Barroso JB, Castillo JM, Muñoz-Rodríguez A (2017) Relationships between spatiotemporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma 305:173–187. https://doi.org/10.1016/j.geode rma.2017.05.037

Daehler CC, Strong DR (1997) Hybridization between introduced smooth cordgrass (*Spartina alterniflora*; Poaceae) and native California cordgrass (*S. foliosa*) in San Francisco Bay, California, USA. Amer J Bot 84:607–611. https://doi.org/10.2307/24458 96

Darby FA, Turner RE (2008) Below- and aboveground biomass of *Spartina alterniflora*: response to nutrient addition in a Louisiana salt marsh. Estuar Coast 31:326–334.
https://doi.org/10.1007/s1223 7-008-9037-8

Di Bella CE, Striker GG, Escaray FJ, Lattanzi FA, Rodríguez AM, Grimoldi AA (2014) Saline tidal flooding effects on *Spartina densiflora* plants from different positions of the saltmarsh. Diversities and similarities on growth, anatomical and physiological responses. Environm Exp Bot 102:27–36.
https://doi.org/10.1016/j.envex pbot.2014.02.009

Ellstrand NC, Schierenbeck KA (2000) Hybridization as a stimulus for the evolution of invasiveness in plants? Proc Natl Acad Sci USA 97:7043–7050.
https://doi.org/10.1073/pnas.97.13.7043

Ellstrand NC, Whitkus R, Rieseberg LH (1996) Distribution of spontaneous plant hybrids. Proc Natl Acad Sci USA 93:5090–5093.
https://doi.org/10.1073/pnas.93.10.5090

Fortune PM, Schierenbeck K, Ayres D, Bortolus A, Catrice O, Brown S, Ainouche ML (2008) The enigmatic invasive *Spartina densiflora*: a history of hybridizations in a polyploidy context. Molec Ecol 17:4304–4316.
https://doi.org/10.1111/j.1365-294X.2008.03916.x

Gallego-Tévar B, Rubio-Casal A, de Cires A, Figueroa E, Grewell BJ, Castillo JM (2018a) Phenotypic plasticity of polyploid plant species promotes transgressive behaviour in their hybrids. AoB Plants 10:ply055.
https://doi.org/10.1093/aobpl a/ply05 5

Gallego-Tévar B, Curado G, Grewell BJ, Figueroa ME, Castillo JM (2018b) Realized niche and spatial pattern of native and exotic halophyte hybrids. Oecologia 188:849–862.
https://doi.org/10.1007/s0044 2-018-4251-y

Groves H, Groves J (1880) *Spartina* × *townsendii* Nobis. Rep Bot Exch Club Soc Brit Isles 1:37

Hall RJ, Hastings A, Ayres DR (2006) Explaining the explosion: modelling a hybrid invasion. Proc Biol Sci 273:1385–1389.
https://doi.org/10.1098/rspb.2006.3473

Hegarty MJ, Hiscock SJ (2005) Hybrid speciation in plants: new insights from molecular studies. New Phytol 165:411–423.
https://doi.org/10.1111/j.1469-8137.2004.01253.x

Hulme PR, Backer S, Kenis M, Klotz S, Kühn I, Minchin D, Nentwig W, Olenin S, Panov V, Pergl J, Pyšek P, Roques A, Sol D, Solarz W, Vilà M (2008) Grasping at routes of biological invasion: a framework for integrating pathways into policy. J Appl Ecol 45:403–414.
https://doi.org/10.1111/j.1365-2664.2007.01442.x

Kern VG, Guarise NJ, Vegetti AC (2008) Inflorescence structure in species of *Spartina* Schreb. (Poaceae: Chloridoideae: Cynodonteae). Pl Syst Evol 273:51–61.
https://doi.org/10.1007/s00606-008-0009-z

Lee AK, Ayres DR, Pakenham-Walsh MR, Strong DR (2016) Responses to salinity of *Spartina* hybrids formed in San Francisco Bay, California (*S. alterniflora* × *foliosa* and *S. densiflora* × *foliosa*). Biol Invasions 18:2207–

2219.
https://doi.org/10.1007/s1053 0-015-1011-3

Lovett Doust L, Lovett Doust J (1982) The battle strategies of plants. New Sci 91:81–84

Mallet J (2007) Hybrid speciation. Nature 446:279–283.

Marchant CJ (1967) Evolution in *Spartina* (Gramineae) I. The history and morphology of the genus in Britain. Bot J Linn Soc 60:1–24.
https://doi.org/10.1111/j.1095-8339.1967.tb00076.x

Marchant CJ (1968a) Evolution in *Spartina* (Gramineae). II. Chromosomes, basic relationships and the problem of *Spartina* × *townsendii* agg. Bot J Linn Soc 60:381–409.
https://doi.org/10.1111/j.1095-8339.1968.tb000 96.x

Marchant CJ (1968b) Evolution in *Spartina* (Gramineae) III. Species chromosome numbers and their taxonomic significance. Bot J Linn Soc 60:411–417.
https://doi.org/10.1111/j.1095-8339.1968.tb000 97.x

Maricle BR, Koteyeva NK, Voznesenskaya EV, Thomasson JR, Edwards GE (2009) Diversity in leaf anatomy, and stomatal distribution and conductance, between salt marsh and freshwater species in the C4 genus *Spartina* (Poaceae). New Phytol 184:216–233.
https://doi.org/10.1111/j.1469-8137.2009.02903.x

Mobberley DG (1953) Taxonomy and distribution of the genus *Spartina*. Retrospective theses and dissertations 12794. Iowa State University Digital Repository. Available at: https://lib.dr.iastate.edu/rtd/12794

Mobberley DG (1956) Taxonomy and distribution of the genus *Spartina*. Iowa State Coll J Sci 30:471–564.

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. Proc Natl Acad Sci USA 98:5446–5451.
https://doi.org/10.1073/pnas.09109 3398

Partridge TR (1987) *Spartina* in New Zealand. New Zealand J Bot 25:567–575

Peterson PM, Romaschenko K, Johnson G (2010) A classification of the Chloridoideae (Poaceae) based on multi-gene phylogenetic trees. Molec Phylogen Evol 55:580–598.
https://doi.org/10.1016/j.ympev.2010.01.018

Raybould AF, Gray AJ, Lawrence MJ, Marshall DF (1991) The evolution of *Spartina anglica* C.E. Hubbard (Gramineae): origin and genetic variability. Biol J Linn Soc 43:111–126.
https://doi.org/10.1111/j.1095-8312.1991.tb00588.x

Renny-Byfield S, Ainouche M, Leitch IJ, Lim KY, Le Comber SC, Leitch AR (2010) Flow cytometry and GISH reveal mixed ploidy populations and *Spartina* nonaploids with genomes of *S. alterniflora* and *S. maritima* origin. Ann Bot (Oxford) 105:527–533.
https://doi.org/10.1093/aob/mcq008

Rieseberg LH, Archer MA, Wayne RK (1999) Transgressive segregation, adaptation and speciation. Heredity 83:363–372.
https://doi.org/10.1038/sj.hdy.6886170

Saarela JM (2012) Taxonomic synopsis of invasive and native *Spartina* (Poaceae, Chloridoideae) in the Pacific Northwest (British Columbia, Washington and Oregon), including the first report of *Spartina* × *townsendii* for British Columbia, Canada. PhytoKeys 10:25–82. https://doi.org/10.3897/phyto keys.10.2734

Sloop CM, Ayres DR, Strong DR (2009) The rapid evolution of selffertility in *Spartina* hybrids (*Spartina alterniflora* × *foliosa*) invading San Francisco Bay, CA. Biol Invasions 11:1131–1144. https://doi.org/10.1007/s10530-008-9385-0

Strong DR, Ayres DR (2013) Ecological and evolutionary misadventures of *Spartina*. Annual Rev Ecol Evol Syst 44:389–410. https://doi.org/10.1146/annur ev-ecolsys-110512-135803

Thompson JD (1990) Morphological variation among natural populations of *Spartina anglica*. In: Gray AJ, Benham PEM (eds) *Spartina anglica*—a research review. Institute of Terrestrial Ecology, London, pp 26–33

Thompson JD (1991) The biology of an invasive plant. What makes *Spartina anglica* so successful? Bioscience 41:393–401. https://doi.org/10.2307/1311746

Tilman D, Wedin D (1991) Plant traits and resource reduction for five grasses growing on a nitrogen gradient. Ecology 72:685–700. https://doi.org/10.2307/2937208

Turland NJ, Wiersema JH, Barrie FR, Greuter W, Hawksworth DL, Herendeen PS, Knapp S, Kusber W-H Li DZ, Marhold K, May TW, McNeill J, Monro AM, Prado J, Price MJ, Smith GF (eds) (2018) International Code of Nomenclature for algae, fungi, and plants (Shenzhen Code) adopted by the Nineteenth International Botanical Congress Shenzhen, China, July 2017. Regnum Vegetabile 159. Koeltz Botanical Books, Glashütten

Vila M, Weber E, D'Antonio CM (2000) Conservation implications by plant hybridization. Biol Invasions 2:207–217. https://doi.org/10.1023/A:10100 03603310

Yao H, Dogra Gray A, Auger DL, Birchler JA (2013) Genomic dosage effects on heterosis in triploid maize. Proc Natl Acad Sci 110:2665–2669. https://doi.org/10.1073/pnas.1221966110

Yoo MJ, Liu X, Pires JC, Soltis PS, Soltis DE (2014) Nonadditive gene expression in polyploids. Annual Rev Genet 48:485–517. https://doi.org/10.1146/annurev-genet-120213-092159

Supplemental material

Table S1 Phenotypic inheritance for 32 plant morphological and anatomical characters of the reciprocal hybrids between native *Spartina maritima* and invasive *S. densiflora*. Phenotypic inheritance types: transgressive vigorous (Tv); transgressive depressed (Td); dominated by *S. densiflora* (D Sd); dominated by *S. maritima* parent (D Sm); dominated by the both parents (D Sd, Sm); intermediate value between both parents (I). Plant traits (code): maximum height of flowered culms (PFH), maximum unflowered culm height (PUH), tuft diameter (PTD), culm density (PCD), number of nodes per culm (PNN), culm diameter (PTD), position of longest leaf on the culm (LLP), sheath length (LSL), blade length (LBL), blade width (LBW), ligule length (LLL), inflorescence length (IIL), terminal spike length (ISL), number of spikes per inflorescence (INS), pedicel length (SPD), lower glume length (SLG), upper glume length (SUG), upper:lower glume length ratio (SGR), upper glume width (SGW), lemma length (SLM), palea length (SPL), number of exerted floral stamens (SNS), number of adaxial ribs (ANR), ratio of major rib count to minor plus medium rib counts (ARR), ratio between the number of aerenchyma channels and the number of depressions between two ribs (AAC), ratio between the number of abaxial sclerenchyma strands and the number of ribs (ASS), length of major ribs length (ARH), width of major ribs (ARW), maximum width of the aqueous parenchyma (ACP), epidermal cell length (AEL), epidermal cell widths (AEW), and stomata density (ADS)

	Spartina x onubensis subsp. *tartessiana*	*Spartina x onubensis* subsp. *onubensis*
PIH	Tv	Tv
PVH	Tv	Tv
PMD	D Sd	I
PTD	D Sd	D Sd
PNN	Tv	D Sm
PSD	D Sd, Sm	D Sd, Sm
LLP	D Sm	I
LSL	I	D Sd
LBL	I	D Sd
LBW	D Sm	Tv
LLL	D Sd	D Sd
IIL	D Sd	D Sd
ISL	Tv	I
INS	D Sm	D Sd
SPD	D Sd, Sm	D Sd, Sm
SLG	I	I
SUG	D Sm	D Sm
SGR	I	I
SGW	Tv	D Sm
SLM	D Sm	I
SPL	D Sm	I
SNS	Td	Td
ANR	D Sm	D Sm
ARR	I	I
AAC	D Sd	D Sd, Sm
ASS	Tv	D Sd
ARH	I	I
ARW	I	I
ACP	I	I
AEL	D Sd	D Sd
AEW	Tv	D Sd
ADS	D Sd	D Sd

Table S2 Analyses of sediment samples from the microenvironments supporting native *S. maritima*, invasive *S. densiflora* and its reciprocal hybrids in the Gulf of Cadiz (Southwest Iberian Peninsula). Values are mean ± SE (minimum, maximum) (n = 13-30). Different letters indicate significant differences between taxa for the same sedimentary factor (ANOVA, P < 0.05)

	Water content (%)	Organic matter content (%)	pH	Conductivity (mS cm^{-1})
S. maritima	45 ± 2^{a}	9 ± 1^{a}	7.3 ± 0.0^{a}	10.8 ± 0.5^{a}
	(24, 66)	(3, 18)	(7.0, 7.8)	(7.8, 16.4)
S. onubensis	53 ± 4^{a}	14 ± 1^{ab}	7.1 ± 0.0^{a}	12.4 ± 0.6^{a}
ssp. tartessiana	(34, 69)	(7, 27)	(6.9, 7.3)	(10.1, 16.4)
S. onubensis	55 ± 4^{a}	19 ± 2^{b}	7.6 ± 0.1^{b}	10.5 ± 1.4^{a}
ssp. onubensis	(41, 69)	(11, 27)	(7.2, 8.1)	(3.4, 16.3)
S. densiflora	52 ± 3^{a}	16 ± 1^{b}	7.3 ± 0.1^{a}	11.7 ± 0.6^{a}
	(24, 80)	(3, 28)	(6.8, 8.0)	(7.5, 16.3)

HERBARIUM UNIVERSITATIS HISPALENSIS

HOLOTYPUS (SEV) SEV 287441

Spartina x onubensis E. Sánchez Gullón, M.D. Infante & B. Gallego subsp. *onubensis*

HUELVA: Palos de la Frontera. Marismas del Tinto
Marisma media.

13/11/2017 37, 225205ºN 6, 911163ºW

Leg.: J.M. Castillo, B. Gallego, M.D. Infante, A.F. Muñoz & E. Sánchez-Gullón

Det.:

Fig. S1 Holotype of *Spartina x onubensis* subsp. *onubensis*

HERBARIUM UNIVERSITATIS HISPALENSIS

HOLOTYPUS (SEV) SEV 287450

Spartina x onubensis subsp. *tartessiana*
E. Sánchez Gullón, B. Gallego & M.D. Infante

HUELVA: Lepe. Marismas del Rio Piedras
Marisma media.

24/11/2017 37, 221501"N 7, 179570"W

Leg.: J.M. Castillo, M.D. Infante & A.F. Muñoz
Det.:

Fig. S2 Holotype of *Spartina x onubensis* subsp. *tartessiana*

3

Fruit set, seed viability and germination of the European native Spartina maritima in Southwest Iberian Peninsula

Publicado en:

M. Dolores Infante-Izquierdo[1], Jesús M. Castillo[2], F. Javier J. Nieva[1], Ioana D. Rotundu[1], Francesca T. David[1], Brenda J. Grewell[3], Adolfo F. Muñoz-Rodríguez[1] (2019).

Wetlands. https://doi.org/10.1007/s13157-019-01188-1

[1] Departamento de Ciencias Integradas, Universidad de Huelva, 21071 Huelva, Spain.
[2] Departamento de Biología Vegetal y Ecología, Universidad de Sevilla, Ap. 1095, 41080 Sevilla, Spain.
[3] USDA-ARS Invasive Species and Pollinator Health Research Unit, University of California, Davis, CA, USA.

Factor de impacto (2018): 1,854. Ecology: 96 de 165, Cuartil 3

Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula

Abstract

Spartina maritima is the only native cordgrass in Europe and Africa and plays an important role in European salt marshes as a primary colonizer. Many aspects of its reproductive biology are unknown. Viable seed production has been described as potentially very low to non-existent. Our primary objective was to evaluate the seed viability of *S. maritima*. We hypothesized that *S. maritima* was capable of producing viable seeds since we observed many new patches of this cordgrass. We analyzed 400 inflorescences in fruiting state from 20 different tussocks in three estuaries in the Southwest Iberian Peninsula and carried out a germination experiment. *Spartina maritima* was able to set moderate numbers of highly viable caryopses. Mature caryopses with embryos were recorded in 12.5 ± 2.9% of total spikelets, with high variation ranging from 0 to 45% among tussocks. Caryopses showed a high level of viability (89.0 ± 4.9%) and final germination fraction (85.3 ± 2.9%). The percentage of spikelets with all their stamens exerted was 38.3 ± 4.5%, being positively correlated with the percentage of mature caryopses with embryo, indicating that tussocks in which inflorescences reached a more advanced state of flowering when fruit set started would ultimately produce more fruits.

Keywords:

Caryopsis viability, Cordgrass, Halophyte, Germination, Saltmarsh conservation, Sexual reproduction

Introduction

The capacity for both vegetative and sexual reproduction is characteristic of many perennial plant species (Barrett 2015). While clonal propagation supports local dynamics such as expansion of established populations, allocation of resources to sexual reproduction is a key process for the long-term persistence and conservation of plant populations since seed set provides them the opportunities for independent and long distance dispersal, and to increase their genetic diversity and ability to adapt to new environments (Wilcock and Neiland 2002; Li et al. 2018). In this sense, genetic diversity in plants is considered advantageous in changing or heterogeneous environments (Philbrick and Les 1996) such as salt marshes (Gedan et al. 2009), especially in a scenario of global climate change and sea level rise (van Wijnen and Bakker 2001).

The *Spartina* (cordgrasses) are one of the most widespread genera in the coastal marshes of the world, where they are predominant (Nieva et al. 2001). *Spartina maritima* (Curtis) Fernald was the only native cordgrass in European and African Atlantic coasts (Mobberley 1956) until the recent hybrids and allopolyploid formation during the nineteenth century (Ainouche et al. 2009). *S. maritima* plays an important role in European salt marshes as a primary colonizer of bare intertidal mudflats through the stabilization of the sediment, thus protecting the coastline from the erosion and facilitating the establishment of other marsh species during ecological succession of vegetation (Castellanos et al. 1994). In this sense, *S. maritima* provides ecosystem services through prevention of erosion and sediment stabilization (Castillo and Figueroa 2009). It is also a useful biotool for phytostabilization of metals in contaminated soils (Cambrollé et al. 2008; Curado et al. 2014). Native plant communities that include *S. maritima* constitute a Habitat of Community Interest according to Directive 92/43/CEE (1320 Spartinion maritimae) in Europe. It appears as species of concern in the Red List of Andalusia (Cabezudo et al. 2005) and in some European coastal regions (Cooper 1993), due to its limited distribution and because populations are decreasing in many coastal areas. *S. maritima* is dwindling in many European coastal areas due to limited dispersion, salt marsh erosion and expansion of exotic species (Wong et al. 2018). The fact that *S. maritima* occupies low elevation tidal marshes (Castellanos et al. 1994), and that very low genetic diversity was recorded within and among populations (Yannic et al. 2004), suggests this cordgrass is highly vulnerable to future changes in the littoral environment predicted as sea level continues to rise. *S. maritima* in Southwest Iberian Peninsula is also threatened by the invasion of the neophyte congener, *Spartina densiflora* Brongn. (Nieva et al. 2001). *S. densiflora* hybridizes reciprocally with *S. maritima* forming transgressive hybrids (Castillo et al. 2010) that show broad ecological niches and alternative zonation patterns (Gallego-Tévar et al. 2018). Also, the formation of hybrids between native *S. maritima* and the introduced *Spartina alterniflora* Loisel., has been described in England (Groves and Groves 1880) and France in both cases with *S. alterniflora* as maternal genome donor (Baumel et al. 2003). In this context, many aspects of the reproductive biology of the *S. maritima* are unknown. Its capacity for seed production has been described as non-existent or very low, suggesting this species allocates resources primarily to vegetative reproduction with rhizomatous spread (Marchant and Goodman 1969; Castellanos et al. 1994; Castillo et al. 2010). However, since trade-offs between clonal and sexual modes of reproduction are linked to both fitness gains in and their dispersal (Boedeltje et al. 2008; Van Drunen and Dorken 2012), improving our knowledge of the sexual reproductive capacity of *S. maritima* is important for the conservation of this threatened European and African native species.

The main goal of this work was to evaluate the ability of *S. maritima* to produce viable seeds, to test caryopsis germination, and to identify environmental factors influencing its fruit set. To achieve these objectives, we evaluated inflorescences from *S. maritima* tussocks distributed in three different estuaries along the Southwest coast of the Iberian Peninsula. To quantify caryopsis germination, we conducted experimental trails under controlled conditions. We hypothesized that *S. maritima* was capable of producing viable seeds since we have observed many new patches of this cordgrass in new forming low marsh areas in the Odiel Marshes (Southwest Iberian Peninsula) during recent decades and their random spatial distribution as well as their similar early development pointed to establishment from seeds.

Materials and methods

Study Sites

The present study was conducted in seven low elevation salt marsh areas located in three estuaries along the coast of the Gulf of Cádiz (Southwest Iberian Peninsula). This coast experiences mesotidal inundation regimes, and the mean sea level is +1.85 m relative to Spanish Hydrographic Zero (SHZ). The tides are semidiurnal and have a mean range of 2.10 m and a mean spring tidal range of 2.97 m, representing 0.40-3.37 m above SHZ. The study area is under a Mediterranean climate with Atlantic influence. The focal taxon, *Spartina maritima*, occurs in low elevation zones of the salt marshes. At higher elevations, other halophytic species co-occur with *S. maritima* such as *Sarcocornia perennis* (Mill.) A.J. Scott (Castellanos et al. 1994) and the invasive *S. densiflora* (Castillo et al. 2008).

Tussock Size and Spatial Pattern

A total of 20 tussocks of *Spartina maritima* were individually marked: 15 tussocks distributed in five salt marshes in the Odiel River Estuary, 2 tussocks within one salt marsh in the Tinto River Estuary, and 3 tussocks in one salt marsh within Piedras Estuary (Fig. 1). In the studied low salt marshes, some *S. maritima* tussocks formed monospecific prairies and others were discrete tussocks (Table 1). The length of major and minor perpendicular axes (m) of each tussock was measured in July 2017. To evaluate spatial pattern and relationships between plant reproductive traits, the distance to the nearest

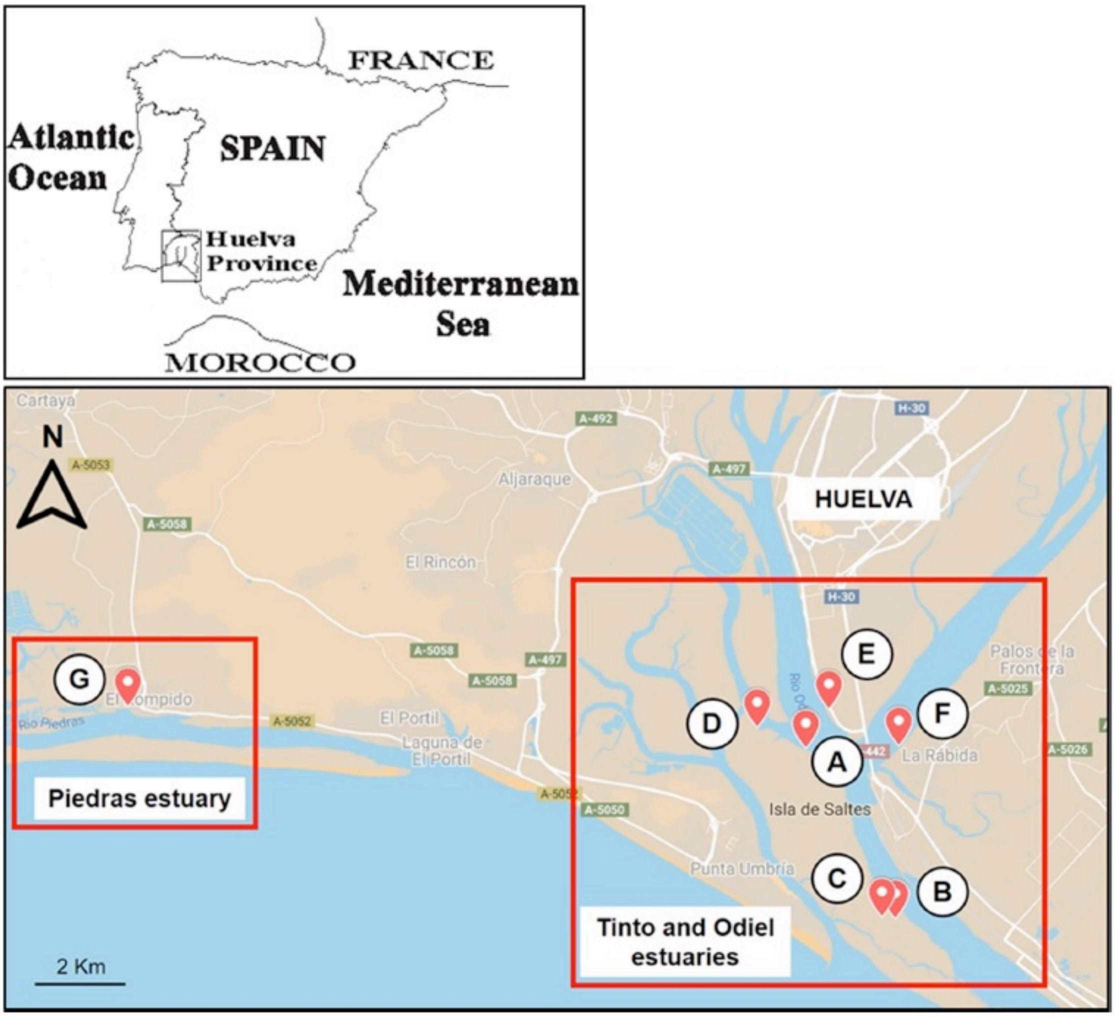


Fig. 1 Maps showing the location of salt marshes where *Spartina maritima* tussocks were sampled on the Iberian Peninsula and on the Gulf of Cádiz: Odiel Estuary: A) Almendral Sandspit (3 tussocks); B) Cabeza Alta Marsh (2 tussocks); C) Cabeza Alta Sandspit (5 tussocks); D) North Saltés Island (1 tussock); E) Huelva Seafront (4 tussocks); Tinto Estuary: F) La Rábida Marsh (2 tussocks); and Piedras Estuary: G) El Rompido Marsh (3 tussocks). Map source: Google maps©2018

neighboring *S. maritima* tussock was measured and recorded for each individually marked tussock in the field.

Sedimentary Environment

Sediments samples were collected randomly within 12 tussocks of *S. maritima* (3 subsamples per tussock, except for the 3 tussocks sampled in the ‘Almendral Sandspit’ where just 1 sample per tussock was collected) in July 2017, when caryopses were observed on *S. maritima* plants. The sedimentary environment was characterized by recording pH, electrical conductivity (mS cm^{-1}), and water and organic matter content (%) (*n* = 12 tussocks). We followed the methodology for collection and analyses of sediment samples as reported by Contreras-Cruzado et al. (2017).

Reproductive Traits

Spartina maritima flowers between May and July in the study areas (Gallego-Tévar et al. 2019a). During July 2017, the density of inflorescences (inflorescence m^{-2}) was recorded by counting the number of inflorescences in 20 × 20 cm plots randomly distributed within each tussock (3-10 plots per tussock depending of the size; n = 20 tussocks) (Table 1). In addition, 20 inflorescences in fruiting state were collected from each tussock and stored at +5 °C until analyses were performed in the laboratory.

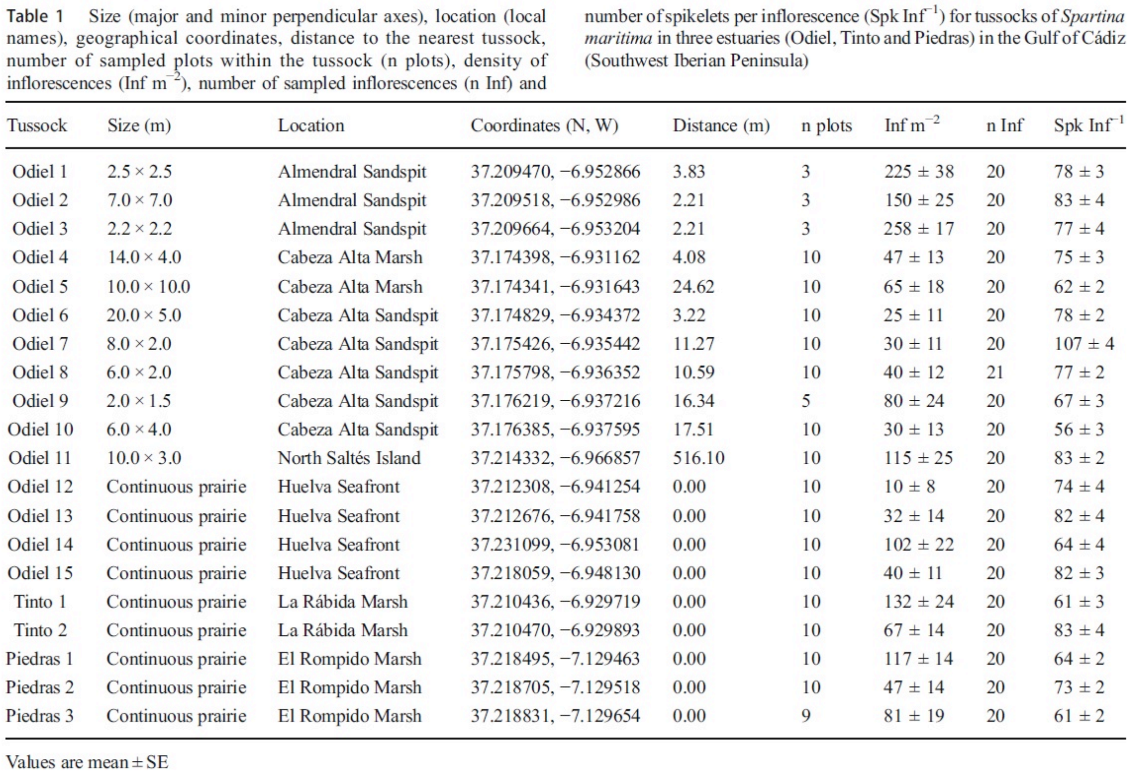


Table 1 Size (major and minor perpendicular axes), location (local names), geographical coordinates, distance to the nearest tussock, number of sampled plots within the tussock (n plots), density of inflorescences (Inf m^{-2}), number of sampled inflorescences (n Inf) and number of spikelets per inflorescence (Spk Inf^{-1}) for tussocks of *Spartina maritima* in three estuaries (Odiel, Tinto and Piedras) in the Gulf of Cádiz (Southwest Iberian Peninsula)

Tussock	Size (m)	Location	Coordinates (N, W)	Distance (m)	n plots	Inf m^{-2}	n Inf	Spk Inf^{-1}
Odiel 1	2.5 × 2.5	Almendral Sandspit	37.209470, −6.952866	3.83	3	225 ± 38	20	78 ± 3
Odiel 2	7.0 × 7.0	Almendral Sandspit	37.209518, −6.952986	2.21	3	150 ± 25	20	83 ± 4
Odiel 3	2.2 × 2.2	Almendral Sandspit	37.209664, −6.953204	2.21	3	258 ± 17	20	77 ± 4
Odiel 4	14.0 × 4.0	Cabeza Alta Marsh	37.174398, −6.931162	4.08	10	47 ± 13	20	75 ± 3
Odiel 5	10.0 × 10.0	Cabeza Alta Marsh	37.174341, −6.931643	24.62	10	65 ± 18	20	62 ± 2
Odiel 6	20.0 × 5.0	Cabeza Alta Sandspit	37.174829, −6.934372	3.22	10	25 ± 11	20	78 ± 2
Odiel 7	8.0 × 2.0	Cabeza Alta Sandspit	37.175426, −6.935442	11.27	10	30 ± 11	20	107 ± 4
Odiel 8	6.0 × 2.0	Cabeza Alta Sandspit	37.175798, −6.936352	10.59	10	40 ± 12	21	77 ± 2
Odiel 9	2.0 × 1.5	Cabeza Alta Sandspit	37.176219, −6.937216	16.34	5	80 ± 24	20	67 ± 3
Odiel 10	6.0 × 4.0	Cabeza Alta Sandspit	37.176385, −6.937595	17.51	10	30 ± 13	20	56 ± 3
Odiel 11	10.0 × 3.0	North Saltés Island	37.214332, −6.966857	516.10	10	115 ± 25	20	83 ± 2
Odiel 12	Continuous prairie	Huelva Seafront	37.212308, −6.941254	0.00	10	10 ± 8	20	74 ± 4
Odiel 13	Continuous prairie	Huelva Seafront	37.212676, −6.941758	0.00	10	32 ± 14	20	82 ± 4
Odiel 14	Continuous prairie	Huelva Seafront	37.231099, −6.953081	0.00	10	102 ± 22	20	64 ± 4
Odiel 15	Continuous prairie	Huelva Seafront	37.218059, −6.948130	0.00	10	40 ± 11	20	82 ± 3
Tinto 1	Continuous prairie	La Rábida Marsh	37.210436, −6.929719	0.00	10	132 ± 24	20	61 ± 3
Tinto 2	Continuous prairie	La Rábida Marsh	37.210470, −6.929893	0.00	10	67 ± 14	20	83 ± 4
Piedras 1	Continuous prairie	El Rompido Marsh	37.218495, −7.129463	0.00	10	117 ± 14	20	64 ± 2
Piedras 2	Continuous prairie	El Rompido Marsh	37.218705, −7.129518	0.00	10	47 ± 14	20	73 ± 2
Piedras 3	Continuous prairie	El Rompido Marsh	37.218831, −7.129654	0.00	9	81 ± 19	20	61 ± 2

Values are mean ± SE

The total number of spikelets was counted in each of the 400 collected inflorescences. Twenty-five spikelets were randomly selected from different inflorescences for each tussock and they were analyzed one by one under magnifying glass after removing glumes, palea and lemma. The fruit set status was classified as mature caryopsis with embryo, caryopsis without embryo, or reabsorbed caryopsis and undeveloped ovaries. A caryopsis was recorded as reabsorbed when growth had been initiated, but resorption of content by the mother plant resulted in a membranous empty sac larger than the original ovary. The percentage of each type of caryopsis was calculated in relation to the total number of analyzed spikelets.

The length of undeveloped ovaries, mature caryopses and their embryo, caryopses without embryo and reabsorbed caryopsis was measured using a ruler under a 10X power magnifying glass. The presence of stamens that had not been exerted during anthesis and remained closed inside the lemma and palea during fruit set was recorded for each analyzed spikelet, and the percentage of spikelets whose stamens were all exerted during the anthesis was calculated. Based on the number of spikelets per inflorescence and the percentage of mature caryopses, the production of mature caryopses per inflorescence was calculated. Then, the density of caryopses (caryopses m^{-2}) was calculated as the product of mature caryopses per inflorescence and the density of inflorescences.

To determine if the low formation of mature caryopsis with embryo was due to lack of pollination and/or fertilization, we analyzed a sample of n = 50 stigmas from 8 inflorescences from a single tussock in Odiel estuary (the tussock 'Odiel 5' that reached the maximum level of fruit set). Each sample was separated by three caryopsis types: mature caryopsis, reabsorbed caryopsis, or undeveloped ovaries. Each stigma was extracted, placed on a microscope-slide and stained in cotton blue in lactophenol. Then, the number of grains of pollen germinated on each stigma surface was counted under a microscope (Fernández-Illescas et al. 2011).

Germination Experiment

A preliminary trial was performed under controlled conditions to establish the optimum conditions for germination of *S. maritima*. Caryopses of the tussock 'Odiel 5' were exposed to three different treatments in distilled water: clean (without glumes, palea and lemma) caryopsis in 12 h light / 12 h dark photoperiod (12 L/12D), clean caryopsis in 24 h dark photoperiod (24D), and complete spikelet in 12 L/12D photoperiod. There were no significant differences in the final germination percentage between these three treatments, but clean caryopses germinated faster than complete spikelets, so we considered working with clean caryopses in the germination experiment (Supplemental Material, Table 1).

Caryopses from three of the 20 *S. maritima* tussocks that had the highest percentages of mature caryopses were used in the germination experiment. Spikelets containing mature caryopses were randomly selected from each of three tussocks, and were then stored at +5 °C for experimental use. Before sowing, we cleaned caryopses by removing the glumes, the palea and the lemma. To prevent contamination of caryopses by fungi, they were surface-sterilized just before use in 5% (v/v) sodium hypochlorite for 10 min and then rinsed with distilled water (Muñoz-Rodríguez et al. 2012). Four replicates of 25 caryopses for each tussock were sown in Petri dishes (9 cm diameter) on two layers of autoclaved filter paper watered with 5 ml of distilled water and sealed with adhesive tape (Parafilm™) to avoid desiccation, and cultivated under temperatures between +20 and + 25 °C and under a 12 h light / 12 h dark photoperiod. The light was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. The numbers of germinated caryopses were recorded every 2 to 3 days for 30 days. A caryopsis was recorded as germinated when coleoptile emergence was observed. At the end of the germination experiment, the viability of each ungerminated caryopsis was tested by incising the embryo using a scalpel and then submerging it in a

1% aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride in darkness at +25 °C for 24 h (Mackay 1972). Then, we recorded the number of red stained embryos as live embryos. The percentage of nonviable and viable caryopses (the sum of germinated and stained), the percentage of germinated caryopses in relation to the viable caryopses, and the days necessary to reach 50% of the final germination percentage (T_{50}) were calculated for every Petri dish (Muñoz-Rodríguez et al. 2012). Also the percentage of dormant caryopses was calculated based on the number of viable caryopses that did not germinate.

Statistical Analysis

Statistical analyses were carried out using the software STATISTICA 8.0 (Statsoft Inc.). Mean deviations were calculated as standard error (SE). The normality and homogeneity of the variance of the data series were checked using the Kolmogorov-Smirnov test and Levene's test, respectively. One-way analysis of variance (ANOVA) followed by Tukey's Honest Significant Difference (HSD) as a post-hoc test were used to compare mean plant traits between *S. maritima* tussocks and salt marsh areas (locations). When data or their transformations (arcsine(x) and $\sqrt{x}$ functions) did not meet homogeneity of variance between groups or normal distribution assumptions for parametric models, non-parametric Kruskal-Wallis H-tests followed by Mann-Whitney U-test as post-hoc tests were used. The relationships between different plant traits and sedimentary factors were established using the Spearman correlation coefficient (ρ). Test results were considered significant at $p \leq 0.05$.

Results

Tussock Size and Spatial Pattern

The area of *Spartina maritima* tussocks varied between 9.4 m^2 for the smallest one and 314.2 m^2 for the largest. From each marked tussock, the distance to the nearest tussock varied between 0.00 m for those tussocks forming continuous prairies after merging with other tussocks, and a maximum 516.10 m of separation for the most isolated tussock (Table 1).

Fruit Set

The density of inflorescences (85 ± 15 inflorescences m^{-2}) varied significantly among *S. maritima* tussocks (Kruskal-Wallis test, $H_{19,173} = 75.48$, $p < 0.0001$) and locations (Kruskal-Wallis test, $H_{6,173} = 48.01$, $p < 0.0001$), ranging from 10 ± 8 to 258 ± 17 inflorescences m^{-2} (Table 1). The number of spikelets per inflorescence ranged from 56 ± 3 to 107 ± 4 (mean number = 74 ± 3 spikelets per inflorescence), and also changed among tussocks (Kruskal-Wallis test, $H_{19,401} = 152.11$, $p < 0.001$) and locations (Kruskal-Wallis test, $H_{6,401} = 37.56$, $p < 0.0001$) (Table 1).

The percentage of mature caryopsis with embryo changed between 0.0 ± 0.0% and 45.0 ± 5.5% (mean 12.5 ± 2.9%), being significantly different among tussocks (Kruskal-Wallis test, $H_{19,401} = 163.10$, $p < 0.0001$) and among locations (Kruskal-Wallis test, $H_{6,401} = 86.65$, $p < 0.0001$) (Fig. 2a). The percentages of caryopsis with embryo and without embryo increased together on the inflorescences of *S. maritima* ($\rho = +0.475$, $p < 0.05$). The density of mature caryopses with embryo (621 ± 145 caryopsis m^{-2}) ranged between 0 ± 0 and 2177 ± 478 caryopsis m^{-2}, also showing significant differences among tussocks (Kruskal-Wallis test, $H_{19,173} = 87.84$, $p < 0.0001$) and among locations (Kruskal-Wallis test, $H_{6,173} = 40.10$, $p < 0.0001$) (Fig. 2b). Additionally, the percentage of caryopsis without embryo ranged from 0.0 ± 0.0% to 3.4 ± 0.8% (mean 1.2 ± 0.3%) (tussocks: Kruskal-Wallis test, $H_{19,401} = 73.17$, $p < 0.0001$; locations: Kruskal-Wallis test, $H_{6,401} = 33.22$, $p < 0.0001$) (Fig. 2a). Finally, 14.3 ± 2.3% of the caryopses were reabsorbed, varying between 1.4 ± 0.5% and 33.2 ± 3.1% (tussocks: Kruskal-Wallis test, $H_{19,401} = 169.16$, $p < 0.001$; locations: Kruskal-Wallis test, $H_{6,401} = 94.14$, $p < 0.001$) (Fig. 2a). The percentage of reabsorbed caryopses increased together with those of caryopses with and without embryo ($\rho = +0.607$, $\rho = +0.394$, respectively, $p < 0.05$).

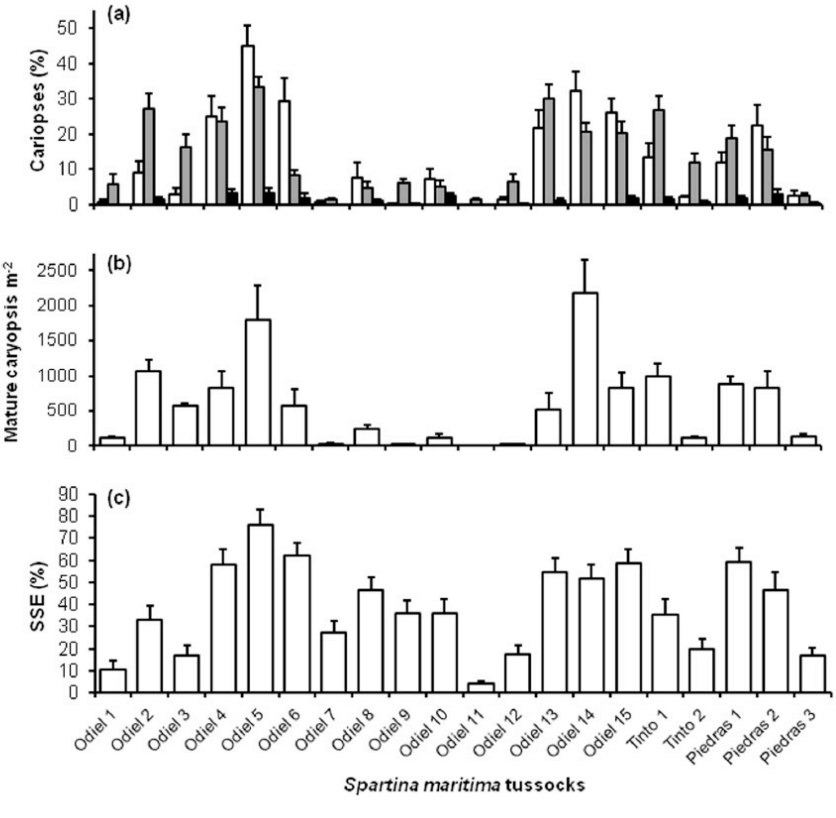
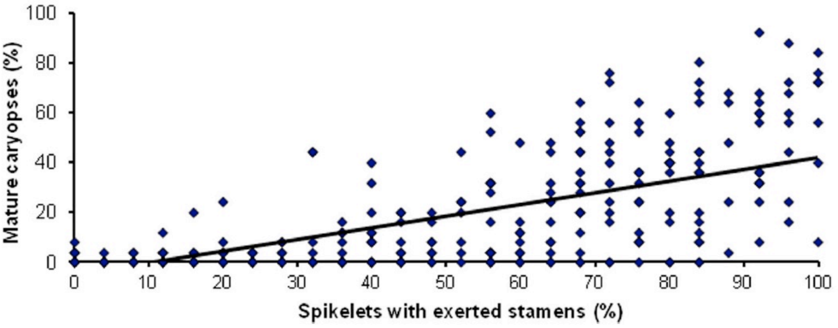


Fig. 2 **a** Percentages of mature caryopses with embryo (white bars), reabsorbed caryopses (grey bars) and caryopses without embryo (black bars); **b** Density of caryopses with embryo (caryopses m^{-2}); **c** Percentage of spikelets with all stamens exerted (SSE). Data are mean ± SEM (n = 20) for different *Spartina maritima* tussocks at three estuaries (Odiel, Tinto and Piedras) in the Gulf of Cádiz (Southwest Iberian Peninsula)

The mean percentage of spikelets with all their stamens exerted was 38.3 ± 4.5%, varying between 4.0 ± 1.1% and 76.2 ± 6.5%, and changing between tussocks (Kruskal-Wallis test, $H_{19,401} = 152.13$, $p < 0.001$) and locations (Kruskal-Wallis test, $H_{6,401} = 87.32$, $p < 0.0001$) (Fig. 2c). This spikelet

Fig. 3 Relationship between the percentage of mature caryopses with embryo and the percentage of spikelets with all their stamens exerted for inflorescences of *Spartina maritima* in the Gulf of Cádiz (Southwest Iberian Peninsula). Regression equation: $y = -4.9 + 0.5x$ ($\rho = +0.749$, $p < 0.05$)



trait was positively correlated with the percentage of caryopses with embryo ($\rho = +0.749$, $p < 0.05$) (Fig. 3), the percentage of caryopses without embryos ($\rho = +0.419$, $p < 0.05$), and with the percentage of reabsorbed caryopses ($\rho = +0.523$, $p < 0.05$).

When plant trait responses were compared to the spatial pattern of tussocks, we did not find significant correlations between measured plant traits and the distance to the nearest neighbor tussocks. However, sample results revealed one exception. The tussock located at the largest distance from any other tussock (Odiel 11 with 516.10 m spatial separation; Table 1) did not produce any mature caryopses or any caryopses without embryo, and had the lowest percentage of reabsorbed caryopses (1.4 ± 0.4%).

Average numbers of pollen grains per stigmas on spikelets varied depending on status of the caryopsis. The average number of pollen grains per stigma for spikelets developing a mature caryopsis was 0.79 ± 0.28 grains, those with reabsorbed caryopses had 0.53 ± 0.21 grains stigma^{-1}, and pollen load where caryopses had undeveloped ovaries was 0.08 ± 0.08 grains stigma^{-1} (Kruskal-Wallis test, $H_{2,50} = 3.55$, $p > 0.05$).

Caryopsis Size

The length of a mature caryopsis with embryo (6.46 ± 0.17 mm) ranged from 4.98 ± 0.45 to 7.97 ± 0.09 mm. The length of the embryo (4.88 ± 0.20 mm) ranged between 3.56 ± 0.52 and 7.17 ± 0.15 mm. Caryopsis without embryo had a length of 6.08 ± 0.21 mm, changing between 4.80 ± 0.20 and 7.06 ± 0.11 mm, and they were longer than reabsorbed caryopsis (4.40 ± 0.14 mm) that ranged from 2.99 ± 0.17 to 5.95 ± 0.10 mm. The length of caryopses with undeveloped ovaries (1.89 ± 0.03 mm) oscillated between 1.67 ± 0.06 and 2.10 ± 0.05 mm (Supplemental Material, Table 2). There were significant differences between the length of the four types of caryopses (Kruskal-Wallis test, $H_{3,1899} = 1565.45$, $p < 0.001$) (Fig. 4). *Spartina maritima* tussocks differed in the length of mature caryopses with embryo (Kruskal-Wallis test, $H_{18,402} = 163.70$, $p < 0.001$), caryopses without embryo (ANOVA test, F = 4.98, d.f. = 93, $p < 0.0001$), reabsorbed caryopses (Kruskal-Wallis test, $H_{19,616} = 182.81$, $p < 0.001$), and undeveloped ovaries (Kruskal-Wallis test, $H_{19,774} = 112.66$, $p < 0.0001$). The percentage of mature caryopsis with embryo was independent of their size ($\rho = +0.500$, $p > 0.05$).

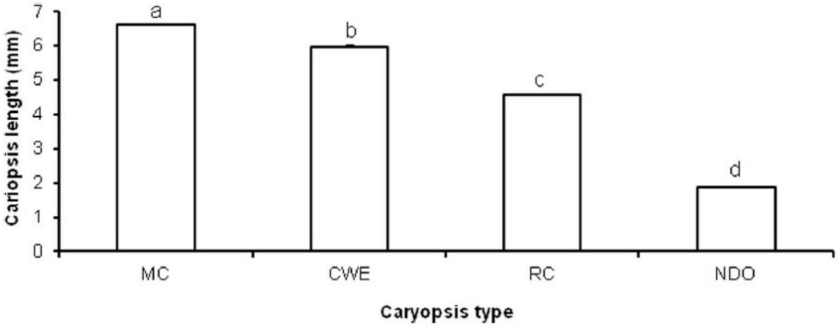
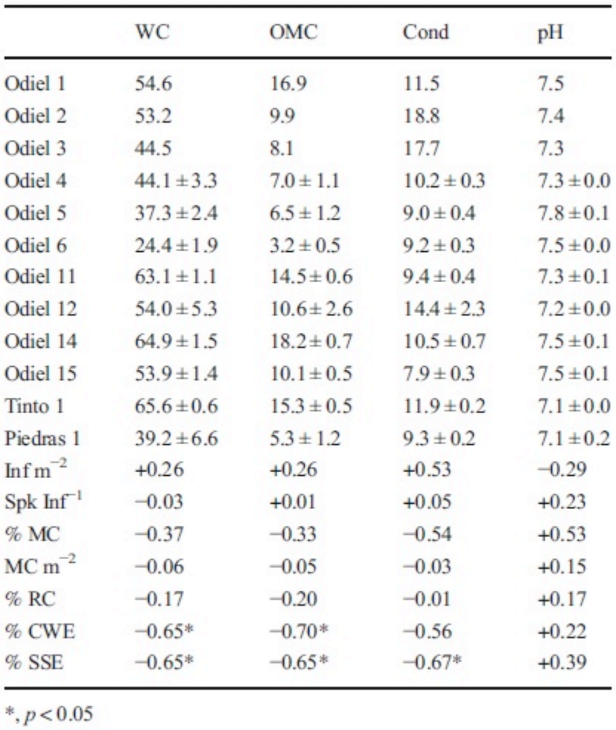


Fig. 4 Mean length (mm) of different caryopses types: mature caryopses with embryo (MC) ($n = 402$), caryopses without embryo (CWE) ($n = 107$), reabsorbed caryopses (RC) ($n = 617$) and undeveloped ovaries (NDO) ($n = 774$). Data are mean ± SE. Different letters indicate significant differences between caryopsis types (Mann-Whitney U-test, $p < 0.05$)

Relationships Between Plant Traits and Sedimentary Environment

The percentage of caryopses without embryo and the percentage of spikelets with all stamens exerted decreased when sediment water content (changing between 24.4 ± 1.9% and 65.6 ± 0.6%) and organic matter content (ranging from 3.2 ± 0.5% and 18.2 ± 0.7%) increased (Table 2). The percentage of spikelets with all stamens exerted also decreased when electrical conductivity increased, reaching values of 18.76 mS cm^{-1}. Every plant trait was independent of sediment pH that varied narrowly between 7.05 ± 0.21 and 7.81 ± 0.11 (Table 2).

Table 2 Water content (WC) (%), organic matter content (OMC) (%), electrical conductivity (Cond) (mS cm^{-1}) and pH of sediments colonized by *Spartina maritima* in three estuaries (Odiel, Tinto and Piedras) in the Gulf of Cádiz (Southwest Iberian Peninsula) (values are mean ± SE; $n = 3$, except $n = 1$ for Odiel 1–3), and their relationships (Spearman correlation coefficient) with fructification factors (density of inflorescences (Inf m^{-2}), number of spikelets per inflorescence (Spk Inf^{-1}), percentage of mature caryopsis with embryo (% MC), density of mature caryopsis with embryo (MC m^{-2}), percentage of reabsorbed caryopsis (% RC), percentage of caryopsis without embryo (% CWE), percentage of spikelets with all stamens exerted (% SSE)) and sedimentary factors



	WC	OMC	Cond	pH
Odiel 1	54.6	16.9	11.5	7.5
Odiel 2	53.2	9.9	18.8	7.4
Odiel 3	44.5	8.1	17.7	7.3
Odiel 4	44.1 ± 3.3	7.0 ± 1.1	10.2 ± 0.3	7.3 ± 0.0
Odiel 5	37.3 ± 2.4	6.5 ± 1.2	9.0 ± 0.4	7.8 ± 0.1
Odiel 6	24.4 ± 1.9	3.2 ± 0.5	9.2 ± 0.3	7.5 ± 0.0
Odiel 11	63.1 ± 1.1	14.5 ± 0.6	9.4 ± 0.4	7.3 ± 0.1
Odiel 12	54.0 ± 5.3	10.6 ± 2.6	14.4 ± 2.3	7.2 ± 0.0
Odiel 14	64.9 ± 1.5	18.2 ± 0.7	10.5 ± 0.7	7.5 ± 0.1
Odiel 15	53.9 ± 1.4	10.1 ± 0.5	7.9 ± 0.3	7.5 ± 0.1
Tinto 1	65.6 ± 0.6	15.3 ± 0.5	11.9 ± 0.2	7.1 ± 0.0
Piedras 1	39.2 ± 6.6	5.3 ± 1.2	9.3 ± 0.2	7.1 ± 0.2
Inf m^{-2}	+0.26	+0.26	+0.53	−0.29
Spk Inf^{-1}	−0.03	+0.01	+0.05	+0.23
% MC	−0.37	−0.33	−0.54	+0.53
MC m^{-2}	−0.06	−0.05	−0.03	+0.15
% RC	−0.17	−0.20	−0.01	+0.17
% CWE	−0.65*	−0.70*	−0.56	+0.22
% SSE	−0.65*	−0.65*	−0.67*	+0.39

*, $p < 0.05$

Germination Experiment

Final germination percentage ranged from 81.4 ± 4.2% to 91.1 ± 3.6%, with little variation among caryopses from sampled tussocks (ANOVA test, F = 1.19, d.f. = 9, $p > 0.05$). The percentage of dormant caryopses ranged from 8.9 ± 3.6% to 18.6 ± 4.2%, without significant differences among tussocks (ANOVA test, F = 1.19, d.f. = 9, $p > 0.05$). The percentage of unviable caryopses changed markedly among the three studied *S. maritima* tussocks from 3.0 ± 1.9% to 20.0 ± 1.6% (ANOVA test, F = 11.95, d.f. = 9, $p < 0.01$) (Fig. 5). Caryopses from tussock Odiel 6 germinated faster than the other marked tussocks in Odiel River Estuary (T_{50}: Odiel 14, 18.7 ± 0.7 days; Odiel 5, 13.1 ± 0.6 days; Odiel 6, 6.2 ± 0.6 days; ANOVA test, F = 89.94, d.f. = 9, $p < 0.0001$). Final germination percentage, T_{50} and seed viability were independent of the size of the caryopses with embryo ($\rho = +1.000$, $p > 0.05$; $\rho = -0.500$; $p > 0.05$; $\rho = -0.500$, $p > 0.05$, respectively).

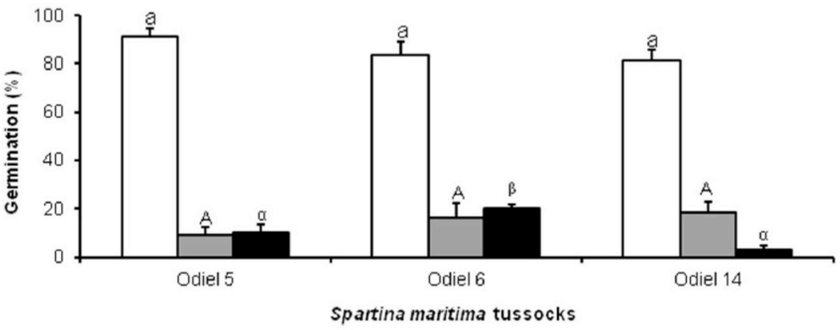


Fig. 5 Percentages of germinated caryopses (white bars), dormant caryopses (grey bars) and non-viable caryopses (black bars) on inflorescences from 3 *Spartina maritima* tussocks in the Gulf of Cádiz (Southwest Iberian Peninsula). Data are mean ± SE ($n = 4$ dishes per tussock). Different letters indicate significant differences between tussocks (HSD test or Mann-Whitney U-test, $p < 0.05$)

Discussion

Our study shows that *Spartina maritima*, native to Europe and Africa, is able to set moderate numbers of highly viable seeds in salt marshes in Southwest Iberian Peninsula. These interesting results contrast with previous studies that described the inability of this halophyte to produce seeds or observations suggesting very low seed set sexual reproductive capacity (Marchant 1967; Marchant and Goodman 1969; Baumel et al. 2001; Castillo et al. 2010). These new findings on reproductive capacity are also relevant for the conservation and recovery of *Spartina maritima*.

The density of inflorescences and the number of spikelets per inflorescence changed between different tussocks of *S. maritima* and varied depending on their salt marsh locations. *S. maritima* tussocks growing in the same salt marsh locations, and sharing the same sedimentary environment, showed contrasting densities of inflorescences, which could be related to genetic differences among the tussocks. However, Yannic et al. (2004) have revealed an extremely low level of genetic variation in French populations of this species and Raybould et al. (1991) in English populations. Nevertheless, a recent study has identified a moderate genetic diversity between *S. maritima* populations in our study area (Gallego-Tévar et al. 2019b), which may be partially explained by sexual reproductive ability. Nieva et al. (2001) found that the invasive congener *Spartina densiflora* showed marked differences in flowering rates between contrasted marsh habitats such as low and high salt marshes. Crosby et al. (2015) likewise observed that flowering stem density in North American *S. alterniflora* varied between different microhabitats, with plants in the less submerged habitats producing fewer flowers and seeds than those in more frequently submerged ones with lower sediment salinity.

In relation with the fecundity of *S. maritima*, Marchant (1967) and Marchant and Goodman (1969) indicated that populations from Britain and Holland failed, at least partially, to form fruits, whereas Mediterranean and African populations were able to set fruits. In this sense, seed production of *S. maritima* has never been observed in France (Baumel et al. 2001). Moreover, Castillo et al. (2010) found no fruits for just one population of *S. maritima* in the Gulf of Cádiz. Nevertheless, our study in three discrete estuaries of the southwest Spain has found that 12.5 ± 2.9% of total spikelets sampled from *S. maritima* contained mature caryopses with embryo. From these data, we can project a production of 621 ± 145 mature caryopses with embryo per m^{-2}. Moreover, the percentage of mature caryopses ranged from 0 to 45% in different tussocks. The recorded spatial differences in fecundity between tussocks growing at the same salt marsh location, and also between different locations, could be explained by pollination success. Davis et al. (2004) demonstrated, in experiments in which they related pollen deposition rates on stigmas with fruit set in *S. alterniflora*, that pollen availability can limit fruit set in wind-pollinated plants when they are in conditions of low pollen flow. In our study, rates of pollen grains on stigmas were similar to those found by Davis et al. (2004) for *S. alterniflora*, which were very low as corresponds to wind-pollinated plants (Friedman and Barret 2009). We found that spikelets that set mature caryopses tended to show more pollen grains per stigma than those that did not develop the ovaries or those that set and then reabsorbed caryopses. Pollen limitation would be especially important if *S. maritima* would be a self-incompatible species (Marchant 1968), as occurs in other *Spartina* species. Daehler (1998) observed a partial self-incompatibility in clones of *S. alterniflora* in San Francisco Bay, recording a marked variation in seed production between individuals and suggesting that the environmental conditions had relatively little effect on seed set. *Spartina pectinata* Link also showed partial self-incompatibility that contributed to poor seed production (Prasifka et al. 2012) and *Spartina patens* (Aiton) Muhl. seemed to be cross fertilized and self-sterile (Lonard et al. 2010). Pollen limitation can also be a limiting factor for those *S. maritima* tussocks located far away from other individuals and/or in the opposite direction in relation to other tussocks than the dominant southwest wind direction in the Gulf of Cádiz. This was supported in our study by the most remote tussock being the only that did not set any mature caryopses and caryopses without embryo, and showing the lowest percentage of reabsorbed caryopses. On the other hand, nutrient availability could also play a role or determine differences in fruit set (Stephenson 1981). Our data suggest this may be the case, since the percentage of caryopses without embryo we detected decreased when sampled from tussocks growing on sediments with higher organic matter contents.

Additionally, the percentage of spikelets with all their stamens exerted increased together with the percentage of mature caryopses with embryo at the tussock level, which may indicate that tussocks in which inflorescences reached a more advanced state of flowering when the fruit set started will produce more fruits. Mullins and Marks (1987) found that an early emergence of inflorescence favored caryopsis formation in *Spartina anglica* C.E. Hubb. Fang et al. (2004) studied the influence of flowering phenology on seed production for *S. alterniflora* in South Louisiana showing that when plants flowered within the peak flowering period had higher seed set. In this sense, the temporal dynamics of flowering are influenced by air temperature (Gallego-Tévar et al. 2019a) and they may be also be influenced by the sedimentary environment since the percentage of spikelets with all the stamens exerted increased where sediment salinity was reduced.

Another interesting result was our documentation of a moderate rate of resorption of the caryopsis content (14.3 ± 2.3%). These reabsorbed caryopses may coincide with the observations by Bromfield (1836), who describes *S. maritima* seeds as seldom well-filled and incapable of germinating, and by Marchant (1967) that found shrivelled caryopses in some spikes in Mediterranean populations of *S. maritima*. Biber and Caldwell (2008) founded many empty, damaged or sterile caryopses for *S. alterniflora*. Shriveled caryopses were also found by Kettenring and Whigham (2009) in *Phragmites australis* Trin., another Poaceae species. Resorption of caryopses that have initiated growth after anthesis could mean that there is absorption of fruits not fully developed at the end of fruiting period. In this case, there should be an inverse correlation between mature fruit set (and the percentage of spikelets with all their stamens exerted) and the rate of reabsorbed caryopses, but our results indicated that these correlations were positive. Thus, it seemed that plants initiated set of more fruits than they could fully develop where availability of critical resources was limiting, resulting in aborted fruits (Stephenson 1981; Martin and Lee 1993). It is also possible that *S. maritima* aborted those fruits with less quality embryos (Sutherland 1986; Burd 1998; Kärkkäinen et al. 1999), since fruit abortion can occur at various stages of development (Li et al. 2008).

The production of caryopses without embryo but with a developed endosperm was also revealed in our study. This developmental response may be caused by lethal alleles or by maternal resource limitations as was previously observed for *Spartina foliosa* Trin. (Daehler and Strong 1997), and for *S. alterniflora* in San Francisco Bay (Daehler and Strong 1994). In this sense, caryopses with aborted embryos were less frequent in cross-pollination than in self-pollination for *S. alterniflora* (Daehler 1999). The production of caryopses without embryo increased together with the percentages of caryopses with embryo and reabsorbed caryopses, and with the percentage of spikelets with all their stamens exerted. These results imply a constant rate of embryo resorption or abortion over the initial fruit set. In Poaceae, the formation of caryopses without embryo has been long recorded in some cultivated cereals like wheat, rye, barley and rice (Lyon 1928a). In double fertilization, the union of the male gametes with egg cell and with the polar nucleus are independent, and the formation of the endosperm starts before embryogenesis (Richards 1997). Thus, the origin of the embryoless seeds may occur in the absence of the fecundation of the egg cell, and as a consequence of genetic problems in the mother plant or the developing embryo (Lyon 1928b; Morinaga and Fukushima 1934; Gustafson 1942; Kärkkäinen et al. 1999). Busey (1976) suggested that the appearance of embryoless seeds in the grass *Eragrostis curvula* could be due to inbreeding depression, which could be the case in *S. maritima* since the formation of embryoless caryopses changed between different tussocks and salt marsh locations. Moreover, both reabsorbed caryopses and caryopses without embryo could be related in *S. maritima* with the fact that the formation of a hybrid with the invader *S. densiflora* has been described (Castillo et al. 2010). This exotic congener species was present in the studied marshes, so the fecundation of *S. maritima* with the exotic pollen could cause the formation of unstable embryos, inducing death. However, Daehler and Strong (1997) found that the embryo abortion rates in the native *S. foliosa* were similar following self-pollination or cross-pollination with the invasive *S. alterniflora* in San Francisco Bay. On the other hand, another study revealed that native *S. foliosa* and invasive *S. alterniflora* showed reproductive competition, since the abundance of *S. alterniflora* pollen overwhelmed stigmas of *S. foliosa* to produce hybrid seeds (Ayres et al. 1999).

Spartina maritima caryopses showed a high level of viability (89.0 ± 4.9%), showing significant differences between tussocks, and a high final germination percentage (85.3 ± 2.9%). In contrast with our study, Bromfield (1836) reported that *S. maritima* could set seed that were seldom well filled and probably incapable to germinate, and that when they did, they were able to germinate in light and dark conditions, as recorded in our work. These results pointed out that *S. maritima* would be able to germinate in dark when buried in sediments and under the canopy of other halophytes. Germinating in buried conditions would be key for this primary colonizer that grows in low marshes with highly dynamic sedimentation (Castellanos et al. 1994). On the other hand, *S. maritima* caryopses germinated when they were sown nude and when they were sown within the spikelet, although the latter germinated more slowly. Thus, the covering elements of the spikelet served to retard the germination, which may ensure that it takes place in optimum environmental conditions for the establishment of seedling such as low salinity levels (Harper 1977; Baskin and Baskin 2014). This behavior has been described previously for other halophytes whose seeds are covered with protective structures such as *Atriplex portulacoides* L. and *Salsola vermiculata* L. (Muñoz-Rodríguez et al. 2017). On the other hand, seedlings from our germination assay stayed alive for at least two months in the Petri dishes (M.D. Infante, personal observation), which contradicts the observations of C.A. Jorgensen, reported by Marchant and Goodman (1969), who indicated that *S. maritima* seeds germinated but seedlings died soon thereafter in the greenhouse.

Our results suggest that *S. maritima* fruits could be used in projects of ecological restoration of salt marshes, which would be more economical than using transplants as has been done in the past (Castillo and Figueroa 2009). However, further studies are needed to analyze the germination and the establishment of *S. maritima* under natural field conditions, since its germination may be affected by different environmental factors such as salinity, anoxia and interactions with other species.

It is possible the very low seed set for *S. maritima* populations from North Europe may be related to the low genetic diversity of this species in that portion of its range (Raybould et al. 1991; Yannic et al. 2004), and to the existence of a selfincompatibility system in this species (Marchant 1968). In this context, it could be interesting to increase the genetic diversity at high latitudes in Europe with *S. maritima* plants translocated from South Europe to favour the sexual reproduction. A facilitated shift to support sexual reproduction mode would facilitate *S. maritima* to response to present sea level rise by dispersing its seeds more frequently and easily to medium and long distances than would be possible by dispersal of rhizomes, which would enable a distributional shift by colonizing new locations as extant populations are flooded by rising tidal waters. Information gleaned from this study can therefore be applied to improve the conservation of native *S. maritima* and preserve biodiversity under changing global climate conditions.

Acknowledgements

We thank to the management of the Odiel Marshes Natural Park for its collaboration. M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte, Spanish Government, for the FPU Grant (Ref. FPU14/06556). The authors thank Dra. Malika Ainouche for her review and comments on the initial manuscript.

References

Ainouche ML, Fortune PM, Salmon A, Parisod C, Grandbastien MA, Fukunaga K, Ricou M, Misset MT (2009) Hybridization, polyploidy and invasion: lessons from *Spartina* (Poaceae). Biological Invasions 11:1159–1173.

Ayres DR, García-Rossi D, Davis HG, Strong DR (1999) Extent and degree of hybridization between exotic (*Spartina alterniflora*) and native (*S. foliosa*) cordgrass (Poaceae) in California, USA determined by random amplified polymorphic DNA (RAPDs). Molecular Ecology 8:1179–1186.

Barrett SC (2015) Influences of clonality on plant sexual reproduction. Proceedings of the National Academy of Sciences 112:8859–8866.

Baskin CC, Baskin JM (2014) Seeds. Ecology, biogeography, and evolution of dormancy and germination. Academic Press, San Diego.

Baumel A, AinoucheML, Levasseur JE (2001) Molecular investigations in populations of *Spartina anglica* C.E. Hubbard (Poaceae) invading coastal Brittany (France). Molecular Ecology 10:1689–1702.

Baumel A, Ainouche ML, Misset MT, Gourret JP, Bayer RJ (2003) Genetic evidence for hybridization between the native *Spartina maritima* and the introduced *Spartina alterniflora* (Poaceae) in south-West France: *Spartina* x *neyrautii* re-examined. Plant Systematics and Evolution 237:87–97.

Biber PD, Caldwell JD (2008) Seed germination and seedling survival of *Spartina alterniflora* Loisel. American Journal of Agricultural and Biological Sciences 3:633–638.

Boedeltje G, QzingWA, Prinzing A (2008) The trade-off between vegetative and generative reproduction among angiosperms influences regional hydrochorous propagule pressure. Global Ecology and Biogeography 17:50–58.

Bromfield WA (1836) A description of *Spartina alterniflora* of Loiseleur, a new British species. In: Hooker WJ (ed) Companion to the botanical magazine 2. Samuel Curtis, London, pp 254–263.

Burd M (1998) "Excess" flower production and selective fruit abortion: a model of potential benefits. Ecology 79:2123–2132.

Busey P (1976) Breeding and cytogenetics of lovegrasses (*Eragrostis* spp.). Ph. D. University of Arizona.

Cabezudo B, Talavera S, Blanca G, Salazar C, Cueto M, Valdés B, Hernández-Bermejo JE, Herrera CM, Rodríguez-Hiraldo C, Navas D (2005) Lista roja de la flora vascular de Andalucía. Consejería de Medio Ambiente. Junta de Andalucía, Sevilla.

Cambrollé J, Redondo-Gómez S, Mateos-Naranjo E, Figueroa ME (2008) Comparison of the role of two *Spartina* species in terms of phytostabilization and bioaccumulation of metals in the estuarine sediment. Marine Pollution Bulletin 56:2037–2042.

Castellanos EM, Figueroa ME, Davy AJ (1994) Nucleation and facilitation in saltmarsh succession: interaction between *Spartina maritima* and *Arthrocnemum perenne*. Journal of Ecology 82:239–248.

Castillo JM, Figueroa ME (2009) Restoring salt marshes using smalls cordgrass, *Spartina maritima*. Restoration Ecology 17:324–326.

Castillo JM, Mateos-Naranjo E, Nieva FJ, Figueroa E (2008) Plant zonation at salt marshes of the endangered cordgrass *Spartina maritima* invaded by *Spartina densiflora*. Hydrobiologia 614:363–371.

Castillo JM, Ayres DR, Leira-Doce P, Bailey J, Blum M, Strong DR, Luque T, Figueroa E (2010) The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Diversity and Distributions 16:547–558.

Contreras-Cruzado I, Infante-Izquierdo MD, Márquez-García B, Hermoso-López V, Polo A, Nieva FJJ, Cartes-Barroso JB, Castillo JM, Muñoz-Rodríguez A (2017) Relationships between spatiotemporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma 305:173–187.

Cooper MA (1993) The status of *Spartina maritima* in Suffolk. Suffolk Naturalists' Society Transactions 29:48–54.

Crosby SC, Ivens-Duran M, Bertness MD, Davey E, Deegan LA, Leslie HM (2015) Flowering and biomass allocation in U.S. Atlantic coast *Spartina alterniflora*. American Journal of Botany 102:669–676.

Curado G, Rubio-Casal AE, Figueroa E, Castillo JM (2014) Potential of *Spartina maritima* in restored salt marshes for phytoremediation of metals in a highly polluted estuary. International Journal of Phytoremediation 16:1209–1220.

Daehler CC (1999) Inbreeding depression in smooth cordgrass (*Spartina alterniflora*, Poaceae) invading San Francisco Bay. American Journal of Botany 86:131–139.

Daehler CC (1998) Self-fertility variation and the reproductive advantage of self-fertility for an invading plant (*Spartina alterniflora*). Evolutionary Ecology 12:553–568.

Daehler CC, Strong DR (1994) Variable reproductive output among clones of *Spartina alterniflora* (Poaceae) invading San Francisco Bay, California: the influence of herbivory, pollination, and establishment site. American Journal of Botany 81:307–313.

Daehler CC, Strong DR (1997) Hybridization between introduced smooth cordgrass (*Spartina alterniflora*; Poaceae) and native California cordgrass (*S. foliosa*) in San Francisco Bay, California, USA. American Journal of Botany 84:607–611.

Davis HG, Taylor CM, Lambrinos JG, Strong DR (2004) Pollen limitation causes an Allee effect in a wind-pollinated invasive grass (*Spartina alterniflora*). Proceedings of the National Academy of Sciences of the USA 101:13804–13807.

Fang X, Subudhi PK, Venuto BC, Harrison SA, Ryan AB (2004) Influence of flowering phenology on seed production in smooth cordgrass (*Spartina alterniflora* Loisel.). Aquatic Botany 80:139–151.

Fernández-Illescas F, Nieva FJJ, de las HerasMA, Muñoz-Rodríguez AF (2011) Dichogamy in Salicornieae species: establishment of floral sex phases and evaluation of their frequency and efficacy in four species. Plant Systematics and Evolution 296:255–264.

Friedman J, Barret SCH (2009) Wind of change: new insights on the ecology and evolution of pollination and mating in windpollinated plants. Annals of Botany 103:1515–1527.

Gallego-Téva B, Curado G, Grewell BJ, Figueroa ME, Castillo JM (2018) Realized niche and spatial pattern of native and exotic halophyte hybrids. Oecologia 188:849–862.

Gallego-Tévar B, Infante-Izquierdo MD, Figueroa E, Nieva FJJ, Muñoz-Rodríguez AF, Grewell BJ, Castillo JM (2019a) Some like it hot: maternal-switching with climate change modifies formation of invasive *Spartina* hybrids. Frontiers in Plant Science 10:1–14.

Gallego-Tévar B, Grewell BJ, Rousseau H, Keller J, Ainouche A, Lima O, Dréano S, Salmon A, Figueroa E, Aïnouche M, Castillo JM (2019b) Genetic structure of *Spartina* hybrids between native *Spartina maritima* and invasive *Spartina densiflora* in Southwest Europe. Perspectives in Plant Ecology, Evolution and Systematics 37:26–38.

Gedan KB, Silliman BR, Bertness MD (2009) Centuries of human-driven change in salt marsh ecosystems. Annual Review ofMarine Science 1:117–141.

Groves H, Groves J (1880) *Spartina* x *townsendii* Nobis. Report of the Botanical Society and Exchange Club of the British Isles 1:37.

Gustafson FG (1942) Parthenocarpy: natural and artificial. Botanical Review 8:599–654.

Harper JL (1977) Population biology of plants. Academic Press, London.

Kärkkäinen K, Savolainen O, Koski V (1999) Why do plants abort so many developing seeds: bad offspring or bad maternal genotypes? Evolutionary Ecology 13:305–317.

Kettenring KM, Whigham DF (2009) Seed viability and seed dormancy of non-native *Phragmites australis* in suburbanized and forested watersheds of the Chesapeake Bay, USA. Aquatic Botany 91:199–204.

Li H, An S, Zhi Y, Yan C, Zhao L, Zhou C, Deng Z, SuW, Liu Y (2008) Protogynous, pollen limitation and low seed production reasoned for the dieback of *Spartina anglica* in coastal China. Plant Science 174:299–309.

Li L, Lan Z, Chen J, Song Z (2018) Allocation to clonal and sexual reproduction and its plasticity in *Vallisneria spinulosa* along a water-depth gradient. Ecosphere 9:e02070.
https://doi.org/10.1002/ecs2.2070.

Lonard RI, Judd FW, Stalter R (2010) The biological Flora of coastal dunes and wetlands: *Spartina patens* (W. Aiton) G.H. Muhlenberg. Journal of Coastal Research 26:935–946.

Lyon ME (1928a) Embryoless seeds in cereals. Science 67:652.

Lyon ME (1928b) The occurrence and behavior of embryoless wheat seeds. Journal of Agricultural Research 36:631–637.

Mackay DB (1972) The measurement of viability. In: Roberts EH (ed) Viability of seeds. Chapman and Hall, London, pp 172–208.

Marchant CJ (1967) Evolution in *Spartina* (Gramineae). I. The history and morphology of the genus in Britain. Journal of the Linnean Society: Botany 60:1–24.

Marchant CJ (1968) Evolution in *Spartina* (Gramineae). II. Chromosomes, basic relationships and the problem of *S*. x *townsendii* agg. Journal of the Linnean Society: Botany 60:381–409.

Marchant CJ, Goodman PJ (1969) *Spartina maritima* (Curtis) Fernald. Journal of Ecology 57:287–291.

Martin ME, Lee TD (1993) Self pollination and resource availability affect ovule abortion in *Cassia fasciculata* (Caesalpiniaceae). Oecologia 94:503–509.

Mobberley DG (1956) Taxonomy and distribution of the genus *Spartina*. Iowa State College Journal of Science 30:471–574.

Morinaga T, Fukushima E (1934) Studies on the haploid plant of *Oryza sativa*. Japanese Journal of Botany 7:75–106.

Mullins PH, Marks TC (1987) Flowering phenology and seed production of *Spartina anglica*. Journal of Ecology 74:1037–1048.

Muñoz-RodríguezAF, Rodríguez-Rubio P, Nieva FJJ, Fernández-Illescas F, Sánchez-Gullón E, SotoHermoso-López JM, Márquez-García B (2012) The importance of bracteoles in ensuring *Atriplex halimus* germination under optimal conditions. Fresenius Environmental Bulletin 21:3521–3526.

Muñoz-Rodríguez AF, Sanjosé I, Márquez-García B, Infante-Izquierdo MD, Polo-Ávila A, Nieva FJJ, Castillo JM (2017) Germination syndromes in response to salinity of Chenopodiaceae halophytes along the intertidal gradient. Aquatic Botany 139:48–56.

Nieva FJJ, Díaz-Espejo A, Castellanos E, Figueroa ME (2001) Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel marshes (SW Spain). Estuarine, Coastal and Shelf Science 52:515–527.

Philbrick CT, Les DH (1996) Evolution of aquatic angiosperm reproductive systems. BioScience 46:813–826.

Prasifka JR, Lee DK, Bradshaw JD, Parrish AS, Gray ME (2012) Seed reduction in prairie cordgrass, *Spartina pectinata* link., by the floret-feeding Caterpillar *Aethes spartinana* (Barnes and McDunnough). Bioenergy Research 5:189–196.

Raybould AF, Gray AJ, Lawrence MJ, Marshall DF (1991) The evolution of *Spartina anglica* C.E. Hubbard (Gramineae): genetic variation and status of the parental species in Britain. Biological Journal of the Linnaean Society 44:369–380.

Richards AJ (1997) Plant breeding systems 2nd. Chapman & Hall, London.

Stephenson AG (1981) Flower and fruit abortion: proximate causes and ultimate functions. Annual Review of Ecology and Systematics 12:253–279.

Sutherland S (1986) Patterns of fruit-set: what controls fruit-flower ratios in plants? Evolution 40:117–128.

Van Drunen WE, Dorken ME (2012) Trade-offs between clonal and sexual reproduction in *Saggitaria latifolia* (Alismataceae) scale up to affect the fitness of entire clones. New Phytologist 196:606–616.

VanWijnen HJ, Bakker JP (2001) Long-term surface elevation change in salt marshes: a prediction of marsh response to future sea-level rise. Estuarine, Coastal and Shelf Science 52:381–390.

Wilcock C, Neiland R (2002) Pollination failure in plants: why it happens and when it matters. Trends in Plant Science 7:270–277.

Wong JXW, Costantini F, Merloni N, Savelli L, Geelen D, Airoldi L (2018) The widespread and overlooked replacement of *Spartina maritima* by non-indigenous *S. anglica* and *S. townsendii* in northwestern Adriatic saltmarshes. Biological Invasions 20:1687–1702.

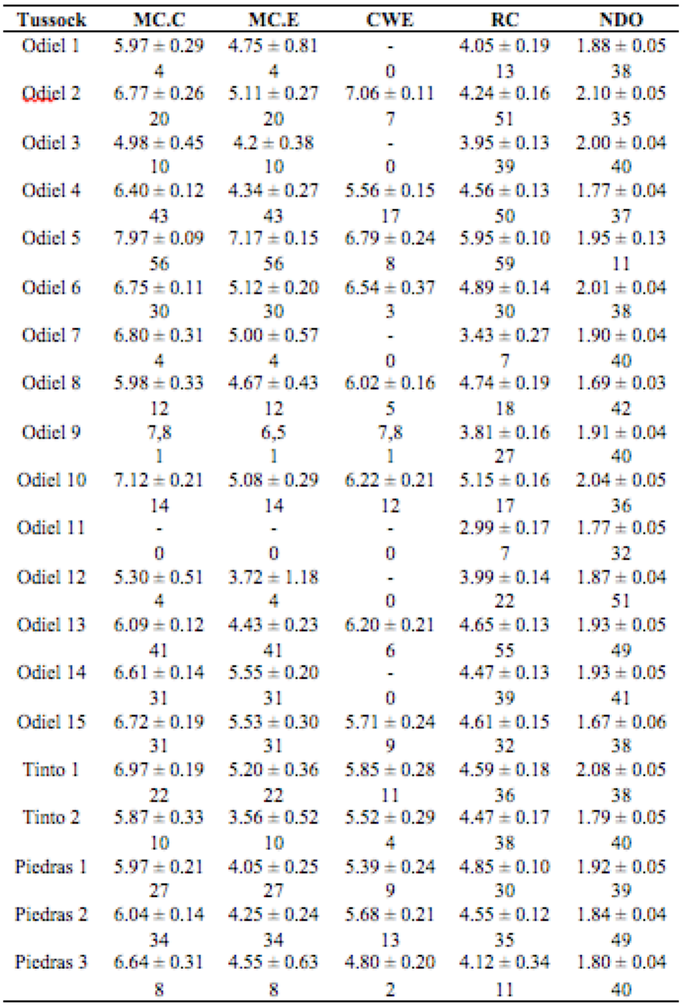
Yannic G, Baumel A, Ainouche M (2004) Uniformity of the nuclear and chloroplast genomes of *Spartina maritima* (Poaceae), a salt-marsh species in decline along the Western European coast. Heredity 93:182–188.

Supplemental material

Table S1 Percentage of non-viable, dormant and germinated caryopses, and days necessary to reach 50% of the final germination percentage (T_{50}) for *Spartina maritima* from the Gulf of Cádiz (Southwest Iberian Peninsula) under three different treatments (clean caryopsis without glumes, palea and lemma with 12 hr light and 12 hr dark photoperiod, clean caryopsis with 24 hr dark photoperiod, and caryopsis within spikelet with 12 hr light and 12 hr dark photoperiod). Values are mean ± SE (n = 4). Different letters indicate significantly differences between treatments for the same caryopsis type (HSD test or Mann-Whitney U-test, $p < 0.05$)

Treatment	% Non-viable	% Dormant	% Germinated	T_{50}
Caryopsis 12L/12D	10.0 ± 3.5^{a}	8.9 ± 3.6^{a}	91.1 ± 3.6^{a}	13.1 ± 0.6^{a}
Caryopsis 24D	14.0 ± 2.6^{a}	4.6 ± 1.8^{a}	95.4 ± 1.8^{a}	-
Spikelets 12L/12D	20.0 ± 4.3^{a}	2.5 ± 2.5^{a}	97.5 ± 2.5^{a}	23.3 ± 1.6^{b}

Table S2 Size (mm) for caryopses types from different *Spartina maritima* tussocks in the Gulf of Cádiz (Southwest Iberian Peninsula): mature caryopsis (MC.C) and its embryo (MC.E), caryopsis without embryo (CWE), reabsorbed caryopsis (RC) and undeveloped ovaries (NDO). Values are mean ± SE and number of sampled caryopsis of each type and tussock (n)



Tussock	MC.C	MC.E	CWE	RC	NDO
Odiel 1	5.97 ± 0.29	4.75 ± 0.81	-	4.05 ± 0.19	1.88 ± 0.05
	4	4	0	13	38
Odiel 2	6.77 ± 0.26	5.11 ± 0.27	7.06 ± 0.11	4.24 ± 0.16	2.10 ± 0.05
	20	20	7	51	35
Odiel 3	4.98 ± 0.45	4.2 ± 0.38	-	3.95 ± 0.13	2.00 ± 0.04
	10	10	0	39	40
Odiel 4	6.40 ± 0.12	4.34 ± 0.27	5.56 ± 0.15	4.56 ± 0.13	1.77 ± 0.04
	43	43	17	50	37
Odiel 5	7.97 ± 0.09	7.17 ± 0.15	6.79 ± 0.24	5.95 ± 0.10	1.95 ± 0.13
	56	56	8	59	11
Odiel 6	6.75 ± 0.11	5.12 ± 0.20	6.54 ± 0.37	4.89 ± 0.14	2.01 ± 0.04
	30	30	3	30	38
Odiel 7	6.80 ± 0.31	5.00 ± 0.57	-	3.43 ± 0.27	1.90 ± 0.04
	4	4	0	7	40
Odiel 8	5.98 ± 0.33	4.67 ± 0.43	6.02 ± 0.16	4.74 ± 0.19	1.69 ± 0.03
	12	12	5	18	42
Odiel 9	7,8	6,5	7,8	3.81 ± 0.16	1.91 ± 0.04
	1	1	1	27	40
Odiel 10	7.12 ± 0.21	5.08 ± 0.29	6.22 ± 0.21	5.15 ± 0.16	2.04 ± 0.05
	14	14	12	17	36
Odiel 11	-	-	-	2.99 ± 0.17	1.77 ± 0.05
	0	0	0	7	32
Odiel 12	5.30 ± 0.51	3.72 ± 1.18	-	3.99 ± 0.14	1.87 ± 0.04
	4	4	0	22	51
Odiel 13	6.09 ± 0.12	4.43 ± 0.23	6.20 ± 0.21	4.65 ± 0.13	1.93 ± 0.05
	41	41	6	55	49
Odiel 14	6.61 ± 0.14	5.55 ± 0.20	-	4.47 ± 0.13	1.93 ± 0.05
	31	31	0	39	41
Odiel 15	6.72 ± 0.19	5.53 ± 0.30	5.71 ± 0.24	4.61 ± 0.15	1.67 ± 0.06
	31	31	9	32	38
Tinto 1	6.97 ± 0.19	5.20 ± 0.36	5.85 ± 0.28	4.59 ± 0.18	2.08 ± 0.05
	22	22	11	36	38
Tinto 2	5.87 ± 0.33	3.56 ± 0.52	5.52 ± 0.29	4.47 ± 0.17	1.79 ± 0.05
	10	10	4	38	40
Piedras 1	5.97 ± 0.21	4.05 ± 0.25	5.39 ± 0.24	4.85 ± 0.10	1.92 ± 0.05
	27	27	9	30	39
Piedras 2	6.04 ± 0.14	4.25 ± 0.24	5.68 ± 0.21	4.55 ± 0.12	1.84 ± 0.04
	34	34	13	35	49
Piedras 3	6.64 ± 0.31	4.55 ± 0.63	4.80 ± 0.20	4.12 ± 0.34	1.80 ± 0.04
	8	8	2	11	40

4

Variation in sexual reproductive capacity among exotic Spartina taxa

Variation in sexual reproductive capacity among exotic *Spartina* taxa

Abstract

Most plant species with both sexual and asexual reproductive modes reach a balance between them. Sexual reproduction can enhance the colonization and spread of invasive plants, including the successful multi-continent invasion of coastal marshes by cordgrasses from the *Spartina* genus. We studied a broad spectrum of sexual reproductive traits in exotic *Spartina* taxa from salt marshes by the Gulf of Cádiz. Density of inflorescences, spikelets per inflorescence, percentage of spikelets with caryopses, and density of caryopses were recorded for *S. patens*, *S. densiflora*, and reciprocal hybrids of invasive *S. densiflora* with native *S. maritima* (*S*. ×*onubensis* subsp. *onubensis* and *S.* ×*onubensis* subsp. *tartessiana*). For *S. patens* and hybrids we also assessed pollen viability, and the percentages of reabsorbed caryopses and spikelets with all stamens exerted. For fertile *S. densiflora*, we evaluated plant sexual traits and characteristics of the sedimentary environment, and conducted germination experiments from discrete habitats long the intertidal gradient (low, middle, high and terrestrialized marshes, and saltpans). We found interannual variation for reproductive traits of *S. densiflora* which also differed among habitats. Plants with highest inflorescence and caryopsis densities occupied low marshes and saltpans, lowest caryopsis production was in low marsh where abiotic stress from frequent inundation was high, while viability of caryopses was highest in middle and high marshes. Our results indicate neither hybrid produced any mature caryopses, due to male sterility. However, male fitness did not explain infecundity of *S. patens* at our study sites, where ca. 80 % of spikelets had all stamens exerted and pollen viability was ca. 60 %. To reduce invasive spread and impact, conservation efforts to protect biodiversity should prioritize rapid eradication of *Spartina* hybrids before they attain sexual reproductive capacity, and removal of *S. densiflora* within two years of establishment before it produces viable caryopses.

Keywords:

Caryopsis, Cordgrass, Halophyte, Hybrid, Invasive species, Self-incompatibility

Introduction

Many perennial angiosperms possess both sexual and asexual reproduction systems (Fischer and van Kluenen 2001; Klimeš et al. 1997). Sexual reproduction via viable seed production provides a mechanism to improve genetic variability and molecular evolution in plant species, while asexual reproduction enables a species to persist and propagate under adverse conditions that limit or preclude sexual reproduction (Barrett 2015). In most plant species, there is a balance between both reproductive systems, depending on their specific or individual characteristics (life cycle, genetics, age, plant size, etc.) and environmental conditions (Yang and Kim 2016; Zhang and Zhang 2007). In fact, under stressful environmental conditions many plant species tend to reduce reliance on vegetative reproduction and increase seed reproductive output (Castillo and Figueroa 2009; Lei 2010; Lieffers and Shay 1981; Yang and Kim 2016). Moreover, polyploidization is a process that supports diversification of plant species, but reduced sexual reproduction can result, and new polyploids rely on vegetative reproduction more than diploid ancestors (Herben et al. 2017).

Many invasive plant species have gained competitive advantages over native species due to their capacity to reproduce vegetatively, such as female individuals of *Elodea canadensis* Michx. in Europe (Barrat-Segretain et al. 2002), or to produce agamospermic seeds, such as triploid taxa of *Taraxacum* F.H. Wigg. (Brock 2009; Shibaike et al. 2002) and the apomictic grass *Cortaderia jubata* (Lemoine) Stapf (Okada et al. 2009). Nevertheless, the added possibility of sexual reproduction, with consequent increased genetic diversity and capacity for ecological adaptation can favor the colonization and spread of invasive plants. For example, the invasion of *Solidago canadensis* L. in China is supported by a mixed reproductive strategy with sexual reproduction contributing significantly to rapid spread and establishment in new habitats (Dong et al. 2006).

In the genus *Spartina* Schreb. (cordgrasses), seed dispersal, with seed referred to as caryopses surrounded by the spikelet parts (Cheplick 1998), is the most important mode of spread (Sloop et al. 2011; Strong and Ayres 2013). However, some *Spartina* taxa may also achieve short and long-distance dispersal by rhizome ramets (Strong and Ayres 2013). Polyploidy is also an important determinant of invasiveness in plants (te Beest et al. 2012), and neopolyploids, especially those of hybrid origin, frequently undergo rapid range expansion (Ainouche et al. 2009). All species of *Spartina* are polyploids, with $n = 10$ base number of chromosomes (Ainouche et al. 2012; Ayres et al. 2008; Fortune et al. 2008; Marchant 1963). Many *Spartina* species have been introduced beyond their native ranges and have successfully invaded coastal marshes around the world (Ainouche et al. 2009; Ainouche and Gray 2016; Strong and Ayres 2013; Zedler and Kercher 2004). Therefore, taxa from the *Spartina* genus within invaded salt marshes provide a good model system to study the role of sexual reproduction among exotic species. In this sense, the relative importance of sexual and asexual reproduction of the invader *Spartina* *alterniflora* Loisel. along the Pacific Coast of China was found to change depending on latitude, tidal zone and the stage of the invasion (Liu et al. 2017). *Spartina anglica* C.E. Hubb. has rapidly expanded worldwide after human-mediated introductions via dispersal of both seeds and rhizomes (Ainouche et al. 2009). Moreover, hybrids between native *Spartina foliosa* Trin. and the exotic *S. alterniflora* formed in San Francisco Bay (California) reproduced sexually showing high seed set, backcrossing with parental species (Ayres et al. 2008b). In contrast, other exotic *Spartina* hybrids do not show sexual reproduction, such as the hybrids between native *S. foliosa* and exotic *S. densiflora* Brongn. in San Francisco Bay (Ayres et al. 2008a).

The focus of this study is a suite of naturalized invasive *Spartina* species (*S. patens* (Aiton) Muhl.*, S. densiflora*) and the reciprocal transgressive hybrids of invasive *S. densiflora* with native *S. maritima* (Curtis) Fernald.: *Spartina ×onubensis* Sánchez Gullón, M.D. Infante & B. Gallego subsp. *onubensis* (□*Spartina densiflora* × □*S. maritima*) and *Spartina ×onubensis* subsp. *tartessiana* Sánchez Gullón, B. Gallego & M.D. Infante (□*Spartina maritima* × □*S. densiflora*) from salt marshes in the Gulf of Cádiz region (Southwest Iberian Peninsula).

S. patens has high production of caryopses in its native range (Silander and Antonovics 1979), however, it has very low flowering rates and rarely sets caryopses in European populations (Baumel et al. 2016; Bertacchi and Lombardi 2014; SanLeón et al. 1999), including those from Southwest of Iberian Peninsula (Castillo et al. 2017). Moreover, Lonard et al. (2010) indicated the existence of self-incompatibility in this species. Thus, *S. patens* seems to have a moderate invasive potential in European coastal marshes and dunes due to its low level of sexual reproduction that limits its dispersion (Castillo et al. 2017). Similarly, no caryopses have been detected in the *Spartina* hybrids from the Gulf of Cádiz (Infante-Izquierdo et al. 2019b), where only asexual reproduction by rhizomes and extravaginal tillers has been documented (Castillo et al. 2010). In contrast, the South American neophyte *S. densiflora* that has invaded European and Pacific North American marshes produces many caryopsis-bearing spikelets that can increase its invasive potential (Kittelson and Boyd 1997; Nieva et al. 2001b).

Since seed production (i.e. specifically, caryopses with spikelet parts), rather than clonal expansion or fragmentation, is key to *Spartina* spread (Strong and Ayres 2013), the main goal of this work was to analyze and compare a broad spectrum of traits related to sexual reproduction in all exotic *Spartina* taxa naturalized in coastal wetlands of the Southwest Iberian Peninsula. Our intent was to improve knowledge of underlying factors that may influence their invasiveness and capacity for successful dispersal, as well as potential barriers that may limit their sexual propagule (caryopsis) production. We hypothesized that self-incompatibility or meiotic problems could be involved in the infecundity of *S. patens* and the two reciprocal hybrids from crosses between *S. maritima* and *S. densiflora*. Some studies have shown that the contribution of sexual reproduction in plants can vary among habitats within marshes (Liu et al. 2017; Xiao et al. 2009). Since *S. densiflora* was the only taxa in our study that produced caryopses, we evaluated differences in sexual reproductive traits from contrasting habitats along the intertidal gradient. We hypothesized that sexual reproduction in *S. densiflora* would be highest in habitats where abiotic-imposed stress is high.

Materials and methods

Studied taxa

We studied sexual reproduction within cordgrass populations of *S. patens*, *S. densiflora*, and the hybrids *S.* ×*onubensis* subsp. *onubensis* and *S.* ×*onubensis* subsp. *tartessiana* distributed in salt marshes along the Gulf of Cádiz (Southwest Iberian Peninsula; Table 1, Fig. 1).

Table 1 Location (local names), code and geographical coordinates for exotic *Spartina patens*, the hybrids *S. × onubensis* subsp. *onubensis* and *S. × onubensis* subsp. *tartessiana*, and invasive *Spartina densiflora* in four estuaries (Odiel, Tinto, Piedras and Guadiana) along the Gulf of Cádiz (Southwest Iberian Peninsula).

Taxon	Location	Code	Coordinates (N, W)
S. patens	Terrón	Sp1	37.22171, -7.18043
	Isla Canela	Sp2	37.17391, -7.38234
	Asperillo	Sp3	37.09273, -6.73366
	Fresneda	Sp4	37.25016, -7.41621
	Odeleite	Sp5	37.35036, -7.44631
S. × onubensis* subsp. *onubensis	Guadiana 1	HSo1	37.19513, -7.40311
	Tinto 1	HSo2	37.22524, -6.91110
	Tinto 2	HSo3	37.22537, -6.91121
	Piedras 3	HSo4	37.21063, -7.16721
S. × onubensis* subsp. *tartessiana	Piedras 1	HSt1	37.21307, -7.17539
	Piedras 2	HSt2	37.22150, -7.17957
	Piedras 4	HSt3	37.21258, -7.17681
	Odiel 1	HSt4	37.18462, -6.95824
	Guadiana 2	HSt5	37.19520, -7.40509
S. densiflora	Calatilla	Sd1	37.25094, -6.96904
	South Bacuta Island	Sd2	37.21883, -6.96418
	North Saltés Island	Sd3	37.20946, -6.95310
	Almendral sand spit	Sd4	37.20671, -6.95430
	Cascajera sand spit	Sd5	37.18919, -6.94390
	Don Claudio marsh	Sd6	37.17436, -6.92848

Spartina patens (2n = 40) is a rhizomatous perennial grass with a high ecological amplitude in its native range in tidal wetlands along the Atlantic Coast of North America, Gulf of Mexico, and Central American and Caribbean coasts (Silander and Antonovics 1979). It colonizes middle to high intertidal marshes, beaches and coastal dunes (authors, personal observations; Silander and Antonovics 1979). It was introduced in Atlantic and Mediterranean European and African marshes before or at the beginning of the nineteenth century (Baumel et al. 2016). *S. patens* has also naturalized and is invasive in marshes from British Columbia to California, along the Pacific Coast of North America (Daehler and Strong 1996; Spicher and Josselyn 1985). Until recently, European populations of *S. patens* were considered as *Spartina versicolor* Fabre, although Mobberley (1956) proposed *S. patens* and *S. versicolor* were the same species based on morphological traits. Recent phylogenetic studies concluded that European and African populations of *S. versicolor* came from North American *S. patens* populations (Baumel et al. 2016; Prieto et al. 2011). In the Iberian Peninsula, *S. patens* is distributed along the Mediterranean, Atlantic and Cantabrian Coasts (Sanz Elorza et al. 2004). Some populations of *S. patens* colonizing saltmarshes along the North Atlantic Iberian Coast are more widespread (SanLeón et al. 1999) than populations along the Mediterranean and Southwestern (Gulf of Cádiz) Coasts (Baumel et al. 2016).

Fig. 1 Map showing the sampling locations for *Spartina patens*, *Spartina densiflora* and hybrids *S.* × *onubensis* subsp. *onubensis* and *S.* × *onubensis* subsp. *tartessiana* in the Guadiana, Piedras, Odiel and Tinto Estuaries (Southwest Iberian Peninsula): A) Sp5; B) Sp4; C) HSo1 and HSt5; D) Sp2; E) Sp1 and HSt2; F) HSt1, HSt3 and HSo4; G) Sd1 (LM, MM and HM); H) Sd2 (LM, MM and HM); I) Sd3 (LM, MM and HM) and Sd4 (SP); J) HSo2 and HSo3; K) HSt4; L) Sd5 (TM); M) Sd6 (SP); and N) Sp3. See Table 1 for population codes. Salt marsh habitats for *S. densiflora*: LM: low marsh, MM: middle marsh, HM: high marsh, SP: saltpan, TM: terrestrialized high marsh. Map source: Google Earth©2019.

Spartina densiflora (2n = 70) is a South American species that has invaded salt marshes in North America, Europe and Africa (Bortolus 2006). *S. densiflora* was introduced to the Southwest Iberian Peninsula centuries ago (Castillo et al. 2010). Since then, its invasion has become a serious ecological problem (Nieva et al. 2001a). *S. densiflora* shows a high tolerance to contrasted environmental conditions and high phenotypic plasticity, which allows it to colonize and thrive in different habitats along the intertidal gradient (Bortolus 2006; Di Bella et al. 2014; Nieva et al. 2001a). This high tolerance to the abiotic environment, together with its strong competitive ability due to its phalanx (dense) clonal growth, can explain the invasion successes of *S. densiflora* (Nieva et al. 2005).

Hybridization between *Spartina* species is very frequent and the formation of hybrids between invasive and native species is well documented (Ainouche et al. 2009; Strong and Ayres 2013). In the Southwest Iberian Peninsula, European native *S. maritima* hybridizes reciprocally with invasive *S. densiflora* forming the transgressive hybrids *S.* ×*onubensis* subsp. *onubensis* (2n = ca. 64, 65, 66) and *S.* ×*onubensis* subsp. *tartessiana* (2n = ca. 92, 94) (Castillo et al. 2010; Infante-Izquierdo et al. 2019b) which have a wide ecological niche that includes growth along the whole intertidal gradient from low to middle-high marshes (Gallego-Tévar et al. 2018). In order to confirm the absence of caryopsis set in *S. patens* and the hybrids, as previously observed in a few populations (Castillo et al. 2010; Castillo et al. 2017; Infante-Izquierdo et al. 2019b), we studied the sexual traits of these taxa from several more populations, and in different years.

Study sites

The study sites included coastal wetlands at the margins of the Gulf of Cádiz (Table 1, Fig. 1), an arm of the Atlantic Ocean in South-western Spain and Southern-most Portugal. Two major rivers, the Guadalquivir and the Guadiana, and several smaller rivers including the Piedras, Odiel, Tinto and Guadalete rivers include estuaries that drain through the Gulf of Cádiz to the Atlantic Ocean.

Three different zones are distinguished in these salt marshes based on the tidal influence and their elevation (Contreras-Cruzado et al. 2017; Packham and Willis 1997). Low marshes (hereafter LM) are defined between Mean High Water Neap (MHWN) and Mean High Water (MHW), middle marshes (hereafter MM) go from Mean High Water (MHW) to Mean High Water Spring (MHWS), and high marshes (hereafter HM) from Mean High Water Spring (MHWS) to the Highest Astronomical Tide (HAT) (Long and Mason 1983). We also sampled at saltpan (hereafter SP), which are non-vegetated depressions that retains water at low tide (Escapa et al. 2015), and at terrestrialized high marsh without tidal influence (hereafter TM).

The study region experiences a warm Mediterranean climate with Atlantic Ocean influences. Annual mean temperature is 18.2 °C, and annual average rainfall is 523 mm (25-year averages, 1984-2010; data from the meteorological station located in Huelva city (37º16'00''N 06º57'00''W - 15 meters above sea level). Rainfall and mean daily temperature for studied years obtained from this meteorological station were 415 mm and 19.1 ºC in the agrometeorological period 2014/2015, 488 mm and 18.5 ºC in 2015/2016, and 598 mm and 18.6 ºC in 2016/2017. The tides along the coast of the Gulf of Cádiz are mesotidal in range. Mean sea level in this area is +1.85 m relative to Spanish Hydrographic Zero (SHZ). Tides are semidiurnal and have a mean range of 2.10 m with a mean spring tidal range of 2.97 m, representing 0.40–3.37 m above SHZ (Castellanos et al. 1994).

Five *S. patens* populations were sampled. Two of them, Sp1 and Sp2, were located in high tidal elevation salt marshes adjacent to coastal dune systems in Piedras and Guadiana Estuaries. Sp3 population was in a coastal sand dune system at the base of a sea cliff. Sp4 and Sp5 populations were established in brackish tidal marshes in Guadiana Estuary (Table 1, Fig. 1). *S. ×onubensis* subsp. *onubensis* was sampled from four marsh populations at middle intertidal elevations in the estuaries of Tinto, Piedras and Guadiana Rivers (Table 1, Fig. 1). *S. ×onubensis* subsp. *tartessiana* was sampled from five populations in low elevation tidal wetlands within the estuaries of Odiel, Piedras and Guadiana Rivers (Table 1, Fig. 1). See Castillo et al. (2010) for description of these hybrid populations. *S. patens* and both *Spartina* hybrids populations were sampled firstly in November 2016 (Sp1, Sp2 and Sp3 for *S. patens*, HSo1 and HSo2 for *S. ×onubensis* subsp. *onubensis* and HSt1 for *S. ×onubensis* subsp. *tartessiana*). In order to confirm the absence of caryopses, these populations were sampled again after one year in November-December 2017 (except Sp3 that was burned during a wild fire), and other populations were added (Sp4 and Sp5 for *S. patens*, HSo3 and HSo4 for *S. ×onubensis* subsp. *onubensis*, and HSt2, HSt3, HSt4 and HSt5 for *S. ×onubensis* subsp. *tartessiana*).

All *S. densiflora* populations were studied in the Odiel Marshes. In these marshes, *S. densiflora* has become very abundant and grows in wide areas displacing native vegetation (Nieva et al. 2001a). In fall 2015, a total of 12 populations of *Spartina densiflora* from 6 different locations were sampled in different habitats along the intertidal gradient: 3 populations in LM, 3 in MM, 3 in HM, 2 in SP and 1 population in TM (Table 1). Since 2015 was a dry year (see above), we sampled again the most representative and abundant *S. densiflora* habitats (LM, MM and HM) in Sd1, Sd2 and Sd3 locations in November 2017 (a rainy agrometeorological year).

Sexual Reproductive Structures

Density of inflorescences per plant surface (inflorescences m^{-2} plant) was calculated by counting the number of inflorescences in randomly distributed squared plots (50 x 50 cm) in patches of *S. patens* and of *S. densiflora* formed by coalescent tussocks (n = 5-10 plots per population of *S. patens*; n = 10 plots per population of *S. densiflora* in MM, HM and TM). For both *Spartina* hybrids and *S. densiflora* populations in LM and SP, in which they grow in discrete tussocks, density of inflorescences was recorded counting the number of inflorescences per tussock and measuring each tussock diameter to calculate its area, using the formula of a circle (n = 5-16 tussocks for *Spartina* hybrids; n = 10 tussocks for *S. densiflora*).

The total number of spikelets (with or without caryopsis) per inflorescence was calculated by randomly collecting inflorescences in fruiting state from each population of the four studied taxa (n = 3-20 inflorescences per population) and storing them at +5 ºC until analysis in the laboratory. Caryopsis set for *S. patens* and both *Spartina* hybrids was recorded by sampling 25 randomly selected spikelets from every inflorescence. These spikelets were analyzed one by one under magnifying glass by removing glumes, palea and lemma from their only floret. As we observed that these three taxa did not set mature caryopses, we counted the number of reabsorbed caryopses and calculated their percentage in relation to the total number of analyzed spikelets following methods we used previously on *S. maritima* (Infante-Izquierdo et al. 2019a). A caryopsis was considered reabsorbed when the ovary growth was initiated but resorption of its content by the mother plant resulted in a membranous empty sac larger than the original ovary. Male fitness of these three taxa was determined by analyzing pollen viability in two populations of *S. patens* (Sp1 and Sp2) and in one population of each hybrid (HSo1 and HSt1) by taking mature anthers from 10 randomly chosen plants. These anthers were fixed in ethylic alcohol and acetic acid in a proportion of 3:1 (Farmer's fixative solution) and opened each one onto a drop of lactophenol cotton blue stain on a glass slide. Then, more than one hundred grains from each plant were observed under light microscopy, and the aborted pollen percentage was calculated (Fernández-Illescas et al. 2010). Stamens development was recorded by observing the presence of anthers that had not been exerted during anthesis and remained inside the palea and lemma during fruit set in each analyzed spikelet. Then, the percentage of spikelets whose stamens were all exerted during anthesis was calculated (Infante-Izquierdo et al. 2019a).

Following methodology of Ayres et al. (2008b), caryopsis set for *S. densiflora* was calculated by counting the total number of spikelets in all collected inflorescences (n = 20 inflorescences per population) and identifying the presence of a caryopsis by pressing every spikelet softly with a finger. We then obtained the number of caryopses per inflorescence and calculated the percentage of spikelets with caryopsis for each sampled population. Caryopsis density (number of caryopses m^{-2} plant) per population was calculated as the product of the mean number of caryopses per inflorescence by the density of inflorescences (n = 10).

Germination experiment for S. densiflora

Spikelets containing a caryopsis of *S. densiflora* were randomly selected from the inflorescences collected in 2015 and stored in dark and dry conditions at room temperature until use. Caryopses were then cleaned (by removing glumes, palea and lemma), surface-sterilized in 5 % (v/v) sodium hypochlorite for 10 min and rinsed with distilled water (Muñoz-Rodríguez et al. 2012). Germination tests were carried out on two layers of autoclaved filter paper humidified with distilled water in Petri dishes (9 cm diameter) sealed with adhesive tape (Parafilm™) to avoid desiccation. Four replicates of 25 caryopses each were sowed for each sampled population. Germination took place at 20-25 ºC and 12h/12h photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. Germination was recorded every 2 or 3 days for 30 days. A caryopsis was considered germinated when its coleoptile emerged.

The viability of non-germinated caryopses was tested using the standard Tetrazolium test (Mackay 1972). The embryos of non-germinated caryopses were incised with a scalpel and submerged in a 1 % aqueous solution of 2, 3, 5 triphenyl tetrazolium chloride at 25 ºC in darkness for 24 hours. Then, caryopses were analysed through a magnifying glass and red stained viable embryos were counted. The percentage of viable caryopses was calculated for each dish, and then the percentages of germinated and dormant caryopses were calculated based on the number of viable caryopses. The days necessary to reach 50 % of the final germination percentage (T_{50}) was calculated following Muñoz-Rodríguez et al. (2012). Mean length of caryopsis and embryo was recorded for each germinated, dormant and non-viable caryopsis.

Sedimentary environment

Because no mature caryopses were observed for *S. patens* and *Spartina* hybrids, the sedimentary environment was sampled only for *S. densiflora* populations in order to analyze the relationships of physico-chemical aspects of the sediment and reproductive traits of *S. densiflora*. Sediments samples were collected randomly in December 2015 using stainless steel tubes of 50 mm diameter and 50 mm height (n = 3 samples per population). Samples were stored in hermetically closed polyethylene bags and stored at -20 ºC until analysis. Sediment electrical conductivity, pH, water content and organic matter content were measured as reported by Contreras-Cruzado et al. (2017). Redox potential (Eh) was measured in the field using a portable apparatus Crison pH/mV meter 25 (Hach Lange Spain, S.L.U., Barcelona, Spain) at the same points where the sediment samples were collected (n = 3).

Statistical analysis

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., USA) considering significant results when $p \leq 0.05$. Normality and homogeneity of variance of data series were tested using the Kolmogorov-Smirnov and the Levene test, respectively. When data or their transformation using $\sqrt{x}$ or arcsine(x) functions had a normal distribution and were homogeneous, means were compared using one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test as the post-hoc test. When data did not achieve normal distribution or homogeneity of variance after being transformed, Kruskal-Wallis H-test and Mann-Whitney U-test as a post-hoc test were applied. These tests were run to compare plant traits among different populations for *S. patens* and both *Spartina* hybrids and to compare plant traits and sedimentary conditions among habitats and years for *S. densiflora*. The relationships between plant traits and sedimentary conditions for *S. densiflora* were evaluated using the non-parametric Spearman correlation coefficient (ρ).

Results

Spartina patens

Inflorescence density was 5 ± 3 inflorescences m^{-2} in *S. patens* populations, without significant differences between populations (Table 2). The number of spikelets per inflorescence was 156 ± 12 spikelets inflorescence^{-1}, showing Sp3 lower values (91 ± 13 spikelets inflorescence^{-1}) than the rest of populations (Table 2). *S. patens* did not produce any mature caryopses in any sampled populations. Percentage of reabsorbed caryopsis was 7.1 ± 1.2 %, being the smallest for Sp3 (Table 2). The percentage of spikelets with all their stamens exerted was 83.8 ± 1.2 %, without significant interpopulation differences (Table 2). Pollen viability was 61.3 ± 7.4 % for Sp1 and 63.3 ± 9.5 % for Sp2.

Table 2 Number of sampled plots (n plots), inflorescence density per plant surface (Inf m^{-2}), number of sampled inflorescences (n Inf), number of spikelets per inflorescence (Spk Inf^{-1}), percentage of spikelets with mature caryopsis (MC Spk^{-1}), percentage of reabsorbed caryopsis per spikelet (RC Spk^{-1}) and percentage of spikelets with all stamens exerted (SSE) for different populations *Spartina patens* and the two *Spartina* hybrids (*S. × onubensis* subsp. *onubensis* and *S. × onubensis* subsp. *tartessiana*). See Table 1 for population codes. Different letters indicate significant differences among populations and years (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). n.s., non-significant; d.f., degrees of freedom. Data are mean ± SE.

Taxon	Code	Year	n plots	Inf m^{-2}	n Inf	Spk Inf $^{-1}$	MC Spk^{-1} (%)	RC Spk^{-1} (%)	SSE (%)
S. patens									
	Sp1	2016	5	2 ± 1	20	158 ± 8^{ac}	0 ± 0	4.0 ± 2.1^{a}	82.2 ± 4.1
	Sp2	2016	5	3 ± 1	20	164 ± 10^{ac}	0 ± 0	12.7 ± 3.0^{bc}	87.4 ± 3.4
	Sp3	2016	-	-	7	91 ± 13^{b}	0 ± 0	2.9 ± 1.4^{ab}	86.9 ± 4.3
	Sp1	2017	10	1 ± 1	20	184 ± 12^{a}	0 ± 0	6.6 ± 1.5^{bc}	79.6 ± 4.6
	Sp2	2017	5	18 ± 13	20	186 ± 8^{a}	0 ± 0	8.4 ± 1.1^{c}	87.2 ± 2.1
	Sp4	2017	5	3 ± 1	20	139 ± 7^{bc}	0 ± 0	9.0 ± 2.5^{bc}	81.2 ± 3.6
	Sp5	2017	5	2 ± 0	18	171 ± 7^{ac}	0 ± 0	6.5 ± 2.3^{abc}	82.0 ± 3.3
		Mean		5 ± 3		156 ± 12	0 ± 0	7.1 ± 1.2	83.8 ± 1.2
		Test		$H_{5,35}$ = 6.33 n.s.		F = 7.65, df = 6 p<0.0001	$H_{6,121}$ = 0.00 n.s.	$H_{6,121}$ = 16.12 p<0.05	F = 0.53, df = 6 n.s.
S. × onubensis* subsp. *onubensis	HSo1	2016	10	30 ± 9	20	173 ± 11^{a}	0 ± 0	1.3 ± 0.6^{a}	0.0 ± 0.0
	HSo2	2016	8	39 ± 7	20	176 ± 11^{a}	0 ± 0	2.4 ± 1.4^{a}	1.0 ± 0.8
	HSo1	2017	5	34 ± 9	20	139 ± 10^{a}	0 ± 0	15.0 ± 5.3^{a}	0.0 ± 0.0
	HSo2	2017	5	84 ± 26	20	182 ± 15^{a}	0 ± 0	1.8 ± 0.6^{a}	0.0 ± 0.0
	HSo3	2017	5	30 ± 5	20	293 ± 23^{b}	0 ± 0	4.6 ± 1.7^{a}	0.2 ± 0.2
	HSo4	2017	5	41 ± 11	19	163 ± 9^{a}	0 ± 0	21.9 ± 3.4^{b}	0.0 ± 0.0
		Mean		43 ± 8		188 ± 22	0 ± 0	7.8 ± 3.5	0.2 ± 0.2
		Test		$H_{5,38}$ = 4.57 n.s		F = 11.35, df = 5 p<0.0001	$H_{5,118}$ = 0.00 n.s.	$H_{5,118}$ = 41.52 p<0.0001	$H_{5,119}$ = 7.07 n.s.
S. × onubensis* subsp. *tartessiana	HSt1	2016	12	1 ± 1^{b}	11	107 ± 7^{b}	0 ± 0	0.0 ± 0.0^{a}	0.0 ± 0.0^{b}
	HSt1	2017	16	6 ± 4^{b}	10	116 ± 13^{b}	0 ± 0	2.4 ± 1.6^{a}	2.4 ± 2.4^{ab}
	HSt2	2017	5	6 ± 3^{ab}	9	81 ±6^{abc}	0 ± 0	3.6 ± 1.7^{a}	0.0 ± 0.0^{b}
	HSt3	2017	5	18 ± 3^{c}	20	64 ± 5^{c}	0 ± 0	0.8 ± 0.4^{a}	0.0 ± 0.0^{b}
	HSt4	2017	5	6 ± 2^{ab}	3	49 ± 15^{ac}	0 ± 0	25.4 ± 9.3^{b}	0.0 ± 0.0^{b}
	HSt5	2017	5	32 ± 22^{ac}	20	91 ± 7^{ab}	0 ± 0	20.2 ± 2.4^{b}	10.6 ± 4.1^{a}
		Mean		11 ± 5		85 ± 10	0 ± 0	8.7 ± 4.5	2.2 ± 1.7
		Test		$H_{5,48}$ = 18.18 p<0.01		F = 7.07, df = 5 p<0.0001	$H_{5,73}$ = 0.00 n.s.	$H_{5,73}$ = 52.57 p<0.0001	$H_{5,73}$ = 22.88 p<0.001

Spartina hybrids

Inflorescences density was 43 ± 8 inflorescences m^{-2} for *S.* ×*onubensis* subsp. *onubensis*, without significant differences between populations (Table 2). The number of spikelets per inflorescence was 188 ± 22 spikelets $inflorescence^{-1}$; plants from HSo3 produced the maximum number of spikelets per inflorescence (293 ± 23 spikelets $inflorescence^{-1}$) (Table 2). *S.* ×*onubensis* subsp. *onubensis* did not produce any mature caryopses and the percentage of those with reabsorbed caryopsis was 7.8 ± 3.5 %, being significantly higher for HSo4 (21.9 ± 3.4 %). The percentage of spikelets with all their stamens exerted was 0.2 ± 0.2 % (Table 2). Pollen viability was 0 % in all the studied stamens.

For the hybrid *S.* ×*onubensis* subsp. *tartessiana*, the density of inflorescences was 11 ± 5 inflorescence m^{-2}, reaching the highest densities in HSt3 and HSt5 populations (Table 2). The number of spikelets per inflorescence was 85 ± 10 spikelets $inflorescence^{-1}$, changing between 49 ± 15 and 116 ± 13 spikelets $inflorescence^{-1}$ (Table 2). This hybrid did not produce any mature caryopses in any of our sampled populations, resulting in a percentage range of reabsorbed caryopses between 0.0 ± 0.0 % and 25.4 ± 9.3 %, (Table 2). On the other hand, the percentage of spikelets with all their stamens exerted was 2.2 ± 1.7 %, being the highest for HSt5 population (10.6 ± 4.1 %) (Table 2). Pollen viability was 0 % in all the studied stamens.

Spartina densiflora sexual reproductive traits

Inflorescence density was higher in 2017 than in 2015 in every habitat (LM: Mann-Whitney U test, U = 168.50, $p < 0.0001$; MM: ANOVA, F = 17.63, $p < 0.001$; HM: ANOVA, F = 8.80, $p < 0.01$) (Fig. 2a). In 2015, the highest inflorescence densities were recorded in low marshes and saltpans (96 ± 19 and 155 ± 19 inflorescences m^{-2}, respectively) (Kruskal-Wallis test: $H_{4,120}$ = 62.83, $p < 0.0001$) (Fig. 2a). In the same way, inflorescence density was around 16 times higher in plants from LM than those in MM and HM in 2017 (Kruskal-Wallis test: $H_{2,89}$ = 56.29, $p < 0.0001$) (Fig. 2a).

The number of spikelets per inflorescence was higher in 2017 than in 2015 in HM (Mann-Whitney U test: U = 642.0, $p < 0.0001$), without showing interannual differences in LM and MM (LM: ANOVA, F = 0.37, df = 1, $p > 0.05$; MM: ANOVA, F = 0.70, df = 1, $p > 0.05$) (Fig. 2b). In 2015, the highest numbers of spikelets per inflorescence was recorded for samples from TM (124 ± 9 spikelet $inflorescence^{-1}$) (Kruskal-Wallis test: $H_{4,221}$ = 9.97, $p < 0.05$) (Fig. 2b). In 2017, HM populations showed more spikelets per inflorescence (149 ± 9 spikelet $inflorescence^{-1}$) than LM and MM (115 ± 5 and 115 ± 6 spikelet $inflorescence^{-1}$, respectively) (Kruskal-Wallis test: $H_{2,180}$ = 12.09, $p < 0.01$) (Fig. 2b).

The percentage of spikelets with caryopsis was similar for plants from MM for both sampling years (Mann-Whitney U test, U = 1715.0, $p > 0.05$), but it was higher in 2017 than in 2015 in LM (Mann-Whitney U test: U = 1197.5, $p < 0.01$). In contrast, this percentage was higher in 2015 than in 2017 in HM (One-way ANOVA: F = 21.28, df = 1, $p < 0.0001$) (Fig. 2c). Percentage of spikelets with caryopsis in 2015 was five times lower in LM (12.0 ± 1.9 %) than in the other habitats, reaching its maximum values in TM (79.6 ± 1.5 %) (Kruskal-Wallis test: $H_{4,221} = 110.23$, $p < 0.001$) (Fig. 2c). In 2017, it was lower in LM than in MM and HM (Kruskal-Wallis test: $H_{2,180} = 47.58$, $p < 0.000$ (Fig. 2c).

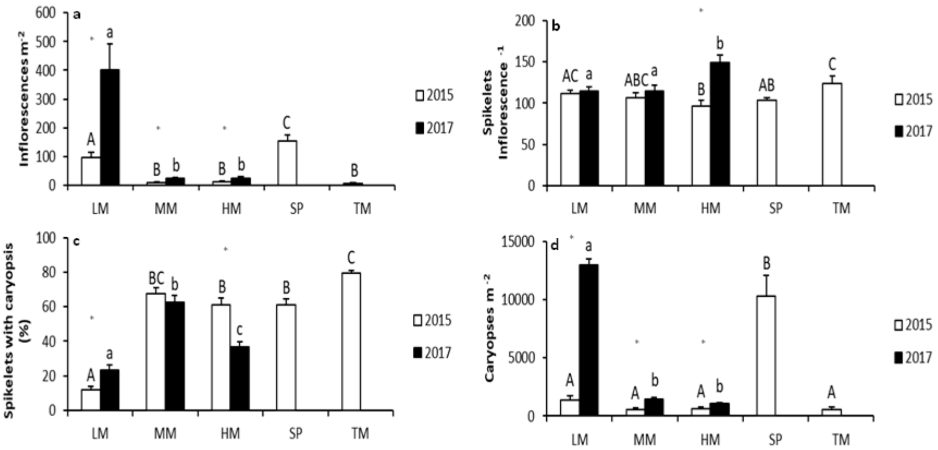


Fig. 2 **a** Inflorescence density (Inflorescences m^{-2}) (n = 10-30), **b** Number of spikelets per inflorescence (Spikelets Inflorescence^{-1}) (n = 20-60), **c** Spikelets with caryopsis (%) (n = 20-60) and **d** Caryopsis density (Caryopsis m^{-2}) (n = 10-30), for different *S. densiflora* habitats (low marsh (LM), middle marsh (MM), high marsh (HM), saltpan (SP) and terrestrialized high marsh (TM)) in Odiel Marshes (Southwest Iberian Peninsula). Different letters indicate significant differences among habitats in the same year (capital letter for 2015 and lowercase letter for 2017) (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). *Significant differences between years within the same habitat. Data are mean ± SE.

Caryopsis density per surface unit was significantly higher in 2017 than in 2015 in plants from LM, MM, and HM (LM: Mann-Whitney U test, U = 117.50, $p < 0.0001$; MM: ANOVA, F = 13.93, $p < 0.001$; HM: ANOVA, F = 4.79, $p < 0.05$) (Fig. 2d). Saltpan populations produced more caryopses per m^2 than other populations in 2015 (Kruskal-Wallis test: $H_{4,120} = 48.64$, $p < 0.0001$) (Fig. 2d). In 2017, plants from LM produced the highest density of caryopses (12979 ± 3409 caryopses m^{-2}) (Kruskal-Wallis test: $H_{2,89} = 31.68$, $p < 0.0001$) (Fig. 2d).

Spartina densiflora caryopsis viability and germination

The highest percentages of viable caryopses produced by *S. densiflora* were recorded in MM and HM zones (87.6 ± 2.2 % and 87.6 ± 2.5 %, respectively), while the lowest percentage was observed in caryopses from SP (54.0 ± 8.6 %) (One-way ANOVA: F = 15.10, df = 4, $p < 0.0001$) (Fig. 3a). Germination fraction was 86.1 ± 2.2 % and dormancy was 13.9 ± 2.2 %, without showing significant differences among habitats (Kruskal-Wallis test: $H_{4,48} = 5.56$, $p > 0.05$) (Fig. 3a). Caryopses of *S. densiflora* from LM, MM and HM germinated more rapidly than those from SP and TM (Kruskal-Wallis test: $H_{4,48} = 19.34$, $p < 0.001$) (Fig. 3b).

Spartina densiflora caryopsis and embryo size

Caryopses of *S. densiflora* were significantly smaller in LM (4.6 ± 0.03 mm) than in the other habitats, and were the largest in TM (5.1 ± 0.03 mm) (Kruskal-Wallis test: $H_{4,1136} = 112.19$, $p < 0.001$) (Fig. 3c). Embryos were also the smallest in LM (4.01 ± 0.03 mm) and were the largest in SP and the TM (4.46 ± 0.03 mm and 4.47 ± 0.03 mm, respectively) (Kruskal-Wallis test: $H_{4,1122} = 131.14$, $p < 0.001$) (Fig. 3c). Caryopsis length did not show significant differences between germinated, dormant and non-viable caryopses of *S. densiflora* (Kruskal-Wallis test: $H_{2,1136} = 2.50$, $p > 0.05$) (Fig. 3d). Nevertheless, dormant caryopses showed larger embryos (4.31 ± 0.06 mm) than germinated (4.26 ± 0.02 mm) and non-viable caryopses (4.19 ± 0.03 mm) (Kruskal-Wallis test: $H_{2, 1122} = 8.25$, $p < 0.05$) (Fig. 3d).

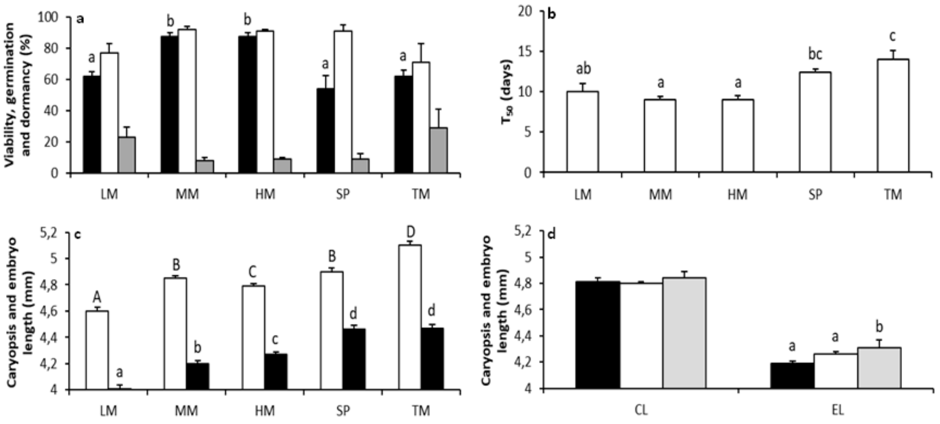


Fig. 3 **a** Percentage of viable caryopses (black bars) (n = 4-12), germination percentage (white bars) (n = 4-12) and dormancy percentage (= 100 - germination) (grey bars) (n = 4-12), **b** Days necessary to reach 50 % of the final germination percentage (T_{50}) (n = 4-12) and **c** Caryopsis (white bars) and embryo (black bars) length (n = 100-300), for different *Spartina densiflora* habitats (low marsh (LM), middle marsh (MM), high marsh (HM), saltpan (SP) and terrestrialized high marsh (TM)). **d** Caryopsis and embryo length (CL and EL, respectively) for non-viable (black bars) (n = 299), germinated (white bars) (n = 746) and dormant (grey bars) caryopsis (n = 93) in Odiel Marshes (Southwest Iberian Peninsula). Different letters indicate significant differences among habitats (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). Data are mean ± SE.

Relationships between sedimentary environment and sexual plant traits

Sediment pH ranged from 5.52 ± 0.06 at TM to 7.45 ± 0.10 at HM (One-way ANOVA: F = 40.76, df = 4, $p < 0.0001$) (Fig. 4a). Sediment Eh was negative in LM and SP (< -60 mV) and positive in the other habitats (> +155 mV) (Kruskal-Wallis test: $H_{4,36} = 26.66$, $p < 0.0001$) (Fig. 4b). Sediment electrical conductivity was the lowest in HM and TM marshes (Kruskal-Wallis test: $H_{4,36} = 20.06$, $p < 0.001$) (Fig. 4c). The most humid sediments were found in MM (63.0 ± 2.1 %), while as expected, the driest sediment was in HM (43.5 ±

8.1 %). However, no significant differences among habitats were found (Kruskal-Wallis test: $H_{4,36} = 9.09$, $p > 0.05$) (Fig. 4d). Sediment organic matter content ranged from 9.1 ± 0.7 % in LM to 37.2 ± 9.8 % in TM (Kruskal-Wallis test: $H_{4,36} = 17.51$, $p < 0.01$) (Fig. 4e). The number of spikelets per inflorescences of *S. densiflora* decreased on more alkaline sediments ($\rho = -0.90$, $p < 0.05$). The percentage of spikelets with caryopsis increased together with sediment Eh ($\rho = +0.90$, $p < 0.05$) and organic matter content ($\rho = +0.90$, $p < 0.05$).

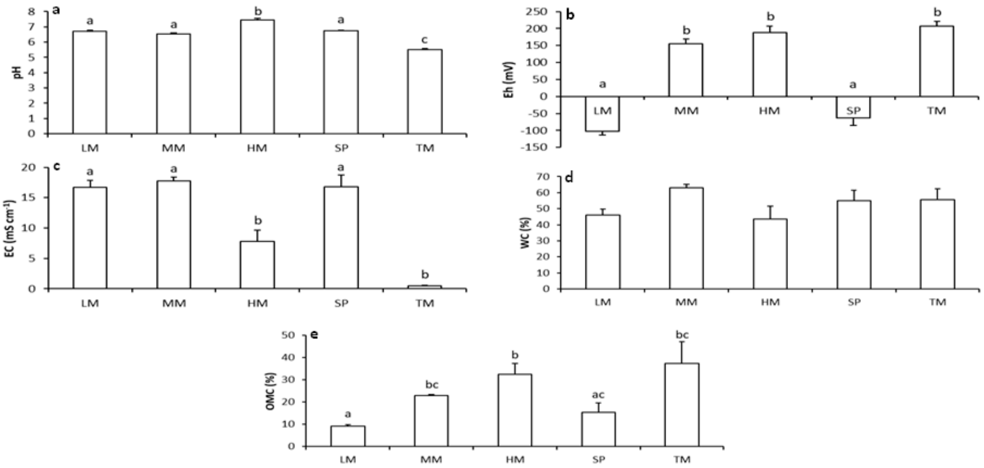


Fig. 4 **a** Sediment pH, **b** Redox potential (Eh), **c** Electrical conductivity (EC), **d** Water content (WC) and **e** Organic matter content (OMC) for different *Spartina densiflora* habitats (low marsh (LM) (n = 9), middle marsh (MM) (n = 9), high marsh (HM) (n = 9), saltpan (SP) (n = 6) and terrestrialized high marsh (TM) (n = 3)) in Odiel Marshes (Southwest Iberian Peninsula). Different letters indicate significant differences among habitats (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). Data are mean ± SE.

Discussion

Our results show that exotic *Spartina patens* and both reciprocal *Spartina* hybrids formed from crosses between native *S. maritima* and invasive *S. densiflora* did not produce any mature caryopses. This was due to male sterility in the case of both hybrids; however *S. patens* showed pollen viability ca. 60 %. For *S. densiflora*, we found interannual variations and also differences among habitats for the reproductive traits. Thus, this invasive cordgrass achieved its highest inflorescence density in LM and SP, its lowest percentage of spikelets with caryopsis in LM, and the highest caryopses viability in MM and HM.

We observed low inflorescences densities for *S. patens* at all sample sites in the Southwest Iberian Peninsula, in agreement with data on populations from this region from a previous time period as reported by Castillo et al. (2017). San León et al. (1999) and Campos et al. (2004) reported that *S. patens* rarely if ever flowers along the North Atlantic and Cantabrian Coasts of the Iberian Peninsula. In contrast, *S. patens* produces abundant number of flowering culms, especially in coastal dunes, in its native range along the Atlantic and the

Gulf Coasts of North America (Silander and Antonovics 1979). Exotic introductions of *S. patens* within the tidal wetlands along the Pacific Coast of North America from San Francisco Estuary, California, to British Columbia also bear inflorescences (Saarela 2012). In California, the density of flowering culms can be high, but is variable among populations, and *S. patens* sets viable caryopses (Grewell, personal obs.). For *S. patens*, the number of spikelets per inflorescence was around a half in dunes than in marshes, that could be related to the lower shoot density in dunes, allowing greater competition with other taxa (Castillo et al. 2017). In any case, the number of spikelets per inflorescence was 50 % less than those recorded along the Tuscany Coast (Italy) by Bertacchi and Lombardi (2014) (sub. *S. versicolor*). In hybrid *S.* ×*onubensis* subsp. *onubensis* the density of inflorescences was four times higher, and the number of spikelets per inflorescence was double than in *S.* ×*onubensis* subsp. *tartessiana* hybrid.

Our findings show inflorescence density within *S. densiflora* tussocks was the highest in LM and SP, in agreement with studies by Nieva et al. (2001a) on *S. densiflora* in Southwest Iberian Peninsula. These differences observed among habitats could be related to the high stress levels experienced by *S. densiflora* on anoxic sediments (low Eh) (Castillo et al. 2000) that occurred in SP and LM. The anoxic environment could explain a reduction in vegetative reproduction, and a corresponding increase to sexual reproduction (Castillo and Figueroa 2009). In this sense, it has been described that many plant species increase investment in sexual reproduction when asexual reproduction is limited by stressful conditions (Lei 2010; Lieffers and Shay 1981). This behavior was pointed out for *S. densiflora* in undisturbed compared to disturbed marshes in its native South American range (Bortolus et al. 2004). Another explanation could be that the increase in salinity at high marshes during spring/summer, as observed by Contreras et al. (2017), would produce salinity stress during the flowering period, thereby reducing sexual investment and increasing resource allocation to vegetative structures, as recorded for other halophytic perennial species (Jefferies et al. 1979). Our data on the number of spikelets per inflorescence for *S. densiflora* revealed certain uniformity among habitats, being similar to those recorded for invasive populations on the Pacific Coast of North America (Kittelson and Boyd 1997).

At our study sites, most sexual reproductive traits showed lower values in the drier year 2015 (415 mm annual precipitation) than observed when rainfall was higher 2017 (598 mm). Negative effects of drought on reproductive traits have been confirmed in other Poaceae species such as cereals (Barnabás et al. 2008; Kato et al. 2008). Thus, the number of spikelets per inflorescence reached maximum values at the acidic TM in 2015, which explained the negative correlation found between this trait and sediment pH. In 2017, the number of spikelets per inflorescence was significantly higher in HM than in LM and MM habitats, suggesting rainfall influence was more evident at upper marsh zones that are less affected by tides. Nevertheless, the lower number of spikelets per inflorescence in HM in 2015 was compensated with higher caryopsis set.

Spartina patens did not produce any mature caryopses in any of our studied populations in all years of study. Nevertheless, the pollen viability and the percentage of spikelets with all stamens exerted were high for *S. patens* (ca. 60 % and 80 %, respectively). This indicates most of spikelets achieved proper development of the male phase. Thus, the male fitness does not explain *S. patens* infecundity at our study sites. The recorded absence of caryopsis set was in agreement with previous studies carried out in some of our studied populations (Castillo et al. 2017), and in other European *S. patens* populations (Baumel et al. 2016; Bertacchi and Lombardi 2014). On the contrary, *S. patens* shows high caryopsis set in its native range, producing around one hundred caryopses per flowered culm (Silander 1979; Silander and Antonovics 1979). Silander and Antonovics (1979) characterized *S. patens* as having a flexible breeding system with different levels of selfing among populations (0-78 %). More recently, Lonard et al. (2010) indicated the existence of self-incompatibility in this species. Since European populations are the results of the introduction from a narrow genetic pool of *S. patens* from North America and they have a very low level of genetic diversity (Baumel et al. 2016), it is possible that our populations were originated from only one self-incompatible individual that expanded asexually. In fact, we found a variable percentage of reabsorbed caryopses in *S. patens* (2.9 to 12.7 %), which has been reported also for other *Spartina* species with low caryopsis sets such as *S. maritima* (Marchant 1967; Infante-Izquierdo et al. 2019a). The resorption of the caryopses could be due to the initial growth of ovaries that have not been fertilized. As in the case of *S. patens*, both studied *Spartina* hybrids were not able to produce caryopses as reported previously by Castillo et al. (2010). In these cases, their sterility was due to problems during pollen development, reflected on unviable pollen and only a few exerted stamens probably due to irregular meiosis (Stebbins 1945).

In contrast to *S. patens* and both *Spartina* hybrids, *S. densiflora* was able to produce many spikelets with caryopsis as reported previously in invasive Californian populations (Kittelson and Boyd 1997). *S. densiflora* caryopsis set was the lowest in LM in both years, which may be the result of high stress levels derived from long flooding periods (Nieva et al. 2001b). In fact, low marshes showed the most anoxic sediments and the lowest nutrients (low organic matter content). This explanation based on the deleterious effects of abiotic stress on *S. densiflora* sexual reproduction success was also supported by recording the smallest caryopses and embryos in LM and the largest in SP and TM habitats. In this sense, Nieva et al. (2001b) found that *S. densiflora* seeds from HM were heavier than in LM. Moreover, tidal submersion can damage sexual organs in the flower, particularly in halophytic perennial grasses (Ungar 1987). Pollination could also be affected by tide inundation in low marshes by wash effect on pollen dispersal and by reducing effective time for exposition of stigmas (Zhang et al. 2013).

The density of caryopses per surface unit, as a result of combining inflorescence density, spikelets per inflorescence and percentage of spikelets with caryopsis, was significantly higher in 2015 in SP and in 2017 at LM, showing a similar pattern to inflorescence density. These results demonstrate the impact of the inflorescence density on caryopses density. In case of LM, the high density of inflorescences compensates the low caryopsis set levels reached in both years. Xiao et al. (2009) found that *S. alterniflora* caryopses density was higher at MM than at LM and HM, which was likely due to the combined effects of tidal influences and interspecific competition.

Caryopsis viability for *S. densiflora* was the highest in MM and HM (ca. 86 %), in the range of value reported for one invasive population in San Francisco Bay, California (79 %) (Kittelson and Boyd 1997). Differences in viability among habitats could be the result of many environmental factors during fruit development and maturation (Grass and Burris 1995; Ungar 1987) such as temperature, anoxia, salinity, competition, etc. Xiao et al. (2009) found low percentages (36 %) of viability for *S. alterniflora* in Yangtze Estuary, reaching the highest percentage at MM.

The relatively high germination percentages of viable caryopses of *S. densiflora* were in accordance with data obtained for this species in other populations in the same region (Mateos-Naranjo and Redondo-Gómez 2016). Caryopses from all marsh elevation zones germinated more rapidly than those from SP and the non-tidal TM. The effect of salt exposure by the tides during fruit ripening in LM and MM, or by the saline spray in HM, could accelerate germination after salinity release (Muñoz-Rodríguez et al. 2017; Rubio-Casal et al. 2003). It is interesting to note that embryo size was significantly higher for dormant than for germinated and non-viable caryopsis. As in our study, the largest seeds of *Erodium brachycarpum* (Godr.) Thell. (Stamp 1990) and *Achnatherum hymenoides* (Roem. & Schult.) Barkw. exhibited the greatest degree of dormancy (Jones and Nielson 1999). Naylor (2003) reported that when variation in seed size exists, large seeds are not dispersed far, ensuring survival at the locality while small seeds have a greater chance of distant dispersal, and larger seeds are more dormant in some species.

The lack of mature caryopsis set in exotic *S. patens* and both *Spartina* hybrids can explain their scarce distribution in contrast to the high caryopsis set of invasive *S. densiflora* that supports its invasiveness and spread (Nieva et al. 2001a). This result, in case of hybrids, aligns with the expectation of reduced sexual reproduction as a consequence of hybridization and polyploidization (Herben et al. 2017), and suggest that *Spartina patens* and the studied hybrids currently rely on vegetative reproduction more than *S. densiflora*. Our results may be viewed as paradoxical for heptaploid *S. densiflora,* yet for which we found the greatest allocation to sexual reproduction. In coastal wetlands, this invasive polyploid has adapted to emphasize seed reproduction that enhances its invasiveness, as its buoyant caryopses float with tidewater and readily establish new populations from seed recruitment. It is possible this high capacity for sexual reproduction may be due to genetic stabilization along its evolution, not reached yet by the studied hybrids.

The hybrid sterility we report in this study coincides with the behaviour of other *Spartina* hybrids such as the hybrid between native *S. foliosa* and exotic *S. densiflora* in San Francisco Estuary (Ayres et al. 2008a), and the hybrids between native *S. maritima* and exotic *S. alterniflora* in Europe (Baumel et al. 2003; Groves and Groves 1880). However, *Spartina* hybrids act as important invaders when they set fertile caryopses. This is the case of *S. anglica,* an allopolyploid species resulting from the sterile hybrid *S. x townsendii* (Ayres and Strong 2001; Gray et al. 1991; Raybould et al. 1991), and fertile reciprocal hybrids between *S. foliosa* and *S. alterniflora* in San Francisco Estuary (Daehler and Strong 1997). These hybrids surpass native *S. foliosa* in flower, pollen and seed production (Ayres et al. 2008b).

The *Spartina* hybrids we evaluated represent a potential threat to native biodiversity. They have already acquired transgressive traits that support invasiveness (Infante-Izquierdo et al. 2019b), and their invasion could accelerate if they acquire the capacity to produce viable caryopsis through allopolyploidization (Baumel et al. 2001). In view of these results, efforts to conserve native biodiversity should be applied to eradicate both *Spartina* hybrids before they become fertile. Control efforts are also warranted for *S. patens* while its populations are few and spatially limited, and before its pollen can reach genetically different plants and set caryopses. Additionally, a management priority should target elimination of the *S. densiflora* invasion. A minimal strategy should be a surveillance and rapid response effort to remove newly invading plants before two years of colonization, since at the two year age they can form and disperse rapidly caryopses via hydrochory, and low tidal elevation marsh habitats are especially vulnerable to further invasion.

Acknowledgments

We thank to the management of the Odiel Marshes Natural Park for their collaboration. M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte of Spanish Government for the FPU Grant (Ref. FPU14/06556).

References

Ainouche M, Chelaifa J, Ferreira S, Bellot A, Ainouche A, Salmon A (2012) Polyploid evolution in *Spartina*: dealing with highly redundant hybrid genomes. In: Soltis PS, Soltis DE (eds) Polyploidy and Genome Evolution. Springer, Berlin, pp 225–243.

Ainouche ML, Fortune PM, Salmon A, Parisod C, Grandbastien MA, Fukunaga K, Ricou M, Misset MT (2009) Hybridization, polyploidy and invasion: lessons from *Spartina* (Poaceae). Biol Invasions 11:1159–1173.

Ainouche M, Gray A (2016) Invasive *Spartina*: lessons and challenges. Biol Invasions 18:2119–2122.

Ayres DR, Strong DR (2001) Origin and genetic diversity of *Spartina anglica* using nuclear DNA markers (Poaceae). Am J Bot 88:1863–1867.

Ayres DR, Grotkopp E, Zaremba K, Sloop CM, Blum MJ, Bailey JP, Anttila CK, Strong DR (2008a) Hybridization between invasive *Spartina densiflora*

(Poaceae) and native *S. foliosa* in San Francisco Bay, California, USA. Am J Bot 95:713–719.

Ayres DR, Zaremba K, Sloop CM, Strong DR (2008b) Sexual reproduction of cordgrass hybrids (*S. alterniflora* x *S. foliosa*) invading tidal marshes in San Francisco Bay. Divers Distrib 14:187–195.

Barnabás B, Jäger K, Fehér A (2008) The effect of drought and heat stress on reproductive processes in cereals. Plant Cell Environ 31:11–38.

Barrat-Segretain MH, Elger A, Sagnes P, Puijalon S (2002) Comparison of three life-history traits of invasive *Elodea canadensis* Michx. and *Elodea nuttallii* (Planch.) H. St. John. Aquat Bot 74:299–313.

Barrett SC (2015) Influences of clonality on plant sexual reproduction. Proc Natl Acad Sci U S A 112:8859–8866.

Baumel A, Ainouche ML, Levasseur JE (2001) Molecular investigations in populations of *Spartina anglica* C. E. Hubbard (Poaceae) invading coastal Brittany (France). Mol Ecol 10:1689–1701.

Baumel A, Ainouche ML, Misset MT, Gourret JP, Bayer RJ (2003) Genetic evidence for hybridization between the native *Spartina maritima* and the introduced *Spartina alterniflora* (Poaceae) in South-West France: *Spartina* x *neyrautii* re-examined. Plant Syst Evol 237:87–97.

Baumel A, Rousseau-Gueutin M, Sapienza-Bianchi C, Gareil A, Duong N, Rousseau H, Coriton O, Amirouche R, Sciandrello S, Duarte B, Caçador I, Castillo JM, Ainouche M (2016) *Spartina versicolor* Fabre: Another case of *Spartina* trans-Atlantic introduction? Biol Invasions 18:2123–2135.

Bertacchi A, Lombardi T (2014) *Spartina versicolor* Fabre in coastal areas of Tuscany (Italy). Contrib Bot XLIX:49–60.

Bortolus A, Laterra P, Iribarne O (2004) Crab-mediated phenotypic changes in *Spartina densiflora* Brong. Estuar Coast Shelf Sci 59:97–107.

Bortolus A (2006) The austral cordgrass *Spartina densiflora* Brong: Its taxonomy, biogeography and natural history. J Biogeogr 33:158–168.

Brock MT (2009) Prezygotic barriers to gene flow between *Taraxacum ceratophorum* and the invasive *Taraxacum officinale* (Asteraceae). Oecol 161:241–251.

Campos JA, Herrera M, Biurrum I, Loidi J (2004) The role of alien plants in the natural coastal vegetation in central-northern Spain. Biodivers Conserv 13:2275–2293.

Castellanos EM, Figueroa ME, Davy AJ (1994) Nucleation and facilitation in saltmarsh succession: interactions between *Spartina maritima* and *Arthrocnemum perenne*. J Ecol 82:239–248.

Castillo JM, Fernández-Baco L, Castellanos EM, Luque CJ, Figueroa ME, Davy AJ (2000) Lower limits of *Spartina densiflora* and *S. maritima* in a Mediterranean salt marsh determined by different ecophysiological tolerances. J Ecol 88:801–812.

Castillo JM, Figueroa E (2009) Effects of abiotic factors on the life span of the invasive cordgrass *Spartina densiflora* and the native *Spartina maritima* at low salt marshes. Aquat Ecol 43:51–60.

Castillo JM, Ayres DR, Leira-Doce P, Bailey J, Blum M, Strong DR, Luque T, Figueroa E (2010) The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Divers Distrib 16:547–558.

Castillo JM, Leira-Doce P, Figueroa E (2017) Biomass and clonal architecture of the cordgrass *Spartina patens* (Poaceae) as an invasive species in two contrasted coastal habitats on the Atlantic coast of the Iberian Peninsula. Pl Ecol Evol 150:129–138.

Contreras-Cruzado I, Infante-Izquierdo MD, Márquez-García B, Hermoso-López V, Polo A, Nieva FJJ, Cartes-Barroso JB, Castillo JM, Muñoz-Rodríguez A (2017) Relationships between spatio-temporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma 305:173–187.

Daehler CC, Strong DR (1996) Status, prediction and prevention of introduced cordgrass *Spartina* spp. invasions in Pacific estuaries, U.S.A. Biol Conserv 78:51–58.

Daehler CC, Strong DR (1997) Hybridization between introduced smooth cordgrass (*Spartina alterniflora*, Poaceae) and native California cordgrass (*Spartina foliosa*) in San Francisco Bay, California, USA. Am J Bot 85:607–611.

Di Bella CE, Striker GG, Escaray FJ, Lattanzi FA, Rodríguez AM, Grimoldi, AA (2014) Saline tidal flooding effects on *Spartina densiflora* plants from diferent positions of the saltmarsh. Diversities and similarities on growth, anatomical and physiological responses. Environ Exp Bot 102:27–36.

Dong M, Lu BR, Zhang HB, Chen JK, Li B (2006) Role of sexual reproduction in the spread of an invasive clonal plant *Solidago canadensis* revealed using intersimple sequence repeat markers. Plant Species Biol 21:13–18.

Escapa M, Perillo GME, Iribarne O (2015) Biogeomorphically driven salt pan formation in *Sarcocornia*-dominated salt-marshes. Geomorphology 228:147–157.

Fernández-Illescas F, Cabrera J, Nieva FJJ, Márquez-García B, Sánchez-Gullón E, Muñoz-Rodríguez AF (2010) Production of aborted pollen in marsh species of Chenopodiaceae: evidence of partial male sterility in Suadeaea and Salsoleae species. Plant Syst Evol 288:167–176.

Fischer M, van Kleunen M (2001) On the evolution of clonal plant life histories. Evol Ecol 15:565–582.

Fortune PM, Schierenbeck K, Ayres D, Bortolus A, Catrice O, Brown S, Ainouche ML (2008) The enigmatic invasive *Spartina densiflora*: A history of hybridizations in a polyploidy context. Mol Ecol 17:4304–4316.

Gallego-Tévar B, Curado G, Grewell BJ, Figueroa ME, Castillo JM (2018) Realized niche and spatial pattern of native and exotic halophyte hybrids. Oecol. 188: 849-862. doi: 10.1007/s00442-018-4251-y

Grass L, Burris JS (1995) Effect of heat stress during seed development and maturation on wheat (*Triticum durum*) seed quality: II. Mitochondrial respiration and nucleotide pools during early germination. Can J Plant Sci 75:831–839.

Gray AJ, Marshall DF, Raybould AF (1991) A century of evolution in *Spartina anglica*. Adv Ecol Res 21:1–62.

Groves H, Groves J (1880) *Spartina* x *townsendii* Nobis. Rep Bot Soc Exch Club Br Isl 1:37.

Herben T, Suda J, Klimešová J (2017) Polyploid species rely on vegetative reproduction more than diploids: a re-examination of the old hypothesis. Ann Bot 120:341–349.

Infante-Izquierdo MD, Castillo JM, Nieva FJJ, Rotundu ID, David FT, Grewell BJ, Muñoz-Rodríguez AF (2019a) Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula. Wetlands. doi.org/10.1007/s13157-019-01188-1

Infante-Izquierdo MD, Gallego-Tévar B, Sánchez-Gullón E, Nieva FJJ, Grewell BJ, Castillo JM, Muñoz-Rodríguez AF (2019b) Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae). Plant Syst Evol. doi.org/10.1007/s00606-019-01591-5

Jefferies RL, Davy AJ, Rudmik T (1979) The growth strategies of coastal halophytes. In: Jefferies RL, Davy AJ (eds) Ecological Processes in Coastal Environments. Blackwell Scientific Publications, Oxford, pp 243–268.

Jones TA, Nielson DC (1999) Intrapopulation genetic variation of seed dormancy in Indian ricegrass. J Range Manage 52: 646–650.

Kato Y, Kamoshita A, Yamagishi J (2008) Preflowering abortion reduces spikelet number in upland rice (*Oryza sativa* L.) under water stress. Crop Sci 48:2389–2395.

Kittelson PM, Boyd MJ (1997) Mechanisms of expansion for an introduced species of cordgrass, *Spartina densiflora*, in Humboldt Bay, California. Estuaries 20:770–778.

Klimeš L, Klimešová J, Hendriks R, van Groenendael J (1997) Clonal plant architecture: a comparative analysis of form and function. In: de Kroon H, van Groenendael J (eds) The ecology and evolution of clonal plants. Backhuys Publishers, Leiden, pp 1–29.

Lei SA (2010) Benefits and costs of vegetative and sexual reproduction in perennial plants: a review of literature. J Ariz-Nev Acad Sci 42:9–14.

Lieffers VJ, Shay JM (1981) The effects of water level on the growth and reproduction of *Scirpus maritimus* var. *paludosus*. Can J Bot 59:118–121.

Liu H, Lin Z, Zhang M, Qi X (2017) Relative importance of sexual and asexual reproduction for range expansion of *Spartina alterniflora* in different tidal zones on Chinese coast. Estuar Coast Shelf Sci 185:22–30.

Lonard RI, Judd FW, Stalter R (2010) The Biological Flora of Coastal Dunes and Wetlands: *Spartina patens* (W. Aiton) G.H. Muhlenberg. J Coast Res 26:935–946.

Long SP, Mason CF (1983) Saltmarsh Ecology. Cambridge University Press, Cambridge.

Mackay DB (1972) The measurement of viability. In: Roberts EH (ed) Viability of Seeds. Chapman and Hall, London, pp 172–208.

Marchant CJ (1963) Corrected chromosome numbers for *Spartina xtownsendii* and its parent species. Nature 199:929.

Marchant CJ (1967) Evolution in *Spartina* (Gramineae). I. The history and morphology of the genus in Britain. J Linn Soc (Bot) 60:1–24.

Mateos-Naranjo E, Redondo-Gómez S (2016) Interpopulation differences in salinity tolerance of the invasive cordgrass *Spartina densiflora*: Implications for invasion process. Estuar Coast 39:98–107.

Mobberley DG (1956) Taxonomy and distribution of the genus *Spartina*. Iowa St Coll J Sci 30:471–574.

Muñoz-Rodríguez AF, Rodríguez-Rubio P, Nieva FJJ, Fernández-Illescas F, Sánchez-Gullón E, Soto JM, Hermoso-López V, Márquez-García B (2012) The importance of bracteoles in ensuring *Atriplex halimus* germination under optimal conditions. Fresen Environ Bull 21:3521–3526.

Muñoz-Rodríguez AF, Sanjosé I, Márquez-García B, Infante-Izquierdo MD, Polo-Ávila A, Nieva FJJ, Castillo JM (2017) Germination syndromes in response to salinity of Chenopodiaceae halophytes along the intertidal gradient. Aquat Bot 139:48–56.

Naylor REL (2003) WEEDS. Weed Seed Biology. In: Thomas B, Murphy DJ, Murray BG (eds) Encyclopedia of Applied Plant Sciences. Academic Press, London, pp 1500-1508.

Nieva FJJ, Díaz-Espejo A, Castellanos EM, Figueroa ME (2001a) Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel Marshes (SW Spain). Estuar Coast Shelf Sci 52:515–527.

Nieva FJJ, Castellanos EM, Figueroa ME (2001b) Effects of light and salinity on seed germination in the marsh invader *Spartina densiflora* Brong., 1829 (Gramineae) from Gulf of Cádiz, Spain. Bol R Soc Esp Hist Nat 96:117–124.

Nieva FJJ, Castellanos EM, Castillo JM, Figueroa ME (2005) Clonal growth and tiller demography of the invader cordgrass *Spartina densiflora* Brongn at two contrasting habitats in SW European salt marshes. Wetlands 25:122–129.

Okada M, Lyle M, Jasieniuk M (2009) Inferring the introduction history of the invasive apomictic grass *Cortaderia jubata* using microsatellite markers. Divers Distrib 15:148–157.

Packham JR, Willis AJ (1997) Ecology of Dunes, Saltmarsh and Shingle. Chapman and Hall, London.

Prieto JAF, Cires E, Sánchez Corominas T, Vázquez VM (2011) Systematics
and management of natural resources: the case of *Spartina* species on European shores. Biologia 66:1011–1018.

Raybould AF, Gray AJ, Lawrence MJ, Marshall DF (1991) The evolution of *Spartina anglica* C.E. Hubbard (Gramineae): origin and genetic variability. Biol J Linn Soc 43:111–126.

Rubio-Casal AE, Castillo JM, Luque CJ, Figueroa ME (2003) Influence of salinity on germination and seeds viability of two primary colonizers of Mediterranean salt pans. J Arid Environ 53:145–154.

SanLeón DG, Izco J, Sánchez JM (1999) *Spartina patens* as a weed in Galician saltmarshes (NW Iberian Peninsula). Hydrobiologia 415:213–222.

Sanz Elorza M, Dana Sánchez ED, Sobrino Vesperinas E (2004) Atlas de las plantas alóctonas invasoras en España. Dirección General para la Biodiversidad, Madrid.

Saarela JM (2012) Taxonomic synopsis of invasive and native *Spartina* (Poaceae, Chloridoideae) in the Pacific Northwest (British Columbia, Washington and Oregon), including the first report of *Spartina × townsendii* for British Columbia, Canada. PhytoKeys 10:25–82.

Shibaike H, Akiyama H, Uchiyama S, Kasai K, Morita T (2002) Hybridization between European and Asian dandelions (*Taraxacum* section *Ruderalia* and section *Mongolica*). J Plant Res 115:321–328.

Silander JA (1979) Microevolution and clone structure in *Spartina patens*. Science 203:658–660.

Silander JA, Antonovics J (1979) The genetic basis of the ecological amplitude of *Spartina patens*. I. Morphometric and physiological traits. Evolution 33:1114–1127.

Sloop CM, Ayres DR, Strong DR (2011) Spatial and temporal genetic structure in a hybrid cordgrass invasion. Heredity 106:547–56.

Spicher D, Josselyn M (1985) *Spartina* (Gramineae) in northern California: distribution and taxonomic notes. Madroño 32:158–167.

Stamp NE (1990) Production and effect of seed size in a grassland annual (*Erodium brachycarpum*, Geraniaceae). Am J Bot 77:874–882.

Stebbins GL (1945) The Cytological Analysis of Species Hybrids. II. Bot Rev 11:463–486.

Strong DR, Ayres DR (2013) Ecological and evolutionary misadventures of *Spartina*. Annu Rev Ecol Evol Syst 44:389–410.

te Beest M, Le Roux JJ, Richardson DM, Brysting AK, Suda J, Kubešovám M, Pyšek P (2012) The more the better? The role of polyploidy in facilitating plant invasions. Ann Bot 109:19–45.

Ungar IA (1987) Population ecology of halophyte seeds. Bot Rev 53:301–334.

Xiao D, Zhang L, Zhu Z (2009) A study on seed characteristics and seed bank of *Spartina alterniflora* at saltmarshes in the Yangtze Estuary, China. Estuar Coast Shelf Sci 83:105–110.

Yang YY, Kim JG (2016) The optimal balance between sexual and asexual reproduction in variable environments: a systematic review. J Ecol Environ 40:12.

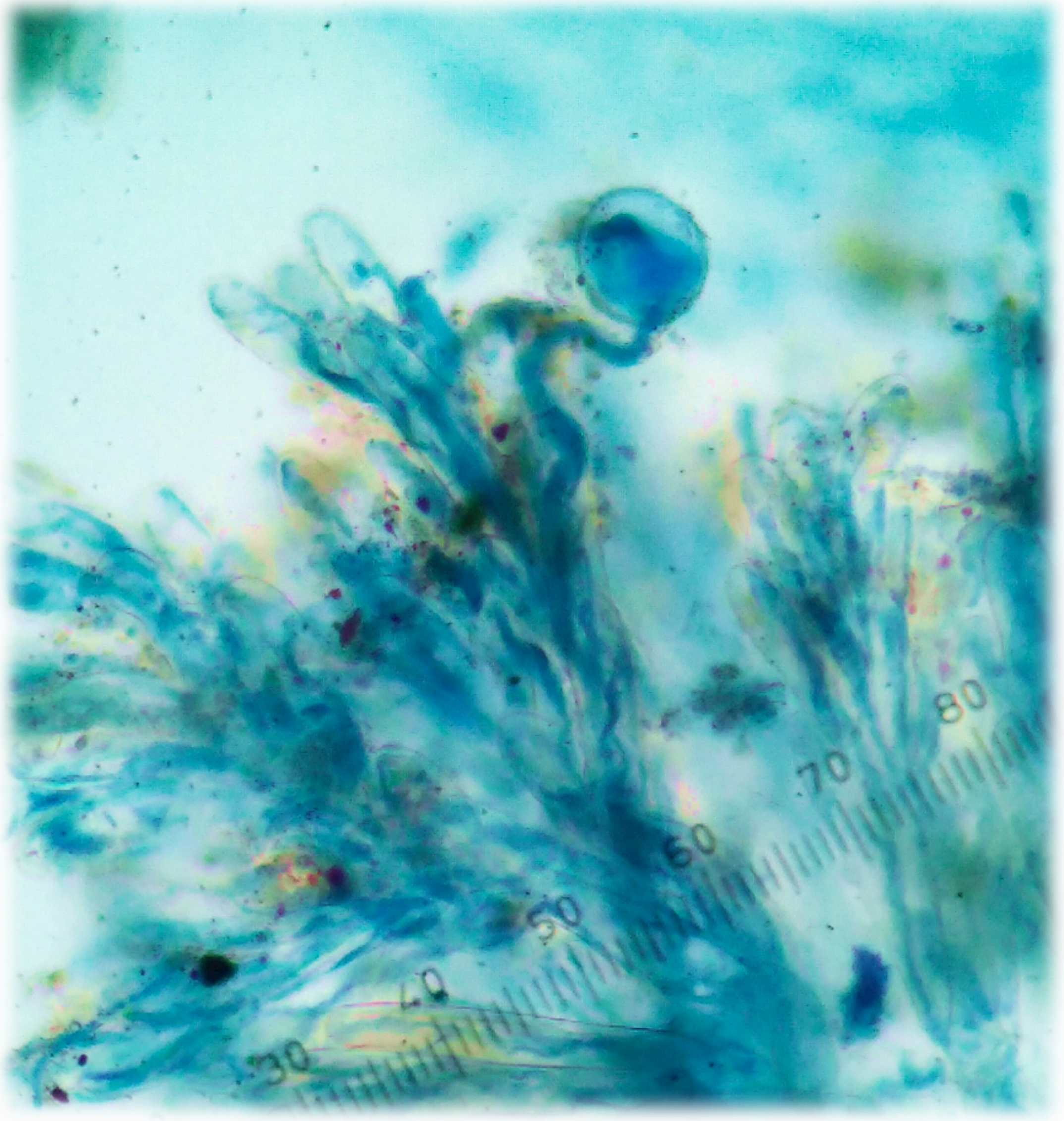
Zedler JB, Kercher S (2004) Causes and consequences of invasive plants in wetlands: opportunities, opportunists, and outcomes. Crit Rev Plant Sci 23:431–452.

Zhang Y, Zhang D (2007) Asexual and sexual reproductive strategies in clonal plants. Front Biol (Beijing) 2:256–262.

Zhang Y, Zhang L, Zhao X, Huang S, Zhao J (2013) Effects of tidal action on pollination and reproductive allocation in an estuarine emergent wetland plant–*Sagittaria graminea* (Alismataceae). PLoS ONE 8:e78956.

5

Is dichogamy responsible of differences in sexual reproduction success between native S. maritima and invasive S. densiflora?



Is dichogamy responsible of differences in sexual reproduction success between native *S. maritima* and invasive *S. densiflora*?

Abstract

• *Background and Aims Spartina maritima* is a threatened species of low salt marshes, native of Europe a North of Africa, with low and variable rates of fruit-set. In contrast, *Spartina densiflora* is a South American invasive species that shows high and regular levels of fruit set. Both species share similar reproductive traits such as spikelet characteristics, wind pollination and protogyny at flower and inflorescence levels. However, the causes of their different reproductive success are unknown, in spite of the interest in conserving *S. maritima* and in controlling *S. densiflora* invasion. This study aims to address this knowledge gap by investigating the reproductive biology of these species.

• *Methods* this reproductive traits were studied for natural populations of both *Spartina* species in tidal salt marshes of Southwest Iberian Peninsula: inflorescences development patterns and degree of overlap between sexual phases, production, viability, liberation, loss of germinability and dispersal of pollen, development and pollination of stigmas, and self-compatibility.

• *Key Results* In both species, stamens and stigmas were exerted from the spikelet early in the morning, and stigmas were receptive for the first two days. Their pollen liberation and dispersal rates were extremely low, and pollen lost its germinability is a few hours, which determined low rates of germinated pollen on stigmas. Both species presented protogyny. *S. maritima* showed only 28% of inflorescences overlaping sexual phases, while in *S. densiflora* this percentage was up to 87%. Both species were self-compatible. *S. maritima* fruit set was associated positively with sexes overlap in its inflorescence.

• *Conclusions* The lower rates of fruit set that presents *S. maritima* in comparison with *S. densiflora* are explained by some disadvantages in its sexual breeding system such as its low rates of pollen dispersal that reduced the chance of crossing, and its low degree of overlap between the two sexual phases in a inflorescence that reduced the chances of geitonogamy. Environmental conditions during flowering may also influence *S. maritima* low fruit set in relation to high temperature and humidity, and tidal immersion that would increase pollen germinability loss, incomplete opening of anthers, and a level of spikelets with non exerted stamens.

Keywords

Spartina maritima, *Spartina densiflora*, Protogyny, Pollen liberation, Pollen dispersion, Outcrossing, Selfing, Geitonogamy

Introduction

The genus *Spartina* is represented in Southwest Iberian Peninsula by three species: the native *Spartina maritima* (Curtis) Fernald, and the invasive *Spartina densiflora* Brongn. and *Spartina patens* (Aiton) Muhl., and by two nothospecies: *Spartina* × *onubensis* Sánchez Gullón, M.D. Infante & B. Gallego and *Spartina* × *onubensis* subsp. *tartessiana* Sánchez Gullón, B. Gallego & M.D. Infante, both resulting from hybridization between *S. maritima* and *S. densiflora* (Infante-Izquierdo et al., 2019b). All these taxa have vegetative reproduction by rhizomes, but seed dispersal is the most important mode of spread (Davis et al., 2004; Sloop et al., 2011; Strong and Ayres, 2013). However, only *S. maritima* and *S. densiflora* produce fertile fruits (Infante-Izquierdo et al., 2019a, in press). *Spartina maritima* is the only native cordgrass in Europe. *S. maritima* is a primary colonizer of bare intertidal mudflats that facilitates ecological succession development (Castellanos et al., 1994). *S. maritima* populations constitute a Habitat of European Community Interest according to Directive 92/43/CEE (1320 *Spartinion maritimae*) and it is considered as a threatened species in some European coastal regions (Cabezudo et al., 2005; Cooper, 1993); its populations are decreasing due to limited dispersion, erosion and expansion of exotic species (Nieva et al., 2001a; Wong et al., 2018). Most aspects of *S. maritima* sexual reproductive biology are unknown, and until recently viable seed production has been described as very low to non-existent (Marchant, 1967; Marchant and Goodman, 1969; Castellanos et al., 1994; Baumel et al., 2001; Castillo et al., 2010). Nevertheless, in a recent study, Infante-Izquierdo et al. (2019a) recorded an average percentage of fruit-set per spikelet of 13%, with high variation among populations, ranging from 0 to 45%. *Spartina densiflora* is native from South America and it is invading European and Pacific North American marshes, producing abundant caryopses that increase its invasive potential (Kittelson and Boyd, 1997; Nieva et al., 2001b).

All *Spartina* species are anemophilous, as most Poaceae species (Connor, 1979). Depending on the density of conspecific plants, pollen limitation had been described in some wind-pollinated species, and it has been reported to reduce fruit set (Friedman and Barrett, 2009), as in the case of *Spartina alterniflora* Loisel. (Davis et al. 2004).

Complete or partial self-incompatibility has been described in some *Spartina* species, such as *S. alterniflora* (Daehler, 1998), *S. pectinata* Link (Prasifka et al., 2012), *S. patens* (Lonard et al., 2010), and *Spartina anglica* C.E. Hubb. (Li et al., 2008). But there is not any study about the presence or absence of self-incompatibility neither in *S. densiflora* nor in *S. maritima*. There are only some suspicions of the existence of self-incompatibility in *S. maritima* as a hypothesis to explain its low fecundity (Marchant, 1968; Infante-Izquierdo et al., 2019a).

Protogyny, present in all species of *Spartina*, acts at flower and inflorescence level, as all the flowers in an inflorescence open firstly as females, exerting their stigmas, in a basipetal sequence from the apex towards the base, and after this their stamens exert in the same sequence (Figure 1). At flower level, protogyny avoid the interference between male function -pollen liberation- and female function -pollen reception- (Lloyd and Webb, 1986; Bertin, 1993; Bertin and Newman, 1993; Barret, 2003), and reduces self-pollination and consequent autogamy (Faegri and van der Pijl, 1979; Griffin et al., 2000). At inflorescence level protogyny reduces the possibility of geitonogamy, and its effects is bigger the lesser is the overlapping between both sexual phases in the same inflorescence (Stout, 1928; Harder et al., 2000). Studies for establishing the efficacy of dichogamy in avoiding self-pollination in a flower or geitonogamy in an inflorescence, or in a whole plant, requires estimating the time interval between female and male phase (Navarro, 1997; Aizen and Basilio, 1995; Bhardwaj and Eckert, 2001; Fernández-Illescas et al., 2011; Valentín-Silva et al., 2015, 2018).

Fig. 1 (A) Receme of *Spartina densiflora* showing florets (B) with exserted stamens on the top, (C) with necrotic stigmas on the middle part, and (D) with florets with exerted fresh stigmas at the base.

The main goal of this work was to look for explanations of the low seed production in *S. maritima*, using as comparison the behavior of invasive *S. densiflora* that shows high fruit-set levels. Infante-Izquierdo et al. (2019a) found that fruit-set levels in *S. maritima* depended on the percentage of spikelets with all their stamens exerted during the flowering period. We expected that other reproductive aspects may be involved in the low and variable fertility of *S. maritima*, so we studied a broad spectrum of traits related to sexual reproduction: efficiency of protogyny, production, viability and dispersion of pollen, receptivity and effective pollination of stigmas, and presence or absence of self-incompatibility. We hypothesized that *S. maritima* would present disadvantages in some of these reproductive aspects in comparison with *S. densiflora*, which would explain their inferior sexual reproduction capacity. Our results are interesting for the conservation efforts of *S. maritima* and it is used as a bio-tool in ecological restoration projects (Castillo & Figueroa, 2009). On the other hand, improving our knowledge about the reproductive biology of invasive species such as *Spartina densiflora* is essential to understand biological invasions mechanisms and to plan invasion control efforts.

Materials and methods

Study species

Spartina maritima (2n = 60) is a rhizomatous perennial, sparsely caespitose with 1.5–12.5 culms dm^{-2} of 30–90 cm tall. It carries racemes 8–15 cm long, with 2–5 spikes bearing unifloral spikelets with 3 stamens (Infante-Izquierdo et al., 2019b). It is a species native from European and African Atlantic Coasts (Mobberley, 1956). *S. maritima* inhabits low salt marshes, where it plays an important role in the ecological succession since it acts as primary colonizer of bare mudflats (Castellanos et al. 1994).

Spartina densiflora (2n = 70) is a rhizomatous perennial, densely caespitose with 14–85 culms dm^{-2} of 50–130 cm tall. It carries racemes 8–29 cm long, with 3–15 spikes bearing unifloral spikelets with 3 stamens (Infante-Izquierdo et al., 2019b). It is a species native from South America that has been reported to be aggressively invading a variety of environments in North America, Europe and Africa (Bortolus, 2006). In the Southwest Iberian Peninsula, *S. densiflora* was introduced accidentally in the sixteenth century (Nieva et al., 2001; Strong and Ayres, 2013). *S. densiflora* can tolerate a wide range of environmental conditions, showing a high phenotypic plasticity, which allows it colonizes different habitats along the intertidal gradient (Nieva et al., 2001, Bortolus, 2006, Castillo et al., 2014).

Study area

This study was done in the Odiel Marshes Natural Park (Huelva, Spain) located at the Gulf of Cádiz, in the Atlantic Coast of South West Iberian Peninsula. The region has Mediterranean climate with Oceanic influences. The annual mean temperature is 18.2 °C, and the annual average rainfall is 523 mm (25-year averages, 1984-2010; data obtained from the meteorological station of Huelva-F Montenegro (ESAND2100000021003B) (37°16'00''N 06°57'00''W, Altitude 15 m).

The tides along the coast of the Gulf of Cádiz are mesotidal in range. Mean sea level in this area is ca. +1.85 m relative to Spanish Hydrographic Zero (SHZ). Tides are semidiurnal and have a mean range of 2.10 m with a mean spring tidal range of 2.97 m, representing 0.40–3.37 m above SHZ (Castellanos et al., 1994).

Our study was done at four low marsh populations of *S. maritima* (Cabeza Alta 1 (CA1), Cabeza Alta 4 (CA4), Colón (COL) and Rábida (RAB)), and at four of middle marsh populations of *S. densiflora* (Almendral (ALM), Acebuchal (ACE), Rábida (RAB) and Don Claudio (DCL)) (Figure 2).

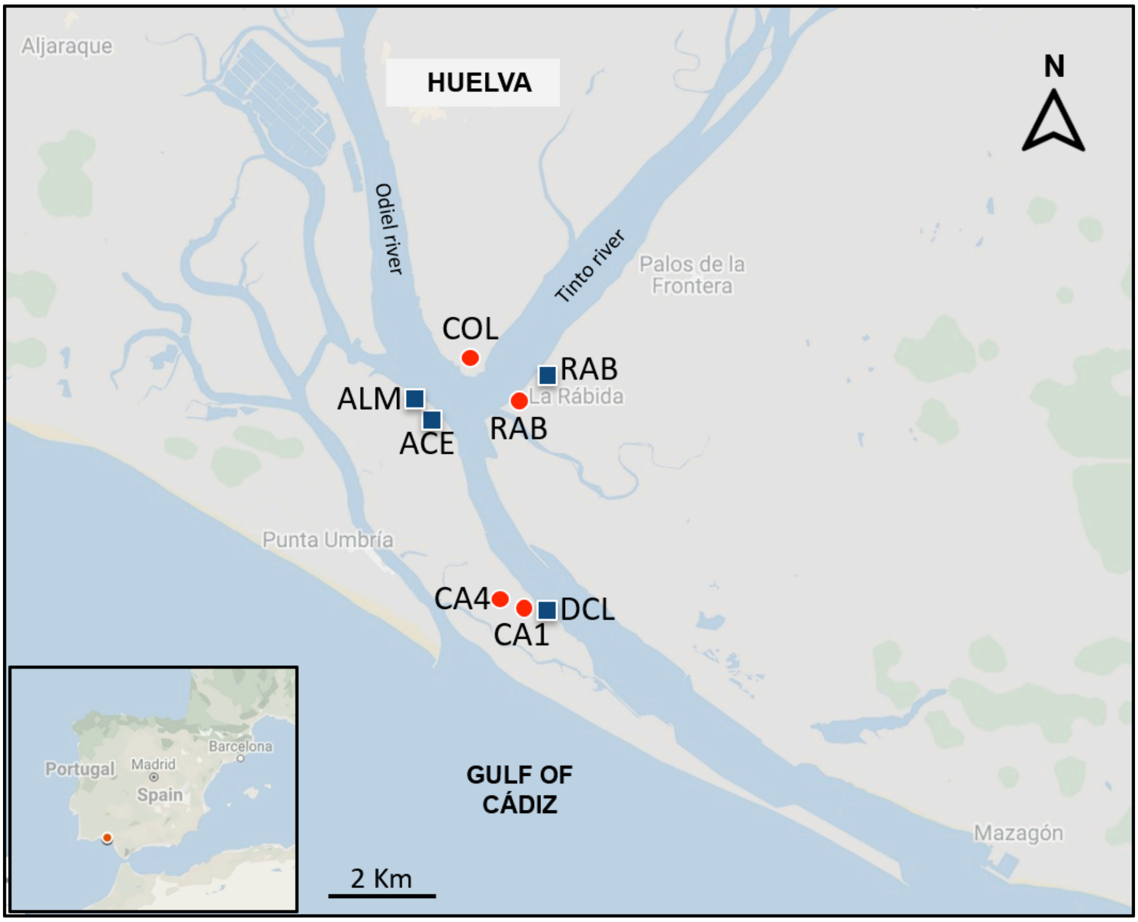


Fig. 2 (A) Map showing the location of the Estuary of Odiel and Tinto Rivers in southwest Iberian Peninsula, and (B) map showing the locations of the studied populations of native *Spartina maritima* (red circles) and invasive *S. densiflora* (blue squares). Source: Google Maps, data from ©2019 Instituto Geográfico Nacional (Spain).

Protogyny at inflorescence level

Inflorescence development was monitored during one week in two populations of *S. maritima* (CA1 and CA4) in June 2017, and in three populations of *S. densiflora,* RAB in August 2016 and ALM and ACE in September 2016. In each population, 30 inflorescences at full anthesis, or near to start, were randomly marked, and measured from the apex of the top spikelet to the apex of the bottom spikelet. Two types of monitoring were conduced: daily, in the evening of each day of the study period (around 18:00 solar local time); and hourly schedule, taking data from inflorescences during two days at four times, early in the morning (around 6:00) (H0), in the mid morning (around 10:00) (H1), in the afternoon (around 14:00) (H2), and in the evening (around 18:00) (H3). Two measures were taken at every sampling (Figure 2): longitude from the top to the apex of the lower spikelet with exposed fresh stigma, and longitude from the top to the apex of the lower spikelet with stamens exerted. Daily and hourly advance of segment with exposed fresh stigmas, and that with exerted stamens, were calculated by the difference in longitude of each segment for each inflorescence between two consecutive days or between two times in a day. For each segment, differences among daily measures were taking into account for analysis only if there was any growth in three consecutive days. To avoid the erratic behaviour of some spikelets during anthesis at the top and, especially, at the base of inflorescences, we only considered the growth of segments between 10-90% of the inflorescence longitude from the top. Once the daily advance of the different sexual segments was calculated, we also calculated the mean estimated period that inflorescences of every species were in every sexual phase; using the mean length of the inflorescence in each population for this analysis. In addition, the percentage of inflorescences in which both sexual phases coincided at least for one day. The mean distance between the last exerted stamen and the last spikelet with fresh stigmas was also recorded. In inflorescences without sex overlap, the days between the last fresh stigmas exertion and the exertion of the first stamen were counted. Finally, due to the interest of knowing environmental factors affecting *S. maritima* fruit set, we collected ripe *S. maritima* inflorescences and the number of spikelets producing caryopses was counted to calculate the percentage of fruit-set per inflorescence.

Pollen-stigma interaction and stigma necrosis

For monitoring the evolution of stigmas, we use the spikelets followed in the protogyny study in the mentioned four populations (*S. maritima*: CA1 and CA4; *S. densiflora:* ALM and ACE). At first, we marked the stigmas that have been exerted between evening of the first day of monitoring (18:00) and morning of the next day (6:00). From these stigmas, we took carefully, with the help of forceps, a sample of 10 whole stigmas from different individuals from each population. Then, stigmas were fixed in a vial with Farmer's fluid (1:3, acetic acid: ethyl alcohol). We repeat this stigmas collection at 6:00 (T0), 10:00 (T4), 14:00 (T8), 18:00 (T12), 18:00 of the next day (T36), and at 18:00 of the third day (T60). Fixed stigmas were carried to the laboratory for analysis. Each stigma was mounted in a drop of cotton blue in lactophenol, on a glass slide covered with a cover slip (Anttila et al., 1998). Slides were gently heated (without boiling) over an alcohol flame to favour the dispersion of non-anchored pollen grains (Fernández-Illescas et al., 2011). Then we observed the stigma under the microscope at x 40 objective, and all the pollen grains that were anchored to stigma through pollen tubes were counted, and the whole stigma length and its necrosis segment were measured., Finally, the percentage of necrosed stigma was calculated.

Pollen production and viability

For record pollen production per anther, 10 mature spikelets with closed anthers (just before anther exertion) were collected from different individuals, from two populations of *S. maritima* (CA1 and CA4) in June 2017, and from two populations of *S. densiflora* (ALM and ACE) in 2106 September. Then, spikelets were fixed, in a vial with Farmer's fluid, At the laboratory, 10 spikelets per species were dissected and an anther from each one was immersed in a vial with 1 ml water with detergent and the anther content was dispersed with a glass rod. Then, we extracted five samples of 10 μl that were placed on slides, and the pollen grains were counted under the microscope at x 40 objective. With these data, the number of pollen grains in each anther and the mean for each population and species were calculated.

To test pollen viability, pollen content of 10 closed anther from each of four mentioned populations and a third *S. densiflora* population (RAB), were dispersed in a drop of cotton blue in lactophenol on a glass slide, stained for several four hours, and covered with a cover slip (Ferguson, 1924). Then we observed a sample of 300 grains in each anther under the microscope at x 40 objective, counting the number of intact pollen grains that were intensely blue coloured (viables), and deformed and/or pale colours grains (non viables). Pollen viability percentage was calculated following Fernández-Illescas et al. (2010).

Pollen liberation and lost of germinability

In the same populations chosen for monitoring protogyny, we selected 10 inflorescences in male phase at the evening (18:00) and all their exerted stamens were removed. Next day, we monitored pollen liberation and pollen germinability in each population (Sm: CA1, 21/6/2017; CA4, 22/6/2017. Sd: ALM, 21/9/2016; ACE, 22/9/2016; RAB, 18/8/2016). As *S. maritima* inflorescences remained some time under tidal waters during high tides, we annotated the hour in which tides reached the inflorescences, that occurred at 11:00 in June 21th and at 12:00 in June 22th.

To study pollen liberation, we collected a sample of 10 stamens (opened in the morning) from 10 inflorescences at 6:00 (T0), 10:00 (T4), 14:00 (T8) and 18:00 (T12). Then, these stamens were placed in a 1.5 ml vial with a drop of Farmer's fluid. On the next day, we complete the vial to 1.5 ml with water at the lab, and recorded pollen grains number in each anther with the same methodology described above.

Pollen longevity was determined by testing the ability of pollen grains to germinate in vitro in a solution of sucrose 20% (0.6 M) and 0.002 M boric acid (Dafni and Firmage, 2000; Tuinstra and Wedel, 2000). Lost of germinability of liberated pollen along the day was monitored for each population collecting opened anthers (exerted from 18:00 to 6:00) at 6:00 (T0), and preserving them, to avoid dispersion, in an open glass tube that was placed in the shade among the plants of the population, attached to their culms. A sample of pollen (3 mm of the solution) was extracted from the tube with the help of a cleaned glass rod, at 10:00 (T4), 14:00 (T8) and 18:00 (T12) and was deposited on a 5 cm diameter glass Petri dish. Petri dishes were conserved, before and after the sowing, sealed with parafilm and in a dark and temperate place. The day after, three subsamples of more that 100 pollen grains were analysed from each petri dish in the laboratory, counting germinated pollen grains. Average percentage of germination was calculated for each Petri dish.

Pollen dispersion

To monitor airborne pollen grains, we used Burkard personal volumetric air samplers with a flow rate of 10 L min^{-1} Petrolatum White as adhesive and an exposition time of 20 min (200 L) (Tormo et al., 2010). Samples were taken in clear days, with wind speed lower than 2 on the Beaufort scale, in three populations of *S. maritima* (COL, 14/7/2016; RAB, 14/7/2016; CA4, 19/7/2016) and in three populations of *S. densiflora* (RAB, 18/8/2016; ALM, 21/9/2016; ACE, 22/9/2016). Samples were taken placing samplers at inflorescences height (Position 1) during three time slots: H1 (6:00-10:00), H2 (10:00-14:00) and H3 (14:00-18:00). During H1, we also took samples in other positions: P2) 50 cm above inflorescences; P3) 100 cm above inflorescences; P4) 5 m from the external limit of the population; P5) 10 m from the population; and P6) at 15 m from the population; last three records were taken in the wind direction. Three samples were taken from each time slot and from each position in each population. Samples were observed under the microscope at x 40 objective, counting the number of *Spartina* pollen grains in the aspired volume. Then, we calculated the number of pollen grains of *Spartina* m^{-3} of air in each sample., Temperature and wind speed were recorded using a pocket digital thermometer and anemometer (AZ 8908 AZ Instrument), at the same time that we took each air sample.

Breeding systems

To evaluate the success of self-pollination, including spontaneous geitonogamy by pollination of flowers with pollen of other flowers of the same inflorescence, we first tried growing plants in a grow room under controlled-environmental conditions at temperatures between +20 °C and 25 °C and a 12 h light/12 h dark photoperiod. For this, rooted stem fragments, from two tussocks of *S. maritima* from CA4 population and from one plant of *S. densiflora* from Don Claudio population (DCL), were transplanted in December 2015. Plants of both species were cultivated on its own soil and watered with tap water. At these conditions, only the plant of *S. densiflora* flowered in June. In this plant we bagged 5 inflorescences using paper bags to test self-pollination. We used 27 non-bagged inflorescences to test forced self-pollination, rubbing them with each other daily. In addition, we tested self-pollination and open-pollination in natural conditions in 3 *S. densiflora*, populations (RAB, ACE y ALM). For self-pollination testing, we used 16 inflorescences bagged with paper bags, and 21 inflorescences exposed to natural pollination were used for open-pollination testing.

As *S. maritima* did not flower at room chamber conditions, and we observed that inflorescences were affected daily by tidal submersion in natural populations (Infante-Izquierdo et al., 2019a), which prevented the use of bags for self-pollination testing, we transplanted rooted stem fragments growing on its own soil from two tussocks of *S. maritima* from CA4 population to undrained containers in December 2017 and 2018, . They were watered with tap water in open-air gardens in the town of Mazagón, more than 10 km from the nearest *S. maritima* population (Figure 1). Inflorescences tested for self-pollination were bagged with paper bags, while those tested for cross-pollination were rubbed, at least three times during their female phase, with inflorescences in male phase collected from natural populations. We repeated these experiment in 2018 and 2019.

Inflorescences used in all these assays were collected when ripening, just before the time of natural spikelet dispersal. The percentage of fruit set was calculated by counting the total number of spikelets in each inflorescence and the number of mature caryopses. In *S. maritima*, the percentage of mature spikelets that retained some un-exerted stamen inside was recorded a symptom that the inflorescence has not complete its flowering before ripening. Since stamens exertion affects fruit set in *S. maritima* (Gallego-Tévar et al., 2019; Infante-Izquierdo et al., 2019a), we calculated the percentage of spikelets with un-exerted stamens for this species.

Statistical analysis

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., USA) considering significant results when $p \leq 0.05$. Normality and homogeneity of variance of data series were tested using the Kolmogorov-Smirnov and the Levene tests, respectively. When data or their transformation using $1/(x+1)$, $\sqrt{x}$, $\ln(x+1)$ and $\arcsin(x)$ functions had a normal distribution and were homogeneous, means were compared using one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test as a post-hoc test. When data had not a normal distribution or showed no homogeneity of variance, Kruskal-Wallis and Mann-Whitney tests were used. The relationships between temperature and wind speed with pollen grains abundance in the air were evaluated using the non-parametric Spearman correlation coefficient (ρ).

Results

Protogyny at inflorescence levels

The advance of fresh stigmas exertion was 1.61 ± 0.21 cm day^{-1} for *S. maritima*, and 1.45 ± 0.12 cm day^{-1} for *S. densiflora* (Mann-Whitney U-test, $p > 0.05$). The advance of stamens exertion was 1.57 ± 0.11 and 2.39 ± 0.14 cm day-^{1} for *S. maritima* and *S. densiflora*, respectively (Mann-Whitney U-test, U = 14217.5, $p < 0.005$). Considering the mean length of measured inflorescences (10.15 ± 0.22 cm for *S. maritima* and 17.98 ± 0.58 cm for *S. densiflora*) (Mann-Whitney test: U = 78.5, $p < 0.001$), the time expended by fresh stigmas exertion (female phase) was 6.31 ± 0.14 and 12.40 ± 0.40 days for *S. maritima* and *S. densiflora*, respectively (Mann-Whitney U-test: U = 37.0, $p < 0.001$). The mean time expended by exertion of anthers (male phase) was of 6.47 ± 0.14 days and 7.52 ± 0.24 days for *S. maritima* and *S. densiflora*, respectively (Mann-Whitney U-test: U = 1273.0, $p > 0.05$).

In *S. maritima*, the percentage of inflorescences with sexual phases overlap was 27.6%. In these inflorescences, the average distance between last exerted stamens and last exerted fresh stigmas was 4.35 ± 0.29 cm. In inflorescences with no sexual phases overlap, the average number of days between both sexual phases, with any exertion neither of new stigmas nor new stamens, was 1.38 ± 0.21 days. The average percentages of fruit-set were 5.00 ± 1.81% for inflorescences with sexual phases overlap, and 1.00 ± 0.49 for inflorescences without overlap (Mann-Whitney test: U = 34.5, $p < 0.01$).

In *S. densiflora*, the percentage of inflorescences with sexual phases overlap was 86.5%, being 8.93 ± 0.32 cm between last exerted stamens and last exerted fresh stigmas. In inflorescences without sexual phases overlap, the average number of days between both sexual phases was 1.40 ± 0.40 days.

In both species, there were significant differences among different slots in the advance of fresh stigmas exertion (Kruskal-Wallis test: $H_{3,156} = 32.14$, $p = 0.0000$ and $H_{3,108} = 49.15$, $p = 0.0000$, respectively), and of stamens exertion (Kruskal-Wallis test: $H_{3,260} = 42.81$, $p = 0.0000$ and $H_{3,211} = 45.52$, $p = 0.0000$, respectively) (Figure 3A). Both species showed the same schedule dynamics: stigmas exertion occured mostly during first slot (H1: 18:00-6:00), and stamens exertion was recorded mainly during first (H0: 18:00-6:00) and second (H1: 6:00-10:00) time slots (Figure 3B).

Pollen-stigma interaction and stigmas necrosis

Considering all the stigmas examined in all time lapses, both species showed no significant difference in stigma length (Mann-Whitney U-test, $p > 0.05$) (Figure 4). Although there were significant differences between some samples collected with different ages, both in *S. maritima* (Kruskal-Wallis test: $H_{(5,136)} = 21.35$, $p < 0.001$) and in *S. densiflora* (Kruskal-Wallis test: $H_{(5,84)} =$

37.19, p = 0.0000) (Figure 4). There seems to be no stigmatic growth during the monitoring time.

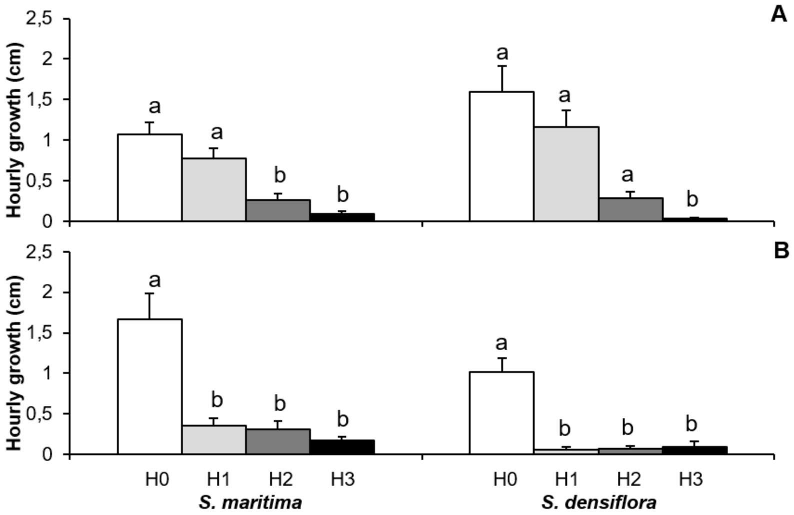


Fig. 3 Growth of segments with (A) exerted stamens and (B) exerted fresh stigmas for native *Spartina maritima* and invasive *Spartina densiflora* in four time slots during the day. Data are mean ± SE. Different letters indicate significant differences among slots (Mann Whitney test, p<0.05). Time slots: H0 (white), 18:00-6:00 solar local time on the previous day; H1 (light grey), 6:00-10:00; H2 (dark grey), 10:00-14:00; H3 (black), 14:00-18:00.

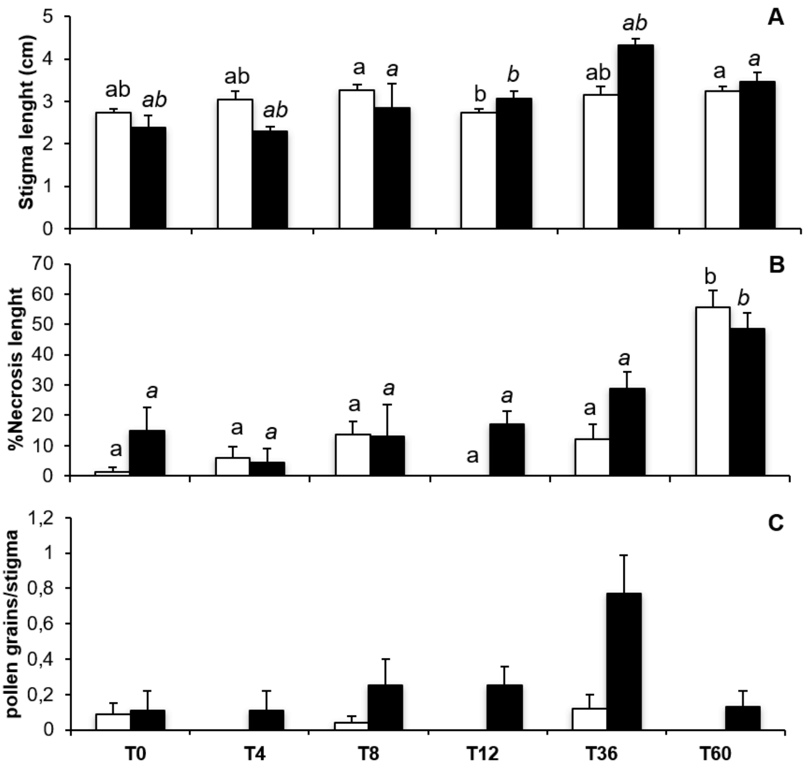


Fig. 4 Temporal changes on (A) stigma length, (B) percentage of stigma necroses length, and (C) number of pollen grains anchored to stigma for native *Spartina maritima* (white) and invasive *S. densiflora* (black). Data are mean ± SE. Different letters indicate significant differences among time periods (T) for each species (Kruskal-Wallis test and Mann Whitney U-test, p < 0.05). Time periods: T0, 6:00 solar local time; T4, 10:00; T8, 14:00; T12,18:00; T36, 18:00 of the next day; T60, 18:00 of the third day.

In both species, necrotic parts appeared on stigmas in the first hours of exertion (T0), but the percentage of necrotic stigma remained below 30% in both species till 36 hours (T36).The percentage of necrotic increased significantly to 55.8% in *S. maritima* and to 48.4% in *S. densiflora* at 60 hours (T60) (Kruskal-Wallis test: *S. maritima* $H_{(5,136)} = 76.76$, $p = 0.0000$; *S. densiflora* $H_{(5,84)}=18.09$, $p < 0.005$) (Figure 4).

Average levels of pollen grains observed anchored to stigmas were 0.039 ± 0.016 for *S. maritima* and 0.333 ± 0.074 for *S. densiflora* (Mann-Whitney test: $U = 5162$, $p < 0.05$) (Figure 4). This variable did not change significantly among time of sampling for any species (Kruskal-Wallis test: $p > 0.05$). *S. densiflora* reached its maximum levels of pollen grains observed anchored to stigmas (0.77 grains stigma^{-1}) at T36 (Figure 5).

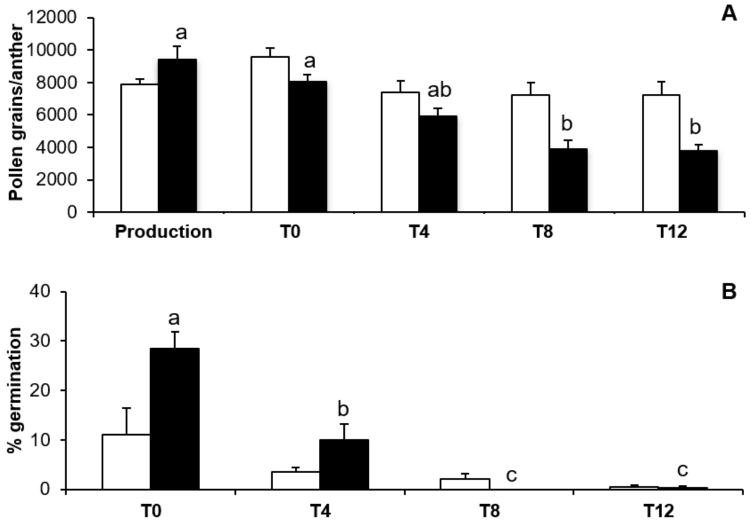


Fig. 5 (A) Pollen production and temporal changes on pollen content per anther and (B) pollen germinability for native *Spartina maritima* (white) and invasive *S. densiflora* (black). Data are mean ± SE. Different letters indicate significant differences among different time periods (T) for each species (Kruskal-Wallis test and Mann Whitney U-test, $p < 0.05$). Time periods: T0, 6:00 solar local time; T4, 10:00; T8, 14:00; T12,18:00.

Pollen production and viability

Pollen production per anther was 7909 ± 297 for *S. maritima* and 9410 ± 833 for *S. densiflora* (ANOVA, $p > 0.05$) (Figure 5). Pollen viability was 89.1% for *S. maritima* and 84.2% for *S. densiflora* (Mann-Whitney U-test, $p > 0.05$).

Pollen liberation and lost of germinability

Pollen content of anthers exposed during a day showed that there were no significant differences neither with initial pollen production nor among samples collected at different times in a day for *S. maritima* (Kruskal-Wallis test, $p > 0.05$). However, pollen liberation from anthers was reduced significantly from T4 for *S. densiflora* (Kruskal-Wallis test: $H_{4,121} = 47.0$, p = 0.0000) (Figure 5).

Pollen germinability showed no significant difference between *S. maritima* (4.29 ± 1.55%) and *S. densiflora* (9.7 ± 2.64%) (Mann-Whitney U-test, $p > 0.05$). Both species showed the same pattern of lost of germinability, being maximum at T0 and decreasing as the hours goes by, reaching values near 0% at T12. However, this reduction showed significant differences in *S. densiflora* (Kruskal-Wallis test: $H_{3,24} = 21.6$, p = 0.0001) (Figure 5).

Pollen dispersal

Neither temperature nor wind speed showed significant correlations with pollen grains abundance in the air in none of the two studied species (Spearman coefficient, $p > 0.05$). In both species, the density of pollen grains in the air was higher at time slot H1 and at position P1 (Figure 6), with an average of 6.16 ± 3.75 pollen grains m^{-3} for *S. maritima* and of 11.11 ± 4.14 pollen grains m^{-3} for *S. densiflora* (Mann-Whitney U-test, $p > 0.05$). There were no significant differences among slots or among positions in none of the species (Kruskal-Wallis test, $p > 0.05$).

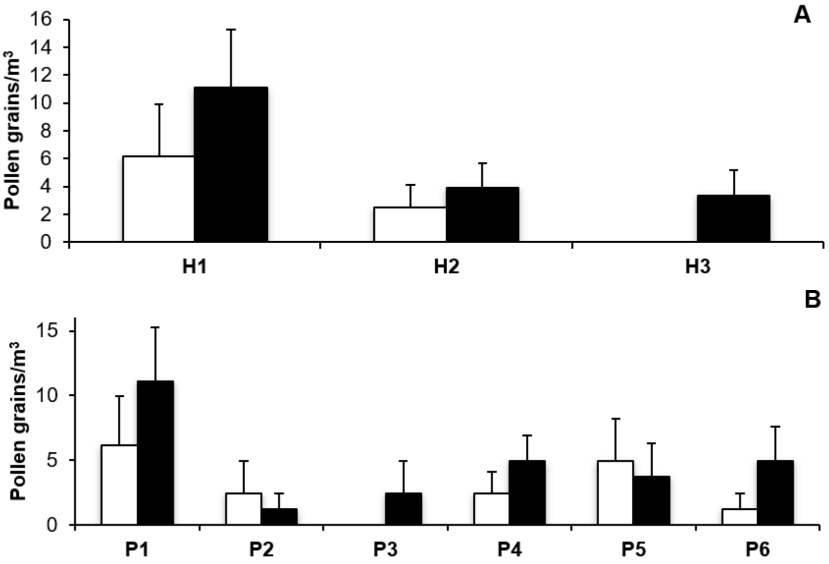


Fig. 6 Densities of pollen grains per m^3 in the air (A) at different time slots (H) and (B) at different spatial locations (P) during H1 slot for native *Spartina maritima* (white) and invasive *S. densiflora* (black). Data are mean ± SE. Time slots: H1 (6:00-10:00); H2 (10:00-14:00); H3 (14:00-18:00). Spatial locations: P1, at inflorescences height; P2, 50 cm above inflorescences; P3, 100 cm above inflorescences; P4, 5 m from the external limit of the population; P5, 10 m from the population; P6, 15 m from the population.

Breeding systems

In 2018. plants of *S. maritima* growing in garden conditions did not set any caryopsis, neither in self-pollination trials nor in cross-pollination ones (Table I), and 66.42 ± 10.35% of spikelets of the inflorescences produced in garden conditions did not exerted their stamens. In 2019, we observed that

56.95 ± 9.88% of spikelets set caryopses in self-pollination conditions, and 66.43 ± 4.06% did it when they were cross-pollinated (Mann-Whitney test: U = 9.00, $p > 0.05$) (Table I), and the percentage of spikelets that had retained some stamens inside was 1.17 ± 0.80%.

The only plant of *S. densiflora* that flowered at grow room conditions produced a percentage of caryopses per spikelet of 37.79 ± 12.36% in self-pollination conditions, and of 45.60 ± 4.77% when self-pollination was forced (one-way ANOVA: $p > 0.05$) (Table I). In fields assays, the fruit-set percentages observed in self-pollination and open-pollination assays also showed no significant differences in none of the studied populations (Mann-Whitney U-test or one-way ANOVA: $p > 0.05$) (Table I).

Table 1 Fruit set (%) obtained in assays of self-pollination, cross-pollination, forced self-pollination and open-pollination in native *Spartina maritima* and invasive *S. densiflora* in different environments and years. Values are mean ± SE. Populations: *S. maritima*: Cabeza Alta 1 (CA1), Cabeza Alta 4 (CA4), Colón (COL) and Rábida (RAB); *S. densiflora*: Almendral (ALM), Acebuchal (ACE), Rábida (RAB) and Don Claudio (DCL).

Population	Environment	Year	Treatment	Tussock	Sample	Fruit set (%)
Spartina maritima						
CA4	Garden	2018	Self-pollination	2	5	0.0 ± 0.0
CA4	Garden	2018	Cross-pollination	1	1	0.0 ± 0.0
CA4	Garden	2019	Self-pollination	2	7	57.0 ± 9.9
CA4	Garden	2019	Cross-Pollination	1	3	66.4 ± 4.1
Spartina densiflora						
DCL	Growth chamber	2016	Self-pollination	1	5	37.8 ± 12.4
DCL	Growth chamber	2016	Forced self-pollination	1	27	45.6 ± 4.8
RB	Field	2016	Self-pollination	2	2	3.1 ± 1.8
RB	Field	2016	Open-pollination	2	2	15.1 ± 6.7
ACE	Field	2016	Self-pollination	4	5	15.1 ± 9.8
ACE	Field	2016	Open-pollination	5	9	50.5 ± 11.4
ALM	Field	2016	Self-pollination	6	9	29.4 ± 10.3
ALM	Field	2016	Open-pollination	6	10	13.8 ± 6.0

Discussion

The rate of advance of stigmas exertion was similar in both species (around 1.5 cm day^{-1}), and the rate of advance of stamens exertion was similar to that of stigmas for *S. maritima*, but faster for *S. densiflora*. These data meant, based on the average inflorescence lengths, 6.5 days of female and male functionality in *S. maritima* and about 15.6 days of female function and 7.1 day of male function in *S. densiflora*.

In both species exertion of stigmas occurred during night and early morning, stigmas did not elongate after their exertion, and necrosis appeared in both species during the first hours after exertion. However, stigmas stayed as non necrotic on most of their length during the first two days. Stigmas necrosis reached ca. 50% in the third day, which represented most of the length appearing outside the spikelet. These results were in accordance with observations of pollen grains on stigmas that reached the maximum in the second day of exposition, presenting few grains in the third day probably due to the effect of necrosis in the breakup of attached grains. This receptivity period for an average segment of 3 cm in which spikelets show functional stigmas was one day lesser than the three days observed in *S. alterniflora* stigmas by Davis et al. (2004).

Both species produced a similar and great amount of pollen, with an average around 8000-9000 grains per anther, higher than 650 and 3395 grains per anther recorded for *S. foliosa* and *S. alterniflora*, respectively (Anttila et al. 1998), and than 509-771 recorded for *S. anglica* (Li et al. 2008).

In both species, exertion of stamens occured during night and morning, and liberation of pollen seemed to occur in the first hours of daytime. Similar results were recorded for *S. alterniflora* pollen shed that was maximum between 8:00 and 10:00 h (Fang et al., 2004), and for many Poaceae species flowering in summer and dispersing their pollen during fresher hours (Muñoz-Rodríguez et al., 2010).

In spite of their similar pollen production, anthers of *S. maritima* still retained most of their pollen amount at the end of the day (around 75%), while in *S. densiflora* disperded a half of its pollen. This higher pollen liberation in *S. densiflora* than in *S. maritima* determined a greater male functionality in the invader, as observed in the invasive *S. alterniflora* with respect to the native *S. foliosa* in California (Anttila et al., 1998).

High air humidity conditions, especially in low mashes where *S. maritima* inhabits could affect anthers opening since it depends on their dehydration (Pacini 1990), and pollen liberation usually occurs under dry conditions that favour pollen dispersal (Whitehead, 1983). Also, heat stress may affect anther dehiscence as occurs in another Poaceae species (Harsant et al., 2013; Arshad et al., 2015; Wang et al., 2018), whicg would lead to reduced pollen dispersal. This negative effect of high temperatures could be greater for

S. maritima than for *S. densiflora*, since the native cordgrass flowers mostly in July, the hottest month in the study area. Maximum pollen densities in the air early in the morning confirmed these results.

From the moment pollen is liberated, pollen viability is reduced mainly due to dehydration, which depends on the vapour pressure of the air (Aylor, 2003). In this sense, both studied species lost its pollen germinability during the first hours after release, which is congruent with observations made by Shi et al. (2009) for *S. alterniflora*, and for other Poaceae species (Dafni and Firmage 2000, Harsant et al., 2013; Sánchez et al., 2014; Arshad et al., 2015; Wang et al., 2018). Again, the higher temperatures during *S. maritima* flowering could explain the lesser degree of pollen germnability recorded for this species compared to *S. densiflora*).

Maximum pollen density in the air early in the morning confirmed these results. The average pollen in the air surrounding the inflorescences of *S. maritima* (6.16 grains/m^3) and *S. densiflora* (11.11 grains/m^3) revealed extremely low pollen dispersal rates. *Atriplex halimus* L. and *Halimione portulacoides* Aellen, two halophytes that share habitat with both studied *Spartina* species in the studied area and bloom in the same period of the year (Fernandez-Illescas et al., 2010), dispersed 2014 and 5105 grains m^{-3}, respectively, (unpublished data). Silander & Antonovics (1979) also evidenced very low pollen dispersal rates for *S. patens*, as they observed that pollen catchment presented a highly leptokurtic dropping from the source, reaching one-half over a distance of 1 m, and only 5% at 4m from source. Li et al. (2008) found that the amount of *S. anglica* pollen decreased along the distance from the flowering plants, without detecting any pollen at 3 m from mother plants.

Except pollen production, the three another studied aspects related to pollen: liberation, lost of viability and dispersal, revealed the existence of a great pollen limitation in the pollination of both studied *Spartina* species. This pollen limitation was the greatest for *S. maritima* in the three mentioned aspects, probably due to its habitat, low marshes with high humidity stressed by tidal submersion, and by its flowering phenology during the hotest month of the year. This pollen limitation explained the low levels of pollen grains anchored to stigmas, that reached a maximum in the second day of exposition. This data is similar as those registered by Infante-Izquierdo et al. (2019a) for *S. maritima* and those observed in pollen limitted *S. alterniflora* (Davis et al. 2004). Pollen limitation is frequent in wind-pollinated plants (Friedman and Barret, 2009), limiting fruit-set (Davis et al., 2004; Infante-Izquierdo et al., 2019a). In this sense, the recorded differences obtained in pollen grains on stigmas between *S. densiflora* and *S. maritima* may explain differences in fruit-set success.

We have shown, for first time, that both studied *Spartina* species are self-compatible, and we did not observe any significant differences in their fruit-set rates, neither between self-pollination assays and cross-pollination assays in *S. maritima*, nor between results of self-pollination assays, forced self-pollination and open-pollination assays in *S. densiflora*. These results indicated that geitonogamy, when possible by the overlap of functional sexual phases in the inflorescence, could compensate the lack of out-crossing, in spite of the existence of pollen limitation (Ashman et al., 2004). Some authors supposed *S. maritima* was self-incompatible (Marchant 1968). Even ourselves in a previous work (Infante-Izquierdo et al., 2019a) invoke the possibility of the existence of self-incompatibility for explaining the very low seed set, if there were any, for some studied *S. maritima* populations (Marchant, 1967; Marchant and Goodman, 1969; Castellanos et al., 1994; Baumel et al., 2001; Castillo et al., 2010). Self-incompatibility in some *Spartina* species is unclear. This is the case of *S. patens*, which is described as self-incompatible by some authors (Lonard et al. 2010), however, Silander and Antonovics (1979) found a large variation in fruit-set per spikelet after self-fertilization in two populations. Also, in self-incompatible *S. alterniflora* there are a great variation among individuals in their capacity to produce self-pollinated seeds (Daehler, 1998, 1999). Therefore, more studies are necessary to clarify if this variation in self-incompatibility also exists in *S. maritima*, since this could then explain, at least partially, the variability on fruit-set levels found among different populations by Infante-Izquierdo et al. (2019a).

In a previous work, Infante-Izquierdo et al., (2019a) revealed a great variation in fruit-set for *S. maritima*, founding that tussocks in which inflorescences reached a more advanced state of flowering (more exerted stamens) when fruit set started produced more caryopses per spikelet. Our present results are in accordance with those as the percentage of spikelets with non-exerted stamens was the highest in 2018, when no caryopses was produced, and the lowest in 2019, when fruit-set reached 57% in self-pollination conditions, and 66% in cross-pollinated inflorescences.

Protogyny has been demonstrated for both studied species. In *S. maritima*, overlap of sexual phases occurred only in 28% of the inflorescences, and in those the distance between last exerted stamens and last exerted fresh stigmas was 4.35 cm. As stigmas remained receptive an average of two days, this meant that in this distance there are an average of 3 cm of spikelets with receptive stigmas, and that real distance between each sex was around 1.35 cm. This short distance together with self-compatibility could explain the greater fruit set in these inflorescences than in those inflorescences (72%) that did not overlap sexual phases, where sexual phases were separated an average of 1.38 neuter days avoiding geitonogamy between spikelets of the same raceme. In contrast, there was an overlap between male and female functions in most *S. densiflora* inflorescences (87%). Thus, avoiding of geitonogamy was supported only by the 8.93 cm average distance between spikelets in different sexual phases. In this case, upper spikelet exhibits their stigmas some days before the first stamens exertion, favouring cross-pollination, however, the lower spikelets exhibits their stigmas when the upper ones had exerted their anthers. Thus, *S. maritima* protogyny at inflorescence level semmed to be more efficient in avoiding geitonogamy than in *S. densiflora*.

Among the wind-pollinated species those that are outcrossing appear to have the highest rates of gene flow, and therefore of pollen dispersal (Hamrick et al., 1995; Ackerman, 2000), so based on the pollen limitation and the incomplete protogyny that both studies species of *Spartina* exhibited, it can be assumed that selfing must play an important role in their breeding systems, explaining the low genetic diversity among individuals recorded for both studies *Spartina* species (Yannic et al., 2004; Castillo et al., 2018).

Moreover, there is a positively correlation between presence of protogyny with the existence of self-compatibility in dichogamous species (Routley et al., 2004). In those cases, protogyny acts mainly avoiding the anther-stigma interference in pollination and not avoiding selfing (Routley et al., 2004; Sargent et al., 2006; Dai and Galloway, 2011; Narbona et al., 2011). In both studied species, selfing was not avoided at all by protogyny, when geitonogamy was allowed by the overlap of both sexual phases in the same inflorescence. Therefore, we can assume that this is the role that protogyny plays in both *Spartina* species. In this way, the reduction of interference that protogyny supposes would allow some cross-pollination, as it is demonstrate through the production of relatively commons reciprocal hybrids between studied species (Infante-Izquierdo et al., 2019b). When overlap of sexual phases exist in an inflorescence, first flowers that open in an inflorescence, the upper ones, have more probabilities for outcrossing.

In any case, reduced pollen dispersal in these species, and in studied *S. patens* (Silander and Antonovics, 1979), caused by its low rate of pollen liberation from its anthers, and its early loss of germinability, determines that these perennials species seem to be in the way of not maintaining an effective outcrossing system, and to let a chance to a delayed selfing (Goodwillie and Weber, 2018). Then, the gains in genetic variability that outcrossing generates, are change by the possibility of fruiting in isolated plants, which it would be very important in a genus that contains a high number of primary colonizers and invasive species (Ainouche et al., 2004, 2009; Fortune et al., 2008; Strong and Ayres, 2014; Ainouche and Gray, 2016). Also, is possible that genetic variation would have a lesser importance in this genus, by the fact that most of their species have a hybrid and polyploidy origin, that gives some advantages maintaining certain level of genetic variability through heterosis and prevent the adverse effects of mutations through redundancy (Miller and Venable, 2000; Comai, 2005). In this way, studied *Spartina* species would respond to the hypotheses of Rosas-Guerrero et al. (2017), who stated that incomplete dichogamy should be favoured in plants without neither strong inbreeding depression nor pollen limitation, as a way to minimize pollen–pistil interference (Bertin, 1993).

Certain level of inbreeding depression has been found in invader *S. alterniflora* in California, a pollen-limited species partially self-incompatible. In the early development of its invasion the inbreeding depression in low density communities could create an Allee effect due to pollen limitation (Davis et al., 2004). But inbreeding depression affected mainly to the selfed progeny of self-incompatible plants, while selfed progeny from maternal plants with high self-fertility carried relatively little genetic load and were likely to survive on open mudflats (Dahler, 1999), and then spread and established vigorous seedlings (Sloop et al., 2011).

The lower fruit set of *S. maritima* in comparison with *S. densiflora* may be explained by some disadvantages characteristics in its sexual breeding system: its lower rates of pollen dispersion that reduces the chance of crossing, and its lower degree of overlap between the two sexual phases in an inflorescence that reduces the chances of geitonogamy. Also, environmental conditions during its flowering, (high temperatures and inmersion by tides and high humidity) may determine a greater degree in loss of pollen germinability, incomplete anthers opening, and a greater level of spikelets in which the stamens do not exert which reduce fruit set.

Acknowledgments

We thank to the management of the Odiel Marshes Natural Park for its collaboration. M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte, Spanish Government, for the FPU Grant (Ref. FPU14/06556).

References

Ackerman, J.D. (2000). Abiotic pollen and pollination: ecological, functional, and evolutionary perspectives. Plant Systematics and Evolution 222: 167-185.

Ainouche, M.L., Baumel, A., Salmon, A., Yannic, G. (2004). Hybridization, polyploidy and speciation in *Spartina* (Poaceae). New Phytologist 161(1): 165-172.

Ainouche, M.L., Fortune, P.M., Salmon, A., Parisod, C., Grandbastien, M.A., Fukunaga, K., Ricou, M., Misset, M.T. (2009). Hybridization, polyploidy and invasion: lessons from *Spartina* (Poaceae). Biological invasions,11(5): 1159-1173.

Ainouche, M., Gray, A. (2016). Invasive *Spartina*: lessons and challenges. Biological Invasions 18(8): 2119-2122.

Aizen, M.A., Basilio, A. (1995). Within and among flower sex-phase distribution in *Alstroerneria aurea* (Alstroemeriaceae). Can. J. Bot. 73: 1986-1994.

Anttila, C.K., Daehler, C.C., Rank, N.E., Strong, D.R. (1998). Greater male fitness of a rare invader (*Spartina alterniflora*, Poaceae) threatens a common native (*Spartina foliosa*) with hybridization. American Journal of Botany, 85(11): 1597-1601.

Arshad, M.S., Farooq, M., Asch, F., Krishna, J.S., Prasad, P.V., Siddique, K.H. (2017). Thermal stress impacts reproductive development and grain yield in rice. Plant physiology and biochemistry 115: 57-72.

Ashman, T.L., Knight, T.M., Steets, J.A., Amarasekare, P., Burd, M., Campbell, D.R., Dudash, M.R., Johnston, M.O., Mazer, S.J., Mitchell, R.J., Morgan, M.T., Wilson, W.G. (2004). Pollen limitation of plant reproduction: ecological and evolutionary causes and consequences. Ecology 85: 2408–2421.

Aylor, D.E. (2003). Rate of dehydration of corn (*Zea mays* L.) pollen in the air. Journal of Experimental Botany 54(391): 2307-2312. DOI: 10.1093/jxb/erg242

Barrett, S.C.H. (2003). Mating strategies in flowering plants: the outcrossing–selfing paradigm and beyond. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences 358(1434): 991-1004.

Baumel, A., Ainouche, M.L., Levasseur, J.E. (2001). Molecular investigations in populations of *Spartina anglica* C.E. Hubbard (Poaceae) invading coastal Brittany (France). Molecular Ecology 10: 1689-1702.

Bertin, R.I. (1993). Incidence of monoecy and dichogamy in relation to self-fertilization in angiosperms. American Journal of Botany 80: 557-583.

Bertin, R.I., Newman, C.N. (1993). Dichogamy in angiosperms. The Botanical Review 59: 112-150.

Bhardwaj, M., Eckert, C.G. (2001). Functional analysis of synchronous dichogamy in flowering rush *Butomus umbellatus* (Butomaceae). American Journal of Botany 88: 2204-2213.

Bortolus, A. (2006). The austral cordgrass *Spartina densiflora* Brong: Its taxonomy, biogeography and natural history. Journal of Biogeography 33: 158-168.

Cabezudo, B., Talavera, S., Blanca, G., Salazar, C., Cueto, M., Valdés, B., Hernández-Bermejo, J.E., Herrera, C.M., Rodríguez-Hiraldo, C., Navas, D. (2005). Lista roja de la flora vascular de Andalucía. Consejería de Medio Ambiente. Junta de Andalucía, Sevilla.

Castellanos, E.M., Figueroa, M.E., Davy, A.J. (1994). Nucleation and facilitation in saltmarsh succession: interaction between *Spartina maritima* and *Arthrocnemum perenne*. Journal of Ecology 82: 239-248.

Castillo, J.M., Ayres, D.R., Leira-Doce, P., Bailey, J., Blum, M., Strong, D.R., Luque, T., Figueroa, E. (2010). The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Diversity and Distributions 16: 547-558.

Castillo, J.M., Gallego-Tévar, B., Figueroa, E., Grewell, B.J., Vallet, D., Rousseau, H., Keller, J., Lima, O., Dréano, S, Salmon, A., Aïnouche, M. (2018). Low genetic diversity contrasts with high phenotypic variability in heptaploid *Spartina densiflora* populations invading the Pacific coast of North America. Ecology and evolution 8(10): 4992-5007.

Castillo, J.M., Grewell, B.J., Pickart, A., Bortolus, A., Peña, C., Figueroa, E., Sytsma, M. (2014). Phenotypic plasticity of invasive *Spartina densiflora* (Poaceae) along a broad latitudinal gradient on the Pacific Coast of North America. American Journal of Botany 101: 448-458.

Castillo, J.M., Figueroa, M.E. (2009). Restoring salt marshes using smalls cordgrass, *Spartina maritima*. Restoration Ecology 17: 324-326.

Connor, H.E. (1979). Breeding systems in the grasses: a survey. New Zealand Journal of Botany 17(4): 547-574.

Cooper, M.A. (1993). The status of *Spartina maritima* in Suffolk. Suffolk Naturalists' Society Transactions 29: 48-54.

Daehler, C.C. (1998). Self-fertility variation and the reproductive advantage of self-fertility for an invading plant (*Spartina alterniflora*). Evolutionary Ecology 12: 553-568.

Daehler, C.C. (1999). Inbreeding depression in smooth cordgrass (*Spartina alterniflora*, Poaceae) invading San Francisco Bay. American Journal of Botany 86(1): 131-139.

Dafni, A., Firmage, D. (2000). Pollen viability and longevity: Practical, ecological and evolutionary implications. Plant Systematics and Evolution 222: 113-132. doi:10.1007/BF00984098

Dai, C., Galloway, L.F. (2011). Do dichogamy and herkogamy reduce sexual interference in a self-incompatible species?. Functional Ecology 25(1): 271-278.

Davis, H.G., Taylor, C.M., Lambrinos, J.G., & Strong, D.R. (2004). Pollen limitation causes an Allee effect in a wind-pollinated invasive grass (*Spartina alterniflora*). Proceedings of the National Academy of Sciences 101(38): 13804-13807.

Faegri, K., van der Pijl, L. (1979). The principles of pollination ecology. Pergamon Press. Oxford.

Fang, X., Subudhi, P.K., Venuto, B.C., Harrison, S.A. (2004). Mode of pollination, pollen germination, and seed set in smooth cordgrass (*Spartina alterniflora*, Poaceae). International Journal of Plant Sciences 165(3): 395-401.

Ferguson, N. (1924). On the determination of the percentage of abortive pollen in plants. Journal of Experimental Biology 2: 65-73.

Fernández-Illescas, F., Cabrera, J., Nieva, F.J.J., Márquez-García, B., Sánchez-Gullón, E., Muñoz-Rodríguez, A.F. (2010). Production of aborted pollen in marsh species of Chenopodiaceae: evidence of partial male sterility in *Suadeaea* and *Salsoleae* species. Plant Systematics and Evolution 288: 167-176.

Fernández-Illescas, F., Nieva, F.J.J., de las Heras, M.A., Muñoz-Rodríguez, A.F. (2011). Dichogamy in Salicornieae species: establishment of floral sex phases and evaluation of their frequency and efficacy in four species. Plant Systematics and Evolution 296: 255-264.

Fernández-Illescas, F., Nieva, F.J.J., Silva, I., Tormo, R., Muñoz, A.F. (2010). Pollen production of Chenopodiaceae species at habitat and landscape scale in Mediterranean salt marshes: An ecological and phenological study. Review of Palaeobotany and Palynology 161(3-4): 127-136.

Fortune, P.M., Schierenbeck, K., Ayres, D., Bortolus, A., Catrice, O., Brown, S., Aïnouche, M.L. (2008). The enigmatic invasive *Spartina densiflora*: a history of hybridizations in a polyploidy context. Molecular Ecology 17(19): 4304-4316.

Friedman, J., Barrett, S.C.H. (2009). Wind of change: new insights on the ecology and evolution of pollination and mating in wind-pollinated plants. Annals of Botany 103: 1515–1527.

Gallego-Tévar, B., Infante-Izquierdo, M.D., Figueroa, E., Nieva, F.J., Muñoz-Rodríguez, A.F., Grewell, B.J., & Castillo, J.M. (2019). Some like it hot: Maternal-switching with climate change modifies formation of invasive *Spartina* hybrids. Frontiers in plant science 10. doi:

10.3389/fpls.2019.00484.

Goodwillie, C., Weber, J.J. (2018). The best of both worlds? A review of delayed selfing in flowering plants. American Journal of Botany 105(4): 641-655.

Griffin, S.R., Mavraganis, K., Eckert, C.G. (2000). Experimental analysis of protogyny in *Aquilegia canadiensis* (Ranunculaceae). American Journal of Botany 87: 1246-1256.

Hamrick, J.L., Godt, M.J.W., Sherman-Broyles, S.L. (1995). Gene flow among plant populations: Evidence from genetic markers. In: Hoch, P.G., Stevenson, A.G. (eds.) Experimental and Molecular Approaches to Plant Biosystematics. 215-232. Missouri Botanical Garden. St. Louis.

Harder, L.D., Barret, S.C.H., Cole, W.W. (2000). The mating consequences of sexual segregation within inflorescences of flowering plants. Proceedings of the Royal Society of London. Series B. Biological Sciences 267: 315-320.

Harsant, J., Pavlovic, L., Chiu, G., Sultmanis, S., Sage, T.L. (2013). High temperature stress and its effect on pollen development and morphological components of harvest index in the C3 model grass *Brachypodium distachyon*. Journal of experimental botany 64(10): 2971-2983.

Infante-Izquierdo, M.D., Castillo, J.M., Nieva, F.J.J., Rotundu, I.D., David, F.T., Grewell, B.J., Muñoz-Rodríguez, A.F. (2019a). Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula. Wetlands (2019). https://doi.org/10.1007/s13157-019-01188-1

Infante-Izquierdo, M.D., Gallego-Tévar, B., Sánchez-Gullón, E., Nieva, F.J.J., Grewell, B.J., Castillo, J.M., Muñoz-Rodríguez, A.F. (2019b). Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae). Plant Systematics and Evolution 305: 531-547.

Infante-Izquierdo, M.D., Muñoz-Rodríguez, A.F., Nieva, F.J.J., Polo-Ávila, A., Sánchez-Gullón, E., Soriano, J.J., Sanjosé, I., Grewel, J., Castillo, J.J. (in press). Variation in sexual reproductive capacity among exotic Spartina taxa.

Kittelson, P.M., Boyd, M.J. (1997). Mechanisms of expansion for an introduced species of cordgrass, *Spartina densiflora*, in Humboldt Bay, California. Estuaries 20: 770-778.

Li, H., An, S., Zhi, Y., Yan, C., Zhao, L., Zhou, C., Deng, Z., Su, W., Liu, Y. (2008). Protogynous, pollen limitation and low seed production reasoned for the dieback of *Spartina anglica* in coastal China. Plant science 174(3): 299-309.

Lloyd, D.G., Webb, C.J. (1986). The avoidance of interference between the presentation of pollen and stigmas in angiosperm. I. Dichogamy.New Zealand J ournal of Botany 24: 135-162.

Lonard, R.I., Judd, F.W., Stalter, R. (2010). The biological Flora of coastal dunes and wetlands: *Spartina patens* (W. Aiton) G.H. Muhlenberg. Journal of Coastal Research 26: 935-946.

Marchant, C.J. (1967), Evolution in *Spartina* (Gramineae). I. The history and morphology of the genus in Britain. Journal of the Linnean Society: Botany 60: 1-24.

Marchant, C.J. (1968). Evolution in *Spartina* (Gramineae). II. Chromosomes, basic relationships and the problem of *S. x townsendii* agg. Journal of the Linnean Society: Botany 60: 381-409.

Marchant, C.J., Goodman, P.J. (1969). *Spartina maritima* (Curtis) Fernald. Journal of Ecology 57: 287-291.

Miller, J.S., Venable, D.L. (2000). Polyploidy and the evolution of gender dimorphism in plants. Science 289: 2335–2338.

Mobberley, D.G. (1956). Taxonomy and distribution of the genus *Spartina*. Iowa State College Journal of Science 30: 471-574.

Muñoz-Rodríguez, A.F., Silva, I., Tormo, R. (2010). Influence of meteorological parameters in hourly patterns of grass (Poaceae) pollen concentrations. Annals of Agricultural and Environmental Medicine 17(1): 87-100.

Narbona, E., Ortiz, P.L., & Arista, M. (2011). Linking self-incompatibility, dichogamy, and flowering synchrony in two *Euphorbia* species: alternative mechanisms for avoiding self-fertilization?. PloS one 6(6) e20668.

Navarro, L. (1997). Is the dichogamy of *Salvia verbenaca* (Lamiaceae) an effective barrier to self-fertilization?. Plant Systematics and Evolution 207: 111-117.

Nieva, F.J.J., Díaz-Espejo, A., Castellanos, E., Figueroa, M.E. (2001a). Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel marshes (SW Spain). Estuarine, Coastal and Shelf Science 52: 515-527.

Nieva, F.J.J., Castellanos, E.M., Figueroa, M.E. (2001b). Effects of light and salinity on seed germination in the marsh invader *Spartina densiflora* Brong., 1829 (Gramineae) from Gulf of Cádiz, Spain. Boletín de la Real Sociedad Española de Historia Natural 96: 117-124.

Pacini, E. (1990). Harmomegathic characters of Pteridophyta spores and Spermatophyta pollen. In: Hesse, M., Ehrendorfer, F. (eds.) Morphology, development, and systematic relevance of pollen and spores. 53-69. Springer. Vienna.

Prasifka, J.R., Lee, D.K., Bradshaw, J.D., Parrish, A.S., Gray, M.E. (2012). Seed reduction in prairie cordgrass, *Spartina pectinata* link., by the floretfeeding Caterpillar *Aethes spartinana* (Barnes and McDunnough). Bioenergy Research 5: 189-196.

Rosas-Guerrero, V., Hernández, D., Cuevas, E. (2017). Influence of pollen limitation and inbreeding depression in the maintenance of incomplete dichogamy in *Salvia elegans*. Ecology and Evolution 7(12): 4129-4134.

Routley, M.B., Bertin, R.I., Husband, B.C. (2004). Correlated evolution of dichogamy and self-incompatibility: a phylogenetic perspective. International Journal of Plant Sciences 165(6): 983-993.

Sánchez, B., Rasmussen, A., Porter, J.R. (2014). Temperatures and the growth and development of maize and rice: a review. Global change biology 20(2): 408-417.

Sargent, R.D., Mandegar, M.A., Otto, S.P. (2006). A model of the evolution of dichogamy incorporating sex-ratio selection, anther-stigma interference, and inbreeding depression. Evolution 60(5): 934-944.

Shi, F., Yuan, Z., He, K. S., Li, J., Guo, S. (2009). The ecological profit in phenology and pollination of alien species *Spartina alterniflora* (Loisel) introduced to coastal wetlands in Northern China. Polish Journal of Ecology 57(2): 283-289.

Silander, J., Antonovics, J. (1979). The genetic basis of the ecological amplitude of *Spartina patens*. Morphometric and physiological traits. Evolution 33: 1114-1127.

Sloop, C.M., Ayres, D.R., Strong, D.R. (2011). Spatial and temporal genetic structure in a hybrid cordgrass invasion. Heredity 106: 547-56.

Stout, A.B. (1928). Dichogamy in flowering plants. Bulletin of the Torrey Botanical Club 55: 141-141.

Strong, D.R., Ayres, D.R. (2013). Ecological and evolutionary misadventures of *Spartina*. Annual Review of Ecology, Evolution, and Systematics 44: 389-410.

Tormo, R., Silva-Palacios, M.I., Gonzalo-Garijo, M.A., Muñoz-Rodríguez, A.F., Fernández-Rodríguez, S., Recio, D. (2010). Use of personal sporetraps to complement continuous aerobiological monitoring. Grana 49: 134-141.

Tuinstra, M.R., Wedel, J. (2000). Estimation of pollen viability in grain *Sorghum*. Crop science 40(4): 968-970.

Valentin-Silva, A., Batalha, M.A., Guimarães, E. (2018). Dynamics of pollen release and stigmatic exposure in neotropical *Piper* species: a possible pattern for the genus. International Journal of Plant Sciences 179(4): 287-295.

Valentin-Silva, A., Coelho, V.P.D.M., Ventrella, M.C., Vieira, M.F. (2015). Timing of pollen release and stigma receptivity period of *Piper vicosanum*: New insights into sexual reproduction of the genus. American Journal of Botany 102(4): 626-633.

Wang, Y., Tao, H., Tian, B., Sheng, D., Xu, C., Zhou, H., Huang, S., Wang, P. (2019). Flowering dynamics, pollen, and pistil contribution to grain yield in response to high temperature during maize flowering. Environmental and experimental botany 158: 80-88.

Whitehead, D.R. (1983). Wind Pollination: Some ecological and evolutionary perspectives. In: Real, L. (ed.) Pollination Biology. 97-108. Academic. New York.

Wong, J.X.W., Costantini, F., Merloni, N., Savelli, L., Geelen, D., Airoldi, L. (2018). The widespread and overlooked replacement of *Spartina maritima* by non-indigenous *S. anglica* and *S. townsendii* in northwestern Adriatic saltmarshes. Biological Invasions 20: 1687-1702.

Yannic, G., Baumel, A., Ainouche, M. (2004). Uniformity of the nuclear and chloroplast genomes of *Spartina maritima* (Poaceae), a salt-marsh species in decline along the Western European coast. Heredity 93: 182-188.

6

Seed viability, spikelet dispersal, seed banks, and seed storage requirements for native and invasive Spartina cordgrasses across salt marsh intertidal gradients

Seed viability, spikelet dispersal, seed banks, and seed storage requirements for native and invasive *Spartina* cordgrasses across salt marsh intertidal gradients

Abstract

Seed banks play an important role in determining the spatial and temporal distribution of halophytes in salt marshes. We tested the ability of native *Spartina maritima* and invasive *S. densiflora* spikelets to disperse by flotation on water with different salinity concentrations, and the longevity of spikelets relative to environmental conditions including dry or wet storage at low or moderate temperatures, and aqueous salinity concentrations from fresh to sea water. We quantified pre-dispersal seed production and pre- and post-dispersal focal *Spartina* spp. presence in seed banks along intertidal gradients within salt marshes at the Gulf of Cádiz (Southwest Iberian Peninsula). *S. densiflora* spikelets, especially from middle and high elevation marshes, showed greater ability than *S. maritima* to remain afloat, which suggests this species could be dispersed over longer distances supporting possible colonization of new areas. Wet-stored *Spartina* seeds were able to maintain viability for months, while seeds under dry storage rapidly lost viability. This decline was most significant for *S. maritima*, and for seeds stored at moderate temperatures. Storage of spikelets under wet and cold conditions optimized spikelet viability. Native *S. maritima* did not establish transient or persistent seed banks, while invasive *S. densiflora* established transient seed banks mainly at higher marsh elevations. Our results on the dynamics of seed dispersal and seed banks and seedling recruitment provide fundamental knowledge that can be applied for conservation of native *S. maritima,* management of invasive *S. densiflor*a, and ecological restoration of tidal salt marshes.

Keywords:

Cordgrass, Plant invasions, Seed dispersal, Seed buoyancy, Seed storage, Tidal wetland, Wetland restoration

Introduction

Soil seed banks play an important role in the establishment, maintenance, regeneration and restoration of vegetation in many plant communities (Bakker et al. 1996; Chang et al. 2001). In salt marshes, seed dispersal and soil seed banks play an important role in determining the spatial and temporal distribution of halophytes (Crain et al. 2008; Egan and Ungar 2000; Rand 2000), and the successional assembly of halophytic communities following disturbance or restoration actions (Dausse et al. 2007; Kottler and Gedan 2019). The role of soil seed banks is known to be particularly more significant for annual taxa than for perennial taxa that can reproduce vegetatively (Ungar 2001).

The presence and abundance of a species in the soil seed bank depends on its seed production, seed dispersal and its longevity in the soil. Regarding longevity in the soil, there are two general types of seed banks: transient and persistent (Fenner and Thompson 2005; Thompson and Grime 1979). This temporal classification of seed banks is useful in the context of exotic plant invasions, because it provides a predictive model for how long a species may persist in soil as a latent source of invasive propagules (Gioria and Pyšek 2016). In the transient seed bank, seeds are renewed annually, with accumulation of viable seeds for only up to one year. Persistent seed banks include live, dormant seeds that are one or more years old. One way for species to survive in stressful environments with high mortality risk is to establish persistent seed banks (Baskin and Baskin 2001). Moreover, in stressful hypersaline environments, buried seeds that form a persistent seed bank represent a significant storage of genetic information (Ungar 2001). Most perennial halophyte species form predominantly transient soil seed banks; most are not known to form persistent seed banks (Hutchings and Russell 1989; Polo-Ávila et al. 2019; Ungar 2001; Wolters and Bakker 2002).

Species with a transient seed bank depend on seed dispersal to maintain diversity and colonize new areas (Polo-Ávila et al. 2019; van den Broek et al. 2005). Since long-term persistent seed banks usually do not exist for perennials in salt marshes, seed dispersal by seawater (hydrochory) also plays an important role in the preservation and ecological restoration of salt-marsh communities (Wolters and Bakker 2002). In this sense, seed buoyancy enhances seed dispersal in aquatic environments, as salt marshes (Elsey-Quirk et al. 2009; van den Broek et al. 2005). Thus, sexual propagules of most salt marsh species are able to float in seawater (Huiskes et al. 1995; Wolters and Bakker 2002). Seeds of some salt marshes can remain afloat for hours, while others may float during months, increasing the chance to disperse further away from their sources (Huiskes et al. 1995). In addition, seeds of halophytes may be influenced by salinity during their dispersal phase, affecting the seed flotation time (Elsey-Quirk et al. 2009). In tidal salt marshes, tidal flooding can disperse and redistribute the seeds locally, affecting to the distributions of local species, or can disperse the seeds over long distances, favouring the colonization of new habitats (Huiskes et al. 1995; Xiao et al. 2016). In addition, seed dispersal seems to be an important factor that affects the spatial distribution of seed banks in salt marshes (Wang et al. 2009).

The spread of exotic species is an important threat to salt marsh conservation (Adam 2002). In this context, the study of soil seed banks and seed dispersal of invasive species is a crucial aspect since our knowledge on seed dynamics in the soil plays an important role in the management of salt marshes, and also may help to improve conservation and restoration actions (Hazelton et al. 2014; Wang et al. 2009; Xiao et al. 2009). Evaluation of soil seed banks can also improve understanding of factors contributing to the invasiveness of species, and the invisibility of resident communities (Gioria et al. 2012).

The genus *Spartina* (cordgrasses, a monophyletic clade of perennial grasses) is abundant in salt marshes and is distributed worldwide in every continent except Antartica (Bortolus et al. 2019). Frequently, exotic *Spartina* species become invasive when introduced to new geographical areas (Ainouche and Gray 2016). In this context, *Spartina maritima* (Curtis) Fernald is primary colonizer in salt marshes and the only European native cordgrass (Marchant and Goodman 1969). In Southwest Iberian Peninsula, this native cordgrass co-occurs with exotic and invasive *Spartina densiflora* Brongn. introduced from South America, which colonizes very contracted habitats along the intertidal gradient (Nieva et al. 2001). In general, *Spartina* species disperse spikelets that are well adapted to float in sea water, allowing them to be transported over long distances (McDonald 2014; Morgan and Sytsma 2013; Xiao et al. 2016). Moreover, *Spartina* species can establish transient seed banks that may vary in seed density and characteristics among different salt marsh habitats (Ungar 2001; Wang et al. 2009; Xiao et al. 2009, 2016).

Until very recently, seed production in *S. maritima* had been described as very low or non-existent (Castellanos et al. 1994; Castillo et al. 2010; Marchant and Goodman 1969), but Infante-Izquierdo et al. (2019a) observed that this species produces a moderate number of caryopses with high seed viability in the Southwest Iberian Peninsula. For this reason, to our knowledge, this is the first work that evaluates seed bank dynamics in *S. maritima*, as well as in *S. densiflora*. In this work, we tested the ability of *S. maritima* and *S. densiflora* spikelets to disperse in water, the spikelet longevity relative to different environmental conditions, and we quantified the pre-dispersal seed production and the seed banks of both cordgrasses along the intertidal gradient. We hypothesized that differences in the ecology behaviour of these species, *S. maritima* as a pioneer species of mudflat soils, and *S. densiflora* as an invader of all habitats along the intertidal gradient, could be affected to a great extend by their seed dispersal and soil seed bank dynamics. In this sense, we hypothesized that both *Spartina* species would form transient, but not long-lived, persistent seed banks. We also postulated that daily submersion of inflorescences in tidal waters would decrease seed buoyancy, especially in more frequently inundated low elevation marshes. Our aim was to evaluate the dynamics of seed dispersal and seed banks to provide fundamental knowledge critical for science-based conservation of native *S. maritima,* and for restoration and management of invasive *S. densiflora*-invaded tidal wetlands.

Materials and methods

Study sites

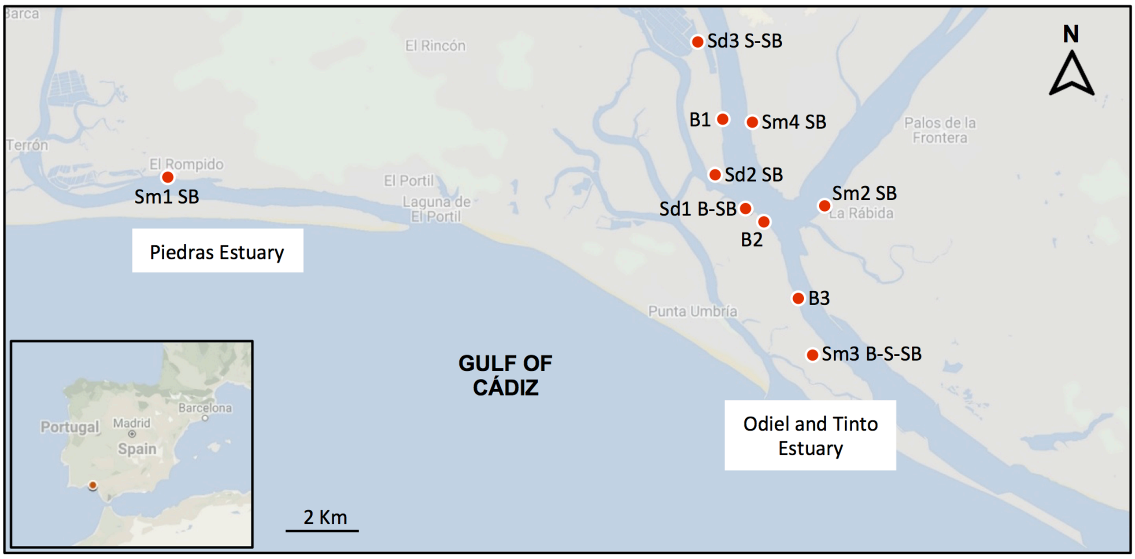


Fig. 1 Sampled points for native *Spartina maritima* (Sm) and invasive *S. densiflora* (Sd) in the Gulf of Cádiz (Southwest Iberian Peninsula). The assays for which locations were sampled (B: buoyancy; S: storage; SB: seed bank) are indicated after the species. The three beaches sampled (B) to study the spikelet dispersion are also indicated (source: Google Maps, data from ©2019 Instituto Geográfico Nacional Spain).

This work was carried out in tidal salt marshes in the estuaries of Odiel, Tinto and Piedras Rivers located along the Gulf of Cádiz (Southwest Iberian Peninsula) (Fig. 1). This area is under a Mediterranean climate with Atlantic influence. The coast of the Gulf of Cádiz is mesotidal and the mean sea level in this area is +1.85 m relative to Spanish Hydrographic Zero (SHZ). The tides are semidiurnal and have a mean range of 2.10 m and a mean spring tidal range of 2.97 m, representing 0.40-3.37 m above SHZ (Castellanos et al. 1994). Native vegetation in salt marshes along the Gulf of Cádiz has been described in previous works (Castellanos et al. 1994; Fernández-Illescas et al. 2010). Different vegetation zones can be distinguished based on tidal influence and soil characteristics (Contreras-Cruzado et al. 2017). Low elevation intertidal marshes (hereafter LM) occur between Mean High Water Neap and Mean High Water, and they are dominated by *Sarcocornia perennis* (Mill.) A.J. Scott and *Spartina maritima*; middle marshes (MM) are located between Mean High Water to Mean High Water Spring, and are dominated by *Sarcocornia fruticosa* (L.) A.J. Scott, *Sarcocornia* hybrids (Figueroa et al. 2003) and *Halimione portulacoides* Aellen; high marshes (HM) are at Mean High Water Spring to Highest Astronomical Tide, and they are colonized by halophytes such as

Arthrocnemum macrostachyum (Moric.) K. Koch, *Suaeda vera* Forssk. ex J. F. Gmel. and *Limoniastrum monopetalum* (L.) Boiss. (Fernández-Illescas et al. 2010; Long and Mason 1983). Native *S. maritima* inhabits LM, and is considered a primary colonizer of bare intertidal mudflats (Castellanos et al. 1994; Infante-Izquierdo et al. 2019a), while exotic and invasive *S. densiflora* invades LM, MM and HM, and with invasive spread, displaces native vegetation (Nieva et al. 2001).

Spikelet buoyancy

Mature spikelets were randomly collected from one *S. maritima* population at LM in August 2017, and from one *S. densiflora* population at LM, MM and HM in November 2017 (Fig. 1). Then, spikelets containing caryopses were randomly selected and stored in paper bags in dark and dry conditions at +5 ºC. Four sets of 25 spikelets from each sampled location and habitat were placed in 500 ml beakers (8 cm diameter), each containing 100 ml of one of three different salt solutions (0.0, 0.3 and 0.6 M NaCl) at +20-25 ºC and a 12h light/dark photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. Floating and sunken spikelets were counted seven times on the first day and twice on the following days during 5 days until practically no spikelets were floating. Beakers were agitated before counting floating and sunken spikelets to simulate wave action that can affect buoyancy (Van den Broek et al. 2005). The percentage of spikelets that remained afloat was calculated over time for each beaker. Sinking rate was calculated as the number of sinking spikelets per hour.

Spikelet storage

Mature spikelets were randomly collected from one *S. maritima* population at LM in August 2017 and from one *S. densiflora* population at HM in November 2016 (Fig. 1). Immediately following collection, spikelets containing caryopses were randomly selected and sowed to establish a control germination before storage. Spikelets were surface-sterilized before sowing in 5% (v/v) sodium hypochlorite for 10 min to prevent fungal contamination, and then rinsed with distilled water (Infante-Izquierdo et al. 2019a; Muñoz-Rodríguez et al. 2012). Four replicates, each with 25 spikelets, were sown in Petri dishes (9 cm diameter) on two layers of autoclaved filter paper, dampened with distilled water, and sealed with adhesive tape (Parafilm™) to avoid desiccation. Germination was carried at +20-25 ºC and a 12h light (60 µmol m^{-2} s^{-1})/dark photoperiod. Germination was recorded every 2 or 3 days for 2 months. Spikelets were considered germinated when the coleoptile emerged. The rest of spikelets were stored for 1, 3, 6 and 12 months in one of five storage treatment conditions: 1) dry at +20 °C); 2) dry at +5 ºC; 3) immersed in distilled water (0 M NaCl) at +5 ºC; 4) immersed in 0.3 M NaCl solution at +5 ºC; and 5) immersed in 0.6 M NaCl at +5ºC. Then, these pre-treated spikelets were sown as reported above. Viability of the caryopses from spikelets that did not germinate was tested using the tetrazolium test (Mackay 1972). For this purpose, the embryo was incised with a scalpel and submerged in a 1% aqueous solution of 2,3,5 triphenyl tetrazolium chloride at +25 °C in darkness for 24 h. Then, red stained viable embryos were counted through a magnifying glass. The percentage of spikelets with viable caryopses (germinated plus dormant), the germination percentage (based on spikelets with viable caryopses) and the days necessary to reach 50% of the final germination percentage (T_{50}) were calculated for each Petri dish (Infante-Izquierdo et al. 2019a; Muñoz-Rodríguez et al. 2012).

Spikelet production and soil seed banks

Both cordgrass species produce spikelets which break off from the mother plant and become a dispersal unit, in which glumes, palea and lemma cover the caryopsis. In our study we quantified the production and the density in the soil of total spikelets, including empty ones, spikelets containing caryopses and spikelets containing viable caryopses. In the soil we have also quantified the density of spikelets with germinating caryopses. We have used the term ‘seed bank’ as a generic term applied to the densities of all these types of spikelets in the soil, in concordance with seed bank literature. To study the dynamics of the seed banks of *S. maritima* and *S. densiflora*, the different phases in spikelet production and dispersal were followed and compared (Fig. 2). Annual spikelet production (ASP) and soil seed bank were studied at LM in four locations for *S. maritima* (Sm1, Sm2, Sm3 and Sm4; Fig.1) and at LM, MM and HM, and adjacent bare mudflats (BM) in three locations (Sd1, Sd2 and Sd3; Fig. 1) for *S. densiflora*.

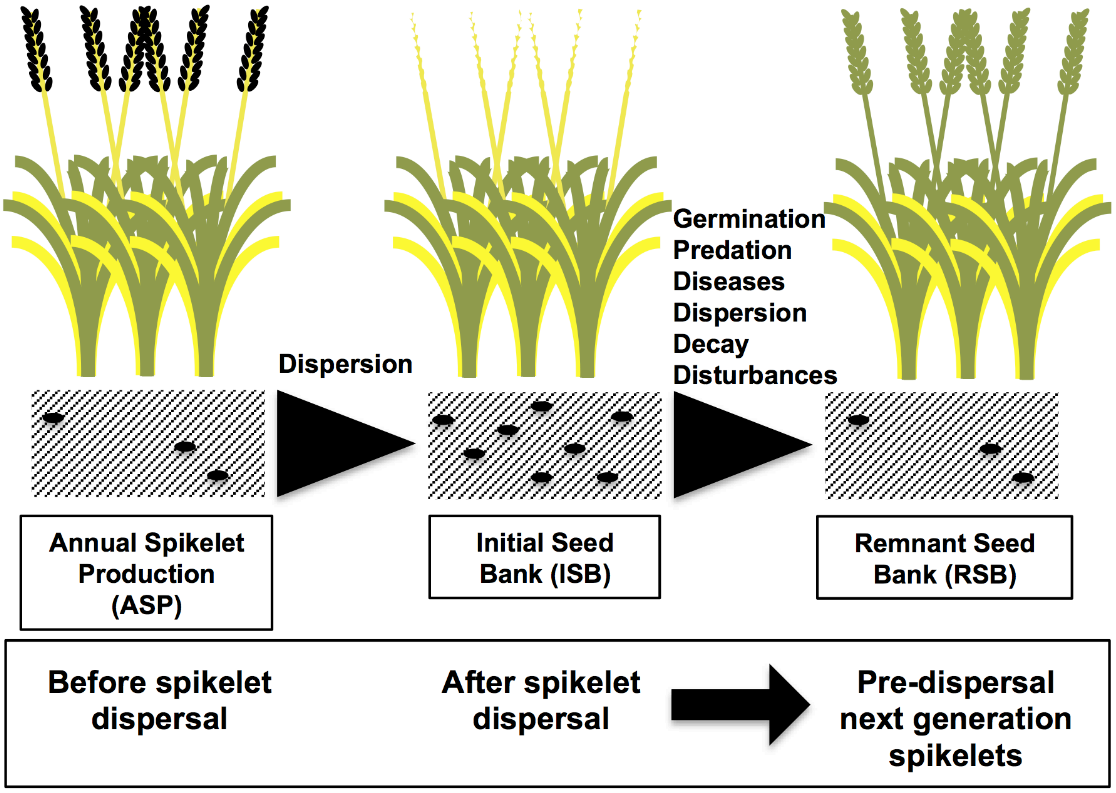


Fig. 2 Scheme showing the different phases in spikelet production and dispersal for *Spartina* species.

Annual Spikelet Production

The production of total spikelets (ASP) and of spikelets with caryopses per m^2 of habitat were calculated for four populations of *S. maritima* in low marshes, and the production of spikelets with viable caryopses per m^2 was calculated for two of those populations, using data obtained from Infante-Izquierdo et al. (2019a). For *S. densiflora*, spikelet production was calculated by counting the number of inflorescences on live plants prior to the onset of primary dispersal. Counts were made in 10 randomly distributed squared plots (50 x 50 cm) in patches of *S. densiflora* formed by coalescent tussocks in MM and HM, while in LM, in which *S. densiflora* grows in discrete tussocks, density of inflorescences was recorded by counting the number of inflorescences in 10 tussocks and measuring each tussock diameter to calculate its area. We randomly collected 20 inflorescences from each *S. densiflora* population and counted the number of total spikelets per inflorescence and the number of spikelets with caryopses, calculating then the density of spikelets with caryopses for each population. Density of spikelets with viable caryopses was calculated as the product of the mean percentage of viability by the density of spikelets with caryopses per population. These data were converted in production per m^2 of habitat by using *S. densiflora* coverage proportion for each habitat and location for the same year, recorded along three 50 m transects in each habitat and location (LM: 0.10 ± 0.02; MM: 0.56 ± 0.09; HM: 0.51 ± 0.08).

Soil seed bank sampling and analysis

S. maritima seed bank was studied in LM at the four studied locations (Fig. 1) in October 2017, just after spikelet dispersal (initial seed bank, ISB). *S. densiflora* seed bank was sampled in LM, MM, HM and BM and at three locations (Fig. 1) in July 2017 (remnant seed bank, RSB) and in January 2018 (ISB). To evaluate presence of *Spartina* spp. in soil seed banks, we randomly collected ten sediment samples at each designated study location and habitat using stainless steel cores (50 mm diameter, 50 mm height) during low tides. We used this model of stainless steel core since previous seed bank studies in salt marshes have reported that most of the seeds accumulates in the first 50 mm of sediments (Coteff and Van Auken 2006; Zepeda et al. 2014).

To analyze spikelet dispersal away from source populations, we have studied the soil seed banks at three sandy beaches located more than 500 m from the nearest *Spartina* population (B1, B2 and B3 in Fig. 1). These beaches were sampled in February 2018, when both species had finished their spikelet dispersal periods. Three zones (bare mudflats, sand beach and high tide line) were sampled in each beach (n = 10-20 sediment samples per zone and beach).

Sediment samples were analysed in the laboratory just after sampling. Spikelets were directly extracted from sediment samples, since methods based on germination are less precise because many seeds may remain dormant and, therefore, the seed bank could be underestimated (Brown

1992; Gross 1990). Thus, each soil sample was washed to reduce the amount of sediment (Wolters and Bakker 2002) over a 1-mm sieve that retained all *Spartina* spikelets (Infante-Izquierdo et al. 2019b). The material that remained in the sieve (sand, gravel, shells, plant wrack and propagules) was placed on a filter paper and, spikelets were counted and extracted under a magnifying glass. Then, glumes, palea and lemma were removed from each spikelet in order to examine the presence or not of caryopsis. Viability of caryopses was tested using the tetrazolium test as described above. Density of the total spikelets (total spikelets m^{-2}), density of spikelets with caryopses (caryopses m^{-2}), density of spikelets with viable caryopses (viable caryopses m^{-2}) and density of spikelets in which caryopses were germinating in the field at sampling moment (germinated caryopses m^{-2}) was calculated for each habitat for ISB and for RSB.

Statistical analysis

Statistical analyses were carried out with STATISTICA 8.0 (StatSoft Inc., USA). Deviation from the mean was calculated as Standard Error (SE). Kolmogorov-Smirnov test and Levene test were used to evaluate the normality and homogeneity of the data series, respectively. Data were screened for normality and homogeneity of variance, and transformed as need using $\sqrt{x}$, $1/(x+1)$, $\ln(x+1)$ or arcsine(x) functions. Seed viability and germinability responses of *S. maritima* and *S. densiflora* to storage period treatments (0, 1, 3, 6, 12 months), spikelet production, and seed bank characteristics were all analysed using one-way ANOVAs with Tukey's Honest Significant Difference (HSD) as post hoc tests. In cases where data transformations did not support use of the parametric ANOVA model, non-parametric Kruskal-Wallis H test with Mann-Whitney U as the post-hoc test were used. Spearman's correlation coefficient (ρ) was used to correlate the percentage of spikelets of *S. maritima* and *S. densiflora* that remained afloat over time. Test results were considered significant when $p \leq 0.05$.

Results

Spikelet buoyancy

In *S. maritima*, there was a significant and linear reduction of floating spikelets over time in the three saline solutions, from 100 ± 0% to 9 ± 1% during the first 24 h, reaching 2 ± 1% at 48 h (Spearman correlations coefficient, 0 M NaCl: $\rho = -0.9591$; 0.3 M NaCl: $\rho = -0.9359$; 0.6 M NaCl: $\rho = -0.9518$; $p < 0.05$) (Fig. 3a). Sinking rate was 2.50 seeds h^{-1} in 0.0 M and 0.6 M NaCl, and 2.44 seeds h^{-1} in 0.3 M NaCl.

The percentage of floating *S. densiflora* spikelets after the first 24 hours was 70 ± 3%. After 70 hours, *S. densiflora* maintained 8 ± 2% of floating spikelets, with 1 ± 0% reduction in buoyancy recorded on the fourth day (94 h). A linear and significant decrease of floating *S. densiflora* spikelets over time was also observed under the three saline solution treatments (Spearman correlations coefficient, 0 M NaCl: ρ = -0.9022; 0.3 M NaCl: ρ = -0.9145; 0.6 M NaCl: ρ = -0.8911; $p < 0.05$) (Fig. 3b), with a sinking rate of 1.25 seeds h^{-1} in 0.0 M and 0.3 M NaCl, and 1.17 seeds h^{-1} in 0.6 M NaCl. *S. densiflora* spikelets from LM, MM and HM showed a significant decrease of floating spikelets over time (Fig. 3c) (Spearman correlations coefficient, LM: ρ = -0.9748; MM: ρ = -0.9206; HM: ρ = -0.9281; $p < 0.05$) with similar sinking rates: 1.27 seeds h^{-1} at HM, 1.22 seeds h^{-1} at MM and 1.18 seeds h^{-1} at LM. However, while the first sunken spikelets from MM and HM were observed at 8.5 h, those from LM started to sink immediately. No *S. maritima* or *S. densiflora* spikelets germinated during the flotation experiment.

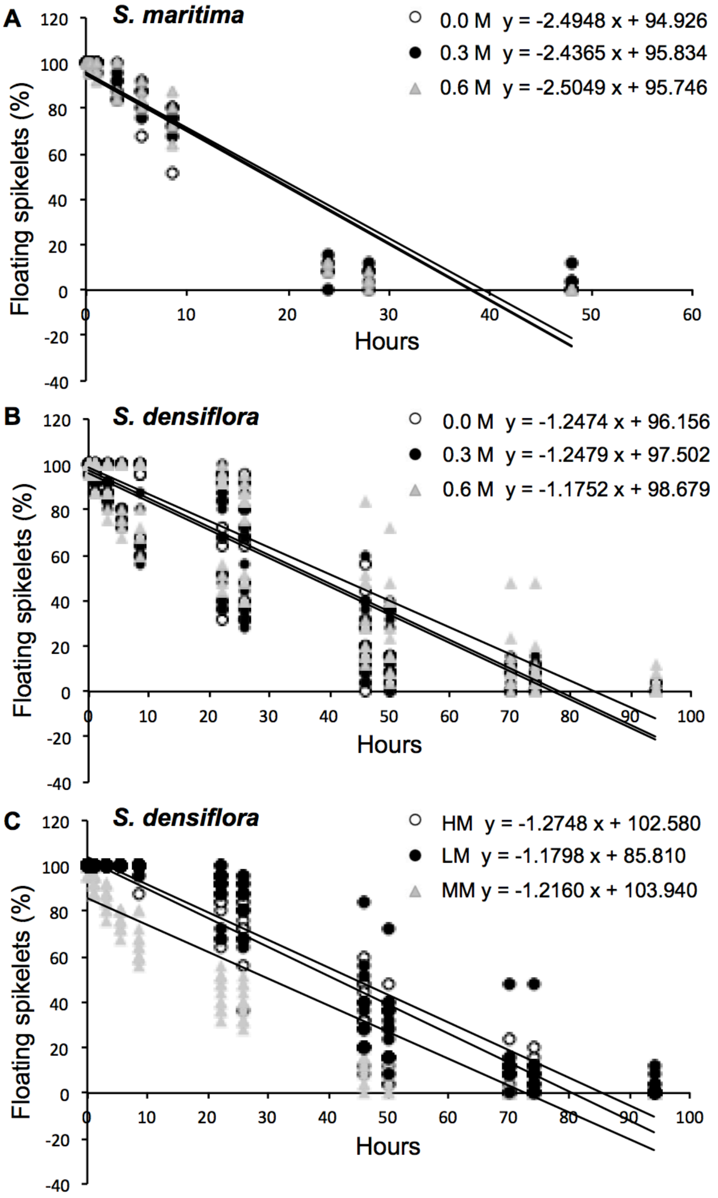


Fig. 3 Percentage of floating spikelets in each beaker over time in different salt solutions of native *S. maritima* (a) and invasive *S. densiflora* (b), and in the different *S. densiflora* habitats (c) (LM: low marsh, MM: middle marsh and HM: high marsh). Regression lines and equations are presented in each case (Spearman correlation coefficient showed in Results).

Spikelet storage

In *S. maritima*, spikelet viability was 80 ± 4% for the control treatment. Spikelets stored dry at +20-25 ºC showed a drastic reduction in viability after 1 month (12 ± 2%), with all spikelets dead after 3, 6 and 12 months in these conditions (Kruskal-Wallis test: $H_{4,20}$ = 18.65, $p < 0.001$) (Fig. 4a). Spikelets dry-stored at +5 ºC maintained 44 ± 4% of viable seeds up to 3 months, although with a significant reduction compared to the control (One-way ANOVA test: F = 110.87, df = 4, $p < 0.0001$) (Fig. 4b). Seeds stored wet at +5 ºC maintained viability for at least 6 months, decreasing after 12 months (Fig. 4c, d, e).

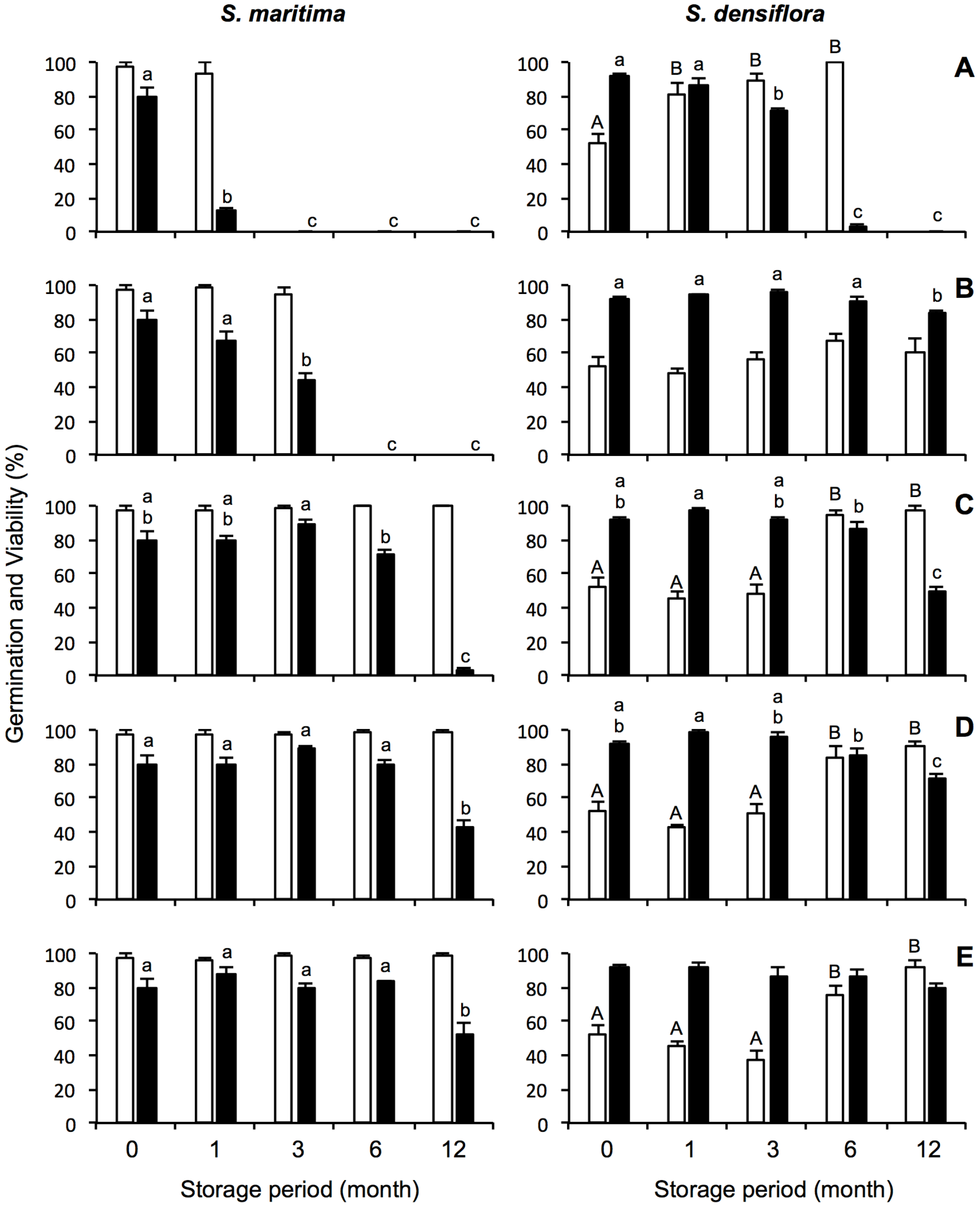


Fig. 4 Germination (white bars) and viability percentages (black bars) for seeds of *S. maritima* and *S. densiflora* stored under different conditions: dry at +20-25 ºC (a), dry at +5ºC (b), wet (0.0 M NaCl) at +5ºC (c), wet (0.3 M NaCl) at +5ºC (d) and wet (0.6 M NaCl) at +5ºC (e); during 0 (control), 1, 3, 6 and 12 months.

This reduction of seed viability over time was more pronounced in distilled water (3 ± 1%) (One-way ANOVA test: F = 56.40, df = 4, $p < 0.0001$), than in 0.3 M and 0.6 M NaCl, in which viability always remained above 40% (One-way ANOVA test: F = 24.34, df = 4, $p < 0.0001$; F = 12.33, df = 4, $p < 0.001$, respectively).

S. maritima viable seed germination was ca. 98% after all storage periods in all conditions (One-way ANOVA or Kruskal-Wallis test, $p > 0.05$) (Fig. 4a, b, c, d, e). *S. maritima* spikelets showed T_{50} values of 23.3 ± 1.6 days for the control treatment. Germination was significantly delayed (higher T_{50}) when spikelets were stored dry at +20-25 ºC for 1 month. However, spikelets stored dry at +5 ºC did not delay their germination (Table 1). In contrast, in the three wet and cold storage conditions, germination was significant and progressively accelerated (lower T_{50}) when storage time increased (Table 1).

Table 1 Days necessary to reach 50% of the final germination percentage (T_{50}) for native *Spartina maritima* and invasive *S. densiflora* spikelets stored under different conditions during 0 (control), 1, 3, 6 and 12 months. Different letters indicate significant differences among storage periods within each storage conditions (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). df, degrees of freedom. Data are means ± SE.

S. maritima	Dry +20ºC	Dry +5ºC	Wet +5ºC 0.0 M NaCl	Wet +5ºC 0.3 M NaCl	Wet +5ºC 0.6 M NaCl
0 month	23.3 ± 1.6^{a}	23.3 ± 1.6^{a}	23.3 ± 1.6^{a}	23.3 ± 1.6^{a}	23.3 ± 1.6^{a}
1 month	37.5 ± 2.9^{b}	23.1 ± 0.6^{a}	14.3 ± 0.7^{b}	14.9 ± 0.9^{b}	18.1 ± 1.7^{ab}
3 months	-	23.6 ± 2.8^{a}	8.3 ± 0.3^{c}	14.8 ± 1.6^{b}	17.5 ± 2.2^{ab}
6 months	-	-	6.5 ± 0.3^{d}	8.0 ± 0.6^{c}	15.5 ± 0.0^{bc}
12 months	-	-	-	7.8 ± 1.0^{c}	9.8 ± 0.8^{c}
	F=20.22, df=1	F=0.02, df=2	F=125.81, df=3	F=28.98, df=4	F=10.97, df=4
	$p < 0.005$	$p > 0.05$	$p < 0.0001$	$p < 0.0001$	$p < 0.0005$
S. densiflora	Dry +20ºC	Dry +5ºC	Wet +5ºC 0.0 M NaCl	Wet +5ºC 0.3 M NaCl	Wet +5ºC 0.6 M NaCl
0 month	22.9 ± 0.6^{a}	22.9 ± 0.6^{a}	22.9 ± 0.6^{a}	22.9 ± 0.6^{a}	22.9 ± 0.6^{a}
1 month	29.5 ± 0.5^{b}	29.4 ± 1.4^{a}	27.6 ± 1.8^{a}	24.4 ± 1.9^{a}	32.3 ± 4.3^{b}
3 months	29.8 ± 1.6^{b}	25.0 ± 1.1^{a}	2.0 ± 0.0^{b}	9.5 ± 3.1^{b}	10.8 ± 1.1^{c}
6 months	-	21.4 ± 3.7^{a}	1.0 ± 0.0^{c}	1.0 ± 0.0^{c}	3.6 ± 0.6^{d}
12 months	-	22.9 ± 3.3^{a}	4.0 ± 0.0^{d}	2.5 ± 0.9^{bc}	4.0 ± 0.0^{d}
	$H_{2,12}=7.57$	F=1.73, df=4	$H_{4,20}=18.33$	$H_{4,20}=16.75$	$H_{4,20}=17.80$
	$p < 0.05$	$p > 0.05$	$p < 0.005$	$p < 0.005$	$p < 0.005$

S. densiflora seed viability was 92 ± 2% for the control treatment. Seeds dry-stored at +20-25 ºC maintained initial viability for 1 month, followed by a 71 ± 1% reduction in viability after 3 months, with senescence of nearly all seeds after 6 months (Kruskal-Wallis test: $H_{4,20} = 17.41$, $p < 0.01$) (Fig. 4a). Spikelets stored dry at +5 ºC maintained initial viability until 6 months, with a reduction in viability to 83% after 12 months (One-way ANOVA test: F = 7.52, df = 4, $p < 0.01$) (Fig. 4b). Spikelets stored wet at +5 ºC maintained initial viability for 6 months (Fig. 4c, d, e). Seed viability was reduced significantly in distilled water and 0.3 M NaCl solution after 12 months (One-way ANOVA test: F = 22.44, df = 4, $p < 0.0001$; F = 19.35, df = 4, $p < 0.0001$, respectively) (Fig. 4c, d), and viability remained constant in 0.6 M NaCl solution (One-way ANOVA test: F = 2.01, df = 4, $p > 0.05$) (Fig. 4e). Viable seed germination was 52 ± 5% for the control sowing, with an initial dormancy level of 48%. Germination of spikelets storage dry at +20-25 ºC for 1 month increased to 81%, being maintained this high for 3 and 6 months (One-way ANOVA test: F = 10.65, df = 3, $p < 0.01$) (Fig. 4a). Germination percentage remained constant during the entire storage period for dry-stored seeds at +5 ºC (One-way ANOVA test: F = 1.85, df = 4, $p > 0.05$) (Fig. 4b). In the three wet and cold storage conditions, initial seed germinability was maintained until 3 months, increasing significantly after 6 or 12 months (Kruskal-Wallis test, 0.0 M: $H_{4,20}$ =14.32, $p < 0.01$; One-way ANOVA test, 0.3 M: F = 18.71, df = 4, $p < 0.0001$; 0.6 M: F=21.32, df=4, $p < 0.0001$) (Fig. 4c, d, e). At the time of spikelet collection (control), T_{50} was 22.9 ± 0.6 days. At dry and +20-25 ºC, germination was significantly delayed (higher T_{50}) after 1 month or more, whereas T_{50} was not significantly affected in dry at +5 ºC (Table 1). In contrast, germination was significantly accelerated (lower T_{50}) in the three wet and cold storage conditions in storage periods longer than 3 months (Table 1). No *S. maritima* and *S. densiflora* spikelet germinated during the storage experiment.

Annual Spikelet Production

Spartina maritima

ASP of *Spartina maritima* from field sampling was 7538 ± 896 total spikelets m^{-2} in Sm1, 8069 ± 1454 total spikelets m^{-2} in Sm2, 1699 ± 760 total spikelets m^{-2} in Sm3, and 6565 ± 1441 total spikelets m^{-2} in Sm4. Production of spikelets with caryopses per m^2 was 889 ± 106 in Sm1, 991 ± 178 in Sm2 562 ± 252 in Sm3, and 2177 ± 478 in Sm4; and the production of spikelets with viable caryopses per m^2 was 450 ± 201 in Sm3 and 2111 ± 463 in Sm4.

Spartina densiflora

ASP showed no significant differences among the three studied *S. densiflora* habitats in any production trait. Production of total spikelets per m^2 ranged from 1623 ± 304 to 6652 ± 1893, production of spikelets with caryopses per m^2 ranged from 533 ± 80 to 1722 ± 476 and production of spikelets with viable caryopses per m^2 oscillated between 304 ± 52 and 1390 ± 380 (Table 2).

Soil seed bank

Spartina maritima

Regarding *S. maritima* seed bank, we only found 153 ± 109 spikelets without caryopses m^{-2} in Sm2 and 51 ± 51 spikelets without caryopses m^{-2} in Sm3 in the ISB. No spikelets with caryopses were recorded in any of the studied *S. maritima* populations neither in any studied *S. densiflora* habitat or beaches.

Spartina densiflora

In the ISB, density of total spikelets was significantly higher in MM and HM than in LM and BM. Density of spikelets with caryopses was significantly higher in soils from LM, MM and HM than in BM. Density of spikelets with viable caryopses increased from BM to HM along the intertidal gradient. Finally, density of spikelets with caryopses germinating in the field at the moment of sampling was significantly higher in HM than in BM, LM and MM (Table 2).

Table 2 Density of total spikelets (total Spk m^{-2}), density of spikelet with caryopses (caryopses m^{-2}), density of spikelets with viable caryopses (Vcaryopses m^{-2}) for invasive *S. densiflora* at the annual spikelet production and initial and remnant seed bank in bare mudflats (BM), low marshes (LM), middle marshes (MM), and high marshes (HM) (n=30). Initial seed bank includes data of spikelets with germinated caryopses (Gcaryopses m^{-2}). Different letters indicate significant differences among habitats (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). Data are means ± SE.

	BM	LM	MM	HM	ANOVA (F) or KW (H) test
Annual spikelet production					
Total Spk m^{-2}	-	6652 ± 1893^{a}	1623 ± 304^{a}	1789 ± 300^{a}	F=2.71, df=2, p>0.05
Caryopses m^{-2}	-	1722 ± 476^{a}	757 ± 124^{a}	533 ± 80^{a}	$H_{2,89}=2.36$, p>0.05
VCaryopses m^{-2}	-	1390 ± 380^{a}	511 ± 80^{a}	304 ± 52^{a}	$H_{2,89}=5.64$, p>0.05
Initial seed bank					
Total Spk m^{-2}	68 ± 32^{a}	475 ± 150^{a}	1783 ± 231^{b}	3922 ± 1101^{b}	$H_{3,120}=60.59$, p<0.0001
Caryopses m^{-2}	17 ± 17^{a}	119 ± 47^{b}	187 ± 57^{b}	340 ± 107^{b}	$H_{3,120}=12.50$, p<0.01
VCaryopses m^{-2}	0 ± 0^{a}	102 ± 45^{b}	119 ± 47^{bc}	306 ± 96^{c}	$H_{3,120}=15.65$, p<0.01
GCaryopses m^{-2}	0 ± 0^{a}	34 ± 24^{a}	17 ± 17^{a}	170 ± 51^{b}	$H_{3,120}=18.48$, p<0.001
Remnant seed bank					
Total Spk m^{-2}	17 ± 17^{a}	51 ± 28^{a}	1681 ± 400^{b}	1358 ± 271^{b}	$H_{3,120}=58.41$, p<0.0001
Caryopses m^{-2}	0 ± 0^{a}	0 ± 0^{a}	17 ± 17^{a}	51 ± 28^{a}	$H_{3,120}=6.16$, p>0.05
VCaryopses m^{-2}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	17 ± 17^{a}	$H_{3,120}=3.00$, p>0.05
GCaryopses m^{-2}	0 ± 0	0 ± 0	0 ± 0	0 ± 0	-

In the RSB, density of total spikelets was significantly higher in MM and HM than in BM and LM. We only found spikelets with caryopses at similar densities in MM and HM. Spikelets with viable caryopses were recorded only in HM. In addition, we did not find any germinated caryopsis in the RSB (Table 2).

In the studied beaches, total density of *S. densiflora* spikelets was higher at the high tide lines than at lower bare mudflats or sand beaches (Kruskal-Wallis test: $H_{2,110} = 21.72$, $p < 0.0001$). Density of spikelets with caryopses, with viable caryopses and with germinated caryopses also showed the highest values at high tide level, but without significant differences among zones (Kruskal-Wallis test, $p > 0.05$) (Table 3).

Table 3 Density of total spikelets, spikelets with caryopses, with viable caryopses and with germinated caryopses for invasive *Spartina densiflora* in soils from different zones at beaches away from source populations. Different letters indicate significant differences among zones (Mann-Whitney U-test or Tukey's HSD test, $p < 0.05$). Data are mean ± SE (n = 30 for bare mudflats and sand beaches; n = 50 for mean high tide line).

	Bare mudflat	Sand beach	High tide line
Total Spikelets m^{-2}	102 ± 45^{a}	34 ± 24^{a}	550 ± 116^{b}
Caryopses m^{-2}	34 ± 24^{a}	0 ± 0^{a}	71 ± 38^{a}
Viable caryopses m^{-2}	34 ± 24^{a}	0 ± 0^{a}	51 ± 30^{a}
Germinated caryopses m^{-2}	0 ± 0^{a}	0 ± 0^{a}	41 ± 28^{a}

Discussion

Our results about seed dispersal potential and soil seed bank dynamics are useful to explain the distribution of *S. maritima* and *S. densiflora* along the intertidal gradient in salt marshes. An important result of this work is confirmation that no seed bank was found for *S. maritima* in either its low marsh populations nor in upper areas in the intertidal gradient. However, in accordance with our hypothesis, *S. densiflora* established a viable transient seed bank in all elevation zones of the marsh. This finding reveals a seed bank life stage of the invader that is present through the salt marsh, and can contribute to its invasiveness and future impacts to the resident native halophyte community. Results of our work show that daily submersion of inflorescences decreases the buoyancy of spikelets and this, together with the exposure to tidal flow, affects spikelet dispersal. This explains why both *Spartina* species were absent or very rare in soil seed banks from low elevation salt marsh as predicted in our hypothesis.

In buoyancy trials, most of the native *Spartina maritima* spikelets sank within the first 24 h in water with different salinities, whereas only c. 30% of *S. densiflora* spikelets sank after 24 h and practically all *S. densiflora* spikelets were submerged after 94 h in water with different salinities. This poor buoyancy ability of *S. maritima* likely contributes to why we did not detect any *S. maritima* seeds in soil seed banks at locations away from their population sites, including sampled *S. densiflora* locations and isolated beaches. The results do provide support for the better dispersal ability of *S. densiflora* which was present in soils seed banks at isolated beaches some distance from sampled or other populations. Moreover, the density of *S. densiflora* spikelets in the seed bank before the germination period (ISB) in HM exceeded that the sum of the density of remnant spikelets and the *in situ* annual production, meaning that the HM was a sink habitat for propagules, whereas LM and MM were propagule production areas from which propagules were exported. Our results coincide with studies on *S. alterniflora* in the Yangtze Estuary (China) that have reported a higher density of spikelets in MM and HM than in LM and bare mudflats (Wang et al. 2009; Xiao et al. 2009). Upper salt marsh zones are less exposed to tides at the same time that halophytes canopies reduce the speed of tidal currents, favouring the deposition of seeds that have been dragged by the tide

(Lambrinos and Bando 2008). In contrast, tidal currents favour the exportation of propagules in low marshes (Wolters and Bakker 2002).

The recorded flotation period for *S. densiflora* spikelets was similar to that found by McDonald (2014). Other cordgrasses showed longer buoyancy periods than *S. densiflora*. For example, *S. alterniflora* and *Spartina patens* (Aiton) Muhl. from the Atlantic and the Gulf Coasts of North America presented flotation times ca. 25 days (Elsey-Quirk et al. 2009) and 50% of spikelets remained afloat after 9 days and 8% after 40 days for South American *Spartina ciliata* Brongn. (Cordazzo and Davy 1994). Moreover, *S. densiflora* spikelets from LM started to sink before than those from MM and HM. This behaviour was also observed by Xiao et al. (2016) in *S. alterniflora*. Waterlogging of *Spartina* spp. propagules can cause loss of buoyancy (Morgan and Sytsma 2013). In this sense, Elsey-Quirk et al. (2009) found that *S. alterniflora* spikelets pre-treated during 30 days with wet stratification sank before than those not pre-treated. The greater ability of *S. densiflora* spikelets, specially from MM and HM, to remain afloat may causes that this species could be dispersed over longer distances favouring the colonization of new areas (McDonald 2014). This could explain the extension of *S. densiflora* invasion from San Francisco Bay to Vancouver Island along the Pacific Coast of North America (Castillo et al. 2014) as well as its invasion from South Portugal to the Strait of Gibraltar along the Gulf of Cádiz (Nieva et al. 2001). In contrast, European native *S. maritima* spikelets capacity to colonize new habitats located at medium and long distances would be more restricted. This limitation in spikelet dispersion may favour population isolation that could explain why *S. maritima* shows a low genetic diversity, especially in North European Marshes (Raybould et al. 1991; Yannic et al. 2004). In agreement with our results, Polo-Ávila et al. (2019) found only one *S. maritima* spikelet in all 420 soil samples analyzed along the whole intertidal gradient. Thus, due to their low buoyancy, most of *S. maritima* spikelets may be deposited on the bottom of salt marsh channels and mudflats, where they could maintain viability for longer due to humidity conditions according to our storage experiments, and where if the elevation is adequate, they could potentially germinate and establish as primary colonizers (Castellanos et al. 1994; Castillo et al. 2000).

In this context and in view of our storage results, wet *Spartina* seeds transported by currents and tides would be able to keep their viability during months in contrast with dry seeds that rapidly lost their viability, especially at moderate temperature and in the case of *S. maritima*, like many aquatic plants with recalcitrant seeds (Biber and Caldwell 2008; Probert and Longley 1989). This behaviour was also observed for *S. alterniflora* seeds that cannot withstand drying at moderate temperature, losing their viability within 40 days (Mooring et al. 1971). Cold mitigated the deleterious effects of dry storage since refrigeration prevents desiccation, being *S. maritima* able to keep some viable seeds until 3 months and *S. densiflora* until 12 months. These results are in accordance with Kittelson and Boyd (1997), who reported that *S. densiflora* caryopses did not lose viability in dry storage at 0 °C, and with Mooring et al.

(1971) who stated that *S. alterniflora* viability was preserved in cold storage but after 8 months viability is lost.

There are numerous endogenous and exogenous types of seed dormancy. *Spartina* spp. have physiological dormancy that inhibits germination (Baskin and Baskin 2001). This dormancy can prevent germination of seeds in unfavourable conditions for seedling growth (Ungar 1978). Dormancy is an important process for establishment of persistent soil seed banks in salt marshes (Ungar 2001), and seeds of many halophytes that disperse during the fall have dormancy mechanisms (Baskin and Baskin 2001). *S. maritima* seeds without spikelet bracts showed dormancy levels that ranged from 9% to 19% (Infante-Izquierdo et al. 2019a), whereas, in the present study, *S. maritima* did not show dormancy sowing the entire spikelet as occurred in our study about germination at different salinities (Infante-Izquierdo et al. 2019c). This means that its seeds were ready to germinate just after being dispersed from the mother plant. In the case of *S. maritima*, its lack of dormancy and its inability to maintain viable seeds covered with bracts for long periods at moderate temperatures supports the characterization of this species as a short distance dispersal, as discussed above, and its inability to establish persistent and even shorter-lived transient seed banks as recorded in this study. The lack of seedling emergence of *S. patens* and *Spartina foliosa* Trin. from seed bank assays also suggests these congeners do not maintain persistent soil seed banks (Baldwin et al. 1996; Hopkins and Parker 1984). *Spartina foliosa* can be an abundant seed producer and quickly colonizes wetland restoration sites, but a low frequency of detection in seed rain and seed banks at San Francisco Estuary is thought to be due to low seed viability, particularly at elevated salinity (Diggory and Parker 2011).

In contrast, *S. densiflora* exhibited dormancy after being dispersed from the mother plant of 47%, which would allow to disperse to long distances and establish seed banks as recorded in our study. This dormancy was broken after 1 month in dry and moderate temperature conditions, accompanied by a decrease in seed viability, and was also broken by storage over 6 months in wet and cold conditions. However, *S. densiflora* dormancy at dispersal moment was retained when spikelets were stored in dry and cold conditions. These results are similar than those recorded previously for *S. patens* (Plyler and Proseus 1996) and *S. alterniflora* (Biber and Caldwell 2008; Wijte and Gallagher 1996; Xiao et al. 2009). Thus, wet and cold conditions were the best for spikelets storage in both cordgrasses, as occurs in *S. alterniflora* (Mooring et al. 1971; Wijte and Gallagher 1996), maintaining seed viability longer, mainly in 0.6 M NaCl solutions, in which high numbers of seeds remained viable during12 months. Moreover, germination was progressively accelerated when storage time increased in these conditions for both species. In this sense, Xiao et al. (2009) observed that a wet and chilling treatment shorten the time of onset seed germination for *S. alterniflora*. Due to its dormancy, *S. densiflora* was able to establish transient seed banks mainly at higher elevations along the intertidal gradient as recorded in this study. Nevertheless, *S. densiflora* was unable to establish permanent seed bank since only 1 spikelet with viable caryopsis remained in a HM soil sample after the germination period. These observations let us to conclude that *S. densiflora* forms transient seed banks, in which viable caryopses are renewed annually. This type of soil seed bank has been reported for other *Spartina* species such as *S. alterniflora* (Wang et al. 2009; Xiao et al. 2009, 2016), *Spartina argentinensis* Parodi (Feldman et al. 2007) and allopolyploid *Spartina anglica* C.E. Hubb. (Ungar and Woodell 1993). The permanence of transient soil seed banks could be for only few days or weeks in species with non-dormant seeds such as *S. maritima*, when the environment conditions are not favourable to maintenance their viability or when there are favourable conditions for germination, or for several months, if they have dormancy (Baskin and Baskin 2001), as is the case of *S. densiflora*. In *S. alterniflora*, Xiao et al. (2009) found that its transient seed bank lasted less than 9 months. We recorded high germination percentages (14-56% of viable caryopses) for dispersed *S. densiflora* spikelets before the beginning of the germination period in the field (ISB). Thus, all these seeds were being subtracted from the transient seed bank during and just after winter rainfalls, that reduce salinity promoting germination as occurs in most halophytes (Infante-Izquierdo et al. 2019c; Keiffer and Ungar 1997; Muñoz-Rodríguez et al. 2017; Ungar 1978, 2001). This first germination window is followed by other germination periods later in the year (J.M. Castillo, personal observation), as *S. densiflora* seeds would lose their dormancy after 6 months in cold and wet conditions. On the other hand, the decay of non-germinated caryopses in the soil bank may be determined by the desiccation that occurs in summer, as reported by Chang et al. (2001), and our results have showed. Other factors that could explain the depletion of the soil seed bank is seed transport by tides (Wang et al. 2009; Xiao et al. 2009) and seed mortality caused by microbial and fungi activity (Wagner and Mitschunas 2008; Xiao et al. 2009), or by seed predation (Espinar et al. 2004; Xiao et al. 2009).

Our results on the dynamics of seed dispersal and seed banks provide fundamental information for the conservation of native *S. maritima* and the management of invasive *S. densiflora*. In this sense, the limited spikelet dispersal and the absence of soil seed banks in native *S. maritima* habitat reveals the need to maintain high levels of genetic variability in populations, which must be consider in wetland restoration works using this species. The limited dispersal ability and the absence of *S. maritima* in soil seed banks, together with the results from other studies that indicate invasive *S. densiflora* is better adapted to salinity changes expected with climate change than native *S. maritima* (Infante-Izquierdo et al. 2019c), suggest that native *S. maritima* is highly vulnerable to future changes in the littoral environment. Therefore, results from this work can be applied to improve the conservation of native *S. maritima* under changing global climate and sea level rise scenario. In this sense, existing natural *S. maritima* populations should be carefully preserved and used as source sites for seeds to actively create new populations. Thus, the limited dispersal and soil seed bank ecology of *S. maritima* suggests revegetation at restored salt marshes will likely require augmentative introductions of *S. maritima* since passive restoration efforts would not be enough. However, like *Spartina foliosa* (Diggory and Parker 2011), this may be locally balanced in some salt marsh areas if annual seed production is sufficient to establish new populations. For invasive *S. densiflora*, whose management in many countries is based on its control or eradication, our results reveal the absence of permanent seed bank, so attempts to manage this invasive plant should focus on eliminating adult plants before annual seed release to reduce augmentation of seed banks. Also, regional weed management strategies are needed to reduce the entry of propagules from other nearby populations that will drive secondary invasions. Ex situ collections and storage of plant species propagules to bank and preserve plant genetic resources for conservation and wetland restoration can provide an important safety net against extinction of native species in the face of climate change, invasive species, and habitat loss (Maunder et al. 2004; Millennium Ecosystem Assessment 2005). The results of storage experiments provide important information about the best way to preserve the seeds of native *S. maritima*, which could be an interesting factor for restoration and conservation actions of this native species. In general, our results add useful knowledge to preserve European salt marshes using *S. maritima* as biotool and to fight exotic *S. densiflora* invasion in North American, European and African marshes.

Acknowledgements

We thank to the management and staff of the Odiel Marshes Natural Park (Andalusia, Spain) for their collaboration. M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte of Spanish Government for the FPU Grant (FPU14/06556).

References

Adam P (2002) Saltmarshes in a time of change. Environ. Conserv. 29:39-61. https://doi.org/10.1017/S0376892902000048

Ainouche M, Gray A (2016) Invasive *Spartina*: lessons and challenges. Biol. Invasions 18:2119-2122. https://doi.org/10.1007/s10530-016-1201-7

Bakker JP, Poschlod P, Strykstra RJ, Bekker RM, Thompson K (1996) Seed banks and seed dispersal: important topics in restoration ecology. Acta Bot. Neerl. 45:461-490. https://doi.org/10.1111/j.1438-8677.1996.tb00806.x

Baldwin AH, McKee KL, Mendelssohn IA (1996) The influence of vegetation, salinity, and inundation on seed banks of oligohaline coastal marshes. Am. J. Bot. 83:470-479. https://doi.org/10.1002/j.1537-2197.1996.tb12728.x

Baskin CC, Baskin JM (2001) Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. Academic Press, San Diego.

Biber PD, Caldwell JD (2008) Seed germination and seedling survival of *Spartina alterniflora* Loisel. Am. J. Agric. Biol. Sci. 3:633-638.

Bortolus A, Adam P, Adams JB et al (2019) Supporting *Spartina*:

Interdisciplinary perspective shows *Spartina* as a distinct solid genus. Ecology 100: e02863.
https://doi.org/10.1002/ecy.2863

Brown D (1992) Estimating the composition of a forest seed bank: a comparison of the seed extraction and seedling emergence methods. Can. J. Bot. 70:1603-1612.
https://doi.org/10.1139/b92-202

Castellanos EM, Figueroa ME, Davy AJ (1994) Nucleation and facilitation in saltmarsh succession: interactions between *Spartina maritima* and *Arthrocnemum perenne*. J. Ecol. 82:239-248. DOI: 10.2307/2261292

Castillo JM, Fernández-Baco L, Castellanos EM, Luque CJ, Figueroa ME, Davy AJ (2000) Lower limits of *Spartina densiflora* and *S. maritima* in a Mediterranean salt marsh determined by different ecophysiological tolerances. J. Ecol. 88:801-812.
https://doi.org/10.1046/j.1365-2745.2000.00492.x

Castillo JM, Ayres DR, Leira-Doce P, Bailey J, Blum M, Strong DR, Luque T, Figueroa E (2010). The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Divers. Distrib. 16:547-558.
https://doi.org/10.1111/j.1472-4642.2010.00673.x

Castillo JM, Grewell BJ, Pickart A, Bortolus A, Peña C, Figueroa E, Sytsma M (2014) Phenotypic plasticity of invasive *Spartina densiflora* (Poaceae) along a broad latitudinal gradient on the Pacific Coast of North America. Am. J. Bot. 101:448-458.
https://doi.org/10.3732/ajb.1400014

Chang ER, Jefferies RL, Carleton TJ (2001) Relationship between vegetation and soil seed banks in an arctic coastal marsh. J. Ecol. 89:367-384.
https://doi.org/10.1046/j.1365-2745.2001.00549.x

Contreras-Cruzado I, Infante-Izquierdo MD, Márquez-García B, Hermoso-López V, Polo A, Nieva FJJ, Cartes-Barroso JB, Castillo JM, Muñoz-Rodríguez A (2017) Relationships between spatio-temporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma 305:173-187.
https://doi.org/10.1016/j.geoderma.2017.05.037

Cordazzo CV, Davy AJ (1994) Seed production, seed size, and dispersal of *Spartina ciliata* Brongniart (Gramineae) in southern Brazilian coastal dunes. Atlantica 16:143-154.

Coteff C, Van Auken OW (2006) Sampling requirements for estimation of the soil seed bank of a west Texas salt marsh. Tex. J. Sci. 58:349-370.

Grain CM, Albertson LK, Bertness MD (2008) Secondary succession dynamics in estuarine marshes across landscape-scale salinity. Ecology 89:2889-2899.

Dausse A, Bonis A, Bouzille, IB, Lefeuvre JC (2007) Seed dispersal in a polder after partial tidal restoration: implications for salt marsh restoration. Appl. Veg. Sci. 11:3-12.
https://doi.org/10.1111/j.1654-109X.2008.tb00199.x

Diggory ZE, Parker VT (2011) Seed supply and revegetation dynamics at restored tidal marshes, Napa River, California. Restor. Ecol. 19:121-130.

https://doi.org/10.1111/j.1526-100X.2009.00636.x

Egan TP, Ungar IA (2000) Similarity between seed banks and above ground vegetation along a salinity gradient. J. Veg. Sci. 11:189-194. https://doi.org/10.2307/3236798

Elsey-Quirk T, Middleton BA, Proffitt CE (2009) Seed flotation and germination of salt marsh plants: the effects of stratification, salinity, and/or inundation regime. Aquat. Bot. 91:40-46. https://doi.org/10.1016/j.aquabot.2009.02.001

Espinar JL, García LV, Figuerola J, Green AJ, Clemente L (2004) Helophyte germination in a Mediterranean salt marsh: Gut-passage by ducks changes seed response to salinity. J. Veg. Sci. 15:315-322. https://doi.org/10.1111/j.1654-1103.2004.tb02267.x

Feldman SR, Alzugaray C, Lewis JP (2007) Relación entre la vegetación y el banco de semillas de un espartillar de *Spartina argentinensis*. Ciencia e Investigación Agraria 34:41-48.

Fenner M, Thompson K (2005) The ecology of seeds. Cambridge University Press, New York.

Fernández-Illescas F, Nieva FJJ, Silva I, Tormo R, Muñoz AF (2010) Pollen production of Chenopodiaceae species at habitat and landscape scale in Mediterranean salt marshes: An ecological and phenological study. Rev. Palaeobot. Palynol. 161:127-136. https://doi.org/10.1016/j.revpalbo.2010.03.006

Figueroa ME, Castillo JM, Redondo S, Luque T, Castellanos EM, Nieva FJJ, Luque CJ, Rubio-Casal AE, Davy AJ (2003) Facilitated invasion by hybridization of *Sarcocornia* species in a salt-marsh succession. J. Ecol. 91:616-626. https://doi.org/10.1046/j.1365-2745.2003.00794.x

Gioria M, Pyšek P (2016) The legacy of plant invasions: changes in the soil seed bank of invaded plant communities. BioScience 66:40-53. https://doi.org/10.1093/biosci/biv165

Gioria M, Pyšek P, Moravcová L (2012) Soil seed banks in plant invasions: Promoting species invasiveness and long-term impact on plant community dynamics. Preslia 84: 327-350.

Gross KL (1990) A comparison of methods for estimating seed numbers in the soil. J. Ecol. 78:1079-1093. DOI: 10.2307/2260953

Hazelton EL, Mozdzer TJ, Burdick DM, Kettenring KM, Whigham DF (2014) *Phragmites australis* management in the United States: 40 years of methods and outcomes. AoB Plants: 6. https://doi.org/10.1093/aobpla/plu001

Hopkins DR, Parker VT (1984) A study of the seed bank of a salt marsh in northern San Francisco Bay. Am. J. Bot. 71:348-355. https://doi.org/10.1002/j.1537-2197.1984.tb12522.x

Huiskes AHL, Koutstaal BP, Herman PMJ, Beeftink WG, Markusse MM, De Munck W (1995) Seed dispersal of halophytes in tidal salt marshes. J. Ecol. 83:559-567. DOI: 10.2307/2261624

Hutchings MJ, Russell PJ (1989) The seed regeneration dynamics of an emergent salt marsh. J. Ecol. 77:615-637.

Infante-Izquierdo MD, Castillo JM, Nieva FJJ, Rotundu ID, David FT, Grewell

BJ, Muñoz-Rodríguez AF (2019a) Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula. Wetlands. https://doi.org/10.1007/s13157-019-01188-1

Infante-Izquierdo MD, Gallego-Tévar B, Sánchez-Gullón E, Nieva FJJ, Grewell BJ, Castillo JM, Muñoz-Rodríguez AF (2019b) Morphological and anatomical evidence supports differentiation of new interspecific hybrids from native *Spartina maritima* and invasive *S. densiflora* (Poaceae, subfamily Chloridoideae). Plant Syst. Evol. 305:531-547. https://doi.org/10.1007/s00606-019-01591-5

Infante-Izquierdo MD, Castillo JM, Grewell BJ, Nieva FJJ, Muñoz-Rodríguez AF (2019c) Differential effects of increasing salinity on germination and seedling growth of native and exotic invasive cordgrasses. Plants 8:372. https://doi.org/10.3390/plants8100372

Keiffer CH, Ungar IA (1997) The effect of extended exposure to hypersaline conditions on the germination of five inland halophyte species. Am. J. Bot. 84:104-111. https://doi.org/10.2307/2445887

Kittelson PM, Boyd MJ (1997) Mechanisms of expansion for an introduced species of cordgrass, *Spartina densiflora*, in Humboldt Bay, California. Estuaries 20:770-778. https://doi.org/10.2307/1352250

Kottler EJ, Gedan K (2019) Seeds of change: characterizing the soil seed bank of a migrating salt marsh. Ann. Bot. https://doi.org/10.1093/aob/mcz133

Lambrinos JG, Bando KJ (2008) Habitat modification inhibits conspecific seedling recruitment in populations of an invasive ecosystem engineer. Biol. Invasions 10:729-741. https://doi.org/10.1007/s10530-007-9165-2

Long SP, Mason CF (1983) Saltmarsh Ecology. Cambridge University Press, Cambridge.

Mackay DB (1972) The measurement of viability. In: Roberts EH (ed) Viability of Seeds. Chapman and Hall, London, pp 172-208.

Marchant CJ, Goodman PJ (1969) *Spartina maritima* (Curtis) Fernald. J. Ecol. 57:287-291. DOI: 10.2307/2258222

Maunder M, Edward O, Guerrant Jr, Havens K, Dixon KW (2004) Realizing the full potential of ex situ contributions to global plant conservation. In: Edward O, Guerrant Jr, Havens K, Maunder M (eds) Ex situ plant conservation: supporting species survival in the wild. Island Press, Washington, pp 389-418.

McDonald K (2014) Tidal seed dispersal potential of *Spartina densiflora* in Humboldt Bay (Humboldt County, California). Doctoral dissertation, Humboldt State University, California.

Millennium Ecosystem Assessment (2005) Ecosystems and human well-being: synthesis. Island Press, Washington DC.

Morgan VH, Sytsma MD (2013) Potential ocean dispersal of cordgrass (*Spartina* spp.) from core infestations. Invasive Plant Sci. Manag. 6:250-259.

https://doi.org/10.1614/IPSM-D-12-00042.1

Mooring MT, Cooper AW, Seneca ED (1971) Seed germination response and evidence for height ecophenes in *Spartina alterniflora* from North Carolina. Am. J. Bot. 58:48-55. https://doi.org/10.1002/j.1537-2197.1971.tb09944.x

Muñoz-Rodríguez AF, Rodríguez-Rubio P, Nieva FJJ, Fernández-Illescas F, Sánchez-Gullón E, Soto JM, Hermoso-López V, Márquez-García B (2012) The importance of bracteoles in ensuring *Atriplex halimus* germination under optimal conditions. Fresenius Environ. Bull. 21:3521-3526.

Muñoz-Rodríguez AF, Sanjosé I, Márquez-García B, Infante-Izquierdo MD, Polo-Ávila A, Nieva FJJ, Castillo JM (2017) Germination syndromes in response to salinity of Chenopodiaceae halophytes along the intertidal gradient. Aquat. Bot. 139:48-56. https://doi.org/10.1016/j.aquabot.2017.02.003

Nieva FJJ, Díaz-Espejo A, Castellanos EM, Figueroa ME (2001) Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel Marshes (SW Spain). Estuar. Coast. Shelf Sci. 52:515-527. https://doi.org/10.1006/ecss.2000.0750

Plyler DB, Proseus TE (1996) A comparison of the seed dormancy characteristics of *Spartina patens* and *Spartina alterniflora* (Poaceae). Am. J. Bot. 83:11-14. https://doi.org/10.1002/j.1537-2197.1996.tb13868.x

Polo-Ávila A, Infante-Izquierdo MD, Soto JM, Hermoso-López V, Nieva FJJ, Castillo JM, Muñoz-Rodríguez AF (2019) Contrasting propagule dispersal and halophyte seed banks along the intertidal gradient. Mar. Ecol. Prog. Ser. 616:51-65. https://doi.org/10.3354/meps12943

Probert RJ, Longley PL (1989) Recalcitrant seed storage physiology in three aquatic grasses (*Zizania palustris*, *Spartina anglica* and *Porteresia coarctata*). Ann. Bot. 63:53-64. https://doi.org/10.1093/oxfordjournals.aob.a087728

Rand T (2000) Seed dispersal, habitat suitability and the distribution of halophytes across a salt marsh tidal gradient. J. Ecol. 88:608-621. https://doi.org/10.1046/j.1365-2745.2000.00484.x

Raybould AF, Gray AJ, Lawrence MJ, Marshall DF (1991) The evolution of *Spartina anglica* C.E. Hubbard (Gramineae): genetic variation and status of the parental species in Britain. Biol. J. Linn. Soc. 44:369-380. https://doi.org/10.1111/j.1095-8312.1991.tb00626.x

Thompson K, Grime JP (1979) Seasonal variation in the seed banks of herbaceous species in ten contrasting habitats. J. Ecol. 67:893-921. DOI: 10.2307/2259220

Ungar IA (1978) Halophyte seed germination. Bot. Rev. 44:233-264. https://doi.org/10.1007/BF02919080

Ungar IA (2001) Seed banks and seed population dynamics of halophytes. Wetl. Ecol. Manag. 9:499-510. https://doi.org/10.1023/A:1012236829474

Ungar IA, Woodell SRJ (1993) The relationship between the seed bank and species composition of plant communities in two British salt marshes. J.

Veg. Sci. 4:531-536.
https://doi.org/10.2307/3236080

van den Broek T, van Diggelen R, Bobbink R (2005) Variation in seed buoyancy of species in wetland ecosystems with different flooding dynamics. J. Veg. Sci. 16:579-586.
https://doi.org/10.1111/j.1654-1103.2005.tb02399.x

Wagner M, Mitschunas N (2008) Fungal effects on seed bank persistence and potential applications in weed biocontrol: a review. Basic Appl. Ecol. 9:191-203.
https://doi.org/10.1016/j.baae.2007.02.003

Wang CH, Tang L, Fei SF, Wang JQ, Gao Y, Wang Q, Chen JK, Li B (2009) Determinants of seed bank dynamics of two dominant helophytes in a tidal salt marsh. Ecol. Eng. 35: 800-809.
https://doi.org/10.1016/j.ecoleng.2008.12.004

Wijte AH, Gallagher JL (1996) Effect of oxygen availability and salinity on early life history stages of salt marsh plants. I. Different germination strategies of *Spartina alterniflora* and *Phragmites australis* (Poaceae). Am. J. Bot. 83:1337-1342.
https://doi.org/10.1002/j.1537-2197.1996.tb13919.x

Wolters M, Bakker JP (2002) Soil seed bank and driftline composition along a successional gradient on a temperate salt marsh. Appl. Veg. Sci. 5:55-62.
https://doi.org/10.1111/j.1654-109X.2002.tb00535.x

Xiao D, Zhang L, Zhu Z (2009) A study on seed characteristics and seed bank of *Spartina alterniflora* at saltmarshes in the Yangtze Estuary, China. Estuar. Coast. Shelf Sci. 83:105-110.
https://doi.org/10.1016/j.ecss.2009.03.024

Xiao D, Zhang C, Zhang L, Zhu Z, Tian K, Gao W (2016) Seed dispersal capacity and post-dispersal fate of the invasive *Spartina alterniflora* in saltmarshes of the Yangtze Estuary. Estuar. Coast. Shelf Sci. 169:158-163.
https://doi.org/10.1016/j.ecss.2015.11.032

Yannic G, Baumel A, Ainouche M (2004) Uniformity of the nuclear and chloroplast genomes of *Spartina maritima* (Poaceae), a salt-marsh species in decline along the Western European coast. Heredity 93:182-188.
https://doi.org/10.1038/sj.hdy.6800491

Zepeda G, Lot A, Nemiga XA, Manjarrez J (2014) Seed bank and established vegetation in the last remnants of the Mexican Central Plateau wetlands: the Lerma marshes. Rev. Biol. Trop. 62:455-472.

7

Differential effects of increasing salinity on germination and seedling growth of native and exotic invasive cordgrasses

Publicado en:

María Dolores Infante-Izquierdo[1], Jesús M. Castillo[2], Brenda J. Grewell[3], F. Javier J. Nieva[1], Adolfo F. Muñoz-Rodríguez[1] (2019).

Plants 8: 372. doi:10.3390/plants8100372

[1] Departamento de Ciencias Integradas. Fuerzas Armadas Ave., Campus El Carmen, Universidad de Huelva, 21071 Huelva, Spain; jimenez@uhu.es (F.J.J.N.); adolfo.munoz@dbasp.uhu.es (A.F.M-R.)
[2] Departamento de Biología Vegetal y Ecología. Universidad de Sevilla, Ap. 1095, 41080 Sevilla, Spain; manucas@us.es
[3] USDA-ARS Invasive Species and Pollinator Health Research Unit, Department of Plant Sciences MS-4, 1 Shields Ave., University of California, Davis, CA 95616, USA; bjgrewell@ucdavis.edu

Factor de impacto (2018): 2,632. Plant Science: 59 de 228, Cuartil 2

Differential effects of increasing salinity on germination and seedling growth of native and exotic invasive cordgrasses

Abstract

Soil salinity is a key environmental factor influencing germination and seedling establishment in salt marshes. Global warming and sea level rise are changing estuarine salinity, and may modify the colonization ability of halophytes. We evaluated the effects of increasing salinity on germination and seedling growth of native *Spartina maritima* and invasive *S. densiflora* from wetlands of the Odiel-Tinto Estuary. Responses were assessed following salinity exposure from fresh water to hypersaline conditions and germination recovery of non-germinated seeds when transferred to fresh water. The germination of both species was inhibited and delayed at high salinities, while pre-exposure to salinity accelerated the speed of germination in recovery assays compared to non-pre-exposed seeds. *S. densiflora* was more tolerant of salinity at germination than *S. maritima*. *S. densiflora* was able to germinate at hypersalinity and its germination percentage decreased at higher salinities compared to *S. maritima*. In contrast, *S. maritima* showed higher salinity tolerance in relation to seedling growth. Contrasting results were observed with differences in the tidal elevation of populations. Our results suggest *S. maritima* is a specialist species with respect to salinity, while *S. densiflora* is a generalist capable of germination of growth under suboptimal conditions. Invasive *S. densiflora* has greater capacity than native *S. maritima* to establish from seed with continued climate change and sea level rise.

Keywords:

Climate change, Dormancy, Odiel Marshes, Quiescent seed, Salinity tolerance, Sea level rise, Radicle

Introduction

Salt marshes are highly stressful environments where halophytes are subjected to high mortality risk [1]. In these habitats, soil salinity is one of the key environmental factors determining vegetation distribution, partially by limiting seed germination and seedling establishment [2]. These phases are crucial in the life cycle of halophytes [3–5]. The general behaviour of halophytic seeds in the presence of salt is well documented [6]. Seeds of most halophytes show optimal germination in freshwater, differing in their germination responses to higher salinities [6–8]. High salinities usually inhibit germination of halophytes, however, some seeds maintain viability and are able to germinate when osmotic stress decreases [9–11].

Estuarine salt marshes are increasingly impacted by biological invasions [12]. Some invasive halophytes show high tolerance to salinity during germination and seedling growth and have colonized a wide range of salt-affected habitats [13–15]. In tidal wetlands, climate change and associated sea level rise are changing estuarine salinity patterns [16]. Local environmental conditions can be highly variable with climate change. Salinity decreases in some salt marshes due to an increase in rainfall, while salinity increases in other locations due to sea level rise and increases in temperature and evapotranspiration rates [17–19]. These environmental changes may modify the ability of native species to colonize new sites as well as the capacity of invasive halophytes to invade them.

Cordgrasses (genus *Spartina*) provide a model halophyte group to study the responses of native and invasive species to environmental conditions since they inhabit salt marshes around the world, and many species have naturalized in habitats beyond their native ranges [20]. Specifically, native *Spartina maritima* (Curtis) Fernald and invasive *Spartina densiflora* Brongn. co-occur in salt marshes along the Gulf of Cádiz (Southwest Iberian Peninsula) [21]. *S. maritima* is the only native cordgrass in European marshes [22], where it is a primary colonizer at low tidal elevations and facilitates the development of ecological succession [23]. Therefore, the conservation of this species is crucial for the maintenance of biodiversity in these ecosystems.

The effects of salinity on seed germination and seedling growth have never been studied for native *S. maritima*. Actually, seed production of this species has been described as very low or non-existent [22–24], but we recently discovered that *S. maritima* in the Southwest Iberian Peninsula produces a moderate number of caryopses (13%) with high variation among tussocks (0%-45%), high viability (89%) and has high germination rates in freshwater (85%) [25]. In contrast, South American *S. densiflora* is one of the three most widely distributed species of the genus and was introduced to the Southwest Iberian Peninsula centuries ago [26]. *S. densiflora* shows high tolerance to environmental variation, including salinity levels [27]. This niche breath has resulted in its colonization of a wide range of different habitats along the intertidal gradient [21,26,28]. Seed production is key to the spread of

Spartina species [20], and the ability of *S. densiflora* to germinate is a recognized determinant for its invasive potential from brackish marshes to hypersaline saltpans [29–31]. To our knowledge, no previous study has evaluated salinity responses of *S. densiflora* seeds that were produced in different habitats along the intertidal gradient. Previous studies of halophytes have found that germination tolerance to salinity depends on environmental conditions in the source habitats where they were produced [9,11,32].

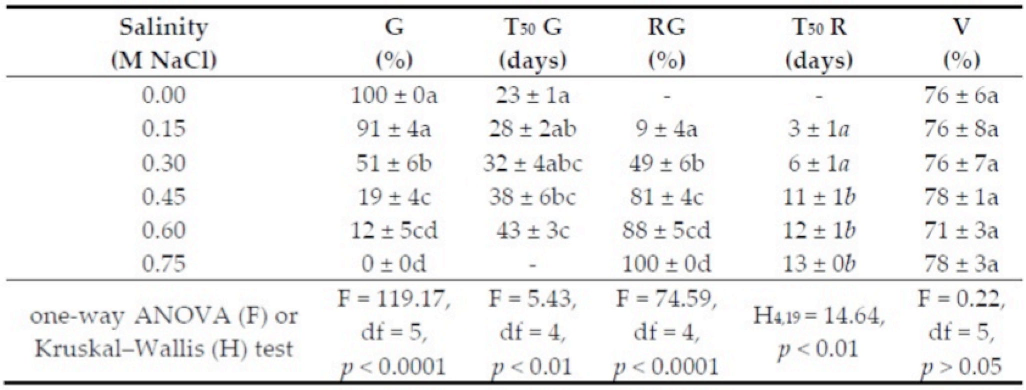
Our main goals were to analyse germination and seedling growth of native *S. maritima* and invasive *S. densiflora* in response to salinity ranging from freshwater to hypersaline levels. We hypothesized that *S. maritima* seeds and seedlings would show high salinity tolerance since the species colonizes low elevation tidal marshes where medium-high salinity levels occur throughout the year [33]. We also hypothesized that *S. densiflora* would show high plasticity in response to salinity since it has invaded a wide range of habitats with contrasted salinity regimes [21]. Germination experiments evaluating the responses of these species to salinity ranging from freshwater to hypersalinity, and recovery of the species after salinity release, were carried out under controlled conditions to test our hypotheses. Results were compared to field conditions where propagules were sourced for the experiments.

Results

Germination Responses to Salinity

Spartina maritima achieved its highest germination percentage (c. 96 %) at low salinity levels between 0.00 and 0.15 M NaCl. Germination rate for the native species decreased at higher salinities. No seed was able to

Table 1. Germination percentage (G), T_{50} of germination (T_{50} G), recovery germination percentage after salt exposure (RG), T_{50} of germination recovery (T_{50} R), and seed viability percentage (V) for native *Spartina maritima* from the Gulf of Cádiz (Southwest Iberian Peninsula) in six salt treatments. Data show mean ± Standard Error (SE) (n = 3–4). Different letters indicate significant differences between treatments (Mann–Whitney U test, in italic, for Kruskal–Wallis or Tukey's Honest Significant Difference (HSD) test, in non-italic, for Analysis of Variance (one-way ANOVA), $p < 0.05$). df (degrees of freedom).



Salinity (M NaCl)	G (%)	T_{50} G (days)	RG (%)	T_{50} R (days)	V (%)
0.00	100 ± 0a	23 ± 1a	-	-	76 ± 6a
0.15	91 ± 4a	28 ± 2ab	9 ± 4a	3 ± 1*a*	76 ± 8a
0.30	51 ± 6b	32 ± 4abc	49 ± 6b	6 ± 1*a*	76 ± 7a
0.45	19 ± 4c	38 ± 6bc	81 ± 4c	11 ± 1*b*	78 ± 1a
0.60	12 ± 5cd	43 ± 3c	88 ± 5cd	12 ± 1*b*	71 ± 3a
0.75	0 ± 0d	-	100 ± 0d	13 ± 0*b*	78 ± 3a
one-way ANOVA (F) or Kruskal–Wallis (H) test	F = 119.17, df = 5, $p < 0.0001$	F = 5.43, df = 4, $p < 0.01$	F = 74.59, df = 4, $p < 0.0001$	$H_{4,19} = 14.64$, $p < 0.01$	F = 0.22, df = 5, $p > 0.05$

germinate at 0.75 M NaCl. T_{50} G (days necessary to reach 50% of the final germination percentage) was increased at salinities higher than 0.30 M NaCl (Table 1).

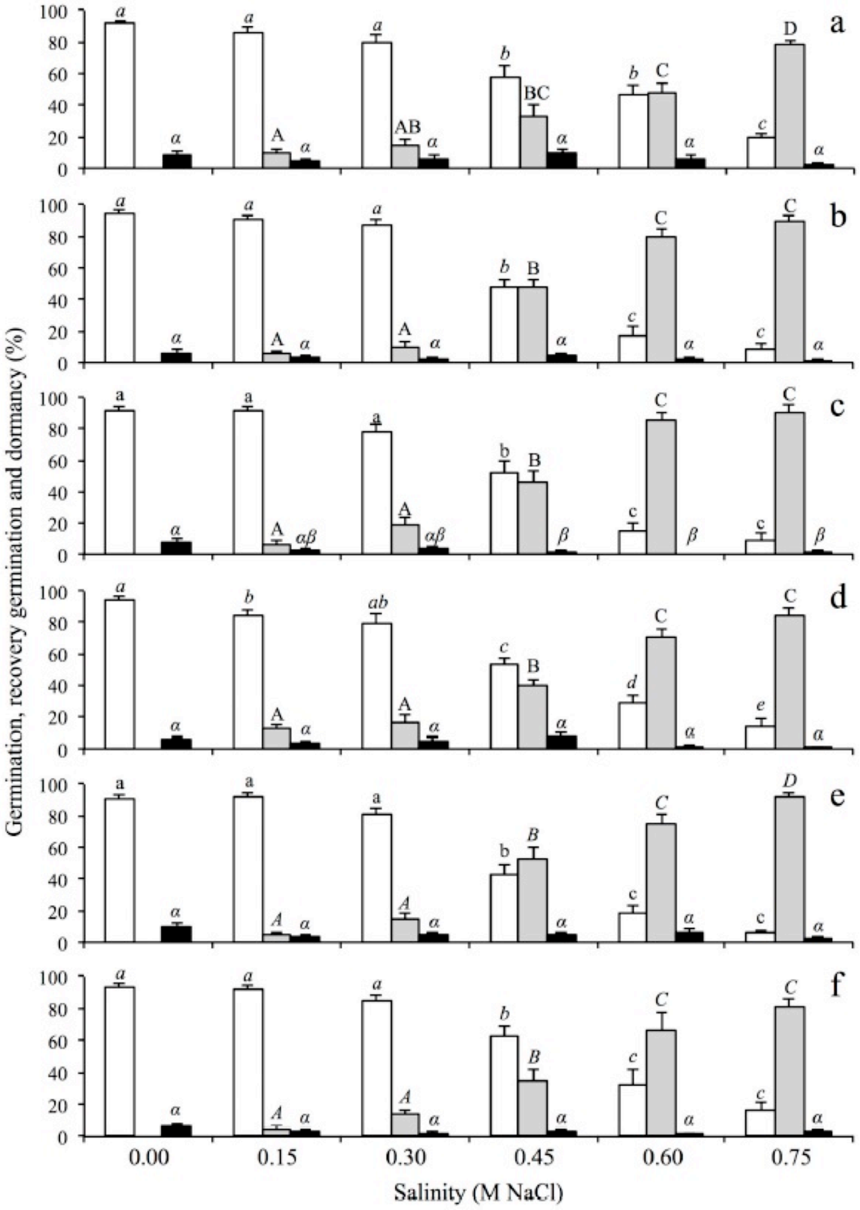
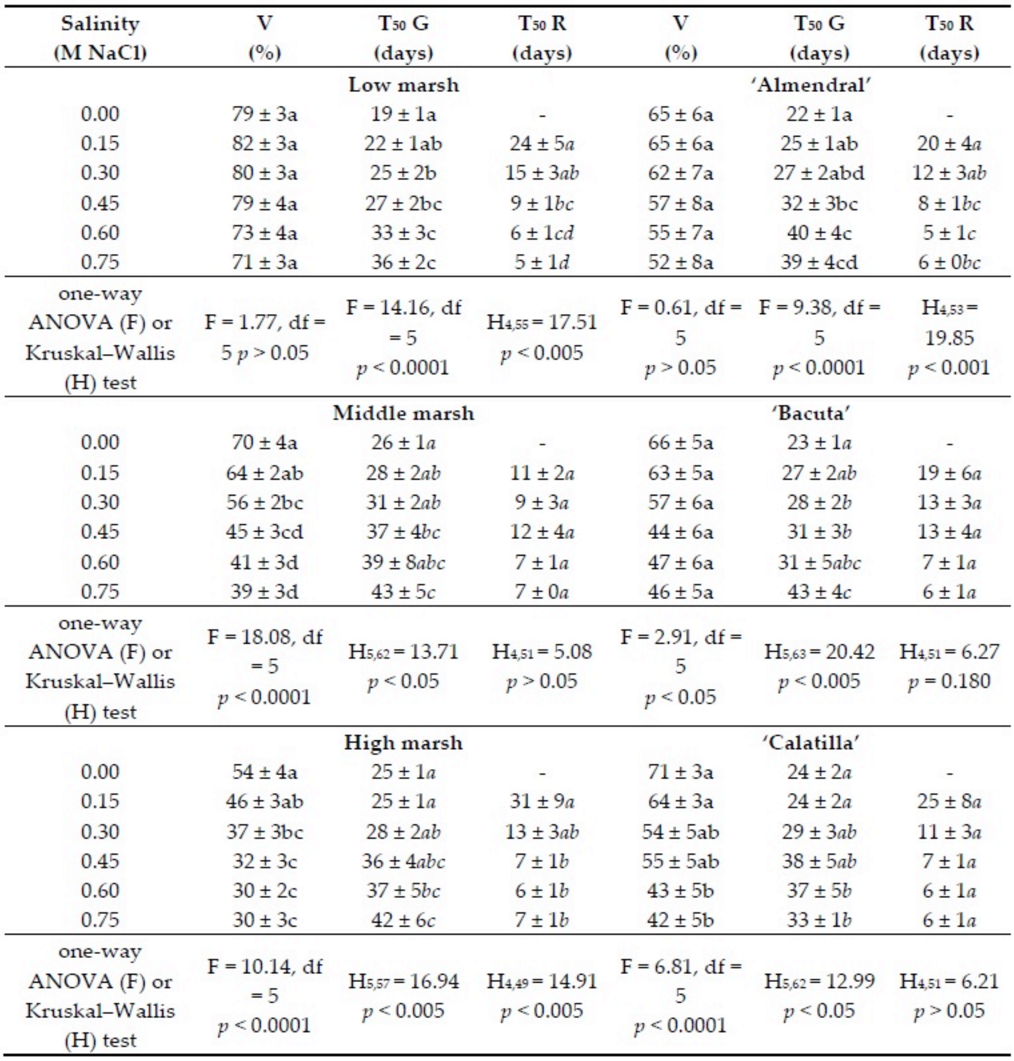


Figure 1. Percentages of germination (white bars), recovery of germination after salt exposure (grey bars) and seed dormancy (black bars) in six salinity treatments for *Spartina densiflora* from three habitats (grouping the three locations in each habitat): (**a**) low marsh (LM), (**b**) middle marsh (MM), (**c**) high marsh (HM), and from three locations (grouping the three habitats in each location): (**d**) Almendral, (**e**) Bacuta, (**f**) Calatilla, in the Odiel Marshes (Southwest Iberian Peninsula). Data are mean ± SE (n = 4). Different letters indicate significant differences among treatments for each trait (Mann–Whitney U test, in italic, for Kruskal–Wallis or Tukey's HSD test, in non-italic, for one-way ANOVA, $p < 0.05$).

Germination percentage for *S. densiflora* decreased significantly in salinities higher than 0.30 M NaCl in seeds from all locations and habitats (Figure 1). However, at higher salinities, the germination percentage of seeds from LM (low marsh) was more than double than those from MM (middle marsh) and HM (high marsh) (Figure 1). Seeds produced in LM showed higher germination (19% ± 3%) than those from MM and HM (c. 9%) at hypersalinity (0.75 M NaCl) (Kruskal-Wallis test, $H_{2,36} = 9.16$, $p < 0.05$) (Figure 1a, b, c).

Increasing salinity decreased the germination speed (higher T_{50} G) for seeds sourced from all marsh elevation zones and locations. Additionally, *S. densiflora* seeds produced at LM germinated 27% faster in freshwater than those from MM and HM (one-way ANOVA, F = 11.03, degrees of freedom (df) = 2, $p < 0.0005$) (Table 2).

Table 2. Comparisons among salt treatments for *Spartina densiflora* in the Southwest Iberian Peninsula. Percentage of viability (V), T_{50} of germination (T_{50} G) and T_{50} of recovery (T_{50} R) in the different salt treatments (0.00, 0.15, 0.30, 0.45, 0.60 and 0.75 M NaCl) for different habitats and locations. Data are mean ± SE (n = 12). Different letters indicate significant differences among treatments for each trait (Mann–Whitney U test, in italic, for Kruskal–Wallis or Tukey's HSD test, in non-italic, for one-way ANOVA, $p < 0.05$). df (degrees of freedom).



Salinity (M NaCl)	**V (%)**	**T_{50} G (days)**	**T_{50} R (days)**	**V (%)**	**T_{50} G (days)**	**T_{50} R (days)**
		Low marsh			**'Almendral'**	
0.00	79 ± 3a	19 ± 1a	-	65 ± 6a	22 ± 1a	-
0.15	82 ± 3a	22 ± 1ab	24 ± 5*a*	65 ± 6a	25 ± 1ab	20 ± 4*a*
0.30	80 ± 3a	25 ± 2b	15 ± 3*ab*	62 ± 7a	27 ± 2abd	12 ± 3*ab*
0.45	79 ± 4a	27 ± 2bc	9 ± 1*bc*	57 ± 8a	32 ± 3bc	8 ± 1*bc*
0.60	73 ± 4a	33 ± 3c	6 ± 1*cd*	55 ± 7a	40 ± 4c	5 ± 1*c*
0.75	71 ± 3a	36 ± 2c	5 ± 1*d*	52 ± 8a	39 ± 4cd	6 ± 0*bc*
one-way ANOVA (F) or Kruskal–Wallis (H) test	F = 1.77, df = 5 $p > 0.05$	F = 14.16, df = 5 $p < 0.0001$	$H_{4,55} = 17.51$ $p < 0.005$	F = 0.61, df = 5 $p > 0.05$	F = 9.38, df = 5 $p < 0.0001$	$H_{4,53} = 19.85$ $p < 0.001$
		Middle marsh			**'Bacuta'**	
0.00	70 ± 4a	26 ± 1*a*	-	66 ± 5a	23 ± 1*a*	-
0.15	64 ± 2ab	28 ± 2*ab*	11 ± 2*a*	63 ± 5a	27 ± 2*ab*	19 ± 6*a*
0.30	56 ± 2bc	31 ± 2*ab*	9 ± 3*a*	57 ± 6a	28 ± 2*b*	13 ± 3*a*
0.45	45 ± 3cd	37 ± 4*bc*	12 ± 4*a*	44 ± 6a	31 ± 3*b*	13 ± 4*a*
0.60	41 ± 3d	39 ± 8*abc*	7 ± 1*a*	47 ± 6a	31 ± 5*abc*	7 ± 1*a*
0.75	39 ± 3d	43 ± 5*c*	7 ± 0*a*	46 ± 5a	43 ± 4*c*	6 ± 1*a*
one-way ANOVA (F) or Kruskal–Wallis (H) test	F = 18.08, df = 5 $p < 0.0001$	$H_{5,62} = 13.71$ $p < 0.05$	$H_{4,51} = 5.08$ $p > 0.05$	F = 2.91, df = 5 $p < 0.05$	$H_{5,63} = 20.42$ $p < 0.005$	$H_{4,51} = 6.27$ $p = 0.180$
		High marsh			**'Calatilla'**	
0.00	54 ± 4a	25 ± 1*a*	-	71 ± 3a	24 ± 2*a*	-
0.15	46 ± 3ab	25 ± 1*a*	31 ± 9*a*	64 ± 3a	24 ± 2*a*	25 ± 8*a*
0.30	37 ± 3bc	28 ± 2*ab*	13 ± 3*ab*	54 ± 5ab	29 ± 3*ab*	11 ± 3*a*
0.45	32 ± 3c	36 ± 4*abc*	7 ± 1*b*	55 ± 5ab	38 ± 5*ab*	7 ± 1*a*
0.60	30 ± 2c	37 ± 5*bc*	6 ± 1*b*	43 ± 5b	37 ± 5*b*	6 ± 1*a*
0.75	30 ± 3c	42 ± 6*c*	7 ± 1*b*	42 ± 5b	33 ± 1*b*	6 ± 1*a*
one-way ANOVA (F) or Kruskal–Wallis (H) test	F = 10.14, df = 5 $p < 0.0001$	$H_{5,57} = 16.94$ $p < 0.005$	$H_{4,49} = 14.91$ $p < 0.005$	F = 6.81, df = 5 $p < 0.0001$	$H_{5,62} = 12.99$ $p < 0.05$	$H_{4,51} = 6.21$ $p > 0.05$

Germination Responses after Salinity Exposure

The germination rate of *Spartina maritima* seeds increased, and the speed of germination decreased significantly during recovery after exposure to higher salinities. T_{50} R (days necessary to reach 50% of the final germination percentage in recovery assays) was reduced after recovery from all levels of salinity exposure than seeds that were germinated in freshwater. The T_{50} reduction indicating an increase in germination speed was most extreme during recovery from 0.15 M NaCl exposure when germination accelerated almost nine times (Table 1). In contrast to *S. densiflora*, no dormant seeds were recorded for *S. maritima* during the recovery experiment. *S. maritima* seed viability was c. 76%, without showing significant differences among salinities (Table 1).

As in the case of *S. maritima*, *S. densiflora* seeds had increased germination rates during recovery following exposure to higher salinities, and this result held for seeds sourced from every location and within-marsh habitat elevation zone (Figure 1). *S. densiflora* seeds tended to have increased germination speeds (lower T_{50} R) during recovery following exposure to higher salinities (Table 2). Seeds from LM exposed previously to hypersalinity showed lower recovery germination percentage (78% ± 3%) and lower T_{50} (5 ± 1 days) than seeds from MM and HM (c. 90% and 7 days, respectively) (Figure 1, Table 2). Dormant *S. densiflora* seeds in freshwater were c. 7% for every habitat and location and tended to decrease with increasing salinity exposure (Figure 1). Seed viability in control treatments were higher for seeds of *S. densiflora* from LM and MM (c. 76%) than those from HM (54% ± 4%) (one-way ANOVA, $F = 12.95$, $df = 2$, $p < 0.0001$). On the other hand, seed viability was not affected by salt treatments in seeds from LM, while in seeds from MM and HM the viability decreased as salinity increased (Table 2). Regarding the three source population locations, there were no significant differences in seed viability at any salinity level for seed sourced from Almendral, whereas viability was reduced at higher salinities for the other two study locations (Table 2).

Initial Seedling Growth Responses to Salinity

S. maritima seedlings had similar cotyledon and first leaf lengths at every salinity level (Figure 2). However, the radicles of *S. maritima* were four times longer at 0.15 M NaCl than radicles that emerged from seeds exposed to other salinity concentrations (Kruskal-Wallis test, $H_{3,59} = 8.66$, $p < 0.05$). Cotyledon, first leaf and radicle of *S. maritima* seedlings growing in freshwater all increased in length in recovery after seeds had been exposed to salinity higher than 0.15 M NaCl (Figure 2).

The cotyledon and first leaf length of *S. densiflora* were reduced as salinity increased in seeds sourced from every study location and elevational habitat (Figure 3). The length of cotyledon and first leaf were shorter for seedlings from LM seed that germinated at salinity higher than 0.30 M NaCl (Figure 3a). In contrast, seedlings from MM and HM seeds displayed this reduction in size at a lower salinity concentration of 0.15 M NaCl (Figure 3b, c). Also, in contrast, salinity had a positive effect on radicle growth at 0.30 M NaCl for seeds coming from LM, though radicle length was reduced at salinities higher than 0.45 M NaCl (Kruskal-Wallis test, $H_{5,205} = 84.93$, $p < 0.0001$) (Figure 3a). This shorter radicle length trait was expressed in salinities higher than 0.30 M NaCl for seeds from MM (Kruskal-Wallis test, $H_{5,147} = 28.99$, $p < 0.0001$) (Figure 3b) and higher than 0.15 M NaCl for seeds from HM (Kruskal-Wallis test, $H_{5,140} = 26.65$, $p < 0.001$) (Figure 3c). In freshwater conditions, *S. densiflora* seedlings emerging from seeds sourced at Almendral and Bacuta locations had 1.3 times, and 1.7 times larger first leaves and radicles respectively, than those from Calatilla (Kruskal-Wallis test, $H_{2,111} = 10.17$, $p < 0.01$; one-way ANOVA test, F = 4.88, df = 2, $p < 0.01$, respectively) (Figure 3d, e, f). Few significant differences, without showing a clear pattern, were recorded for seedling responses during the recovery experiment for all previous salinity exposures (Figure 3).

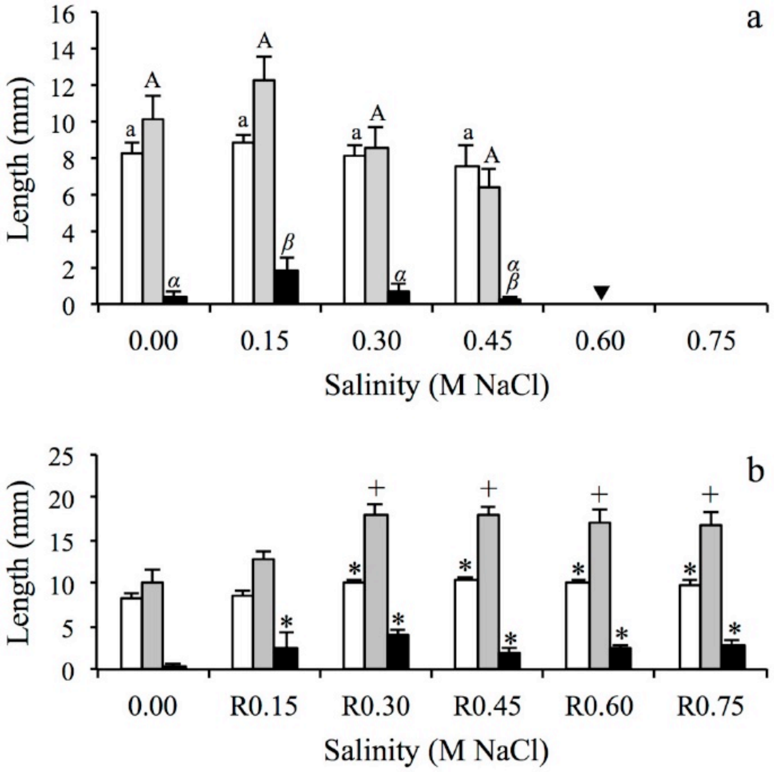


Figure 2. Cotyledon (white bars), first leaf (grey bars) and radicle length (black bars) for seedlings of *Spartina maritima* germinated: (**a**) in six salt treatments and (**b**) in the recovery (R) assays after salinity exposure. Data show mean ± SE (n = 5–13). Different letters indicate significant differences between salinity treatments (Mann–Whitney U test, in italic, for Kruskal–Wallis or Tukey's HSD test, in non-italic, for one-way ANOVA, $p < 0.05$). Asterisks and plus sign indicate significant differences

Discussion

Our hypotheses were partially confirmed by the outcomes of the experiments. As expected, germination rates of seeds from native *S. maritima* expressed high tolerance to salinity, but tolerance of this native species was lower than that of salinity levels tolerated by invasive *S. densiflora*. In this circumstance, *S. densiflora* has a higher capacity for phenotypic plasticity in response to salinity. However, invasive seedling trait responses to salinity suggest a lower tolerance to salinity during the initial 15 days of seedling growth than the more vigorous initial growth of native *S. maritima*.

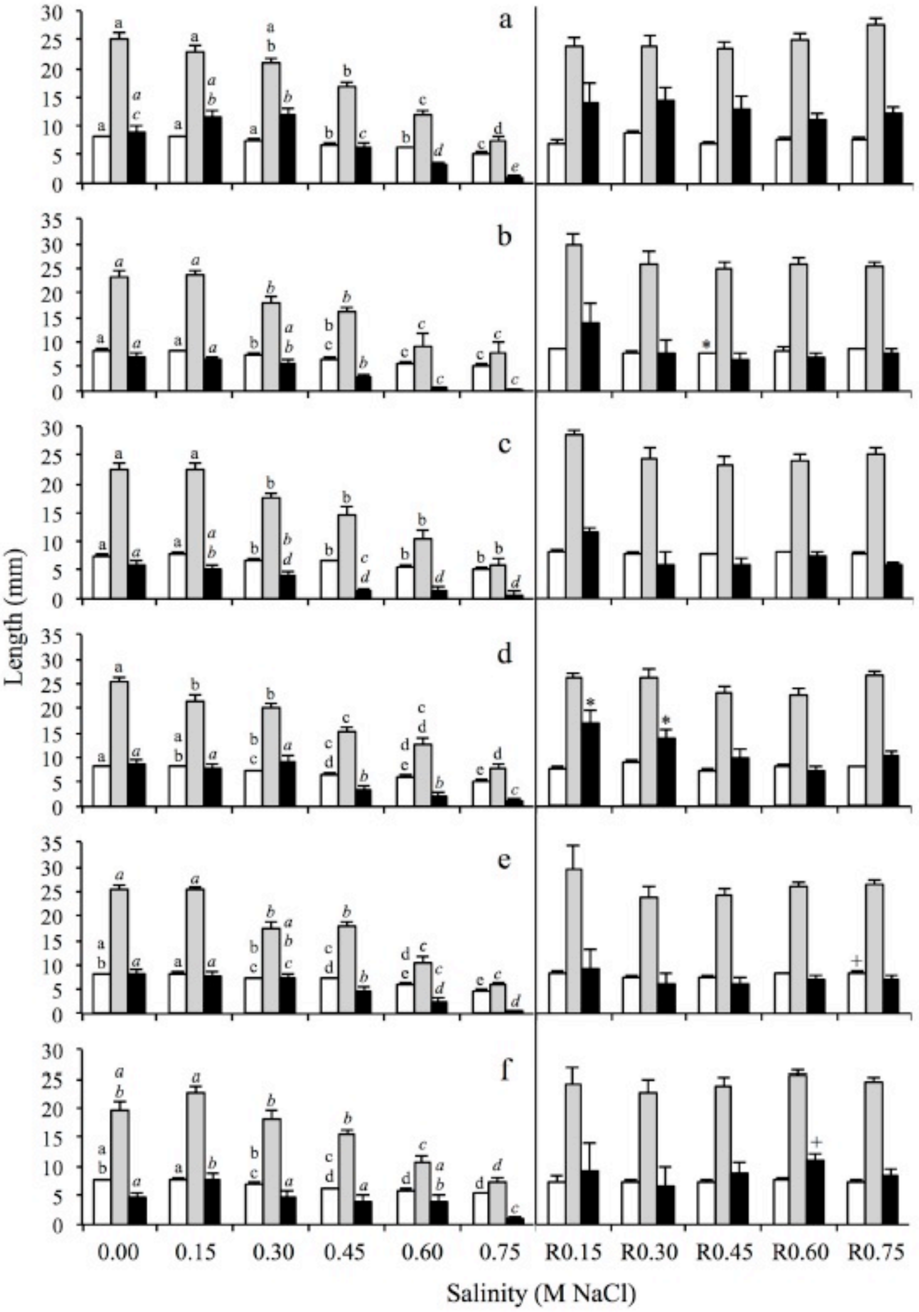


Figure 3. Cotyledon (white bars), first leaf (grey bars) and radicle length (black bars) from seedlings of *Spartina densiflora* germinated in six salinity treatments (left) and in the recovery assays after salinity exposure (R) (right) coming from seeds collected from three habitats (grouping the three locations in each habitat): (**a**) low marsh, (**b**) middle marsh, (**c**) high marsh, and from three locations (grouping the three habitats in each location): (**d**) Almendral, (**e**) Bacuta. (**f**) Calatilla), in the Odiel Marshes (Southwest Iberian Peninsula). Data show mean ± SE (n = 3–40). Different letters indicate significant

Some salinity responses were common to both studied cordgrasses. For example, high seed viability after salinity exposure was recorded for both *Spartina* species, as it has commonly been observed for many halophyte species [9]. Also, elevated salinity concentrations inhibited and delayed germination for both studied *Spartina* species [8]. This seed quiescence prevents seed germination under stressful conditions [1,5]. Decreases in germination rates have been reported for the congener *Spartina alterniflora* Loisel. at salinities higher than 0.20-0.40 M NaCl in the native and invasive range [34,35], however some authors recorded high germination (> 90%) even at hypersalinity [15]. Germination of *Spartina ciliata* Brongn. from Brazil was reduced at salinities higher than 0.20 M NaCl, totally inhibited at seawater concentration, and speed of germination increased after salinity exposure [36]. In addition, exposure to salinity followed by recovery after freshening accelerated germination for *S. maritima* and *S. densiflora* in our study, which has been reported previously for other halophyte species from a range of functional groups [8,37–39]. The observed stimulation of germination speed and rate after salinity exposure can provide windows of opportunity for seeds to germinate and quickly establish when salinity is sporadically and temporarily reduced by precipitation events or other sources of freshwater inflow [10,40].

While both studied cordgrasses showed high tolerance to salinity and shared some common germination and initial seedling trait responses, each also expressed distinctly contrasting responses. Germination rates and speed of germination under increasing NaCl concentrations indicated *S. densiflora* had higher germination tolerance to salinity than *S. maritima*. *S. maritima* germination was completely inhibited and *S. densiflora* was able to germinate at hypersalinity. Moreover, germination percentage decreased from 0.15 M NaCl up for *S. maritima* and from 0.30 M NaCl up for *S. densiflora*. As in our study, invasive *S. densiflora* in Humboldt Bay (California) showed reductions in seed germination at salinities higher than 0.30 M NaCl [30]. Some authors recorded total germination inhibition at 1.00 M NaCl and at 0.70 M NaCl for invasive *S. densiflora* in the Gulf of Cádiz [29,31]. The great salinity tolerance of *S. densiflora* was also reflected on its rapid germination after being pretreated at increasing salinities, whereas this study is the first to document the opposite response for native *S. maritima*. Seed quiescence of *S. densiflora* did not alter its initial seedling growth, in contrast to responses of *S. maritima*, in which seedlings were longer after saline pre-treatments compared to control. In addition to the capacity for seed quiescence under stressful conditions, the response of *S. densiflora* indicates a degree of physiological seed dormancy (< 10%). In addition, the radicle and first leaf of *S. densiflora* always emerged earlier than those of *S. maritima*. This suggests *S. densiflora* seeds were able to remain dormant in stressful saline environments without damaging the quality of the embryo, and then had the capacity to germinate later when salinity stress was reduced, providing multiple opportunities for establishment [1,10,39].

Its higher germination tolerance to salinity, faster germination after being pre-treated at increasing salinities and the presence of seed dormancy help to explain that *S. densiflora* is able to invade a wide range of habitats along the intertidal gradient [21] including hypersaline saltpans [3]. Invasive *S. densiflora* germination showed higher salinity tolerance than native *S. maritima*, but the autochthonous species showed higher salinity tolerance in relation to early seedling growth. Negative effects of salinity on seedling growth have been reported previously for invasive *S. densiflora* in Humboldt Bay (California) where seedling height decreased at salinities higher than 0.20 M NaCl [30], for invasive *S. alterniflora* in China [35], where shoot height decreased from 0.20 M NaCl up and radicle length from 0.10 M NaCl up, and for native *S. ciliata* in Brazil [36], with shoot and radicle being smaller at salinities higher than from 0.05 M NaCl.

Besides the general comparison between both *Spartina* species, contrasted responses to salinity in seed viability, germination rate and speed, seed dormancy and seedling growth were also recorded among native and invasive species and among *S. densiflora* populations along the intertidal gradient. These differences in germination and seedling trait responses between *S. maritima* and *S. densiflora* and for seeds sourced among contrasting *S. densiflora* habitats could be attributed to local adaptation to contrasted environments [41], or to pre-adaptive conditioning determined by the maternal environment during seed development [42–44]. Salinity responses from different *S. densiflora* locations along the Odiel-Tinto Estuary (grouping LM, MM and HM elevations at each location) may support the pre-adaptive conditioning hypothesis. Supporting this idea, seed viability was high and independent of exposure to salinity concentrations for seeds from Almendral, but it decreased as salinity increased for seeds from Bacuta and Calatilla locations. Almendral is the nearest location to the coastline (12,500 m), whereas Bacuta and Calatilla are located along a tidal gradient 1800 m and 5500 m inland from Almendral, respectively. Thus, seeds ripening in low elevations and in locations closer to the coastline are more frequently exposed to tidal flooding and salt spray than those at higher elevations and more inland locations on the intertidal gradient [45]. Differentiated environmental conditions may acclimate seeds to salt stress in LM and closer to the sea, protecting their embryo from being killed due to ion toxicity at high salinities [2]. Furthermore, the invasive *S. densiflora* populations have low genetic diversity in North American and European marshes [46,47], which also supports that differences recorded along the intertidal gradient would likely be due to phenotypic plasticity rather than to genetic adaptation. Pre-adaptive conditioning determined by maternal stress conditions could increase survivorship and germination under high salinities, which may suppose an advantage for offspring in conditions similar to those experienced by the parents [42–44]. Moreover, salinity acclimation would facilitate survivorship during hydrochorus dispersal of buoyant seeds with sea water currents [48]. Other authors have observed that salt tolerance in halophyte germination is related to the duration and intensity of their exposure to salts in field conditions [9,11,32,49]. As in our study, *Iris hexagona* Walter and *Suaeda aralocaspica* (Bunge) Freitag and Schütze growing in high salinities produced seeds that had higher germination rates and speeds of germination when seeds were exposed to different salinity concentrations than seeds produced in low salinity environments [37,50]. Furthermore, other environmental factors such as temperature, photoperiod, soil moisture and nutrients availability can influence seed viability, germinability and dormancy [1,51–54].

Materials and methods

Study Area and Plant Material

The plant propagules evaluated in this study were sourced from the Odiel Marshes by the Gulf of Cádiz, in the Southwest Iberian Peninsula. The coast of the Gulf of Cádiz is mesotidal and the mean sea level in this area is +1.85 m relative to Spanish Hydrographic Zero (SHZ). The tides are semidiurnal and have a mean range of 2.10 m and a mean spring tidal range of 2.97 m, representing 0.40-3.37 m above SHZ. This area is under a Mediterranean climate with Atlantic influence, with +18.2 °C as the annual mean temperature [23]. Native vegetation in salt marshes along the Gulf of Cádiz has been described in previous works [23,33,55].

Inflorescences in fruiting stage were randomly collected from *S. maritima* and *S. densiflora* tussocks in the Odiel Marshes. Since native *S. maritima* colonizes mainly low elevations in the tidal frame [23], inflorescences were collected in August 2017 from a low marsh at the location known locally as Ludovico (37.174341N, –6.931643W; See a site description in previous work [23]) (Figure 4). *S. densiflora* invades LM, MM and HM in the Gulf of Cádiz [21], so its inflorescences in fruiting stage were collected from those three habitats at each of three different locations distributed from close to the estuary inlet to more inland areas (Almendral: 37.209699N, –6.953506W; Bacuta: 37.218836N, –6.964066W; Calatilla: 37.250382N, –6.969434W) in November 2016 (Figure 4). Marsh habitats were distinguished based on tidal influence and soil characteristics [33]. LM were defined between Mean High-Water Neap (MHWN) and Mean High Water (MHW), MM went from Mean High Water (MHW) to Mean High Water Spring (MHWS), and HM from Mean High Water Spring (MHWS) to Highest Astronomical Tide (HAT) [56]. In all sampled salt marshes, *S. densiflora* has become very abundant, displacing native vegetation [21]. Spikelets containing caryopses were randomly selected from collected inflorescences and stored in paper bags in dark and dry conditions at +5 °C until use.

Salinity Germination Experiment

Before sowing, in September 2017 in the case of *S. maritima* and in December 2016 in the case of *S. densiflora*, the spikelets of both *Spartina* species were surface sterilized in 5% (v/v) sodium hypochlorite for 10 min to prevent fungal contamination and then rinsed with distilled water [25,39]. Four replicates with 25 spikelets each were sown, for each habitat and location, on two layers of autoclaved filter paper watered with six different salt treatments (sodium chloride puriss pro analysis >99.5%, Sigma-Aldrich; 0.00 (control), 0.15, 0.30, 0.45, 0.60 and 0.75 M NaCl) in Petri dishes (9 cm diameter) sealed with adhesive tape (Parafilm™) to avoid desiccation. Sodium chloride was chosen as the salt to be investigated since it is by far the most prevalent major salt dissolved in the Odiel estuary water [57]. This salinity range was chosen to include salinities from freshwater (0.0 M NaCl) to sea water (0.60 M NaCl), and hypersalinity (0.75 M NaCl). The dishes were maintained during 2 months under controlled-environmental conditions in a plant grow room, at temperatures between 20 °C and 25 °C and a 12 h light/12 h dark photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m-2 s-1. During this time, germination was recorded every 2 or 3 days. A seed was considered germinated when the coleoptile emerged.

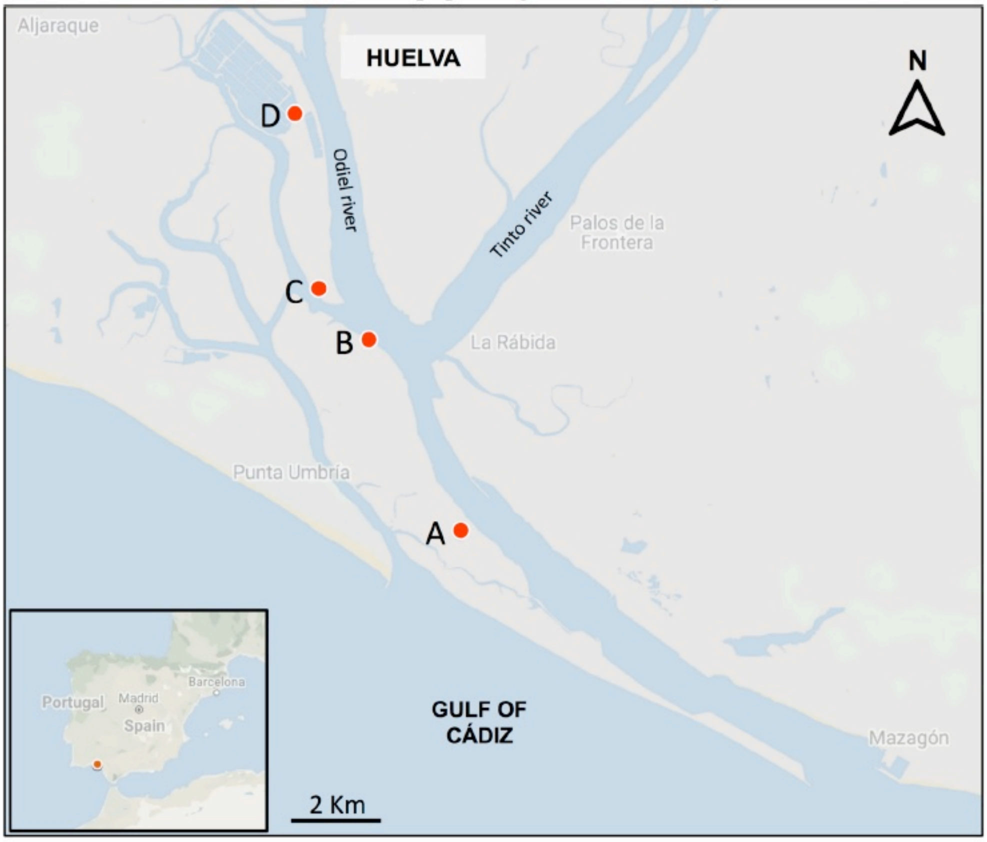


Figure 4. Map of the Odiel Marshes (Southwest Iberian Peninsula) showing (A) the location where inflorescences in the fruiting stage of native *Spartina maritima* were collected from a low marsh, and the three locations (B, Almendral; C, Bacuta; D, Calatilla) where inflorescences of invasive *S. densiflora* were collected from low, middle, and high marshes. (Source: Google Maps, data from ©2019 Instituto Geográfico Nacional Spain).

Post-Salinity Exposure Recovery Experiment

Spikelets that did not germinate during the 2 months salinity exposure trials were rinsed with distilled water and sown in new Petri dishes with distilled water to assess post-salinity exposure recovery. Germination was recorded every 2 or 3 days for 2 months. Seed viability of the spikelets that did not germinate during the recovery experiment was tested using the Tetrazolium test [58]. For this purpose, the embryo was incised with a scalpel and submerged in a 1% aqueous solution of 2,3,5 triphenyl tetrazolium chloride at 25 °C in darkness for 24 h. Then, red-stained viable embryos were counted through a magnifying glass.

The percentage of viable seeds (germinated plus dormant seeds) was calculated for each Petri dish. The germination rates (percentage) for viable seeds at different salinities, and the recovery germination percentage after salt exposure were then calculated. Seeds that did not germinate during the salinity treatments, but germinated in the recovery experiment, were considered quiescent seeds. Seed dormancy percentage was calculated for each Petri dish using the number of viable seeds that did not germinate at the end of the recovery experiment. In addition, the days necessary to reach 50% of the final germination percentage was calculated for each Petri dish in both the germination experiment (T_{50} G) and the recovery experiment (T_{50} R) [25,39].

Initial Seedling Growth

To evaluate the effects of salinity exposure and post-salinity recovery on initial seedling growth, the cotyledon, first leaf and radicle length of 1-7 seedlings per Petri dish (n = 4 Petri dishes per treatment) were measured under a magnifying glass using a ruler [59]. These data were recorded 15 days after germination in both experiments to assess initial growth of both *S. maritima* and *S. densiflora* seedlings.

Statistical Analysis

Statistical analyses were carried out with STATISTICA 8.0 (StatSoft Inc., USA) considering significant results when $p \leq 0.05$. Deviation to the mean was calculated as Standard Error (SE). The normality of the data series was tested with Kolmogorov-Smirnov test and the homogeneity of variance using the Levene test. When data or their transformations (using $\sqrt{x}$, $1/(x + 1)$ or arcsine(x) functions) had a normal distribution and presented homeostasie, differences in germination parameters and seedling measurements between different salinities were analysed using one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test as post-hoc test. If data series did not have a normal distribution or homogeneity of variance after transformation, we evaluated response differences using a non-parametric Kruskal-Wallis H-test and a Mann-Whitney U post-hoc test.

Conclusions

Together, our results provide new information on seed germination and early seedling life stage characteristics of native Spartina maritima in comparison to responses of co-occurring invasive South American S. densiflora to increasing estuarine salinity changes driven by global warming and sea level rise. At these life stages, critical to survival and establishment, S. maritima displayed a specialist strategy by germinating primarily under salinity concentrations that support survival and optimal initial seedling growth. This strategy is in accordance with field observations with S. maritima colonizing mostly only stressful low salt marshes. In contrast, invasive S. densiflora behaved as a generalist species [21] and showed the capacity to germinate and produce seedlings under a wide range of salinity concentrations. However, it presented its optimum seedling growth at freshwater and light brackish conditions, with sub-optimum seedling growth at higher salinities. This behaviour is in agreement with S. densiflora adult individuals showing high phenotypic plasticity for many traits and opportunistically colonizing a wide range of habitats along the intertidal gradient, though often as sub-optimal phenotypes for the conditions [46]. In view of these results, invasive S. densiflora seems to be better prepared than native S. maritima to tolerate salinity changes provoked by climate change and sea level rise [16]. Conservation priorities to provide a future habitat for S. maritima and other native tidal wetland flora should consider preservation of undeveloped uplands for accommodation space above current high water levels for estuarine marsh transgression with sea level rise, and immediate implementation of invasive plant management, as successional development of new tidelands will favour aggressive alien colonizers such as S. densiflora [60]. Considering this work, wetland restoration strategies should consider seed and seedling life stage responses under changing environmental conditions to support recruitment and establishment of S. maritima.

Funding

This research was funded by Ministerio de Educación, Cultura y Deporte of Spanish Government for the FPU Grant (FPU14/06556).

Acknowledgements

We thank the management and staff of the Odiel Marshes Natural Park for its collaboration.

References

1. Ungar, I.A. Population ecology of halophyte seeds. Bot. Rev. **1987**, 53, 301–334, doi:10.1007/BF02858320.
2. Ungar, I.A. Seed germination and seed-bank ecology of halophytes. In Seed Development and Germination; Kigel, J., Galili, G., Eds.; Marcel Dekker: New York, NY, USA, **1995**; pp. 599–628.

3. Rubio-Casal, A.E.; Castillo, J.M.; Luque, C.J.; Figueroa, M.E. Nucleation and facilitation in salt pans in Mediterranean salt marshes. J. Veg. Sci. **2001**, 12, 761–770, doi:10.2307/3236863.
4. Ungar, I.A. Seed banks and seed population dynamics of halophytes. Wetl. Ecol. Manag. **2001**, 9, 499–510, doi:10.1023/A:1012236829474.
5. Song, J.; Feng, G.; Tian, C.Y.; Zhang, F.S. Strategies for adaptation of *Suaeda physophora*, *Haloxylon ammodendron* and *Haloxylon persicum* to a saline environment during seed germination stage. Ann. Bot. **2005**, 96, 399–405, doi:10.1093/aob/mci196.
6. Gul, B.; Ansari, R.; Flowers, T.J.; Khan, M.A. Germination strategies of halophyte seeds under salinity. Environ. Exp. Bot. **2013**, 92, 4–18, doi:10.1016/j.envexpbot.2012.11.006.
7. Woodell, S.R.J. Salinity and seed germination patterns in coastal plants. Vegetatio **1985**, 61, 223–229, doi:10.1007/BF00039828.
8. Muñoz-Rodríguez, A.F.; Sanjosé, I.; Márquez-García, B.; Infante-Izquierdo, M.D.; Polo-Ávila, A.; Nieva, F.J.J.; Castillo, J.M. Germination syndromes in response to salinity of Chenopodiaceae halophytes along the intertidal gradient. Aquat. Bot. **2017**, 139, 48–56, doi:10.1016/j.aquabot.2017.02.003.
9. Keiffer, C.H.; Ungar, I.A. The effect of extended exposure to hypersaline conditions on the germination of five inland halophyte species. Am. J. Bot. **1997**, 84, 104–111, doi:10.2307/2445887.
10. Khan, M.A.; Ungar, I.A. Effects of thermoperiod on recovery of seed germination of halophytes from saline conditions. Am. J. Bot. **1997**, 84, 279–283, doi:10.2307/2446089.
11. Khan, M.A.; Gulzar, S. Light, salinity, and temperature effects on the seed germination of perennial grasses. Am. J. Bot. **2003**, 90, 131–134, doi:10.3732/ajb.90.1.131.
12. Zedler, J.B.; Kercher, S. Causes and consequences of invasive plants in wetlands: Opportunities, opportunists, and outcomes. Crit. Rev. Plant Sci. **2004**, 23, 431–452, doi:10.1080/07352680490514673.
13. Weber, E.; D’Antonio, C.M. Germination and growth responses of hybridizing *Carpobrotus* species (Aizoaceae) from coastal California to soil salinity. Am. J. Bot. **1999**, 86, 1257–1263, doi:10.2307/2656773.
14. Dethier, M.N.; Hacker, S.D. Physical factors vs. biotic resistance in controlling the invasion of an estuarine marsh grass. Ecol. Appl. **2005**, 15, 1273–1283, doi:10.1890/04-0505.
15. Yuan, Z.; Shi, F. Ecological adaptation strategies in alien species: Effects of salinity, temperature and photoperiod on *Spartina alterniflora* Loisel. seed germination. Pol. J. Ecol. **2009**, 57, 677–684.
16. Stralberg, D.; Brennan, M.; Callaway, J.C.; Wood, J.K.; Schile, L.M.; Jongsomjit, D.; Kelly, M.; Parker, V.T.; Crooks, S. Evaluating tidal marsh sustainability in the face of sea-level rise: A hybrid modeling approach applied to San Francisco Bay. PLoS ONE **2011**, 6, e27388, doi:10.1371/journal.pone.0027388.
17. Pennings, S.C.; Bertness, M.D. Using latitudinal variation to examine effects of climate on coastal salt marsh pattern and process. Curr. Top. Wetl. Biogeochem. **1999**, 3, 100–111.

18. Scavia, D.; Field, J.C.; Boesch, D.F.; Buddemeier, R.W.; Burkett, V.; Cayan, D.R.; Fogarty, M.; Harwell, M.A.; Howarth, R.W.; Mason, C.; et al. Climate change impacts on US coastal and marine ecosystems. Estuaries **2002**, 25, 149–164, doi:10.1007/BF02691304.
19. Gilman, E.L.; Ellison, J.; Duke, N.C.; Field, C. Threats to mangroves from climate change and adaptation options: A review. Aquat. Bot. **2008**, 89, 237–250, doi:10.1016/j.aquabot.2007.12.009.
20. Strong, D.R.; Ayres, D.R. Ecological and evolutionary misadventures of *Spartina*. Annu. Rev. Ecol. Evol. Syst. **2013**, 44, 389–410, doi:10.1146/annurev-ecolsys-110512-135803.
21. Nieva, F.J.J.; Díaz-Espejo, A.; Castellanos, E.M.; Figueroa, M.E. Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel Marshes (SW Spain). Estuar. Coast. Shelf Sci. **2001**, 52, 515–527, doi:10.1006/ecss.2000.0750.
22. Marchant, C.J.; Goodman, P.J. *Spartina maritima* (Curtis) Fernald. J. Ecol. **1969**, 57, 287–291. doi:10.2307/2258222
23. Castellanos, E.M.; Figueroa, M.E.; Davy, A.J. Nucleation and facilitation in saltmarsh succession: Interactions between *Spartina maritima* and *Arthrocnemum perenne*. J. Ecol. **1994**, 82, 239–248, doi:10.2307/2261292.
24. Castillo, J.M.; Ayres, D.R.; Leira-Doce, P.; Bailey, J.; Blum, M.; Strong, D.R.; Luque, T.; Figueroa, E. The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Divers. Distrib. **2010**, 16, 547–558, doi:10.1111/j.1472-4642.2010.00673.x.
25. Infante-Izquierdo, M.D.; Castillo, J.M.; Nieva, F.J.J.; Rotundu, I.D.; David, F.T.; Grewell, B.J.; Muñoz-Rodríguez, A.F. Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula. Wetlands **2019**, doi:10.1007/s13157-019-01188-1.
26. Bortolus, A. The austral cordgrass *Spartina densiflora* Brong: Its taxonomy, biogeography and natural history. J. Biogeogr. **2006**, 33, 158–168, doi:10.1111/j.1365-2699.2005.01380.x.
27. Castillo, J.M.; Rubio-Casal, A.E.; Redondo, S.; Álvarez-Lopez, A.A.; Luque, T.; Luque, C.; Nieva, F.J.J.; Castellanos, E.M., Figueroa, M.E. Short-term responses to salinity of an invasive cordgrass. Biol. Invasions **2005**, 7, 29–35, doi:10.1007/s10530-004-9626-9.
28. Di Bella, C.E.; Striker, G.G.; Escaray, F.J.; Lattanzi, F.A.; Rodríguez, A.M.; Grimoldi, A.A. Saline tidal flooding effects on *Spartina densiflora* plants from different positions of the salt marsh. Diversities and similarities on growth, anatomical and physiological responses. Environ. Exp. Bot. **2014**, 102, 27–36, doi:10.1016/j.envexpbot.2014.02.009.
29. Nieva, F.J.J.; Castellanos, E.M.; Figueroa, M.E. Effects of light and salinity on seed germination in the marsh invader *Spartina densiflora* Brong., 1829 (Gramineae) from Gulf of Cádiz, Spain. Bol. R. Soc. Esp. Hist. Nat. **2001**, 96, 117–124.
30. Kittelson, P.M.; Boyd, M.J. Mechanisms of expansion for an introduced species of cordgrass, *Spartina densiflora*, in Humboldt Bay, California. Estuaries **1997**, 20, 770–778, doi:10.2307/1352250.

31. Mateos-Naranjo, E.; Redondo-Gómez, S. Interpopulation differences in salinity tolerance of the invasive cordgrass *Spartina densiflora*: Implications for invasion process. Estuar. Coast. **2016**, 39, 98–107, doi:10.1007/s12237-015-9956-0.
32. Redondo, S.; Rubio-Casal, A.E.; Castillo, J.M.; Luque, C.J.; Álvarez, A.A.; Luque, T.; Figueroa, M.E. Influences of salinity and light on germination of three *Sarcocornia* taxa with contrasted habitats. Aquat. Bot. **2004**, 78, 255–264, doi:10.1016/j.aquabot.2003.11.002.
33. Contreras-Cruzado, I.; Infante-Izquierdo, M.D.; Márquez-García, B.; Hermoso-López, V.; Polo, A.; Nieva, F.J.J.; Cartes-Barroso, J.B.; Castillo, J.M.; Muñoz-Rodríguez, A. Relationships between spatio-temporal changes in the sedimentary environment and halophytes zonation in salt marshes. Geoderma **2017**, 305, 173–187, doi:10.1016/j.geoderma.2017.05.037.
34. Wijte, A.H.; Gallagher, J.L. Effect of oxygen availability and salinity on early life history stages of salt marsh plants. I. Different germination strategies of *Spartina alterniflora* and *Phragmites australis* (Poaceae). Am. J. Bot. **1996**, 83, 1337–1342, doi:10.1002/j.1537-2197.1996.tb13919.x.
35. Li, R.; Shi, F.; Fukuda, K. Interactive effects of salt and alkali stresses on seed germination, germination recovery, and seedling growth of a halophyte *Spartina alterniflora* (Poaceae). S. Afr. J. Bot. **2010**, 76, 380–387, doi:10.1016/j.sajb.2010.01.004.
36. Cordazzo, C.V. Effects of salinity on seed germination, seedling growth and survival of *Spartina ciliata* Brong. Acta Bot. Bras. **1999**, 13, 317–322, doi:10.1590/S0102-33061999000300012.
37. Van Zandt, P.A.; Mopper, S. The effects of maternal salinity and seed environment on germination and growth in *Iris hexagona*. Evol. Ecol. Res. **2004**, 6, 813–832.
38. Zia, S.; Khan, M.A. Effect of light, salinity, and temperature on seed germination of *Limonium stocksii*. Can. J. Bot. **2004**, 82, 151–157, doi:10.1139/b03-118.
39. Muñoz-Rodríguez, A.F.; Rodríguez-Rubio, P.; Nieva, F.J.J.; Fernández-Illescas, F.; Sánchez-Gullón, E.; Soto, J.M.; Hermoso-López, V.; Márquez-García, B. The importance of bracteoles in ensuring *Atriplex halimus* germination under optimal conditions. Fresen. Environ. Bull. **2012**, 21, 3521–3526.
40. Pujol, J.A.; Calvo, J.F.; Ramírez-Diaz, L. Recovery of germination from different osmotic conditions by four halophytes from southeastern Spain. Ann. Bot. **2000**, 85, 279–286, doi:10.1006/anbo.1999.1028.
41. Greipsson, S.; Davy, A.J. Germination of *Leymus arenarius* and its significance for land reclamation in Iceland. Ann. Bot. **1994**, 73, 393–401, doi:10.1006/anbo.1994.1049.
42. Wulff, R.D. Environmental maternal effects on seed quality and germination. In Seed Development and Germination; Kigel, J., Galili, G., Eds.; Marcel Dekker: New York, NY, USA, **1995**, pp. 491–505.
43. Donohue, K.; Schmitt, J. Maternal environmental effects in plants: Adaptive plasticity? In Maternal Effects as Adaptations; Mousseau, T.A., Fox, C.W., Eds.; Oxford University Press: New York, NY, USA, **1998**, pp. 137–158.

44. Galloway, L.F. Maternal effects provide phenotypic adaptation to local environmental conditions. New Phytol. **2005**, 166, 93–100, doi:10.1111/j.1469-8137.2004.01314.x.
45. Silvestri, S.; Defina, A.; Marani, M. Tidal regime, salinity and salt marsh plant zonation. Estuar. Coast. Shelf Sci. **2005**, 62, 119–130, doi:10.1016/j.ecss.2004.08.010.
46. Castillo, J.M.; Gallego-Tévar, B.; Figueroa, E.; Grewell, B.J.; Vallet, D.; Rousseau, H.; Keller, J.; Lima, O.; Dréano, S.; Salmon, A.; et al. Low genetic diversity contrasts with high phenotypic variability in heptaploid *Spartina densiflora* populations invading the Pacific coast of North America. Ecol. Evol. **2018**, 8, 4992–5007, doi:10.1002/ece3.4063.
47. Gallego-Tévar, B.; Grewell, B.J.; Rousseau, H.; Keller, J.; Ainouche, A.; Lima, O.; Dréano, S.; Salmon, A.; Figueroa, E.; Aïnouche, M.; et al. Genetic structure of Spartina hybrids between native *Spartina maritima* and invasive *Spartina densiflora* in Southwest Europe. Perspect. Plant Ecol. Evol. Syst. **2019**, 37, 26–38, doi:10.1016/j.ppees.2019.02.001.
48. Xiao, D.; Zhang, C.; Zhang, L.; Zhu, Z.; Tian, K.; Gao, W. Seed dispersal capacity and post-dispersal fate of the invasive *Spartina alterniflora* in saltmarshes of the Yangtze Estuary. Estuar. Coast. Shelf Sci. **2016**, 169, 158–163, doi:10.1016/j.ecss.2015.11.032.
49. Ungar, I.A. Effects of the parental environment on the temperature requirements and salinity tolerance of *Spergularia marina* seeds. Bot. Gaz. **1988**, 149, 432–436, doi:10.1086/337736.
50. Wang, L.; Baskin, J.M.; Baskin, C.C.; Cornelissen, J.H.C.; Dong, M.; Huang, Z. Seed dimorphism, nutrients and salinity differentially affect seed traits of the desert halophyte *Suaeda aralocaspica* via multiple maternal effects. Bmc Plant Biol. **2012**, 12, 170. doi:10.1186/1471-2229-12-170.
51. Fenner, M. The effects of the parent environment on seed germinability. Seed Sci. Res. **1991**, 1, 75–84, doi:10.1017/S0960258500000696.
52. Gutterman, Y. Maternal effects on seeds during development. In Seeds: The Ecology of Regeneration in Plant Communities, 2nd ed.; Fenner, M., Ed.; CABI Publishing: Wallingford, UK, **2000**; pp. 59–84.
53. Baskin, C.C.; Baskin, J.M. Seeds. Ecology, Biogeography, and Evolution of Dormancy and Germination; Academic Press: San Diego, CA, USA, **1998**; p. 666.
54. Luzuriaga, A.L.; Escudero, A.; Pérez-García, F. Environmental maternal effects on seed morphology and germination in *Sinapis arvensis* (Cruciferae). Weed Res. **2006**, 46, 163–174, doi:10.1111/j.1365-3180.2006.00496.x.
55. Fernández-Illescas, F.; Nieva, F.J.J.; Silva, I.; Tormo, R.; Muñoz, A.F. Pollen production of Chenopodiaceae species at habitat and landscape scale in Mediterranean salt marshes: An ecological and phenological study. Rev. Palaeobot. Palynol. **2010**, 161, 127–136, doi:10.1016/j.revpalbo.2010.03.006.
56. Long, S.P.; Mason, C.F. Saltmarsh Ecology; Blackie: Glasgow and London, UK, **1983**; p. 160.
57. Barba-Brioso, C.; Fernández-Caliani, J.C.; Miras, A.; Cornejo, J.; Galán, E. Multi-source water pollution in a highly anthropized wetland system

associated with the estuary of Huelva (SW Spain). Mar. Pollut. Bull. **2010**, 60, 1259–1269, doi:10.1016/j.marpolbul.2010.03.018.

58. Mackay, D.B. The measurement of viability. In Viability of Seeds; Roberts, E.H., Ed.; Chapman and Hall: London, UK, **1972**; pp. 172–208.
59. Márquez-García, B.; Márquez, C.; Sanjosé, I.; Nieva, F.J.J.; Rodríguez-Rubio, P.; Muñoz-Rodríguez, A.F. The effects of heavy metals on germination and seedling characteristics in two halophyte species in Mediterranean marshes. Mar. Pollut. Bull. **2013**, 70, 119–124, doi:10.1016/j.marpolbul.2013.02.019.
60. Grewell, B.J.; Baye, P.R.; Fiedler, P.L. Shifting mosaics: Vegetation of Suisun Marsh. In Suisun Marsh: Ecological History and Possible Futures; Moyle, P.B., Manfree, A.D., Fiedler, P.L., Eds.; University of California Press: Berkeley, CA, USA, **2014**; pp. 65–102.

8

Effects of metals on germination and early seedling growth in native Spartina maritima and invasive S. densiflora

Effects of metals on germination and early seedling growth in native *Spartina maritima* and invasive *S. densiflora*

Abstract

Seed germination and seedling establishment are the most sensitive stages to abiotic stress in the plant cycle. Metal exposition of seeds can affect germination and seedling growth. We analyzed the effects of Cu, Zn and Ni on seed germination and early seedling growth of native *Spartina maritima* and invasive *S. densiflora* from tidal marshes in the Southwest Iberian Peninsula. In addition, we analyzed metals effects on *S. densiflora* seeds coming from polluted and non-polluted estuaries. Germination percentage of both *Spartina* species was not affected by any metal at any tested concentration (from 0 to 2000 μM). However, *S. maritima* seedlings were more tolerant to metals than *S. densiflora*, since the increase in Cu, Zn and Ni had negative effects on the early seedling growth of *S. densiflora*, mainly on the radicle length, while *S. maritima* seedlings were not affected. Comparing our results to metal concentrations in the field, we expect invasive *S. densiflora* seedling development may be impacted in most metal-polluted areas in Odiel and Tinto Estuaries. *S. densiflora* seedlings coming from seeds from Tinto Estuary showed higher tolerance to metals than those from Odiel and Piedras Estuaries.

Keywords:

Abiotic stress, Cordgrass, Metal tolerance, Phytoremediation, Pollution

Introduction

Seed germination and seedling establishment are crucial in the life cycle of plants (Ungar 2001). They are the most sensitive stages to abiotic stresses such as metal pollution because defence mechanisms have not fully developed (Liu et al. 2005; Ahsan et al. 2007). However some metals such as Cu, Mn, Co, Cr, Ni and Zn are essential elements for plant metabolism by participating in redox reactions and as integral part of several enzymes, they can become toxic when they are bioavailable in high concentrations (Munzuroglu & Geckil, 2002, Nagajyoti et al. 2010). Thus, metals are among major environmental pollutants and their toxicity has a high impact on plants and, consequently, on the ecosystem (Nagajyoti et al. 2010). For example, metal exposure of growing plants may reduce or inhibit seed germination (Kranner & Colville 2011, Sethy & Ghosh 2013). Germination can be affected by metals in two ways: direct toxicity and/or water uptake inhibition (Kranner & Colville 2011). In this context, one of the most used methods to test metal toxicity in plants is the seed germination test (Munzuroglu & Geckil 2002).

Halophytes have mechanisms of adaptation that allow them to survive to environmental stresses, such as high salt concentrations and exposure to heavy metals (Van Oosten & Maggio 2015). Thus, halophytes offer a great potential for phytoremediation and phytostabilization of heavy metal polluted soils since they have the ability to accumulate heavy metals in its tissues (Manousaki & Kalogerakis 2011, Van Oosten & Maggio 2015). Some halophytes species had been analyzed for their tolerance to metals during adult growth (Mateos-Naranjo et al. 2008a,b, Manousaki & Kalogerakis 2011, Redondo-Gómez et al. 2011, Van Oosten & Maggio 2015). Thus, many adult halophytes have a great capacity to accumulate metals in its roots, avoiding metal translocation to photosynthetic tissues (Redondo-Gómez 2013). But the effects of metals on their germination and seedling growth have been little studied (Williams et al. 1994, Márquez-García et al. 2013). *Spartina* species (cordgrasses) mostly inhabit salt marshes around the world, and many of them have successfully invaded many areas beyond their native ranges (Strong & Ayres 2013, Ainouche & Gray 2016). For this reason, cordgrasses provide a good model halophyte group to study the responses of native and invasive species to altered environmental conditions such as sediments polluted with metals. Adult *Spartina* plants can tolerate and bioaccumulate high levels of metals in its tissues, and therefore, they can be adequate biotools in phytoremediation projects (Redondo-Gómez 2013). In salt marshes along the Gulf of Cádiz (Southwest Iberian Peninsula), native *Spartina maritima* (Curtis) Fernald, the only native cordgrass in European marshes (Marchant & Goodman 1969), co-occurs with invasive *Spartina densiflora* Brongn., introduced from South America centuries ago (Nieva et al. 2001). Seeds of some *Spartina* species, such as *S. densiflora*, are able to germinate in highly metal-polluted sediments (Curado et al. 2010, Mateos-Naranjo et al. 2011). In *Spartina alterniflora* Loisel., Hg, Cd, Pb and Zn stimulated early germination at low salinities, whereas metals had negative effects on germination at high salinities (Mrozek 1980, Mrozek & Funicelli 1982). In *S. densiflora*, Curado et al. (2010)

analyzed the germination and establishment in acidic and metal-polluted sediments collected along the Tinto River (Southwest Iberian Peninsula), observing that final germination decreased with pH and higher Al and Cr concentrations, and that germination was the rapidest and growth decreased in the most polluted sediments. Mateos-Naranjo et al. (2011) analyzed the differences in metal tolerance between *S. densiflora* populations from polluted and unpolluted estuaries founding that seeds of all populations were able to germinate even in the most contaminated soils and that seedling growth and survival decreased in high metal concentrations. To our knowledge, there is no information in the literature about the effects of metals on seed germination and early seedling growth of native *S. maritima*, since until recently seed production in this species has been described as very low or inexistent (Marchant & Goodman 1969, Castellanos et al. 1994, Castillo et al. 2010), but in a recent study we recorded that *S. maritima* produced a moderate number of caryopses with high germination rates in Southwest Iberian Peninsula (Infante-Izquierdo et al. 2019a).

Our main goal was to analyze and compare the effects of metals on seed germination and early seedling growth of native *S. maritima* and invasive *S. densiflora* from tidal marshes in the Southwest Iberian Peninsula. In contrast to previous studies that germinates seed directly on polluted sediments coming from the field, we analyzed the effects of each metal separately and in different concentrations. In addition, in *S. densiflora* we analyzed metals effects on seeds coming from polluted and non-polluted estuaries. We hypothesized that native *S. maritima* would show greater tolerance to metals than *S. densiflora* at least in Odiel and Tinto Estuaries, since these estuaries have been affected by natural metal pollution for at least 4500 years (Davis et al. 2000, Leblanc et al. 2000, Pérez-López et al. 2011). This old pollution has even led to speciation processes, such as the case of the endemic heather *Erica andevalensis* Cabezudo & J. Rivera (Márquez-García et al. 2009). In contrast to *S. maritima*, *S. densiflora* is an invasive species native from South America that was introduced around the sixteenth century to the Southwest Iberian Peninsula (Nieva et al. 2001, Strong & Ayres 2013), and therefore has had less time to adapt to the polluted environment of Odiel and Tinto Estuaries.

Materials and methods

Study sites and plant material

The present work was carried out along the coast of the Gulf of Cádiz in Southwest Iberian Peninsula. Fruiting inflorescences from three populations of invasive *S. densiflora*, from at least ten different tussocks per population, colonizing middle marshes and located in different estuaries with different degree of pollution by heavy metals (Odiel Estuary (37.209102 N, -6.952582 W), Tinto Estuary (37.213830 N, -6.925824 W) and Piedras Estuary (37.213804 N, -7.174761 W)) were collected in November 2016. Fruiting inflorescences were collected in August 2017, from at least ten different tussocks, in one population of native *Spartina maritima* colonizing a low salt marsh in the metal-polluted Odiel Estuary (37.174343 N, -6.931640 W). Once in the laboratory, spikelets containing caryopses were randomly selected and stored in cold (+5 ºC) conditions until use.

The estuaries of Odiel and Tinto rivers are among the most polluted areas by metals in the world (Nelson and Lamothe 1993, Davis et al. 2000, Sainz et al. 2004). This strong metal contamination is the results of mining pollution by acid mine drainage in the Iberian Pyrite Belt that is crossed by the drainage basins of Odiel and Tinto Rivers. Metal pollution is also coming from industrial discharges and urban effluents (Fernández-Caliani et al. 1997, Ruiz 2001, Borrego et al. 2002, Galán et al. 2003, Sainz et al. 2004). Many studies have analyzed metal loads in sediments in Odiel and Tinto Marshes (Fernández-Caliani et al. 1997, Elbaz-Poulichet et al. 1999, 2001, Borrego et al. 2002, Achterberg et al. 2003; Braungardt et al. 2003, Galán et al. 2003; González-Pérez et al. 2008; Mateos-Naranjo et al. 2011). According to these studies, the maximum concentrations of metals registered in sediment solution were: >1000 μM Fe and Al; >500 μM Zn and Cu; 50-500 μM Mn; < 50 μM As, Co, Pb, Ni, Cd and Hg. The accumulation, mobility and bioavailability of metals depend on physical and chemical properties of soils, as texture, pH, salinity, redox potential, organic matter content, etc. (Williams et al. 1994, Gambrell et al. 1994). In this sense, some metals dissolved in the Estuaries of Odiel and Tinto precipitate as salinity increases, being trapped in the sediments, while others remain in the solution and, therefore, are more mobile, such as Cu, Mn and Zn (Elbaz-Poulichet et al. 2001; Borrego et al. 2002, Achterberg et al. 2003; Braungardt et al. 2003). In contrast, neighboring Piedras Estuary is very low polluted, with sediment metals concentrations < 400 ppm Ni, Zn, Cu, Cr, Pb, As, Cd and Co (Ruiz 2001, Redondo-Gómez et al. 2009, Mateos-Naranjo et al. 2011).

Germination experiments

Germination experiments were carried out in January 2017 for *S. densiflora* and in October 2017 for *S. maritima*. To prevent fungal contamination, spikelets were surface-sterilized in 5% (v/v) sodium hypochlorite for 10 min and then rinsed with distilled water (Muñoz-Rodríguez et al. 2012; Infante-Izquierdo et al. 2019a). For each species and population, four replicates of 25 spikelets were sown for each metal and concentration on Petri dishes (9 cm diameter) with two layers of autoclaved filter paper adding different treatments solutions: distilled water (control), solution containing 100, 250, 500, 1000 and 2000 μM Cu (as $CuSO_45H_2O$), Zn (as $ZnSO_47H_2O$) or Ni (as $NiSO_46H_2O$). Metals and concentrations were chosen based on the metal composition and their levels registered in the waters and soils of the source estuaries (Fernández-Caliani et al. 1997, Elbaz-Poulichet et al. 1999, 2001, Borrego et al. 2002, Achterberg et al. 2003; Braungardt et al. 2003, Galán et al. 2003; González-Pérez et al. 2008; Mateos-Naranjo et al. 2011), and because of the impact of these contaminants in plants and environment (Kranner & Colville 2011, Nagajyoti et al. 2010). The of the metals tested in this study was dissolved in distilled water. We used sulphates because it is the most abundant chemical form in the source estuaries (Barba-Brioso et al. 2010), and because the toxicity of chloride ion might have inhibitory effects on seed germination (León et al. 2005). Petri dishes were sealed with adhesive tape (Parafilm™) to avoid desiccation. The experiments were done under controlled-environmental conditions at temperatures between +20-25 °C and a 12h/12h photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 µmol m^{-2} s^{-1}. Spikelets were exposed to treatments for 2 months, and germination was recorded every 3 or 4 days. A spikelet was considered germinated when the coleoptile emerged. Final germination percentage and days necessary to reach 50% of the final germination (T_{50}) were calculated for each dish.

Early seedling growth

To study the effects of metals on initial seedling growth, we measured cotyledon, first leaf and radicle length for 1-5 seedlings per Petri dish under a magnified glass using a ruler 15 days after germination (n = 4 Petri dishes per treatment).

Statistical analysis

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., USA). Deviation to the mean was calculated as Standard Error (SE). Normality and homogeneity of variance of the data series were tested using Kolmogorov-Smirnov test and Levene test, respectively. When data or their transformations (using $\sqrt{x}$, 1□(x+1), arcsine(x) and ln (x) functions) showed normal distribution and homogeneity of variance, one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test as post-hoc test were used to compare germination and seedling parameters among treatments. Otherwise, we used a non-parametric Kruskal-Wallis H-test and a Mann-Whitney U-test as post-hoc test. In all statistical analysis, we considered significant results when $p \leq 0.05$.

Results

Effects of metals on seed germination

In *S. maritima*, none tested metal affected final germination percentage, which was c. 70% (Table 1). Germination was significantly accelerated (lower T_{50}) in all Cu concentrations c. 3-5 days respect to the control treatment. Germination speed in all Zn and Ni concentrations (c. 27 days) did not show significant differences with the control (Table 1).

In *S. densiflora*, there were no significant differences in germination percentage and germination speed among spikelets sourced from Odiel, Tinto and Piedras estuaries. Among treatments, germination percentage was significantly higher at 250 µM Ni than at 1000 and 2000 µM Ni for spikelets from Tinto Estuary, and at 500 µM Zn than at 2000 µM Zn for spikelets from Piedras Estuary (Table 2).

Effects of metals on early seedling growth

In *S. maritima* seedlings, length of cotyledon and first leaf were not significantly affected by any metal concentration (One-way ANOVA or Kruskal-Wallis test, $p > 0.05$) (Fig. 1a, 2a, 3a). *S. maritima* radicle length showed significant differences among different Cu concentrations, showing its maximum length at 100 µM Cu (Kruskal-Wallis test: $H_{5,115} = 12.56$, $p < 0.05$; Man-Whitney U-test, $p < 0.05$) (Fig. 1a).

Table 1 Germination percentage (G) and days necessary to reach 50% of the final germination percentage (T_{50}) for native *S. maritima* spikelets sown in different concentrations of Cu, Zn and Ni. Data show are mean ± SE (n = 4). Different letters indicate significant differences among concentrations within each heavy metal (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

Treatment	Concentration (µM)	G (%)	T_{50} (days)
Control	0	71.0 ± 7.2a	28.5 ± 0.0a
$CuSO_45H_2O$	100	65.0 ± 2.5a	25.1 ± 1.1b
	250	75.0 ± 4.4a	24.0 ± 0.0b
	500	64.0 ± 1.6a	23.2 ± 0.7b
	1000	64.0 ± 6.7a	24.0 ± 0.0b
	2000	65.0 ± 7.7a	25.1 ± 1.1b
One-way ANOVA or Kruskal-Wallis test		F = 0.68, df = 5, $p > 0.05$	$H_{5,24} = 14.77$, $p < 0.05$
Control	0	71.0 ± 7.2a	28.5 ± 0.0a
$ZnSO_47H_2O$	100	66.0 ± 3.8a	26.6 ± 0.9a
	250	69.0 ± 3.4a	25.9 ± 1.1a
	500	65.0 ± 1.9a	25.1 ± 1.1a
	1000	71.0 ± 6.6a	27.7 ± 1.3a
	2000	74.0 ± 3.5a	30.2 ± 1.7a
One-way ANOVA		F = 0.50, df = 5, $p > 0.05$	F = 2.58, df = 5, $p > 0.05$
Control	0	71.0 ± 7.2a	28.5 ± 0.0a
$NiSO_46H_2O$	100	71.0 ± 3.4a	25.1 ± 1.1a
	250	72.0 ± 6.3a	27.4 ± 3.2a
	500	72.0 ± 3.6a	27.7 ± 1.3a
	1000	81.0 ± 3.4a	29.2 ± 0.4a
	2000	75.0 ± 5.3a	29.4 ± 0.9a
One-way ANOVA or Kruskal-Wallis test		F = 0.58, df = 5, $p > 0.05$	$H_{5,24} = 6.20$, $p > 0.05$

In *S. densiflora*, cotyledon length was not diminished in any concentration of the three tested metals for any populations (Fig. 1,2,3b,c,d). Radicle length was significantly affected by all metals for spikelets sourced from all estuaries. Thus, a significant reduction on radicle length between 55-68%

was recorded with increasing Cu concentration over 100 μM Cu for Odiel and Piedras populations and over 250 μM Cu for Tinto population (Kruskal-Wallis test, $p < 0.0001$) (Fig. 1b,c,d). Moreover, radicle length decreased between 56-69% over 500 μM Zn (One-way ANOVA test, $p < 0.0001$) (Fig. 2b,c,d), and between 69-83% over 100 μM Ni for the three studied populations (One-way ANOVA test or Kruskal-Wallis test, $p < 0.0001$) (Fig. 3b,c,d). First leaf length was significantly shorter than the control just for seedlings from Odiel Estuary exposed to 100 and 2000 μM Cu (One-way ANOVA test: $F = 3.13$, $df = 5$, $p < 0.05$) (Fig. 1b). In addition, first leaf length was reduced at concentrations higher than 500 μM Ni for seedlings from Odiel and Piedras (One-way ANOVA test or Kruskal-Wallis test, $p < 0.0001$) (Fig. 3b,d) and higher than 1000 μM Ni for seedlings coming from Tinto Estuary (Kruskal-Wallis test: $H_{5,118} = 35.91$, $p < 0.0001$) (Fig. 3c). Exposition to Zn did not affect significantly first leaf size for any population (One-way ANOVA test or Kruskal-Wallis test, $p > 0.05$) (Fig. 2b,c,d).

Table 2 Germination (G) and days necessary to reach 50% of the final germination percentage (T_{50}) for invasive *S. densiflora* spikelets from three different estuaries (Odiel, Tinto and Piedras) sown in different concentrations of Cu, Zn and Ni. Data show are mean ± SE (n = 4). Different letters indicate significant differences among concentrations within each metal and each estuary (Tukey's HSD test, $p < 0.05$).

Treatment	Concentration (μM)	Odiel Estuary		Tinto Estuary		Piedras Estuary	
		G (%)	**T_{50} (days)**	**G (%)**	**T_{50} (days)**	**G (%)**	**T_{50} (days)**
Control	0	51.0 ± 5.3a	27.6 ± 0.9a	58.0 ± 5.3a	28.7 ± 3.4a	60.0 ± 2.3a	24.0 ± 1.7a
$CuSO_45H_2O$	100	43.0 ± 5.7a	31.4 ± 2.8a	60.0 ± 5.9a	27.2 ± 0.8a	69.0 ± 7.0a	24.0 ± 2.3a
	250	48.0 ± 5.9a	30.2 ± 2.3a	58.0 ± 6.2a	26.1 ± 1.8a	63.0 ± 5.0a	24.4 ± 2.0a
	500	44.0 ± 2.8a	28.2 ± 4.0a	60.0 ± 4.3a	27.4 ± 3.0a	64.0 ± 6.7a	25.5 ± 0.5a
	1000	41.0 ± 7.7a	29.1 ± 3.4a	60.0 ± 2.8a	24.0 ± 0.6a	67.0 ± 5.3a	21.1 ± 1.5a
	2000	37.0 ± 6.2a	26.4 ± 0.8a	64.0 ± 4.3a	25.2 ± 1.8a	56.0 ± 7.8a	22.7 ± 3.0a
One-way ANOVA		$F = 0.74$, $df = 5$, $p > 0.05$	$F = 0.52$, $df = 5$, $p > 0.05$	$F = 0.20$, $df = 5$, $p > 0.05$	$F = 0.60$, $df = 5$, $p > 0.05$	$F = 0.62$, $df = 5$, $p > 0.05$	$F = 0.57$, $df = 5$, $p > 0.05$
Control	0	51.0 ± 5.3a	27.6 ± 0.9a	58.0 ± 5.3a	28.7 ± 3.4a	60.0 ± 2.3ab	24.0 ± 1.7a
$ZnSO_47H_2O$	100	43.0 ± 1.9a	27.7 ± 3.0a	62.0 ± 4.8a	25.9 ± 0.9a	63.0 ± 4.7ab	21.1 ± 1.3a
	250	43.0 ± 5.7a	30.9 ± 4.0a	56.0 ± 3.3a	28.2 ± 2.0a	60.0 ± 4.9ab	23.0 ± 1.1a
	500	38.0 ± 3.5a	27.0 ± 1.9a	64.0 ± 2.3a	29.2 ± 2.1a	77.0 ± 2.5a	22.1 ± 1.8a
	1000	30.0 ± 6.0a	32.1 ± 1.7a	62.0 ± 1.1a	30.0 ± 2.1a	63.0 ± 3.0ab	24.6 ± 2.5a
	2000	39.0 ± 3.4a	24.5 ± 0.9a	53.0 ± 3.8a	27.1 ± 1.5a	58.0 ± 4.8b	22.6 ± 1.8a
One-way ANOVA		$F = 2.33$, $df = 5$, $p > 0.05$	$F = 1.37$, $df = 5$, $p > 0.05$	$F = 1.29$, $df = 5$, $p > 0.05$	$F = 0.48$, $df = 5$, $p > 0.05$	$F = 3.18$, $df = 5$, $p < 0.05$	$F = 0.52$, $df = 5$, $p > 0.05$
Control	0	51.0 ± 5.3a	27.6 ± 0.9a	58.0 ± 5.3ab	28.7 ± 3.4a	60.0 ± 2.3a	24.0 ± 1.7a
$NiSO_46H_2O$	100	46.0 ± 4.2a	28.4 ± 1.3a	62.0 ± 2.0ab	30.0 ± 0.9a	64.0 ± 3.6a	21.5 ± 1.3a
	250	41.0 ± 8.4a	27.9 ± 3.6a	72.0 ± 4.0a	28.2 ± 1.9a	63.0 ± 4.4a	25.1 ± 2.3a
	500	51.0 ± 7.5a	27.4 ± 0.4a	54.0 ± 2.0ab	27.5 ± 1.6a	58.0 ± 5.3a	24.0 ± 1.3a
	1000	51.0 ± 11.9a	30.4 ± 0.7a	52.0 ± 4.0b	28.7 ± 1.6a	62.0 ± 3.5a	24.5 ± 1.3a
	2000	34.0 ± 2.6a	32.0 ± 2.4a	46.0 ± 6.2b	28.4 ± 1.3a	50.0 ± 2.6a	24.1 ± 2.5a
One-way ANOVA		$F = 0.91$, $df = 5$, $p > 0.05$	$F = 1.02$, $df = 5$, $p > 0.05$	$F = 4.56$, $df = 5$, $p < 0.01$	$F = 0.18$, $df = 5$, $p > 0.05$	$F = 1.86$, $df = 5$, $p > 0.05$	$F = 0.48$, $df = 5$, $p > 0.05$

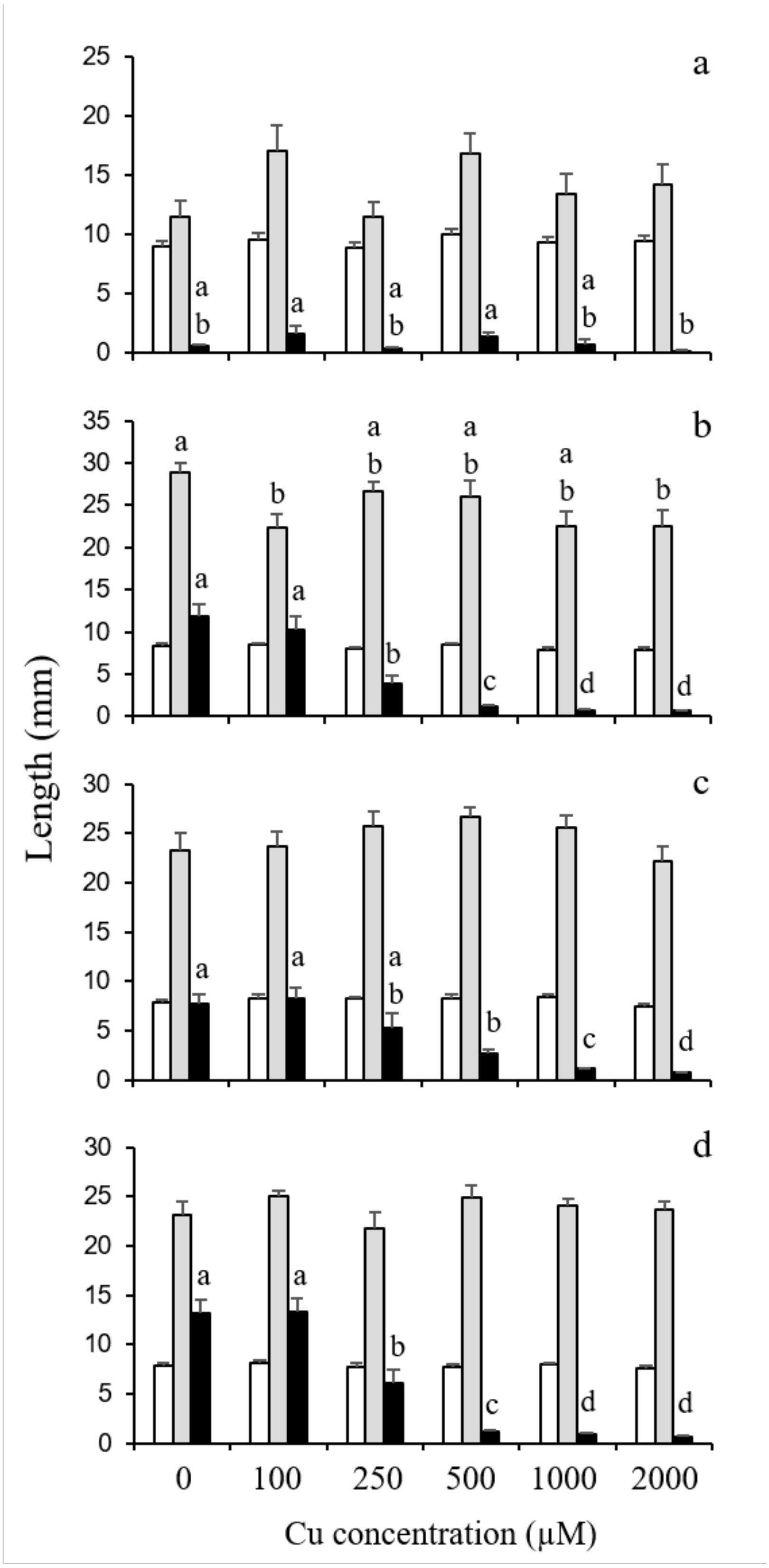


Figure 1. Cotyledon length (white bars), first leaf length (grey bars) and radicle length (black bars) for native *Spartina maritima* seedlings from Odiel Estuary (a), and invasive *S. densiflora* seedlings from Odiel (b), Tinto (c) and Piedras (d) Estuaries exposed to different concentrations of Copper. Data show mean ± SE (n = 16-20). Different letters indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

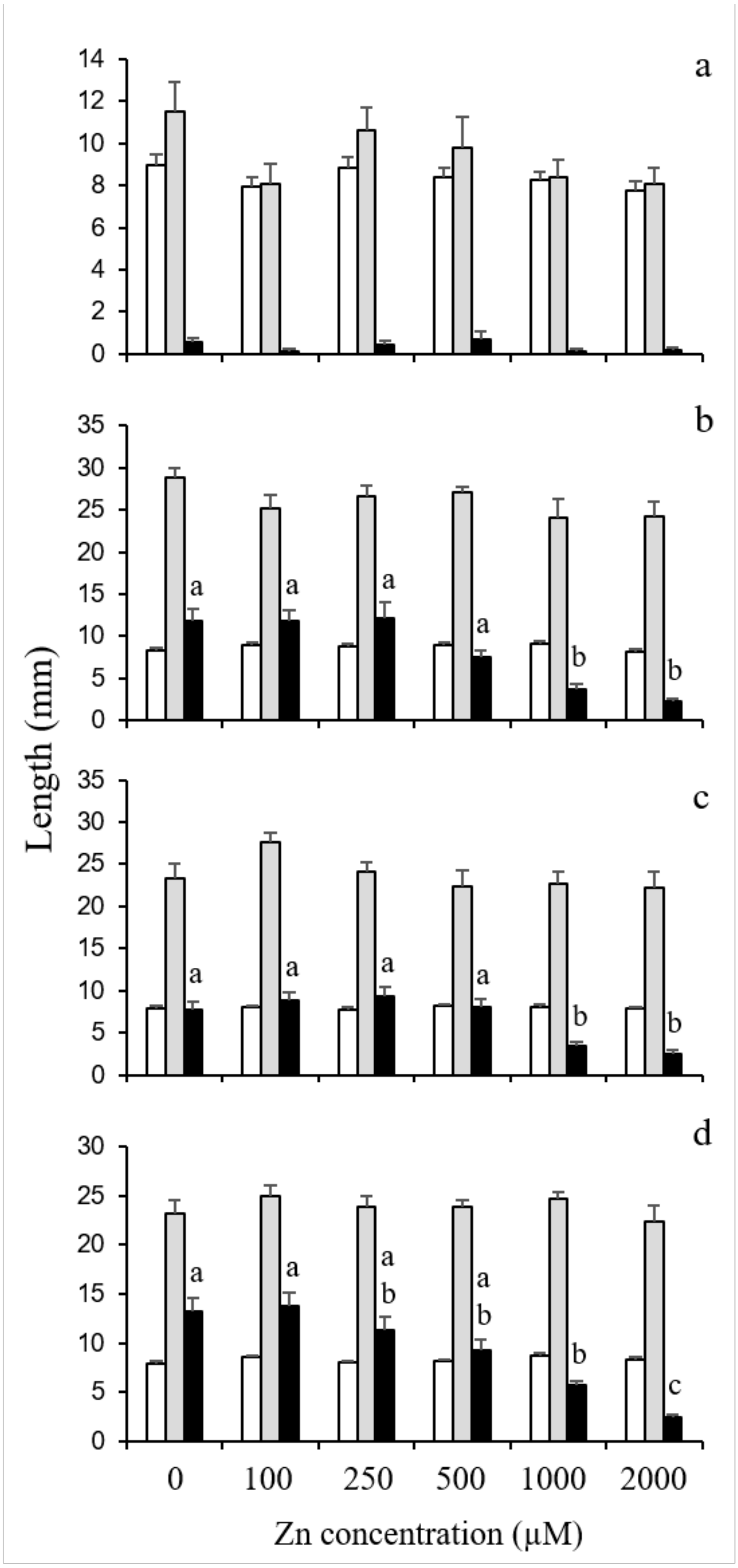


Figure 2. Cotyledon length (white bars), first leaf length (grey bars) and radicle length (black bars) of native *Spartina maritima* from Odiel Estuary (a) and invasive *S. densiflora* seedlings from Odiel (b), Tinto (c) and Piedras (d) Estuaries exposed to different concentrations of Zinc. Data show mean ± SE (n = 16-20). Different letters indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

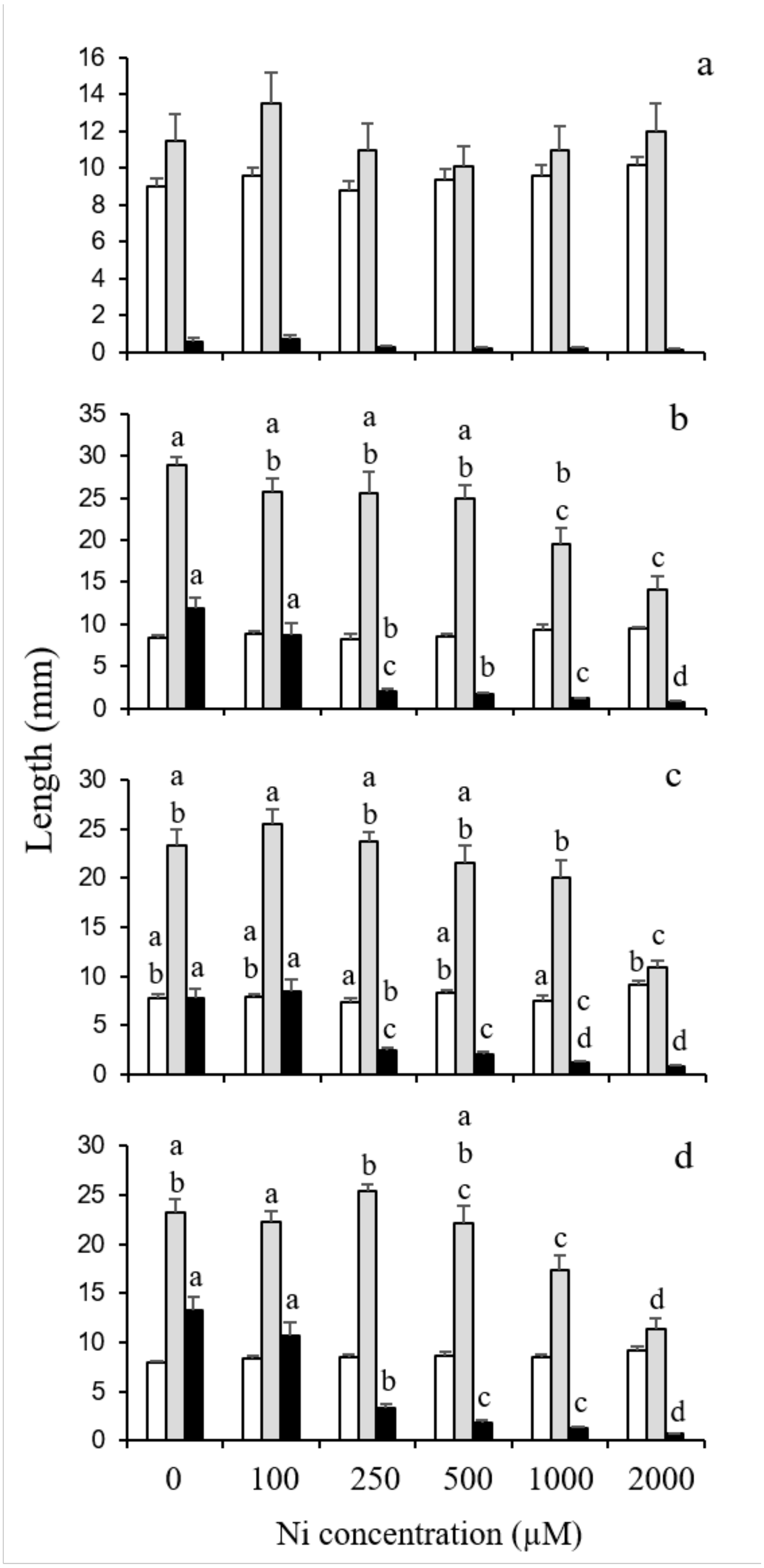


Figure 3. Cotyledon length (white bars), first leaf length (grey bars) and radicle length (black bars) of native *Spartina maritima* from Odiel Estuary (a) and invasive *S. densiflora* seedlings from Odiel (b), Tinto (c) and Piedras (d) Estuaries exposed to different concentrations of Nickel. Data show mean ± SE (n = 10-20). Different letters indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

Discussion

As we hypothesized, native *Spartina maritima* showed greater tolerance to metals than invasive *S. densiflora* in relation to early seedling growth, since neither *S. maritima* nor *S. densiflora* seed germination were affected by any of the tested metals. As in other studies, seedling phase was more sensible to metals exposure than seed germination (Li et al. 2005, Ahsan et al. 2007, Curado et al. 2010).

Although seed coat may act as a barrier to metal uptake (Munzuroglu & Geckil, 2002, Li et al. 2005, Kranner & Colville 2011), seed germination and seedling growth can be diminished by high metal concentrations (Williams et al. 1994, Kranner & Colville 2011). In our study, both *Spartina* seeds behaved as metal-tolerant, since their germination percentage was not affected even at high metal concentrations (Kranner & Colville 2011). Similarly to our results, high concentrations of Cu and Zn (up to 2000 *µ*M) did not affect seed germination in the halophytes *Salicornia ramosissima* J. Woods and in *Atriplex halimus* L. (Márquez-García et al. 2013). Curado et al. (2010) found that *S. densiflora* seeds germinated in high metal-polluted sediments even in acidic conditions (pH ca. 2), although final germination was reduced at the highest concentrations in fresh water conditions along the Tinto River. Mateos-Naranjo et al. (2011) found that *S. densiflora* seeds showed high germination percentages even in the most contaminated soils in the Odiel Estuary. As these studies and our own study show, halophytes germination is usually highly tolerant to metals. Thus, many of the mechanisms that allow halophytes to deal with high salinity, including synthesis of organic solutes and efficient antioxidative systems, may confer tolerance to other abiotic stresses such as high metals concentrations (Manousaki & Kalogerakis 2011, Van Oosten & Maggio 2015). Even so, some halophytes germination is sensitive to metal pollution. For example, Márquez-García et al. (2013) found that Ni inhibited *Salicornia ramosissima* germination even at low concentrations (10 *µ*M Ni), and Sharma et al. (2011) observed *Salicornia brachiata* Miq. germination was reduced up from 50 µM Ni.

However final germination percentage did not change at increasing metal concentration, *S. maritima* germination was significantly accelerated in the presence of Cu. In this sense, germination acceleration occurred in *S. densiflora* seeds at metal-polluted and acidic conditions (Curado et al. 2010). In some species, Cu-enrichment during seed development accelerates germination , which could be attributed to an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plants exposed to metals, causing a slightly enhanced level of oxidative stress that stimulates germination (Kranner & Colville 2011), but an excess of Cu may also causes osmotic stress, inhibiting water uptake by seeds and their germination (Ahsan et al. 2007, Kranner & Colville 2011). For example, in *Oryza sativa* L., a glycophyte from the Poaceae family, Cu had an adverse effect on seed germination that was totally inhibited at 1500 *µ*M Cu (Ahsan et al. 2007).

In our study, *S. maritima* seedlings development was not affected by any of the tested metals. *S. maritima* seedlings showed very small radicles, even smaller than those of seedlings obtained from spikelets previously exposed to salt conditions (Infante-Izquierdo et al. 2019b). This reduced radicle development may imply a low capacity for metals uptake, reducing their toxic effects. In contrast, *S. densiflora* seedlings produce longer radicles and their development was negatively affected by metals, being radicle the most impacted organ. In this sense, most *Spartina* species reduce metal translocation to photosynthetic tissues by accumulating them in the belowground biomass (Redondo-Gómez 2013). Curado et al. (2010) found that aerial and subterranean growth rate of *S. densiflora* seedlings decreased in more metal-polluted and acidic sediments from Tinto Estuary. The higher metal tolerance of native *S. maritima* seedlings in comparison to invasive *S. densiflora* may be explained by their contrasted habitats along the intertidal gradient since *S. maritima* is a colonizer of low tidal elevations, where metal pollution is higher than at medium elevations colonized by *S. densiflora* (Luque et al. 1999).

The exposition of plants to excess Cu, Zn or Ni produces oxidative stress and inhibits metabolic functions such as photosynthesis, water uptake and pigment synthesis, damages membrane integrity and reduces growth (Fernandes & Henriquez 1991, Nagajyoti et al. 2010). Seed coat permeability to different metals depends on the physical and chemical properties of metals (Kranner & Colville 2011) and, in contrast to other metals that are accumulated mainly in the seed coat cells, Ni can be translocate across the seed coat and accumulate in embryo cells and scutellum (Seregin & Kozhevnikova 2005). This may explain why Ni was the tested metal whose negative effects on seedling growth were noticed at lower concentrations (from 250 μM Ni for *S. densiflora* radicle), being the only metal reducing the first leaf length for all *S. densiflora* populations. Our results were in accordance with Márquez-García et al. (2013) that recorded roots of *Atriplex halimus* and *Salicornia ramosissima* reducing its growth at lower concentrations of Ni (above 100 μM Ni) than Cu and Zn (at 250 and 1000 μM, respectively). There have been many reports on the toxic effects of Ni on germination and seedling growth in plants (Yusuf et al. 2011). For example, Sharma et al. (2011) observed that *S. brachiata* shoot and root length decreased up to 400 µM Ni. In two rice cultivars, Maheshwari & Dubey (2008) found that the exposition of seeds up to 200 μM of Ni reduced root and shoot length.

Comparing our results to metals concentration in the field, we expect invasive *S. densiflora* seedling development may be impacted in most metal-polluted areas in Odiel and Tinto Estuaries. Thus, maximum concentrations of Cu registered in Odiel and Tinto Estuaries was 745 μM (Elbaz-Poulichet et al. 1999) and, in our study, radicle length was reduced from 100 μM Cu and first leaf was shortened over 100 μM Cu for Odiel seedlings. Moreover, maximum Zn concentrations in Odiel and Tinto Estuaries are ca. 900 μM (Elbaz-Poulichet et al. 2001) and we recorded shortened *S. densiflora* radicles at concentrations higher than 500 μM Zn, and *S. densiflora* radicle and first leaf length were reduced over 250 and 500 μM Ni, respectively, whereas maximum concentration has been recorded in 2.8 μM Ni in Odiel and Tinto Estuaries (Braungardt et al. 2003).

Finally, it is interesting to point out that *S. densiflora* seedlings coming from seeds from Tinto Estuary showed higher tolerance to metals than those from Odiel and Piedras Estuaries. This higher tolerance was reflected in radicle length reduction over 100 μM Cu for Odiel and Piedras seedlings and over 250 μM Cu for Tinto seedlings, and first leaf length reduction over 500 μM Ni for Odiel and Piedras seedlings and over 1000 μM Ni for Tinto seedlings. These differences in metals tolerance may be explained because *S. densiflora* seedlings are exposed to higher metal concentrations that are more bioavailable (at lower pH) in Tinto than in Odiel and Piedras Estuaries (Mateos-Naranjo et al. 2011). The existence of *S. densiflora* ecotypes in relation to seedling metal tolerance is agree with Waddell & Kraus (1990) who found different *S. alterniflora* seedling responses to Cu exposure between polluted and non-polluted estuaries.

We can conclude that seeds of native *S. maritima* and invasive *S. densiflora* tolerate very high concentrations of Cu, Zn and Ni during germination, and that *S. maritima* seedlings are more tolerant to metals than *S. densiflora*. Invasive *S. densiflora* showed negative effects on seedling growth, mainly in the radicle, with increasing metal concentrations, being the seedling coming from seeds from Tinto Estuary more tolerant to metals than those from Odiel and Piedras Estuaries.

Acknowledgements

We thank to the management and staff of the Odiel Marshes Natural Park for its collaboration. M.D. Infante-Izquierdo acknowledges to Ministerio de Educación, Cultura y Deporte of Spanish Government for the FPU Grant (Ref. FPU14/06556).

References

Achterberg, E.P., Herzl, V.M., Braungardt, C.B., Millward, G.E. (2003). Metal behaviour in an estuary polluted by acid mine drainage: the role of particulate matter. Environmental Pollution 121: 283-292. https://doi.org/10.1016/S0269-7491(02)00216-6

Ahsan, N., Lee, D.G., Lee, S.H., Kang, K.Y., Lee, J.J., Kim, P.J., Yoon, H.S., Kim, J.S., Lee, B.H. (2007). Excess copper induced physiological and proteomic changes in germinating rice seeds. Chemosphere 67: 1182-1193. https://doi.org/10.1016/j.chemosphere.2006.10.075

Ainouche, M., Gray, A. (2016). Invasive *Spartina*: lessons and challenges. Biological Invasions 18: 2119-2122. https://doi.org/10.1007/s10530-016-1201-7

Barba-Brioso, C., Fernández-Caliani, J.C., Miras, A., Cornejo, J., Galán, E. (2010). Multi-source water pollution in a highly anthropized wetland system associated with the estuary of Huelva (SW Spain). Marine Pollution Bulletin 60: 1259-1269.
https://doi.org/10.1016/j.marpolbul.2010.03.018

Borrego, J., Morales, J., De la Torre, M., Grande, J. (2002). Geochemical characteristics of heavy metal pollution in surface sediments of the Tinto and Odiel river estuary (southwestern Spain). Environmental Geology 41: 785-796.
https://doi.org/10.1007/s00254-001-0445-3

Braungardt, C.B., Achterberg, E.P., Elbaz-Poulichet, F., Morley, N.H. (2003). Metal geochemistry in a mine-polluted estuarine system in Spain. Applied Geochemistry 18: 1757-1771.
https://doi.org/10.1016/S0883-2927(03)00079-9

Castellanos, E.M., Figueroa, M.E., Davy, A.J. (1994) Nucleation and facilitation in saltmarsh succession: interactions between *Spartina maritima* and *Arthrocnemum perenne*. Journal of Ecology 82: 239-248. DOI: 10.2307/2261292

Castillo, J.M., Ayres, D.R., Leira-Doce, P., Bailey, J., Blum, M., Strong, D.R., Luque, T., Figueroa, E. (2010). The production of hybrids with high ecological amplitude between exotic *Spartina densiflora* and native *S. maritima* in the Iberian Peninsula. Diversity and Distributions 16: 547-558.
https://doi.org/10.1111/j.1472-4642.2010.00673.x

Curado, G., Rubio-Casal, A.E., Figueroa, E., Castillo, J.M. (2010) Germination and establishment of the invasive cordgrass *Spartina densiflora* in acidic and metal polluted sediments of the Tinto River. Marine Pollution Bulletin 60: 1842-1848.
https://doi.org/10.1016/j.marpolbul.2010.05.022

Davis Jr, R.A., Welty, A.T., Borrego, J., Morales, J.A., Pendon, J.G., Ryan, J.G. (2000). Rio Tinto estuary (Spain): 5000 years of pollution. Environmental Geology 39: 1107-1116.
https://doi.org/10.1007/s002549900096

Elbaz-Poulichet, F., Braungardt, C., Achterberg, E., Morley, N., Cossa, D., Beckers, J.M., Nomérange, P., Cruzado, A., Leblanc, M. (2001). Metal biogeochemistry in the Tinto-Odiel rivers (Southern Spain) and in the Gulf of Cadiz: a synthesis of the results of TOROS project. Continental Shelf Research 21: 1961-1973.
https://doi.org/10.1016/S0278-4343(01)00037-1

Elbaz-Poulichet, F., Morley, N.H., Cruzado, A., Velasquez, Z., Achterberg, E.P., Braungardt, C.B. (1999). Trace metal and nutrient distribution in an extremely low pH (2.5) river-estuarine system, the Ria of Huelva (south-west Spain). Science of the Total Environment 227: 73-83.
https://doi.org/10.1016/S0048-9697(99)00006-6

Fernandes, J.C., Henriques, F.S. (1991). Biochemical, physiological, and structural effects of excess copper in plants. The Botanical Review 57: 246-273.
https://doi.org/10.1007/BF02858564

Fernández-Caliani, J.F., Muñoz, F.R., Galán, E. (1997). Clay mineral and heavy metal distributions in the lower estuary of Huelva and adjacent Atlantic shelf, SW Spain. Science of the Total Environment 198: 181-200. https://doi.org/10.1016/S0048-9697(97)05450-8

Galán, E., Gómez-Ariza, J.L., González, I., Fernández-Caliani, J.C., Morales, E., Giráldez, I. (2003). Heavy metal partitioning in river sediments severely polluted by acid mine drainage in the Iberian Pyrite Belt. Applied Geochemistry 18: 409-421. https://doi.org/10.1016/S0883-2927(02)00092-6

Gambrell, R.P. (1994). Trace and toxic metals in wetlands-a review. Journal of Environmental Quality 23: 883-891. doi:10.2134/jeq1994.00472425002300050005x

González-Pérez, J.A., De Andrés, J.R., Clemente, L., Martín, J.A., González-Vila, F.J. (2008). Organic carbon and environmental quality of riverine and off-shore sediments from the Gulf of Cádiz, Spain. Environmental Chemistry Letters 6: 41-46. https://doi.org/10.1007/s10311-007-0107-0

Infante-Izquierdo, M.D., Castillo, J.M., Nieva, F.J.J., Rotundu, I.D., David, F.T., Grewell, B.J., Muñoz-Rodríguez, A.F. (2019a). Fruit set, seed viability and germination of the European native *Spartina maritima* in Southwest Iberian Peninsula. Wetlands. https://doi.org/10.1007/s13157-019-01188-1

Infante-Izquierdo, M.D., Castillo, J.M., Grewell, B.J., Nieva, F.J.J., Muñoz-Rodríguez, A.F. (2019b). Differential Effects of Increasing Salinity on Germination and Seedling Growth of Native and Exotic Invasive Cordgrasses. Plants 8: 372. https://doi.org/10.3390/plants8100372

Kranner, I., Colville, L. (2011). Metals and seeds: biochemical and molecular implications and their significance for seed germination. Environmental and Experimental Botany 72: 93-105. https://doi.org/10.1016/j.envexpbot.2010.05.005

Leblanc, M., Morales, J.A., Borrego, J., Elbaz-Poulichet, F. (2000). 4,500-year-old mining pollution in southwestern Spain: long-term implications for modern mining pollution. Economic Geology 95: 655-662. https://doi.org/10.2113/gsecongeo.95.3.655

Léon, V., Rabier, J., Notonier, R., Barthelemy, R., Moreau, X., Bouraïma-Madjèbi, S., Viano, J., Pineau, R. (2005). Effects of three nickel salts on germinating seeds of *Grevillea exul* var. *rubiginosa*, an endemic serpentine proteaceae. Annals of botany 95: 609-618. https://doi.org/10.1093/aob/mci066

Li, W., Khan, M.A., Yamaguchi, S., Kamiya, Y. (2005). Effects of heavy metals on seed germination and early seedling growth of *Arabidopsis thaliana*. Plant growth regulation 46: 45-50. https://doi.org/10.1007/s10725-005-6324-2

Liu, X., Zhang, S., Shan, X., Zhu, Y. G. (2005). Toxicity of arsenate and arsenite on germination, seedling growth and amylolytic activity of wheat. Chemosphere 61: 293-301. https://doi.org/10.1016/j.chemosphere.2005.01.088

Luque, C.J. Castellanos, E.M., Castillo, J.M., González, M., González-Vilches, M.C., Figueroa, E.M. (1999). Metals in halophytes of a contaminated estuary (Odiel saltmarshes, SW Spain). Marine Pollution Bulletin 38: 49-51.

Maheshwari, R., Dubey, R.S. (2008). Inhibition of ribonuclease and protease activities in germinating rice seeds exposed to nickel. Acta Physiologiae Plantarum 30: 863. https://doi.org/10.1007/s11738-008-0192-0

Manousaki, E., Kalogerakis, N. (2011). Halophytes present new opportunities in phytoremediation of heavy metals and saline soils. Industrial & Engineering Chemistry Research 50: 656-660. https://doi.org/10.1021/ie100270x

Marchant, C.J., Goodman, P.J. (1969) *Spartina maritima* (Curtis) Fernald. Journal of Ecology 57: 287-291. DOI: 10.2307/2258222.

Márquez-García, B., Márquez, C., Sanjosé, I., Nieva, F.J.J., Rodríguez-Rubio, P., Muñoz-Rodríguez, A.F. (2013) The effects of heavy metals on germination and seedling characteristics in two halophyte species in Mediterranean marshes. Marine Pollution Bulletin 70: 119-124. https://doi.org/10.1016/j.marpolbul.2013.02.019

Márquez-García, B., Muñoz-Rodríguez, A.F., Córdoba, F. (2009). Germination requirements of *Erica andevalensis*, an endemic species restricted to acid and metal/metalloid enriched soils of the Iberian Pyrite Belt (SW Iberian Peninsula). Fresenius Environmental Bulletin 18: 2268-2274.

Mateos-Naranjo, E., Redondo-Gómez, S., Cambrollé, J., Figueroa, M.E. (2008a). Growth and photosynthetic responses to copper stress of an invasive cordgrass, *Spartina densiflora*. Marine Environmental Research 66: 459-465. https://doi.org/10.1016/j.marenvres.2008.07.007

Mateos-Naranjo, E., Redondo-Gómez, S., Cambrollé, J., Luque, T., Figueroa, M.E. (2008b). Growth and photosynthetic responses to zinc stress of an invasive cordgrass, *Spartina densiflora*. Plant Biology 10: 754-762. https://doi.org/10.1111/j.1438-8677.2008.00098.x

Mateos-Naranjo, E., Andrades-Moreno, L., Redondo-Gómez, S. (2011) Comparison of germination, growth, photosynthetic responses and metal uptake between three populations of *Spartina densiflora* under different soil pollution conditions. Ecotoxicology and environmental safety 74: 2040-2049. https://doi.org/10.1016/j.ecoenv.2011.06.019

Mrozek Jr, E. (1980). Effect of mercury and cadmium on germination of *Spartina alterniflora* Loisel seeds at various salinities. Environmental and Experimental Botany 20: 367-377. https://doi.org/10.1016/0098-8472(80)90229-4

Mrozek, E., Funicelli, N.A. (1982). Effect of zinc and lead on germination of *Spartina alterniflora* Loisel seeds at various salinities. Environmental and Experimental Botany 22: 23-32. https://doi.org/10.1016/0098-8472(82)90005-3

Munzuroglu, O., Geckil, H. (2002). Effects of metals on seed germination, root elongation, and coleoptile and hypocotyl growth in *Triticum aestivum* and *Cucumis sativus*. Archives of Environmental Contamination and

Toxicology 43: 203-213.

Muñoz-Rodríguez, A.F., Rodríguez-Rubio, P., Nieva, F.J.J., Fernández-Illescas, F., Sánchez-Gullón, E., Soto, J.M., Hermoso-López, V., Márquez-García, B. (2012). The importance of bracteoles in ensuring *Atriplex halimus* germination under optimal conditions. Fresenius Environmental Bulletin 21: 3521-3526.

Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M. (2010). Heavy metals, occurrence and toxicity for plants: a review. Environmental chemistry letters 8: 199-216.
https://doi.org/10.1007/s10311-010-0297-8

Nelson, C.H., Lamothe, P.J. (1993). Heavy metal anomalies in the Tinto and Odiel river and estuary system, Spain. Estuaries 16: 496-511.
https://doi.org/10.2307/1352597

Nieva, F.J.J., Díaz-Espejo, A., Castellanos, E.M., Figueroa, M.E. (2001). Field variability of invading populations of *Spartina densiflora* Brong. in different habitats of the Odiel Marshes (SW Spain). Estuarine, Coastal and Shelf Science 52: 515-527.
https://doi.org/10.1006/ecss.2000.0750

Pérez-López, R., Nieto, J.M., López-Cascajosa, M.J., Díaz-Blanco, M.J., Sarmiento, A.M., Oliveira, V., Sánchez-Rodas, D. (2011). Evaluation of heavy metals and arsenic speciation discharged by the industrial activity on the Tinto-Odiel estuary, SW Spain. Marine Pollution Bulletin 62: 405-411.
https://doi.org/10.1016/j.marpolbul.2010.12.013

Redondo-Gómez, S. (2013). Bioaccumulation of heavy metals in *Spartina*. Functional Plant Biology 40: 913-921.
https://doi.org/10.1071/FP12271

Redondo-Gómez, S., Andrades-Moreno, L., Mateos-Naranjo, E., Parra, R., Valera-Burgos, J., Aroca, R. (2011). Synergic effect of salinity and zinc stress on growth and photosynthetic responses of the cordgrass, *Spartina densiflora*. Journal of Experimental Botany 62: 5521-5530.
https://doi.org/10.1093/jxb/err234

Redondo-Gómez, S., Cantos, M., Mateos-Naranjo, E., Figueroa, M.E., Troncoso, A. (2009). Heavy metals and trace element concentrations in intertidal soils of four estuaries of SW Iberian Peninsula. Soil & Sediment Contamination 18: 320-327.
https://doi.org/10.1080/15320380902772646

Ruiz, F. (2001). Trace metals in estuarine sediments from the southwestern Spanish coast. Marine Pollution Bulletin 42: 481-489.
https://doi.org/10.1016/S0025-326X(00)00192-2

Sainz, A., Grande, J.A., De la Torre, M.L. (2004). Characterisation of heavy metal discharge into the Ria of Huelva. Environment International 30: 557-566.
https://doi.org/10.1016/j.envint.2003.10.013

Seregin, I.V., Kozhevnikova, A.D. (2005). Distribution of cadmium, lead, nickel, and strontium in imbibing maize caryopses. Russian Journal of Plant Physiology 52: 565-569.
https://doi.org/10.1007/s11183-005-0084-8

Sethy, S.K., Ghosh, S. (2013). Effect of heavy metals on germination of seeds. Journal of natural science, biology, and medicine 4: 272. doi: 10.4103/0976-9668.116964

Sharma, A., Gontia-Mishra, I., Srivastava, A.K. (2011). Toxicity of heavy metals on germination and seedling growth of *Salicornia brachiata*. Journal of phytology 3: 33-36.

Strong, D.R., Ayres, D.R. (2013). Ecological and evolutionary misadventures of *Spartina*. Annual review of ecology, evolution, and systematics 44: 389-410. https://doi.org/10.1146/annurev-ecolsys-110512-135803

Ungar, I.A. (2001). Seed banks and seed population dynamics of halophytes. Wetlands Ecology and Management 9: 499-510. https://doi.org/10.1023/A:1012236829474

Van Oosten, M.J., Maggio, A. (2015). Functional biology of halophytes in the phytoremediation of heavy metal contaminated soils. Environmental and experimental botany 111: 135-146. https://doi.org/10.1016/j.envexpbot.2014.11.010

Waddell, D.C., Kraus, M.L. (1990). Effects of $CuCl_2$ on the germination response of two populations of the saltmarsh cordgrass, *Spartina alterniflora*. Bulletin of environmental contamination and toxicology 44: 764-769. https://doi.org/10.1007/BF01701800

Williams, T.P., Bubb, J.M., Lester, J.N. (1994). Metal accumulation within salt marsh environments: a review. Marine pollution bulletin 28: 277-290. https://doi.org/10.1016/0025-326X(94)90152-X

Yusuf, M., Fariduddin, Q., Hayat, S., Ahmad, A. (2011). Nickel: an overview of uptake, essentiality and toxicity in plants. Bulletin of environmental contamination and toxicology 86: 1-17. https://doi.org/10.1007/s00128-010-0171-1

9

Conclusions/Conclusiones

1. Reciprocal hybrids of *Spartina maritima* and *S. densiflora* have unique morphological and anatomical characteristics that can be used to distinguish them from their parental species and each other, and they were described as two new nothotaxa, *Spartina* × *onubensis* subsp. *onubensis* and *Spartina* × *onubensis* subsp. *tartessiana*.

   Los híbridos recíprocos entre *Spartina maritima* y *S. densiflora* presentan características morfológicas y anatómicas que se pueden utilizar para distinguirlos de sus especies parentales y entre sí, los cuales han sido descritos como dos nuevos nothotaxa, *Spartina* × *onubensis* subsp. *onubensis* y *Spartina* × *onubensis* subsp. *tartessiana*.

2. Caryopses formation in *Spartina maritima* was firstly observed in populations of Southwest Iberian Peninsula, with a fruit set average of 13%, and with high variation among tussocks (0%-45%). This percentage increased together with the percentage of spikelets with all their stamens exerted, which may indicate that tussocks in which inflorescences reached a more advanced state of flowering when the fruit set started will produce more fruits.

   La formación de cariopsis en *Spartina maritima* se ha observado por primera vez en poblaciones del Suroeste de la Península Ibérica, con un promedio de fructificación del 13%, y con una elevada variación entre clones (0%-45%). Este porcentaje aumentó junto con el porcentaje de espiguillas con todos los estambres exertos, lo que podría indicar que los clones en los que las inflorescencias alcanzaron un estado de floración más avanzado cuando comenzó la fructificación, producirán más frutos.

3. Caryopses of *S. maritima* showed high viability (89%) and high germination rates in freshwater (85%).

   Las cariopsis de *S. maritima* mostraron una elevada viabilidad (89%), así como altas tasas de germinación en agua dulce (85%).

4. *Spartina densiflora* showed interannual variation and differences among habitats for reproductive traits, achieving its highest inflorescence and caryopsis densities in low marshes and saltpans; its lowest caryopsis production in low marshes, where abiotic stress from frequent inundation was high; and the highest caryopses viability in middle and high marshes.

   Spartina densiflora mostró variaciones interanuales y diferencias entre hábitats en los parámetros reproductivos estudiados, alcanzando la mayor densidad de inflorescencias y de cariopsis en las zonas de marisma baja y en las cubetas hipersalinas, la producción más baja de cariopsis en zonas de marisma baja, donde el estrés abiótico debido a las frecuentes inundaciones es alto, y la mayor de viabilidad de cariopsis en zonas de marisma media y alta.

5. *Spartina patens* did not produce any mature caryopses in the Southwest Iberian Peninsula, even though around 80% of spikelets had all stamens exerted and its pollen viability was around 60%. Both reciprocal *Spartina* hybrids showed total male sterility and they did not produce caryopses.

   Spartina patens no produjo ninguna cariopsis madura en el Suroeste de la Península Ibérica, a pesar de que alrededor del 80% de las espiguillas tuvieron todos los estambres exertos y la viabilidad del polen fue de aproximadamente el 60%. Ambos híbridos mostraron una esterilidad masculina total y no produjeron cariopsis.

6. Protogyny has been demonstrated for *Spartina maritima* and *S. densiflora*, but while in *S. maritima* the overlap of sexual phases occurred only in 28% of the inflorescences, in *S. densiflora* there was an overlap between male and female functions in most inflorescences (87%).

   La protoginia ha sido demostrada en *Spartina maritima* y *S. densiflora*, pero mientras que en *S. maritima* la superposición de las fases sexuales se produjo solo en el 28% de las inflorescencias, en *S. densiflora* hubo una superposición entre las funciones masculinas y femeninas en la mayoría de las inflorescencias (87%).

7. *S. maritima* and *S. densiflora* showed similar pollen production per anther. However, at the end of one day of anther exposition, *S. maritima* anthers still retained inside around 75% of their pollen, while *S. densiflora* dispersed a half of its pollen. In both species the average pollen in the air surrounding the inflorescences revealed extremely low pollen dispersal rates, that explained the low levels of pollen grains observed anchored to stigmas. Moreover, both species lost pollen germinability during the first hours after release.

   S. maritima y *S. densiflora* mostraron una producción de polen por antera similar. Sin embargo, al final de un día de exposición de las anteras, las de *S. maritima* aún conservaban alrededor del 75% de su polen, mientras que *S. densiflora* dispersó la mitad de su polen. En ambas especies, el promedio de polen en el aire que rodea las inflorescencias reveló unas tasas de dispersión de polen extremadamente bajas, lo que explica los bajos niveles de granos de polen observados anclados a los estigmas. Además, ambas especies perdieron la germinabilidad del polen durante las primeras horas después de la liberación.

8. It has been shown, for the first time, that *S. maritima* and *S. densiflora* are self-compatible, and no significant differences were observed in their fruit set rates, neither between self-pollination and cross-pollination assays in *S. maritima*, nor between self-pollination, forced self-pollination and open-pollination assays in *S. densiflora*.

   Se ha mostrado, por primera vez, que *S. maritima* y *S. densiflora* son autocompatibles, y no se observaron diferencias significativas en sus tasas de fructificación, ni entre los ensayos de autopolinización y polinización cruzada en *S. maritima*, ni entre los ensayos de autopolinización, autopolinización forzada y polinización abierta en *S. densiflora*.

9. *S. densiflora* spikelets, especially from middle and high elevation marshes, showed greater ability to remain afloat than *S. maritima*, which suggests *S. densiflora* could be dispersed over longer distances supporting possible colonization of new areas, in contrast to native *S. maritima*, whose dispersion would be more restricted.

   Las espiguillas de *S. densiflora*, especialmente las de zonas de marisma media y marisma alta, mostraron una mayor capacidad para flotar que *S. maritima*, lo que sugiere que *S. densiflora* podría dispersarse a distancias más largas haciendo posible la colonización de nuevas áreas, al contrario que *S. maritima*, cuya dispersión sería más restringida.

10. Wet-stored *S. maritima* and *S. densiflora* seeds were able to maintain viability for months, mainly in salt solutions, while seeds under dry storage rapidly lost viability, especially at moderate temperature, with a more pronounced effect on *S. maritima*.

    Las semillas de *S. maritima* y *S. densiflora* almacenadas sumergidas mantuvieron la viabilidad durante meses, sobre todo en soluciones salinas, mientras que las semillas almacenadas en seco perdieron rápidamente la viabilidad, especialmente a temperaturas moderadas, con un efecto más acusado en *S. maritima*.

11. No seed bank was found for *S. maritima* neither in its low marsh populations nor in upper areas in the intertidal gradient. However, *S. densiflora* established a transient seed banks mainly at higher marsh elevations.

    No se encontró banco de semillas de *S. maritima* ni en sus poblaciones de marisma baja ni en zonas más elevadas del gradiente mareal. Sin embargo, *S. densiflora* estableció bancos de semillas transitorios principalmente en las zonas más elevadas de la marisma.

12. *S. maritima* and *S. densiflora* seed germination was inhibited and delayed at high salinities, but *S. densiflora* tolerate higher salinity levels than *S. maritima*. Pre-exposure to salinity accelerated the germination in both species. It was observed dormancy in *S. densiflora* seeds.

    La germinación de *S. maritima* y *S. densiflora* se inhibió y retrasó a salinidades elevadas, pero *S. densiflora* fue más tolerante a salinidades altas que *S. maritima*. En los ensayos de recuperación, la salinidad aceleró la germinación. Se observó la existencia de dormancia en las semillas de *S. densiflora*.

13. *S. maritima* showed higher salinity tolerance than *S. densiflora* in relation to seedling growth. In *S. maritima*, salinity did not affect seedling growth, at least up to 0.45 M NaCl, and salinity pre-exposure up to 0.75 M NaCl stimulated the growth of seedling in fresh water. In *S. densiflora*, salinity did not have negative effects on seedling growth up to 0.15 M NaCl, showing higher salinity tolerance seedlings coming from seeds from low marshes than from middle and high marshes.

    S. maritima mostró mayor tolerancia a la salinidad que *S. densiflora* respecto al crecimiento de las plántulas. En *S. maritima*, la salinidad no afectó al crecimiento de las plántulas, al menos hasta 0.45 M NaCl, y la preexposición a la salinidad hasta 0.75 M NaCl estimuló el crecimiento de las plántulas en agua dulce. Sin embargo, en *S. densiflora*, la salinidad no tuvo efectos negativos sobre el crecimiento de las plántulas hasta 0.15 M NaCl, mostrando mayor tolerancia a la salinidad las plántulas procedentes de semillas de marisma baja que de marismas medias y altas.

14. Seeds of *S. maritima* and *S. densiflora* tolerated very high Cu, Zn and Ni concentrations (up to 2000 μM) during germination.

    Las semillas de *S. maritima* y *S. densiflora* toleraron concentraciones muy altas de Cu, Zn y Ni (hasta 2000 μM) durante la germinación.

15. *S. maritima* seedlings did not show negative effects at least up to 2000 μM Cu, Zn and Ni. Growth of *S. densiflora* seedlings was not affected at concentrations up to 100 μM Cu, 500 μM Zn and 100 μM Ni, showing higher tolerance to metals seedlings coming from seeds from Tinto Estuary than those from Odiel and Piedras Estuaries.

    Las plántulas de *S. maritima* no mostraron efectos negativos al menos hasta 2000 μM de Cu, Zn y Ni. El crecimiento de las plántulas de *S.*

densiflora no se vio afectado a concentraciones de hasta 100 μM de Cu, 500 μM de Zn y 100 μM de Ni, mostrando una mayor tolerancia a los metales las plántulas procedentes de semillas del Estuario del Tinto que las de los Estuarios del Odiel y del Piedras.