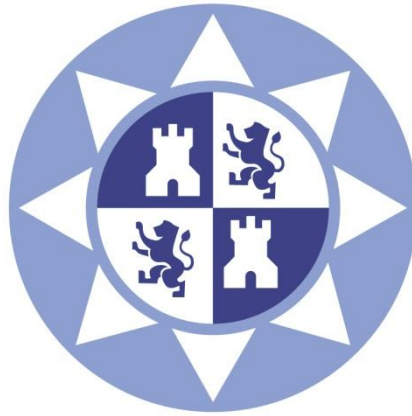Universidad Politécnica de Cartagena

Departamento de Producción Vegetal

Biological and Ecological Traits of *Anthemis chrysantha* J. Gay (Asteraceae), a Critically Endangered Species

Mayra Aguado López

2012

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2012

Directores

María José Vicente Colomer

Juan José Martínez Sánchez

Universidad
Politécnica
de Cartagena

DT-16

CONFORMIDAD DE SOLICITUD DEAUTORIZACIÓN DE DEPÓSITO DE TESIS DOCTORAL POR EL/LA DIRECTOR/A DE LA TESIS

D. Juan José Martínez Sánchez y Dª. María José Vicente Colomer, Directores de la Tesis doctoral "Biological and ecological traits of *Anthemis chrysantha* J. Gay (Asteraceae), a *Critically Endangered* species".

INFORMAN:

Que la referida Tesis Doctoral, ha sido realizada por Dª. Mayra Aguado López dando nuestra conformidad para que sea presentada ante la Comisión de Doctorado, para ser autorizado su depósito.

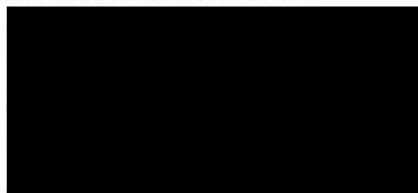La rama de conocimiento por la que esta tesis ha sido desarrollada es:

- [x] Ciencias
- [ ] Ciencias Sociales y Jurídicas
- [ ] Ingeniería y Arquitectura

En Cartagena, a 5 de Septiembre de 2012

LOS DIRECTORES DE LA TESIS

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COMISIÓN DE DOCTORADO

Universidad
Politécnica
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DT-17

CONFORMIDAD DE DEPÓSITO DE TESIS DOCTORAL POR LA COMISIÓN ACADÉMICA DEL PROGRAMA

D. Francisco Artés Hernández, Presidente/a de la Comisión Académica del Programa Técnicas Avanzadas en Investigación y Desarrollo Agrario y Alimentario (TAIDAA).

INFORMA:

Que la Tesis Doctoral titulada, "Biological and ecological traits of *Anthemis chrysantha* J. Gay (Asteraceae), a *Critically Endangered* species", ha sido realizada por Dª. Mayra Aguado López, bajo la dirección y supervisión del Dr. Juan José Martínez y la Dra. María José Vicente Colomer.

En reunión de la Comisión Académica de fecha --4/6/12. visto que la mencionada tesis doctoral tiene acreditados los indicios de calidad, requeridos para el depósito de tesis doctorales, regulados en el artículo 32 del Reglamento de Estudios Oficiales de Máster y Doctorado de la UPCT, y la autorización del Director de la misma, se acordó dar la conformidad para que a dicha tesis le sea autorizado, por la Comisión de Doctorado, su depósito.

La Rama de conocimiento por la que esta tesis ha sido desarrollada es:

x Ciencias
☐ Ciencias Sociales y Jurídicas
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En Cartagena, a 5 de septiembre de 2012

EL PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA

Fdo: Francisco Artés Hernández

COMISIÓN DE DOCTORADO

RECONOCIMIENTOS

Proyectos

Durante la realización de esta tesis doctoral he disfrutado de varias becas asociadas a algunos de los proyectos de investigación en los que he participado y dentro de los cuales se engloban los estudios llevados a cabo en este trabajo:

- Estudios básicos para la elaboración de los Planes de Recuperación de *Anthemis chrysantha* y *Astragalus nitidiflorus.* Consejería de Industria y Medio Ambiente de la Comunidad Autónoma de la Región de Murcia. De junio de 2006 a febrero de 2008.

- Realización de Estudios Relativos a Especies de la Flora Silvestre Amenazada. Consejería de Agricultura y Agua de la Comunidad Autónoma de la Región de Murcia. De abril de 2008 a diciembre de 2010.

- Desarrollo Científico–Tecnológico para la Conservación de los Recursos Fitogenéticos de la Región de Murcia. Consejería de Universidades, Empresa e Investigación de la Comunidad Autónoma de la Región de Murcia. De noviembre de 2008 a diciembre de 2011.

- Estudio de la dinámica de los bancos de semillas de dos especies vegetales en peligro crítico de la Región de Murcia. Fundación Séneca de la Región de Murcia. De enero de 2010 a diciembre de 2012.

Artículos científicos

Algunos de los resultados obtenidos en la presente tesis ya han sido publicados o enviados para su publicación en revistas científicas internacionales:

Aguado, M., Martínez-Sánchez, J.J., Reig–Armiñana, J., García–Breijo, F.J., Franco, J.A., Vicente, M.J., 2011. Morphology, anatomy and germination response of heteromorphic achenes of *Anthemis chrysantha* J. Gay (Asteraceae)*, a critically endangered species. Seed Sci. Res. 21, 283–294.

Aguado, M., Vicente, M.J., Miralles, J., Franco, J.A., Martínez-Sánchez, J.J., 2012. Aerial seed bank and dispersal traits in *Anthemis chrysantha* (Asteraceae), a critically endangered species. Flora 207, 275–282.

Aguado, M., Vicente, M.J., Franco, J.A., Martínez–Sánchez. The role of the soil seed bank in the unpredictable habitat of *Anthemis chrysantha* J. Gay*, a critically endangered species. Submited to Fora.

Congresos

Parte de los estudios realizados a lo largo de estos años han sido presentados en los siguientes congresos como comunicaciones escritas (pósters):

Aguado M., Miralles J., Franco J.A., Bañón S., Martínez-Sánchez J.J. & Vicente M.J. Transitory aerial seed bank in *Anthemis chrysantha* (*Compositae*), an endemic species from SE Spain. Póster. Biodiversity Hotspots in the Mediterranean Area: species, communities and landscape level. 45º International Congress of SISV & FIP. Libro de resúmenes. ISBN: 978-88-904296-06. University of Cagliari, Italia. Cagliari, Italia. 22–29 junio de 2009.

Aguado M., Miralles J., Vicente M.J., Bañón S., Franco J.A., & Martínez-Sánchez J.J. Autoecology and life history of *Astragalus nitidiflorus*, a critically endangered species of SE Spain. Póster. Biodiversity Hotspots in the Mediterranean Area: species, communities and landscape level. 45º International Congress of SISV & FIP. Libro de resúmenes. ISBN: 978-88-904296-06. University of Cagliari, Italia. Cagliari, Italia. 22–29 junio de 2009.

Aguado, M., Segura, F., Aznar, L., Franco, J.A., Martínez-Sánchez, J.J. & Vicente, M.J. Comportamiento germinativo de los aquenios heteromórficos de *Anthemis chrysantha* (Compositae). Póster. IV Congreso de Biología de la Conservación de Plantas. Libro de resúmenes. Almería, España. Septiembre de 2009.

Aguado, M., Vicente, M.J., Miralles, J., Franco, J.A., Martínez-Sánchez, J.J. Patrones de dispersión espacial de *Anthemis chrysantha* J. Gay (Asteraceae). Póster. V Congreso de Biología de la Conservación de Plantas. Libro de resúmenes. Es Mercadal, Menorca, España. Septiembre de 2011.

Aguado, M., Segura, F.J., Robles, J., Aznar, L., Vicente, M.J., Martínez-Sánchez, J.J. Efecto de la lluvia sobre la dispersión temporal de los aquenios de *Anthemis chrysantha* J. Gay (Asteraceae). Póster. V Congreso de Biología de la Conservación de Plantas. Libro de resúmenes. Es Mercadal, Menorca, España. Septiembre de 2011.

Proyectos fin de carrera dirigidos

Estudio de la diversidad genética de *Anthemis chrysantha*, una especie endémica *En Peligro de Extinción*, mediante marcadores moleculares ISSR. Alumna: Dolores Desirée Naveira Ruiz. 30 de septiembre de 2011. Escuela Técnica Superior de Ingeniería Agronómica. Universidad Politécnica de Cartagena.

AGRADECIMIENTOS

Llega el difícil momento de recopilar cuatro años de trabajo e ilusión en esta tesis, y no puedo dejar de recordar a todos los que me han ayudado en esta apasionante tarea y dejar constancia de mi agradecimiento.

En primer lugar quiero dar las gracias a mis directores de tesis, Juan José Martínez ("Juanjo") y María José Vicente, por confiar en mí y brindarme esta oportunidad. Han puesto toda su experiencia, su pasión y, claro, también su paciencia a lo largo de estos años, incluidas vacaciones. Son unos grandes jefes, pero aún son mejores personas.

Como el de ellos, he sentido en todo momento el apoyo de la Universidad Politécnica de Cartagena (UPCT) y, en especial, de la Escuela de Agrónomos (ETSIA). Empezaré por los profesores, que siempre han estado dispuestos a ofrecerme su ayuda: José Antonio Franco, un estímulo para la curiosidad tanto en los congresos como en el trabajo de campo, que resultaron tan interesantes como salpicados de anécdotas que hicieron más grato el trabajo; Pablo Bielza, gracias al cual comprendo un poco mejor el mundo de la estadística, del que nunca dejan de surgir dudas; Pepe Álvarez, cuyos análisis de suelo y explicaciones me han resultado tan útiles, como por ejemplo el trabajo con la pasta saturada; Sebastián Bañón, nuestro asesor logístico en cámaras, anemómetros y otros aparatos e inagotable compañero de andanzas en el Congreso de Cagliari; Encarna Conesa, entusiasta en las rutas biotecnológicas y las prácticas; Juan Antonio Martínez, al quite con las tinciones y los mecheros, siempre con su gran sentido del humor; y el resto de docentes del Departamento de Producción Vegetal, que han mostrado siempre interés por mi trabajo.

Asimismo, no quisiera pasar por alto la ayuda de Esteban Jordán, de los trabajadores de la Finca Tomás Ferro, y del Departamento de Ciencia y Tecnología Agraria, que puso a mi disposición sus laboratorios.

Y como no nombrar a mis compañeros becarios, ahora también amigos. El trabajo diario con Ely y Fran ha sido fundamental, ellos no necesitan leerse esta tesis; la conocen al dedillo. Raquel y Diana me han enseñado la importancia de intercambiar opiniones y conocimientos entre colegas, y que las pausas del café pueden ser muy

productivas. Julián e Izaskun, ya doctores, han contribuido con el peso de su veteranía. Manolo, compañero de despacho dispuesto siempre a echar una mano. Desirée y Antonio, cuyos proyectos fin de carrera han servido como aportación a mi tesis. María José, que demostró su constancia en el conteo de semillas. Marina y Almudena, con las que compartí los primeros momentos. Los compañeros de "enfrente", las chicas "de suelos", etc.

Debo reconocer el respaldo de la Comunidad Autónoma de la Región de Murcia, que bajo diferentes direcciones generales ha hecho posible que este trabajo, y muchos otros sobre especies vegetales amenazadas, sea viable económicamente. En particular, quiero citar al equipo de trabajo que más estrechamente ha colaborado con nosotros: Miguel Ángel Carrión, Laura Aznar, Jesús Robles y los agentes ambientales Juan y Pepe, de la brigada móvil.

Para mí es un motivo de alegría poder compartir con mis amigos este paso en mi carrera. Siempre los he sentido cerca: mis biólogas (Patri, Sara, Ana, Eva, Irene, Laura, y María), los tupper de pasillo nos unieron y ahora ni el tiempo ni la distancia nos separan; mis amigas de toda la vida (Lucía, Mariví, Helen, Leticia, Ana Yensi, Lorena, Vanesa y Tania) , creo que ahora puedo deciros que terminé de estudiar, ¿o no?; Vanessa, Pedro Ignacio y Rosabel; y los santomeranos (Antonio, Bea, José Ángel, Ortuño, Carmina, Estherilla, Amparín, Francisco José, Ismael, Bea, Ramón, Ana, Mario...podría seguir hasta terminar ¡echando un Alboroke!), por acogerme desde el primer día.

Y mucho más que agradecimiento siento hacia mi familia (necesitaría otra tesis para detenerme en cada uno de sus miembros): hacia mi familia murciana, ahora legalmente pero desde el principio de corazón; y hacia mi familia toledana, mis tíos y primos, mi abuelo, mi yaya, mi hermana y mis padres. Ellos me han aguantado, escuchado, apoyado y animado durante toda mi vida en cada proyecto que he emprendido. No habría llegado hasta aquí sin mis padres, que me han enseñado el valor de las cosas y cómo conseguirlas.

Por último, quiero destacar al responsable en gran medida de embarcarme en esta aventura, el sol de mi vida desde que le conocí, mi aliciente y mi aliento: mi marido, José Alberto.

ABSTRACT

Anthemis chrysantha (Asteraceae) is a winter annual plant with erect habit which reaches a height of 30 cm. Its emergence period begins in autumn, flowering occurs from early March to late May, and fruits mature mostly in June, after that the plant dies. This species is endemic to North Africa and Cartagena (Murcia, southeast of Spain) and it is classified as *Critically Endangered* according to the International Union for Conservation of Nature (IUCN) categories, and as *En Peligro de Extinción* in the Catálogo Regional de Flora Silvestre Protegida de la Región de Murcia. Due to this fact and the little prior knowledge about the species, the aim of this thesis is to study biological ad ecological traits of *A. chrysantha* in order to establish appropriate measures for its conservation. Thus, this work includes the study of: (1) morphology, anatomy and germination behavior of the heteromorphic achenes of *A. chrysantha*; (2) the spatial and temporal dispersal traits of the achenes and the implication of their dispersal strategy for the species; (3) the ability of the species to form a soil seed bank and the role that it can play in the maintenance of the population in an arid and unpredictable environment; (4) the level of genetic diversity, including the genetic variation within and between natural populations of the species, using ISSR markers; and (5) two different introduction methods, transplant of plants grown in a greenhouse and direct achene sowing, for testing them regarding to the survival and the establishment of the populations.

According to the obtained results from the conducted tests, *A. chrysantha* produces two morphs of achenes: white and dark achenes which differ in size, weight, anatomy, and germination behavior. White achenes germinate in high percentages at different conditions, but dark achenes do not germinate. Dark achenes have dormancy due to the thickness of their pericarp, which makes difficult the entry of water and also prevents germination by mechanical restriction. This work also demonstrates the two achenes type remain on the dead mother plant from the fruiting period (early summer) to late spring forming a seasonal aerial seed bank, and rain is necessary to release the achenes from the capitula (ombrohydrochory), which are mainly dispersed at short distances (atelechory). The aerial seed bank is an effective trait to ensure the maintenance of this species in its unpredictable habitat, but *A. chrysantha* has also the ability to form a persistent soil seed bank (PSB), with fluctuations of achene density due to the variability in annual rainfall and where dark dormant achenes are largely responsible for the permanent fraction. A PSB plays a crucial role in the maintenance of the annual population of this endangered plant and the study, carried out during five

consecutive years, highlights its importance in dry years. In addition, the genetic diversity study of the Spanish populations shows that both at the species and population level the genetic variation of *A. chrysantha* is high, with a high within–population variability and a low, differentiation between populations. The ISSR markers have detected genetic variation among Spanish and Algerian *A. chrysantha* populations, which could support the proposal of Sánchez et al. (2002) for recognizing the rank of subspecies, at least, for the Spanish populations. Finally, the two introduction methods seem to be valid for the establishment of *A. chrysantha,* in spite of the reproductive failure after two years due to the dry spring in 2012. However, if the next years are conducive, the formation of a possible PSB could provide dramatic populations recovery and this fact could be of great interest for the species conservation.

In conclusion, this work goes into the biology of this threatened species and we hope that it will become a very useful tool for establishing appropriate measures for the species conservation when the Regional Administration carry out its recovery plan.

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GENERAL INTRODUCTION

1. Systematics and description of the species

The family Asteraceae is one of the largest families of flowering plants with about 1,100 currently accepted genera and 25,000 species (Heywood, 1977). It is of worldwide distribution particularly in semiarid region of the tropics and subtropics. Most members are evergreen shrubs or subshrubs or perennial rhizomatous herbs; biennial and annual herbs are also frequent. It is generally accepted that the Asteraceae are a "natural" family with well established limits and a basic uniformity of floral structure imposed on all members by the common possession of characters such as the aggregation of the flowers into capitula and the special features of the stamens and corolla (Zareh, 2005). *Anthemis* L. (tribe Anthemideae) is the second largest genus within the Asteraceae, with more than 210 species. The total geographical range of *Anthemis* encompasses most of western Eurasia, the Mediterranean Region and a small part of eastern Africa, but the main center of diversity is found in southeastern Asia (Oberprieler, 2001). Only 62 species of this genus are distributed in Europe, 14 of them in the Iberian Peninsula.

One of these species is *Anthemis chrysantha* J. Gay, a winter annual plant which has an erect habit, is corymbosely branched, and reaches a height of 30 cm. Its leaves are fleshy, broadly ovate, 1 to 2 pinnatisect, and the lobes are usually oblong to obovate, obtuse or rounded, not mucronate. The capitula of the species (with yellow flowers on a rather convex disc of 12–25 mm in diameter) have peduncles up to 6 cm in length. The receptacle is hemispherical to oblong-ovoid, rounded at the apex, with receptacular bracts longer than the achenes (Tutin et al., 1980). Achenes are obconical and small (shorter than 2 mm in size and between 0.15 and 0.30 mg in weight), with ten granular ribs and a denticulate rim or sometimes a short pappus (0.3–0.5 mm in length).

Regarding the phenological cycle of the species (Picture 1), in *A. chrysantha* the emergence period begins with the first autumn rains, stretching into spring if weather conditions are favorable. Flowering occurs from early March-late May, and fruits mature mostly in June. The plants die in summer, but persist in a dried state in the habitat for several months after death (Aguado et al., 2012).

Anthemis chrysantha is endemic to North Africa (coast of Algeria) and to South-eastern Spain (coast of Cartagena, Murcia) (Picture 2).

Picture 1. Phenological cycle of *Anthemis chrysantha*: seedling (A), young plant in pot (B), plants in the wild before flowering (C), plants during flowering (D) and plants during fruiting before death (E).

Picture 2. Location of *Anthemis chrysantha* in South-eastern Spain and the North of Algeria.

Some authors (e.g. Fernandes, 1983; Oberprieler, 1998) have found differences between Spanish and Algerian material, although nowadays they are not recognized as different taxa. However, Sánchez et al. (2002) proposed the recognition of the rank of subspecies, at least, for the Spanish populations. The Spanish population (Picture 3A) is different to the Algerian (Picture 3B), presenting: generally more divided leaves, longer capitulum peduncles (up to 6 cm), bigger capitula (up to 25 mm diameter), and generally longer achenes and a more developed pappus.

Picture 3. Herbarium sheet of *Anthemis chrysantha* from La Azohía, Cartagena (A) and from Great Habiba Island, Algeria (B). Dry material belongs to the UPCT Herbarium (A) and the Natural History Museum Herbarium (Paris) (B).

2. Background and justification

Anthemis chrysantha has been found on the coast of Algeria, in the Oran Department (Habibas Islands and Kristel) and Mostaganem Department, occupying sands, cliffs, and grasslands next to the sea (Battandier, 1988; Battandier and Trabut, 1902), but data about these Algerian populations barely exist. In Cartagena, four populations are known. Here, the oldest known localities were the Escombreras Island (37º33´35´´N, 0º58´08´´W) and La Azohía (37º33´8´´N, 1∘10´22´´W) (Picture 4A); later, the species was detected in the continental zone of Escombreras and La Muela.

The Escombreras continental population was eliminated by the work of the new port in Escombreras; the last time the species was observed in this population was in 1998. The La Muela population dates from 1996 and it has been not detected since its discovery. Thus, since the late 1990s only two populations have remained in Europe, occupying an area of around 0.02 km^2. The populations of La Azohía and the Escombreras Island, although having high numbers of individuals (about 40,000 and 12,000, respectively), are very limited in terms of extension, their present areas being around 0.005 and 0.015 km^2, respectively (Picture 4B and 4C). This limited distribution and its status of endemism led to *A. chrysantha* being classified first as *Endangered* (Sánchez et al., 2002) and later as *Critically Endangered* (Sánchez et al., 2004) according to the International Union for Conservation of Nature (IUCN) categories. Besides, it is catalogued as *En Peligro de Extinción* in the Catálogo Regional de Flora Silvestre Protegida de la Región de Murcia (Decreto 50/2003 BORM 131).
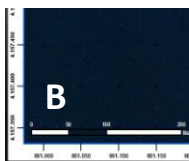
Picture 4. Geographical distribution of *Anthemis chrysantha* in Spain (A): La Azohía (B) and Escombreras Island (C) populations.

Currently, the main threat to the population of La Azohía is trampling, because its habitat is crossed by a highly frequented path. Besides, the site colonized by the species is next to housing development. In the Escombreras Island, the main threat is the construction of the port in Escombreras, above mentioned, that may increase contact with the continent and it will lead to colonization by other species, probably more competitive.

Despite the degree of threat to this species, there are few references to its distribution and the plant communities in which it appears and no reports on its biology and ecology, prior to the commencement of this doctoral thesis. However, in order to establish appropriate measures for the conservation of this species, we thought it necessary to determine its reproductive biology and ecology.

3. Features of the distribution area in Cartagena

The Region of Murcia lies within the scope of the Baetic Ranges, which are divided in three areas: Pre-baetic, Sub-baetic and Baetic, with materials that belong to each one. In general, the coastal design is rugged; however, there are some areas with softer lines such as the Mazarrón coast. From La Azohía to Cartagena, where *A. chrysantha* grows, the coast is very rugged again and has high cliffs of great beauty, with carbonates from the Alpujárride. To the east Escombreras Island, the materials from the Alpujárride are separated from the coast by quarzitic shales, from the Nevado-Filábride, causing strong coast reliefs (Mas et al., 1986).

Regarding the climatic conditions, this Region presents the characteristics of the semi-arid subtropical Mediterranean climate. Precipitation is low throughout the regional territory (approximately 300–350 mm per year), but frequently concentrated in periods such as spring (April) and autumn (October), the summer being predominantly dry. However, within the region, we can distinguish three rainfall areas, as well: sub-humid (defined by rainfall of around 500 mm), semi-arid (between 500 and 300 mm), and arid (300 mm or less). Cartagena lies within the arid area. The Region of Murcia, like the whole of southeastern Spain, is characterized by rainfall irregularity among years. Thus, in the interval from 1692 to 1985, were recorded averages of 88 mm in 1945 and 765 mm in 1884, even 1000 mm in mountainous areas (Mas et al., 1986). Among dry years, 1961, 1978, 1983, and 1984 were particularly dry, especially 1978 in

Cartagena, with less than 100 mm. Regarding temperature, the region has an annual average temperature of 18ºC, with hot summers (absolute maxima of 40ºC) and mild winters (11ºC average temperature in January and December). More specifically, the local area of Cartagena is characterized by the even milder temperatures. The mean annual air temperature is 17.6ºC, and the mean maximal and minimal temperatures are 23.4 and 6ºC, respectively, lying within the Thermo-Mediterranean bioclimatic level (Rivas–Martínez, 1987).

The vegetation landscape found by primitive man in Cartagena was probably extensive areas of *Pistacia lentiscus* L., *Chamaerops humilis* L., *Ziziphus lotus* (L.) Lam., *Periploca angustifolia* Labill., *Pinus halepensis* Mill., and *Tetraclinis articulata* (Vahl) Masters. In the watercourses there was *Nerium oleander* L., in salt marsh areas *Tamarix boveana* Bunge. and *Tamarix canariensis* Willd., and so on. The destruction of that old vegetation has given rise to the current vegetation and many of the processes that originated from the massive destruction (erosion, decline in rainfall, desertification…) are largely irreversible (Mas et al, 1986). Despite all of this, the Region of Murcia is squarely in the territorial area of the Mediterranean basin, a hot spot of world plant-diversity. In the Mediterranean basin there are nearly 25,000 species of vascular plants, value which represents at least 10% of the world plant diversity. In the Region of Murcia, plants with flowers (Angiosperms) encompass virtually all of the vascular flora, with more than 1,900 species. More than 50% of the flora is in only 8 families, the more diversified being the Asteraceae, Poaceae, and Leguminosae (11.5%, 9.2%, and 8.8% of the total, respectively). The species that set this region apart from others are those with a smaller geographic range, which is called, although not rigorously, the endemic component. Within this endemic component, the majority corresponds to elements of Iberian distribution (19%), such as *Ferula loscosii,* and Ibero-African elements (12.7%) such as *Tetraclinis articulata* (Vahl.) Masters and *Anthemis chrysantha* J. Gay, while some species have their sole population in Murcia, such as *Astragalus nitidiflorus* Jiménez Mun. et Pau*, Cistus heterophyllus* Desf. subsp. *carthaginensis* (Pau) Crespo & Mateo*,* or *Limonium carthaginense* (Rouy) C. E. Hubb. & Sandwith**,** which are endemic to Cartagena (Martínez–Sánchez et al, 2008).

Anthemis chrysantha grows in terofitic meadows developed in thyme and halonitrophilous scrubs, with a great influence of the sea. The main species we can find in these habitats, with *Anthemis chrysantha*, are: *Asteriscus maritimus* (L.) Less.*, Ferula communis* L.*, Frankenia corymbosa* Desf.*, Limonium cossonianum* Kuntze*, Lotus edulis*

L*., Lycium intricatum* L*., Salsola oppositifolia* Desf., *Mesembryanthemum nodiflorum* L*., Sedum sediforme* (Jacq.) Pau*, Silene secundiflora* Otth in DC., and *Sonchus tenerrimus* L. (Sánchez et al., 2004).

Populations of *A. chysantha* in Cartagena grow on lithosols; the plants can even grow in fissures when the bedrock has been fully exposed due to the loss of soil. Soil analysis for the La Azohía population (Table 1) showed a high pH and a very high electrical conductivity ($EC_{1:5}$). According to Alarcon (2007) the soil is extremely saline (> $2dS·m^{-1}$), but it should be noted that the samples for this analysis were taken in summer, when the soil is very dry. However, in summer the species is dead and other analyses performed in winter (Aguado et al., data not published) indicated that the soil is less saline when the plant is growing. Taking into account the $CaCO_3$ content, the soil in La Azohía is very calcareous (Alarcon, 2007).This area has high amounts of assimilable phosphorus (P_2O_5), total nitrogen (TN), and organic carbon (OC). Besides, the C/N ratio is high, which may be due to a low mineralization and the shallowness of the soil, which prevents its scroll to the profile. The cation-exchange capacity (CEC) is high because it depends on the organic matter and the clay, which determine the capacity of the soil in this area to retain cationic nutrients. The proportions of clay, silt, and sand give the soil a loam to sandy loam texture.

Table 1. Characteristics of representative soil samples from the natural population of *Anthemis chrysantha* in La Azohía.

Sample	Real pH	Potential pH	$EC_{1:5}$ ($dS·m^{-1}$)	$CaCO_3$ (%)	Assimilable P ($mgP·Kg^{-1}$)	TN ($g·Kg^{-1}$)	OC ($g·Kg^{-1}$)	C/N	Clay (%)	Silt (%)	Sand (%)	CEC ($mEq·100g^{-1}$)
LA 1	8 24	7 53	2 24	34 33	39 58	2 46	36 21	14 73	23 45	29 84	46 71	15 8
LA 2	8 12	7 6	3 17	32 37	33 07	2 11	51 27	24 24	24 52	26 91	48 57	12 8
LA 3	8 33	7 6	1 4	39 07	38 93	1 98	65 23	32 89	13 52	22 06	64 42	46 9
Average	8.23	7.58	2.27	35.25	37.19	2.19	50.90	23.95	20.5	26.27	53.23	25.17
Deviation	0.11	0.04	0.89	3.44	3.59	0.25	14.51	9.08	6.07	3.93	9.73	18.88

4. Endangered species conservation

Native plants have been declining at an alarming rate. They face an ever-increasing range of threats, from the fragmentation of their habitats to pressures resulting from agriculture, forestry, and urban sprawl. Climate change and the spread of invasive alien species are additional threats. Conservation management oriented to the

conservation of species is a consequence of the finding that the rate of species extinction is reaching levels not seen throughout history and that man is mainly responsible for this situation. However, the starting point (i.e., what are the current species extinction rates?) is an aspect of enormous controversy. Probably the most important effort in this sense comes from the IUCN Red List Programme. Thus, scientists (including botanists, taxonomists, and ecologists) are working hard to catalogue and describe the biology of plants throughout the world before they disappear, often using a method called Rapid Ecological Assessment. This activity is especially important in highly diverse regions of the globe with high numbers of rare species. However, uncertainty remains high, especially if we take into account that the material evaluated so far is a small fraction of the existing biodiversity and is far from representing the necessary basic knowledge (Escudero et al., 2002). Global efforts are underway to protect the remaining areas on earth that harbor high diversity of both common and rare plants (called biodiversity hotspots). The goal is to provide habitat in perpetuity for as many species of plants as possible. Another approach is to educate people about the economic, utilitarian, and intrinsic values of biodiversity. Finally, national and international legislation is necessary to prevent the trafficking of rare species such as orchids. For example, the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) prohibits or limits the trade of almost 28,000 species of rare plants worldwide (Farnsworth and Sarkar, 2012).

In Spain, starting from the 4/89 Nature Conservation Law, public regional administrations have been obliged to establish recovery plans for threatened species, since the 1990s. Since then, some scientists have tackled research lines about inventories of endangered species (for example, Bañares, Blanca, Güemes, and Moreno Sáiz); about genetic diversity (Caujapé–Castells and Sosa); and about the biology and ecology of threatened species, in which many scientists are working in Spain, perhaps following Iriondo and Escudero.

In this context, since 2005, we have been studying the ecology and biology of some endangered species endemic to the Southeast of Spain, such as *Anthemis chrysantha*. Thanks to the Consejería de Agricultura y Agua of the Comunidad Autónoma de la Región de Murcia and others organisms, like the Fundación Séneca of the Región de Murcia, some important projects have been carried out and the data obtained from them will be crucial for the future recovery of plants of the studied species.

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GENERAL OBJECTIVES

The high degree of threat of *Anthemis chrysantha* amply justifies its study in depth, however until the beginning of this work there were only few references to its distribution and the plant communities in which it appears, and no reports on its biology and ecology. For that, the goal of this study is to know the biological traits of the species, in order to establish appropriate measures for its conservation. This fact means to get the following specific aims:

1. To study the germination behavior of *A. chrysantha*.

2. To determine the spatial and temporal dispersal traits of the achenes and the implication of their dispersal strategy for the species.

3. To evaluate the ability of the species to form a soil seed bank and to know the role that it can play in the maintenance of the population in an arid and unpredictable environment.

4. To assess the level of genetic diversity including the genetic variation within and among natural populations of the species and provide elementary information for future conservation strategies.

5. To check different introduction methods for the studied species and to evaluate them according to the obtained results about the survival and the establishment of the introduced populations in the first two years.

CHAPTER 1

Morphology, anatomy and germination response of heteromorphic achenes of *Anthemis chrysantha* J. Gay (Asteraceae)*,* a critically endangered species.

Abstract

This study demonstrated that *Anthemis chrysantha*, a *Critically Endangered* annual plant, produces two morphs of achenes: white and dark achenes which differ in size, weight, anatomy, and germination behavior. Fresh white achenes germinated at all temperatures assayed from 10 to 25°C in both continuous darkness and 12–h photoperiod, ranging between 24% at 25°C in darkness and 89% at 12/20°C in light, while fresh dark achenes did not germinate at any temperature. To identify differences in dormancy type between the two morphs, germination of dry-stored achenes, and achenes stratified at 5 or 25°C for two months were tested in both darkness and light at 5, 15 and 12/20°C for dry-stored and warm-stratified (25°C) achenes; and at 15, 25 and 12/20°C for cold-stratified (5°C) achenes. Of white achenes, 90% germinated during the cold stratification period. In general, dry storage and warm stratification did not increase germination compared to fresh achenes. However, dark achenes did not germinate in any conditions. Dark achene dormancy was only broken by mechanical scarification or by excising the embryo (germination reached 71%). An anatomical study showed that the mesocarp of dark achenes had no intercellular spaces and was much thicker and stronger than for white achenes, making the entry of water difficult, and also preventing germination by mechanical restriction. This study demonstrated that the causes of dormancy in the dark achenes should be sought in the thickness of their pericarp physically impeding germination and making imbibition of water difficult.

1. Introduction

The morphology and physiological behavior of seeds are important features for understanding the pattern of seasonal and spatial distributions of species (Silvertown and Doust, 1993; Imbert, 2002). Many species of Asteraceae are known for producing two or more different morphs of their achenes within a single plant (Larson and Kiemnec, 1997; Imbert, 2002; Brändel, 2004), which is generally associated with adaptation to unstable environments (Stebbins, 1974; Harper, 1977; Venable, 1985). Seed polymorphism can affect characteristics such as dispersal capacity, seed dormancy, predation, germinability and seedling competition (Harper, 1977; Imbert et al., 1996; Brändel, 2004).

In general, dormancy is considered a mechanism to avoid germination in periods that are favorable for germination but unfavorable for subsequent seedling establishment (Vleeshouwer et al., 1995). According to Nikolaeva (1977) there are two general types of organic seed dormancy. In endogenous dormancy, some characteristic of the embryo prevents germination; whereas in exogenous dormancy, some characteristics of structures - e.g. endosperm (sometimes perisperm), seeds coats or fruits walls - covering the embryo prevents germination (Baskin and Baskin, 1998). Nikolaeva (1977) sorted the different types of organic seed dormancy and defined physical exogenous dormancy as when the seed (fruit) coats were impermeable to water. Most types of dormancy were broken by warm and/or cold stratification, but not physical exogenous dormancy.

There have been studies on morphology of the capitulum and the fruit to establish phylogenetic classifications within the Asteraceae (Chehregani and Mahanfar, 2007; Kreitschitz and Vallés, 2007), and some have described relationships between fruit morphology and germination capacity (Porras and Muñoz, 2000; Imbert, 2002; Brändel, 2004, Sun et al., 2009), but very few have addressed germination in the genus *Anthemis* (Gealy et al., 1985; Rashid et al., 2007).

Anthemis L. is the second largest genus in Asteraceae (tribe Anthemidae), with > 210 species in the Mediterranean region, southwest Asia, and eastern Africa (Oberprieler, 2001); about 62 species are distributed in Europe (Fernandes, 1976), and 14 in the Iberian Peninsula. One of these species is *A. chrysantha* J. Gay, an annual plant endemic to North Africa and the southeast of the Iberian Peninsula. It is only found on the Algerian coast and in Europe only on the coast of Cartagena (Murcia,

southeast Spain; Bañares et al., 2004). In Cartagena, four populations were known, but since the late 1990s only two remain, occupying an area of < 2 ha. *Anthemis chrysantha* grows in terophytic meadows affected by the sea winds, between halophytic thyme-bushes. It was first classified as *Endangered* (Sánchez et al., 2002) and later as *Critically Endangered* (Sánchez et al., 2004) according to the IUCN categories. It is currently protected by a Murcia regional law (BORM, 2003).

Despite the degree of threat to this species, there are few references to its distribution and the plant communities in which it appears (Sánchez et al., 2004), and no scientific papers on its biology and ecology. In general, to establish appropriate measures for conservation of a species it is necessary to determine its reproductive biology and ecology. One of the most important aspects of reproductive biology of a species, especially in arid and unpredictable environments, is the response of seed germination (Gutterman, 1993).

Since the existence of heteromorphic achenes is not mentioned in the botanical description of *A. chrysantha* (Tutin et al., 1980), the aims of the present study were to (a) explore morphological and anatomical variability in achenes; (b) identify possible differences in dormancy and germination behavior; and (c) determine possible adaptive advantages of this variation in *A. chrysantha* achenes.

2. Material and Methods

2.1. Plant material

The capitula of *A. chrysantha* (with yellow flowers on a rather convex disc of 12–25 (30) mm in diameter), have peduncles up to 6 cm in length (Sánchez et al., 2002). The receptacle is hemispherical to oblong-ovoid, rounded at the apex (Tutin et al., 1980). Each capitulum contains about 100–130 achenes, with receptacular bracts between them, arranged in several rows (7–12) on the disc. Achenes are obconical, generally shorter than 2 mm, with ten granular ribs and a denticulate rim or sometimes a short crenulate auricle (pappus). Under a stereoscopic microscope, two principal morphs of achenes are distinguishable within one capitulum (Picture 1): the rows of the upper section containing elongated, almost white achenes (white achenes hereafter), while the achenes of the basal rows are brown-black (dark achenes hereafter) and harder than white achenes. Finally, the last basal line is composed of achenes from ligules (only 8–

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10 per capitula), which are similar to white achenes but frequently are empty, and were not studied in this work. The proportion of white and dark achenes in a capitulum is about 70 and 30%, respectively. The percentage of empty white and dark achenes is very variable from one capitulum to another, but the average in the dark achenes is always lower than in white achenes (about 10 and 24%, respectively). A representative sample of freshly-matured achenes was collected from La Azohía (Cartagena, Murcia; 37°33′8″ N; 1°10′22″ W; altitude 30 m). This area has a semi-arid Mediterranean climate characterized by irregular rainfall and a harsh dry summer period. Annual mean precipitation is around 300 mm, and mean annual temperature is 17°C. August is the warmest month, with an average temperature of 24.9°C and a maximum of 42°C. The coldest month is January, with an average temperature of 10.6°C and the minimum always > 0°C. For collection, a central capitulum of similar maturation state was harvested in July 2009 from > 400 plants. Achenes were separated according to the white or dark type using a stereoscopic microscope (Olympus SZ61), and stored for 6–7 d at room temperature before germination studies.
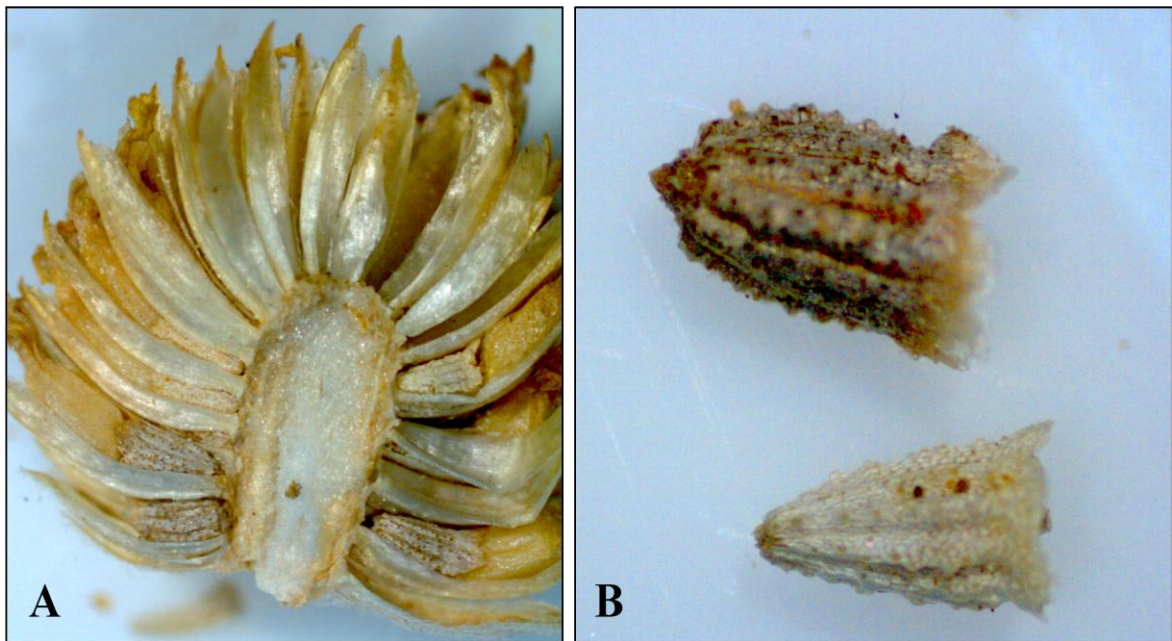
Picture 1. Longitudinal section of one capitulum of *Anthemis chrysantha* (A) and detail of the two principal achene types contained inside (B) observed by stereoscopic microscope.

2.2. Morphological characterization of achenes and embryos

Using the stereoscopic microscope with a micrometer, the length, width and pappus length were determined for 50 randomly selected achenes of each type. After measuring the achenes, the pericarp was removed to determine the length and width of the embryos. Similarly, to estimate the mass of the achenes and embryos, 50 achenes of each type were weighed using a Mettler Toledo XP56 electronic microbalance (with 0.001 mg precision).

2.3. Achene germination tests

2.3.1. Effect of temperature and light on the germination of fresh achenes

To determine the influence of light and temperature on achene germination, 100–achene lots of each type were incubated at each of the following constant temperature regimes: 10, 15, 20, 25, and an alternating temperature regime at 12ºC in darkness and 20°C in light. Each 100–achene lot was distributed into four replicates of 25 achenes. Each replicate was incubated in a 9–cm–diameter Petri dish, on a double layer of filter paper moistened with 4 mL of distilled water, in germination chambers (Sanyo MLR–351H, Osaka, Japan) with a digital temperature and light control system (± 0.1°C, cool white fluorescent light of 20,000 lux). At each temperature regime, achenes were tested for germination in both continuous darkness and light. Darkness treatments were obtained by wrapping Petri dishes in a double layer of aluminum foil. Achenes were checked every 2–3 d, with protrusion of the radicle the criterion for successful germination. Achenes incubated in darkness were checked in a dark room using the stereoscopic microscope with the light source (lamp type EKE 21 V/150 W halogen; illumination intensity 5.7 Mio lux) covered with a double layer of cellophane (blue and green) acting as a light filter. The experiments in both light and darkness were terminated after 37 d.

2.3.2. Effects of stratification and dry storage on achene germination

Simultaneously, each achene type was subjected to a wet-cold stratification treatment at 5°C or a wet-warm stratification at 25°C for two months in darkness. One

achene lot was preserved dry at room temperature during the same period. Then, four replicates of 25 dark achenes each from cold stratification were incubated at 15, 25 and 12/20°C; white achenes from the cold stratification could not be incubated at these temperatures due to the high germination during the stratification period (c.a. 90%). Both the warm-stratified and the dry-stored achenes were incubated at 5, 15 and 12/20°C. All treatments were tested for germination in both continuous darkness and light. Achene germination was checked every 2–3 d during 37 d.

At the end of the germination period, the germination percentage and the mean time to germination (MTG) were calculated for all treatments. The latter was determined using the following formula (Brenchley and Probert, 1998): MTG = $(\sum_i n_i \times d_i)/N$; where n is the number of achenes germinated at day i, d is the incubation period in days, and N is the total number of achenes germinated in the treatment.

2.4. Role of pericarp on embryo germination and water absorptions

Four replications of 25 embryos (without pericarps) from each type of achene were incubated in the abovementioned germination chambers in light at 15, 20 and 12/20°C. Embryo germination was checked every 2–3 d for 37 d. To determine if the hardness of the dark achene pericarp could affect embryo germination, four replications of 25 dark achenes with their fruit coats scarified slightly at the basal end (radicle end) or at the apical end (cotyledons emergence)were incubated in light at 12/20°C for 37 d. At the end of the scarification tests, embryos from ungerminated achenes were checked for viability on the basis of embryo appearance, paying special attention to colour and turgidity (Copete et al., 2009). Those achenes which showed a good appearance were tested for viability using the tetrazolium test at 1%.

In order to determine if water seeped through the achene pericarp and was absorbed by the embryos, five 20–achene lots of each type were weighed. They were then maintained in 9–cm–diameter Petri dishes (one lot per dish), on a double layer of filter paper moistened with 4 mL of distilled water, at room temperature for 36 h. Then the lots of achenes were weighed again, the embryos were excised from the achenes and weighed also. As the results obtained could be due to a water-impermeable fruit coat in dark achenes, two further experiments were conducted. Firstly, 50 dark achenes were weighed individually (with 0.001 mg precision), maintained in a tube with 0.5 mL of distilled water at room temperature for 14 d, and then weighed again. The embryos were

excised from the achenes and were also weighed. Then, 30 achenes of each type (i.e. to compare dark and white) were incubated individually at room temperature for 7 d, in aqueous solutions at 50% of fuchsin basic-carbol to determine if the solution penetrated into the embryos.

2.5. Anatomical characterization of achenes

Two lots of each achene type were studied using a Scanning Electron Microscope (Hitachi S–3500N, Singapore) with backscattered electron detector, working in low vacuum mode (70 Pa). Conditions were accelerating voltage of 15 kV and working distance of 15 mm.

Separately, two lots of each achene type were cut superficially to facilitate the later process of infiltration. Some of the samples of each lot were fixed under vacuum with 2% glutaraldehyde, washed with 0.1 M phosphate buffer (pH 7.4), and dehydrated in an ethanol series (50, 70, 96% and followed by $2 \times 100\%$), and infiltrated in Histosec® paraffin (Merck, Darmstadt, Germany) at 56–58°C. Isoamyl acetate was used as an intermediary solvent between the ethanol and the paraffin, the infiltration time was 60 min (60°C) and the blocks were cut into 8–μm sections with an Anglia Scientific microtome (Ontario, Canada). After removing paraffin with xylene and tissue rehydration in ethanol series, the samples were dyed with safranin and fast green (Johansen, 1940). Other samples of each lot were included after fixation in Spurr resin (Spurr, 1969). Semifine cuts (1.5 μm) were done with a Sorvall MT 5000 Ultra Microtome (Girald–Dupont,Wilmington, DE, USA) with glass blades (45°) obtained from a special glass (Glass Strips 6.4 mm from Leica) in a knifemaker (Reichert–Jung, Wien, Austria). These samples were dyed with toluidine blue (Ruzin, 1999) at 1%. To observe the preparations, an Olympus Provis AX–70 light field microscope (Capovani Brothers Inc, Scotia, New York, USA) was used, and pictures taken with an Infinity 2 CCD digital camera (Lumenera Corp, Ottawa, Canada) and treated with image acquisition software 'Infinity Analyze'.

2.6. Statistical analysis

A multivariate analysis of variance (MANOVA) was used to evaluate the effects of the different assay treatments on each type of achene and embryo. Data were analyzed with the software SPSS 13.0 for Windows (SPSS Inc, Chicago, Illinois, USA). When significant main effects existed, differences were tested by Tukey's multiple comparison test at 95% confidence. Percentage germination data were arcsine square-root transformed to meet the MANOVA requirements.

3. Results

3.1. Morphological characterization of achenes and embryos

Dark achenes had significantly greater length, width, weight and pappus length than white achenes. However, embryos from the two types of achenes were only significantly different in width, with the dark being wider (Table 1).

Table 1. Morphological characteristics of achene types and embryos of *Anthemis chrysantha*.

Morphometric characteristics	Achenes		Embryos	
	White	Dark	White achenes	Dark achenes
Pappus length (mm)	0.36 ± 0.02^a	0.50 ± 0.03^b		
Length (mm)	1.33 ± 0.02^a	1.39 ± 0.02^b	1.09 ± 0.01^a	1.09 ± 0.01^a
Width (mm)	0.68 ± 0.01^a	0.75 ± 0.02^b	0.48 ± 0.01^a	0.50 ± 0.01^b
Mass (mg)	0.142 ± 0.004^a	0.313 ± 0.012^{bA}	0.110 ± 0.002^a	0.116 ± 0.003^{aA}
Mass after 14 days water imbibition (mg)	-	0.349 ± 0.013^A	-	0.154 ± 0.004^B
Mass before 36 h water imbibition (mg)*	3.480 ± 0.086^{aA}	6.280 ± 0.153^{bA}	2.504 ± 0.071^{aA}	2.340 ± 0.040^{aA}
Mass after 36 h water imbibition (mg)*	7.540 ± 0.774^{aB}	7.680 ± 0.242^{aB}	4.300 ± 0.138^{bB}	2.460 ± 0.068^{aA}

Means (± standard error) within a row with different lowercase letters are significantly different from each other, within achene /embryo columns, and means within a column with different uppercase letters are significantly different from each other within achene /embryo columns (Tukey test; $p < 0.05$). Means are from 50 achenes or embryos.

*Means from five 20–achenes lots.

3.2. Achene germination tests

3.2.1. Effect of temperature and light on the germination of fresh achenes

There were significant differences for the three factors considered (achene type, temperature and light) and their interactions regarding achene germination ($P < 0.05$). Dark achenes occasionally reached 2–3% germination at 15 and 25°C in light but generally did not germinate. Thus, each type of achene was analyzed separately.

White achenes germinated in all treatments assayed, with a germination range of 24–89% (Figure 1A). In light, at constant temperatures, there were significant differences in germination, ranging between 68% at 15°C and 51% at 10°C (Figure 1A). The alternating regime of 12/20°C increased the germination percentages significantly ($P = 0.000$), reaching 89% (Figure 1A). However, the MTG was not significantly different at 12/20°C compared to 10, 15 and 20°C; germination was slowest at 25°C (Table 2). In darkness, the germination percentages at constant temperatures were significantly lower than those reached at 12/20°C (c.a. 72%; $P = 0.000$). The lowest germination was also obtained at 25°C, although not significantly different to 20 and 15°C treatments (Figure 1A). As in light treatments, the highest MTG value in darkness was at 25°C (Table 2). In all temperature regimes, the achene germination in light was higher than in darkness (Figure 1A). The MTG values obtained in darkness were significantly lower than in light, except at 20 and 25°C for which values were similar (Table 2).

3.2.2. Effects of stratification and dry storage on the germination

White achenes germinated at such high percentage (c.a. 90%) during the cold stratification that it was not possible to perform subsequent germination tests due to the insufficient remaining achenes. Dark achenes neither germinated during cold stratification nor in the subsequent germination testing at 15, 25 and 12/20°C.

Dark achenes did not germinate during warm stratification, while white achenes germinated little (8%). After warm stratification, there were significant effects on germination of the remaining white achenes due to the interaction between temperature and light ($P = 0.036$). White achenes germinated at high percentages without significant

differences between light and darkness. In light, the percentages ranged from 63% at 5°C to 90% at 12/20°C, with significant differences ($P = 0.010$; Figure 1B). In darkness there were no significant differences between incubation temperature regimes, with germination values of range 82–87% (Figure 1B). Both in light and darkness, the lowest MTG value was at 12/20°C and the highest at 5°C (Table 2).Warm-stratified dark achenes only germinated at 12/20°C in light, with a low final percentage of c.a. 5% (data not shown).

Table 2. Mean time germination (MTG; days) of fresh white achenes, warm-stratified and dry-stored white achenes of *Anthemis chrysantha* incubated at different conditions of temperature and illumination.

Temperature	Fresh achenes		Warm stratified achenes		Dry stored achenes	
	Light	Darkness	Light	Darkness	Light	Darkness
5°C	-	-	17.39 ± 0.247^{Ca}	18.74 ± 0.316^{Cb}	20.38 ± 1.292^{Ba}	16.99 ± 0.582^{Ba}
10°C	9.37 ± 0.453^{Ab}	7.42 ± 0.530^{ABa}	-	-.	-	-
15°C	9.22 ± 0.909^{Ab}	6.35 ± 0.257^{ABa}	6.72 ± 0.332^{Ba}	6.94 ± 0.131^{Ba}	8.29 ± 0.503^{Ab}	5.78 ± 0.690^{Aa}
20°C	7.87 ± 0.905^{Aa}	9.48 ± 1.353^{Ba}	-	-	-	-
25°C	18.72 ± 1.428^{Ba}	16.58 ± 1.217^{Ca}	-	-	-	-
12/20°C	$7.28\pm0,724^{Ab}$	5.35 ± 0.186^{Aa}	5.26 ± 0.480^{Aa}	4.57 ± 0.324^{Aa}	6.54 ± 0.119^{Aa}	6.41 ± 1.083^{Aa}
F	24.856	27.245	327.744	779.569	87.919	59.896
P	0.000	0.000	0.000	0.087	0.000	0.000

Means (± standard error) within a column that have a different uppercase letter are significantly different from each other, and means within a row that have different lowercase letter are significantly different from each other, within each achene treatment (Tukey test; $p < 0.05$).

There was no effect of light, temperature or their interaction on percentage germination of white achenes stored dry at room temperature, with a range of 58–80% (Figure 1C). However, the MTG values were affected by light ($P = 0.007$) and temperature ($P = 0.000$). Only at 15°C was germination faster in darkness than in light. Both in light and darkness the highest MTG value was at 5°C (Table 2). Dry-stored dark achenes did not germinate at any temperature.

Compared to fresh achenes, only dry-stored white achenes incubated at 15°C (both in light and darkness), and warm-stratified white achenes incubated at the same temperature in darkness showed significantly higher germination percentage ($P = 0.043$ and $P = 0.001$, respectively, in light and darkness). However, nearly all dark achenes remained dormant and did not germinate at any temperature after two months of stratification treatments or dry storage.
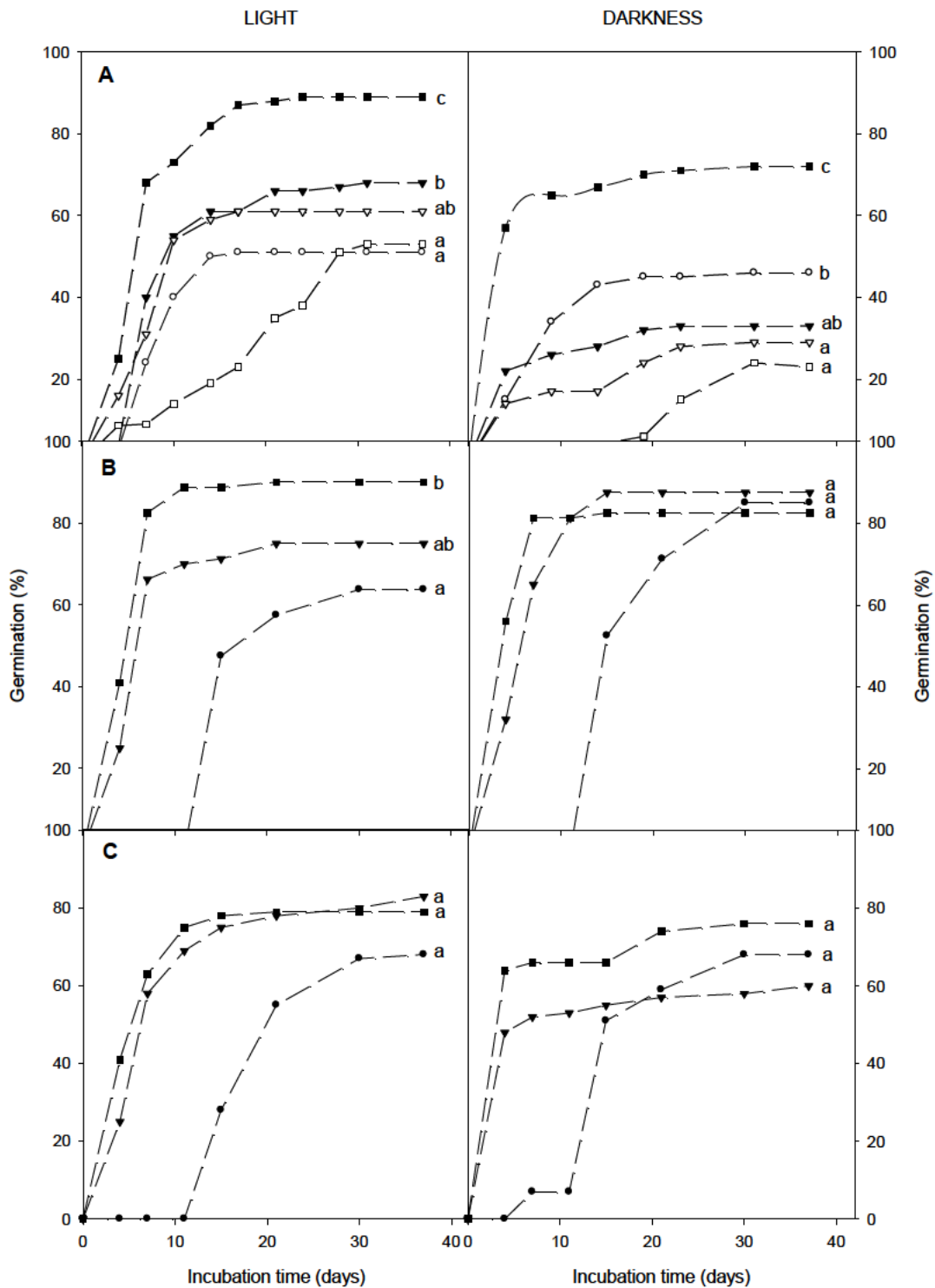
Figure 1. Germination of freshly-matured white achenes (A), after wet-warm stratification (B) and after dry storage (C) of *Anthemis chrysantha* at 5ºC (closed circles), 10ºC (open circles), 15ºC (closed triangles), 20ºC (open triangles), 25ºC (open squares) and 12/20ºC (closed squares) under light (left) or darkness (right).

3.3. Role of pericarp on embryo germination and water absorption

While dark achenes did not germinate during whole-achene germination experiments, excised embryos from dark achenes showed a high germination response of 57–71%, with no significant differences between incubation temperatures.

Excised embryos from white achenes also germinated similarly at all temperatures. However, their germination was always significantly higher than for embryos from dark achenes ($P = 0.002$ at 15°C, $P = 0.002$ at 20°C and $P = 0.011$ at 12/20°C; Figure 2), with range of 83–94%. The MTG in embryos from white achenes was lower than for dark achenes, except at 15°C (Table 3).

Table 3. Mean time germination (MTG; days) of embryos from white and dark achenes of *Anthemis chrysantha* incubated under different conditions of temperature.

Temperature (ºC)	White achenes	Dark achenes
15	9.44±0.36^{Ba}	10.29±0.58^{Aa}
20	5.42±0.40^{Aa}	12.95±1.81^{Ab}
12/20	6.28±0.16^{Aa}	11.11±0.28^{Ab}
F	42.622	1.496
P	0.006	0.275

Means (± standard error) within a column that have a different uppercase letter are significantly different from each other, and means within a row that have different lowercase letter are significantly different from each other (Tukey test; $p < 0.05$).

The germination percentage of the dark achenes scarified at the basal end was 72±4.0% similar to that obtained when the embryos were completely excised; and significantly higher ($P = 0.012$) than for apical end scarified achenes (39%). However, when ungerminated achenes were checked for viability a 22% of them were non-viable.

After achene imbibition for 36 h, the weight of embryos of white achenes increased significantly (c.a. 72%; $P = 0.000$), while embryos from dark achenes showed no increase in weight. However, after 14 d of incubation the average weight of embryos from dark achenes increased significantly ($P = 0.000$), but only by 34.5%.

In addition, when the dark achenes were incubated in the dye, the color stain was seen in the embryo, mainly both at the radicle and at the opposite end, with the rest of

the embryo not stained. However, embryos from white achenes were completely stained.
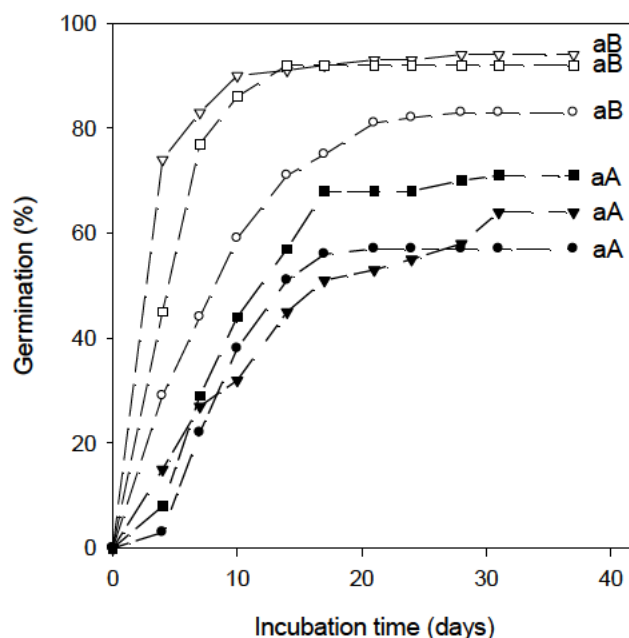
Figure 2. Germination of freshly-matured embryos of *Anthemis chrysantha* from white (open symbols) and dark (closed symbols) achenes, at 15ºC (circles), 20ºC (triangles) and 12/20ºC (squares) under light. Uppercase letters show the presence (different letter) or absence (the same letter) of statistical differences between each achene type and lowercase letters within the same achene type.

3.4. Anatomical characterization of achenes

Both types of achenes were obconic (Pictures 2A and 3A), glabrous, with 10 ribs (Pictures 2D and 3D) and usually scabrous. The ribs were almost equal and the ornamentation of the intercostal gaps was reticulate (Pictures 2A and 3A). The anatomical study showed that the dark achene wall (pericarp) was generally thicker and stronger than the white achene wall (Pictures 2E and 3F).

The white achene pericarp was 16–40 μm in thickness in the intercostal gaps and 50–80 μm in the ribs (Picture 2E). The exocarp consisted of flat epidermal cells with cellulosic walls that were not very thick. The outer tangential wall had a not very thick cuticle (0.8–0.9 μm). The cell content was very uniform, with a thick vacuole filling most of the cytosol, and was full of many microcrystals in suspension (asterisks in

Pictures 2C, 2F–2H). The middle layer (mesocarp) consisted of a single cell type - bulky cells that left some intercellular spaces (Pictures 2E–2H). The primary wall was thin and cellulosic (dark blue when stained with toluidine blue), while the secondary wall was a little thicker (light blue when stained with toluidine blue) (Pictures 2G and 2H). The rib area contained mechanical tissue-fibers that were not very developed, mixed with some tracheids (Pictures 2E and 2F). Only epidermal and parenchymal cells were found in intercostal gaps (arrowheads in Picture 2E). A thin layer of cells formed the endocarp.

The dark achene pericarp had a thickness of 30–40 μm in the intercostal gaps and 85–115 μm in the ribs (Pictures 3E and 3F). The histology of dark achenes showed, from outside to inside, an exocarp of flat epidermal cells with cellulosic walls that were not very thick. The outer tangential wall had a thick cuticle (1.5–2.5 μm). The cell content was very similar to that of white achenes with a large vacuole that filled most of the cytosol and full of many microcrystals in suspension (asterisks in Pictures 3C, 3E and 3H). Below this area, an external mesocarp consisted of 2–3 very sclerotic cell layers of thick-walled, isodiametric cells with little lumen. The walls of these cells were very lignified (dyed red by double staining with safranin-fast green and light blue with toluidine blue). There were few intercellular spaces, which made this layer a considerable and highly reinforced insulation. To the inside was the internal mesocarp of fibers arranged longitudinally in the direction of the achene (Pictures 3E and 3H). These fibers, especially abundant in ribs (Picture 3F), were sclerenchyma fibers with very little cell lumen, occupied largely by cube-shaped crystals (arrowheads in Picture 3H) located in rows along the cell. This internal mesocarp consisted of 8–10 layers of cells with no intercellular spaces. Among the fibers, some tracheids were distinguished toward the center and were probably no longer functional in mature achenes. Finally, there was a thin layer of cells forming the endocarp.
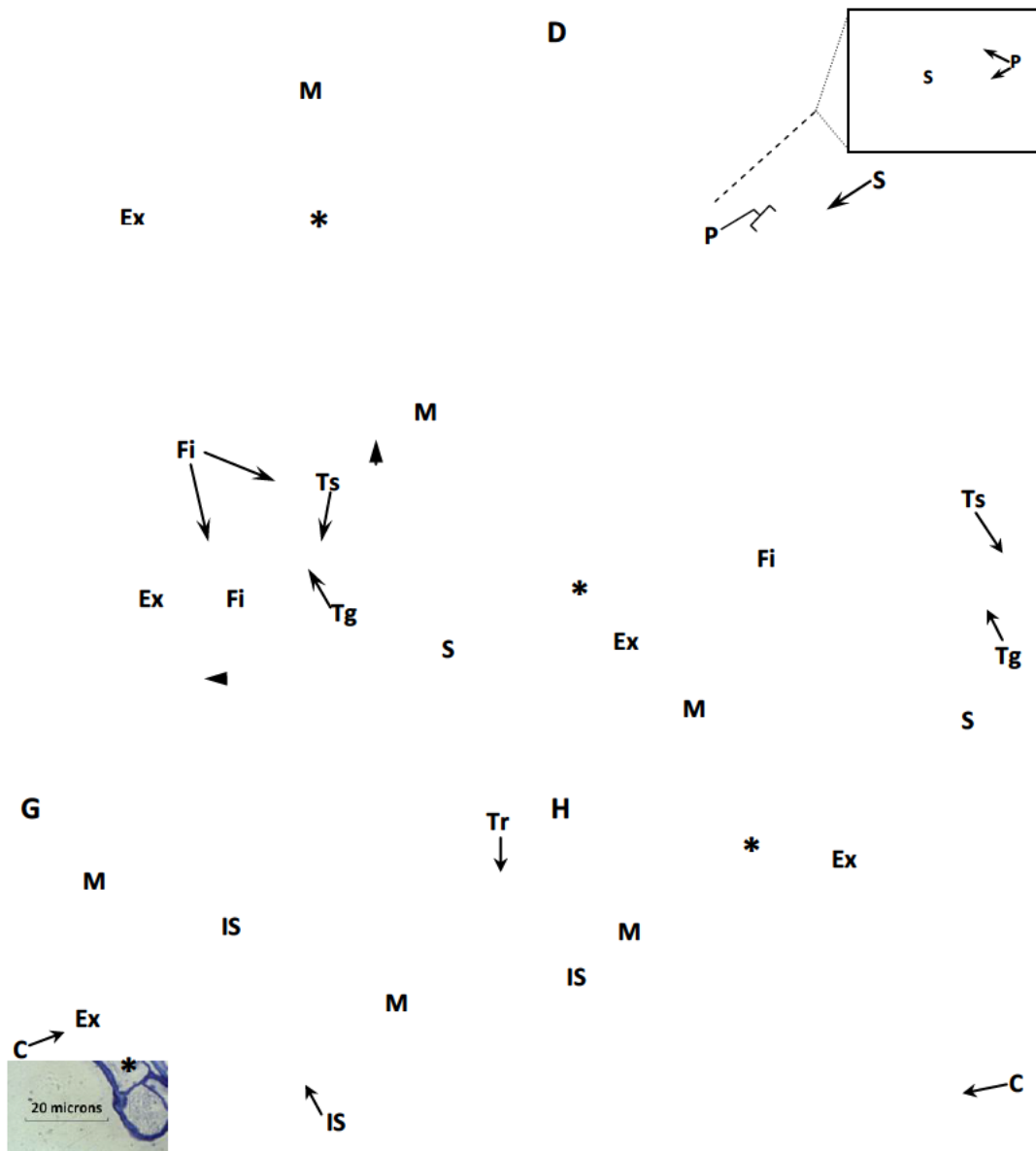
Picture 2. White achenes of *Anthemis chrysantha*. **A.** Micrograph of a white achene observed by SEM. Note the external morphology of pericarp showing ribs and intercostal gaps. **B.** Micrograph of a longitudinal section of an achene observed by SEM. **C.** Micrograph of a longitudinal section of a white achene observed by SEM. Detail of pericarp showing the

exocarp and mesocarp. Asterisks show microcrystals in suspension. **D**. Micrograph of a longitudinal section of a white achene observed by light microscopy (LM). Box: detail of a transversal section. Both sections stained with toluidine blue. Bars: 200 µm. **E**. Micrograph of a transversal section stained with toluidine blue and observed by LM. Black arrowhead show the intercostals gaps. Bar: 40 µm. **F**. Micrograph of a transversal section stained with toluidine blue (detail of a rib) and observed by LM. Asterisks show microcrystals in suspension. Bar. 20 µm. **G**. Micrograph of a longitudinal section of pericarp stained with toluidine blue and observed by LM. Bar: 20 µm. **H**. Micrograph of a longitudinal section of a rib stained with toluidine blue and observed by LM. Asterisks show microcrystals in suspension. Bar: 20 µm. **Abbreviations**: **P**, pericarp; **Ex**, exocarp; **M**, mesocarp; **En**, endocarp; **Fi**, fibers (sclerenchyma); **S**, seed; **Ts**, testa; **Tg**, tegmen; **C**, cuticle; **IS**, intercelular spaces; **Tr**, tracheids.
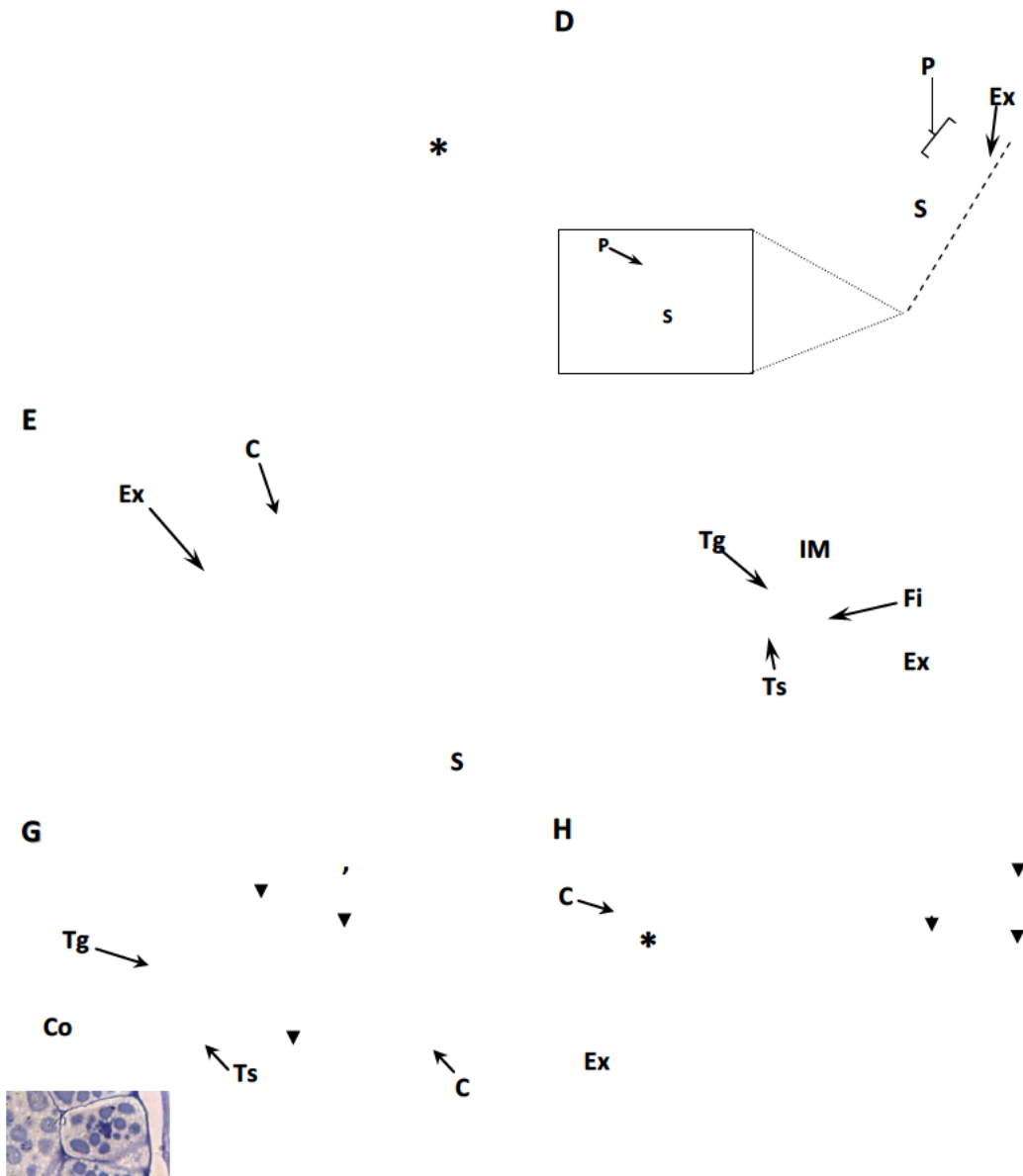
Picture 3. Dark achenes of *Anthemis chrysantha*. **A**. Micrograph of a dark achene observed by SEM. Note the external morphology of pericarp showing ribs and intercostal gaps. **B**. Micrograph of a longitudinal section of a dark achene observed by SEM. **C**. Micrograph of a longitudinal section of dark achene observed by SEM. Detail of pericarp showing the exocarp,

the external and the internal mesocarp. Asterisks show microcrystals in suspension. **D**. Micrograph of a longitudinal section of a dark achene observed by light microscopy (LM). Box: detail of a transversal section. Both sections stained with toluidine blue. Bars: 200 μm. **E**. Micrograph of a longitudinal section of a dark achene stained with safranin-fast green and observed by light microscopy (LM). Asterisks show microcrystals in suspension. **F.** Micrograph of a transversal section stained with toluidine blue and observed by LM. Note the external mesocarp and internal mesocarp in the ribs and intercostal gaps. Bar: 40 μm. **G**. Micrograph of a transversal section stained with toluidine blue (detail) and observed by LM. Arrowheads show cube-shaped crystals. Bar. 20 μm. **H**. Micrograph of a longitudinal section of pericarp stained with toluidine blue and observed by LM. Asterisks show microcrystals in suspension. Arrowheads show cube-shaped crystals located in rows along the cell. Bar: 20 μm. **Abbreviations**: **P**, pericarp; **Ex**, exocarp; **M**, mesocarp; **EM**, external mesocarp; **IM**, internal mesocarp; **En**, endocarp; **Fi**, fibers (sclerenchyma); **S**, seed; **Ts**, testa; **Tg**, tegmen; **C**, cuticle; **IS**, intercelular spaces; **Tr**, tracheids; **Co**, cotyledon cells.

4. Discussion

The present study demonstrated morphological differences in the achenes of *A. chrysantha*: basal dark achenes were slightly larger than upper white achenes in length, width, weight and pappus length. Dimorphism also occurs in many other species of Asteraceae (Imbert, 2002), although the morphological differences in *A. chrysantha* were not as visually distinct as for other species of the genus. In *A. chia*, for example, the achenes differ in presence or absence of the wing (Feinbrun–Dothan and Zohary, 1978; Imbert, 2002), and in *A. arvensis* the achenes are clearly different in size (Ellis and Ilnicki, 1968 cited in Baskin and Baskin, 1998). However, the anatomical study showed defined differences between the two achene morphs in *A. chrysantha*. The pericarp of dark achenes was thicker and stronger than that of white achenes. These differences in the fruit wall were most pronounced in the mesocarp. In dark achenes the mesocarp consisted of 2–3 external sclerotic cells with very thick lignified walls, and 8–10 layers of internal cells with few intercellular spaces, making this layer a considerable and highly reinforced insulation. The mesocarp of white achenes was much thinner than the dark achenes, and had intercellular spaces. These differences in the fruit wall may explain the different behavior of the achenes in the water absorption experiment; the embryos of dark achenes absorbed no water after 36 h of imbibitions while embryos of white achenes increased their weight by 72%. However, 7 d after immersing the dark

achenes in the dye solution, staining was observed in some areas of the embryos, although water penetrating after 14 d imbibition only increased the embryo weight by 34%. Thus, dark achenes were not water impermeable but had very slow absorption rates, so that only when soil moisture conditions were very high and prolonged, would water penetrate into the embryo. However, such conditions of soil saturation are rare in the habitat of *A. chrysantha*.

Morphological variations in the achenes may represent different dispersal ability (Greene and Johnson, 1989; Brändel, 2004). In *A. chrysantha* there are differences in the temporal dispersion pattern, for example the white achenes located in the upper rows of the capitula are dispersed some time before the basal dark achenes, as occurs in *Bidens pilosa* (Rocha, 1996) also of the Asteraceae. However, we have no data on the ability of spatial dispersion of the species, and future studies are needed to describe it in detail. In relation to this issue, and based on personal observations, we suggest that achene dispersion occurs by the patter of raindrops in the capitulum (ombrohydrochory *sensu* Gutterman, 1990) and that the presence of bracts in the receptacle between achenes may play an important role in dispersion. Pappus may have little importance in the spread of this species due to its short length which, together with the small size of the achenes, would not contribute to secondary dispersion, as occurs in several species of *Anthemis* (Gutterman, 1990; Chehregani and Mahanfar, 2007). Moreover, variation in diaspore morphology is usually correlated with diverse patterns of dormancy and germination (Venable et al., 1987; Brändel, 2004; Sun et al., 2009). In the present study these morphological and anatomical differences were related to the different behavior of the germination response of the two achene types. White achenes germinated in high percentages in many conditions, while basal dark achenes were strictly dormant and did not germinate under any of the test conditions. When fresh white achenes were incubated, the highest germination percentages were at 12/20 °C, both in light (89 ± 0.65%) and darkness (72 ± 1.85), and the mean germination time was short (5–7 d). The germination rate at constant temperatures (within 10–25°C) was lower than that at 12/20°C, although it was also relatively high (51–68%). When white achenes were stratified at 5°C, the germination was very high (90%). In general, cold stratification, warm stratification or dry storage did not improve the germination response of fresh achenes when temperature was optimal. However, at 15°C, dry storage or warm stratification improved germination. The fact that the white achenes germinated at high percentages at 12/20°C and at 5°C suggests that achenes can germinate in both autumn

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and the coldest winter months (December-January). Field observations have shown that plants emerge from late September to March, typical of a winter annual species. Alternating temperatures of 12/20°C were similar to the temperatures reached in southeast Spain in autumn, when *A. chrysantha* begins to germinate. Moreover, the ability to germinate over a range of average temperatures is common in Asteraceae (Baskin and Baskin, 1998; Schütz et al., 2002). In most temperature conditions, light appears to improve germination of fresh achenes. In many studies on germination models of plant groups coexisting in the same habitat, there are always some species that germinate better under light than in darkness (Schütz et al., 2002; Khan and Gulzar, 2003; Copete et al., 2009). This light-mediated germination mechanism may favor germination of seeds located near the surface in areas of soil disturbance and may restrict germination of seeds buried deeper in the soil, giving very little chance of successful emergence of seedlings (Grime, 1979; Milberg et al., 2000). However, darkness did not inhibit germination when temperatures were optimal (72 and 90% at 12/20 and 5°C in darkness, respectively), contrary to expectations for most species with small seeds (Grime 1979; Milberg et al., 2000). After warm stratification or dry storage, white achenes germinated independently of light/darkness conditions.

In Asteraceae, the non-germination of some achene types could be due to the presence of inhibitors in surrounding tissues (Beneke et al., 1993), thick pericarp (McEvoy, 1984; Tanowitz et al., 1987) or innate dormancy (Negbi and Tamari, 1963) as cited by Sun et al. (2009). The embryo germination test showed that embryos from fresh dark achenes reached significant germination percentages (57–71%), although lower than for embryos from fresh white achenes. Additionally, the scarification test showed that when the dark achenes were scarified at the basal end the germination percentage was similar (72%) to that for completely excised embryos. However, when these were scarified on the apical end the germination reached 39%. This indicates that the pericarp of the dark achenes was primarily a mechanical constraint to radicle emergence. The fact that the germination of embryos excised from achenes was not higher than the scarified achenes rules out the presence of inhibitors in the pericarp. Therefore, the causes of dormancy in the dark achenes should be sought in the thickness of their pericarp - physically impeding germination and making imbibition of water difficult - as shown in other Asteraceae species (McEvoy, 1984; Tanowitz et al., 1987). The hypothesis of physiological dormancy can be rejected, since dark achenes did not increase their germination response after being subjected to wet cold and warm

stratification (e.g. Baskin and Baskin, 1998; Baskin et al., 2000, 2004). Moreover, if we must taking to account that around 22% of dark achenes are non-viable, almost viable achenes germinated when they were scarified at the basal end. This percentage of non-viable dark achenes can be explain the differences in germination between those and white achenes showed in Figure 2.

In Asteraceae, the difference in mass of fruits is mainly due to differences in embryo size, but in a few species the difference is also due to pericarp structure (Venable and Levin, 1985; Beneke et al., 1992; Imbert et al., 1999). However, our study showed no difference in weight between embryos from white and dark achenes (Table 1); therefore the greater weight of the dark achenes (Table 1) was due to the reinforced structure of their pericarp. Similar observations were made by Ellis and Ilnicki (1968) in *A. arvensis*, and they attributed the lower germination of larger achenes to pericarp hardening due to increased lignification. Thus, according to these authors and many others (Forsyth and Brown, 1982; Tanowitz et al., 1987; Mohamed–Yasseen et al., 1994; Sun et al., 2009), the thickness and structure of the pericarp plays an important role in germination, possibly due to differences in imbibition time, oxygen exchange and leakage of germination inhibitors. The embryos of white achenes were not dormant, and the loosely-structured pericarp did not prevent the passage of water through it.

Differences in dormancy levels of achenes on the same plant enable plants to spread their offspring in time (Venable, 1985). The results of our study suggest that the strategy and ecological adaptation of *A. chrysantha* are similar to those of most other heteromorphic species (Brändel, 2004; Sun et al., 2009), especially those in dry habitats (Gutterman, 1993). The different capacity of germination of white achenes (temperature dependent) and the complete dormancy of dark achenes ensures that not all dispersed achenes germinate at the same time. White achenes germinate quickly after a pulse of dispersion, especially if caused by rain. The delay of dispersal and germination of the dark achenes constitute a very safe means of reproduction and an available achene reserve on/in soil of an unpredictable habitat, and thus increases the probability of persistence of an *A. chrysantha* population, as described by Sun et al. (2009) for *Garhadiolus papposus* of the Asteraceae. Moreover, delayed germination and seed storage in the soil increases the separation of genotypes in time and space (Baker, 1974), which may be particularly advantageous in the Asteraceae, where self-incompatibility is very frequent and would lead to a reduction of fertile progeny (Pandey, 1960).

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The results of the present study suggest the hypothesis that the presence of a large seed bank in the soil would play an important role in the population dynamics of *A. chrysantha*. Therefore, future studies should investigate the role of *A. chrysantha* seed banks in their habitats, as well as the mechanisms of achene dispersion in time and space.

Acknowledgements

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CHAPTER 2

Aerial seed bank and dispersal traits in *Anthemis chrysantha* (Asteraceae), a critically endangered species.

Abstract

Mature plant density and fruit production were monitored in the main population of four successive cohorts of the endangered winter annual herb *Anthemis chrysantha* (Asteraceae) in southeastern Spain. Experiments were conducted with artificial rainfall and a wind tunnel to determine the temporal and spatial dispersal pattern of the species and the relationship with rain and wind.

The population fluctuations of *A. chrysantha* observed during 2006–2009 could be explained by the rainfall amount and seasonal distribution, which in turn influenced the production of achenes. This study demonstrated the formation of a seasonal aerial achene bank that remained on the dead plant from the fruiting period (early summer) to late spring. We experimentally demonstrated that rain was necessary to release the achenes from the capitula (ombrohydrochory), with wind having no effect on dispersal. The pattern of released achenes was related to rainfall events. The main timing of dispersal was during autumn, decreasing the aerial bank by around 80% by December. However, in March, when the next cohort was flowering, some achenes remained on old capitula (up to 1,700 achenes per m^2 in some years). White non-dormant achenes were first released, and dark dormant achenes were released later. The non-dormant achenes on the soil surface could germinate quickly (2 d) after receiving as a minimum as 10 mm of rainfall at autumn temperatures. Most achenes dispersed very short distances (atelechory): 75% of them landed beneath the plant canopy and only 7% landed > 30 cm from the canopy edge. Results suggest that seasonal aerial seed bank is an effective trait to ensure the maintenance of this species in its unpredictable habitat.

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1. Introduction

Annual plant populations in unpredictable habitats are often subject to large fluctuations over time, mainly due to the amount of available water being spatially and temporally highly variable (Brown, 2002). Often, these plants have specific strategies to increase their reproductive success. In some plants (e.g. Asteraceae) the production of two or more seed or fruit morphs with different germinative behaviors within a single plant seems to be a good adaptive strategy in unpredictable habitats (Larson and Kiemnec, 1997; Imbert, 2002; Brändel, 2004). Another strategy allowing reduction of environmental risks is retaining mature seeds on the dead mother plant as a protected long-term seed bank (Gutterman, 2000). After dispersal, the seeds are harvested and consumed by insects, birds and other animals, thus species that protect their seeds have a survival advantage (Gutterman and Guinott, 1994). Long-term seed retention (> 1 year), i.e. serotiny, has been well documented in plants from arid to Mediterranean regions, however short-term (< 1 y) seed retention, or bradychory, has received much less attention (Bastida et al., 2010).

An aerial seed bank could also favor a gradual dispersal of seeds. In some species the dead mother plants are the main seed bank that periodically releases a portion of the seeds with rain, over a period of many years (Gutterman, 1993). Annual species, inhabiting deserts or arid zones, frequently disperse seeds by ombrohydrochory (Gutterman, 1990), a special form of hydrochory which occurs primarily in environments where rain has a determining role in plant life. Ombrohydrochory is based on dispersal by water drops which cause the activation of the ballistic mechanism in the plant. The pulse of dispersal, which coincides with rainfall, may have implications for possible gradual germination. Fractional germination is another strategy that enables desert annuals to persist in harsh environments in which survival and fitness are highly variable from year to year (Pake and Venable, 1996). Germination of small portions of the large seed-bank following small amounts of rain during mild winter temperatures, when further rainfall is likely, is an opportunistic germination strategy of some plants (Gutterman, 2000).

Dispersal mechanisms are related to dispersal distance, and usually seed dispersed by ombrohydrochory have reduced distances (atelechory) (Vittoz and Engler (2007). Both temporal and spatial seed dispersal can influence population biology. If

temporal dispersal can promote a gradual germination, spatial distribution will not only determine the distribution of a species, but also the environmental variability encountered by its seedlings and saplings and thus, ultimately, the probability of new adults into the population (Gómez et al., 2004). Reduced dispersal promotes persistence in areas where conditions, although variable, are conducive to germination and establishment. By contrast, reduced dispersal prevents possible expansion of the range of the species (Tanowitz et al., 1987). Field observations suggest that bradychory could occur in *Anthemis chrysantha* J. Gay, an endemism of the southeastern Iberian Peninsula and the Algerian coast, and which is critically endangered. Field observations suggest that raindrops are responsible for the gradual fall of achenes in the capitula of *A. chrysantha*. To get a deeper understanding of specific survival strategies of *A. chrysantha*, the aims of this study were to (a) determine the importance over time of a possible transitional aerial seed bank; (b) understand the role that physical meteorological agents (e.g. rain and wind) could play in the dispersal of the achenes; (c) study the temporal and spatial dispersal of the achenes; and (d) discuss on the importance of physical and biotic factors in the establishment of annual cohorts from year to year.

2. Material and methods

2.1. Plant material

The genus *Anthemis* is the second largest genus in Asteraceae (tribe Anthemidae), and has been an interesting differentiation process in the last 12 Myr in the circum-Mediterranean region, reaching the colonization of a wide diversity of ecological niches (Lo Presti et al., 2009; 2010). In fact, the genus *Anthemis* has more than 210 species in the Mediterranean region, southwest Asia, and eastern Africa (Oberprieler, 2001); about 62 species are distributed in Europe and 14 in the Iberian Peninsula.

Among these species, *Anthemis chrysantha* is a winter annual plant with erect habit, corymbosely branched, and reaching a height of 30 cm. The emergence period begins with the first autumn rains, stretching into spring if weather conditions are favorable. Flowering occurs from early March to late May, and fruits mature mostly in

June. The plants die in summer, but persist in a dried state in the habitat for several months after death (Picture 1). The capitula, appearing in the extremities of the erect branches, are long-pedunculate and hard when dry. The receptacle is hemispherical to oblong-ovoid, rounded at the apex, with receptacular bracts longer than achenes. Achenes are obconical, small (shorter than 2 mm in size and between 0.15 and 0.30 mg in weight), with ten granular ribs and a denticulate rim or sometimes a short pappus (0.3 to 0.5 mm in lengh). These achenes are heteromorphic with a different position in the capitulum. The rows of the upper section containing elongated, almost white achenes (referred to as white achenes hereafter), while the achenes of the basal rows are brown-black (dark achenes hereafter) and harder than white achenes. Finally, the last basal line is composed of achenes from ligules (only 8–10 per capitulum). Each capitulum contains about 100–130 achenes, with receptacular bracts between them. The proportions of white and dark achenes in a capitulum are about 70 and 30%, respectively. The morphological differences between the two achene types are correlated with variations in their germinability. White achenes germinate to high percentages in many conditions, while basal dark achenes are strictly dormant (Aguado et al., 2011; Chapter 1).
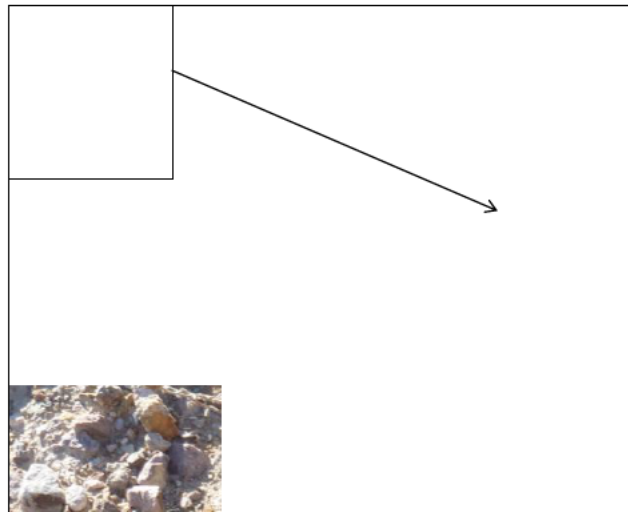
Picture 1. Dead plant of *Anthemis chrysantha* and detail of two capitula which had dispersed some achenes.

2.2. Study site

Anthemis chrysantha is only found on the Algerian coast and the coast of Cartagena (Murcia, southeastern Spain); the latter is the only European location (Bañares et al., 2004). In Cartagena, four populations were known, but since the late
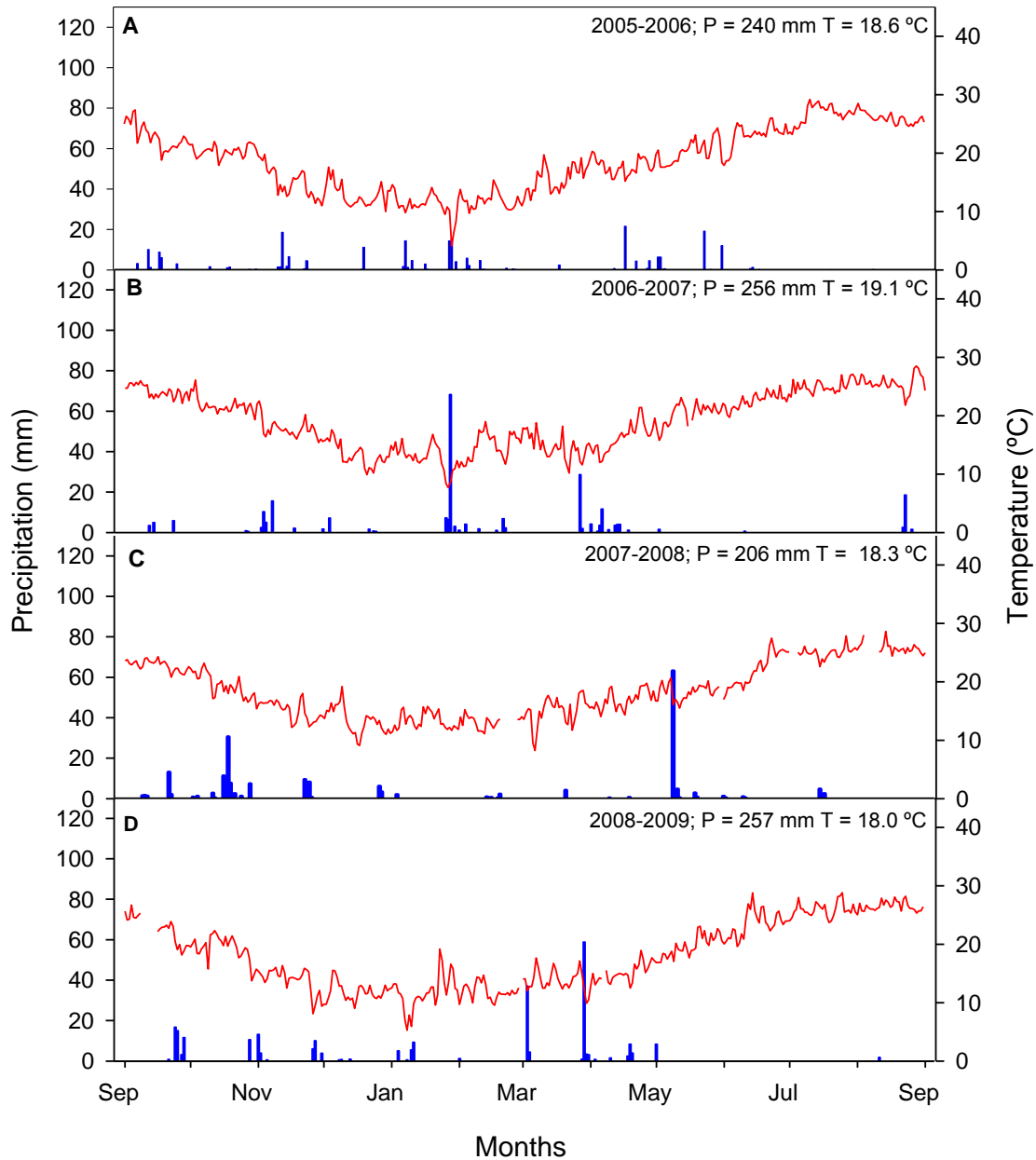
Figure 1. Daily precipitation (bars) and mean daily temperatures (lines) from September 2005 to August 2006 (A), September 2006 to August 2007 (B), September 2007 to August 2008 (C), and September 2008 to August 2009 (D) in the studied area. *P* = total precipitation in each period; *T* = mean temperature for each period.

1990s only two remain, occupying an area of < 2 ha. One of these is located in La Azohía (Cartagena, Murcia; 37º33′8″N; 1º10′22″W; altitude 30 m.a.s.l.), with a semi-arid Mediterranean climate characterized by irregular rainfall and a long dry summer. The mean annual rainfall for a 30–y period is around 300 mm, and the mean annual air temperature is 17.6ºC, the warmest month is August and the coldest month is January (monthly mean 24.9 and 10.4ºC, mean maximal 28.9 and 15.3ºC, and mean minimal temperatures 23.4 and 6ºC, respectively).

Precipitation and temperature during the study period (2006–2009) are shown in Figure1. Daily climatic data were provided by the Agricultural Information Service of Murcia (SIAM) from the Cañada Gallego Meteorological Station, located about 15 km from La Azohía. The temperature regime was similar in all years of the study period, but there were differences between years in both the total amount of annual rainfall and its temporal distribution. The driest year was 2008, with only 9.3 mm of rainfall during the flowering period of the plant, compared to 49.7, 81.4 and 126.9 mm in the same period in 2006, 2007 and 2009, respectively. In 2009, 107.6 mm of rain fell in March.

2.3. Seed production and aerial seed bank

The study was carried out in the natural population of *A. chrysantha* in La Azohía, from late June 2006 to late March 2009. To determine seed production and the significance of the aerial seed bank, 50 plots (1 m × 1 m) were selected along five transects, each of 100 m in length. Transects were designed perpendicular to the line of maximum slope on the site colonized by the species (0.5 ha). To estimate seed production, plants of the annual cohort within these plots were counted in June, when all individuals had flowered, and they were subsequently sampled in December of the same year and in March of the following year, with the aim of determining the aerial seed bank evolution. In the sampling of June, all individuals in the plots and the total number of capitula per plant were counted; the same was counted in December and March, but dead plants without capitula or capitula without achenes were not counted. At each sampling date, 30 mature capitula were collected from plants growing outside plots, and taken to the laboratory where the number of achenes per capitulum was counted using a stereoscopic microscope (Olympus SZ61). The achenes were counted

by separating white from dark from 2007 onward. Finally, we estimated the number of achenes per m^2 using the following expression:

Total no. achenes·m^{-2} = (no. plants·m^{-2}) × (no. capitulum·plant^{-1}) × (no. achenes·capitulum^{-1})

2.4. Achene dispersal studies

To determine the role the wind plays in dispersal of achenes, 30 intact mature capitula were individually exposed to a wind of 50 km·h^{-1} for 10 min; for that, we designed a wind tunnel (Picture 2) consisting of a polyethylene horizontal cylinder (43 cm in length and 4.5 cm in diameter) fixed to a metal support column. A small slit was made along the cylinder to place the capitulum in the middle of the wind tunnel. At the end of the tunnel a sheet with double-sided adhesive was placed to fix the dispersed achenes from each capitulum exposed. A hair dryer (230 V, 1400 W), adapted to the entrance of the tunnel, produced the airstream. Wind speed was monitored with a flow anemometer (PCE Group; model PCE–007).
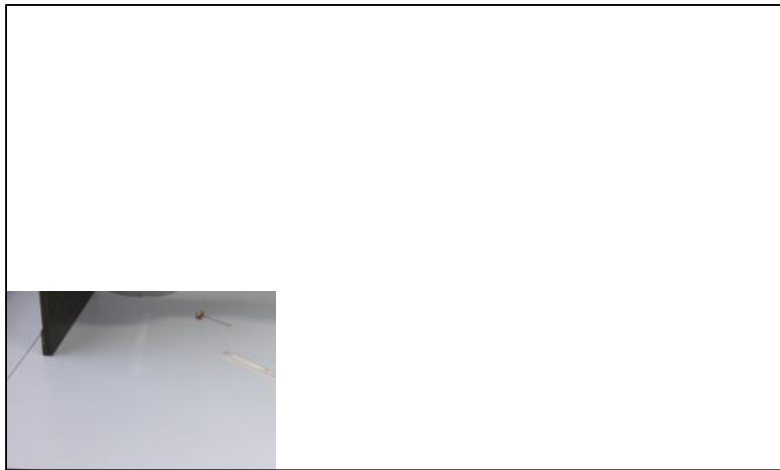
Picture 2. Wind tunnel designed to determine the role of the wind in achene dispersal.

To study the role of the rain in achene dispersal, 60 mature capitula were separated from mother plants, and secured tightly to a wood rod of 25 cm in length. This rod was firmly anchored on a 9–cm–diameter pot (200 cm^3 volume) filled with a heavy substrate of sand and gravel. To collect the released achenes from each capitulum and to prevent their falling into the pot (making them impossible to count) the pots was

covered with an inverted plastic funnel (11–cm diameter and 14–cm high) and placed on a tray (43.5 cm × 27.5 cm × 7.5 cm) with drainage, but covered with a nylon gauze to avoid loss of achenes (Picture 3). Thirty of these trays were placed on a metal table outdoors at the Agricultural Experimental Station of the Technical University of Cartagena, located about 30 km from La Azohía, from September 2008 (at the beginning of the rainy season) to May 2009 (when all capitula were empty). Another 30 trays were used as controls protected from rain inside a greenhouse. After each rain event, the achenes released into trays from each capitulum were counted.

Picture 3. Structure designed to determine the role of the rain in achene dispersal.

To estimate the seed dispersal distance caused by rain drops, three whole plants (about 100–130 capitula each) were individually subjected to a simulated rainfall event. Each plant was placed in the center of a padded area of polyurethane foam of 3.5 m × 3.5 m. The foam allowed the water to drain but retained the achenes at the exact point where they fell. The mean diameters of the projected area of the aerial part of each plant (obtained from two perpendicular diameters measured in each) were 32, 26.5 and 46 cm for plants P1, P2 and P3, respectively. The rain simulator consisted of a nozzle at a height 2.40 m at the top of a greenhouse structure. The nozzle was connected to a polyethylene tube delivering a water flow rate of 0.283 L·s^{-1}. The intensity of rain was 30 L·h^{-1}, achieved by alternating periods of rain and no rain of 1.74 and 58.26 s, respectively, using a solenoid coupled to a sprinkler timer. The intensity was checked before the trial by five rain gauges distributed uniformly over this surface. The water

from the nozzle fell onto a circular surface of about 0.7 m in diameter. The nozzle had 50 holes of 1 mm in diameter each. Thus, each plant was exposed to simulated rain consisting of drops of uniform size. Achene dispersal gradients resulting from rain splash were studied by measuring the horizontal dispersal of achenes from plants placed under the rain generator. Achene dispersal was assessed by placing a graduated methacrylate layer on the foam (Picture 4) to facilitate the counting of achenes per cm^2.
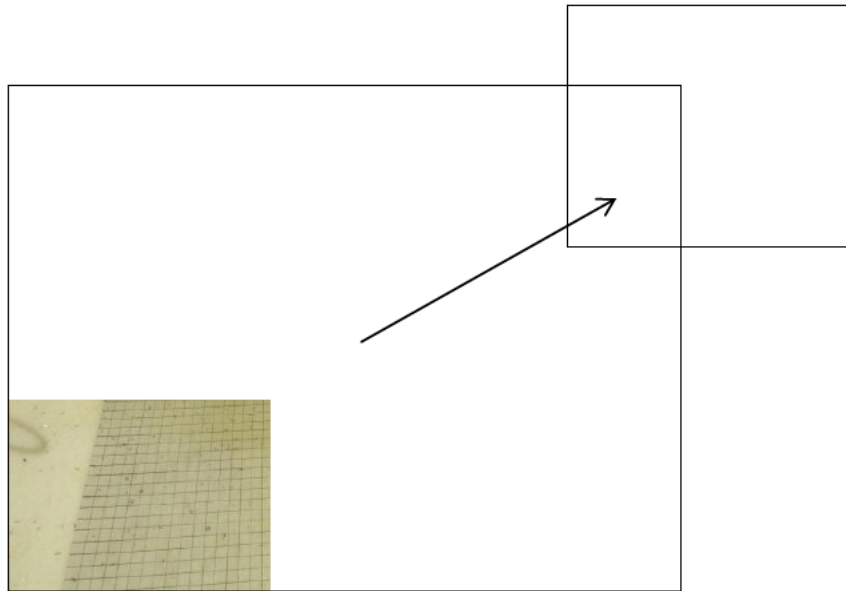
Picture 4. The graduated methacrylate layer on the foam padded area of polyurethane.

2.5. Minimum rainfall to stimulate germination

To determine the minimum amount of rain required for *A. chrysantha* achene germination, irrigation doses equivalent to rainfall of 5, 7, 10, 15 and 20 mm were tested on achenes buried at 0.5 cm deep or placed on the soil surface. For each dose and depth a 100–achene lot was used: sown as four replicates of 25 achenes each in pots of 7.5 cm in diameter and 5 cm deep. These pots were filled with sterilized soil obtained from the habitat of the species. For each depth, four replicates of 25 achenes each watered until saturation, were used as controls. The trial was carried out in a laboratory at room temperature (18–20ºC). All achenes used in this experiment were white, as dark achenes are dormant and rarely germinate (Aguado et al., 2011; Chapter 1).

2.6. Statistical analysis

A multivariate analysis of variance was used to evaluate the behavior of the aerial seed bank. Data were analyzed with the software SPSS 19.0 for Windows (SPSS Inc, Chicago, USA). When significant main effects existed, differences were tested by Tukey's multiple comparison tests at $P < 0.05$. The rain dispersal pattern of the achenes and relationships between plant density and production of capitulum per plant were analyzed using simple linear regression in Sigma Plot 8.0 (SPSS Inc).

3. Results

3.1. Seed production and aerial seed bank

The achene number on capitula per m^2 in late June ranged from $2{,}032 \pm 586$ in 2008 to $24{,}870 \pm 5{,}054$ in 2007. The achene production in the 2008 cohort was significantly lower than that in the 2006, 2007 and 2009 cohorts (Table 1). The average of capitula produced per plant ranged from 1.4 in 2008 to 8.6 in 2006, and the mean number of achenes per capitulum ranged from 47.3 in 2008 to 137.9 in 2009. Both capitula number per plant and achenes per capitulum had the lowest values in the 2008 cohort (Table 1). The plant number per m^2 sampled in June did not change significantly in 2006, 2008 and 2009 cohorts; with range about 20–40. However, in June 2007 the population density was much higher, reaching average values of 80 plants per m^2 (Table 1). Only in the 2007 cohort, when density of plants was very high, there was a negative correlation between this parameter and the production of capitula per plant (Figure 2A).

In each annual cohort, the achene number per capitulum, capitula per plant and plant density containing at least one capitulum with remaining achenes decreased during June-March. By December, 68–83% of achenes produced in June had been released. Thus, the achene number on capitula per m^2 decreased from the fruiting season (June) until the following spring (March), i.e. in cohort 2006 from $19{,}834 \pm 3{,}928$ to 201 ± 45 (Table 1). Most achenes contained in capitula collected in June were white, but in subsequent sampling the proportion of white achenes remaining in the capitula decreased, so that by March most achenes contained were dark (Table 1).

Table 1. Variation in achene number and achene type per capitulum, capitulum number per plant, plant number per m^2 and achene number per m^2 (mean ± standard error) in the studied population of *Anthemis chrysantha* for 2006–2009 cohorts.

Parameters	Samplings	*2006 Cohort*	*2007 Cohort*	*2008 Cohort*	*2009 Cohort*	
Achenes per capitulum	June	110.3±3.84Bc	127.1±5.21BCb	47.3±8.96Ab	137.9±4.69Cc	F = 45.623; P = 0.000
	December	18.6±3.17Ab	24.2±3.94Aa	15.4±2.42Aa	26.1±4.57Ab	F = 1.859; P = 0.140
	March	3.2±1.33Aa	20.0±2.68Ba	7.0±1.65Aa	6.9±1.30Aa	F = 16.238; p = 0.000
	F	378.603	221.064	15.239	336.539	
	P	0.000	0.000	0.000	0.000	
% of white achenes per capitulum	June	-	72.8±3.18Ab	87.7±3.36Ab	56.4±2.58Ab	F = 2.078; P = 0.131
	December	-	-	47.5±5.39Ba	14.2±2.40Aa	F = 21.298; P = 0.000
	March	-	27.0±4.29Aa	40.0±7.13Aa	26.7±5.84Aa	F = 1.184 ; P= 0.312
	F	-	57.134	31.431	26.376	
	P	-	0.000	0.000	0.000	
Capitula per plant	June	8.6±0.52Db	2.4±0.09Bb	1.4±0.05Aa	3.1±0.11Cb	F = 226.784; P = 0.000
	December	5.5±0.40Ca	1.7±0.02Aa	1.9±0.12Ab	2.6±0.10Bb	F = 250.563; P = 0.000
	March	4.5±0.28Ba	1.6±0.02Aa	1.7±0.11Aab	1.6±0.09Aa	F = 228.041; P = 0.000
	F	22.783	83.238	11.392	15.007	
	P	0.000	0.000	0.000	0.000	
Plants·m^{-2}	June	20.9±4.15Aa	82.6±16.80Ba	30.2±8.71Ab	44.4±8.19Ab	F = 6.679; P = 0.000
	December	17.1±3.38Aa	76.9±11.31Ba	5.6±1.54Aa	11.6±2.74Aa	F = 26.354; P = 0.000
	March	14.2±3.19Aa	54.3±7.62Ba	2.2±0.73Aa	4.5±1.02Aa	F = 32.660; P = 0.000
	F	0.660	1.435	8.904	18.029	
	P	0.519	0.241	0.000	0.000	
Achenes on capitula·m^{-2}	June	19,834.1±3,928.85Bb	24,870.5±5,054.77Bb	2,032.1±586.22Ab	16,469.6±3,232.44Bb	F = 7.430; P = 0.000
	December	1,734.5±342.47ABa	3,129.0±460.07Ca	165.1±45.80Aa	863.0±203.65ABa	F = 19.955; P = 0.000
	March	201.6±45.10Aa	1,731.3±242.61Ba	26.0±8.69Aa	64.0±16.45Aa	F = 37.827; P = 0.000
	F	10.502	19.559	10.886	24.467	
	P	0.000	0.000	0.000	0.000	
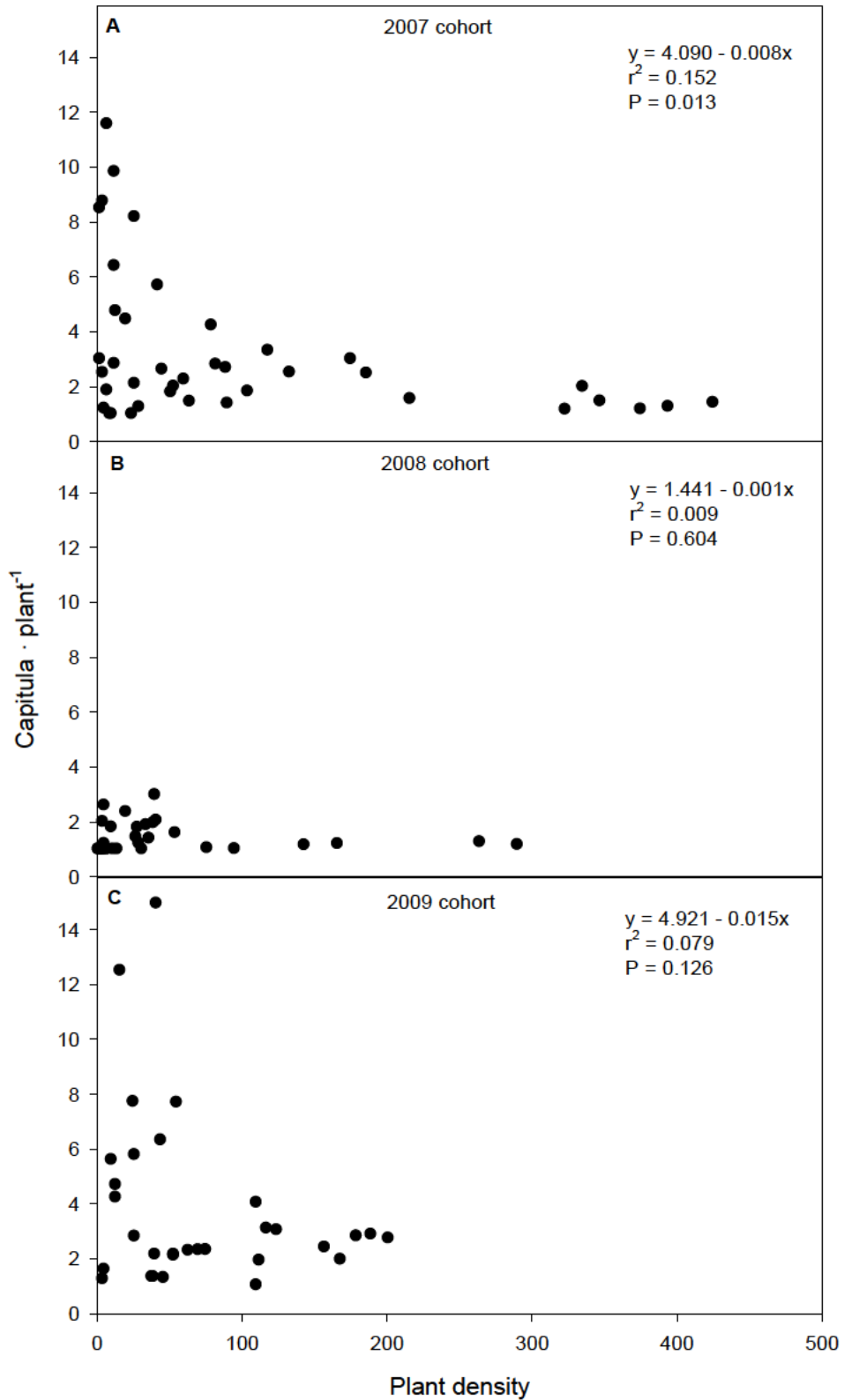
Figure 2. Capitula per plant as a function of plant density in 2007 cohort (A), 2008 cohort (B), and 2009 cohort (C).

3.2. Achene dispersal studies

There was no achene dispersal in any of the 30 capitula exposed in the wind tunnel. During the exposure period of capitula to natural rainfall (from 25 September 2008 to 13 May 2009) there were 27 rainfall events (511.2 mm in total). Most capitula showed an extended period of achene release, up to early May, when all capitula were empty or had fallen (Figure 3). Achene release was cued by rainfall and, although a minimum amount of rain was able to disperse achenes, the main dispersal events occurred with high precipitation (> 40 mm; Figure 3A). Most achene release occurred before December (87 and 66% of white and dark achenes, respectively). In this period (with 15 rainfall events) there was a positive correlation between achene dispersal and the intensity of rain ($r = 0.928$, $P = 0.000$ for white achenes; and $r = 0.612$, $P = 0.015$ for dark; Figure 4A). However, during December-May, coinciding with the low number of capitula and achenes which remained in pots, there was a low dispersal despite the high rainfall (Figure 3B). In this second period (with 12 rainfall events) there was no correlation between released achenes and intensity of rain ($r = 0.350$, $P = 0.265$ for white achenes; and $r = 0.231$, $P = 0.475$ for dark; Figure 4B).

More white than dark achenes were released for most of the achene dispersal period, especially in the first rainfall events (until the 21st event). However, in the last six precipitation events most dispersed achenes were dark (Figure 3A).

There was no achene release from capitula protected from rain in a greenhouse, used as a control, during the nine months of the study (September 2008 to May 2009).

The average number of achenes dispersed per plant in the artificial rainfall event was $1,080 \pm 482.5$. The achene dispersal gradient showed that $74.9 \pm 0.17\%$ of them landed beneath the plant canopy (77.2, 53.1 and 94.8% for plants P1, P2 and P3, respectively). Moreover, a mean of only 7% of achenes landed > 30 cm from the canopy edge. The maximum distances achieved were 99, 131 and 45 cm from the canopy edge for plants P1, P2 and P3, respectively.
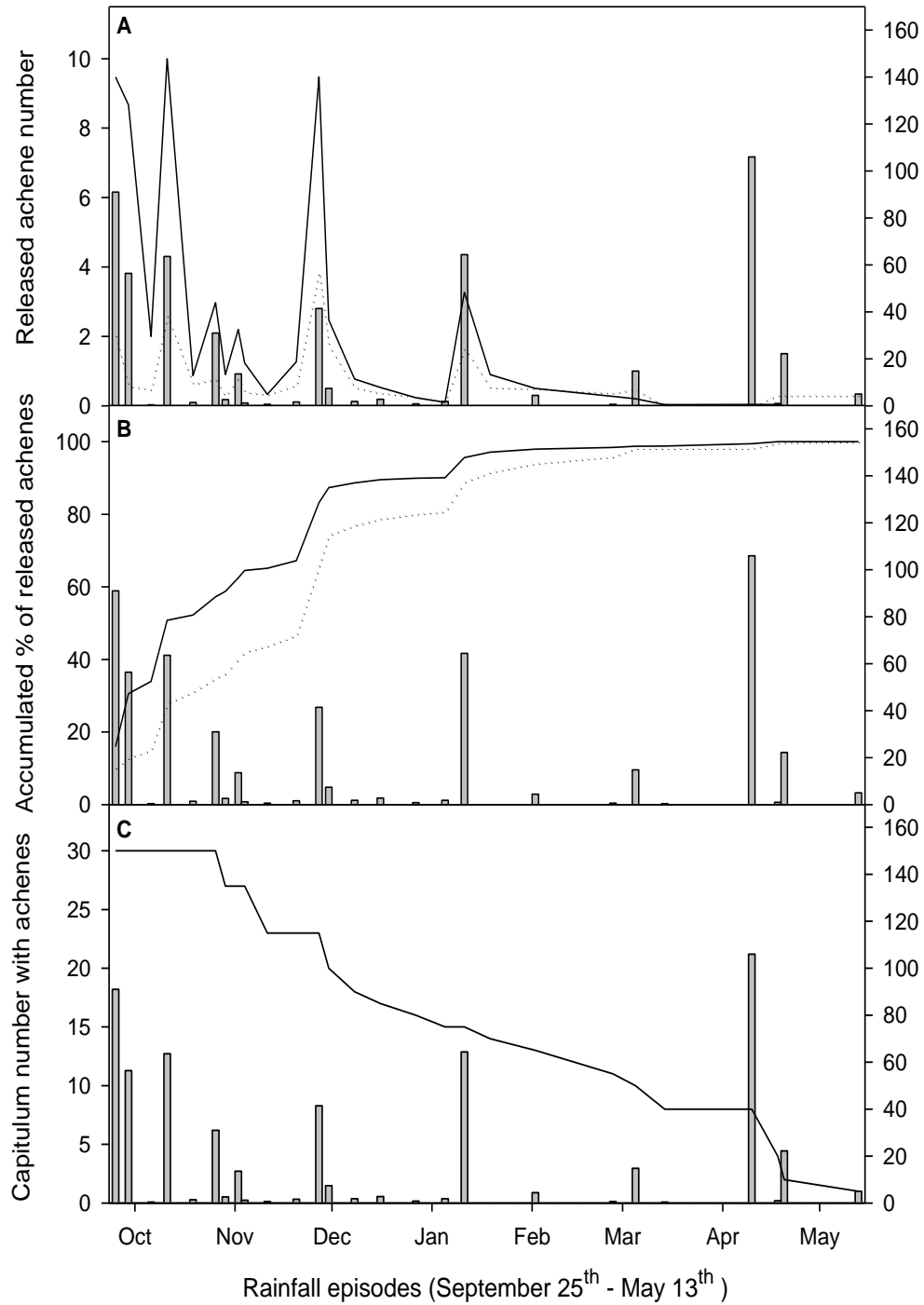
Figure 3. (A) Precipitation (bars) and released achene number for each rainfall event during the studied period (25 September to 13 May). (B) Precipitation (bars) and accumulated percentage of released achenes for each rainfall event during the studied period (25 September to 13 May). (C) Precipitation (bars) and number of capitula with achenes for each rainfall event during the studied period (25 September to 13 May). Solid line represents white achenes and dashed line represents dark achenes.
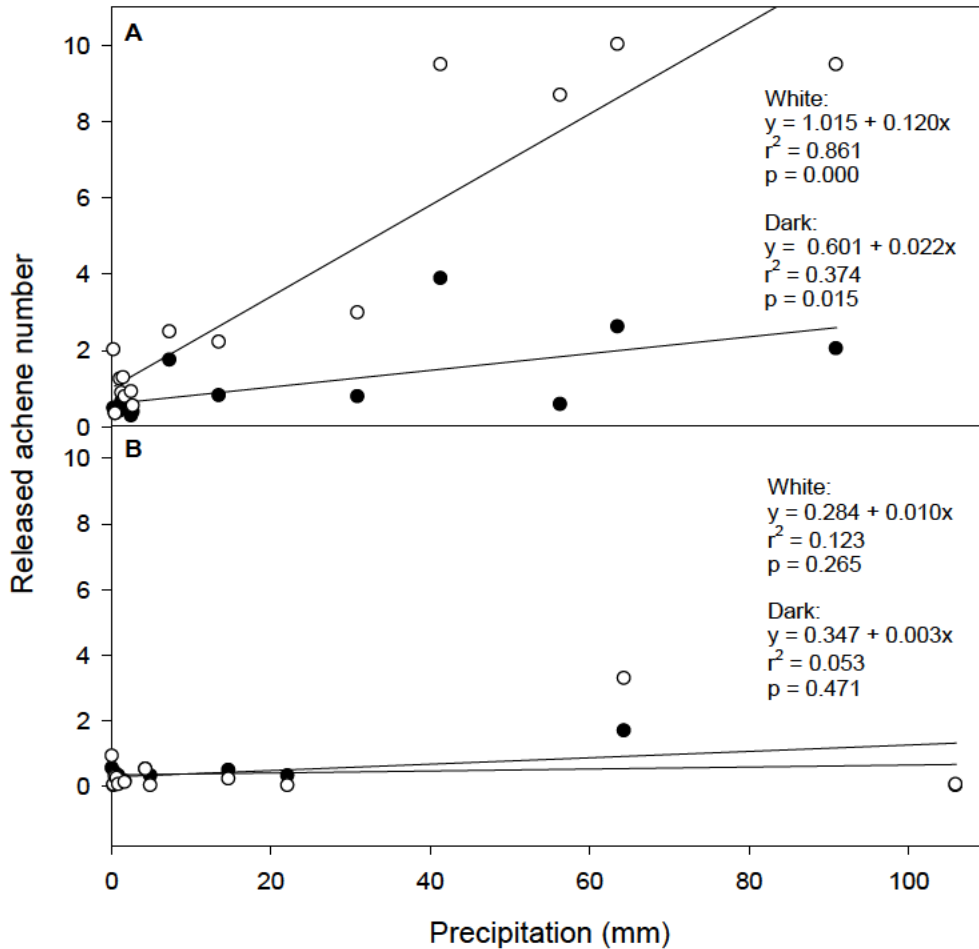
Figure 4. Number of released white (open circles) and dark (closed circles) achenes as a function of precipitation in the first 15 rainfall events (A) or in the last 12 rainfall events (B) of the experimental period Sept 2008 through May 2009.

3.3. Minimum rainfall to stimulate germination

The minimum amount of water required for white achene germination was 10 mm, reaching germination percentages of 8 and 1% for achenes on the soil surface or at 0.5 cm deep, respectively. The germination response increased with increased amount of water provided up to 20 mm, when > 80% of achenes on the soil surface, and around 20% of those buried, had germinated. There were no statistically significant differences between the control treatment (watered until saturation) and the 20–mm treatment. Dark achenes are dormant as showed Aguado et al. (2011) (Chapter 1).

4. Discussion

The data of plant density in each studied cohort showed year-to-year changes in the individual numbers of the *A. chrysantha* population. Changes of density in wild populations occur in response to a confluence of factors, such as nutrient and water availability or climatic conditions (Bishir and Namkoong, 1992). In the present case the rain distribution during the study period seemed to be the main factor determining plant density, as it affected both the survival of plants and seed production. Thus, the high density of fruiting plants in the 2007 cohort could be explained by the high production of achenes in the previous cohort (Table 1) and by the regular distribution of rainfall during the life cycle of the 2007 cohort (Figure 1B), both during the growing season (autumn-winter) and the flowering period (February-April). In the 2008 cohort, autumn rainfall was sufficient for seedling recruitment and establishment of plants. So, density of the 2008 cohort was not significantly different to the 2006 and 2009 cohorts. However, the spring drought in 2008 (only 9.3 mm of rain fell during February-April; Figure 1C) caused high mortality of plants before they reached fruiting, and the final density in June 2008 was lower than in 2007. Additionally, these low precipitation values during the fruiting period caused low production of achenes, thus possibly conditioning the emergence of the next cohort. Hence, plant density in the 2009 cohort was lower than in 2007, despite the high precipitation during the life cycle of the 2009 cohort (Figure 1D). In short, the population fluctuations of *A. chrysantha* can be explained by the distribution of rainfall and the total annual amount, which in turn influenced the production of seeds.

The availability of soil water for plants depends on rainfall and also on competition. This study showed that the number of capitula produced by an *A. chrysantha* plant was plant density-dependent, as has been observed in other Asteraceae (Ruiz de Clavijo and Jiménez, 1998); however, in the present case only from a very high threshold of density. In fact, only in cohorts from years with good precipitation (i.e. 2007) a very high plant density caused low capitula production (Figure 2A). Density is not the only factor affecting capitula formation but also the amount of rainfall and its overall distribution in spring. Thus, when spring rainfall was low (i.e. 2008) plants produced few capitula although plant density was low (Figure 2B). In the 2009 cohort, the production of capitula per plant was lower than expected if we consider the

low density of plants and the high spring precipitation. This fact can be explained by the abundant precipitation in March 2009 (107.6 mm; Figure 1D) that led to a delayed emergence of many seedlings, which had no time to achieve good vegetative growth and produced only one capitulum during the flowering period (Figure 2C).

With the exception of the poor population results in 2008, the lower production of capitula in years of high plant density for all cohorts was offset by greater numbers of plants per m^2. This somewhat maintained a balance in achene production from year-to-year.

The results of this study clearly demonstrate the existence of a transitional aerial seed bank constituted by mature achenes remaining on dead plants from the fruiting period (early summer) to late spring. Achenes were retained on dead plants and, when these plants are protected from weathering, were not dispersed (dried plants kept in the laboratory indefinitely retained intact capitula). Although achenes persisted on dead plants for several months, the main timing dispersal is during autumn, decreasing the aerial bank by around 80% by December (Table 1). During winter the number of achenes released from each capitulum gradually reduced; during December-March only 3.1–17.2% of the initial content of achenes were released, due to fewer achenes left in capitula. However, in March, when the next cohort was flowering, there were still some achenes in old capitula (up to 1,700 achenes per m^2 in 2007; Table 1), with most of them being dark.

In the same way, the temporal dispersal experiment with natural rainfall showed that at maturity achenes were gradually released over several months, but most achene release occurred before December. Moreover, there was a positive correlation between the achene dispersal and rain intensity during this period. During December-May there was no significant relationship due to the low number of achenes remaining on capitula. In addition, in agreement with field samplings, both achene types were gradually released, but white achenes were dispersed early, especially in the first autumn rainfall.

Although originally the pappus was thought to be a structure favoring wind dispersal of diaspores to greater distances (Llorens et al., 2009), our study demonstrated that the wind was not a primary dispersal agent of the achenes of *A. chrysantha*, and it is likely that wind has no significance in the secondary spread either. In fact, the achenes of this species have not a papus-based wind dispersal strategy, as demonstrates the short length of their pappus (Aguado et al., 2011; Chapter 1), as occurs in several species of

Anthemis (Gutterman, 1990; Chehregani and Mahanfar, 2007). In addition, the achenes are small, which reduces their chance of secondary dispersal.

The release of achenes from the dead mother plants over time was closely related to rainfall events, indicating ombrohydrochoric dispersal. This is a special form of hydrochory where diaspores are propelled by the action of rain falling on the plant structure (Parolin, 2006). In fact, the skeletal structure of the dried plants indicates an adaptation for dispersal by ombrohydrochory: dried and hardened capitula face upward on long terminal-peduncles or achenes protected by receptacular bracts (this hinders the release of the achenes by gravity or wind, but can catapult achenes when bracts are hit by raindrops). The position of capitula on the skeletons, as well as rainfall, determines the number of seed liberated at a time (Friedman et al., 1978). Thus, achenes are protected in the dry and hardened capitula after maturation until the rain disperses them. This dispersal mechanism has been observed in other Asteraceae, some of them taxonomically very close to *A. chrysantha* (e.g. *A. pseudocotula* Boiss), in which achenes are protected after maturity in spring by bracts of the inflorescence; the achenes are dispersed when inflorescences are crushed during winter rain. This mechanism is very important in protecting seeds during summer, the season when the seed collectors are very active (Gutterman, 1990). Seed dispersal triggered by rain also provides an additional advantage, because the activity of seed predators decreases during a rainfall (Gutterman, 2000). This protection strategy of seed dispersal seems to be common to all ombrochoric plant species in arid zones of the Mediterranean region (Gutterman, 1990).

Additionally, data from the achene germination experiment showed that achenes on the soil surface could germinate quickly (2 d) after receiving a minimum as 10 mm of artificial rainfall, but most of them germinated with 20 mm. The latter was the amount of rainfall with which increased numbers of achenes were released from capitula in our experiment of temporal dispersal with natural rainfall. The coupling of seed dispersal and germination, which is the result of the highly complex fruit construction, reduces the possibility of secondary dispersal of seeds, and thus reduces gene-flow distances and potentially promoting isolation in populations in space (Klak et al., 2004). This may be one reason why this species is a local endemic with restricted geographic distribution. It has been argued that reduced dispersal promotes persistence in areas where conditions, although variable, are conductive to germination and establishment (Harper, 1977; McEvoy, 1984). By contrast, moderate to long distance

dispersal events provide the possibility of germination in new sites and the consequent expansion of the species range (Tanowitz et al., 1987). In *A. chrysantha*, about 75% of achenes released in an intense rainfall event landed beneath the plant canopy, and the remaining fraction landed in the immediate vicinity of the mother plant, as measured experimentally.

It seems that short dispersal distance is typical of ombrochoric plants. Vittoz and Engler (2007) identified seven 'dispersal types' with similar dispersal distances that were related to dispersal modes; ombrohydrochory had lower dispersal distances (50% of seed were dispersed at < 10 cm, and 99% at < 1 m). This phenomenon of limited dispersal (atelechory) is a mechanism by which seeds remain in the habitat in which the species is competitive (Van Rheede and Van Rooyen, 1999). Aguado et al. (2011) (Chapter 1) showed that white achenes of *A. chrysantha* were not dormant and germinated over a wide range of temperature, and concluded that these achenes had no means to prevent immediate germination. These facts suggest that the timing of germination is regulated by duration and degree of moisture and the presence of moderate soil temperatures. Aguado et al. (2011) (Chapter 1) also showed that, although it germinated over a wide range of temperature, the highest germination percentages of *A. chrysantha* were at 15–20ºC and the mean time to germination was around 5–7 d. These data indicate that germination usually takes place in autumn as for most early colonizing species of the thermophilous Mediterranean area (Herranz et al., 2002; Copete et al., 2009). So, the greater dispersal of achenes observed in autumn-winter (Figure 3B) supports the establishment of annual cohorts from year to year. However, heterocarpy is a reproductive strategy frequently found in Asteraceae (Ruíz de Clavijo, 2005). In *A. chrysantha*, as we mentioned above, two morphs of achenes with different germination patterns have been described (Aguado et al., 2011; Chapter 1). White achenes, occupying most of the capitulum in the top rows, show early dispersal; however, dark achenes, located in basal rows of the capitulum, are dispersed later and so remain much longer in the aerial seed bank than do white achenes. In June, 13–44% of achenes on the capitula were dark; however, in March the proportion was around 70%. Taking into account that these dark achenes are dormant (Aguado et al., 2011; Chapter 1), the fraction of achenes produced by *A. chrysantha* that fail to germinate ensure the presence of a soil seed bank. This the fractional dispersal strategy of white achenes, with high capacity to germinate quickly after rain, is accompanied by a long-

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term dispersal over time strategy of dark achenes incorporated into the soil seed bank (Tanowitz et al., 1987; Rocha, 1996; Imbert et al., 1997). Delayed germination of a fraction of a plant's progeny buffers it from the consequences of near or complete reproductive failure in unfavorable years (Westoby, 1981; Pake and Venable, 1996; Ruíz de Clavijo, 2005).

In conclusion, *A. chrysantha* forms an aerial seed bank on dead plants that favors gradual seed dispersal by ombrohydrochory and gradual germination during autumn-winter, depending on rainfall. The atelechory linked to ombrochory confer capabilities for persistence in habitats of *A. chrysantha*, but not for range extension. However, with the elimination of wind as a likely dispersal agent, it is necessary to study the roles that other secondary agents may play in *A. chrysantha* dispersal. The findings of the present study can be applied in the design of governmental programs to introduce new populations (sowing date and planting of individuals containing an aerial seed bank), although further studies are needed concerning the seed bank that *A. chrysantha* can produce in the soil.

Acknowledgements

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CHAPTER 3

The role of the soil seed bank in the unpredictable habitat of *Anthemis chrysantha* J. Gay, a critically endangered species.

Abstract

The ability of *A. chrysantha* to form a soil seed bank (SB) was investigated in order to understand better the adaptation of this rare annual species to its arid and unpredictable natural habitat. The natural population SB was analyzed in five consecutive plant cohorts (2006–2010) by sampling at two different times: in May, after the germination period, and in October, after the first dispersal episodes due to the rainfall. Besides, to determine the persistence in the soil of the two achene morphs (white and dark achenes), an artificial SB was created where achenes were buried and exhumed successively after each season, during two years. In all the cohorts studied, the seedling emergence from May samples indicated the existence of a persistent seed bank (PSB). Moreover, the dark achenes were largely responsible for the permanent fraction because, after two years of burial in the artificial SB, 85% of them remained apparently healthy without germinating, versus 9.88% of white achenes. Both types of buried achenes exhibited an annual conditional dormancy/non-dormancy cycle, induced by low winter temperatures. The PSB dynamic appeared to oscillate between the minimum values at the end of the germination period in spring (up to 2,000 achenes per square meter) and the maximum values of the dispersal episodes in early autumn (up to 6,000 achenes per square meter), with fluctuations of achene density due to the variability in annual rainfall. Hence, the SB showed a decline due to the failure of fruiting in the 2008 cohort, caused by drought, although this low value (ca. 560 achenes per square meter) was able to establish the following population. Our study highlights the importance of the PSB, which, in "bad" years, may be critical for the persistence of this species.

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1. Introduction

Information about the ecology of germination allows us to assess the potential persistence ability of seeds in the soil, and it is of special interest when studying threatened plant species (Herranz et al., 2003). Following dispersal, seeds may germinate immediately but, as a survival strategy, not all of them germinate during the year following their spread. Thus, every year, a substantial amount of seeds can remain dormant on the ground, for a period that can last years or even decades, to form a soil seed bank (SB, hereafter) (Fenner, 1985). Soil seed banks have been classified as transient or persistent in accordance with the time that seeds remain viable in the soil (Thompson and Grime, 1979); later, Walck et al. (1996) suggested that they should be described in terms of germination. Thus, transient seed banks (TSB, hereafter) are those in which all seeds either germinate or lose viability within the same year of production. In persistent seed banks (PSB, hereafter), no seeds, or a variable fraction, germinate during the first year and the remaining seeds retain viability for additional years (Fenner and Thompson, 2005). According to Grime (1979), most of the SB consists of buried seeds; however, some seeds are on the soil surface or in the litter, duff, or humus (Baskin and Baskin, 1998). Buried seeds experience lower rates of predation than seeds that remain on the soil surface (Hulme, 1994, 1998) and are also less exposed to germination-promoting stimuli, such as light and alternating temperatures. Studies about SBs and germination in Mediterranean ecosystems are numerous (Baskin and Baskin, 1998), but these SBs display characteristics that distinguish them from the SBs of other regions of the world (Figueroa and Jaksic, 2004). They are important for the structure, dynamics, and spatiotemporal distribution of Mediterranean communities of plants (Ortega et al., 1997; Parker et al., 1989; Peco et al., 1998). In many populations of Mediterranean plants, the SBs are the main source of new recruits and largely determine the future composition of the established plant community (Paker and Kelly, 1989). Similarly, SBs are posited to be critical to desert annual populations which persist in variable and unpredictable environments (Cohen, 1966; Pake and Venable, 1996). In arid ecosystems, the SB is characterized by high spatial and temporal variability (Thompson, 1987), mainly due to the scarcity and irregular patterns of precipitation found in arid regions, which leads to the potential for spatial variability in processes important to the storage of germinable seeds (Pungnaire and Lazaro, 2000).

Anthemis chrysantha J. Gay is a winter annual herb endemic to the Southeast of the Iberian Peninsula and the Algerian coast. The Southeast of Spain has a semi-arid Mediterranean climate, characterized by irregular rainfall and a long, dry summer period. This species has specific strategies to increase its reproductive success, such as heteromorphic achenes which differ in their germination behavior (Aguado et al., 2011; Chapter 1), and it also forms an aerial seed bank with short-term (< 1 year) seed retention (Aguado et al., 2012; Chapter 2).

Species with an aerial seed bank have few, if any, viable seeds in the soil (the seeds are frequently non-dormant and germinate or lose viability quickly) after they are dispersed (Baskin and Baskin, 1998). However, the production of achenes with different germination behaviors by *A. chrysantha* (as mentioned above) leads us to think that this species could also form a SB and that it is important to better understand the management of the species. The study of the SB traits is crucial in the case of endangered species because of the ecological advantages that they may confer in the context of population dynamics (Herranz et al., 2003). Persistent seed banks permit species to explore environmental variability in time, allow maintenance of biodiversity in communities and genetic diversity in populations, can protect populations from local extinction when above-ground vegetation is removed, and hence are important for restoration and conservations purposes (Arroyo et al., 2006). Also, knowledge of the SB persistence as well as the processes involved in the SB dynamics is needed, to assess the potential response of *A. chrysantha* to drought events.

Taking into account the above, the general aim of the study was to evaluate the ability of *A. chrysantha* to form a SB and to elucidate the role that this possible SB can play in the maintenance of the population in an arid and unpredictable environment. This information represents a crucial step in the design of a management program that can contribute effectively to the conservation of this threatened species. More specifically, the goals of this study were to: (a) characterize a possible SB of the species, both from the quantitative and persistence point of view, (b) determine the role of both achene types in the formation of the SB, and (c) discuss the ecological advantages that a SB can confer in the population dynamics context.

2. Material and methods

2.1. Plant material and study site

A. chrysantha is a winter annual plant whose germination period begins with the first autumn rains and stretches into early spring if the weather conditions are favorable. Flowering occurs from early March to late May, and the fruits mature mostly in June. The plants die in summer, but persist in a dried state in the habitat for several months after the end of their phenological cycle (Aguado et al., 2012; Chapter 2). The species produces heteromorphic achenes with different positions in the capitulum (Aguado et al., 2011; Chapter 1). The rows of the upper section contain elongated, white achenes (referred to as white achenes hereafter), while the achenes of the basal rows are brown-black (dark achenes hereafter) and harder than the white achenes. Finally, the last basal line is composed of achenes from ligules (only 8–10 per capitulum). Each capitulum contains about 100–130 achenes, white achenes always being more numerous than the dark ones in the capitula (ca. 70% white and 30% dark). The morphological differences between the two achene types are correlated with variations in their germinability. White achenes germinate to high percentages in many conditions, while dark achenes are strictly dormant (Aguado et al., 2011; Chapter 1). Both achene types are protected in the dry and hardened capitula after maturation, until rainfall releases them gradually (Aguado et al., 2012; Chapter 2).

The study site was located in La Azohía (Cartagena, Murcia; 37°33′8″N; 1°10′22″W; altitude 30 m), where one of the two Spanish populations of *A. chrysantha* grows (Aguado et al., 2011; Chapter 1). This area has a semi-arid Mediterranean climate characterized by irregular rainfall and a harsh, dry summer period. The annual mean precipitation is around 300 mm, and the mean annual temperature is 17.6°C. August is the warmest month, with an average temperature of 24.9°C and the coldest month is January, with an average temperature of 10.39°C. During the period studied (from 2006 to 2010), the temperature regime was similar in all years, but there were differences among years in both the total annual rainfall and its temporal distribution (Figure 1). The driest year was 2008, with only 9.3 mm of rainfall during the flowering period of the plant - compared with 49.7, 81.4, 126.9, and 102.6 mm in the same period in 2006, 2007, 2009, and 2010, respectively.

To simulate an artificial SB, achenes of *A. chrysantha* were collected from this population in July 2008 and were stored at room temperature during the summer.
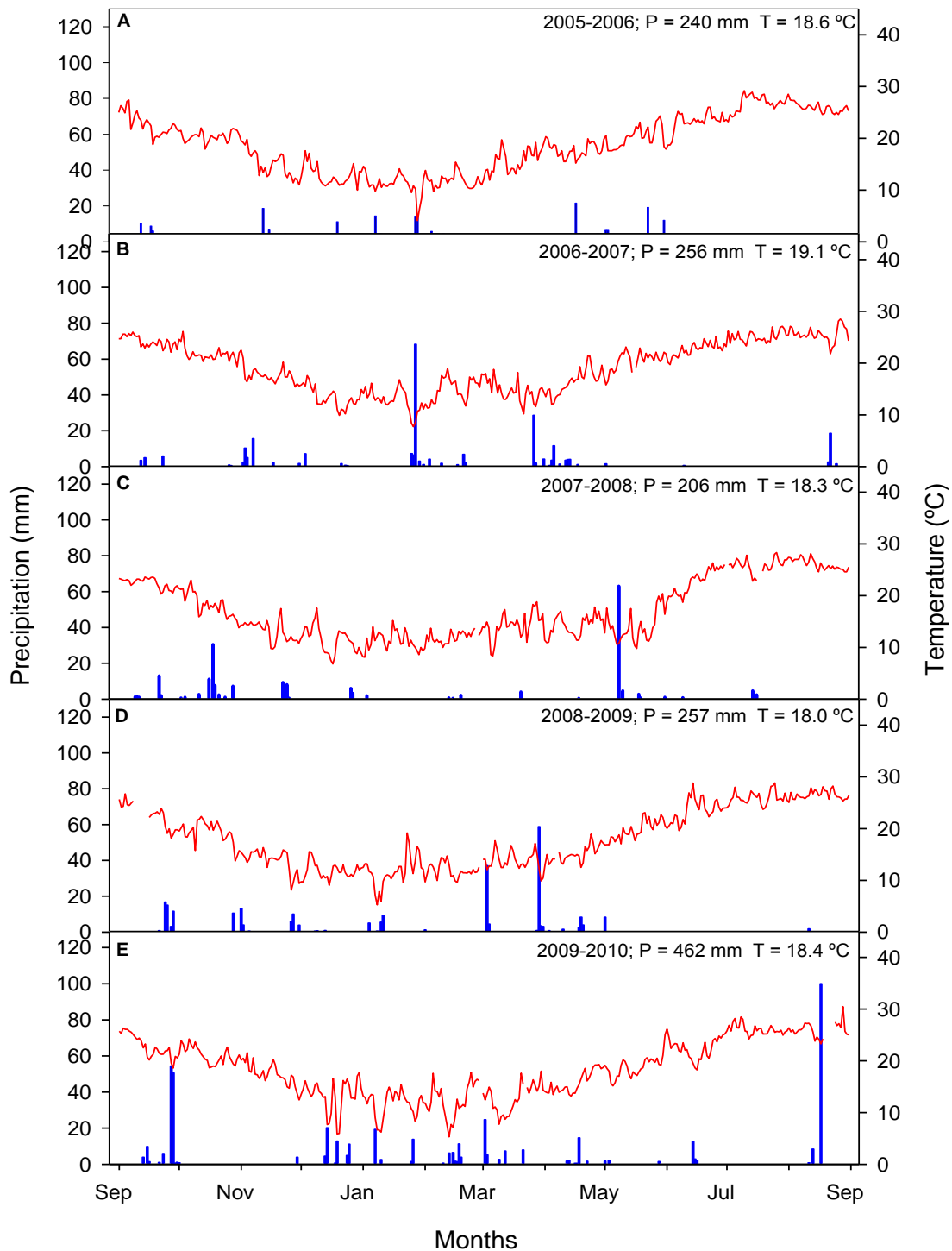
Figure 1. Daily precipitation (bars) and mean daily temperatures (lines) from September 2005 to August 2006 (A), September 2006 to August 2007 (B), September 2007 to August 2008 (C), September 2008 to August 2009 (D), and September 2009 to August 2010 (E) in the studied area. P = total precipitation in each period; T = mean temperature for each period.

2.2. Analysis of the natural soil seed bank

The soil seed bank of the natural population of *A. chrysantha* was analyzed for five consecutive plant cohorts (from 2006 to 2010), in soil samples collected in late spring (May), after the germination period and before dispersal of the achenes. This sampling date was determined on the basis of Thompson and Grime's (1979) concept of PSB, in order to quantify the persistent fraction of the SB, which overlaps between two consecutive phenological cycles. In addition, to discover the dynamic of the SB, another soil sampling was performed in October, when the first achene dispersal episodes due to rainfall have occurred and the germination period has barely started (Aguado et al., 2012; Chapter 2). In each sampling, thirty 10 cm x 10 cm random soil samples were collected from a 5–cm–deep soil layer. In the samplings carried out in October, often some seedlings had emerged and their numbers were counted for inclusion in the final estimation of achenes per square meter for each sample.

The samples were carried in plastic bags to the laboratory, where they were dried completely. Large soil aggregates were broken up manually, in order to homogenize the soil samples, and big stones and vegetative plant material were removed with a 4–mm–mesh sieve. Once the samples were processed, they were stored in the laboratory until analysis.

The achene content was assessed using the seedling emergence method (indirect method) because it is more accurate and less time-consuming than physical separation, since small seeds are likely to escape visual detection (Thompson et al., 1997; Ferrandis et al., 2001). For this, in October of each year, soil samples from May and October were scattered over 3–cm–deep sterile peat in 38 cm x 28 cm x 6.5 cm black plastic seedling trays and placed on metal tables in a non-heated mesh greenhouse at the Agricultural Experimental Station of the Technical University of Cartagena, located about 30 km from La Azohía (Picture 1). The thickness of the soil sample layer in the trays was around 0.5 cm, to facilitate the seedling emergence. The trays were watered periodically, to keep the samples constantly moist, and they were checked for seedling emergence every month. Emerged seedlings were removed as soon as they were identified. The seedling emergence of *A. chrysantha* was registered from October of each sampling year until the end of the second germination period (18 months).
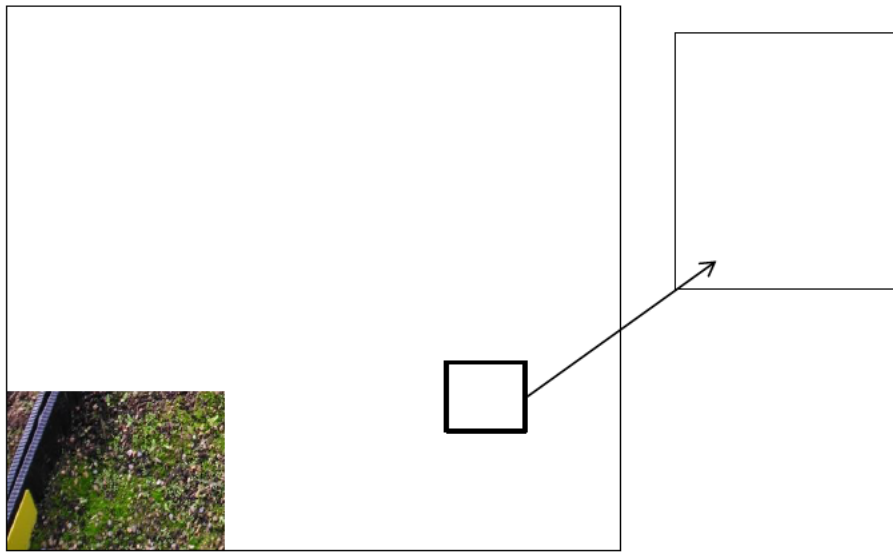
Picture 1. Soil samples from La Azohía in which germinated achenes of *Anthemis chrysantha*.

2.3. Artificial soil seed bank analysis

To identify the persistence in the soil of the two achene types, an artificial SB was created by following some experimental seed burial protocols (Ferrandis et al., 1999a; Herranz et al., 2003). Samples of 100 undamaged achenes of each type were put into 0.1–mm–mesh nylon bags (5 cm x 5 cm), filled with 0.25–mm–sieved soil from the habitat of the species, in order to prevent the achenes from sticking to each other at high humidity. Sixteen lots of 4 bags each (replicates) were prepared for each achene type. Eight lots were buried at a depth of 2 cm and the other lots at 7 cm, in trays (42 cm x 29 cm x 7.5 cm) with drainage holes, containing soil from the habitat mixed with peat (1:1) (Picture 2). The trays were placed in a non-heated mesh greenhouse, so that they received only the natural rainfall. This experiment was designed for two consecutive years, beginning in September 2008 and ending in September 2010. At the end of each season, one lot of each achene type was dug out and carried to the laboratory, where the fraction of ungerminated and physically-undamaged achenes (apparently-healthy, exhumed achenes hereafter) was counted using a stereoscopic microscope (Olympus SZ61).

In order to reveal the germination behavior of buried achenes under optimal conditions, a germination test was conducted on exhumed achenes which were apparently healthy. Achenes from each bag were incubated in a 9–cm–diameter Petri

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dish, on a double layer of filter paper moistened with 4 ml of distilled water, at 12/20ºC and 12 h daily photoperiod in a germination chamber (Sanyo MLR–351H, Osaka, Japan) with a digital temperature and light-control system (± 0.1°C, cool white fluorescent light of 20,000 lx). These are the optimal germination conditions for this species, according to Aguado et al. (2011) (Chapter 1). The germination test lasted for one month. The achenes were checked every week; radicle protrusion was the criterion for successful germination. Achenes that did not germinate were checked under the stereoscopic microscope to confirm that the embryo was healthy.
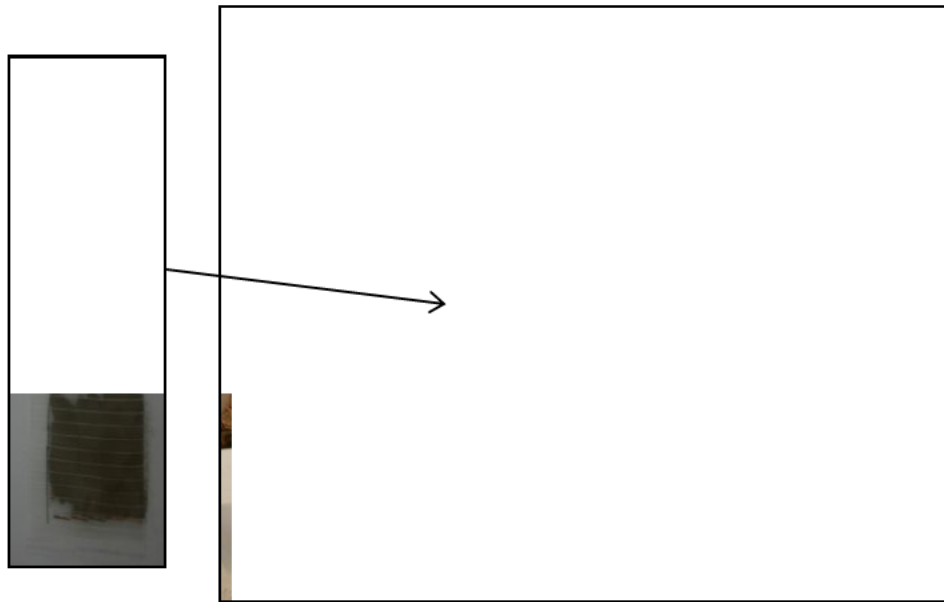
Picture 2. Nylon bags with achenes inside buried in a tray.

2.4. Statistical analysis

A multivariate analysis of variance was used to evaluate the evolution of the artificial and natural SBs. The data were analyzed with the software SPSS 19.0 for Windows (SPSS Inc., Chicago, USA). The data normality and homocedasticity were checked, and the percentages of apparently-healthy, exhumed achenes and later germinated achenes were arcsine transformed. When significant main effects existed, differences were tested by Tukey's multiple comparison test at $P < 0.05$.

3. Results

3.1. Analysis of the natural soil seed bank

Although strong fluctuations in the number of emerged seedlings were observed when comparing the May and October samples, lower achene numbers in the soil were found in the May sampling. The values ranged from 564 to 2,096 achenes per square meter (Table 1). The amount of achenes in the soil sampled in October was always significantly higher than in May, with the exception of the year 2008 (Table 1). In all the sampled years (2006–2010), most of the seedlings observed in the soil samples emerged during the first germination period following sampling, but a small proportion of achenes remained in the soil and emerged during the second period (Table 1). In both periods, most seedlings emerged in the autumn (85.13 to 99.05%), with a small fraction in winter (0.05 to 14.04%) and very few in spring (0 to 1.39%).

The dynamics of the SB over the sampled years showed a fall in the achenes content during three consecutive samplings: October 2008 and May and October 2009 (Table 1). The seed number had recovered from this fall in May 2010; this date and October 2010 exhibited values similar to the range of May and October of 2006 and 2007.

3.2. Artificial soil seed bank analysis

The percentage of apparently-healthy, exhumed achenes was significantly affected by the factors season ($P = 0.000$) and achene type ($P = 0.000$), and their interaction ($P = 0.006$). Depth of burial did not affect the values, so the data are shown without differentiating between depths. Apparently-healthy, dark achenes recovered after soil-burial were abundant, 85% of buried achenes remaining in the bag after two years (Table 2). On the other hand, the percentage of apparently-healthy, white achenes decreased progressively along the seasons: 50% of buried achenes remaining in the bag after the first autumn, 14.17% after the second autumn, and only 9.88% after the second summer (two years after burial) (Table 2).

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Table 1. Emergence of *Anthemis chrysantha* seedlings (mean number per square meter ± standard error) from soil samples collected in May and October in 2006–2010 cohorts, cultivated from October of each sampling year until the end of the second germination period. Different uppercase letters for values within a column indicate a significant difference from each other, and different lowercase letters for values within a row indicate a significant difference (Tukey test; $p < 0.05$). * Samples were cultivated only during the first period.

Cohort	May Samples			October Samples			F	P
	First Period	Second Period	Total	First Period	Second Period	Total		
2006*	1,143.26 ± 157.96	-	1,143.26 ± 157.96Aab	5,631.06 ± 604.59	-	5,631.06 ± 604.59Bb	53.146	0.000
2007	1,151 ± 184.83	60.13 ± 17.27	1,211.37 ± 190.87Aab	5,627.02 ± 709.15	572.24 ± 93.28	6,199.26 ± 771.69Bb	39.370	0.000
2008	1,839 ± 237.64	257.46 ± 70.68	2,096.94 ± 289.97Bc	828.53 ± 125.37	317.16 ± 76.27	1,145.69 ± 162.37Aa	8.193	0.006
2009	554.52 ± 111.55	10.00 ± 7.35	564.52 ± 111.11Aa	1,166.67 ± 200.82	203.33 ± 74.51	1,370.00 ± 266.31Ba	7.792	0.007
2010	1,295.78 ±208.07	66.02±24.07	1,361.80 ± 217.30Abc	4,395.98 ±654.31	146.34±46.63	4,542.31 ± 657.68Bb	21.085	0.000
			F = 7.373			F = 19.040		
			$P = 0.000$			$P = 0.000$		

Table 2. Variation of the apparently-healthy achenes percentage over time in both achene types of *Anthemis chrysantha*. Means (± standard error) within a row with different uppercase letters are significantly different from each other; means within a column with different lowercase letters are significantly different from each other (Tukey test; $P < 0.05$).

Achene Type	Autumn 1st Year	Winter 1st Year	Spring 1st Year	Summer 1st Year	Autumn 2nd Year	Winter 2nd Year	Spring 2nd Year	Summer 2nd Year	
White Achenes	50.38±2.82Ca	39.50±3.70BCa	42.13±2.18Ca	21.50±7.13ABa	14.17±8.17Aa	6.63±2.72Aa	10.38±2.01Aa	9.88±2.36Aa	$F = 16.506$ $P = 0.000$
Dark Achenes	93.50±2.26Bb	95.25±1.25Bb	82.25±7.03ABb	87.00±4.04ABb	81.00±3.78ABb	68.50±9.47Ab	86.57±3.717Bb	85.00±4.41ABb	$F = 2.954$ $P = 0.011$
	$F = 95.813$ $P = 0.000$	$F = 148.312$ $P = 0.000$	$F = 23.767$ $P = 0.000$	$F = 58.061$ $P = 0.000$	$F = 49.324$ $P = 0.000$	$F = 43.757$ $P = 0.000$	$F = 137.791$ $P = 0.000$	$F = 156.382$ $P = 0.000$	

Table 3. Variation of the germination percentage (of apparently-healthy, exhumed achenes) along the seasons in both achene types of *Anthemis chrystanta*. Means (± standard error) within a row with different uppercase letters are significantly different from each other; means within a column with different lowercase letters are significantly different from each other (Tukey test; $P < 0.05$).

Achene Type	Autumn 1st Year	Winter 1st Year	Spring 1st Year	Summer 1st Year	Autumn 2nd Year	Winter 2nd Year	Spring 2nd Year	Summer 2nd Year	
White Achenes	1.19±1.19Aa	3.04±1.13ABa	12.97±3.17ABa	76.39±15.49Ca	27.82±10.46ABa	4.13±3.14ABa	30.45±8.03Ba	90.69±5.07Cb	$F = 20.481$ $P = 0.000$
Dark Achenes	1.40±0.74Aa	0.80±0.39Aa	7.47±2.02ABa	50.46±6.47CDa	23.84±5.14BCa	2.71±1.36Aa	23.22±6.79BCa	54.00±11.77Da	$F = 20.913$ $P = 0.000$
	$F = 0.375$ $P = 0.550$	$F = 2.507$ $P = 0.136$	$F = 0.512$ $P = 0.486$	$F = 3.207$ $P = 0.095$	$F = 0.046$ $P = 0.834$	$F = 0.020$ $P = 0.890$	$F = 0.419$ $P = 0.529$	$F = 9.441$ $P = 0.008$	

Regarding the later germination behaviour of the apparently-healthy achenes, the germination percentage was, in the same way, affected significantly by season (P = 0.000), achene type (P = 0.003), and their interaction (P = 0.005). Depth was a non-significant factor and had no interaction with the other factors. In most seasons for each achene type with healthy external appearance, after they were recovered only a small fraction germinated when incubated for a month in a growth chamber at 12/20ºC and 12 h photoperiod. But, achenes recovered after the two summers germinated more than in the other seasons (76.39 and 50.46% of white and dark achenes, respectively, after the first summer and 90.69 and 54%, respectively, after the second summer; Table 3).

4. Discussion

The emergence of seedlings in the soil samples collected in late spring (May), after the germination period and before the beginning of seed dispersal from the capitula produced that year, indicates the formation of a PSB by this species. Another finding that supports this idea is that, although most achenes contained in the soil samples collected in the natural population germinated during the first germination period following sampling, some viable achenes remained in the SB for at least another germination period (Table 1). The persistent fraction of the SB is also of considerable size (between 1,000 and 2,000 achenes per square meter in most years), comparable in magnitude to that reached by other species such as those of the Cistaceae, a family whose seeds form large SBs in Mediterranean ecosystems (Ferrandis et al., 1999a, b).

The sampling conducted in October, when some achenes had dispersed from dead plants which had died in the summer, indicates the recovery of the SB, which reached values of up to 4,500 to 6,000 achenes per square meter. With the autumn and winter rains, some of these achenes in the soil will germinate, depleting the SB in late spring. Thus, the dynamic of the SB ranges between the minimum values at the end of the spring and the maximum values in early autumn (after dispersal starts but before germination; Figure 2). According to many authors (Kalisz and McPeek, 1993; Pake and Venable, 1996; Gutterman, 2000, Fox et al., 2006), a PSB allows species to form new cohorts of individuals even if the seed production had failed the previous year. This has been verified in the case of *A. chrysantha*, due to the occurrence of an extremely-dry spring during the study period. Figure 2 shows a break in the "normal" dynamic of

the SB caused by a fall in the values of soil achene density from October 2008 to October 2009, recovering in May 2010. This is explained by the low rainfall in spring 2008: only 9.3 mm fell during the flowering period of the species compared with 49.7, 81.4, 126.9, and 102.6 mm in the same period in 2006, 2007, 2009, and 2010, respectively (Figure 1). The flowering and fruiting of *A. chrysantha* were affected by this period, failing the reproductive success of the species (Aguado et al., 2012; Chapter 2) and, therefore, preventing the recharge of the soil SB in October 2008. The seedling emergence from October 2008 to March 2009 further accentuated the depletion of the SB in May 2009 (throughout the entire study period, the lowest values of the SB were found at this time; Figure 2). However, this seedling emergence from October 2008 to March 2009 - from the permanent fraction of the SB - allowed the establishment of a new annual plant cohort (the 2009 cohort in Table 1) that grew in a wet flowering season and was able to recover the "normal" values of achenes per square meter in the SB (May 2010). Despite the decrease in the SB achene density due to the failure of the fruiting success caused by drought, the minimum values were maintained at around 564 achenes per square meter and they were able to perpetuate the population, annually. In spite of this dry period which affected the dynamics of the SB (as discussed previously), the size of the population did not show significant oscillations among most of the five years studied (Aguado et al., 2012; Chapter 2), because the SB could stabilize the population dynamics by damping the oscillations in population size to stable equilibrium (MacDonald and Watkinson, 1981; Pacala, 1986; Kalisz and McPeek, 1993). This also supports the notion that the SB represents a good survival strategy for *A. chrysantha*, as for other winter annual species (Venable and Brown, 1988; Arroyo et al., 2006; Copete et al., 2009), and that it is essential for ensuring its conservation, since it allows the re-establishment and maintenance of populations after years with poor seed set, without the necessity for an external seed source (Baskin and Baskin, 1978, 2000, Copete et al., 2009). This important role of the SB in "bad" years can be minimized in "good" years by the role of the transitional aerial seed bank, described by Aguado et al. (2012) (Chapter 2). Although the aerial bank persists on dead plants for several months, the main period of achene dispersal is during the autumn; this decreases the aerial bank by around 80% by December, with a positive correlation between the achene dispersal and rain intensity during this period. Therefore, in years with good reproductive success, the transitional aerial seed bank (up to 24000 achenes per square meter) could

be the main source that establishes the following cohort. However, in dry years, the aerial seed bank does not form due to reproductive failure and the establishment of the new cohort depends on the SB.
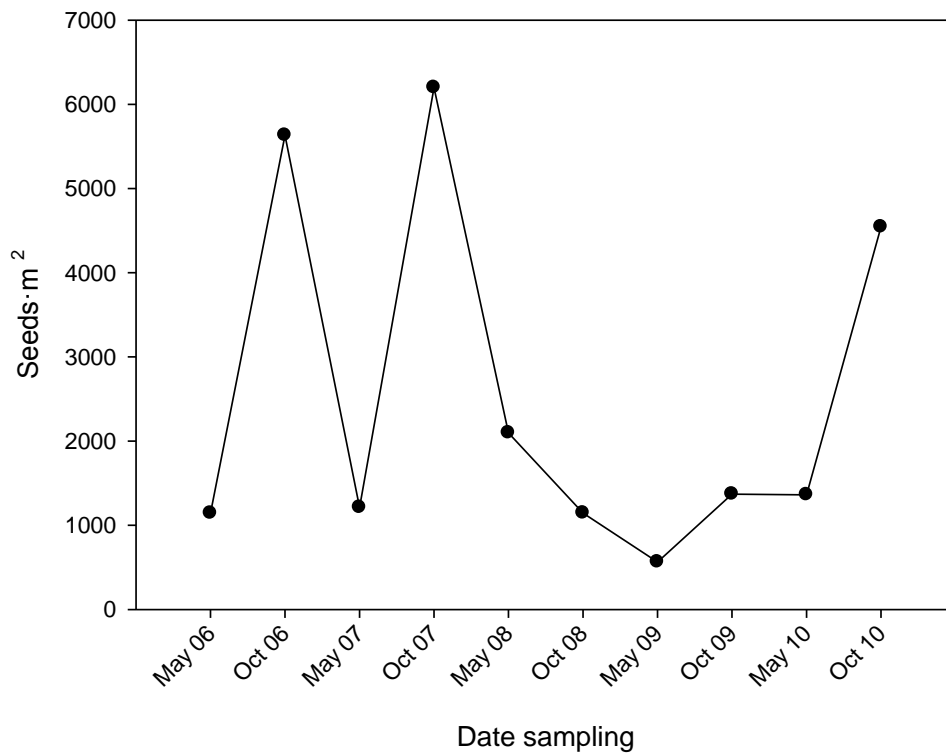
Figure 2. Dynamics of the natural soil seed bank of *Anthemis chrysantha* during the studied years. The sum of the emerged achenes per square meter in both emergence periods studied is represented for each study year.

The results obtained from the soil-burial experiment also demonstrate the ability of *A. chrysantha* to form a PSB, since a large proportion of the buried seeds were recovered and were able to germinate after two complete phenological cycles in the soil. The behavior of the dark and white buried achenes in the artificial SB shows that the dark achenes are largely responsible for the permanent fraction of the SB: after two years of burial, 85% of the dark achenes remained apparently healthy without germinating. However, 21% of the white achenes remained apparently healthy before the second autumn after burial and a small portion of them (ca. 10%) had not germinated after two phenological cycles; so, they will also contribute - although to a lesser extent than the dark achenes - to the PSB, as hypothesized by Aguado et al. (2011) (Chapter 1), taking into account that a small fraction of the white achenes did not germinate under optimal conditions. The germination behaviour observed in this work

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is similar to that observed by Aguado et al. (2011) (Chapter 1) in the laboratory: a small proportion of white achenes seem to have conditional, non-deep physiological dormancy which could be broken by dry and hot periods, as noted by Herranz et al. (2003) for two winter annual *Sisymbrium* species. Dark achenes have a pericarp-mediated dormancy which physically prevents radicle emergence and also hampers imbibition of water. In fact, the germination of dark achenes under optimal conditions of light and temperature in the laboratory is usually negligible compared to the high germination of the white achenes (Aguado et al., 2011; Chapter 1). These differences in achene dormancy levels from the same plant enable plants to separate their genotypes in time and space (Pandey 1969; Venable, 1985) and it occurs frequently in other heteromorphic species of the Asteraceae (Brändel, 2004; Sun et al., 2009).

The germination tests carried out with apparently-healthy achenes exhumed periodically from the artificial SB at the end of each season, during the two years studied, show that the seasons affect achenes dormancy. Thus, buried *A. chrysantha* achenes exposed to natural, seasonal variations in temperature exhibit an annual conditional dormancy/non-dormancy cycle: their entry into secondary dormancy is induced by low winter temperatures (to prevent germination in dry and hot months) and they come out of conditional dormancy with the high temperatures of summer (so that the achenes are ready to germinate with the first rains of autumn). This conditional dormancy/non-dormancy cycle has been found also in many facultative winter annual species (Roberts and Nielson, 1982; Baskin and Baskin, 1989, 2000; Copete et al., 2009).

Since the germination observed in the natural SB experiment was concentrated in autumn and winter, while spring percentages were around 1% of the total accumulated germination recorded during the study, *A. chrysantha* can be considered a facultative winter annual. In fact, in some years during the study period, some seedlings emerged in early spring (April) in their natural habitat, although they did not fruit in summer and died without reproductive success.

In conclusion, *A. chrysanta* has the ability to form a PSB, which plays a crucial role in the maintenance of the annual population of this endangered species in its unpredictable habitat with occasional dry years. The SB formation is favored by the production of heteromorphic achenes with different germination behavior, which is a specific trait to ensure that some of them remain in the SB for a number of years. Low

winter temperatures in the study area may induce the achenes which remained in the soil to enter dormancy, preventing summer germination. After the hot and dry summer periods, the achenes lose their dormancy and germinate mostly with the first autumn rains.

Also, *A. chrysanta* is a facultative winter annual species whose annual population success is particularly sensitive to spring rain. When the spring is dry, the reproductive success of the population fails and consecutive dry years could deplete the SB. Therefore, for the persistence of *A. chrysantha* population could ultimately depend on the frequency of years with a wet flowering season.

Acknowledgements

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CHAPTER 4

Assessment of genetic diversity in the critically endangered

Anthemis chrysantha **(Asteraceae) using ISSR markers.**

Abstract

Anthemis chrysantha is an endangered species endemic to the Algerian coast and the coast of Cartagena (Murcia, SE Spain). In order to obtain information that may contribute to better management and conservation of this species in Spain and to determine if the morphological variability between Spanish and Algerian material is attributable to genetic variation, ISSR markers were used to determine the genetic structure of the remaining Spanish populations and to assess phylogenetic relationships between the Spanish and Algerian populations. Both at the species and population level, the genetic diversity indices show that the genetic variation of *A. chrysantha* is high. The mean gene diversity (H_E) was estimated to be 0.289 within populations and 0.343 at the species level. All the analyses performed to assess the genetic structure of *A. chrysantha* in Spain indicate a high within-population variability (82%) and a low, albeit significant, differentiation between populations ($\Phi_{ST} = 0.177$), the population size and breeding system being the major factors in explaining these levels of genetic variability. The high genetic connectivity found between the remaining Spanish populations ($G_{ST} = 0.157$) is evidence that these populations were connected in the past and were only recently isolated, and that not enough time has passed for this species to show more genetic divergence. On the other hand, ISSR markers have detected genetic variation among *A. chrysantha* populations with different geographical origins. The UPGMA clustering shows the Spanish populations grouping together, with the Algerian population separated into the other group with 100% bootstrap support. Eleven ISSR markers obtained in this study can be considered as Algerian population-linked markers. All the data indicate a clear geographic tendency in the distribution of the genetic variability, which supports the recognition of the rank of subspecies, at least, for the Spanish populations. The implications of these results for the conservation of the species are discussed.

1. Introduction

Anthemis L. is the second largest genus in the Asteraceae (tribe Anthemideae), with more than 210 species that occur in the Mediterranean region, western Eurasia and eastern Africa (Oberprieler, 2001). About 62 species of this genus are distributed in Europe (Fernandes, 1976); 14 of them appear in the Iberian Peninsula. One of these species is the annual plant *Anthemis chrysantha* J. Gay that is endemic to North Africa and the Southeast of the Iberian Peninsula. It has a restricted distribution in the Algerian coast and the coast of Cartagena (Murcia, SE Spain); the latter is its only European location (Bañares et al., 2004). Some authors (e.g. Fernandes, 1983; Oberprieler, 1998) have found morphological differences between Spanish and Algerian material and Sánchez et al. (2002) proposed the recognition of the rank of subspecies, at least, for the Spanish populations, although nowadays they are not recognized as different taxa. To date, it is not known if this morphological variability is attributable to genetic variation. In the coast of Cartagena, four populations were known. The oldest known localities were the Escombreras Island and La Azohía; later, it was detected in the continental zone of Escombreras and La Muela. The Escombreras continental population was eliminated in 1998 by the work on the new port of Escombreras. The La Muela population dates from 1996 and it has not been detected since its discovery. Thus, since the late 1990s, only two populations remain in Cartagena, less than 20 km apart and occupying an area of around 0.02 km^2. Both Spanish populations, although growing in small areas, about 0.015 km^2 in the Escombreras Island and 0.005 km^2 in La Azohía, have a high number of individuals (about 12000 and 40000, respectively). Its narrow distribution and status of endemism led to *A. chrysantha* being classified first as *Endangered* (E) (Sánchez et al., 2002) and later as *Critically Endangered* (CR) (Sánchez et al., 2004), according to the International Union for Conservation of Nature (IUCN) categories. Climate warming, loss of habitat by urbanization, and excessive trampling in the areas where it grows are now the threats to its survival in the Spanish populations. To date, basic research has been carried out on its life traits and ecological characteristics (Aguado et al., 2011, 2012; Chapter 1 and Chapter 2), but not on its genetic diversity and structure.

The extinction rates of wild species are increasing, directly and indirectly in response to human activities, and conservation plans for endangered species have been suggested from various biological perspectives (Primack, 1993; Bowles and Whelan,

1994; Pickett et al., 1997). In endangered species of high conservation priority, knowledge of the genetic variation within and among populations is essential for future management strategies, especially decisions that involve the foundation of new populations or the restoration of populations that have suffered extensive damage or extirpation (Fleishman et al., 2001). Moreover, preserving the genetic diversity of endangered species is one of the primary goals in conservation strategies, because the long-term survival and evolution of species depend on the maintenance of sufficient genetic variability within and among populations to accommodate new selection pressures brought about by environmental changes (Barrett and Kohn, 1991). Empirical studies have shown that fitness is correlated positively with high levels of genetic variation both within species (Fischer et al., 2003; Hensen and Oberprieler 2005) and among species within ecosystems (Reusch et al., 2005). Positive relationships between population size, plant fitness, and within-population genetic diversity have been found too (Leimu et al., 2006), tending to be stronger for rare than for common species.

The Inter-simple sequence repeat (ISSR) markers system uses repeat-anchored primers to amplify DNA sequences between two inverted SSRs (Zietkiewicz et al., 1994). Because of higher annealing temperatures and longer sequences of ISSR primers, they can yield reliable and reproducible bands, and the cost of the analyses is lower than that of some other markers such as AFLPs. Although the ISSR technique has some problems as a dominant marker, it has proved an effective tool in addressing problems of systematics and hybridization, as well as in population genetics (Reddy et al., 2002). ISSR markers have been used successfully to determine the genetic diversity of other narrow endemic species of the Asteraceae (Luan et al., 2006; Ramp Neale et al., 2008; Jeong et al., 2012; Lopez and Barreiro, 2012).

Anthemis chrysantha is currently protected in Spain by a regional law (BORM, 2003). Therefore, in order to obtain information that may contribute to better management and conservation of this protected narrow endemic in Spain, in the present study we used ISSR markers to: (i) determine what levels of genetic diversity are present within and between the Spanish populations of *A. chrysantha*; and (ii) assess the genetic relationships of the Spanish and Algerian populations of the species. Finally, based on this molecular information, some specific conservation measures for the species will be suggested.

2. Materials and methods

2.1. Plant material

Anthemis chrysantha is a winter annual plant which has an erect habit, is corymbosely branched, and reaches a height of 30 cm. The capitula have peduncles up to 6 cm in length, with yellow flowers on a rather convex disc of 12–25 (30) mm in diameter (Sánchez et al., 2004). The emergence period begins with the first autumn rains, stretching into spring if weather conditions are favorable. Flowering occurs from early March to late May, and fruits mature mostly in June. The plants die in summer, but persist in a dried state in the habitat for several months after death. The species has heteromorphic achenes which differ in their position in the capitulum and their germination behavior (Aguado et al., 2011; Chapter 1). *Anthemis chrysantha* grows in terofitic meadows developed in thyme and halonitrophilous scrubs, with a great influence of the sea, on litosoils, lying within the Thermo-Mediterranean level and with a semi-arid Mediterranean climate characterized by irregular rainfall and a long, dry summer.

The plant material used in the study includes a total of 65 individuals of *A. chrysantha* from the two remaining Spanish populations, in La Azohía and Escombreras Island, and 15 individuals collected from one Algerian population, in Kristel (Picture 1). *Anthemis chrysantha* has been found on the coast of Algeria, in the Oran Department (Habibas Islands and Kristel) and the Mostaganem Department, occupying sands, cliffs, and grasslands next to the sea (Battandier, 1898; Battandier and Trabut, 1902). However, it is probable that these are isolated populations and that some populations have disappeared due to the development of the coast, as is the case for the Tip of the Salamander, in Mostaganem (personal observation). The details of the sampled populations are shown in Table 1. Spanish and Algerian plant material was collected in February 2009 and June 2011, respectively. Young leaf material from selected plants was collected from each population and stored at –20ºC with silica gel in plastic bags until DNA extractions were carried out.

Picture 1. Locations of the *Anthemis chrysantha* populations in Southeastern Spain and Northern Algeria.

Table 1. Sampling details of the *Anthemis chrysantha* populations used in the present study.

Population	Latitude and longitude	Population size	Sample size
La Azohía (Spain)	37º33´8´N, 1º10´22´W	~40000	41
Escombreras Island (Spain)	37º33´35´N, 0º58´08´W	~12000	24
Kristel (Algeria)	35º49´27´´ N, 0º28´59´W	*	15

*Data unknown

2.2. Extraction of total DNA and ISSR amplification

To determine the levels of genetic diversity within and between Spanish populations of *A. chrysantha*, total genomic DNA was extracted individually from young leaves of 65 individuals, using a ''Danapure. Plantas y hongos'' extraction kit (GeneDan, Barcelona, Spain). The DNA concentration was assessed spectrophotometrically and by 1% agarose gel electrophoresis. The DNA samples were then diluted to 20 ng/μL, prior to polymerase chain reaction (PCR) amplification. A total of 30 ISSR primers (UBC primers set #9, University of British Columbia, Vancouver, Canada) were tested for DNA amplification. Eight primers were chosen for ISSR analyses of genetic diversity, based on band reproducibly (Table 2). The PCR

reactions were carried out using a single primer at a time, in 20 µL of reaction mixture containing 40 ng of template DNA, 1x reaction buffer, 200 µM of each of the four dNTPs, 1 U of Taq DNA polymerase (ECOGEN, S.R.L.), 1.5 mM MgCl$_2$, and 0.5 µM of primer. Amplification was performed using a thermal cycler (Bio-Rad, California, USA) programmed for an initial denaturation step of 4 min at 94 ºC followed by 35 cycles of 45 s at 94 ºC, 45 s at the specific annealing temperature, and 90 s at 72 ºC, ending with a final extension step of 7 min at 72 ºC. Each reaction was repeated at least twice, and only reproducible bands were scored. The ISSR markers were resolved on 1.5% agarose gels, with ethidium bromide detection (Picture 2). Gels were documented using a gel documentation and image analyser (Vilber Lourmat, Germany).

Table 2. The ISSR primers used and analysis of ISSR-generated banding patterns in the study of genetic diversity within and between the Spanish populations of *Anthemis chrysantha*.

Primers	Sequence (5´— 3´)	Annealing temperature (ºC)	No. of bands scored	No. of polymorphic bands
UBC 825	(AC)$_8$T	49.2	8	5
UBC 826	(AC)$_8$C	53.3	9	7
UBC 840	(GA)$_8$(CT)T	48.2	12	7
UBC 850*	(GT)$_8$(CT)C	55.2	12	12
UBC 855	(AC)$_8$(CT)T	57.1	8	5
UBC 857C	(AC)$_8$(CT)GC	63.6	12	11
UBC 857G*	(AC)$_8$(CT)GG	63.4	10	9
UBC 864	(ATG)$_7$	58.1	8	6
Average	-	-	9.87	7.75
Total	-	-	79	62

*ISSR primers not used in the intraspecific relationships study.

To assess the genetic relationship of the Spanish and Algerian populations of *A. chrysantha*, three bulk DNA samples (one per population) were prepared by pooling an equal amount of fresh leaf tissue of 15 individuals from each population. Bulked samples of fresh leaf tissue were ground in liquid nitrogen and genomic DNA was extracted following the CTAB protocol of Doyle and Doyle (1987). The DNA concentration was assessed spectrophotometrically and by 1% agarose gel electrophoresis. The bulk DNA samples were then diluted to 20 ng/µL, prior to polymerase chain reaction (PCR) amplification. Six of the eight primers used in the previous study were chosen for ISSR analyses (Table 2), using the same PCR procedure mentioned above. A DNA marker present in one bulk sample but absent in the others was considered to be a potential population-linked marker.
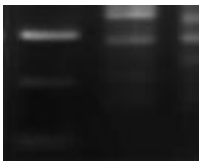
Picture 2. The banding pattern obtained in 12 individuals from the La Azohía population with the ISSR primer UBC 840, resolved on 1.5% agarose gel with ethidium bromide detection.

2.3. Data analysis

The ISSR bands were used to assign loci for each primer and were scored as presence (1) or absence (0). Only data from intensely stained, unambiguous, clear bands were used for statistical analysis. The band presence/absence data matrix was analyzed by POPGENE version 1.32 (Yeh et al., 1997), making the assumption that the populations are in Hardy-Weinberg equilibrium at these ISSR marker loci. The genetic diversity within and between the Spanish populations was measured by the percentage of polymorphic bands (PPBs), the observed number of alleles (A_o), the effective number of alleles (A_e), Nei´s (1973) gene diversity (H_E, also called expected heterozygosity), and Shannon´s information index (SI) (Lewontin, 1972). The within-population genetic diversity (H_{pop}), total genetic diversity (H_{sp}), within-population gene diversity (H_S), total gene diversity (H_T), genetic differentiation coefficient among populations (G_{ST}), Nei´s (1972) genetic identity, and genetic distance (D) between Spanish individuals were also calculated. A dendrogram was constructed based on Nei´s genetic distance (D) by an unweighted pair-group method of cluster analysis using arithmetic averages (UPGMA), using the software NTSYS pc2.02 (Rohlf, 1998). The amount of gene flow between Spanish populations was estimated as $Nm = (1/G_{ST} - 1)/4$ (Slatkin and Barton, 1989). The additional measurement of partitioning genetic variation was obtained with the

hierarchical analysis of molecular variance (AMOVA) analysis, using GENALEX 6 (Peakall and Smouse, 2006) with 9999 permutations.

To visualize the genetic relationship among the Spanish and Algerian populations, a dendrogram was constructed based on Nei´s genetic distance (D) between populations by UPGMA, using the software NTSYS pc2.02 (Rohlf, 1998). To test the correlation between Nei´s genetic distance (D) between populations and the geographic distances (in km) between populations, a Mantel test was performed using GENALEX 6 (Peakall and Smouse, 2006).

3. Results

3.1. Genetic diversity within the Spanish populations

The eight ISSR primers chosen for this analysis produced a total of 79 bands ranging from 300 to 1500 bp, corresponding to an average of 9.87 bands per primer (Table 2). The percentage of polymorphic bands (PPB) was 68.35% for the La Azohía population and 62.03% for Escombreras Island, while at the species level this value was 78.48% (Table 3). The average effective number of alleles per locus (A_e) was 1.5212 at the population level and 1.6251 at the species level (Table 3). The average gene diversity (H_E) was estimated to be 0.289 within populations and 0.3427 at the species level. The Shannon´s index (SI) was 0.4351 for the La Azohía population and 0.3904 for Escombreras Island, with a value of 0.4917 at the species level (Table 3). The Escombreras Island population had the lowest genetic variation, as shown in Table 3.

Table 3. Genetic variability within the Spanish populations of *Anthemis chrysantha*, detected by ISSR analysis.

Population	PPB (%)	A_o	A_e	H_E	SI
La Azohía	68.35	1.6835 ± 0.4681	1.5658 ± 0.4168	0.3051 ± 0.2160	0.4351 ± 0.3039
Escombreras Island	62.03	1.6203 ± 0.4884	1.4965 ± 0.4133	0.2724 ± 0.2195	0.3904 ± 0.3116
Average	65.19 ± 4.47	1.6519 ± 0.0447	1.5312 ± 0.0490	0.2888 ± 0.0231	0.4127 ± 0.0317
Species	78.48	1.7848 ± 0.4136	1.6251 ± 0.3685	0.3427 ± 0.1899	0.4917± 0.2668

PPB, percentage of polymorphic bands; A_o, number of alleles per locus; A_e, effective number of alleles per locus; H_E, Nei´s genetic diversity (assuming Hardy-Weinberg equilibrium); SI, Shannon´s information index.

3.2. Diversity between the Spanish populations

The coefficient of genetic differentiation between populations (G_{ST}) was 0.1573 (Table 4), indicating that about 16% of the total ISSR variation occurred between populations. The ratio of genetic diversity between populations as estimated by Shannon´s information index was 0.1605 (Table 4). The AMOVA analysis showed that 82% of the total variation occurred within populations and only 18% between populations ($P = 0.001$) (Table 5). The nearly similar Φ_{ST} from the AMOVA analysis (Table 5) and the G_{ST} from the PopGene analysis demonstrate the robustness of the ISSR markers used in this study. The estimated number of migrants per generation (Nm) was 1.342 (Table 4), which suggests that the gene flow in *A. chrysantha* was moderately low. The UPGMA dendrogram revealed that the 65 individuals from the two Spanish populations were clustered into two main groups with 100% bootstrap support (Figure 1). All individuals from the La Azohía population were clustered into one group, while the individuals from Escombreras Island were clustered into the other group.

Table 4. Genetic differentiation within and between the Spanish populations of *Anthemis chrysantha*.

Shannon´s information index		Nei´s gene diversity	
H_{pop} (S.D)	0.4127 (0.0317)	H_S	0.2888 (0.0231)
H_{sp} (S.D)	0.4917 (0.2668)	H_T	0.3427 (0.1899)
H_{pop}/H_{sp}	0.8394	H_S/H_T	0.8427
$(H_{sp} - H_{pop})/H_{sp}$	0.1605	G_{ST}	0.1573
-		Nm	1.342

H_{pop}, within-population genetic diversity; H_{sp}, total genetic diversity; H_{pop}/H_{sp}, ratio of genetic diversity within population; $(H_{sp} - H_{pop})/H_{sp}$, ratio of genetic diversity between populations. H_S, within-population gene diversity; H_T, total gene diversity; H_S/H_T, ratio of gene diversity within population; G_{ST}, genetic differentiation coefficient; Nm, gene flow estimated from G_{ST}; S.D., the standard deviation.
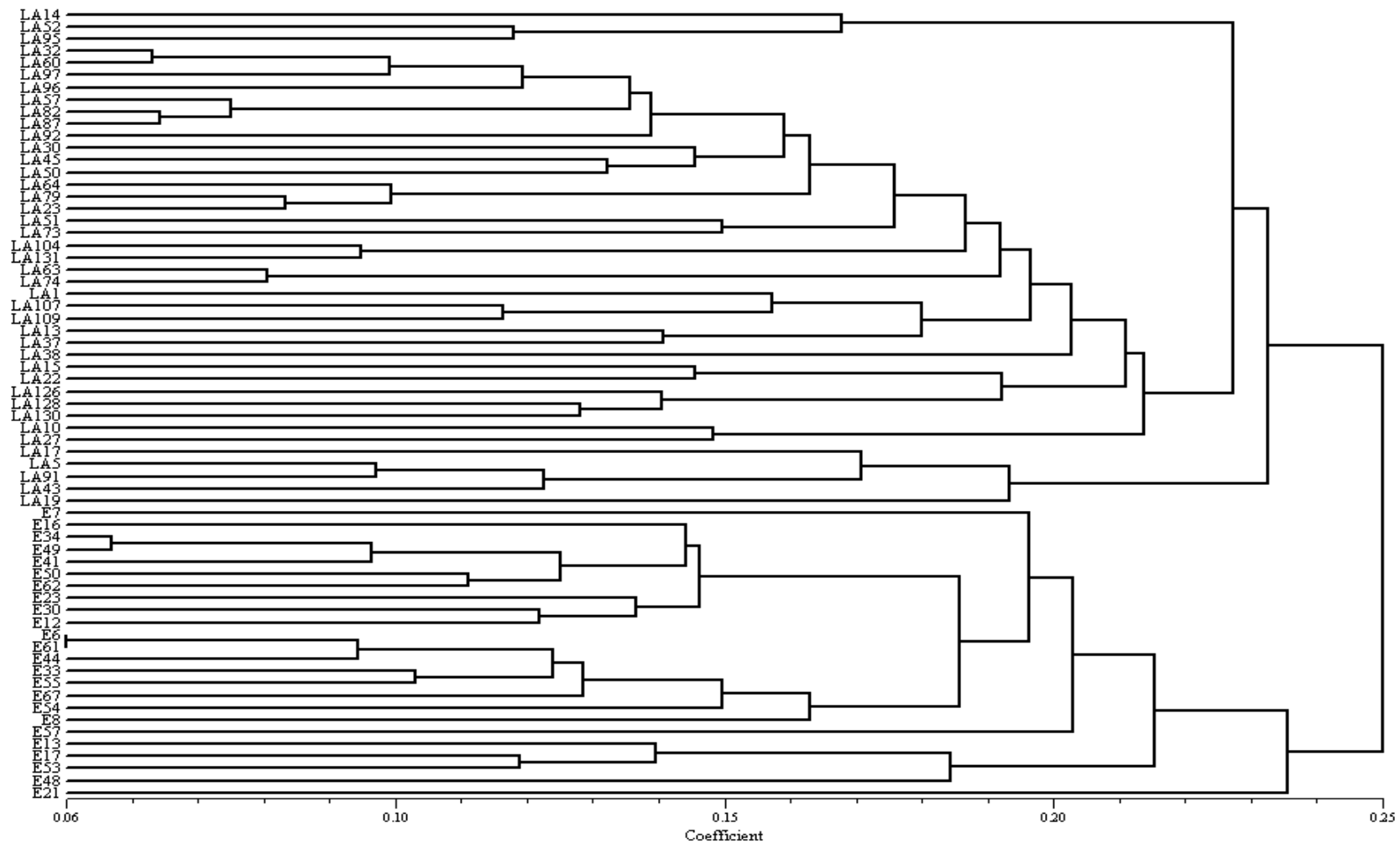
Figure 1. Dendrogram of the 65 individuals from the Spanish populations of *Anthemis chrysantha*, using UPGMA. Individuals from the La Azohía and Escombreras Island populations are designated as LA and E, respectively.

Table 5. Analysis of molecular variance (AMOVA) within and between the Spanish populations of *Anthemis chrysantha*.

Source of variation	d.f.	SSD	MSD	Variance component	Percentage	Φ_{ST}	*P* value
Between populations	1	81.178	81.178	2.324	18%	0.177	
Within populations	63	681.960	10.825	10.825	82%		0.001

d.f., degrees of freedom; SSD, sums of squares; MSD, mean square deviations; variance component estimates; percentage of total variance (% total) contributed by each component and the significance of the variance (*P* value). Significance test after 9999 permutations.

3.3. Intraspecific phylogenetic relationships

The six ISSR primers chosen for this analysis (Table 2) produced a total of 53 bands ranging from 300 to 1500 bp, corresponding to an average of 8.8 bands per primer (data not shown). Eleven DNA markers present in the Algerian bulk DNA sample but which were absent in the two Spanish bulk samples could be considered as Algerian population-linked markers. However, only two DNA markers could be considered as La Azohía population-linked markers, and just one for the population of Escombreras Island. The UPGMA method of clustering was carried out to estimate the phylogenetic relationships among the three populations, using Nei´s unbiased genetic distance matrix (Table 6). The three populations of *A. chrysantha* were separated into two groups with 100% bootstrap support: the two Spanish populations were clustered in one group, while the population from Algeria was separated into another group (Figure 2). The examination of the relationship between the geographical and Nei´s genetic distances - using a Mantel test - was not significant ($R^2 = 0.9525$, $P > 0.05$).

Table 6. Nei´s (1978) unbiased estimates of genetic distance and the pairwise geographical distances (km) between Spanish and Algerian populations of *Anthemis chrysantha*.

Population	La Azohía	Escombreras	Algeria
La Azohía	-	0.216	0.365
Escombreras	17.8	-	0.325
Algeria	198.7	193.2	-

The genetic distance is shown above the diagonal and the geographical distance below the diagonal.

100

99

ESCOMBRERAS▪

0.11
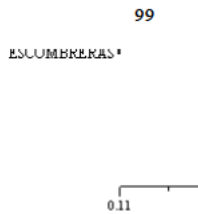
Figure 2. Dendrogram of the three populations of *Anthemis chrysantha* using UPGMA. The numbers marked on the branches are bootstrap values (%) out of 1,000 bootstrappings.

4. Discussion

At the species level, the genetic diversity indices (PPB: 78.48%, A_e: 1.6251, H_E: 0.3427, SI: 0.4917) based on ISSR markers show that the genetic variation of *A. chrysantha* was high. Although, generally, species with small geographic ranges tend to maintain less genetic diversity than geographically widespread species (Hamrick and Godt, 1989), exceptions are not uncommon (López–Pujol et al., 2002; Godt et al., 2005; Luan et al., 2006). The estimate of Nei´s genetic diversity in this study (0.3427) for *A. chrysantha* is higher than the mean values reported for random amplification of polymorphic DNA (RAPD) markers in other annual species (0.13) (Nybom, 2004). However, the results obtained in this species are consistent with some traits of its life history, such as population size and breeding system. *Anthemis chrysantha,* although growing in small areas (≤ 0.02 km^2), forms large-scale populations. Population genetic theory predicts that larger populations tend to maintain higher allelic diversity than smaller ones (Hedrick, 1985; Ellstrand and Elam, 1993).

On the other hand, the breeding system is a major factor in explaining levels of genetic variability at both the species and population level (Nybom and Bartish, 2000), with selfing taxa being the least diverse and outcrossing taxa the most diverse. Thus, the breeding system is a species characteristic that, among others (e.g. seed dispersal and life forms), determines the influence of habitat fragmentation on the overall genetic structure of a plant population (Young et al., 1996). The mating system of *A. chrysantha* has not been investigated yet in detail, but it can be assumed to involve a process of

cross pollination. This species is described as generalist entomophilous (Sánchez et al., 2004), its inflorescence consisting of one flowering head having between 100 and 130 flowers, with a long flowering period. However, we cannot discard the expression of a mixed mating system with differences in outcrossing rates between floret types, as have been documented in many heterocarpic Asteraceae (Cheptou et al., 2001; Gibson and Tolimson, 2002).

All the analyses performed to assess the genetic structure of the *A. chrysantha* populations in Spain (i.e the ratio of genetic diversity among populations as estimated by Shannon´s information index, the coefficient G_{ST}, and AMOVA analysis with Φ_{ST} statistics) produced similar patterns: they all indicated a high within-population variability and a low, albeit significant, differentiation between populations. Thus, of the total gene diversity (H_E), which was 0.3427, the within-population genetic diversity accounts for about 82%, while the among-population genetic diversity represents only a small fraction of the total H_E. Like *A. chrysantha*, higher levels of genetic diversity within populations and lower differentiation between populations have been reported for many species, Asteraceae included (Luan et al., 2006; Petros et al., 2007). This seems to be a common pattern in outcrossing plants, whereas selfing and clonal plants would be more likely to reveal a contrasting pattern (Cole, 2003; Nybom, 2004).

The low G_{ST} value obtained (0.1573) indicates considerable genetic connectivity between populations of *A. chrysantha*, which could be explained by medium-high levels of genetic flow, or a recent species fragmentation (Caujapé–Castells, 2006). Species with a limited potential for gene flow show more differentiation among populations than do species with high levels of gene flow (Hamrick et al., 1991). In population genetics, a value of gene flow (Nm) < 1.0 is generally regarded as the threshold beyond which significant population differentiation occurs (Slatkin, 1987), although a migration rate of 0.5 was considered sufficient by Ellstrand and Elam (1993) to overcome the diversifying effects of random drift. Our ISSR-based estimate of migration, Nm = 1.342, suggests that there is still a certain amount of gene flow between Spanish populations of this species, and that genetic drift has not yet had a major influence on these populations. Since seed dispersal is limited to beneath the plant canopy or in the immediate vicinity of the mother plant, and discarding the secondary dispersal of seeds (Aguado et al., 2012; Chapter 2), we assume that gene flow should occur through pollinators. However, since the distance that separates the populations (> 17 km) exceeds the foraging radius of their pollinators, we do not believe that gene flow is

currently occurring between the sampled populations. It is possible that these populations were connected in the past and were isolated only recently (the La Muela population - located between them - has not been detected since 1996), and that not enough time has passed for this species to show more genetic divergence among the remaining populations. Therefore, we conclude that the high genetic connectivity found between the Spanish populations is the result of a recent genetic isolation by habitat fragmentation, rather than an effective gene flow.

It is possible that the populations of *A. chrysantha* in Spain have good fitness and evolutionary potential, taking into account the size of these populations and the high genetic variation found at both the species and population level (Reed and Frankham, 2003; Leimu et al., 2006). In fact, several reproduction fitness measures reported in studies by Aguado et al. (2011, 2012) support this assumption: *A. chrysanta* is a plant with high reproductive output (about 100-130 achenes per head with hundreds of heads per square meter) that produces two types of achenes, with differing germination behavior, which are retained on the dead mother plant as a protected, short-term (< 1 year) aerial seed bank. These are specific strategies to increase its reproductive success and to ensure its maintenance in the unpredictable environment that this species inhabits (Gutterman, 2000; Imbert, 2002; Brändel, 2004). Moreover, this species has the ability to form a persistent seed bank, which plays a crucial role in the maintenance of the annual population of this species in occasional dry years (Aguado et al., data not published; Chapter 3). Finally, it is possible that a seed bank contributes to the high levels of within-population genetic diversity and low levels of differentiation found in *A. chrysantha* (Ramp Neale et al., 2008).

Based on the genetic analysis and taking into account that larger populations are less vulnerable to genetic drift or inbreeding depression than smaller ones (Boyce, 1992), we can conclude that the habitat loss and the population size reduction are the main risks of local extinction for *A. chrysantha*. The close relationships between population size, plant fitness and genetic variation (Leimu et al., 2006) suggest that the maintenance of population size is fundamental for plant evolution and conservation.

In this study, ISSR markers proved to be a method useful for the assessment of the phylogenetic relationships among populations of *A. chrysantha*. Fifty-three ISSR loci were sufficient to detect variation and to differentiate *A. chrysantha* populations with different geographical origins. Although no significant correlation was found between the geographical and Nei´s genetic distances, the UPGMA clustering shows the

Spanish populations grouping together, with the Algerian population separated into the other group in 100% of the dendrograms, indicating a clear geographic tendency in the distribution of the genetic variability. Moreover, the high number of Algerian population-linked markers found in this study supports the assumption of this tendency. On the other hand, as occurred in the case of the Asteraceae *Lasthenia conjugens* (Ramp Neale et al., 2008), it is possible that the utilization in the Mantel test of Bayesian estimates of genetic differentiation among populations instead of Nei´s genetic distance gave rise to differences in significance between the two Mantel tests. This may be explained by the fact that the Bayesian estimate is a better estimator of genetic divergence when dominant markers such as ISSRs are used, because the loci are not assumed to be in Hardy-Weinberg equilibrium, which is an assumption of traditional statistics (Holsinger et al., 2002).

In this case, genetic polymorphisms between the Algerian and Spanish populations may be indicative of evolutionary adaptation, which plays a key role in the survival of a population in a changing environment (Stevens et al., 2007). Moreover, this genetic variability may be correlated with the morphological variability observed between the Spanish and Algerian material, which would support the proposal of Sánchez et al. (2002) to recognize the rank of subspecies, at least, for the Spanish populations. Some studies based on molecular data have reconstructed the phylogeny of the tribe Anthemideae (Watson et al., 2000) and the genus *Anthemis* (Lo Presti and Oberprieler, 2009), but *A. chrysantha* was not included in these studies. Our analysis of the intraspecific genetic relationships of this species is the first study carried out on its phylogeny at the infraspecific level.

From the results reported here, it is possible to draw inferences on the conservation of *A. chrysantha* in Spain. Severe habitat loss ascribed to anthropogenic pressures caused local extinction of this species in the past century. Thus, *in situ* conservation strategies should be adopted to protect and maintain its remaining populations. Anthropogenic habitat destruction should be prevented, because natural populations need to be kept at a size sufficient to retain genetic diversity and minimize their risk of extinction. Further management strategies that involve foundation of new populations could be adopted in order to connect the remaining isolated populations of this species. This decision would limit the loss of genetic connectivity due to the current interrupt of gene flow among the remaining populations. However, high levels of genetic polymorphisms should be maintained in these new populations to ensure the

success of conservation. For this, periodic restorations must be carried out, increasing the number of plants in the new populations to maintain an effective population size.

In addition, the conservation strategy for this species should include complementary *ex situ* methods. The low amount of genetic differentiation among populations indicates that a considerable amount of the overall genetic variation of the species could be captured when sampling a larger number of plants from a limited number of populations, rather than smaller collections from many different sites (Ma et al., 2008). In the case of *A. chrysantha*, taking into account that only two populations remain in Spain, the samples for *ex situ* conservation must be collected from all Spanish populations of this species, the sample size of each population being as large as possible so as to capture most of the detected genetic variability.

Acknowledgements

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CHAPTER 5

Introductions in the wild of *Anthemis chrysantha* J. Gay. Methodological assays.

Abstract

Anthemis chrysantha, a critically endangered species, was introduced in the wild by two different methods in 2010. Introductions were conducted in Cartagena, in two places with similar conditions to the natural habitat of the species: in Cala Salitrona, using the transplant method, and in La Podadera, by achene sowing.

Soil samples for each introduced population were analyzed and, during the whole period of study, the temperature and precipitation regimes were monitored. Plant density and reproductive success, measured by achene production or fruitfulness, were determined in two consecutive plant cohorts for each introduction. The data show that transplanting and achenes sowing were valid methods which allowed the establishment of at least two consecutive plant cohorts. The plants introduced in Cala Salitrona gave a high achene production and these achenes were dispersed by atelechory, giving the first plant cohort. In the same way, achenes sowed in La Podadera germinated and consolidated the first plant cohort in this area. These first plant cohorts, both in Cala Salitrona and in La Podadera, produced achenes in June 2011. However, due to the dry spring in 2012, the second plant cohorts, which emerged in autumn 2011, died when they were in bloom and before they could produce achenes in the two places. Since the studied species has the ability to form persistent soil seed bank (Chapter 3), the following years will be crucial in determining whether the introduced populations will be able to establish again, overcoming the reproductive failure of 2012.

1. Introduction

The high rate of disappearance of plant species or increase of the level of threat makes necessary the adoption of conservation programs that prevent the loss of biodiversity in general. Humans may have accelerated the rate of extinction by 100 to 1,000 times the natural rate (Ricketts et al., 2005), causing during the last few centuries extinctions of species the ecological impacts of which are difficult to evaluate. The introduction of populations can be a useful conservation tool (Falk et al., 1996), because increasing the number of populations must increase the probability of a species' persistence over a given time period (Colas et al., 2008). So, the value of species reintroduction has been acknowledged increasingly in international treaties and legislation; hence, much effort has been made in that sphere (Godefroid at al., 2011). Introduction is the deliberate release of organisms into the wild for the purpose of establishing a new population (introduction), re–establishing an extirpated population (reintroduction), or augmenting a critically small population (reinforcement). In reintroductions, plants are managed out of their natural environment at any time and the aim is to reverse a process of population decline or reintroduce specimens of a species where previously extinguished, in order to create new populations.

Anthemis chrysantha J. Gay is a winter annual herb belonging to the family Asteraceae. It is only found on the Algerian coast and the coast of Cartagena (Murcia, Southeastern Spain); the latter is the only European location (Bañares et al., 2004). In Cartagena, four populations were known, but since the late 1990s only two remain, occupying an area around 0.02 km^2: the populations of La Azohía and the Escombreras Island. Although these populations have high numbers of individuals (about 40,000 and 12,000, respectively), they are very limited in terms of extension, their present areas being about 0.005 and 0.015 km^2, respectively. This extremely restricted distribution and its status of endemism meant that *A. chrysantha* was first classified as *Endangered* and later as *Critically Endangered* (Sánchez et al., 2004), according to the International Union for Conservation of Nature (IUCN) categories. Besides, it is catalogued as *En Peligro de Extinción* in the Catálogo Regional de Flora Silvestre Protegida de la Región de Murcia (Decreto 50/2003 BORM 131).

The species *Anthemis chrysantha* grows in terofitics meadows developed in thyme and halonitrophilous scrubs, with a great influence of the sea. The main species we can find in these habitats, with *Anthemis chrysantha*, are: *Asteriscus maritimus* (L.)

Less*., Ferula communis* L*., Frankenia corymbosa* Desf*., Limonium cossonianum* Kuntze*, Lotus edulis* L*., Lycium intricatum* L*., Salsola oppositifolia* Desf*., Mesembryanthemum nodiflorum* L*., Sedum sediforme* (Jacq.) Pau*, Silene secundiflora* Otth in DC*.,* and *Sonchus tenerrimus* L. (Sánchez et al., 2004).

Although the primary goal of an introduction or reintroduction is to increase the number of populations, the ultimate goal is the establishment of viable, self–sustaining populations (Griffith et al., 1989; Maunder, 1992; Gordon, 1996; Menges, 2008), and has been regarded as an extinction–prevention strategy for plant species for at least 100 years (Armstrong and Seddon, 2007). Successful introduction requires knowledge about the taxonomy, reproductive biology, demography, horticulture, and ecology of the introduced species (Kleiman, 1989; Armstrong and Seddon, 2007). Besides, in order to understand the reason for a possible failure of population establishment after introduction, it is crucial to compare the population dynamics of introduced and natural populations surveyed simultaneously (Colas et al., 2008). However, most introductions attempts have failed, and the causes remain unknown (Ren et al, 2010).

This study analyzes *A. chrysantha* introductions by focusing on the two different methods used: direct transplant of the mature plants in the field and achene sowing on the soil. Thus, the general aim of the work is to evaluate both introduction methods according to the results obtained concerning the survival and the establishment of the introduced populations in the first two years. Regarding the introduction by plantation, the specific aims were i) to analyze survival and achene production of the introduced plants, ii) to quantify the first natural cohort from the achenes produced *in situ*, and iii) to observe the dispersal pattern of that cohort. In the introduction by sowing, the specific aims were i) to analyze survival and achene production of the first plant cohort from the achene sowing, and ii) to quantify the second natural cohort grown. However, this study shows only the first steps of the monitoring of the introductions because the following years will be crucial for determining whether the introductions established could be self–sustaining populations.

2. Material and methods

2.1. The studied species

Anthemis chrysantha is only found on the Algerian coast and the coast of Cartagena (Murcia, southeastern Spain). Cartagena has a semi–arid Mediterranean climate characterized by irregular rainfall and a harsh, dry summer period. The annual mean precipitation is around 300 mm, and the mean annual temperature is 17ºC. August is the warmest month, with an average temperature of 24.9ºC and a maximum of 42ºC. The coldest month is January, with an average temperature of 10.6ºC and a minimum always > 0ºC (Aguado et al, 2011; Chapter 1). The emergence period of *A. chrysantha* begins with the first autumn rains, stretching into spring if the weather conditions are favorable. Flowering occurs from early March–late May and fruits mature mostly in June. The plants die in summer, but persist in a dried state in the habitat for several months after death (Aguado et al, 2012; Chapter 2).

2.2. Experimental introductions

In summer 2009, achenes were collected from one of the natural populations of the species, in La Azohía (Cartagena, Murcia; 37∘33´8´´N, 1∘10´22´´W; altitude 30 m), and plants were obtained in a greenhouse in the Tomás Ferro Experimental Station (Cartagena, Murcia). From those plants, two different introductions of the species were carried out.

2.2.1. Introduction by plantation

The place for the introduction was proposed by the Consejería de Agricultura y Agua de la Comunidad Autónoma de la Región de Murcia, near Cala Salitrona (Picture 1) (Cartagena, Murcia; 37º 33´10.6´´ N, 1º 07´33.6´´ W; altitude 25 m), on the basis of its apparent similarity to La Azohía regarding the influence of the sea, orientation, and slope. The surrounding mountain has vegetation composed of esparto grass (*Stipa tenacissima* L.) and rosemary (*Rosmarinus officinalis* L.), but the selected hillside, where the introduction was made (Picture 2), is more bare and has mainly thyme brushes with *Frankenia corymbosa* Desf., *Phagnalon saxatile* (L.) Cass., *Asteriscus*

maritimus L., *Limonium insigne* (Coss.) Kuntze., and some splashes of *Stipa tenacissima* L. and *Salsola genistoides* Juss. ex Poir. in Lam. plants.
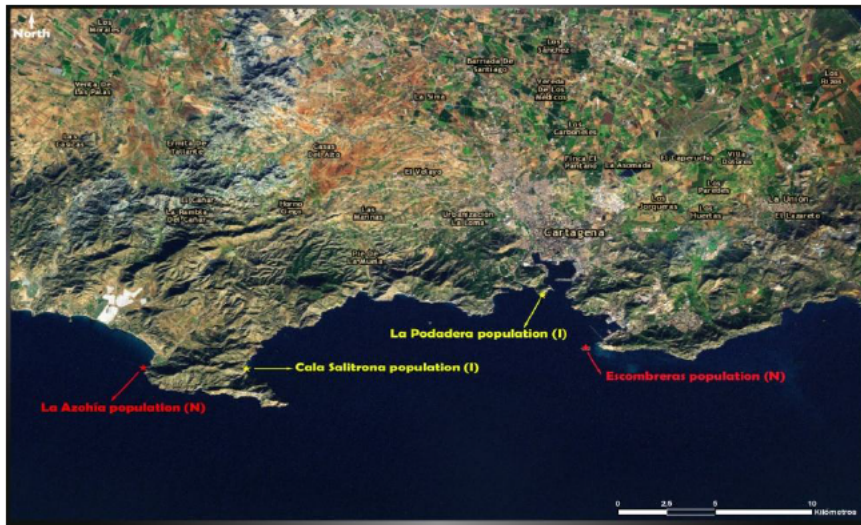
Picture 1. Geographical location of the introduced populations (I), in Cala Salitrona and in La Podadera, and natural populations (N) on the coast of Cartagena.

The introduction of the species was carried out in March 2010. To obtain the plants needed for the introduction, achenes were sown in November of the previous year in 250–mL–volume and 9–cm–diameter pots, which contained peat. The pots were kept in a greenhouse in the Tomás Ferro Experimental Station until their transport to the field, just before the plants started to bloom.
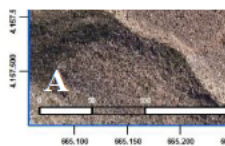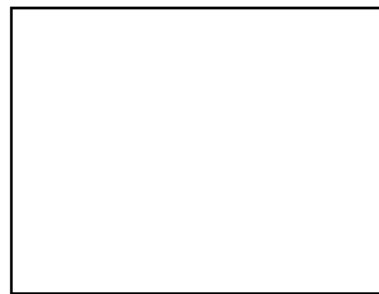
Picture 2. Detail of the geographical location in Cala Salitrona (A) and selected site in Cala Salitrona (B).

Then, once in the selected site, a total of 40 plants were planted in 8 plots of 5 plants each. The plants were about 40 cm apart and each plot was at least 3 meters away from the others. A single irrigation was made in order to ensure the plant establishment.

A soil sample was taken from each plot and analyzed in the laboratory by the Agro Chemistry, Substrate and Soil Technology and Management Research Group (Agriculture Science and Technology Department, Higher Technical School of Agricultural Engineering, Technical University of Cartagena). The real pH was estimated following Peech (1965), making a 1:1 suspension in water with air–dried soil sifted to 2 mm. The potential pH was estimated also in a 1:1 suspension, but with KCl solution. The electrical conductivity ($EC_{1:5}$) was measured in a 1:5 solid:liquid suspension. Calcium carbonate ($CaCO_3$) was determined by the method of Bernad (Muller and Gastner, 1971), using 4 N HCl to dissolve it. Assimilable phosphorus (P_2O_5) was estimated according to Watanable and Olsen (1965), based on the extraction of phosphorus with 0.5 M $NaHCO_3$ (Olsen and Dean, 1965). Total nitrogen (TN) was determined by the modified Kjeldahl method (Bremmer and Mulvaney, 1982). The percentage of organic matter (OM) was determined on the basis of the loss by calcination (LOI) at 550 ° C (Bengtsson and Enell, 1986). Then, the organic carbon content (OC) was obtained using the expression C_{total} = OM % / 1.84 (Iglesias and Pérez, 1992). The particle size was determined by the Bouyoucos Method (Filgueira et al., 2006) with samples sieved to 2 mm. The cation–exchange capacity (CEC) was estimated by saturation of the soil exchange complex with ammonium (NH_4^+), which was evaluated by distillation after its displacement by sodium (Na^+).

The precipitation and temperature were checked during the study period (2010–2012). Daily climatic data were provided, by the Agricultural Information Service of Murcia (SIAM), from the Cañada Gallego Meteorological Station, located about 22 km to the West of Cala Salitrona. Roche Meteorological Station is also located about 19 km to the East of Cala Salitrona but, according to the isohyets, Cañada Gallego Station fits better the Cala Salitrona conditions. The data are shown in Figure 1.

After the introduction, Cala Salitrona was visited in early June 2010, when the plants were in bloom, for evaluation of the survival of the introduced plants. Then, in late June the site was visited again and 30 capitula selected randomly from all the plots were carried to the laboratory in order to determine the achene number and then to estimate the achene production of the introduced plants. In September and November 2010, a new monitoring was performed, respectively, in order to check the achene

dispersal and the possible seedling emergence due to the autumn rainfall. In May 2011, when the new plant cohort was in flower, we determined the plant number per plot and measured the distance of each plant from the nearest mother plant. In early July, a last monitoring was made, to check the success of fruiting. In December 2011, after the autumn rainfall, we counted the seedlings of the next generation or plant cohort. In May 2012, plant number was determined again as well as the plant survival in spring and the achene production.
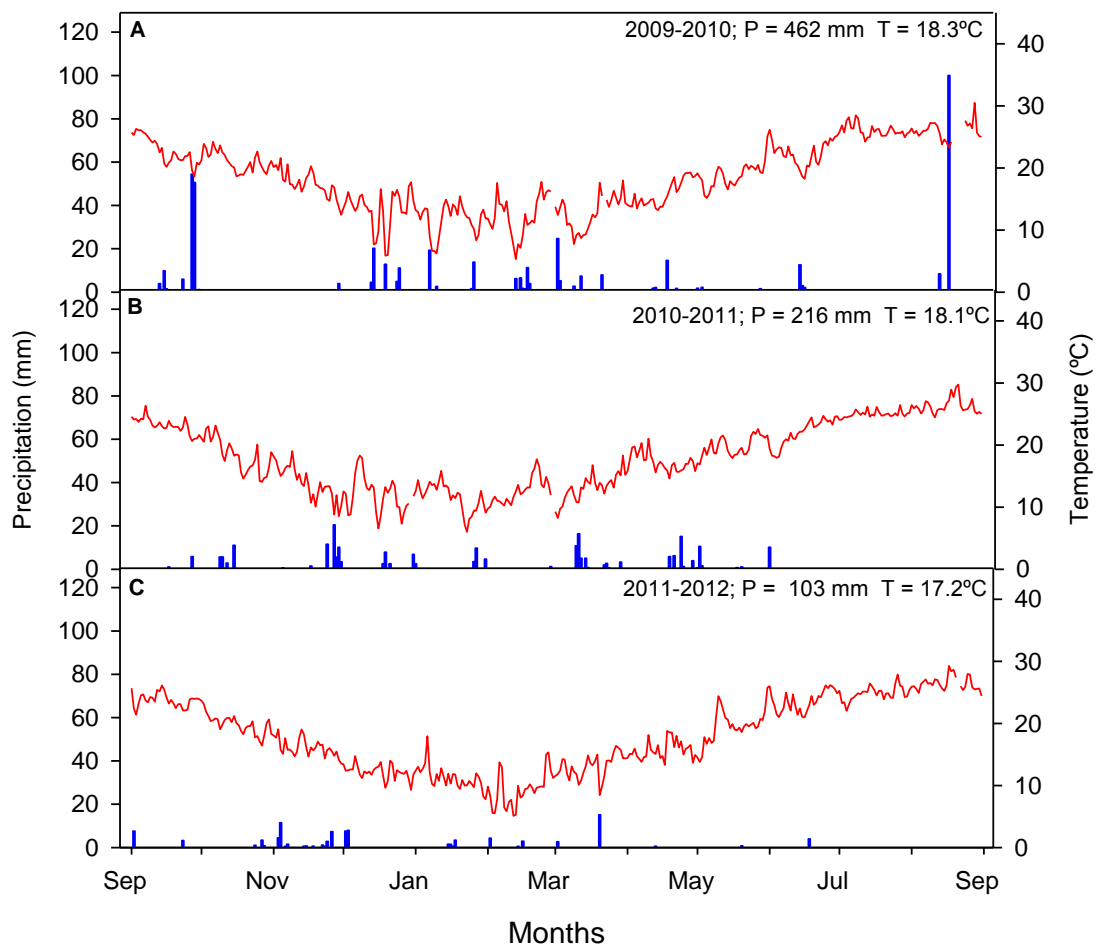
Figure 1. Daily precipitation (bars) and mean daily temperatures (lines) from September 2009 to August 2010 (A), September 2010 to August 2011 (B), and September 2011 to August 2012 (C) at the Cañada Gallego Station. *P* = total precipitation in each period; *T* = mean temperature for each period.

2.2.2. Introduction by achene sowing

The site was proposed by the Consejería de Agricultura y Agua de la Comunidad Autónoma de la Región de Murcia, following criteria similar to those described in

Introduction by plantation. In this area there are scrubs of *Suaeda vera* Forssk. ex J.F. Gmel., *Salsola oppositifolia* Desf., and *Atriplex halimus* L. In the clearings of the scrub grow *Asteriscus maritimus* L., *Limonium cossonianum* Kuntze, *Sedum sediforme* (Jack.) Pau, *Sonchus oleraceus* L., *Frankenia corymbosa* Desf., *Dactylis glomerata* L., *Lotus edulis* L., *Plantago coronopus* L., and *Sonchus tenerrimus* L.

This introduction was carried out in October 2010, near La Podadera Fort (Cartagena, Murcia; 37°35′03.5′′ N, 0°59′14.7′′ W; altitude 20 m) (Picture 1). Twelve 1-m-diameter circular plots, tagged correctly, were established. In each plot, achenes contained within 50 capitula from plants grown in the greenhouse were dispersed by hand, giving 5,000–6,000 achenes per plot (Picture 3).
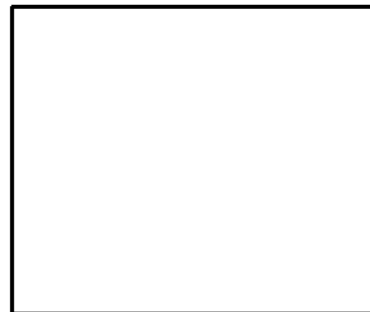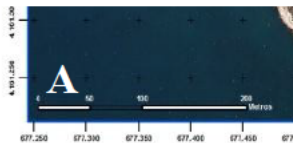
Picture 3. Detail of the geographical location in La Podadera Fort (A) and selected site in La Podadera Fort (B).

A soil sample was taken from some randomly selected plots and analyzed in the laboratory by the Agro chemistry, Substrate and Soil Technology and Management Research Group, as described in the *Introduction by plantation*.

The precipitation and temperature were checked during the study period (2009–2012). The daily climatic data, provided by the Agricultural Information Service of Murcia (SIAM), were from the Roche Meteorological Station, located about 7 km to the East of La Podadera, and are shown in Figure 2.
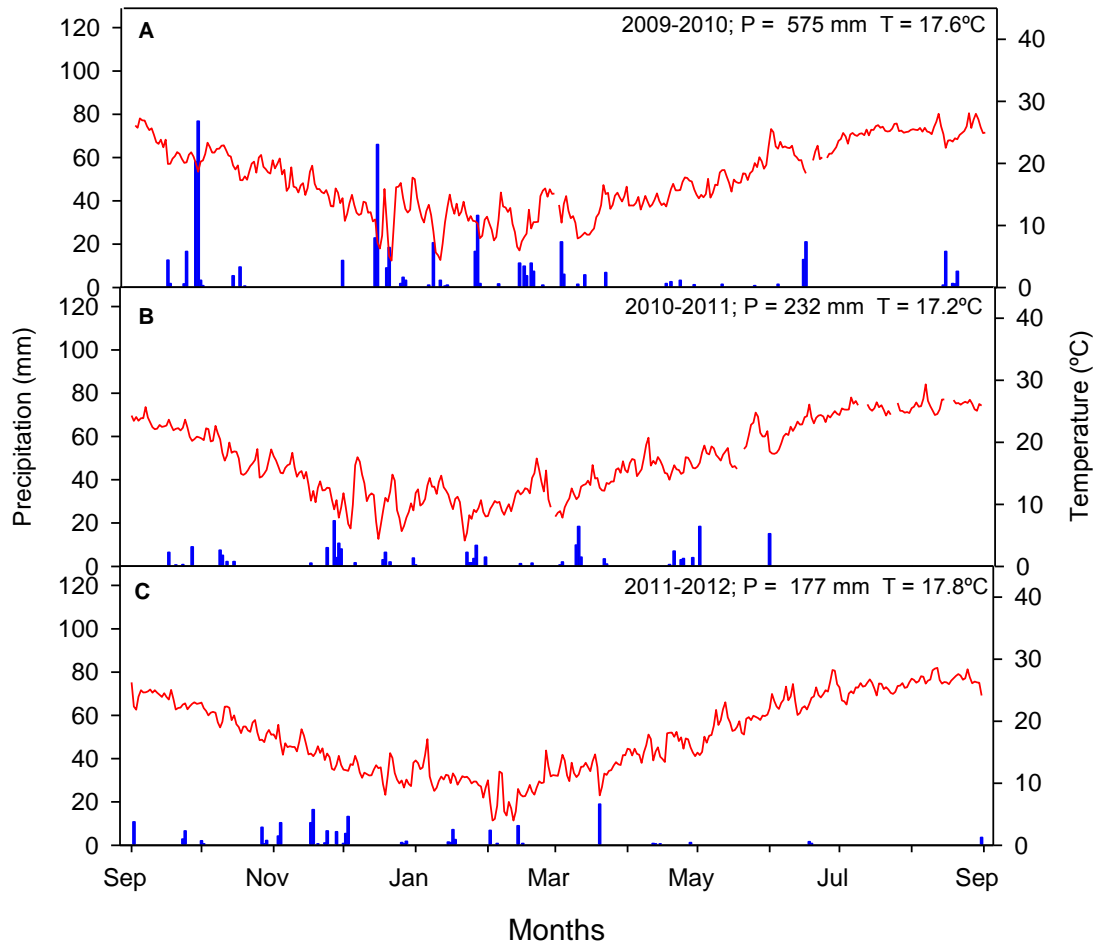
Figure 2. Daily precipitation (bars) and mean daily temperatures (lines) from September 2009 to August 2010 (A), September 2010 to August 2011 (B), and September 2011 to August 2012 (C) at the Roche Station. P = total precipitation in each period; T = mean temperature for each period.

In this introduction carried out by achene sowing in October 2010, the monitoring of the seedling emergence was estimated in December. Thus, four randomly–selected subplots of 10 x 10 cm were selected in each sown plot and the seedling number was counted in order to estimate plant density. In May 2011, plant density was estimated again, but in 10 randomly-selected subplots, to obtain more-accurate data. Besides, the capitula number per plant was counted in these plants. Finally in July, when the achenes were mature, three capitula were collected in the most of the plots and their achenes were counted. Then, the total achene production per plot was calculated.

After the autumn 2011 and the first rainfall episodes, we visited the population to check the seedling emergence of the new plant cohort, but the census of the

population was estimated in late April 2012, when all the plants were flowering and the survival possibilities were higher. Finally, in early July 2012 a last monitoring was performed, to check the success of fruiting.

3. Results

3.1. Introduction by plantation

The analysis of soil samples from Cala Salitrona (Table 1) showed a high pH, but similar to the natural population soil. The $EC_{1:5}$ indicated that the soil is not saline (Alarcon, 2007), but in La Azohía the value is much higher - although these samples were taken in summer when the soil was very dry. According to the $CaCO_3$ content, the soil is moderately calcareous compared to La Azohía, which is very calcareous (Alarcon, 2007). In Cala Salitrona, phosphorus that could be assimilated by plants (P_2O_5), total nitrogen (TN), and total organic carbon (OC) are very low; the latter indicates a low organic matter content as well. The C/N ratio is also very low and indicates very low formation of humus and predominance of the mineralization of the low organic matter content. The texture in Cala Salitrona is sandy-loam to sandy-clay-loam. Due to the low value of organic matter and the high proportion of sand, the cation–exchange capacity (CEC) is very low too. Thus, the soil of the selected area is less favorable than that of La Azohia for vegetation development. In fact, the vegetation of the area is less than in the natural population, and the soil type is different also, being petric calcisols.

The monitoring carried out in June 2010 showed that all the plants survived the transplanting, producing on average 15–49 capitula per plant, depending on the plot (Table 2). Each capitulum produced on average 122.87±24.21 achenes (Table 3), being the proportion 63.29% and 36.71% for white and dark achenes, respectively. Both values were whitin the range of those obtained in the natural population in La Azohía (Aguado et al., 2012; Chapter 2), but the unviable white achenes was high in the introduced plant (29.53%). The achene production per plot was estimated from the apparently viable achenes and ranged between 7,228 and 22,509 (Table 2), being the average achene production 11,769.19±4,138.65.

Table 1. Characteristics of representative soil samples from the natural population of *Anthemis chrysantha* in La Azohía and the introduced population in Cala Salitrona.

Sample	Real pH	Potential pH	EC$_{1:5}$ (dS·m^{-1})	CaCO$_3$ (%)	Assimilable P (mgP·kg^{-1})	TN (g·kg^{-1})	OC (g·kg^{-1})	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mEq·100g^{-1})
LA 1	8.24	7.53	2.24	34.33	39.6	2.46	36.21	14.73	23.45	29.84	46.71	15.80
LA 2	8.12	7.60	3.17	32.37	33.1	2.11	51.27	24.24	24.52	26.91	48.57	12.80
LA 3	8.33	7.60	1.40	39.07	38.9	1.98	65.23	32.89	13.52	22.06	64.42	46.90
Average LA	**8.23**	**7.58**	**2.27**	**35.25**	**37.19**	**2.19**	**50.90**	**23.95**	**20.50**	**26.27**	**53.23**	**24.17**
Deviation LA	**0.11**	**0.04**	**0.89**	**3.44**	**3.59**	**0.25**	**14.51**	**9.08**	**6.07**	**3.93**	**9.73**	**18.88**
CS 1	8.93	8.67	0.31	6.79	1.18	0.15	0.79	5.12	17.45	22.06	60.50	1.30
CS 2	9.28	8.40	0.20	20.51	4.25	0.17	1.23	7.36	15.99	18.24	65.78	1.30
CS 3	8.80	8.28	0.20	22.41	2.38	0.25	1.20	4.80	13.99	19.52	66.50	2.40
CS 4	8.83	7.99	0.20	11.67	1.85	0.41	4.50	11.140	15.99	17.52	66.50	3.40
CS 5	8.83	8.11	0.26.	16.56	3.61	0.85	8.61	10.15	9.99	19.44	70.57	3.20
CS 6	8.69	8.31	0.16	14.77	1.97	0.36	6.41	17.69	3.99	13.44	82.57	1.40
CS 7	8.57	8.14	0.17	13.97	2.33	0.70	-	-	7.99	13.44	78.57	2.10
CS 8	8.76	7.90	0.30	32.94	3.61	0.60	-	-	24.06	23.23	52.71	4.80
Average CS	**8.84**	**8.23**	**0.22**	**17.45**	**2.65**	**0.44**	**3.79**	**9.37**	**13.68**	**18.36**	**67.96**	**2.49**
Deviation CS	**0.20**	**0.24**	**0.06**	**5.74**	**1.16**	**0.26**	**3.25**	**4.82**	**5.08**	**2.87**	**7.50**	**0.97**

Table 2. Capitula number per plant and viable achene production from the introduced plants and offspring monitoring (plant number) of the two studied cohorts, in Cala Salitrona.

Plot	Introduced plants		1st plant cohort	2nd plant cohort	
	Jun 2010		May 2011	Dec 2011	May 2012
	Capitula/plant (total capitula)	Achenes	Plants	Plants	Plants
1	17.20±5.89 (86)	7,869.00±2,694.98	2	30	0
2	49.20±10.96 (246)	22,509.00±5,015.84	52	16	0
3	32.60±21.81 (163)	14,914.50±9,979.37	159	20	10
4	27.80±14.13 (139)	12,718.50±6,465.17	56	6	0
5	22.40±5.73 (112)	10,248.00±2,620.16	256	13	0
6	21.60±3.58 (108)	9,882.00±1,636.80	196	20	0
7	15.80±5.45 (79)	7,228.50±2,493.27	143	53	1
8	19.20±4.82 (96)	8,784.00±2,203.61	78	3	0
Average	25.73	11,769.19	117.75	20.13	1.38
Deviation	10.98	4,138.65	85.10	15.76	3.50

Neither in September nor in November 2010 was seedling recount performed, because the capitula remained intact on the dead mother plants, so no seedling emergence was observed. However, in May 2011 we took a census of the new plant cohort and plant density ranged between 2, in the first plot, and 256 plants, in plot number five (Table 2). We also observed that an average of about 75% of the first cohort had grown in the shadow of the dead mother plant, less than 30 cm from it, and only 2.31% grew towards other directions (against the slope, generally) of the hillside (Table 4). The mean dispersal distance of the seedlings with slope was 78.57±62.45 cm, and the maximum was 270.29±89.47 on average. However, the mean and maximum dispersal distances reached against the slope by the seedlings were only 16.82±24.19 and 30.71±34.09 cm, respectively (Table 4). In July 2011, we saw that all the plants recorded in May, which belonged to the first cohort, had produced achenes.

In autumn 2011 a new plant cohort was established and in December seedling emergence was observed in all the plots, ranging between 3 and 53 plants per plot with an average of 20.13 plants. In May 2012 we found only two plots with live plants; there were 10 in plot number three and only 1 in plot number 7 (Table 2). However, all these plants were small and without vigor and when the site was visited in July they had died without producing achenes.

Table 3. Recount of the different achene types from the sampled capitula of *Anthemis chrysantha* in June 2010 in Cala Salitrona.

Capitula	White achenes	Dark achenes	Achenes from ligules	Total achenes	Unviable white achenes	Total viable achenes
1	87	61	7	155	16	139
2	50	38	9	97	31	66
3	47	51	11	109	31	78
4	44	57	7	108	28	80
5	76	71	9	156	17	139
6	77	72	8	157	8	149
7	78	56	9	143	8	135
8	129	15	9	153	61	72
9	63	68	7	138	9	129
10	111	59	9	179	49	130
11	69	53	6	128	29	99
12	89	19	9	117	89	28
13	43	69	8	120	19	101
14	87	15	6	108	56	52
15	64	50	7	121	10	111
16	56	71	7	134	41	93
17	33	84	8	125	12	113
18	56	39	6	101	10	91
19	72	31	7	110	54	56
20	74	5	8	87	8	79
21	81	9	8	98	55	43
22	24	27	6	57	4	53
23	85	15	8	108	44	64
24	82	22	8	112	40	72
25	68	42	9	119	31	88
26	74	48	8	130	22	105
27	110	6	8	124	6	98
28	86	35	11	132	57	75
29	78	41	7	126	26	100
30	75	53	6	134	15	107
Average	72.27	42.73	7.87	122.87	29.53	91.50
Deviation	22.64	22.38	1.33	24.21	21.08	30.93

Table 4. Seedling dispersal distance from the mother plants of *Anthemis chrysantha* introduced in Cala Salitrona.

Plot	Plant number	Dispersed seedling proportion (%)			Mean dispersal distance (cm)		Maximum dispersal distance (cm)	
		Under mother	With the slope	Other directions	With the slope	Other directions	With the slope	Other directions
1	2	100.00	0.00	0.00	-	-	-	-
2	52	63.46	34.62	1.92	193.80	0.00	404.00	0.00
3	159	84.81	14.56	1.27	121.50	3.50	341.00	5.00
4	56	62.50	37.50	0.00	67.50	0.00	142.00	0.00
5	256	86.33	12.89	0.78	76.30	65.00	320.00	67.00
6	196	60.71	35.71	3.57	5.80	29.14	212.00	81.00
7	143	88.11	11.19	0.70	31.20	0.00	220.00	14.00
8	78	53.85	35.90	10.25	53.90	20.13	253.00	48.00
Average		74.97	22.80	2.31	78.57	16.82	270.29	30.71
Deviation		16.74	14.71	3.41	62.45	24.19	89.47	34.09

3.2. Introduction by achene sowing

Analysis of the soil samples from La Podadera (Table 5) showed that the area has a high pH, similar to the natural population, and the $EC_{1:5}$ indicates that is not saline (Alarcon, 2007), as in Cala Salitrona. Taking into account the $CaCO_3$ content, the soil is very calcareous (Alarcon, 2007), similar to La Azohía. Phosphorus that could be assimilated by plants (P_2O_5) is extremely high, more than at the natural population site. Besides, the total nitrogen (TN), organic carbon (OC), and C/N ratio are high, but similar to the values obtained in La Azohía. However, the cation–exchange capacity (CEC) is very low compared to La Azohía, due to the low clay and high sand proportion, although it is higher than in Cala Salitrona. The texture of the samples from La Podadera is mainly sandy loam. Like in La Azohía, in La Podadera we find lithosols with high organic matter but here they are altered; in fact, some selected plots are in areas where soil developed on old constructions.

Estimated plant number of the population in early winter (2010) ranged between 314 plants in plot number seven and 1,315 plants in plot number two, the average being 572.69±266.58 plants per plot (Table 6). However, in May 2011, plant density decreased to 124.52±67.74 plants per plot. The plants were small with no more than 3 capitula in most plots; only plots four and ten reached an average of 6 and 5 capitula per plant, respectively. Each capitulum had an average of 78.84±44.17 achenes, being the proportion 72.57% and 27.43% for white and dark achenes, respectively (Tabla 7). Achene production per plot, estimated from apparently viable achenes, was on average 36,821.70±32,844.28, which equates to around 46,883 achenes per square meter, but the deviation was very high due to the high differences between plots (Table 6). Both achene production and achene type proportion were within the range of data obtained in Cala Salitrona and La Azohía populations. The proportion of the unviable white achenes was similar to Cala Salitrona (16.58%).

Table 5. Characteristics of representative soil samples from the natural population of *Anthemis chry*santha in La Azohía and the introduced population in La Podadera.

Sample	Real pH	Potential pH	EC$_{1:5}$ (dS·m^{-1})	CaCO$_3$ (%)	Assimilable P (mgP·kg^{-1})	TN (g·kg^{-1})	OC (g·kg^{-1})	C/N	Clay (%)	Silt (%)	Sand (%)	CEC (mEq·100g^{-1})
LA 1	8.24	7.53	2.24	34.33	39.6	2.46	36.21	14.73	23.45	29.84	46.71	15.80
LA 2	8.12	7.60	3.17	32.37	33.1	2.11	51.27	24.24	24.52	26.91	48.57	12.80
LA 3	8.33	7.60	1.40	39.07	38.9	1.98	65.23	32.89	13.52	22.06	64.42	46.90
Average LA	**8.23**	**7.58**	**2.27**	**35.25**	**37.19**	**2.19**	**50.90**	**23.95**	**20.50**	**26.27**	**53.23**	**24.17**
Deviation LA	**0.11**	**0.04**	**0.89**	**3.44**	**3.59**	**0.25**	**14.51**	**9.08**	**6.07**	**3.93**	**9.73**	**18.88**
LP 1	8.46	8.20	0.28	43.97	20.80	1.18	25.04	21.31	3.99	26.22	69.79	2.59
LP 2	8.23	8.07	1.41	24.29	89.23	1.47	54.47	37.16	0.82	23.39	75.79	9.46
LP 4	8.31	7.97	0.84	27.58	51.16	3.85	71.34	18.55	2.75	31.39	65.86	8.69
LP 5	8.38	7.92	0.43	34.14	40.36	1.66	49.82	30.08	0.82	23.32	75.86	5.26
LP 7	8.52	7.97	0.66	31.63	91.03	2.67	66.23	24.83	2.82	37.24	59.94	7.93
LP 11	8.18	7.52	0.66	22.99	77.74	4.78	113.30	23.69	2.75	33.32	63.94	20.88
Average LP	**8.35**	**7.94**	**0.71**	**30.77**	**61.74**	**2.60**	**63.37**	**25.94**	**2.32**	**29.14**	**68.53**	**9.14**
Deviation LP	**0.13**	**0.23**	**0.39**	**7.73**	**28.70**	**1.45**	**29.31**	**6.71**	**1.26**	**5.72**	**6.48**	**6.29**

Table 6. Monitoring of plant number in the two studied cohorts (December 2010 and December 2011), and estimation of the achene production in the first cohort, in La Podadera Fort.

Plot	1st plant cohort					2nd plant cohort		
	Dec 2010	May 2011	May 2011	Jul 2011	Jul 2011	Dec 2011	Apr 2012	Jul 2012
	Plants	Plants	Capitula/plant	Achenes/capitulum	Achene production	Plants	Plants	Plants
1	333.80	196.35	1.41±0.87	22.67±3.98	6,284.12±3,873.75	25	7	0
2	1,315.55	125.66	1.13±0.34	5.33±2.50	753.51±228.78	7	0	0
3	510.51	39.27	1.40±0.55	-	-	8	2	0
4	510.51	141.37	6.22±9.40	56.40±12.83	49,611.45±74,923.55	110	50	0
5	726.50	39.27	1.80±1.79	-	-	30	16	0
6	647.96	86.39	1.55±0.69	12.00±5.21	1,602.14±712.77	3	0	0
7	314.16	62.83	1.75±1.04	-	-	12	3	0
8	490.88	274.89	3.09±3.85	98.00±21.11	83,126.74±103,796.01	124	61	0
9	530.15	109.96	3.29±1.86	92.00±25.12	33,239.34±18,791.73	273	107	0
10	412.34	164.93	5.48±3.64	96.00±20.13	86,706.06±57,659.89	221	98	0
11	667.59	164.93	3.05±2.45	95.67±20.77	48,087.93±39,439.62	12	1	0
12	412.34	86.39	3.09±3.51	82.33±15.88	21,984.06±24,935.24	19	0	0
Average	572.69	124.52	3.02	62.27	36,821.70	70.33	28.75	0.00
Deviation	266.58	67.74	1.60	39.03	32,844.28	92.33	40.08	0.00

In December 2011, we observed the emergence of seedlings of the second plant cohort with an average of 70.33±92.33 plants per plot, but there were high differences between the plots: two of them reached more than 200 plants and three plots had less than 10. In the next monitoring, in late April 2012, plant density had decreased, being only 28.75±40.08 plants per plot on average. However, in early July we could not find any plants. All the plants died before their biological cycle had completed and hence were not fruitful.

Table 7. Recount of the different achene types from the capitula of *Anthemis chrysantha* sampled in June 2010, in La Podadera.

Plot	White achenes	Dark achenes	Achenes from ligules	Total achenes/capitulum	Unviable white achenes	Total viable achenes/capitulum
1	13.67±2.89	9.33±4.16	3.00±1.73	26.00±3.87	3.33±1.53	22.67±3.98
2	10.00±9.85	0.33±0.58	0.33±0.58	10.67±2.71	5.33±3.06	5.33±2.50
3	-	-	-	-	-	-
4	58.20±30.17	23.00±21.84	4.40±5.41	85.60±11.69	29.10±12.15	56.40±12.83
5	-	-	-	-	-	-
6	29.33±4.62	1.33±2.31	0.67±1.15	31.33±8.77	19.33±12.06	12.00±5.21
7	-	-	-	-	-	-
8	60.00±8.19	41.00±24.64	8.66±1.15	109.67±20.18	11.67±5.51	98.00±21.11
9	72.33±2.52	26.33±21.39	8.00±0.00	106.67±22.01	14.67±14.57	92.00±25.12
10	62.00±19.31	50.67±30.55	10.67±1.15	123.33±16.60	27.33±16.56	96.00±20.13
11	61.67±18.93	51.33±30.57	10.00±2.00	123.00±17.11	27.33±16.56	95.67±20.77
12	50.00±9.54	34.00±24.64	9.33±2.31	93.33±15.36	11.00±8.89	82.33±15.88
Average	46.36	26.37	6.12	78.84	16.58	62.27
Deviation	22.84	19.66	4.04	44.17	9.74	39.03

4. Discussion

The survival data show that all *A. chrysantha* plants withstood the transplanting in the introduction made in Cala Salitrona, completing their lifecycle with normal fruitfulness. The achene number per capitulum and the proportions of white and dark achenes were similar to the natural population in La Azohía. From these achenes the recruitment of the first plant cohort originated, which in general was high (an average of 117 plants in May 2011) (Table 2). As expected, the achene dispersal, from the capitula of the transplanted plants, which formed the first cohort did not occur until the first autumn rains at the end of November. From the achene maturation to the first rainfall events the achenes remained in the capitula on the dead mother plants. Thus, the strategy of this species with respect to forming an aerial seed bank, described in Aguado

et al. (2012) (Chapter 2), has persisted. We also showed that this species has a short-distance dispersal mechanism called atelechory, typical of ombrochoric plants. The results obtained in this study on the introduction by transplanting, regarding dispersal distance, confirm those obtained with plants in pots and an artificial rainfall simulator (Aguado et al., 2012; Chapter 2). In that experiment, 74.9% of the achenes landed beneath the plant canopy and in the first cohort of this introduction the proportion of plants which grew under the mother plant was exactly the same (Table 4), showing that the species behaves in a similar way in the wild. Atelechory is a strategy by which seeds remain in the habitat in which the species is competitive and enable the plants to persist in a determinate area, forming groups, if the environmental conditions are good. However, around 23% of the first cohort could be dispersed with the slope, reaching a maximum distance of 404 cm from the mother plants, while around 2% of the plants could be dispersed in other directions (against the slope, generally), reaching a maximum distance of 81 cm. This would let the introduced plants, in the long-term, form a continuous, big group in the area. In autumn 2011, the second plant cohort was established, but the average plant number per plot was 20; so the seedling recruitment of the second plant cohort was low compared to the first. The rainfall accumulated from the 1st of September to 31st of December 2011 was less than in the same period in 2010 (around 64 and 83 mm in 2011 and 2010, respectively), but its distribution in time was similar. However, the low recruitment in the second plant cohort cannot be explained by this lower rainfall. The decline in the recruitment of the second plant cohort was not surprising since other studies about plant species introductions show that the success rates of the introduced populations decline with time (Godefroid et al., 2011). In fact, it is known that the introduction success should be evaluated in the long-term. After December 2011, the next winter and spring were also dry (Figures 1 and 2) and the plants died due to lack of water before they could complete their lifecycle, so the second cohort will not produce offspring.

In the introduction carried out by achene sowing, the first census - in early winter (2010) - estimated 573 plants per plot, although the deviation was high (Table 6). However, an average of only about 10% of the total sown achenes germinated, indicating that a high fraction of them could remain in the soil without germination or loss. The mean plant number decreased to 125 plants per plot in spring 2011. In spite of this, the achene production and the proportion of each achene type in this first cohort were within the range of those obtained in the natural population, in La Azohía, and in

the introduced population in Cala Salitrona. In December 2011, we observed again the decline in the recruitment of the second plant cohort, which had a lower plant number than the previous one, with an average of 70 plants per plot (Table 6). Besides, the plant number decreased with time: the average in April was 29 plants per plot and in July all the plants had died without becoming fruitful, again due to lack of rainfall in the spring, so this was another "lost" plant cohort.

The reproductive failure in 2012 in both the Cala Salitrona and La Podadera populations was linked intimately to the dry meteorological conditions in spring but not to intrinsic causes arising from the population; but, this does not have to mean the total failure of the introductions carried out in these areas. After an introduction, it is crucial to compare the population dynamics of the introduced and natural populations surveyed simultaneously. Thus, in Chapter 3 we demonstrated that when the spring is dry the reproductive success of the population fails, but we also demonstrated the ability of *A. chrysantha* to form a persistent soil seed bank (PSB). That happened in 2012 in both places, where the spring was too dry and the plants died without becoming fruitful but a fraction of the dispersed achenes, from both the introduced and the first plant cohort, could remain in the soil without germinating, starting the formation of the seed bank of the species in the introduced populations.

In fact, a PSB plays a crucial role in the maintenance of the annual population of this endangered species living in an unpredictable habitat with occasional dry years. In the two introduced populations, some of the plots had high achene production, especially at La Podadera where we also observed some favorable microenvironments, giving us hope about the introductions. The formation, in La Podadera and/or Cala Salitrona, of a possible PSB could provide dramatic population recovery, if the coming years are conducive. This happened before in the natural population of the species in La Azohía (Chapter 3), where the PSB was able to reestablish the population after the very-dry spring of 2008. However, consecutive dry years could deplete the possible and young seed bank of the species in both Cala Salirona and La Podadera. Therefore, for *A. chrysantha*, persistent continuation of its populations could depend ultimately on the frequency of years with a wet flowering season. Thus, it is necessary to continue monitoring both introductions for some years more, paying special attention to the possible seed bank. Due to the dry spring, this year will give us the opportunity to check if the introduction techniques used have allowed the species to form a PSB in just two years. Besides, we will know if these possible PSBs will be enough to ensure the

continuity of the population after a reproductive failure, since that is what happens in the natural population according to the data presented in Chapter 3.

Some aspects we think interesting to discuss are those pointed out by Godefroid et al. (2011). For example: is the number of individuals introduced enough to ensure the population success? The results obtained by these authors highlight a positive relationship between the number of individuals introduced and their survival. Demographic and genetic theories both predict that the persistence time of a population increases with the initial size (Robert et al., 2007). Simulation studies have shown that demographic stochasticity is only important in populations of 50 or fewer individuals (Menges, 1991), and that is supported by Godefroid et al. (2011). Regarding the introduction by plantation in Cala Salitrona, although we introduced only 40 individuals we thought that an annual plant, such as *A. chrysantha*, would have a high probability of survival to complete its lifecycle. In other words, we knew that these annual plants die in summer but also that they would release a high number of achenes, which would lead a large first plant cohort. A different aspect is that the introduction of few individuals can lead to loss of genetic diversity due to inbreeding depression or post-introduction genetic drift (e.g., Frankham et al., 2002; Pierson et al., 2007), and smaller populations are generally less capable of adapting to novel environments (Redd et al., 2003). However, this problem can be solved during the establishment phase of the population, by making periodical reinforcements. When considering the achene sowing, we found that most restoration projects have included a very limited number of propagules - an average of 1,500, according to the review by Godefroid at al. (2011) - mainly due to the fact that introduction often involves rare species for which numerous propagules are not available and is a labor-intensive and costly process. However, in our case, the natural populations of *A. chrysantha* produce a very high number of achenes; in fact, we used around 60,000 achenes which led to a plant cohort of around 1,500 individuals (124 plants per plot, on average) that were productive.

Another aspect to discuss is if it is better to use plants or seeds. Various published studies have shown the benefit of using plants instead of seeds (e.g. Milton et al., 1999, Drayton and Primack, 2000; Jusaitis et al., 2004; Maschinski and Duquesnel, 2006; Guerrant and Kaye, 2007; Menges, 2008): seed germination is often scarce and, in any case, gives rise to seedlings, which are the most vulnerable stage of the plant lifecycle (Primack and Drayton, 1997). Plants transplanted in Cala Salitrona, in an environment that we think now is unfavorable, could survive, flower, and become

fruitful without problems. This fact suggests or supports the idea that it would be better to use plants instead of seeds. However, as introduced populations must perpetuate themselves in time, when working with annual plants, although at the introduction young plants are used, there will always be a critical recruitment phase from seeds every year. In the case *of A. chrysanta*, if young pre-flowering plants are introduced, the formation of the aerial seed bank will allow the gradual release of achenes with the episodes of rain, the rest remaining in the capitula of the dead plants waiting for favorable conditions for germination. In fact, we still observed in July 2012 the presence of a minimum aerial seed bank in dead plants introduced in 2010 in Cala Salitrona. However, if achenes are sown directly, there may be a loss of the beneficial effects that the reserve of achenes in the aerial seed bank represents or, in other words, the favourable strategy of the fractioned germination in time in the unpredictable habitat (Aguado et al., 2012) (Chapter 2). By contrast, since this species produces a high achene number in its natural population, direct sowing allows us to carry out low-cost introductions with a high genetic diversity.

Furthermore, there are some traits which may make species good or poor candidates for introductions, such as longevity, mating system, dispersal ability, competitive ability, vegetative reproduction, seed bank persistence, wind pollination, plant size, growth rate, and genetic diversity (Godefroid et al., 2011). Taking into account the survival strategies of the species studied in this thesis, such as the production of heteromorphic achenes with different germination behavior, transitional aerial seed bank and ombrohydrochory (that favor a gradual germination of the achenes in an optimal environment), atelechory (that promotes persistence in areas where conditions are conductive to germination and establishment), and persistent soil seed bank (that allows the species to form new cohorts of individuals after a failed achene production), and considering the results of these introductions in the wild, we think that *A. chrysantha* has many of the traits needed to make it a good candidate for introductions.

Acknowledgments

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GENERAL CONCLUSIONS

Anthemis chrysantha is a *Critically Endangered* species but this thesis shows it has many survival strategies that let it to maintain in the unpredictable habitat in which it occurs.

The first chapter demonstrates morphological differences in the achenes of *A. chrysantha*: basal dark achenes are slightly larger than upper white achenes in length, width, weight and pappus length. Besides, the pericarp of dark achenes is thicker and stronger than that of white achenes. These morphological and anatomical differences are related to the different behavior of the germination response of the two achene types. White achenes germinate in high percentages in many conditions, while dark achenes are strictly dormant and do not germinate due to their pericarp physically impeding germination and making imbibition of water difficult. This fact ensures that not all dispersed achenes germinate at the same time, which is an ecological adaptation of this species similar to those of most other heteromorphic species.

The second chapther clearly verifies the existence of a transitional aerial seed bank constituted by mature achenes remaining on dead plants from the fruiting period (early summer) to late spring. Besides, this work also shows that autumn and winter rainfall is necessary to release the achenes from the capitula, being the ombrohydrochory the main dispersal mechanism of the species. The achenes are gradually released over several months at short dispersal distance, typical of ombrochoric plants, and this phenomenon of limited dispersal is called atelechory, a mechanism by which seeds remain in the habitat in which the species is competitive. The wind is not a primary dispersal agent of the achenes of *A. chrysantha*.

In the third chapter we demonstrate the existence of a persistent seed bank (PSB), whose dynamic ranges between the minimum values at the end of the spring and the maximum values in early autumn, after dispersal starts but before germination. Dark achenes are largely responsible for the permanent fraction, which is of considerable size. Besides, this study verified the PSB represents a good survival strategy for *A. chrysantha*, playing a crucial role in the maintenance of the annual population of this endangered species in its unpredictable habitat, due to the occurrence of an extremely–dry spring during the study period, when the PSB allowed the species to form new cohorts of individuals after a failed seed production.

The fourth chapter shows that ISSR markers have proved to be an effective tool for to determine the genetic structure of Spanish populations and to assess phylogenetic relationships among Spanish and Algerian populations. Both at the species and

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population level, the genetic diversity indexes showed that genetic variation of *A. chrysantha* is high, with a high within–population variability and a low, albeit significant, differentiation between populations. The high genetic connectivity found between remaining Spanish populations is evidence of that these populations were connected in the past time, and that not enough time has passed for this species to show more genetic divergence. On the other hand, ISSR markers have detected genetic variation among Spanish and Algerian *A. chrysantha* populations, what could support the recognition of the rank of subspecies, at least, for the Spanish populations.

The fifth chapter shows that transplant and achene sowing are valid methods which let establish at least two consecutive plant cohorts. Reproductive failure in 2012 in both Cala Salitrona and La Podadera population does not have to be the total failure of the introductions carried out those areas. Since the studied species has a PSB, next years will be crucial for determining whether the introduced populations will be able to establish again, overcoming the reproductive failure of 2012.

Take into account the specific conclusions obtained from the different chapters of this doctoral thesis, a general conclusion can be reached, namely that this species has no intrinsic threat. In other words, from the biological and ecological points of view, the species is adapted perfectly to its habitat, having a range of very interesting adaptations. Perhaps, one of the main questions which remains unanswered, and which we have to address in future research, is why the species has not been able to colonize other coastal habitats next to the natural populations. However, if it is a matter of lack of propagules in these areas or a very concrete specificity towards certain microhabitats, this could be resolved by tracking the artificial introductions in new habitats.

We hope that the conclusions of our studies can serve to aid the recovery plan for *A. chrysantha,* which will be the basic tool to protect the species. However, new lines of research could be included in this plan, to determine: (i) the relationships with other plant species which cohabit with *A. chrysantha*, regarding competition, facilitation, or invasion by exotic species; (ii) the secondary dispersal mechanisms of the species; and (iii) why the species is unable to colonize new habitats. Moreover, the monitoring of the introductions carried out in this work has to be performed for some years more and new introductions should be undertaken, taking into account the knowledge acquired in the previous ones, such as the detection of microhabitats within selected sites, in order to resolve the question mentioned above and to ensure the survival of the species.

Also, lines of action have to be proposed in the future recovery plan for *A. chrysantha* and the regional administration should adopt them, knowing that the main threats to the species may be the result of human actions, such as building and trampling.