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EFFECTS ON SOIL AND ALTERNATIVES FOR BIOLOGICAL CONTROL OF THE INVASIVE PLANT *CARPOBROTUS EDULIS*

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Effects on soil and alternatives for biological control of the invasive plant *Carpobrotus edulis*

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1. Introduction

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1. Introduction

1.1. BIOLOGICAL INVASIONS

The distribution of a species is limited by their capability to overcome physical barriers to dispersal, by their environmental requirements (e.g. climate, soil, water and food availability) and biotic constraints (e.g. predation, competition, diseases) (Boulangeat et al., 2012, Guisan and Thuiller, 2005, Mott, 2010). Species distribution is dynamic, changing with environmental conditions (which can lead to regional extinctions or expansions), evolution and modifications of physical barriers (leading to vicariance, when populations are separated, or to dispersal, when they disappear) (Holt, 2003, McNeely, 2001, Sanmartín, 2009). The limitations on species distribution has led to the high biodiversity of the planet (McNeely, 2001).

Human activity significantly impacts species distribution, decreasing their range (and even causing their extinction) or increasing their distribution, either directly through transportation or indirectly due to changes in the environment and climate (McNeely, 2001, IUCN, 2017a). These species that are deliberately or accidentally introduced outside their native range are called alien species (DAISIE, 2018). From the arrival of an alien species to the new ecosystem until its consideration as invasive, three different stages can be differentiated (see Fig. 1.1 and Table 1.1 for further details):

a. introduced or casual species: when they have been transported by humans outside their natural range (Richardson et al., 2000),
b. naturalized or established species: they survive and regularly reproduce in the new environment, being able to sustain populations on its own (Richardson et al., 2000),
c. invasive species: those whose introduction or spread threatens or negatively affects the ecosystems as indicated by the European_Parliament_and_Council (2014).

It is usually assumed that around 10 % of the imported species become introduced, 10 % of the introduced species become naturalized and 10 % of these naturalized species become invasive (Williamson and Fitter, 1996) (Fig. 1.1).

Stage	ALIEN	INTRODUCED CASUAL	NATURALIZED ESTABLISHED	INVASIVE
Process	transported outside native range[2]	released or escaped[2]	established viable populations[2]	spread outside released area[2]
Advantageous traits or situations	utility or association to humans[2,5], hitchhikers[5], seed longevity[5], wide native range[5]	original from a similar environment[2], generalist[2], high number of individuals released[2,3,5], fast growth[5]	asexual reproduction or hybridization[3], rapid adaptation by epigenetic changes[3], behavioural changes[2], high genetic variation[3], fast growth[5], efficient resource use[5]	better competitor[3], absence of enemies[3], phenotypic plasticity[5], rapid evolutionary changes[3], good dispersal ability[5], high fecundity[5], short generation time[5], habitat connectivity[5], anthropogenic disturbance[5]
Limitations	no capacity to survive long-distance transport[5], no association with humans[5]	no local adaptations[3], absence of a suitable niche[2]	population bottleneck[3], migratory behavior[2], small populations are vulnerable to stochastic events[2]	no out-competition of natives[3], slow spread[5], isolation[5]
Management	prevention[4]	eradication[4]	eradication, containment[4]	eradication, mitigation[4]

Dispersal barrier[1]
Survival barrier (abiotic factors of local environment)[1,5]
Reproduction & biotic barriers[1,5]
Dispersal & environmental barriers (abiotic and biotic)[1]

Fig. 1.1. Stages of an alien species, from its introduction into a new area to its invasion: barriers to overcome, processes, positive and negative traits and situations and management options for each step. References: [1]Richardson et al. (2000); [2]Duncan et al. (2003); [3]Allendorf and Lundquist (2003); [4]Blackburn et al. (2011); [5]Theoharides and Dukes (2007).

Table 1.1 Approximate number of naturalized species in the world and percentage of naturalized species within each group, with respect to the total number of known species.

	Vascular plants	Fishes	Reptiles	Amphibians	Birds	Mammals	Marine species
nº naturalized	>13000[1]	>200[2]	>185[3]	>80[3]	>200[4]	>65[5]	1800[6]
nº total	316143[7]	34797[8]	10711[9]	6260[10]	10000[11]	5488[12]	212000[13]
% naturalized	4.2	0.57	1.7	1.3	2.0	1.2	0.86

[1]van Kleunen et al. (2015); [2]Lever (2002); [3]Lever (2003), [4]Lever (2005); [5]Lever (1985); [6]Ahyong et al. (2018); [7]The_Plant_List (2013); [8]Eschmeyer and Fong (2018); [9]Uetz (2018); [10]IUCN (2017b); [11]IUCN (2018a); [12]IUCN (2018b); [13]Bouchet (2006).

The introduction of alien species began long ago. Humans spread outside Africa to other continents, since 100,000 years ago, has been linked to the transportation of domesticated species used for feeding or protection (e.g. dogs, pigs, wheat) and other stowaway species (e.g. rats, diseases, parasites) across their dispersal barriers (McNeely, 2001, Mack et al., 2000). With the development of trading and long-distance traveling humans' capacity to move species outside their native range increased (McNeely, 2001), especially after late 15th century with the sailing voyages between Europe and America. The beginning of the 16th century entailed the commencement of a new period in biological invasions due to its facilitation by changes in demography, agriculture, commerce and industry and the beginning of colonialism and exploration voyages (Hulme, 2009, Preston et al., 2004). Species were transported for explorers and colonists for feeding, medicines or even nostalgic and aesthetic reasons (Mack, 2001, Lever, 2005). The introduction of alien species increased in the 19th century, with the Industrial Revolution, due to the more intensive international trade and migrations (Hulme, 2009). Military activities have also led to the introduction of species to new regions since the 19th century due to the transportation of cattle (to feed armies), plants (which were used for producing natural remedies or materials) or by inadvertently transporting other species (McNeely, 2001, Wearn, 2016). Also, to control accidentally-introduced pests, other species have been introduced to control the former, resulting in some cases in new invasions (McNeely, 2001).

Currently, terrestrial transport networks, migration, importations and tourism are ligated to the introduction of alien plants (Vilà and Pujadas, 2001). The globalization of the economy since the 20th century has enhanced the demand of new species (McNeely, 2001). Foreseeable, invasions will be facilitated by new intercontinental transportation networks (with higher volumes of merchandise that will travel further distances), global change, increase of human activities in the Artic due to loss of ice (such as shipping, mineral exploitations, fisheries or tourism), refugee movement and military transport (Ricciardi et al., 2017). It is expected that the use of new crops (and their pathogens), new farmed species (such as insects) and soil bacteria and fungi (to increase crop production) will entail new invasions (Ricciardi et al., 2017).

Invasive species are a worldwide problem and pose one of the major threats to biodiversity (European_Commission, 2011, McGeoch et al., 2010, UNEP, 2002) as well as to the structure and functioning of ecosystems (Ehrenfeld, 2010, Hernández et al., 2014, Lake and Leishman, 2004, Le Maitre et al., 2011). Invasive plants can affect natives by modifying water and fire regimes, soil nutrients availability and ecosystem energy budgets (Mack et al., 2000). Invasive animals can affect natives by predation, competition or alteration of the invaded habitat (Mack et al., 2000). The introduced species can be sometimes very competitive due to the absence of coevolution with the native species (e.g. the brown tree snake in Hawaii) (McNeely, 2001). Since the 16th century, large extinctions of species are related to alien predators and diseases (Bellard et al., 2016, Loehle and Eschenbach, 2011), being alien species considered by some authors as the second most common cause of species extinctions (Bellard et al., 2016). However, extinctions are usually the result of a set of causes (which add to the effect of the invasion), and in some cases the contribution of alien species to extinctions might be overrated as the invasion by aliens and the extinction of natives can be triggered by the same anthropogenic disturbances (Pyšek et al., 2017). Around 27% and 62% of plant and vertebrate extinctions, respectively, are believed to be related to alien species (Bellard et al., 2016). Aliens affect more importantly amphibians, reptiles and mammals, for which the introduction of new species is the major cause of extinction, and to island endemisms (Bellard et al., 2016). Almost one fourth of the threatened or nearly threatened species are affected by invasive species (Maxwell et al., 2016).

Alien invaders can also have economic, social and human health impacts (Pimentel et al., 2005, Vitousek et al., 1996). For instance, microbial pathogens'

invasions, favoured by tourism, global commerce and changes in climate or environmental conditions can affect biodiversity and human's economy and health (Ricciardi et al., 2017). Pathogens can rapidly undergo genetic changes and our capacity to detect and identify them is limited (Ricciardi et al., 2017). Invasive alien species' damage has been estimated as € 12.5 billion per year in the EU (European_Commission, 2011), more than € 100 billion per year in the USA, more than € 10 billion in Australia, around € 6 billion in South Africa (Pimentel et al., 2001) and globally could reach € 1200 billion per year (Saad et al., 2009). However, damage estimations are highly variable depending on the methods used and the number of species included (Marbuah et al., 2014).

In order to fight the increasingly concerning problem of invasive species, improving the knowledge on them is crucial and, consequently, several programs such as the GISP (Global Invasive Species Programme) and DAISIE (Delivering Alien Invasive Species Inventories for Europe) have been created. In addition, their control and eradication is the 5th target of the 2020 Biodiversity strategy (European_Commission, 2011).

1.2. INVASIVE PLANTS

Around 4 % of the vascular plants in the Earth have become established outside their native range (van Kleunen et al., 2015). In Spain, 12% of the flora is invasive (Sanz-Elorza et al., 2004) and over € 50 million have been expended, mainly in removal of invaders, from 1997 to 2007 (Andreu and Vilà, 2007). Galicia (NW Spain) exceeds the national average, with 14% of its flora being invasive (Romero-Buján, 2007).

1.2.1. Species invasiveness and habitat invasibility

That an alien plant becomes invasive or not in a given habitat mainly depends on the interaction between the characteristics of the habitat and the species (Alpert et al., 2000, Rodríguez-Echeverría, 2010). Despite the importance of the early identification of potential invaders (Gibson et al., 2011), to date much remains to learn regarding this matter (Fuentes-Ramírez et al., 2011).

Plants invasiveness is commonly related with high dispersion and competition capacities, generalist characteristics and the ability to exclude natives (Lorenzo et al., 2010, Fuentes-Ramírez et al., 2011, Gibson et al., 2011, Hui et al., 2011, Daehler, 2003, Parker, 2001). Another factor of invasive

species success is a lower regulation of invasive populations by predators, parasites and other enemies ("enemy release hypothesis"), which derives from the lack of specialist consumers, low impact from specialist predators of native species and less pressure from generalist predators in comparison to native species (Keane and Crawley, 2002, Maron et al., 2014). Needing to expend less resources in defence, the invasive plant can allocate more resources to growth and reproduction, obtaining a competitive advantage (Callaway and Ridenour, 2004). Several experiences seem to corroborate this hypothesis, although exceptions have also been found (Keane and Crawley, 2002, Daehler, 2003). Another explanation for invaders success is the "novel weapon hypothesis": the absence of a coevolution between invasive and native plants can be a competitive advantage for the invader, as natives may be more susceptible to allelopathic or antimicrobial substances exuded by the invader's roots (Callaway and Ridenour, 2004).

Habitats most prone to invasions, for instance islands, riverbanks and coastal areas, have usually low levels of competition, altered disturbance regimes, low environmental stress or a combination of these factors (Alpert et al., 2000, Carboni et al., 2010). Frequently, invasive plants take advantage of disturbances, particularly those that diminish competition and increase the amount of unused resources (Alpert et al., 2000, Lake and Leishman, 2004, Daehler, 2003, Werner et al., 2008). However, disturbances by themselves may not be sufficient to enhance invasion if they are not accompanied by an increase in nutrient availability (Lake and Leishman, 2004). Among the main adaptations of invasive species to increased fertility are rapid growth, higher competitive ability than natives, high leaf area and less leaf construction cost (Alpert et al., 2000, D'Antonio and Meyerson, 2002, Daehler, 2003, Lake and Leishman, 2004). These adaptations to high resources availability suggest they will be benefited by an increase in atmospheric CO_2 concentration (Manea and Leishman, 2011). It has been suggested that invasibility and the deviation of the disturbance regime from the natural one may be directly related and, thus, even the suppression of a disturbance could lead to invasion in some cases (see Alpert et al. (2000) and references therein).

Under low-nutrient conditions, invasive species rarely overcome natives (Daehler, 2003), although exceptions have been found (Funk, 2013). A high capacity of competition for limiting resources in resource-poor environments is associated with an easier acquisition of resources, a higher efficiency in their use and a more efficient conservation (Kurokawa et al., 2010, Werner et al., 2008, Funk and Vitousek, 2007, Morris et al., 2011, Funk, 2013, Sardans et

al., 2016), as well as an increase in fertility through N_2 fixation, rapid decomposition of litter or modification of fire regime (Funk and Vitousek, 2007, Saad et al., 2009).

1.2.2. Effects of the invasion

1.2.2.1. Effects on economy, society and human health

Although in some cases the introduction of alien plants can produce economic benefits and important human services (e.g. crops), invasive species can negatively affect the economy in several ways, such as loss of ecosystem services, impacts on human health or expenses derived from the control of the invasion and the restauration of the invaded habitat (Sharma et al., 2005). Usually, the introduction of new species is not accompanied by a cost-benefit analysis (McNeely, 2001) and the economic impacts of aliens are usually not assessed due to the absence of quantitative data of their impacts or to the difficulty of including nonmarket costs (Duncan et al., 2004, Evans, 2003).

Alien plants can affect human health by producing toxins [e.g. giant hogweed (*Heracleum mantegazzianum* Somm. & Lev.) (Nielsen et al., 2005)] or allergens [e.g. common ragweed (*Ambrosia artemisiifolia* L.) (Bohren, 2006)]. For instance, allergy costs due to common ragweed are predicted to be around 300-350 million € per year in the next years (Richter et al., 2013). The effects of invasive plants on ecosystem services are rarely quantified, despite of their importance for human society (Charles and Dukes, 2007). They include provisioning (food, fiber, etc.), cultural (such as recreation or cultural heritage), supporting (e.g. maintenance of nutrient and water cycles) and regulatory (e.g. regulation of air quality) services (Charles and Dukes, 2007, Vilà and Hulme, 2017). For example, negative effects on crop production have been reported by *Cirsium arvense* (L.) Scop. in North America, producing yield losses of around 45 % (Vilà and Hulme, 2017).

1.2.2.2. Effects on the environment

Invasive plant can affect both abiotic - such as water and fire regimes and soil nutrient status, and biotic characteristics - like native species biodiversity (Brooks et al., 2004, Le Maitre et al., 2002, Enright, 2000, Ehrenfeld, 2003, Ehrenfeld, 2010, Vilà et al., 2006); in some cases enhancing their own invasion (Ehrenfeld, 2003). The effects of an invasive species can vary depending on the characteristics of the invaded ecosystem (Ehrenfeld, 2003, Dassonville et al., 2008).

Fire regimes can be altered through a higher fuel load input or flammability, as it is the case of wattles (*Acacia* spp.) in South Africa (Van Wilgen and Richardson, 1985), eucalypt (*Eucalyptus globulus* Labill) in Portugal (Fernandes et al., 2011) or saltcedar (*Tamarix* spp.) in the USA (Zouhar, 2003). Indirect effects of increasing forest fires can be soil erosion and nutrient loss in soils or water eutrophication (Castro-Díez and Alonso, 2017, Chamier et al., 2012).

Invasive plants can alter water regimes by reducing stream flows through high water use, as it is the case of wattles in South Africa (Le Maitre et al., 2002) or eucalypt in NW Spain (Rodríguez-Suárez et al., 2011). This high water uptake can derive in reduction of water quality, as was found in saltcedar invaded areas where groundwater salinity vastly increased (Nagler et al., 2008). Alien plants can also alter fluvial geomorphic processes (Castro-Díez and Alonso, 2017): wattles can increase riverbanks erosion because of the absence of adaptations to floods (D'Antonio and Meyerson, 2002), whilst the giant reed (*Arundo donax* L.) and the saltcedar (*Tamarix* sp.) trap sediments with their extensive radicular systems increasing floods severity (Sanz-Elorza et al., 2004, Manners et al., 2014).

Variations in soil nutrients by invasive species are related to differences from natives in tissue composition, phenology and productivity among others (Ehrenfeld, 2003, Ehrenfeld, 2010, Kurokawa et al., 2010, Liao et al., 2008, Lee et al., 2016). The nature and intensity of the effects not only depend on the invasive species, but also on the invaded habitat (Ehrenfeld, 2003, Castro-Díez et al., 2012, Dassonville et al., 2008, Saad et al., 2009, Vilà et al., 2006).

Although generalizations cannot be made, invasive species tend to produce more biomass than natives, speed nutrient cycles and lead to higher levels of C, N, P and cations (Ehrenfeld, 2003, Ehrenfeld, 2010, Liao et al., 2008), particularly in low-nutrient sites. Contrastingly, in high-nutrient sites they tend to lower nutrient levels, apparently leading to an homogenisation of soil characteristics (Dassonville et al., 2008).

Vilà et al. (2011) propose that by the time soil fertility has been affected, major ecological impacts have already occurred. Modifications in resources induced by invasive species can generate alterations in the microbial community and vice versa, which may in turn have consequences over plant species (Lazzaro et al., 2014).

The effects of alien plants on soil nutrient cycles have been scarcely studied, although they should be taking into account when evaluating the consequences of the invasion (Ehrenfeld, 2003). Invasive plants tend to increase C and N pools and fluxes, especially those which are associated with N_2-fixing symbionts and woody plants (Liao et al., 2008). Corbin and D'Antonio (2004) suggest that when invasive species not only affect soil N fluxes, but also N pools, restoration difficulty would be expected to increase. The N cycle is fundamental for the maintenance of plant productivity (Gruber and Galloway, 2008) and is mostly driven by microorganisms (Knops et al., 2002). Changes in plant species can alter the N cycle through different pathways (see Fig. 1.2):

1) Differences in canopy architecture can lead to variations in wet or dry atmospheric depositions, and therefore in the N inputs (Knops et al., 2002). Lower N depositions can increase microbial NO_3^- immobilization (Berntson and Aber, 2000).
2) Increases in the fire regime produced by some exotic plants can induce N losses through volatilization (Neary et al., 1999) or NO_3^- leaching, as N mineralization is promoted and the temporary decrease in plant biomass reduces the capacity of N uptake (Mack et al., 2001). Also, fire may affect asymbiotic N_2 fixation, reducing N inputs (Mack et al., 2001). To a lesser extent, some N can be lost by erosion (Neary and Overby, 2006).
3) Associations of invasive plants with symbiotic N_2-fixing microorganisms have been widely studied (Castro-Díez et al., 2014). These associations can increase N pools and nitrification in environments where native species could not associate with these symbionts (Vilà et al., 2011), leading to the largest effect on soil N cycle (Liao et al., 2008, Vilà et al., 2011, Castro-Díez et al., 2014, Ehrenfeld, 2003). However, soil N pools may not increase due to other factors that diminish microbial activity, as a reduction in the quality or the amount of litter (Castro-Díez et al., 2012, González-Muñoz et al., 2013).
4) Plants may influence asymbiotic N_2 fixation, although the processes by which they can alter it are still unclear (Knops et al., 2002).
5) Differences in the timing and capacity of N uptake of the plant can influence N losses through NO_3^- leaching (Knops et al., 2002).
6) Variations between species in root exudates and root turnover may alter C availability for microorganisms (Grayston et al., 2001). Quantity and quality of C inputs can alter N mineralization rate

(Schmidt et al., 1997, Cheng et al., 2011) and DNRA (dissimilatory NO_3^- reduction to ammonium) (Chen et al., 2015, Lu et al., 2015, Hardison et al., 2015). A higher mineralization could increase the NO_3^- pool and therefore N losses by leaching (Knops et al., 2002). Contrarily, production of secondary metabolites can inhibit nitrification (Thorpe and Callaway, 2011).

7) Changes in soil microbiota composition or activity (Knops et al., 2002, Wang et al., 2015) can alter autotrophic nitrification (through changes in ammonia oxidizing bacteria composition (Hawkes et al., 2005)), heterotrophic nitrification (Zhang et al., 2011), denitrification (Dassonville et al., 2011), N losses (Knops et al., 2002) or NH_4^+ immobilization (Hawkes et al., 2005).
8) Alterations of herbivore behaviour can impact N redistribution (Frank and Evans, 1997) or soil microorganisms (Hamilton and Frank, 2001).
9) Differences in litter quality may affect the N cycle (Chapman et al., 2006). A lower C:N ratio of the litter from the invasive plant can lead to a higher release of N from its decomposition, which may give a competitive advantage of invasive plants over natives in nutrient acquisition and promote a positive feedback (Li et al., 2017). Thus, invasive plants that produce litter with higher N content and lower C:N can increase inorganic N pools and mineralization rates (Lee et al., 2016). This is especially common in annual plants and grasses (Parker and Schimel, 2010, Piper et al., 2015, Stark and Norton, 2015). On the other hand, exotic species with high lignin:N and C:N ratios such as trees and perennial plants (Castro-Díez et al., 2012) can reduce mineralization. In contrast, Knops et al. (2002) propose that the effects of plant litter variations may be limited because most of the N provided by the litter is immobilized by microorganisms creating a time lapse between litter contribution and N release in soil through organic matter decomposition, preventing a positive feedback from litter to plant growth. However, their review mostly focused on grasslands, which may have lower variation in litter quality between species (explaining the little effect of plant species on the soil N cycle), and this microbial bottleneck may happen only in some situations (Chapman et al., 2006).

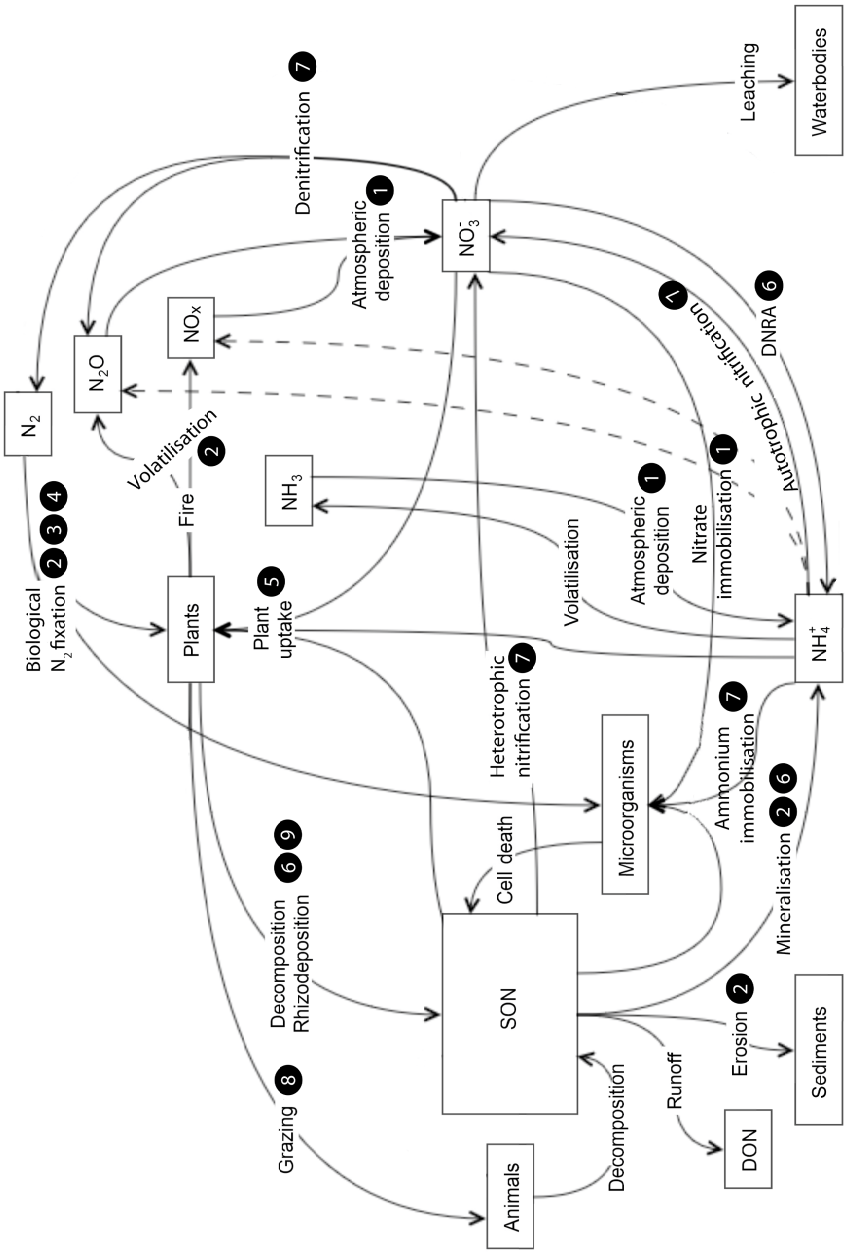

Fig. 1.2. Impacts of an invasive plant in the N cycle. The numbers in the black circles refer to the enumerated pathways of the text above. Figure adapted from Fernández-Fernández (2017).

Changes of invasive plants on the N cycle can affect the availability of N to other species (Vilà et al., 2011), the emission of greenhouse-effect gases (increased nitrification can lead to higher release of NO) (Barnard et al., 2005) or can increase the leaching of eutrophication compounds (as NO_3^-) (Knops et al., 2002).

Usually, invasions result in higher N pools (total N, NH_4^+) and faster N transformation rates (mineralization, nitrification) in the soil (Lee et al., 2016). However, much variability between the effects of invasive species is found (Wang et al., 2015). Also, the effect of an invasive species depends on the characteristics of the invaded habitat (Scott et al., 2001, Wang et al., 2015). As studies have been predominately focusing on a few species (Hulme et al., 2013), there is a need of studying the effects of more plant species. Besides, there are very few studies about the effect of invasive plants on gross N cycles, despite their importance to correctly interpret the effects of these plants on the soil N cycle (Piper et al., 2015).

1.2.2.3. Effects on the biota

The invasion of alien plants can alter the composition of native communities (Castro-Díez and Alonso, 2017) and reduce species richness, diversity and evenness (Hejda et al., 2009).

Alien plants can drive the extinction of native plants through competition, although this process on its own does not produce extinctions (Sax and Gaines, 2008). Other processes which can produce native species decline include: producing disturbances or altering disturbance regime, transforming the habitat or interactions between those processes (Downey and Richardson, 2016). Evidences of causality in extinctions by invasive plants are more difficult to gather than for animals (Pyšek et al., 2017). The ability to escape detrimental situations through dormant stage, the long-term effect of competition, the effects of management interventions and the deficiency of appropriate data can partly explain this lack of evidences (Pyšek et al., 2017, Downey and Richardson, 2016). Also, previous stages to a species extinction (such as decrease in population size or local extinctions) are not always considered when studying the effect of invasive plants (Downey and Richardson, 2016).Therefore, the impact of alien plants on natives should not be undervalued when considering management interventions (Downey and Richardson, 2016, Pyšek et al., 2017, Baider and Florens, 2011).

Plant invasion can also affect both fitness and growth of animal species, as well as the production, abundance, diversity and behaviour of animal

communities (Vilà et al., 2011). Thus, alien plants can reduce species richness and abundance of Lepidoptera (Burghardt et al., 2010), decrease species richness and change composition of leaf-litter invertebrates (Nguyen et al., 2016) or host less food available for insectivorous birds (Narango et al., 2017). Also, soil microorganism communities composition can be altered by the invasion (Wolfe and Klironomos, 2005, Mummey and Rillig, 2006).

The invasion of an exotic species or its impact on the environment may facilitate the entry or establishment of other exotic species, which is commonly referred as secondary invasions (O'Loughlin and Green, 2017). Plants' secondary invasions can be facilitated by exotic plants that increase resources availability, such as soil N [by plants associated with N_2-fixing symbionts, e.g. Hughes and Denslow (2005)] or light [through native species suppression, e.g. Flory and Bauer (2014)]. Exotic plants can also increase the presence of exotic animals. For instance, invasion of streams shores by eucalypt can increase the abundance of alien fishes (Oliveira et al., 2016).

1.2.3. Control methods of invasive plants

Prevention of invasive species introduction and establishment is considered the best approach to avoid plant invasions (Myers et al., 2000, Catford et al., 2012, Monaco and Genovesi, 2014). Generating risk maps (Hulme, 2009) and identifying future invaders (Mack et al., 2000) can help to tackle the problem from the beginning. Nevertheless, once an alien plant is established, several methods can be applied (see Table 1.2). Control methods can be implemented to eradicate the species, but also with the objectives of lowering their density, hindering their spread or introducing natural enemies (Myers et al., 2000). However, when the invasion has been triggered by environmental changes, focusing on environmental conditions can be the best approach (Catford et al., 2012, Bakker and Wilson, 2004).

Cost-effect analysis can be used to choose how to control an alien plant (Rajmis et al., 2016), and are compulsory in control programs of the EU (EU-regulation 1143/2014). The environmental impacts that these control methods may produce (see Table 1.2) [particularly when they involve large land areas (Myers et al., 2000)] should also be taking into account when deciding which method should be used. Citizen participation is increasingly used, as it helps to detect, survey and manage invasive species with a lower cost and the additional benefit of raising public awareness (Ricciardi et al., 2017).

Table 1.2.a. Mechanical control methods to manage invasive plants, with their main advantages and limitations.

Method	Description	Advantages	Limitations
Hand-pulling or digging	For herbaceous and small woody species[1]; removes both the aerial and below-ground biomass[1]	Usually high effectiveness[2]; prevents re-infestation by roots[1]	Usually needs long-term control[2]; site disturbance and creation of open niches can favour secondary invasions[2], increase erosion risk[3], expose soil to higher N mineralization[4] and hamper native species recolonization[4]; time and money-consuming (limiting in large-scale application)[2]
Cutting or mowing	Mow or cut invasive plants regularly[1]	Constrain growth by removing photosynthetic tissues[1]	Site disturbance can favour secondary invasions[2]; long-term application (up to 5 years)[1]
Burning	Prescribed fires	Depletes seed bank by inducing germination[5]; cheap[2]	Usually reduces native biomass and increases invasive biomass[2]; may affect the native seed bank and induce sprouting of invasive pyrophyte plants[6]; low effectiveness[2], risk of uncontrolled fires[8]
Water manipulation	Flooding invaded areas[8]; restoring tidal flows[12]		Only applicable when water levels can be manipulated[8]
Debarking	Strip the bark in species with trunk[7]	Appropriate for sensitive areas[7], usually prevents resprouting[7]	High cost[7]
Litter removal		Avoid germination[5], allelopathy[9], effects on soil microbiota[9] and properties[10]	
Native revegetation	Establishing native plants that outcompete the invasive[11]	Native species can self-regenerate; does not produce an early-successional state[11]	Usually only slight increase in native density[2]
Tilling, scalping	Effective with annuals and shallow-rooted perennials[12]	Avoid germination[5]	Risk of higher erosion and soil N mineralization, hampering native species recolonization[4]; can damage native vegetation[12]; only affects upper part of roots[13]
Freezing	Applying liquid nitrogen	Does not leave residues[14]	Only partial death in dense mats[14]
Suffocation, smoothering	Covering of seedlings and weeds with a thick plastic sheet[1], mulch[8] or carpet[8]		Site disturbance can favour secondary invasions[2]; long-term application (2 years)[1]; not specific[1]

Table 1.2.b. Chemical (white background) and biological (grey background) control methods to manage invasive plants, with their main advantages and limitations.

Method	Description	Advantages	Limitations
Herbicides	Application on leaves or cut stems[1]	High effectiveness[2]	Low or none specificity[1]; usually needs long-term control[2]; alters soil bacteria communities[15] or symbiont microorganisms of natives[16]; development of resistance[17]
Soil amendments	Addition of sucrose or sawdust to reduce nutrient availability[2]		Time and money-consuming (limiting in large-scale application)[2]; can affect natives[18]
Activated carbon	For exotic plants which use allelochemicals and are favoured by high soil nutrient availability[19]	Reduces competitive advantages of some exotics[19]	Changes native plant communities[19]
Classical	Targets exotic plants by introducing its natural enemies[20]	Cost-effectivenes[21], long-term effect[2]	Unexpected impacts on non-target species[22]
New association	Targets native plants (or exotics with unknown origin) with the natural enemies of related species[20]		

[1]Mattrick (2006); [2]Kettenring and Adams (2011); [3]D'Antonio and Meyerson (2002); [4]Yelenik et al. (2004); [5]Wilson et al. (2011); [6]Le Maitre et al. (2011); [7]Silva and Marchante (2012); [8]Wisconsin_Department_of_Natural_Resources (2016); [9]Marchante et al. (2011); [10]Conser and Connor (2009); [11]Simmons (2005); [12]Oehler (2006); [13]Lebo (2007); [14]Leach and Dawson (2000); [15]Souza-Alonso et al. (2015); [16]Weidenhamer and Callaway (2010); [17]Evans (2013); [18]Haubensak et al. (2004); [19]Kulmatiski and Beard (2006); [20]Van Driesche et al. (2008); [21]Shaw et al. (2014); [22]Cory and Myers (2000).

1.2.3.1. Biological control

One of the hypothesis to explain invasive species success is enemy release (Keane and Crawley, 2002, Maron et al., 2014). Therefore, introducing enemies of the invasive plants can help counteracting this competitive advantage. Biological control consists mostly on the introduction of potential predators or parasites of the invasive species (Shaw et al., 2014), although it can also involve the increase or maintaining of an existing enemy (McEvoy and Coombs, 1999). It has been done for more than 100 years, especially in developed countries. Experiences in developing countries are often dependent on the research of control agents in developed countries (Shaw et al., 2014).

Agents are often looked up for in the area of origin of the invasive species (Shaw et al., 2014). Once found, the safeness of its introduction in the new ecosystem is studied and, if approved, it is released in the invaded area, which should be monitored and evaluated (Shaw et al., 2014). However, there are few studies of the effectiveness of the biological control agent (Shaw et al., 2014, Morin et al., 2009, Myers et al., 2009), whilst most of the funding is spent on looking for potential agents, doing host specificity tests and releasing the agents (Myers et al., 2009).

Biological control success depends on the stage of invasion, being more effective on plants in an early phase of invasion (Zimmermann and Neser, 1999). The outcome of biocontrol also depends on the target plant traits, usually being aquatic and asexual plants easier to control (Paynter et al., 2012). Biocontrol tends to also be more successful with plants that are not considered major weeds in their native area (Paynter et al., 2012). Herbivores tend to weaken their host, instead of immediately killing it (Van Driesche et al., 2010). Therefore, unlike mechanical and chemical tools, biological control may take longer to be effective (Van Driesche et al., 2010, McFadyen, 2000).

The main advantages of biological control are its cost-effectiveness, its sustainability and its long-term effect, as when it is successful the species will reproduce and spread by itself after its introduction (Shaw et al., 2014, DiTomaso et al., 2017). Biological control is usually applied jointly with mechanical, chemical or other control techniques, allowing a reduction in the use of chemicals (Moran et al., 2013, Van Driesche et al., 2010, Olckers, 2004, Lake and Minteer, 2018). The efficacy of mechanical and chemical control tools without risking re-infestation is limited to small

or isolated areas (Van Driesche et al., 2010). In contrast, biological control can be efficient in larger areas without repeated application of the treatment (Van Driesche et al., 2008).

Biological control can affect ecosystems through impact on native species (Van Driesche et al., 2010). Non-target effects on native species due to a lack of specificity are more likely when there are native congeneric relatives of the invasive plant (Van Driesche et al., 2010, Louda et al., 2003). However, non-target effects are rare and the perceived risks seems to be frequently magnified in a subjective way (Seastedt, 2015). Most of these undesirable effects are due to introductions performed decades ago (Strong and Pemberton, 2000, Simberloff and Stiling, 1996), some of them targeting native plants (Seastedt, 2015). To decrease the risk of non-target attacks, it is recommendable to take into account the environmental conditions of the treated area and the dispersal capacities and adaptive change of biocontrol agents, to look for non-target indirect effects, and to do long-term studies and life table analysis (Louda et al., 2003). Also, studying the genetic diversity of control agents can optimize the control, by choosing the most adequate population, which can vary according to the environmental conditions of the area (Gaskin et al., 2011, Rauth et al., 2011).

1.2.3.2. Restoration of invaded habitats

To facilitate the recovery of the previous ecosystem properties, several actuations can be performed. According to the Society for Ecological Restoration, restoration can be defined as "the process of assisting the recovery of an ecosystem that has been degraded, damaged, or destroyed" (SER, 2016). Restoration includes abiotic manipulation (Le Maitre et al., 2011) and regeneration with native species by plantation or sowing (Richardson et al., 2007). Once the species is introduced the possibility of restoring the affected area must be considered (Myers et al., 2000) because removing the exotic species might not be enough due to the legacy effects produced in the ecosystem (D'Antonio and Meyerson, 2002, Nsikani et al., 2018). However, the high cost involving restoration makes necessary to select areas that are most affected or where management has the highest probability of success (D'Antonio and Meyerson, 2002).

Management success depends on species characteristic such as reproductive biology, size and persistence of the seed bank or propagule dispersal (Panetta, 2009, Myers et al., 2000), as well as habitat properties,

decreasing with higher nutrient concentration (Daehler, 2003). Furthermore, the longer the area has been invaded, the more complicated the restoration might be, as the seed bank becomes larger (D'Antonio and Meyerson, 2002), changes in soil properties and microbial community are more important (Marchante et al., 2008, Souza-AlonsoGuisande-Collazo et al., 2015) and invasive species distribution widens (Myers et al., 2000). Restoration efficiency depends as well on the understanding of the processes by which invasive species alter the environment (Le Maitre et al., 2011).

Some restoration labours might even enhance exotic species invasion through disturbances of the area, leading to secondary invasions (D'Antonio and Meyerson, 2002, Le Maitre et al., 2011). Consequently, prevention of further invasions must be made (Myers et al., 2000). The complexity of controlling invasion and restoration side effects highlights the importance of taking a preventive approach.

1.3. *CARPOBROTUS EDULIS* AS AN INVADER

1.3.1. Biology and native distribution of the species

The genus *Carpobrotus* (Phylum Magnoliophyta; Class Magnoliopsida; Order Caryophyllales; Family Aizoaceae; Subfamily Ruschioideae) comprises approx. 13 species of perennial succulent plants original mostly from South Africa and Australia (Waycott, 2016, Malan and Notten, 2006). *Carpobrotus* species are prostrate plants with creeping branches, extensive root system, sharp 3-angled green (or purple tinged) leaves, solitary flowers with 3-5 petals (purple, pink, yellow or white) on erect and short shoots, and fleshy indehiscent fruits (Hartmann, 2002). Plants from this genus grow mostly in coastal areas (although they can be also found in sandy to marshy areas inland) with Mediterranean or temperate climates (Hartmann, 2002). They can grow in dry and salty soils of preferentially sunny areas, but they cannot tolerate temperatures below -6 °C (GEIB, 2006). In drought or salt stress situations, they can induce crassulacean acid metabolism (CAM) (Robert et al., 2013).

Carpobrotus edulis (L.) N.E. Br. (1926) and *Carpobrotus acinaciformis* (L.) L. Bolus (1927) [commonly called ice plant, Hottentot fig, Cape fig, sour fig (Eng.); uña de gato (Esp.); herba do coitelo (Gal.)] are both South African species widely naturalized in Mediterranean climates. The native

range of *Carpobrotus edulis* includes both coastal and inland slopes of Northern (from Namaqualand), Western and Eastern Cape (Malan and Notten, 2006). It is easily recognized by its yellow flowers, whilst all other *Carpobrotus* species have pink/purple flowers (Waycott, 2016). *Carpobrotus acinaciformis* inhabits coastal sand dunes in Western Cape, from Saldanha to Mossel Bay, where it is native (Malan and Notten, 2006). Besides for the flowers colour, it can be differentiated from *C. edulis* in the shape of the leaves (the cross section of the middle of the leaves is equilateral in *C. edulis* and isosceles in *C. acinaciformis*) (Gonçalves, 1990).

Carpobrotus edulis can hybridize with other *Carpobrotus* species (Weber and D'Antonio, 1999, Waycott, 2016), including *C. acinaciformis*, both in South Africa (Wisura and Glen, 1993) and in the introduction areas (Suehs et al., 2004), or even with other genera from the Aizoaceae family (Chinnock, 1972). The introgressed hybrid complex between *C. edulis* and *C. acinaciformis* is cited as *C.* affine *acinaciformis* (Suehs et al., 2004, Delipetrou, 2006).

1.3.2. Introduction and invasiveness

Introductions of *Carpobrotus* species have been mainly of *C. edulis* and *C.* aff. *acinaciformis* (Table 1.3). In Europe, ice plants were firstly introduced in the 17th century (1680-1690) through Holland, Belgium and England (Wisura and Glen, 1993, Mulder, 2003, Robert et al., 2013). Widespread introductions and naturalization along the Mediterranean coast began at the end of the 19th century (Robert et al., 2013, Badalamenti et al., 2016). In Spain, the first record was in the NW coast, in Baiona in 1892 (Lázaro-Ibiza, 1900). In the United States *C. edulis* is present since its introduction in California in the 1900s (D'Antonio, 1993).

References for Table 1.3 (next page): [1]Marchante et al. (2014); [2]Sanz-Elorza et al. (2004); [3]FCBN (2018); [4]Vilà et al. (2006); [5]Julve (2017); [6]Flora_Italiana (2018); [7]Seljak (2010); [8]Stancic et al. (2008); [9]Trinajstic (1998); [10]Stešević et al. (2017); [11]Arianoutsou et al. (2010);[12]Washburn and Frankie (1985); [13]Robert et al. (2013); [14]Dufour-Dror (2012); [15]Dufour-Dror (2013); [16]Livshits et al. (1988); [17]Delipetrou (2006); [18]EDIT (2018); [19]Vivrette (2012a); [20]Vivrette (2012b); [21]EDDMapS (2018); [22]Wunderlin et al. (2018); [23]Gobierno_Mexico (2018); [24]Naturalista (2018); [25]Tropicos (2018); [26]Ríos et al. (2010); [27]Sotes et al. (2015); [28]Couto and Cardoso (2018); [29]Parker (2008); [30]Atlas_of_Living_Australia (2018); [31]Florence (2004); [32]Meyer (2000).

Table 1.3. Presence of *Carpobrotus edulis* (Ce), *Carpobrotus* aff. *acinaciformis* (Ca), *Carpobrotus aequilaterus* (Haw.) N.E.Br. (Cq) and *Carpobrotus chilensis* (Molina) N.E. Br. (Cc) outside their native range. Key: Int., introduced; Inv., invasive; Nat., naturalised.

Country	Regions	Species	Status	Ref.
Portugal	Mainland coast, Madeira & Açores islands	Ce, Ca	Inv.	1
Spain	Mainland coast, Balearic & Canary islands	Ce, Ca	Inv.	2
France	Mainland coast, Corse & Hyères islands	Ce, Ca	Inv.	3-5
Italy	Mainland coast, Sicily & Sardinia islands	Ce,Ca,Cq	Inv.	6
Malta	-	Ce	Nat.	3
Slovenia	-	Ca	-	7
Croatia	Mainland coast, Korcula islands	Ce, Ca	Inv.	8,9
Montenegro	-	Ce	Nat.	10
Albania	-	Ce	-	3
Greece	Mainland, Crete & Lesbos islands	Ce, Ca	-	4,11
Germany	-	Ce	-	12
U.K.	Mainland coast, Channel & Scilly islands	Ce, Ca	Nat.	13
Ireland	Eastern coast	Ce	Nat.	13
Netherland	Locally in the Western coast	Ce	Int.	13
Belgium	Not naturalised; ornamental along the coast	Ce	Nat. Risk	13
Israel	-	Ce, Ca	Inv., Nat.	14,15
Russia	-	Ce	-	16
Turkey	-	Ce	-	17
Syria	-	Ce	-	17
Lebanon	-	Ce	-	17
Cyprus	-	Ce, Ca	Nat.	18
USA	Coast of California, locally in Florida	Ce, Ca	Inv., Nat.	19-22
Mexico	Tijuana, Cancún, Ciudad de México	Ce	-	23,24
Guatemala	-	Cc	-	24
Bolivia	La Paz	Ce	-	25
Uruguay	San Luis, Punta Ballena	Ce	-	26
Chile	Valparaíso, Biobío	Ce	Nat.	27
Brazil	Rio Grande do Sul, Santa Catarina, Mato Grosso	Ce	-	24,28
Argentina	-	Ce	-	17
Tunisia	-	Ce	-	29
Algeria	-	Ce	-	17
Morocco	-	Ce	-	17
Libya	-	Ce	-	17
Cape Verde	-	Ce	-	29
St. Helena	-	Ce	-	17
Australia	Tasmania, SE and SW Australian coast	Ce, Cc	-	30
N. Zealand	-	Ce	-	30
Polynesia	Tahiti island (French Polynesia)	Ce	-	31
Pitcairn	-	Ce	Inv. Risk	32

Alien *Carpobrotus* species are present in temperate coastal areas of Eurasia, America, Africa and Oceania (Table 1.3). They colonize dunes, cliffs and disturbed environments (Campos et al., 2004, Maltez-Mouro et al., 2010, D'Antonio, 1993), including burnt areas (D'Antonio et al., 1993). In Europe, they are primarily naturalized in the coastal areas of Southern Europe, both in the Atlantic façade and the Mediterranean basin (see Table 1.3). *Carpobrotus edulis* is also present in central and Northern Europe, although in a lower extent (Table 1.3).

Introductions of *Carpobrotus edulis* and its hybrid *C.* aff. *acinaciformis* can respond to different purposes:

a. Ornamental (Campos et al., 2004, Maltez-Mouro et al., 2010, Sanz-Elorza et al., 2004), being this one of its major attractiveness.
b. Prevention of erosion by stabilizing embankments (Chenot et al., 2018), dunes and slopes (Campos et al., 2004, Maltez-Mouro et al., 2010), being extensively used in highways cuts in California (Donaldson et al., 1978). However, their shallow roots limit its utility in steep unstable slopes (Pierce, 1994).
c. Medical use, using leaves for its antibacterial (van der Watt and Pretorius, 2001, Chokoe et al., 2008) or antifungal (Omoruyi et al., 2014) activity. They are used as a laxative or lotion for scalds and burns, to treat dysentery, as gargle for sore throats or to treat jellyfish stings (Pierce, 1994).
d. Culinary use, as jam can be made from its fruits (Pierce, 1994) and leaves can be used to preserve food (Omoruyi et al., 2014).
e. Fire prevention, using the plants as firebreaks, which was advised for protecting houses in California by the Fire Department (Pierce, 1994).

The ice plant *C. edulis* and its hybrid *C.* aff. *acinaciformis* are considered among the most invasive alien species in Europe (DAISIE, 2018) and Spain (GEIB, 2006). The higher competition capacity of the ice plant compared to natives is attributed to different characteristics:

a. Dense mats formation (Maltez-Mouro et al., 2010) of up to 50 cm in depth (Ruffino et al., 2015), which leads to the elimination of native species by competition (Campos et al., 2004).
b. Rapid growth (approx. 40 cm per year), which facilitates the colonization of new habitats (Traveset et al., 2008b).

c. Inhibition of native plant germination, apparently through release of allelopathic substances during necromass decomposition (Novoa et al., 2012).
d. Reproduction both by sexual means, with a production of >1000 seed per fruit for *C. edulis* and >350 seeds per fruit for *C.* aff. *acinaciformis* (Suehs et al., 2004) that have and average viability of 2 years (D'Antonio, 1990b); and asexual means, with rooting nods (Delipetrou, 2006).
e. Endozoochory in the invaded areas, being dispersion and germination increased through scarification by rabbits, rats or deers (D'Antonio, 1990b, Novoa et al., 2012, Bourgeois et al., 2005).
f. Clonality with capacity of labour division in patchy environments (Roiloa et al., 2014) and putative belowground communication that can promote compensation responses to herbivory (Rodríguez et al., 2018).
g. Rapid adaptive evolution in the invaded areas (Roiloa et al., 2016).
h. Higher competitiveness with low or moderate soil salinity, which promotes shoot elongation (Varone et al., 2017).
i. Different traits than native plants, which can help to explain the ecological effects of the ice plant (Badalamenti et al., 2016).
j. Morphological plasticity to light availability (Fenollosa et al., 2017), which allows the ice plant to rapidly colonize heterogeneous habitats (Traveset et al., 2008b).

1.3.3. Effects of the invasion

Carpobrotus edulis and its hybrid *C.* aff. *acinaciformis* are considered ecosystem engineers, as they can alter both the abiotic and biotic properties of the ecosystem, turning these changes to their favour (Conser and Connor, 2009, Molinari et al., 2007). *Carpobrotus edulis* invasion seems to be influenced by the habitat (D'Antonio, 1993), so its effects on the ecosystem may be context-specific (Vilà et al., 2006). As the effects of *Carpobrotus* spp. on the invaded ecosystems can remain even after the removal of the plant, they can significantly influence restoration success (Conser and Connor, 2009).

1.3.3.1. Effects on biodiversity and ecosystems

The invasion of ice plants alters the structure of the native plant community towards randomness, similarly to the effect of other disturbances (Santoro et al., 2012). The α-species richness and the diversity decrease with the invasion (Badalamenti et al., 2016, Jucker et al., 2013). In invaded dunes, the net productivity of the ecosystem can decrease (Maltez-Mouro et al., 2010). Ruderal plant species appear to be favoured by the invasion (Santoro et al., 2012), whilst the perennial community of transition dunes seems to be the most affected due to the higher propagule pressure of the ice plant in the transition dune (Carboni et al., 2010). Also, plant species related to grasslands are scarce in invaded areas (Badalamenti et al., 2016). *Carpobrotus edulis* can affect pollination of native plants through competition or facilitation, depending on the plant species and varying with the ecological conditions and time (Moragues and Traveset, 2005, Vilà et al., 2009). Ice plants can also decrease germination, survival, growth and reproduction of native plants (Conser and Connor, 2009, de la Peña et al., 2010).

Carpobrotus edulis hybridizes with native *Carpobrotus* species in Australia (hybrid with *Carpobrotus rossii* (Haw.) Schwantes) (Waycott, 2016) and in California (hybrid with the supposedly native *C. chilensis*) (Vilà et al., 1998, Vilà and D´Antonio, 1998). Hybridization with native plants can lead to genetic contamination of the native populations, hybrid vigour or a change in the ecological function of the *Carpobrotus* plants (Waycott, 2016).

The invasion by ice plant can increase both bacterial and fungal biomass, affect the structure of soil microbiota and favor fungal growth (Badalamenti et al., 2016), favouring in turn the invasion (de la Peña et al., 2010).

Invasions by *Carpobrotus* spp. can affect animal communities. It can decrease the occurrence of some reptile species (e.g. *Chalcides striatus* in NW Spain) due to a lower suitability of the characteristics of the invaded habitat (Galán, 2008), and reduce both abundance and species richness of insects through a decrease in microhabitat heterogeneity (Orgeas et al., 2007). As the ice plants produce fruits when food is scarce for herbivorous animals in California (at the end of the dry season, when there are no annuals, grasses or forbs and the perennial plants are under water stress),

the invasion by *Carpobrotus* species can increase the presence of mammals that predate their fruits (D'Antonio, 1990b).

1.3.3.2. Effects on water and light resources

The ice plant *C. edulis* can affect water availability (Molinari et al., 2007) and compete with native species for soil water resources, affecting the morphology and growth of the native plants (D'Antonio and Mahall, 1991). Also, salinity can increase with the invasion by *Carpobrotus* spp., limiting water availability for native plants due to an alteration of the soil water potential (Novoa et al., 2014). However, other studies found increases or non-significant effects on soil moisture in the invaded areas, which they related to a lower soil temperature, radiation and wind or to the higher organic matter content (Novoa et al., 2014, Novoa et al., 2013). The invasion by ice plants can reduce the sun radiation that reaches the soil surface (both in invaded dunes and coastal scrubs) hindering native plant regeneration due to a lower establishment of seedling and a lower re-sprouting of plants from basal meristems (Molinari et al., 2007).

1.3.3.3. Effects on soil properties

Studies on how ice plants alter soil properties have mostly focused on pH, organic matter, and total and available N (Table 1.4). The effect of the invasion on soil pH is variable depending on the invaded ecosystem, but *Carpobrotus* spp. invasion tends to lower pH in most cases (see Table 1.4). There is still controversy on how these alien plants can alter soil pH, which in some cases is related to a higher necromass production than native plants (Table 1.4). Soil acidification can lead to inhibition of nitrification, leaching of Ca and Mg (Conser and Connor, 2009) and increase of P availability (Novoa et al., 2014), persisting this acidification for years after the alien plant removal (Conser and Connor, 2009). When decreasing soil pH, the invasion by ice plant is accelerating the natural acidification of soils that is produced during succession (Conser and Connor, 2009). Effects of ice plant invasion on soil total and available N are also variable (Table 1.4). Studies on the effect of the invasion on the N cycle may help to elucidate the mechanisms by which *Carpobrotus* spp. can alter the concentrations of the different N species. Both soil pH and N can affect ice plant reproduction, being flower density of *C. edulis* negatively related to soil pH and positively related to soil N (Traveset et al., 2008a).

Ice plants can alter nutrient availability, for instance through changes in soil pH (Novoa et al., 2014, Molinari et al., 2007) and differences in quantity and quality of necromass production (Novoa et al., 2014, Santoro et al., 2011). However, studies about the effect of ice plants on soil nutrient availability are scarce and focused exclusively on macronutrients (Table 1.4). The effects of ice plants on macronutrient availability are variable, so they might depend on the characteristics of the invaded habitat (Table 1.4). Further studies on the effects of *Carpobrotus* invaders on macronutrients can help explain how they are conditioned by habitat properties. Also, studies on the effect of ice plant on micronutrients and trace elements are indispensable, due to their importance as essential nutrients or toxic substances (Williams and Fraústo da Silva, 2000).

The changes produced in soils invaded by ice plants seem to benefit the invasion (Conser and Connor, 2009, Fenollosa et al., 2016), and can remain after *Carpobrotus* removal (Novoa et al., 2013) hampering habitat restoration and leading to secondary invasions by ruderal nitrophilous plants (Santoro et al., 2012, Santoro et al., 2011, Novoa et al., 2013). The most sensitive habitat to the changes in edaphic characteristics triggered by *Carpobrotus* spp. invasion might be fore dunes, where soil properties can change the most due to their low content in nutrients and organic matter (Santoro et al., 2011, Novoa et al., 2014).

1.3.4. Control methods

Control of *Carpobrotus* spp. invasion is usually done mechanically or with herbicides (Sanz-Elorza et al., 2004). Secondary effects of the different control methods should be taken into account, especially when the invaded habitats are dunes, which are vulnerable to erosion and usually have endemic or endangered species (Carta et al., 2004). Another limitation of control methods is their cost. For instance, mechanical removal of *Carpobrotus* spp. can cost around 4 € $\cdot m^{-2}$ (Scalera et al., 2017).

In order to enhance restoration of the invaded ecosystem, it has been suggested that *Carpobrotus* spp. necromass should be removed to avoid both the persistence of its effects on soil properties (Novoa et al., 2013) and the inhibitory effect on the germination of native plants (Generalitat_Valenciana, 2014). However, necromass removal increases the risk of soil erosion and can even hinder the recolonization by native plants (Chenot et al., 2018). To prevent erosion when removing ice plants, contour strips of *Carpobrotus* can be placed along the contour lines in steep

areas (Chenot et al., 2018). This treatment should be accompanied of periodical trimming and flower cutting (Chenot et al., 2018).

Table 1.4 Review of the effects on soil properties found in studies regarding the invasion by ice plants (+, increase; =, no significant differences; -, decrease; avail., available), with details of the invaded ecosystem, the studied *Carpobrotus* species (Ce, *C. edulis*; Ca, *C.* aff. *acinaciformis*) and the proposed processes by which the invasion alters soil properties.

Variable	Effect	Ecosystem	Species	Possible causes	Ref.
pH	+	fixed dune (postfire)	Ce	low native pH	1
	=	dune	Ce	-	1,2
	=	fixed dunes	Ca	similar litter abundance and plant cover than native vegetation	3
	-	scrub, chaparral, grassland,	Ce	salt uptake, H^+ exudation, organic acids produced during litter decomposition	2
	-	fore dune, urban	Ce	production of organic acids, nitrification	1,4
	-	shifting dune	Ca	higher OM content	3
NaCl	+	dune, urban	Ce	seaweed and marine debris accumulation	1
	=	dune	Ce	-	1
organic matter	+	dune	Ce, Ca	higher litter production than natives	1,3
	=	fixed dune	Ce, Ca	similar litter production than natives	1,3
total N	+	dune	Ca	higher litter production than natives	3
	=	fixed dune	Ca	similar litter production than natives	3
NH_4^+ NO_3^-	+	urban	Ce	higher OM input than natives	1
	=	dune	Ce	-	1
	-	fixed dune (postfire)	Ce	similar OM input than natives, higher N accumulation in *Carpobrotus* litter or lower NH_3 volatilisation due to lower pH	1
avail. P	+	dune, urban	Ce	OM input, lower pH	1
	=	dune	Ce	-	1
avail. Ca	=	dune	Ce	-	5
	-	grassland	Ce	-	2
avail. Na	+	various	Ce	-	2
	-	dune	Ce	-	5
avail. Mg	-	dune	Ce	-	5
avail. K	+	dune	Ce	-	5

References (Ref.): [1]Novoa et al. (2014); [2]D'Antonio (1990a); [3]Santoro et al. (2011); [4]Conser and Connor (2009); [5]Winsemius (2013).

1.3.4.1. Mechanical control

Hand-pulling is widely used to control ice plants, as their roots are very shallow and plants can be easily removed (Carta et al., 2004, GEIB, 2006). For large mats, rolling them up as a carpet can facilitate the removal (PIER, 2013). When ice plants are present in extensive monocultures, solarisation (e.g. covering the area with plastic sheets) can be applied, with the advantages of minimal soil disturbance and mulch production (MARM, 2011, The_Bay_Foundation, 2016). Other alternatives of mechanical control can be removal with tractors or prescribed fires, but both options are very destructive and cannot be applied in sensitive areas (such as dunes) where damage to endemic or endangered species and soil erosion must be avoided (Carta et al., 2004).

Mechanical methods are time and manpower consuming and lead to a problem of biomass management (Carta et al., 2004). The high water content of the plant increases the weight/volume ratio of the removed plant, increasing in turn the transportation costs (GEIB, 2006). Allowing the plants to dry facilitates transportations, but increases the risk of reinvasion (GEIB, 2006). Mechanical removal of the plants leads to low recolonization by native plants (Carta et al., 2004) and colonization by alien vegetation (GEIB, 2006). Although seed germination of *C.* aff. *acinaciformis* seems to be negligible, recolonization by vegetative growth of surrounding plants can be produced (Carta et al., 2004).Therefore, the use of prescribed fires (reaching temperatures above 100 °C) to decrease the seedbank (Delipetrou, 2006) does not seem an effective way of avoiding recolonization. Prescribed fires would also imply losses in the native seedbank that would hinder the ecosystem restoration (Le Maitre et al., 2011). To prevent reinvasions, by *Carpobrotus* spp. or other alien plants, it is necessary to monitor, maintain and restore the invaded area (Carta et al., 2004, Chenot et al., 2018). The presence of native vegetation can prevent the re-establishment of *Carpobrotus* spp., because these invasive plants have a lower performance in shadowed areas (Sanz-Elorza et al., 2004).

1.3.4.2. Chemical control

Glyphosate (min. 0.3g · m^{-2}) and chlorflurenol can be used to control *Carpobrotus* spp. (ISSG, 2008, MARM, 2011, Generalitat_Valenciana, 2014). To increase the efficacy of the herbicide, artificial acidification or the addition of a 1% surfactant can be done to break the plants cuticle

(Fagúndez and Beiras, 2007). The death of the treated plants can be slow and, therefore, the risk of resprouting remains during one month (GEIB, 2006). As these herbicides have a broad spectrum, chemical control should not be applied when *Carpobrotus* spp. cohabit with native plants (GEIB, 2006), because they can reduce their germination (Generalitat_Valenciana, 2014).

To minimize the effect over native vegetation, herbicide application should be carried out in winter (MARM, 2011). As with mechanical control, after application of herbicides the reintroduction with native vegetation is necessary (MARM, 2011).

1.3.4.3. Potentiality for biological control

Carpobrotus spp. is hardly affected by herbivory, diseases or competition in the invaded areas (D'Antonio, 1993, Maltez-Mouro et al., 2010, Donaldson et al., 1978) and seems to be benefited by enemy release, as found for soil pathogens (Van Grunsven et al., 2009). Therefore, biological control could provide an effective long-term alternative to current control, although it has not been implemented yet.

1.3.4.3.1 Enemies in the native and introduced areas

In their native range, ice plants can be affected by bacteria or fungi (i.e. *Botrytis* sp., *Phytophthora crypotogea* Pethybr. & Laff., *Pythium aphanidermatum* (Edson) Fitzp.) in humid and shady areas (Malan and Notten, 2006, Schmalzer and Hinkle, 1987). However, their use as biocontrol agents is limited by the need of flooding (Schmalzer and Hinkle, 1987). In invaded areas, *Carpobrotus edulis* can also be infected by the soil-borne fungus *Verticillium dahliaea* Kleb. (McCain and Farnham, 1974, ISSG, 2008, Schmalzer and Hinkle, 1987), which has been studied as a potential biocontrol agent of other invasive plants (Davis et al., 2011). Also, the fungi *Phomopsis* sp., *Fusarium* spp., *Macrophomina* sp. and *Pestolotia* sp. were found on infected tissues of *C. edulis* in California (Schmalzer and Hinkle, 1987, Campoy et al., in press, MacDonald et al., 1984, MacDonald et al., 1983). Another potential pathogenic fungi, namely cytrids (O. Chytridiales), have been observed to be favoured by invasions of *C. edulis* (de la Peña et al., 2010). However, it seems that this fungus only decreases the fitness of native plants (de la Peña et al., 2010). In *Carpobrotus glaucescens* (Haw.) Schwantes and other plants from the Aizoaceae family [i.e. *Dorotheanthus bellifórmis* (Burm.) and *Tetragonia tetragonioides* (Pall.)

Kuntze], the fungus *Sclerotinia sclerotiorum* (Lib.) de Bary can produce symptoms (Cother, 2000, Saharan and Mehta, 2008a). This fungus has been widely studied as a mycoherbicide for the control of different invasive plants (Table 1.5).

Scale insects from the families Diaspididae and Coccidae can feed on *Carpobrotus* spp. The Diaspididae species [*Aonidia mesembryanthemi* Brain; *Entaspidiotus lounsburyi* (Marlatt)] associated with *Carpobrotus* spp. (including *C. edulis*) have been reported in the native area (Prinsloo, 1995, Williams, 1963, Sethusa et al., 2014). From the Coccidae family, *Ceroplastes* sp. has been reported in the native area in *Carpobrotus* sp. plants (Prinsloo, 1995). Another two Coccidae species, *Pulvinariella mesembryanthemi* (Vallot) and *Pulvinaria delottoi* Gill, are present both in South Africa (where they are native) and outside their native range (where *C. edulis* and *C.* aff. *acinaciformis* are alien species) (Washburn and Frankie, 1985) (pers. obs.).

Leaf or fruit herbivory by snails [such as *Theba pisana* (Müller)] has been found both in the native (where *T. pisana* is invasive) (Malan and Notten, 2006, van Elden et al., 2015) and invasive (Preston and Sell, 1988, Holyoak and Holyoak, 2016) areas of *C. edulis*. In South Africa, flowers and leaves of *Carpobrotus* spp. are also predated by baboons [*Papio ursinus* (Kerr) (Davidge, 1978)], different rodent species, porcupines [*Hystrix africaeustralis* (Peters)], springbok [*Antidorcas marsupialis* (Zimmermann)] and humans (Vilà et al., 2009, Wisura and Glen, 1993). In the invaded area, predators of *C. edulis* include mammals such as: kangaroos [*Macropus irma* (Jourdan)*; Macropus fuliginosus* (Desmarest)] in Australia (Wann and Bell, 1997), deers (*Odocoileus hemionus* Rafinesque) (D'Antonio, 1990b) and rodents (*Sylvilagus bachmanii* Waterhouse, *Spermophilus beecheyi* Richardson, *Lepus californicus* Gray) (D'Antonio, 1990b) in California and rodents in Europe (*Oryctolagus cuniculus* L., *Rattus rattus* L.) (Novoa et al., 2012, Bourgeois et al., 2005). However, predators can enhance the invasion by promoting an increase in shoot growth of *C. edulis* to compensate the herbivory (Rodríguez et al., 2018), or in some cases by increasing the germination and dispersal of *Carpobrotus* spp. seeds through endozoochory (Novoa et al., 2012, D'Antonio, 1990b, Bourgeois et al., 2005).

1.3.4.3.2. *Sclerotinia sclerotiorum* as a potential biocontrol agent

Sclerotinia sclerotiorum (Phylum Ascomycota, class Discomycetes, order Helotiales, family Sclerotiniaceae) is a cosmopolitan fungus from temperate areas, which has a broad host range. It can infect more than 500 plant species worldwide, including trees, shrubs and grasses (Saharan and Mehta, 2008a).

The fungus *S. sclerotiorum* produces airborne ascospores, which are the primary form of inoculum, and infects plants preferentially through petals, early symptoms of the disease being usually brown lesions and cottony patches of mycelium (Saharan and Mehta, 2008b). In decaying parts of the infected plant, *S. sclerotiorum* can produce sclerotia (able to survive 5-10 years in the soil), which can form apotecia and produce ascospores (Saharan and Mehta, 2008b) (Fig. 1.3).

Many studies have addressed the potential of *S. sclerotiorum* to control invasive plants, especially in New Zealand (see Table 1.5). To favour the infection, the mycoherbicide should be applied when moisture is high (Saharan and Mehta, 2008c). Also, pathogenicity can be enhanced by increasing the production of oxalic acid in *S. sclerotiorum* by adding sodium succinate to the growth media (Cessna et al., 2000, Briere et al., 2000). Although in some cases this fungus was able to seriously damage weeds, the high cost of its use discouraged its implementation (Saharan and Mehta, 2008c). The dispersal of *S. sclerotiorum* ascospores, which is essential to determine the safety zone for its application as a mycoherbicide, is approximately 250 m depending on wind speed (BourdôtBaird et al., 2006, De Jong et al., 2002).

From *S. sclerotiorum*, the mycoherbicide Hyakill™ was developed to control the invasive water hyacinth (*Eichhornia crassipes* (Martius) Solms Laubach), although it was not admitted for commercialization by the European Patent Office (apparently due to the low specificity of *S. sclerotiorum*) (Dagno et al., 2012). A related species, *Sclerotinia minor* Jagger, is the base of a commercial mycoherbicide to control dandelion (*Taraxacum officinale* Webber) (Dagno et al., 2012).

Although *S. sclerotiorum* can infect some Aizoaceae species (Cother, 2000, Saharan and Mehta, 2008a), its potential as a control agent has not been studied yet. One main advantage of its application is that its use as a mycoherbicide would not mean the introduction of a new alien species, as

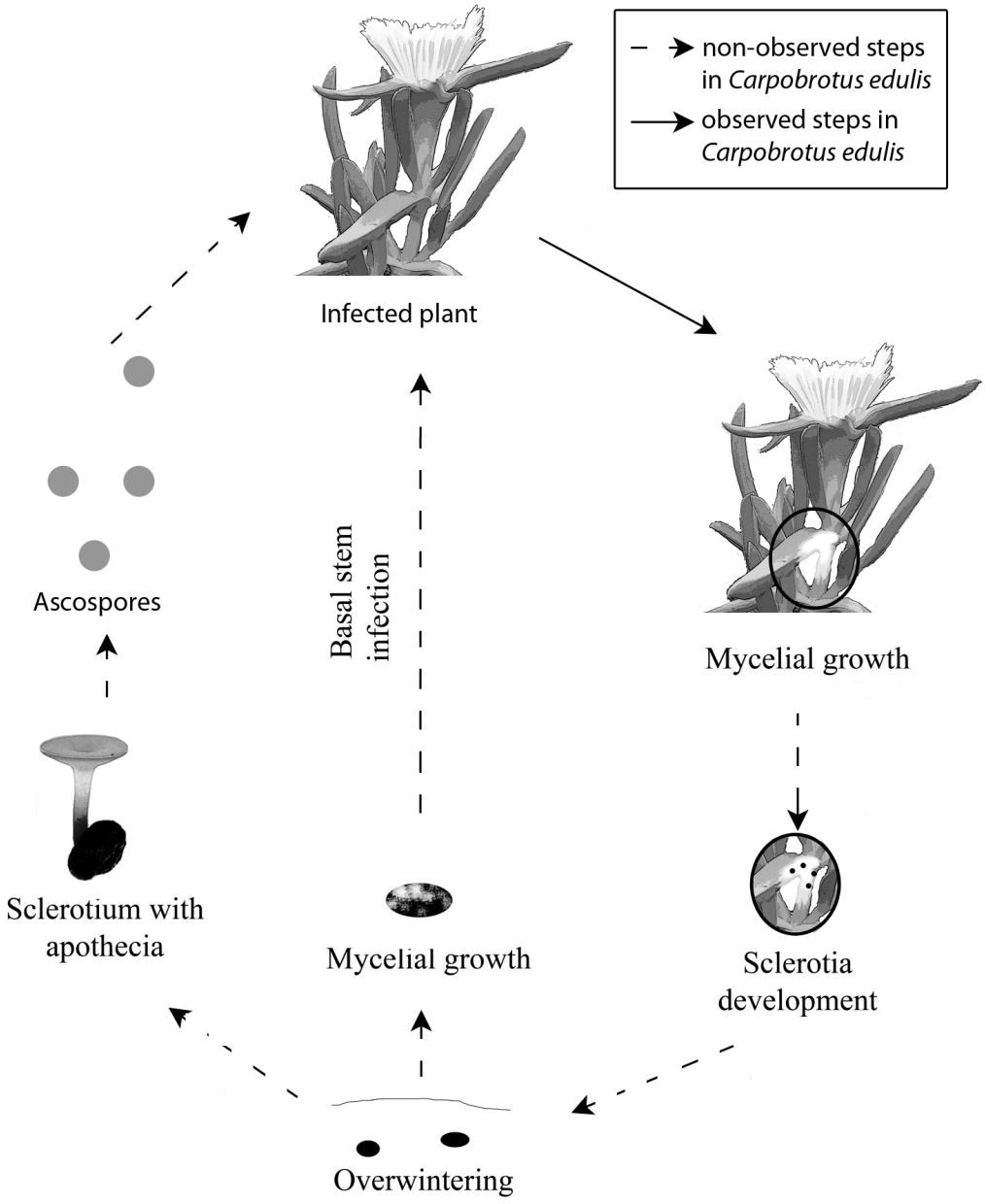

Fig 1.3. Cycle of *Sclerotinia sclerotiorum* based on the infection of cultivated plants. The formation of sclerotia has not been found in diseased tissues of *Carpobrotus edulis* (*pers. obs.*). Modified and adapted to *C. edulis* from Johnson and Atallah (2014).

the fungus is present worldwide. Also, it has already been widely studied as a biocontrol agent in other species, so the mycoherbicide formulation and the application procedures have already been optimized.

1.3.4.3.3. *Pulvinariella mesembryanthemi* as a potential biocontrol agent

The South African *P. mesembryanthemi* (Phylum Arthropoda, class Insecta, order Hemiptera, family Coccidae), commonly called cottony pigface scale, is a highly monophagous parasite of *Carpobrotus* spp. (Washburn and Frankie, 1985). The accidental introduction of infested iceplants outside their native range seems to be the reason of the presence of *P. mesembryanthemi* in temperate coastal areas from America, Africa, Eurasia and Oceania (Table 1.6).

The scale insect *P. mesembryanthemi* reproduces exclusively by parthenogenesis, although in occasions males are produced (apparently as a relictic feature) (Pesson, 1941, Washburn and Frankie, 1985). Mature females produce ovisacs (waxy sacs with up to 2500 eggs) (Washburn and Frankie, 1985). The new-born scales are mobile and they look for suitable feeding sites, where they settle losing mobility (Washburn and Frankie, 1985). For long-distance dispersal, they can be transported by wind (reaching up to 190 km per generation) or zoochory (e.g. humans, dogs) (Washburn and Frankie, 1981). Immature scales go through three instar before maturing (forth instar) and producing ovisacs (Washburn and Frankie, 1985). In California, *P. mesembryanthemi* has two cycles per year (bivoltine), although in some favourable conditions (warmer temperatures) it can have 3 cycles per year. In the laboratory its fastest growth is reached at 24.5 °C with 3-4 months per generation (Washburn and Frankie, 1985). Growth rate of *P. mesembryanthemi* is also favoured by higher nitrogen and water availability for the host plant (Washburn et al., 1987).

The South African *Pulvinaria delottoi* is a semi-cryptic species, with very little morphological differences with *P. mesembryanthemi* (e.g. the lateral setae, curve and shorter in *P. mesembryanthemi* and straight and longer in *P. delottoi*). Both species differ in feeding habits (*P. mesembryanthemi* feeds preferentially on younger leaves and *P. delottoi* prefers older leaves) and life cycle duration (*P. delottoi* only has 1 cycle per year in California) (Washburn and Frankie, 1985, Washburn and Frankie, 1981). Outside its native range, *P. delottoi* has only been described in the

Table 1.5. List of plants for which *Sclerotinia sclerotiorum* has been studied as a biocontrol agent, method of inoculation chosen, country of study and effectiveness. Key: G, greenhouse; F, field.

Target species	Method of inoculation	Country	Effectivity
Chrysanthemoides monilifera (L.) Norl.	mycelium spray	Australia	Plant death after 12-32 days (G)
Centaurea diffusa Lam	mycelium-on-wheat	Canada	Not sufficient, need of recurrent infections (F)
Cirsium arvense (L.) Scop.	mycelium-on-wheat	N. Zealand	Death and rotting of roots, lower seedbank (F)
Cirsium vulgare L.	mycelium-on-wheat	N. Zealand	Sufficient (G)
Taraxacum officinale Webber	mycelium-on-wheat	N. Zealand	Low effect on growth (G)
Rannunculus acris L.	mycelium-on-wheat, ascospores	N. Zealand	Complete plant infection (G), death and loss of fitness (F)
Senecio jacobaea L.	mycelium-on-wheat	N. Zealand	Sufficient (G)
Carduus tenuiflorus Curt.	mycelium-on-wheat	N. Zealand	Not sufficient (G)
Carduus nutans L.	mycelium-on-wheat	N. Zealand	Sufficient (G)
Crepis capillaris (L.) Wallr.	mycelium-on-wheat	N. Zealand	Low effect on growth (G)
Hypochaeris radicata	mycelium-on-wheat	N. Zealand	Low effect on growth (G)
Rumex obtusifolius L.	mycelium-on-wheat	N. Zealand	Low effect on growth (G)
Pistia stratiotes L.	mycelium-on-barley	N. Zealand	Plant death after 54 days (G)
Alternanthera philoxeroides (Mart.) Griseb.	mycelium-on-barley	N. Zealand	Shoot necrosis (G)
Tradescantia fluminensis Vell.	mycelium-on-barley	N. Zealand	Shoot necrosis (G)

References: [1]Cother (2000); [2]Mortensen and Hogue (1992); [3]Bourdôt and Harvey (1994); [4]Green et al. (1993); [5]Verkaaik et al. (2004); [6]Pottinger et al. (2008); [7]Bourdôt et al. (2006); [8]Waipara et al. (1993); [9]Waipara et al. (2006)

United Kingdom (Salisbury et al., 2011) and California (Washburn and Frankie, 1985).

In the 70s in California, both *P. mesembryanthemi* and *P. delottoi* produced considerable damages in introduced populations of *C. edulis*, especially in heavily infested plants (Washburn and Frankie, 1985, Donaldson et al., 1978). Their success can be explained by suboptimal growth-conditions of ice plants (drought and low temperatures) and predator release (Washburn and Frankie, 1985, Donaldson et al., 1978). At the time, the scale insects were considered an economic pest for its capacity of producing decline and death on ice plants and they released predators and parasites to control *P. mesembryanthemi* (Washburn et al., 1985, Tassan et al., 1982). However, the use of these scale insects as biological control agents was considered for coastal habitats (Washburn and Frankie, 1985) and their potential as biological control agent outside the United States has been repeatedly suggested (ISSG, 2008, Fagúndez and Beiras, 2007). Nevertheless, their suitability as biocontrol agents of *Carpobrotus* spp. has not been studied so far.

Pulvinariella mesembryanthemi has three clear advantages to be used as a biocontrol agent of *Carpobrotus* spp.:

a. It feeds preferentially and almost exclusively on *Carpobrotus* spp.;
b. It has already been introduced accidentally with the plant in areas where *Carpobrotus* spp. is invasive, so its use as a control agent would not entail the introduction of a new species in invaded ecosystems;
c. It can produce massive death in *C. edulis*, as recorded in the 70's in California.

However, the existence of mechanism of defence in *Carpobrotus* spp. can threaten control success. Death of *Carpobrotus* plants or plant parts with high densities of scales is considered a major scale mortality factor (Washburn et al., 1985). As scale insects are sessile (except at the crawlers stage), this could result in mortality of them and less available feeding sites for the next generation. Moreover, *Carpobrotus* avoids the formation of new tissues when supporting high scale densities, which limits population growth (Washburn et al., 1985).

Table 1.6. Countries where the presence of *Pulvinariella mesembryanthemi* has been recorded outside its native range.

Continent	Country	Region	Ref.
America	USA	California	1
	Argentina	Buenos Aires	2
	Chile	-	3
Africa	Egypt	Alexandria	4
	Algeria	-	5
	Zimbabwe	Odzani	6
Eurasia	Spain	Mainland, Canary islands	7, 8
	France	-	8
	Great Britain	-	8
	Netherlands	(in greenhouses)	9
	Greece	Crete island	10
	Italy	Mainland, Sicily & Sardinia islands	11
	Malta	-	8
	Slovenia	-	12
	Portugal	Mainland, Madeira island	13, 14
	Turkey	-	15
Oceania	Australia	Mainland, Tasmania island	16, 17
	New Zealand	North and South islands	18

References (Ref.): [1]Washburn and Frankie (1985); [2]Granara de Willink and Claps (2003); [3]Kondo and Gullan (2010); [4]Hall (1922); [5]Balachowsky (1927); [6]Hodgson (1967); [7]Douglas (1887); [8]Pellizzari and Germain (2010); [9]Jansen (2000); [10]Kozar et al. (1991); [11]Longo et al. (1995); [12]Seljak (2010); [13]Franco et al. (2011); [14]Vieira et al. (1983); [15]Cebeci and Selmi (2004); [16]Hebert (2010); [17]Qin and Gullan (1992); [18]Hodgson and Henderson (2000).

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2. Objectives

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2. Objectives

Carpobrotus edulis (ice plant) invades dunes, rocky areas and cliffs all along the Galician coast (NW Iberian Peninsula). Extensive invasions of this plant are also found in other temperate coastal areas around the world. Ice plants engineer the invaded ecosystems altering their biotic and abiotic properties, in some cases persistently after plant removal, facilitating their own invasion. Therefore, studying the effects of *C. edulis* in the invaded ecosystem can help to discern the mechanisms by which this alien plant outcompetes the native vegetation and may help to optimize habitat restoration. Especially variable are the effects of the invasion on soil properties, as they seem to be dependent on the initial characteristics of the invaded habitat. Also, studies on the chemical and physicochemical effects of *C. edulis* on the soil have been mostly done in dunes and focused on pH and macronutrients. There is an information gap on the effects of the invasion on micronutrients, despite their importance for plant nutrition or toxicity. Furthermore, the effects on the N cycle have not been studied yet. Knowing how *C. edulis* affects the different soil N fluxes can help to disentangle how this alien plant alters the availability and concentration of one of the most commonly limiting nutrients for plants.

Another loose end in the study of *C. edulis* is the search for alternatives to mechanical and chemical control. Ice plants usually colonizes vulnerable habitats, such as dunes and coastal areas, where it is primordial to avoid eroding the soil and affecting native species (especially those endemic or endangered). Also, invaded cliffs have difficult access for applying mechanical or chemical control to *C. edulis* and can serve as dispersion sources of the plant. For these reasons, it is necessary to find out an alternative of control which does not produce disturbances in the habitat and that is self-sustainable, such as biological control. *Carpobrotus edulis* has few enemies on its invaded areas, so introducing a new parasite or predator could counteract this competitive advantage over native plants. The fungus *Sclerotinia sclerotiorum* is one potential candidate for biocontrol because its use as a mycoherbicide has already been studied for several invasive species. Moreover, it can infect some species of the same family as *C. edulis* (Aizoaceae), although its suitability to control *C. edulis* has never been tested. Another potential biocontrol agent is the scale insect *Pulvinariella mesembryanthemi*, a very specific parasite of *Carpobrotus* species that can produce the mortality of

the plant at high densities. It has been accidentally introduced with *C. edulis* in different areas worldwide where the plant is invasive. As the abiotic and biotic interactions of *P. mesembryanthemi* in these alien areas are conditioning its abundance and phenology, their study can give vital information about its suitability as a biocontrol agent in the introduced areas. Characteristics of *P. mesembryanthemi* like performance under different environmental conditions or impact on the plant can vary across populations due to genetic differences, hence selecting the most appropriate populations would improve the success of *P. mesembryanthemi* as biocontrol of *C. edulis*.

Taking all these considerations into account, the following objectives were proposed:

- To evaluate the effects of *C. edulis* invasion on the chemical and physico-chemical properties of necromass and soils (0-5 cm and 5-10 cm depth) of invaded and non-invaded dunes and rocky areas in NW Iberia (Chapter 3).
- To determine the impacts of *C. edulis* invasion on the soil N cycle (gross N fluxes) of invaded and non-invaded soils (0-5 cm and 5-10 cm layers) of rocky areas from NW Iberia (Chapter 3).
- To assess the effects of the fungus *S. sclerotiorum* and the insect *P. mesembryanthemi*, separately or combined, on the mortality and performance (growth, biomass and physiological indexes) of native and non-native (NW Iberia) *C. edulis* plants (Chapter 4).
- To study the effect of abiotic (temperature, precipitation, irradiation) and biotic (host, predators, parasites, mutualists) factors on the population dynamics and phenology of *P. mesembryanthemi* in NW Spain (Chapter 5).
- To determine the intra- and inter-populations genetic variability of native and non-native *P. mesembryanthemi*, as well as the original population and number of colonizing events of the alien populations through a worldwide phylogeographic analysis (Chapter 6).

3. Effects of *Carpobrotus edulis* invasion on main litter and soil characteristics and on soil gross N fluxes

This chapter (text, tables and figures) is based on the following journal articles[1,2]:

Vieites-Blanco, C. & González-Prieto, S. J. (2017) Effects of *Carpobrotus edulis* invasion on main litter and soil characteristics in backdune and rocky coastal habitats with oceanic climate. *Plant and Soil,* **425,** 363-374. doi: 10.1007/s11104-018-3598-5

Vieites-Blanco, C. & González-Prieto, S. J. (2018) Effects of Carpobrotus edulis invasion on soil gross N fluxes in rocky coastal habitats. *Science of The Total Environment,* **619-620,** 966-976. doi: 10.1016/j.scitotenv.2017. 11.154

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3. Effects of *Carpobrotus edulis* invasion on main litter and soil characteristics and on soil gross N fluxes

3.1. INTRODUCTION

Soils are the base of all terrestrial ecosystems and where the different compartments [biosphere, detritosphere (dead organic matter), geosphere, hydrosphere and atmosphere] interact (Voroney, 2007). Therefore, it is essential to study the impact of invasive plants on soil properties. This research is particularly needed for *C. edulis*, as the changes it produces in soil characteristics can benefit its own invasion (Conser and Connor, 2009) and remain after *C. edulis* removal, hindering restoration (Novoa et al., 2013) and leading to secondary invasions by ruderal nitrophilous plants (Santoro et al., 2011, Novoa et al., 2013, Santoro et al., 2012).

The most studied soil properties in *C. edulis* invaded areas have been organic matter, salinity and humidity, which increase; as well as pH and inorganic N, which are differently affected depending on the invaded area (Vilà et al., 2006, Novoa et al., 2013, Novoa et al., 2014, Conser and Connor, 2009). Less studied have been other macronutrients, for which increases (P, Na, K), decreases (Ca, Na, Mg) or no significant changes (P, Ca, Na, Mg) have been observed (Novoa et al., 2014, D'Antonio, 1990, Winsemius, 2013). As far as we know, the effect of *C. edulis* on micronutrients and trace element availability has not been studied until now, despite their importance for the soil-plant system as essential nutrients or toxic elements above a given threshold (Williams and Fraústo da Silva, 2000). While Cr, I, Se, Si, V and W can also be essential for some organisms, the most widespread and important micronutrients, listed in a decreasing order of concentration in living organisms, are: Fe > Mn > Zn, B > Cu >> Co, Mo and Ni (Hoppert, 2011, Grusak et al., 2016, Williams and Fraústo da Silva, 2000). As constituents of metalloenzymes and other proteins, these eight elements are essential in metabolic processes as respiration, photosynthesis or N_2 fixation (Hoppert, 2011, Grusak et al., 2016, Williams and Fraústo da Silva, 2000), which play a key role even at the ecosystem level.

Likewise, the use of plant and soil ^{13}C and ^{15}N isotopic signatures has been scarce in plant invasions despite being a basic and powerful tool in environmental sciences that provides valuable information on water, C and N cycles (Dawson et al., 2002, Robinson, 2001). Although these analyses have been used in some studies of other invasive species (Li et al., 2017, Msanne et al., 2017), and despite being *C. edulis* a facultative CAM (Crassulacean Acid

Metabolism) species with a ^{13}C isotopic signature different from that of C-3 plants (Herrera, 2009), we have not found references on C and N isotopic signatures in *C. edulis* invaded areas. Of particular importance are the invaders' effects on the N cycle, as N is the most widespread limiting nutrient in ecosystems (Vitousek and Howarth, 1991, Galloway et al., 2004). Impacts of invasive plants on the soil N cycle can persist after their removal (Elgersma et al., 2011). This is particularly true when invasions involve changes in microbial communities (Elgersma et al., 2011) or in N stock and availability, affecting the recolonization patterns and the restoration of the ecosystem (Corbin and D'Antonio, 2004), and increasing the risk of secondary invasions by ruderal nitrophilous plants (Novoa et al., 2013, Santoro et al., 2012, Santoro et al., 2011). Invasive plants can modify the soil N cycle through their impacts on soil microbial communities, litter decomposition and soil properties (Wang et al., 2015, Schaeffer et al., 2012, Laughlin, 2011). These changes can be driven by differences in phenology, leaf traits, plant litter composition, N use, N residence time, interaction with herbivores, symbiosis with native or co-introduced N_2-fixing bacteria, effects on soil microbiota structure and activity, and effects on the microclimate (Mack and D'Antonio, 2003, Knops et al., 2002, Laughlin, 2011, Laungani and Knops, 2009, Corbin and D'Antonio, 2004, Castro-Díez et al., 2014, Lee et al., 2017). The modifications of the N cycle can be influenced by the characteristics of the invaded site and the invasive species, being often stronger in mild climates and islands (Castro-Díez et al., 2014) and when the traits of the invasive plant differ from those of the native flora (Castro-Díez et al., 2014, Lee et al., 2017).

As net N fluxes are the result of several counteracting processes (Murphy et al., 2003), they do not adequately reflect the impacts of invasive plants (Piper et al., 2015). Therefore, studies on the effect of invasive plants on the gross N fluxes are necessary to fully understand how the invasion is disturbing the N cycle. However, these studies are scarce and mostly focussed on annual grasses (Booth et al., 2003, Hawkes et al., 2005, Parker and Schimel, 2010, Piper et al., 2015, Schaeffer et al., 2012, Stark and Norton, 2015), with only a few exceptions for perennial grasses (Thorpe and Callaway, 2011), leguminous plants and trees (Laungani and Knops, 2012). The impact of *C. edulis* on gross N fluxes has not been studied yet, despite having this alien invasive species site-dependent effects over N compounds (Novoa et al., 2014, Santoro et al., 2011), and being still controversial the interpretation of the processes involved in these changes.

Carpobrotus edulis can colonize dunes, cliffs and disturbed environments (Campos et al., 2004, Maltez-Mouro et al., 2010, D'Antonio et al., 1993), including burnt areas (D'Antonio et al., 1993). Habitat characteristics seem to condition the impact of *C. edulis* over soil properties, since induced changes of the invasive plant on some soil characteristics vary between sites (Molinari et al., 2007, Novoa et al., 2014), as it has also been reported for other invasive species. Poorer soils, with low content in nutrients and organic matter, appear to be more sensitive and where soil properties will change more (Santoro et al., 2011, Novoa et al., 2014), and most studies of *C. edulis* effects on soil properties focus on dunes. However, the other frequently invaded habitats, coastal cliffs and rocky areas, have been less studied.

Therefore, the aim of this chapter was to compare *C. edulis* invaded and non-invaded areas to evaluate the effects of *C. edulis* on: a) the main characteristics of the necromass and topsoil; and b) the soil gross N rates. For the first part, we measured 24 topsoil (0-5 and 5-10 cm layers) variables in two dunes and two rocky areas from the Atlantic coastline of NW Iberia: water holding capacity (WHC), humidity, pH, electrical conductivity (EC), NH_4^+-N, NO_3^--N, $\delta\ ^{13}C$, $\delta\ ^{15}N$, organic C, organic N, and available Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P and Zn. Also, in necromass, the total content of the latter 16 elements was measured as well as their amounts on a surface basis and the $\delta\ ^{13}C$ and $\delta\ ^{15}N$ isotopic signatures. For the second part, we used a paired ^{15}N labelling experiment and the state-of-the-art *Ntrace* compartment model (Müller et al., 2007) to estimate up to 8 gross N rates in the two rocky areas.

3.2. Material and Methods

3.2.1. Site and sampling description

Among 23 sites with well-established *C. edulis* populations (8 on dunes; 15 on rocks), two back dune and two rocky representative study sites of the areas invaded by *C. edulis* were selected across the Atlantic coastline of NW Iberia (Table 3.1).

In September 2015, for each location, necromass and soil (0-5 and 5-10 cm depth) were separately sampled in 10 randomly distributed 15x15 cm squares under native vegetation and another 10 under *C. edulis*. Soil subsamples were mixed into a composite sample per site and depth and the same was done with the necromass. In the laboratory, necromass was dried at 55 ^oC, weighted, triturated and homogenized. The soils were sieved (< 2 mm), homogenized and then divided into fresh subsamples, which were kept

at 4 ºC for inorganic N measurements and the paired ^{15}N labelling experiment, and air-dried subsamples for the other analyses. Sub-samples of dried necromass and soils were finely ground (< 100 µm) for chemical analysis in a planetary ball mill (Retsch PM100, Germany, with cups and balls of zirconium oxide).

Table 3.1. Main characteristics of the four study sites with invaded (*Carpobrotus edulis*) and non-invaded plots. Key: MAT, mean annual temperature; MAP, mean annual precipitation.

	Punta Nariga	**Sálvora Island**	**Pragueira**	**Moledo**
Country	Spain	Spain	Spain	Portugal
Province	A Coruña	A Coruña	Pontevedra	Viana do Castelo
UTM coordinates (29T grid zone)	0507346(x) 4796386(y)	0498894(x) 4701427(y)	0511201(x) 4695464(y)	0511011(x) 4632650(y)
Latitude	43º19'13'' N	42º27'55'' N	42º24'41'' N	41º50'44'' N
Longitude	8º54'34'' W	9º0'49'' W	8º51'50'' W	8º52'3'' W
Altitude	35-40 m asl	20-25 m asl	15-20 m asl	3-5 m asl
Distance to the sea	70-80 m	65-95 m	70-85 m	110-130 m
Parent material	Coarse grained two micas granitoid		Coastal dune	
Soil type[1]	Umbric Leptosol		Eutric Arenosol	
Dominant natural vegetation	*Ulex europaeus* L., *Erica vagans* L. and *Armeria pubigera* (Desf.)	*Armeria pubigera*	*Cistus* sp.	Gramineae and *Cistus* sp.
Natura 2000 Network site	Yes	Yes	No (in the border)	Yes
Time of invasion	15-20 years	80 years	6-8 years	> 15 years
MAT[2]	---------- 14-16 ºC----------			
MAP[2]	---------- 1400-1800 mm ----------			

[1] IUSS Working Group (2014); [2]AEMET-IMP (2011).

3.2.2. Soils and necromass analysis

Soil water-holding capacity (WHC) was measured in a Richards' membrane-plate extractor at a pressure of 10 kPa. Soil pH was measured in a 1:2.5 soil:solution ratio, both in water and 0.1 M KCl, with a pH-meter (Metröhm, Switzerland). Electrical conductivity (EC) was measured in soil extracts (1:5 soil:water ratio) with an EC meter (Metröhm, Switzerland). Soil humidity was determined by drying soil samples at 105 ºC for 5 h.

Total C and total N of soils and necromass, as well as their ^{13}C and ^{15}N isotopic signatures, were measured in ground samples with an elemental analyser (Carlo Erba, Milano, Italy) coupled on-line with an isotopic ratio mass spectrometer (Finnigan Mat, delta C, Bremen, Germany). In the back dune soils, organic C and organic $\delta\ ^{13}C$ were also determined after $CaCO_3$ removal with the 'capsule method' using 20% HCl (Brodie et al., 2011). An elemental reference material (Soil 3 from Eurovector, Milano, Italy) and isotopic standards [IAEA-C-6 and IAEA-CH-7 (for $\delta\ ^{13}C$) or IAEA-N1 and IAEA-N2 (for $\delta\ ^{15}N$), alternately, from the International Atomic Energy Agency, Vienna, Austria] were included in each set of 10 samples to check the accuracy of the results; if necessary, drift correction was made against internal standards during the run.

Inorganic N species were extracted with 2 M KCl (1:5 soil:solution ratio). The mixture was shaken for 1 h and filtered through glass microfiber filters (Whatman GF/A, Ø 125 mm). Ammonium and nitrate were sequentially liberated with two consecutive microdiffusions (55 ^{o}C, 72 h) from 50 mL aliquots placed in 500 mL glass jars, by adding respectively MgO (0.2 g) and MgO (0.2 g) plus Devarda's alloy (0.4 g). Both N forms were trapped as NH_3 into 10 mL of 0.004 M H_2SO_4 in a Teflon bottle suspended in the glass jar. Measurement was made by back titration of the H_2SO_4 excess with 0.004 M NaOH. Three blanks and three standards (NH_4NO_3) were included in each batch to subtract N from reagents and to check for N recovery.

Soil available nutrients and trace elements (Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P and Zn) where extracted with a mixture of 1 M NH_4Ac and 0.005 M DTPA (soil:solution ratio of 1:5). The mixture was shaken for 2 h, filtered through cellulose paper (Filter-Laboratory 1242, Ø 90 mm) and analysed with a simultaneous ICP-OES (Varian Vista Pro, Mulgrave, Australia). A calibration curve prepared with certified standards of all elements was measured beforehand and one of the calibration solutions was routinely included in each set of 30 samples as a quality control and, when necessary, the calibration curve was measured again. In order to measure the total nutrient and trace element content of necromass, aliquots of 500 mg were digested in a high performance digestion unit (Milestone 1200 Mega, Sorisole, Italy) for 55 min with 8 mL of 65% HNO_3 and 25 mL of 30% H_2O_2. Blanks and reference materials (hay powder No. 129, Community Bureau of References, EU; apple leaves No. 1515, National Institute of Standards and Technology, USA) were also included in each digestion batch to subtract elements from reagents and to check for element recovery. Once cooled, the

solutions were filtered through quantitative filter paper (Filter-laboratory 1242, Ø 90-mm), transferred to 25 mL volumetric flasks, made to volume with water and analysed by ICP-OES as previously described for soils.

Samples analyses were always done in duplicate (in triplicate for inorganic N analysis), and the mean was used for the statistical analysis.

3.2.3. Soil incubation and gross N transformation rates

Gross N transformation rates were estimated for the rocky areas (see Table 3.1). Before starting the experiment, soils were wetted to slightly below 70% of their water holding capacity with the wetting system described in Gómez-Rey and González-Prieto (2013), which allows easily wetting highly hydrophobic soils.

A paired ^{15}N labelling experiment, with $^{15}NH_4NO_3$ and $NH_4{}^{15}NO_3$ as tracers and four incubation times (0.5 h, 1, 3 and 7 days), was conducted. Aliquots equivalent to 30 g of dry soil were placed in a total of 192 centrifuge bottles (250 ml): two invaded soils, two non-invaded soils, two soil depths, two ^{15}N-tracers, four incubation times and three replicates. The aliquot of soil corresponding to each bottle was deposited as 4 successive layers, each of which received 1 mL of ^{15}N-tracer solution added uniformly over the soil surface with an automatic pipette (i.e. 4 mL per bottle), equivalent to an N addition of 1 mg kg^{-1} dry soil with a ^{15}N excess fraction of 49 %. After labelling, soils were incubated at 25 ^{o}C in darkness and, then, an extraction-diffusion method was used for NH_4^+-N and NO_3^--N quantification. Inorganic N species were extracted and measured as mentioned above (section 2.2.). After the two titrations (for NH_4^+ and NO_3^-), the resulting $(NH_4)_2SO_4$ solutions were evaporated to dryness at 60 °C in a vacuum oven (Memmert VO400, PM400) for obtaining $(NH_4)_2SO_4$ crystals. To accelerate the drying process, the oven was alternatively under vacuum (15 kPa) and atmospheric pressure. In order to trap possible traces of atmospheric NH_3, the incoming air was passed through a column of activated charcoal. The $(NH_4)_2SO_4$ crystals were packed into tin capsules and analysed for ^{15}N. The soil remaining in the flasks and the filters during the extraction procedure were washed with deionized water until no chlorides were detected (silver nitrate test), oven-dried at 105 ^{o}C, finely ground (< 100 µm) and packed into tin capsules for organic N analyses.

The ^{15}N enrichment of NH_4^+-N and NO_3^--N, as well as the organic N content and its ^{15}N enrichment were measured with an elemental analyser

(Carlo Erba CNS 1508) coupled on-line with an isotopic ratio mass spectrometer (Finnigan Mat, delta C, Bremen, Germany). An elemental reference material (Soil 3 from Eurovector, Milano, Italy) and an isotopic standard (IAEA-N1, IAEA-N2, IAEA 305-A, IAEA 305-B and IAEA 311, alternately, from the International Atomic Energy Agency, Vienna, Austria) were included in each set of 10 samples to check the accuracy of the results; if necessary, drift correction was made against internal standards during the run.

3.2.4. ^{15}N-tracing model

To quantify gross N transformation rates, a *Ntrace* compartment model (set up in Simulink and summoned by the MCMC optimisation routine programmed in MatLab; The Math Works Inc.) with different N pools and possible N transformations was suggested as a start point (Rütting and Müller, 2007, Müller et al., 2007).

No evidences of abiotic NH_4^+ fixation were found and, consequently, only two pools of inorganic N (exchangeable NH_4^+, exchangeable NO_3^-) were considered for the modelling. Following Nelissen et al. (2012), the soil organic nitrogen (SON) was compartmentalized in a microbially easily available fraction (SON_{lab}, 1% of total SON) and a more difficult to mineralize fraction (SON_{rec}, 99% of total SON) as this often helps to better model the mineralization rate of SON. The initial pool sizes and ^{15}N abundance for NH_4^+ and NO_3^- were estimated by extrapolating back to t=0 the measurements from the first two soil extractions (t=0.5 hours and t=1 day) (Müller et al., 2004).

Depending on the considered soil, the model was run 7-15 times changing both the number of rates and the combinations of them until the most fitting model to our data was found. In all soils, we checked from the simplest model, with only the most usually considered gross N rates (mineralization, nitrification, NH_4^+ and NO_3^- immobilization), to the most complex *Ntrace* model possible with our data, which would include: a) M_{SONrec}, mineralization of SON_{rec} to NH_4^+; b) I_{NH4rec}, immobilization of NH_4^+ to SON_{rec}; c) M_{SONlab}, mineralization of SON_{lab} to NH_4^+; d) I_{NH4lab}, immobilization of NH_4^+ to SON_{lab}; e) O_{SON}, oxidation of SON to NO_3^- or heterotrophic nitrification [NO_3^- production from SON without mixing into the free soil NH_4^+ pool; see Barraclough and Puri (1995) for assumptions and possible limitations]; f) O_{NH4}, oxidation of NH_4^+ to NO_3^- or autotrophic nitrification; g) I_{NO3}, immobilization of NO_3^- to SON; and h) D_{NRA},

dissimilatory nitrate reduction to ammonium. *Ntrace* is also able to model N losses by denitrification and NH_3 volatilisation, but these rates were discarded taking into account that the recovery of the added ^{15}N showed nil or negligible N losses. All transformations were described by first order kinetics, except M_{SONrec} and O_{SON} which followed zero order kinetics. If the values yielded by the model for a certain rate were close to zero, they did not follow a normal distribution and the inclusion of the rate did not improve the performance of the model, the rate was not retained in the model following the Akaike information criterion (Staelens et al., 2012). According to this, M_{SONrec} was not considered in any of the modelled soils, being the other N rates retained at least in one of the studied soils.

The parameters of the selected transformation rates were estimated using a Markov chain Monte Carlo (MCMC) method by fitting the model values to the measured contents and ^{15}N enrichments of NH_4^+ and NO_3^- (Müller et al., 2007). The optimisation procedure results in a probability density function (PDFs) from which parameter averages and standard deviations were calculated (Müller et al., 2007). For transformations following first order kinetics, average rates were calculated by integrating gross N rates over the experimental period divided by the total time (Rütting and Müller, 2007). Net N transformations were calculated from the obtained gross rates: net ammonification [$M_{SONlab} - (I_{NH4lab} + I_{NH4rec})$], net nitrification [$(O_{NH4} + O_{SON}) - (D_{NRA} + I_{NO3})$] and net N mineralization (sum of both net rates).

3.2.5. Statistical analysis

Due to the highly contrasting characteristics of soils from back dune and rocky habitats, the differences in soil properties between invaded (X_{inv}) and non-invaded (X_{nat}) sites were standardized by the value of the non-invaded site (X_{nat}), as:

$$X_{stand} = \frac{X_{inv} - X_{nat}}{X_{nat}}$$

and significant differences between the standardized variable (X_{stand}) and 0 where tested with a one-sample *t*-test. Data on necromass characteristics were analysed by the paired t-test. The normality assumption was tested in both cases with the Shapiro-Wilk test, and when not accepted, data were subjected to Cox-Box transformations or to the Tukey's ladder of powers. Statistically significant differences where established at $P < 0.05$.

A Principal Component Analysis (PCA, based on the correlation matrix, to extract the factors, plus Varimax rotation with Kaiser normalization) was performed to assess the relationships among the studied variables, in soils and necromass separately, and whether samples are grouped together according to vegetation cover (native plants or *C. edulis*). The anti-image correlation matrix (comprising the negative values of the partial correlation coefficients) was analysed to detect and discard the variables less suitable for the factor analysis, aiming to improve the Kaiser-Meyer-Olkin measure of sampling adequacy and the Barlett's test of sphericity. The statistical package IBM SPSS Statistics 23 was used in all analysis.

For the gross N fluxes, standard errors of means were calculated based on autocorrelation as described in Harmon and Challenor (1997). Due to the high number of iterations used in the model, the usual statistical tests cannot be used (Rütting et al., 2010). Alternatively, statistical significance in differences between treatments was tested by an overlap of the 85% confidence intervals (CI) (Payton et al., 2000, Rütting et al., 2010).

3.3. RESULTS

3.3.1. Soil characteristics

Substrate seemed to determine the effect of *C. edulis* on soil pH, measured either in water or KCl, which increased around 0.6 units in invaded dune soils and decreased more than 1.1 units in invaded rocky soils ($P < 0.05$, Fig. 3.1; see Supplementary Material SM Tables 3.1 and 3.2). Compared to soils under native vegetation, the NO_3^--N content decreased by 60-74% in all invaded soils ($P < 0.001$, Fig. 3.1 and SM Tables 3.1 and 3.2), whilst the other SOM related variables (organic C, total N and NH_4^+-N) decreased by 34-39% in dune soils ($P < 0.01$) but not in soils over rocks, where NH_4^+-N even increased (P= 0.057, Fig. 3.1 and SM Tables 3.1 and 3.2). Regarding nutrients and trace elements, *C. edulis* invasion led to a decrease in available Co (-55 to -95%, significant only in dune soils), as well as in Mg, Cu and Zn (-35 to -54%, significant only in soils over rocks) (Fig. 3.1 and SM Tables 3.1 and 3.2). Depending on the substrate, *C. edulis* invasion had significant contrasting effects on available B (+14% in invaded dunes; -15% in invaded rocks) and Fe (-45% in invaded dunes; +45% in invaded rocks). Besides, *C. edulis* invaded soils over rocks became depleted in Na (-12%) and enriched in Ni (+51%). We found no significant differences between *C. edulis* soils and native vegetation

soils in WHC, humidity at sampling, EC, $\delta^{15}N$, $\delta^{13}C_{organic}$, C/N ratio, and available Al, Ca, K, Mn, Mo and P.

The best PCA with soil properties (KMO measure of sampling adequacy= 0.754; Bartlett's test of sphericity $P < 0.001$) included pH_{H2O}, EC, WHC, total N, NH_4^+-N, organic C and available Al, B, Fe, K, Mg, Mo and P concentrations (SM Table 3.3a). This PCA extracted two factors that jointly explained 86% of the variance (76.3% by Factor 1 and 9.9% by Factor 2). Factor 1 was mostly determined by total N, organic C, WHC, and available B, K and Mg, with factor loadings higher than +0.71. The positive arm of Factor 2 was mostly determined by available Al, Fe, Mo and P with factor loadings higher than +0.76, and to a lesser extent by EC and NH_4^+-N (factor loadings of +0.69 and+0.67, respectively), whilst pH_{H20} (-0.837) was the only variable in the negative arm.

The plane of these two factors discriminated the samples according to substrate, plant cover and depth (Fig. 3.2): a) soils from rocky areas and from dunes formed two differentiated groups in the graph, and wider differences between invaded and non-invaded soils were found for rocky areas; b) in rocky areas, invaded soils showed lower loadings in Factor 1 and higher loadings in Factor 2 than non-invaded ones; c) invaded dune soils showed also lower loadings in Factor 1 (except for the Moledo topsoil) but lower loadings in Factor 2 than non-invaded ones; and d) for each sampling site and vegetation, soils from 0-5 cm showed higher loadings in Factor 1, except Pragueira soil, and bigger effects of *C. edulis* were found in the topsoil.

Several elements were significantly correlated with soil pH, either positively (available Ca) or negatively (WHC, EC, total C, total N, $\delta^{15}N$, NH_4^+ and available Al, Fe, K, Mo, Na, Ni and P) (Table 3.2).

3.3.2. Necromass characteristics

After *C. edulis* invasion, an alien litter layer was deposited over the native litter layer leading to an average necromass accumulation under *C. edulis* in dunes and rocky areas two and four times higher respectively ($P < 0.05$, Fig. 3.1 and SM Table 3.1) than under native vegetation. Both the amount of necromass and its C content showed a significant strong negative correlation with soil pH (r= -0.739 and -0.808, respectively; $P < 0.01$).

In the necromass of the invaded areas, the concentration of Al (P= 0.052), Fe and Cu were significantly lower than in native necromass, while that of B, Ca and Na were higher (Fig. 3.3); levels of K were also higher in necromass from invaded rocky habitats, although lower in dunes (SM Table 3.4).

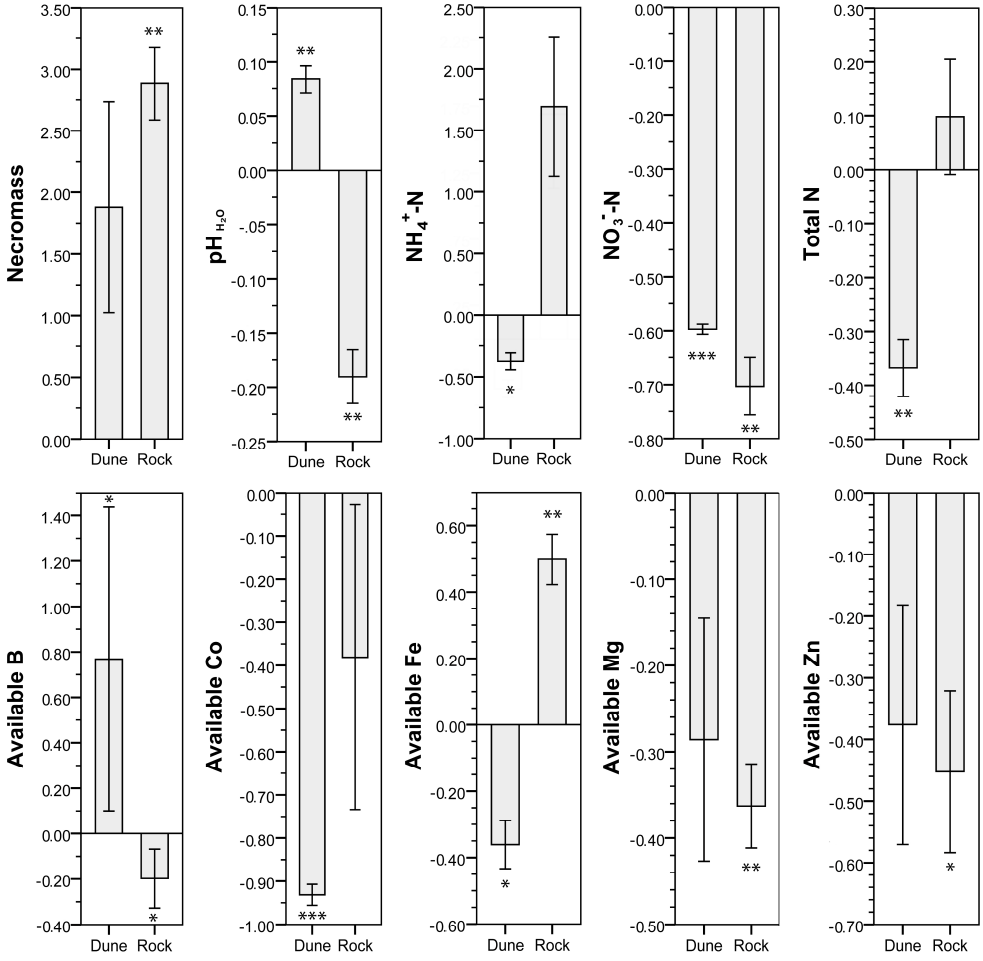

Fig. 3.1. Standardized value [(I-A)/A] ± standard error and significance of differences between the standardized variable and 0 (one-sample *t*-test: * P <0.05; ** P <0.01; *** P <0.001) for some of the most representative studied soil properties for dunes and rocky sites. Key: A, autochthonous vegetation; I, invasive vegetation.

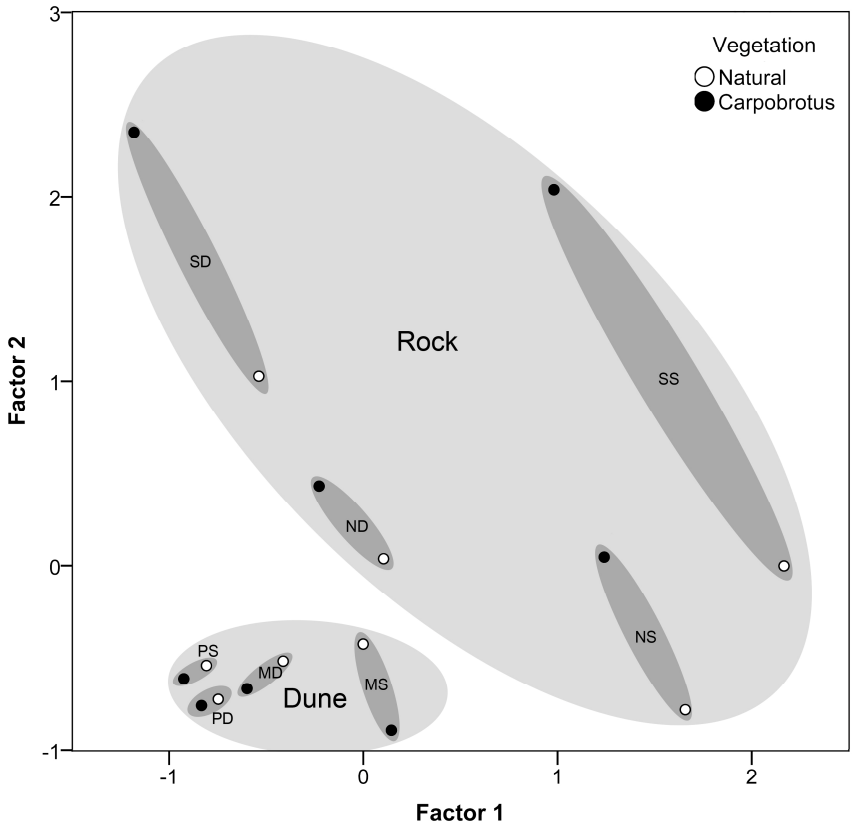

Fig. 3.2. Score plots from the principal component analyses (PCA) performed with soil data for each habitat type and deepness. Key: Moledo 0-5 cm layer (MS), Moledo 5-10 cm layer (MD), Nariga 0-5 cm layer (NS), Nariga 5-10 cm layer (ND), Pragueira 0-5 cm layer (PS), Pragueira 5-10 cm layer (PD), Sálvora 0-5 cm layer (SS) and Sálvora 5-10 cm layer (SD).

When expressed as amount of nutrients per surface unit (SM Table 3.5), compared to non-invaded areas, necromass under *C. edulis* accumulated significantly more B in back dunes, more Al and Na in rocky habitats and more Ca and Mn in both habitats, these differences being completely explained by the higher necromass amount. No significant differences were found for the other studied elements.

The best PCA with necromass properties (KMO= 0.614; Bartlett's test of sphericity $P < 0.01$) included the concentrations of Al, Co, Cu, Fe, Mg, Ni and Zn (SM Table 3.3b). This PCA extracted two factors that jointly explained 83% of the variance (66.3% and 16.9%, respectively, by Factor 1 and 2). Factor 1 was determined by all trace elements considered, with factor loadings higher than +0.70, whereas Factor 2 was determined by Mg and to a lesser extent by Co and Cu (factor loadings of +0.99, -0.42 and -0.42, respectively).

Table 3.2. Pearson Correlation values and significance (* P <0.05; ** P <0.01; *** P <0.001; n=16) between soil pH and accumulated necromass and the analyzed soil properties. Note: WHC, water holding capacity; EC, electrical conductivity.

	pH_{H2O}	pH_{KCl}	Necromass
Necromass	-0.739**	-0.613*	1.000
WHC	-0.771**	-0.770**	0.421
Humidity	-0.073	-0.088	-0.238
EC	-0.902***	-0.819***	0.710**
Total N	-0.829***	-0.759***	0.581*
$\delta^{15}N$	-0.607*	-0.565*	0.369
NH_4^+-N	-0.687**	-0.531*	0.722**
NO_3^--N	-0.234	-0.213	-0.356
Organic C	-0.808***	-0.766**	0.528*
$\delta^{13}C$	-0.128	-0.148	0.031
Available Al	-0.862***	-0.750***	0.756***
Available B	-0.372	-0.225	0.370
Available Ca	0.852***	0.932***	-0.492
Available Co	0.163	0.145	-0.456
Available Cu	-0.147	-0.127	-0.339
Available Fe	-0.832***	-0.647**	0.702**
Available K	-0.731**	-0.673**	0.536*
Available Mg	-0.488	-0.440	0.081
Available Mn	0.053	-0.039	-0.418
Available Mo	-0.834***	-0.706**	0.669**
Available Na	-0.668**	-0.603*	0.456
Available Ni	-0.505*	-0.505*	0.131
Available P	-0.696**	-0.456	0.582*
Available Zn	-0.063	-0.100	-0.411

The plane of these two factors discriminate the samples according to both substrate and plant cover (Fig. 3.4): a) except native necromass in Nariga site, necromass over rocky substrates have positive loadings on Factor 2 while those over dunes have ever negative loadings; and b) in all cases, necromass from invaded sites had more negative loadings on Factor 1 and (except for Moledo site) more positive loadings on Factor 2 than native necromass.

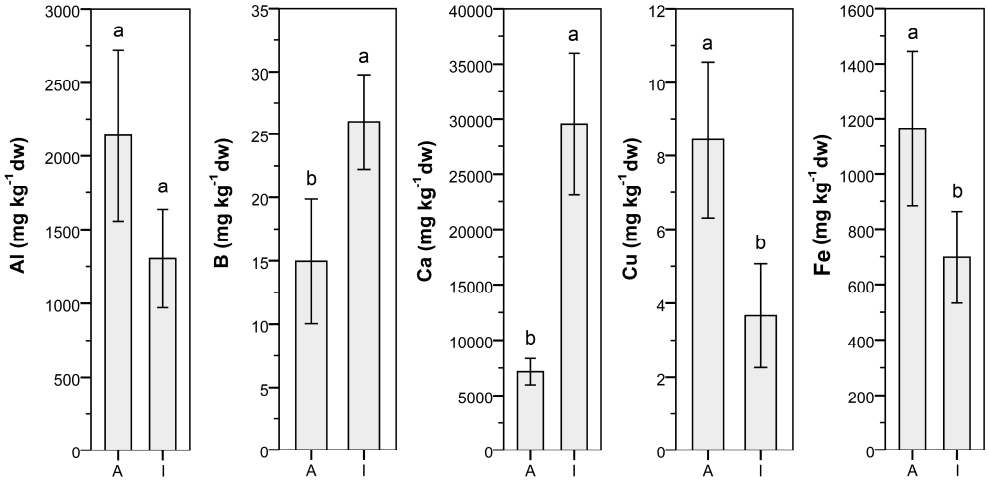

Fig. 3.3. Mean value ± standard error for some of the most representative studied necromass properties under autochthonous (A) and invasive (I) vegetation. Different letters (a, b) indicate significant differences (paired *t*-test; P <0.05) within type of vegetation. For Al, the significance (P= 0.052) was close to the considered threshold.

3.3.3. N pools

In soils from rocky areas, N pools varied with the invasion. The initial NH_4^+-N pool was usually higher in the *C. edulis* invaded soils than in those under native vegetation, differences being wider for the 0-5 cm layer (Figs. 3.5-3.8). While the amount of NH_4^+-N decreased during the incubation in the uninvaded soils (irrespectively of soil depth), it showed contrasting tendencies in the invaded soils: increase in the surface layer and decrease in the sub-surface one (Figs. 3.5-3.8). Conversely, the initial NO_3^--N pool was higher in soils under native vegetation than in the *C. edulis* invaded soils and its size always increased during the incubation (Figs. 3.5-3.8).

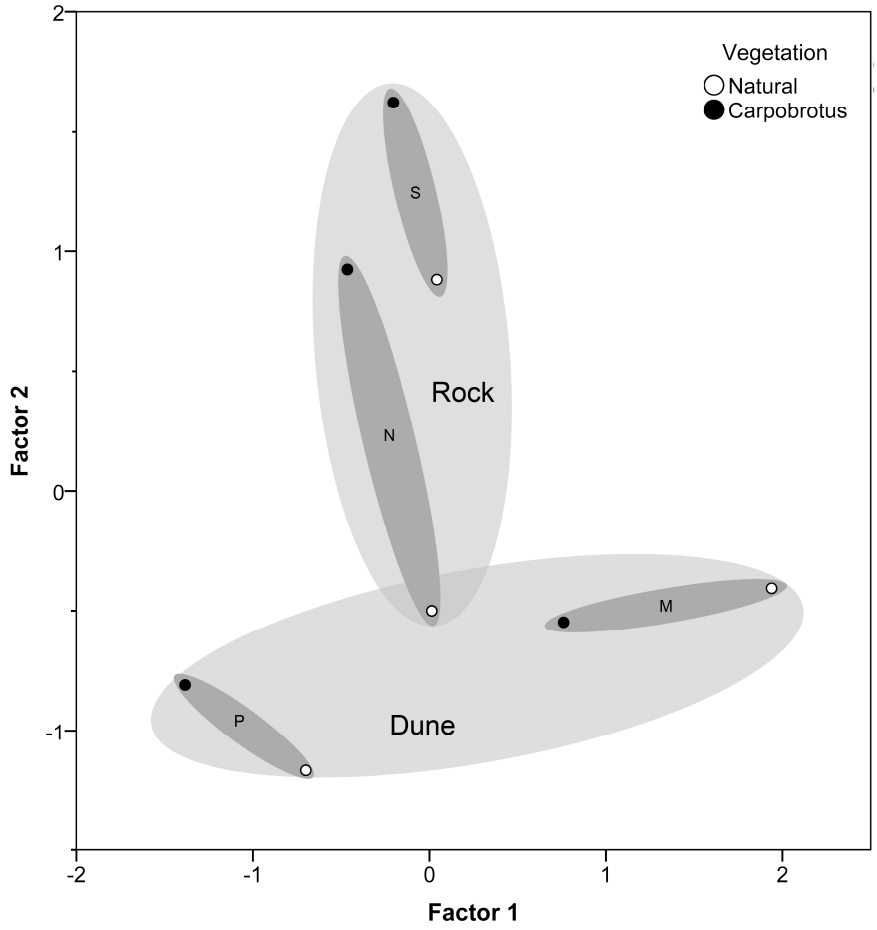

Fig. 3.4. Score plots from the principal component analyses (PCA) performed with necromass data for each habitat type and deepness. Key: Moledo (M), Nariga (N), Pragueira (P) and Sálvora (S) sites.

3.3.4. Gross N fluxes

In general, there was a good fit between the measured and the *Ntrace* modelled data of the size and ^{15}N abundance of the NH_4^+-N and NO_3^--N pools (see Figs. 3.5-3.8).

Irrespectively of vegetation cover and soil depth, no satisfactory adjustments were obtained for models with two organic N pools (SON_{lab} and SON_{rec}: 1% and 99% of total SON, respectively) and two mineralization rates (M_{SONlab} and M_{SONrec}, with first and zero order kinetic, respectively). In all

cases, the best fittings were obtained when only the mineralization of the labile fraction (M_{SONlab}) was considered, because the values yielded by *Ntrace* for M_{SONrec} were not normally distributed and its inclusion never improved model fitting; consequently, this rate was not included in the finally selected models. Except in the Nariga soil under native vegetation, the M_{SONlab} was significantly higher in the topsoil than in the 5-10 cm layer (Fig. 3.9a). The effect of *C. edulis* invasion in the M_{SONlab} rate was site- and depth-dependent, not showing a clear trend.

The gross immobilization of NH_4^+ to recalcitrant N (I_{NH4rec}) was only modelled for surface soils under native vegetation, accounting for more than half of the total NH_4^+ immobilization in these soils (Fig. 3.9b).

As for M_{SONlab}, except in the Nariga soil under native vegetation, the gross immobilization of NH_4^+ to labile N (I_{NH4lab}) was significantly higher in the topsoil than in the 5-10 cm layer (Fig. 3.9a,b). *Carpobrotus edulis* invasion increased the I_{NH4lab} rate in surface soils (2.81 to 3.07x; 1.19x when considering I_{NH4rec}+I_{NH4lab}) and it had contrasting effects within sites in the 5-10 cm layer (0.79x for Nariga, 4.48x for Sálvora).

Regarding NO_3^--N production processes, gross oxidation of recalcitrant N (O_{Nrec}, i.e. heterotrophic nitrification) was only modelled in the uninvaded surface soil of Nariga site (Fig. 3.9c), the inclusion of this rate in the model being necessary because it improves by 23% the misfit function. Both the ammonium oxidation (O_{NH4}, i.e. gross autotrophic nitrification) and the total gross nitrification rate (O_{NH4}+O_{Nrec}) decreased strongly (0.26 to 0.20x) in the 0-5 cm soil layer of the invaded areas, and moderately (0.61 to 0.79x) in the 5-10 cm layer. These rates decreased with depth in native soils, while the reverse was true in invaded soils (Fig. 3.9c).

In most soils, the immobilization of NO_3^- to recalcitrant N (I_{NO3}) was not well modelled by *Ntrace* and it had to be discarded because its probability density functions (PDFs) did not follow a normal distribution and its sampling accuracy (monitored with the Gelman's R test) was not acceptable; moreover, the inclusion of I_{NO3} in the model did not improve the misfit function. The I_{NO3} rate was only modelled in Sálvora soils under *C. edulis*, even being the dominant gross NO_3^- consumption process in its surface layer (Fig. 3.9d). Conversely, the dissimilatory reduction of NO_3^- to NH_4^+ (D_{NRA}) was the exclusive gross NO_3^- consumption process in the other soils (Fig. 3.9d), strongly decreasing in invaded topsoils (0.07 to 0.04x), and to a lesser extend in invaded deep soils (0.18 to 0.70x).

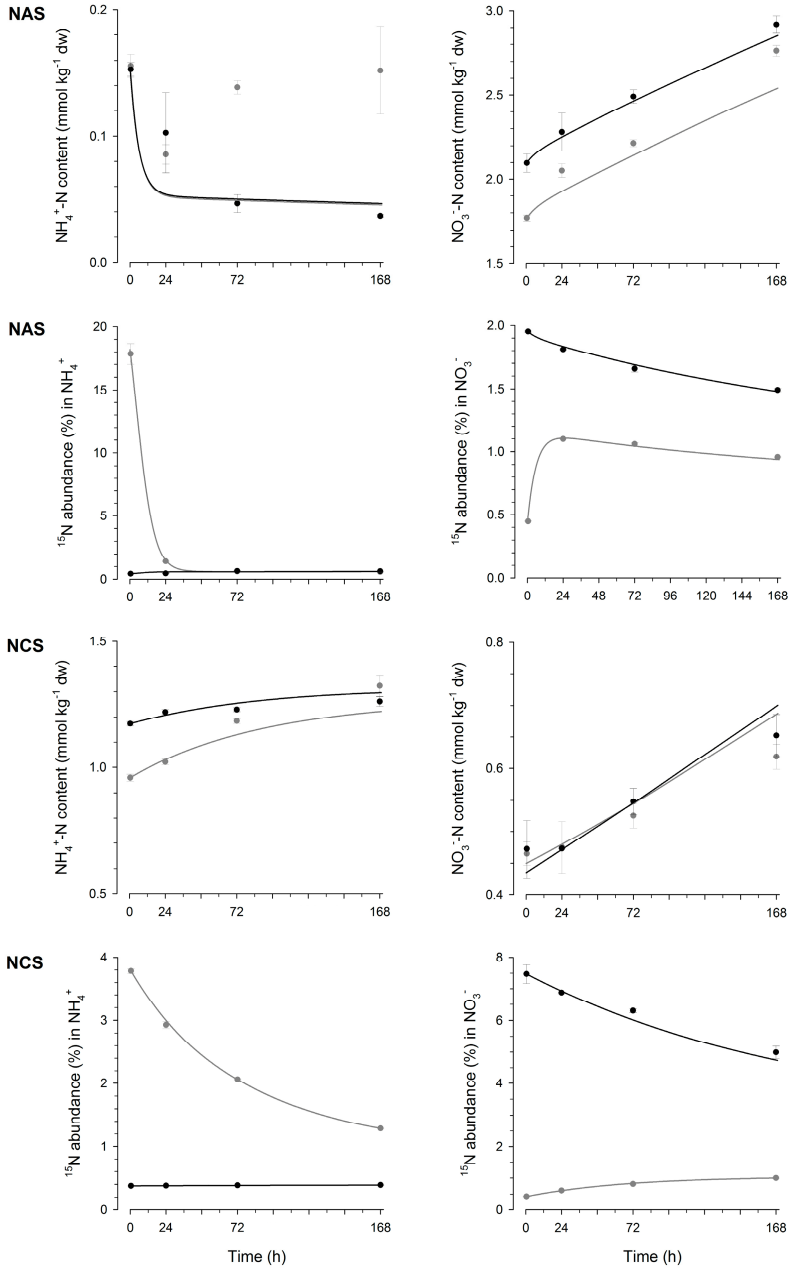

Fig. 3.5. Amount and ^{15}N abundance of NH_4^+-N and NO_3^--N (mean ± s.e.) in Nariga soils (0-5 cm), under autochthonous vegetation (NAS) and *Carpobrotus edulis* (NCS), during the aerobic incubation. Lines show the fit of the model to the experimental data. Light grey points or lines refer to the $^{15}NH_4NO_3$ experiment, whilst black points or lines refer to the $NH_4{}^{15}NO_3$ experiment.

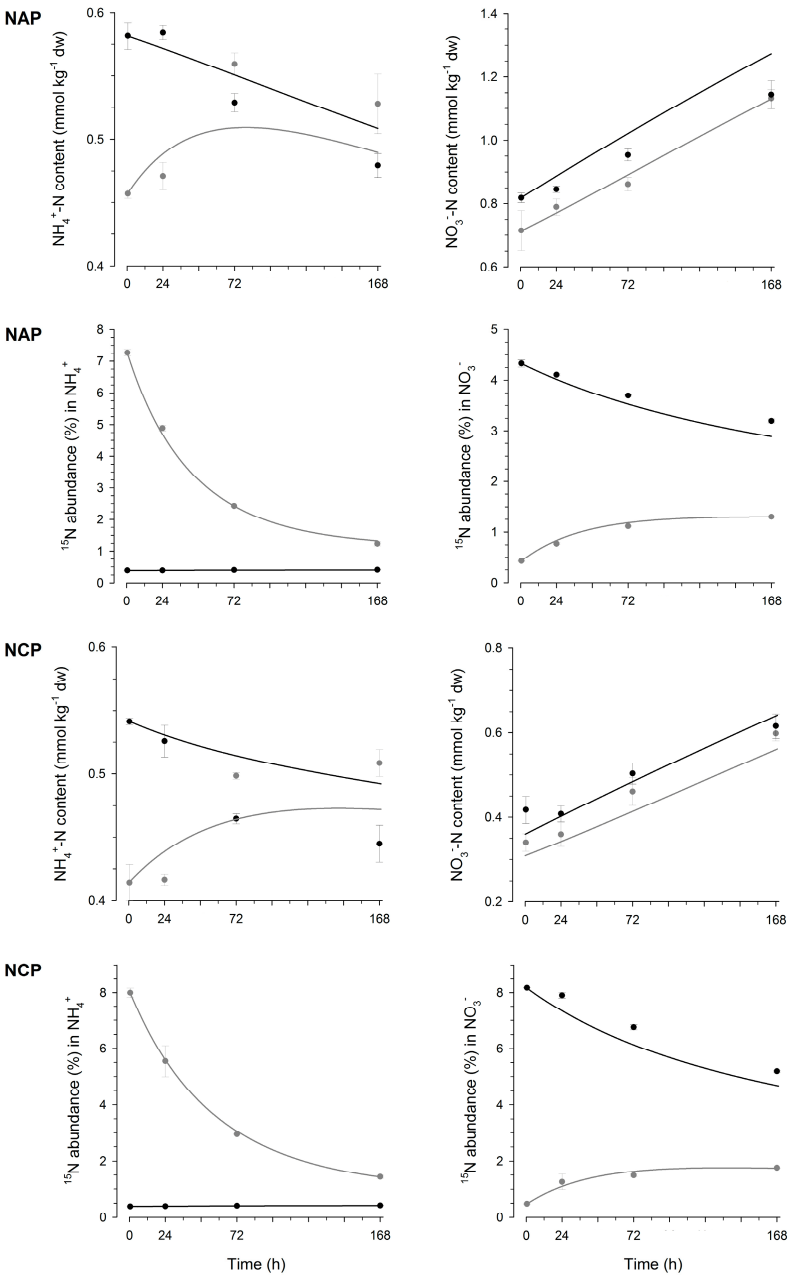

Fig. 3.6. Amount and ^{15}N abundance of NH_4^+-N and NO_3^--N (mean ± s.e.) in Nariga soils (5-10 cm), under autochthonous vegetation (NAP) and *Carpobro-tus edulis* (NCP), during the aerobic incubation. Lines show the fit of the model to the experimental data. Light grey points or lines refer to the $^{15}NH_4NO_3$ experiment, whilst black points or lines refer to the $NH_4{}^{15}NO_3$ experiment.

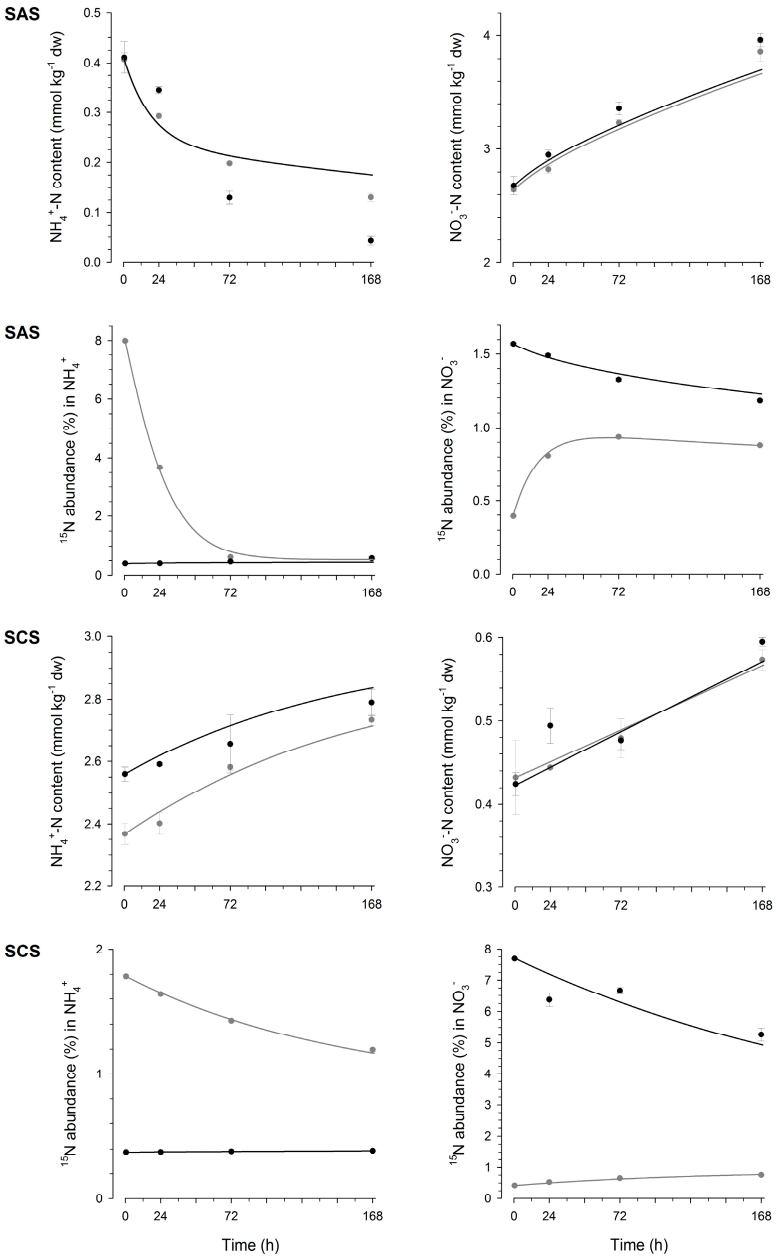

Fig. 3.7. Amount and ^{15}N abundance of NH_4^+-N and NO_3^--N (mean ± s.e.) in Sálvora soils (0-5 cm), under autochthonous vegetation (SAS) and *Carpobrotus edulis* (SCS), during the aerobic incubation. Lines show the fit of the model to the experimental data. Light grey points or lines refer to the $^{15}NH_4NO_3$ experiment, whilst black points or lines refer to the $NH_4{}^{15}NO_3$ experiment.

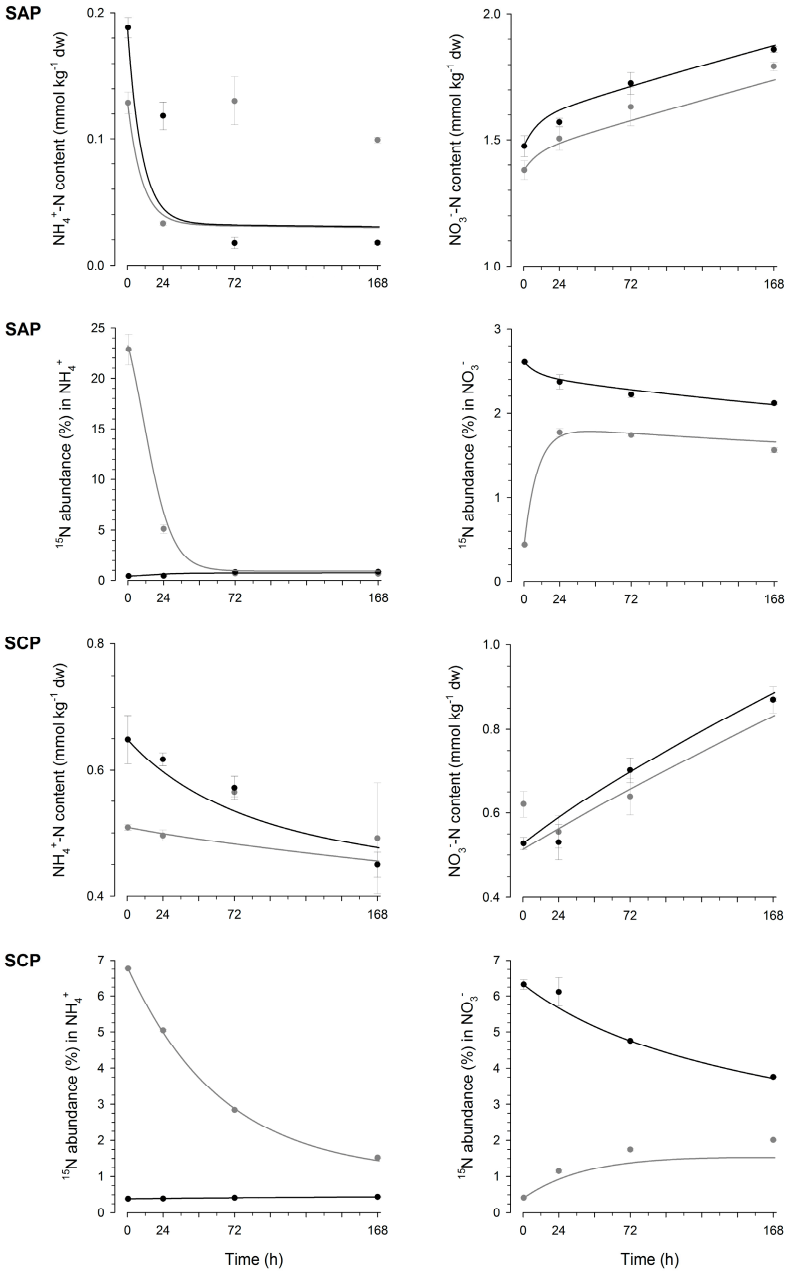

Fig. 3.8. Amount and ^{15}N abundance of NH_4^+-N and NO_3^--N (mean ± s.e.) in Sálvora soils (5-10 cm), under autochthonous vegetation (SAP) and *Carpobro-tus edulis* (SCP), during the aerobic incubation. Lines show the fit of the model to the experimental data. Light grey points or lines refer to the $^{15}NH_4NO_3$ experiment, whilst black points or lines refer to the $NH_4{}^{15}NO_3$ experiment.

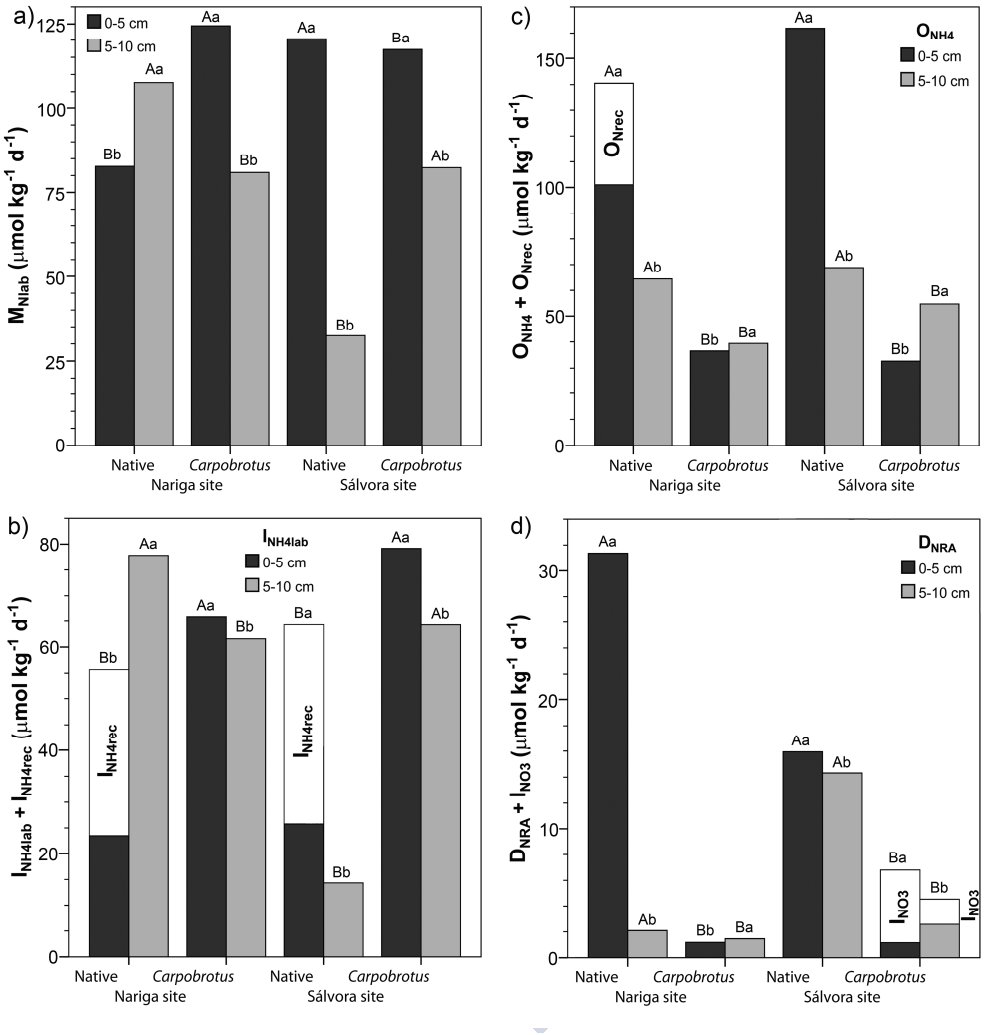

Fig. 3.9. *Ntrace* modelled gross fluxes in the 0-5 cm and 5-10 cm soil layers under native vegetation and *Carpobrotus edulis* in Nariga and Sálvora sites: (a) mineralization of labile SON to NH_4^+ (M_{SONlab}); (b) immobilization of NH_4^+ to recalcitrant (I_{NH4rec}) and to labile SON (I_{NH4lab}); (c) autotrophic (O_{NH4}) and heterotrophic (O_{SON}) nitrification; and (d) dissimilatory NO_3^- reduction to NH_4^+ (D_{NRA}) and NO_3^- immobilization to SON (I_{NO3}). Significant differences - tested by an overlap of the 85% confidence intervals (Payton *et al.*, 2000; Rutting *et al.*, 2010) - is shown by different capital letters for the vegetation types (native or *C. edulis*), and lowercase letters for soil layers (0-5 or 5-10 cm).

In *C. edulis* invaded soils (and in Nariga deep soil under native vegetation) gross immobilization was the dominant NH_4^+ consumption process, exceeding its oxidation rate by 1.18-2.42x, especially in the surface layer, being found the opposite trend in the other native soils (0.55 to 0.21x). Gross production of NO_3^- always exceeded gross consumption, both in uninvaded and invaded soils (3.22 to 31.05x). Gross consumption of NH_4^+ exceeded its gross production (1.23 to 1.78x), except for the surface invaded soils (0.82 to 0.94x).

3.3.5. Net N fluxes

There was not a clear effect of *C. edulis* invasion on the net ammonification rate, but the net nitrification rate decreased in invaded soils, especially in surface soils (0.18 to 0.32x). Overall, net mineralization moderately decreased in invaded soils (0.32 to 0.69x), except for the light decrease in Sálvora deep soil (0.94x).

3.4. DISCUSSION

The results corroborated that the effects of *C. edulis* invasion on soil properties are context-specific, due to differences in the initial characteristics of the habitat (Molinari et al., 2007, Novoa et al., 2014) and to variations in the production of necromass (Molinari et al., 2007). Necromass accumulation in invaded rocky areas was much higher than in invaded dunes, being this variable strongly and negatively correlated to soil pH. Thus, the invasion increased pH in dune soils and decreased pH in soils from rocky areas. Seemingly, in invaded rocky areas, the higher accumulated necromass led to a higher release of organic acids during its decomposition that reduced soil pH (Novoa et al., 2014, Santoro et al., 2011). In invaded dunes, where a lower accumulation of alien necromass was found, other factors (such as Ca uptake by *C. edulis* from deeper soil layers than native plants) may have prevailed, resulting in an increase of soil pH. The invasion by *C. edulis* may lead to different vegetation community successions in dunes and rocky areas through its effect on soil pH: in the studied dunes, the pH increases, contrarily to the natural acidification over the years of dunes (Anwar-Maun, 2009); while in rocky areas and other dunes (Novoa et al., 2014, Santoro et al., 2011) the pH decreases, accelerating natural soil acidification. Overall, according to the PCA, soil properties from rocky areas (with more accumulated necromass and an older invasion) were more affected by *C.*

edulis invasion than those from back dunes (with a lower necromass accumulation and a more recent invasion). This contradicts other studies where the poorer soils (fore dunes) where more affected than richer soils (back dunes) (Novoa et al., 2014, Santoro et al., 2011). Also, the invasion affected more intensively soil properties of the uppermost soil layer (0-5 cm) than those of the 5-10 cm layer (only the decrease of the highly mobile NO_3^- was significant), which agrees with the supposition that *C. edulis* alters soil properties mainly through its necromass production.

Soil pH is one of the main factors determining nutrient availability (Molinari et al., 2007, Fageria et al., 2002). Therefore, the different effect of *C. edulis* invasion in soil pH of dunes and rocky areas resulted in contrasting effects on some nutrients availability (such as Fe), which decreased in invaded dunes and increased in invaded rocky areas. Moreover, available Al, K, Ni and P showed significant negative correlations with pH, and different trends along Factor 2 in the PCA (with positive loading of Al, Fe and P) were found when comparing invaded and native soils of dunes and rocky sites. The decrease in Co availability in invaded dune soils [likely triggered by the pH increase, Macías Vázquez and Calvo de Anta (2009)] and the decrease in Cu, Mg and Zn availability in invaded rocky areas [probably due to the pH decrease, Fageria et al. (2002)], may derive in micronutrient deficiencies in plants and microorganisms, as they are fundamental for several essential metabolic processes (Kamnev et al., 2002, Nicholas et al., 1962, Williams and Fraústo da Silva, 2000).

The differences in necromass chemical composition between invaded and non-invaded areas showed a lower accumulation of trace elements (lower uptake of the potentially toxic Al and lower requirement or higher resorption from senescent tissues of the micronutrients Co, Ni, Fe, Cu and Zn) and a higher concentration of some macronutrients (Mg, Ca) in the necromass of *C. edulis* than in that of native species. This result can partly explain the capacity of *C. edulis* to outcompete native vegetation. Sodium was higher in necromass from invaded than non-invaded areas, but available Na was lower in the invaded soils. Therefore, *C. edulis* seems to be accumulating salt in its tissue [like other Aizoaceae plants (Agarie et al., 2007, Delnavaz Hashemloian et al., 2010, Weber and D'Antonio, 1999)], as an adaptation to salinity.

In dunes, the invaded soils showed significantly lower organic C and total N, contrarily to other studies of *C. edulis* (Novoa et al., 2014, Santoro et al., 2011, Vilà et al., 2006) and despite the higher necromass accumulation in

the invaded soils. This decrease in organic C and N could be related to an inhibition of microbial decomposers by the antibacterial compounds of *C. edulis* (van der Watt and Pretorius, 2001) or to the higher C/N ratio of *C. edulis* necromass compared to that of native vegetation, which may have reduced necromass mineralization and incorporation into the soil organic matter (Packham et al., 2001). This reduction in soil N and organic C goes in the opposite way of natural succession in dunes (Anwar-Maun, 2009, Jones et al., 2008), and seems to be reflected in a lower inorganic N as NH_4^+ and NO_3^-. The effect of *C. edulis* in soil NH_4^+, as in other studies (Novoa et al., 2014), was site dependant, decreasing in invaded dunes but increasing in invaded rocky areas. In rocky areas, *C. edulis* invasion decreased soil NO_3^- concentration, possibly through a reduction of biological nitrification by the acidification of soil (Bramley and White, 1990), leading to an increase in soil NH_4^+. Although *C. edulis* is a facultative CAM species, the ^{13}C results (which did not differ significantly in soil and plants from invaded and non-invaded areas) pointed to a low contribution of CAM metabolism to the fixed C, as also found by Herrera (2009).

The effects of *C. edulis* invasion on soil N cycle were reflected both in N stocks and fluxes and, even though effects on the N fluxes were more intense in the 0-5 cm soil layer, they were also found in the 5-10 cm layer. While in other studies the invasion by alien plants (whose necromass has higher N content and lower C/N ratio than that of native plants) results in higher net nitrification and mineralization (Parker and Schimel, 2010, Piper et al., 2015, Stark and Norton, 2015), in the *C. edulis* invaded soils decreases in net nitrification and mineralization rates were found (which may be linked to the slightly lower N concentration and slightly higher C/N ratio of *C. edulis* necromass). The lower net mineralization of *C. edulis* invaded soils can lead to a decrease in N availability, which may alter the composition of plant communities (Eviner and Chapin, 2003).

In the models obtained with *Ntrace*, gross N mineralization was only included for the labile N pool (and not for the recalcitrant N) and did not varied between soils of invaded and non-invaded areas (whose vegetation did not differ in C/N ratio from *C. edulis* and which was perennial as the invader). Contrastingly, other studies showed an increase in gross N mineralization, when comparing annual invasive grasses (with low C/N ratio) to perennial native grasses (with higher C/N ratio) (Booth et al., 2003, Hawkes et al., 2005, Parker and Schimel, 2010, Piper et al., 2015, Stark and Norton, 2015).

In *C. edulis* invaded soils, heterotrophic bacteria seemed to outcompete nitrifying microbiota for ammonium, as NH_4^+ immobilization was greater than autotrophic nitrification. Immobilization of NH_4^+ was higher in most invaded soils than in non-invaded soils, as also reported for other invasive plants (Bengtsson et al., 2003, Hawkes et al., 2005, Laungani and Knops, 2012), probably enhanced by the decrease in available N derived from the higher recalcitrance of *C. edulis* necromass. Autotrophic nitrification decreased in invaded soils, which may be caused by an inhibition of the nitrifying communities through secondary metabolites of *C. edulis* [as found by Thorpe and Callaway (2011) for *Centaurea stoebe*] or due to the soil acidification (Prosser, 1990) triggered by *C. edulis*. Other invasive plants increased autotrophic nitrification by changing soil microbiota composition or plant coverage (Booth et al., 2003, Hawkes et al., 2005, Parker and Schimel, 2010).

Immobilization of NO_3^- was only found in Sálvora invaded topsoils, while dissimilatory reduction of NO_3^- (D_{NRA}) was the sole NO_3^- consuming process in the other soils. D_{NRA} may have occurred in anaerobic microsites (Pett-Ridge et al., 2006, Rütting et al., 2011), which were depleted in O_2 through organic matter mineralization (Norton and Stark, 2011, Tiedje et al., 1984), as the C content of the soils from rocky areas was high. The D_{NRA} was lower in the invaded soils (especially in the topsoil layer), where substrate (NO_3^-) availability and NH_4^+ nitrification were also lower than in non-invaded soils.

3.5. SYNTHESIS

Carpobrotus edulis can change many soil properties, benefiting its own invasion and hampering the restoration of the invaded habitats. Some of its effects on soils were previously known but there was a knowledge gap about the effects in rocky areas, micronutrients and N cycle.

We compared invaded vs non-invaded paired plots in two dunes and two rocky areas by measuring 18 variables in litter and 24 in soils (0-5 and 5-10 cm layers). Additionally, for the rocky areas, we estimated the gross N fluxes by using a paired ^{15}N labelling experiment and a *Ntrace* compartment model.

Invasion effects on soil properties increased with the accumulated alien necromass, decreased with soil depth and are substrate-dependent: soil pH,

Al, Fe and P increased in dunes, while these variables and Mg, Cu and Zn decreased in rocky sites. *Carpobrotus* necromass is richer in Mg and Ca and poorer in Al, Co, Cu, Fe, Ni and Zn than native necromass.

The invasion also altered the gross N fluxes. *Carpobrotus edulis* generally increased NH_4^+ immobilization (I_{NH4}, 1.19-4.48x), presumably due to a lower N availability for the microbiota. The invasion also decreased autotrophic nitrification ($O_{NH4,}$ 0.20-0.79x), either by a direct effect over soil microbiota or by the acidification triggered by *C. edulis*. The lower NO_3^- availability of invaded soils could explain their lower dissimilatory nitrate reduction (D_{NRA}, 0.04-0.70x) compared to native soils. Both D_{NRA} and O_{NH4} were more affected in the 0-5 cm layer, but the invasion also significantly affected N rates in the 5-10 cm layer. Overall, net nitrification and mineralization generally decreased in the invaded soils.

Invader effects on soils are largely mediated by its necromass, which has contrasting characteristics with the autochthonous necromass. *Carpobrotus edulis* ability to discriminate against Al-uptake, while favouring Mg and Ca uptake, and its lower requirement (or higher resorption) of key micronutrients (Co, Cu, Fe, Ni, Zn) than native vegetation could partly explain its invasiveness. This study also shows that the invasion of *C. edulis* alters soil gross and net N fluxes in a 0-10 cm depth through its effects on soil properties and microbiota.

SM Table 3.1a. Raw and standardized [(I-A)/A] values (mean ± s.e), *t*-test value and significance (* P <0.05; ** P <0.01; *** P <0.001) of the statistical analysis for the studied soil properties under autochtonous (A) and invasive (I) vegetation, globally (d.f.=7). Data are given in the following units: accumulated necromass, kg m^{-2}; water holding capacity (WHC), g H_2O kg^{-1} dw; humidity, %; electrical conductivity (EC), mS cm^{-1}; organic C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; $\delta\ ^{15}N$ and $\delta\ ^{13}C$, ‰; and other elements, mg kg^{-1} dw.

	Globally			
	A	I	(I-A)/A	*t*-value
Necromass	1.38 ± 0.25	4.52 ± 1.12	2.38 ± 0.46	3.39*
WHC	306 ± 70	287 ± 65	-0.09 ± 0.06	1.15
Humidity	13.5 ± 3.0	13.5 ± 3.6	-0.12 ± 0.10	1.23
pH_{H2O}	6.65 ± 0.28	6.36 ± 0.56	-0.05 ± 0.05	1.00
pH_{KCl}	4.73 ± 0.41	4.47 ± 0.72	-0.09 ± 0.08	1.13
EC	114 ± 29	145 ± 47	0.20 ± 0.20	1.01
Total N	3.53 ± 0.96	3.45 ± 1.10	-0.13 ± 0.10	1.30
$\delta\ ^{15}N$	3.64 ± 1.01	3.95 ± 0.93	1.39 ± 1.34	1.04
NH_4^+-N	2.45 ± 0.64	5.41 ± 2.68	0.66 ± 0.47	1.40
NO_3^--N	13.4 ± 3.3	4.16 ± 0.90	-0.65 ± 0.03	20.30***
Organic C	61.6 ± 16.1	60.9 ± 17.9	-0.12 ± 0.11	1.12
$\delta\ ^{13}C$	-27.1 ± 0.3	-27.1 ± 0.2	0.00 ± 0.01	0.19
C/N	18.8 ± 1.3	18.7 ± 1.8	-0.01 ± 0.05	0.13
Available Al	33.7 ± 10.4	44.9 ± 18.7	0.07 ± 0.22	0.33
Available B	0.089 ± 0.018	0.084 ± 0.016	0.29 ± 0.36	4.73**
Available Ca	1442 ± 352	1429 ± 424	-0.06 ± 0.13	0.44
Available Co	0.049 ± 0.012	0.007 ± 0.003	-0.66 ± 0.19	3.38*
Available Cu	0.578 ± 0.195	0.334 ± 0.106	-0.31 ± 0.07	4.40**
Available Fe	167 ± 68	224 ± 98	0.07 ± 0.17	0.40
Available K	80.6 ± 22.9	87.3 ± 29.8	-0.03 ± 0.11	0.25
Available Mg	294 ± 91	176 ± 51	-0.32 ± 0.07	4.61**
Available Mn	8.23 ± 2.57	6.49 ± 2.75	-0.19 ± 0.10	1.84
Available Mo	0.093 ± 0.036	0.104 ± 0.042	553 ± 553	-0.67
Available Na	219 ± 75	190 ± 67	-0.12 ± 0.08	1.63
Available Ni	0.119 ± 0.037	0.144 ± 0.039	79.1 ± 78.8	1.53
Available P	4.93 ± 0.68	4.89 ± 0.94	-0.05 ± 0.07	0.76
Available Zn	8.12 ± 3.40	3.37 ± 1.61	-0.41 ± 0.11	3.78**

SM Table 3.1b. Raw and standardized [(I-A)/A] values (mean ± s.e), *t*-test value and significance (* P <0.05; ** P <0.01; *** P <0.001) of the statistical analysis for the studied soil properties under autochtonous (A) and invasive (I) vegetation, separately for dunes (d.f.= 3). Data are given in the following units: accumulated necromass, kg m^{-2}; water holding capacity (WHC), g H_2O kg^{-1} dw; humidity, %; electrical conductivity (EC), mS cm^{-1}; organic C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; $\delta^{15}N$ and $\delta^{13}C$, ‰; and other elements, mg kg^{-1} dw.

	Dune			
	A	I	(I-A)/A	*t*-value
Necromass	0.864 ± 0.322	1.66 ± 0.13	1.88 ± 0.86	2.20
WHC	148 ± 52	155 ± 69	-0.08 ± 0.13	0.66
Humidity	15.3 ± 5.1	16.9 ± 6.09	-0.27 ± 0.15	0.00
pH_{H2O}	7.16 ± 0.40	7.74 ± 0.35	0.08 ± 0.01	6.56**
pH_{KCl}	5.50 ± 0.60	6.10 ± 0.74	0.11 ± 0.03	3.12
EC	54.5 ± 27.0	28.6 ± 10.6	-0.15 ± 0.25	0.60
Total N	1.47 ± 0.64	0.915 ± 0.434	-0.37 ± 0.05	6.92**
$\delta^{15}N$	1.76 ± 1.25	2.46 ± 1.38	2.79 ± 2.65	1.05
NH_4^+-N	1.28 ± 0.18	0.776 ± 0.090	-0.38 ± 0.07	5.55*
NO_3^--N	10.8 ± 4.8	4.23 ± 1.79	-0.60 ± 0.01	64.21***
Organic C	30.6 ± 15.3	20.2 ± 11.3	-0.38 ± 0.06	6.69**
$\delta^{13}C$	-27.0 ± 0.5	-27.2 ± 0.3	0.01 ± 0.03	0.40
C/N	21.2 ± 2.2	20.4 ± 3.6	-0.05 ± 0.10	0.51
Available Al	10.8 ± 4.8	4.15 ± 0.97	-0.36 ± 0.20	1.83
Available B	0.056 ± 0.019	0.064 ± 0.005	0.77 ± 0.67	3.55*
Available Ca	2158 ± 429	2323 ± 535	0.06 ± 0.16	0.36
Available Co	0.076 ± 0.009	0.005 ± 0.002	-0.93 ± 0.02	38.39***
Available Cu	0.75 ± 0.36	0.403 ± 0.194	-0.29 ± 0.15	1.90
Available Fe	40.1 ± 19.2	22.1 ± 9.7	-0.36 ± 0.07	4.98*
Available K	32.8 ± 13.2	22.3 ± 10.3	-0.24 ± 0.13	1.84
Available Mg	154 ± 70	93.1 ± 47.1	-0.29 ± 0.14	2.03
Available Mn	9.48 ± 4.62	9.64 ± 5.24	-0.06 ± 0.06	1.03
Available Mo	0.004 ± 0.004	0.011 ± 0.011	1106 ± 1107	-
Available Na	49.2 ± 12.7	40.5 ± 10.8	-0.12 ± 0.16	0.73
Available Ni	0.13 ± 0.07	0.124 ± 0.070	157 ± 158	0.79
Available P	3.85 ± 0.46	3.19 ± 0.58	-0.19 ± 0.10	1.77
Available Zn	11.5 ± 6.6	4.60 ± 3.26	-0.38 ± 0.19	1.94

SM Table 3.1c. Raw and standardized [(I-A)/A] values (mean ± s.e), *t*-test value and significance (* P <0.05; ** P <0.01; *** P <0.001) of the statistical analysis for the studied soil properties under autochtonous (A) and invasive (I) vegetation, separately for rocky sites (d.f.= 3). Data are given in the following units: accumulated necromass, kg m^{-2}; water holding capacity (WHC), g H_2O kg^{-1} dw; humidity, %; electrical conductivity (EC), mS cm^{-1}; organic C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; δ ^{15}N and δ ^{13}C, ‰; and other elements, mg kg^{-1} dw.

	Rock			
	A	I	(I-A)/A	*t*-value
Necromass	1.9 ± 0.0	7.37 ± 0.61	2.88 ± 0.29	9.81**
WHC	465 ± 60	420 ± 59	-0.10 ± 0.03	2.85
Humidity	11.8 ± 3.72	10.1 ± 3.7	0.03 ± 0.10	2.40
pH_{H2O}	6.14 ± 0.21	4.98 ± 0.27	-0.19 ± 0.02	7.72**
pH_{KCl}	3.96 ± 0.15	2.83 ± 0.25	-0.29 ± 0.06	5.08*
EC	174 ± 30	261 ± 37	0.55 ± 0.20	2.70
Total N	5.60 ± 1.04	5.99 ± 1.07	0.10 ± 0.11	0.90
δ ^{15}N	5.52 ± 0.93	5.44 ± 0.81	0.00 ± 0.06	0.01
NH_4^+-N	3.62 ± 0.98	10.0 ± 4.4	1.69 ± 0.56	3.01
NO_3^--N	16.0 ± 4.8	4.1 ± 0.8	-0.70 ± 0.05	13.24**
Organic C	92.5 ± 17.9	101 ± 16	0.14 ± 0.10	0.95
δ ^{13}C	-27.1 ± 0.4	-27.0 ± 0.3	-0.01 ± 0.01	1.95
C/N	16.4 ± 0.3	17.1 ± 0.7	0.04 ± 0.03	4.44
Available Al	56.6 ± 11.6	85.6 ± 23.0	0.50 ± 0.26	0.81
Available B	0.122 ± 0.019	0.103 ± 0.029	-0.20 ± 0.13	1.08*
Available Ca	726 ± 226	536 ± 156	-0.17 ± 0.22	32.83
Available Co	0.021 ± 0.009	0.009 ± 0.005	-0.38 ± 0.35	6.59
Available Cu	0.406 ± 0.166	0.265 ± 0.108	-0.34 ± 0.01	1.65***
Available Fe	293 ± 103	427 ± 132	0.50 ± 0.08	7.47**
Available K	128 ± 27	152 ± 35	0.19 ± 0.11	1.69
Available Mg	432 ± 145	259 ± 71	-0.36 ± 0.05	0.36**
Available Mn	6.98 ± 2.93	3.35 ± 1.10	-0.32 ± 0.19	3.31
Available Mo	0.182 ± 0.030	0.198 ± 0.046	0.05 ± 0.14	5.04
Available Na	388 ± 84	339 ± 76	-0.13 ± 0.04	2.02*
Available Ni	0.108 ± 0.029	0.163 ± 0.040	0.56 ± 0.11	3.45*
Available P	6.01 ± 1.07	6.59 ± 1.35	0.08 ± 0.04	2.02
Available Zn	4.79 ± 1.85	2.18 ± 0.71	-0.45 ± 0.13	3.45*

SM Table 3.2a. Raw and standardized [(I-A)/A] values (mean ± s.e), *t*-test value and significance (* P <0.05; ** P <0.01; *** P <0.001) of the statistical analysis for the studied soil properties under autochtonous (A) and invasive (I) vegetation, for the soil 0-5 cm layer (d.f.= 3). Data are given in the following units: water holding capacity (WHC), g H_2O kg^{-1} dw; humidity, %; electrical conductivity (EC), mS cm^{-1}; organic C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; δ ^{15}N and δ ^{13}C, ‰; and other elements, mg kg^{-1} dw.

	0 - 5 cm soil layer			
	A	I	(I-A)/A	*t*-value
WHC	369 ± 122	351 ± 112	-0.07 ± 0.11	0.66
Humidity	12.1 ± 5.8	10.7 ± 5.6	0.00 ± 0.16	2.39
pH_{H2O}	6.60 ± 0.35	6.26 ± 0.83	-0.06 ± 0.09	0.65
pH_{KCl}	4.70 ± 0.45	4.56 ± 0.96	-0.05 ± 0.13	0.41
EC	130 ± 52	174 ± 83	0.33 ± 0.32	1.03
Total N	4.60 ± 1.68	4.38 ± 1.93	-0.17 ± 0.12	1.36
δ ^{15}N	3.40 ± 1.68	3.02 ± 1.77	0.07 ± 0.15	0.45
NH_4^+-N	2.77 ± 1.17	8.04 ± 5.26	1.02 ± 0.84	1.21
NO_3^--N	17.8 ± 5.4	4.89 ± 1.59	-0.70 ± 0.05	13.21**
Organic C	81.3 ± 27.3	78.0 ± 31.0	-0.18 ± 0.14	1.27
δ ^{13}C	-27.5 ± 0.4	-27.5 ± 0.3	0.00 ± 0.03	0.09
C/N	17.8 ± 1.7	17.7 ± 2.7	-0.02 ± 0.07	0.27
Available Al	26.1 ± 9.5	37.6 ± 22.0	0.17 ± 0.39	0.44
Available B	0.101 ± 0.033	0.105 ± 0.028	0.53 ± 0.67	3.40*
Available Ca	1600 ± 379	1661 ± 536	0.02 ± 0.21	0.08
Available Co	0.050 ± 0.023	0.006 ± 0.004	-0.74 ± 0.23	3.24*
Available Cu	0.684 ± 0.310	0.444 ± 0.191	-0.32 ± 0.04	8.78**
Available Fe	205 ± 128	261 ± 173	0.06 ± 0.23	0.25
Available K	107 ± 41	121 ± 53	0.03 ± 0.15	0.22
Available Mg	422 ± 160	258 ± 83	-0.33 ± 0.06	5.72*
Available Mn	12.3 ± 3.9	9.21 ± 5.13	-0.32 ± 0.17	1.82
Available Mo	0.108 ± 0.063	0.110 ± 0.070	-0.23 ± 0.26	-0.90
Available Na	291 ± 138	261 ± 121	-0.06 ± 0.06	0.93
Available Ni	0.142 ± 0.059	0.155 ± 0.058	0.06 ± 0.23	0.27
Available P	5.69 ± 0.91	5.67 ± 1.33	-0.03 ± 0.08	0.40
Available Zn	10.5 ± 5.6	5.31 ± 3.01	-0.46 ± 0.08	5.44*

SM Table 3.2b. Raw and standardized [(I-A)/A] values (mean ± s.e), *t*-test value and significance (* P <0.05; ** P <0.01; *** P <0.001) of the statistical analysis for the studied soil properties under autochtonous (A) and invasive (I) vegetation, for the soil 5-10 cm layer (d.f.= 3). Data are given in the following units: water holding capacity (WHC), g H_2O kg^{-1} dw; humidity, %; electrical conductivity (EC), mS cm^{-1}; organic C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; $\delta^{15}N$ and $\delta^{13}C$, ‰; and other elements, mg kg^{-1} dw.

	5 - 10 cm soil layer			
	A	I	(I-A)/A	*t*-value
WHC	244 ± 74	224 ± 68	-0.11 ± 0.07	1.48
Humidity	14.9 ± 2.7	16.2 ± 4.7	-0.25 ± 0.10	0.21
pH_{H2O}	6.69 ± 0.50	6.46 ± 0.88	-0.05 ± 0.07	0.68
pH_{KCl}	4.76 ± 0.75	4.37 ± 1.21	-0.13 ± 0.11	1.13
EC	97.8 ± 33.9	116 ± 56	0.07 ± 0.26	0.27
Total N	2.47 ± 0.86	2.52 ± 1.13	-0.10 ± 0.19	0.56
$\delta^{15}N$	3.88 ± 1.39	4.89 ± 0.56	2.72 ± 2.67	1.02
NH_4^+-N	2.13 ± 0.67	2.82 ± 1.13	0.30 ± 0.50	0.61
NO_3^--N	9.00 ± 2.83	3.43 ± 0.95	-0.60 ± 0.02	28.90***
Organic C	41.8 ± 13.7	43.8 ± 18.4	-0.07 ± 0.19	0.23
$\delta^{13}C$	-26.7 ± 0.3	-26.7 ± 0.1	0.00 ± 0.01	0.10
C/N	19.9 ± 2.2	19.8 ± 2.7	0.01 ± 0.09	3.03
Available Al	41.3 ± 19.5	52.2 ± 33.4	-0.03 ± 0.26	0.72
Available B	0.077 ± 0.017	0.062 ± 0.008	0.04 ± 0.37	1.66
Available Ca	1284 ± 646	1199 ± 720	-0.13 ± 0.18	2.09
Available Co	0.048 ± 0.012	0.009 ± 0.004	-0.57 ± 0.35	0.28
Available Cu	0.472 ± 0.271	0.224 ± 0.089	-0.31 ± 0.15	0.49
Available Fe	129 ± 66	187 ± 117	0.08 ± 0.28	2.27
Available K	54.5 ± 17.8	53.8 ± 24.5	-0.09 ± 0.19	0.67
Available Mg	165 ± 46	94.9 ± 26.6	-0.32 ± 0.14	0.21
Available Mn	4.15 ± 2.20	3.78 ± 2.01	-0.07 ± 0.10	1.35
Available Mo	0.078 ± 0.046	0.099 ± 0.056	1107 ± 1107	2.75
Available Na	147 ± 60	120 ± 53	-0.19 ± 0.14	0.58
Available Ni	0.096 ± 0.051	0.133 ± 0.059	158 ± 157	1.70
Available P	4.17 ± 0.96	4.11 ± 1.38	-0.08 ± 0.13	0.58
Available Zn	5.70 ± 4.35	1.44 ± 0.73	-0.37 ± 0.22	1.70

SM Table 3.3.a. Factor loadings of the rotated component matrix from the principal component analysis (PCAs) performed with soil data for each locality and deepness. Note: WHC, water holding capacity; EC, electrical conductivity.

	Component	
	1	2
pH_{H2O}	-0.398	-0.837
WHC	0.849	0.447
EC	0.648	0.687
Total N	0.803	0.582
NH_4^+-N	0.459	0.672
Organic C	0.839	0.497
Available Al	0.101	0.951
Available B	0.705	0.345
Available Fe	0.480	0.835
Available K	0.857	0.405
Available Mg	0.934	0.133
Available Mo	0.535	0.800
Available P	0.435	0.761

SM Table 3.3.b. Factor loadings of the rotated component matrix from the principal component analysis (PCA) performed with necromass data for each locality and deepness.

	Component	
	1	2
Total Al	0.966	-0.072
Total Co	0.696	-0.418
Total Cu	0.870	-0.422
Total Fe	0.901	0.031
Total Mg	-0.004	0.992
Total Ni	0.827	-0.078
Total Zn	0.897	0.031

SM Table 3.4. Mean values ± standard error for the studied necromass properties under autochthonous (A) and invasive (I) vegetation, globally (d.f.=3) and separately for dunes and rocky sites (d.f.=1), and *t*-test value and significance (* P <0.05; ** P <0.01;*** P <0.001) of the correspondent statistical analysis. Data are given in the following units: total C and N, g kg^{-1} dw; C/N ratio, g g^{-1}; $\delta^{15}N$ and $\delta^{13}C$, ‰; and other elements, mg kg^{-1} dw.

	Globally			Dune			Rock		
	A	I	*t*-value	A	I	*t*-value	A	I	*t*-value
Total N	12.2 ± 1.4	9.51 ± 2.00	1.65	10.6 ± 1.9	6.37 ± 1.00	4.95	13.9 ± 1.5	12.6 ± 1.8	0.37
$\delta^{15}N$	2.00 ± 2.54	3.49 ± 1.76	0.98	-0.99 ± 2.08	1.83 ± 1.09	0.89	4.99 ± 4.06	5.14 ± 3.45	0.26
Total C	414 ± 26	384 ± 7	1.4	394 ± 1	377 ± 10	2.14	433 ± 58	391 ± 8	0.83
$\delta^{13}C$	-27.5 ± 1.2	-27.9 ± 0.3	0.01	-26.4 ± 2.5	-28.3 ± 0.1	0.57	-28.5 ± 0.5	-27.5 ± 0.2	2.94
C/N	34.8 ± 3.5	46.3 ± 9.8	1.70	38.4 ± 6.9	60.8 ± 11.0	5.35	31.1 ± 0.8	31.7 ± 5.2	0.13
Al	2138 ± 582	1304 ± 332	3.13	2335 ± 1398	1368 ± 792	1.60	1940 ± 12	1239 ± 167	4.51
B	15.0 ± 4.9	26.0 ± 3.8	3.47 *	8.79 ± 1.06	19.5 ± 0.3	13.95 *	21.1 ± 8.2	32.5 ± 0.5	1.47
Ca	7158 ± 1183	29533 ± 6441	4.21 *	9024 ± 644	40560 ± 1331	45.96 *	5293 ± 1012	18506 ± 1979	13.66 *
Co	0.536 ± 0.060	0.501 ± 0.149	0.35	0.588 ± 0.094	0.623 ± 0.318	0.16	0.484 ± 0.085	0.378 ± 0.046	2.72
Cu	8.43 ± 2.12	3.67 ± 1.40	6.37 **	10.8 ± 4.0	5.09 ± 2.79	4.72	6.08 ± 0.31	2.24 ± 0.11	9.03
Fe	1164 ± 281	698 ± 165	3.73 *	1228 ± 603	771 ± 373	1.99	1100 ± 317	625 ± 114	2.35
K	1531 ± 135	1641 ± 219	0.45	1638 ± 266	1323 ± 280	22.72 *	1424 ± 125	1960 ± 87	14.14 *
Mg	2122 ± 362	2690 ± 521	2.11	1674 ± 222	1832 ± 283	2.59	2570 ± 580	3549 ± 270	3.17
Mn	116 ± 23	213 ± 91	1.65	118 ± 57	295 ± 192	1.72	114 ± 0	131 ± 3	6.57
Mo	3.67 ± 0.95	2.12 ± 0.51	2.72	3.63 ± 2.28	1.68 ± 1.09	1.63	3.71 ± 0.43	2.56 ± 0.03	2.52
Na	1085 ± 251	1991 ± 309	4.61 *	699 ± 184	1470 ± 24	3.72	1471 ± 215	2512 ± 176	2.67
Ni	2.55 ± 0.54	1.90 ± 0.23	1.12	2.95 ± 1.03	1.70 ± 0.37	1.90	2.15 ± 0.59	2.11 ± 0.33	0.05
P	749 ± 61	571 ± 82	2.73	815 ± 51	540 ± 107	4.96	682 ± 104	601 ± 164	1.37
Zn	61.3 ± 18.2	64.5 ± 20.8	0.43	74.2 ± 40.5	67.7 ± 50.7	0.63	48.4 ± 3.4	61.3 ± 3.4	1.90

SM Table 3.5. Mean values ±standard error for the studied necromass properties under autochthonous (A) and invasive (I) vegetation, globally (d.f.=3) and separately for dunes and rocky sites (d.f.=1), and *t*-test value and significance (*** P <0.001; ** P <0.01; * P <0.05) of the correspondent statistical analysis. Except for total N and C (g m^{-2}), all data are expressed in mg m^{-2}.

	Globally			Dune			Rock		
	A	I	*t*-value	A	I	*t*-value	A	I	*t*-value
Total N	17.2 ± 5.6	52.7 ± 26.9	1.56	8.11 ± 4.31	10.3 ± 0.4	0.55	26.3 ± 2.6	95.2 ± 26.9	2.34
Total C	581 ± 170	1753 ± 668	2.15	341 ± 221	628 ± 139	3.49	821 ± 100	2877 ± 357	4.5
Al	2459 ± 706	5487 ± 2037	2.23	1237 ± 96	2012 ± 867	0.80	3680 ± 24	8961 ± 77	99.90 **
B	24.2 ± 11.6	136 ± 62	2.18	8.18 ± 5.81	32.4 ± 6.8	23.75 *	40.3 ± 16.1	240 ± 38	9.18
Ca	9108 ± 2492	103157 ± 25547	3.89 *	8152 ± 5590	67741 ± 15442	6.05	10065 ± 2047	138574 ± 34189	4.00
Co	0.686 ± 0.178	1.83 ± 0.54	2.49	0.456 ± 0.247	0.930 ± 0.325	0.83	0.916 ± 0.150	2.74 ± 0.06	8.75
Cu	9.30 ± 1.67	12.1 ± 3.2	0.92	7.08 ± 2.56	7.53 ± 2.97	0.08	11.5 ± 0.4	16.7 ± 3.2	1.41
Fe	1409 ± 476	2943 ± 1209	2.02	725 ± 165	1158 ± 368	0.81	2094 ± 627	4727 ± 1504	3.00
K	1983 ± 506	8232 ± 3585	1.95	1266 ± 684	2105 ± 33	1.17	2699 ± 203	14359 ± 1432	7.13
Mg	3105 ± 1173	14702 ± 7181	1.91	1322 ± 742	2948 ± 128	2.65	4889 ± 1161	26457 ± 5750	4.70
Mn	143 ± 43	695 ± 186	3.53 *	70.1 ± 16.6	426 ± 222	1.49	217 ± 3	963 ± 118	6.43
Mo	4.46 ± 1.54	10.6 ± 4.9	1.85	1.86 ± 0.05	2.43 ± 1.26	0.44	7.06 ± 0.90	18.9 ± 2.5	7.44
Na	1648 ± 693	10395 ± 4627	2.22	501 ± 231	2447 ± 519	6.77	2795 ± 443	18343 ± 1365	16.88 *
Ni	3.02 ± 0.81	9.30 ± 4.27	1.57	1.98 ± 0.76	2.69 ± 0.06	0.88	4.07 ± 1.07	15.9 ± 4.7	2.06
P	986 ± 261	2735 ± 1318	1.54	676 ± 411	861 ± 1	0.45	1297 ± 214	4609 ± 1844	2.03
Zn	66.6 ± 14.9	276 ± 113	2.06	41.5 ± 6.4	95.8 ± 62.1	0.79	91.7 ± 5.3	456 ± 90	3.81

3.6. REFERENCES

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4. Biocontrol potential of *Sclerotinia sclerotiorum* and *Pulvinariella mesembryanthemi* on *Carpobrotus edulis*

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4. Biocontrol potential of *Sclerotinia sclerotiorum* and *Pulvinariella mesembryanthemi* on *Carpobrotus edulis*

4.1. INTRODUCTION

As explained in Chapter 1, the ice plant *Carpobrotus edulis* is a highly invasive plant which engineers the invaded ecosystem, altering abiotic and biotic factors on its favour (Conser and Connor, 2009, Molinari et al., 2007). The extensive impacts on the invaded areas highlight the need of using effective control methods. Campaigns aimed at controlling and eradicating *Carpobrotus* sp. have been implemented across Europe in the last decades. The associated control costs have occasionally been quantified, amounting to almost €3 million in Spain between 2002 and 2007, around €2.5 million in Italy since 2010, and €167,000 in France between 2009-2013 (Campoy et al., in press, Scalera et al., 2017). Attempts to control *C. edulis* currently involve pulling out plants by hand or using herbicides (Delipetrou, 2006). However, these methods are quite expensive (Ruffino et al., 2015) and can negatively affect the invaded environment (usually dunes, which are vulnerable to erosion) and the native species, some of which are endemic or endangered (Carta et al., 2004). Biological control represents a promising, possibly effective and sustainable, alternative to the current control methods (Shaw et al., 2014). However, as far as we know, no previous studies have considered the potential use of biological agents to control *C. edulis*.

The invasive plant *C. edulis* is scarcely affected by predation or competition in the areas where it has been introduced (Maltez-Mouro et al., 2010). *Sclerotinia sclerotiorum* (Lib.) de Bary, a generalist fungus, has been observed to have negative effects on some other members of the Aizoaceae family (Saharan and Mehta, 2008), including *Carpobrotus glaucescens* (Haw.) Schwantes (Cother, 2000). However, its effectiveness to control plants of the Aizoaceae family has not yet been tested. Use of this fungus as a mycoherbicide on several invasive plants has been widely studied (Cother, 2000, De Jong et al., 1999, Green et al., 1998), usually with the mycelium (Green et al., 1998, Cother, 2000). The ascospores can be dispersed as far as 250 m, giving an idea of the safety zone for application (Bourdôt et al., 2001, De Jong et al., 2002).

Among the native predators of this plant, the South African scale insect *Pulvinariella mesembryanthemi* (Vallot) is a highly specific parasite of *Carpobrotus* spp. (Miller and Miller, 2003, Miller et al., 2005). Its potential value as a biological control agent has been reported by several authors (Washburn and Frankie, 1985, Fagúndez and Beiras, 2007). However, to the best of our knowledge, no studies have evaluated this potential yet. This insect may have been introduced accidentally with infested plants outside its native range, as it is currently found in different coastal temperate regions of Europe, Northern and Southern America, Northern Africa and Oceania (Cebeci and Selmi, 2004, Granara de Willink and Claps, 2003, Gómez-Menor Ortega, 1954). When present in high numbers, *P. mesembryanthemi* can cause considerable damage to *C. edulis*, as observed in plantations in California (Donaldson et al., 1978, Washburn and Frankie, 1985). It is exclusively parthenogenic (the males that are occasionally produced do not reproduce) and can be univoltine or bivoltine, depending on the environmental conditions (Washburn and Frankie, 1985). Active dispersion of *P. mesembryanthemi* is limited to the first stages of their life cycle, and long-distance dispersion is mediated by wind (Washburn and Frankie, 1981).

Synergistic effects between biocontrol agents are scarcely observed (Xu et al., 2011). However, studying the combined effects of different potential biocontrol agents is important. Different organisms can interact directly or indirectly, with different net effects on the target plants (Morris et al., 2007, Campanella et al., 2009). In the case of *C. edulis*, the combination of *P. mesembryanthemi* and *S. sclerotiorum* appears promising, as each species targets different parts of the plant: thus, the insect prefers to attack younger leaves (Washburn and Frankie, 1985), while the fungus appears to infect more successfully the older leaves (*pers. obs.*).

Invasive plants can undergo rapid adaptive evolution in the new environments, thus altering the interactions they have with antagonists (Müller-Schärer et al., 2004, Prentis et al., 2008). The susceptibility of the plant to the biocontrol agents may depend on the plant genotype, which may vary between the invaded area and the native area (Paterson et al., 2009, Hinz and Schwarzlaender, 2004). In *C. edulis*, there are some evidences of differences between native plants from South Africa and introduced plants in the Iberian Peninsula regarding division of labour capacity (Roiloa et al., 2016). These differences may be due to an

evolutionary adaptation of the plant to the invaded area (Roiloa et al., 2016).

The aim of this study was to test the individual and combined effects of two potential biocontrol agents - the insect *P. mesembryanthemi* and the fungus *S. sclerotiorum* - on *C. edulis* plants from the native area of distribution and from invaded areas. We hypothesise that the biocontrol capacity of both biological agents together will be greater than that of each individual as a result of synergistic effects. Likewise, we predicted that *C. edulis* plants from the invaded areas will be more sensitive to both agents as these plants are unlikely to have previously encountered the pathogens. To this end, we evaluated the effects on short-term (physiological indexes) and long-term (survival, growth, biomass allocation) estimators of plant performance commonly used as indicators of fitness (Traveset et al., 2008). To our knowledge, this is the first study investigating the biological control of *C. edulis*.

4.2. Material and Methods

4.2.1. Plant material

Samples of plants from four native populations of *C. edulis* growing in the Cape Region (South Africa) and another four invasive populations in NW Iberia (Table 4.1), all growing in coastal dune systems, were used in the study. In August 2015, 24 uniformly-sized ramets with 2-3 nodes were removed from the stock plants in each population and transplanted into individual 5 L plastic pots containing a 1:1 mixture of sand and peat as substrate. The initial total fresh weight of the ramets used (measured on November 2015) was 16.13±0.42 g for native plants and 27.69±1.36 g for introduced plants. Plants were maintained in a greenhouse in Santiago de Compostela (Spain; 45.874° N; 8.559° W) under controlled conditions (22 ± 2 °C, natural photoperiod) and were watered regularly throughout the experiment to field capacity. Within each population, plants were randomly allocated to one of four treatments, which were equally represented (six plants per treatment): 'C', control (untreated) plants; 'S', plants inoculated with the fungus *S. sclerotiorum*; 'P', plants artificially infested with the insect *P. mesembryanthemi*; 'PS', plants treated with both *S. sclerotiorum* and *P. mesembryanthemi*. After the application of the treatments, the plants were randomly relocated 3 times during the experiment.

Table 4.1. Populations of the plant *C. edulis* used in this study. Information about the origin of the plants (native or introduced) and coordinates (in decimal degrees) is provided.

Origin	Country	Sampling location	Latitude	Longitude
Native	South Africa	Kleinmond	34.34˚ S	18.99˚ E
	South Africa	Hawston	34.40˚ S	19.12˚ E
	South Africa	Fish Hoek	34.15˚ S	18.44˚ E
	South Africa	Cape Point	34.36˚ S	18.50˚ E
Introduced	Portugal	Caminha	41.87˚ N	8.86˚ W
	Spain	O Grove	42.46˚ N	8.94˚ W
	Portugal	Viana do Castelo	41.68˚ N	8.83˚ W
	Spain	Viveiro	43.67˚ N	7.60˚ W

4.2.2. Fungal isolate and inoculation

The *S. sclerotiorum* isolate used in the study was extracted from *Brassica napus* L. growing in Salcedo, Pontevedra, Spain (42.255° N, 8.384° W, 20 m asl) and provided by the Misión Biológica de Galicia (CSIC).

Sclerotia were first grown on potato dextrose agar (PDA, Fluka) in Petri dishes (diameter 9 cm), and sub-cultures were established (also on PDA) from 1.5 cm diameter discs of fresh mycelial growth. In October 2016, plants were inoculated with 1.5 mm diameter discs of fresh mycelium from the sub-cultures. Each disc was placed in a recently cut leaf tip of the second node of the main ramet and was covered with a pipette tip to maintain humidity.

After one month, a second inoculation was made in the stem (in the first node of the ramet) to ensure the introduction of the fungus in the plants. To this end, a small cut was made in the stem, and fresh mycelium was inserted. The incision was covered with Parafilm® to maintain the humidity. Inoculated leaves that did not exhibit symptoms of infection were reinoculated with fresh mycelium.

At the end of the experiment, one year after the first inoculation, small slices of leaves were removed from the surviving inoculated plants to test for the presence of *S. sclerotiorum*. To isolate the fungus, the tissue was dipped in 2% bleach for approx. 5 seconds and rinsed with deionized water, before being transferred to potato dextrose agar (PDA) medium and incubated at room temperature (20-22 °C).

4.2.3. Insect source and infestation

Individual specimens of *P. mesembryanthemi* were collected from Punta Nariga (Spain, 43.230° N; 8.910° W) and bred in the greenhouse under controlled conditions (22 ± 2 °C, natural photoperiod). The mean number (±SE) of eggs produced by each female was 333 (± 12).

Two mature ovisacs were used to infest each plant in October 2016, by placing them on the first node and on a secondary node of the ramet, respectively.

After one month, plants were checked for live crawlers and settlers, and plants in which none of the ovisacs had hatched were infested with another two ovisacs. Only one of the plants (which died after 2.5 months) was not infested with *P. mesembryanthemi* after the second infestation.

4.2.4. Insect cycle

The number of insects on each life stage (instar 1 to 4 and ovisacs; as described by Washburn and Frankie (1985)) per plant was counted every 3-4 weeks. The life cycle of the insect for the treatments 'P' (*Pulvinariella*) and 'P+S' (*Pulvinariella* + *Sclerotinia*) and the different life stages were represented. Survival was also calculated for each date on which the number of insects was counted.

4.2.5. Physiological measures and visual rating

All ecophysiological measures were determined on fully developed and healthy leaves of the main ramet of each plant, before (- 1 day) and after (1week; 3 weeks; 1 month; 1.5 months and then monthly until 1 year) the first inoculation and infestation.

Leaf spectral reflectance (300-1100 nm) was measured with a portable spectrometer (UniSpec Spectral Analysis System; PP Systems, Haverhill, MA, USA). The following indexes were calculated from the reflectance data: (1) the photochemical reflectance index 'PRI' (R531-R570)/(R531+R570), where R is reflectance and the subscript refers to the wavelength in nanometers, which is inversely correlated with the zeaxanthin content (a photoprotective pigment) and directly correlated with photosynthetic radiation-use efficiency and net CO_2 uptake (Gamon et al., 1997 , Peñuelas et al., 1995a); (2) the chlorophyll index 'CHL-NDI' (R750-R705)/(R750+R705), which is directly proportional to the chlorophyll content of leaves (Richardson et al., 2002, Gitelson et al., 1996);

(3) the structural independent pigment index 'SIPI' (R800-R445)/(R800-R680), related to the carotenoid/chlorophyll ratio (Peñuelas and Inoue, 1999); (4) the water index 'WI' (R970/R900), used to estimate plant water concentration (Peñuelas et al., 1997); and (5) the normalised phaeophytinization index NPQI (R415-R435)/(R415+R435), indicative of chlorophyll degradation to phaeophytin (Barnes et al., 1992).

Chlorophyll fluorescence parameters were measured by the saturation pulse method (Screiber et al. 1998) with a portable pulse-amplitude-modulated fluorometer (MINI-PAM photosynthesis yield analyser; Walz, Effeltrich, Germany). The leaves were allowed to adapt to darkness for 30 minutes, and the maximum quantum yield of photosystem II (F_v/F_m) was then estimated as the ratio $(F_m-F_0)/F_m$, where F_m and F_0 are respectively the maximal and minimal fluorescence yield. F_v/F_m is indicative of the photosynthetic performance of plants (Schreiber et al., 1998) and is correlated with the amount of carbon gained per unit of light absorbed (Bolhàr-Nordenkampf and Öquist, 1993), as well as with photoinhibition of photosynthesis and zeaxanthin production (Baker, 2008, Falbel et al., 1994, Leverenz et al., 1992).

In addition, on the same dates that the reflectance and fluorescence measurements were made, the plant performance was assessed using a visual rating based on a 1-9 scale, where 1 indicates a perfectly healthy plant and 9 a dead plant.

4.2.6. Growth rate and dry mass allocation

At the end of the experiment, plants were oven-dried at 60 ^{o}C to constant weight to enable the determination of the dry weight of the aerial part (leaves + shoots) and for the roots. The root/aerial biomass ratio was then calculated. A randomly-selected sample of fresh plants was weighted, immediately after being removed from their pots and then after drying to enable calculation of the water content of the plants.

The shoot length was also measured, and nodes and leaves were counted both at the beginning and at the end of the experiment, as estimators of plant growth. The increase in the number of nodes (points of leaf insertion in the shoots) has been found to be closely correlated with growth of *C. edulis* (Traveset et al., 2008). The internodal distance was estimated as the total shoot length of the plant divided by the number of nodes.

4.2.7. Statistical analysis

All analyses were performed with R statistical software (*https://www.r-project.org/*).

Differences in leaf spectral reflectance and fluorescence indexes were analysed by linear mixed models via restricted maximum likelihood (REML) and post hoc tests, using the 'lme4', 'lsmeans' and 'multcomp' packages. The inclusion of random factors in the model (population, origin or subject) was conditioned by the AIC values (Akaike, 1974). The random effect of population nested within origin was therefore never included, while the subject always improved the model. When the analysis of variance (ANOVA) test indicated a significant difference, the following were included as fixed factors in the model (following the minimal adequate model): treatment ('C', control plants; 'S', *S. sclerotiorum* inoculated plants; 'P', *P. mesembryanthemi* infested plants; 'PS', plants treated with both *S. sclerotiorum* and *P. mesembryanthemi*), date, origin (native or non-native populations) and their interactions. The assumptions of linearity, homoscedasticity and normality were checked by plotting the model residuals. Only measurements made after the application of the treatments (from 1 week to 12 months) were used in the analysis.

A negative binomial mixed model was applied for visual rating and for the number of insects on each plant (total and per life stage: instars 1st-4th and ovisacs), with subject as a random factor and treatment or origin as fixed factors. The significance of the factors was checked with the Wald test. For those factors indicated to be significant, post-hoc tests were conducted. The analyses were done using the 'R2admb', 'glmmADMB', 'survey' and 'multcomp' packages.

A general lineal model was used to analyse insect survival and variables related to plant growth and biomass (growth in shoot length, increase in number of nodes, increase in number of leaves, final dry aerial biomass, final dry root biomass, root/aerial biomass rate, water content), with treatment, origin and their interaction as fixed factors. The assumptions of the model were checked, and when not met, the Tukey's ladder of powers was applied to the variables (i.e. the square root was applied to root biomass; the decimal logarithm to the root: aerial biomass rate; the square and cube were applied to some of the dates in the survival analysis).

The non-parametric Kaplan-Meier procedure (Kaplan and Meier, 1958) was used to construct the plant survival curves. To study the relative effect of the treatments, plant origin and their interaction on plant survival, a Cox proportional hazards regression model (Cox, 1972) was used after checking the assumption of proportional hazard (Grambsch and Therneau, 1994). The log rank test (Harrington and Fleming, 1982) was used to check the homogeneity of survival functions between treatments. For pairwise comparisons, the log-rank test was applied, with Bonferroni correction (Bonferroni, 1936). Survival analyses and representations were conducted using the 'survival' and 'survminer' packages.

Correlations between variables were tested using the Pearson's product moment correlation coefficient.

4.3. RESULTS

4.3.1. Visual observations and recovery of fungus

At the end of the experiment (1 year), the surviving plants of the initial 48 per treatment were: 37 fungus-infected plants ('S'), 17 insect-infested plants ('P') and 18 plants treated with both agents ('PS'). Within the surviving plants, visual examination revealed death of some plant parts in 3 fungus-infected plants ('S'), in 9 insect-infested plants ('P') and in 4 plants treated with both agents ('PS'). At the end of the experiment, most of the insect-infested plants (i.e. 'P' and 'PS' treatments) were found to be affected by black mould (82 and 83 % of the plants, respectively). The inoculated fungus was only recovered from a few plants one year after the first inoculation.

4.3.2. Insect life cycle and survival

The insect underwent two complete life cycles and one partial cycle during the experiment, and the duration of the life cycle under the particular greenhouse conditions was 4-6 months (Fig. 4.1). The origin of the plant (i.e. from native or introduced populations) and the treatment ('P' or 'P+S') did not seem to affect the duration of the life cycle (Fig. 4.1). Some male specimens of the insect were occasionally found on a few plants.

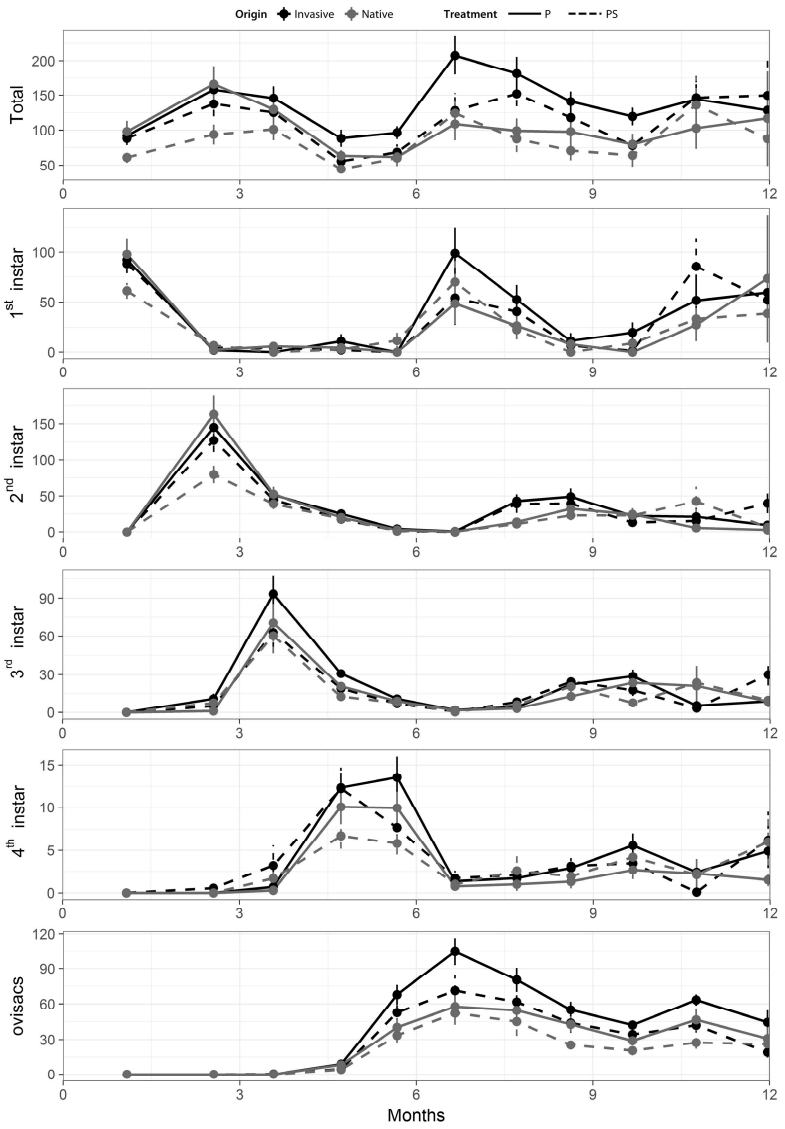

Fig. 4.1. Mean number ± SE of *P. mesembryanthemi* insects per plant throughout the year of the experiment (October 2016 - October 2017). The different graphs represent the total number of insects, the insects at the different growth stages (1st, 2nd, 3rd, 4th instars and ovisacs). Black and grey lines represent respectively plants from invasive and native populations. Solid lines represent treatment 'P' (plants infested with the insect) and dashed lines represent treatment 'PS' (plants infested with the insect and inoculated with the fungus).

The total numbers of *P. mesembryanthemi*, as well as the number of insects on 1st, 2nd and 3rd instar and ovisacs per plant, were significantly higher in insect-only infested plants ('P') than in plants in which both agents were combined ('PS') (Table 4.2). Significantly greater total numbers of insects per plant, as well as of 2nd and 4th instar and of ovisacs, were found on the introduced plants than on native plants (Table 4.2). Survival of the insect on the plants did not vary depending on the treatment ('P', 'PS') or the origin of the plant ('N', 'I'), and the interaction Treatment*Origin was only significant after 10 months ($F_{1,42}$=4.80, p =0.034; non-significant post-hoc interactions, p >0.05).

Table 4.2. Results of the Wald test for the mixed negative binomial models of the number of *P. mesembryanthemi* insects (total and for each of the different stages: 1st to 4th instars and ovisacs) counted on the *C. edulis* plants in the experiment, with treatment and plant origin as fixed factors. The table includes the numerator (nDF) and denominator (dDF) degrees of freedom, the F-value and p-value of the tests, and the mean ± SE for the total number of insects counted throughout the experiment per plant, for each treatment (P, plants infested with the insect; PS, plants both infested with the insect and inoculated with the fungus) and plant origin (native, introduced). Significant differences (at p ≤0.05) across treatments or origins are indicated by different letters.

	Treatment						Plant origin					
	nDF	dDF	F	p	P	PS	nDF	dDF	F	p	Native	Introduced
Total	1	78	7.22	0.009	1108 ± 81^{a}	824 ± 90^{b}	1	78	7.63	0.007	749 ± 61^{b}	1183 ± 92^{a}
1st	1	77	3.99	0.049	282 ± 28^{a}	204 ± 24^{b}	1	77	3.33	0.071	198 ± 23^{a}	290 ± 28^{a}
2nd	1	78	7.45	0.008	322 ± 26^{a}	236 ± 22^{b}	1	78	4.98	0.028	236 ± 22^{b}	322 ± 26^{a}
3rd	1	78	4.11	0.046	172 ± 14^{a}	129 ± 15^{b}	1	78	3.25	0.075	125 ± 13^{a}	176 ± 16^{a}
4th	1	78	1.49	0.227	33 ± 3^{a}	27 ± 3^{a}	1	78	9.18	0.003	23 ± 3^{b}	37 ± 3^{a}
ovisac	1	78	3.85	0.053	300 ± 33^{a}	202 ± 28^{b}	1	78	10.6	0.002	166 ± 23^{b}	335 ± 34^{a}

The mean number of insects per plant from the first life cycle of *P. mesembryanthemi* was positively correlated with the insect settlement (number of 1st instars) in the subsequent cycles (Pearson's correlation=0.255, t_{77}=2.31, p =0.023).

4.3.3. Leaf spectral reflectance, chlorophyll fluorescence and visual rating

The 'treatment' factor was significant for all the indexes included in the analysis, except for WI (Table 4.3). Both treatments with *P.*

mesembryanthemi infestation ('P' and 'PS') yielded lower values of PRI and higher values of SIPI than the control (untreated) plants ('C'). For the other indexes (CHL-NDI, F_v/F_m, NPQI), only the treatment in which both agents were applied together ('PS') produced significantly lower values than the control (no treatment) (Table 4.4). There were no significant differences between 'P' and 'PS' or between 'S' and 'C' (Table 4.4).

Table 4.3. Mixed-model results from *C. edulis* experiment describing the variation in the reflectance indexes for the fixed factors and their interactions. The results of a type III ANOVA with Satterthwaite approximation for the numerator (nDF) and denominator (dDF) degrees of freedom are shown. 'Treat', treatment applied to the plant; 'Or', origin of the plant.

	PRI				CHL-NDI				SIPI			
Effect	nDF	dDF	F	P	nDF	dDF	F	P	nDF	dDF	F	P
Treat	3	148	20	<0.001	3	149	3.4	0.020	3	154	26.6	<0.001
Date	13	1756	76	<0.001	13	1750	79	<0.001	13	1752	116	<0.001
Or	1	167	1.2	0.270	1	191	0.5	0.460	1	175	0.1	0.716
Treat*Or	3	166	0.4	0.730	3	191	1.2	0.296	3	175	1.5	0.228
Treat*Date	39	1754	6.7	<0.001	39	1749	6.3	<0.001	39	1751	5.7	<0.001
Treat:Or:Date	52	1756	1	0.455	52	1749	1.6	0.008	4	1752	1.2	0.116

	F_v/F_m				WI				NPQI			
Effect	nDF	dDF	F	P	nDF	dDF	F	P	nDF	dDF	F	P
Treat	3	137	3.4	0.020	3	147	0.8	0.472	3	151	6.7	<0.001
Date	12	1602	676	<0.001	13	1749	329	<0.001	13	1756	132	<0.001
Or	1	139	0	0.842	1	164	20.8	<0.001	1	175	15.7	<0.001
Treat*Or	3	137	0.4	0.757	3	163	2.2	0.094	3	174	1	0.399
Treat*Date	36	1600	4	<0.001	39	1748	4.3	<0.001	39	1755	3.3	<0.001
Treat:Or:Date	4	1599	0.8	0.832	4	1749	0.6	0.983	4	1755	0.9	0.582

For all spectral reflectance and fluorescence indexes, the time since the start of the experiment ('Date') was a significant factor (Table 4.3), although no clear trend was observed (Figs. 4.2, 4.3). The variations in the indexes throughout the year of the experiment were similar for all treatments (Figs. 4.2, 4.4). However, for all variables the effect of the treatment was time-dependent (significant 'Treat*Date' interaction; Table 4.3). No differences between treatments were found after 1 week for any of the indexes studied (Fig. 4.2, 4.3, SM4.1). The values were lower in plants inoculated with *S. sclerotiorum* than in the control (untreated) plants

for CHL-NDI after 3 weeks and 3.5 months and for PRI after 3.5 and 5 months (2 and 3.5 months, respectively, from the second inoculation done in shoots) (SM4.1, Figs. 4.2, 4.3).

Table 4.4. Mean values ± SE for the different calculated reflectance indexes for the control (untreated) (C), *S. sclerotiorum* – inoculated (S), *P. mesembryanthemi* - infested (P) and both infested and inoculated (PS) *C. edulis* plants. Across rows, different letters represent significant differences ($p \leq 0.05$) between treatments according to the post-hoc test.

Variable	C	S	P	PS
PRI	0.029 ± 0.001^{a}	0.025 ± 0.001^{a}	0.020 ± 0.002^{b}	0.015 ± 0.002^{b}
CHL-NDI	0.142 ± 0.003^{a}	0.132 ± 0.003^{ab}	0.137 ± 0.003^{ab}	0.123 ± 0.003^{b}
SIPI	0.451 ± 0.013^{b}	0.473 ± 0.013^{b}	0.586 ± 0.017^{a}	0.605 ± 0.018^{a}
F_v/F_m	0.828 ± 0.002^{a}	0.824 ± 0.002^{ab}	0.820 ± 0.002^{ab}	0.817 ± 0.002^{b}
WI	1.023 ± 0.002^{a}	1.020 ± 0.002^{a}	1.024 ± 0.002^{a}	1.023 ± 0.002^{a}
NPQI	0.354 ± 0.001^{a}	0.353 ± 0.001^{ab}	0.351 ± 0.001^{ab}	0.347 ± 0.002^{b}

A positive and significant effect of *P. mesembryanthemi* was detected for F_v/F_m after 1.5 months and for NPQI after 6 months (SM 4.1). In the plants artificially infested with *P. mesembryanthemi* ('P' and 'PS'), the F_v/F_m, PRI and WI values were lower, but the SIPI values were higher from 2.5, 5, 6 or 5 months (in specific dates) respectively after the beginning of the experiment (Fig. 4.2; SM 4.1). For CHL-NDI and NPQI, no significant effects of the insect were found until the 11th month (Fig. 4.3; SM4.1).

Compared with the controls, only the combination of both agents ('PS') negatively affected ($p \leq 0.05$) *C. edulis* plants after 3 weeks (for PRI, NPQI), 1 month (for SIPI, PRI), 5 months (for NPQI and F_v/F_m), 7 months (for WI, NPQI) and 9 months (for PRI) from the beginning of the experiment (Fig. 4.2; SM 4.1).

The values of WI and NPQI were significantly higher ($p < 0.001$) in the native plants than in the introduced plants, although the interaction with 'treatment' was not significant for any of the variables. The triple interaction 'Treatment*Origin*Date' was only significant for CHL-NDI. Nevertheless, the 'Treatment*Origin' interaction did not vary significantly with 'Date' ($p > 0.05$).

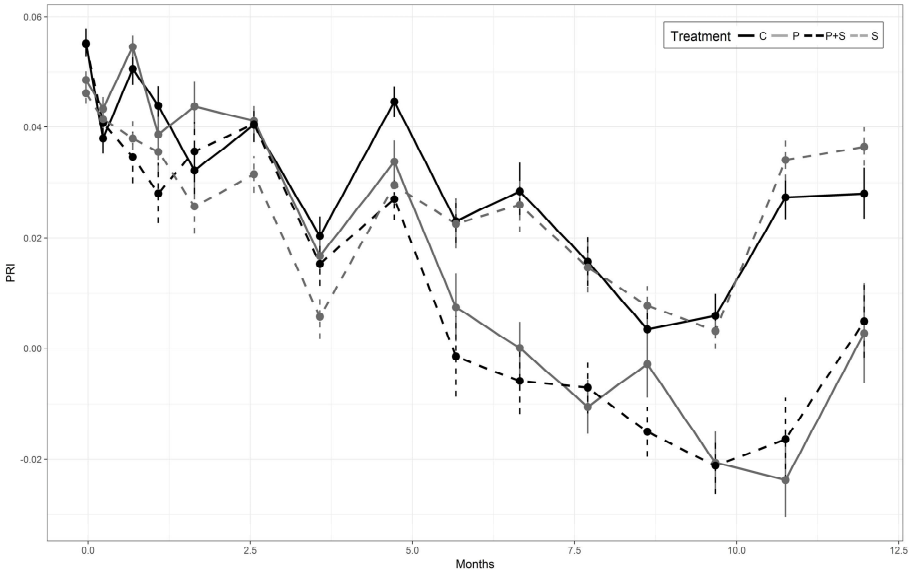

Fig. 4.2. Mean ± SE of the photochemical reflectance index 'PRI' of *C. edulis* plants throughout the year of the experiment (October 2016 - October 2017). Different lines represent different treatments: control, untreated plants ('C', black solid line), fungus-inoculated plants ('S', grey dashed line), insect-infested plants ('P', grey solid line) and plants treated with both agents ('PS', black dashed line).

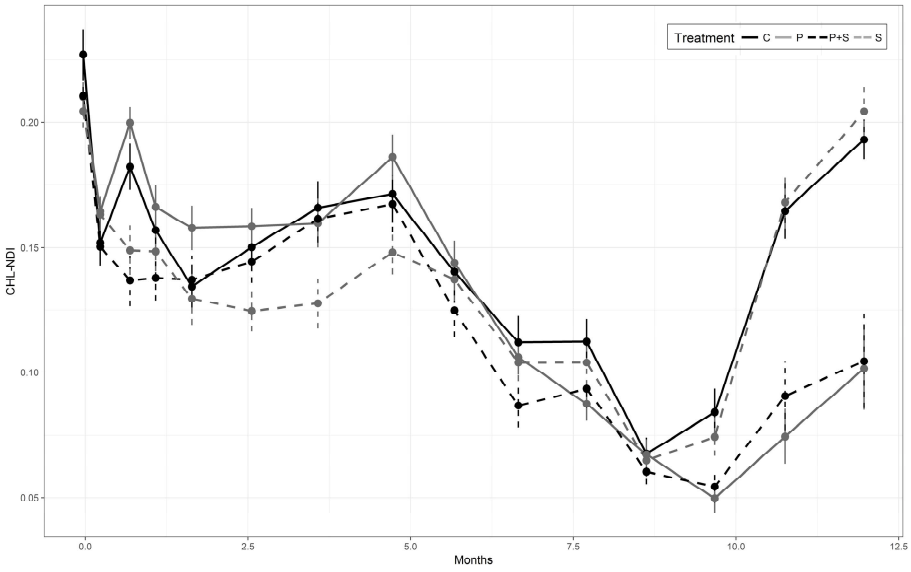

Fig. 4.3. Mean ± SE of the chlorophyll index 'CHL-NDI' of *C. edulis* plants throughout the year of the experiment (October 2016 - October 2017). Different lines represent different treatments: control, untreated plants ('C', black solid line), fungus-inoculated plants ('S', grey dashed line), insect-infested plants ('P', grey solid line) and plants treated with both agents ('PS', black dashed line).

The treatment applied to the plant significantly affected the visual rating ($F_{3,2381}$=23.80, p <0.001), and the plants artificially infested with the insect ('P','PS') were less healthy than the control plants (p <0.001). The origin of the plant did not significantly affect the visually rated appearance (p >0.05).

4.3.4. Plant survival and reproduction

The log-rank test revealed that the treatment variable significantly affected the survival functions, while the plant origin was not significant (Table 4.5). Survival was only lower than in control plants in plants affected by treatments including insect infestation ('P' and 'P+S') (p <0.001, log-rank test) (Fig. 4.4; Table 4.6). However, there was no difference in plant survival between 'P' and 'P+S' treatments (p =1.000, log-rank test) (Fig. 4.4). Most of the mortality of plants treated with *P. mesembryanthemi* ('P' and 'P+S') occurred between 6 and 10 months, and in both cases survival fell below 50% one year after infestation with insects (Fig. 4.4).

Table 4.5. Survival analysis of *C. edulis* with the log-rank test for the 4 treatments (untreated control, infection with *S. sclerotiorium*, infestation with *P. mesembryanthemi*, combination of both agents), 2 plant origins (native or introduced) and the interaction of both factors (Treat * Or).

Source	χ^2	df	p
Treatment	46.0	3	<0.001
Origin	3.1	1	0.077
Treat * Or	1.8	3	0.624

The interactions between the different treatments and the origin of the plant (native or invasive populations) did not significantly affect plant survival (Table 4.5).

Throughout the year of the trial, only 10 plants produced fruits (3 plants from the 'C' treatment, 4 from the 'P' treatment, 2 from the 'S' treatment and 1 from the 'PS' treatment), all of which were sterile (without seeds).

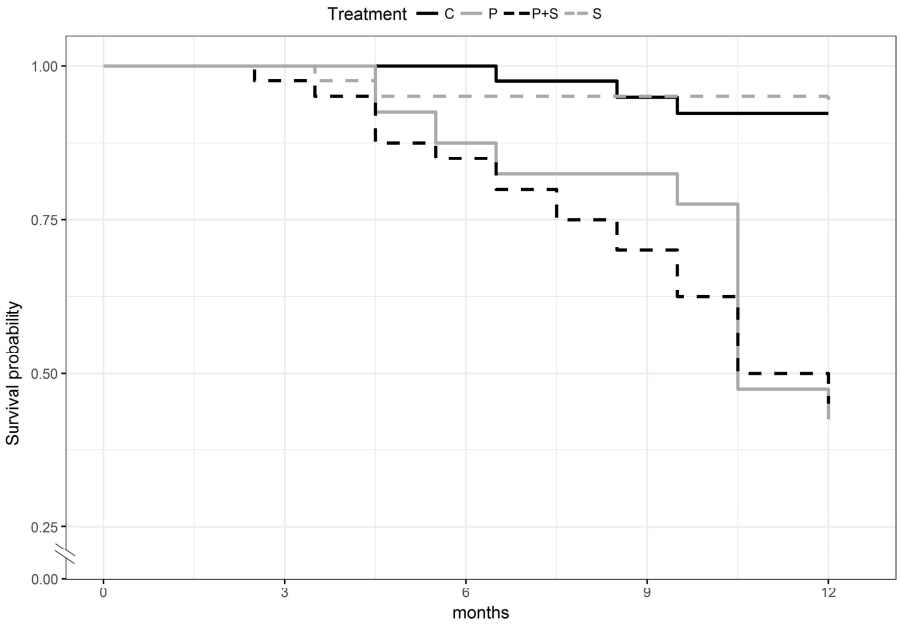

Fig. 4.4. Kaplan-Meier survival plot showing the proportion of *Carpobrotus edulis* plants surviving the different treatments ('C' control, untreated plants, 'S' plants infected with the fungus *Sclerotinia sclerotiorum*, 'P' plants infested with the insect *Pulvinariella mesembryanthemi* and 'P+S' plants treated with both the insect and the fungus) during 12 months (October 2016 - October 2017).

Table 4.6. Hazard ratios (HR) and the lower (LCI) and upper (UCI) confidence intervals for the effects of the different treatments ('S' plants infected with the fungus *S. sclerotiorum*, 'P' plants infested with the insect *P. mesembryanthemi* and 'P+S' plants treated with both the insect and the fungus) on *C. edulis* plants, estimated by the Cox regression model. The Wald statistic value (z) and the corresponding p-value are presented. Note: HR > 1 increase in hazard, HR<1 reduction in hazard, HR=1 no effect.

Source	HR	LCI	UCI	z	p
S	0.97	0.20	4.82	-0.04	0.972
P	9.81	2.94	32.71	3.71	<0.001
P+S	10.11	3.02	33.82	3.76	<0.001

4.3.5. Growth rate and dry mass allocation

The increase in shoot length and node and leaf numbers in the plants throughout the trial was significantly higher for the 'Control' and 'Sclerotinia' treatments than for the two treatments in which plants were infested with the insect ('P' and 'PS') (Table 4.7), and the increases were higher for the introduced than for the native plants (Table 4.8).

Table 4.7. Mean values ± SE for the different biomass and growth estimators from *C. edulis* experiment in untreated control (C), *S. sclerotiorum* – inoculated (S), *P. mesembryanthemi* - infested (P) and both infested and inoculated (PS) plants. Across rows, different letters represent significant differences ($p \leq 0.05$) between treatments according to the post-hoc test.

Variable	C	S	P	PS
Shoot growth (cm)	24.2 ± 1.8^{a}	25.7 ± 1.5^{a}	10.8 ± 1.6^{b}	12.3 ± 1.8^{b}
Increase in node number	23 ± 1^{a}	24 ± 1^{a}	13 ± 1^{b}	13 ± 1^{b}
Internodal distance (cm)	1.4 ± 0.04^{a}	1.34 ± 0.03^{a}	1.42 ± 0.05^{a}	1.34 ± 0.04^{a}
Increase in leaf number	66 ± 4^{a}	69 ± 3^{a}	51 ± 4^{b}	49 ± 4^{b}
Final dry aerial biomass (g)	25.9 ± 1.6^{a}	23 ± 1.3^{a}	19 ± 1^{b}	17.3 ± 1.4^{b}
Final dry root biomass (mg)	1098 ± 103^{a}	1173 ± 127^{a}	327 ± 40^{b}	341 ± 42^{b}
Root : Aerial biomass rate	0.042 ± 0.003^{a}	0.046 ± 0.004^{a}	0.019 ± 0.002^{c}	0.027 ± 0.003^{b}
Water content (g)	60.5 ± 3.1^{a}	57.7 ± 2.9^{ab}	46.4 ± 5.4^{ab}	43.9 ± 6.2^{b}

Table 4.8. Mean values ± SE for the different biomass and growth estimators in native and introduced *C. edulis* plants. Different letters in a row represent significant differences ($p \leq 0.05$) between treatments according to the post-hoc test.

Variable	Native	Introduced
Growth in shoot length (cm)	15.65 ± 1.31^{b}	21.52 ± 1.46^{a}
Increase in number of nodes	16.00 ± 1.00^{b}	20.00 ± 1.00^{a}
Internodal distance (cm)	1.33 ± 0.03^{b}	1.41 ± 0.03^{a}
Increase in number of leaves	52.00 ± 2.00^{b}	65.00 ± 3.00^{a}
Final dry aerial biomass (g)	17.26 ± 0.73^{b}	25.60 ± 1.05^{a}
Final dry root biomass (mg)	654.0 ± 73.0^{b}	839.0 ± 81.0^{a}
Root / Aerial biomass rate	0.035 ± 0.003^{a}	0.032 ± 0.002^{a}
Water content (g)	52.2 ± 2.68^{a}	56.35 ± 3.35^{a}

Table 4.9. Results of a type III ANOVA with Satterthwaite approximation for the numerator (nDF) and denominator (dDF) degrees of freedom describing the variation in the biomass and growth estimators for the fixed factors and its interactions. 'Treat', treatment applied to the plant; 'Or', origin of the plant.

	Growth in shoot length				Increase node number				Internodal distance				Increase in leaf number			
Effect	nDF	dDF	F value	P	nDF	dDF	F value	P	nDF	dDF	F value	P	nDF	dDF	F value	P
Intercept	1	132	527	<0.001	1	141	810	<0.001	1	149	4532	<0.001	1	144	1164	<0.001
Treat	3	132	23.3	<0.001	3	141	23.1	<0.001	3	149	1.0	0.392	3	144	9.7	<0.001
Or	1	132	13.7	<0.001	1	141	11.0	0.001	1	149	4.0	0.047	1	144	16.0	<0.001
Treat:Or	3	132	0.5	0.718	3	141	0.4	0.748	3	149	0.8	0.478	3	144	1.4	0.246

	Final dry aerial biomass				Final dry root biomass				Root / Aerial biomass				Water content			
Effect	nDF	dDF	F value	P	nDF	dDF	F value	P	nDF	dDF	F value	P	nDF	dDF	F value	P
Intercept	1	142	1407	<0.001	1	140	1066	<0.001	1	143	7132	<0.001	1	60	728	<0.001
Treat	3	142	12.0	<0.001	3	140	31.6	<0.001	3	143	24.9	<0.001	3	60	3.7	0.016
Or	1	142	55.4	<0.001	1	140	6.6	0.011	1	143	0.3	0.571	1	60	2.8	0.098
Treat:Or	3	142	1.2	0.303	3	140	0.7	0.525	3	143	2.2	0.088	3	60	0.4	0.753

However, there was no significant interaction between the plant origin and the treatment (Table 4.9). Final dry weights of both the aerial parts and the roots were significantly lower for the insect-infested plants ('P' and 'PS') than for the control plants ('C'), and lower for the native than for the introduced plants (Table 4.7). The root / aerial biomass ratio was also significantly lower in both treatments with the insect than in the 'Control' and '*Sclerotinia*' treatments, although they also differed from each other (the ratio was lower for 'P' than 'PS': p =0.052) (Table 4.7). The origin of the plant did not significantly affect the root / aerial biomass ratio (Table 4.8). The water content of the plants at the end of the experiment was only affected by the treatment, and it was significantly lower in the fungus-inoculated + insect-infested plants 'PS' than in the control plants. The internodal distance was higher in introduced than in native plants (Table 4.7), but did not vary depending on the treatment applied (Table 4.9).

The maximum number of insects counted in a plant throughout the trial was positively and significantly correlated with the final dry weight of plant aerial and root biomass, shoot growth, increase in number of nodes and leaves, increase in internodal distance and initial fresh weight of the biomass (Table 4.10).

Table 4.10. Pearson's correlation coefficients between maximum number of insects per plant and the biomass and growth parameters of *C. edulis* plants (aerial and root dry biomass, root/aerial dry biomass ratio, shoot growth, increase in the number of nodes and leaves, internodal distance and initial fresh biomass).

	r	t	df	p
Aerial Biomass vs. Maximum nº insects	0.498	5.07	78	<0.001
Root Biomass vs. Maximum nº insects	0.272	2.50	78	0.015
Root/aerial ratio vs. Maximum nº insects	- 0.021	- 0.19	74	0.854
Shoot growth vs. Maximum nº insects	0.341	3.21	78	<0.001
Node nº increase vs. Maximum nº insects	0.348	3.28	78	0.002
Internodal distance vs. Maximum nº insects	0.251	2.29	78	0.025
Increase in no. leaves vs. Maximum nº insects	0.252	2.30	78	0.024
Init. fresh biomass vs. Maximum nº insects	0.422	3.89	70	<0.001

4.4. DISCUSSION

4.4.1. Plants treated with the fungus

The fungus *S. sclerotiorum* by itself (treatment 'S') had only very brief, short-term effects on the ecophysiological parameters of *C. edulis* considered, with some decreases in chlorophyll content and photosynthetic-radiation use efficiency (as suggested by the CHL-NDI and F_v/F_m indexes, respectively) in the first half of the year of the experiment. This may indicate that the plants were able to recover quickly from the infection and were only significantly affected for very short periods of time. Fungal pathogens vary in their effects on plants and can produce necrotic or chlorotic diseases (Malthus and Madeira, 1993). In this case, the reflectance values suggest that *S. sclerotiorum* produces leaf chlorosis, as observed by Lorenzen and Jensen (1989) for mildew-infected leaves and in contrast to the findings reported by Malthus and Madeira (1993) for plants infected with *Botrytis fabae*.

Sclerotinia sclerotiorum did not significantly affect plant survival one year after inoculation, either alone (treatments 'C' and 'S' did not have significantly different effects) or when combined with the insect (treatments 'P' and 'PS' did not have significantly different effects). This fungus usually kills plants within a month (Cother, 2000, Waipara et al., 1993, Bourdôt and Harvey, 1994), and therefore it was not expected to affect plant survival even in the longer term. This agent did not significantly affect any of the growth and biomass indicators considered.

Overall, the fungus *S. sclerotiorum* only significantly affected some short-term estimators of fitness at specific times, but it did not alter any of the long-term estimators measured (survival, growth and dry mass allocation). However, the efficiency of *S. sclerotiorum* infection varies depending on climate conditions and is favoured by high humidity and moderate temperatures of around 20 °C (Berg and Lentz, 1968). Therefore, growth of the fungus may be higher in the field than in greenhouse or laboratory conditions, especially in the warm humid months of spring and autumn.

4.4.2. Plants treated with the insect

Considering all dates, the insect *P. mesembryanthemi* significantly decreased the photosynthetic-radiation use efficiency (as suggested by the

PRI) of the plants. However, the effects of the insect on the plant varied over time.

A positive and occasional effect of the insect on photosynthetic performance (F_v/F_m) was observed at the beginning of the experiment. This was similar to the observed increase in photosynthetic efficiency in *Ilex aquifolium* plants infested with scale insects (Retuerto et al., 2004), which was attributed to an imbalance between source and sink tissues in the host plants caused by the insect feeding, promoting compensatory photosynthesis in the plants.

The first symptoms of negative effects of the insect *P. mesembryanthemi* were observed from 5 months after the infestation (with the exception of the lower F_v/F_m observed after 2.5 months), coinciding with the most advanced life stages during the first life cycle (4^{th} instar or ovisac). Infestation with the insect affected all of the indexes considered at some point during the trial. The insect generally took longer to affect the plant than the fungus (2.5 months for F_v/F_m; 5 months for WI and SIPI; 6 months for PRI and 10-11 months for the other indexes), but the symptoms lasted longer. However, the F_v/F_m values were close to 0.8 throughout the year of the trial and for all four treatments. This indicates that the plants were affected by a low level of stress during the experiment (Traveset et al., 2008, Maxwell and Johnson, 2000), even for the treatments including insects ('P' and 'PS'). Like the fungus, the insect seemed to induce chlorophyll degradation (inferred by the decrease in PRI, CHL-NDI and NPQI). Chlorosis has previously been observed in plants infested by other sap-sucking insects such as mites (Peñuelas et al., 1995b) . The reflectance indexes show that the insect *P. mesembryanthemi* was also able to decrease the water content of the leaves (WI) and the photosynthesis efficiency (PRI) at some points during the trial. Water depletion of the plants could be caused by intense insect feeding (Washburn et al., 1985).

In contrast to the effect of the fungus, the insect *P. mesembryanthemi* affected *C. edulis* plants in both the short-and the long-term. Survival decreased greatly in the second half of the year-long trial. The increase in mortality in the treatments infested by *P. mesembryanthemi* ('P' and 'P+S) from the 6^{th} month coincided with the end of the first cycle of the insect, when the insects were fully grown and were probably feeding more intensively on the plant. Furthermore, growth of the plants infested with the insect was lower in terms of shoot length, number of nodes and leaves and aerial and root dry biomass. Although the infestation initially

enhanced plant photosynthesis (as indicated by the F_v/F_m), the decrease in photosynthetic performance found from 2.5 months after the infestation and the loss of sugars due to feeding seemed to have a detrimental effect on plant growth.

Washburn et al. (1985) also observed lower plant growth in plants infested with *P. mesembryanthemi*, probably due to a reduction in the plant capacity to obtain resources and increased vulnerability to stress (Vranjic and Ash, 1997). In fact, extensive mortalities of *P. mesembryanthemi* - infested *C. edulis* plants in the field have been associated with stress conditions of drought and freezing temperatures (Donaldson et al., 1978, Washburn and Frankie, 1985). Although insect feeding sometimes lowered the plant water index, the water content of the plants at the end of the experiment was not significantly different from that of the control (untreated) plants. As plants were watered regularly throughout the trial, they were able to replenish the water lost due to insect feeding. However, in nature, *P. mesembryanthemi* infestation could increase the vulnerability of *C. edulis* to drought conditions. The root / aerial biomass ratio was lower in the plants infested with *P. mesembryanthemi* than in the control plants. The higher proportion of photosynthetic tissue suggests (as indicated by the F_v/F_m index) a measure that compensates for the carbohydrates lost due to insect feeding.

The decrease in plant growth produced by the infestation could also be detrimental to the next generations of insects as they would have less material on which to feed (Washburn et al., 1985). Nevertheless, we observed a positive correlation between the mean density of insects in the first life cycle and the number of new settled insects (1st instar) in the subsequent cycles. In contrast to the findings of Washburn et al. (1985) and despite the insect being a deterrent to plant growth, we found that the *C. edulis* plants supporting the highest density of insects were those with the largest aerial and root dry biomass, shoot growth and increase in number of leaves. The findings may reflect that the density of insects on the plants is limited by the size of the plant because of competition for feeding space. Therefore, bigger plants would support larger numbers of insects. The significant and positive correlation between the initial fresh biomass weight and maximum number of insects per plant corroborates this supposition. The maximum insect density on plants was also positively correlated with the internodal distance, suggesting an exploratory

strategy and faster spread of the plants most affected by insects (Traveset et al., 2008, Doust, 1981).

In around half of the plants that were only treated with the insect and that survived until the end of the trial, death of the plant parts supporting high densities of *P. mesembryanthemi* was observed. This could be a defence mechanism of the plant, as it increases the insect mortality and decreases the number of offspring (Washburn et al., 1985). Scale insects are particularly strongly affected by the death of plant tissues as their limited mobility restricts their capacity to spread to healthy plant parts (Washburn et al., 1985).

Most of the insect-infected plants were affected by black sooty mould, which has previously been associated with the accumulation of honeydew excreted by scale insects (Bokonon-Ganta et al., 2002). The presence of the mould may lead to a decrease in the level of photosynthesis, as observed in mango trees infested with mealybugs (Bokonon-Ganta et al., 2002). On the other hand, this fungus has also been attributed to increased mortality of scale insects (Collins and Scott, 1982). As ants reduce the accumulation of honeydew (Bach, 1991), the high level of colonization by mould observed in the greenhouse-grown plants might not occur in the field, due to the natural presence of ants.

The male specimens of *P. mesembryanthemi* occasionally observed throughout the trial were probably derived from the same female on each plant, as they occurred close together on the plants (Pesson, 1941).

4.4.3. Plants treated with the combination of the fungus and the insect

Only when the insect and fungus were applied together, plant chlorophyll content and photosynthetic performance were significantly lower than in control plants, suggesting that the combination of both agents may be more effective than the application of either agent by itself. This is consistent with the findings of other studies in which the combined use of an insect and a fungus proved more effective than the use of each biocontrol agent alone (e.g. Caesar (2003) in *Euphorbia sp*.). Nevertheless, the effects of the combination of both agents on the ecophysiological parameters were temporary, and we did not observe any long-term synergetic effects, as treatments 'P' and 'PS' did not produce any significant differences in survival, biomass or growth of the plants.

The higher densities of insects observed in treatment 'P' than in 'PS' indicate a possible detrimental effect of fungal infection on the insect. Insect survival was not affected by the treatment, but the number of 1st instar stages was already higher in plants in treatment 'P'. This may indicate greater success of the insect colonization in 'P' plants than in 'PS' plants. The emergence of the insects from the first life cycle (after approx. 4 weeks) coincides with the highest effect of the fungus on the chlorophyll content of leaves (as suggested by the CHL-NDI after approx. 3 weeks). The lower chlorophyll content of the 'PS' plants due to the effect of the fungus may negatively affect settlement of the insects, possibly due to lower quality sap, with consequently lower densities of insects. *Carpobrotus edulis* can produce antifungal compounds (Omoruyi et al., 2014), and inoculation of the plants with *S. sclerotiorum* may have induced production of such substances. This, in turn, may have affected *P. mesembryanthemi*, explaining the lower densities in treatment 'PS'. For instance, saponins, which are present in *C. edulis* (Omoruyi et al., 2014), are both antifungal and insecticidal compounds (De Geyter et al., 2007). In a study combining an insect and a fungus for plant biocontrol, Campanella et al. (2009) also observed indirect competition between both agents, which was detrimental to the insect.

The higher densities of insects found in 'P' plants may explain the higher biomass allocation to the aerial parts (and therefore to photosynthetic tissues) than in plants treated with both agents ('PS'), as they would have to cope with greater loss of sugars through insect feeding.

In this case, the combination of both agents did not appear to be beneficial for controlling *C. edulis*, as the fungus did not have long-term repercussions on the plants and it had a detrimental effect on the settlement of the insects on the plants.

4.4.4. Plant origin (native or non-native populations)

Although plant origin affected some of the reflectance indexes, no 'Treatment*Origin' interaction was observed. The reflectance indexes therefore did not indicate any differences between native and introduce plants in the vulnerability to the biocontrol agents.

The native plants showed lower growth (as estimated by the lower increase in shoot length and node and leaf number) and lower aerial and root dry biomass. However, the native plants were not more susceptible

to the fungus or insect attack than the introduced plants, as the 'Treatment*Origin' interaction for all these variables was not significant. Native plants also had lower internodal distances than the introduced plants, which suggests a slightly different, more explorative strategy, in non-native plants.

The introduced plants were infested by greater total numbers of insects per plant. This did not appear to be the result of any differences in settlement success, as there was no difference in the number of 1st instar stages. The greater success of the insect on the invasive plants may be explained by the larger biomass of introduced plants than of natives plants, which would imply more space for feeding. Also, plant defence is usually lower in exotic populations than in native populations, which can benefit the performance of specialist insects (Hinz and Schwarzlaender, 2004).

4.5. SYNTHESIS

In a preliminary attempt to find a biological control of *Carpobrotus edulis*, we evaluated the potential use of the fungus *Sclerotinia sclerotiorum* and the insect *Pulvinariella mesembryanthemi* as biocontrol agents.

We carried out a greenhouse-experiment to evaluate the effects of both agents, separately and together, on short-term (physiological) and long-term (survival, growth, biomass) estimators of plant performance. We compared the susceptibility to both agents in plants originating from native and non-native areas.

The fungus had immediate and negative, but short-lasting, effects on chlorophyll content and photosynthetic-radiation use efficiency. No significant effects on plant survival, growth and biomass were observed after one year.

Artificial infestation with the insect increased photosynthetic performance and decreased the root/aerial biomass ratio of the plants, suggesting a counteractive response to insect feeding. After 5 months, the reflectance parameters were found to be negatively affected. Only half of the infested plants survived for a year, and the growth and biomass were lower in the surviving plants than in untreated (control) plants. In half of the surviving plants, the most heavily-infested parts died. The insect-infested plants were usually also infected by black mould.

The density of insects was lower in native plants and when both biocontrol agents were used together. Nevertheless, no long-term synergetic effects of the insect and fungus were observed, and the susceptibility of native and introduced plants was not different. Therefore, use of the insect seems to be the best strategy for controlling *C. edulis*, as it decreases plant growth and increases plant mortality and susceptibility to stress.

4.6. SUPPLEMENTARY MATERIAL

SM 4.1. (*next pages*) Mean ± SE for the different calculated reflectance indexes from *C. edulis* experiment in control (C), *P. mesembryanthemi* - infested (P), *S. sclerotiorum* – inoculated and both infested and inoculated (PS) plants for the 14 times reflectance was measured throughout the experiment. Different letters in a row represent significant differences between treatments for each time of measurement (weeks or months since the beginning of the experiment) according to the post-hoc test. Significance was set at $p \leq 0.05$.

		1 week (13.10.2016)	3 weeks (27.10.2016)	1 month (08.11.2016)	1.5 months (25.11.2016)	2.5 months (23.12.2016)	3.5 months (23.01.2017)	5 months (27.02.2017)
		mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
PRI	C	0.038 ± 0.003^{a}	0.051 ± 0.003^{ab}	0.044 ± 0.004^{a}	0.032 ± 0.004^{ab}	0.040 ± 0.003^{a}	0.020 ± 0.004^{a}	0.045 ± 0.003^{a}
	S	0.041 ± 0.002^{a}	0.038 ± 0.004^{bc}	0.036 ± 0.004^{ab}	0.026 ± 0.005^{b}	0.032 ± 0.003^{a}	0.006 ± 0.004^{b}	0.030 ± 0.003^{b}
	P	0.043 ± 0.002^{a}	0.055 ± 0.002^{a}	0.039 ± 0.003^{ab}	0.048 ± 0.006^{a}	0.041 ± 0.003^{a}	0.017 ± 0.003^{ab}	0.034 ± 0.004^{ab}
	PS	0.041 ± 0.002^{a}	0.035 ± 0.005^{c}	0.028 ± 0.005^{b}	0.036 ± 0.005^{ab}	0.041 ± 0.003^{a}	0.015 ± 0.004^{ab}	0.027 ± 0.004^{b}
CHL-NDI	C	0.152 ± 0.009^{a}	0.182 ± 0.009^{a}	0.157 ± 0.010^{a}	0.134 ± 0.009^{a}	0.150 ± 0.009^{ab}	0.166 ± 0.010^{a}	0.171 ± 0.011^{ab}
	S	0.163 ± 0.007^{a}	0.149 ± 0.010^{b}	0.149 ± 0.008^{a}	0.130 ± 0.010^{a}	0.125 ± 0.008^{b}	0.128 ± 0.010^{b}	0.148 ± 0.009^{b}
	P	0.164 ± 0.006^{a}	0.200 ± 0.006^{a}	0.166 ± 0.009^{a}	0.153 ± 0.010^{a}	0.159 ± 0.007^{a}	0.160 ± 0.010^{a}	0.186 ± 0.009^{a}
	PS	0.150 ± 0.007^{a}	0.137 ± 0.010^{b}	0.138 ± 0.009^{a}	0.137 ± 0.010^{a}	0.144 ± 0.008^{ab}	0.162 ± 0.010^{a}	0.167 ± 0.011^{ab}
SIPI	C	0.243 ± 0.040^{a}	0.258 ± 0.037^{a}	0.227 ± 0.039^{b}	0.308 ± 0.041^{a}	0.315 ± 0.035^{a}	0.797 ± 0.017^{a}	0.411 ± 0.038^{b}
	S	0.277 ± 0.029^{a}	0.307 ± 0.038^{a}	0.292 ± 0.043^{ab}	0.302 ± 0.034^{a}	0.347 ± 0.034^{a}	0.851 ± 0.023^{a}	0.502 ± 0.023^{ab}
	P	0.235 ± 0.032^{a}	0.309 ± 0.030^{a}	0.354 ± 0.036^{ab}	0.251 ± 0.071^{a}	0.352 ± 0.029^{a}	0.849 ± 0.021^{a}	0.594 ± 0.024^{a}
	PS	0.199 ± 0.039^{a}	0.385 ± 0.051^{a}	0.424 ± 0.058^{a}	0.341 ± 0.046^{a}	0.318 ± 0.046^{a}	0.832 ± 0.017^{a}	0.603 ± 0.035^{a}
F_v/F_m	C	(no data)	0.814 ± 0.006^{a}	0.805 ± 0.006^{a}	0.811 ± 0.006^{b}	0.892 ± 0.007^{a}	0.825 ± 0.007^{a}	0.847 ± 0.006^{a}
	S		0.808 ± 0.005^{a}	0.798 ± 0.006^{a}	0.819 ± 0.006^{b}	0.888 ± 0.008^{a}	0.828 ± 0.006^{a}	0.836 ± 0.006^{ab}
	P		0.824 ± 0.006^{a}	0.807 ± 0.006^{a}	0.846 ± 0.005^{a}	0.850 ± 0.005^{b}	0.820 ± 0.006^{a}	0.831 ± 0.004^{ab}
	PS		0.809 ± 0.007^{a}	0.796 ± 0.006^{a}	0.843 ± 0.006^{a}	0.845 ± 0.005^{b}	0.811 ± 0.006^{a}	0.820 ± 0.005^{b}
WI	C	1.068 ± 0.003^{a}	1.054 ± 0.005^{a}	1.075 ± 0.005^{a}	1.064 ± 0.004^{a}	1.063 ± 0.005^{a}	0.962 ± 0.003^{a}	1.055 ± 0.005^{a}
	S	1.065 ± 0.003^{a}	1.051 ± 0.004^{a}	1.064 ± 0.005^{a}	1.060 ± 0.005^{a}	1.058 ± 0.004^{a}	0.960 ± 0.003^{a}	1.044 ± 0.004^{a}
	P	1.066 ± 0.003^{a}	1.053 ± 0.004^{a}	1.060 ± 0.003^{a}	1.073 ± 0.005^{a}	1.055 ± 0.003^{a}	0.970 ± 0.002^{a}	1.028 ± 0.004^{b}
	PS	1.071 ± 0.004^{a}	1.042 ± 0.004^{a}	1.057 ± 0.004^{a}	1.067 ± 0.005^{a}	1.055 ± 0.004^{a}	0.970 ± 0.002^{a}	1.028 ± 0.004^{b}
NPQI	C	0.369 ± 0.004^{a}	0.357 ± 0.003^{a}	0.369 ± 0.003^{a}	0.356 ± 0.002^{a}	0.373 ± 0.003^{a}	0.358 ± 0.004^{a}	0.381 ± 0.003^{a}
	S	0.372 ± 0.003^{a}	0.351 ± 0.002^{ab}	0.368 ± 0.002^{a}	0.351 ± 0.002^{a}	0.376 ± 0.003^{a}	0.355 ± 0.003^{a}	0.376 ± 0.003^{a}
	P	0.378 ± 0.003^{a}	0.347 ± 0.002^{ab}	0.367 ± 0.003^{a}	0.356 ± 0.003^{a}	0.370 ± 0.003^{a}	0.348 ± 0.003^{a}	0.371 ± 0.002^{ab}
	PS	0.377 ± 0.002^{a}	0.344 ± 0.003^{b}	0.362 ± 0.003^{a}	0.354 ± 0.003^{a}	0.370 ± 0.003^{a}	0.346 ± 0.004^{a}	0.360 ± 0.003^{b}

		6 months (28.03.2017)	7 months (27.04.2017)	8 months (29.05.2017)	9 months (26.06.2017)	10 months (28.07.2017)	11 months (30.08.2017)	12 months (06.10.2017)
		mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE	mean ± SE
PRI	C	0.023 + 0.004^{a}	0.028 + 0.005^{a}	0.016 + 0.004^{a}	0.003 + 0.004^{a}	0.006 + 0.004^{a}	0.027 + 0.004^{a}	0.028 + 0.005^{a}
	S	0.023 ± 0.004^{a}	0.026 ± 0.005^{a}	0.015 ± 0.005^{a}	0.008 ± 0.004^{a}	0.003 ± 0.003^{a}	0.034 ± 0.004^{a}	0.036 ± 0.003^{a}
	P	0.007 ± 0.006^{b}	0.000 ± 0.005^{b}	-0.011 ± 0.005^{b}	-0.003 ± 0.006^{ab}	-0.021 ± 0.006^{b}	-0.024 ± 0.007^{b}	0.003 ± 0.009^{b}
	PS	-0.001 ± 0.007^{b}	-0.006 ± 0.005^{b}	-0.007 ± 0.005^{b}	-0.015 ± 0.004^{b}	-0.021 ± 0.005^{b}	-0.016 ± 0.007^{b}	0.005 ± 0.007^{b}
CHL-NDI	C	0.140 ± 0.011^{a}	0.112 ± 0.011^{a}	0.112 ± 0.009^{a}	0.068 ± 0.006^{a}	0.084 ± 0.010^{a}	0.165 ± 0.011^{a}	0.193 ± 0.008^{a}
	S	0.137 ± 0.010^{a}	0.104 ± 0.007^{a}	0.104 ± 0.009^{a}	0.065 ± 0.006^{a}	0.074 ± 0.008^{a}	0.168 ± 0.010^{a}	0.204 ± 0.011^{a}
	P	0.144 ± 0.009^{a}	0.106 ± 0.007^{a}	0.087 ± 0.007^{a}	0.067 ± 0.006^{a}	0.050 ± 0.006^{a}	0.074 ± 0.011^{b}	0.102 ± 0.017^{b}
	PS	0.125 ± 0.011^{a}	0.087 ± 0.009^{a}	0.094 ± 0.008^{a}	0.060 ± 0.005^{a}	0.054 ± 0.006^{a}	0.091 ± 0.014^{b}	0.105 ± 0.019^{b}
SIPI	C	0.681 ± 0.028c	0.367 ± 0.053^{b}	0.697 ± 0.027^{c}	0.524 ± 0.047^{b}	0.602 ± 0.048^{b}	0.400 ± 0.034^{b}	0.520 ± 0.034^{b}
	S	0.724 ± 0.020^{bc}	0.375 ± 0.048^{b}	0.737 ± 0.043^{bc}	0.496 ± 0.050^{b}	0.633 ± 0.045^{b}	0.300 ± 0.040^{b}	0.515 ± 0.023^{b}
	P	0.871 ± 0.038^{a}	0.712 ± 0.034^{a}	0.875 ± 0.034^{ab}	0.761 ± 0.076^{a}	0.783 ± 0.074^{a}	0.954 ± 0.072^{a}	0.811 ± 0.071^{a}
	PS	0.862 ± 0.049^{ab}	0.823 ± 0.043^{a}	0.941 ± 0.038^{a}	0.806 ± 0.047^{a}	0.902 ± 0.092^{a}	0.854 ± 0.086^{a}	0.675 ± 0.045^{ab}
F_v/F_m	C	0.802 ± 0.007^{a}	0.813 ± 0.006^{a}	0.797 ± 0.007^{ab}	(no data)	0.826 ± 0.005^{a}	0.855 ± 0.005^{a}	0.842 ± 0.004^{a}
	S	0.796 ⊥ 0.005^{a}	0.808 ⊥ 0.005^{a}	0.787 ⊥ 0.007^{b}		0.828 ⊥ 0.004^{a}	0.850 ⊥ 0.005^{a}	0.834 ⊥ 0.005^{a}
	P	0.791 ± 0.006^{a}	0.802 ± 0.007^{a}	0.800 ± 0.007^{ab}		0.820 ± 0.005^{a}	0.821 ± 0.007^{b}	0.820 ± 0.009^{a}
	PS	0.781 ± 0.006^{a}	0.801 ± 0.005^{a}	0.818 ± 0.008^{a}		0.821 ± 0.008^{a}	0.835 ± 0.009^{ab}	0.833 ± 0.006^{a}
WI	C	0.987 ± 0.003^{a}	1.030 ± 0.010^{ab}	0.977 ± 0.004^{c}	1.014 ± 0.003^{a}	0.985 ± 0.003^{a}	1.005 ± 0.003^{a}	0.976 ± 0.003^{ab}
	S	0.980 ± 0.002^{a}	1.038 ± 0.009^{a}	0.979 ± 0.004^{bc}	1.013 ± 0.004^{a}	0.978 ± 0.003^{a}	1.010 ± 0.003^{a}	0.970 ± 0.003^{a}
	P	0.996 ± 0.004^{a}	1.009 ± 0.005^{bc}	0.993 ± 0.002^{ab}	1.015 ± 0.004^{a}	0.983 ± 0.004^{a}	0.994 ± 0.003^{a}	0.985 ± 0.005^{ab}
	PS	0.995 ± 0.004^{a}	1.002 ± 0.005^{c}	0.998 ± 0.002^{a}	1.001 ± 0.004^{a}	0.985 ± 0.003^{a}	0.994 ± 0.003^{a}	0.987 ± 0.005^{b}
NPQI	C	0.352 ± 0.005^{b}	0.366 ± 0.004^{a}	0.348 ± 0.004^{a}	0.340 ± 0.004^{a}	0.315 ± 0.005^{a}	0.342 ± 0.004^{a}	0.322 ± 0.004^{a}
	S	0.358 ± 0.004^{ab}	0.367 ± 0.004^{a}	0.339 ± 0.005^{a}	0.334 ± 0.005^{a}	0.318 ± 0.005^{a}	0.345 ± 0.004^{a}	0.323 ± 0.004^{a}
	P	0.365 ± 0.004^{a}	0.354 ± 0.003^{ab}	0.344 ± 0.004^{a}	0.336 ± 0.004^{a}	0.294 ± 0.006^{b}	0.312 ± 0.009^{b}	0.310 ± 0.006^{a}
	PS	0.358 ± 0.004^{ab}	0.342 ± 0.005^{b}	0.340 ± 0.006^{a}	0.330 ± 0.005^{a}	0.285 ± 0.007^{b}	0.319 ± 0.009^{b}	0.309 ± 0.008^{a}

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5. Population dynamics and potential distribution of *Pulvinariella mesembryanthemi* in an area invaded by its host *Carpobrotus edulis*

5.1. INTRODUCTION

Between the 17th and the 20th centuries, *Carpobrotus edulis* and *Carpobrotus* aff. *acinaciformis* were introduce in temperate coastal areas worldwide (Wisura and Glen, 1993, Preston and Sell, 1988, D'Antonio et al., 1993). In the invaded ecosystems, *Carpobrotus* spp. seem to be rather released from predators, parasites and diseases (Van Grunsven et al., 2009, Donaldson et al., 1978, Maltez-Mouro et al., 2010). Nevertheless, the South African insect *Pulvinariella mesembryanthemi* (Vallot) has been reported outside its native range, in areas invaded by its host plant *C. edulis* (including NW Spain, *pers. obs.*), apparently introduced accidentally with the plant (Washburn and Frankie, 1985). This insect is a soft scale (from the *Coccidae* family) that feeds mostly on Aizoaceae plants from the *Carpobrotus* genus (Washburn and Frankie, 1985). In California, during the 70s and 80s, *P. mesembryanthemi* caused major damages on *C. edulis* plantations (Donaldson et al., 1978). However, in order to preserve the plantations, the insect was effectively controlled after the introduction of Hymenoptera parasitic wasps and Coccinellidae predators from South Africa (Tassan et al., 1982). From another point of view, this soft scale insect has been proposed for the control of *C. edulis* where this plant is invasive (Fagúndez and Beiras, 2007, Washburn and Frankie, 1985), although so far – and to the best of our knowledge- it has not been implemented as a biocontrol agent.

This insect is parthenogenetic without sexual reproduction, even though some males are occasionally produced (Washburn and Frankie, 1985, Pesson, 1941). Mature females (4th instar) produce waxy ovisacs where they lay their eggs (up to 2,500) (Washburn and Frankie, 1985). When the eggs hatch, the new-born insects are in the only mobile stage of the species and they search feeding sites (Washburn and Frankie, 1985). However, long-distance dispersal is restricted to wind transport (Washburn et al., 1985). Afterwards, they pass through three immature instars, which have highly limited active mobility, and finally they mature and form the ovisacs (Washburn and Frankie, 1985).

To our knowledge, the population dynamics of this species was only studied in an introduced area in California, where it was found to be bivoltine (or even trivoltine in one of the studied populations) (Washburn and Frankie, 1985). According to these authors, in California they have one reproductive episode in spring and other in autumn, each of them with a duration of 6 to 12 weeks, although in laboratory optimal conditions (i.e. 24.5 °C) the insect can complete a generation in 3-4 months (Washburn and Frankie, 1985). High temperatures and sun exposure accelerate the development and ovisac formation, although during winter, the insects continue the development (Washburn and Frankie, 1985).

Field studies are crucial for the assessment of population dynamics in sessile scale insects, as they are usually very influenced by seasonal factors and by mutualists (ants), parasites and predators (Jha et al., 2009). These interactions with the environment could affect their potential as biocontrol agents.

In Spain, the invasion by *Carpobrotus* species began in Galicia (NW Spain) in the late 19th century (Lázaro-Ibiza, 1900). In this region, *C. edulis* and *C.* aff. *acinaciformis* are currently widespread along the Galician coast *(pers. obs.)*. In several *Carpobrotus* invaded areas of Galicia we found the presence of *P. mesembryanthemi*, which was firstly cited in Spain in the late 19th century (Douglas, 1887). The distribution of *P. mesembryanthemi* in Galicia is more restricted than that of their host plants (*pers. obs.*), which may be due to a limitation either by dispersal or by the environmental conditions. The suitability of a biocontrol agent to an area where biocontrol is aimed can be predicted with distribution models (Sun et al., 2017). The biocontrol candidate should cover an extensive range of the invasive plant distribution (Sun et al., 2017).

Therefore, we aim (1) to model the potential distribution of the invasive *Carpobrotus* spp. and its proposed biocontrol agent *P. mesembryanthemi* in an invaded area (NW Spain), and (2) to study the population dynamics of the scale insect *P. mesembryanthemi* in this area, taking into account climatic (temperature, precipitations, irradiation) and biotic factors (host plant, predators, parasites and mutualists), which can affect its abundance and phenology.

5.2. Material and Methods

5.2.1. Predicted habitat suitability of *Carpobrotus* spp. and *P. mesembryanthemi*

Field surveys along the Galician coast were carried out to detect the presence of *C. edulis* (or its hybrid *C.* aff. *acinaciformis*) and *P. mesembryanthemi*. The distribution of both *Carpobrotus* species was assumed to be restricted to coastal areas (Fagúndez and Beiras, 2007). To create the models, 50 environmental variables, in the form of digital raster maps with a resolution of 250 m, have been used (Supplementary Material SM Table 5.1) (Rodríguez-Lado et al., 2018).

The maximum entropy (MaxEnt) software version 3.3.3e (Phillips et al., 2006) and the 'dismo' version 1.1-4 were used to model the spatial distribution of both *C. edulis* and *P. mesembryanthemi*. For evaluation of the *Carpobrotus* spp. model, 35 presence records were used as training data, and the remaining 15 were used for validation. For *P. mesembryanthemi*, models were calibrated using 10 presence records as training data, and the remaining 4 to validate. The models were calibrated using a random set of 84 locations within the studied region which define the background environmental conditions. The accuracy of the models was estimated with the area under the curve (AUC). The contribution of each environmental variable was evaluated by jackknifing. The variables were considered independently to measure their relative and absolute contribution to the model. The potential distribution maps were processed with ArcGIS 10.5.1 (ESRI, Redlands, CA, USA).

5.2.2. Population dynamics of *P. mesembryanthemi*

Between 2015 and 2017, four localities from Galicia (Coruña, Sálvora, Nariga, Mera; see Table 5.1 for details) were chosen to study the population dynamics of the scale insect *P. mesembryanthemi*, the influence of parasites, predators and mutualists, and its effect over the host plant *C. edulis*. In each locality, one to four study sites of 20 m^2 were stablished, selected by the presence of mats of *C. edulis* infested by *P. mesembryanthemi*.

These populations of *P. mesembryanthemi* were regularly examined every 1-2 months (depending on the dynamics of the population in that period). The study of the *P. mesembryanthemi* populations started between

Table 5.1. Main characteristic and mean temperature and mean annual rainfall of the last 10 years (mean±SE) of the localities included in this study.

	Nariga	Sálvora	Coruña	Mera
Study areas	N1, N2, N3, N4	S1, S2, S3, S4	C1, C2,C3	M1
Province		A Coruña		
UTM coordinates (29T grid zone)	507309(x) - 4796386(y)	498894(x) - 4701427(y)	545369(x)- 4803242(y)	553432(x) - 4803426(y)
Geographical coordinates	43º19'13'' N - 8º54'34'' W	42º27'55'' N - 9º0'49'' W	43º22'49" N - 8º26'26" W	43º22'54" N - 8º20'25" W
Altitude (m asl)	35-40	20-25	20-30	6
Distance to sea (m)	80-120	55-130	40-60	12
Orientation of the study areas	W or S	W or S	NW	SE
Mean temperature (˚C)	13.4 ± 0.3	15.1 ± 0.3	14.2 ± 0.3	14.2 ± 0.3
Annual rainfall (mm)	944 ± 70	1208 ± 97	681 ± 47	681 ± 47

July and October of 2015 and ended in October 2017 (for the Sálvora sites) or December 2017 (for the other sites).

Within each study site, we checked the following parameters aided by a quadrat of 50x50 cm, which was thrown undirected 10 times in each survey:

a. Number of *P. mesembryanthemi* insects in each development stage [as described by Washburn and Frankie (1985)]: ovisacs, nymphs (1st-3rd instar) and adults (4th instar).
b. Number of *P. mesembryanthemi* parasitized by Hymenopteran wasps per developmental stage; number and developmental stage of scales predators (Coccinellidae) and presence of mutualists (ants).
c. Effects of the infestation on the visual aspect of *C. edulis* (evaluated with a subjective visual scale of 1-9 where 1 would correspond to a perfectly healthy plant and 9 to a death plant), fruit abortion (% with respect to the sum of aborted and healthy mature fruits) and necromass (% of the surface of the quadrat occupied by *C. edulis* necromass).
d. Presence of other parasites (mealybugs, spittlebugs, aphids), predators (rabbits, rats, snails) or diseases (virus symptoms) of the host plant.

Climatic data was collected from nearby meteorological stations (*http://www.meteogalicia.gal/*) to study the effect of temperature, precipitation and irradiation on the abundance and population dynamics of *P. mesembryanthemi* and its predators and parasites. The temperature and irradiation means and the rainfall sum of the 30 days before each field survey data collection for the different localities were calculated.

The non-parametric Kruskal-Wallis test with a *post hoc* Dunn test were used to study the factors affecting *P. mesembryanthemi* density, its parasitism and the host plant. Only data from 2016 and 2017 was used in this analysis, as it was the time period in which we had data of all location so we could compare them. Spearman's correlation test was used to evaluate the influence of meteorological and biotic factors on *P. mesembryanthemi* density, parasitism, ants' presence and the host plant. Significance was set at $P \leq 0.05$ in all the analysis. All tests were conducted using IBM SPSS Statistics 23.

5.3. RESULTS

5.3.1. Predicted habitat suitability of Carpobrotus *spp.* and *P. mesembryanthemi*

The predictive maps for *Carpobrotus* spp. and *P. mesembryanthemi* revealed the most suitable areas for these species included the entire Galician coast in both cases, with little differences between the potential distribution of the alien plant and the insect (Fig. 5.1). The model accuracy was evaluated by using the area under the curve (test AUC ± SE) for the models of *Carpobrotus* spp. (AUC=0.828±0.036) and *P. mesembryanthemi* (AUC=0.859±0.035) (see SM Fig. 5.1, SM Fig. 5.2). The AUC of the training and test data are similar, which indicates a good performance of the model predictions.

The Maximum Entropy model (MaxEnt) and the Jackknife test revealed that the major contributing factors determining *Carpobrotus* spp. distribution were altitude (70.0 %), the thermal regime of the soil (10.8 %), the number of consecutive days with moist soil and Temp > 8 °C (7.9 %) and the maximum temperature in January (5.2 %) (see SM Table 5.1). The first three contributing variables accounted for 88.7 % of the explanatory ability of the model. The variable with the highest gain when used alone was the altitude, while the number of consecutive days with moist soil and Temp > 8 °C was the variable that included a higher rate of explanatory performance that was not present in the other variables (SM Fig. 5.3).

The variables that contributed the most to the *P. mesembryanthemi* model were the number of consecutive days with moist soil and Temp > 8 °C (72.2 %), the continentality index compensated by altitude (9.2 %), the ombrothermic index (from May to August) (4.6 %) and the minimum temperature in January (4.5%) (see SM Table 5.1). The top three contributing variables sum a cumulative contribution rate of 86.0 %. For the *P. mesembryanthemi* model, altitude was both the variable with the highest gain when used alone and the variable with valuable amounts of explanatory information that were not present in the other variables (SM Fig. 5.4).

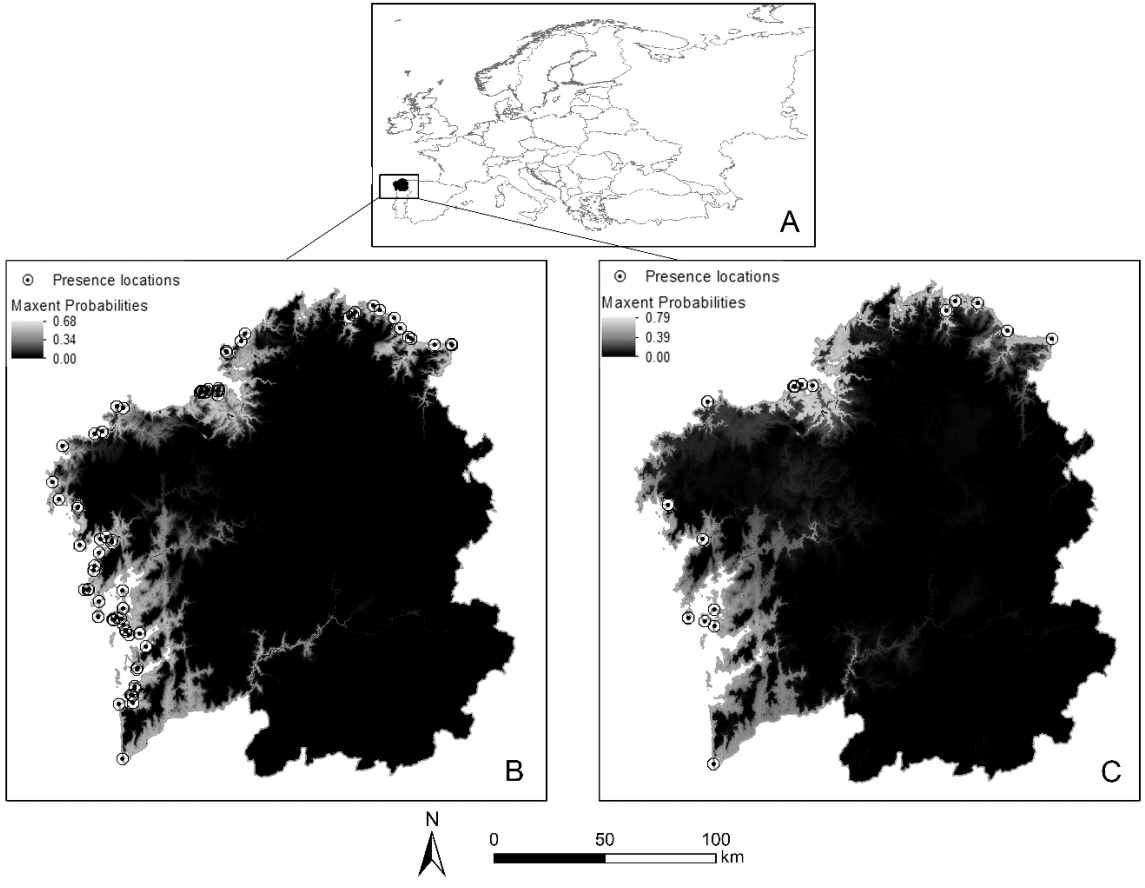


Fig. 5.1. Known occurrence points and probability of occurrence (predicted by MaxEnt algorithm) of *Carpobrotus* spp. (B) and *P. mesembryanthemi* (C) in Galicia. Map A shows the location of Galicia in Europe.

5.3.2. Population dynamics of *P. mesembryanthemi*

5.3.2.1. Phenology and abundance of P. mesembryanthemi

Insect density was significantly affected by locality, season and meteorological parameters (mean temperature, cumulative precipitation and mean irradiation from the last 30 days) (Table 5.2). Mera was the most heavily infested locality, followed by Nariga (Table 5.3). The abundance of the insect was also the highest in summer and when mean temperatures were higher than 16 °C, the cumulative precipitation of the last month was lower than 20 L $\cdot$ m^{-2} and irradiation was higher than 2000 W $\cdot$ m^{-2} (Table 5.3). When only taking into account 1st to 4th instars, irradiation was not significant (SM Table 5.2). However, ovisacs were significantly affected by irradiation (SM Table 5.2), being more abundant when the mean irradiation was over 2000 W $\cdot$ m^{-2} (Table 5.4). All the other studied factors were significant both for immatures and ovisacs (data not provided). Correlations between abundance of *P. mesembryanthemi* and temperature, rainfall and irradiation were of 0.16, -0.42 and 0.21 respectively (Table 5.5).

Table 5.2. Results of the Kruskal-Wallis test to assess the effect of locality, sampling season, mean temperature, cumulative rainfall and mean irradiation of the past month on *Pulvinariella mesembryanthemi* density (individuals $\cdot$ m^{-2}) from December 2015 to December 2017.

	ALL			MERA			SÁLVORA			CORUÑA			NARIGA		
Factors	stat.	d.f.	sign.	stat.	d.f.	sign.	stat.	d.f.	sign.	stat.	d.f.	sign.	stat.	d.f.	sign.
Locality	63.18	3	<0.001		-			-			-			-	
Season	44.56	3	<0.001	8.50	3	0.037	12.79	3	0.005	22.00	3	<0.001	30.86	3	<0.001
Temperature	21.84	2	<0.001	1.07	2	0.587	3.18	2	0.204	12.66	2	0.002	33.91	2	<0.001
Rainfall	20.94	2	<0.001	0.49	2	0.781	4.48	2	0.107	12.16	2	0.002	13.78	2	0.001
Irradiation	9.13	2	0.010	2.54	2	0.281	1.67	2	0.434	6.13	2	0.047	44.25	2	<0.001

Table 5.3. Pairwise comparisons between levels of the different factors [locality, season, mean temperature (˚C), cumulative rainfall (mm) and mean irradiation (W · m^{-2}) of the last 30 days] for the *P. mesembryanthemi* density (individuals · m^{-2}) from December 2015 to December 2017. The mean rank value, the arithmetic mean and the standard error (mean±SE) are included. Different letters correspond to significant differences by Dunn's post- hoc test at p=0.05.

		ALL		MERA		SÁLVORA		CORUÑA		NARIGA	
	Levels	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE
Locality	Mera	202.1a	534.0 ± 86.6								
	Sálvora	81.9c	27.8 ± 13.0								
	Coruña	91.6c	23.4 ± 7.0								
	Nariga	124.9b	70.6 ± 23.7								
Season	spring	73.5b	29.18 ± 13.4	7.0[a]	317.8 ± 41.8	24.8[b]	1.9 ± 1.4	14.1[b]	0.4 ± 0.2	27.1[b]	5.3 ± 2.0
	summer	152.7a	167.9 ± 33.8	11.5[a]	562.5 ± 98.3	47.3[a]	84.9 ± 42.0	42.1[a]	59.6 ± 23.0	57.5[a]	206.2 ± 65.8
	fall	101.7b	48.52 ± 22.9	7.3[a]	423.3 ± 196.2	30.0[ab]	2.7 ± 1.4	29.9[a]	20.8 ± 7.4	27.9[b]	6.2 ± 1.4
	winter	108.2b	124.3 ± 65.6	17.3[a]	1396 ± 431.1	40.3[ab]	6.9 ± 3.0	30.2[ab]	6.4 ± 2.6	28.5[b]	4.5 ± 1.0
Temp.	9.0-11.9	90.6b	45.98 ± 23.3	10.8[a]	542.4 ± 174.8	29.2[a]	8.0 ± 5.1	20.9[b]	3.5 ± 2.0	23.7[b]	3.5 ± 0.7
	12-15.9	98.7b	101.2 ± 41.8	11.8[a]	860.8 ± 268.5	34.6[a]	2.8 ± 0.9	23.3[b] b	6.3 ± 3.4	29.1[b]	6.3 ± 1.5
	16-20	136.0a	118.1 ± 23.7	8.7[a]	407.5 ± 84.8	40.9[a]	54.2 ± 27.0	38.0[a]	48.3 ± 15.2	56.0[a]	178.6 ± 57.7
Rainfall	0-19.9	144.5a	120.2 ± 32.8	11.7[a]	567.7 ± 102.1	46.1[a]	20.0 ± 6.9	42.3[a]	66.7 ± 28.4	49.3[a]	92.7 ± 57.5
	20-99.9	110.1b	109.8 ± 30.7	9.6[a]	680.7 ± 248.6	34.1[a]	48.0 ± 27.1	27.3[b]	14.3 ± 4.2	40.4[a]	101.5 ± 40.9
	100-250	88.1b	45.95 ± 26.0	11.0[a]	639.3 ± 257.2	33.3[a]	2.1 ± 0.6	18.1[b]	1.9 ± 1.2	24.3[b]	3.9 ± 0.8
Irrad.	400-1199	96.9b	94.56 ± 37.9	12.6[a]	899.2 ± 263.3	35.8[a]	5.3 ± 2.6	24.0[a]	6.3 ± 2.8	24.0[b]	4.0 ± 0.8
	1200-1999	117.2ab	92.79 ± 24.9	8.0[a]	382.7 ± 88.1	31.4[a]	3.4 ± 1.6	31.1[a]	34.5 ± 11.9	41.3[a]	109.5 ± 56.5
	2000-2800	127.1a	98.42 ± 26.5	11.0[a]	538.4 ± 142.5	39.5[a]	54.0 ± 27.0	39.0[a]	48.7 ± 32.7	51.3[a]	121.4 ± 54.0

Table 5.4. Pairwise comparisons between levels of mean irradiation (W · m^{-2}) of the last 30 days for ovisacs and 1st-4th instars of *Pulvinariella mesembryanthemi* (individuals · m^{-2}) for the different localities between December 2015 and December 2017. The mean rank value, the arithmetic mean and the standard error (mean±SE) are included. Different letters correspond to significant differences by Dunn's post- hoc test at p=0.05.

		ALL		MERA		SÁLVORA		CORUÑA		NARIGA	
	Irradiation	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE
ovisacs	400-1199	87.30[b]	1.96 ± 0.86	8.94[a]	18.45 ± 6.71	28.50[b]	0.00 ± 0.00	22.94[b]	0.12 ± 0.08	23.87[b]	0.24 ± 0.16
	1200-1999	105.39[b]	5.96 ± 2.51	11.06[a]	38.56 ± 16.22	32.66[b]	0.15 ± 0.13	27.95[b]	0.50 ± 0.27	31.55[b]	3.30 ± 2.08
	2000-2800	151.01[a]	8.10 ± 2.39	13.67[a]	62.80 ± 42.55	44.42[a]	1.45 ± 0.57	49.61[a]	3.13 ± 1.00	59.50[a]	12.00 ± 2.61
instars	400-1199	106.12[a]	92.79 ± 37.26	12.67[a]	880.8 ± 261.0	37.75[a]	5.30 ± 2.56	26.37[a]	6.83 ± 2.73	30.45[b]	3.73 ± 0.76
	1200-1999	123.18[a]	86.83 ± 23.46	7.88[a]	344.2 ± 77.98	33.28[a]	3.30 ± 1.46	31.43[a]	33.98 ± 11.77	47.00[a]	106.2 ± 55.80
	2000-2800	109.10[a]	90.31 ± 25.11	11.00[a]	475.6 ± 116.6	37.17[a]	52.50 ± 26.89	31.22[a]	45.56 ± 32.35	38.40[ab]	109.4 ± 51.78

In Mera, *P. mesembryanthemi* was clearly bivoltine, with one short cycle (5 months) approximately from June to November (with a peak in June) and other longer cycle (7 months) from November to June (with a peak in November-December) (Fig. 5.2). Insect density appeared to decrease year after year (Fig. 5.2). For each year, the number of insects appears to be slightly higher in the winter-spring cycle than in the summer-fall cycle (Fig. 5.2). Insect abundance was not significantly affected by season, mean temperature, cumulative precipitation or irradiation (Table 5.2; SM Table 5.2).

Table 5.5. Spearman's rank correlation test (corr. coef., correlation coefficient; p, significance; N, number of cases) between the abundance of *Pulvinariella mesembryanthemi* (ovisacs, instars or all life stages; individuals $\cdot m^{-2}$) and mean temperature (°C), cumulative rainfall (mm) and mean irradiation ($W \cdot m^{-2}$) of the preceding 30 days.

		Temperature			Rainfall			Irradiation		
	Locality	corr. coef.	p	N	corr. coef.	p	N	corr. coef.	p	N
ovisacs	All	**0.457**	<0.001	286	**- 0.526**	<0.001	273	**0.521**	<0.001	283
	Mera	0.173	0.337	33	**- 0.400**	0.028	30	**0.514**	0.004	30
	Sálvora	**0.575**	<0.001	107	**- 0.637**	<0.001	107	**0.646**	<0.001	107
	Coruña	**0.652**	<0.001	65	**- 0.553**	<0.001	65	**0.621**	<0.001	65
	Nariga	**0.612**	<0.001	81	**- 0.539**	<0.001	81	**0.698**	<0.001	81
instars	All	**0.278**	<0.001	273	**- 0.326**	<0.001	273	0.082	0.176	273
	Mera	0.089	0.659	27	0.059	0.770	27	- 0.207	0.300	27
	Sálvora	**0.404**	<0.001	100	**- 0.506**	<0.001	100	**0.302**	0.002	100
	Coruña	**0.421**	<0.001	65	**- 0.269**	0.030	65	0.039	0.756	65
	Nariga	**0.340**	0.002	81	- 0.172	0.125	81	0.010	0.932	81
all stages	All	0.156	0.010	273	**- 0.416**	<0.001	273	**0.207**	0.001	273
	Mera	0.109	0.589	27	0.021	0.920	27	- 0.167	0.406	27
	Sálvora	**0.439**	<0.001	100	**- 0.518**	<0.001	100	**0.362**	<0.001	100
	Coruña	**0.509**	<0.001	65	**- 0.382**	0.002	65	0.191	0.127	65
	Nariga	**0.593**	<0.001	81	**- 0.435**	<0.001	81	**0.348**	0.001	81

In the other localities (Sálvora, Coruña, Nariga) the insect had a summer-fall cycle (between approx. June and October) and a winter-spring cycle (between approx. November and June) (Figs. 5.3-5.5). However, the density of the populations deeply dropped in winter and in some cases the existence of a winter cycle was not clear (e.g. winter of 2015 in Sálvora or winter 2016 in Nariga; see Figs. 5.3-5.5), so the insect might be overwintering in an immature stage. In the year 2016, the density of the insect was by far the lowest from the studied period in Sálvora and Nariga.

In Sálvora, insect abundance was dependent on the season (being higher in summer than in spring), but not on the meteorological variables (Table 5.3). In Coruña and Nariga insect density was significantly affected by season (being the highest in summer and fall or in summer respectively), mean temperature (being higher with temperatures over 16 °C), cumulative rainfall (being higher when it was below 20 or 100 mm in the last month, respectively) and mean irradiation (being higher when it was over 1200 $W \cdot m^{-2}$ in Nariga) (Table 5.3). Ovisacs were more abundant when irradiation was higher than 2000 $W \cdot m^{-2}$ in Sálvora, Coruña and Nariga (Table 5.4). Irradiation only affected significantly the abundance of the instar stages in Nariga (Tables 5.4). Correlations between abundance of *P. mesembryanthemi* and temperature, rainfall and irradiation for Sálvora, Coruña and Nariga were higher for ovisacs than for instars, especially for irradiation (Table 5.5).

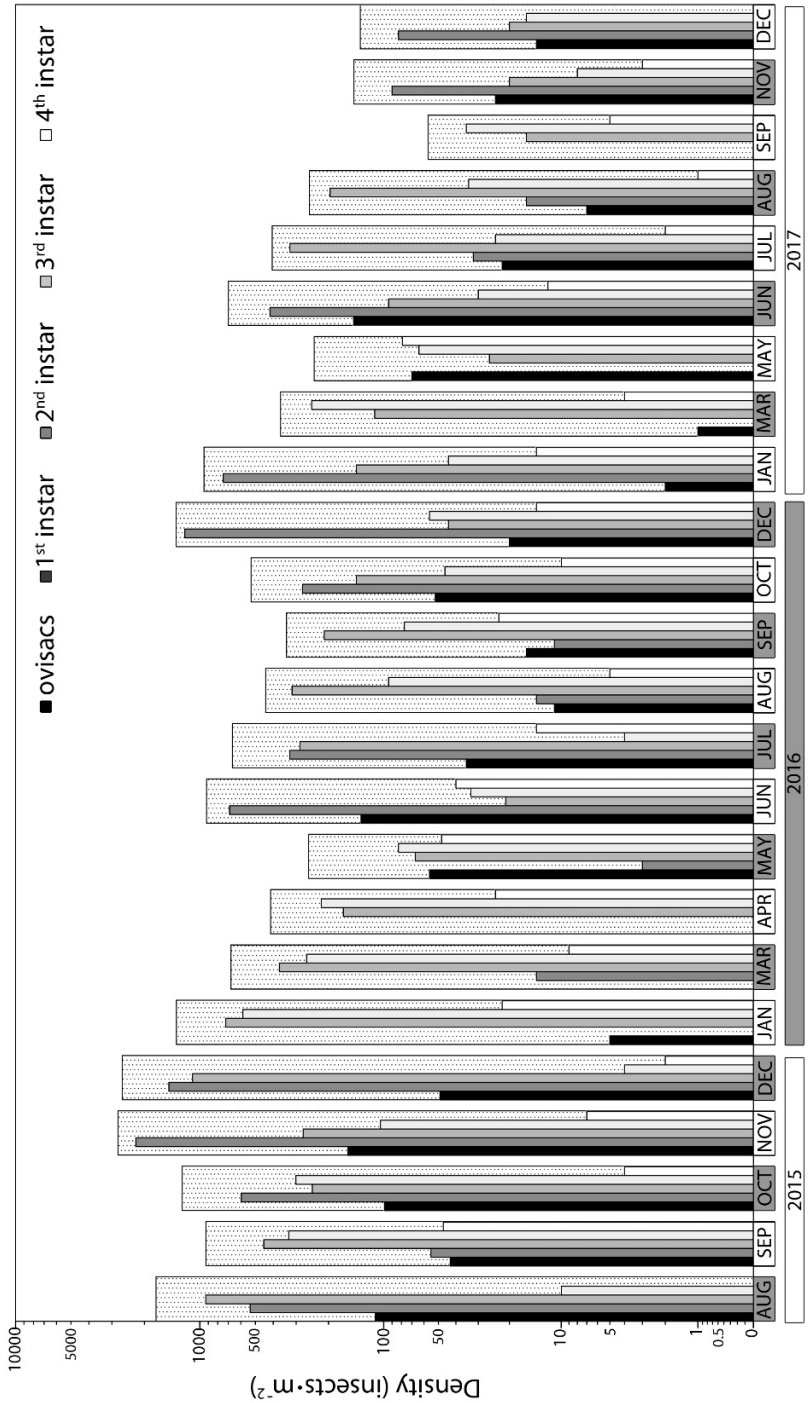


Fig. 5.2. Density of *Pulvinariella mesembryanthemi* (individuals · m^{-2}) in Mera's study area from August 2015 to December 2017. Narrow columns represent the density of the different life stages (ovisacs and 1st to 4th instars). Wide dotted columns represent the total number of *Pulvinariella mesembryanthemi* per m^2. Note that the Y-axis is in a logarithmic scale and that not all months are represented (sampling was done monthly or bimonthly).

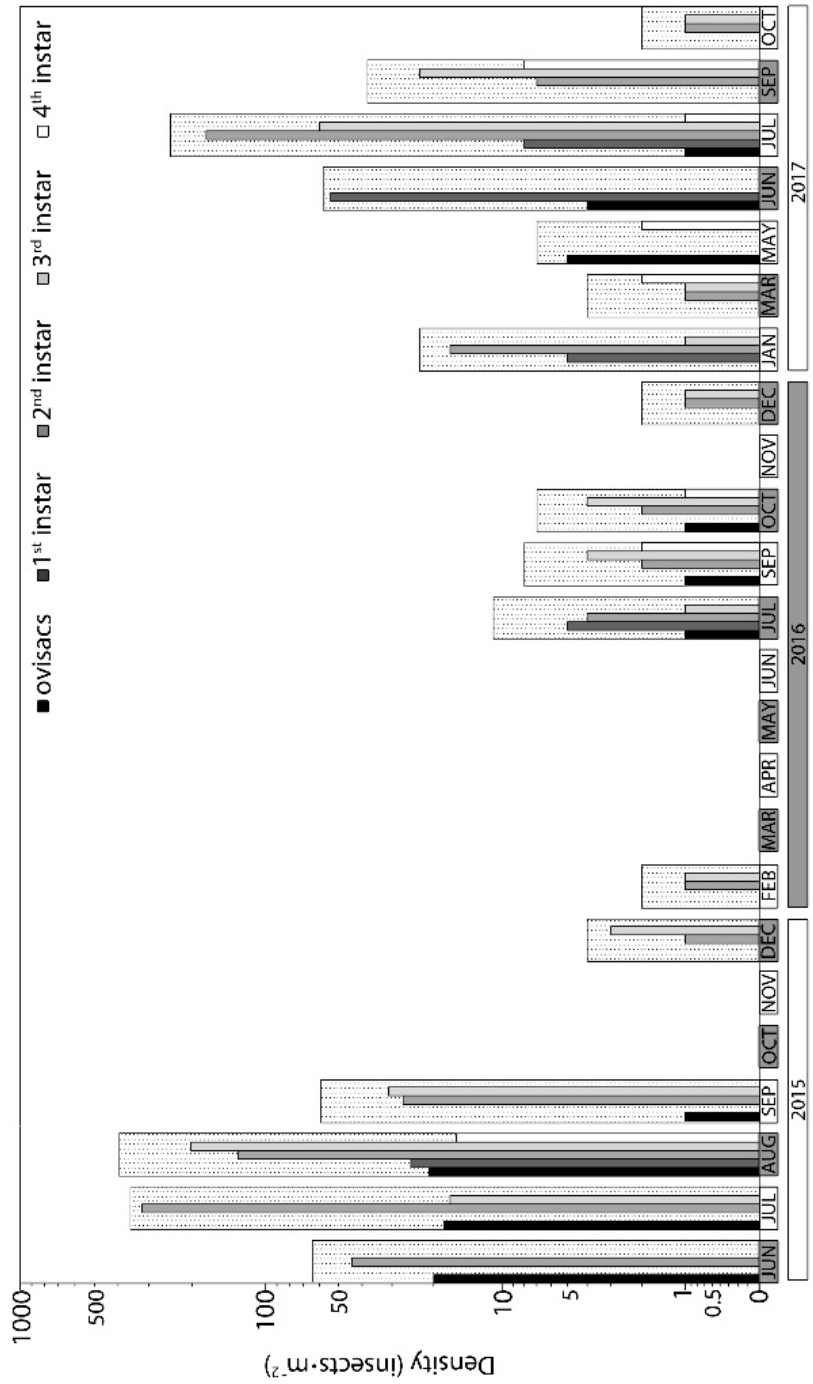


Fig. 5.3. Mean density of *Pulvinariella mesembryanthemi* (individuals · m^{-2}) in Salvorá's study areas from June 2015 to October 2017. Narrow columns represent the density of the different life stages (ovisacs and 1st to 4th instars). Wide dotted columns represent the total number of *Pulvinariella mesembryanthemi* per m^2. Note that the Y-axis is in a logarithmic scale and that not all months are represented (sampling was done monthly or bimonthly).

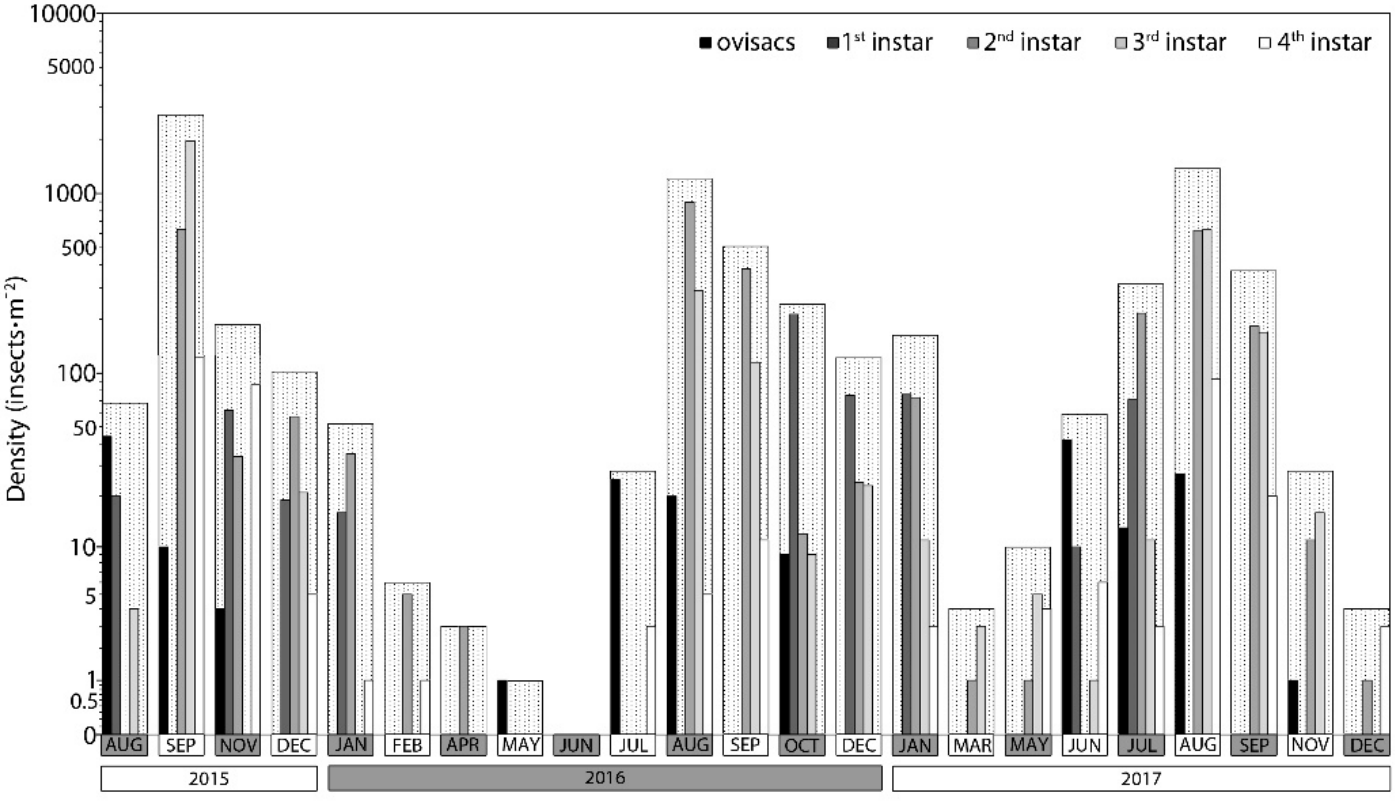


Fig. 5.4. Mean density of *Pulvinariella mesembryanthemi* (individuals · m^{-2}) in Coruña's study areas from August 2015 to December 2017. Narrow columns represent the density of the different life stages (ovisacs and 1st to 4th instars). Wide dotted columns represent the total number of *Pulvinariella mesembryanthemi* per m^2. Note that the Y-axis is in a logarithmic scale and that not all months are represented (sampling was done monthly or bimonthly).

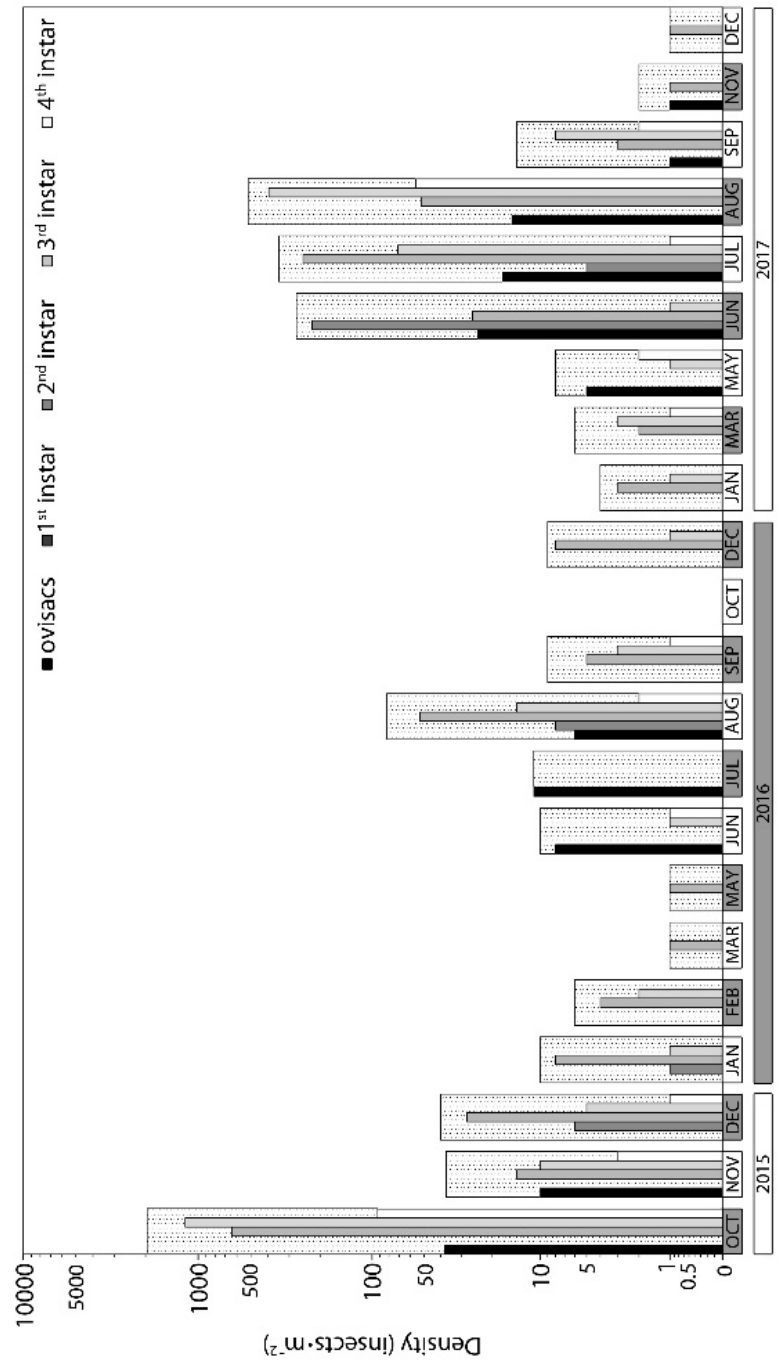


Fig. 5.5. Mean density of *Pulvinariella mesembryanthemi* (individuals · m^{-2}) in Nariga's study areas from October 2015 to December 2017. Narrow columns represent the density of the different life stages (ovisacs and 1st to 4th instars). Wide dotted columns represent the total number of *Pulvinariella mesembryanthemi* per m^2. Note that the Y-axis is in a logarithmic scale and that not all months are represented (sampling was done monthly or bimonthly).

5.3.2.2. Biotic interactions with P. mesembryanthemi

Parasitism of *P. mesembryanthemi* by Chalcidoidea wasps was found in all the localities throughout the study period. The percentage of parasitism depended on the site, the development stage of the insect, the season, mean temperature and cumulative rainfall (SM Table 5.3). Mera was the locality with highest parasitism (SM Table 5.4) and where parasitized individuals were found throughout all the study period (Fig. 5.6). Although parasitized individuals of all stages (from 1st to 4th instar) were observed, the 3rd and 4th instars where the most affected (SM Table 5.4). Parasitism was the highest in summer and fall seasons (SM Table 5.4). High temperatures and low precipitations were also related to a higher parasitism (SM Table 5.4). The density of *P. mesembryanthemi* immatures was positively correlated with the percentage of parasitism (SM Table 5.5). Mean temperature and cumulative rainfall were significantly but weakly correlated with parasitism (SM Table 5.5). We observed holes in the scales corresponding to wasp emergences mostly on the ovisac stage, although some emergences occurred in immature scales.

In Mera, the parasitized scales seemed to develop, in some cases, slower than their non-parasitized congeners. Thus, in the cycles beginning in summer from 2016 and 2017, the 2nd, 3rd and 4th instars achieved their highest density quicker (1-2 months earlier) when they were not parasitized (Fig. 5.6). However, for the winter cycles of 2016 and 2017, this pattern was only found for the 3rd (in 2017) and 4th instar (in both years) (Figs. 5.6). The cycles of parasitized and non-parasitized individuals of *P. mesembryanthemi* were not represented for the other localities because of the much lower number of insects.

Predation of *P. mesembryanthemi* by Coccinellidae insects was punctually found in Nariga and Coruña (by *Chilocorus bipustulatus* (L.)) and Sálvora (by *Exochomus* sp. and *Nephus quadrimaculatus* (Hbst.)). The Coccinellidae presence was most intense in Nariga, where it concurred with the peaks on *P. mesembryanthemi* density (Fig. 5.7). In Sálvora, predators were only found during the first year (Fig. 5.8), whilst their presence in Coruña was very sporadic.

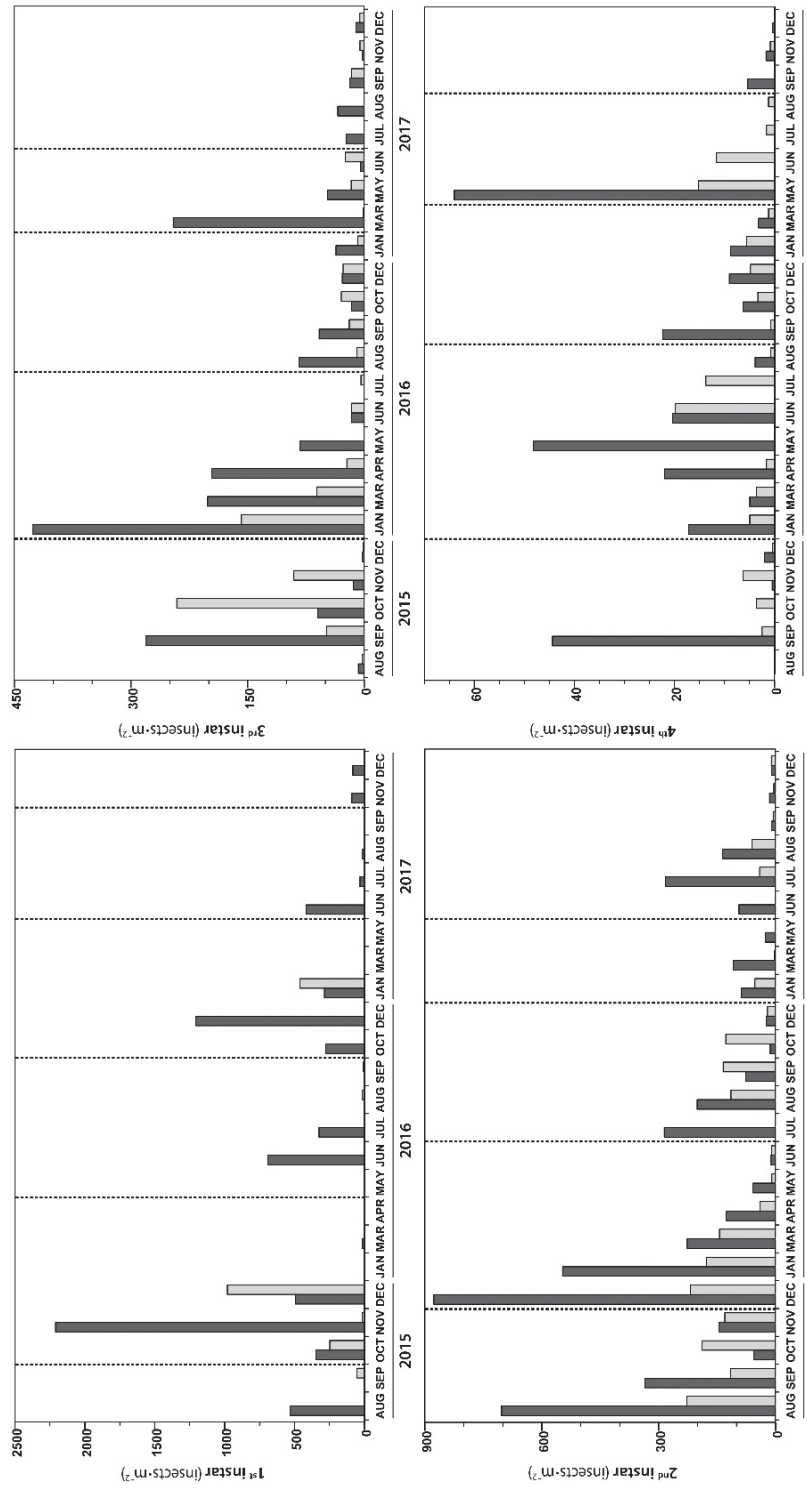


Fig 5.6. Density of non-parasitized (dark gray) and parasitized (light gray) *P. mesembryanthemi* in Mera's study area. Dashed lines approximately separate the different life cycles of the insect from August 2015 to December 2017.

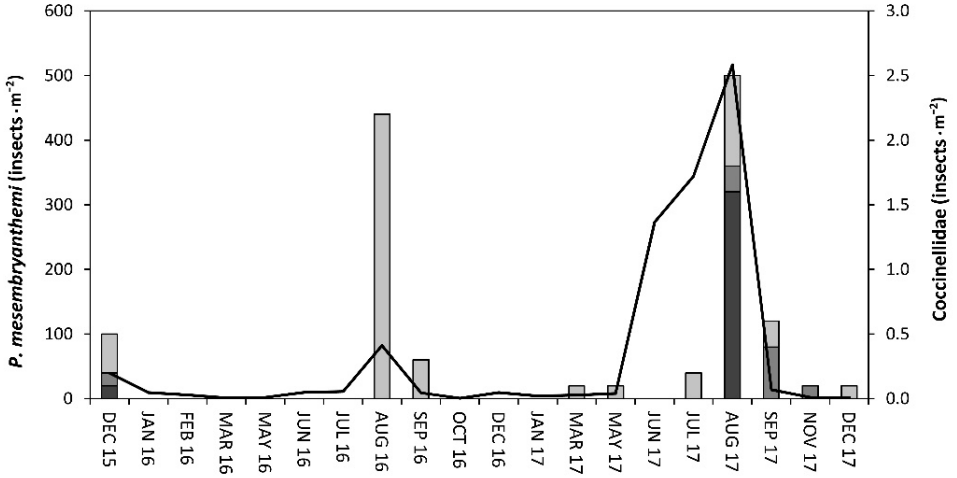


Fig. 5.7. Mean density of *P. mesembryanthemi* (black line) and its Coccinellidae predators [columns: dark gray in larvae stage, medium gray in pupae stage, light gray in adult stage] in Nariga's study areas from December 2015 to December 2017.

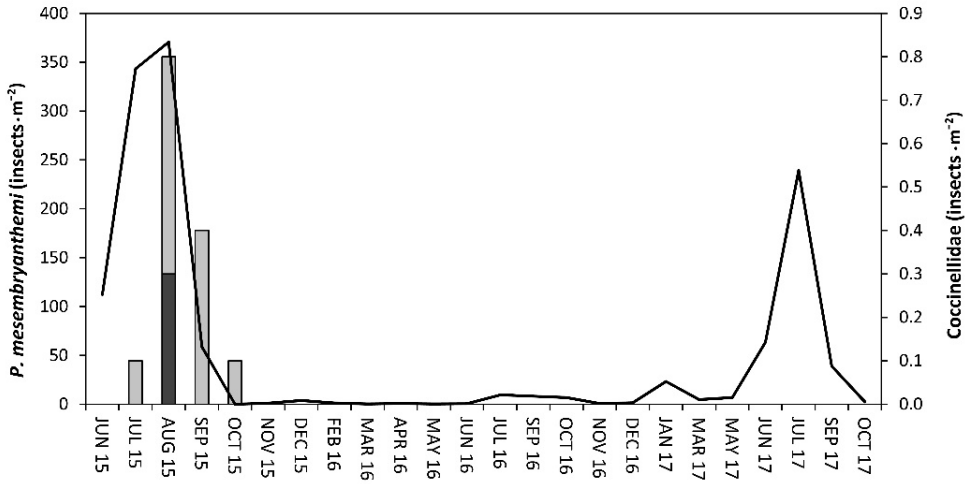


Fig 5.8. Mean density of *P. mesembryanthemi* (black line) and its Coccinellidae predators [columns: dark gray in larvae stage, light gray in adult stage] in Sálvora from June 2015 to October 2017.

Ant presence was positively correlated with *P. mesembryanthemi* presence in Mera, Sálvora and Coruña, but no significant correlation was found between ant presence and parasitism percentage for any of the localities (Table 5.6). Sooty mould was sporadically found in heavily-infested plants (from 700 to 1900 immatures $\cdot m^{-2}$) of Sálvora, Mera (in August 2015) and Nariga (in October 2015).

Table 5.6. Spearman's rank correlation test (coef., correlation coefficient; p, significance; N, number of cases) between ants' presence and *Pulvinariella mesembryanthemi* (PME) presence, Hemiptera Sternorrhynca presence or instars parasitism percentage in the four studied localities.

Ants vs.:	Mera			Sálvora			Coruña			Nariga		
	coef.	p	N	coef.	p	N	coef.	p	N	coef.	p	N
PME	**0.588**	0.001	27	**0.305**	0.003	95	**0.297**	0.016	65	0.137	0.223	81
Hemiptera	**0.576**	0.002	27	**0.517**	<0.001	95	**0.534**	<0.001	65	0.182	0.104	81
Instar parasit.	-0.185	0.356	27	0.032	0.801	63	0.031	0.834	49	0.049	0.681	74

5.3.2.3. Effects of P. mesembryanthemi *on* C. edulis

Pulvinariella mesembryanthemi abundance significantly affected its host visual aspect, percentage of necromass and percentage of aborted fruits (Table 5.7), having the plants with lower densities of the insect a healthier aspect and a lower proportion of necromass and aborted fruits (Table 5.8). Insect abundance was positively and significantly correlated with plant's visual aspect (Spearman's rho coefficient=0.160, N=273, p=0.008), percentage of necromass (Spearman's rho coefficient=0.285, N=273, p<0.001) and percentage of aborted fruits (Spearman's rho coefficient=0.274, N=158, p<0.001). Climatic factors also affected these three plant parameters (Table 5.7), having plants healthier aspect when cumulative rainfall was intermediate and having lower necromass and aborted fruit percentages when temperature and irradiation were low and rainfall high (Table 5.8).

Table 5.7. Results of the Kruskal-Wallis test (stats., test statistic; d.f., degrees of freedom; p, significance) of the effects of mean temperature, cumulative rain and mean irradiation of the preceding month and *Pulvinariella mesembryanthemi* abundance (PME) on *Carpobrotus edulis* plants' visual aspect (evaluated with a subjective scale), percentage of necromass and percentage of aborted fruits.

Factors	Visual aspect score			% necromass			% aborted fruits		
	stats.	d.f.	p	stats.	d.f.	p	stats.	d.f.	p
Temperature	1.71	2	0.425	15.46	2	**<0.001**	5.06	2	0.080
Rainfall	20.37	2	**<0.001**	14.94	2	**0.001**	24.22	2	**<0.001**
Irradiation	0.07	2	0.967	15.73	2	**<0.001**	37.61	2	**<0.001**
PME	9.31	2	**0.010**	35.93	2	**<0.001**	10.82	2	**0.004**

Table 5.8. Pairwise comparisons between levels of the different factors [mean temperature (°C), cumulative rainfall (mm) and mean irradiation ($W \cdot m^{-2}$) of the last 30 days and *Pulvinariella mesembryanthemi* abundance (PME) (individuals $\cdot m^{-2}$)] for *Carpobrotus edulis* visual aspect (subjective scale of 1-9 where 1 would correspond to a perfectly healthy plant and 9 to a death plant), % of necromass and % of aborted fruits. The mean rank value, the arithmetic mean and the standard error (mean±SE) are included. Different letters correspond to significant differences by Dunn's post- hoc test at p=0.05.

		Visual aspect (score)		Necromass (%)		Aborted fruits (%)	
Factor	Levels	mean rank	mean ± SE	mean rank	mean ± SE	mean rank	mean ± SE
Temp	9.0-11.9	147.3^{a}	4.4 ± 0.2	118.9^{b}	20.4 ± 4.9	56.2^{a}	12.5 ± 10.0
	12.0-15.9	148.7^{a}	4.4 ± 0.1	123.3^{b}	22.5 ± 2.0	74.0^{a}	30.3 ± 7.4
	16.0-20.0	135.5^{a}	4.4 ± 0.1	159.6^{a}	30.7 ± 1.4	84.9^{a}	24.5 ± 3.2
Rainfall	0-19.9	154.2^{a}	4.6 ± 0.1	170.8^{a}	33.1 ± 1.9	101.2^{a}	34.5 ± 4.9
	20.0-99.9	119.9^{b}	4.2 ± 0.1	129.0^{b}	25.3 ± 1.6	70.7^{b}	21.7 ± 4.1
	100-250	171.2^{a}	5.0 ± 0.2	128.1^{b}	23.9 ± 3.6	53.4^{b}	9.3 ± 6.1
Irradiation	400-1199	143.5^{a}	4.3 ± 0.1	116.7^{b}	19.4 ± 2.0	47.0^{c}	8.1 ± 4.6
	1200-1999	141.7^{a}	4.5 ± 0.1	146.7^{a}	30.4 ± 2.1	77.1^{b}	22.0 ± 4.9
	2000-2800	140.5^{a}	4.4 ± 0.1	161.6^{a}	31.0 ± 1.6	101.5^{a}	37.3 ± 4.7
PME	0-19	127.4^{b}	4.1 ± 0.1	118.6^{b}	22.9 ± 1.5	69.2^{b}	21.0 ± 4.2
	20-79	140.1^{ab}	4.5 ± 0.2	140.6^{b}	30.3 ± 3.0	82.9^{ab}	23.4 ± 6.9
	>80	163.5^{a}	4.7 ± 0.1	189.7^{a}	35.2 ± 1.9	94.3^{a}	32.7 ± 5.1

5.3.2.4. Biotic interactions with C. edulis

Regarding the potential predators of *C. edulis*, there were evidences of rabbit (*Oryctolagus cuniculus* (Linnaeus, 1758)) and rat (*Rattus rattus* (Linnaeus, 1752)) presence on Sálvora and Nariga, respectively. Also, snails (i.e. *Theba pisana* (Müller, 1774), *Helix* spp.) were found recurrently on *C. edulis* plants in the four localities included in the study, being more abundant in Mera.

Eaten fruits of *C. edulis* were found in all localities, being fruit predation much higher in Sálvora (48%) than in the other localities (3.5 - 26.4 %). Seeds of *C. edulis* were found on rabbit dejections. In Sálvora, fruits were preferentially predated in the drier periods (Fig. 5.9). In this locality, flowers were sporadically predated.

Other Hemiptera insects were found feeding on *C. edulis* plants: aphids (*Aphis* sp.) (in Sálvora, Coruña and Nariga, and very sporadically in Mera), spittlebugs (*Philaenus spumarius* (Linnaeus, 1758)) (in Sálvora, and sporadically in Coruña and Nariga) and mealybugs (*Pseudococcus* sp.) (in Mera). Aphids and mealybugs belong, like *P. mesembryanthemi*, to the Sternorrhyncha suborder. When the presence of either of these three Sternorrhyncha insects was taken into account, Spearman's rho coefficient explained around half of the variance in ants' presence from Mera, Sálvora and Coruña (Table 5.6). Flower distortion, reduction in flower size, deformation and chlorotic patterns in leaves were observed on *C. edulis* plants in Sálvora and Nariga.

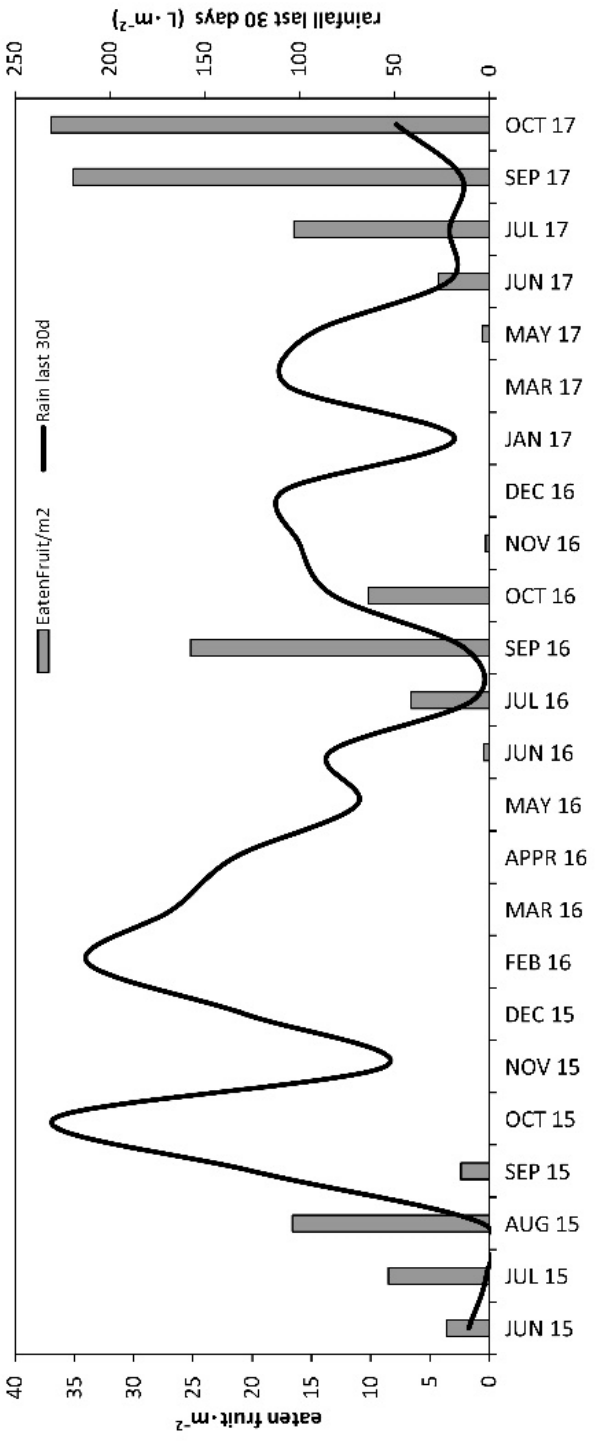


Fig. 5.9. Abundance of predated fruits (grey columns) in Sálvora from June 2015 to October 2017 and cumulative rainfall in the preceding 30 days to each sampling date (black line).

5.4. DISCUSSION

5.4.1. Predicted habitat suitability of *Carpobrotus* spp. and *P. mesembryanthemi*

The MaxEnt models for *C. edulis* and *P. mesembryanthemi* had high Area under the Curve values (AIC>0.8), so both models achieve a good prediction performance (Araújo et al., 2005).

The potential distribution of *Carpobrotus* spp. was highly dependent on variables related to air and soil temperature (altitude, thermal regime of soil, number of consecutive days with moist soil and temperatures above 8 °C, maximum temperature in January), as expected for the intolerance of the plant to temperatures below -6 °C (GEIB, 2006).

The predicted distribution of *P. mesembryanthemi* was much dependent on soil water availability (number of consecutive days with moist soil and Temp > 8 °C, ombrothermic index from May to August). A higher water availability for the host plant is related to an increase in growth rate, ovisac length, fecundity and development rate of *P. mesembryanthemi* (Washburn et al., 1987). Soil and air temperature (number of consecutive days with moist soil and Temp > 8 °C, continentality, minimum temperature in January) also determined the distribution of the insect. Accordingly, the development of *P. mesembryanthemi* is highly dependent on temperature, being its growth rate the fastest at 24.5 °C (Washburn and Frankie, 1985).

Although *Carpobrotus* spp. invasion has been reported in Galicia only in the coast (Fagúndez and Beiras, 2007), the models of both *Carpobrotus* spp. and *P. mesembryanthemi* predicted their presence also inland, in some river valleys. Therefore, along with the environmental variables included in the model, other factors not included here such as salinity (as low to moderate soil salinity increases the competitiveness of *Carpobrotus* spp. (Varone et al., 2017)), biotic interactions, dispersal or host plant presence (in the case of *P. mesembryanthemi*) may also determine the distribution of the studied species.

Suitable habitats for *Carpobrotus* spp. and *P. mesembryanthemi* largely overlap. Therefore, the much more limited current distribution of *P. mesembryanthemi* than *Carpobrotus* spp. seems to be due to a limited dispersal. The insect *P. mesembryanthemi* has only active mobility in the

first stages of development, although long-distance dispersal is limited to wind and zoochory (Washburn and Frankie, 1981).

5.4.2. Phenology and abundance of *P. mesembryanthemi*

In the studied populations of NW Spain, *P. mesembryanthemi* is bivoltine, as in California (Washburn and Frankie, 1985). Although in optimal conditions the insects can have up to 4 generations per year (Washburn and Frankie, 1985), even in Mera (where the density of the insect was the highest throughout the year) no more than 2 generations per year were observed. As in California, at the start of each life cycle the density of *P. mesembryanthemi* peaked. The highest densities of each cycle were recorded both in summer and in winter. Contrastingly, in California the abundance of this species peaked in spring and fall (Washburn and Frankie, 1985).

In Mera, the development speed was higher in the summer cycle than in the winter cycle, which is in accordance to the higher temperatures found in summer that accelerate the development (Washburn and Frankie, 1985). This was the only studied area where we found high densities of insects throughout all the year. The special emplacement of this area, highly protected from wind and highly isolated due to its SE orientation, could explain why insect density did not drop in winter as in the other localities. Therefore, the meteorological data we associated to this area may not be representative of its microclimate, even though the meteorological station is quite close to it (approx. 3 km). Summer-fall cycles were slightly faster than winter-spring cycles, as expected due to the temperature-dependency of the insect's development speed (Washburn and Frankie, 1985).

The number of scale insects in Mera decreased every consecutive year from 2015 to 2017. This decline, especially in 2017, may be related to the high *P. mesembryanthemi* densities in the previous years. In fact, Washburn et al. (1985) also found drastic drops in *P. mesembryanthemi* populations after several generations with high densities. In Mera the abundance of ovisacs was not related to irradiation and it was the only study area were ovisacs were found almost all year round with no dependency of the insect on the seasons or meteorological conditions. This could be attributed to the sheltered location of this population, which may soften winter conditions.

In Sálvora, Nariga and Coruña the population density decreased drastically in winter, when temperatures dropped. The increase in irradiation and temperature in summer likely promotes the formation of ovisacs and therefore the start of a new cycle. Consequently, the highest insect densities (corresponding to the beginning of the cycle) were found in summer, when temperatures were high and precipitations low. This is in accordance to other studies of scale insects which were favoured by high temperatures and low precipitations (Cid et al., 2006, Retuerto et al., 2004) and whose ovisac formation depended on sun exposure and temperature (Washburn and Frankie, 1985). Unlike in Mera population, ovisacs seemed to be even more determined by meteorological conditions than instars, as they had stronger correlations with temperature, rainfall and irradiation.

5.4.3. Biotic interactions with *P. mesembryanthemi*

Parasitism was positively related to *P. mesembryanthemi* abundance and to high temperatures, which can favour scales' parasites (Kapranas and Tena, 2015, Wright and Kerr, 1988), and low precipitation. Accordingly, parasitism was higher in summer and fall, coinciding with the summer-fall cycle of *P. mesembryanthemi*, which had higher densities than the winter-spring cycle in three of the four localities, and also higher temperatures and lower precipitations. Parasitism, as *P. mesembryanthemi* abundance, was the highest in Mera. Nariga, which was the second locality with the highest *P. mesembryanthemi* density, had nevertheless a slightly lower parasitism than Coruña ($p=0.055$). This could be due to the lower mean temperatures of Nariga compared to Coruña which could be hindering the parasites. In turn, the lower parasitism of Nariga would explain that the abundance of *P. mesembryanthemi* was higher there than in Coruña and Sálvora. The preference of parasites towards more developed individuals of *P. mesembryanthemi* (3rd and 4th instars) is in accordance with the higher suitability for parasitoid development of bigger scale insects (Kapranas and Tena, 2015).

Parasitism can be an important cause of death in scale insects, either directly by being parasitized or indirectly due to wasp feeding (Kapranas and Tena, 2015). They act also as density-dependent regulatory agents (Wakgari and Giliomee, 2001). It has also been proposed that parasitism could decelerate *P. mesembryanthemi*'s development (Washburn and Frankie, 1985). In Mera, we found a clear delay of the parasitized individuals with respect to the non-parasitized ones, especially in summer

and in the most advanced instar stages. In winter this effect over the parasitized scales was less clear, perhaps due to the slower growth of the wasp under colder conditions (Wright and Kerr, 1988). Although parasitized individuals were also found in the periods with the lowest temperatures, the faster development of the scale insect with respect to the wasp could reduce their mortality (Wright & Kerr, 1988). This can explain why in Mera the insect densities are similar to the summer densities (or even slightly higher for the same year) despite a faster growth of *P. mesembryanthemi* under warmer conditions (Washburn and Frankie, 1985).

Coccinellidae predators can also considerably reduce scale insect populations (Farooq-Ahmad, 2012, Mani and Krishnamoorthy, 1990). In Sálvora and Nariga (and very rarely in Coruña) these potential predators of *P. mesembryanthemi* were present, apparently associated to abundance peaks of *P. mesembryanthemi*. The much lower abundance of *P. mesembryanthemi* found in Sálvora and Nariga in 2016 (with respect to 2015 and 2017) might be related to these predators (predation in Sálvora was most intense in summer-fall 2015). Therefore, the presence of these Coccinellidae might be affecting *P. mesembryanthemi* abundance, although a longer study period would be needed to ascertain it.

Ants are widely associated to scales because they feed on honeydew (Buckley, 1987), a liquid excreted by scale insects which contains oligosaccharides (Bogo and Mantle, 2000). In our study we found a positive relation between their presence and the abundance of *P. mesembryanthemi* in all the localities except for Nariga. This correlation was much stronger in Mera, perhaps because it was the only studied locality where ants had this food source available all year and where we expect (based on its sheltered location) that the soil temperature did not drop much in winter, whereas in the other localities the presence of *P. mesembryanthemi* was scarce during winter and spring. This relationship between ants and scales is described as a mutualism, as ants can protect scales from predators or parasites (Buckley, 1987). However, in the case of *P. mesembryanthemi*, Majer (1982) did not find a reduction of predation. Neither did we find a negative significant correlation between ants' presence and *P. mesembryanthemi* parasitism.

Sooty mould was found in heavily infested plants, corresponding in Sálvora and Nariga with the highest mean densities of the scale insects recorded in those places. The presence of mould has been related to honeydew accumulation (Bach, 1991). This fungus is also favoured by

high relative humidity (Cid et al., 2006), as it was found in these places when the mould was spotted (mean relative humidity of 87-93 %). This formation of sooty mould has been referred as one significant limiting factors to the viability of high densities of scale insects (Majer, 1982). Ants have been seen to remove honeydew, thus avoiding the formation of sooty mould and allowing high densities of the *P. mesembryanthemi* (Majer, 1982, Collins and Scott, 1982). However, in these specific cases, ants' attendance was not enough to prevent the formation of mould. The presence of sooty mould can also affect the host plant through a reduction of photosynthesis (Washburn and Frankie, 1985) and fruit production (Bokonon-Ganta et al., 2002) or a more rapid leaf abscission (Bach, 1991).

5.4.4. Effects of *P. mesembryanthemi* on *C. edulis*

Pulvinariella mesembryanthemi worsen plants' aspect and increased necromass and aborted fruit proportions the most when its abundance was high. In fact, sap-sucking insects can limit fruit production (Crawley, 1989), which may explain the increase in aborted fruits with respect to total fruit production. However, correlations between *P. mesembryanthemi* abundance and plants' aspect, necromass and aborted fruit percentages were low, as some climatic factors were also altering them. Low water availability seemed to increase necromass and fruit abortion.

5.4.5. Biotic interactions with *C. edulis*

Snails (i.e. *Theba pisana*, *Helix* spp.) were recurrently present on *C. edulis* plants all over the year. Snails can predate this plant, as found for *Carpobrotus dimidiators* in its native habitat (van Elden et al., 2015), and may also act as pollinators (Preston and Sell, 1988).

Other potential predators [i.e. rats (*Rattus rattus*) and rabbits (*Oryctolagus cuniculus*)] were found. Rabbits predated *C. edulis* fruits in Sálvora, which enhances seed germination (D'Antonio, 1990, Novoa et al., 2012). Almost half of the predated fruits in Sálvora were found in the driest periods, presumably due to the scarcity of juicy food then.

Aphids (*Aphis* sp.), spittlebugs (*Philaenus spumarius*) and mealybugs (*Pseudococcus* sp.) were also found feeding on *C. edulis* plants. They are sap-sucking insects as *P. mesembryanthemi*, and therefore capable of transmitting viruses between plants (Nault, 1997). As *P. mesembryanthemi*, aphids and mealybugs are frequently associated to ants, which predate the honeydew they excrete (Styrsky and Eubanks, 2007). In Sálvora and

Coruña, the three Sternorrhyncha insects (*P. mesembryanthemi*, aphids, mealybugs) helped to explain ants' presence in their host plant.

Although genetic mutation can produce yellow or white colours (i.e. variegation) in reproductive or vegetative organs, the malformations on leaves and flowers we also observed in Sálvora and Nariga suggest that these symptoms can be caused by a viral infection (Valverde et al., 2012).

Nevertheless, these other antagonist of *C. edulis* did not clearly affect the plant in a substantial way.

5.5. SYNTHESIS

The South-African scale insect *Pulvinariella mesembryanthemi* has been accidentally introduced in temperate coastal areas worldwide with its host plants, the aggressive invader *Carpobrotus edulis* and its hybrids, which have been favoured by enemy-release outside their native range. Due to the feeding specificity of *P. mesembryanthemi*, this insect is a potential biocontrol agent.

Predicting habitat suitability of *P. mesembryanthemi* and *Carpobrotus* spp. is especially important for determining the adequacy of using the insect as a biocontrol agent in a region. The geographic distribution of *Carpobrotus* spp. and *P. mesembryanthemi* in Galicia (NW Spain) was predicted by using the maximum entropy algorithm (MaxEnt). The MaxEnt model indicates that the potential distribution of the species overlapped, being both mainly present along the coast. *Carpobrotus* spp. distribution was mainly influenced by altitude (70%) and soil thermal regime (11%), while *P. mesembryanthemi* distribution was majorly determined by the consecutive days with moist soil and Temp>8°C (72%) and continentality (9%).

Through a 2-year field study in several localities invaded by *C. edulis*, we examined the population dynamics of *P. mesembryanthemi* taking into account climatic factors and biotic interactions with other insects and the host plant. In the studied localities, this scale was bivoltine, reaching its highest densities in the warmer, more irradiated and drier months of summer and sharply decreasing in abundance in winter. Only in a specially sheltered locality (Mera) the insect densities were maintained high all year.

Pulvinariella mesembryanthemi infestation worsened visual aspect of its host plant and increased necromass and aborted fruits proportions. However, Chalcidoidea parasites and Coccinellidae predators could limit *P. mesembryanthemi* abundance, and therefore restrict the efficiency of using *P. mesembryanthemi* as a biocontrol agent. Chalcidoidea parasitism affected predominately the biggest scales (especially when *P. mesembryanthemi* density and temperatures were high and rainfall low), slowing down scales development in summer. The excretion of honeydew by these scales attracted ants, and in exceptional cases led to mould fouling.

Carpobrotus edulis plants were also attacked by fruit predators (which, nonetheless, helped seed dissemination and germination), other sap-sucking insects (i.e. aphids, mealybugs, spittlebugs) and viruses, although with no evident severe consequences.

5.6. SUPPLEMENTARY MATERIAL

SM Table 5.1. (*next page*) Variables included in the model and estimated relative contributions (percent contribution, PC; permutation importance, PI) for *Carpobrotus* spp. (CSP) and *Pulvinariella mesembryanthemi* (PME).

		CSP		PME	
Code	Variable	PC	PI	PC	PI
DEM	Digital Elevation Model	70.0	88.6	0.9	61.8
str_class	Thermal regime of the soil	10.8	0.0	0.0	0.0
Bio8mst	Consecutive days with moist soil and T> 8 °C	7.9	0.0	72.2	9.1
T_01max	Maximum temperature in January	5.2	0.0	0.0	0.0
Io	Annual ombrothermic index	2.4	4.5	0.0	0.0
Landuse	Land use	1.9	1.4	4.2	2.3
P_07_06	Mean precipitation in June	0.7	2.2	0.0	0.0
T_01min	Minimum temperature in January	0.3	0.0	4.5	0.0
Ios3	Ombrothermic index (June-August)	0.3	0.0	0.0	0.0
AWB	Annual water balance	0.2	0.4	0.0	0.0
P_02_06	Mean precipitation in February	0.1	0.0	0.0	0.0
It	Heat index	0.1	2.4	0.1	0.0
T_11_06	Mean temperature in November	0.1	0.0	0.5	0.0
P_10_06	Mean precipitation in October	0.0	0.0	0.0	0.0
T_12_06	Mean temperature in December	0.0	0.4	0.0	0.0
Ic_Alt	Continentality index compensated by altitude	0.0	0.0	9.2	16
P_04_06	Mean precipitation in April	0.0	0.0	0.0	0.0
T_04_06	Mean temperature in April	0.0	0.0	0.0	0.0
T_05_06	Mean temperature in May	0.0	0.0	0.0	0.0
T_01_06	Mean temperature in January	0.0	0.0	0.0	0.0
T_00_06	Annual mean temperature	0.0	0.0	0.0	0.0
SWB	Summer water balance	0.0	0.0	0.0	0.0
SMRdry	Consecutive days with dry soil after summer solstice	0.0	0.0	0.0	0.0
P_12_06	Mean precipitation in December	0.0	0.0	0.0	0.0
P_11_06	Mean precipitation in November	0.0	0.0	0.0	0.0
T_06_06	Mean temperature in June	0.0	0.0	0.0	0.0
P_09_06	Mean precipitation in September	0.0	0.0	0.0	0.0
P_08_06	Mean precipitation in August	0.0	0.0	0.2	1.4
T_07_06	Mean temperature in July	0.0	0.0	0.0	0.0
P_06_06	Mean precipitation in June	0.0	0.0	0.0	0.0
P_05_06	Mean precipitation in May	0.0	0.0	0.0	0.0
P_03_06	Mean precipitation in March	0.0	0.0	1.2	9.5
T_08_06	Mean temperature in August	0.0	0.0	0.0	0.0
P_01_06	Mean precipitation in January	0.0	0.0	0.0	0.0
P_00_06	Mean annual precipitation	0.0	0.0	0.0	0.0
T_09_06	Mean temperature in September	0.0	0.0	0.0	0.0
T_10_06	Mean temperature in October	0.0	0.0	0.0	0.0
Ios4	Ombrothermic index (May-August)	0.0	0.0	4.6	0.0
WTRmst	Consecutive days with moist soil after winter solstice	0.0	0.0	0.0	0.0
Ios2	ombrothermic index (July-August)	0.0	0.0	0.0	0.0
YRdry	Cumulative days with dry soil	0.0	0.0	0.0	0.0
T_03_06	Mean temperature in June	0.0	0.0	0.0	0.0
T_02_06	Mean Temperature in February	0.0	0.0	0.2	0.0
Ic	Continentality index	0.0	0.0	0.0	0.0
YRmd	Cumulative days with partially moist soil	0.0	0.0	0.0	0.0
YRmst	Consecutive days with moist soil	0.0	0.0	0.0	0.0
Bio5mst	Cumulative days with moist soil and Temp > 8 °C	0.0	0.0	0.0	0.0
Bio5md	Cumulative days with partially moist soil and Temp > 8 °C	0.0	0.0	2.1	0.0
Bio5dry	Cumulative days with dry soil and Temp > 5 °C	0.0	0.0	0.0	0.0
YRmst2	Cumulative days with moist soil	0.0	0.0	0.0	0.0

SM Table 5.2. Results of the Kruskal-Wallis test (including stats., test statistic.; p, significance) (d.f.=2, degrees of freedom for all the cases) of the effects of mean irradiation of the past month on ovisacs and 1st-4th instars of *Pulvinariella mesembryanthemi* (individuals $\cdot m^{-2}$) of all the data together and separately by locality between December 2015 and December 2017.

	ALL		MERA		SÁLVORA		CORUÑA		NARIGA	
stage	stats.	p	stats.	p	stats.	p	stats.	p	stats.	p
ovisacs	49.48	**<0.001**	1.56	0.459	16.31	**<0.001**	25.87	**<0.001**	44.25	**<0.001**
instars	2.87	0.239	2.8	0.246	0.51	0.773	1.32	0.516	7.19	**0.027**

SM Table 5.3. Results of the Kruskal-Wallis test of the effect of different studied factors on the percentage of instars of *Pulvinariella mesembryanthemi* that are parasitized: locality of study, developmental stage of the insect (from 1st to 4th instar) and season; mean temperature, cumulative rain and mean irradiation of the previous 30 days to the measure. The table includes the statistic, the degrees of freedom (d.f.) and the significance of the test.

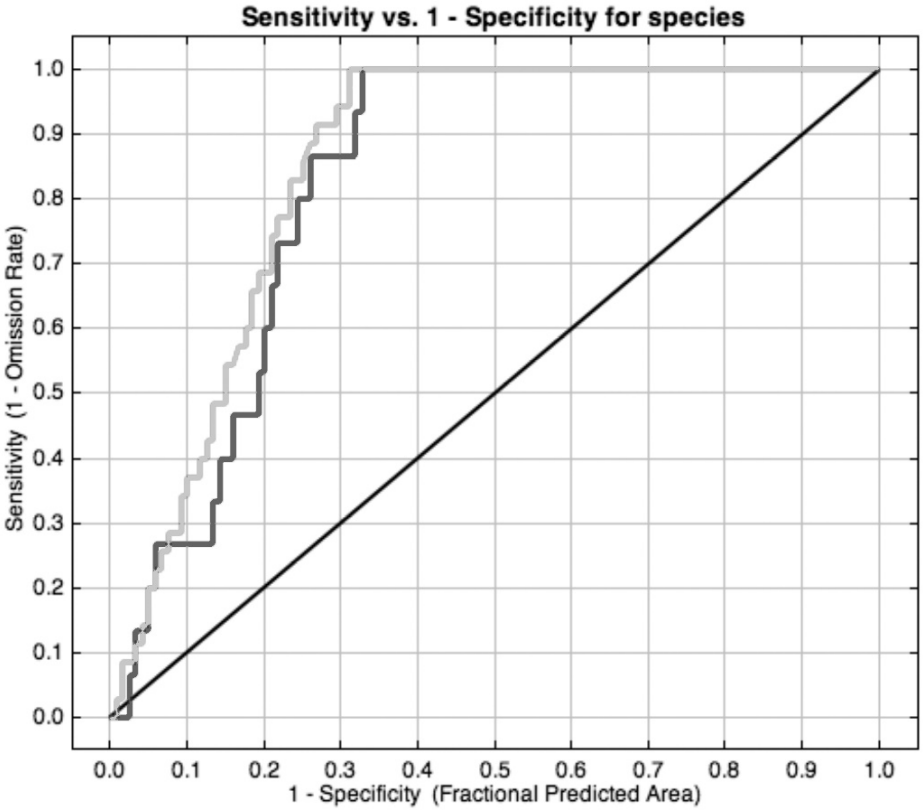
Factors	stats.	d.f.	p
Locality	32.01	3	**<0.001**
Stage	22.52	3	**<0.001**
Season	27.69	3	**<0.001**
Temperature	21.17	2	**<0.001**
Rainfall	21.22	2	**<0.001**
Irradiation	5.77	2	0.056

SM Table 5.4. Pairwise comparisons between levels of the different factors [locality, season, mean temperature (°C), cumulative rainfall (mm) and mean irradiation ($W \cdot m^{-2}$) of the last 30 days] for the *Pulvinariella mesembryanthemi* parasitism (%) of 1st to 4th instars between December 2015 and December 2017. The mean rank value, the arithmetic mean and the standard error (mean±SE) are included. Different letters correspond to significant differences by Dunn's post- hoc test at p=0.05.

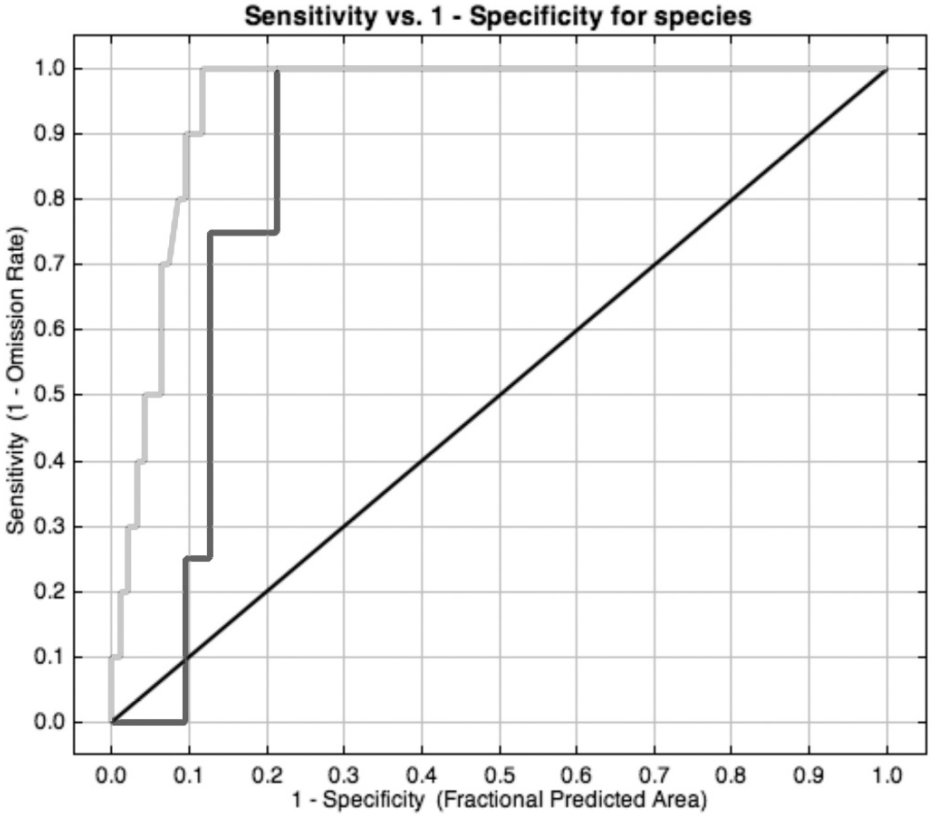
Factor	Levels	mean rank	mean ± SE
Locality	Mera	141.60[a]	31.17 ± 3.59
	Sálvora	87.14[b]	19.12 ± 3.60
	Coruña	89.04[b]	13.40 ± 2.71
	Nariga	74.63[b]	5.54 ± 1.42
Stage	1st instar	157.15[b]	7.66 ± 3.67
	2nd instar	191.78[b]	9.18 ± 1.72
	3rd instar	225.72[a]	17.11 ± 2.51
	4th instar	231.82[a]	24.09 ± 3.60
Season	spring	166.20[b]	4.37 ± 2.02
	summer	224.32[a]	16.26 ± 2.50
	fall	232.47[a]	23.95 ± 3.09
	winter	176.43[b]	7.65 ± 1.80
Mean temperature (°C)	9.0-11.9	186.91[b]	11.41 ± 2.65
	12-15.9	185.33[b]	9.61 ± 1.69
	16-20	233.31[a]	20.33 ± 2.40
Cumulative rainfall (mm)	0-20	216.75[a]	18.39 ± 3.03
	20-100	222.62[a]	16.99 ± 1.99
	100-250	163.95[b]	6.28 ± 1.93
Irradiation ($W \cdot m^{-2}$)	400-1199	197.07[a]	13.64 ± 2.03
	1200-1999	225.46[a]	17.47 ± 2.56
	2000-2800	203.38[a]	14.07 ± 2.75

SM Table 5.5. Spearman's rank correlation test between the parasitism percentage of *Pulvinariella mesembryanthemi* instars and instars abundance (individuals · m^{-2}) and meteorological parameters [mean temperature (°C), cumulative rainfall (mm) and mean irradiation (W · m^{-2}) of the preceding 30 days].

	Corr. Coef.	p	N
Instar abundance	**0.524**	<0.001	213
Temperature	**0.307**	<0.001	213
Rainfall	**-0.240**	<0.001	213
Irradiation	0.116	0.09	213

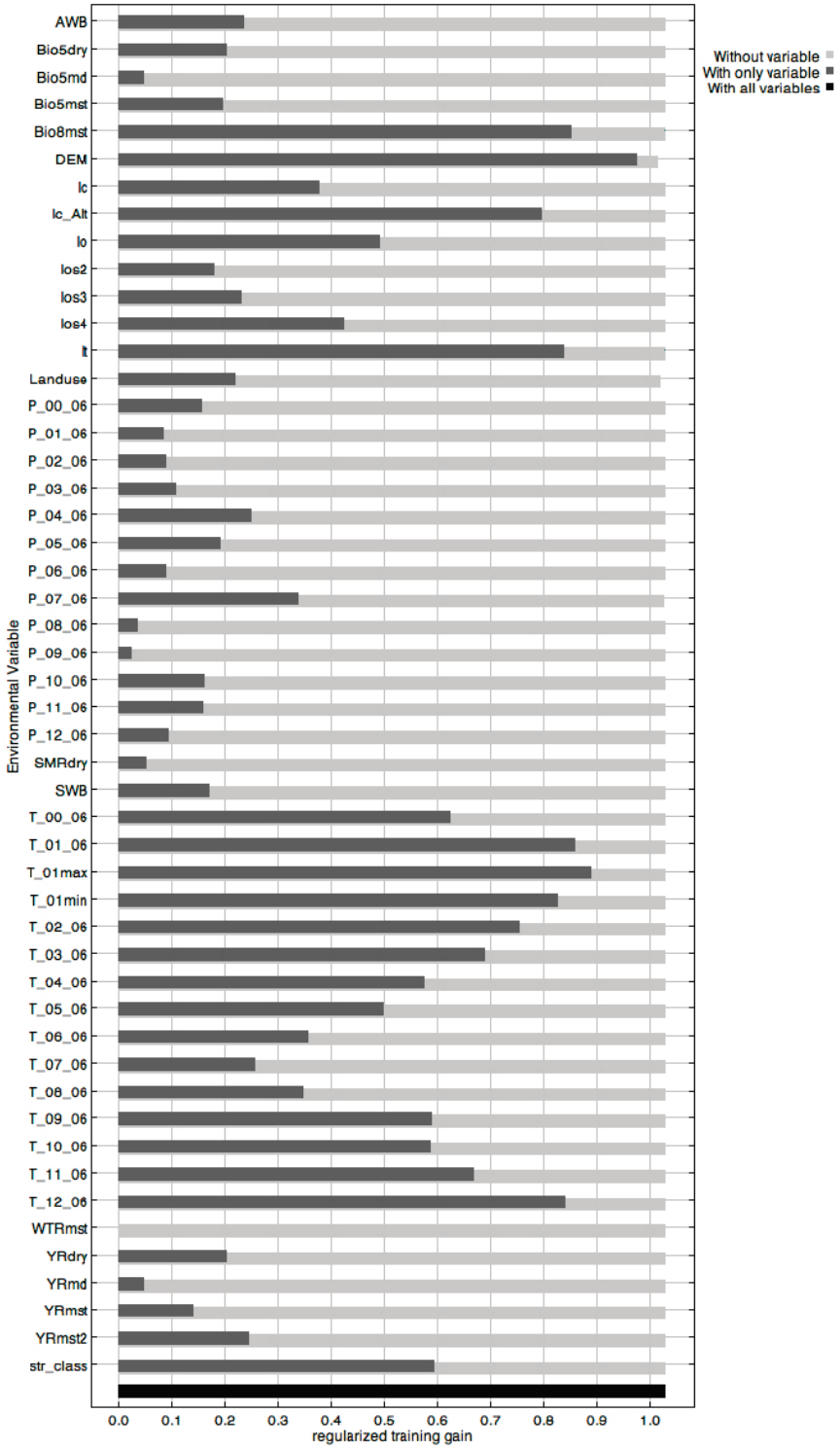


SM Fig. 5.1. Receiver operating characteristic (ROC) curve and Area under the Curve (AUC) values for MaxEnt model of *Carpobrotus* spp. The black line indicates random prediction (AUC = 5), the light grey line training data (AUC= 0.851), and the dark grey line the test data (AUC = 0.828).



SM Fig. 5.2. Receiver operating characteristic (ROC) curve and Area under the Curve (AUC) values for MaxEnt model of *Pulvinariella mesembryanthemi*. The black line indicates random prediction (AUC = 0.5), the light grey line training data (AUC= 0.945), and the dark grey line the test data (AUC = 0.859).

SM Fig. 5.3. (*next page*) Jackknife test results of variable importance for *Carpobrotus* spp. Grey bars consider individual environmental variables and the black bar considers all environmental variables. Within the grey bars, those that are light grey show information given by a variable that is not given by the others, and those that are dark grey show if the variable gives useful information by itself. For the environmental variables, the abbreviations correspond to those used in SM Table 5.1.


AWB
Bio5dry
Bio5md
Bio5mst
Bio8mst
DEM
lc
lc_Alt
lo
los2
los3
los4
lt
Landuse
P_00_06
P_01_06
P_02_06
P_03_06
P_04_06
P_05_06
P_06_06
P_07_06
P_08_06
P_09_06
P_10_06
P_11_06
P_12_06
SMRdry
SWB
T_00_06
T_01_06
T_01max
T_01min
T_02_06
T_03_06
T_04_06
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T_08_06
T_09_06
T_10_06
T_11_06
T_12_06
WTRmst
YRdry
YRmd
YRmst
YRmst2
str_class
Environmental Variable
0.0
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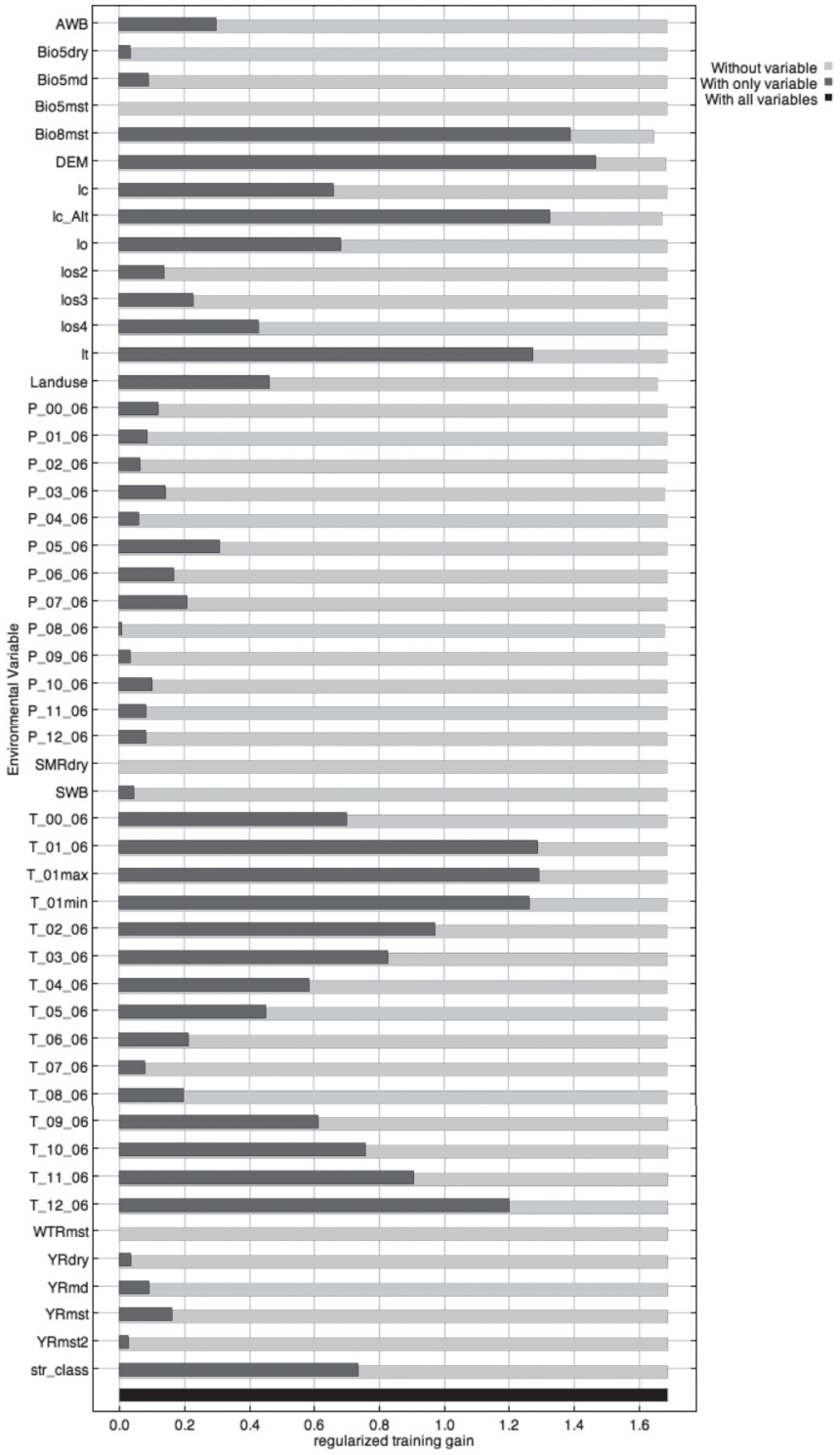
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
regularized training gain
Without variable
With only variable
With all variables


AWB
Bio5dry
Bio5md
Bio5mst
Bio8mst
DEM
lc
lc_Alt
lo
los2
los3
los4
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Landuse
P_00_06
P_01_06
P_02_06
P_03_06
P_04_06
P_05_06
P_06_06
P_07_06
P_08_06
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SWB
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T_10_06
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T_12_06
WTRmst
YRdry
YRmd
YRmst
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str_class
Environmental Variable
0.0
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0.4
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1.0
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1.4
1.6
regularized training gain
Without variable
With only variable
With all variables

SM Fig. 5.4. (*previous page*) Jackknife test results of variable importance for *Pulvinariella mesembryanthemi.* Grey bars consider individual environmental variables and the black bar considers all environmental variables. Within the grey bars, those that are light grey show information given by a variable that is not given by the others, and those that are dark grey show if the variable gives useful information by itself. For the environmental variables, the abbreviations correspond to those used in SM Table 5.1.

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6.1. INTRODUCTION

The scale insect *Pulvinariella mesembryanthemi* (Vallot) (Family: Coccidae, Order: Hemiptera), which parasites *C. edulis*, presents itself as a potential biological control agent because of its high host specificity (Miller et al., 2005, Miller and Miller, 2003) and its capability of producing severe damages to the plant (Washburn and Frankie, 1985, Donaldson et al., 1978). This insect, as well as its host plant (*Carpobrotus* spp.), is original from South Africa (Washburn and Frankie, 1985), from where it may have been accidentally introduced in several parts of the world along with infested plants. Currently, it is present in Southern Europe (Gómez-Menor Ortega, 1954, Kozar et al., 1991, Pellizzari and Germain, 2010, Seljak, 2010, Vieira et al., 1983), Turkey (Cebeci and Selmi, 2004), Argentina (Granara de Willink and Claps, 2003), USA (Miller et al., 2005), New Zealand (Hodgson and Henderson, 2000) and Australia (Collins and Scott, 1982). The introduction of the host plant to these areas was mostly done at the end of the 19th century or beginning of the 20th (Lázaro-Ibiza, 1900, D'Antonio et al., 1993, New-Zealand-Plant-Conservation-Network, 2014).

Cryptic species complexes are common in scale insects, but frequently ignored by morphological identification (Andersen et al., 2010). In the case of *P. mesembryanthemi*, the semi-cryptic species *Pulvinaria delottoi* Gill has been cited in California (Washburn and Frankie, 1985) and the UK (Salisbury et al., 2011). They are almost morphologically identical, but with different feeding habits and life cycle duration: *P. mesembryanthemi* prefers younger leafs and has two reproductive events per year, while *P. delottoi* usually feeds on older leaves and has one generation per year in Southern California (Washburn and Frankie, 1985, Washburn and Frankie, 1981). Their reproduction seems to be exclusively parthenogenic, being able of producing up to 2400 hatches per ovisac, with an average fecundity of 350-800 crawlers per individual and higher for *P. delottoi* than for *P. mesembryanthemi* (Washburn and Frankie, 1985). Although *P. mesembryanthemi* males have been observed, no sexual reproduction has been recorded and the presence of male individuals has been related to a relictic species feature (Washburn and Frankie, 1985). Active dispersion of these two species is restricted to the first stages

(crawlers and 1st instar). This highlights the role of passive dispersion, by animals and especially by wind, with a dispersion rate which could reach 190 km per generation (Washburn and Frankie, 1981).

Scale insects, as other sap-sucking Hemiptera, tend to have different bacterial endosymbionts which are still barely studied (Gruwell et al., 2007). Usually, they are vertically transmitted (from the mother to the progeny) (Bing et al., 2013, Li et al., 2011b), and are useful for phylogeny reconstructions (Andersen et al., 2010) and insect identification (Mathenge et al., 2015). However, to the extent of our knowledge, no studies about the existence of these endosymbionts in *P. mesembryanthemi* have been done.

Genetic studies of potential biological control agents are highly recommended in order to uncover cryptic species complexes or intraspecific entities (Gaskin et al., 2011, Rosen, 1986), which can affect control success by having different host specificity or aggressiveness towards the plant (Rauth et al., 2011). They can also give information about the minimal number of individuals that should be introduced to maintain diversity (Rauth et al., 2011).

To compare interspecific genetic diversity and to unravel cryptic biodiversity, the COI gene is frequently used (Bekker et al., 2016, Porco et al., 2012, Mesquita Fonseca et al., 2017). Classical primers used for insect COI sequencing have failed for some scale insects, sequencing instead endosymbionts of the insect, so new primer sets have been developed for this group (Park et al., 2010). Also, due to the difficulties encountered for COI gene sequencing in scale insects, the nuclear large subunit ribosomal RNA gene (LSU rRNA, 28S) has been used sometimes as a substitute of the COI as a barcode region (Schroer et al., 2008).

The aim of this study was to accomplish a phylogeographic analysis of the insect *P. mesembryanthemi* to obtain information about the intra- and inter-population variability, which could be helpful for future studies on the convenience of using this species as a biocontrol agent on its natural host *C. edulis.* We also intended to determine the origin of the exotic populations in South Africa and the number of colonizing events of *P. mesembryanthemi* outside its native range.

6.2. MATERIAL AND METHODS

6.2.1. Sample collection and preservation

Samples were taken from 18 populations worldwide (11 from the Iberian Peninsula, 2 from the Canary Islands, 1 from New Zealand and 3 from South Africa) (for collection information, see SM Table 6.1a and Fig. 6.1) during 2016-2017. The samples were taken from females preferentially in the 4th instar as described by Washburn and Frankie (1985). Careful visual inspection of the samples was done to detect parasitized individuals, which were discarded. Specimens were preserved in 70 % to 96% ethanol and stored at -20 °C.

6.2.2. DNA extraction, amplification and sequencing

DNA was extracted from whole individuals with the E.Z.N.A. ® Tissue DNA kit (Omega bio-tek). A mitochondrial and a ribosomal gene were amplified. The primers LepF and LepR (Hajibabaei et al., 2006) were used in a first attempt to amplify the 5′ end of the mytochondrial cytochrome *c* oxidase I (COI) of the insect. However, it amplified the COI fragment of an α-Proteobacteria, endosymbiotic of the scale insect, instead of the insect itself. A new primer, PcoF1 (Park et al., 2010) was tried in conjunction with the LepR, managing to amplify a COI fragment of the insect. The D2 and D3 expansion segments of the nuclear large subunit ribosomal RNA gene (LSU rRNA, 28S) were amplified with the pair of primers s3660/A335 (Schroer et al., 2008).

The PCR was performed in a total volume of 20 mL comprising 2.5 mL (5.0 mL when amplifying the endosymbionts) of nucleic acid solution (10-35 ng/mL) and 17.5 mL of mix (4 mL of 5x Buffer, 25 mM $MgCl_2$, 2.5 mM dNTPs, 8 pmol of each primer and 0.8 U of GoTaq DNA Polimerase). The PCR was performed in a Biorad thermocycler, in the following conditions for the endosymbiont COI fragment: one cycle of 1 min at 94ºC; 5 cycles of 30 s at 94 ºC, 1 min at 45 ºC and 1 min at 72 ºC; other 30 cycles as the former but with an annealing temperature of 50 ºC; 5 min at 72 ºC; held at 4 ºC. In order to target the scale insect genes, the conditions where as described by Park et al. (2010) when using the pair of primers PcoF1/LepR and as in Schroer et al. (2008) for the 28S fragment. PCR products were visualized on 0.5 % agarose gel, purified with SureClean Kit and sent to Genewiz Inc. for dideoxy chain termination sequencing.

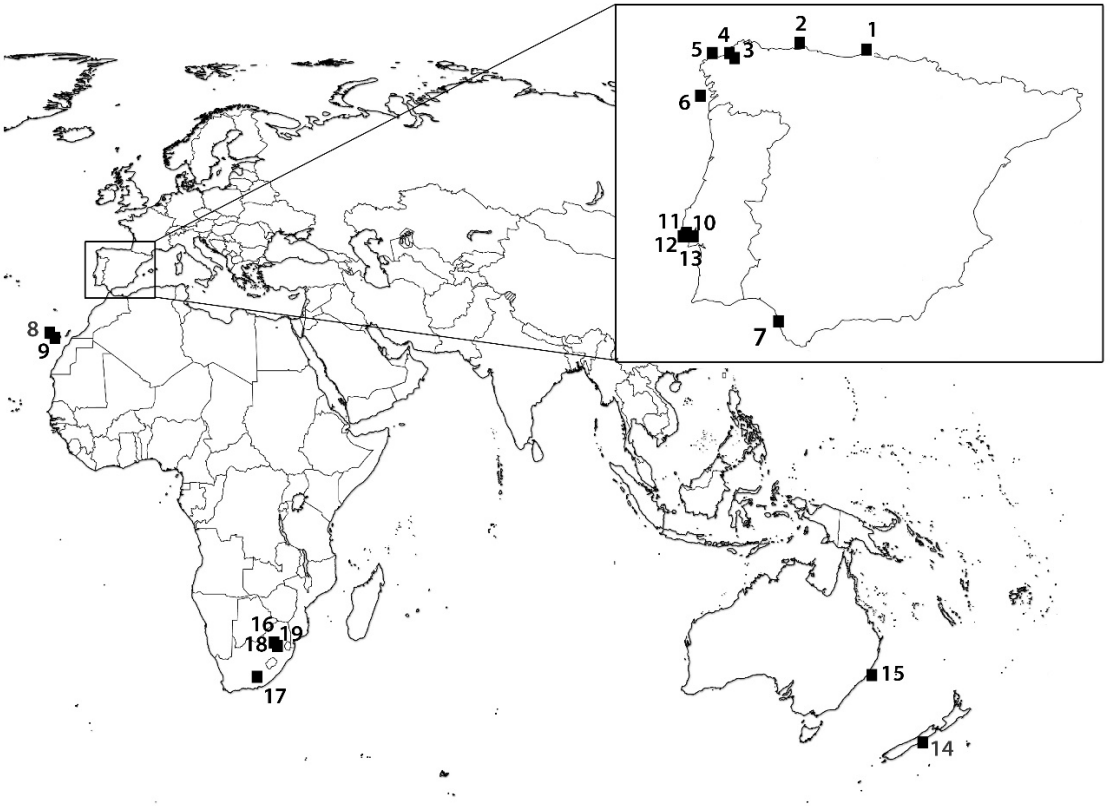


Fig. 6.1. Origin of populations of *Pulvinariella mesembryanthemi* used in this study. SPAIN: 1- Santander (STD), 2- Asturias (AST), 3- Oleiros (MER), 4- A Coruña (CNH), 5- Punta Nariga (NAR), 6- Sálvora island (SAL), 7- Cádiz (CAD), 8- Tenerife island (TEN), 9- La Palma island (LPA). PORTUGAL: 10- Lisboa (LTE), 11- Cascais (CCS), 12- Estoril (EST), 13- Costa de Caparica (CAP). NEW ZEALAND: 14- North New Brighton (NZL). AUSTRALIA: 15- New South Wales (AUS). SOUTH AFRICA: 16,18,19- Gauteng (SA1, SA3, SAP), 17-Eastern Cape (SA2).

The COI fragment of *P. mesembryanthemi* was sequenced for one individual of each location, except for the South African populations where 8 (for SA2 and SA3) or 7 (for SA1) individuals were sequenced. For the 28S fragment of *P. mesembryanthemi*, one individual of the populations NZL, AST, SA1, SA3, SA2 (see SM Table 6.1b) were sequenced. The COI fragment of the endosymbiont was sequenced for one individual of the populations AST, NAR, TEN, CCS, AUS and SA1 (see SM Table 6.1b).

6.2.3. Phylogenetic analysis

Besides the sequenced samples, sequences of the insect and its endosymbiont obtained from Bold System (*www.boldsystem.org*) were included in the analysis (see SM Table 6.1b for accession numbers). The COI sequences of *P. mesembryanthemi* from Chile which are available in the NCBI (accession numbers: KY085884- KY085888, KY085331-KY085334) were not included in the analysis as they seemed to be misidentified – the plants from which they were sampled do not match their suitable host plants (Washburn and Frankie, 1985) and their similarity with other COI sequences of *P. mesembryanthemi* obtained in this study and from the Bold System are quite low (16-18 % uncorrected p-distances).

After checking sequences identity with the BLAST tool from the NCBI website, forward and reverse sequences were assembled and edited with Sequencher 4.0.5 (Sequencher®) and then aligned with ClustalW Multiple Aligment function (Thompson et al., 1994) through BioEdit v.7.0.5.2 (Hall, 1999). For the analysis, only unique haplotypes (which diverged at least in one nucleotide from the others) were included.

The most suitable models of DNA substitutions to the data for each dataset were found with jModelTest 2.1.10 (Darriba et al., 2012, Guindon and Gascuel, 2003). Those models were then used to infer the phylogenetic trees by Maximum Likelihood (ML) with PhyML 3.0 (Guindon et al., 2010), and by Bayesian Inference (BI) with MrBayes 3.2 (Huelsenbeck and Ronquist, 2001). For the BI analysis with the concatenated dataset we used for each partition (COI, 28S gene fragments) the best-fit model corresponding to the COI or 28S data, respectively. Phylogenetic trees were also inferred by Maximum Parsimony (MP) with PAUP* 4.0a152 (L. Swofford, 2002). MP analysis were performed with a heuristic search with TBR (tree bisection reconnection) branch swapping of 100 random sequence addition starting trees, and branch support was obtained from

1000 pseudo-replicates of nonparametric bootstrap. ML analysis were performed with SPR (sub-tree pruning and re-grafting) branch swapping of 10 random starting trees, and branch support was obtained from 1000 bootstrap pseudo-replicates. For the BI, posterior probabilities were estimated by a Metropolis-coupled Markov chain Monte Carlo sampling algorithm, and 2,000,000 generations were sampled every 1,500 generations. Stationary of the likelihood scores with the generation time was checked with TRACER v1.6 (Rambaut et al., 2018). Other nine Coccidae species and one Diaspididae species were used as outgroups in the *P. mesembryanthemi* trees. For the endosymbionts trees, other five species (five of them Proteobacteria, one Heterokontophyla and another Ochrophyta) were used.

Pairwise divergences were calculated through the most suitable model for each dataset (as found with jModelTest) and the Kimura two-parameter (K2P) model with PAUP*. The K2P distances were included for comparison with other studies. Descriptive parameters such as haplotype and nucleotide diversity were calculated with DNASP 5.10 (Librado and Rozas, 2009).

Analysis were done with the COI and 28S gene fragments of *P. mesembryanthemi* both individually and concatenated [using Concatenator v 1.0.1. (Pina-Martins and Paulo, 2008) to combine the sequences]. For the endosymbiont, analyses were only done with the COI gene fragment.

6.3. RESULTS

Based on the similarity results obtained with the BLAST tool from the NCBI website, all the exotic scale insects that were sequenced for this study belonged to the species *P. mesembryanthemi* and not to the semi-cryptic species *P. delottoi* or any other unknown cryptic species. Also, no heterozygotic individuals were found for the mitochondrial and ribosomal sequences.

6.3.1. *Pulvinariella mesembryanthemi* populations: COI gene

Sequencing of *P. mesembryanthemi* COI gene with the PcoF1/LepR primers resulted on a 679 bp fragment with an average GC composition of 25% (SM Table 6.2). No gaps were found in the COI fragments.

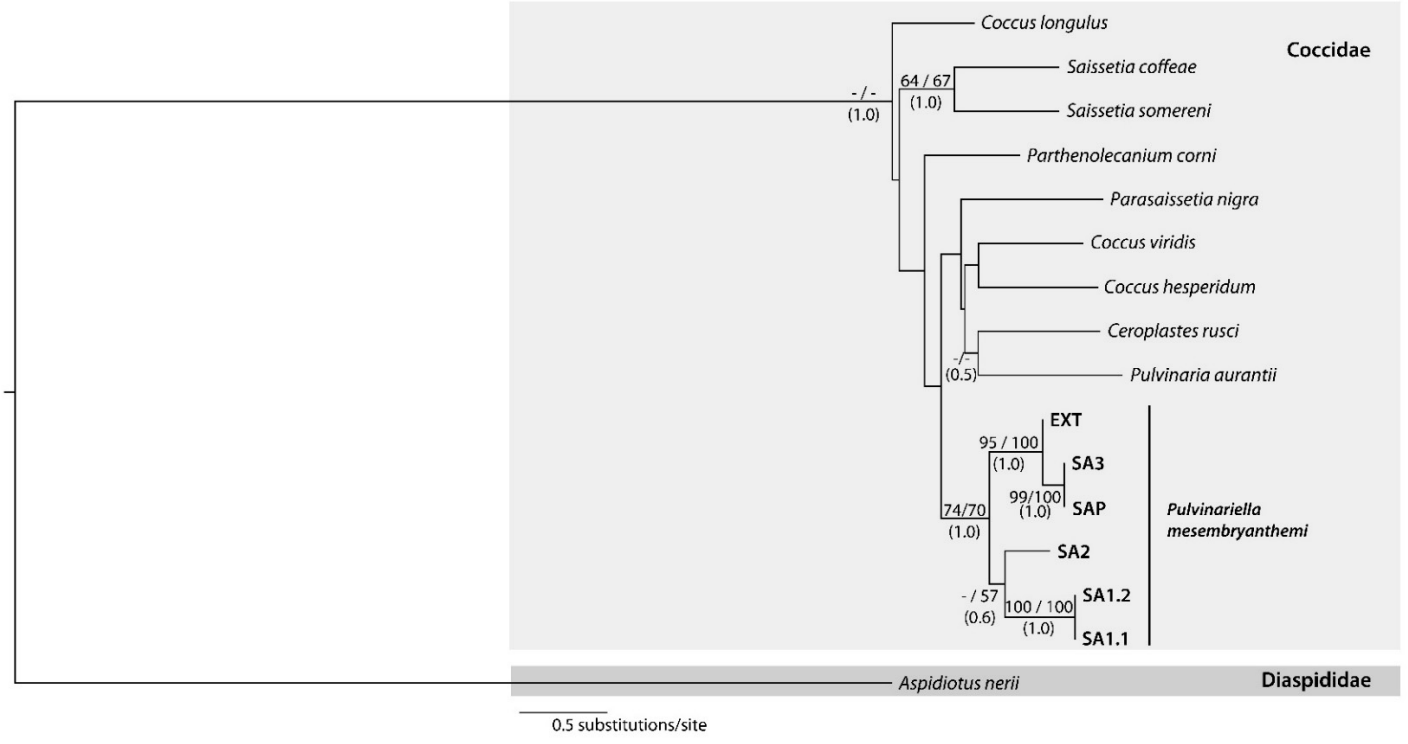


Fig. 6.2. Maximum likelihood tree based on COI data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1b). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent, in this order, the bootstrap support values (≥50 %) of maximum likelihood and maximum parsimony. Numbers within parenthesis represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.

Haplotype diversity for the COI gene was high in the native area (Hd=0.757), where 5 different haplotypes were found, whilst only one haplotype was found in the introduced area (EXT) (SM Table 6.2; Fig. 6.2). In the native area, there were 129 polymorphic sites, all of them parsimony informative; and 141 mutations (SM Table 6.2). The only native population where intra-population variability was found (of the three native populations in which 7-8 samples were studied) was SA1, with 2 haplotypes (SA1.1, SA1.2).

The distances calculations showed that the sole exotic haplotype found for *P. mesembryanthemi* was most similar to two South African populations from Pretoria (SAP, SA3), having a 3 and 5% of TrN model and K2P distances from them for this gene fragment (Tables 6.1, 6.2a). The most divergent South African populations from the exotic ones were also from Pretoria (SA1.1, SA1.2), and had a 12-13 % of TrN distance (15 % K2P distance) (Table 6.2a). The South African population from Eastern Cape Province (SA2) had an intermediate TrN distance of 11 % (13 % K2P distance) (Table 6.2).

Table 6.1. Parameters of the nucleotide substitution models used for each dataset [from data of *Pulvinariella mesembryanthemi* and the included outgroups (see SM Table 6.1, SM Table 6.3) and from data of *P. mesembryanthemi* 's endosymbiont and the included outgroups (Symbiont) (see SM Table 6.1, SM Table 6.4)]. Key: shape parameter of the gamma distribution (Γ), proportion of invariable sites (*I*), base frequencies, transition/transversion ratio (*ti/tv*) and substitution rates.

	Pulvinariella mesembryanthemi			Symbiont
Dataset	COI	28S	COI+28S	COI
Model	TrN+*I*+Γ	HKY+Γ	GTR+*I*+Γ	GTR+Γ
Γ	0.5050	0.3550	0.4530	0.6190
I	0.4680	0.0000	0.1760	0.0000
freq. A	0.4196	0.1537	0.2632	0.2511
freq. C	0.1445	0.2853	0.2188	0.1551
freq. G	0.0371	0.3400	0.2129	0.1908
ti/tv	-	1.9721	-	-
A-C	1.0000	-	0.9538	1.9082
A-G	25.4553	-	3.9748	4.0862
A-T	1.0000	-	4.2129	3.9314
C-G	1.0000	-	2.8473	1.8657
C-T	48.9725	-	14.4075	5.6215

Table 6.2a. Pairwise distances for the COI data of *P. mesembryanthemi* and the included outgroups (see SM Table 6.1 and 6.3). Distances within *P. mesembryanthemi* samples are marked in bold. Corrected distances calculated with the best-suited model for each dataset (see Table 6.1) are shown below the matrix diagonal and Kimura 2-parameter (K2P) distances are shown above diagonal.

COI gene		AN	CR	CH	CL	CV	SC	SS	PN	PA	PC	**SA1.1**	**SA1.2**	**SA3**	**SAP**	**SA2**	**EXT**
Aspidiotus nerii	AN	-	0.30	0.35	0.31	0.33	0.32	0.32	0.32	0.33	0.32	0.37	0.36	0.37	0.37	0.34	0.35
Ceroplastes rusci	CR	0.30	-	0.17	0.17	0.16	0.16	0.16	0.15	0.16	0.15	0.17	0.17	0.18	0.18	0.15	0.17
Coccus hesperidum	CH	0.38	0.13	-	0.18	0.18	0.20	0.20	0.20	0.18	0.20	0.19	0.19	0.22	0.22	0.17	0.20
Coccus longulus	CL	0.33	0.13	0.14	-	0.17	0.18	0.20	0.18	0.18	0.16	0.20	0.20	0.19	0.19	0.16	0.18
Coccus viridis	CV	0.36	0.12	0.14	0.14	-	0.19	0.18	0.19	0.19	0.17	0.20	0.20	0.20	0.20	0.18	0.20
Saissetia coffeae	SC	0.35	0.13	0.18	0.16	0.15	-	0.16	0.19	0.18	0.2	0.19	0.19	0.23	0.23	0.19	0.24
Saissetia somereni	SS	0.35	0.13	0.19	0.20	0.16	0.14	-	0.20	0.21	0.19	0.18	0.18	0.21	0.22	0.18	0.20
Parasaissetia nigra	PN	0.35	0.12	0.16	0.15	0.18	0.16	0.18	-	0.18	0.17	0.19	0.18	0.19	0.19	0.19	0.18
Pulvinaria aurantii	PA	0.35	0.13	0.14	0.14	0.16	0.16	0.24	0.16	-	0.20	0.20	0.19	0.21	0.21	0.18	0.19
Parthenolecanium corni	PC	0.38	0.12	0.19	0.15	0.14	0.20	0.18	0.15	0.19	-	0.18	0.18	0.18	0.18	0.16	0.17
Pulvinariella mesembryanthemi	**SA1.1**	0.48	0.13	0.15	0.17	0.17	0.16	0.16	0.15	0.19	0.16	-	**0.003**	**0.17**	**0.17**	**0.13**	**0.15**
	SA1.2	0.47	0.13	0.15	0.17	0.17	0.16	0.16	0.15	0.18	0.16	**0.002**	-	**0.17**	**0.17**	**0.12**	**0.15**
	SA3	0.48	0.16	0.19	0.16	0.17	0.24	0.20	0.17	0.21	0.15	**0.14**	**0.15**	-	**0.002**	**0.16**	**0.05**
	SAP	0.47	0.16	0.19	0.16	0.17	0.24	0.21	0.17	0.21	0.15	**0.15**	**0.15**	**0.001**	-	**0.16**	**0.05**
	SA2	0.39	0.12	0.14	0.12	0.16	0.16	0.17	0.13	0.14	0.13	**0.10**	**0.10**	**0.14**	**0.15**	-	**0.13**
	EXT	0.42	0.13	0.17	0.15	0.17	0.26	0.20	0.14	0.17	0.15	**0.12**	**0.13**	**0.03**	**0.03**	**0.11**	-

Table 6.2b. Pairwise distances for the 28S data of *P. mesembryanthemi* and the included outgroups (see SM Table 6.1 and 6.3). Distances within *P. mesembryanthemi* samples are marked in bold. Corrected distances calculated with the best-suited model for each dataset (see Table 6.1) are shown below the matrix diagonal and Kimura 2-parameter (K2P) distances are shown above diagonal.

28S gene		AN	CR	CH	CL	CV	SC	SS	PN	PA	PC	**SA1**	**SA3**	**SA2**	**EXT**
Aspidiotus nerii	AN	-	0.31	0.27	0.28	0.29	0.27	0.28	0.27	0.34	0.25	0.24	0.24	0.26	0.24
Ceroplastes rusci	CR	0.68	-	0.12	0.11	0.18	0.13	0.10	0.10	0.20	0.13	0.20	0.20	0.20	0.20
Coccus hesperidum	CH	0.52	0.15	-	0.09	0.17	0.10	0.08	0.08	0.12	0.07	0.18	0.18	0.18	0.18
Coccus longulus	CL	0.56	0.14	0.11	-	0.18	0.11	0.08	0.07	0.17	0.1	0.18	0.18	0.19	0.19
Coccus viridis	CV	0.58	0.28	0.27	0.28	-	0.22	0.18	0.18	0.24	0.14	0.17	0.17	0.17	0.17
Saissetia coffeae	SC	0.51	0.17	0.12	0.15	0.38	-	0.05	0.10	0.17	0.11	0.20	0.20	0.21	0.20
Saissetia somereni	SS	0.57	0.13	0.10	0.09	0.28	0.06	-	0.07	0.17	0.08	0.18	0.18	0.18	0.18
Parasaissetia nigra	PN	0.52	0.12	0.10	0.08	0.28	0.12	0.08	-	0.15	0.08	0.17	0.17	0.18	0.17
Pulvinaria aurantii	PA	0.76	0.31	0.15	0.25	0.43	0.25	0.25	0.21	-	0.14	0.25	0.25	0.26	0.25
Parthenolecanium corni	PC	0.45	0.17	0.08	0.12	0.20	0.14	0.10	0.10	0.18	-	0.16	0.16	0.16	0.15
	SA1	0.42	0.33	0.28	0.29	0.26	0.32	0.27	0.25	0.45	0.22	-	**0.000**	**0.02**	**0.002**
Pulvinariella mesembryanthemi	**SA3**	0.42	0.33	0.28	0.29	0.26	0.32	0.27	0.25	0.45	0.22	**0.000**	-	**0.02**	**0.002**
	SA2	0.46	0.32	0.28	0.29	0.25	0.35	0.27	0.26	0.46	0.23	**0.02**	**0.02**	-	**0.02**
	EXT	0.41	0.33	0.28	0.29	0.26	0.33	0.27	0.25	0.45	0.22	**0.002**	**0.002**	**0.02**	-

Table 6.2c. Pairwise distances for the COI and 28S data of *P. mesembryanthemi* and the included outgroups (see SM Table 6.1 and 6.3). Distances within *P. mesembryanthemi* samples are marked in bold. Corrected distances calculated with the best-suited model for each dataset (see Table 6.1) are shown below the matrix diagonal and Kimura 2-parameter (K2P) distances are shown above diagonal.

COI + 28S		AN	CR	CH	CL	CV	SC	SS	PN	PA	PC	**SA1**	**SA3**	**SA2**	**EXT**
Aspidiotus nerii	AN	-	0.31	0.31	0.29	0.31	0.29	0.30	0.29	0.33	0.28	0.30	0.30	0.30	0.29
Ceroplastes rusci	CR	0.75	-	0.14	0.13	0.17	0.14	0.13	0.12	0.18	0.14	0.19	0.19	0.18	0.19
Coccus hesperidum	CH	0.75	0.20	-	0.13	0.17	0.14	0.13	0.13	0.14	0.12	0.19	0.20	0.18	0.19
Coccus longulus	CL	0.72	0.19	0.18	-	0.18	0.14	0.13	0.11	0.18	0.12	0.19	0.19	0.17	0.18
Coccus viridis	CV	0.77	0.27	0.30	0.30	-	0.20	0.18	0.18	0.22	0.15	0.18	0.18	0.17	0.18
Saissetia coffeae	SC	0.73	0.22	0.21	0.21	0.39	-	0.10	0.13	0.17	0.14	0.20	0.21	0.20	0.22
Saissetia somereni	SS	0.77	0.18	0.19	0.19	0.32	0.13	-	0.13	0.19	0.13	0.18	0.19	0.18	0.19
Parasaissetia nigra	PN	0.72	0.17	0.18	0.16	0.34	0.20	0.18	-	0.16	0.12	0.18	0.18	0.17	0.17
Pulvinaria aurantii	PA	0.85	0.29	0.20	0.27	0.39	0.28	0.31	0.26	-	0.16	0.23	0.23	0.21	0.22
Parthenolecanium corni	PC	0.67	0.20	0.18	0.18	0.25	0.23	0.19	0.17	0.25	-	0.17	0.16	0.16	0.16
Pulvinariella mesembryanthemi	**SA1**	0.40	0.30	0.30	0.30	0.30	0.4	0.31	0.30	0.40	0.30	-	**0.07**	**0.07**	**0.07**
	SA3	0.70	0.33	0.34	0.32	0.30	0.42	0.35	0.30	0.44	0.25	**0.09**	-	**0.08**	**0.02**
	SA2	0.70	0.28	0.28	0.27	0.28	0.36	0.32	0.27	0.37	0.24	**0.08**	**0.10**	-	**0.07**
	EXT	0.63	0.30	0.32	0.30	0.30	0.43	0.34	0.27	0.40	0.25	**0.08**	**0.03**	**0.09**	-

Also, the Pretoria populations SA1 and SA3 had a 14-15 % genetic TrN distance (17 % K2P distance), slightly higher than the distance between the Pretorian SA1 and the one from Eastern Cape Province SA2 (10 % TrN and 12-13 % K2P distances) (Table 6.2a). The highest similarities between *P. mesembryanthemi* haplotypes corresponded to the pairs of haplotypes SA3 - SAP (TrN and K2P distance of 0.1 and 0.2 %) and SA1.1 - SA1.2 (TrN and K2P distance of 0.2-0.3%) (Table 6.2a). The most similar outgroup species to *P. mesembryanthemi* was the scale insect *Ceroplastes rusci* (Table 6.2a).

Regarding the topology of the trees for the *P. mesembryanthemi* samples, it was quite similar for all methods (Fig. 6.2, SM Fig. 6.1, SM Fig 6.5). All the haplotypes were grouped in a clade with a high support from BI posterior probabilities, but with low support from ML and MP bootstrap values (Fig. 6.2). A clade formed by the exotic populations and the Pretorian populations SA3 and SAP was highly supported by both bootstrap values and by the BI posterior probability (Fig. 6.2). The three trees grouped the haplotypes SA1.1 and SA1.2 with SA2, although with a low bootstrap value (Fig. 6.2).

6.3.2. *Pulvinariella mesembryanthemi* populations: 28S gene

A 820 bp fragment with an average GC composition of 61 % resulted when the s3660/A335 primers were used (SM Table 6.2). The 28S sequenced region did not show variability between exotic population, but it distinguished 2 haplotypes in the South African populations (SA1, SA2) which were different from the exotic one (SM Table 6.2; Fig. 6.3). There were 18 polymorphic sites, only 2 of them parsimony informative (SM Table 6.2). Native populations had a haplotype diversity of 0.67 and 16 mutations (SM Table 6.2). From the analysed sequences of *P. mesembryanthemi* only one South African population (SA2) showed indels (SM Table 6.2).

From the distances calculations, the most similar populations to the exotics were the South African ones from Pretoria (SA1, SA3; with 0.2% HKY model and K2P distances), whilst the most different one was the South African population from Eastern Cape Province (2.2 % distance with both methods) (Tables 6.1, 6.2b). The distance between the Pretorian haplotype (SA1) and SA2 was of 2.0 % (with both methods) (Table 6.2b).

Trees estimated by Maximum Likelihood (Fig. 6.3), Maximum Parsimony (SM Fig. 6.2) and Bayesian Inference (SM Fig. 6.6) included a

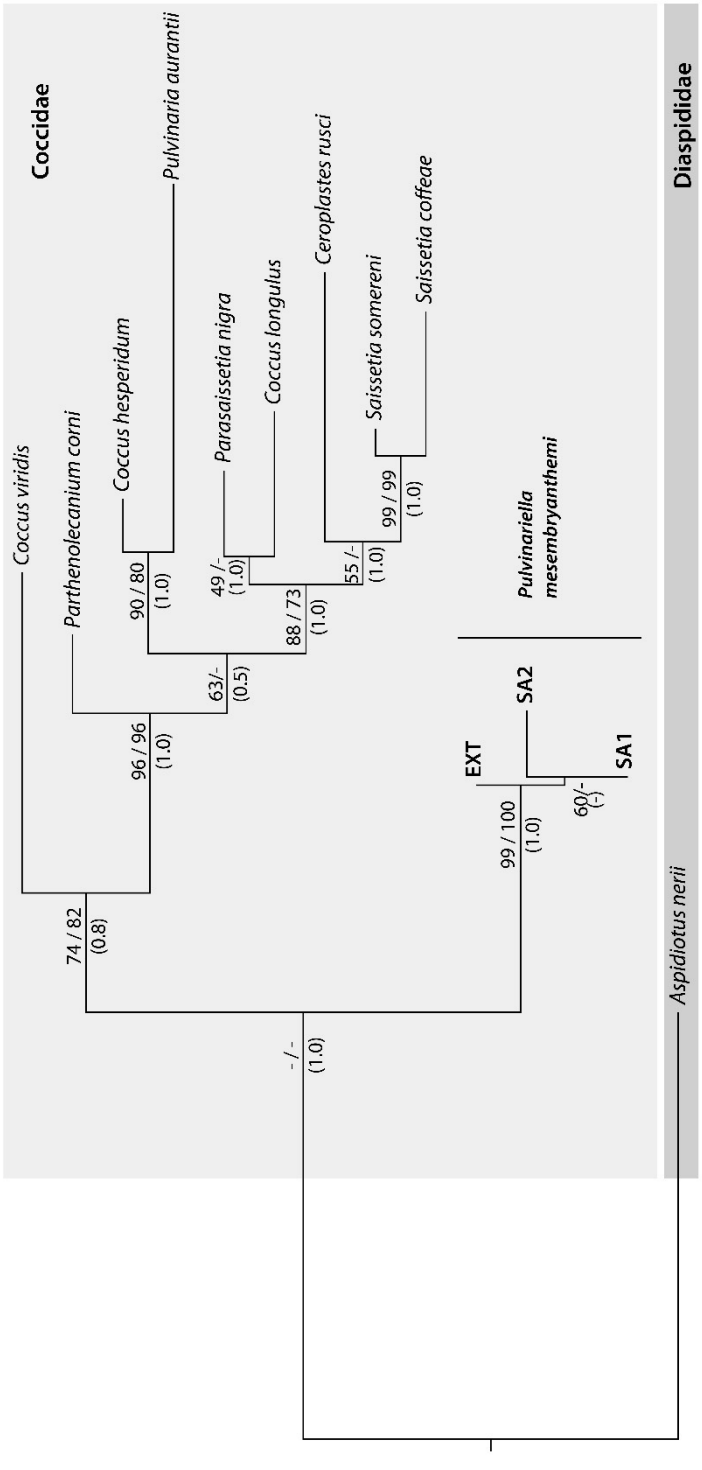


Fig. 6.3. Maximum likelihood tree based on 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1b). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent, in this order, the bootstrap support values (≥50 %) of maximum likelihood and maximum parsimony. Numbers within parenthesis represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.

monophyletic assemblage for the *P. mesembryanthemi* haplotypes, which was highly supported by bootstrap and posterior probability (Fig. 6.3).

The *P. mesembryanthemi* exotic haplotype (EXT) was nested with the native haplotype SA1 in the MP and BI trees, although with low support (SM Fig. 6.2, SM Fig. 6.6). However, the ML tree nested the two native haplotypes (SA1, SA2) with a low support (Fig. 6.3).

6.3.3. *Pulvinariella mesembryanthemi* populations: COI+28S genes

The most similar population to the exotic ones was the South African SA3, from Pretoria (Table 6.2c), whilst no much difference in distance was found for SA1 and SA2 (Table 6.2c). The most similar outgroup species to *P. mesembryanthemi* was *Parthenolecanium corni* (Table 6.2c).

As in the 28S trees, *P. mesembryanthemi* was recovered as a clade with a strong support of all methods (Fig. 6.4). The topology for the *P. mesembryanthemi* haplotypes varied between methods (Fig. 6.4, SM Fig. 6.3, SM Fig. 6.7). Independently of the method, there was a high support for a clade with the exotic haplotype (EXT) and one of the Pretorian haplotypes (SA3) (Fig. 6.4). There was a high support of ML bootstrap value and BI posterior probability for a clade between these two haplotypes (EXT, SA3) and the other Pretorian haplotype (SA1) (Fig. 6.4). However, the MP tree nested SA1 and SA2 haplotypes with a very low support (<50%) (SM Fig. 6.3).

6.3.4. Endosymbiont: COI gene

One band of 633 bp was obtained when the extracted DNA samples of *P. mesembryanthemi* were sequenced with the pair of primers LepF/LepR. However, when searched by BLAST in NCBI (*http://www.ncbi.nih.gov/BLAST/*), the highest similarity was with an α-Proteobacteria, a secondary endosymbiont of the aphid *Sitobian miscanthi* (NCBI –HQ645970; Li et al. 2011). The amplification of the COI region of this endosymbiont did not show any variability between exotic and native populations, except for the Australian population, which slightly differed from the others due to one single mutation.

The SA2 population was not included in this analysis because, when using the LepF/LepR primers, a parasitoid of the insect was sequenced instead. When that sequence was searched in the BLAST tool of the NCBI (*https://blast.ncbi.nlm.nih.gov/*), a match of 84 % similarity with two genera

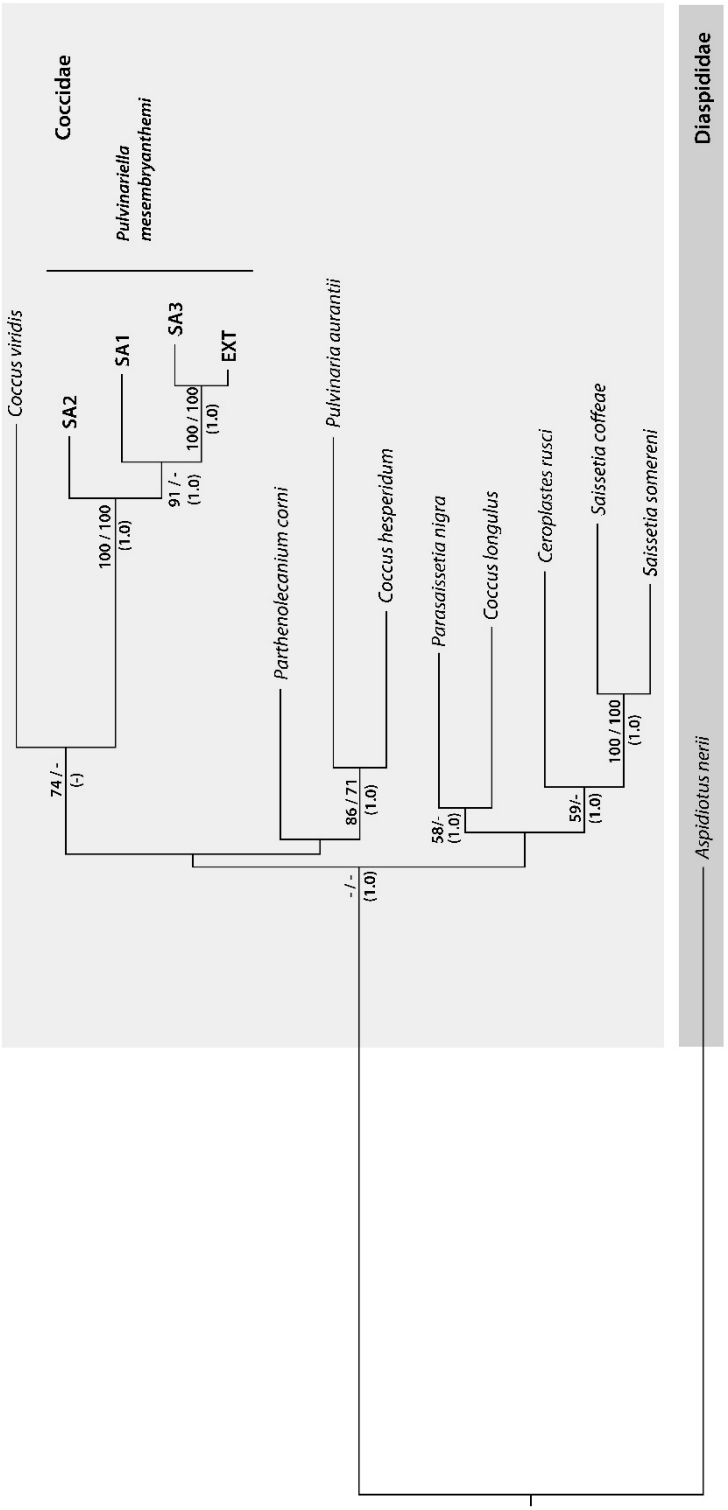


Fig. 6.4. Maximum likelihood tree based on the concatenation of COI and 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent, in this order, the bootstrap support values (≥50 %) of maximum likelihood and maximum parsimony. Numbers within parenthesis represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.

of the Chalcidoidea parasitic wasps (*Encarsia* sp. and *Encyrtidae* sp.) was found.

The two *P. mesembryanthemi* haplotypes that were found only differed a 0.3 % model corrected distance (Tables 6.1, 6.3). This *P. mesembryanthemi* endosymbiont was related the most to *S. mischanthi* endosymbiont (6 % GTR distance), and then to *Wolbachia* sp. and *Orientia tsutsugamushi* (Table 6.3).

Table 6.3. Pairwise distances for the COI data of *P. mesembryanthemi* 's endosymbiont and the included outgroups (see SM Table 6.1, SM Table 6.4). Distances within *P. mesembryanthemi* samples are marked in bold. Corrected distances calculated with the best-suited model for each dataset (see Table 6.1) are shown below the matrix diagonal and Kimura 2-parameter (K2P) distances are shown above diagonal.

		KS	LD	W	OT	SMLS	S_PME.1	S_PME.2
Kuckuckia spinosa	KS	-	0.22	0.38	0.46	0.46	0.43	0.43
Lagenidium deciduum	LD	0.33	-	0.35	0.35	0.38	0.32	0.32
Wolbachia sp.	W	0.68	0.60	-	0.32	0.42	0.30	0.30
Orientia tsutsugamushi	OT	0.99	0.65	0.50	-	0.29	0.30	0.30
Sitobion miscanthi endosymbiont	SMLS	0.88	0.70	0.89	0.45	-	0.06	0.06
Pulvinariella mesembryanthemi endosymbiont	S_PME.1	0.77	0.49	0.45	0.46	0.06	-	**0.003**
	S_PME.2	0.77	0.49	0.45	0.47	0.06	**0.003**	-

The two haplotypes of *P. mesembryanthemi* endosymbiont were grouped with a high support of the three tested methods (Fig. 6.5, SM Fig. 6.4, SM Fig. 6.8). The three Hemiptera endosymbiont haplotypes (two from *P. mesembryanthemi* and one from *S. miscanthi*) were nested with a high support of both bootstrap values and posterior probability (Fig. 6.5). The maximum likelihood tree was the only one to group the Hemiptera endosymbionts with *Wolbachia* sp. (a Collembola endosymbiont), although with a very low support (53%) (Fig. 6.5). With all methods, all the Proteobacteria included in the analysis were grouped with a very high support (Fig. 6.5).

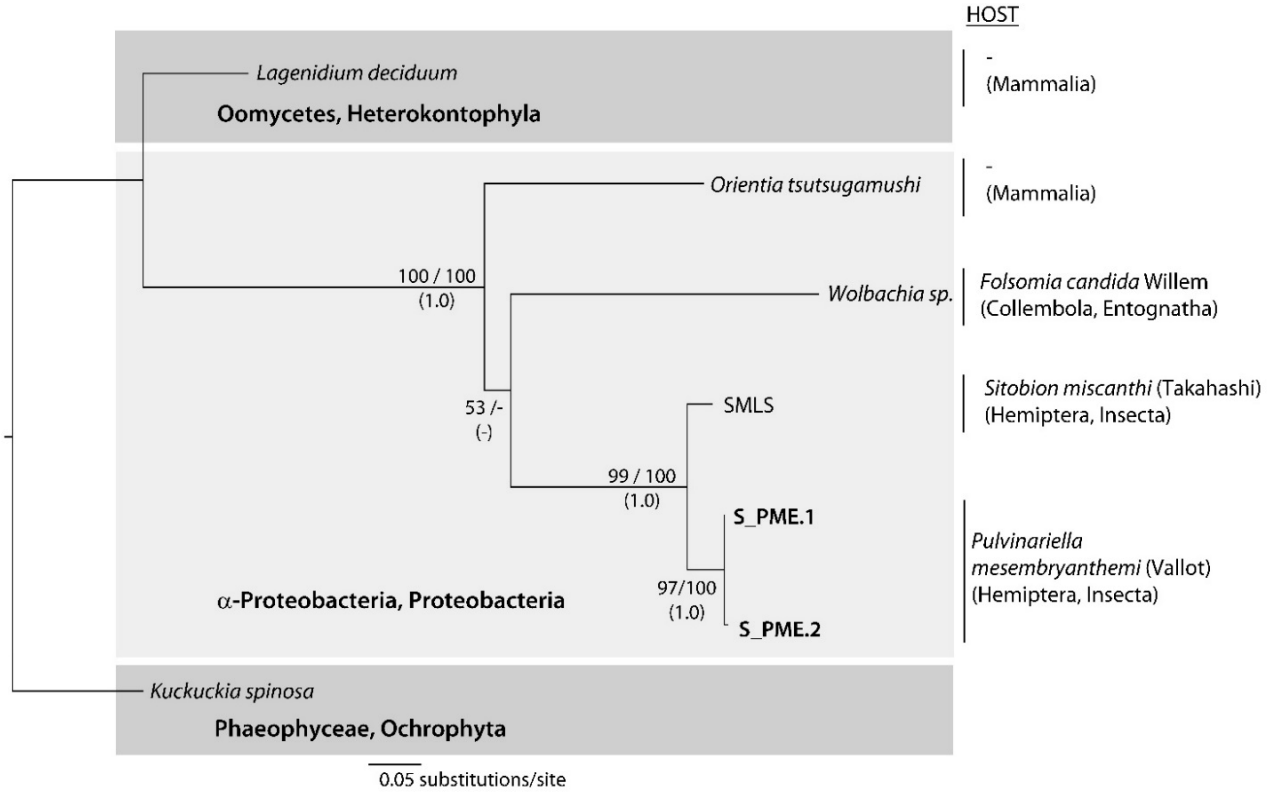


Fig. 6.5. Maximum likelihood tree based on COI data of the *Pulvinariella mesembryanthemi* endosymbiont and its closest taxa. Samples from the *P. mesembryanthemi* endosymbiont have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Kuckuckia spinosa*. On the left side of the figure, the class and phylum of the taxa are indicated, whereas in the right side the host species and their order and class are showed. The numbers next to the nodes represent, in this order, the bootstrap support values (≥50 %) of maximum likelihood and maximum parsimony. Numbers within parenthesis represent the Bayesian posterior probability (≥0.5). See SM Table 6.4 for NCBI codes.

6.4. DISCUSSION

6.4.1. *Pulvinariella mesembryanthemi* populations

The exotic populations of *P. mesembryanthemi* included in the analysis did not show any variability between populations for the two sequenced gene fragments, even when comparing populations from Europe and Oceania. The absence of genetic variability for the studied genes suggest that there was one single genotypic and geographic origin from South Africa of the different exotic populations of the insect, from where it expanded to the different areas, and that they did not diverged since then.

This lack of genetic variability in the introduced range has also been seen in studies of other scale insects (Schroer et al., 2008). Also, if we take into account that *P. mesembryanthemi* has only parthenogenetic reproduction (Nur, 1963, Pesson, 1941), even a single female might have been the origin of the exotic populations. In addition, the parthenogenetic reproduction together with the fairly recent introduction outside the native range [the first records of the insect in Europe and Oceania are from the 1980s (Vieira et al., 1983, Collins and Scott, 1982)] might have contributed to the genetic homogeneity of the species in the introduced area.

As this insect has a very high host specificity (Washburn and Frankie, 1985), it is probable that the same *Carpobrotus* sp. plants that hosted the original insect population were dispersed to the different exotic localities included in this study.

The origin of the exotic populations of *P. mesembryanthemi*, seems to be near Pretoria, since the native population SA3 was the most similar to them. However, a wider sampling in South Africa would be necessary to determine the precise origin of the introduced insects. Apparently, none of the native populations we included in this study was the origin of the exotic ones, as the genetic distances between them were quite high for the COI gene fragment.

Contrarily to what happened with *P. mesembryanthemi* exotic populations, native populations showed between samples variability and even, for one of the populations, slightly different haplotypes within it. The much higher variability between- than within- native populations may be due to reproductive isolation derived from the parthenogenetic reproduction (Roderick, 1996) and the limited mobility of the insect.

However, this results may be misleading due to the low number of analysed individuals.

Curiously, for the COI gene fragment, a slightly higher genetic distance was found between SA1 and SA3 populations (both from Pretoria and separated by approximately 12 km) than between SA1 and SA2 populations (separated roughly 900 km). This could be explained by a combination of limited mobility (as this insect is mostly sessile; Washburn and Frankie (1985)) and asexual reproduction, which would explain the higher differentiation between SA1 and SA3 populations; and occasional long-distance mobility [that could be attributed to wind; Washburn and Frankie (1981)], which could explain the higher similarity between SA1 and SA2 populations. This combination of the two factors have also been suggested for other scale insects (Cook and Rowell, 2007).

Pairwise distances between the studied populations were quite high in some cases. Amouroux et al. (2017) delimited the intra-specific K2P distance for the COI 5′ gene fragment to 0-2% and for the 28S gene fragment to 0-0.6% for Coccidae. Only the haplotype pairs SA3-SAP and SA1.1-SA1.2 (for COI) and SA1-EXT (for 28S) lay within these intervals. Amouroux et al. (2017) also found a gap region (from 2.0 to 12.5 % K2P distance) for their Coccidae samples between the intra-specific and inter-specific distances. However, in our COI samples the K2P distance between the exotic haplotype and SA3 or SAP is of 5%. The K2P distances between SA1 or SA2 and the rest of haplotypes would lie in the inter-specific interval found by Amouroux et al. (2017) for the 5′ COI region. Distances between SA2 and SA1 or EXT would also lie within the inter-specific interval found in Amouroux et al. (2017) for the 28S gene fragment. Another study on Coccidae found a wider intra-specific interval (0-4.2% K2P distance for COI and 0-1.25 % for 28S), but a much narrower gap (4.2-4.6% for COI) between intra-specific and inter-specific distances (Wang et al., 2015). The high genetic distance found between SA2 and the other *P. mesembryanthemi* haplotypes for both the COI and 28S gene fragments suggests the existence of a cryptic species complex, although a much more thorough sampling and morphological comparisons would be needed to check this possibility. Although the intra-specific and inter-specific intervals of the COI barcode region are usually well separated (Hebert et al., 2003), in some cases they can overlap (Davison et al., 2009). Moreover, divergences can be high in sedentary species with geographic isolation (Hebert et al., 2003).

As much higher genetic diversity was found in native areas compared to exotic areas of the insect, optimization experiments for the use of *P. mesembryanthemi* as a biocontrol agent should test native populations, where most of the genetic diversity can be found, and consequently, more chances of finding differences on host range, climate adaptability or capability of damaging the plant would be found.

6.4.2. Endosymbiont

Symbiosis of Hemiptera insects with α-Proteobacteria have been documented for several other species (Mathenge et al., 2015, Brady et al., 2014, Bing et al., 2013, Li et al., 2011b). In this study, the presence of an α-Proteobacteria endosymbiont in *P. mesembryanthemi* was confirmed by the sequences obtained with the pair of primers LepF/LepR.

The closest specimen found in the NCBI database was the endosymbiont SMLS (*Sytobian miscanthi* L type symbiont), which is as α-Proteobacteria from the Rickettsiaceae family (Li et al., 2011a, Li et al., 2011b). They were very closely related, with a similarity for the COI fragment of 95 %. SMLS was found to give some fitness advantages (Li et al., 2016) and to be widely present in the populations of its host insect (Li et al., 2011b), which belongs to the Hemiptera order as *P. mesembryanthemi*. In our study, endosymbiont genetic material was amplified from all the populations we tried (5 of them exotic and 2 native), with the exception of the SA2 population (although this could be related to a low specificity of the primers or of the extraction technique, as a parasitoid was sequenced in its place).

The sequenced endosymbionts of the exotic populations were identical for the chosen gene fragment to the native ones, with the exception of one exotic population that had a small difference. The variability of the endosymbiont was therefore much more reduced than the variability found for *P. mesembryanthemi*. This results are in accordance to the low genetic diversity between populations that Li et al. (2011b) also found for SMLS, and which they related to a recent and frequent intraspecific transfer.

The α-Proteobacteria endosymbionts found in Hemiptera insects are closely related to Rickettsiaceae bacteria (Bing et al., 2013, Brady et al., 2014, Mathenge et al., 2015). For instance, Li et al. (2011a) found a very close relationship between SMLS and the Rickettsiaceae bacteria *Orientia tsutsugamushi* (94% of similarity for the 16S sequence, although probably

from different genera), whilst in our case the similarity between our endosymbiont and a COI sequence from *O. tsutsugamushi* (GBBAC698-15) was of 75%.

6.5. SYNTHESIS

The South African scale insect *Pulvinariella mesembryanthemi* was introduced worldwide in several coastal areas with Mediterranean climate, probably through infested plants of *Carpobrotus* sp. Its high host specificity and its capacity to produce severe damages in the invasive *Carpobrotus* sp. plants makes this insect a potential biocontrol agent. In order to test the efficiency and host range of insects used for biocontrol, population genetic studies can help to unravel cryptic complexes and intraspecific diversity. In this study we performed a phylogeographic analysis including native and exotic populations of *P. mesembryanthemi*, through Sanger sequencing of mitochondrial (cytochrome *c* oxidase I, COI) and ribosomal (D2-D3 expansion segments of the large subunit ribosomal RNA gene 28S) gene fragments. Accidentally, an endosymbiont was sequenced with one of the pair of primers we used.

The exotic populations of the insect did not show any variability among populations for both studied genes, which suggest a common origin of all studied introduced populations. Contrastingly, native populations showed a fairly high variability. Also, the Gauteng populations (from NE South Africa) were phylogenetically the closest to the exotic ones, which suggests that the exotic populations could came from somewhere near there.

An endosymbiont of *P. mesembryanthemi* was detected, and the sequenced COI gene was similar to that of the Rickettsiaceae family from the α-Proteobacteria, and close to other insect endosymbionts. To the best of our knowledge, this was the first mention of this endosymbiont in *P. mesembryanthemi*, although α-Proteobacteria endosymbionts have been reported for other sap-sucking insects.

6.6. SUPPLEMENTARY MATERIAL

SM Table 6.1a. Samples of *Pulvinariella mesembryanthemi* used in this study: code, collection details, host plant (*Ce*, *Carpobrotus edulis; Ca, Carpobrotus aff. acinaciformis; Csp., Carpobrotus* sp.) are indicated. Key of collectors: CV, Cristina Vieites-Blanco; IS, Iñigo Sánchez-García; JA, Jesús R. Aboal Viñas; JG, José Rafael González López; KM, Kate McCombs; ML, Margarita Lema-Márquez; MS, M. T. Sethusa; PH, Paul D. N. Hebert; SG, Serafín González-Prieto; SN, Stefan Neser.

Code	Country, Region	Latitude-Longitude	Altitude (m asl)	Host	Collect by
STD	Spain, Santander	43.47ºN, 3.78ºW	13	*Ca*	CV
AST	Spain, Asturias	43.60ºN, 5.95ºW	23	*Csp.*	JG
MER	Spain, Oleiros	43.38ºN, 8.34ºW	11	*Ca*	ML,CV
CNH	Spain, A Coruña	43.38ºN, 8.44ºW	29	*Ce*	ML,CV
NAR	Spain, Punta Nariga	43.32ºN, 8.91ºW	25	*Ce*	ML,CV
SAL	Spain, Sálvora Island	42.47ºN, 9.01ºW	25	*Ca,Ce*	ML,CV
CAD	Spain, Cádiz	36.63ºN, 6.16ºW	22	*Csp.*	IS
TEN	Spain, Tenerife Island	28.48ºN, 16.31ºW	517	*Csp.*	JA
LPA	Spain, La Palma Island	28.66ºN, 17.92ºW	297	*Ce*	SG
LTE	Portugal, Lisboa	38.76ºN, 9.17ºW	99	*Ce*	CV
CCS	Portugal, Cascais	38.70ºN, 9.47ºW	40	*Ca,Ce*	CV
EST	Portugal, Estoril	38.70ºN, 9.39ºW	14	*Ca*	CV
CAP	Portugal, Costa da Caparica	38.61ºN, 9.22ºW	5	*Ce*	CV
NZL	N. Zealand, Christchurch	43.49ºS, 172.72ºE	7	*Ce*	KM
AUS	Australia, New South Wales	31.06ºS, 153.05ºE	37	-	PH
SA1	S. Africa, Pretoria	25.79ºS, 28.31ºE	1376	*Csp.*	SN
SA2	S. Africa, Eastern Cape	33.13ºS, 24.37ºE	434	*Ce*	SN
SA3	S. Africa, Pretoria	25.72ºS, 28.22ºE	1298	*Csp.*	SN
SAP	S. Africa, Pretoria	25.60ºS, 28.35ºE	-	-	MS

S.M. Table 6.1b. On the first column, codes of the samples of *Pulvinariella mesembryanthemi* used in this study. the right side of the table, the accession number (AUS and SAP in *www.boldsystems.org*; the other in *https://www.ncbi.nlm.nih.gov*), the number of samples used and the number and code of the obtained haplotypes are presented for each gene (COI and 28S).

	COI			28S			Endosymbiont COI		
Code	Accession no.	No.	Haplot.	Accession no.	No.	Haplot.	Acces. no.	No.	haplot.
STD	MH317111	1	EXT	-	0		-	0	
AST	MH317120	1	EXT	MH317125	1	EXT	MH317129	1	S_PME.1
MER	MH346333	1	EXT	-	0		-	0	
CNH	MH317119	1	EXT	-	0		-	0	
NAR	MH317118	1	EXT	-	0		MH317131	1	S_PME.1
SAL	MH317117	1	EXT	-	0		-	0	
CAD	MH317122	1	EXT	-	0		-	0	
TEN	MH317121	1	EXT	-	0		MH137133	1	S_PME.1
LPA	MH317115	1	EXT	-	0		-	0	
LTE	MH317112	1	EXT	-	0		-	0	
CCS	MH317114	1	EXT	-	0		MH317130	1	S_PME.1
EST	MH317113	1	EXT	-	0		-	0	
CAP	MH317116	1	EXT	-	0		-	0	
NZL	MH317123	1	EXT	MH317124	1	EXT	-	0	
AUS	HMAS125.11	1	EXT	-	0		HMAS126.11	1	S_PME.2
SA1	MH317088-094	7	SA1.1-1.2	MH317126	1	SA1	MH317132	1	S_PME.1
SA2	MH317103-110	8	SA2	MH317128	1	SA2	-	0	
SA3	MH317095-102	8	SA3	MH317127	1	SA1	-	0	
SAP	SIBI521.11	1	SAP	-	0		-	0	

SM Table 6.2. Descriptive parameters of the *Pulvinariella mesembryanthemi* sequences used in this study for the exotic populations, native populations (South African) and all together (exotic+native). Key: Hd (haplotype diversity), π (nucleotide diversity).

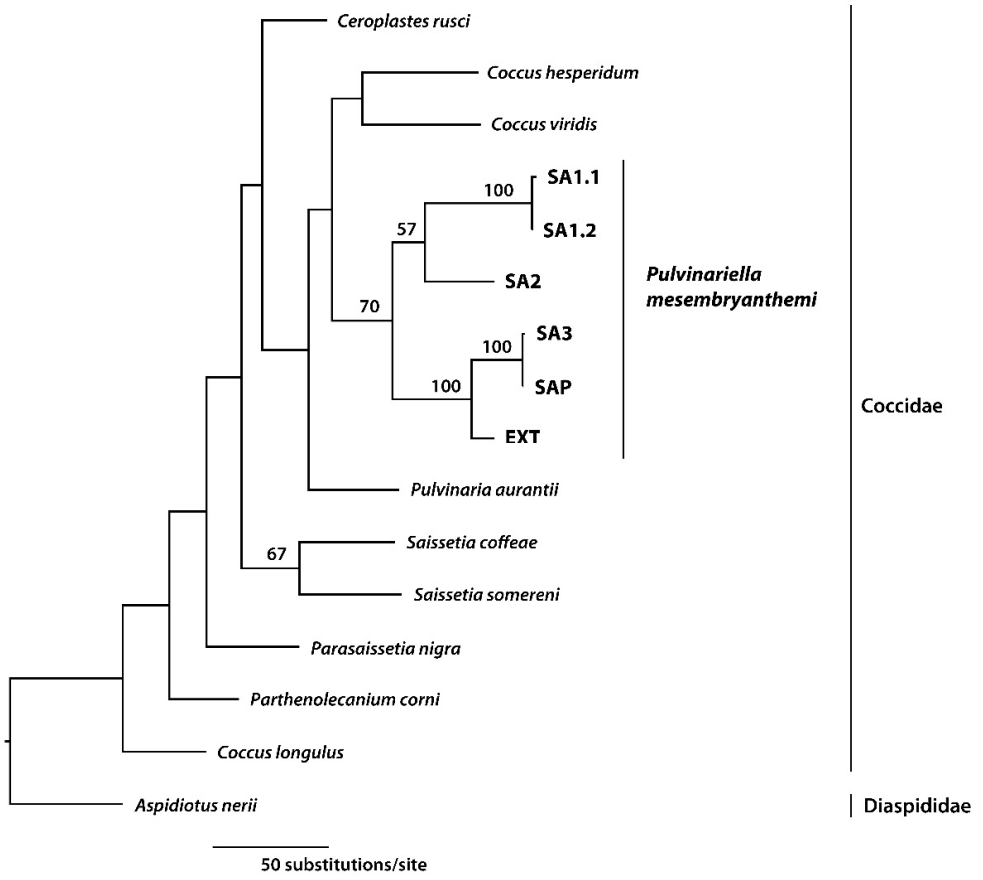
	PME exotic			PME native			PME exotic + native		
Parameters	COI	28S	COI + 28S	COI	28S	COI + 28S	COI	28S	COI + 28S
Sequenced taxa	16	2	2	24	3	3	40	5	5
Fragment size (bp)	679	820	1499	679	825	1504	679	825	1504
Parsimony informative sites	0	0	0	129	0	0	122	2	53
S (polymorphic sites)	0	0	0	129	16	145	122	18	147
Eta (t no. of mutations)	0	0	0	141	16	157	138	18	164
h (no. haplotypes)	1	1	1	5	2	3	5	3	4
Hd (± SD)	0	0	0	0.76 ± 0.05	0.67 ± 0.31	1.00 ± 0.27	0.75 ± 0.04	0.80 ± 0.03	0.90 ± 0.16
π (± SD)	0	0	0	0.10 ± 0.01	0.01 ± 0.01	0.07 ± 0.02	0.08 ± 0.01	0.01 ± 0.01	0.05 ± 0.01
Indel sites	0	0	0	0	5	0	0	5	0
Average indel length	0	0	0	0	1.67	0	0	1.67	0
Indel haplotypes	0	0	0	0	2	0	0	2	0
Indel haplotype diversity	0	0	0	0	0.67	0	0	0.4	0
G + C content	25	61	-	25	61.3	-	24.9	61.2	-

SM Table 6.3. Species name and accession number (from the NCBI, *www.ncbi.nlm.nih.gov*) of the scale insect COI and 28S gene fragments included in the analysis of *Pulvinariella mesembryanthemi*.

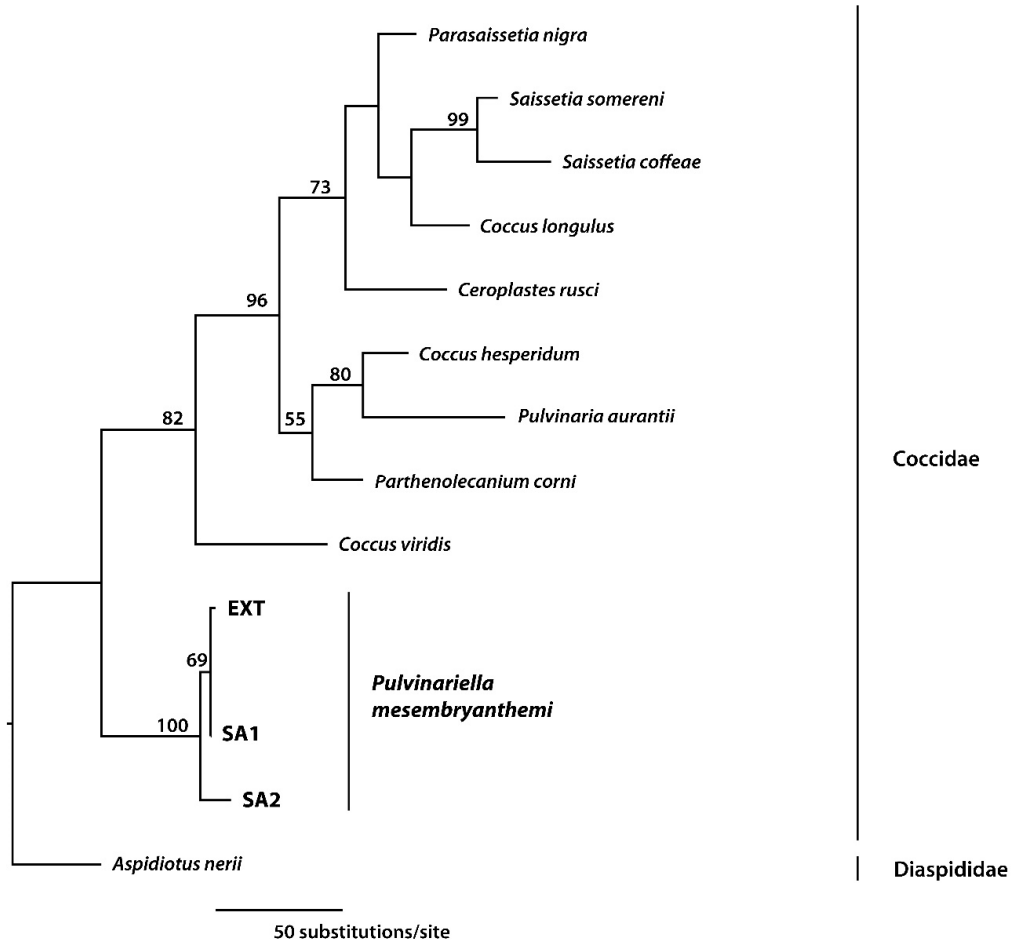
Species	COI	28S
Ceroplastes rusci (Linnaeus, 1758)	KJ919960	JQ651327
Coccus hesperidum (Linnaeus, 1758)	GU936950	JX645350
Coccus longulus (Douglas, 1887)	JX853907	JX866693
Coccus viridis (Green,1889)	GU936953	JX645351
Parasaissetia nigra (Nietner, 1861)	KY927696	KY927608
Parthenolecanium corni (Bouche, 1844)	KP189848	KY085852
Pulvinaria aurantii (Cockerell, 1896)	KP189885	KP189549
Saissetia coffeae (Walker, 1852)	JX845480	JX645353
Saissetia somereni (Newstead,1910)	KY927540	KY927550
Aspidiotus nerii (Bouché, 1833)	KY085366	KY085669

SM Table 6.4. Species name and accession number (from the NCBI, *www.ncbi.nlm.nih.gov*) of COI gene fragments close to *Pulvinariella mesembryanthemi* 's endosymbiont.

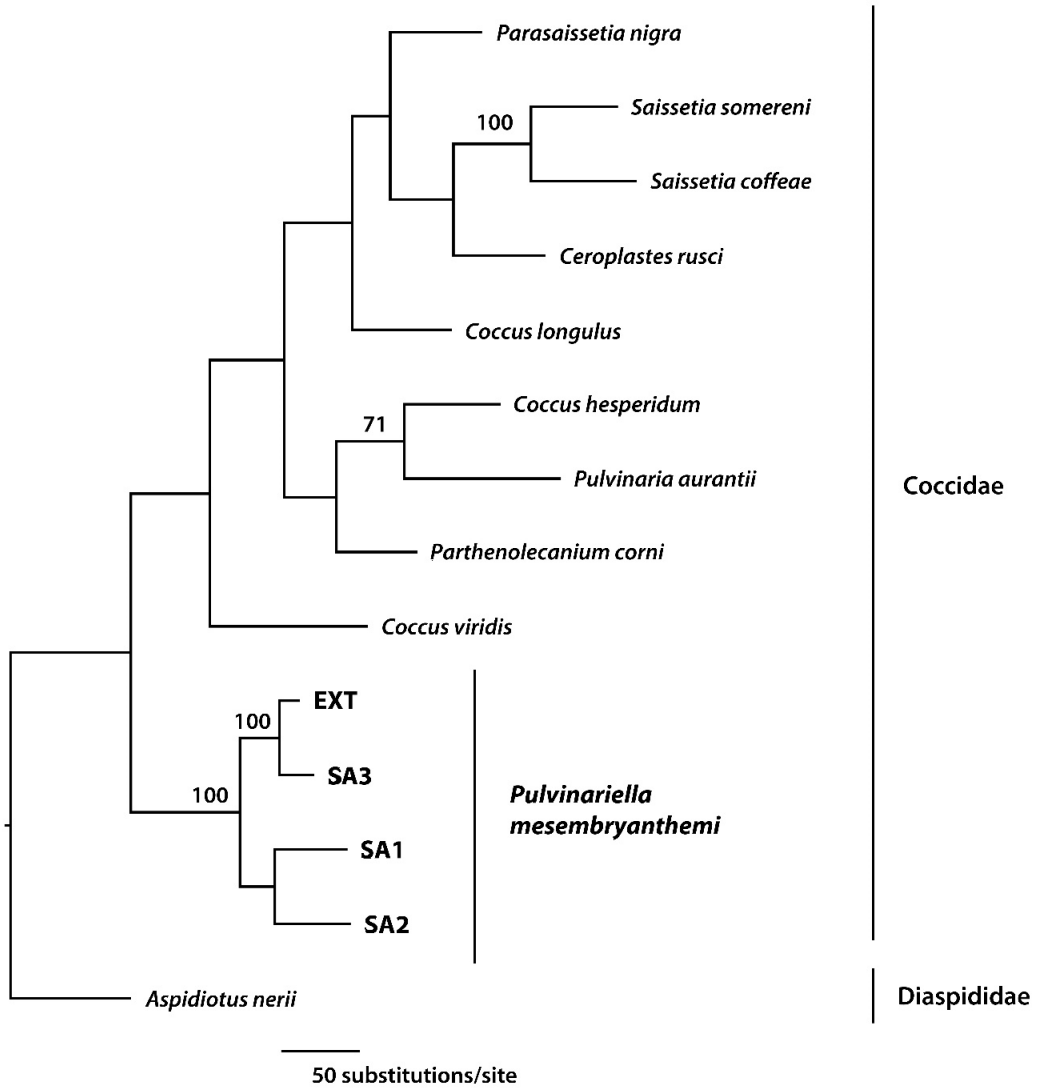
Species	COI
Kuckuckia spinosa (Kuetzing) Kornmann, 1958	LM995336
Lagenidium deciduum Chi Y. Chen, Grooters, Spies, de Cock, Levesque, 2013	KC741455
Orientia tsutsugamushi (Hayashi 1920) Tamura et al. 1995	GBBAC698-15
SMLS (*Sitobion miscanthi* L type symbiont)	HQ645970
Wolbachia sp.	CP015510



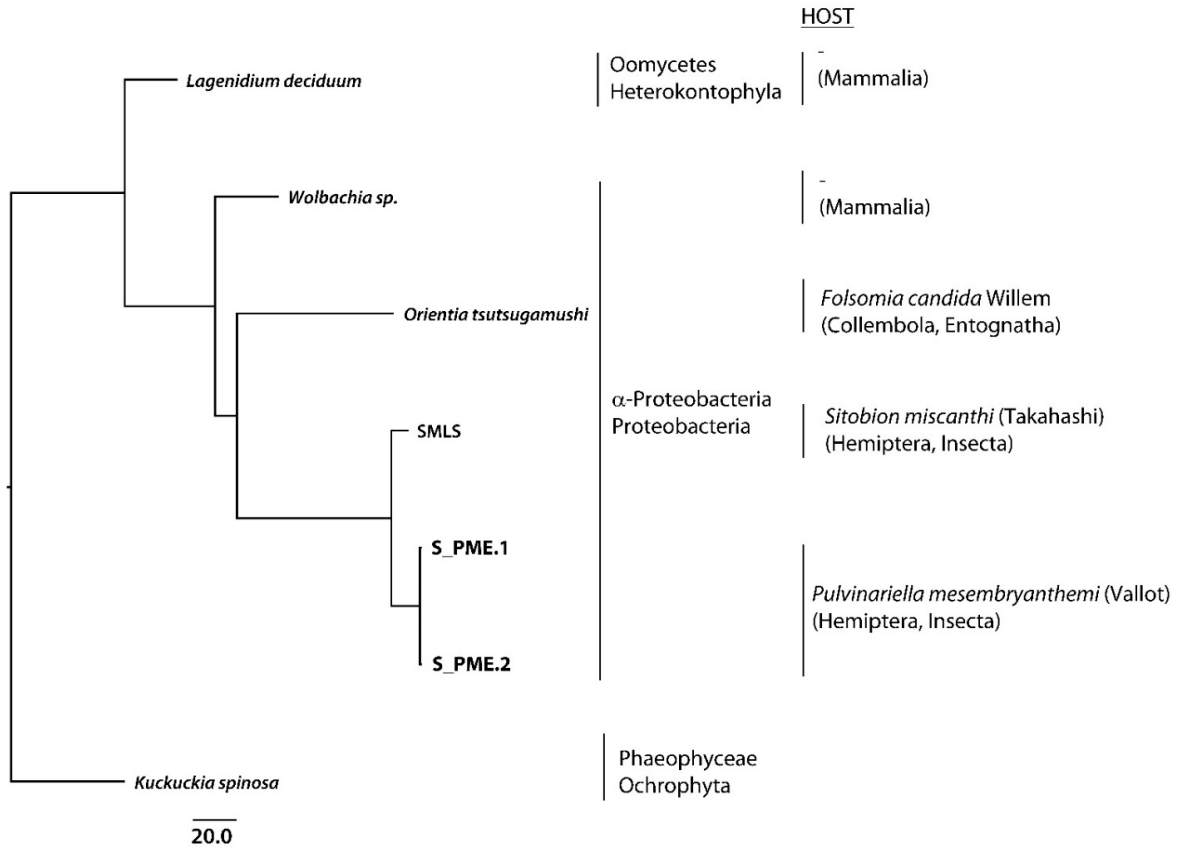
SM Fig. 6.1. Maximum parsimony tree based on COI data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent the bootstrap support values (≥50 %) of maximum parsimony. See SM Table 6.3 for NCBI codes.



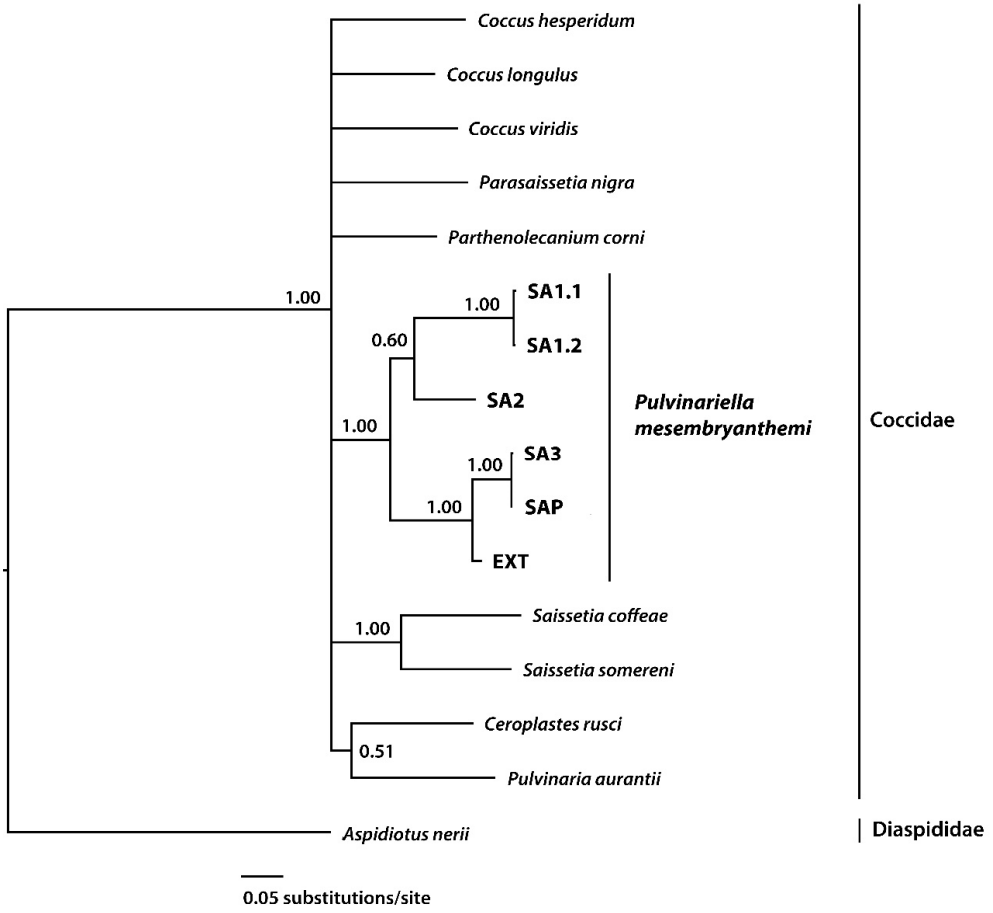
SM Fig. 6.2. Maximum parsimony tree based on 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent the bootstrap support values (≥50 %) of maximum parsimony. See SM Table 6.3 for NCBI codes.



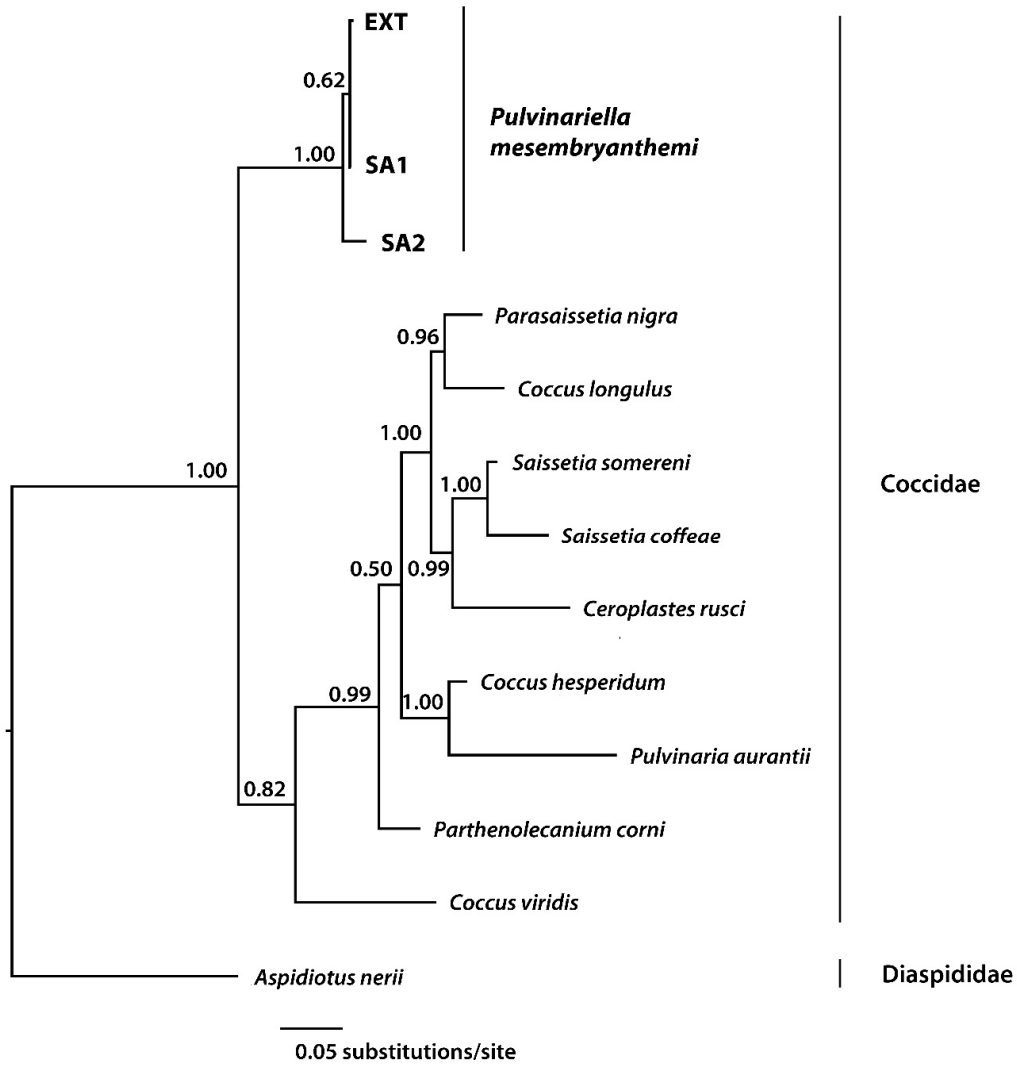
SM Fig. 6.3. Maximum parsimony tree based on the concatenation of COI and 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent the bootstrap support values (≥50 %) of maximum parsimony. See SM Table 6.3 for NCBI codes.



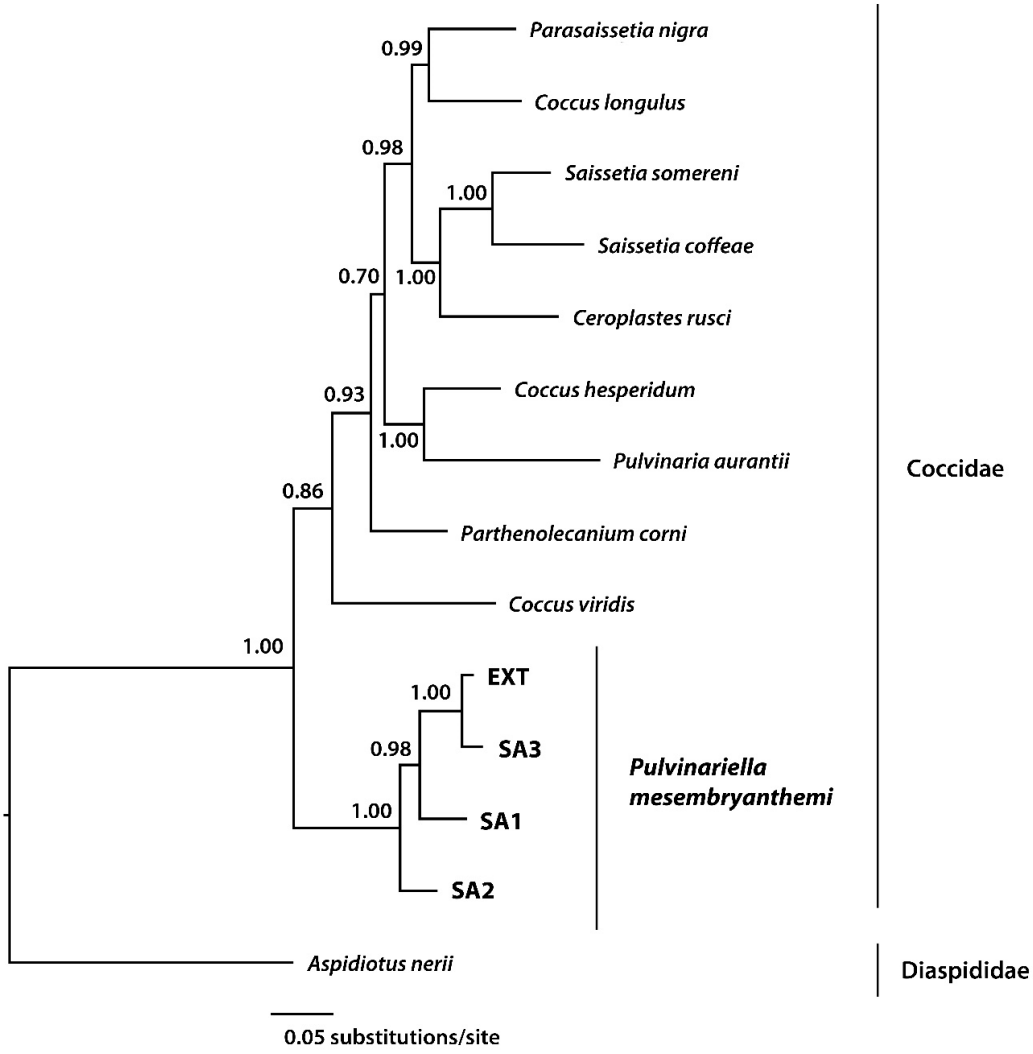
SM Fig. 6.4. Maximum parsimony tree based on COI data of the *Pulvinariella mesembryanthemi* endosymbiont and its closest taxa. Samples from the *P. mesembryanthemi* endosymbiont have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Kuckuckia spinosa*. On the right side of the figure, first the class and phylum of the taxa, and then the host species and its order and class are indicated. The numbers next to the nodes represent the bootstrap support values (≥50 %) of maximum parsimony. See SM Table 6.4 for NCBI codes.



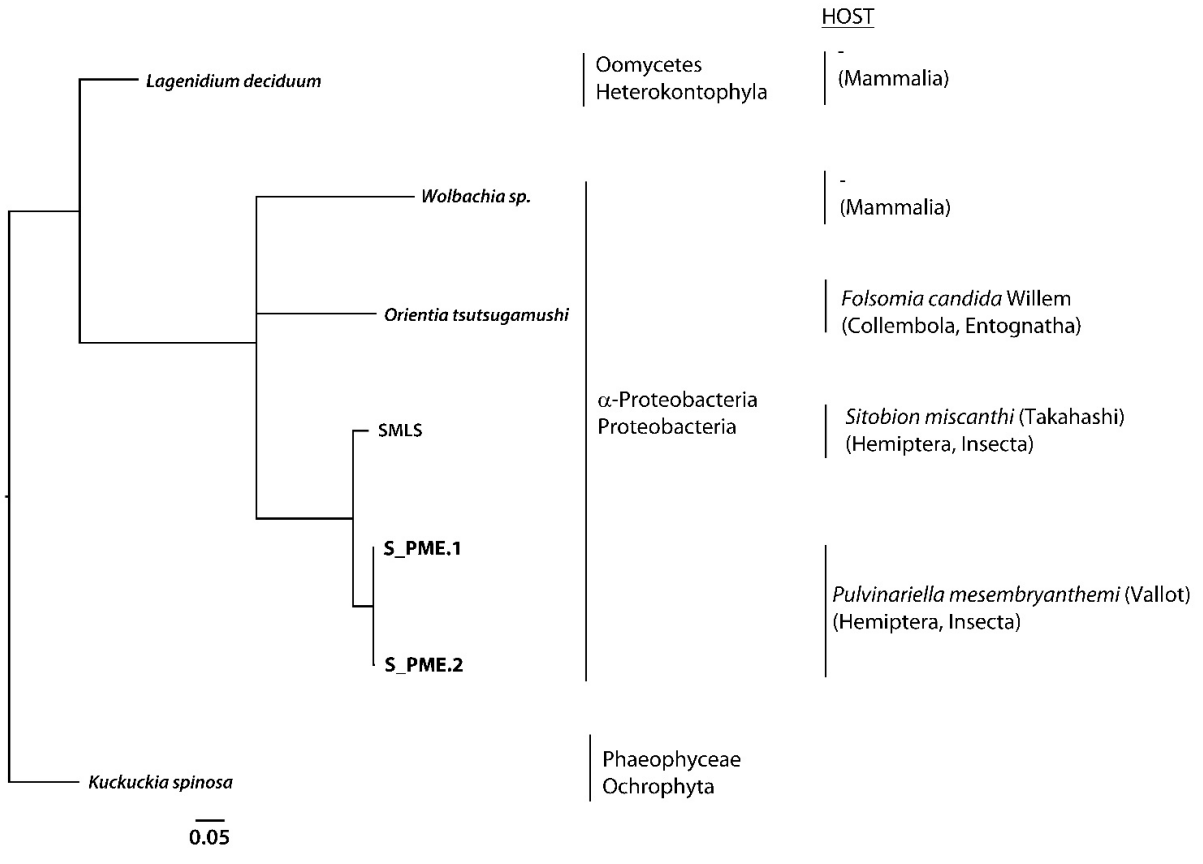
SM Fig. 6.5. Bayesian inference tree based on COI data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.



SM Fig. 6.6. Bayesian inference tree based on 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the family of the scale insects are indicated. The numbers next to the nodes represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.



SM Fig. 6.7. Bayesian inference tree based on the concatenation of COI and 28S data of scale insects. Samples from *Pulvinariella mesembryanthemi* have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Aspidiotus nerii*. On the right side of the figure, the families of the scale insects are indicated. The numbers next to the nodes represent the Bayesian posterior probability (≥0.5). See SM Table 6.3 for NCBI codes.



SM Fig. 6.8. Bayesian inference tree based on COI data of the *Pulvinariella mesembryanthemi* endosymbiont and its closest taxa. Samples from the *P. mesembryanthemi* endosymbiont have been marked in bold and the name of the haplotype is presented (see SM Table 6.1). The tree was rooted with *Kuckuckia spinosa*. On the right side of the figure, first the class and phylum of the taxa, and then the host species and its order and class are indicated. The numbers next to the nodes represent the Bayesian posterior probability (≥ 0.5). See SM Table 6.4 for NCBI codes.

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7. Conclusions

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7. Conclusions

7.1. Effect on soils of *Carpobrotus edulis*

1. The invasion by *C. edulis* alters soil properties, especially in rocky sites (richer in alien necromass) and the uppermost layer suggesting that this invader mostly affects soil characteristics through necromass accretion, which is strongly and negatively correlated with soil pH, and consequently alters nutrient availability.

2. The differences in necromass characteristics from invaded and non-invaded areas could be related with some competitive advantages derived from *C. edulis* physiology, such as a restriction of Al uptake, higher uptake of macronutrients and less requirements (or higher resorption from senescent tissues) of micronutrients. Consequently, more research is needed on nutrient uptake and reallocation after senescence to better understand the invasive potential of *C. edulis*.

3. Through its effect over soil chemical and biological properties, the invasion of rocky coastal habitats by *C. edulis* modifies the pools and fluxes (gross and net) of N in the topsoil (0-10 cm), impacting the N cycle. The NO_3^--N pool decreases and that of NH_4^+-N increases in the invaded soils. Overall, net mineralization decreases after the invasion, which limits the availability of soil N to plants, with unknown consequences at the level of plant community.

4. The main NH_4^+ consuming process was immobilization in invaded soils and autotrophic nitrification in non-invaded soils. These results are likely a consequence of the reduction of N availability triggered by the invasion, which increased NH_4^+ immobilization, and the inhibition of nitrifiers activity due to soil acidification or *C. edulis* exudates, which decreased autotrophic nitrification.

5. Although it is usually associated to anaerobic conditions, the dissimilatory reduction to ammonium was the exclusive or dominant NO_3^- consumption rate in most of our C-rich soils. The oxygen depletion in microsites usually found in organic matter rich soils could create the adequate conditions for the dissimilatory NO_3^- reduction to ammonium, its decrease in the invaded soils being likely explained by the lower NO_3^- availability.

6. Restoration of invaded ecosystems with *C. edulis* should be preferentially done in the first stages of the invasion, when soil effect would be lighter, and should include the elimination of necromass to avoid legacy effects on soil.

7.2. BIOLOGICAL CONTROL OF *CARPOBROTUS EDULIS*

1. The fungus *Sclerotinia sclerotiorum* can infect *C. edulis*, generating chlorosis and reducing the photosynthetic-radiation use efficiency of the plant in greenhouse conditions. These effects are brief and with no long-term consequences on *C. edulis* survival and growth. However, the effects of the fungus on the field may be more intense, as the infection efficiency of *S. sclerotiorum* depends on the environmental conditions.

2. The insect *Pulvinariella mesembryanthemi* can affect *C. edulis* in greenhouse conditions (especially after 6 months), inducing chlorosis and reducing photosynthetic performance, survival and growth. The infested plants counteract the attack increasing the photosynthetic performance in the short term and increasing the proportion of photosynthetic tissue. As a possible defence mechanism, the most heavily infested plant parts usually die, which may hinder the insect dispersion to non-infested parts of the plant.

3. The combination of *S. sclerotiorum* and *P. mesembryanthemi* only produces synergetic effects on plant performance on the short-term and has detrimental effects on the insect colonization success. Therefore, the application of *P. mesembryanthemi* alone seems the best option for *C. edulis* control. Also, the origin of the plant (native or non-native) does not affect the effectiveness of these two tested biocontrol agents, although infested native plants reach lower *P. mesembryanthemi* densities in greenhouse conditions.

4. The modelled potential distribution of *P. mesembryanthemi* in Galicia (NW Spain) covers all the coast, similarly to *Carpobrotus* spp. Therefore, the more restricted distribution of the insect than the plant may derive from low dispersal capacity or biotic factors not taken into account in the model, and not from limitations in habitat suitability. The distribution of both the insect and the host plant are dependent on soil and air temperature, being both limited by low temperatures. Also, *P.*

mesembryanthemi is favoured by high soil moisture, as its growth and performance is dependent on the water content of *C. edulis*.

5. In NW Spain, *P. mesembryanthemi* has two cycles per year, reaching the highest density in summer. Only in specially sheltered locations the insect maintains high densities all year, as the climate conditions of the region cause a heavy drop of the insect populations in winter.

6. Although *C. edulis* is also attacked by other sap-sucking insects, fruit predators and viruses, only high densities of *P. mesembryanthemi* seem to negatively impact *C. edulis* in the field. The insect *P. mesembryanthemi* has parasites and predators in NW Spain, which can limit its population growth despite the presence of potentially mutualistic ants.

7. Worldwide, the exotic populations of *P. mesembryanthemi* seem to come from a single geographic origin within its native area, which would explain the low genetic variability found for the studied genes. However, native populations of *P. mesembryanthemi* are genetically much more variable, so studies on the optimization of the control should preferentially focus on South African populations.

8. The insect *P. mesembryanthemi* has an endosymbiont, very close to the Rickettsiaceae family (α-Proteobacteria), which has a very low genetic variability, even when comparing populations from the native and non-native range of *P. mesembryanthemi*.

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8. Resumen general

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8.1. LA PLANTA INVASORA *CARPOBROTUS EDULIS*

Carpobrotus edulis (L.) N.E. Br. y su complejo de híbridos *Carpobrotus* aff. *acinaciformis* (L.) L. Bolus (nombres comunes: uña de gato, hierba del cuchillo, uña de león, bálsamo, diente de león, higo marino, higo del Cabo), son plantas crasuláceas originarias de Sudáfrica y pertenecientes a la familia Aizoaceae (Phylum Magnoliophyta; Clase Magnoliopsida; Orden Caryophyllales) (Delipetrou, 2006, Suehs et al., 2004). Están presentes, como exóticas naturalizadas o invasoras, en zonas costeras templadas de Eurasia, África, América y Oceanía (Parker, 2008) donde pueden colonizar dunas, acantilados y áreas perturbadas (Campos et al., 2004, D'Antonio, 1993, Maltez-Mouro et al., 2010). En España, se citó por primera vez en Baiona (Galicia) en 1892 (Lázaro-Ibiza, 1900), y actualmente se encuentra ampliamente naturalizada con carácter invasor a lo largo de la costa peninsular española y en las islas Baleares y Canarias (Sanz-Elorza et al., 2004). Las introducciones fuera de su área nativa responden principalmente a motivos ornamentales y de estabilización de dunas o taludes, pero también a usos médicos o culinarios o utilización como cortafuegos (Pierce, 1994, Campos et al., 2004).

La alta capacidad invasora de la uña de gato se relaciona con una mayor capacidad competidora que las plantas nativas de los ecosistemas invadidos, derivada de la formación de densas matas de hasta 50 cm de altura (Maltez-Mouro et al., 2010, Ruffino et al., 2015), su rápido crecimiento (Traveset et al., 2008), la inhibición de la germinación de plantas nativas (Novoa et al., 2012), su reproducción por medios sexuales (con alta producción de semillas por fruto: >1000 en *C. edulis* y >350 en *C. aff. acinaciformis*) (Suehs et al., 2004) y asexuales (con capacidad de división de tareas en ambientes heterogéneos y comunicación subterránea frente a ataques de herbivoría) (Roiloa et al., 2014, Rodríguez et al., 2018), la endozoocoria en zonas invadidas (que incrementa la dispersión y germinación de semillas) (Novoa et al., 2012), una rápida adaptación evolutiva (Roiloa et al., 2016), adaptaciones a condiciones de salinidad edáfica (Varone et al., 2017), o la plasticidad morfológica frente a la disponibilidad de luz (Fenollosa et al., 2017).

Carpobrotus spp. es considerado un ingeniero ecosistémico, ya que puede alterar tanto factores bióticos (biodiversidad vegetal y animal, estructura de comunidades vegetales y microbianas, polinización, hibridación con plantas nativas) como abióticos (disponibilidad de agua y luz, propiedades del suelo) de los ecosistemas invadidos en su propio beneficio (Molinari et al., 2007, Badalamenti et al., 2016, Carboni et al., 2010, Vilà et al., 2009, Waycott, 2016, de la Peña et al., 2010, Orgeas et al., 2007). Los efectos sobre las propiedades del suelo de esta planta invasora dependen del tipo de hábitat invadido (Novoa et al., 2014, Santoro et al., 2011, Winsemius, 2013) y pueden persistir después de la retirada de la planta, dificultando la restauración de la zona (Conser y Connor, 2009). Los estudios del efecto de *Carpobrotus* spp. en el suelo se han centrado principalmente en ambientes dunares y en la disponibilidad de macronutrientes (N, P, Ca y Na), el pH y la materia orgánica, dejando de lado los efectos sobre la disponibilidad de micronutrientes [pese a su importancia como nutrientes esenciales o elementos tóxicos por encima de un cierto umbral (Williams and Fraústo da Silva, 2000)] o sobre el ciclo del N [considerado uno de los nutrientes más frecuentemente limitantes en los ecosistemas (Vitousek y Howarth, 1991, Galloway et al., 2004)].

El control de las invasiones de uña de gato se realiza generalmente mediante el arrancado manual o el uso de herbicidas (Sanz-Elorza et al., 2004). Estos métodos no pueden ser utilizados en zonas poco accesibles (p. ej. acantilados), vulnerables a la erosión o con especies endémicas o protegidas (p. ej. dunas) (Carta et al., 2004). Una posible alternativa sería la introducción de agentes de control biológico, como depredadores, patógenos o enfermedades de *Carpobrotus* spp., que contrarresten la ventaja de esta exótica de encontrarse menos afectada por enemigos naturales que las especies nativas (Van Grunsven et al., 2009, Maltez-Mouro et al., 2010). El hongo generalista y cosmopolita *Sclerotinia sclerotiorum* (Lib.) de Bary (Helotiales: Sclerotiniaceae), ampliamente estudiado como micoherbicida de otras invasoras (Saharan y Mehta, 2008b), puede producir síntomas en plantas de la familia Aizoaceae (Cother, 2000, Saharan y Mehta, 2008a). No obstante, de acuerdo con la revisión bibliográfica realizada, las posibilidades de este hongo como agente de control biológico sobre la uña de gato no han sido todavía estudiadas. El insecto escama *Pulvinariella mesembryanthemi* (Vallot) (Hemiptera: Coccidae), de origen sudafricano (pero ampliamente

extendido en zonas invadidas por *Carpobrotus* spp.), parasita casi exclusivamente plantas de este género (Washburn y Frankie, 1985). Cuando se encuentra en altas densidades es capaz de producir daños importantes y una elevada mortandad sobre *Carpobrotus* spp., especialmente si las condiciones ambientales son subóptimas para la planta (Washburn y Frankie, 1985).

Por todo ello, es necesario proceder al estudio de:

1) El efecto de la uña de gato sobre las propiedades del suelo en distintos tipos de hábitat invadido, con especial hincapié en los efectos sobre la disponibilidad de micronutrientes y en el ciclo de N que han sido ignorados hasta el momento.

2) Las posibles alternativas para el control biológico de esta invasora, considerando el efecto de factores bióticos y abióticos sobre su viabilidad y eficiencia.

8.2. ESTUDIO DE LOS EFECTOS DE *C. EDULIS* EN EL SUELO

Para determinar el efecto de *C. edulis* en suelos de distintos tipos de hábitat (dunas o zonas rocosas del NO ibérico) y en distintas profundidades (capas de 0-5 y 5-10 cm), se compararon propiedades físico-químicas y químicas en suelos (capacidad de campo, humedad, pH, conductividad eléctrica, NH_4^+-N, NO_3^--N, $\delta\,^{13}C$, $\delta\,^{15}N$, C orgánico, N orgánico y Al, B, Ca, Co, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P y Zn disponibles) y necromasa (contenido total de los últimos 16 elementos) de áreas invadidas y no invadidas por *C. edulis*. En las zonas rocosas, se estimaron además los flujos brutos de N en el suelo (0-5 y 5-10 cm) realizando un experimento con marcaje pareado ($^{15}NH_4{}^{14}NO_3$ y $^{14}NH_4{}^{15}NO_3$) y el modelo *Ntrace* (Müller et al., 2007).

Los efectos de *C. edulis* sobre las propiedades del suelo son más intensos en las zonas rocosas y en la capa más superficial del suelo (0-5 cm), lo que sugiere que la invasora afecta a las características del suelo principalmente a través de la acumulación de necromasa. Estos efectos varían según el tipo de hábitat invadido. En zonas rocosas invadidas, la acumulación de necromasa supera ampliamente aquella de zonas

dunares, lo que podría explicar el diferente efecto de *C. edulis* en el pH: con incrementos en dunas (posiblemente por una mayor absorción de Ca de zonas profundas del suelo que las especies nativas) y descensos en zonas rocosas (probablemente por una mayor producción de ácidos orgánicos procedentes de la descomposición de necromasa). No obstante, en otros ambientes dunares *C. edulis* acidifica mayoritariamente los suelos, posiblemente por producción de ácidos orgánicos (D'Antonio, 1990, Santoro et al., 2011, Conser y Connor, 2009). Esta diferencia en el efecto de *C. edulis* en el pH de suelos dunares y rocosos tiene como consecuencia efectos variables sobre la disponibilidad de nutrientes: en zonas rocosas invadidas aumenta la disponibilidad de Fe y disminuye la de Cu, Mg y Zn; mientras que en dunas invadidas disminuye la disponibilidad de Fe y Co. La reducción en la disponibilidad de estos nutrientes podría conllevar deficiencias en plantas y microorganismos (Williams y Fraústo da Silva, 2000). En dunas invadidas por *C. edulis*, al contrario que en otros estudios (Novoa et al., 2014, Santoro et al., 2011) así como en la sucesión natural de dunas (Anwar-Maun, 2009, Jones et al., 2008) y a pesar del incremento en necromasa acumulada, el C orgánico y el N total disminuyen en el suelo. La producción por parte de *C. edulis* de compuestos antibacterianos (van der Watt y Pretorius, 2001) podría estar inhibiendo la descomposición microbiana de su necromasa y reduciendo su humificación e incorporación en la materia orgánica edáfica. Asimismo, la necromasa de esta exótica presenta una mayor relación C/N que la de plantas nativas, lo que podría reducir la mineralización (Packham et al., 2001).

La composición de la necromasa varía entre zonas invadidas y no invadidas por *C. edulis*, presentando la necromasa exótica una menor concentración de Al (elemento potencialmente tóxico) y de algunos micronutrientes (Fe, Cu). Esto podría reflejar una ventaja competitiva de *C. edulis*, que restringiría la absorción de Al más eficientemente y requeriría menos micronutrientes (o los reabsorbería más eficientemente de tejidos senescentes). Además, la mayor acumulación de Na en la necromasa con respecto a las nativas podría ser una adaptación a la salinidad, al igual que en otras plantas de la misma familia (Weber y D'Antonio, 1999, Delnavaz Hashemloian et al., 2010).

La invasión por *C. edulis* altera los flujos y reservas de N en la capa de 0-10 cm de suelo, especialmente en los primeros 5 cm. La invasión reduce

las tasas netas de nitrificación y mineralización, lo que podría conllevar un descenso en la disponibilidad de N y, con ello, un cambio en la composición de las comunidades vegetales (Eviner y Chapin, 2003). La mineralización bruta no varía significativamente con la invasión, probablemente por las pequeñas diferencias en la relación C/N de la necromasa y en la forma de vida de la vegetación nativa e invasora, que es perenne en ambos casos.

La nitrificación autótrofa desciende en suelos invadidos (posiblemente por inhibición de las comunidades microbianas por exudación de metabolitos secundarios o acidificación del suelo), mientras que la inmovilización de amonio aumenta (probablemente por una mayor recalcitrancia de la necromasa de *C. edulis*). Consecuentemente, la invasión por *C. edulis* conlleva que la inmovilización de amonio supere a la nitrificación autótrofa.

En los suelos estudiados (tanto invadidos como no invadidos) el mayor consumo de nitrato fue por reducción catabólica [a pesar de que este proceso está habitualmente asociado a suelos anaerobios (Pandey et al., 2016)], aparentemente producida en micrositios anaerobios (Rütting et al., 2011), y siendo ésta superior en zonas no invadidas por *C. edulis*, donde la disponibilidad de nitrato para substrato de este proceso era mayor.

Los resultados obtenidos enfatizan la necesidad de restaurar los ecosistemas invadidos por *C. edulis* en los primeros estadios de la invasión, para evitar que los efectos en el suelo se intensifiquen, así como la importancia de eliminar la necromasa exótica para evitar efectos heredados en el suelo.

8.3. POTENCIAL DE *SCLEROTINIA SCLEROTIORUM* Y *PULVINARIELLA MESEMBRYANTHEMI* PARA CONTROL BIOLÓGICO DE *C. EDULIS*

Se estudió el efecto individual y combinado del insecto *P. mesembryanthemi* y del hongo *S. sclerotiorum* en plantas de *C. edulis* procedentes de su área de distribución nativa (Sudáfrica) y exótica (NO ibérico). Para ello, se evaluaron los efectos en la planta a corto (índices fisiológicos derivados de medidas de fluorescencia y reflectancia foliar) y largo plazo (supervivencia, crecimiento, relación biomasa

aérea/subterránea) en un experimento de invernadero de un año con un diseño factorial [8 procedencias de *C. edulis* (4 nativas sudafricanas; 4 invasoras ibéricas) x 4 tratamientos (control; infestación con *P. mesembryanthemi*; inoculación con *S. sclerotiorum*; inoculación e infestación combinados) x 6 réplicas].

Las plantas de *C. edulis* infectadas con *S. sclerotiorum* mostraron descensos transitorios y a corto plazo en el contenido de clorofila y la eficiencia en el uso de radiación fotosintética [sugeridos por el índice clorofílico (CHL-NDI) y el rendimiento cuántico máximo del fotosistema II (F_v/F_m), respectivamente], que no conllevaron efectos significativos en la supervivencia o crecimiento. Sin embargo, la capacidad de infección de este hongo está condicionada por factores climáticos (Berg y Lentz, 1968) y en el campo podría verse favorecido por una mayor humedad ambiental.

Pulvinariella mesembryanthemi desencadenó en la planta (aparentemente para contrarrestar los efectos de la infestación) un incremento transitorio del rendimiento fotosintético [como sugieren los índices F_v/F_m y NPQI (índice de feofitinización normalizado)] y un descenso de la relación biomasa radicular/aérea. También, como un posible mecanismo de defensa, en la mitad de las plantas con *P. mesembryanthemi* se observó la muerte de las partes más intensamente infestadas. Esto podría ser especialmente perjudicial para *P. mesembryanthemi* por su limitada movilidad (Washburn y Frankie, 1985), que podría dificultar la colonización de plantas sanas. La infestación redujo la supervivencia (especialmente después de los 6 meses, cuando *P. mesembryanthemi* estaba en estadios avanzados de desarrollo) y el crecimiento de las plantas al cabo de un año.

El origen de *C. edulis* (procedencia nativa o exótica) no afectó a su susceptibilidad a los agentes de control estudiados, pese a lo que cabría esperar dadas las evidencias de adaptación evolutiva encontradas en plantas de zonas invadidas con respecto a las nativas (Roiloa et al., 2016). El uso combinado del hongo y el insecto para controlar *C. edulis* sólo produjo efectos sinérgicos a corto plazo, no teniendo efecto sobre la supervivencia y el crecimiento. Además, la combinación de los agentes fue perjudicial para el establecimiento de *P. mesembryanthemi* en la planta. Por ello, el uso individual de este insecto parece ser la mejor estrategia de

control biológico de *C. edulis.* Como línea de investigación futura, se considera preciso realizar experimentos con *P. mesembryanthemi* en condiciones de campo para estudiar posibles interacciones bióticas y abióticas de este insecto en condiciones naturales.

8.4. DISTRIBUCIÓN POTENCIAL Y DINÁMICA DE POBLACIÓN DE *P. MESEMBRYANTHEMI* EN ÁREAS INVADIDAS POR *C. EDULIS*

Para que un agente de control biológico sea efectivo en una zona, es necesario que su distribución potencial y la de la planta invasora que se quiere controlar se solapen ampliamente (Sun et al., 2017). Por ello, se modelizó la distribución potencial en Galicia (NO España), en base a factores climáticos, tanto de *P. mesembryanthemi* como de su planta huésped *Carpobrotus* spp. mediante un algoritmo de máxima entropía (MaxEnt). Además, teniendo en cuenta que los estudios de campo son especialmente importantes en el caso de insectos sésiles como *P. mesembryanthemi* [que se ven particularmente afectados por factores estacionales y bióticos (Jha et al., 2009)], se realizó un estudio de dinámica de poblaciones. Para ello, se siguió la dinámica de 4 poblaciones gallegas de *P. mesembryanthemi* durante 2 años y se estudió su relación con factores climáticos (temperatura, precipitación, irradiación) y bióticos (planta huésped, depredadores, parásitos y mutualistas).

Las distribuciones potenciales de *Carpobrotus* spp. y *P. mesembryamthemi* abarcan toda la costa gallega y se solapan ampliamente, por lo que la distribución del insecto parece estar limitada por dispersión o interacciones bióticas y no por factores climáticos (incluidos en el modelo). Ambas especies se ven favorecidas por una mayor temperatura aérea y edáfica. Además, la distribución de *P. mesembryanthemi* parece estar relacionada con la humedad del suelo, posiblemente porque su crecimiento y fecundidad aumentan con un mayor contenido de agua en la planta huésped (Washburn et al., 1987).

En Galicia, el insecto *P. mesembryanthemi* tiene dos ciclos por año y su abundancia depende de factores climáticos, alcanzando máximos poblacionales en verano y descendiendo drásticamente en invierno. Sólo en una de las poblaciones (situada en un enclave especialmente

resguardado) el tamaño poblacional se mantiene más o menos constante a lo largo del año. A pesar de que en condiciones de campo *C. edulis* se ve sometida a depredación (caracoles *Theba pisana* Müller y *Helix* sp.; ratas *Rattus rattus* L.; conejos *Oryctolagus cuniculus* L.) y parasitismo por otros organismos (hemípteros *Aphis* sp., *Pseudococcus* sp. y *Philaenus spumarius* L.), sólo las infestaciones por *P. mesembryanthemi* parecen afectarla significativamente tanto a nivel reproductivo (número y aspecto sanitario de flores y frutos) como vegetativo (vigor y aspecto sanitario de las plantas evaluado a través de una escala visual subjetiva). Sin embargo, las poblaciones de *P. mesembryanthemi* podrían estar limitadas por parasitismo (Hymenoptera: Chalcidoidea) y depredación (Coleoptera: Coccinellidae, como *Chilocorus bipustulatus* (L.), *Exochomus* sp. y *Nephus quadrimaculatus* (Hbst.)). A pesar de que la presencia de hormigas estaba ligada a la de *P. mesembryanthemi* [al igual que en otros Hemiptera, de los que se consideran mutualistas (Buckley, 1987)], éstas no parecen evitar la presencia de parásitos y depredadores.

8.5. VARIABILIDAD GENÉTICA DE POBLACIONES DE *P. MESEMBRYANTHEMI* A ESCALA MUNDIAL

Los estudios genéticos de agentes de control biológico permiten optimizar su efectividad al descubrir complejos crípticos de especies (Gaskin et al., 2011), que pueden tener distinta especificidad y agresividad hacia la planta objetivo (Rauth et al., 2011). Por ello, se realizó un análisis filogeográfico [de poblaciones nativas (Sudáfrica) y exóticas (Europa, Oceanía) de *P. mesembryanthemi*] a partir de un fragmento de gen mitocondrial (citocromo *c* oxidasa subunidad I, COI) y de otro ribosómico (segmentos de expansión D2-D3 de la subunidad grande del gen 28S), con el fin de calcular la variabilidad intra- e inter-poblacional y determinar el origen y el número de eventos colonizadores de las poblaciones exóticas.

Las poblaciones exóticas no muestran variabilidad genética para ninguno de los dos genes estudiados (incluso comparando poblaciones de Europa y Oceanía), mientras que las nativas son muy variables (existiendo incluso variabilidad intrapoblacional en una de las poblaciones estudiadas). Por ello, búsquedas posteriores de la población más adecuada para su uso como control biológico deberían centrarse en las poblaciones

sudafricanas, donde se encuentra la mayor parte de la variabilidad de la especie. Además, estos resultados sugieren que las poblaciones exóticas proceden de una única población sudafricana de *P. mesembryanthemi*. La reproducción partenogenética de este insecto (Nur, 1963) junto con la relativamente reciente introducción en las áreas exóticas [las primeras citas de la especie en Europa y Oceanía datan de los años 80 (Collins y Scott, 1982, Vieira et al., 1983)] pueden explicar que se mantuviera esta homogeneidad genética en las poblaciones no nativas de *P. mesembryanthemi*. La variabilidad encontrada en las poblaciones nativas de *P. mesembryanthemi* está por encima del límite intraespecífico delimitado en estudios de insectos de la familia Coccidae (Amouroux et al., 2017, Wang et al., 2015), por lo que podría tratarse de un complejo críptico de especies.

Adicionalmente, se secuenció un endosimbionte de *P. mesembryanthemi,* próximo a la familia Rickettsiaceae (α-Proteobacteria) y a los endosimbiontes de otros insectos Hemiptera, que presentaba una muy baja variabilidad genética (incluso comparando poblaciones del área nativa y exótica). La presencia de endosimbiontes en Hemiptera es común (Mathenge et al., 2015, Li et al., 2011) y parece estar relacionada con un mayor rendimiento del huésped (Li et al., 2016).

8.6. BIBLIOGRAFÍA

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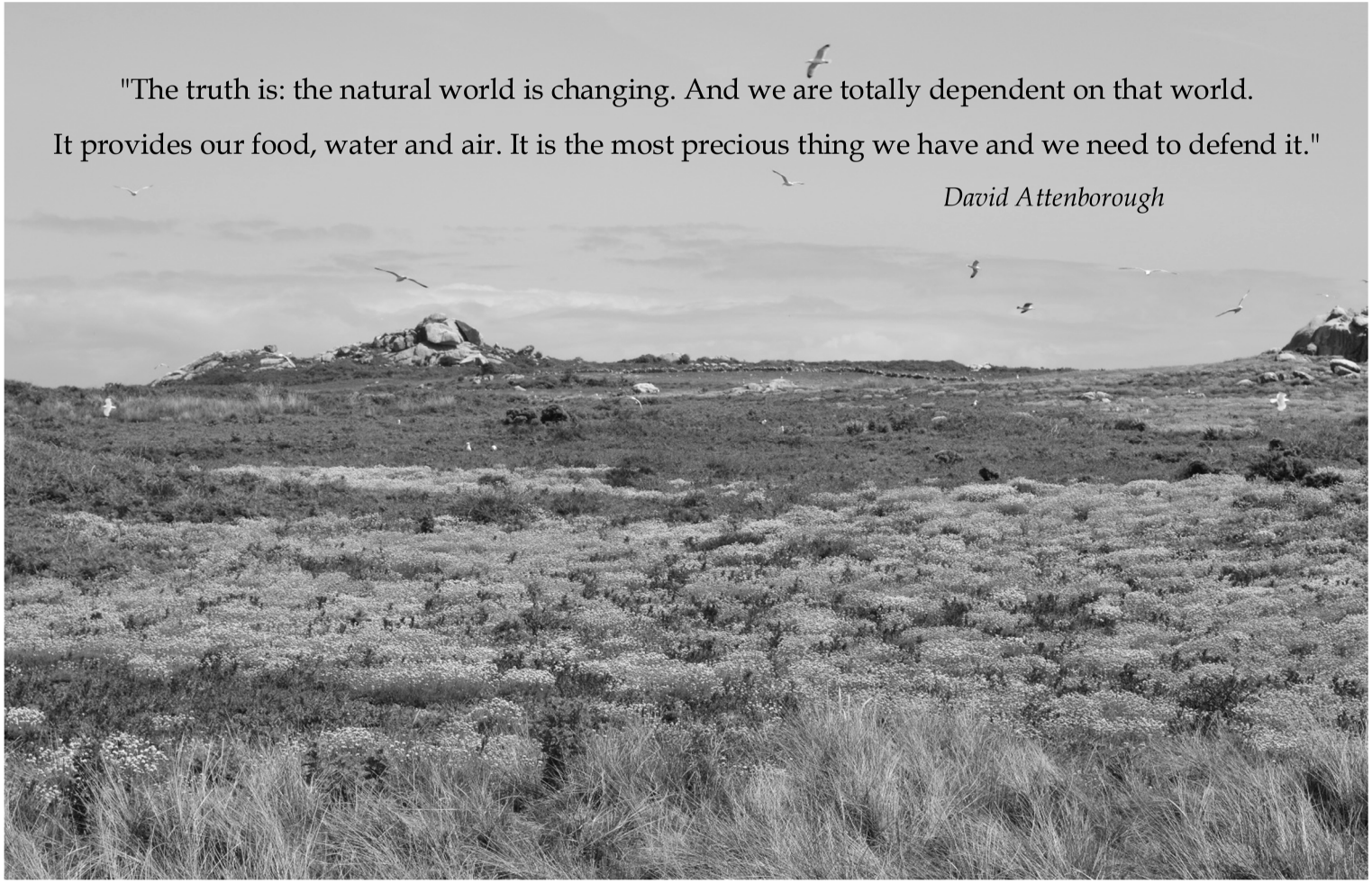
"The truth is: the natural world is changing. And we are totally dependent on that world.
It provides our food, water and air. It is the most precious thing we have and we need to defend it."
David Attenborough