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Evolution of invasiveness: the case study of the invasive *Oxalis pes-caprae* in the Mediterranean basin.

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia – Especialização em Investigação em Ecologia, realizada sob a orientação científica do Professor Doutor João Carlos Mano Castro Loureiro e da Doutora Sílvia Raquel Cardoso Castro Loureiro (Universidade de Coimbra).

Daniela Susana Rodrigues Tavares

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“Sei agora como nasceu a alegria,

como nasce o vento entre barcos de papel,

como nasce a água ou o amor

quando a juventude não é uma lágrima.”

Eugénio de Andrade

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Resumo

Processos rápidos de evolução desempenham muitas vezes um papel chave no processo de invasão por plantas exóticas. *Oxalis pes-caprae*, uma espécie geófita nativa da África do Sul, tornou-se uma invasora persistente e problemática, encontrando-se largamente distribuída em várias partes do mundo, particularmente em regiões de clima Mediterrânico. O objectivo desta Tese foi avaliar alterações evolutivas em populações de *O. pes-caprae* na área invadida da bacia do Mediterrâneo ocidental, onde a espécie foi introduzida na segunda metade do século XVIII. Para tal, foi avaliada a existência de diferenças de origem genética em características da planta determinantes no ciclo de vida entre populações invasoras (do oeste Mediterrâneo) e nativas (Sul-Africanas) através de uma experiência de estufa com plantas de ambas as áreas, a crescer em condições controladas, sozinhas ou em competição com *Trifolium repens*. As características da planta estudadas incluíram o tempo de emergência, o início da floração, a biomassa aérea, a quantidade de ácido oxálico nas folhas, a fluorescência clorofílica, a sobrevivência e a produção final de bolbos. Plantas da área invadida emergiram mais cedo, floriram mais tarde e produziram mais biomassa aérea e um maior número de bolbos do que as plantas da África do Sul. Para além disso, embora a competição interespecífica não tenha afectado qualquer das características estudadas em *O. pes-caprae*, independentemente da proveniência, o crescimento de *T. repens* foi significativamente mais afectado por plantas da área invadida do que por plantas nativas. Estes resultados constituem uma forte evidência da ocorrência de diferenciação genética, indicando uma mudança rápida em direcção a um fenótipo com maior potencial invasor em populações Mediterrânicas. Sugere-se que acontecimentos ligados à introdução da espécie e uma rápida evolução adaptativa após a introdução, possivelmente associada a uma realocação de recursos da defesa para o crescimento e reprodução na ausência de inimigos naturais, possam ter contribuído de forma independente ou em conjunto para esta divergência genética.

Palavras-chave: biogeografia comparativa; capacidade competitiva; evolução da capacidade de invasão; hipótese EICA; invasões biológicas; *Oxalis pes-caprae*; planta invasora.

Abstract

Rapid evolutionary processes often play key roles in determining the course of plant invasions. *Oxalis pes-caprae,* a geophyte native to South Africa, has become a persistent, troublesome and widespread invasive weed in several areas of the world, particularly in regions with a Mediterranean climate. The objective of this thesis was to assess evolutionary change in *O. pes-caprae* populations from the invaded range of the western Mediterranean basin, where the species was introduced at the second half of the eighteenth century. For this, genetically based differences in life-history traits between invasive (western Mediterranean basin) and native (South African) populations were tested for in a greenhouse experiment with plants from both ranges growing under controlled conditions, alone or in competition with *Trifolium repens*. The life-history traits studied included emergence time, beginning of flowering, aboveground biomass, amount of oxalic acid in the leaves, chlorophyll fluorescence parameters, survival and final bulb production. Plants from the invaded region emerged earlier, began flowering later and produced more aboveground biomass and offspring bulbs when compared to South African plants. Furthermore, although interspecific competition had no significant effect on any life-history trait of *O. pes-caprae* regardless of provenance, *T. repens* growth was more severely affected by invasive plants than by their native conspecifics. These results provide strong evidence for genetic differentiation, indicating a rapid change toward a phenotype with higher invasive potential in invasive populations. It is suggested that founder events and rapid post-introduction adaptive evolution, possibly associated with a reallocation of resources from defense to growth and reproduction in the absence of natural enemies, may have contributed, independently or in concert, to this divergence.

Key words: biological invasion; comparative biogeography; competitive ability; EICA hypothesis; evolution of invasiveness; invasive plant; *Oxalis pes-caprae*.

1. Introduction

1.1. Biological invasions

A biological invasion can be defined as a multistage process that occurs when a species is transported from its native range to a novel region in which it is able to survive and reproduce, establish viable populations, and then spread widely (Richardson *et al*. 2000). Each stage of the process is associated with barriers that a taxon must overcome to ultimately become invasive (Richardson *et al*. 2000; Mitchell *et al*. 2006). The first and possibly the most evident of these barriers is the geographical one, which is generally overcome through human assistance. Humans exchange thousands of species between different areas both intentionally and inadvertently (Vitousek *et al*. 1997; Nentwig 2007); and while most introduced species become locally extinct soon after their arrival at the new region, a small part may establish and become invasive, frequently disrupting the structure and functioning of native communities (Mack *et al*. 2000; Levine *et al*. 2003). Indeed, as a result of the intensification of human transport and commerce, invasion became a widespread phenomenon, including organisms of all taxonomic groups and affecting nearly all types of ecosystems and habitats (Vitousek *et al*. 1997; Pyšek *et al*. 2008). Nevertheless, the role of humans in increasing the extent and frequency of biological invasions goes beyond purely acting as dispersal agents. For example, farming and horticulture are known to facilitate the establishment of non-indigenous species by protecting them from stochastic processes until they are capable of forming self-perpetuating populations, and are strongly linked with subsequent invasion events (Mack 2000; Dehnen-Schmutz *et al.* 2007). Additionally, human-caused disturbances associated with agriculture and urban development can also play a major role in promoting the spread of alien species in the new areas (Hobbs and Huenneke 1992; Kercher and Zedler 2004).

Charles Elton was one of the first researchers to recognize the real impacts of biological invasions, deeming them as “one of the great historical convulsions in the world’s fauna and flora” on his book *The Ecology of Invasions by Animals and Plants* (1958). In fact, such changes in the distribution of the Earth’s biota are far from being harmless to the environment. Invaders can affect the role and abundance of native species in a community (even leading to extinctions) and modify ecosystem properties such as productivity, nutrient cycling, hydrology, carbon sequestration, fire regimes and plant-pollinator interactions (Vitousek *et al*. 1996; Mack *et al*. 2000; Levine *et al*. 2003, Ferrero *et al.* 2013). Consequently, they are now considered one of the main contributors to the loss of biodiversity (Mack *et al*. 2000; Sala *et al*. 2000) and to the homogenization of the world's ecosystems (Nentwig 2007). Biological invasions can also pose serious risks to human health as some invaders act as direct agents or vectors of human diseases, while others produce allergenic substances, are poisonous and/or cause injuries (Pimentel *et al*. 2001, 2005; Belmonte and Vilà 2004). Furthermore, the disruption of ecosystem services fostered by invasive species bears strong negative socioeconomic and cultural impacts (Vilà *et al*. 2009; Pyšek and Richardson 2010). Kettunen *et al*. (2009) estimated the total monetary costs of invasive species in Europe to be at least €12.5 billion per year (excluding costs of epidemic human diseases); however, since economic impacts are only documented for about 13% of over 10,000 alien species found in Europe (Vilà *et al*. 2009), this amount is clearly underestimated. In another study, Pimentel *et al*. (2001) reported that the economic damages associated with invasions by alien species in the United States, United Kingdom, Australia, South Africa, India, and Brazil sum more than US$ 336 billion annually. Assuming similar costs worldwide, the study estimated that damages from invasive species would represent nearly 5% (US$ 1.4 trillion per year) of the gross world product (GWP). The most affected economic sectors include agriculture, forestry, fisheries, aquaculture, health, recreation and tourism (Pimentel *et al*. 2000; Vilà *et al*. 2009; Pyšek and Richardson 2010). In agriculture, the effects of invasive alien pests (e.g., alien weeds, invertebrate pests and plant pathogens) are particularly severe, with the financial costs of alien pest control and yield losses contributing greatly to the total economic impact of invasions (Pimentel *et al*. 2005; Kettunen *et al*. 2009).

In order to reduce the harmful effects of biotic invaders, many countries have launched integrated management strategies focused on prevention, early detection and rapid response, containment, mitigation and restoration (Pyšek and Richardson 2010). These national strategies are very important, but an effective management of the invasion problem also requires international cooperation (Hulme *et al*. 2009; Pyšek and Richardson 2010; Keller and Perrings 2011). Some international treaties such as the World Trade Organization Agreement on the Application of Sanitary and Phytosanitary Measures (SPS), the International Plant Protection Convention (IPPC) and the Convention on Biological Diversity (CBD) aim to reduce the introduction and spread of invasive species (Perrings *et al*. 2005; Hulme *et al*. 2009). However, in many cases the commitment to these agreements is not fully followed up by action (Hulme *et al*. 2009).

In Europe, the recognition that uncoordinated approaches are not efficient and can frequently undermine the efforts made by some countries to tackle invaders, led the European Commission to publish a proposal for a *Regulation of the European Parliament and of the Council on the prevention and management of the introduction and spread of invasive alien species* in 2013 (European Commission 2013). This regulation could represent an important step towards a common European approach to the problem of invasive species; however, reducing the impacts of biological invasions on a global scale will require a worldwide strategy that allows for coordinated action.

1.2. Plant invasions

Invasive plants are one of the taxonomic groups of invaders with greater economic and environmental impacts (Pimentel *et al*. 2005; Vilà *et al*. 2009). Numerous studies have shown that invasive plant species can radically change the abundance of native species in a community and completely alter ecosystem processes, transforming the ecosystems both above- and belowground (Mack *et al*. 2000; Hierro and Callaway 2003). According to the DAISIE (Delivering Alien Invasive Species Inventories for Europe) database, over half of the 12,122 alien species occurring in Europe are terrestrial plants. Nonetheless, the number of alien plant species known to have an ecological or economic impact is disproportionally small (Vilà *et al*. 2009). Similarly, despite accounting for almost half of the species case studies on biological invasions, alien plants seem to be less intensively researched than would be expected given their greater number (Pyšek *et al*. 2008). An explanation for these numbers may be that only a relatively small fraction of naturalized plant species become invasive pests that threaten biological diversity and ecosystem services, and are therefore likely to become the subject of a study (Pyšek *et al*. 2008).

Richardson *et al*. (2000) conceptualized the plant invasion process, defining three key phases - introduction, naturalization and invasion - based on the barriers that a species has to overcome (Figure 1). Throughout introduction a plant species is, deliberately or accidentally, moved beyond its native range by humans, being transported across a major geographical barrier. Many introduced alien species survive as casuals. These individuals may overcome the abiotic and biotic barriers to survival at the introduction site and even reproduce sporadically, but fail to sustain self-replacing populations, depending on repeated introductions to persist. However, a small fraction of the introduced species overcomes not only the local environmental barriers to survival, but also the barriers to regular reproduction, becoming naturalized. At this point, alien species have the ability to produce offspring consistently and their persistence does not rely on recurrent re-introductions. Some of these naturalized plants may eventually surmount regional dispersal barriers and environmental barriers (abiotic and biotic) in a wider area, recruiting large numbers of reproductive offspring in areas distant from the introduction site(s). The few species that possess this potential to spread over a considerable area are considered invasive and can often be found in disturbed communities. Further invasion of natural, undisturbed habitats usually requires that different environmental barriers are overcome.

Despite the effort made by Richardson *et al*. (2000) to define the different stages in the invasion process in a clear and precise manner and thus lessen the inconsistency in the use of terms and concepts in invasion ecology, some other authors have also suggested different key phases and/or terminology (e.g., introduction, naturalization, facilitation, increased distribution and stabilization, Marchante 2001; transport, establishment and spread, Sakai *et al*. 2001; transport, colonization, establishment and landscape spread, Theoharides and Dukes 2007). During the process of invasion the introduced species frequently passes through a lag phase in the population growth and range expansion before the progression from naturalized to invader (Mack *et al*. 2000; Theoharides and Dukes 2007; Thuiller *et al*. 2007). This lag phase varies in duration (it may be very brief or last for decades) and is followed by a phase of rapid exponential population growth that may be triggered by a specific event or phenomenon (e.g., the introduction of a mutualist such as a pollinator or seed disperser, a rapid adaptive evolution of the exotic species itself, and/or natural or anthropogenic habitat disturbances) (Marchante 2001; Maron *et al*. 2004). Ultimately, an invasion reaches its last stage and the invader’s population growth rate stabilizes (Mack *et al*. 2000; Marchante 2001).

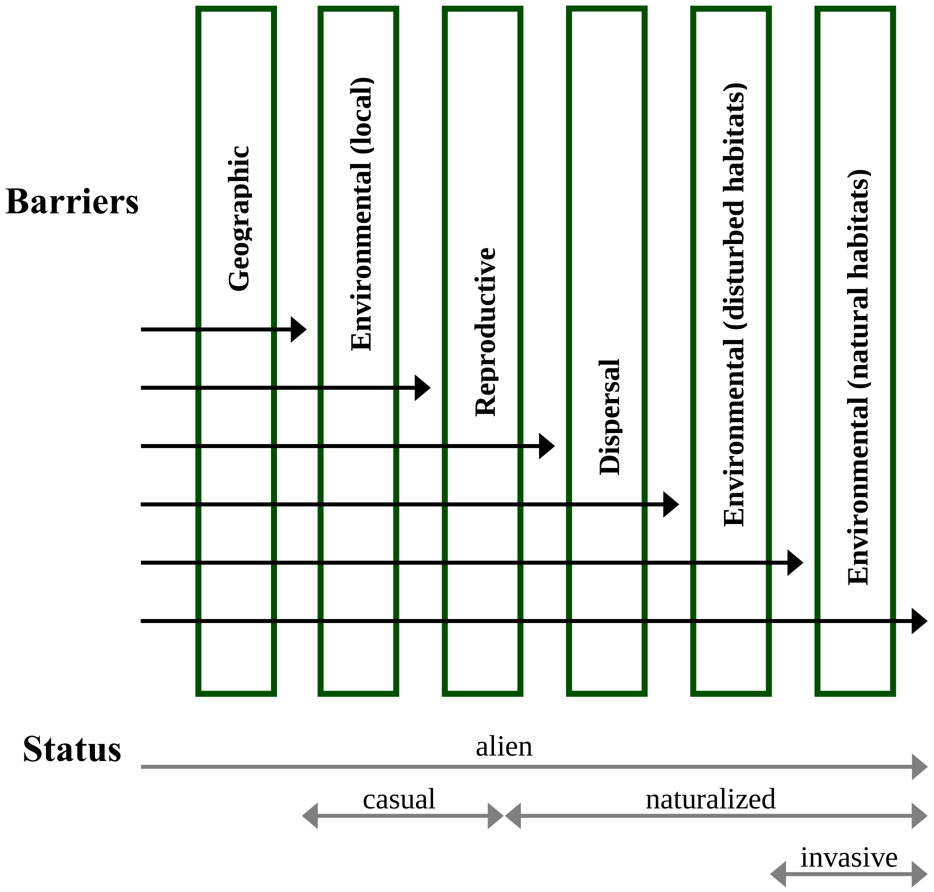

Figure 1. Schematic representation of the major barriers that a plant species has to overcome to become invasive, including the status of the plant in each phase (adapted from Richardson *et al.* 2000).

1.3. Leading hypotheses for exotic plant success

One of the main purposes of invasion ecology is to understand the role of intrinsic species attributes, human activities, and environmental factors in explaining successful invasions. Understanding why some exotics succeed while others fail to establish is essential for choosing appropriate management measures and for predicting future invasions (Keane and Crawley 2002; Pyšek and Richardson 2007). Traits that promote invasiveness in plant species include tolerance to a broad range of environmental conditions, potential for rapid evolutionary change, ability to reproduce sexually and asexually, high dispersal efficiency, high competitive ability, polyploidy, and the possession of novel biochemical weapons (Rejmánek 2000; Sakai *et al.* 2001; Pyšek and Richardson 2007; Callaway *et al.* 2008; Pandit *et al.* 2011). Certain plant traits associated with small genome sizes, such as short generation time, small seed size and high relative growth rate of seedlings may also predispose species to rapid range expansion (Rejmánek 2000; Pandit *et al.* 2014). Another important determinant of invasiveness is the initial purpose of the introduction (Thuiller *et al.* 2006). Many invasive plant species, including some of the worst pests, were introduced intentionally and cultivated for specific purposes (Mack *et al.* 2000; Pyšek *et al.* 2002). Ornamental horticulture, for example, is considered a major driver of plant invasion (Reichard and White 2001; Dehnen-Schmutz *et al.* 2007). Pyšek *et al.* (2002) reported that, in the Czech Republic, nearly half of the alien flora consists of intentionally introduced species, and 53% of these were first brought in for ornamental purposes. The same pattern was found in Germany, where more than half of the deliberately introduced non-indigenous plant species came in as ornamentals (Kühn and Klotz 2002 cited in Dehnen-Schmutz *et al.* 2007). Finally, the characteristics of the invaded ecosystem are also crucial in determining the course of an invasion. These include the climate, the pattern of anthropogenic disturbance, the absence of natural enemies and availability of potential mutualistic partners, as well as the biological diversity of the recipient community (Thuiller *et al.* 2006).

The major hypotheses for invasion success consider some of the above-mentioned intrinsic species attributes and extrinsic factors, and their interactions, capturing a variety of mechanisms thought to be involved in the invasion process. The main hypotheses for invasion success are presented below.

1. Empty niche hypothesis: certain exotics may take advantage of “empty niches” in the recipient communities, accessing resources that no local species makes use (Roché *et al.* 1994; Hierro *et al.* 2005; Stachowicz and Tilman 2005). The successful establishment of these exotic species is therefore determined by the opportunities provided by the recipient community and by the exotic species ability to exploit such opportunities (Shea and Chesson 2002; MacDougall *et al.* 2009). Consequently, the susceptibility of a community to invasion is expected to increase in conditions of resource enrichment (theory of fluctuating resource availability, Davis *et al.* 2000).

2. Disturbance hypothesis: anthropogenic disturbances (or intensification of natural disturbances) are assumed to promote invasion either by directly increasing

resource availability or by disrupting the priority effects and competitive interference of native flora (Hobbs and Huenneke 1992; Corbin and D'Antonio 2004; Kercher and Zedler 2004). In either case, potential invaders must have some advantage over native species (Shea and Chesson 2002; MacDougall *et al*. 2009). That advantage could be, for example, a high colonization ability or a critical adaptation to types and intensities of disturbance that are novel even to native ruderals (Mack *et al*. 2000; Shea and Chesson 2002; Hierro *et al*. 2005). Common disturbances that may contribute to the success of exotic plants include cultivation, uncontrolled grazing, altered fire regimes, altered hydrology and erosion, nutrient enrichment, and climate change (Mack *et al*. 2000; Norton *et al*. 2007).

3. Species richness hypothesis: communities with high biodiversity may be more resistant to invasion than species-poor communities (Elton 1958). Theoretically, more diverse communities have a more efficient use of resources and less "empty niches", which makes them less invasible (Shea and Chesson 2002; Hierro *et al*. 2005). However, while some experimental plant community studies have supported this idea (e.g., Naeem *et al*. 2000; van Ruijven *et al*. 2003; Fargione and Tilman 2005), large-scale observational studies have demonstrated that species-rich systems tend to be more heavily invaded (Levine and D'Antonio 1999; Stohlgren *et al*. 1999; Hierro *et al*. 2005). This inconsistency suggests that, although diversity tends to reduce invasibility at the neighborhood level, other ecological factors co-varying with diversity may be more important in determining community-level patterns (Levine 2000).

4. Enemy release hypothesis: upon introduction to a novel region, many exotic species are released from suppression by their specialist herbivores and pathogens (Mack *et al*. 2000; Keane and Crawley 2002; Mitchell and Power 2003; DeWalt *et al*. 2004). This can translate into a substantial advantage over resident species that may be disproportionately burdened by natural enemies, and may enable exotics to increase in density and distribution area (Torchin and Mitchell 2004; Hierro *et al*. 2005).

5. Propagule pressure hypothesis: as the number of introduction events and/or the number of individuals introduced into the system (i.e. propagule pressure) increases, the probability of establishment and invasion is expected to increase (Lockwood *et al*. 2005; Von Holle and Simberloff 2005; Colautti *et al*. 2006; Eschtruth and Battles 2009). Propagule pressure may reflect, for example, human preferences for certain exotic

species and the extent of trade between particular countries/regions (Lockwood *et al*. 2005).

6. Novel weapons hypothesis: some invaders succeed because they possess novel biochemical weapons that species in recipient communities have never encountered (Callaway and Aschehoug 2000; Callaway and Ridenour 2004; Cappuccino and Arnason 2006). Introduced plants engage in new, non-coevolved interactions with resident competitors, herbivores and pathogens, which have not evolved adaptations to deal with the novel compounds that these plants may produce. Hence, phytochemicals that are relatively ineffective against well-adapted natural enemies, may function as powerful allelopathic, antifungal, antiherbivore and/or antimicrobial agents in the new range granting an advantage to the introduced species (Bais *et al*. 2003; Callaway *et al*. 2008; Verhoeven *et al*. 2009; Schaffner *et al*. 2011).

7. Evolution of invasiveness hypothesis: adaptive evolutionary changes, founder effects, and hybridization cause genetic differentiation between native and introduced populations and may play an important role in the success of invasive plant species (Lee 2002; Stockwell *et al*. 2003; Blair and Wolfe 2004; Bossdorf *et al*. 2005; Zou *et al*. 2008; Barney *et al*. 2009). The evolution of increased competitive ability (EICA) hypothesis proposed by Blossey and Nötzold (1995) has been particularly influential in this context, arguing that after release from specialist enemies, exotics will shift resource allocation from defense to growth and fecundity, i.e., traits that confer competitive advantage in the new range.

It is important to note that these hypotheses are not mutually exclusive and in many cases are intimately correlated, as it is evident by the descriptions above. Also, their validity varies across species. For example, the possession of novel weapons might be important in some invasion processes, while other factors, such as disturbance, may play a key role in other cases. As the advantages and disadvantages of different traits have the potential to interact in most invasion processes, it is fundamental to have a holistic perspective and consider the effects of all potential factors.

1.4. Evolution of invasiveness

As suggested by a growing number of studies, rapid evolutionary processes may be fundamental in determining the course of plant invasions (Sakai *et al*. 2001; Lee 2002; Blair and Wolfe 2004; Maron *et al*. 2004; Dlugosch and Parker 2008; Zou *et al*. 2008; Barney *et al*. 2009; Hahn *et al*. 2012). Rapid evolution of invasive species has been attributed to several causes, including founder effects, genetic drift, hybridization, adaptive evolution, or any combination of these processes (Lee 2002; Bossdorf *et al*. 2005; Prentis *et al*. 2008). Although genetic bottleneck is often associated with reduced population fitness as a result of inbreeding depression, in some cases, the loss of genetic variation during founder events leads to evolution by genetic drift in newly established populations (Bossdorf *et al*. 2005). Furthermore, introduction into new environments often comprises marked changes in selection pressures that may drive adaptive evolutionary changes in invading populations (Sakai *et al*. 2001; Bossdorf *et al*. 2005). Adaptive evolution appears to be common in plant invaders, and may occur for any ecological trait that enhances their performance in recipient communities (Lee 2002; Bossdorf *et al*. 2005). Dlugosch and Parker (2008) demonstrated that even founding populations with diminished genetic variation may still adapt rapidly under new selection regimes. Novel selective forces may be related with both abiotic and biotic factors; in the latter case, adaptive evolution may occur not only in response to newly encountered resident species, but also in response to the absence of natural neighbors, in particular antagonists (Lee 2002; Hierro *et al*. 2005).

The evolution of increased competitive ability (EICA) hypothesis suggests that, in the absence of natural enemies, natural selection will favor less defended, but highly competitive individuals (Blossey and Nötzold 1995). Eventually, this will lead to genetic differentiation between introduced and native populations, with introduced plants presenting reduced resistance to natural enemies and increased growth (or competitive ability) when compared to plants from native populations (Blair and Wolfe 2004; Zou *et al*. 2008).

A direct way of testing for genetically based differences between introduced and native conspecifics is to grow plants from both ranges in a common environment, using propagules from numerous populations sampled widely across each region. If native and introduced plants growing under identical conditions differ significantly, there is evidence for genetic differentiation.

1.5. Study system: *Oxalis pes-caprae* L.

Oxalis L. (Oxalidaceae) is a cosmopolitan genus of over 800 species, extremely variable in morphology and ecology, with two main centers of diversification, one in Central and South America and the other in southern Africa (Marks 1956; Emshwiller and Doyle 1998). The genus contains some of the very few non-monocot angiosperm groups that form true bulbs (Oberlander *et al*. 2009). While the American *Oxalis* taxa exhibit a wide range of growth forms (only ± 15% are bulbous), all native southern African species are bulb-forming geophytes (Oberlander *et al*. 2009). Many bulbous *Oxalis* species are invasive in other parts of the world, a fact that is often attributed to their great ability to spread vegetatively through the production of bulbils (Luo *et al*. 2006; Vilà and Gimeno 2006; Castro *et al*. 2007).

Oxalis pes-caprae L., a perennial bulbous geophyte native to South Africa, has become a persistent, troublesome and widespread invasive weed in several areas of the world (Rappa 1911; Ornduff 1987; Vilà *et al*. 2006a). More specifically, *O. pes-caprae* has spread widely across regions with a Mediterranean climate (similar to that of its native range), i.e., the Mediterranean basin, western and southern Australia, western South America and California; but has also been recorded in Pakistan, India, China, Japan, the South Island of New Zealand, and Florida (Lambdon 2006). In most of these regions, the species was introduced for ornamental purposes (Rappa 1911; Lambdon 2006) and, subsequently, escaped from cultivation, successfully invading open habitats, mostly in disturbed areas such as old fields, pastures, tree groves, orchards, field margins and roadsides (Figure 2; Gimeno *et al.* 2006). Occasionally, it can also be found in more shaded and less disturbed habitats like shrublands and forests (Gimeno *et al*. 2006).

Oxalis pes-caprae is considered a highly noxious invader with adverse impacts on native species diversity, as well as, with harmful effects on agriculture and livestock production. Dense infestations can have a severe impact on native ground-flora, suppressing smaller plants and limiting the growth of seedlings (Blood 2001), and, in cultivated areas, this species can lead to significant yield losses. For example, in Western Australia, *O. pes-caprae* has been found to reduce the yield of oats by 87% and of wheat by 75% (Parsons and Cuthbertson 2001). It is also harmful in pastures due to the production of oxalic acid, a strong organic acid that is toxic to livestock when consumed in large quantities (James 1972; Libert and Franceschi 1987). Oxalate poisoning associated with the ingestion of this plant is known to have caused substantial losses of sheep and cattle in some pastoral areas (James 1972; Lambdon 2006). In its native range both weedy and non-weedy populations were observed (Ornduff 1987).

Figure 2. Field invaded by *Oxalis pes-caprae*. Photo credit: Joana Costa.

This species is heterostylous with trimorphic flowers (short-styled, S-morph; mid-styled, M-morph; and long-styled, L-morph; Figure 3) and a self- and intramorph-incompatibility system, which implies that legitimate pollinations are only possible between individuals with different floral morphs (Ornduff 1987; Castro *et al*. 2007). Additionally, *O. pes-caprae* is a polyploid species with reported diploid ($2n = 2x = 14$ chromosomes), tetraploid ($2n = 4x = 28$) and pentaploid ($2n = 5x = 35$) individuals (Ornduff 1987; Castro *et al*. 2013a). In South Africa, the species reproduces both sexual and asexually and is represented by all floral morphs and cytotypes, although pentaploid individuals seem to be extremely rare (Michael 1964; Ornduff 1987; te Beest *et al*. 2012). The scenario is completely different in the invaded range, where the pentaploid short-styled morphotype (5*x* S-morph), which reproduces mainly asexually through the production of bulbs, is clearly predominant (Ornduff 1987; Castro *et al*. 2007; 2013a).

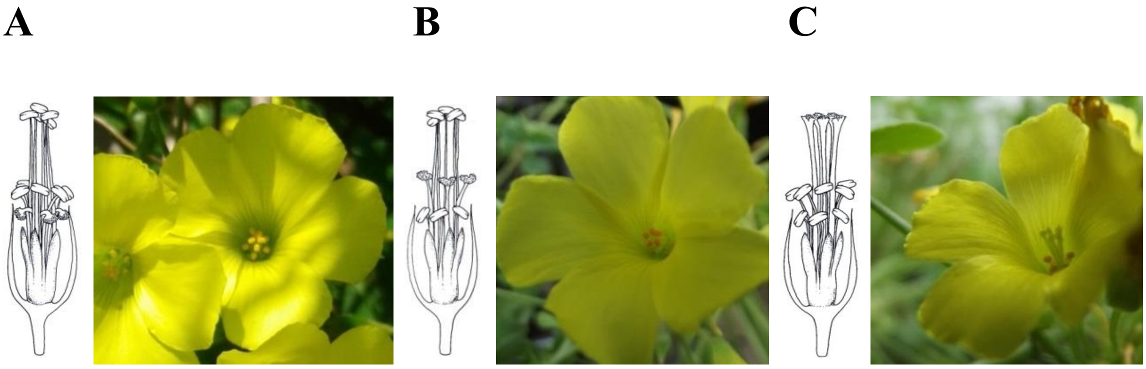

Figure 3. Floral morphs of *Oxalis pes-caprae*: **A.** short-styled, **B.** mid-styled and **C.** long-styled. Photo credit: Lucie Mota. Illustrations: Sílvia Castro.

In the invaded region of the Mediterranean basin, *O. pes-caprae* has, for a long time, been reported to reproduce exclusively via asexual reproduction as a result of founder events after the introduction of the 5*x* S-morph alone (Ornduff 1987; Rottenberg and Parker 2004; Vilà *et al*. 2006a). However, in the last years, the existence of mixed populations containing other floral morphs and cytotypes (namely, 4*x* S-morph, 4*x* M-morph and 4*x* L-morph) and the occurrence of sexual reproduction have been described in the western part of the basin (Castro *et al*. 2013a; Costa *et al*. 2014). A partial breakdown in the intramorph-incompatibility system, allowing the 5*x* S-morph to reproduce sexually, has also been observed and may be one of the mechanisms involved in the occurrence of the other floral morphs in this area (Costa *et al*. 2014).

Despite this, the 5*x* S-morph remains the predominant form in the Mediterranean region and asexual reproduction continues to be the only known form of reproduction in most of the invasive populations (Vilà *et al*. 2006a; Castro *et al*. 2013a). The species' ability to spread vegetatively, assured by a profuse production of bulbs and a combination of shoot elongation and root contraction ability that distributes the bulbs along a distance of up to 47 cm (Galil 1968; Pütz 1994), has been considered the major determinant of its success throughout the whole invasion process (Vilà and Gimeno

2006). In the past, the most important method of dispersal was the intentional propagation of the plant in gardens, from where it escaped to agricultural areas (Parsons and Cuthbertson 2001). Currently, the species is no longer cultivated and long distance spread is mostly achieved through soil movement in agriculture and gardening (Parsons and Cuthbertson 2001; Gimeno *et al*. 2006).

Except for the above-mentioned factors, little is known about what makes *O. pes-caprae* such a successful invader. Therefore several unexplored issues could be involved with the success of this species, such as the release from natural enemies, the production of toxic compounds (e.g., oxalic acid characteristic of the genus) to which resident species have not yet evolved resistance, and particularly the high potential for rapid evolutionary changes already evident by the changes observed in its sexual system after invasion. These factors may be key elements in its invasion process, making *O. pes-caprae* an excellent study system to address evolutionary questions and their contribution to invasion success.

1.6. Objectives

The general objective of this thesis was to better understand the mechanisms involved in the successful invasion of the South African geophyte *Oxalis pes-caprae* in the western Mediterranean basin. For this, evolutionary change in invasive populations, particularly regarding the species competitive ability, was assessed in a greenhouse experiment with plants from the native and invaded ranges growing under controlled conditions, with and without interspecific competition. *Trifolium repens* L. was chosen as the resident competitor because it commonly co-occurs with *O. pes-caprae* in invaded agricultural systems.

In order to test for genetically based differences between invasive and native populations, the following traits were measured in *O. pes-caprae*: emergence time, beginning of flowering, aboveground biomass, amount of oxalic acid in leaf extracts, chlorophyll fluorescence parameters, survival and final bulb production. Aboveground biomass, survival and chlorophyll fluorescence parameters were also measured in *T. repens* for a better understanding of the effects of competition with *O. pes-caprae* plants from both areas.

It was hypothesized that plants from the invaded region would have a higher competitive ability than those from the native range, producing a greater amount of aboveground biomass and more offspring (measured as number of bulbs). Furthermore, if there was a trade-off between investment in growth and in defence as predicted by the EICA hypothesis, plants from the invaded area would also be expected to produce less oxalic acid (oxalate provides protection against herbivores and pathogens, Yoshihara *et al*. 1980; Libert and Franceschi 1987) than native plants.

This constitutes the first study testing evolutionary changes in invasive populations of *O. pes-caprae* regarding competitive ability, and it benefits from considering not only plants from the invaded range, but also populations from the native area, enabling to fully understand the evolutionary changes that occurred since this species was introduced in the western Mediterranean basin at the second half of the eighteenth century (Rappa 1911; Signorini *et al*. 2011, 2013).

2. Materials and Methods

2.1. Study species

Oxalis pes-caprae L. (Oxalidaceae) is a perennial geophyte, up to 30-40 cm high, with a true bulb that annually sends out a subterranean stem from which a rosette of leaves arises (Sánchez-Pedraja 2008). During vegetative growth, the species develops a fleshy contractile root, which later in the season pulls the offspring bulbs produced in the axillary buds of the underground stem to deeper soil horizons (Pütz 1994). Mature plants produce terminal umbellate cymes with yellow, actinomorphic flowers (Sánchez-Pedraja 2008). The flowers are tristylous, presenting two whorls of five anthers and one whorl of five stigmas, arranged in three levels according to the floral morph of each individual (S-morph, M-morph or L-morph; Figure 3) (Ornduff 1987; Castro *et al*. 2007). In the invaded region of the Mediterranean basin, the peak of vegetative growth and flowering occur from winter to early spring, and leaves completely senesce before the end of spring (Verdaguer *et al*. 2010). Offspring bulbs develop to final size after the aboveground part of the plant senesces (Vilà *et al*. 2006a; Verdaguer *et al*. 2010). Bulbs remain dormant in summer, sprouting in autumn (Vilà *et al*. 2006a).

2.2. Bulb collection

In February and March 2010, bulbs of *O. pes-caprae* were collected from 39 distinct populations in the invaded range of the western Mediterranean basin. All the populations were located in highly-invaded areas, distributed along a latitudinal transect from La Coruña province (Spain) to Essaouira province (Morocco). Sampling was particularly intensive in the Estremadura province (Portugal), where trimorphic populations are dominant (Castro *el al*. 2013a). This procedure allowed to collect bulbs from 5*x* S-morph, 4*x* S-morph, 4*x* M-morph and 4*x* L-morph individuals, i.e., all floral morphs and cytotypes found so far in this invaded range. In South Africa, bulbs were harvested from 33 populations in the Western and Northern Cape provinces during August 2011. Collection sites were chosen to span the broad latitudinal and longitudinal distribution of the species within the native area, extending from Namaqualand to the Cape Peninsula and eastwards along the Indian Ocean coast to the Mossel Bay area. Despite this extensive sampling effort, pentaploid individuals proved once more to be extremely rare in this range (4 plants from a total of 990 plants sampled). Consequently, only bulbs from 4*x* S-morph, 4*x* M-morph and 4*x* L-morph plants were collected in sufficient quantity to be included in this comparative study.

In order to remove potential maternal effects, bulbs from the invaded and native ranges were grown in the nurseries of the Botanical Garden of the University of Coimbra for three and two generations, respectively.

2.3. Greenhouse experiment

To determine if there is a genetic component that contributes to the differences between plants of *O. pes-caprae* from the native and the invaded range, a greenhouse experiment with plants from both areas and plants of *Trifolium repens,* growing alone or in competition, was performed in the greenhouse facilities of the Botanical Garden of the University of Coimbra (Figure 4).

In August 2013, at the end of the second and third generation of plants from the native and invaded area, respectively, all the offspring bulbs were harvested and stored in identified paper envelopes. The bulbs were then subjected to a careful selection process based on the following criteria: (a) the selected bulbs would have to have a similar weight, (b) two bulbs would have to be selected from each mother plant, and (c) within each area, bulbs would have to represent all forms (i.e., all cytotype-floral morph combinations) in equal proportion. The bulbs were weighed and the initial bulb weight recorded. This selection resulted in a group of 144 pairs of bulbs with a similar weight (0.38 ± 0.11 g, mean ± SD), of which 63 belonged to the native area and 81 to the invaded area, representing 12 and 23 populations, respectively (Appendix 1). Bearing in mind that it is impossible to determine both maximum aboveground biomass and final bulb production in the same individual (Sala *et al*. 2007; Verdaguer *et al*. 2010), for each mother plant one of the two selected bulbs was assigned for harvesting at the time of peak aboveground biomass (set 1), and the other bulb for harvesting at the end of the experiment (set 2), when offspring bulbs were fully developed. This approach was based on the procedure developed by Sala *et al*. 2007 and Verdaguer *et al*. 2010 for *O. pes-caprae*. Afterwards, the 144 pairs of bulbs were randomly distributed among the following competition treatments: control, one individual of *O. pes-caprae* growing in each pot; low competition, a single individual of *O. pes-caprae* growing with two plants of *T. repens*; and high competition, a single individual of *O. pes-caprae* growing with six plants of *T. repens*. A total of 27 pairs of bulbs from the invaded range (6 pairs of 4*x* S-morph, 7 of 4*x* M-morph, 7 of 4*x* L-morph and 7 of 5*x* S-morph) and 21 from the native range (7 pairs of 4*x* S-morph, 7 of 4*x* M-morph and 7 of 4*x* L-morph) were assigned to each of the competition treatments (Figure 4). Bulbs from the same pair, i.e., from the same mother plant, were always designated to the same treatment and as they were harvested at different times, it was possible to gather information of the aboveground biomass and final bulb production for the same genotype.

On September 26, 2013, *O. pes-caprae* bulbs were planted 2.5-3.0 cm below the soil surface in 1-L plastic pots (8.6 × 8.6 × 21.5 cm) filled with a mixture of standard soil and sand (1:1). Several *T. repens* seeds (purchased from an horticultural shop) were sown on the soil surface in all the pots designated to the low and high competition treatments and in 42 (21 × 2 sets) additional pots assigned to a control treatment that consisted of *T. repens* growing without interspecific competition (Figure 4). After germination, seedlings were thinned out to two per pot in the low competition treatment and in the control of *T. repens*, and to six per pot in the high competition treatment. The control treatment of *T. repens* enabled to evaluate the effects of competition with *O. pes-caprae* plants from both areas on *T. repens* development by comparing it with the low competition treatment in which *T. repens* plants were grown with *O. pes-caprae*.

Summarizing, from *O. pes-caprae* perspective, this experimental design consisted of two crossed factors (area: native, invaded; and competition: control, low competition, high competition) and two sets, while from *T. repens* perspective it consisted of one factor (competition: control, competition with *O. pes-caprae* from the native area and competition with *O. pes-caprae* from the invaded area) and two sets. In total, the experiment involved 330 pots [((27+21) × 3 treatments × 2 sets) + (21 × 2 sets)].

Pots were completely randomized at the beginning of the experiment, except for the ones with *T. repens* growing alone, which were maintained together to prevent the effects of shade generated by *O. pes-caprae* plants, and re-randomized five weeks after planting; in the following months, the leaves of neighboring plants became intertwined and it was no longer possible to move the pots without damaging the plants. The greenhouse temperature was set at 20°C, but fluctuated to some degree depending on the external temperature, with minimum and maximum temperatures reaching 13°C and 28°C, respectively. The plants were grown under a natural day/night light cycle, and watered regularly until two months before the final harvest. The emergence time and the beginning of flowering were assessed for each *O. pes-caprae* plant and recorded in days after the beginning of the experiment (September 26, 2013).

On January 15 and 16, 2014, at the time of peak of *O. pes-caprae* aboveground biomass, all the plants from set 1 were harvested. The aboveground part of each *O. pes-caprae* and *T. repens* plant was cut at the soil surface, placed in a paper bag identified with the plant code, dried at 68°C for 48 hours, and weighed. Root biomass was not assessed because in the pots assigned to the low and high competition treatments, roots of the two species were too closely interwoven and hard to differentiate, making the separation unfeasible. Plants in set 2 were left intact and remained in the same conditions as before until the beginning of March; then watering was gradually reduced until it stopped. The cessation of watering served to mimic the natural Mediterranean conditions and accelerate bulb maturation. On April 23 and 24, 2014, when *O. pes-caprae* offspring bulbs were completely developed, the harvesting of the belowground biomass was conducted using set 2. Each pot was emptied and bulbs were harvested, counted, dried as described above, and weighed. At this point, *T. repens* plants had become water-stressed and, therefore, no measurements were taken for this species.

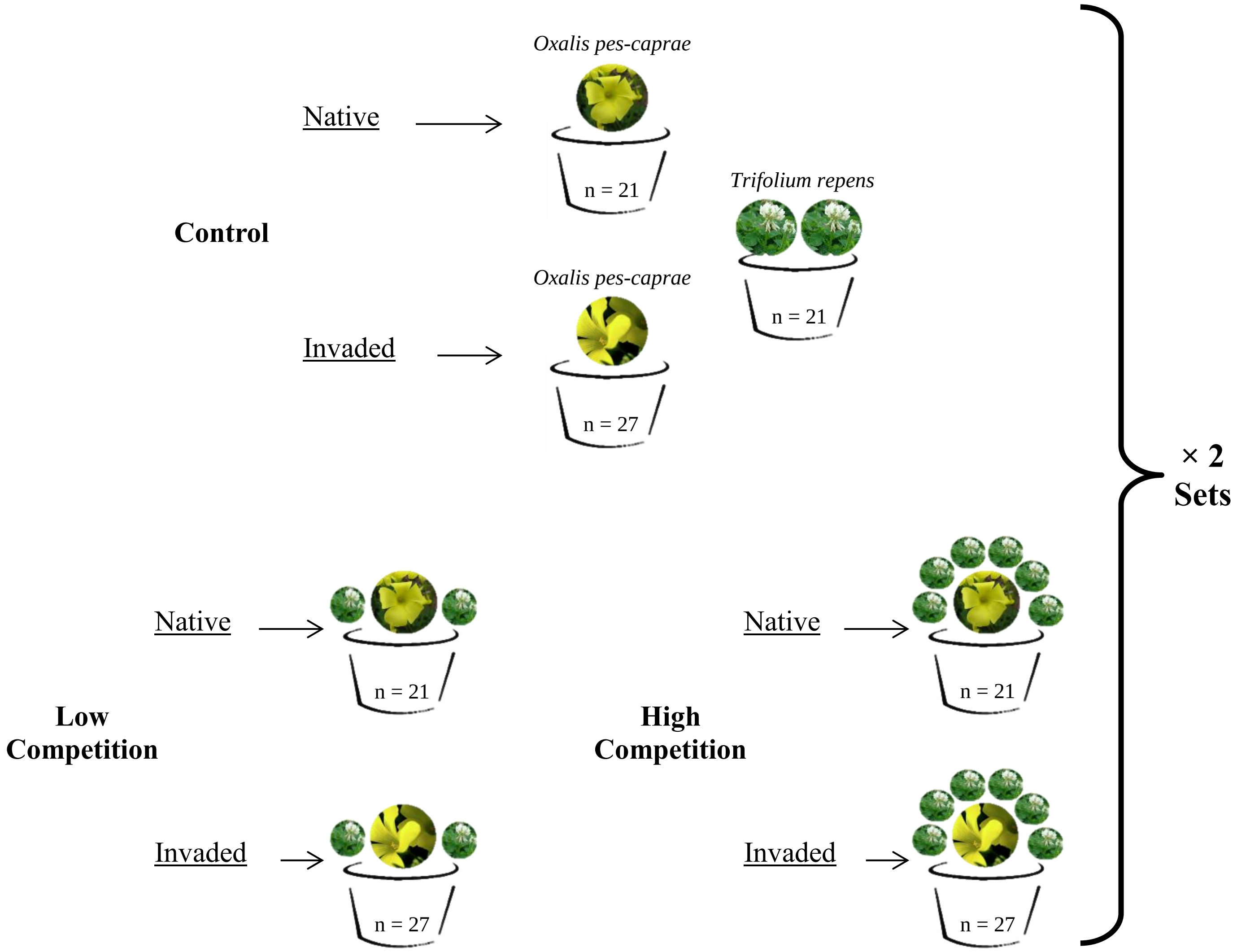

Figure 4. Schematic representation of the experimental design showing the factors included in the study: area (*Oxalis pes-caprae* from the native area and from the invaded area) and competition (control, and low and high competition with *Trifolium repens*). Sample sizes (n) are also provided. The entire design was replicated to harvest the plants at two different developmental stages (see *Greenhouse experiment* section for more details).

2.4. Oxalic acid quantification

Oxalic acid content was measured in the leaves of *O. pes-caprae* plants assigned to the final harvesting (set 2). In total, 21 plants from the native area and 35 from the invaded area, representing all forms and competition treatments, were analyzed.

On February 4, 2014, leaf samples of 2.5-5.0 g per plant were collected, identified and stored in a deep-freezer at -80°C until analysis; although oxalic acid quantification was only performed for a subgroup of 56 plants, leaves were collected from all the individuals in set 2 so that an equal stress would be applied to each one of them. Oxalic acid quantifications were made in the Department of Chemistry of the University of Coimbra. To extract the oxalic acid, leaf samples were grinded in 20 mL of 0.5 M HCl in methanol (MeOH) 1:1 ratio, and subsequently shaken for 1 h. After extraction, samples were filtered and a 10 mL volume of each filtrate was evaporated to dryness at 30-35°C using a vacuum rotary evaporator. Then, a 10-15 mg aliquot of each extract was suspended in 4 mL of diethyl ether containing 1 M methylmalonic acid (internal reference standard) and treated with diazomethane for the esterification of carboxyl groups. After staying overnight at 4°C, the suspensions were evaporated to dryness under a stream of nitrogen.

Oxalic acid detection and quantification was performed using gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed with an Agilent Technologies 7820A GC System coupled to a 5975 MSD operating in electron ionization mode with an ionization potential of 70 eV. Chromatographic separation was carried out using a capillary column HP5-MS (30 m × 250 µm × 0.25 µm). Prior to injection into the GC-MS system, samples were dissolved in 1 mL of dichloromethane. The injection volume was 1 µL and helium was used as carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 250°C and the transfer line at 280°C. The oven temperature program consisted of an initial temperature of 80°C held for 2 min, followed by a ramp of 20°C/min to 290°C, and a final hold at 290°C for 4 min. Total run time per sample was 16.5 min. The ion source and quadrupole temperatures were 230°C and 150°C, respectively. The amount of oxalic acid in leaf extracts was determined using its peak area and the peak area of the internal standard as follows: (peak area oxalic acid/peak area internal standard × 100)/amount of extract (g).

2.5. Chlorophyll fluorescence

Chlorophyll fluorescence parameters were measured by the saturation pulse method (Schreiber *et al*. 1998) with a portable fluorometer (MINI-PAM photosynthesis yield analyzer; Walz, Effeltrich, Germany). A pulse of saturating light (>4000 µmol photons m^{-2} s^{-1}, 0.8 s pulse length, actinic white light) was applied through an optical fiber at an angle of 60° relative to the sample and a distance of 12 mm from the leaf. Measurements were taken on the upper surface of a fully expanded leaf of each plant of *O. pes-caprae* and *T. repens* of set 1 approximately one month before the harvesting time.

The maximum quantum yield of photosystem II (PSII) was assessed as the ratio $F_v/F_m = (F_m - F_0)/F_m$ (Bolhàr-Nordenkampf *et al*. 1989), where F_0 and F_m are the minimal and maximal fluorescence yields of a dark-adapted sample, respectively, with all PSII reaction centers fully open, i.e., all primary acceptors oxidized. This parameter was measured after a 30 min period of dark adaptation. The F_v/F_m ratio provides an estimate of the efficiency of excitation energy capture by open PSII reaction centers (Butler and Kitajima 1975). An increase in F_0 can be interpreted as reduced effectiveness of energy transport from antenna chlorophyll *a* to reaction centers of PSII and/or as a malfunction of the latter (Briantais *et al*. 1986).

2.6. Statistical analysis

For *O. pes-caprae*, descriptive statistics (mean and standard deviation) were calculated for emergence time, starting date of flowering, aboveground biomass, number of bulbs produced, total bulb biomass, oxalic acid content in leaf extracts and chlorophyll fluorescence parameters.

Differences among areas and competition treatments in emergence time, beginning of flowering and number of offspring bulbs were evaluated using generalized linear models (GLM) with a Poisson distribution and a log link function. A similar approach was used for the probabilities of flowering and survival, but with a binomial distribution and logit link function; for aboveground biomass, a Gaussian distribution and identity link function were used, while for total bulb biomass and oxalic acid content in leaf extracts, a gamma distribution and inverse link function were employed. Area and competition were defined as fixed factors for all the analyses. Initial bulb weight was used as a covariate for emergence time, and emergence time as a covariate for all the other response variables. Data on phenological variables (emergence time, starting date of flowering and probability of flowering) were taken from set 1;

emergence time from set 2 was used as a covariate for variables measured in set 2 (survival, number of bulbs, total bulb biomass and oxalic acid in leaf extracts). When a significant area × competition interaction was found, multiple comparison tests among competition levels were performed within each area.

Differences in emergence time, beginning of flowering, aboveground biomass, number of bulbs produced, total bulb biomass and oxalic acid content in leaf extracts among distinct *O. pes-caprae* forms within each area were analyzed using nested generalized linear models with morph and cytotype combined nested within area. Error distributions and link functions were set as mentioned above and, when results were significant, multiple comparison tests were performed within each area.

Differences in *O. pes-caprae* chlorophyll fluorescence parameters were assessed using competition and area as main factors in two-way ANOVAs, followed by LSD tests. F_v/F_m was raised to 10 and F_0 was log10-transformed before the analyses, to meet the assumptions of normality and homoscedasticity.

For *T. repens*, descriptive statistics were calculated for aboveground biomass, probability of survival and chlorophyll fluorescence parameters and are presented as the mean and standard deviation. GLM followed by multiple comparison tests were used to test for the effect of competition with *O. pes-caprae* on the aboveground biomass and probability of survival of *T. repens* (competition was used as fixed factor). Aboveground biomass was fitted to a gamma distribution with an inverse link function, while the probability of survival was adjusted to a binomial distribution with a logit link function. Differences in *T. repens* chlorophyll fluorescence parameters between competition treatments were evaluated using one-way ANOVAs, followed by LSD tests when results were significant. F_v/F_m was raised to 10 before the analysis to achieve normality and homoscedasticity.

The GLM analyses were performed using the pscl and multcomp packages of the R 2.14.2 software. Statistical tests for chlorophyll fluorescence parameters were performed with IBM SPSS Statistics 19.0 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. *Oxalis pes-caprae*

3.1.1. Phenological traits

The time of emergence varied significantly among *O. pes-caprae* plants from different areas ($\chi^2_{1,137} = 263.34$, $P < 0.0001$), with plants from the invaded range emerging earlier than plants from the native range (Figure 5A and B). For the invaded area, emergence was very condensed in time, reaching 79.2% within one week after planting and a maximum of 98.7% after three weeks. On the other hand, native *O. pes-caprae* plants presented more variable emergence time, with only 42.9% of the plants having emerged within the first week of the experiment and a maximum emergence of 98.4% being reached nine weeks after planting, only (Figure 5B). There were no significant differences in time of emergence between competition treatments, nor with the area × competition ($\chi^2_{2,137} = 0.35$, $P = 0.840$ and $\chi^2_{2,137} = 3.78$, $P = 0.151$, respectively; Figure 5A).

Plants from the invaded region began flowering significantly later than plants from the native range ($\chi^2_{1,91} = 102.77$, $P < 0.0001$; Figure 5C and D), remaining vegetative for a longer period of time. Competition with *T. repens* did not significantly influence the beginning of flowering ($\chi^2_{2,91} = 5.48$, $P = 0.064$; Figure 5C). However, there was a significant area × competition interaction ($\chi^2_{2,91} = 14.87$, $P < 0.001$) and the analyses by area showed that, within the native region, plants in the low competition treatment began flowering earlier than plants in the control and high competition treatments ($P < 0.05$; Figure 5C). The probability of flowering did not differ among provenances or competition treatments (area: $\chi^2_{1,137} = 2.47$, $P = 0.116$; competition: $\chi^2_{2,137} = 2.32$, $P = 0.314$; area × competition: $\chi^2_{2,137} = 1.34$, $P = 0.510$).

Contrary to what is expected for a tristylous species, both emergence time and beginning of flowering were significantly different among distinct plant forms within each area ($\chi^2_{5,137} = 40.73$, $P < 0.0001$ and $\chi^2_{5,91} = 62.11$, $P < 0.0001$, respectively; Figure S1A and B in Appendix 2).

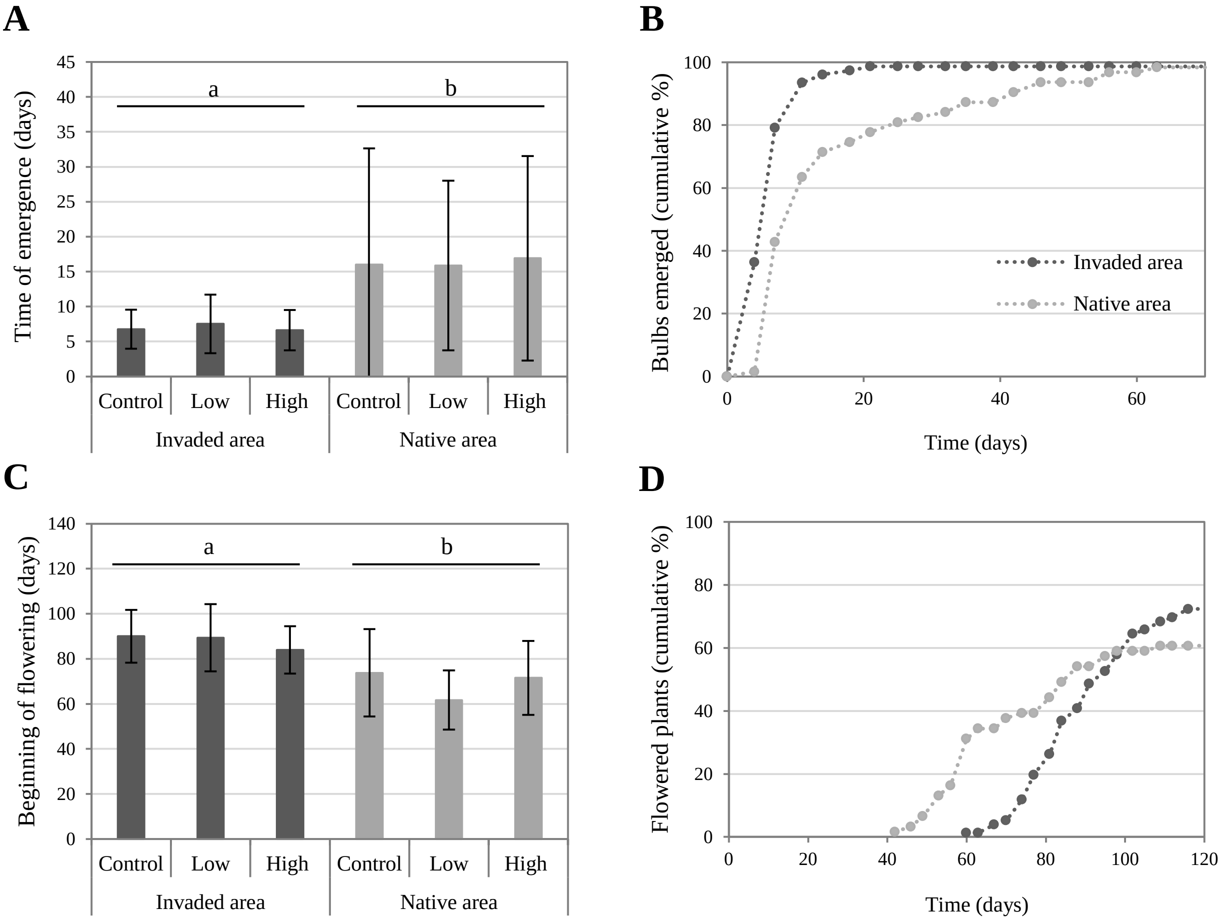

Figure 5. Phenological traits of *Oxalis pes-caprae* plants in set 1: **A.** mean (± standard deviation) time of emergence of plants from the invaded and native areas grown alone (Control), and under low and high competition (Low and High, respectively); **B.** cumulative percentage of bulbs emerged as a function of time for the invaded and native areas; **C.** mean (± standard deviation) starting date of flowering for plants from the invaded and native areas growing without competition, and under low and high competition; **D.** cumulative percentage of flowered plants as a function of time for the invaded and native areas. In **A** and **C**, different letters denote significant differences between areas. Time is given in days after the beginning of the experiment. Invaded area - dark grey, native area - light grey; Control - no competition, Low - low competition, High - high competition.

3.1.2. Growth, survival, asexual reproduction and chemical defense

Oxalis pes-caprae aboveground biomass differed significantly among areas, being higher in plants from the invaded region ($\chi^2_{1,132} = 37.93$, $P < 0.0001$; Figure 6A). Competition with *T. repens,* however, did not significantly affect this response variable

($\chi^2_{2,132}$ = 1.25, P = 0.103; Figure 6A). Further, the area × competition interaction did not reach significance ($\chi^2_{2,132}$ = 1.51, P = 0.064).

The proportion of plants dying before finishing their cycle was 4.84% for the native and 9.21% for the invaded area. No statistically significant differences were observed in survival between areas or competition levels (area: $\chi^2_{1,138}$ = 1.64, P = 0.200; competition: $\chi^2_{2,138}$ = 4.45, P = 0.108; area × competition: $\chi^2_{2,138}$ = 1.85, P = 0.396).

Concerning final bulb production, significant differences between native and introduced *O. pes-caprae* plants were obtained for the number of bulbs produced, with the latter producing a greater number of bulbs ($\chi^2_{1,122}$ = 6.27, P = 0.012; Figure 6B), but not for total bulb biomass ($\chi^2_{1,115}$ = 2.96, P = 0.100; Figure 6C). There were no statistically significant differences in these response variables between competition levels ($\chi^2_{2,122}$ = 5.77, P = 0.056 for number of bulbs and $\chi^2_{2,115}$ = 2.65, P = 0.299 for total bulb biomass) and no interactive effects of area and competition were detected ($\chi^2_{2,122}$ = 0.61, P = 0.738 for number of bulbs and $\chi^2_{2,115}$ = 0.52, P = 0.788 for total bulb biomass) (Figure 6B and C). Despite this, it is worth noting that the number of bulbs tended to increase with competition (Figure 6B).

The amount of oxalic acid in leaf extracts was highly variable and, although plants from the native region tended to produce more of this compound, no significant differences were found among areas or competition levels (area: $\chi^2_{1,52}$ = 4.96, P = 0.148; competition: $\chi^2_{2,52}$ = 3.01, P = 0.531; area × competition: $\chi^2_{2,52}$ = 8.74, P = 0.158; Figure 6D).

As expected, the aboveground biomass, number of bulbs produced, total bulb biomass, and leaf content of oxalic acid did not vary significantly among distinct plant forms within each area ($\chi^2_{5,134}$ = 1.29, P = 0.641; $\chi^2_{5,122}$ = 10.07, P = 0.073; $\chi^2_{5,115}$ = 5.94, P = 0.349 and $\chi^2_{5,52}$ = 20.53, P = 0.121, respectively; Figure S1C-F).

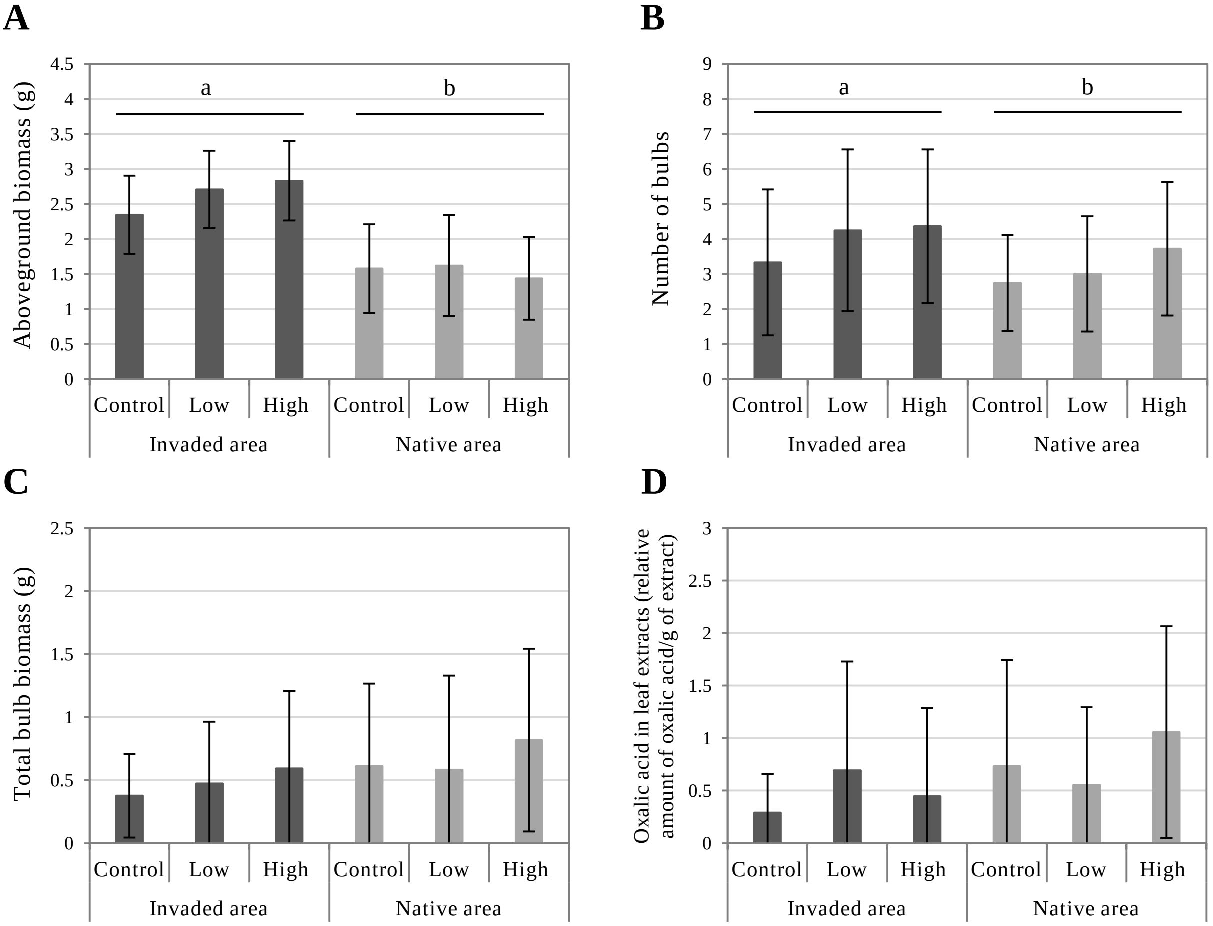

Figure 6. Mean values (± standard deviation) of **A.** aboveground biomass (g), **B.** number of bulbs, **C.** total bulb biomass (g) and **D.** oxalic acid in leaf extracts (relative amount of oxalic acid/g of extract) of *Oxalis pes-caprae* plants from the invaded (dark grey) and native (light grey) areas growing alone (Control), under low competition (Low), and under high competition (High). Different letters denote significant differences among areas. Aboveground biomass was measured in plants from set 1, while number of bulbs, total bulb biomass and oxalic acid in leaf extracts were measured in plants from set 2.

3.1.3. Chlorophyll fluorescence

In *O. pes-caprae*, chlorophyll fluorescence parameters (F_v/F_m and F_0) were not significantly different between areas ($F_{1,132}$ = 0.34, P = 0.561 for F_v/F_m and $F_{1,132}$ = 0.05, P = 0.830 for F_0) or competition treatments ($F_{2,132}$ = 1.50, P = 0.227 for F_v/F_m and $F_{2,132}$ = 1.72, P = 0.183 for F_0) (Figure 7A and B). Nonetheless, a significant area × competition interaction was observed for both F_v/F_m and F_0 ($F_{2,132}$ = 4.19, P = 0.017 and

$F_{2,132}$ = 5.55, P = 0.005, respectively). Within the native area, F_v/F_m was significantly higher in the high competition than in the control treatment ($P < 0.05$; Figure 7A), whereas F_0 was lower in the high competition than in the control ($P < 0.05$; Figure 7B). Despite this difference, all mean values of F_v/F_m were within the optimal range for this parameter (0.75-0.85, Björkman and Demmig 1987), and therefore, results must be interpreted with caution.

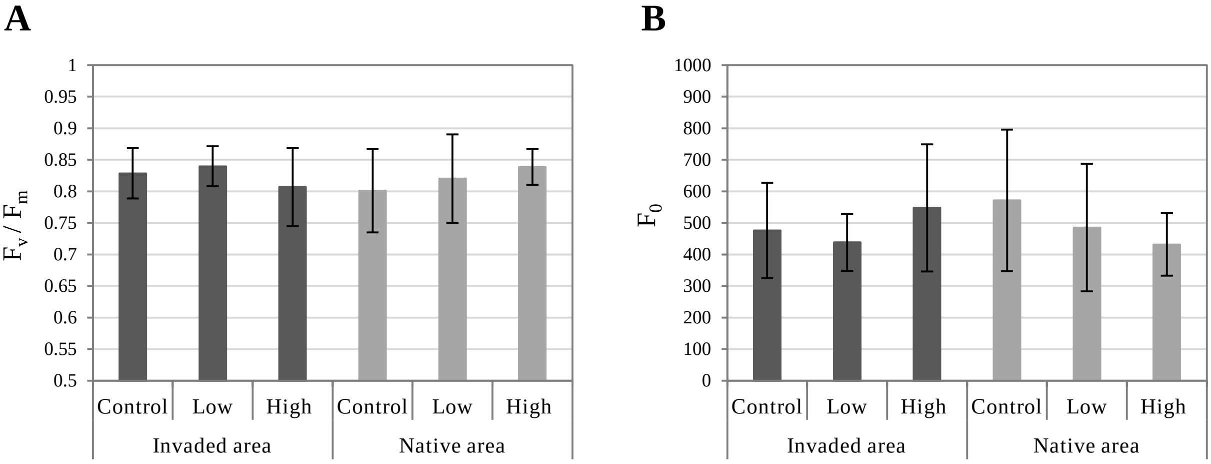

Figure 7. Chlorophyll fluorescence parameters for *Oxalis pes-caprae* plants from the invaded (dark grey) and native (light grey) areas growing alone (Control), under low competition (Low), and under high competition (High): mean values (± standard deviation) of **A.** F_v/F_m and **B.** F_0. Measurements were taken from plants in set 1.

3.2. *Trifolium repens*

3.2.1. Growth and survival

Trifolium repens aboveground biomass differed significantly among all competition treatments ($\chi^2_{2,68}$ = 92.67, $P < 0.0001$), with plants in the control presenting the highest values, plants competing with *O. pes-caprae* from the native area having low but intermediate values, and plants competing with *O. pes-caprae* from the invaded area presenting the lowest values ($P < 0.05$; Figure 8A).

Mortality tended to be greater in the presence of competition with *O. pes-caprae*: 11.90% and 20.37% for *T. repens* growing with *O. pes-caprae* plants from the native and invaded area, respectively, compared to 2.38% for the control (survival under different competition treatments: $\chi^2_{2,137} = 8.30$, $P = 0.016$). However, the multiple comparison test revealed only marginal significant differences between the control and competition with *O. pes-caprae* from the invaded area treatments, with the latter having lower survival values ($P = 0.066$; Figure 8B).

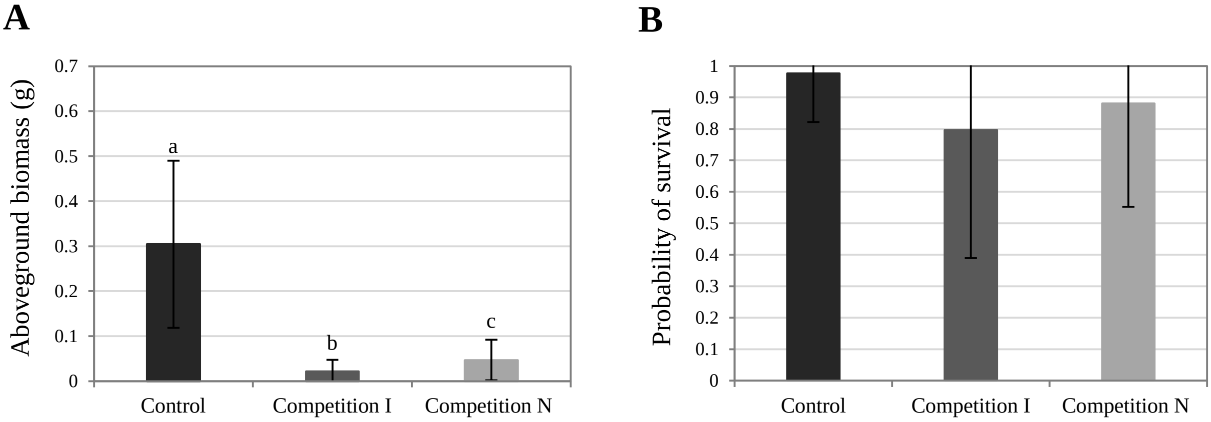

Figure 8. Mean values (± standard deviation) of **A.** aboveground biomass (g) and **B.** probability of survival of *Trifolium repens* plants grown alone (Control), and in competition with *Oxalis pes-caprae* plants from the invaded (Competition I) and native (Competition N) areas. Means with different letters differed significantly at $P < 0.05$. Measurements were taken from plants in set 1 at the first harvesting.

3.2.2. Chlorophyll fluorescence

In *T. repens*, the maximum quantum yield of PSII (F_v/F_m) was not significantly affected by competition with *O. pes-caprae* plants from either area ($F_{2,59} = 2.10$, $P = 0.131$; Figure 9A). F_0, on the other hand, was significantly different between competition treatments ($F_{2,59} = 3.99$, $P = 0.024$), being higher for plants in competition with *O. pes-caprae* than for those in the control treatment ($P < 0.05$; Figure 9B). However, no differences were found in these parameters between competition with plants from the native and plants from the invaded area (Figure 9B).

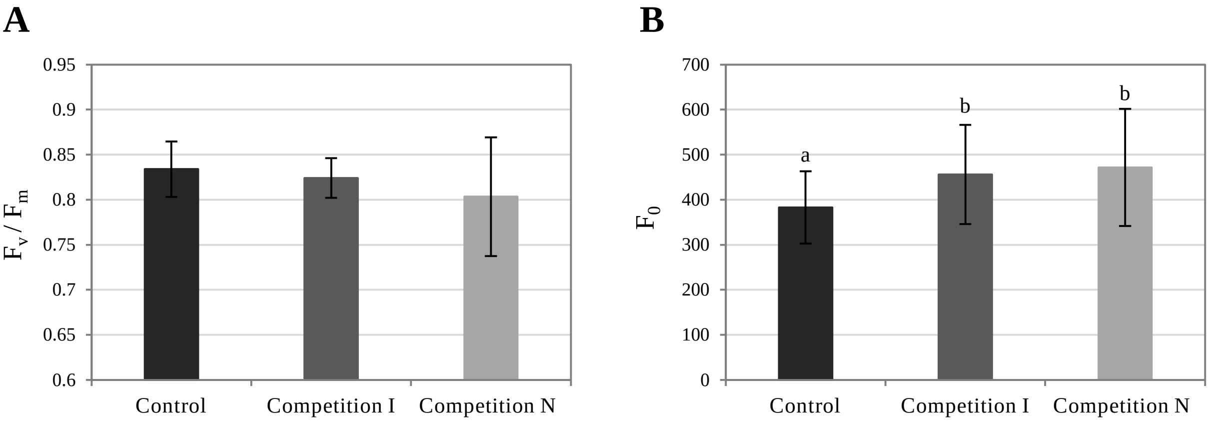

Figure 9. Chlorophyll fluorescence parameters for *Trifolium repens* plants grown alone (Control), and in competition with *Oxalis pes-caprae* plants from the invaded (Competition I) and native (Competition N) areas: mean values (± standard deviation) of **A.** F_v/F_m and **B.** F_0. Means with different letters differed significantly at $P < 0.05$. Measurements were taken from plants in set 1.

4. Discussion

The results of the present study demonstrate significant genetic based differences in life-history traits between native and invasive populations of *O. pes-caprae*. Plants from the invaded region emerged earlier, began flowering later and produced more aboveground biomass and offspring bulbs when compared to South African plants. Furthermore, although interspecific competition had no significant effect on *O. pes-caprae* plants regardless of their provenance, *T. repens* growth was more severely affected by invasive plants than by their native conspecifics. These patterns are consistent with previous studies that have demonstrated that, when grown in a common environment, plants from invasive populations perform better than those from native populations (Blair and Wolfe 2004; Brown and Eckert 2005; Ridenour *et al*. 2008; Zou *et al*. 2008; Barney *et al*. 2009; Hahn *et al*. 2012).

Phenological traits, in particular emergence and flowering time, are highly responsive to environmental change and, consequently, to range shifts (Fitter and Fitter 2002; Franks *et al*. 2007; Donohue *et al*. 2010), and can evolve rapidly during the course of an invasion (Weber and Schmid 1998; Lee 2002; Ridley and Ellstrand 2010). Indeed, genetically determined differences in these phenological traits between native and introduced populations of the same species have been demonstrated by several authors (e.g., Blair and Wolfe 2004; Dlugosch and Parker 2008; Barney *et al*. 2009). In the present study, the earlier emergence and later onset of flowering of *O. pes-caprae* plants from invasive populations are in accordance with the assumption that plants evolve enhanced fitness in their invaded range. Early emergence is assumed to provide a competitive advantage (for example, when competition for light is intense) and to benefit growth and fecundity by allowing plants to attain a larger size before reproduction and/or increase their reproductive period (Verdú and Traveset 2005; Donohue *et al*. 2010). Early flowering, on the other hand, may come at the cost of reduced size at maturity, which often translates into lower fecundity later in life and lower overall reproductive output (Geber 1990; Weber and Schmid 1998; Colautti *et al*. 2010).

Similarly, the change towards increased growth and production of clonal propagules in invasive populations observed in this study is consistent with the patterns found in other invasive species (Blossey and Nötzold 1995; Blair and Wolfe 2004; Jakobs *et al*. 2004; Brown and Eckert 2005; Dlugosch and Parker 2008; Zou *et al*. 2008; Barney *et al*. 2009). Growth and reproductive performance are important components of plant fitness, and clonal reproduction, in particular, has been regarded as one of the determinants of success in *O. pes-caprae* (Vilà *et al*. 2006a; Vilà and Gimeno 2006; Verdaguer *et al*. 2010). However, the production of a greater number of offspring bulbs by invasive plants was not translated into a higher total bulb biomass (i.e. invasive plants produce more but smaller bulbs). This result is particularly interesting if we consider that parent bulb size has been shown to be important in nutrient-deficient soils; still it was also observed that it has little influence on plant growth and fecundity under favorable conditions (Sala *et al*. 2007; Verdaguer *et al*. 2010). Therefore, in the fertile agricultural soils that *O. pes-caprae* tends to occupy in the invaded range, the supposed disadvantage of having smaller bulbs is likely to have low to no effects; instead the production of a large number of smaller bulbs, equally capable of growing and reproducing prolifically, can enhance the spread of *O. pes-caprae* without any additional costs.

A number of comparative studies have supported the prediction that invading populations are more vigorous, inferring that increased growth and fecundity confer greater competitive ability (Blossey and Nötzold 1995; Jakobs *et al*. 2004; Brown and Eckert 2005). Nonetheless, fewer studies have investigated the competitive abilities of native and invasive populations by including interspecific competition in the experimental design. Investigating if native and introduced populations respond differently to the presence of a competitor is fundamental to assess whether phenotypic differences actually translate into enhanced competitive ability (Bossdorf *et al*. 2005; Barney *et al*. 2009). In the present study, although there were no differences in competitive responses among *O. pes-caprae* plants from different provenances, their competitive effects varied significantly. Plants from the invaded area diminished *T. repens* growth more severely than plants from the native area, which may be indicative of a greater competitive ability. Barney *et al*. (2009) found that under interspecific competition with *Solidago canadensis* L., invasive (North American) populations of *Artemisia vulgaris* L. performed better than native (European) ones. Similarly, invasive plants of *Centaurea maculosa* Lam. from North American populations were demonstrated to be less affected by competition with *Pseudoroegneria spicata* (Pursh) Á. Löve and *Festuca idahoensis* Elmer, and produced stronger competitive effects than plants from Europe, where the species is native (Ridenour *et al*. 2008). However, other studies have also reported no differences in competitive ability between introduced and native populations regardless of changes observed in growth (Vilà *et al*. 2003; Blair and Wolfe 2004; Blumenthal and Hufbauer 2007).

Competition with *T. repens* had no negative effect on any of the life-history traits measured in *O. pes-caprae*. Actually, *O. pes-caprae* plants from both areas tended to produce a higher number of offspring bulbs in the presence of *T. repens* (as indicated by the marginally significant competition effect) and, in the case of the plants from the invaded range, there was a slight tendency for the aboveground biomass to increase in the same manner (as reflected by the marginal significance of the area × competition interaction). One possible explanation for this might be the occurrence of below-ground nitrogen transfer from *T. repens*, which is a N_2-fixing legume that already proved to be an efficient N donor (Pirhofer-Walzl *et al*. 2012), to *O. pes-caprae*. Still, despite the competitive superiority demonstrated here, previous studies of invasive *O. pes-caprae* populations indicate that the competitive responses and effects of this invader may depend on the identity of the competitor. Sala *et al*. (2007) found that competition with *Lolium rigidum* Gaudin had a strong negative effect on invasive *O. pes-caprae* plants. However, a comparison with native populations was not included in that experiment.

The finding that native and invasive *O. pes-caprae* plants growing in a common environment differed significantly in many life-history characters provides strong evidence for genetic differentiation, with the observation of a change towards a more aggressive phenotype in invasive populations. Now it is important to understand whether this divergence is the result of factors associated with founder events or if it is due to rapid post-introduction evolution (or a combination of these two non-exclusive processes). For a long time, the 5*x* S-morph, which reproduced exclusively asexually, was the only known form in the invaded region of the Mediterranean basin, indicating that colonization events may have been accompanied by strong founder effects. Under such circumstances, it is not possible to rule out the possibility that invasive populations were founded by a small subset of native plants of aggressive/vigorous genotypes, not necessarily representative of the native genotypes. Indeed, the initial motivation for *O. pes-caprae* introduction provides some support for this hypothesis. As *O. pes-caprae* was introduced for ornamental purposes (Rappa 1911), it would not be surprising that the first colonists had been chosen based in their vigor. In other studies, human-mediated selection of ornamental plants with traits that are associated with invasiveness has been suggested to increase the risk of invasion of some species (Chrobock *et al*. 2011).

Interestingly, of the three cytotypes (2*x*, 4*x* and 5*x*) known for this species (Ornduff 1987; Krejčíková *et al*. 2013), the most rare cytotype in the native region, i.e., the pentaploid, was the one thought to have been initially introduced and that has subsequently spread widely in the invaded range (Ornduff 1987; Castro *et al*. 2013a). Although the pre-adaptation of certain cytotypes has been suggested to partly explain the success of some invasive species (Treier *et al*. 2009; Thébault *et al*. 2011; te Beest *et al*. 2012), in this study, tetraploid and pentaploid plants from the invaded range did not differ in any of the traits measured. This is in contrast to the general assumption that ploidy level may influence several traits associated with invasiveness, such as size and competitive ability. Indeed, in the literature, in some cases polyploids have been characterized to be larger and more competitive than the corresponding diploids (Maceira *et al*. 1993; Ni *et al*. 2009; te Beest *et al*. 2012), but not always (Sakai and Suzuki 1955; Sakai and Utiyamada 1957; Garbutt and Bazzaz 1983; Münzbergová 2007; Collins *et al*. 2011). Furthermore, these differences seem to be more common when diploids are compared to tetraploids and become less evident in higher ploidy levels (Stebbins 1940). Still, in the future, it would be interesting to assess if the different *O. pes-caprae* cytotypes differ in other traits related to invasive potential, such as tolerance to disturbances. As for now, considering the observed similarity between pentaploid and tetraploid individuals within the invaded area, as well as the weedy character presented by tetraploids in the native range (Ornduff 1987), both cytotypes might possess similarly aggressive invasive traits.

It is puzzling, however, that the rarest cytotype was the one chosen to be used as an ornamental, especially if it did not possess any distinctive trait. In a previous study using plants from the same populations used here, Castro *et al*. (2013b) found that 5*x* S-morph individuals produced bigger flowers when compared to all tetraploid forms from the invaded and native areas. This could have been one of the distinctive traits that led to its introduction as an ornamental. Still, the real events that have occurred during the introduction of this species remain unknown, and stochastic events cannot be ruled out from the introduction scenario. It is important to note that the greater investment in the production of sexual structures by the 5*x* S-morph was not a determinant of its success throughout the invasion process and, until the recent discovery of the partial breakdown of its morph-incompatibility system (Costa *et al.* 2014), it could even be considered to be mal-adaptive (Verdaguer *et al*. 2010). Indeed, the changes in the sexual system (Castro *et al*. 2013a; Costa *et al*. 2014) together with the observed similarity between different cytotypes and morphotypes within the invaded region and the differences with the 4*x* plants from the native area (results herein), provide further support for the hypothesis that the tetraploid forms recently reported in the invaded range have originated in this region as a result of a partial breakdown in the morph-incompatibility system of the 5*x* S-morph.

Therefore, despite the important role that founder events may have played in the invasion process of *O. pes-caprae*, the patterns revealed in the present experiment indicate that this species also presents a high potential for rapid evolution. The shifts in phenology and the changes toward increased competitive ability, growth, and production of asexual propagules in invasive plants of *O. pes-caprae* may be, at least partly, explained by post-introduction evolutionary changes. Invasive populations experience markedly different selection pressures in the new environment and in the presence of standing genetic variation or new mutations may undergo rapid evolutionary adaptation (Prentis *et al*. 2008). Vegetatively reproducing populations are generally assumed to be genetically more homogeneous and, therefore, less likely to evolve (Sakai *et al*. 2001). However, Rottenberg and Parker (2004) have surprisingly detected considerable genetic variability in several asexual populations of *O. pes-caprae* composed exclusively by 5*x* S-morph individuals, proposing mutations and genome rearrangements as possible explanations. Although the existence of genetic variation is considered a requirement for adaptation, in the context of invasion, adaptive evolution has been demonstrated to occur even in cases where it would seem very unlikely, due genetic bottlenecks (Dlugosch and Parker 2008).

As referred above, until now, the most striking evidence of an evolutionary change in this invader was the partial breakdown of its morph-incompatibility system, with the subsequent appearance of sexual reproduction and possibly the emergence of new forms in the invaded region of the western Mediterranean basin (Castro *et al*. 2013a; Costa *et al*. 2014), in comparison with the fully functional heteromorphic incompatibility system in populations from the native range (Ornduff 1987; Castro *et al*. personal communication). Also, Vilà and Gimeno (2006) found a genetically based higher propagation potential (i.e., greater production of bulbs) in Mediterranean insular populations compared to neighboring mainland populations, suggesting adaptive evolution as one of the possible explanations. All together, these findings indicate that *O. pes-caprae* (namely, the 5*x* S-morph) may have a great potential for rapid evolutionary change and support the hypothesis that the overall better performance of invasive plants in this experiment is a result of rapid post-introduction adaptation.

This potential evolutionary change affecting life-history traits related to invasiveness could be associated with a reallocation of resources from defense to growth and reproduction in the absence of specialist enemies, as predicted by the evolution of increased competitive ability (EICA) hypothesis (Blossey and Nötzold 1995). The fact that plants from the invaded area produced more aboveground biomass and offspring bulbs than those from the native range provides partial support for the EICA hypothesis. However, the quantification of oxalic acid in leaf extracts failed to reveal the predicted decrease in defensive compounds. One possible explanation for this is that the production of this chemical defense may be induced by herbivory, which was not included in the experimental design. Further, oxalic acid is presumed to have additional functions within the plant, including pH regulation, osmoregulation, regulation of internal calcium levels and protection against allelochemicals (Libert and Franceschi 1987; Weir *et al*. 2006). Therefore, to better assess the EICA hypothesis, further investigations should consider different levels of herbivory by specialist and generalist herbivores in native and invasive populations to evaluate both plant resistance and tolerance.

To conclude, this Thesis suggests that founder events and evolutionary forces may have contributed, independently or in concert, to the genetic differentiation between native and invasive populations of *O. pes-caprae*, leading to the appearance of a phenotype with higher invasive potential. To discriminate between these hypotheses, the patterns of colonization are currently being investigated using molecular markers. Additionally, the similarity between the different cytotypes and morphotypes found within the invaded region implies that all these forms have the potential to behave as aggressive invaders with negative impacts on the resident flora. *Oxalis pes-caprae* often invades anthropogenic habitats where it has the potential to suppress species of economic and conservation value, leading to significant losses. This is particularly dramatic considering that, by opposition to the general belief, the ruderal communities where this species occurs represent a valuable and unique element of the Mediterranean flora, with many endemic and vulnerable plants (Vilà *et al*. 2006b).

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6. Appendices

Appendix 1. Characterization of the native (South African) and invasive (Western Mediterranean) populations of *Oxalis pes-caprae* used in the experiment.

Table S1. Collection localities, geographical coordinates, and distribution of floral morphs and cytotypes for the South African *Oxalis pes-caprae* populations used in the greenhouse experiment.

Population	**Geographical coordinates**		**Floral morphs (%)**			**Cytotype composition**		
			S	**M**	**L**	**S**	**M**	**L**
ZA: Doringbos	32°06'59.9"S	19°03'05.2"E	18	41	41	4*x*	4*x*	4*x*
ZA: Gouda	32°13'11.5"S	18°58'26.5"E	48	29	23	4*x*	4*x*	4*x*
ZA: Dwarskersbos	32°36'33.5"S	18°19'03.5"E	18	37	45	4*x*	4*x*	4*x*
ZA: Porteville	32°44'00.7"S	18°54'35.8"E	28	34	38	4*x*	4*x*	4*x*
ZA: Langebaan	33°03'29.7"S	18°04'43.0"E	25	17	58	4*x*	4*x*	4*x*
ZA: Riebbek Wes	33°13'13.3"S	18°43'15.5"E	37	42	21	4*x* (2*x*)	4*x* (2*x*)	4*x*
ZA: Yzerfontein	33°20'58.7"S	18°09'18.1"E	46	22	32	4*x*	4*x*	4*x*
ZA: Oudtshoorn	33°32'49.6"S	21°50'36.7"E	5	56	39	4*x*	4*x*	4*x*
ZA: Worcester	33°33'40.3"S	19°54'04.3"E	23	37	40	4*x*	4*x*	4*x*
ZA: Paarl	33°41'04.4"S	18°45'52.1"E	20	73	7	4*x*	4*x*	4*x*
ZA: Barrydale	33°47'14.8"S	21°08'39.1"E	40	23	37	4*x*	4*x*	4*x*
ZA: Suurbraak	34°03'28.1"S	20°35'31.6"E	31	16	53	4*x*	4*x*	4*x*
ZA: Riversdal	34°04'38.9"S	21°14'39.1"E	21	14	65	4*x*	4*x*	4*x*
ZA: Mossel Bay	34°05'39.9"S	22°03'24.0"E	29	51	20	4*x*	4*x*	4*x*
ZA: Cape Point	34°09'24.8"S	18°26'06.0"E	9	76	15	4*x* (5*x*)	4*x*	4*x*
ZA: Caledon	34°10'57.7"S	19°24'09.5"E	81	11	8	4*x*	4*x*	4*x*
ZA: Botrivier	34°13'24.0"S	19°11'59.6"E	4	70	26	4*x*	4*x*	4*x*
ZA: Witsand	34°15'07.1"S	20°59'33.4"E	35	41	24	4*x*	4*x*	4*x*
ZA: Gouritsmond	34°17'42.2"S	21°49'21.4"E	32	38	30	4*x*	4*x*	4*x*
ZA: Bredasdorp	34°18'07.7"S	20°12'12.8"E	46	21	33	4*x*	4*x*	4*x*
ZA: Stilbaai	34°21'14.5"S	21°25'00.2"E	38	41	21	4*x*	4*x*	4*x*
ZA: Standford	34°27'22.3"S	19°35'02.8"E	60	3	37	4*x*	4*x*	4*x*
ZA: Elim	34°35'57.7"S	19°45'33.4"E	21	78	1	4*x*	4*x*	4*x*

Country: ZA, South Africa. Floral morphs: S, short-styled morph; M, mid-styled morph; L, long-styled morph. Cytotypes: 2*x*, diploid; 4*x*, tetraploid; 5*x*, pentaploid. Latitude and longitude are given in degrees, minutes and seconds. Floral morphs are given in percentage. Rare cytotypes are presented in parentheses.

Table S2. Collection localities, geographical coordinates, and distribution of floral morphs and cytotypes for the Mediterranean *Oxalis pes-caprae* populations used in the greenhouse experiment.

Population	Geographical coordinates		Floral morphs (%)			Cytotype composition		
			S	M	L	S	M	L
SP: Baiona	42º06'42.2"N	8º49'40.4"W	100	0	0	5*x*	-	-
PT: Praia de Mira	40º27'15.4"N	8º46'45.3"W	100	0	0	5*x*	-	-
PT: Coimbra	40º12'21.2"N	8º25'25.7"W	100	0	0	5*x*	-	-
PT: Colares I	38º48'45.2"N	9º28'23.7"W	48	13	39	4*x*, 5*x*	4*x*	4*x*
PT: Colares II	38º48'01.0"N	9º28'03.7"W	28	22	50	4*x*, 5*x*	4*x*	4*x*
PT: Colares III	38º47'51.8"N	9º28'34.6"W	19	18	63	4*x*, 5*x*	4*x*	4*x*
PT: Troia	38°29'29.8"N	8°54'23.2"W	95	0	5	5*x*	-	4*x*
PT: Melides	38°07'50.6"N	8°46'57.7"W	100	0	0	5*x*	-	-
PT: Almograve	37°38'53.1"N	8°47'19.2"W	100	0	0	5*x*	-	-
PT: Armação de Pêra	37°04'51.4"N	8°17'12.1"W	100	0	0	5*x*	-	-
MA: Moulay-Bousselham	34°52'32.6"N	6°17'49.9"W	69	0	31	5*x*	-	4*x*
MA: Essaouira	31°29'43.3"N	9°45'38.3"W	96	0	4	5*x*	-	4*x*

Countries: SP, Spain; PT, Portugal; MA, Morocco. Floral morphs: S, short-styled morph; M, mid-styled morph; L, long-styled morph. Cytotypes: 4*x*, tetraploid; 5*x*, pentaploid. Latitude and longitude are given in degrees, minutes and seconds. Floral morphs are given in percentage.

Appendix 2. Life-history traits of the different *Oxalis pes-caprae* forms within each area.

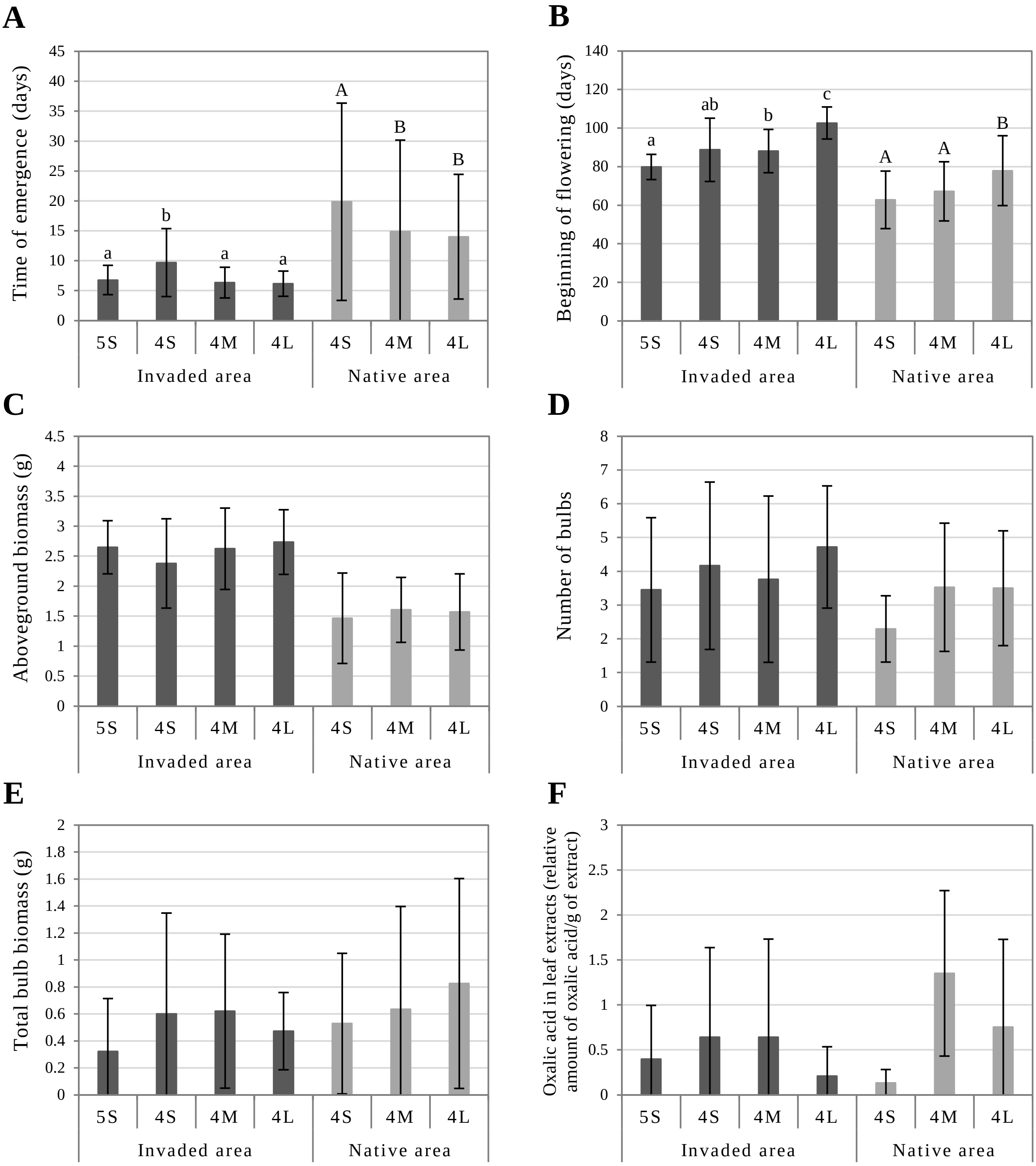

Figure 1S. Mean values (± standard deviation) of **A.** time of emergence (days), **B.** beginning of flowering (days), **C.** aboveground biomass (g), **D.** number of bulbs, **E.** total bulb biomass (g) and **F.** oxalic acid in leaf extracts (relative amount of oxalic acid/g of extract) for the different *Oxalis pes-caprae* forms within the invaded and native areas. Different lower and upper case letters indicate statistically significant differences at $P < 0.05$ among forms within the invaded and native areas, respectively. Time of emergence, beginning of flowering and aboveground biomass were measured in plants from set 1, while number of bulbs, total bulb biomass and oxalic acid in leaf extracts were measured in plants from set 2. Time is given in days after the beginning of the experiment. *Oxalis pes-caprae* forms: 5S - pentaploid short-styled, 4S - tetraploid short-styled, 4M - tetraploid mid-styled and 4L - tetraploid long-styled. Invaded area - dark grey, native area - light grey.