UNIVERSITÀ
DI PAVIA

SCUOLA DI ALTA FORMAZIONE DOTTORALE
MACRO-AREA SCIENZE E TECNOLOGIE

Dottorato di Ricerca in Scienze della Terra e dell’Ambiente

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Competition or facilitation? Studies of wetland plant species in natural environment and agroecosystems for future translocation perspectives

Anno Accademico 2020-2021
Ciclo XXXIV

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To people, places, experiences
that inspired me and brightened my days

To new unknown extraordinary adventures

Abstract

Competition is one of the major factors that rules plant communities. It explains coexistence, species diversity maintenance and ecosystem functioning; moreover, it acts with different intensity both in time and space according to the species involved and resource availability. In particular, it is crucial to understand whether different species may compete or be facilitated in the exploitation of the available resources.

Competition is also the major driver of biological invasions, which are the primary cause of biodiversity loss. Invasive species are constantly increasing the extinction rate of native species and a valid tool that has been used in the last decades to preserve species threatened with extinction is plant conservation translocation. To obtain positive outcomes through the creation of self-sustaining populations in the translocated sites, knowledge of species biology and their ecological requirements is necessary for successful translocations, jointly with the long-term monitoring and site management.

This work aims to understand the competitive ability of two wetland policy species typical of natural environments and agroecosystems (i.e., *Marsilea quadrifolia* L. and *Kosteletzkya pentacarpos* (L.) Ledeb.) versus their invasive potential competitors and the recipient community, respectively, for future translocation perspectives. In addition, the competitive ability is addressed from the invasive' point of view, with a focus on the performance of the invasive *Bunias orientalis* L. in its recipient native grassland community.

The experimental studies show that competition between plants is extremely various and depends on several factors that may significantly affect species' responses, basing not only on the resources for which they compete (e.g., light, water, nutrients) but also on the presence or absence of the local vegetation. Moreover, competition is species-specific dependent and changes with changing of the environment.

The study of the competition must then be addressed to understand the dynamics occurring among species, especially in disturbed and anthropized habitats such as wetlands and grasslands where invasive species are abundant and highly competitive but also several threatened species and diverse native communities still occur.

General statement

The thesis is structured according to the experimental studies that primarily tested the competitive ability of two wetland policy species (i.e., *Marsilea quadrifolia* L. and *Kosteletzkya pentacarpos* (L.) Ledeb.), for their future translocation perspectives. The thesis is divided by target species; the former chapter on *M. quadrifolia* includes also research studies that have been addressed during my PhD program and contribute to the long-term conservation of the species. Except for sub-chapter 4 on *M. quadrifolia*, the thesis is a collection of manuscripts that have been published to indexes journals (ISI), have been submitted or are in preparation for submission. I have personally planned, performed and written a significant portion of all these works. The thesis is organized as follow:

Target species: *M. quadrifolia* L.

1. Original paper: **Corli, A.,** Rossi, G., Orsenigo, S., Abeli, T., 2021. Biological flora of Central Europe: *Marsilea quadrifolia* L. Perspective in Plant Ecology, Evolution and Systematics, 53, 125641. (Published; https://doi.org/10.1016/j.ppees.2021.125641)
2. Original paper: **Corli, A.,** Orsenigo, S., Gerdol, R., Bocchi, S., Smolders, A.P., Brancaleoni, L., Caffi, M.T., Abeli, T., Rossi, G., 2021. Coexistence of rice production and threatened plant species: testing *Marsilea quadrifolia* L. in N-Italy. Paddy and Water Environment. (Published; https://doi.org/10.1007/s10333-021-00840-z)
3. Original paper: **Corli, A**., Orsenigo, S., Gerdol, R., Brancaleoni, L., Abeli, T., Rossi, G., 2021. A mesocosm experiment testing the competition between the invasive *Heteranthera reniformis* Ruiz & Pav. and the endangered fern *Marsilea quadrifolia* L. occurring in paddy fields. (Sumbitted to Aquatic Botany on 18th November 2021)
4. Research project: Preserving biodiversity in agro-ecosystems: conflict or opportunity? The CLOVER Project

Target species: *Kosteletzkya pentacarpos* (L.) Ledeb.

5. Original paper: **Corli, A.,** Orsenigo, S., Gerdol, R., Brancaleoni, L., Abeli, T., Rossi, G., 2021. Relative importance of site selection and aftercare in the reintroduction of the policy species *Kosteletzkya pentacarpos* (L.) Ledeb. (In preparation)

In the experience abroad I took part during my PhD program, I collaborated in a research project addressing the competitive ability of the invasive *Bunias orientalis* L. in its recipient native grassland community. Therefore, the work is also included in the thesis.

Target species: *Bunias orientalis* L.

6. Original paper: **Corli, A.**, Walter, J., Sheppard, C.S., 2021. Invasion success of *Bunias orientalis* (warty cabbage) in grasslands: a mesocosm experiment on the role of hydrological stress and disturbance. Frontiers in Ecology and Evolution (Published; https://doi.org/10.3389/fevo.2021.625587)

Introduction

Competition as major factor influencing plant communities

In the past 100 years, ecological theories and empirical studies have focused widely on the importance of competition in plant communities, that helps in explaining coexistence and evolution of traits, in maintaining species diversity, in understanding ecosystems functioning and biological invasions (Wright et al., 2013; Aschehoug et al., 2016).

Competition is the negative effect on plant growth and/or plant fitness caused by the presence of neighbors, usually through the reduction of resources availability. Competition by a plant is expressed through either the capture of resources before its neighbors or the investment in structures that enable the plant to acquire resources in higher demand, resulting in trade-offs such as a greater shoot growth at the expense of root growth (Aschehoug et al., 2016).

The outcome of competition is the combined effect of both plant's competitive ability (i.e., the ability of the species to suppress a neighbor) and the plant's competitive response (i.e., the ability of the species to tolerate a neighbor's competitive effects; Goldberg, 1990). However, since plants typically interact within communities in a multispecies neighborhood, the outcome of competition is influenced by the presence of the other species (Aschehoug & Callaway, 2015). Within this scenario, species can be potentially favored when competitive interactions are weak or excluded when competitive interactions are strong enough, with competition playing an important role in influencing plant distributions at spatial scales also in response to climate change (Tylianakis et al., 2008).

Understanding species coexistence in plant communities is particularly challenging since most plant species use a small number of limiting resources in similar ways (Silvertown, 2004). Plants mainly compete for light, water and soil nutrients (principally nitrogen and phosphorus), depending upon the species requirement and the environment. In brief, preemption of light (Craine & Dybzinski, 2013) is achieved by a plant positioning its leaves between its neighbors and the light source by growing taller, earlier and/or faster, with direct effects on plants phenology, height and relative growth rate. Plants unable to experience these traits may express a shade avoidance syndrome, that enables shaded leaves to be positioned in patches of improved light quality, or a shade tolerance syndrome (Pierik et al., 2013). In addition, competition for light affects competitive ability with a great investment in leaf area to maximize light collection (Craine & Dybzinski, 2013), resulting in reduced net carbon gain per individual plant. Competition for water mainly regards either an investment in traits that minimize competition through rapid uptake (low water use efficiency) or by higher competitive ability through higher water use efficiency (Craine & Dybzinski, 2013). Competition for soil nutrients is more complex than competition for light or water because of various mechanisms among different nutrients uptake. Acquiring soil nutrients creates depletion zones around roots (Tinker & Nye, 2000), that are critical for soil resource acquisition, particularly for nutrients such as phosphorus that has low diffusion rates and is not acquired via mass flow. Preemption of soil nutrients may require active proliferation of fine roots and root hairs into resource patches (Kembel & Cahill, 2005), giving the potential of competitive advantages. However, competition for nutrients may not simply be a race to grow more roots: root development and patch exploitation can also be regulated by whole-plant nutrient status (Pierik et al., 2013), meaning that root proliferation-resource pool capture relationship is not always size dependent.

Competition within plant communities can also result in positive effects, implying facilitative interactions (Wright et al., 2013). Together with competition, facilitation is one of the major forces driving community structure (Bertness & Callaway, 1994) and it is defined as the positive association between two or more plants, where the presence of one plant is beneficial to the growth, reproduction and survival of another plant in its proximity (Callaway, 2007); facilitation can in turn affect local species richness and promote changes in the interaction networks among the facilitated species (Soliveres et al., 2015). Competition and facilitation may occur simultaneously on the same plant and the net observable outcome is the result of the combination of both positive and negative interactions (Brooker & Callaghan, 1998).

Understanding the balance between the interactions on species coexistence has been a major focus in plant ecology over the last decades (Bertness & Callaway, 1994), with environmental gradients contributing to understand the contradictory predictions of the major theories of competition among plants (Maestre et al., 2009). The Resource Ratio hypothesis (Tilman, 1982) predicts that the role of competition should remain constant across environmental gradients, although the focal resources may vary; thus, plants coexist if they have a trade-off in their requirements for two essential resources and if the ratio at which these resources are supplied varies in space (Tilman, 1982). In contrast, in the Life History Trade-off hypothesis (Grime, 1973), the author proposed a theoretical triangular scheme of competitor, stress tolerator and ruderal plants, where competition is predicted to be stronger in high-productivity and low-stress areas, whereas is weaker in low-productivity and high-stress areas. The basic distinction between Tilman's theory and Grime's theory is on the focus of the hypothesis, with the former author supporting competition' intensity (i.e., absolute impact) and the second author its importance (i.e., impact relative to the environment; Brooker et al., 2005).

Following Grime's model, the Stress Gradient Hypothesis has been later postulated (Bertness & Callway, 1994): the balance between competition and facilitation may be tipped one way or another depending on environmental context, with great influence of stress, disturbance and consumer pressure. Under mild environmental conditions, competition is considered as the leading interaction, because higher resource availability may enhance biomass production and reduce the presence of suitable sites for establishment (Connell, 1983). Conversely, under stressful or disturbed environmental conditions (e.g., Soliveres & Maestre, 2014) facilitation commonly occurs, allowing species not tolerant to stress or disturbance to persist in extreme habitats; consequently, an increase in biodiversity may be observed (e.g., Cavieres et al., 2014), with nurse plants being able to facilitate seedlings establishment by ameliorating abiotic conditions but also by increasing soil nutrient levels (e.g., Anthelme et al., 2012). However, a given level of environmental stress does not have the same effect on all species within a community, and this depends on the level of stress tolerance of each species (Soliveres et al., 2015). The Stress Gradient Hypothesis has been supported by numerous studies in many ecosystems (Callaway, 2007) and refined according to new outcomes of experimental studies (Maestre et al., 2009).

The role of competition in biological invasions

Resource competition is a key process regulating plant community dynamics (e.g., Grime, 1973; Tilman, 1982) and has long been considered as a major mechanism determining the success of species in biological invasions (Elton, 1958; Levine et al., 2004; Vilà & Weiner, 2004).

Biological invasion is the deliberate or inadvertent movement of species (i.e., alien species; synonyms: exotic plants, non-native plants, nonindigenous plants) outside of their native range as a result of human activity, with the establishment of self-sustaining populations and spread into the new recipient habitats (Elton, 1958; Richardson et al., 2000). Some alien species may reproduce regularly to form self-replacing populations over many life cycles without direct intervention by humans (i.e., naturalized species); a subset of these may spread rapidly over substantial distances from introduction sites and produce reproductive offspring in very large numbers (i.e., invasive species; Richardson et al., 2000; Blackburn et al., 2011), being responsible of negative impacts on the economy, environment and health (Vilà et al., 2011). So far, almost 4% of the extant vascular plant species (van Kleunen et al., 2015), and similar proportions of birds and mammals (Blackburn et al., 2014), have established wild populations beyond their native range, with invasive species spreading widely and rapidly (Blackburn et al., 2011).

Biological invasions vary among regions across the globe (Pyšek et al. 2017; Bradley et al., 2019): basing on several experimental studies, islands are more susceptible than mainland areas (Dawson et al., 2017) and regions in the southern hemisphere are more subjected to biological invasions than areas in the northern hemisphere (van Kleunen et al., 2015). In addition, biomes in the New World, both in temperate and mediterranean-type climates, are more invaded than those in arid and warm climates (van Kleunen et al., 2015; Pyšek et al., 2017), with the extent of biological invasions varying in relation to current and historical human impact (Pyšek & Richardson, 2010).

Among the best-studied direct drivers, biological invasions are mainly influenced by climate change, land-use change, pollution, global trade, human disturbance (Hulme, 2017) but also by the facilitative effect of other alien species through invasional meltdown (Redding et al., 2019). Human actions worse thus the strength, the direction and the rate of invasiveness through indirect drivers, that influence both directs and other indirect drivers such as economic, demographic, governance, technological and cultural processes.

If native plants have tightly adapted within their environment during their biological history in respect to the biotic community and edaphic factors (e.g., evolution of root system, leaf-area and shoot allocation, mutualistic relationships such as with pollinators, symbiosis between roots and microbiota; Richardson et al., 2000), alien species act in a different way. As posit the Enemy Release hypothesis (Keane & Crawley, 2002), alien plants establish and spread in the recipient communities thanks to their lack of local adaptation, with regard especially to the natural enemies such as herbivores. In line, this lack of adaptation within the recipient community may not only have ecological consequences, but evolutionary responses as well. The Evolution of Increased Competitive Ability hypothesis suggests that a decrease in enemy attack on alien plants may select for increased competitive ability because plants can allocate resources to growth or reproduction (or both) instead of defence (Blossey & Nötzgold, 1995).

Alien species but especially invasives express a superior capacity to compete for resources (Levine et al., 2004; Vilà & Weiner, 2004) and they acquire disproportionately more resources than native species (e.g., Funk, 2013), forming easily monospecific stands. Moreover, they can extirpate both functionally distant species but also similar species, depending on the intensity of competitive ability (i.e., hierarchical functional trait differences) and niche similarity (i.e., non-directional functional trait differences) to the native community (Kraft et al., 2015). On the other hand, when alien species experience the same competitive ability of native species, the former can compete more intensively with those native species that show a similar niche (functional similarity), with other natives being excluded (Cavender-Bares et al., 2009). In both cases, native and alien species can co-exist via niche complementarity if they are sufficiently different and use distinct resources (functionally distant) (Mayfeld & Levine, 2010). On the other side, a natural barrier for alien species to become invasive is guarantee through the biotic resistance of native species (Richardson et al., 2000).

In recent decades, a multitude of plant-species traits and environmental characteristics have been identified to promote naturalization and invasiveness, with invasive species being characterized by fast growth, early flowering (Pyšek & Richardson, 2007; van Kleunen et al., 2010), high fecundity, large native range and habitat affiliation in the native range (Pyšek et al., 2015). It is also suggested that invasive species are often phenotypically plastic, which enables them to grow and reproduce in a wide range of environmental conditions (Rejmánek et al., 2005) and broaden their habitat niche in the invaded range (Hejda et al., 2009). Following Grime's CSR categories, ruderals (R), competitors (C) and the intermediate stage between C and R are prevalent among invasive species, while stress-tolerators (S) are under-represented among invasive species (Lambdon et al., 2008). However, experimental studies have also revealed that the factors associated with plant invasions are stage- (e.g. Dawson et al., 2017) or context-dependent (van Kleunen et al., 2015) and the response of native community may change.

Impacts of biological invasion: consequences at biodiversity level

Besides being an outcome of globalization, biological invasions are considered the major driver of global biodiversity change (Pyšek et al., 2020) and have become a defining feature of the Anthropocene. Despite risk assessment, early detection, eradication plans and management actions have been widely applied and implemented (e.g., Regulation No. 1143/2014 of the European Parliament), the expansion rate of alien and especially invasive species continue to increase (Pyšek et al., 2017; Seebens et al., 2021), with negative effect on biodiversity and human livelihood (Vilà et al., 2011; Shackleton et al., 2019): alien species but especially invasives affect native species richness and abundance (Vilà et al., 2011; Gallardo et al., 2016). They modify the phylogenetic and functional diversity of the recipient communities and trophic networks, increasing in turn native species risk extinction. Of note, one out of five plant species recorded as extinct on the IUCN Red List report invasive species as the primary cause of their extinction (IUCN, 2015). In addition, invasive species have broken down biogeographical realms altering ecosystem functioning and provision of ecosystem services (Vilà & Hulme, 2017) such as ecosystem productivity, nutrient and contaminant cycling, hydrology, and disturbance regimes (Blackburn et al., 2019). The impacts of some invasive species go beyond changes to the environment, since they can negatively affect production in agriculture, forestry, aquaculture or fisheries, as well as they can be a concern for human well-being (Vilà & Hulme, 2017) and health (e.g., *Ambrosia artemisiifolia* L., *Heracleum mantegazzianum* Sommier & Levier).

Therefore, prevention and management of biological invasions is critical, both for the conservation of plant species threatened with extinction as well as for the maintenance of high level of biodiversity in natural communities, with invasive species being able to increase rare species extinction rate and reduce biodiversity, respectively.

The importance of plant translocation in biodiversity conservation

Threatened species management is increasingly involving several forms of conservation to secure viable metapopulations and reverse local extinctions, with human actions having contributed to accelerating the rate of species extinction by 100- to 1000-times the natural rate in the last century (Thuiller, 2007).

The best place to conserve plant biodiversity is in the wild, where many species occur in viable populations and can persist in their natural habitats with the associated ecological interactions (Mc Naughton, 1989). However, because of habitat loss and degradation, as well as the presence of transient seed bank and/or plants dispersal-limited (Clark et al., 2007), spontaneous recovery of rare plants may be constrained by the absence of propagules occurring in the restored sites. In this case, plant conservation translocation (i.e., the intentional human-mediated movement of organisms from one location to another for conservation purposes; IUCN, 2013) is a valid tool to preserve species threatened with extinction.

Conservation translocations include: (i) population reinforcement – that is the intentional movement of an organism into an existing population of conspecifics, with the purpose of increasing the population size, the genetic variability, or the representation of specific demographic stages or groups; (ii) reintroduction – that is the intentional release of a new population in an area within the historical native range of the target species from which it has disappeared, with the aim of re-establishing a viable population of the focal species; (iii) conservation introduction – that is the intentional release of an organism outside its indigenous range to avoid extinction (i.e., 'Assisted colonization'; IUCN, 2013) or to perform a specific ecological function (i.e., 'Ecological replacement'; IUCN, 2013). Plant translocation aims at reducing the extinction risks for threatened species through the establishment of resilient and self-sustaining plant populations in areas in the absence of naturally occurring propagules (e.g., Seddon et al., 2007; Ren et al., 2014), enhancing a species survival with a long-term perspective (Godefroid et al., 2011; Commander et al., 2018) and ensuring that threats that cause the decline in wild populations can be ameliorated in the recipient reintroduction site (Dalrymple et al., 2012).

The potential of plant translocation to contribute to the recovery of threatened species is significant, and to achieve its goal, identifying and removing threats that caused the decline and extinction of the target species is a fundamental prerequisite. To translocate efficiently, a detailed understanding of the species' biology (i.e., plant growth, breeding system, seed production, viability and dispersal capacities, patterns of genetic diversity and structure) and ecology (i.e., ecological niche requirements) is needed (Godefroid et al., 2011). A successful translocation is more reachable in protected sites and when the site is prepared accurately in the pre-translocation phases and is monitored and managed as well in the post-translocation phases i.e., aftercare; moreover, its success is enhanced when plant translocation is associated with an integrated and well-developed conservation plan (e.g., Albrecht et al., 2011).

In recent decades there has been a global proliferation in the number of plant translocations (Godefroid et al., 2011; Dalrymple et al., 2012). However, although several international treaties and legislations are in place to reduce the loss of biodiversity (e.g., the Convention on Biological Diversity, the Bern Convention, the Global Strategy for Plant Conservation, the European Strategy for Plant Conservation, the Gran Canaria Declaration on Climate Change and Plant Conservation, and the European ''Habitat" Directive 92/43/EEC), many translocations are relatively high costly and have failed to establish viable populations (Bottin et al., 2007; Drayton & Primack, 2012). Reasons for failure are manifolds (e.g., Godefroid et al., 2011; Commander et al., 2018) and include the lack of knowledge of species biology and habitat requirement, with a consequent choose of the inadequate recipient site. This is one of the mayor constrain for rare species, with almost half of the translocation programs not having sufficient knowledge on either the ecological requirements or breeding system of the species that are translocated (49% and 43%, respectively; Godefroid et al., 2016). For example, recent reviews highlighted that survival, flowering and fruiting rates of translocated plants were generally low (on average 52%, 19% and 16%, respectively; Godefroid et al., 2011), with plants achieving reproductive maturity between 19-49% depending on the type of propagule used (Dalrymple et al., 2012). Moreover, there is a lack of documentation and spread of the outcomes and protocols and an overly optimistic evaluation of success based on short-term results, with insufficient monitoring following plant translocation (i.e., the monitoring ceases after 4-5 years, with a consequent lack of a post-translocation phase that include the long-term monitoring and site management and activities, also known as aftercare). Therefore, to guarantee a long-term successful translocation, is crucial to perform, develop, manage and spread each of the phases that this conservation tool included.

Plant conservation and invasion in disturbed and anthropized habitats

Plant conservation is highly important in disturbed and anthropized habitats such as wetlands and grasslands, where several threatened species and diverse native communities occur; here, the elevate anthropogenic activities increase the probability of both species extinction rate and invasiveness capacity, reducing in turn species and habitat biodiversity.

Despite international recognition as enormously important for biodiversity, ecosystem health and human well-being, wetlands continue to be degraded and destroyed faster than any other terrestrial ecosystem (Laurance et al., 2012), with around 85% of wetland area been lost since the 1700s (Davidson et al., 2014). Many causal factors have been associated with wetland loss and degradation including vegetation clearing and drainage for agriculture, infrastructure expansion, pollution, global climate change, but also biological invasions. In this context, several species with high conservation value rely directly or indirectly on wetlands for survival and urgent actions are needed to preserve them to the extinction.

Grasslands are also valuable habitats supporting a huge amount of biodiversity that have been accumulated during millennia of low intensity land use (Habel et al., 2013); of note, European grasslands include the most species-rich plant communities, with temperate grasslands holding world records in the number of vascular plant species (Wilson et al., 2012). As wetlands, grasslands offer key ecosystem services, balancing the concentrations of global atmospheric greenhouse gases, protecting soil and water resources, providing livestock forage and therefore meat and milk for human consumption (O’Mara, 2012). Nevertheless, they are one of the most sensitive ecosystems to global climate change drivers (Seastedt and Pyšek, 2011), where anthropogenic influence accelerated their biodiversity decline over the last decades, being among the Europe’s most threatened ecosystem (Janssen et al., 2016). Indeed, as a consequence of abandonment and changing of traditional management toward habitat intensification, European grasslands have experienced a decrease in species richness and changes in competitive regimes, being also increasingly disturbed by frequent mowing or grazing (Stampfli et al., 2018). Also, grasslands have one of the highest proportion of habitats with an unfavorable and deteriorating conservation status, with biological invasions acting as a drivers of change (Vitousek et al., 1997).

Biological invasions are thus a key driver of biodiversity loss in both ecosystems, where invasive species can shift those ecosystems to alternative stable states less diverse where they dominate, benefitting disproportionately both from competition and disturbance (Smith and Knapp, 1999). To better understand the dynamics occurring within species (i.e., interspecific relationships) both for translocation perspectives of threatened species occurring in this context as well as in view of biological invasions from the invasive species' point of view, it is necessary to study and address this topic with experimental studies both in common-garden as well as in the wild. In the former case, experimental manipulations can help in understanding the adaptation of invasive species to their new environment and the response of threatened species or native community along either environmental gradients (i.e., different water table depth; Wei et al., 2017; Walter, 2018) or different community composition (i.e., species cultivated in co-occurrence and in monoculture condition; Sheppard & Burns, 2014; Sheppard & Brendel, 2021). However, both in view of the creation of self-sustaining population of threatened species for translocation perspectives as well as to understand whether the balance between species may be maintained, experimental studies must be addressed long-term to understand the dynamics occurring within species. In this case, the best place to perform the experiment is in the wild, where the environmental variables are also taken into account in determining species performance.

Aims and structure of the work

The main scope of this work is to understand the entity of interspecific interactions focusing on the competition ability of two wetland policy species typical of natural environments and agroecosystems (i.e., *Marsilea quadrifolia* L. and *Kosteletzkya pentacarpos* (L.) Ledeb.) versus their alien potential competitors and the recipient community, respectively, for future translocation perspectives. Moreover, since for the development of a successful conservation plan for the species, ecological requirement and knowledge of species biology are needed, this work includes experimental studies that help in increasing species survival for the final goal. In addition, the competitive ability is addressed from the invasive' point of view, with a focus on the performance of the invasive *Bunias orientalis* L. in its recipient native grassland community. In detail, the thesis is divided by target species with the relative experimental studies and it is organized as follow:

Target species: *M. quadrifolia* L.:

1. Original paper: I conducted an accurate literature research on several aspects regarding the species, ranging from taxonomy and morphology to species distribution and habitat requirements, as well as life cycle and conservation aspects.
2. Original paper: I studied the effect of different farming systems of paddy fields (conventional, organic, in transition from conventional to organic), on the species, where species growth and

performance was monitored. This is the semi-natural habitat where the species occurs in Italy. Cultivating the species in organic rice farms represents an opportunity for preserving this endangered species in areas of intensive agriculture, offering a valid chance to combine nature conservation and productivity, also through possible income for farmers.

3. Original paper: I studied the effect of competitive ability between the target species and the invasive monocot *Heteranthera reniformis* Ruiz & Pav. in a mesocosm experiment. Species were cultivated both in monoculture and in association at different density, also in presence of rice plants. Data on vegetative growth, reproduction, ecophysiology and functional traits were collected. Cultivating *M. quadrifolia* in context where fertilization is reduced and *H. reniformis* occurs as well, such as in low input and organic farming systems, may represent an opportunity for preserving *M. quadrifolia* in its semi-natural environment. Nature conservation and productivity may thus coexist and be promoted by specific funds (see CLOVER Project).
4. Research project: I collaborated in managing the activities of the CLOVER Project which has specific actions aim at demonstrating the best practices to conserve *M. quadrifolia* in rice fields (i.e., *on farm* cultivation during the growing season and *ex situ* conservation of rhizomes during the growing season) and in wet meadows listed in Habitat Directive (i.e., effect of mowing and grazing on species performance and survival)

Target species: *Kosteletzkya pentacarpos* (L.) Ledeb.

5. Original paper: I investigated the importance of site selection and aftercare in the reintroduction of the species focusing on the presence/absence of natural vegetation to see whether species may compete for resources. Environmental data, plant growth information, data on vegetative and reproductive performance, phenological data as well as ecophysiological traits were acquired. Environmental characteristic of the sites affected the performance of the species to much a greater extent than interactions with co-existing vegetation and site management as well.

Target species: *Bunias orientalis* L.

6. In a three-years mesocosm experiment I studied the invasion success of the species in its native recipient grassland community. The establishment and growth of plants was compared with bare soil with varying hydrological conditions (from waterlogged to dry soil moisture), mimicking the predicted changes in precipitation patterns under climate change. Invasion success of the species in grasslands will not increase if precipitation patterns change toward more extreme events. However, disturbance that creates bare soil patches might favor *B. orientalis* under drought conditions.

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Target species: *Marsilea quadrifolia* L.

Biological flora of Central Europe: Marsilea quadrifolia L.

Corli, A., Rossi, G., Orsenigo, S., Abeli, T., 2021. Biological flora of Central Europe: *Marsilea quadrifolia* L. Perspective in Plant Ecology, Evolution and Systematics, 53, 125641. (Published; https://doi.org/10.1016/j.ppees.2021.125641)

Abstract

Marsilea quadrifolia L. is a leptosporangiate aquatic fern which has a played key role in the evolutionary history of plants. It is characterized by heterospory, the ancestral progressive trait that led to the evolution of seeds. The species has creeping, fleshy, adventitious roots containing multiple rhizomes. From the rhizomes a four-leaf clover grows above the water level with a long petiole, at the base of which the sporocarps containing spores are located. Its life cycle is characterized by alternation of generations; reproduction occurs either sexually or by vegetative propagation. The species grows in wet habitats containing shallow water. In the natural environment this includes lakes and small rivers; in agricultural areas it can be found in ditches and rice fields. The species can tolerate nutrient rich waters and because of its phytoremediation properties is capable of partially counteracting the negative effects induced by a moderate organic enrichment of sediments. It has been harvested for centuries in Asian countries as both a food source and for ethnobotanical use in Ayurvedic medicine. Supposed medicinal properties include antibacterial, diuretic, depurative, cytotoxic and antioxidant effects, but these require further investigation and testing.

M. quadrifolia has a widespread distribution, occurring throughout central-southern Europe and extending from Eurasia to tropical and temperate areas of eastern Asia and North America, where it is considered a non-native species. Despite its wide distribution, in its home range the species is threatened with extinction and has already been locally extirpated in several European countries. As a result, it is listed as “Vulnerable” in the European Union Red List due to its scattered distribution and declining population. Habitat loss and degradation, excessive water eutrophication, and agricultural practices such as the use of herbicides, mechanization and simplified rotation are the main threats to the species.

As it is listed in Appendix I of the Bern Convention and in Annexes II and IV of Directive 92/43/EEC as a strictly protected species, *in situ* and *ex situ* conservation activities have been conducted in most European countries. Reintroduction, cultivation in botanical gardens and in vitro propagation are the most commonly applied conservation methods.

Keywords Marsileaceae · leptosporangiate fern · plant conservation · water management · ethnobotanical uses

1. Taxonomy and morphology

1.1 Taxonomy and evolution

Systematic position. Pteridophyta
Division. Pteridophyta
Class. Polypodiopsida
Order. Salviniales (Marsileales)
Family. Marsileaceae
Genus. *Marsilea*
Species. *Marsilea quadrifolia* L.

Marsilea quadrifolia L. Sp. Pl. 2: 1099 (1753), commonly named European water clover but also four-leaf clover, water shamrock and pepperwort, is an aquatic fern with creeping, fleshy, adventitious roots containing multiple rhizomes (Benson et al., 2004, Figure 1). The species owes its name to Carl Linnaeus (1753) who named it in honor of the Italian botanist and naturalist Luigi Ferdinando Marsili (1658-1730), founder of the "Istituto delle Scienze di Bologna". The epithet *quadrifolia* (lat. '*quadrifolius*, *-a*, *-um*', *quadri* = four, *folium* = leaves) indicates the plant has four leaflets resembling clover leaves.

Ferns (*Polypodiopsida*) are one of the oldest groups of plants on Earth, with fossil records dating back to the middle Devonian period (383-393 million years ago) (Smith et al., 2006; Taylor et al., 2009). They are the second most diverse group of vascular plants, with ~10,600 species (PPG I, 2016). Among extant ferns, the vast majority belong to the subclass Polypodiidae, leptosporangiate ferns (sporangia arise from a single epidermal cell rather than from a cell group as in eusporangiate), whose success has been associated with the evolution of epiphytism (Lehtonen et al., 2017).

Most ferns are homosporous but the aquatic ferns in the order *Salviniales* Link are characterized by heterospory, which is defined as "the most iterative key innovation" in plant evolution (Bateman & DiMichele, 1994) as it led to the evolution of seeds. These ferns produce microspores and a few megaspores, developing male and female gametophytes respectively. The order *Salviniales* includes two families, *Salviniaceae* Martinov and *Marsileaceae* Mirb. (Nagalingum et al., 2008), with the latter being unique among ferns for the production of heterospores within long-persistent structures called sporocarps.

The *Marsileaceae* family includes three genera with an estimated 61 species (PPG I, 2016) and are poorly represented in the fossil record (Pryer, 1999). *Marsilea* L. (Sp. Pl. 2: 1099. 1753) has an estimated 55 species and is one of the oldest genera known from the Dakota Formation (early Cenomanian period, ~100 million years ago) of Kansas (Skog & Dilcher, 1992). *Pilularia* L. (Sp. Pl. 2: 1100. 1753) has 5 species with cosmopolitan distribution whereas the monotypic *Regnellidium* Lindm. (Ark. Bot. 3(6):2. 1904) is endemic to southeastern Brazil and Argentina and, like *Pilularia*, has been known to exist since the Eocene period (~40 million years ago) (Collinson, 1991).

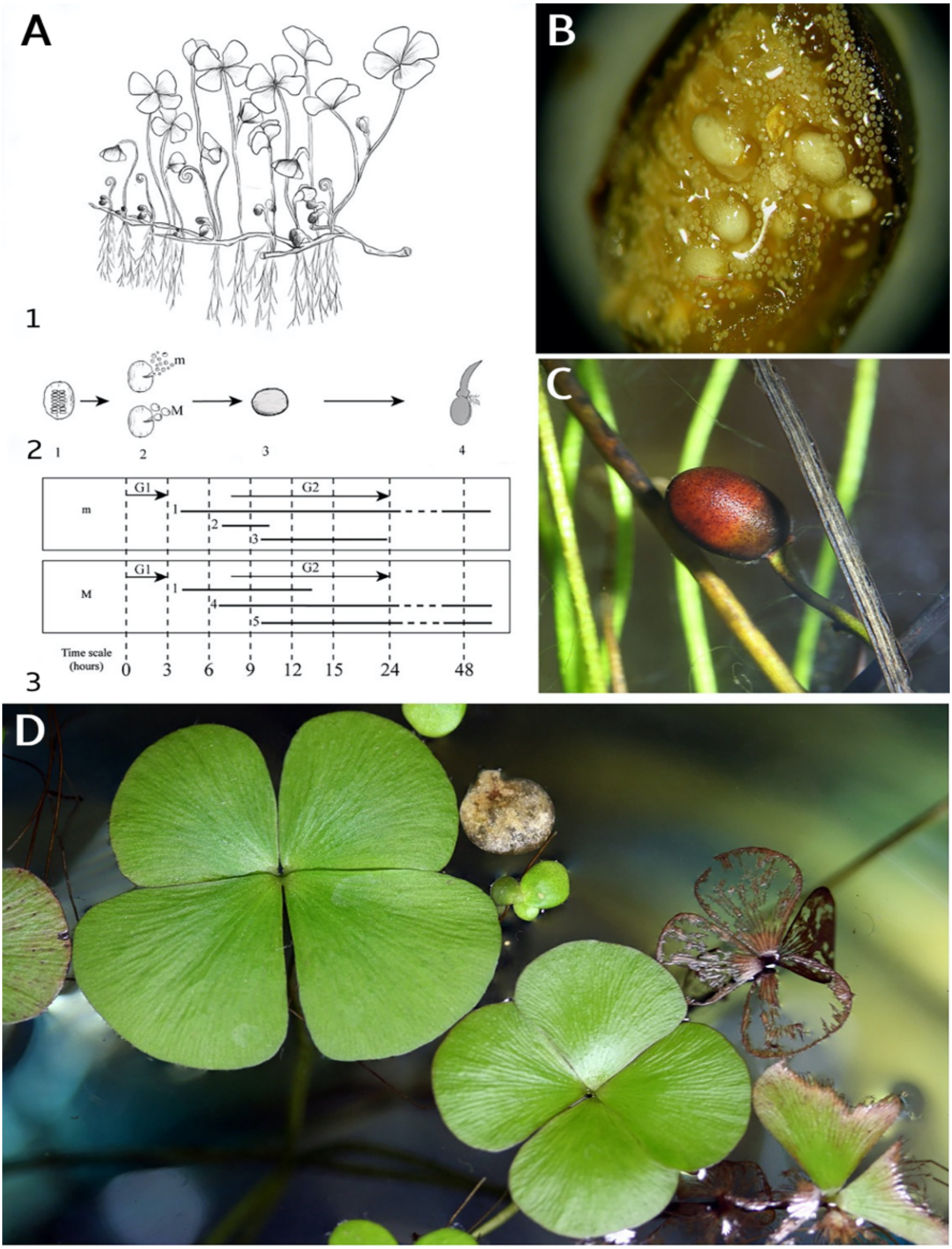

Figure 1. *Marsilea quadrifolia L. A) (1) Species illustration by Elena Bracchi. (2) Reproductive biology of the species: 1) vertical section of sporocarp; 2) release of microspores (m) and megaspore (M) from the sporangia; 3) fertilization; 4) formation of embryo sporophyte. (3) Sexual reproduction time points starting with the time of spore release (time 0 h) and ending with embryo formation (48 h): 1) spores floating at water surface; 2) mature male gametophyte; 3) free-swimming sperm cells; 4 fully developed sperm lake; 5) mature female gametophyte. G1 = gelatinous layer expanding; G2 = gelatinous layer disintegrating. B) Sporocarp with microspores and macrospores (from Bassanini, 2008, unpublished data, Bachelor Thesis). C) Sporocarp. D) Leaves of the species (photograph by Thomas Abeli).*

The genus *Marsilea* is one of the most widely distributed among ferns, with the greatest diversity being found in Africa. It has a cosmopolitan distribution, although it is uncommon in cool-temperate regions and oceanic islands (Nagalingum et al., 2007). Plants can be either true aquatic or semi-aquatic: the former are characterized by glabrous leaves and fleshy rhizomes and colonize permanent water bodies; the latter have hairy leaves and tough, fibrous rhizomes and prefer seasonal wetlands (Jacono & Johnson, 2006).

Understanding the relationship within the genus is highly problematic due to morphological plasticity, with wetness, leaf size and shape, hairiness, petiole length and trichomes making consistent identification and classification difficult (Johnson, 1986). Consequently, most taxonomic characters are found on the sporocarp or, for a higher degree of accuracy, using molecular data.

The first systematic analysis of the *Marsilea* genus dates back to almost 150 years ago, when Braun recognized 13 groups of species (Braun, 1871). Later, taxonomic studies were undertaken on a regional basis, examining species from Africa, Australia, India and the New World (Nagalingum et al., 2007), but the first phylogeny of Marsileaceous ferns was not published until 1999 (Pryer, 1999). Based on DNA sequencing data, the phylogeny of *Marsilea* has a basal dichotomy with two robustly supported groups. According to Nagalingum et al. (2007), *Marsilea quadrifolia* belongs to Group II, the most geographically spread and diverse subgroup, informally named “Marsilea”. A revisited analysis using four plastid regions from 223 accessions divided the two Groups into 12 Clades, with *M. quadrifolia* listed in Clade F (Whitten et al., 2012).

In contrast with the plasticity of leaf characters, sporocarp characteristics are important for species delimitation. These include the number of sporocarps attached to a petiole, the attachment point of the sporocarp and stalk, the number of sori, and the number of mega- and micro-sporangia per sorus (Nagalingum et al., 2007). Sporocarps are therefore taxonomically useful because they are generally consistent across varying environmental conditions. However, the over-reliance on sporocarp characters for plant identification is quickly realized when sterile specimens are encountered (Nagalingum et al., 2007).

1.2 Morphology and anatomy

1.2.1 Rhizome

M. quadrifolia has a creeping, cylindrical rhizome, 0.5-1 m in length, which is rooted at the nodes and anchored to the ground. The rhizome is partly or completely subterranean and is capable of indefinite growth. It is dichotomously branched either axillary or laterally and distinguished into short nodes and long internodes. Transversally, the rhizome is characterized by epidermis, cortex and central stele. The epidermis is a continuous layer of thickly cuticularized parenchymatous cells without any stomata. The cortex is divided into three regions: the outer part is composed of compact, parenchymatous tissue and may be one to several cells thick; the middle cortex has large lacunae with aerenchymatous tissue; and the inner cortex has thick-walled sclerenchymatous cells (fibres).

Beneath the cortex there is compact parenchymatous tissue which acts as storage, containing starch (Bercu, 2004) and, occasionally, tannin cells (Bendre and Kumar, 2010). The central stele is an amphiphloic solenostele i.e., the center is occupied by pith, with xylem surrounded on both sides by rings of phloem (Bercu, 2004).

1.2.2 Roots

One or more adventitious roots develop at each node on the underside of the rhizome, but they can also emerge from the internodal region (Bendre & Kumar, 2010). Primary roots are short-lived and replaced by adventitious roots developing in acropetal succession, i.e., the youngest root near the growing tip of the rhizomes. As for the rhizome, roots are characterized transversally by epidermis, cortex, and stele. The epidermis shows the same structure of the rhizome. In contrast with rhizome morphology, the outer layer is aerenchymatous, the middle layer is parenchymatous, whereas the inner layer is thick-walled, sclerenchymatous, internally bound by successive layers of endodermis and pericycle. The stele is bound by an endodermis and pericycle. The xylem consists of two large metaxylem tracheids and two protoxylem tracheids and may be exarch or diarch. Although highly characteristic of flowering plants, the roots of *M. quadrifolia* show vessels (Schneider & Carlquist, 2000), an example of parallel evolution in vascular tissues.

1.2.3 Leaves and petiole

Leaves experience circadian and phototropic movements (Kao & Lin, 2010), with leaflets able to adjust their angle and azimuth in response to changes in the position of the sunlight. Leaflets exhibit diaphototropic movements (orientation of the lamina is perpendicular to incident light) in the morning and late afternoon, and paraphototropic movements (orientation of the lamina is parallel to incident light) at noon.

Leaves develop heterophylly in order to withstand moisture constraints (Wu & Kao, 2011). In submerged plants, leaflet margins are entire, with long and flexible petiole floating over the surface of the water, whereas in emergent plants, leaflets are crenate, with short and rigid petiole (Johnson, 1986). The species displays the C3 photosynthetic pathway (Lin et al., 2007). Stomata of *M. quadrifolia* are smaller ($101\ \mu m^2 \pm 6\ \mu m^2$) than other ferns and have the smallest width to length ratio (K*übarsepp et al., 2020).* Aquatic leaves are epistomatic, i.e., stomata lie on the upper epidermis, whereas terrestrial leaves are amphistomatic, i.e., stomata are equally distributed on both surfaces. In these leaves, trichomes are on the adaxial and abaxial surfaces (Wu & Kao, 2011) in order to reduce water loss and to reflect light to help protect the plant against the damaging effects of photoinhibition (Wu & Kao, 2009).

1.2.4 Sporocarps

The plants have 1-12 mm stalks called peduncle (or pedicel) that are dichotomously branched, bearing two to five sporocarps (Figure 1C). Sporocarps are thick, oval, glabrescent, and bean shaped (3-5 mm in diameter), with small basal teeth and bisporangiate (Benson et al., 2004).

The sporocarp originate early in the ontogeny of the leaf, long before the appearance of the lamina. A young sporocarp is characterized by the tip developing horizontally. This then enlarges, creating two rows of soral mother cells on the ventral side that give rise to two rows of clusters of sporangia (*sori*). With this development, the marginal cells withdraw and form soral canals which are lined on the inner face of a tissue called *inducium*. Each sorus has a band of fertile tissue (receptacle) where sporangial initials arise (Bendre & Kumar, 2010).

As the species is heterosporous, each sorus produces micro- and mega-sporangia. Microsporangia develop from the sides whereas megasporangia develop from the top of the fertile tissue. Early stages of development are similar, with differentiation only at the spore mother cell stage. Initially, the number of spores is the same in both micro and mega-sporangia. Then, in mega-sporangium all spores except one disintegrate. The surviving megaspore enlarges and almost fills the sporangial cavity. In micro-sporangium all the spores are functional. Mature sporocarps are composed of up to two valves, with a thick wall including a gelatinous ring that extends into the sporocarp cavity. Sori (from 2 to 20) are arranged parallel to the pedicel in two rows. Each sorus is encased in a thin *inducium* and has a row of mega-sporangia on top and two rows of microsporangia towards the sides (Bendre & Kumar, 2010).

Due to the controversial nature of the morphological structure of sporocarps, two main hypotheses on their development have been suggested:

1. Laminar hypothesis or leaf segment theory**:** based on the vascular supply to the peduncle as well as within the sporocarp, this structure is considered to be a modification of a leaf segment rather than an entire leaf.
2. Petiolar hypothesis or whole leaf theory (Johnson, 1933): the sporocarp is the modification of the whole leaf (lamina and petiole) where the marginal cells develop into sporangia instead of leaflets.

Unlike the related Salviniaceae, where sporocarps occur on submerged leaves, in the Marsileaceae family they are derived from a highly modified sorus (Taylor et al., 2009).

2. Biology and phenology

2.1 Life cycle

The life cycle of *M. quadrifolia* is characterized by the alternation of generations, i.e., alternate succession of haploid gametophyte and diploid sporophyte (Johnson, 1986). More often, however, vegetative propagation through the division of rhizomes occurs.

2.2 Reproduction and development

Vegetative propagation occurs by means of rhizomes. Sexual reproduction takes place via sporocarps and the release of micro- and megaspores (Schneider & Pryer, 2002, Figure 1B). Gametophyte generation is completed within 24 hours, and the first roots and shoots appear within three days (Figure 1A). Spore germination occurs between June and September (Marchetti, 2004) after the rupture of the sporocarp wall, a structure that allows the sporocarp contents to be hydrated.

Sexual reproduction occurs in the water and the release of spores, fertilization and development of the embryo takes approximately 48 hours (Schneider & Pryer, 2002). Within three months a young sporophyte develops into a mature plant producing sporocarps (Johnson, 1986).

2.2.1 Dehiscence of the sporocarp

The sporocarp wall is very thick and, unless injured by external agents, it may remain closed for a long time. As soon as an injury occurs, the sporocarp absorbs water and splits open along the ventral suture. The gelatinous ring protrudes (G1, Figure 1A3) and breaks, extending out like a long gelatinous band that carries the sori in the sorophore (G2, Figure 1A3). The sori then break their connection with the sorophore causing the sporangia and sporangial wall to rupture and release the spores (Reddy, 2001).

2.2.2 Gametophyte

In Marsileaceae the gametophytes are endosporic, that is they develop enclosed, or almost so, in the spore wall. Microspores are globose (Ø 0.06-0.075 mm, Figure 2) with two wall layers and a nucleus surrounded by starch-rich cytoplasm. Germination is very rapid and is completed within 10-12 hours, with the release of antherozoids (Ferrarini et al., 1986). The megaspore is ellipsoidal to oval in shape (0.41×0.36 - 0.8×0.5 mm, Figure 2) with a small papilla protruding at one end. Externally it is characterized by the endospore followed by an inner layer of epispore, prismatic layer, outer epispores (where the nucleus is situated) and a storage part rich in starch, oil and albuminous granules (Reddy, 2001). As with the male gametophyte, the development is rapid and is completed within 12-15 hours.

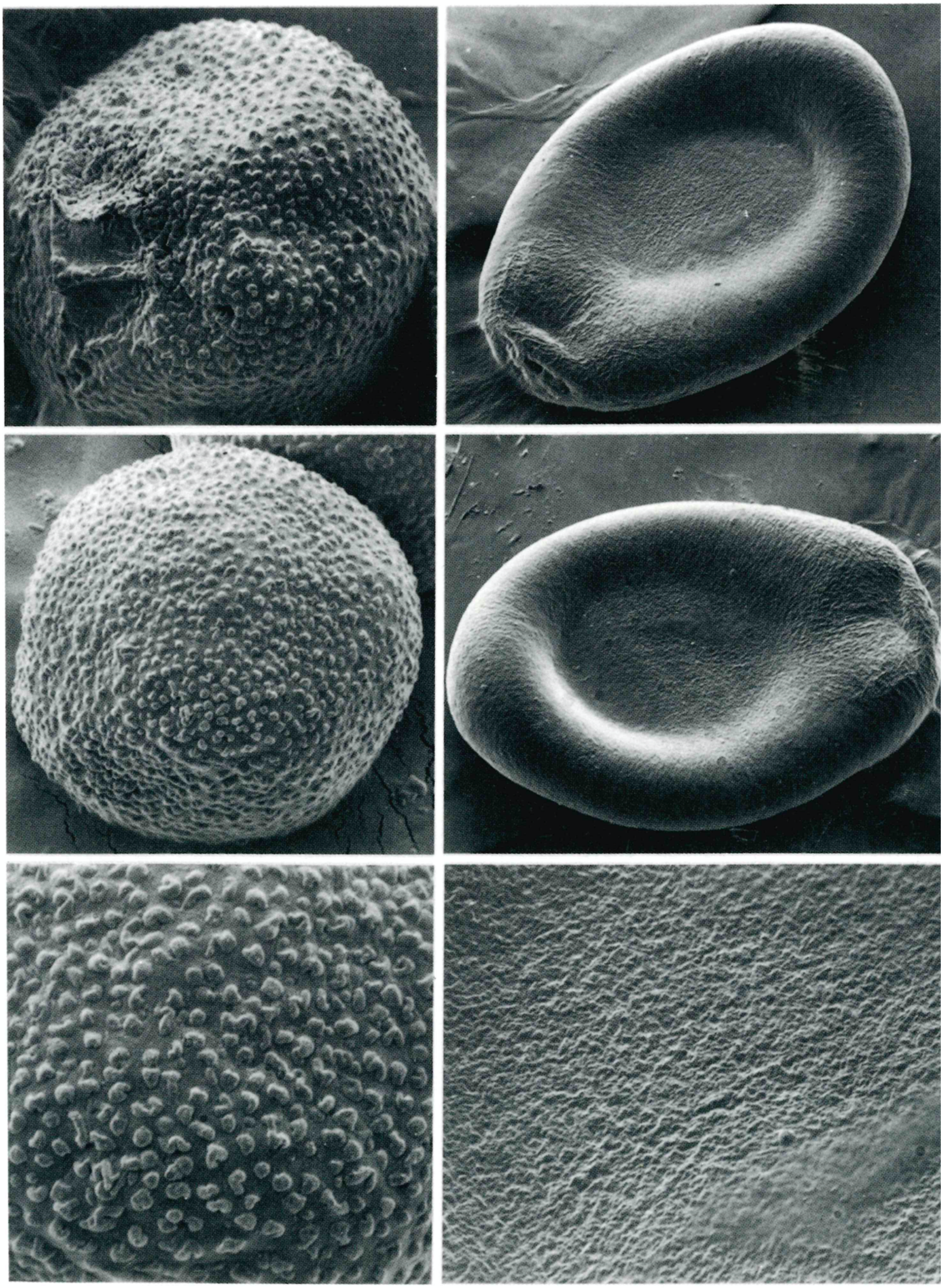

Figure 2. Microspore (left side) and macrospore (right side) detail on Marsilea quadrifolia (from Ferrarini et al., 1986; The use of this figure has been authorized by Webbia Editorial Office).

2.2.3 Development of the embryo

Similar to the development of gametophytes, embryogenesis is very rapid, with the first leaf germinating within 2-4 days after fertilization. The first division of the zygote is influenced by gravity and is parallel to the long axis of the archegonium. Since the female gametophyte is prone, the zygote forms an upper and a lower cell, with the former producing stems and leaves and the latter producing shoots and roots. Fertilization takes place at the air/water interface (Figure 1A2): the aquatic form of the sporophytes has smooth leaves and float in water, whereas the terrestrial form is anchored to the substrate (Reddy, 2001).

The species has a strong dependence on high moisture levels for sexual reproduction because it has flagellate gametes and external fertilization. However, sporocarps are highly resistant to desiccation and can “germinate” (the sporocarp wall ruptures and the spores are released) even after 100 years of dormancy (Gupta, 1962; Pistoja et al., 2006).

2.3 Species dispersal

Dispersal in *M. quadrifolia* is not clearly documented, although other congeneric species are clearly dispersed by waterfowl (Serviss and Peck, 2008). According to Johnson (1986), dispersal in *Marsilea* differs from most ferns because biotic rather than abiotic agents are involved. Wind dispersal is not possible because of both the size and weight of sporocarps, in addition to them being submerged. Conversely, hydrochory and zoochory play a key role, since reproduction exclusively occurs in the water (Strat, 2015). Sporocarps can be dispersed by aquatic birds as well as herbivorous mammals and fish (Benson et al., 2004), with dispersal occurring over several hundred kilometers (Holm et al., 1997).

2.4 Genetic data

M. quadrifolia (2n=40; Lesho, 1994) has tiny chromosomes ranging from 0.8 to 2.5 µm (Marcon et al., 2003) and a DNA C-value of 541 Mbp (Hanson and Leich, 2002).

Selected populations of the species (six from Italy and one from Switzerland) have been demonstrated to suffer from low genetic variability, with Nei’s gene diversity ranging from 0.025 to 0.036, 20.24% of overall polymorphic loci, and a mean percentage of polymorphic loci of 8.91 (Bruni et al., 2013). However, analysis of the distribution of genetic variation suggested that four populations were characterized by different genetic traits, which may characterize different conservation units. Similarly, the analysis conducted by Janiak et al. (2014) on three Polish populations from botanical gardens and four natural populations (two from Slovakia, one from France and one from Germany) showed a very low level of DNA polymorphism, both within and between populations, and overall, only 12.6% of AFLP loci were polymorphic. These values are very low when compared to the data available for other fern species.

3. Distribution and habitat requirements

3.1 Previous and current distribution

M. quadrifolia has a widespread global distribution: it is native to Eurasia and considered an alien species in North America (Benson et al., 2004). Populations have been reported in the following countries and regions (Strat, 2012; Hassler, 2020): Afghanistan, Albania, Austria, Azerbaijan, Bangladesh, Bhutan, Bosnia and Herzegovina, Bulgaria, Cambodia, Canada, China, Croatia, France, Georgia, Germany, Greece, Hungary, India, Iran, Italy, Japan, Kazakhstan, Malaysia, Moldova, Montenegro, Netherlands, North Caucasus (Russia), North Macedonia, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, South Korea, Spain, Sri Lanka, Switzerland, Tajikistan, Turkey, Ukraine, USA and Uzbekistan (Figure 3).

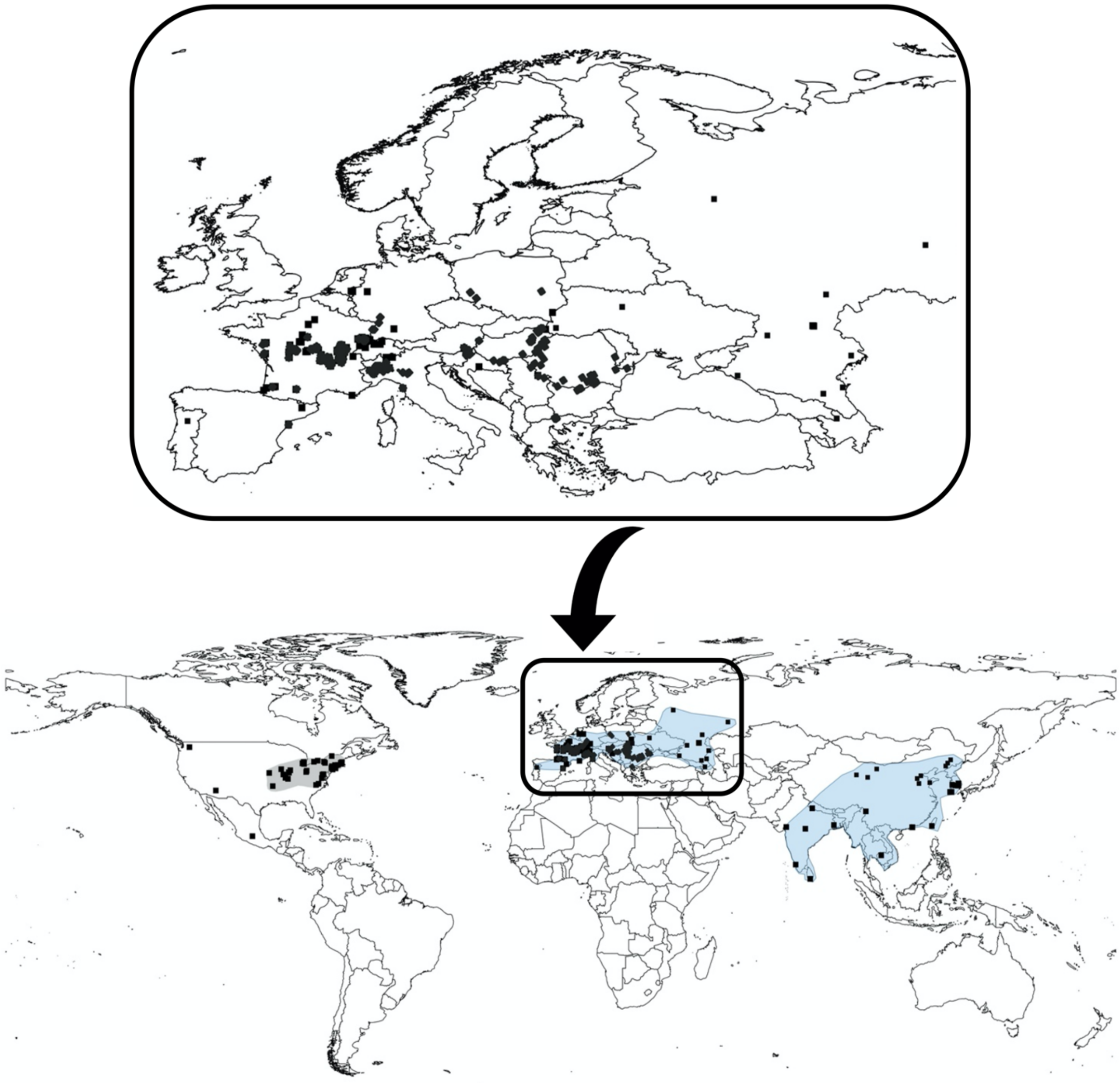

Figure 3. *Distribution of Marsilea quadrifolia over the past 20 years (2000-2020). Information are obtained from the following sources: National Red Lists, European Environmental Agency, GBIF.*

In Europe, *M. quadrifolia* has been reported in several localities listed in Table S1. Other congeneric species are also present in Europe: *M. strigosa* Willd. restricted to the Mediterranean basin; *M. batardae* Launert, endemic to the Iberian Peninsula; and *M. azorica* Launert & Paiva, endemic to the Azores. Although these species are clearly differentiated, *M. quadrifolia* may be erroneously confused with *M. strigosa.* The main differences are in the shape of the sporocarps and the hairiness of the leaves. *M. quadrifolia* is characterized by ellipsoid, not sulcate, pedicellate sporocarps and has glabrous leaves. Conversely, *M. strigosa* has more or less sessile obovoid sporocarps and is usually caespitose, with hairy or glabrous leaves (Tutin et al., 1964).

In the past, *M. quadrifolia* was quite common in its range and gained the name "pig pasture weed" (Poschlod et al., 2005). However, at the end of the 20th century it became rare in its natural European range as a consequence of habitat loss and use of herbicides (Bruni et al., 2013; Estrelles et al., 2001a, see also chapter 4).

In Asia (Figure 3), *M. quadrifolia* is considered to be a weed, especially in rice fields (Holm et al., 1997; Strat, 2012).

The species is spreading in North America, where it is an alien species with invasive behavior in some regions. It was first recorded in Connecticut in 1862 as an ornamental aquatic plant. Then, it spread throughout New England, Ontario, the Midwest to Ohio and Missouri, and south to Georgia, being documented in the following States: Arkansas, Connecticut, Delaware, Georgia, Illinois, Indiana, Iowa, Kansas, Kentucky, Maine, Maryland, Massachusetts, Michigan, Missouri, New Jersey, New York, North Carolina, Ohio, and Pennsylvania, and also in Ontario, Canada (USDA, 2018). Eradication campaigns are ongoing in North America.

3.2 Ecological requirements and ecophysiological characteristics

M. quadrifolia is a slightly eutrophic-rooted aquatic species, able to tolerate different nutrient levels (Schneider-Binder, 2014). Bolpagni and Pino (2017) found that growth peaked in slightly eutrophic waters, and persisted, though inhibited, under elevated nutrient levels. It does not tolerate high concentrations of nutrients and pollutants, even though it has been demonstrated to be able to remove nitrites from contaminated waters (Rawat et al., 2012). The species is ubiquitous in many soil types, from sandy to loamy soils that differ in pH value and organic matter richness (Husák & Oťahelová, 1986; Estrelles et al., 2001b). It prefers soft waters, frequently measured as deficient in calcium carbonate (Oberdorfer, 2001).

Original data from an Italian study reported that *M. quadrifolia* grows in soil with a slightly basic pH (8.12-8.25), with this finding also supported by a Spanish study (Estrelles et al., 2001b). Conversely, an experimental study in the Latorika River, Slovakia, showed that the species was able to grow under slightly acid conditions (Husák & Oťahelová, 1986). *M. quadrifolia* grows in soils with electrical conductivity of 290-372 µS, total phosphorous 78.72-160.76 ppm, phosphate concentration 99.38-150.43 ppm, ammonium concentration 0.37-1.30 ppm, nitrate concentration 1.0-1.4 ppm, total nitrogen 9.88-13.08 ppm, total carbon 92.86-130.87 ppm, calcium 42.97-54.99 ppm, and potassium 19.21-32.39 pm (Corli et al., unpublished data). In addition, the species has been found growing under the following environmental conditions: sand 41.72%, loam 40.01%, clay 18.27%, fine soil texture, organic matter 5.97%, CO_2^{3-} 22.04%, NO_{3-} 0.54 mgN/100g, NH_{4+} 0.67 mgN/100g, P 11 mgP/100g, and K^+ 23 mgK/100g (Estrelles et al., 2001b), but also under P 55 ppm, K^+ 290 ppm, and Mg 250 ppm (Husák & Oťahelová, 1986).

The plastic response of the leaves to the environment contributes to the ability of *M. quadrifolia* to occupy a continuum of habitats, ranging from terrestrial soils to continuously flooded substrates (Wu & Kao, 2011). The species grows best in areas where annual daytime temperatures are within the range of 20-28°C but it can tolerate temperatures between 14-32°C. When dormant, the plant can survive to temperatures of -15°C, but young shoots can be severely damaged at 0°C.

Experimental studies demonstrated that *M. quadrifolia* can tolerate concentrations up to 450 and 4 μM of ammonium and phosphate respectively in sediment pore water (Bolpagni & Pino, 2017) and low to medium salinity levels (Schneider-Binder, 2014 but also 0.76 mS/cm, Estrelles et al., 2001b). Optimal growth occurs at a water level that fluctuates within the limits of +/-0.12 m, although the species can tolerate depths > 0.5 m (Husák & Oťahelová, 1986).

Chlorophyll content has been evaluated as 0.189±0.004 μg cm^{-2}, with a photosaturated photosynthetic rate of 12.29±1.31 μmol $m^{-2}s^{-1}$ and stomatal conductance of 0.139±0.004 mol $m^{-2}s^{-1}$ (Wu & Kao, 2011). In addition, water use efficiency has been evaluated as 8.35±0.46 μmol CO_2 $mmol^{-1}H_2O$ (Wu & Kao, 2011), with chlorophyll fluorescence at 0.76±0.01 (Wu & Kao, 2009).

M. quadrifolia is a pioneer, heliophilous species. Light disturbances and open spaces caused by human intervention allows the species to establish without competition (e.g., in old pig pastures, Schneider-Binder, 2014). During the course of succession, the plant is quickly eliminated by competition with overshadowing species (Dehondt et al., 2005). However, a recent study in Italian rice fields (Corli et al., 2021) demonstrated that the species exhibits higher growth potential under rice canopy than in completely open areas where other weeds such as *Heteranthera reniformis* Ruiz & Pav., *Ammannia coccinea* Rottb. and *Cyperus microiria* Steud. limit the species growth.

3.3 Habitat and associated plant communities

M. quadrifolia habitats are mainly shallow permanent lakes, edges of ponds, small rivers with slow water flow, oxbow lakes, and flood channels. Man-made water bodies such as ditches, fishponds, rice fields, clay-pits, areas of gravel exploitation, and artificial lakes are also colonized by the species (Dehondt et al., 2005; Gentili et al., 2010; Schneider-Binder, 2014).

M. quadrifolia is found in plant communities belonging to the class *Littorelletea uniflorae* Braun-Blanq. & Tüxen, which includes floating or submerged aquatic vegetation, alliance *Eleocharition acicularis* Pietsch. It can be found alongside *Elatine triandra* Schkuhr, *Limosella aquatica* L., *Cyperus fuscus* L., *Juncus bulbosus* L. and *Lindernia procumbens* (Krock.) Philcox in communities of the alliance *Nanocyperion flavescentis* Koch ex Libbert 1932, or with *Utricularia australis* R.Br., *Trapa natans* L., *Myriophyllum spicatum* L. and *Phragmites australis* (Cav.) Trin. ex Steud. (Dehondt et al., 2005) in communities of the class *Potamogetonetea pectinati* Klika. Moreover, the species occurs with *Azolla filiculoides* Willd., *Salvinia natans* (L.) All., *Hydrocharis morsus-ranae* L., *Lemna trisulca* L. and *Lemna minor* L. in the class *Lemnetea minoris* Tuxen (Schneider-Binder, 2014). Under terrestrial conditions the species has been observed with *Juncus maritimus* L., *Limonium meyeri* (Boiss.) Kuntze, *Pulicaria dysenterica* (L.) Bernh., *Trifolium fragiferum* L., *Mentha pulegium* L., *Cynodon dactylon* (L.) Pers. and *Tamarix ramosissima* Ledeb. (Schneider-Binder, 2014).

According to the Habitat Directive 92/43/EEC, *M. quadrifolia* is present in the following habitats of community interest,: “Oligotrophic waters containing very few minerals of sandy plains (*Littorelletalia uniflorae*)” (code 3110); “Oligotrophic waters containing very few minerals generally on sandy soils of the West Mediterranean, with *Isoëtes* spp.” (code 3120); and “Oligotrophic to mesotrophic standing waters with vegetation of the *Littorelletea uniflorae* and/or of the *Isoëto-Nanojuncetea*” (code 3130).

4. Uses of the plant

4.1 Ethnobotanical uses

M. quadrifolia has been harvested for centuries for its ethnobotanical importance and as a source of food (Ripa et al., 2009). In India, it has been used in the local Ayurvedic medicine by the *Vaidyas* (people who practice Ayurveda) for thousands of years (Joy et al., 2019). In particular, it is used by the Indian tribal communities of Kadars, Pulaiyars, Malasars, Malaimalasars, Anamailais hills, and Western Ghats (Ramachandran, 2007). Here, the plant is called *Sunishannaka* and references can be traced back to the Vedic era (1500-1100 BCE). *M. quadrifolia* is popularly known as *Neeraral or Nalila thamara* in Kerala; its name *Sushni saag* in colloquial Bengali means “don’t sleep” (Soni & Singh, 2012). Ethnobotanical uses and suggestions from different tribes and communities are summarized in Table S2.

4.2 Food source and commercial value

As a food, *M. quadrifolia* has a relatively high nutritional value and is important for food security in times of drought or poor harvest (Tanaka, 1976). The species is rich in vitamins, minerals, trace elements, dietary fiber and proteins. Nitrogen and phosphorous content in the leaves have been assessed at 2.02-2.69% and 0.402-0.638% respectively (Corli et al., unpublished data). Chemical composition and nutritional values vary between seasons: dry matter ranges from 9.2 to 22.8% and crude protein content from 24.5-36.2%, with lower ash (7.5-15.8%) and mineral content compared to other plants. The plant contains a β-carotene range from 2.1 to 2.8 mg/100g; 0.7-1.4% (dry weight) Sodium; 1.1-2.8% Potassium; and an average of 0.5% Calcium and Phosphorus (Dewanji et al., 1993). In addition, the plant has higher amounts of thiamine, riboflavin, and vitamin C than other species (Jadhao & Wadekar, 2010).

The commercial value of the species is difficult to estimate as it is abundantly, and freely, available in the environment where is not endangered (i.e., Asia). In India and Laos, the leaves and petioles are sold in local markets. The species is also cultivated as an ornamental plant for aquaria and garden ponds.

4.3 Medicinal properties

Several experimental studies have been conducted to demonstrate the medicinal properties of *M. quadrifolia*. Anti-inflammatory properties (Vijayalakshmi et al., 2015) and antioxidant activity (Zahan et al., 2011) have been observed on methanolic extracts of the plant (Table 1).

Phytocompound	**Leaf (mg/g)**	**Stem (mg/g)**
Phenolic	8.34±0.92	7.31±0.46
Flavonoids	7.46±0.64	6.45±0.68
Alkaloids	6.12±0.51	5.89±0.61
Tannins	6.58±0.72	6.07±0.56
Saponins	5.32±0.48	6.30±0.58

Table 1. *Phytocompounds assessed in Marsilea quadrifolia leaf and stem (from Gopalakrishnan & Udayakumar, 2017).*

The species has demonstrated anti-stress potential. By testing various acute stress experimental models on mice (e.g., swimming endurance, anoxic tolerance and restraint stress test), Singh and Yadav (2014) noted that extracts of *M. quadrifolia* significantly reduced the immobility timing and increased the swimming endurance time, post motor function and clinic convulsion timing in anoxic tolerance tests as compared to a control group. The extracts also significantly reversed the behavioral and biochemical alterations in restraint stress. Moreover, a dose of 500 mg/kg produced reduced irritability, reactivity and grooming in mice (Tripathys & Madhuri, 2013).

M. quadrifolia has been shown to exhibit antidiabetic properties (Zahan et al., 2011). A dose of 300 mg/kg body weight supplied to rats reverted the level of the activities of hexokinase, pyruvate kinase and glucose- 6-phosphate dehydrogenase (Karikalan & Rajangam, 2018), showing a glucose level reduction of 47.57% in alloxan induced rats (Zahan et al., 2011).

Aerial parts of *M. quadrifolia* have shown antimalarial effects in vitro. Aqueous and methanol-aqueous extract (1:1) showed 70% and 67% cell growth respectively when the chloroquine-resistant *Plasmodium falciparum* strain FCR-3 was applied, and 84% and 89% cell growth respectively on mouse mammary FM3A cells served as a model host (le Tran et al., 2003).

Antimicrobial activity of leaf extracts has also been reported (Mathangi & Prabahakaran, 2012). 5 mg of both leaf and stem extracts showed highest antimicrobial activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* and *Streptococcus pyogenes*. Antifungal activity against *Aspergillus niger*, *A. flavus*, *A. terreus*, *Trichoderma viride* and *Fusarium solani* was clear when diethyl ether was used (Gopalakrishnan and Udayakumar, 2014). In addition, the species has been used to heal eye and skin diseases (Gopalakrishnan & Udayakumar, 2017) as well as to reduce body heat and thirst (Manjula & Mythili, 2012).

M. quadrifolia had a large impact on brain disorders and activity. Marsiline (an ester of 1-triacontanol and hexacosanoic acid) showed anti-epileptic efficacy (Snehunsu et al., 2013) and plant ethanolic extracts improved learning and memory in mice, also influenced by the antioxidant property and presence of steroids (Ashwini et al., 2012). The species has anti-cholinesterase potential (Bhadra et al., 2012) and since scopolamine inducing amnesia was reversed by *M. quadrifolia*, it is possible that the beneficial effect on learning and memory is due to facilitation of cholinergic transmission in the mouse brain (Ashwini et al., 2012).

4.4 Environmental uses

M. quadrifolia has an economic value for wetland restoration thanks to its phytoremediation properties (Miranda et al., 2014; Jiang et al., 2018). Specifically, the species is used for nutrient uptake from freshwater lakes (Khan and Shah, 2010) and for phytoremediation against heavy metals such as vanadium, arsenic, cadmium, chromium, mercury, and lead in the sediments and waters of mining areas (Jiang et al., 2018). In combination with *Cynodon dactylon* L. and other aquatic macrophytes (*Phragmites australis* and *Ceratophyllum demersum* L.), *M. quadrifolia* exhibited high uptake capacity of heavy metals, especially in the roots (Rai, 2009).

M. quadrifolia is also able to treat sewage and is the main bioagent in SHEFROL (SHEet Flow Root Level), a bioreactor capable of utilizing macrophytes with which sewage is given primary, secondary, and tertiary treatment in a single stage process. The species can treat the sewage with varying strengths in the chemical oxygen demand, from 600 to 1800 mg/L and to the extent of > 80% at a hydraulic retention time of just 4.5 h. In this way, a total removal of biological oxygen demand and suspended solids is achieved, and total Kjeldahl nitrogen, soluble phosphorous, and heavy metal zinc can be removed (Abbasi et al., 2018). In addition, dying or harvested plants that have been removed to reduce overgrowth where the species is invasive can be utilized in anaerobic digestion to obtain energy in the form of flammable CH_4-CO_2 (Abbasi & Abbasi, 2012). The plants can be also converted into organic fertilizer through vermicomposting (Hussain et al., 2016).

5. Conservation

5.1 Threats

M. quadrifolia is mainly threatened by human activities that result in habitat loss and degradation. Drainage of wetlands, management changes of fishponds, and agriculture intensification or abandonment are responsible for its population decline throughout Europe (Dehondt et al., 2005). Water eutrophication and pollution, and changes in agricultural practices such as the application of herbicides, are responsible for the species extirpation in Spain (Moreno et al., 2008), and the cause of a strong reduction in its range in Italy (Bruni et al., 2013; Rossi et al., 2016).

Since *M. quadrifolia* has been largely considered a weed, especially in rice fields in Europe (Bruni et al., 2013; Corli et al., 2021), several experimental studies have been conducted to understand the effect of herbicides (Aida et al., 2004; Luo & Ikeda, 2007; Bruni et al., 2013; Raj & Syriac, 2016; Kathiresan et al., 2019). The species demonstrated high sensitivity to a wide range of herbicides, even at low concentration, with different mechanisms of action (e.g., the interruption of fatty acid or pigment biosynthesis, the inhibition of enzymes involved in amino acid biosynthesis, and the disruption of photosynthesis; Table S3).

Vegetation succession and competition with alien species negatively influences the presence of *M. quadrifolia*. In Europe, it competes with alien plant species such as *Heteranthera reniformis* Ruiz et Pavon (Pistoja et al., 2006), *Eleocharis obtusa* (Willd.) Schult. (Gentili et al., 2010), and *Azolla filiculoides* Lam. (Strat, 2012). In addition, the species is competitively displaced by rush stands, reed (*Phragmites australis*), reed-mace (*Typha angustifolia* L.) and by floating vegetation, decreasing, or even disappearing in some locations. In addition, the monitoring of a reintroduced population in Germany showed there was a high level of competition between *M. quadrifolia* and *Potamogeton nodosus* Poir.

M. quadrifolia is one of the hosts of the North European aphid *Rhopalosiphum nymphaeae* Linnaeus (Albrecht, 2015) and the North polyphagous aphid *Myzus persicae* Sulzer. An association with mosquitoes of the *Culex vishnui* Theobald subgroups may also occur (Victor et al., 1991). The species is a food source for several animals: the leathery sporocarps are eaten by waterfowl and the leaves are grazed by pigs and cattle (Poschlod et al., 2005). In addition, the species is predated by the nutria (*Myocastor coypus* Molina) and the Louisiana crayfish (*Procambarus clarkii* Girald). Mycorrhizal fungi have been found in the roots, where vesicular colonization occurs with species belonging to the *Glomus* genera (Radhika & Rodrigues, 2007).

Even though new scattered and/or occasional growing sites of *M. quadrifolia* have recently been reported (Pistoja et al., 2006), its presence is extremely variable and it is vulnerable to water level fluctuations, overgrowth of other aquatic species, and agricultural practices.

5.2 Conservation status

M. quadrifolia is listed as “Least Concern” in the global IUCN Red List (Gupta, 2011). However, the distribution of the species in Europe is restricted and it is characterized by fragmented populations (Janiak et al., 2014). For this reason, the species has been previously assessed as “Near Threatened” (Bilz et al., 2011) and, more recently, as “Vulnerable” (García Criado et al., 2017) at the European level. The species is included in Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats/Bern Convention (1979) as a strictly protected species, and also in Annexes II and IV of the Habitat Directive 92/43/EEC among the species requiring designation of Special Areas of Conservation. The typical habitat of *M. quadrifolia* (code 3130 -

"oligotrophic to mesotrophic standing waters with vegetation of *Littorelletea uniflorae* and/or *Isoëto-Nanojuncetea*") is strictly protected under the Habitat Directive (Strat, 2015).

The species is listed as threatened with extinction in the National Red Lists of several European countries (Schneider-Binder, 2014; Table 2) and occurs in 106 Natura 2000 sites (European Environmental Agency, 2020). Protection measures at the local level are also in place, for example in Italy (Lombardy regional Law n. 10/2008).

In comparison, in southeast Asia, *M. quadrifolia* is considered to be an undesirable weed (Luo and Ikeda, 2007), especially in rice fields (Holm et al., 1997; Strat, 2012). The exception being in Japan (Environment Agency of Japan, 2000) where the species has been observed to coexist with rice in paddy fields that are managed using a traditional farming system with no pesticides or fertilizer; in contrast to conventional farming systems where the species is no longer found (Nemoto & Otsuka, 2014).

Red List Categories	RE	CR	EN	VU	NT	References
Albania			X			Vangjeli, 2017
Austria			X*			*"1r!" = Threatened with extinction. Differences at regional scale according to the National Red List (Niklfeld & Schratt-Ehrendorfer, 1999)
Bosnia-Herzegovina				X		Đug et al., 2013
Bulgaria		X				Ivanova & Tzonev, 2011
Carpathians				X		Witkowski et al., 2003
Croatia			X			Nikolić, 2005
France					X	UICN France, 2018
Germany	X					Metzing et al., 2018
Greece			X			Phitos et al., 2009
Hungary			X			Barina et al., 2007
Italy			X			Rossi et al., 2016
Japan				X		Environmental Agency of Japan, 2020
Lower Volga and Lower Don (Russia)			X			Shcherbakov & Lyubeznova, 2017
Moldova		X				Duca et al., 2015
North Caucasus (Russia)			X	X		Shcherbakov & Lyubeznova, 2017. The species is "VU" in Daghestan and "EN" in Krasnodar
North Macedonia			X			The National Red List of North Macedonia, 2019.
Poland	X					Kaźmierczakowa et al., 2016
Portugal		X				Carapeto et al., 2020
Romania			X			Oltean et al., 1994
Serbia		X*				Radulović et al., 2012. * National Conservation Status, Strictly Protected
Slovakia			X			Feráková et al., 2001
Slovenia			X*			*"V" according to the Rules on the inclusion of endangered plant species in the Red List (Official Gazette of the Republic of Slovenia, no. 82/02 and no. 42/10)
Spain	X					Moreno et al., 2008
Switzerland		X				Moser et al., 2002
Ukraine				X		Ukrainian Red Book of plants, 2020

Table 2. *Red List categories of Marsilea quadrifolia in countries and regions. RE = Regionally Extinct, CR = Critically Endangered, EN = Endangered, VU = Vulnerable, NT= Near Threatened.*

5.3 Conservation activities

In Europe, most populations of *M. quadrifolia* occur within protected areas such as Special Areas of Conservation (SAC), in application of the Habitat Directive. In countries where the species is locally extirpated or threatened, many efforts have been made to reintroduce it back into the wild. For example, reintroduction attempts have been conducted in the Delta of the Ebro River in Spain (Estrelles et al., 2001a), in the Po Plain in Italy (Orsenigo et al., 2016), in Switzerland (Nöel et al., 2011; Schneider-Binder, 2014), in Poland near Przemysl (Kaźmierczakowa et al., 2016) as well as in agroecosystems in Japan (Osawa et al., 2013).

The implementation and monitoring of reintroductions has been promoted by the EU LIFE funding scheme in Germany (LIFE04 NAT/DE/000025 “Rheinauen Karlsruhe - Living Rhine floodplain near Karlsruhe”), France (LIFE94 NAT/F/00854 “Management plan for the aquatic habitats of the Dombes”), Portugal (LIFE02 NAT/P/008480 “Mares temporaires - Conservation of Mediterranean temporary ponds”) and Italy (LIFE07 NAT/IT/000499 “Pianura parmense - Actions for the bird species of EU interest in the Natura 2000 sites in the lowlands of Parma (Italy)” and LIFE03 NAT/IT/000109 “LOMELLINA - Conservation of alder woods in Lomellina area's SIC”).

Ex situ conservation activities include *in vivo* (cultivation in botanical gardens) and *in vitro* (micropropagation and cryopreservation) methods (Strat, 2014). *M. quadrifolia* is currently hosted in 74 Botanic Gardens worldwide (BGCI, 2020). The main disadvantages of using *in vivo* conservation are the small size of *ex situ* populations, and clonal multiplication which reduces the genetic variation of *ex situ* stands.

In vitro propagation has largely been used to reduce the risk of extinction (Rolli et al., 2013).

Cryopreservation through the storage of spores at subzero temperatures, including under liquid nitrogen at -196°C, could be a valid tool to maintain the species (Ballesteros and Pence, 2018). Storage of spores allows the preservation of large quantities of germplasm with high genetic variation stored in small spaces (Ibars & Estrelles, 2012). Trials of cryopreservation in *M. quadrifolia* have been performed at the Botanical Garden of Warsaw (Poland) and Universidad Politécnica de Madrid (Spain) and tested at the University of Pavia (Italy, unpublished data). However, so far, no protocols have been developed for the long-term *ex situ* conservation of the species, even though other studies on ferns have reported successful spore conservation for *Cyathea australis* (R.Brown) Domin and *Osmunda regalis* L. respectively (Mikuła et al., 2009; Magrini & Scoppola, 2012).

Interestingly, the long viability of sporocarps is indicated by the return of the species to sites where it has not been seen for long periods of time. The reappearance, after decades, of *M. quadrifolia* as a result of the excavation of small ponds for amphibians has been reported in Italy and Switzerland and demonstrates the ability of the species to produce a soil bank of sporocarps. Sporocarps are highly resistant to drought and can “germinate” even after long periods, provided a suitable environment is re-established (Pistoja et al., 2006). Proposed management of *M. quadrifolia* cannot ignore the agricultural and semi-natural habitats in which the species grows. Experimental studies have demonstrated that the species can coexist with rice in organic paddy fields and is also able to survive under conventional farming with reduced herbicide use (Corli et al., 2021). The conservation of the species in an agricultural context could reconcile its ecological requirements with the needs of stakeholders such as farmers, landowners and local policymakers. An opportunity exists to combine nature conservation and agricultural production in areas such as the Po Plain, Italy, Spain and France. This may offer new sources of income for farmers through, for example, the specific funds provided by the EU Common Agricultural Policy (CAP) from the Rural Development Programme for those willing to contribute to the conservation of a vulnerable species.

6. Inspirations for further research

Several experimental studies have already been conducted in *M. quadrifolia*, including morphological, ecophysiological and medicinal studies. However, to gain further insight into *M. quadrifolia* conservation needs, it may be appropriate to focus on the secondary habitat of the species, i.e., rice paddies. In this context, information is lacking within the communities where *M. quadrifolia* grows, such as the relationship with other invasive weeds as well as with rice. Indeed, even though it has been reported that the species competes with the invasive *Heteranthera reniformis* (Pistoja et al., 2006), experimental studies addressing this topic are lacking. This is extremely relevant in Europe, where the species is threatened with extinction and its conservation is mandatory in accordance with the Habitat Directive. In addition, since the species is mainly restricted to agricultural areas, the collaboration of landowners and farmers is key for its conservation. The relationship between *M. quadrifolia* and rice should be investigated to understand if the former might be threatened by farming and whether rice yield is lowered when *M. quadrifolia* co-occurs in a field. The identification of potential benefits that the species may provide to farmers (e.g., ecosystem services, access to funds for biodiversity conservation, more effective marketing strategies) could provide a stimulus for their active collaboration in *M. quadrifolia* conservation.

Funding The Grant of Excellence Departments, MIUR-Italy (ARTICOLO 1, COMMI 314 – 337 LEGGE 232/2016) is gratefully acknowledged for the support to one of the authors (Prof. Thomas Abeli).

Acknowledgments The authors are grateful to Dr. A. Efremov (Omsk State Pedagogical University, Russia) for implementing data on species distribution in Asia. We would like to thank Fiona Jane White (University of Pavia) for revising the English.

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Supplementary Materials

Table S1*. European countries and related current and past localities of the species.*

Country	Current and past localities
Albania	Shkodra Lake and Small Prespa in northern Albania (Vangjeli, 2017)
Austria	Styria, Southeastern has been reconfirmed recently whereas the former sites in Carinthia, Upper Austria, and Burgenland are likely to be extinct (Magnes, 2016)
Bosnia Herzegovina	Neretva and Trebišnjica River Basin (Jasprica & Carić, 2002)
Bulgaria	Northeast part of the Danubian Plain, Valley of Struma River and Thracian Lowla (Ivanova & Tzonev, 2011)
Croatia	Upper Sava Valley and in Črnec Polje (Hulina, 1998)
France	Pays de la Loire (Nantes, Angers and La Roche-sur-Yon), Centre val De Loire (Chartres, Châteauroux, Bourges and Orléans), Auvergne Rhône-Alpes (Moulins, Clermont-Ferrand, Saint Étienne and Bourg-en-Bresse), Bourgogne-Franche-Comté (Mâcon, Côte-d'Or, Vesoul, Lons-le-Saunier and Belfort), Grand Est (Colmar), Nouvelle-Aquitaine (Poitiers and Mont-de-Marsan) (Tela Botanica, 2018). In the past, the species was also present southern and northern to the actual occurrences: in Rennes (Bretagne), Laval and Le Mans Pays de La Loire, Blois (Centre-Val de Loire) and Strasbourg (Grand Est) in the North France whereas occurred in Pau (Nouvelle-Aquitaine), Tarbes, Auch and Montpellier (Occitanie), Toulon and Nice (Provence-Alpes-Côte D'Azur) in the South (Tela Botanica, 2018).
Germany	Upper Rhine (reintroduced population; Schneider-Binder, 2014). In the past, the species occurred in the middle and northern Upper Rhine as well as in Karlsruhe
Greece	Lake Kerkini (Prefecture of Serres) (Dimopoulos et al., 2013)
Hungary	Between the Danube and the Tisza/Tisa rivers
Italy	Northern part (Lombardy, Piedmont, Emilia-Romagna, Veneto, Tuscany); species occurrence decreased in the last decades, with 25 populations attested (Gentili et al., 2010)
Montenegro	The species has never been reconfirmed (Petrović et al., 2008) despite numerous botanical investigations in Skadar Lake
Netherlands	Bank of the river Waal (Drok and Weeda, 1999) and brook in the vicinity of Venray (Bremer, 2007)
North Macedonia	The four populations recorded in Novaci, Dojran, Kochani and Monospitovo Swamp were never confirmed in the last ten years (The National Red List of North Macedonia, 2019)
Moldova	The species has never been reconfirmed recently (Duca et al., 2015) despite the past occurrence in the district of Ștefan-Vodă (Emerald Network)
Poland	Kuźnia Rybnicka and Wisła Wielka on the site of Goczałkowice Lake (Rostański,1976) and Lower Silesia (Puławy and Bolestraszyce) (reintroduced population; Janiak et al., 2014)
Portugal	Duero valley (Moreno-Saiz et al., 2015)
Romania	Sulina, Gârla Magearu, Canalul Rusca, and Sfântu Gheorghe villages (Strat, 2012)
Serbia	Low riverbanks and Moravica canals (Ljevnaić et al., 2016)
Slovakia	Laborec and Uh rivers in the area of Bodrog (Botanix, 2012)
Slovenia	Podvinci site and in Savinja Valley (Martinčič, 2010)
Spain	Wetland of the Ebro's Delta (reintroduced population; Estrelles et al., 2001; Aedo et al., 2012). In the past was largely present in the area
Switzerland	Les Grangettes (mouth of the Rhône in Lake Geneva), Fribourg, Bernese, Ajoie in Bonfol, Miécourt and Vendlincourt (Käsermann and Moser, 1999). The species was introduced several times into the ponds of Bonfol, but without lasting success. In all other localities known from older data, the species has disappeared (Schneider-Binder, 2014)
Ukraine	Transcarpathia (Latorytsia River Basin), west Polissya (Shatsky lakes), Dniester river watershed (Zalishchyky, Ternopil region) and Danube lakes (Ukrainian Red Book of plants, 2020)

Table S2. *Main recipes to heal diseases and for culinary reasons in the Indian traditional medicine. * Bangladesh. ** China tradition.*

Plant part used	Uses	Preparation
Whole plant	Lactogen	Create a paste with crushed *Centella asiatica* and apply around the nipple twice daily for 7 days (Shahidullah et al., 2009)
Whole plant	Heal peptic ulcer	Mix 20 g with milk and take twice daily up to 3 weeks (Nazar et al., 2008)
Leaves	Febrifuge	Cook and take twice daily for 2 days (Shanmugam et al., 2012)
Leaves	Heal rheumatism	Squeeze and apply to tendons twice daily for 2-3 days (Hossan et al., 2009)*
Petiole, leaves	Reduce hypertension, sleep disorders and headache	Cook with oil, salt and masalas (Soni & Singh, 2012)
Petiole, leaves	Soporific effect, physical and mental relax. Reduce hypertension and other nervous disorders	Cook 250 g with 50 ml warm mustard oil and garlic (2-5 g), cover with bamboo basket with seasoning 4-5 g desi masala (red chilies, turmeric, coriander and zeera) and salt. Consume in the evening (Soni & Singh, 2012)
Whole plant	Heal cough and convulsive condition of leg and muscles	Mix juice with garlic (Soni & Singh, 2012)
Fresh shoots	Heal cough and respiratory troubles	Create juice (Soni & Singh, 2012)
Whole plant	Relieve sleep disorder and hypertension	Create juice or paste to apply on the head (Soni & Singh, 2012)
Whole fresh plant	Antiepileptic	Mix 10 g with 100 g of curd from black cow’s milk. Take orally once daily in empty stomach for one month (Soni & Singh, 2012)
Young leaves	Anti-migraine	Crush two drops in the nostrils twice daily (Soni & Singh, 2012)
Whole plant	Heal infantile diarrhea	Crush with sugar candy or honey (Sen & Behera, 2008)
Young stems, leaves	Famine food	Cook (Tanaka, 1976)
Spores	Culinary purpose	Grind spores and mix with bread flour (Cribb & Cribb, 1976)
Leaves	Treat snakebite	Create juice (Duke and Ayensu, 1985) **

Table S3*. Chemicals, doses and results of Marsilea quadrifolia exposure to herbicides in various experimental studies.*

Chemical	Substance group and mode action	Dose	Results
Bensulfuron-methyl: methyl a-[4,6-dimethoxypyrimi-din-2-ylcarbamoylsulfamoylmethyl]-o-toluate	Sulfonylurea. Selective pre-emergence and post-emergence herbicide. Inhibitor of acetolactate synthase.	0.75, 2.5, 7.5, 25 and 75 g/ha	Reduction in leaf leaf/internode growth and dry matter production after 20 days exposures. The highest doses caused plant necrosis (Aida et al., 2004)
Bensulfuron-methyl: methyl a-[4,6-dimethoxypyrimi-din-2-ylcarbamoylsulfamoylmethyl]-o-toluate	Sulfonylurea. Selective pre-emergence and post-emergence herbicide. Inhibitor of acetolactate synthase.	0.003, 0.03, 0.3, 3, and 30 mmol/L	Inhibition of the relative growth rate, with differences among herbicides. For bensulfuron-methyl and simetryn, even the lowest concentration inhibited the RGR during the 10 days treatment and the degree of inhibition increased with increasing herbicide concentrations. For mefenacet and thiobencarb, significant inhibition during treatment was observed at concentrations higher than that for bensulfuron-methyl and simetryn (Luo and Ikeda, 2007)
Mefenacet: 2-benzothiazol-2-yloxy-N-methylacetanilide	Oxyacetamide. Selective pre- and early post-emergence control of weeds. Inhibitor of cell division and growth.	0.006, 0.06, 0.6, 6, and 60 mmol/L	
Simetryn: N ,N -diethyl-6-methylthio-1,3,5-triazine-2,4-diamine	Triazine. Selective systemic used for broad-leaved weed and grass control. Inhibitor of photosynthesis (photosystem II).	0.004, 0.04, 0.4, 4, and 40 mmol/L	
Thiobencarb: S-4-chlorobenzyl diethyl[thiocarbamate]	Thiocarbamate. Selective pre-emergence and early post-emergence herbicide for weed control. Inhibitor of acetyl-CoA Carboxylase.	0.005, 0.05, 0.5, 5 and 50μmol/L	
Glyphosate: 2-[(phosphonomethyl) amino]acetic acid)	Phosphonoglycine. A broad-spectrum systemic herbicide. Inhibitor of 5-enolpyruvylshikimate-3-phosphate synthase.	10 mg/L	The species is sensitive to a wide range of herbicides that have different mechanisms of action and clear differences in the per cent survival. Gliphosate: TQ = 38%; T1 = 80%; T2 = 96%; Clincher: TQ = 2.3%; T1 = 24%; T2 = 58%; Aura: TQ = 2.3%; T1 = 18%; T2 = 36%; Viper: TQ = 4.7%; T1 = 40%; T2 = 63%; Command: TQ = 35%; T1 = 85%; T2 = 76%; Aura + Dash: TQ = 0%; T1 = 4,7%; T2 = 15%; Gulliver: TQ = 47%; T1 = 78%; T2 = 82%; Most Micro: TQ = 76%; T1 = 80%; T2 = 80%. *herbicides tested at TQ concentration, T1 1:100 and T2 1:1000 (Bruni et al., 2013)
Clincher. 100 g contain: Cyhalofop-butile 20,92% (200 g/l), coformulants and solvents, Hydrocarbons, C10, aromatics, <1% naphthalene Hydrocarbons, C10-C13, aromatics, <1% naphthalene	Aryloxyphenoxypropionate. Systemic post-emergence herbicide for the control of grassy weeds.	0.5 mg/L	
Aura: 200 g/L profoxydim (active constituent) + 743 g/L liquid hydrocarbon (solvent)	Ciclohexenone. Selective post-emergence control of barnyard grasses (*Echinochloa* spp.) and silvertop grass (*Leptochloa fusca*). Inhibitor of acetyl coA carboxylase.	10mg/L	
Viper. 100 g contain: 10g Penoxsulam 2.14% (= 20 g/L) + coformulants, 1,2-benzisothiazol-3-(2H)-one	Sulfonamide and Triazolopyrimidine. Selective post-emergence control of weeds. Inhibitor of AcetoLactate Synthetase.	20 ml/L	
Command. 100 g contain pure Clomazone + coformulants 30.74 g (=360 g/L)	Iso-oxazolidinone. Selective pre and post-emergence control of weeds. Inhibitor of chlorophyll and carotenoids synthesis.	0.5 mg/L	
Aura + Dash (adjuvant property. 100 g contain: methyl oleate and methyl	Ciclohexenone. Selective post-emergence control of barnyard grasses (*Echinochloa* spp.) and	20 mg/L	

palmitate 37.5 g (348.75 g/l), coformulants, solvent naphtha, oxirane, methylpolymer with oxirane, mono C10-16-alkyl ethers, phosphates)	silvertop grass (*Leptochloa fusca*). Inhibitor of acetyl coA carboxylase.		
Gulliver. 100 g contain: pure Azimsulfuron 50g + coformulants	Sulfonylurea. Selective early post emergence control of aquatic broadleaf and sedge weeds. Inhibitor of AcetoLactate Synthetase.	15 mg/L	
Most Micro: pure Pendimetalin 31.7 g(=365 g/l) + coformulants, solvents)	Dinitroaniline. Selective pre-emergence control of weeds. Inhibitor of germination of seeds and development of the small buds of the most important annual weeds.	20 mg/L	
Bispyribac sodium (Sodium 2,6-bis((4,6-dimethoxypyrimidin-2-yl)oxy)benzoate) + metamifop	Aryloxyphenoxypropionate. Selective post-emergence herbicide for the control of a wide range of weeds, in particular *Echinochloa crus-galli*. Inhibitor of the branched amino acid biosynthesis and, in addition with Metamifop, ACCase.	70, 80 and 90 g/ha	The herbicide reduced the density and dry weight of weeds. Application of bispyribac sodium+metamifop at 90 g/ha registered the highest weed control efficiency of 96.62%. Mortality: 93.66; 95.47; 96.62% respectively (Raj and Syriac, 2016)
Butachlor (N-(Butoxymethyl)-2-chloro-N-(2,6-diethylphenyl)acetamide)	Chloroacetamide. Selective pre-emergence herbicide. Inhibitor of germination processes.	1.5 kg/ha	The application of bensulfuron-methyl 0.06 + pretilachlor 0.6 kg/ha was significantly superior to the other treatments. Bensulfuron-methyl 0.06 + pretilachlor 0.6 Kg/ha offered effective control of weeds. Butachlor weed density: S1 = 8.7; S2 = 9.7; Bensufuron- methyl + pretilachlor weed density: S1 = 8.3; S2 = 1.1; Bxadiargyl weed density: S1 = 5.7; S2 = 4.2. * weed density (quadrat of 0.25 m^2) determined in two seasons (S1 and S2). (Kathiresan et al., 2019)
Bensufuron- methyl + pretilachlor: methyl a-[4,6-dimethoxypyrimi- din-2-ylcarbamoylsulfamoylmethyl]-o-toluate + 2-Chloro-N-(2,6-diethylphenyl)-N-(2-propoxyethyl)acetamide	Sulfonylurea + Chloroacetamide. Selective pre-emergence and post-emergence herbicide. Inhibitor of acetolactate synthase + Inhibitor of VLCFA (inhibition of cell division).	0.6 kg/ha	
Oxadiargyl (5-tert-butyl-3-[2,4-dichloro-5-(prop-2-ynyloxy)phenyl]-1,3,4-oxadiazol-2(3H)-one)	Oxidiazole. Pre-emergence oxadiazolone herbicide for grass and broad-leaved weed control. Inhibitor of protoporphyrinogen oxidase (PPO).	0.07 kg/ha	

Coexistence of rice production and threatened plant species: testing Marsilea quadrifolia L. in N-Italy

Corli, A., Orsenigo, S., Gerdol, R., Bocchi, S., Smolders, A.P., Brancaleoni, L., Caffi, M.T., Abeli, T., Rossi, G., 2021. Coexistence of rice production and threatened plant species: testing *Marsilea quadrifolia* L. in N-Italy. Paddy and Water Environment. (Published; https://doi.org/10.1007/s10333-021-00840-z)

Abstract

In the past, the aquatic pteridophyte *Marsilea quadrifolia* L. was considered as a weed in paddy fields of southern Europe. The systematic use of herbicides as a crucial component of intensive agronomic approach has led to a dramatic decline in *M. quadrifolia* populations in European countries, mostly in the Mediterranean area. However, the introduction in recent years of sustainable rice cultivation practices has allowed partial recovery. We present here the results of a research aimed at analyzing the effects of farming typologies in respect to *M. quadrifolia* growth in the Po Valley area. After having transplanted *M. quadrifolia* swards in rice fields belonging to different farming systems, we monitored its growth and diffusion. Our results showed that *M. quadrifolia* has higher vegetative performance in organic farms, even though it can survive in conventional fields with reduced herbicide supply. Differences in water chemistry have limited effects on *M. quadrifolia* performance, because of its wide ecological amplitude. The shady conditions provided by rice canopy create micro-habitats suitable for *M. quadrifolia* growth. Cultivating *M. quadrifolia* in organic rice farms represents an important opportunity for preserving this endangered species in areas of intensive agriculture. This can, meanwhile, represent a valid opportunity to combine nature conservation and productivity offering a new possible income for farmers.

Keywords agroecology · biodiversity · EU common agricultural policy · plant conservation · reintroduction

1. Introduction

Many plant and animal species associated with agricultural environments experienced severe population declines during the last century (Meyer et al., 2013; Arbeiter et al., 2018). Among these, four leaf clover [*Marsilea quadrifolia* L. (Marsileaceae)] can be considered as an emblematic species. This aquatic pteridophyte grows in slightly eutrophic waters, including river oxbows, temporary ponds and rice paddies (Gentili et al., 2010). In the past, for a long period, it was considered as a weed in paddy fields of southern Europe (Viggiani et al., 2003). European rice farmers, with the so-called Green Revolution starting from 1950s, modified rice agrotechniques by introducing new practices like mechanization, use of chemicals and simplified rotation (Ferrero & Vidotto, 2010; Hill et al., 1991). This led to a drastic decline of *M. quadrifolia* that is now considered in unfavorable conservation status in most European countries (EEA, 2019), including Italy (Rossi et al., 2016). Concerns about its conservation status led to the inclusion of *M. quadrifolia* in Annexes II and IV of the EU Directive 92/43/EC “Habitats” and in the Annex I of Bern Convention, the primary legal instruments driving species protection in Europe (Supplementary Material 1). Species and habitats listed in the Directive 92/43/EC require a strict protection and their long-term survival should be applied in the Natura 2000 network, a system of areas for the conservation of the flora and fauna stretching across all EU countries, through the establishment of Special Areas of Conservation (SAC). However, in highly anthropized regions SAC are in contact or included in areas of intensive agriculture.

In the Po Valley (Northern Italy), a region hosting more than 50% of the European rice production area with total production of 1,512,228 tons per year (Kraehmer et al., 2017; Enterisi, 2018), new spontaneous occurrences of *M. quadrifolia* were recently recorded in some rice farms. These new records are likely due to improved environmental conditions related to organic or low-input farming practices (Hazra et al., 2018) or changed management practices in conventional farms, as planned by the European Directive on the sustainable use of herbicides (Directive 2009/128/EC; Supplementary Material 1). Despite improved environmental conditions in rice farms, natural spreading of *M. quadrifolia* to pre-green revolution levels may be jeopardized by landscape fragmentation and limited to farms adopting agroecological principles and practices.

Reintroduction of *M. quadrifolia* in agricultural areas may represent an opportunity to improve the conservation status of the species but could create conflicts with farmers. Attempts to recover viable populations have been conducted in rice fields of the Ebro Delta Natural Park, Spain (Estrelles et al., 2001).

The EU Common Agricultural Policy (CAP) provides specific funds from the Rural Development Programme (Supplementary Material 1) for farmers willing to contribute to the conservation of habitats and species (Paracchini et al., 2015). Although these measures are considered not yet sufficient (Pe’er et al., 2020), in some cases they are very effective because they transform potential conflicts between nature conservation and productivity into new income chances for farmers. With the aim to stimulate the adoption of specific measures for the conservation of *M. quadrifolia* [and other species from similar habitats like *Isoetes malinverniana* Ces. & De Not. and *Lindernia procumbens* (Krocker) Philcox] in areas of intense agriculture, we explored the possibility of successfully growing *M. quadrifolia* 'on farm' in rice paddies. Specifically, we aimed to answer the following questions: (i) can environmental conditions in rice farms promote land sharing between agriculture activity and *Marsilea quadrifolia*? (ii) Is the performance of *M. quadrifolia* affected by the farming system typology? (iii) How *M. quadrifolia* is affected by the presence of rice plants? Answering those questions can help to set up and apply a conservation plan for *M. quadrifolia* in southern Europe.

2. Materials and methods

2.1 Farm description and cultivation techniques

We cultivated *M. quadrifolia* in four wet rice farms with differing farming systems in the province of Pavia (Po Valley, N-Italy): two organic farms, one conventional farm and a farm in transition from conventional to organic farming (hereafter called 'transitional'). According to the Council Regulation (EC) n. 834/2007 on organic production and labelling of organic products and following updates (Supplementary Material 1) and its transposition in the Italian Law (last update Ministerial Decree n. 3286/2016), organic farms are recognized and distinguished from conventional farms because they mainly adopt fertilization with natural fertilizer of animal origin or with organic matter and maintain/enhance soil fertility and biological activity through multi-annual crop rotation. More in general, organic agriculture involves complex systems, regulated by long-term biological processes (e.g., humus formation) and non-linear effects, then the effectiveness of an agricultural practice is site- and time- specific. On the other hand, conventional agriculture involves standardized techniques, applied in simplified and specialized cropping systems managed with a short-term approach and based on the fast action of external inputs (Duru et al., 2015; Orlando et al., 2020).Transitional farms are those that have been converted from conventional managing system but are still not recognized as organic farms. During the experimental cultivation of *M. quadrifolia*, farmers managed the fields with normal agro-techniques (as they usually do), including application of fertilizers and herbicides in the conventional farm. Herbicide treatments varied between years, depending on weeds (Table S1). The rice cultivars were Ronaldo in the organic farms, Baldo in the transitional one and Selenio in the conventional farm; the slightly differences between the three varieties did not affect significantly the performance of *Marsilea quadrifolia.*

2.2 Experimental cultivation of M. quadrifolia

The experimental cultivation of *M. quadrifolia* was conducted in 2017 in eight rice paddies, two in each farm, whereas in 2018 the cultivation was restricted to six rice paddies. Six 20 × 20 cm potted swards of *M. quadrifolia* were placed along a transect in each paddy field. Before the swards were placed in the fields, the shoots were cut to a height of ca. 1 cm to standardize the initial biomass. In 2017, *M. quadrifolia* swards were placed in early July after the first (pre-sowing) and the second (post-emergence) herbicide treatments in the conventional farm had been supplied in the conventional farm. In 2018, the swards were again placed in the field at the end of May just after rice sowing, so that *M. quadrifolia* was subjected to the whole spectrum of herbicide treatments. In 2018, once swards were placed in the field, rice was removed to create a 1 m^2 open area all around the swards, to evaluate the micro-environmental conditions that can affect growth of *M. quadrifolia* in paddy fields (sunny vs shaded). As *M. quadrifolia* spread well beyond the border of open areas under the rice canopy, the growth performance of *M. quadrifolia* was evaluated both in the open areas and under the rice canopy.

2.3 Data collection and statistical analyses

Total biomass of *M. quadrifolia* was collected at the end of September in both years in a 3-m radius circular area around each sward, oven-dried at 100 °C for 24 h and weighed in the laboratories of the University of Pavia. One soil sample and two water samples were collected in 2017 in each paddy field at the time of swards placement and analyzed by the Vassanelli Food and Drink analysis s.r.l., Bussolengo (Italy) and at the University of Nijmegen, respectively (Table S2). In 2018, temperature and irradiance were recorded at 15-min intervals by data loggers (Hobo, Onset Bourne, MA, USA) during the week preceding rice harvest (13th-20th September) in open areas and under rice canopy. The data loggers were placed 1-m above ground. At the beginning of September, chlorophyll fluorescence ($F_v\ F_m\ ^{-1}$) was determined in the field on *M. quadrifolia* with a modulated fluorometer (Opti Sciences, OS1-FL, Tyngsboro, MA, USA) both in open areas and under rice canopy. Leaf samples of *M. quadrifolia* were collected and used to determine spectrophotometrically (UV–Vis spectrophotometer, Pharmacia Biotech Ultrospec, 2000) the concentration of chlorophyll *a*, chlorophyll *b* and total carotenoids using the extinction coefficients of Lichtenthaler (1987). Since *M. quadrifolia* did not survive herbicide application in the conventional farm, only the organic farms and the transitional farm were considered. Data on biomass were log-transformed and statistically analyzed by one-way ANOVAs with farming system as explanatory variable and swards and fields as covariates. Comparison between farming systems was performed with Fisher's LSD post hoc test. Data on temperature, irradiance, chlorophyll fluorescence, pigment concentrations and pigment ratios were statistically analyzed by one-way ANOVAs with micro-environment (sunny vs shaded) as the explanatory variable. Stepwise multiple discriminant analysis was performed both for water and soil chemistry in relation to farming system. The statistical analyses were carried out with R v3.6.1 (R Core Team 2019) and STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1 Plant biomass, water chemistry and soil chemistry

Total biomass of *M. quadrifolia* was highest in organic farms both in 2017 and in 2018 (Figure 1). The ANOVAs revealed no significant differences in plant biomass among farming systems in 2017 ($F_{2,43}$=3.12; *p*=0.054), whereas differences were significant in 2018 ($F_{2,31}$=32.54; *p*<0.001). It is noteworthy the effects of covariates were not significantly different except in 2017 ($F_{1,43}$=8.11, *p*=0.007). In 2018, M. *quadrifolia* did not survive in the conventional farm. Discriminant analysis revealed differences among farming systems in terms of water chemistry ($F_{1,14}$=8.81, *p*<0.001; *Wilk's* Λ = 0.010). Water chemistry in the transitional farm differed strongly from both the conventional farm and the organic farms which mirrored in a sharp separation of the two groups across the first discriminant axis (Figure 2). Indeed, water in the transitional farm was richer in dissolved ions, especially HCO_3^-, NO_3^- and SO_4^{-2}, and thus presented higher electrical conductivity (Table S2). Conversely, soil chemistry did not differ significantly among farming systems ($F_{10,2}$=1.11, *p*<0.56; Wilk's Λ = 0.023; Table S2).

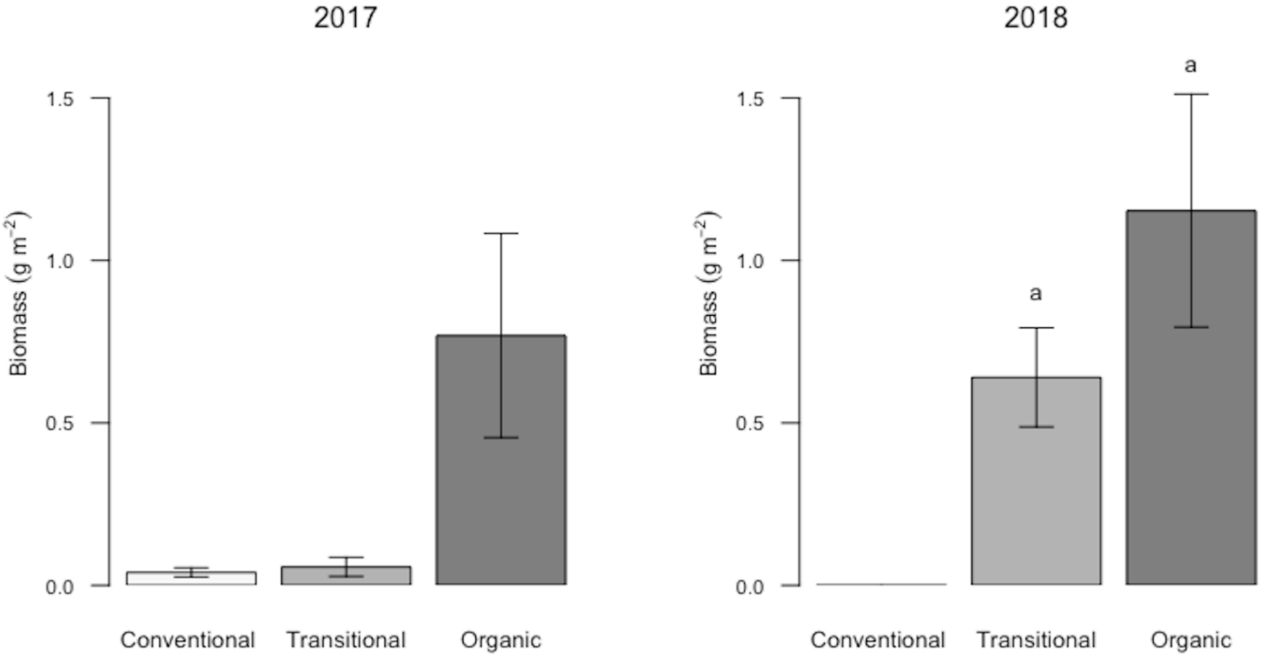

Figure 1. *Mean (+SE) total biomass of M. quadrifolia collected from different rice farming systems. Lowercase letters indicate significant (p < 0.05) differences between farming systems, according to post hoc Fisher's LSD tests.*

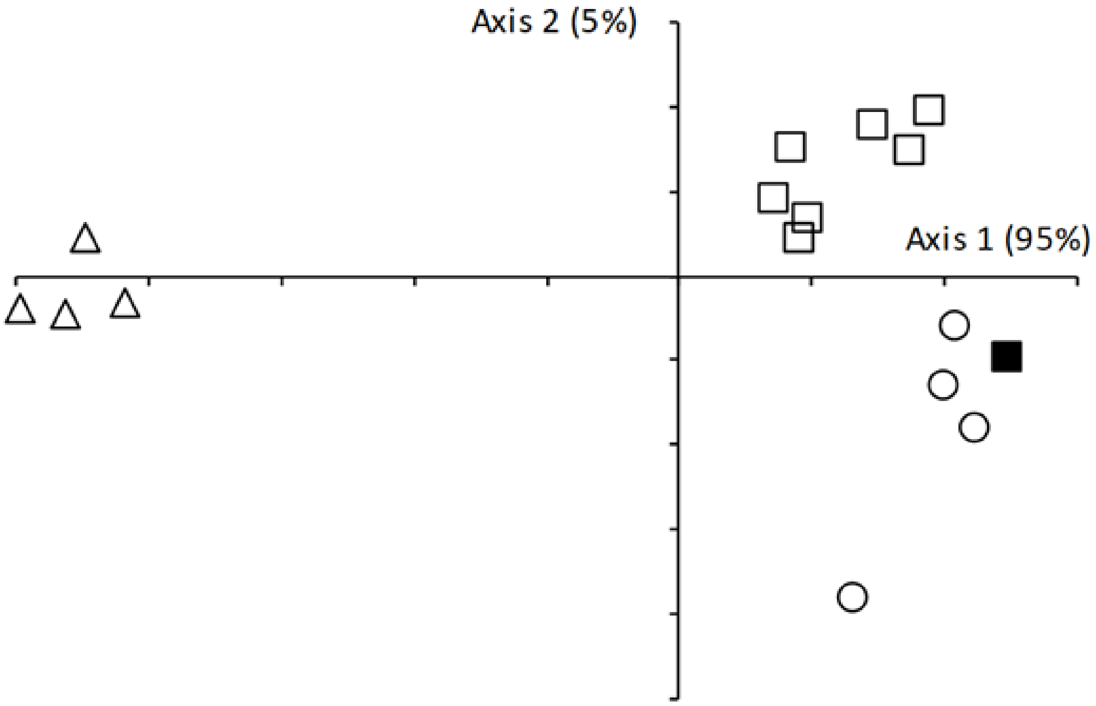

Figure 2. *Plot scores along the first two axes of stepwise multiple discriminant analysis of water chemistry data (the percentage of variance accounted for by each axis is in parenthesis. Circles: conventional farms. Triangles: transitional farms. Squares: organic farm (the black square indicates the only misclassified case).*

3.2 Micro-environment and photosynthetic efficiency

Temperature differed significantly between micro-environments ($F_{1,22}$=33.32; p<0.001) with higher mean temperature (23.70±0.17 °C) in open areas compared to under rice canopy areas (22.15±0.21 °C). Irradiance also differed significantly between micro-environments ($F_{1,22}$ =49.33; p<0.001), with more than double mean values in open areas (101,104±7621 kW) than under rice canopy (41,656±3681 kW). Chlorophyll fluorescence was significantly higher under rice canopy compared to open areas (Table 1). Photosynthetic pigment concentrations and pigment ratios did not differ between micro-environments (Table 1).

	Open areas	**Under rice canopy**
$F_v\ F_m\ ^{-1}$	**0.69±0.01**	**0.76±0.01 ****
Chlorophyll a+b (µg/g DW)	17.9±2.2	20.0±2.7
Carotenoids (µg/g DW)	4.1±0.5	3.8±0.4
Chlorophyll a / Chlorophyll b	3.02±0.05	2.91 ±0.03
Chlorophyll a+b / Carotenoids	4.67±0.33	5.01 ±0.23

Table 1. *Mean (+SE) values of $F_v\ F_m\ ^{-1}$, concentrations and ratios of photosynthetic pigments in M. quadrifolia leaves from open areas and under rice canopy. Bold characters indicate significant differences in one-way ANOVAs (p < 0.01).*

4. Discussion

Our study showed that cultivating *M. quadrifolia* in organic rice farms represents an important opportunity for preserving this endangered species in areas of intensive agriculture. In the two organic farms *M. quadrifolia* showed better performance than in both the transitional and conventional farms. It is noteworthy that the experiment was performed in a real rice production context, without the possibility to fully standardize the methodology among farms and years. Although this could represent a potential flaw in our experimental design, at the same time it strengthens our conclusions and provide more reliable information than a completely standardized (but far-from-reality) design.

In the conventional farm, *M. quadrifolia* survived herbicide application in 2017 but not in 2018. This was likely due to the effect of the pre-sowing herbicide treatment to which *M. quadrifolia* was exposed in 2018 but not in 2017. Indeed, the species is sensitive to a broad range of herbicides, but type and strength of the effects vary among them. Aura is considered as the most harmful herbicide, already at 1:100 and 1:1000 dilutions, whereas Clincher One and Viper do not preclude survival of *M. quadrifolia* (Bruni et al., 2013). Glyphosate used in the pre-sowing treatment 2018 should not be as harmful as the abovementioned chemicals (Bruni et al., 2013), but its application in an early development phase (mid-May) may have had a more detrimental effect leading to the die-off of the introduced *M. quadrifolia*.

Our results demonstrate that *M. quadrifolia* prefers organic rice fields but can also survive in conventional farms with reduced supply of herbicides. Increasing public concern about the use of chemicals in the EU resulted in important Directives like 2009/128/EC - and following updates 2019/782/EC - with the aim to reduce the use of chemicals and to improve their selectiveness (e.g., toward more aggressive weeds; Lamichhane et al., 2016). This could open new interesting opportunities for land sharing between threatened species and farm production. Although we found local differences in the chemical features of the water filling the experimental paddies, mainly due to the distance between farms and to the different management of water supply, effects of these differences on growth of *M. quadrifolia* were overall poor. Previous studies also reported wide ecological amplitude of this species with respect to water chemistry (Bolpagni & Pino, 2017). Indeed, *M. quadrifolia* is a rooted aquatic fern tolerating rather high nutrient levels both in the water and in the soil (Abbasi et al., 2018).

M. quadrifolia exhibited unexpected higher growth potential under rice canopy than in open areas (independently by the used rice cultivar), which suggests that higher *M. quadrifolia* performance is found inside the paddy than in open areas at the edge of a field. Long-term acclimation of vascular plants to light level relies primarily on adjustment of the photosynthetic machinery. Our study revealed poor, if any, capacity of *M. quadrifolia* to adjust pigment composition to light level. Lower chlorophyll fluorescence values testify that *M. quadrifolia* underwent some degree of stress in full-light habitats. Hence, shadow cast by rice canopy creates a suitable micro-environment for *M.*

quadrifolia. Furthermore, open areas are quickly colonized by exotic invasive weeds like *Heteranthera reniformis* Ruiz & Pav., *Ammannia coccinea* Rottb. and *Cyperus microiria* Steud. This is an important finding suggesting that a reintroduction of *Marsilea quadrifolia* in areas of intensive agriculture may have more chances of success if the species is planted under the rice canopy, especially in organic farms (on farm reintroduction).

Since *M. quadrifolia* is listed in the Directive 92/43/CEE and in the Bern Convention, its conservation is mandatory in the European Union. Consequently, a conservation management plan for this fern is urgent and cannot disregard the agricultural and semi-natural context in which this species grows. Considering that rice fields are recognized worldwide as surrogate habitats for wetland species (Lawler, 2001), and many rice farms in the crop district of northwestern Italy are included in protected areas (e.g., the only Special Protection Area “Garzaie della Lomellina” extends for 30.940 ha), such a conservation plan should consider simultaneously the ecological requirements of the species and the needs of stakeholders (i.e., farmers, landowners, local policymakers). We suggest that ad hoc agro-environmental compensations or incentives from the EU Rural Development Plan (RDP) should support farmers willing to reintroduce and maintain *M. quadrifolia*, at least in organic rice farms. For example, in Lombardy Region (N-Italy) specific funds from the Rural Development Programme (RDP 2014–2020) were available to farmers for maintaining wet meadows listed in Directive 92/43/EC in a ‘favorable’ conservation status, but also rice fields and wet habitats if relevant for nature conservation. There are of course several open questions related to: (i) level of competition and consequent effects on farm productivity of *M. quadrifolia*, (ii) interactions between *M. quadrifolia* and other alien invasive species, (iii) effects on *M. quadrifolia* of frequent crop rotations in organic farms, and (iv) possibility that selective herbicides may allow *M. quadrifolia* to thrive also in conventional farms. Incentives like those proposed here may benefit several arable species currently threatened or at risk of extinction (Meyer et al., 2013), like *Isoëtes malinverniana* Ces. & De Not. and *Lindernia procumbens* (Krocker) Philcox in our experimental area.

Funding Open Access funding provided by Università degli Studi di Pavia. The Grant of Excellence Departments, MIUR-Italy (ARTICOLO 1, COMMI 314 – 337 LEGGE 232/2016), is gratefully acknowledged for the support to one of the authors (Prof. Thomas Abeli).

Acknowledgements The authors would like to thank the farmers R. Caimo Duc (Candia Lomellina), E. Bianchi (Torre de Negri), C. Carturan (Candia Lomellina) and A. Paravicini † (Bereguardo) that kindly offered their farms for this experimentation. Thanks are also due to: L. Tomasi, M. Canella, A.W. Rossi, M. Marangon, F. Ferrari, M.C. Mariani and S. Lodetti for their support to field work. We are also grateful to P. Cauzzi (University of Pavia) for *Marsilea quadrifolia* cultivation in Pavia Botanical Garden. The results of this research will be illustrated as best practices in project CLOVER (Agroecosistemi e Conservazione in Lombardia di specie vegetali rare di Direttiva Habitat), RDP of Lombardy.

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Supplementary Materials

Supplementary Material 1

COUNCIL DIRECTIVE 92/43/EEC of 21 May 1992 on the conservation of natural habitats and of wild fauna and flora also known as “HABITAT DIRECTIVE”

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31992L0043&from=ENCouncil

CONVENTION on the conservation of European wildlife and natural habitats also known as “BERN CONVENTION”

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:21979A0919(01)&from=EN

DIRECTIVE 2009/128/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 21 October 2009 establishing a framework for Community action to achieve the sustainable use of pesticides

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009L0128&from=EN

REGULATION (EU) No 1305/2013 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 17 december 2013 on support for rural development by the European Agricultural Fund for Rural Development (EAFRD) and repealing Council Regulation (EC) No 1698/2005

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32013R1305&from=EN

COUNCIL REGULATION (EC) No 834/2007 of 28 June 2007 on organic production and labelling of organic products and repealing Regulation (EEC) No 2092/91

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32007R0834&from=IT

COMMISSION REGULATION (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control

https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008R0889&from=IT

Table S1. *Herbicide treatments supplied during the experimental cultivation in the conventional farm in 2017 and 2018.*

Year	1° treatment	2° treatment	3° treatment
2017	Clincher One 1200 g/ha - Aura 450 g/ha (end of May)	Most Micro 1995 g/ha - Command 300 g/ha (early June)	Viper 3000 g/ha (early July)
2018	Glyfos Dakar 3000 g/ha - Codacide 900 g/ha - Novatec 900 g/ha (mid-May)	Most Micro 1995 g/ha - Command 300 g/ha (end of May)	Clincher One 1200 g/ha - Aura 450 g/ha (mid-June)

Clincher One: Cialofop-butile 20.02% (200 g/l)

Aura: 20 g Profoxydim

Command: Clomazone 30.7% (360.0 g/l)

Viper: Penoxsulam 1.6% (=16 g/l) Triclopyr-butotilpuro 16.7%

Coformulated q.s to 100 g with 1,2-benzisothiazol-3-(2H)-one.

Codacide: Rapeseed Oil (CAS 8002-13-9)

Glyfos dakar: Glyphosate Acid pure

Table S2. *Mean ± SE of the main variables evaluated from soil and water samples in different farming systems.*

Water variables

Variable	HCO_3^- (μmol/l)	NO_3^- (μmol/l)	Fe (μmol/l)	PO_4^{3-} (μmol/l)	SO_4^2 (μmol/l)	EC (μs/cm)	pH
Organic	1090±205	26.54±6.15	4.97±1.20	4.10±0.87	252±19	164±10	8.40±0.17
Transitional	3311±185	103.10±4.47	6.49±2.70	4.08±0.86	371±2	368±4	7.46±0.06
Conventional	952±88	17.18±11.06	4.87±0.35	2.16±0.14	202±2	172±20	8.85±0.13

Soil variables

Variable	Nitrogen (% DW w/w)	PO_4^{3-} (mg/kg)	C/N (% DW w/w)	Clay (% DW w/w)	pH
Organic	0.13±0.01	33.2±9.6	9.8±3.1	6.88±0.63	4.88±0.47
Transitional	0.15±0.01	33.5±1.3	9.4±0.5	8.75±1.25	5.60±0.65
Conventional	0.15±0.01	21.3±0.4	8.2±0.1	8.75±1.25	4.05±0.05

A mesocosm experiment testing the competition between the invasive species Heteranthera reniformis Ruiz & Pav. and the endangered fern Marsilea quadrifolia L. occurring in paddy fields

Corli A., Gerdol, R., Orsenigo, S., Rossi, G., Abeli, T., Brancaleoni, L. (Submitted to Aquatic Botany on 18th November 2021)

Abstract

Biological invasion is one of the most severe threat to biodiversity, with invasive alien species altering ecosystem functioning and services. Once invasive species arrive in the recipient community, they can easily spread and outcompete native species thanks to their colonization ability, commonly due to competition for resources. On the other side, native species can limit plant invasiveness thanks to their biotic resistance, especially when both species display similar traits when occupying human-disturbed habitats.

We present here the results of a mesocosm experiment aimed at understanding the competitive ability of the threatened fern *M. quadrifolia* L. and the invasive aquatic vascular plant *H. reniformis* Ruiz & Pav., two species that can coexist in paddy fields. The two species were cultivated both in monoculture and in mixed culture at different density, also in presence of rice plants. Data on vegetative growth, reproduction, ecophysiology and functional traits were collected.

Contrarily to what we expected, our results showed that *M. quadrifolia* expressed high competitive ability towards the other plants in absence of high nutrient loads typically found in rice fields, with *H. reniformis* maintaining some of the traits typical of invasive species. The performance of the threatened species was also density-dependent, whereas rice and *H. reniformis* had the best performance in monoculture condition.

Cultivating *M. quadrifolia* in organic farming systems, where fertilizer input is in slower amount than conventional farms and is slow release, may represent an opportunity for preserving *M. quadrifolia* in its semi-natural environment, where the invasive species *H. reniformis* also occurs.

Keywords competitive ability · mesocosm experiment · aquatic plants · policy species · agroecosystems

1. Introduction

Determining factors associated with the naturalization and invasiveness of alien species is a central theme in plant ecology (van Kleunen et al., 2015). Species-specific traits such as growth rate, competitiveness and dispersal ability as well as environmental conditions, disturbance and diversity of the recipient community are, among many, the primary factors influencing alien species establishment and spread. Although many alien species have reduced impact when reaching new locations, some of them become invasive. When this happens, alien species pose threats to ecosystem functioning and services (Vilà et al., 2011). Invasive alien species, hereafter IAS, have been introduced mainly through the global horticultural trade, with growing concern raised over the last century of the unintended and sometimes severe consequences of biological invasion (Simberloff et al., 2013). Of note, 3.9% of the world's vascular plant species have been introduced, established and naturalised outside their native ranges by humans (van Kleunen et al., 2015). Risk assessments, early detection, management actions and eradication plans have been widely implemented (e.g., Regulation No 1143/2014 of the European Parliament). Nevertheless, the expansion rate of IAS continues to increase, with negative impacts on biodiversity but also on economic and social aspects of human well-being (Vilà et al., 2011).

IAS tend to outcompete native species due to better performance of functional traits related to growth rate, resource acquisition and reproduction (propagule pressure or vegetative reproduction; van Kleunen et al., 2015). Hence, IAS often exhibit faster vegetative growth, earlier and extended flowering (Pyšek & Richardson, 2007) as well as 'soft leaves, fast growth' strategy, as noted both in terrestrial and aquatic vascular flora (Lukács et al., 2017). Traits promoting invasiveness are strongly related to leaf traits: IAS experience rapid carbon capture through high specific leaf area (i.e., ratio of leaf area to leaf dry mass, SLA) and leaf area ratio (i.e., ratio of total leaf area to dry mass of the entire plant; Pyšek & Richardson, 2007), with SLA scaling positively with mass-based light saturated photosynthetic rate and with leaf nitrogen (N) concentration (Lukács et al., 2017). Conversely, high SLA scales negatively with leaf longevity and leaf dry matter content (i.e., ratio of dry leaf mass to fresh leaf mass, LDMC). IAS are thus characterized by higher SLA and lower LDMC, whereas plants with an efficient conservation of nutrients are characterized by opposite strategy (i.e., lower SLA and higher LDMC; Li et al., 2005).

Competition is the most commonly invoked mechanism by which IAS impact native species in the recipient ecosystems (Levine et al., 2003). Plants compete predominantly by nutrient and/or light acquisition, both influencing the structure and development of plant communities. In addition, species respond differently in relation to population density (Zhang & Tielbörger, 2020). Differences in competitive ability between invasive and native species are both the result of gradual directional changes in competitive interactions between species and a priori differences resulting from species traits and propagule pressure. Indeed, alien species tend to have stronger human association, hence they may be better adapted to human-modified environments (Buckley & Catford, 2016). However, it could also be that native species typical of human-disturbed habitat display similar traits as invasive species, with native and invasive co-occurring species occupying the same ecological niche (Thompson & Davis, 2011). A key natural barrier for non-native species to become invasive is manifested through the biotic resistance of native species (Richardson et al., 2000), resulting in lower invasion success by alien species in the recipient community. Nevertheless, the mechanisms that drive such resistance are still unsolved. Thus, understanding whether invasive species benefit from higher interspecific competitive ability and explaining how biotic resistance by native species can prevent species invasiveness, are both of primary importance for biodiversity conservation and management.

Italy is characterized by a broad range of ecosystems and habitats (Capotorti et al., 2020). However, the extremely high rates of human perturbations have led to a strong loss of natural and semi-natural habitats, especially in lowlands, where collapse of the traditional agro-sylvo-pastoral practice has occurred (Bolpagni & Piotti, 2016). This is the case of the Po Valley area (N-Italy), one of the most productive croplands in the northern Hemisphere, where the landscape is fragmented by civil and industrial settlements and intensive agriculture (van Donkelaar et al., 2010). Despite the habitat quality reduction, this area still hosts important plant species of high conservation value like *Marsilea quadrifolia* L., commonly known as water-clover fern. This species, native to Eurasia, was considered in the past as a weed in paddy fields of southern Europe (Viggiani et al., 2003). However, *M. quadrifolia* populations experienced a drastic decline in the last decades, because of habitat loss and degradation as well as modified rice cultivation (i.e., mechanization, use of chemicals and simplified rotation; Ferrero & Vidotto, 2010). In addition, *M. quadrifolia* seems to be negatively affected by the spread of the invasive alien monocot *Heteranthera reniformis* Ruiz & Pav. (Gentili et al., 2010), native to North and Central America (Csurhes, 2016).

M. quadrifolia is legally protected at the EU level, listed in the Annexes II and IV of the EU Directive 92/43/EEC "Habitats" and Annex I of the Bern Convention (Corli et al., 2021a); the species is classified as "Vulnerable" in Europe according to the IUCN criteria (Garcìa Criado et al., 2017) and as "Endangered" in the Italian Red List (Rossi et al., 2016). Reintroduction attempts have been performed in conventional paddy fields with low success (e.g., Orsenigo et al., 2016), probably because *M. quadrifolia* is sensitive to a wide range of herbicides (Bruni et al., 2013). Recent experimental studies conducted in organic rice fields in Italy demonstrated that *M. quadrifolia* grows in this artificial habitat if the application of herbicide is null or very reduced (Corli et al., 2021b) giving a gleam of hope for species conservation in paddy fields, where the needs of stakeholders (i.e., farmers, landowners, local policymakers; Corli et al., 2021b) needs also to be considered. However, the problematic relationship between the threatened *M. quadrifolia* and the invasive *H. reniformis* has also to be taken into account and addressed. Indeed, *H. reniformis* is considered one of the most important weeds in Italian paddy fields (Ferrero & Vidotto, 2007), being able to reduce rice yield by 65%. *H. reniformis* can be managed only by chemicals, both with pre-seeding as well as post-emergence application (Ferrero & Vidotto, 2007). The management of *H. reniformis* does not imply conservation attention in conventional farms where *M. quadrifolia* is rare because of its sensitiveness to herbicides (Bruni et al., 2013). In contrast the presence of *H. reniformis* may be a limiting factor for M. quadrifolia in organic farms where ,in the absence of herbicides, H. reniformis spreads constantly in the entire rice field.

Thus, understanding whether the invasive species *H. reniformis* may outcompete *M. quadrifolia* especially in organic farms is paramount in view of the conservation of *M. quadrifolia* (Rossi et al., 2013) and for the development of an Action Plans for *M. quadrifolia* (i.e., Lombardy Action Plan; Rossi et al., 2019). At the same time, it is important to understand to what extent the performance of rice may be influenced by the presence of both species.

To shed light on the competition between *M. quadrifolia* and *H. reniformis* in organic rice cultivation, we set up a mesocosm experiment where *M. quadrifolia* and *H. reniformis* were cultivated both in monoculture and in mixed culture at different densities. The aim of the study is to better understand competitive relationships between species since the colonization performance of invasive species is density dependent (Dalle Fratte et al., 2019). Additionally, our study aims to evaluate whether competition also exists with rice (*Oryza sativa* L.), at least in the initial stages of plant development (before inflorescence emergence stage). To address this, rice plants were also cultivated both in monoculture and in presence of the other two species.

We collected data on vegetative growth, reproduction, ecophysiology and functional traits on the three species aiming to answer these questions: i) to what extent does the level of competition affect species growth and performance? ii) how the colonization performance of the invasive species *H. reniformis* on the threatened species *M. quadrifolia* is affected by density? iii) to what extent rice performance is affected by the presence of “weeds”? We hypothesized that i) *M. quadrifolia* is negatively affected by the presence of *H. reniformis* and ii) the magnitude of negative effects of competition on *M. quadrifolia* is proportionally related to the density of the invasive species *H. reniformis*; moreover, iii) rice is negatively affected by *H. reniformis* but not by *M. quadrifolia*. Answering these questions will help us understand *M. quadrifolia* biology in the context of agro-ecosystems, with the purpose to develop a robust conservation plan considering both the surrounding community and the needs of stakeholders.

2. Material and methods

2.1 Study species

Marsilea quadrifolia L. is a perennial aquatic fern (Corli et al., 2021a). The species is typical of wetlands with shallow water; it grows both in natural habitats such as shallow lakes, ponds and slow-flowing rivers as well as in semi-natural and artificial areas, like canals, ditches and rice fields (Gentili et al., 2010). *M. quadrifolia* is characterized by a creeping rhizome, rooted at the nodes and anchored to the ground with one or more adventitious roots (Bendre & Kumar, 2010). A long petiole develops above the water level, characterized by four leaflets with triangular shape resembling a clover. The leaves develop heterophylly to withstand moisture constraints (Wu & Kao, 2011): in submerged plants, leaflet margins are entire with long and flexible petiole floating over the surface of water whereas in emergent plants leaflets are crenate, with a short rigid petiole (Johnson, 1986). At the base of the petiole, the plants have 1-12 mm stalks named peduncle dichotomously branched, bearing two to five sporocarps, the reproductive structure of the species. The life cycle of the species consists of the alternation of generations, i.e., alternate succession of haploid gametophyte and diploid sporophyte (Johnson, 1986). *M. quadrifolia* reproduces both vegetatively by the division of rhizome or sexually, with the release of micro- and megaspores from sporocarps (Schneider & Pryer, 2002).

M. quadrifolia has a widespread distribution, being native to Eurasia; the species is considered as a weed in Asia and as an alien species in North America (Benson et al., 2004). In the past, the species was quite common in its native range and gained the name of “pig pasture weed” (Poschlod et al., 2005). However, at the end of the 20th century *M. quadrifolia* became rare in its European range as a consequence of habitat loss and degradation, changing of agrotechniques towards mechanization, use of chemicals and simplified rotation (Ferrero & Vidotto, 2010). In addition, vegetation succession from *Phragmites australis* (Cav.) Trin. ex Steud. and competition with alien species such as *Eleocharis obtusa* (Willd.) Schult. together with the presence of *H. reniformis* Ruiz & Pav. have been observed (still to be confermed) to negatively affect species performance (Gentili et al., 2010); *M. quadrifolia* is also a food source for native and alien animals such as waterfowls, nutria (*Myocastor coypus* Molina) and the Louisiana crayfish (*Procambarus clarkii* Girald; Gentili et al., 2010).

H. reniformis Ruiz & Pav. is an annual or facultatively perennial aquatic monocot growing either submerged or floating on the water surface (Csurhes, 2016). This species, 20-50 cm tall, can grow submerged or floating, with procumbent stems that either creep along the mud or float. Leaves are sessile, forming basal rosettes; the inflorescence is a 2-10-flowered raceme (Horn, 2002). Flowers are white to pale blue, arranged in clusters; each flower has 6 petals and 3 stamen and it open about three hours after sunrise and wilt by early afternoon. The fruit is a capsule with 8 to 14 small winged seeds less than 1 mm long (Csurhes, 2016). *H. reniformis* reproduces vegetatively through stem fragmentation and root production as well as sexually by producing many seeds which can survive for long periods in the soil seed bank, enabling species reappearance when the environmental conditions are optimal (Hill, 2006). Noteworthy, *H. reniformis* is considered as one of the main aquatic weeds in European paddy fields, thanks to its massive reproductive capacity and biomass production (Bianchini et al., 2010). In Italy the species was first reported in 1962 (Pirola, 1968) in the Pavia province, N-Italy, when it became one of the most frequent weeds in rice paddies (Sgattoni et al., 1989).

2.2 Experimental design

The mesocosm experiment was carried out in summer 2019 in the Botanical Garden of the University of Ferrara, Italy (44°50′30.85″ N, 11°37′20.94″ E, 9 m a.s.l.). The plant material used in the experimental cultivation consisted in: *M. quadrifolia* swards reproduced vegetatively from one *ex situ* population coming from Pavia area already cultivated at the Botanical Garden of Pavia University, Lombardy; *H. reniformis* seedlings collected from one rice field in the province of Pavia; rice seedlings (variety Ronaldo; main characteristics: mean height 78 cm, earing time 92 days, maturation 150 days; Enterisi, 2021) grown from seeds kindly provided by a farmer from the province of Pavia, where *M. quadrifolia* naturally occurs in the organic farm. Rice seeds were sown in rounded pots filled with commercial organic soil on 20th April 2019 (i.e., 66 days before transplanting), according to the sowing period for that specific cultivar. The experimental cultivation lasted 99 days, from 24th June to 1st October 2019. On 24th June, the plant material was cleaned and transplanted in rectangular pots (50 × 40 × 25 cm) filled with commercial organic soil mixed with sand and peat. Water level was kept at 2 cm above soil level throughout the cultivation period. Other plants occurring in the pots, especially algae and weed seedlings were manually removed every three days. The cultivation design is shown in Figure 1, with each of the nine cultivation unit (hereafter "treatment") shaving 5 replicated pots, with 45 pots in total.

The amounts of plants used in monocultures were set preliminarily and arbitrarily: we selected 12 individuals/monoculture pot for both *M. quadrifolia* and *H. reniformis* and 40 individuals/monoculture pot for rice (mean initial height: 10 cm, phenological stage: tillering), considering rice density in rice fields. Since *M. quadrifolia* is a rhizomatous species, an individual was conventionally defined as a 10-cm long rhizome stretch. The mixed cultures contained different proportions of the species as reported in in Figure 1.

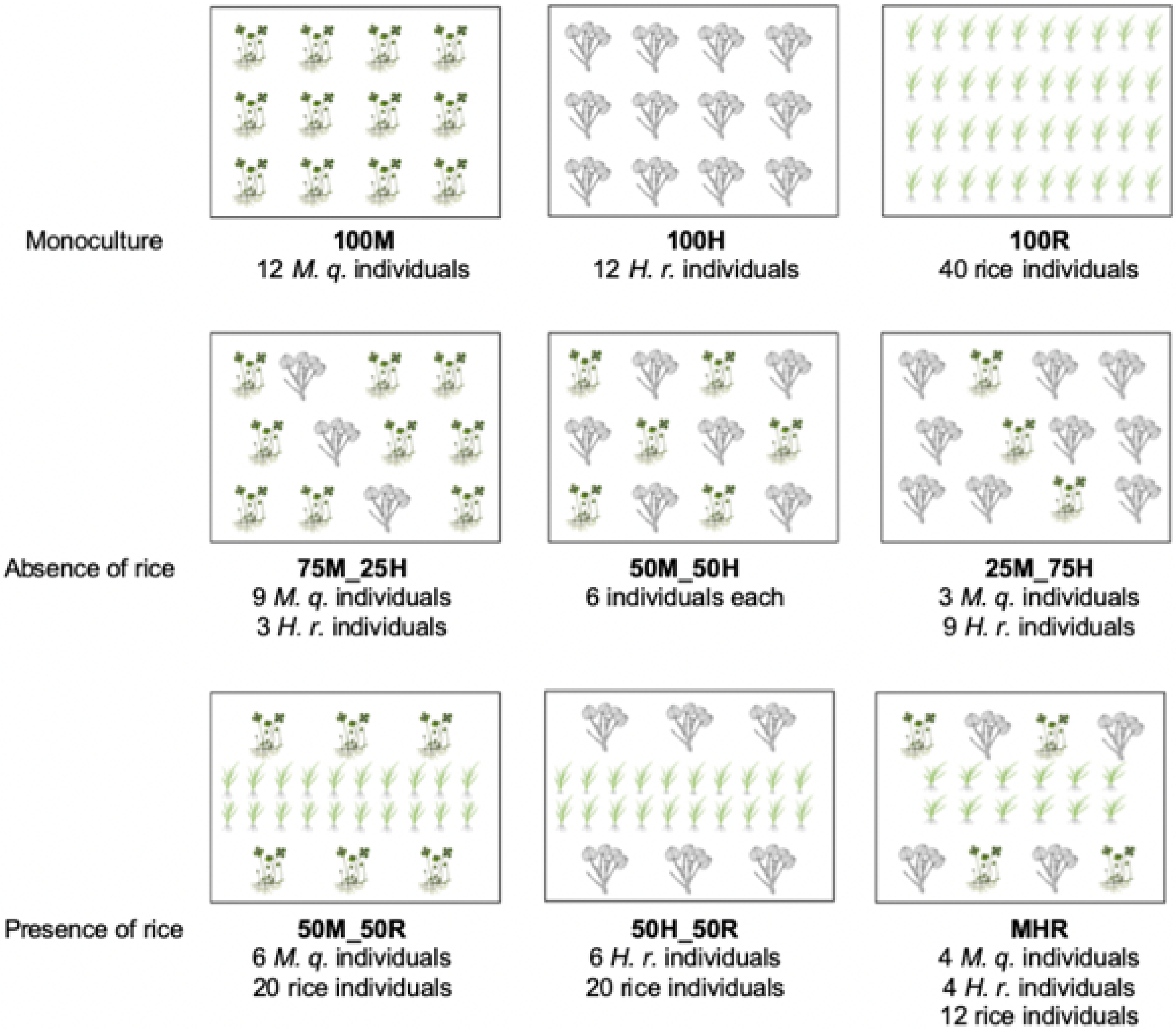

Figure 1. *Experimental design: focus on plants disposition and plant proportion inside the pots. Species cultivation in monoculture (cultivation: 100M, 100H, 100R), in absence of rice (cultivation: 75M_25H, 50M_50H, 25M_75H), in presence of rice (cultivation: 50M_50R, 50H_50R, MHR). The number of plants used in the experiment are specified below each scheme. M and* M.q. *refers to* Marsilea quadrifolia, *H and* H.r. *refers to* Heteranthera reniformis, *R refers to rice.*

2.3 Study limitation

The focus of the experiment was to investigate competition between *M. quadrifolia* and *H. reniformis*. To do so, we performed a mesocosm experiment without reproducing accurately the environmental conditions occurring in paddy fields where the two species typically grow. We did not supply fertilisers, because we wanted to investigate how M. quadrifolia and H. reniformis mutually compete without external inputs of nutrients. This choice, along with the use of shallow pots, negatively affected development of rice, a species that normally requires a big amount of N to obtain effective yield. Consequently, rice did not produce any inflorescence and yield in our experiment, which makes the results for this crop poorly reliable. Nevertheless, we decided to present the results for rice because they still provide important information although limited to the early developmental stage of the plant. In particular the fastest radical system development occurs at the end of the tillering stage, parallel to maximum percentage increase of plant mass and highest nutrient uptake (Tinarelli, 1986).

2.4 Data collection and elaboration

Growth in mass was determined based on total plant biomass: total fresh initial biomass (both belowground and aboveground biomass) was measured at the beginning of the experiment by weighing the plants immediately before placing the plant material in the pots. Total fresh final biomass was measured at the end of the experiment by weighing the plants immediately after the final harvest. Relative growth rate (RGR) was calculated on fresh biomass as follow:

$$RGR = \frac{(Biomass_t - Biomass_0)}{Day_t - Day_0}$$

Where *t* refers to the end of the experiment and *0* at the beginning.

RGR for each species per pot was converted into the Competitive Interaction (CI) index, that provides information on competitive interactions between species in a mixed culture by comparing their growth in mixed and pure cultures (Fowler, 1982). The CI index for species *i* in mixed culture with species *j* was calculated as:

$$CI_{ij} = \frac{RGR_{ij}}{p * RGR_i}\,; \qquad CI_{ji} = \frac{RGR_{ji}}{q * RGR_j}$$

where RGR*ij* is the RGR of species *i* growing with species *j*, RGR*ji* is the RGR of species *j* growing with species *i*, RGR*i* is the RGR of species *i* growing in monoculture, RGR*j* is the RGR of species *j* growing in monoculture, *p* is the initial proportion of species *i* in mixed culture and *q* the initial proportion of species *j* in mixed culture, with (*p* + *q*) = 1. Specifically, a CI*ij* = 1 indicates that species *i* performs equally in mixed culture with species *j* as it does in monoculture. A CI*ij* > 1 means that species *i* performs better in mixed culture rather than in monoculture (i.e., species *i* is a superior competitor than species *j*). A CI*ij* < 1 means that species *i* performs better in monoculture than in mixed culture (i.e., species *i* an inferior competitor than species *j*; Snyder et al., 1994).

Ecophysiological and functional traits were periodically assessed during the experimental period. Leaf chlorophyll content (Chl) and chlorophyll fluorescence (Fv/Fm) were determined five times under the same light conditions and time of day during the peak season (from 9th August to 1st October) in three healthy leaves per species per pot. Chl was determined with a chlorophyll content meter (CCM-200; Opti Sciences, Tyngsboro, MA, USA). Fv/Fm was determined with a modulated fluorometer (OS1-FL; Opti Sciences, Tyngsboro, MA, USA). Leaf samples were collected at the end of the sampling period (1st October) for each species in each pot and used to determine functional traits. For this purpose, 12 leaves for *M. quadrifolia* and 5 leaves for both *H. reniformis* and rice were collected and scanned. The leaves were weighed, both fresh and dry (after oven-drying at 40°C for

48h), to calculate specific leaf area (SLA, i.e., leaf area/leaf dry mass) and leaf dry matter content (LDMC, i.e., dry leaf mass/fresh leaf mass). Foliar N and phosphorus (P) contents were determined by colorimetric analysis of three replicated leaf samples per pot, previously dried at 70°C. The chemical analyses were carried out with an autoanalyzer (Systea Flowsys, Anagni, Italy) in the Laboratory of Plant Ecology of Ferrara University.

Reproductive performance was determined for *M. quadrifolia* and *H. reniformis*. The reproductive performance of rice could not be determined because the rice plants did not reach even the inflorescence emergence stage. The number of sporocarps of *M. quadrifolia* was estimated by counting sporocarps in a 10-cm2 subsample of the *M. quadrifolia* sward in each pot at the end of the cultivation period. The number of fruits per pot of *H. reniformis* were counted during the cultivation period three times, on 30th August, on 9th September, and on 1st October.

2.5 Statistical analyses

The data on CI index, SLA, LDMC and foliar N and P contents were analysed by two-way factorial ANOVAs, with species (factor with 3 levels: *M. quadrifolia*, *H. reniformis*, rice) and cultivation (factor with 9 levels: 100M, 100H, 100R, 75M_25H, 50M_50H, 25M_75H, MHR, 50M_50R, 50H_50R) as explanatory variables. In a first step, we tested whether the interaction between species and cultivation was significant. As the three species differed strongly from each other because of intrinsic differences in terms of most traits, separate one-way ANOVAs were used for each species when the interaction was significant, where the response variable was tested against cultivation. Repeated measures two-way ANOVAs with species and cultivation as between-subject factors and time (factor with 5 levels, i.e., the five sampling dates) as within-subject factor was performed for Chl. The number of sporocarps of *M. quadrifolia* was analysed by a one-way ANOVA with cultivation as explanatory variable whereas the number of ripe fruits of *H. reniformis* was analysed by a two-way factorial ANOVAs with cultivation and time (factor with 3 levels, i.e., the three sampling dates) as explanatory variable. Significance of differences between cultivation were assessed by Tukey's post-hoc honest significant difference method (HSD) tests. The statistical analyses were performed with R 3.6.1 (R Core Team, 2019).

3. Results

Rice performance was constrained by small cultivation pots and the absence of fertilization (mean final height: 61 cm), limiting the reliability of the comparison in the experiment. However, results on the species are reported, with major importance given to the performance of *M. quadrifolia* and *H. reniformis.*

3.1 Competitive interaction index

The CI index was significantly affected by species and treatment (Table 1), with *M. quadrifolia* reporting the highest CI index (Figure 2). *M. quadrifolia* showed higher CI index in mixed culture with the other species than in monoculture. Indeed, the CI index for *M. quadrifolia* was always > 1 in mixed culture, which means that *M. quadrifolia* performed better in terms of mass growth in presence of *H. reniformis* and rice than alone. Noteworthy, *M. quadrifolia* exhibited higher CI index with increasing density of *H. reniformis*, with highest CI index when *M. quadrifolia* density was lowest (i.e., 25M_75H; Figure 2). *H. reniformis* grew better in monoculture than in mixed culture. *H. reniformis* was strongly negatively affected by the presence of *M. quadrifolia*, as documented by the CI index always < 1 in mixed culture in especially with *M. quadrifolia* (Figure 2). Rice also was negatively affected by the presence of *M. quadrifolia* (Figure 2).

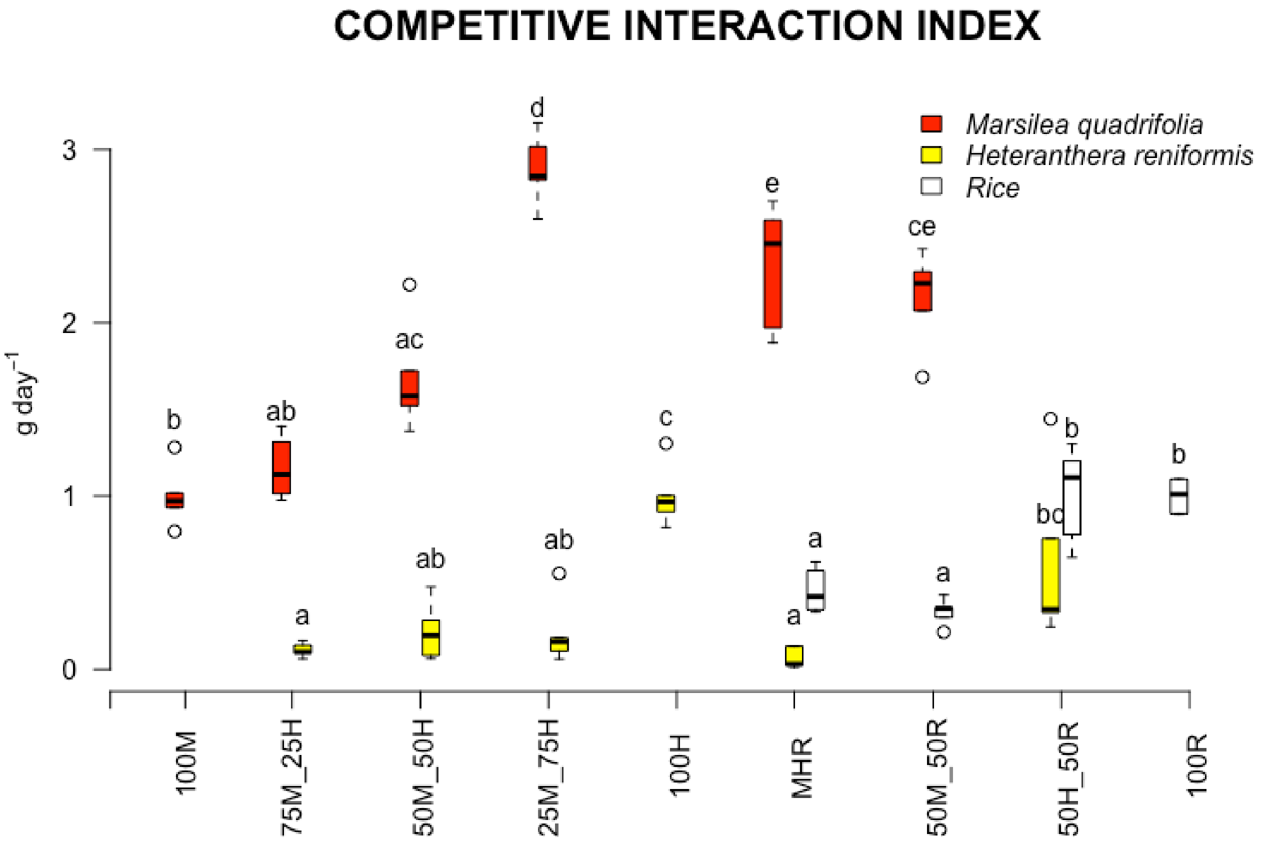

Figure 2. *Competitive interaction index of the species. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot condition within the same species according to the Tukey's post-hoc HSD tests.*

3.2 Ecophysiological and functional traits

Leaf chlorophyll content (Chl) was significantly affected by species, treatment, time and their interactions (Table S1), with highest values for *H. reniformis*, especially in monoculture. For *M. quadrifolia* Chl did not vary in relation to treatment whereas Chl for rice was negatively affected by the presence of *M. quadrifolia*, showing the lowest value in 50M_50R. Chl changed through time, with higher values at the beginning of the cultivation period for *M. quadrifolia* and *H. reniformis* (Fig.

A1). H. reniformis presented a second Chl peak at the end of the cultivation period. Conversely, Chl for rice was lowest at the beginning of the cultivation period, then slightly increasing through time (Figure S1).

Chlorophyll fluorescence always presented optimum values (with mean Fv/Fm 0.766 ± 0.002 for *M. quadrifolia*, 0.772 ± 0.002 for *H. reniformis* and 0.760 ± 0.002 for rice), which indicates that the plants never experienced physiological stress during the experimental cultivation.

SLA was significantly affected by species and treatment, with a significant interaction between these two factors (Table 1). *H. reniformis* presented the highest SLA, with higher value in mixed culture than in monoculture (Figure 3). SLA of *M. quadrifolia* increased as its density decreased in mixed culture with *H. reniformis*, with highest SLA at 25M_75H (Figure 3). Conversely, *M. quadrifolia* presented lower SLA in mixed culture with rice. Rice showed the lowest SLA, with slightly higher value in mixed culture with *M. quadrifolia* (50M_50R; Fig. 2B).

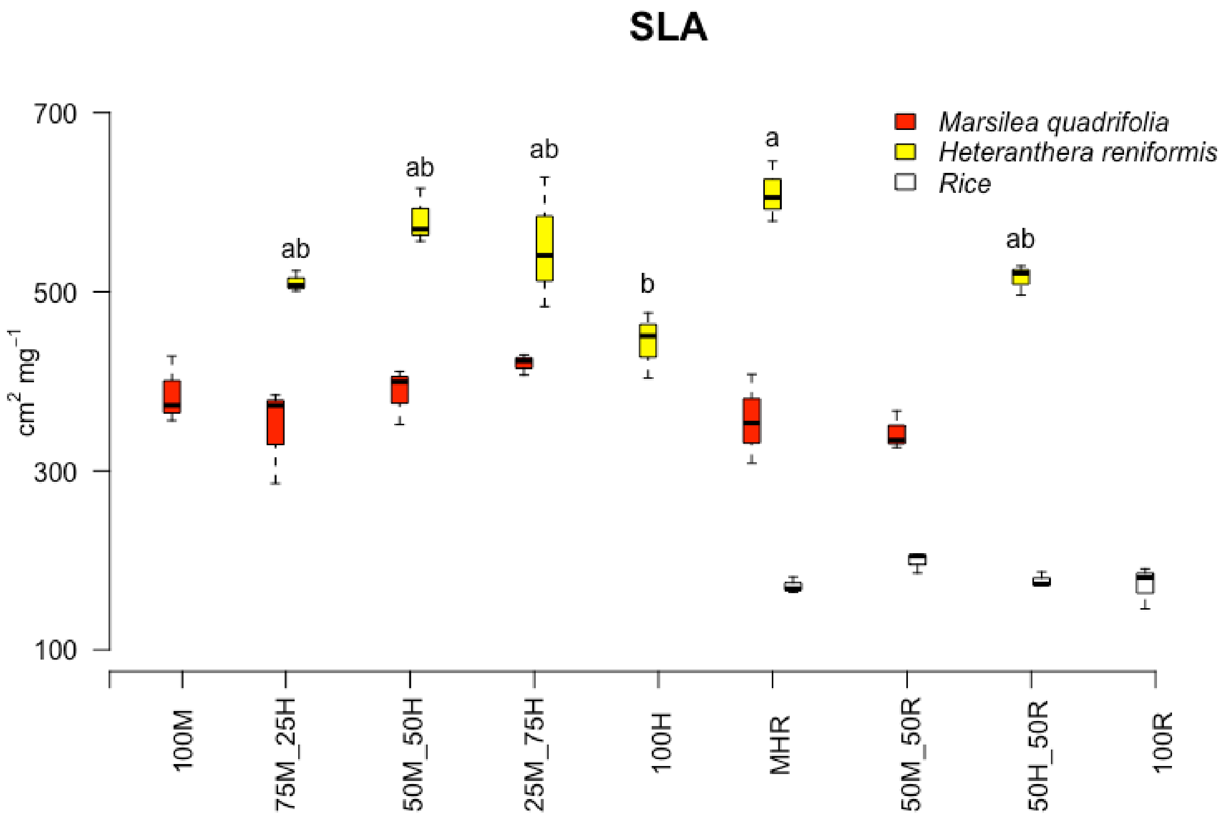

Figure 3. *Specific Leaf Area (SLA) of the species. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot condition within the same species according to the Tukey's post-hoc HSD tests.*

LDCM also was significantly affected by species and treatment with a significant interaction between these two factors (Table 1). In line with its inverse relation with SLA, LDMC was highest in rice (Figure 4), with higher values in mixed culture with both *M. quadrifolia* and *H. reniformis* than in monoculture. LDMC of *M. quadrifolia* varied little in relation to treatment except for a slightly higher value when growing in mixed culture with rice. Conversely, LDMC of *H. reniformis* was significantly higher in monoculture than in mixed culture (Figure 4).

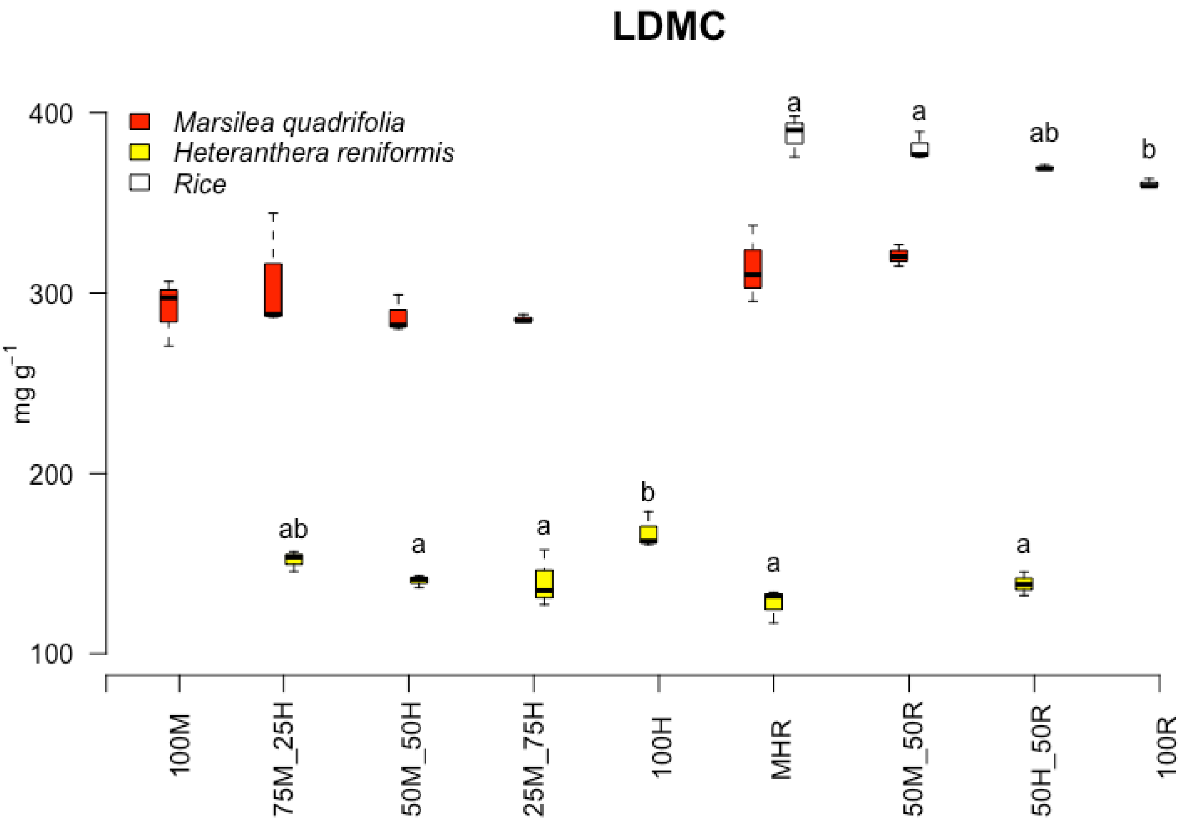

Figure 4. *Leaf Dry Matter Content (LDMC) of the species. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot condition within the same species according to the Tukey's post-hoc HSD tests.*

Foliar N content was significantly affected by species and treatment, with no significant interaction (Table 1; Figure 5). *H. reniformis* exhibited the highest foliar N content, with higher values when growing at highest density in mixed culture with *M. quadrifolia* (25M_75H; Figure 5). On the contrary, the presence of rice negatively influenced the foliar N content of *H. reniformis*, especially in 50H_50R. *M. quadrifolia* leaves had higher N concentration when growing at lowest density in mixed culture with *H. reniformis* (25M_75H). As for *H. reniformis*, *M. quadrifolia* was negatively influenced by rice in term of foliar N content. Rice presented the lowest foliar N content, with lower values in mixed culture with *H. reniformis* (50H_50R; Figure 5).

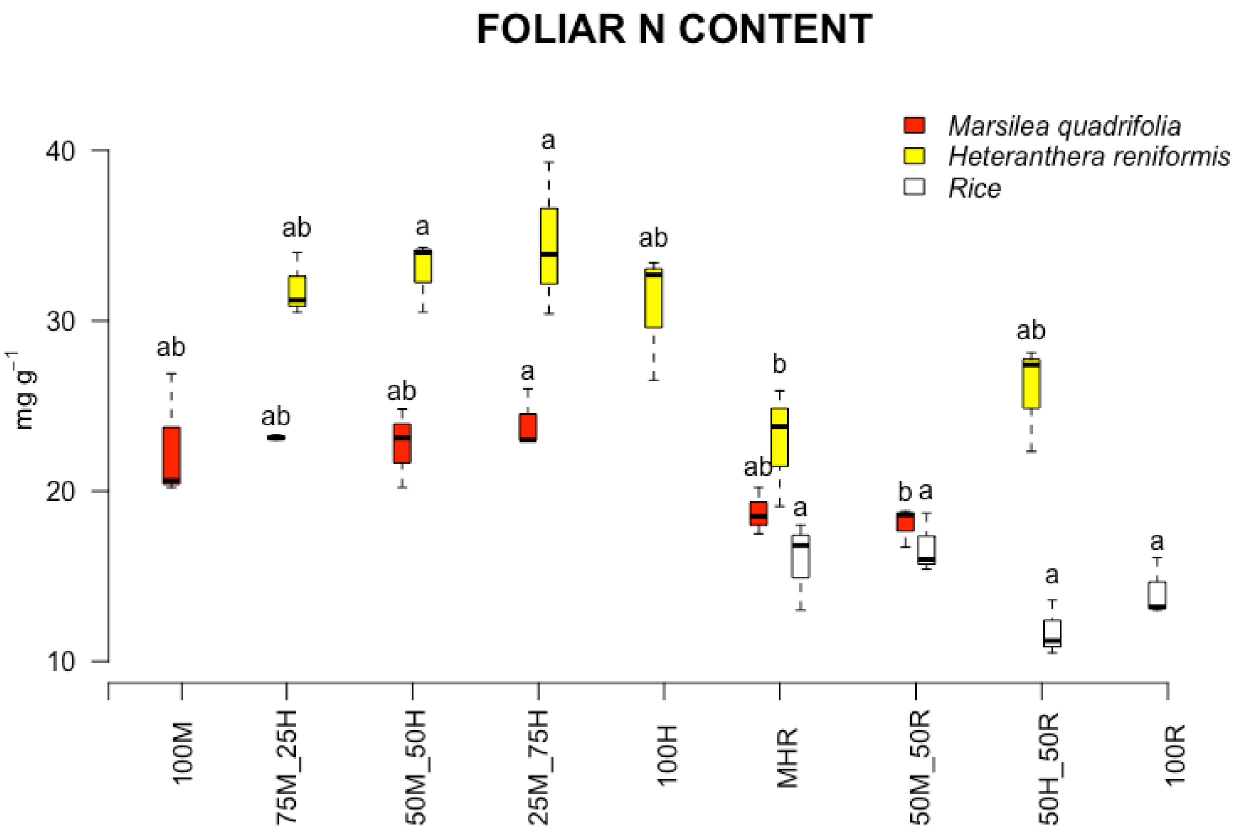

Figure 5. *Foliar nitrogen content of the species. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot condition within the same species according to the Tukey's post-hoc HSD tests.*

Foliar P content was significantly affected by species but not by treatment (Table 1), with higher values for *H. reniformis* (mean foliar P concentration: 0.59±0.02 mg g^{-1}) and *M. quadrifolia* (mean foliar P concentration: 0.52±0.03 mg g^{-1}) compared with rice (mean foliar P concentration: 0.18±0.04 mg g^{-1}).

	Species	**Treatment**	**Species × Treatment**	**One-way ANOVA**
CI	$F_{2,64}$=162.896; ***p*<0.001**	$F_{8,64}$=46.914; ***p*<0.001**	$F_{5,64}$=3.661; ***p*=0.006**	*M. quadrifolia:* $F_{5,24}$=4.60; ***p*=0.004** *H. reniformis*: $F_{5,24}$ =33.12; ***p*<0.001** Rice: $F_{3,16}$=94.51; ***p*<0.001**
SLA	$F_{2,32}$=395.065; ***p*<0.001**	$F_{8,32}$=5.23; ***p*<0.001**	$F_{5,32}$=3.194; ***p*=0.019**	*M. quadrifolia*: $F_{5,12}$=1.88; *p*=0.172 *H. reniformis*: $F_{5,12}$ =6.78; ***p*=0.003** Rice: $F_{3,8}$=2.37; *p*=0.147
LDMC	$F_{2,32}$=1243.782; ***p*<0.001**	$F_{8,32}$=3.697; ***p*=0.004**	$F_{5,32}$=2.712; ***p*=0.037**	*M. quadrifolia*: $F_{5,12}$=1.97; *p*=0.155 *H. reniformis*: $F_{5,12}$=6.37; ***p*=0.004** Rice: $F_{3,8}$=8.68; ***p*=0.007**
N	$F_{2,32}$=130.33; ***p*<0.001**	$F_{8,32}$=7.088; ***p*<0.001**	$F_{5,32}$=1.849; *p*=0.131	*M. quadrifolia*: $F_{5,12}$=4.35; ***p*=0.017** *H. reniformis*: $F_{5,12}$=5.57; ***p*=0.007** Rice: $F_{3,8}$=3.72; ***p*=0.061**
P	$F_{2,32}$=39.226; ***p*<0.001**	$F_{8,32}$=0.769; *p*=0.632	$F_{5,32}$=0.995; *p*=0.436	*M. quadrifolia*: $F_{5,12}$=0.6; *p*=0.701 *H. reniformis*: $F_{5,12}$=1.97; *p*=0.156 Rice: $F_{3,8}$=0.88; *p*=0.49

Table 1. *Two-way ANOVAs with species and treatment as explanatory variable for competitive interaction (CI) index, specific leaf area (SLA), leaf dry matter content (LDMC), foliar nitrogen (N) and phosphorus (P) content. Significant value (p<0.05) highlighted in bold. In the last column, results of one-way ANOVA for each species.*

3.3 Reproductive performance

The number of sporocarps of *M. quadrifolia* did not vary in relation to treatment ($F_{5,24} = 0.698$; p=0.63, Figure S2). Conversely, the number of ripe fruits produced by *H. reniformis* was affected by treatment ($F_{5,72} = 74.55$; p<0.001), time ($F_{2,72} = 19.9$; p<0.001) and their interaction ($F_{10,72} = 6.22$; p<0.001). The highest number of ripe fruits was observed in monoculture while the lowest number of fruits was recorded in mixed culture with *M. quadrifolia* (Figure 6). The highest number of fruits was recorded at the beginning of September, with only fruits in monoculture increasing by the end of the cultivation period (Figure S3).

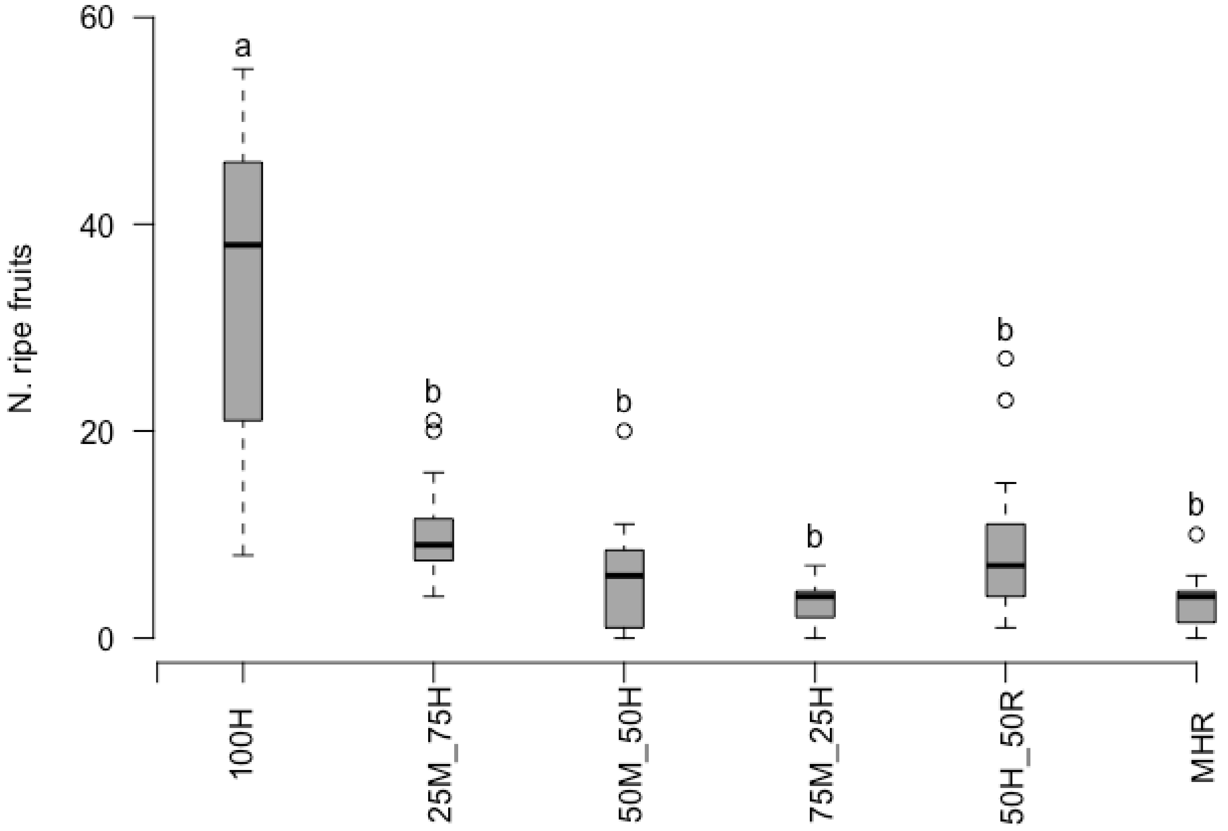

Figure 6. *Number of ripe fruits observed in* H. reniformis*. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot composition according to the Tukey's post-hoc HSD tests.*

4. Discussion

Contrarily to our first hypothesis and the outcome of several experimental studies (e.g. Michelan et al., 2018; Bradley et al., 2019), our study showed that in absence of the high nutrient loads typically found in rice fields, the endangered fern *M. quadrifolia* was not negatively affected by the presence of the invasive species *H. reniformis.* The performance of *M. quadrifolia* was density-dependent and increased with the increasing of the co-occurring species. On the other hand, *H. reniformis* growth was constrained by the presence of *M. quadrifolia*, with the invasive species having the greatest performance in monoculture condition and being not influenced by the density.

The high competitive ability of *M. quadrifolia* in terms of vegetative growth was likely determined by its pioneer ability (Dehondt et al., 2005) that allowed individuals to quickly establish at the beginning of the experiment. Moreover, the well-developed rooting system of *M. quadrifolia,* that consists in a dense belowground network of rhizomes and roots (Csurhes, 2016), helped *M. quadrifolia* to outcompete *H. reniformis,* as normally happens with most competitors (Benson et al., 2004) through the formation of monospecific stands. The highest biomass of *M. quadrifolia* was also influenced by its ability to adjust the angle of the floating leaflets to optimize access to sunlight, thus promoting early growth and shading potential competitors, like *H. reniformis*, in the undergrowth (Wu & Kao, 2011).

H. reniformis, although possessing some of the traits typical of alien species such as high SLA, Chl and foliar N concentration (Lukács et al., 2017), did not show the expected (Richardson et al., 2000) superior competitive ability towards *M. quadrifolia*. In particular, the performance of the invasive species was not density-dependent, with the performance of vegetative biomass being not in line with the higher value of functional traits. Although *H. reniformis* might have been constrained by light availability, it showed high level of plasticity, since it is largely demonstrated that ferns normally report higher value of SLA than monocots. The link between SLA and LDMC was also maintained (Cornelissen et al., 2003) within the three species; *M. quadrifolia* and rice had higher tissue density associated with a nutrient-conservation habit (i.e., higher LDMC; Li et al., 2005) whereas *H. reniformis,* acting as an annual species in paddy fields although it can live longer in other environments (Csurhes, 2016), presented consistently lower LDMC. Nevertheless, our results could have been altered by the controlled conditions we applied, that might be often in contrast to what normally occurs in the field (Poorter et al., 2016).

Our unexpected results may be related to the absence of fertilizer that we have deliberately decided not to supply. Indeed, the goal of our experiment was not to recreate the environmental conditions typical of agroecosystems but rather to investigate the performance of an endangered species vs. an invasive species. The absence of fertilizer affected primarily the performance of rice. As already mentioned, rice did not reach even the inflorescence stage. Moreover, the lack of fertilizer supply influenced the competitive interactions between *M. quadrifolia* and *H. reniformis* in our mesocosm experiment, that was opposite to what normally occur in agroecosystems, both organic and conventional farms, thus altering the performance of both species. However, as suggested by the foliar N content of the two species, *H. reniformis* kept its invasive ability in terms of the nutrient acquisition. Rice also maintained its ability to be a strong accumulator of N as normally occurs in paddy fields, even if with reduced amount than observed in other experimental studies (Yang et al., 2016; Hou et al., 2020). These features are also mirrored in the two co-occurring species. In mixed culture with rice, *M. quadrifolia* and *H. reniformis* presented lower foliar N content, which demonstrates the ability of rice plants to acquire more N than the two co-occurring species. However, rice brought about asymmetric competition for N with the associated plants with a stronger reduction of foliar N content in *H. reniformis* than in *M. quadrifolia.* A possible explanation consists in the presence of arbuscular mycorrhizae that are often hosted in *M. quadrifolia* roots (Radhika & Rodrigues, 2007; Mane et al., 2017). Following the trend of reduced foliar N content, *M. quadrifolia* also exhibited lower SLA than observed in other experimental studies (Wu & Kao, 2011), but had Fv/Fm values comparable to those observed both in rice Italian paddy fields below rice canopy (Corli et al., 2021b) and in experimental studies (e.g., Wu & Kao, 2009).

Nitrogen is an important nutrient affecting rice yield (Jiang et al., 2020) that cannot be ignored since insufficient N supply leads to smaller leaves, lower leaf Chl, less biomass production, and consequently, reduced grain yield and quality (Berenguer et al., 2009; Saberioon et al., 2014). No matter which management paddy fields have, either chemical or organic fertilizers must be supplied in agroecosystems to guarantee an adequate and financially sustainable crop yield. In conventional farming systems, fertilization takes place with urea (EU Regulation 2019/1009/EC) while in organic farms the supply of fertilizer is mainly supplied with manure (EU Regulation 2018/148/EC). Urea, a white crystalline solid containing 46% nitrogen as an animal feed additive and fertilizer, is a fast-release fertilizer with rapid absorption whereas manure is characterized by 0.3-0.5% N and slow release, since N has first to be mineralized. This implies that the N availability differs among farming systems, with lower amount in organic farms.

The low performance of rice plants might have been also influenced secondarily by the plant density we applied. Indeed, dramatic variation in phenological stages, as for example the lack of grain formation occurs when the units of investment limit (i.e., number of plants/m^2) are not maintained, especially when the density of population is higher. In this case, both direct (between individuals of the same species) or indirect (for available resources, i.e., radiation, water, nutrients etc.) competition limits species growth (Caporali & Pignatti, 1995).

Despite the abovementioned limitations, our experiment demonstrated a good performance of *M. quadrifolia* also with the co-occurrence of *H. reniformis* in condition of low N supply with slow release. This leads to a gleam of hope for *M. quadrifolia* conservation in contexts where fertilization is reduced, such as in low input and organic farming systems. In addition, thanks to the long-term persistence of sporocarps in the soil (Pistoja et al., 2006), the "cultivation" of *M. quadrifolia* in organic farms may be guaranteed even longer than two years, the maximum consecutive period of rice cultivation on the same field, according to the rotation rules established by the Regulation of Organic Farming. This study highlights thus the contribution that agricultural environment may give to species conservation: *ad-hoc* agro-environmental compensations or incentives from the EU Rural Development Plan (RDP) may help farmers operating in this context willing to contribute to biodiversity and conservation in agro-ecosystems. This is the case the CLOVER Project (https://clover.unipv.it/), where specific funds are available from the Rural Development Programme (RDP 2014-2020) in Lombardy Region (N-Italy) for farmers willing to maintain rice fields and wet habitats relevant for nature conservation. Doing so, a trade-off between plant management strategies and field productivity is promoted, thanks also to participatory approach between researchers and local farmers (Orlando et al., 2020).

5. Conclusion and future perspectives

Even if *M. quadrifolia* is considered a threatened species in Italy, our mesocosm experiment demonstrated that the species still behaves as a pioneer species, able to quickly colonize open habitats where it is competitively superior to invasive species like *H. reniformis*. On the other hand, the invasive species H. reniformis maintained some of the functional traits typical of invasive alien species but did not show high performance in terms of vegetative growth when fertilizer is not supplied. Therefore, M. quadrifolia can be cultivated in organic farms to guarantee its conservation and partially limit the spread of other invasive species, difficult to handle in such contexts where herbicides are not allowed. This action may be sustained by EU compensation from the Rural Development Plan (RDP), where nature conservation and productivity are mutually connected. However, to develop a robust conservation plan for the species, a better understanding of relationships among species is required. Future research should consider the natural dynamics of species within their natural agroecosystem, with experimental studies considering crop yield, seed/spore banks and the timing of species establishment in the field.

Funding The Grant of Excellence Departments, MIUR-Italy (ARTICOLO 1, COMMI 314 – 337 LEGGE 232/2016), is gratefully acknowledged for the support to one of the authors (Prof. Thomas Abeli).

Acknowledgements The authors would like to thank Dr. Fabrizio Negrini and Mr. Fausto Molinari (University of Ferrara) for technical assistance during cultivation in the Botanical Garden and Dr. Roberta Marchesini who performed the chemical analyses. We are also grateful to Dr. Paolo Cauzzi (University of Pavia) for *M. quadrifolia* cultivation in Botanical Garden of Pavia. The authors would like to thank the farmer R. Caimo Duc from the farm “Azienda Agricola Terre di Lomellina”, Candia Lomellina, Pavia, for having provided the rice seeds.

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Supplementary Materials

Table S1. *Repeated measured two-way ANOVAs with species and treatment as between-subject factors and time as within-subject factor performed for leaf chlorophyll content. Significant value (p<0.05) highlighted in bold. In the last column, results of one-way ANOVA for each species.*

	Chl	**Results for Chl**
Species	$F_{2,1115}$=72.398; **p <0.001**	
Treatment	$F_{8,1115}$=7.961; **p <0.001**	*M. quadrifolia*: $F_{5,420}$=0.95; p=0.447 *H. reniformis*: $F_{5,420}$=5.31; **p<0.001** Rice: $F_{3,280}$=19.69; **p<0.001**
Day	$F_{4,1115}$=2.514; **p=0.04**	*M. quadrifolia*: $F_{4,420}$=11.18; **p<0.001** *H. reniformis*: $F_{4,420}$=15.99; **p<0.001** Rice: $F_{4,280}$=6.77; **p<0.001**
Species × Treatment	$F_{5,1115}$=4.794; **p <0.001**	
Species × Day	$F_{8,1115}$=9.177; **p <0.001**	
Treatment × Day	$F_{32,1115}$=1.564; **p=0.024**	*M. quadrifolia*: $F_{20,420}$ =1.87; **p=0.012** *H. reniformis*: $F_{20,420}$=1.02; p=0.437 Rice: $F_{12,280}$=1.78; **p=0.050**
Species × Treatment × Day	$F_{20,1115}$=1.415; p=0.105	

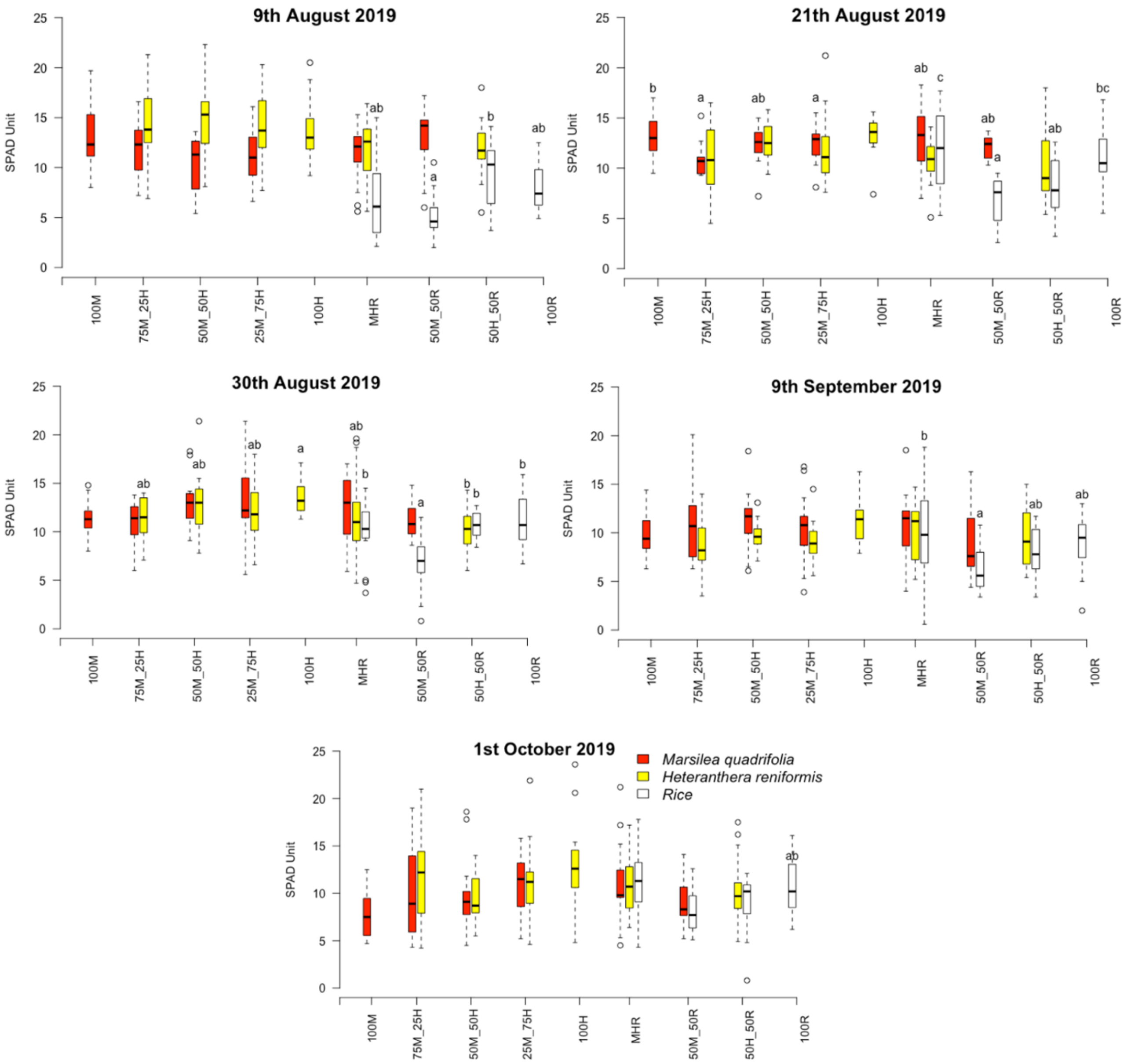

Figure S1. *Leaf chlorophyll content recorded for species through time. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot composition according to the Tukey's post-hoc HSD test.*

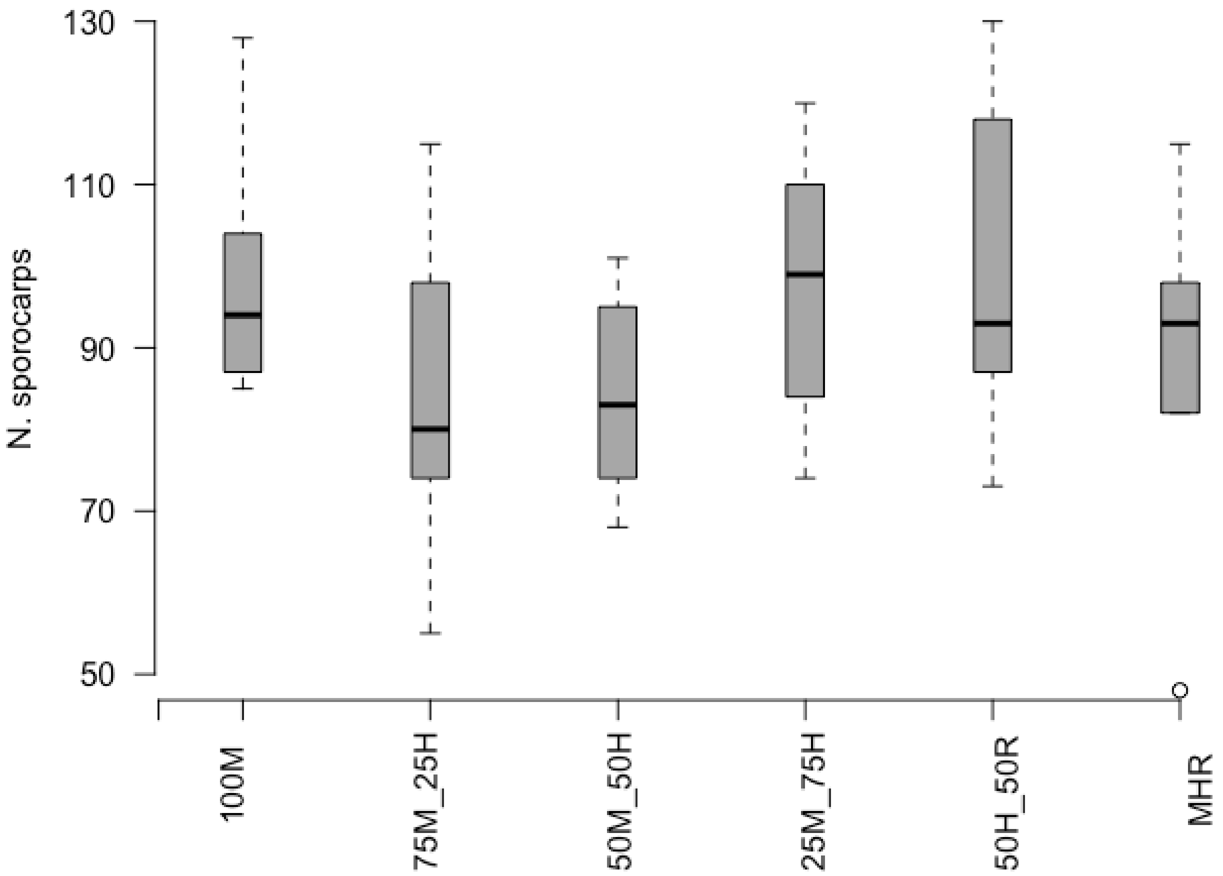

Figure S2. *Number of sporocarps observed in 10-cm*^{2} *of* M. quadrifolia *within pots. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers.*

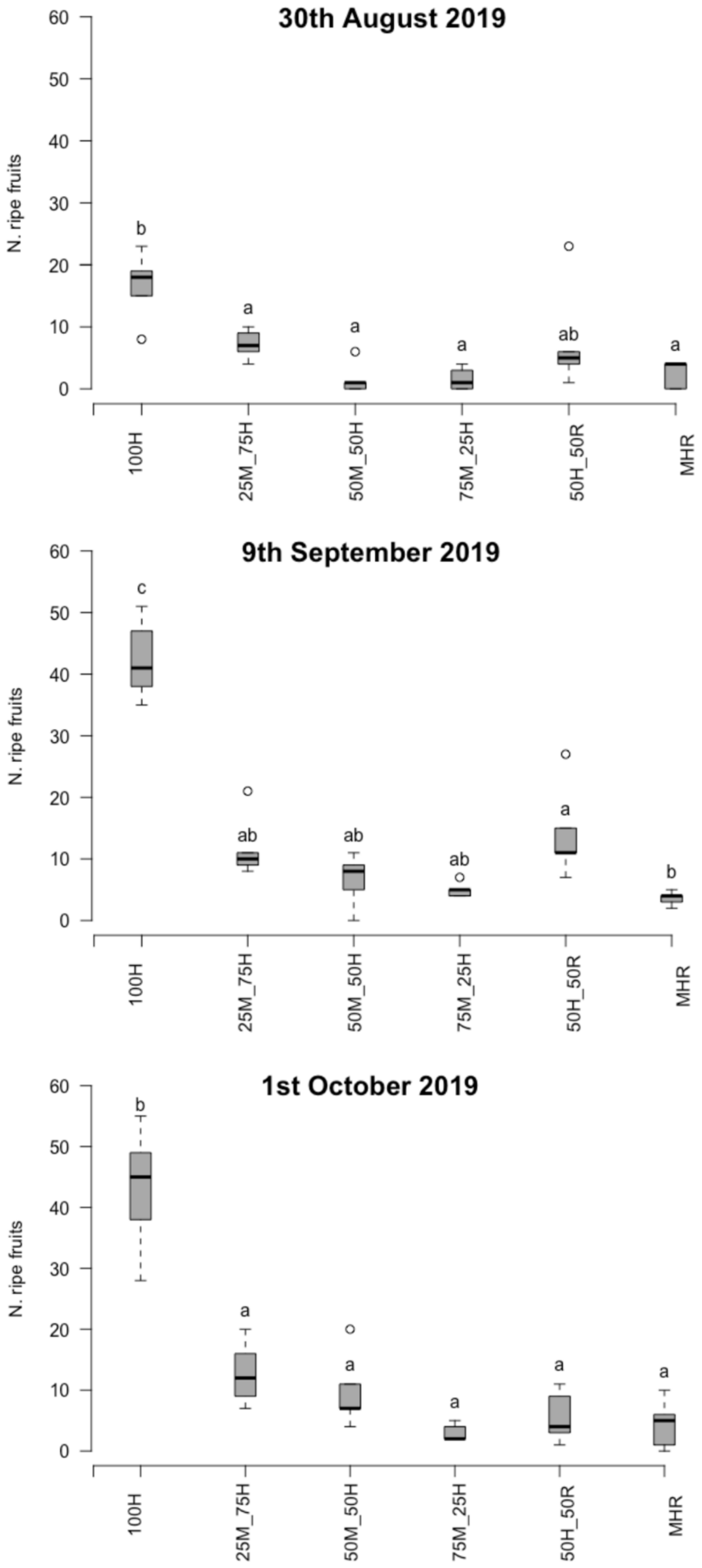

Figure S3. *Number of ripe fruits observed in H. reniformis. Treatments are shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among pot composition according to the Tukey's post-hoc HSD test.*

Preserving biodiversity in agro-ecosystems: conflict or opportunity? The CLOVER Project

In line with the previous and current studies on *M. quadrifolia* and the importance of its conservation in agroecosystems, the CLOVER Project can be seen as the continuum of research topics on the species. The CLOVER Project (https://clover.unipv.it/), with the name being the acronymous of "agroecosistemi e Conservazione in LOmbardia di specie VEgetali Rare di Direttiva Habitat (i.e., "Agroecosystems and conservation of rare plant species of Habitat Directive in Lombardy"), aims at spreading theoretical and practical knowledge to farmers and stakeholders operating within the Natura 2000 Network and Protected Areas or in adjacent area in Lombardy Region, NW-Italy, of the best practices that allow the coexistence between nature conservation and agricultural activities without any lost yield, rather with additional incomes. Among the activities, the project aims at maintaining species with high conservation value such as *M. quadrifolia* and habitat rich in biodiversity in agroecosystems, focusing on pastures, wet meadows, wetlands and rice paddies. The Project has been funded by the Rural Development Program (RDP 2014-2020) of the Lombardy Region, in line with the EU Common Agricultural Policy (CAP); the project last for 24 months, from June 2020 to May 2022.

Of note, the Project has specific actions on the *on farm* cultivation of *M. quadrifolia* in rice fields and in the maintenance of wet meadows listed in Habitat Directive where species with high conservation value such as *M. quadrifolia* occur. I am personally collaborating in managing the activities with the head of the Project, Dr. Simone Orsenigo, Department of Earth and Environmental Sciences, University of Pavia.

1. **Good management practices in rice fields and *on farm* conservation of *Marsilea quadrifolia***

1.1 Introduction

This action aims at demonstrating the best practices that allow a correct management of paddy fields with no yield reduction, jointly with the conservation of the threatened species *Marsilea quadrifolia.* It consists in the *on farm* cultivation of *M. quadrifolia* during the vegetative season (June-September 2021) and in the *ex-situ* conservation of rhizomes during the winter season (from October 2021). The final goal is to spread knowledge to rice farmers operating within Natura 2000 Network on how to manage *M. quadrifolia* cultivation for conservation purpose and its coexistence with rice crop. Since it has been demonstrated that *M. quadrifolia* is not negatively affected by the presence of rice plants, rather it grows better under rice canopy (Corli et al., 2021), during the *on farm* cultivation, ecophysiological data on species growth and rice productivity information have been obtained to address the following question: (i) to what extent the presence of *M. quadrifolia* affects rice performance and productivity? Basing on previous findings, we hypothesed that the threatened species should not negatively affect the performance of the crop species. Answer this question will help in developing a conservation plan for the species in rice fields to be also extended in analogous context.

1.2 Materials and methods

1.2.1 Study area

The action is performed at the farm “Terre di Lomellina” (102 m a.s.l.), Candia Lomellina, Pavia province, Lombardy region, NW-Italy. The farm is located within the Special Protection Area IT2080501 “Garzaie della Lomellina” and the Special Area of Conservation IT2080005 “Garzaia della Rinalda”. Noteworthy, this farm hosts since 2015 a natural population of the species in the canals and in the rice fields (Figure 1).

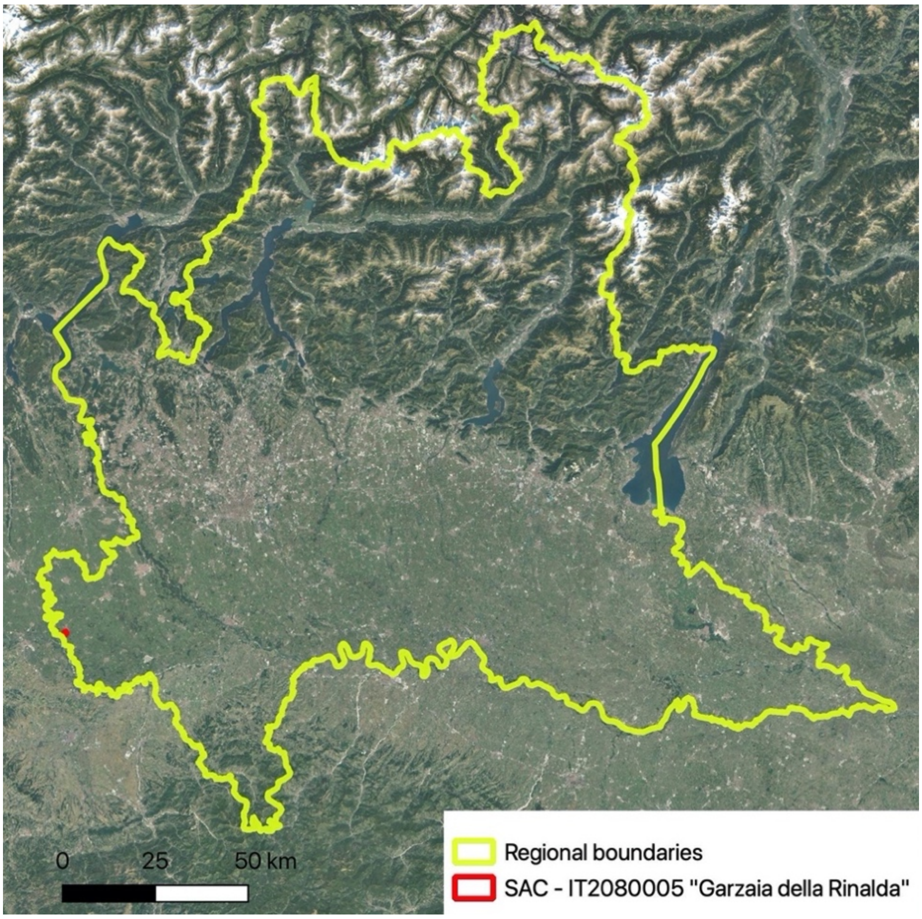

Figure 1. *Location of the study area in Lombardy region, NW-Italy.*

The farm is characterized by an organic farming system, therefore bound at crop rotation as requested by the EU Regulation 2018/148/EC. A correct rotation involves a maximum of two consecutive years of rice cultivation, with the possibility of growing rice for the third consecutive year alternating with two cycles of main crops of different species (Ministerial Decree n. 3286/2016). In the farm, crop rotation is guaranteed with several crop species; a typical rotation used by the farmer is the following: bean (*Phaseolus vulgaris* L.), ryegrass (*Lolium perenne* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), bean, ryegrass, rice, with the latter remaining for only one crop cycle.

The selected rice field is an area of 3.8 ha, cultivated with rice Ronaldo variety. Basing on the Legislative Decree n.131/2017, the variety is characterized by a long grain, also known as "lungo A" (i.e., long A), 6.4 mm long, with a mean final height of 78 cm and maturation after 150 days (Enterisi, 2021). The variety is also highly resistant to intertwined as well as fungi (i.e., *Pyricularia oryzae* Cavara, *Bipolaris oryzae* (Breda de Haan) Shoemaker), and tolerate cold condition both in the germination as well as in the inflorescence stage.

1.2.2 Experimental design

Rice seeds were sown on 9th May 2021. Seeds were sown in rows at 15 cm distance; weed management was guaranteed twice with grooming, on 15th May and on 25th May. On 20th June, water was added to the field. On 23th June 2021, 3 macroplots (4 × 10 m) were identified and delimited with wooden sticks in the rice field (Figure 2A). Basing on the spreading ability of *M. quadrifolia* observed in an experimental cultivation (Corli et al., 2021), 9 swards (20 × 20 cm) of the species coming from an *ex situ* population from the Botanical Garden of Pavia University were selected and scattered randomly for each plots, with a total of 27 swards (Figure 2B; plot with presence of *M. quadrifolia*, henceforth "presence"). 3 macroplots with the same dimension were individuated in the adjacence of the fenced macroplots; in this case, rice plants were growing in the absence of *M. quadrifolia* (plot with no *M. quadrifolia,* henceforth "absence"). Macroplots were displayed in the middle of the field to avoid the effect of water inflow. Note that because of crop rotation, in 2021 the available field cultivated with rice is adjacent to the border of the SAC but not included (Figure 2C).

***Figure 2**. A) Macroplots within the rice field. B)* Marsilea quadrifolia *sward cultivated in the rice field C) Focus on rice field and* Marsilea quadrifolia *cultivation area.*

To see the effect of *M. quadrifolia* on rice performance and whether the threatened species may affect rice growth inducing ecophysiological stress, chlorophyll content was determined 7 times during the field season at 15 days' interval (from 9th July to 1st October 2021) on both species. Chlorophyll content was determined on 20 healthy leaves/species in both macroplots.

On 1st October, *M. quadrifolia* swards were removed from the field and transferred to the Botanical Garden of the University of Pavia for the *ex situ* conservation during the winter season for the next *on farm* cultivation and demonstrative action foreseen in in summer 2022. On the same day, to see the effect of *M. quadrifolia* on rice reproductive performance and whether the threatened species may affect rice productivity, the upper parts of rice plants including grains were cut from three 1 x 1 m^2 plot within macroplots both in absence and presence of *M. quadrifolia*. Rice grains were cleaned and stored in the dry room of the University of Pavia for three days at constant 20°C temperature and constant relative humidity at 20%. On 5th October, total weight/1 m^2, total number of seeds/1 m^2 and thousand seed weight/1 m^2 were determined by using a fine balance.

1.2.3 Statistical analysis

Data on rice chlorophyll content was analysed by two-way ANOVAs performed with macroplots (factor with 2 levels: presence, absence) and time (factor with 7 levels: i.e., the 7 sampling dates) as explanatory variables. Data on *M. quadrifolia* chlorophyll content was analyzed by one-way ANOVA performed with time as explanatory variable. Data on rice productivity (total weight, total number of seeds, thousand seeds weight) were analyzed by one-way ANOVA performed with macroplots as explanatory variable. Significance of differences between cultivation were assessed by Tukey's post-hoc honest significant difference method (HSD) tests. The statistical analyses were performed with R 3.6.1 (R Core Team, 2019).

1.4 Preliminary results

Chlorophyll content was significantly affected by time, with higher value at the beginning of the field monitoring and a decrease towards the vegetative season (Table 1; Figure 3). Although the effect of macroplots was not significant in determining variation in chlorophyll content on rice plants in presence or absence of *M. quadrifolia*, chlorophyll content was significantly higher in rice plants growing with *M. quadrifolia* at the end of the field season (Table 1).

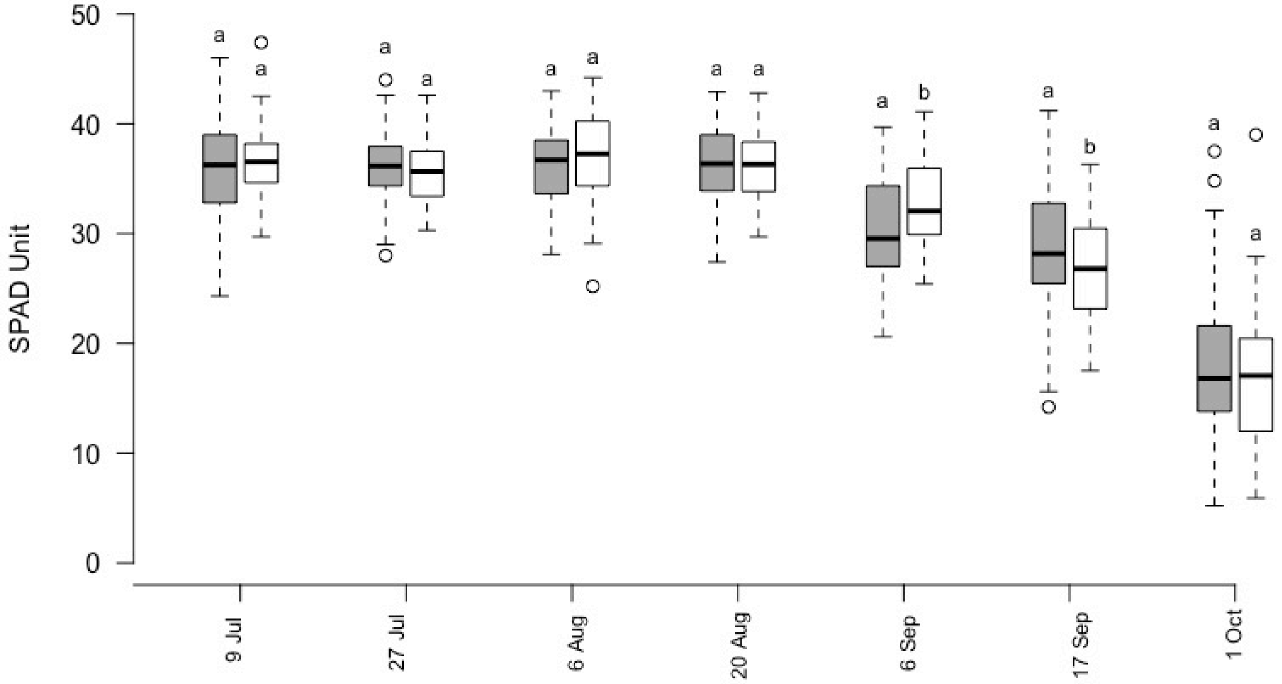

***Figure 3.** Chlorophyll content of rice plants in presence of* M. quadrifolia *(grey color) and in its absence (white color). Time is shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences between plots according to the Tukey's post-hoc HSD tests.*

Response variables	Plot	Time	Plot x Time
Chl Rice	$F_{1,826}$=0.314; p=0.575	$F_{6,826}$=308.451; **p<0.001**	$F_{6,826}$=3.588; **p<0.001**
Chl *Marsilea*		$F_{6,413}$ = 15.26; **p<0.001**	
Total weight	$F_{1,16}$=0.097; p=0.76		
1000 seeds weight	$F_{1,16}$=1.779; p=0.201		
Total n. seeds	$F_{1,16}$=0.461; p=0.507		

Table 1. *Results from ANOVAs performed on rice chlorophyll content,* M. quadrifolia *chlorophyll content, total rice weight on 1 m^2, thousand seeds weight on 1m^2 and total number of seeds on 1m^2, with plot and time as explanatory variables. Significant value (p<0.05) are highlighted in bold character.*

Overall, chlorophyll content attested no stress in both plants, with lower content in *M. quadrifolia* leaves than rice (mean *M. quadrifolia:* 24.9±0.23; mean rice 34.07±0.17), highlighting the actual species-specific differences. Of note, *M. quadrifolia* chlorophyll content was significantly affected by time, with a significant decrease in content during the vegetative period (Table 1; Figure 4).

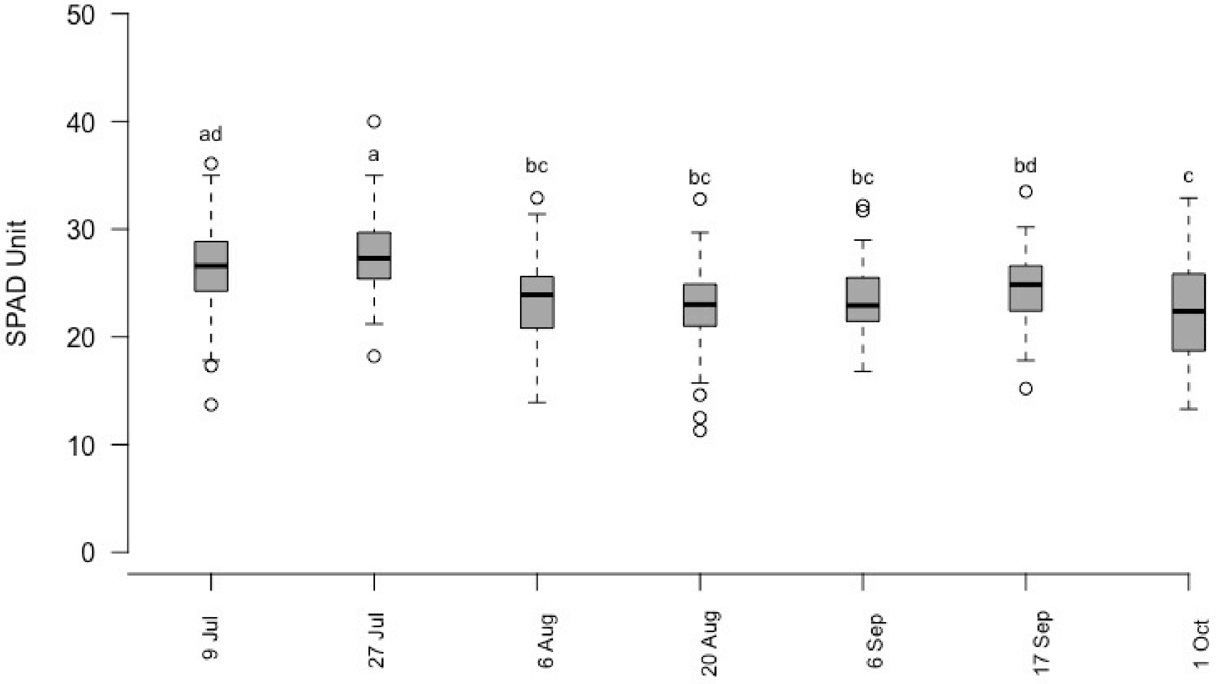

Figure 4. *Chlorophyll content of* M. quadrifolia *through time. Time is shown on x-axis. Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. Letters indicate significant differences among time according to the Tukey's post-hoc HSD tests.*

Although the results on rice productivity were not significant for the assessed variables (Table 1), the presence of *M. quadrifolia* lead to higher rice productivity, with higher total weight (mean presence: 753.01±46.46 g; mean absence: 736.01±28.80 g) and higher number of seeds (mean presence: 22,663.22±1262.55; mean absence: 21,651.22±791.16). Of note, thousand seeds weight was slightly lower in the presence of *M. quadrifolia* (mean presence 33.13±0.33 g; mean absence: 34.00±0.57 g).

4.5 Discussion and future perspectives

The results of the experiment demonstrated that the presence of *M. quadrifolia* does not negatively affects the performance of rice plants, both from an ecological point of view as well as from the main aspect the farmers might be worried, i.e., rice productivity. Rather, although results were not significant, the presence of the threatened species guaranteed higher rice productivity in terms of total weight and number of seeds per m^2. The results are promising since they suggest and highlight for the first time the possibility of conservation of a threatened species in agroecosystem without any reduced performance of the crop species, rather possibly with an increase in crop yield, contributing to the co-existence between nature conservation and productivity. However, this experiment is still ongoing and species performance need to be further addressed and monitored the next year, when the *on farm* cultivation will be reproduced and knowledge on how to manage *M. quadrifolia* conservation in rice fields will be spread to farmers willing to contribute to biodiversity in rice fields. In addition, to have robust outcomes, the experiment should be applied to other paddy fields.

2. Good management practices and mowing of wet meadows

2.1 Introduction

This action aims at demonstrating the best practices that allow a correct management of wet meadows (i.e., grazing and mowing) that allow the maintenance of high level of biodiversity and the conservation of species occurring within them, such as *M. quadrifolia*. The final goal is to spread knowledge to farmers operating within Natura 2000 Network on the correct management of wet meadows that allow the conservation of policy species within this context.

In the area, the farmer might have found unknowingly the right balance between the management of wet meadow with the usual agronomic practices (i.e., grazing and mowing) and nature conservation, allowing the optimal growth of *M. quadrifolia*; grazing and mowing guarantee both high level of nitrogen, to whom the species is adapted (Schneider-Binder, 2014) and limiting level of competition with the surrounding community as well as the reduced progress of vegetation that would normally occur without the periodical removal, necessary for the species establishment as trait of pioneer species, (Schneider-Binder, 2014). The species was studied in experimental plot where was monitored the effect of mowing and grazing during the vegetative season. The following question was addressed: (i) to what extent the effect of grazing and mowing affects *M. quadrifolia* growth and survival? Basing on the current scenario, we supposed that the effect of both agronomic practices should favors species survival. Answer this question will help in developing a reintroduction plan for the species in wet meadows to be also extended in analogous context.

2.2 Materials and methods

2.2.1 Study area

The action is performed at the farm “Bio Angus” (201 m a.s.l.), Sorico, Como province, NW-Italy. The organic livestock farm is located within the Nature Reserve “Pian di Spagna - Lago di Mezzola”, included in the Natura 2000 Network in SPA/SAC “Pian di Spagna e Lago di Mezzola IT2040042/ IT2040022” (Figure 5).

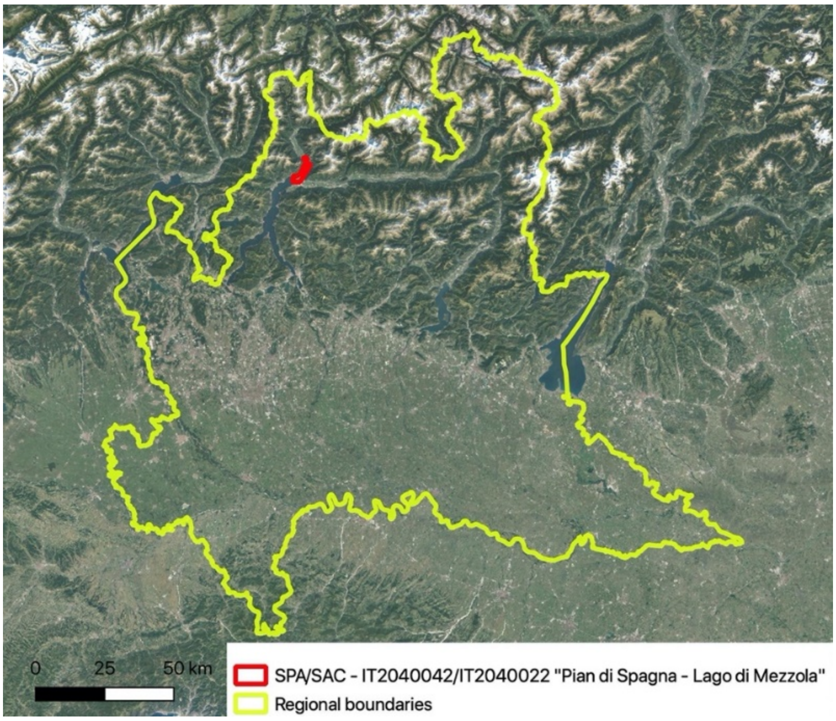

Figure 5. *Location of the study area in Lombardy region, NW-Italy.*

The territory of the Nature Reserve is characterized by a small plain situated between Mezzola Lake and the northern part of the Como Lake, where reeds, wet meadows and cultivated areas are part of the landscape (Osio & Tartarini, 2020). In this context, a population of *M. quadrifolia* of about 1 ha has been discovered in 2018 (Rossi G., personal observation) and it is considered as the largest population in Lombardy and Italy. The species grows in a wet meadow belonging to the farm, 300 m from the Como Lake shore (Figure 6), where Black Angus breed for meat production graze during the year, except for summertime (from the beginning of June to middle September) when they are moved to the alpine pasture. The meadow is framed in the habitat “6510 Lowland hay meadows (*Alopecurus pratensis*, *Sanguisorba officinalis*)” and 6410 “Molinia meadows on calcareous, peaty or clayey-silt-laden soils (*Molinion caeruleae*)” according to the Annex I of Habitat Directive. The meadow is periodically flooded as a consequence of both climatic conditions and the management of the water level of the lake; it is managed by the farmer with harrowing (two times/year, in March and November) and mowing (one time/year, August), with a facultative chopping before mowing. Note that because of adverse climatic conditions, mowing was postponed on 21st September 2021.

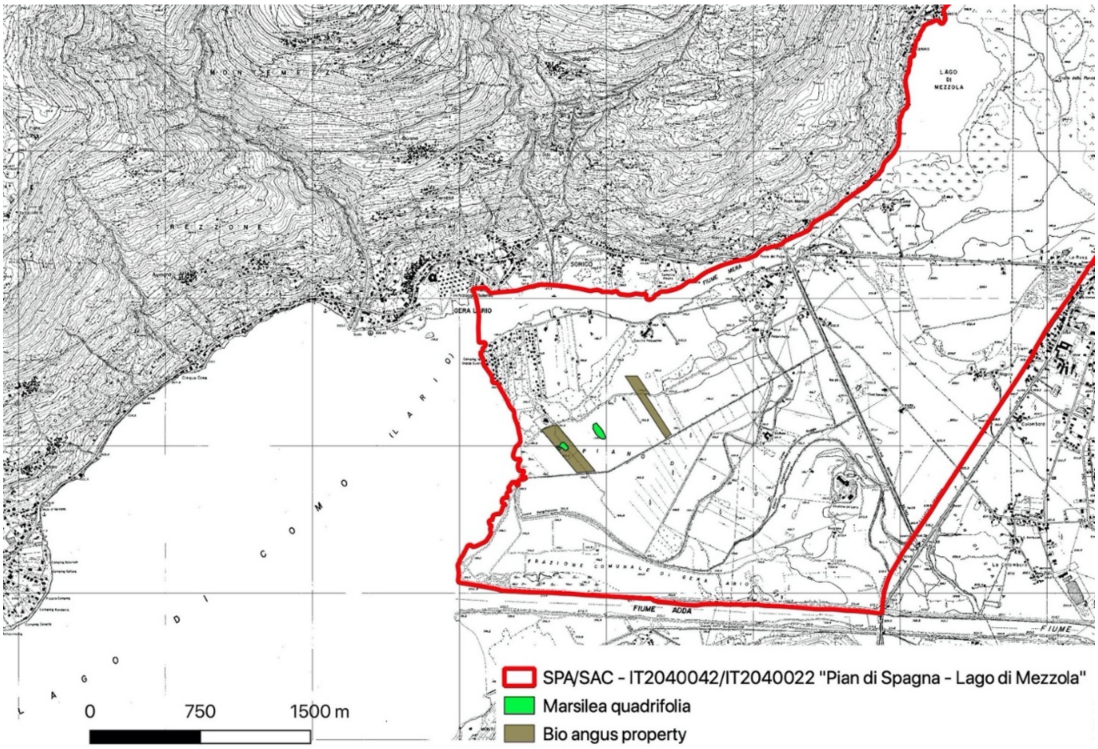

Figure 6. *M. quadrifolia cultivation area. Of note, the population used in Action is the one within the Bio Angus property. The other population has been discovered during a survey in summer 2021.*

2.2.2 Experimental Design

Aiming to understand to what extent growing and mowing may affect *M. quadrifolia* growth and survival, the effect of both agronomic practices was analyzed, reproducing all of the available combinations between them; 4 plots were identified, 5 replicates each, with a total of 20 plots (Table 1).

Plot	Main description	Details
Control	simultaneous effect of mowing and grazing (actual regime managed by the farmer)	random area with the presence of cattle (from the 5th May to 8th June and from late September onward) and mowed (on 21th September)
Mowing	effect of mowing	fenced area mowed
Grazing	effect of grazing	random area with the presence of cattle but not mowed
No management	absence of mowing and grazing	fenced area not mowed

Table 1. *Plot reproduced and relative description.*

In detail, basing on the distribution of the species, on 21th April 2021 two electric fences were used to fence two areas in correspondence of the greatest presence of the species, in order to exclude previously cattle (i.e., “No management” plot). Areas had different dimensions (12 × 25 m, 6 x 12 m, Figure 7A). On 5th May, cattle entered the wet meadow (i.e., “Grazing”; part of “Control” plot). On 8th June, cattle were transferred to the mountain pasture. On 21st September, mowing was performed both within half of the two fenced area (i.e., “Mowing” plot) and outside the fenced area (i.e., “Control” plot) to have more randomized plots.

2.2.3 Data monitoring and analysis

Aiming to understand how the management of wet meadow can influence *M. quadrifolia* growth and survival, we acquired information on the occurring vegetation and species abundance among plots by using a 1 × 1 m wooden square plot (Figure 6B). Two vegetation surveys were scheduled; the former survey was performed on 1st July after the fencing time and before mowing to see the differences between the fenced area and not-fenced area (i.e., presence/absence of grazing). 10 replicates were obtained inside the fences and 10 replicates outside the fenced area, with a total of 20 replicates. To see the effect of mowing, the second survey was scheduled at the beginning of September; however because of adverse climatic conditions that delayed this agronomic practice, vegetation survey was not performed this year.

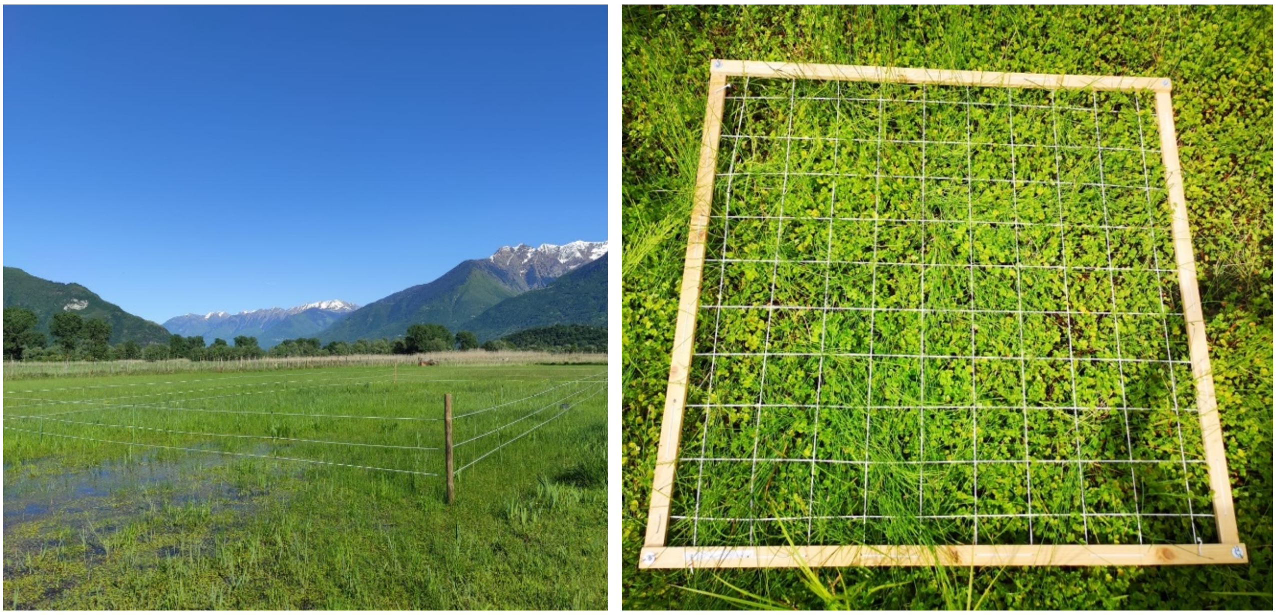

***Figure 7**. A) A fenced area. B) Wooden square plot for vegetation survey and species abundance evaluation.*

As an indication of environmental conditions, water level was also evaluated; water table depth was measured with a graduated PVC pipe at 15 days' intervals in 6 points close to *M. quadrifolia* occurrence; water table depth was measured 10 times from 26th April to 21th September.

2.3 Preliminary results

The first vegetation survey highlighted a total of 30 species (Table 2). Outside the fenced area, species number within plots varied between 5 and 14, with a minimum of 30% species cover and maximum of 104%, with species occupying multiple layers (Table 3). Cover percentage changed between plots, with the highest area occupied by *Marsilea quadrifolia* L.*,* *Agrostis stolonifera* L.*,* *Carex elata* All.*,* *Carex flava* L. and *Equisetum palustre* L. Conversely, the number of the species occurring within plots in the fenced area varied between 4 and 14; the species cover was higher than in plots where cattle was present, with a minimum cover of 62% and a maximum cover percentage of almost 150% (Table 4). In this case, the highest cover was occupied by *Marsilea quadrifolia* L., *Equisetum palustre* L., *Juncus articulatus* L., *Carex flava* L. and *Trifolium hybridum* L.

About *M. quadrifolia,* the species was present in 8 to 10 plots both in the fenced (i.e., presence of grazing) and in the adjacent (i.e., absence of grazing) area. In the first case, its coverage varied between 1 to 90 whereas in the second case, its coverage varied between a single leave (i.e., +) to 96%.

Water table fluctuated during the growing period, with a homogeneous trend among the 6 sampling points (Figure 8). Water table depth was negative (i.e., below soil ground) on average in the early season; then, water level sharply increased by the end of May. From June to the end of the monitoring, water table was on average positive (i.e., soil was flooded).

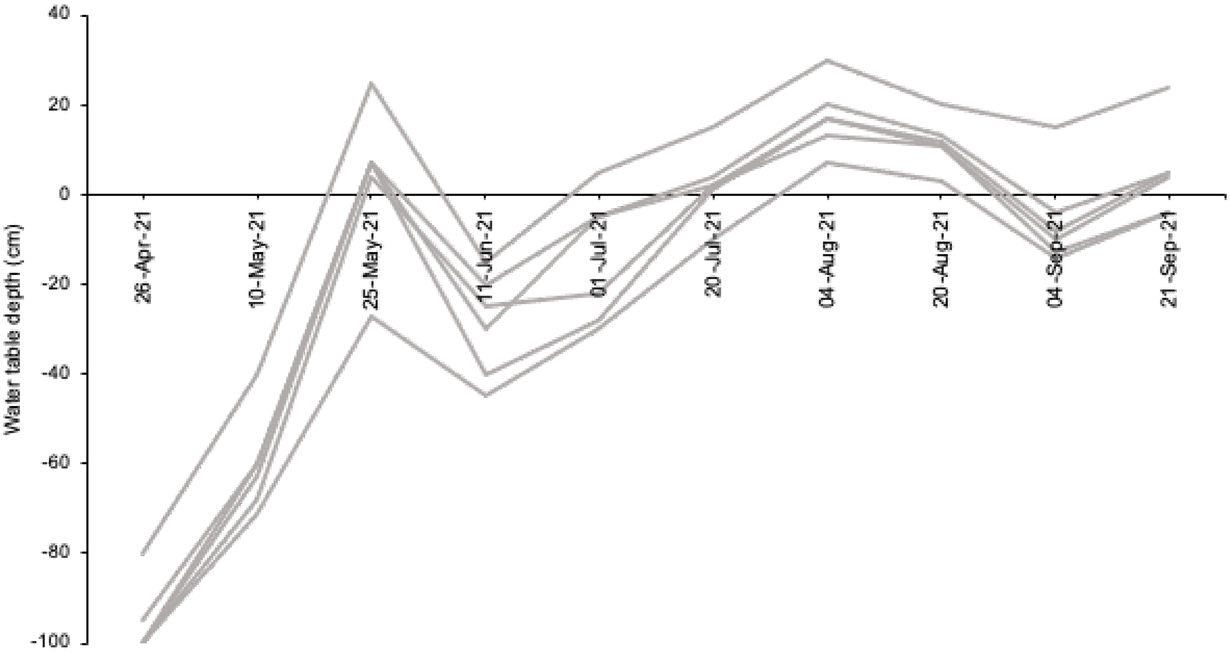

Figure 8. *Water table depth monitored during the growing season in the 6 monitoring points. Dates are shown on x-axis.*

Species	**Family**
Agrostis stolonifera L.	Poaceae
Alisma lanceolatum With.	Alismataceae
Alisma plantago-aquatica L.	Alismataceae
Alopecurus aequalis Sobol.	Poaceae
Bidens *tripartita* L.	Asteraceae
Carex elata All.	Cyperaceae
Carex flava L.	Cyperaceae
Carex hirta L.	Cyperaceae
Centaurium pulchellum (Sw.) Druce.	Gentianaceae
Eleocharis uniglumis (Link) Schult.	Cyperaceae
Equisetum palustre L.	Equisetaceae
Galium palustre L.	Rubiaceae
Gratiola officinalis L.	Plantaginaceae
Iris pseudacorus L.	Iridaceae
Juncus articulatus L.	Juncaceae
Lysimachia vulgaris L.	Primulaceae
Lythrum salicaria L.	Lythraceae
Marsilea quadrifolia L.	Marsileaceae
Mentha spicata L.	Lamiaceae
Myosotis laxa subsp. *caespitosa* (Schultz) Hyl. ex Nordh.	Boraginaceae
Persicaria *hydropiper* L.	Polygonaceae
Persicaria minor (Hudson) Opiz	Polygonaceae
Phragmites australis (Cav.) Trin. ex Steud.	Poaceae
Ranunculus acris L.	Ranunculaceae
Rorippa pyrenaica (Lam.) Rchb.	Brassicaceae
Schoenoplectus mucronatus L.	Cyperaceae
Trifolium campestre L.	Fabaceae
Trifolium hybridum L.	Fabaceae
Veronica scutellata L.	Plantaginaceae

Table 2. *List of the species occurring in the fenced area and in the not-fenced area.*

Replicate 1		**Replicate 2**		**Replicate 3**		**Replicate 4**		**Replicate 5**	
Name	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**
A. stolonifera	15	*A. lanceolatum*	1	*A. stolonifera*	1	*E. uniglumis*	7	*A. lanceolatum*	1
C. flava	12	*A. stolonifera*	10	*C. flava*	40	*E. palustre*	0.5	*A. stolonifera*	20
E. uniglumis	7	*E. uniglumis*	1	*E. palustre*	3	*M. quadrifolia*	90	*E. uniglumis*	2
E. palustre	1	*E. palustre*	1	*G. officinalis*	1	*P. minor*	0.5	*G. officinalis*	8
G. officinalis	8	*G. officinalis*	4	*J. articulatus*	5	*S. mucronatus*	0.5	*J. articulatus*	5
J. articulatus	5	*J. articulatus*	7	*M. laxa*	3			*L. salicaria*	+
M. quadrifolia	45	*M. quadrifolia*	75	*P. australis*	3			*M. quadrifolia*	50
P. minor	1	*P. australis*	2	*R. acris*	5			*M. laxa*	+
T. campestre	+	*S. mucronatus*	1	*T. hybridum*	20			*P. minor*	5
								S. mucronatus	8
Total	**95%**	**Total**	**102%**	**Total**	**81%**	**Total**	**98.5%**	**Total**	**99%**
Replicate 6		**Replicate 7**		**Replicate 8**		**Replicate 9**		**Replicate 10**	
Name	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**
A. lanceolatum	1	*A. lanceolatum*	+	*A. stolonifera*	25	*A. lanceolatum*	+	*A. stolonifera*	+
A. stolonifera	10	*A. stolonifera*	25	*C. elata*	25	*A. p.-aquatica*	0.5	*C. flava*	5
E. uniglumis	1	*C. elata*	15	*C. flava*	1	*A. aequalis*	10	*E. palustre*	1
E. palustre	1	*C. flava*	20	*E. uniglumis*	3	*C. flava*	12	*G. officinalis*	5
G. officinalis	4	*E. uniglumis*	0.5	*E. palustre*	15	*E. uniglumis*	1	*J. articulatus*	10
J. articulatus	7	*E. palustre*	2	*G. officinalis*	2	*E. palustre*	2	*M. quadrifolia*	1
M. quadrifolia	75	*G. officinalis*	1	*J. articulatus*	5	*G. officinalis*	0.5	*M. spicata*	+
P. australis	2	*J. articulatus*	1	*M. laxa*	5	*L. salicaria*	+	*M. laxa*	3
S. mucronatus	1	*M. quadrifolia*	30	*P. minor*	3	*M. quadrifolia*	40	*P. australis*	1
V. scutellata	2	*P. minor*	1	*P. australis*	2	*M. laxa*	0.5	*R. acris*	2
		P. australis	0.5	*R. acris*	+	*P. minor*	2	*T. hybridum*	2
		V. scutellata	1.5	Naked soil	10	*P. australis*	1	*V. scutellata*	+
						S. mucronatus	0.5		30
						V. scutellata	20		
Total	**104%**	**Total**	**97.5%**	**Total**	**96%**	**Total**	**90%**	**Total**	**30%**

Table 3. *Species name and species abundance expressed in percentage outside the fenced area. + refers to a single specimen within the plot. The total percentage cover is expressed below each replicate. Note that below 100% refers to the presence of naked soil, above 100% refers to multiple layers of vegetation.*

Replicate 1		**Replicate 2**		**Replicate 3**		**Replicate 4**		**Replicate 5**	
Name	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**
A. stolonifera	5	*A. stolonifera*	3	*A. stolonifera*	1	*E. uniglumis*	10	*A. lanceolatum*	+
B. tripartita	0.5	*B. tripartita*	+	B. spp	+	*E. palustre*	10	*A. p.-aquatica*	1
C. flava	12	*C. flava*	10	*C. flava*	15	*M. quadrifolia*	95	*B. tripartita*	+
E. uniglumis	15	*E. uniglumis*	+	*E. uniglumis*	10	*P. minor*	+	*C. flava*	4
J. articulatus	2	*E. palustre*	40	*E. palustre*	4			*E. uniglumis*	5
M. quadrifolia	96	*I. pseudacorus*	25	*G. officinalis*	+			*E. palustre*	2
M. laxa	+	*J. articulatus*	2	*M. quadrifolia*	90			*G. officinalis*	5
P. hydropiper	+	*M. laxa*	20	*P. minor*	3			*J. articulatus*	2
P. minor	15	*P. australis*	+	*S. mucronatus*	10			*M. quadrifolia*	90
								P. minor	8
								P. australis	3
								R. pyrenaica	+
								S. mucronatus	3
Total	**145.5%**	**Total**	**100%**	**Total**	**133%**	**Total**	**115%**	**Total**	**123%**

Replicate 6		**Replicate 7**		**Replicate 8**		**Replicate 9**		**Replicate 10**	
Name	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**	**Name**	**Abundance (%)**
A. stolonifera	7	*A. stolonifera*	1	*A. stolonifera*	1	*A. stolonifera*	2	*A. stolonifera*	+
C. hirta	3	*B.* spp.	2	*B.* spp.	2	*A. p.-aquatica*	+	*C. flava*	15
C. flava	25	*C. flava*	25	*C. flava*	25	*C. flava*	10	*C. pulchellum*	+
E. uniglumis	1	*E. palustre*	25	*E. uniglumis*	1	*E. uniglumis*	1	*E. uniglumis*	2
E. palustre	10	*G. officinalis*	+	*E. palustre*	3	*E. palustre*	1	*E. palustre*	10
G. officinalis	2	*J. articulatus*	13	*G. officinalis*	5	*G. officinalis*	1	*G. palustre*	+
J. articulatus	33	*L. salicaria*	5	*J. articulatus*	5	*J. articulatus*	20	*J. articulatus*	30
M. quadrifolia	3	*M.* spp.	5	*L. salicaria*	3	*L. salicaria*	2	*L. vulgaris*	+
M. laxa	1	*M. laxa*	15	*M. quadrifolia*	45	*M. quadrifolia*	10	*L. salicaria*	1
P. minor	3	*R. acris*	10	*M. laxa*	10	*M. laxa*	+	*M. quadrifolia*	+
P. australis	2	*S. mucronatus*	1	*P. australis*	3	*P. minor*	7	*M. laxa*	7
S. mucronatus	10	*T. hybridum*	5	*R. acris*	5	*P. australis*	1	*P. australis*	5
						S. mucronatus	5	*R. acris*	+
						V. scutellata	2	*T. hybridum*	25
Total	**100%**	**Total**	**107%**	**Total**	**108%**	**Total**	**62%**	**Total**	**95%**

Table 4. *Species name and species abundance expressed in percentage inside the fenced area. + refers to a single specimen within the plot. The total percentage cover is expressed below each replicate. Note that below 100% refers to the presence of naked soil, above 100% refers to multiple layers of vegetation.*

2.4 Discussion and future perspectives

Although the results are preliminary and still data needs to be acquired to understand to what extent the effect of mowing and grazing can affect the growth and performance of *M. quadrifolia* in wet meadows, our data suggests that *M. quadrifolia* is highly present and not negatively affected by the presence of cattle that graze in the wet meadow. Nevertheless, information on the effect of mowing on the species growth are missing and will be acquired the next year. Moreover, the fluctuation of the water table depth does not negatively affect the performance of the species.

The outcome of this Action is promising since it suggests that the agronomic techniques of wet meadow that the farmer applied for managing its farm guarantee the survival and growth of a species of high conservation interest. However, this study must be further addressed and will continue during next years in order to better understand the effect of the agronomic techniques and water level fluctuation on *M. quadrifolia* growth, with the purpose to extend this study in similar context.

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Target species: *Kosteletzkya pentacarpos* (L.) Ledeb.

Relative importance of site selection and aftercare in the reintroduction of the policy species Kosteletzkya pentacarpos (L.) Ledeb.

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Abstract

Plant translocation is a valid tool commonly used for the conservation of many threatened species, with the main objective to establish long-term resilient and self-sustaining plant population. Although several plant translocations have been performed and are ongoing, some of them have not succeeded: the main causes of failure are both in the pre-translocation phases (e.g., recipient site selection) and post-translocation phases (e.g., plant and site management), where the choice of the erroneous site, the lack of post-translocation monitoring (e.g., understanding of competitive effect with natural community and invasive species) and the insufficient adaptive management (e.g., through weeding, fencing) lead to unsuccessful translocation.

We present here the results of a study on both pre-translocation and post-translocation phases on the performance of the halophyte herb *Kosteletzkya pentacarpos* (L.) Ledeb, focused on the relative importance of site suitability and aftercare. The species was studied in two different sites that represent opposite ends of the ecological niche of the species (freshwater and brackish sites), both in plots with the natural cover of the local vegetation (mainly *Phragmites australis* (Cav.) Trin. ex Steud. and *Juncus* L. spp.) and in plots where the natural community has been cleared. Environmental data as well as data on plant growth, ecophysiology and reproductive performance were obtained.

K. pentacarpos performed better in the brackish site, experiencing greater vegetative growth and reproductive performance, especially in the presence of the local vegetation. On the other hand, plants growing in the freshwater site had lower performance and were more negatively affected by the local vegetation.

Our study demonstrated that environmental characteristic of the sites affected the performance of the species to much a greater extent than interactions with co-existing species and site management as well; this highlighted that a selection of the suitable recipient site is crucial for guarantee a successful translocation.

Keywords policy species · interspecific relationship · halophyte herb · post-translocation management · site-effect · translocation

1. Introduction

Plant translocation in its many forms (reintroduction, conservation introduction and population reinforcement; IUCN, 2013) is a valid tool for the conservation of many threatened plant species (Abeli & Dixon, 2016). The primary objective of any translocation is the establishment of resilient and self-sustaining populations, enhancing species survival with a long-term perspective (Guerrant & Kaye, 2007; Godefroid et al., 2011). Moreover, ensuring that threats that cause the declines in wild populations can be ameliorated in the recipient reintroduction site is necessary for a successful plant translocation (Dalrymple et al., 2012).

Hundreds of plant translocations have been documented and several are currently ongoing worldwide (e.g., Fenu et al., 2019; Soorae, 2021; Abeli et al., 2021), supported by guidelines rooted in best conservation practices and experimental evidence (e.g., IUCN, 2013; Rossi et al., 2013; Commander et al., 2018; CPC, 2019). However, translocations are challenging actions with an high risk of failure, with only a few having succeeded in establishing self-sustaining populations (Godefroid et al., 2011; CPC, 2019).

Both pre-translocation phases (e.g., recipient site selection) and post-translocation phases (e.g., plant and site management) are key aspects of successful translocations. Pre-translocation phases include knowledge on species biology and habitat requirement, environmental conditions, but also functional ecosystem processes (CPC, 2019): the more that is known about the target species' habitat requirements and biology, the greater the probability of success. The selection of the most suitable site has been demonstrated to be a crucial step in this phase for determining a successful translocation (Abeli & Dixon, 2016; Reiter et al., 2017). Nevertheless, although in most translocations pre-translocation phases received great attention (Godefroid et al., 2016), post-translocation site management has received little attention, which may explain why early successes observed within 5 years after translocation are often followed by a decrease in plant performance over time (e.g., Drayton & Primack, 2012). Indeed, once a target species is released in the recipient community, its establishment depends on several factors such as the interaction between the target species and the recipient community (CPC, 2019), that involves for example hybridization, pollinator availability, competition (e.g., LaBar et al., 2014; Laguna et al., 2016). Therefore, emphasis should also be given to the post-translocation phases, that include the long-term monitoring of translocated plants and post-release site management i.e., aftercare; these phases have been demonstrated to positively impact post-translocation establishment and survival of plants (Bontrager et al., 2014; Daws & Koch, 2015). In some extreme cases continuous post-translocation management is the only possibility to maintain reintroduced populations (Adamski et al., 2020) Indeed, competition with the natural communities, weeds, herbivory, below average rainfall are just a few examples of unexpected threats that may affect translocation outcomes and need to be controlled through adaptive management of the out-planting site (e.g., through weeding, fencing, irrigation, respectively). Particularly, weeds removal and native and/or invasive plant management has proved to effectively maintain translocated population viable (Bontrager et al., 2014; Daws & Koch, 2015; Tischew et al., 2017; Commander et al., 2018).

Competition is a strong driving factor in wetlands (Merlin et al., 2015), where plant communities commonly consist of extensive stands dominated by one or a few species forming a dense tall coverage (Weiher & Keddy, 1995). In this context, knowledge of the relationship between dominant species and other species is extremely important for understanding vegetation dynamics, especially if the resulting competitive effect of the dominant species may reduce the establishment and growth opportunity for non-dominant species, with negative consequences for reintroduced threatened plants.

In this study we focused on the pre- and post-translocation phases on *Kosteletzkya pentacarpos* (L.) Ledeb. performance, commonly known as seashore mallow. This species is a perennial halophilous herb belonging to Malvaceae family, occurring in southeastern USA, Western Asia and Southern Europe, where it grows in brackish to saline coastal wetlands (Pino & De Roa, 2007; Ercole et al., 2013). *K. pentacarpos* is listed as Vulnerable (VU) in the European Union Red List (Bilz et al., 2011) as well as included in the Annexes II and IV of the EU Directive 92/43/EEC “Habitats” and in Annex I of the Bern Convention (Rossi et al., 2016). Reintroduction attempts have been performed in Europe, with experimental translocations in Spain (Llobregat Delta, Catalonia, Pino & De Roa, 2007), Corsica (Fenu et al., 2019) and Italy (“Bosco della Mesola” Natural Reserve, Ferrara; Brancaleoni et al., 2018). In Italy the species is classified as Critically Endangered (Rossi et al., 2016). Here, several seashore mallow populations were known in six regions at the beginning of the 20th century; however, because of land use change and habitat fragmentation, the species experienced a drastic decline, being currently limited to the only area of the Delta of the Po River in two regions, Veneto and Emilia-Romagna (Ercole et al., 2013).

The performance of species populations might be affected both by abiotic and biotic factors: although the species is heliophilous (Snell, 2018), seedling emergence of Spanish translocated populations reported shade tolerance during the first two years, being also facilitated by the canopy (Pino et al., 2007; Pino & De Roa, 2007). In addition, an experimental study conducted in Delaware, United States, showed that the species is intermittently dispersed amongst the dominant marsh communities, not likely to compete with other wetland halophytes rather facilitating their recruitment or hinder the progress of migrating wetland halophytes when salinity and waterlogging increased (Voutsina et al., 2015). Nevertheless, despite the increasing dynamics the species reported long-term (Pino et al., 2007), *K. pentacarpos* survival is constrained by its reproductive ability, with the species reporting a reduction in fecundity as a result from biotic agent and recruitment owing to habitat limitation; moreover, the species depends on a fluctuating seed output and is characterized by a transient seed bank (Pino et al., 2007). Lastly, environmental characteristics of sites might affect species growth, with waterlogging condition negatively altering the performance of the species (Zhou et al., 2011).

One of the problems that makes difficult the management and reintroduction of *K. pentacarpos* is the dynamics of natural vegetation in which the species embedded, with contrasting trends in the studied populations. Indeed, an experimental translocation performed in Spain reported an increase species dynamic over nine years of study (Pino et al., 2007), whereas other studies (e.g. Voutsina et al., 2015) highlighted that its decrease is strictly linked with its reproductive ability. In addition, the species is not characterized by an invasive character (Voutsina et al., 2015; Qin et al., 2015), being found intermittently dispersed amongst the dominant marsh communities, not likely to compete with or hinder the progress of migrating wetland halophytes when salinity and waterlogging increased (Voutsina et al., 2015).

Therefore, this study aims to investigate the relative importance of site suitability and aftercare on performance of two reintroduced populations of seashore mallow at the “Bosco della Mesola” Natural Reserve (Italy) (Brancaleoni et al., 2018). The species has been studied in two different sites that represent opposite ends of the ecological niche of the species (freshwater and brackish sites), both in plots with the natural cover of the local vegetation (mainly *Phragmites australis* (Cav.) Trin. ex Steud. and *Juncus* L. spp.) and in plots where the local vegetation has been cleared. The following questions were addressed: (i) to what extent the performance of the species is affected by environmental characteristics of the site and by post-translocation vegetation management? (ii) what is the effect of the local vegetation on species performance i.e., do the species compete for available resources? We hypothized that: (i) the species should grow better in the brackish site, as previously noted and removal of vegetation should guarantee a better performance of the species (ii) seashore mallow should be negatively affected by competitive co-occurring species. Answers will help to improve the knowledge on species requirement and ecological needs for a long-term monitoring, guaranteeing the survival of the translocated species.

2. Material and methods

2.1 Study species

Kosteletzkya pentacarpos (L.) Ledeb. is a perennial plant 1-2 meter high. The leaves are half wide as long, typically triangular with spreading basal lobes (hastate). The flowers are pink and may be solitary on axial line or in cyme, leafy panicles. The fruits are black capsules with five compartments that contain one seed each. The species flowers from July to September, while fruiting occurs from August to October (Ercole et al., 2013). The seeds are dispersed by barochory and hydrochory; they are equipped with air sacs that ensure flotation (Poljakoff-Mayber et al., 1992), with waterproof teguments allowing survival even in saline habitats. Although the species is characterized by rapid growth and high adult longevity, studies conducted on Spanish populations demonstrated that species reproduction is limited both by transient and shallow soil seed bank and by depletion of fecundity with age, with a decrease in seed production after some years (Pino & De Roa, 2007; Pino et al., 2007). Seed viability is also jeopardized by parasites: insects are responsible for seed abortion (e.g., *Oxycarenus lavaterae* Fabricius 1787, *Crocidosema plebejana* Zeller 1847; Voutsina et al., 2015) and fungi may live on seed surface (Monés, 1998). The species is diploid, with 2n=38 (Blanchard, 2012).

Across its range, seashore mallow grows in brackish to saline, nutrient-rich habitats. It also occurs in coastal wetlands, river deltas, and estuaries (Ercole et al., 2013; Pino & De Roa, 2007). The species is dependent on edaphic humidity and salinity, living at the edge of coastal lagoons or water bodies close to the sea on humid, sub-salty, sandy or loamy, acidic, neutral or basic, occasionally flooded soils. In addition, it grows in sunny areas or with scarce tree cover (Abeli et al., 2016). The species grows in natural communities mainly characterized by reeds, rushes, river mouths and subsalt wetlands along the coast with *Phragmites australis* and *Juncus maritimus* Lam., referable to the alliance *Juncion maritimi* Br.-Bl. ex Horvatic 1934 (Pino & De Roa, 2003).

Seashore mallow populations are not equally distributed among its range: in North America and Asia the species occurs abundantly, whereas European populations are characterized by narrow distribution in small isolated patches along the seaside marshes of the coasts of the Caspian, Black and Mediterranean Seas (Kikvidze & Ohsawa, 2001; Figure 1; see "Taxonomic and genetic debate" in the Supplementary Materials for details). High agricultural and urban pressures (e.g., land reclamation, drainage) have caused the loss of many of seashore mallow stations in the last few decades in Europe.

Figure 1. *Distribution map of* K. pentacarpos *over the last 10 years (2011-2021). Data obtained from GBIF, 2021.*

2.2 Study area

The experimental study was carried out at the "Bosco della Mesola" Nature Reserve, Northern Italy, a European Union Site of Community Importance (IT4060015; 44°50′N, 12°15′E, 1088 ha, 0-2.8 m a.s.l.). The study area lies on a dune system consisting of sand dunes and dune slacks with NE-SW orientation. The area is prevalently covered with woodlands that represent a relic of ancient coastal forests (Piccoli & Gerdol, 1984). The woodlands present transitional features between Mediterranean and Central European forest types, with a xerophilic *Quercus ilex* community on high, more recent arid dunes, a mesophilic *Carpinus betulus–Quercus robur* community on flat, more ancient and less arid dunes and *Populus alba–Fraxinus angustifolia* community on dune slacks (Gerdol et al. 1985).

The Nature Reserve represents the main area hosting seashore mallow populations in the Emilia-Romagna region, where its presence was confirmed in 2014 after a long period without any records (Brancaleoni et al., 2018). The Nature Reserve is in an area of reclaimed land with a dense network of canals supplying fresh water to inland sector. In the south-eastern peripheral sector, the local aquifer is in connection with the sea and is therefore characterized by salt water. The entire coastal area in this region is subjected to subsidence, which results in increasing levels of salt-water intrusion towards the inland sector of the Nature Reserve.

2.3 Experimental design

Preliminarily, an experimental reinforcement has been performed in 2015 in an existing natural population occurring in Goara, a site that represent the optimum ecological requirement of the species (Brancaleoni et al., 2018). Moreover, the species was also reintroduced in Elciola site, a site representing opposite ends of the ecological niche of the species (Brancaleoni et al., 2018).

Goara site, about 300 m^2, receives brackish water from the surrounding lagoon. Here, the dominant species is *Juncus* spp. (*J. acutus* L. and *J. litoralis* C. A. Mey.). The Goara site is also characterized by *Calystegia sepium* (L.) R.Br., *Phragmites australis*, *Samolus valerandi* and *Tripolium pannonicum* (Jacq.) Dobrocz. Elciola site, about 100 m^2, is a freshwater site. Here, the dominant species is *Phragmites australis*. The vegetation in the Elciola site is also characterized by *Cyperus fuscus* L., *Juncus articulatus* L., *Mentha aquatica* L., *Samolus valerandi* L., *Schoenoplectus tabernaemontani* (C.C. Gmel.) Palla and *Erianthus ravennae* (L.) P.Beauv.

Aiming to understand the most suitable site allowing species survival and growth, experimental plots were set up in 2019 at the two sites, where local vegetation was either maintained or removed. The sites were primarily surveyed in May, prior to setting up the experimental plots, to identify all the seashore mallow individuals occurring at each of the two sites. We found a total of 36 individuals, 13 individuals at Elciola and 23 at Goara. At each site, we set up two plots where the natural community was either maintained (control plot) or removed (removal plot). In the removal plots the aboveground biomass of all plants was cut and removed at the beginning of June. Plant regrowth was periodically trimmed and removed at 20-day intervals during the growing season. In Elciola, 6 individuals were selected in the control plot and 7 in the removal plot; in Goara, 12 individuals were selected in the control plot and 11 in the removal plot (Figure 2). The control plot was characterized by the reinforced population, the removal plot by the natural population.

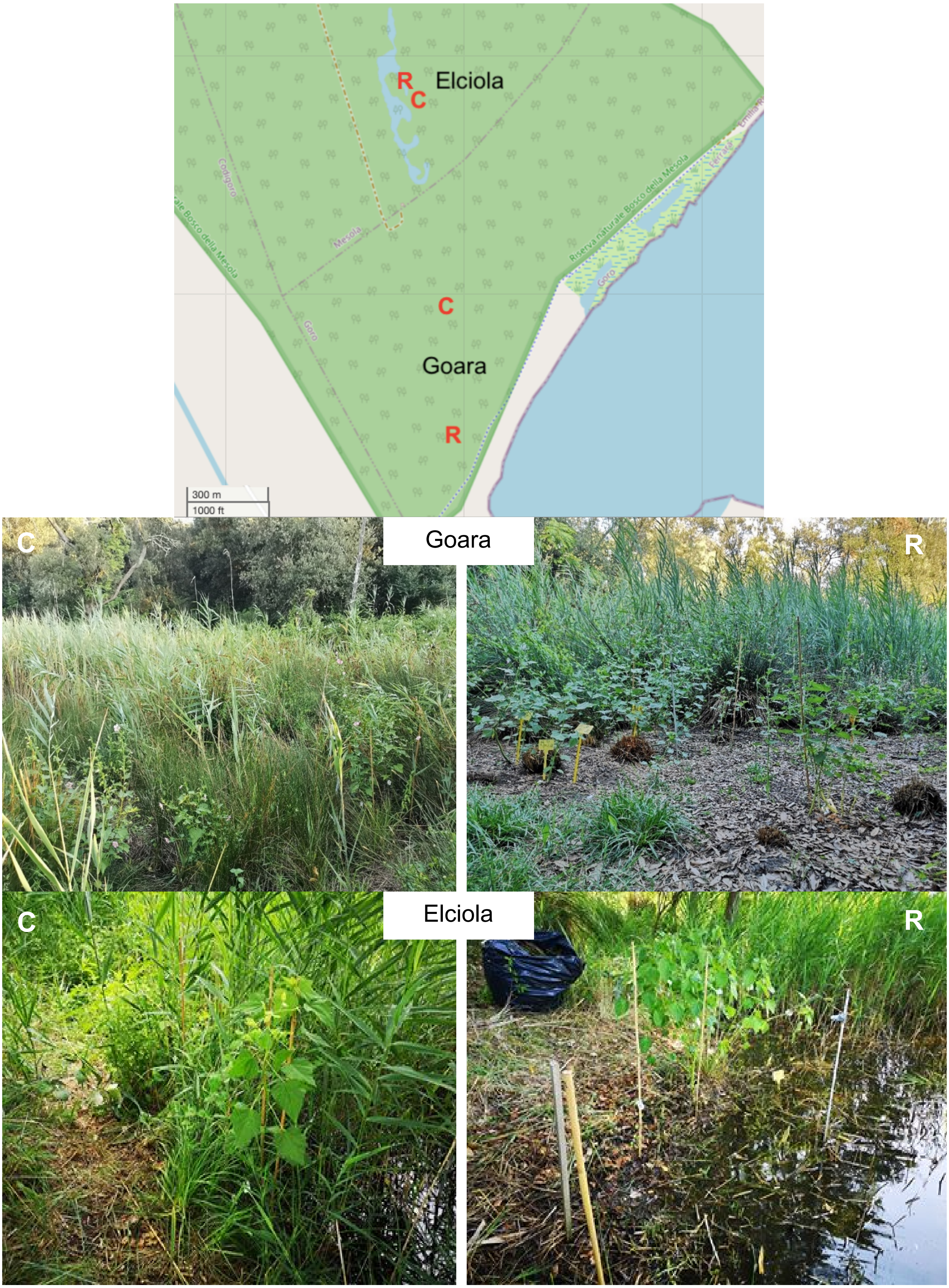

Figure 2. *Study site and focus on plot area. C refers to “control plot”, R refers to “removal plot”.*

2.4 Response variables

The plots were surveyed at about 20-day intervals during the growing period and lasted for 127 days, from 18th June to 23rd October 2019; environmental data as well as data on plant growth, ecophysiology and reproductive performance of seashore mallow individuals were obtained.

Air temperature and irradiance were recorded at 15-min interval by data loggers (Hobo, Onset Bourne, MA, USA) placed at 75 cm height in each site and plot. Water table depth was measured with a graduated PVC pipe in Elciola and Goara. As the two experimental plots in Elciola were very close to each other, water table was measured at an only pipe in between. In Goara two pipes were used, one for each plot.

Height and stem diameter of all seashore mallow individuals were measured 7 times from the beginning of the experiment until plants reached the fruits ripening stage with a measuring tape and manual calliper, respectively. The diameter was measured at the first (lowest) node. Number of branches was counted on each plant. From 18th July to the end of the experiment, plant senescence was visually assessed according to the BBCH phenology scale (BBCH 90-99, Table S1) that records senescence based on leaf abscission (Meier, 2001).

Leaf chlorophyll content (Chl), chlorophyll fluorescence (Fv/Fm) and net CO_2 exchange rate were determined in three to five healthy leaves from each plant by enclosing one leaf in a leaf chamber. Chl and Fv/Fm were determined 7 times during the peak growing season (from 18th June to 16th September) by using a chlorophyll content meter (CCM-200; Opti Sciences, Tyngsboro, MA, USA) and a modulated fluorometer (OS1-FL; Opti Sciences, Tyngsboro, MA, USA), respectively. Net CO_2 exchange rate was determined once on 18th July, by using an open infrared gas analysis system (LCA-4; ADC Bio-Scientific Ltd, Hoddesdon, Herts, UK) at saturating photon flux density (>1000 μmol photons m^{-2} s^{-1}) and at an air temperature of about 30°C.

Reproductive performance was assessed by recording the phenological phases of blossoming (BBCH 50-59; 18th June-1st October), flowering (BBCH 60-69; 18th July-1st October), fruit development (BBCH 70-79; 18th July-23rd October) and fruit ripening (BBCH 80-89; 18th July-23rd October), according to the BBCH scale (Meier, 2001, Table S1). Number of ripe fruits was counted and collected when available. Since at the end of the growing season we noticed several aborted fruits in the Goara plants, this number was also counted.

Further data on ecophysiological traits and environmental variables were collected in 2020. On the 31st of July, two leaves from each plant the fourth and the fifth leaves from the apex) were collected to determine the foliar nutrient contents. Concentrations of total nitrogen (N) and total phosphorus (P) were determined by colorimetric analysis with the sodium salicylate (Scheiner, 1974) and the molybdenum blue (Crouch & Malmstadt, 1967) method, respectively. Five replicated soil samples were collected from the top 5-cm layer at each site, using a stainless steel cylindrical corer (inner diameter 6.6 cm). Five replicated water samples were also collected with a syringe and 100 ml were filtered. The soil and water samples were taken to the Laboratory of Plant Ecology of Ferrara

University and freezed before analysis. Concentrations of extractable nitrate (N-NO_3), ammonium (N-NH_4) and phosphate (P-PO_4) were determined in soil and water samples through colorimetric analyses at 420-, 690- and 700-nm wavelengths, respectively, using a micro-flow automated continuous-flow analyser (Systea Flowsys, Anagni, Italy). In addition, salinity and electrical conductivity (EC) were measured in water samples with a conductivity-meter (Crison CM 35, L'Hospitalet de Llobregat, Spain). A subsample of leaves was used to determine stable isotope discrimination of leaf carbon ($\delta^{13}C$) and leaf nitrogen ($\delta^{15}N$). Samples were ground to a fine powder and measured with an elemental analyzer (EA 1110, Carlo Erba, Milan, Italy), coupled online with an isotope ratio mass spectrometry (delta Plus XP, ThermoFinnigan, Bremen, Germany) at the Isotopic Geochemistry Laboratory of Parma University.

2.5 Data elaboration and statistical analyses

Data on height, diameter, number of branches, Chl, Fv/Fm and number of ripe fruits were statistically analyzed by repeated measured two-way ANOVAs with site (factor with 2 levels: Elciola, Goara) and plot (factor with 2 levels: control, removal) as between-subject factors and time (factor with 3 to 8 levels, depending on the dates on which the response variable was measured) as within-subject factor. Data on net CO_2 exchange rate, foliar N and P concentrations, $\delta^{13}C$ and $\delta^{15}N$ were statistical analyzed by two-way factorial ANOVAs with site and plot as fixed factors. Data on soil and water chemistry (N-NO_3, N-NH_4, P-PO_4, salinity and electrical conductivity) were analyzed by one-way ANOVA, with site as explanatory variable. The number of aborted fruits collected in Goara was analyzed by two-way ANOVAs, where plot and time were used as explanatory variables.

To visualize the phenological data, a Cumulative Stage Development method was employed, based on the comparative method of Cornelius et al. (2011) that determines the progression of the phenological phase development. Briefly, the onset dates of phenological phases were determined with the help of summation curves. At each sampling date (t), relative frequency values from 0 to 1 at stage i ($xi(t)$) were added to the relative frequency values in the following stages ($x(t,k)$).

$$CF_i(t) = x_i(t) + \sum_{k=i+1}^{p} x(t,k)$$

The resulting cumulative frequency values (CF) per sampling date and phenological phase were then linearly interpolated over time (Table S1). Onset date was defined as the point in time when 50% of all individuals were at the stage of interest.

The Statistical analyses were performed with R 3.6.1 (R Core Team, 2019) and STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1 Environmental data

Mean air temperature during the growing season was almost 1°C higher in Goara (24.04±0.30°C) than in Elciola (23.41±0.27°C). Air temperature was unaffected by plot, with only very slightly higher values in the control plots (mean Goara: 24.10±0.45°C; mean Elciola: 23.42±0.36°C) than in the removal plots (mean Goara: 23.98±0.40°C; mean Elciola: 23.39±0.39°C). Irradiance was almost double in Goara than in Elciola; removal of vegetation implied higher irradiance in Elciola whereas this trend was not observed in Goara (Figure S1).

Water table fluctuated during the growing period, with different trends in the two sites depending on hydraulic management. Water table depth was positive (i.e., the soil was flooded) in Elciola except at the end of the growing season. Conversely, water table depth was negative (i.e., below soil ground) at both plots in Goara, with lower value in the removal plot where the vegetation was removed (Figure 3). Notably, at the end of the growing season water-table depth was negative in both sites.

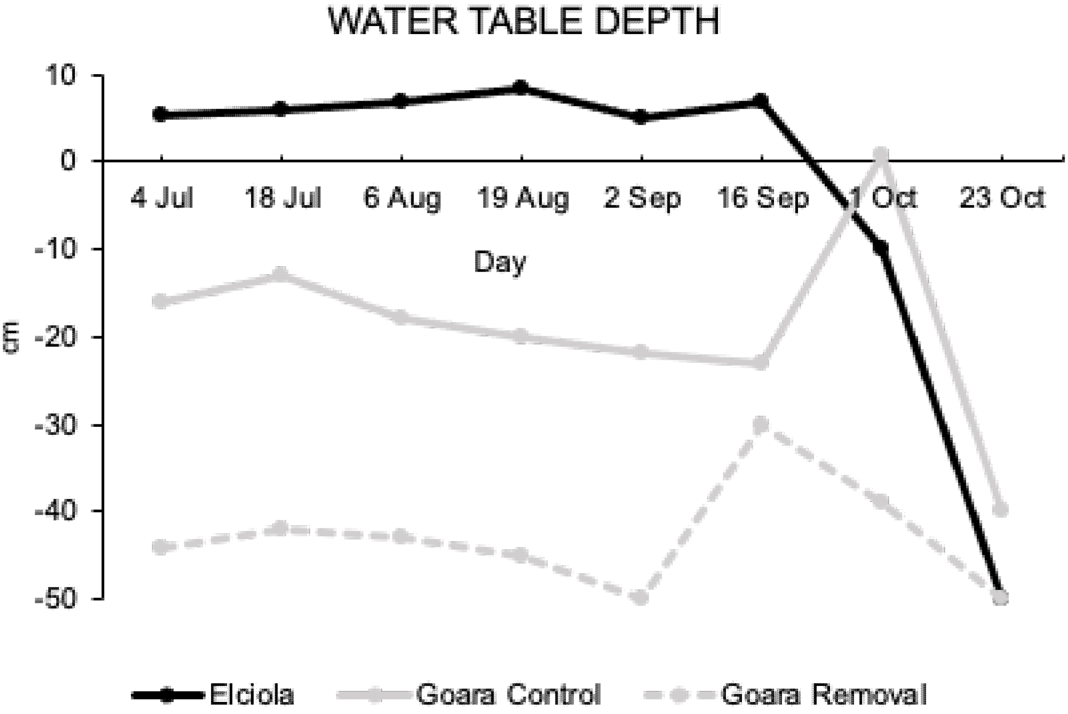

***Figure 3**. Water table depth monitored during the growing season in sites and plots.*

Concentrations of N-NO_3, N-NH_4, P-PO_4 in soil as well as concentrations of N-NH_4, P-PO_4, salinity and electrical conductivity were significantly higher in Goara than in Elciola (Table 1, Table S2). Overall, the soil and water in Goara were characterized by a higher content of nitrate, ammonium, phosphate as well as salinity and electrical conductivity.

3.2 Plant growth

Plant height was significantly affected by treatment, with seashore mallow plants being taller in the control plots in both sites (Table 1, Figure 4). The Goara plants were overall taller, even if the main effect of site was not significant (Table 1).

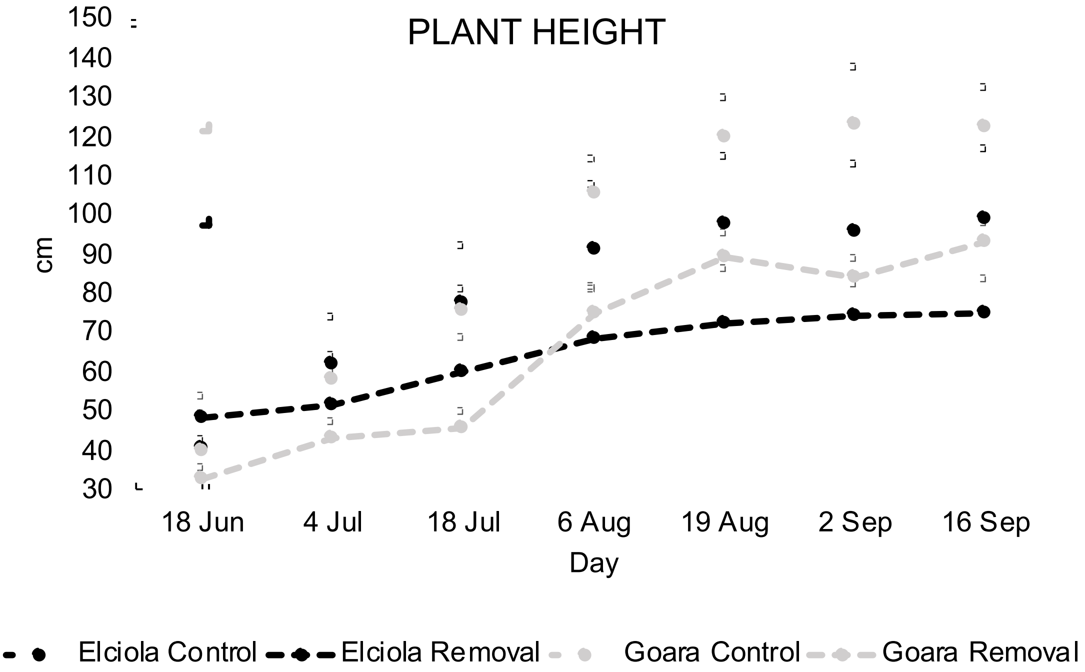

Figure 4. *Mean (± SE) plant height recorded during the monitoring time on* K. pentacarpos *individuals.*

Stem diameter and number of branches were significantly affected by site but not by treatment (Table 1). In general, plants in control plots reached the greatest size, and Goara plants were bigger, with higher number of branches, than Elciola plants (Figure 5, Figure 6).

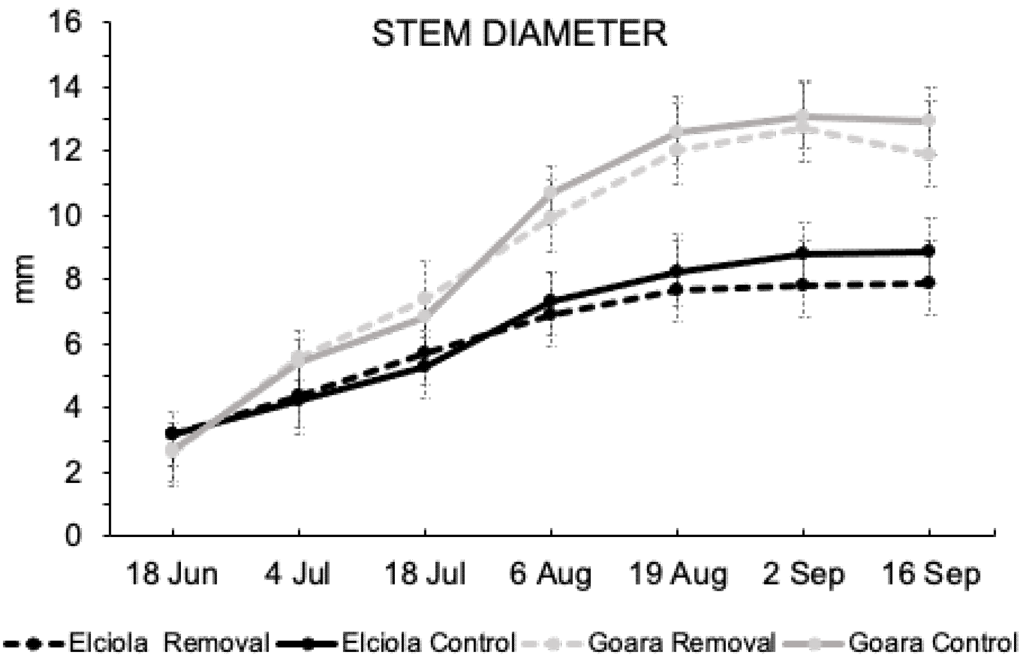

Figure 5. *Mean (± SE) plant diameter recorded during the monitoring time on* K. pentacarpos *individuals.*

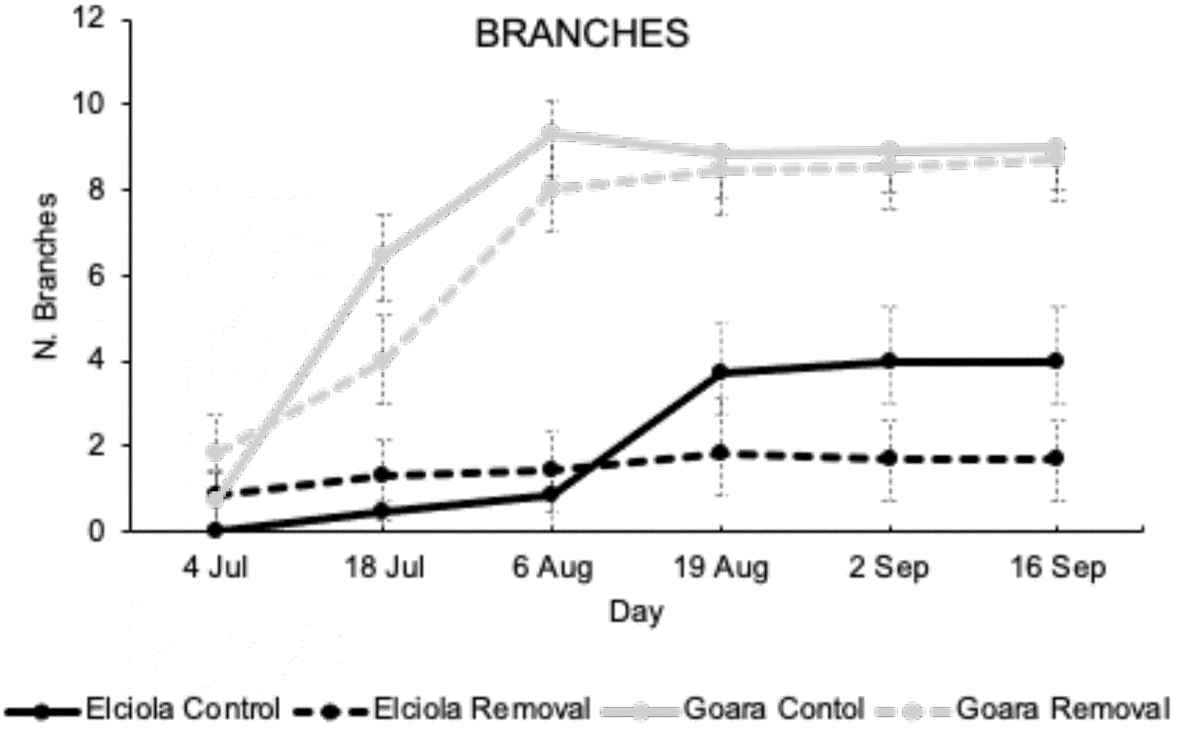

Figure 6. *Mean (± SE) number of branches recorded during the monitoring time on* K. pentacarpos *individuals.*

	Site	**Plot**	**Site × Plot**	**Time**	**Time × Site**	**Time × Plot**	**Time × Site × Plot**
N-NO$_3$ soil	$F_{1,8}$=20.05; **$p<0.001$**						
N-NH$_4$ soil	$F_{1,8}$=29.32; **$p<0.001$**						
P-PO$_4$ soil	$F_{1,8}$=55.54; **$p<0.001$**						
N-NO$_3$ water	$F_{1,8}$=0.08; p=0.786						
N-NH$_4$ water	$F_{1,8}$=4.68; **p=0.062**						
P-PO$_4$ water	$F_{1,8}$=16.16; **p=0.004**						
Salinity	$F_{1,8}$=177.9; **$p<0.001$**						
EC	$F_{1,8}$=198.2; **$p<0.001$**						
Height	$F_{1,27}$=0.50; p=0.483	$F_{1,27}$=4.96; **p=0.034**	$F_{1,27}$=0.25; p= 0.617	$F_{6,162}$=107.47; **$p<0.001$**	$F_{6,162}$=9.67; **$p<0.001$**	$F_{6,162}$=6.24; **$p<0.001$**	$F_{6,162}$=0.35; p=0.908
Diameter	$F_{1,27}$=7.95; **p=0.009**	$F_{1,27}$=0.12; p=0.735	$F_{1,27}$=0.01; p=0.981	$F_{6,162}$=97.12; **$p<0.001$**	$F_{6,162}$=9.93; **$p<0.001$**	$F_{6,162}$=0.72; p=0.630	$F_{6,162}$=0.05; p=0.999
N. Branches	$F_{1,33}$=83.73; **$p<0.001$**	$F_{1,33}$=1.39; p=0.247	$F_{1,33}$=0.01; p=0.948	$F_{5,165}$=37.01; **$p<0.001$**	$F_{5,165}$=11.95; **$p<0.001$**	$F_{5,165}$=1.70; p=0.137	$F_{5,165}$=2.74; **p=0.021**
Chl	$F_{1,65}$=31.13; **$p<0.001$**	$F_{1,65}$=0.01; p=0.955	$F_{1,65}$=0.01; p=0.975	$F_{6,390}$=6.96; **$p<0.001$**	$F_{6,390}$=4.24; **$p<0.001$**	$F_{6,390}$=1.23; p=0.287	$F_{6,390}$=4.47; **$p<0.001$**
Net CO$_2$	$F_{(2,90)}$=125.62; **$p<0.001$**	$F_{(2,90)}$=9.89; **p=0.002**	$F_{(1,90)}$=5.91; **p=0.017**				
Foliar N	$F_{1,17}$=4.52; **p=0.047**	$F_{1,17}$=0.04; p=0.836	$F_{1,17}$=0.88; p=0.361				
Foliar P	$F_{1,17}$=79.47; **$p<0.001$**	$F_{1,17}$=0.58; p=0.457	$F_{1,17}$=0.42; p=0.524				
δ^{13}C	$F_{1,23}$=0.81; p=0.378	$F_{1,23}$=0.59; p=0.451	$F_{1,23}$=4.20; **p=0.052**				
δ^{15}N	$F_{1,21}$=25.30; **$p<0.001$**	$F_{1,21}$=0.10; p=0.749	$F_{1,21}$=18.68; **$p<0.001$**				
N. Fruits	$F_{1,66}$=4.53; **p=0.037**	$F_{1,66}$=0.75; p=0.389	$F_{1,66}$=1.07; p=0.304	$F_{4,66}$=4.87; **p=0.002**	$F_{4,66}$=1.99; p=0.105	$F_{4,66}$=0.93; p=0.451	$F_{3,66}$=0.36; p=0.779

Table 1. *Results from statistical analyses on the response variables. One-way ANOVA with site as explanatory variable for soil and water nitrate (N-NO$_3$), ammonium (N-NH$_4$), phosphate (P-PO$_4$), water salinity and water electrical conductivity (EC). Repeated measured two-way ANOVAs with site and plot as between-subject factors and time as within-subject factor performed for plant height, stem diameter, number of branches, chlorophyll content (Chl), number of ripe fruits. Factorial ANOVAs with site and plot as explanatory variable performed for net CO$_2$ exchange rate, foliar nitrogen (N) and phosphorous (P) content and isotopes concentration (δ^{13}C and δ^{15}N). Significant value ($p<0.05$) are highlighted in bold character.*

3.3 Ecophysiological traits

Chlorophyll content differed significantly between sites with higher Chl in the Goara plants (Table 1; Figure 7). Chlorophyll content was unaffected by plot but changed significantly through time (Table 1), with higher Chl at the beginning of the growing season and in August; the lowest values were observed in mid-July. Chlorophyll fluorescence presented optimum Fv/Fm at both sites (mean Fv/Fm in Elciola: 0.761±0.006; mean Fv/Fm in Goara 0.750±0.006), suggesting that plants did not experience environmental stress independently of treatment.

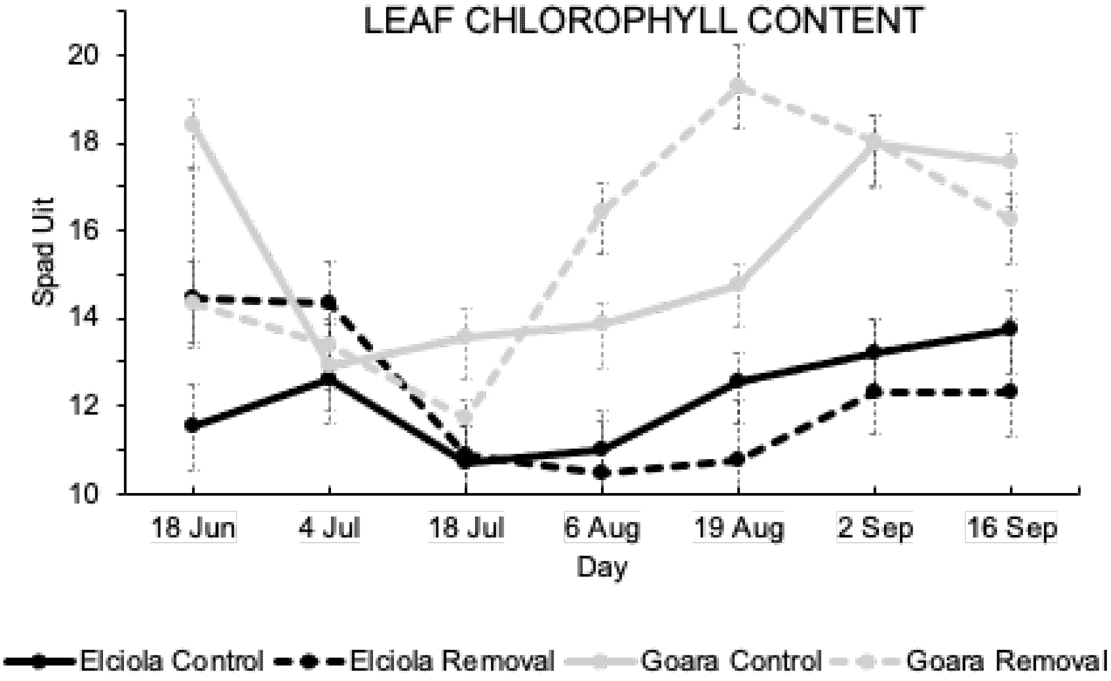

Figure 7. *Mean (± SE) chlorophyll content recorded during the monitoring time on* K. pentacarpos *individuals.*

Net CO_2 exchange rate was significantly affected by site and plot, with a significant site × plot interaction (Table 1). Net CO_2 exchange rate was overall higher values in Goara. However, while net CO_2 exchange rate in Goara was higher in the removal plot, it did not differ in relation to plot in Elciola (Figure 8).

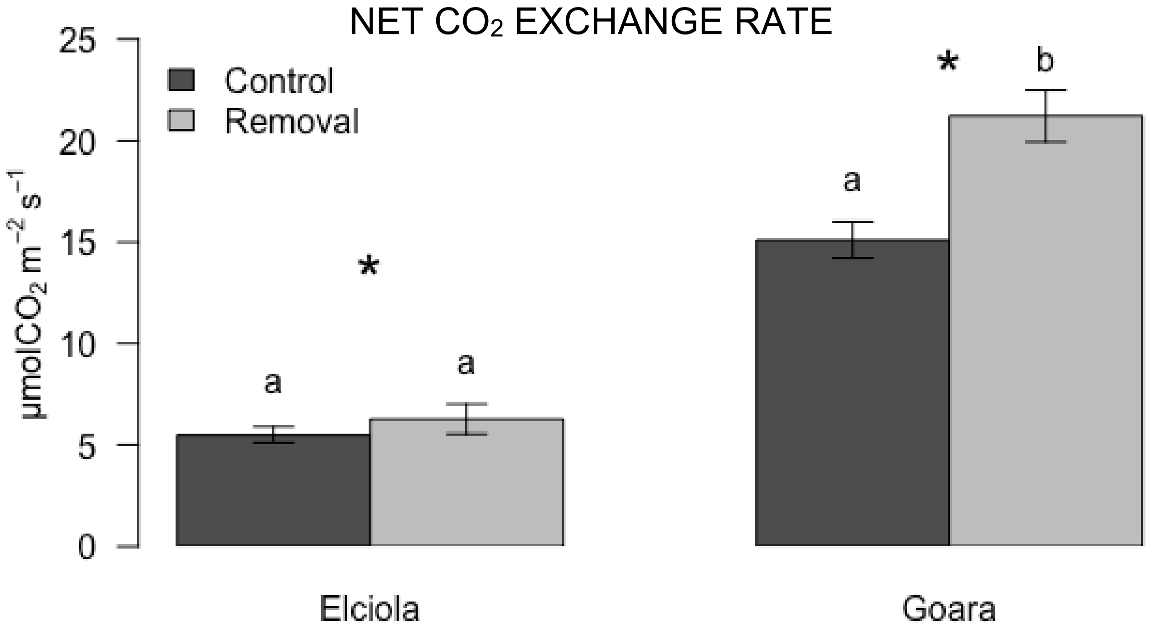

Figure 8. *Mean (± SE) net CO_2 exchange rate recorded on* K. pentacarpos *individuals. Letters indicate significant differences among plot; asterisks indicate significant differences between according to the Tukey's post-hoc HSD tests.*

Foliar N (Figure 9) and P concentrations were significantly higher in Goara than in Elciola plants (Table 1), but were unaffected by plot.

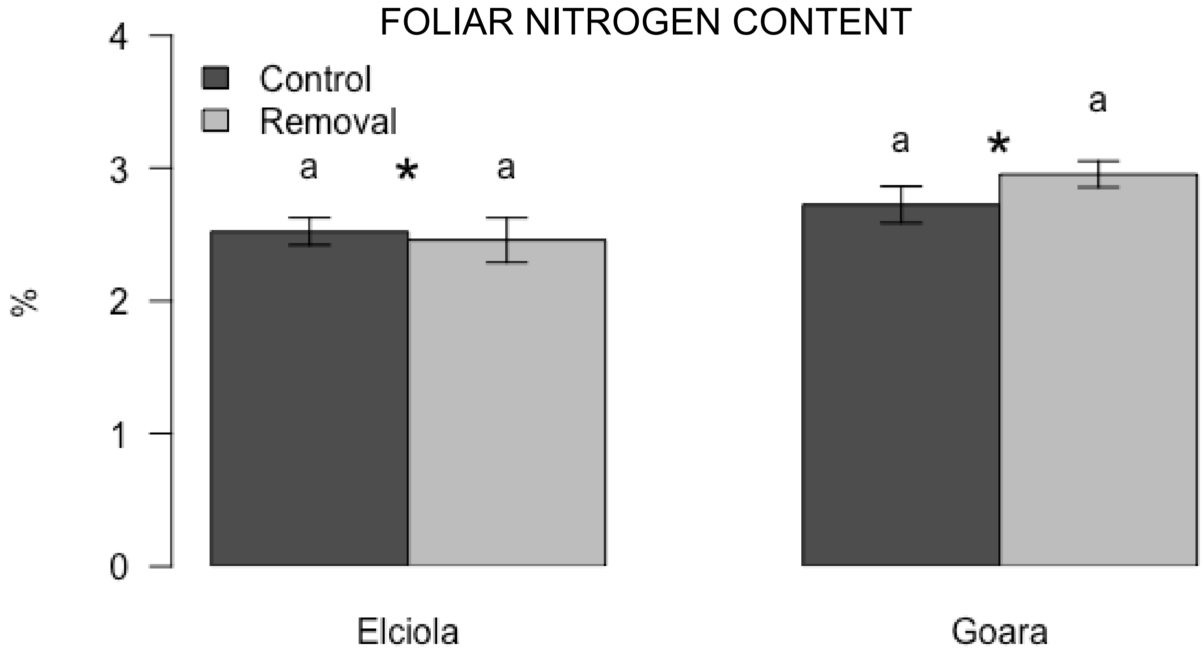

Figure 9. *Mean (± SE) foliar N content of* K. pentacarpos *individuals. Letters indicate significant differences among plot; asterisks indicate significant differences between according to the Tukey's post-hoc HSD tests..*

Foliar $\delta^{13}C$ isotope composition was not affected by site and plot (Table 1). Conversely, foliar $\delta^{15}N$ was significantly higher in Goara, especially in the removal plot (Table 1, Figure 10).

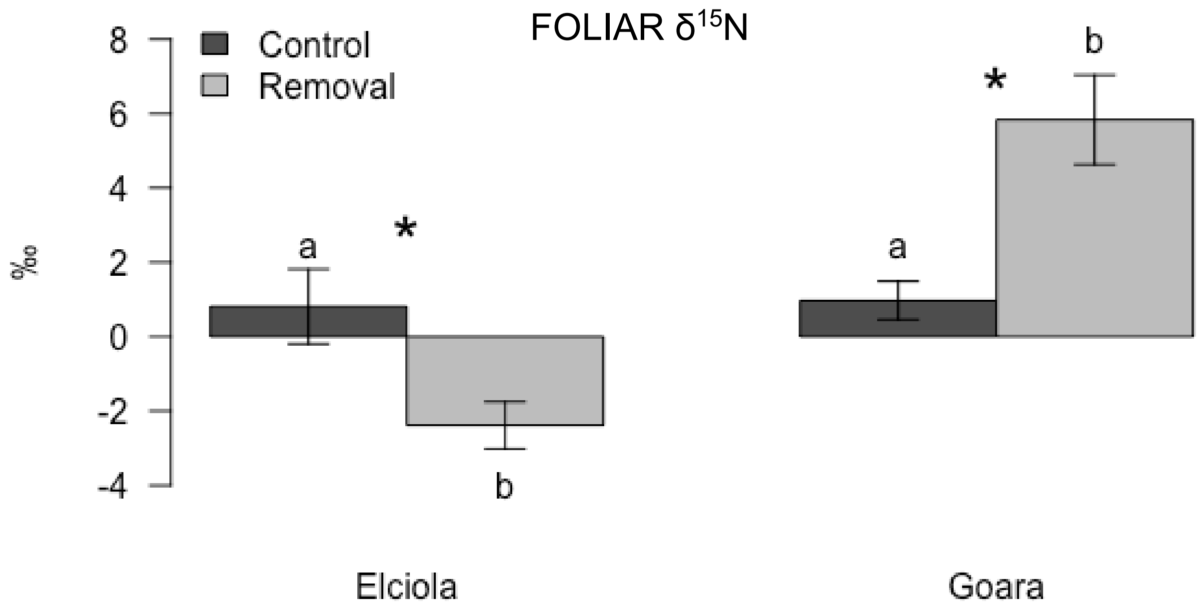

Figure 10. *Mean (± SE) foliar δ^{15}N content on K. pentacarpos individuals. Letters indicate significant differences among plot; asterisks indicate significant differences between according to the Tukey's post-hoc HSD tests.*

3.4 Reproductive performance

The number of ripe fruits was significantly affected by site and time, with higher amount of ripe fruits in Goara than in Elciola (mean number of ripe fruits in Goara: 17.41±2.09; mean number of ripe fruits in Elciola: 6.62±1.72), especially at the beginning of September, with no differences between plots.

The number of aborted fruits did not differ significantly with plot in Goara ($F_{1,20}$ = 1.450; p = 0.242), whereas it was significantly influenced by time ($F_{2,40}$ = 6.5532; p = 0.003). The interaction plot × time was also significant ($F_{2,40}$ = 5.409; p = 0.008). The number of aborted fruits in the control plots increased at the end of the growing season, while the number of aborted fruits in the removal plots was lower and remained stable over time.

3.5 Phenology

The phenological phases were faster in Goara than in Elciola. This applied especially to senescence, with the Goara plants experiencing greater faster leaf abscission in shorter time independent of plot (Figure 11; Table S3). Conversely, the Elciola plants exhibited considerable phenological differences in relation to plot, with later start of blossoming, flowering as well as with fruiting in the control plots (Figure S2A and Figure S2B; Table S3).

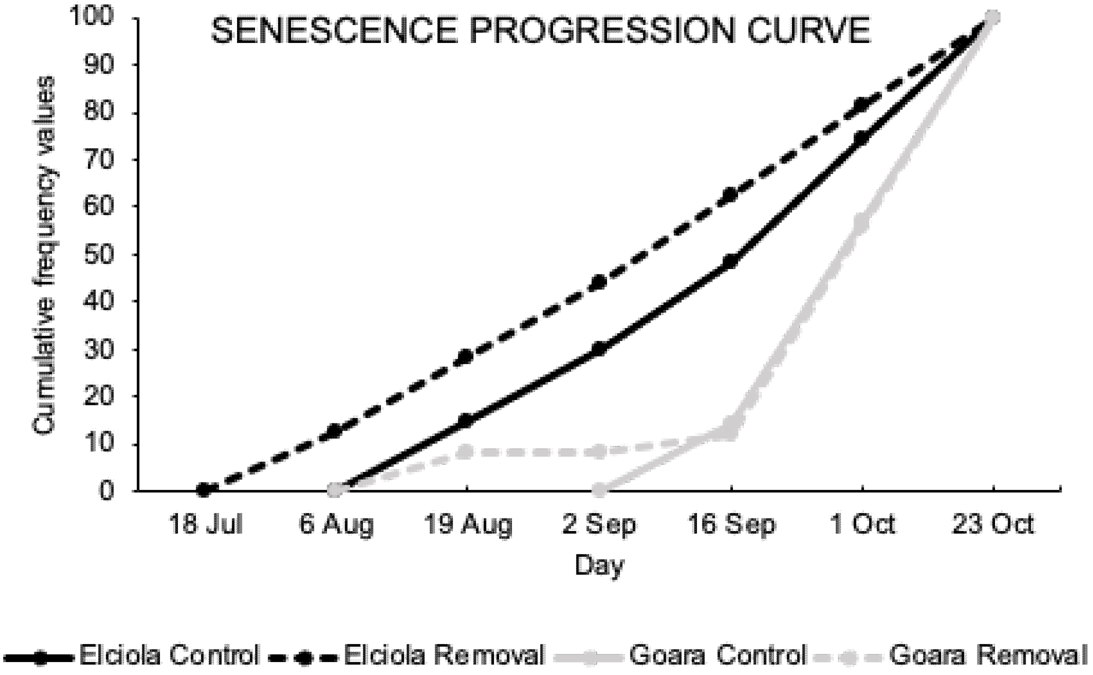

Figure 11. *Progression curves calculated by methods of Cumulative Stage Development on growth stages for BBCH-9 i.e. senescence.*

4. Discussion

Our study demonstrated that environmental characteristic of recipient sites affected the performance of translocated populations of seashore mallow to a greater extent than the site management.

4.1 Effects of site characteristic on species performance

Seashore mallow performed better in Goara than in Elciola, in terms of both vegetative growth and reproduction. This outcome is also in line with our expectation. Indeed, a natural population of the species was already present in Goara whereas plants have never been observed in Elciola.

Goara plants presented higher height, greater stem diameter, number of branches, Chl, net CO_2 exchange rate and more ripe fruits than the Elciola plants. In addition, the Goara plants had higher foliar N and P contents and higher, i.e., more positive, leaf $\delta^{15}N$. The phenological phases, especially plant senescence, were faster and occurred later in Goara. Faster leaf abscission and premature senescence of cotyledons in the Goara plants represents an adaptation for providing adequate energy supply for the regulation of saline conditions (Blits & Gallagher, 1990), as noted in the translocation experiment carried out in 2015 (Brancaleoni et al., 2018).

Seashore mallow performance was also enhanced by higher irradiance, warmer temperature and deeper water table in Goara compared with Elciola. Our results thus supported previous studies that diagnosed seashore mallow as a heliophilous species (Snell, 2018), with irradiance being a major factor limiting its growth. At low light level, seashore mallow competes for light either through shade avoidance or by developing shade tolerance mechanisms, especially by decreasing photosynthetic capacity (Gruntman et al., 2017). This is what happened in Elciola, where in shaded conditions the plants developed shade tolerance at seedling emergence, as also reported in a translocation experiment in Spain (Pino & De Roa, 2007).

As a halophilous herb, seashore mallow prefers soils with moderate salt content. This aspect was already noted in the previous translocation performed in 2015 (Brancaleoni et al., 2018) and confirmed again in the current study. Moreover, seashore mallow had the greatest performance in conditions of higher nitrate, ammonium and phosphate content both at soil and water level. The species has indeed been demonstrated to be able to grow on saline mesotrophic to eutrophic soil thanks to both cellular-level and whole plant-level mechanisms (Li et al., 2006), with the high levels of nutrients, especially N, helping in acquire more biomass and produce fruits (Moser et al., 2013; Abeli et al., 2017). In addition, seashore mallow does not perform well in sites with flooded soils, where oxygen deficiency might occur, with roots or rhizomes suffering from the periodical or prolonged complete (anoxia) or partial (hypoxia) absence of oxygen (Yordanova et al., 2003). Consequently, the oxygen-dependent pathways are limited, especially the energy-generating system, the functional interrelation between roots and shoots is disturbed, and both carbon assimilation and photosynthate utilization are suppressed (Vartapetian & Jackson, 1997). Root hypoxia suppresses chlorophyll accumulation thus hindering leaf photosynthetic activity (Ladygin, 2004). Seashore mallow exposed to 40 days of waterlogging showed high mortality rates, reduced size of chloroplast, with additional alterations of mitochondria, thylakoid lamellae and plastoglobules (Zhou et al., 2011). Despite our results showed no significant results both for chlorophyll content and net CO_2 exchange rate, waterlogging condition in Elciola led to a decrease in species performance probably in line with these findings. Even if seashore mallow has been demonstrated to produce significant quantities of fine roots, that enhance the soil shear strength and increase soil stability in coastal areas (Halchak et al., 2011), the flooded condition in Elciola might have indeed negatively affected the plants which were not solidly anchored to the soft soil. The higher leaf $\delta^{15}N$ in as well as the higher foliar N and P contents in the Goara plants might be due to an arbuscular mycorrhizal (AM) colonization and/or by phosphate-solubilizing microorganisms, such as *Glomus mossea* and *Mortiella* sp., already found in seashore mallow roots (Zhang et al., 2014), whereas in condition of reduced stability of soil such as Elciola, the mycorrhizal system or a structured rhizosphere cannot be solidly developed.

4.2 Effects of the natural community on species performance

Post-translocation phases aimed at reducing the competitive effect of the local vegetation on seashore mallow performance had marginal effects on the target species, thus suggesting that there was overall poor evidence of competition or facilitation between seashore mallow and the co-existing species. Nonetheless, seashore mallow plants exhibited improved performance in the presence of the local vegetation in both sites, especially in Goara. Positive interactions between seashore mallow and co-existing species have been also reported in a Spanish site, where the canopy enhanced survival of seashore mallow seedlings during the first year of life (Pino & De Roa, 2007). Moreover, positive interaction of seashore mallow in presence of *S. patens* and *B. halimifolia* have been also demonstrated in Delaware, USA (Voutsina et al., 2015), where the natural recruitment increased when seashore mallow was cultivated as transitional crop. Our results suggest that the seashore mallow plants in Goara could be partly facilitated by the presence of *Juncus* spp., even if no data strictly related with this aspect has been evaluated; nevertheless, studies on the facilitative role of reeds already demonstrated that the plant is able to act as nurse plants (Núñez et al., 2009). Consistently, several of the seashore mallow individuals in Goara grew inside the rush tussocks and the removal of *Juncus* spp. led to a decrease in foliar N content. On the other side, although *K. pentacarpos* growing in Elciola reported higher plant growth in the presence of the local vegetation, seashore mallow might have expressed some degree of competition with *Phragmites australis* that was reflected in the lower net CO_2 exchange rate. This finding is in line with expectations of higher competition in the sites with milder environmental conditions (Bertness & Callway, 1994) but also with the ecology of the reed. Indeed, the life-cycle strategy of dominant plants is commonly based on effective vegetative regeneration mechanisms, which are one of the main causes of their high growth rate and colonisation ability (Clevering, 1995); in addition it has been largely demonstrated that the species possesses a competitive advantage over most of the coexisting species (Holdredge et al., 2010), reporting for example high nitrogen (N) uptake capacity (Mozdzer and Zieman, 2010), ventilation efficiency (Tulbure et al., 2012) and salt tolerance (Vasquez et al., 2005).

However, species performance in both sites and plots was highly influenced by microsite condition, with higher differences between plots in Goara and a negative effect of species performance in Elciola because of the higher water level. Indeed, plants growing in control plot in Goara were more affected by the canopy effect of holm oak than plants in the removal plot, jointly with the sun exposure from east to west and the more developed structure of rushes in the control plot. Conversely, plots environmental conditions were more homogeneous in Elciola since they were adjacent to each other.

There is also overall poor evidence of competition for light at both sites. Even if removing the local vegetation reduced significantly plant height in both sites, removal did not affect any of the other response variables considered. A possible explanation can be found in the intrinsic differences between the two sites in terms of the light conditions created by the local vegetation of the plot and the canopy effect of the surrounding vegetation. Indeed, both in Elciola and in the plot where the vegetation was removed in Goara, holm oaks were present at the borders of the plot and their canopy might have been reduced the light intensity. In addition, the removal of vegetation differently influenced seashore mallow plants in both sites: if the removal of natural vegetation implied more light availability in Elciola as expected, this trend was not seen in Goara, where the removal of vegetation was not in line with the expected increase in irradiance. Noteworthy, the removal of vegetation influenced nitrate and phosphate content in Goara, with an increase in the concentration probably because of the reduced filter effect guaranteed by the removal of reeds and rushes (Gerdol et al., 2014); on the contrary, this was not clear in Elciola.

Nevertheless, data on soil nutrient did not reflect data on plant nutrients, where higher nutrient in Goara plants leaves were not in line with the reduced nutrients in the site. Removal of *Phragmites australis* might have affected seashore mallow phenology in Elciola, by advancing the phenological phases as a consequences of the increased irradiance been demonstrated to accelerate phenological processes in seashore mallow (e.g., Navas et al., 1997).

4.3 Study limitation and effect of site management on environment

In this experiment we faced with a natural community that implied a limited number of plants, different recipient communities as well as different plants age and environmental context, impossible to handle completely. Moreover, performance of seashore mallow plants might have been influenced by age; however, since we faced both with a translocated population and a natural population, we had no information whether how old were the species we monitored. For example, it has been reported that population dynamics decrease through years and there is a strict link between reproductive ability of the plant and the presence of parasites (Voutsina et al., 2015), as might have happened in Goara site, where plants reported several aborted seeds.

5. Conclusions and future perspectives

A brackish site characterized by moderate light availability and deep water table depth has to be considered as a good candidate for translocation of *K. pentacarpos*, with the natural community (so far, only *Juncus* spp. can be demonstrated) helping partly in facilitating species performance. More generally, our study demonstrate that the selection of the suitable recipient site is crucial for guarantee a successful translocation, with the post-translocation removal of competing vegetation reporting limited advantage in terms of plant establishment and performance.

Acknowledgements We are particularly grateful to Dr. Giovanni Nobili, Mr. Mauro Menghini, and the staff of the former State Forestry Corps (presently CUTFAA) for permissions and valuable support during the experiment within the Riserva Naturale Bosco della Mesola. The authors would like to thank Dr. Roberta Marchesini (University of Ferrara) and Dr. P. Iacumin (University of Parma) who performed the chemical analyses.

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Supplementary Materials

Table S1 *Phenological stage, main description and related principal growth stage basing on the BBCH-scale (extracted from Meier, 2001).*

Stage	Description	Principal growth stage
5	Inflorescence emergence (main shoot) / heading	51 - Inflorescence or flower buds visible; Beginning of heading 55 - Half of inflorescence emerged (middle of heading) 59 - Inflorescence fully emerged (end of heading)
6	Flowering (main shoot)	61 - Beginning of flowering: 10% of flowers open 65 - Full flowering: 50% of flowers open, first petals may be fallen 69 - End of flowering: fruit set visible
7	Development of fruit	71 - 10% of fruits have reached final size or fruit has reached 10% of final size; fruit is inside the calix 75 - 50% of fruits have reached final size or fruit has reached 50% of final size; fruits are green, calix is open 79 - Nearly all fruits have reached final size, fruits protrude from the calix
8	Ripening or maturity of fruit and seed	81 - Beginning of ripening or fruit coloration 85 - Advanced ripening or fruit coloration; fruits brownish/blackish 89 - Fully ripe: fruit shows fully-ripe color, beginning of fruit abscission
9	Senescence, beginning of dormancy	91 - Shoot development completed; foliage still green 95 - 50% of leaves fallen 99 - Plant resting or dormant; end of leaves falling

Table S2. *Mean (± SE) concentrations of nitrate ($N-NO_3$), ammonium ($N-NH_4$), phosphate ($P-PO_4$) extracted from soil and water samples, water salinity and water electrical conductivity (EC) determined in both sites.*

	Response variable	**Elciola**	**Goara**
Soil	$N-NO_3$ (ppm)	0.958±0.143	3.790±0.748
	$N-NH_4$ (ppm)	9.578±2.761	81.442±12.980
	$P-PO_4$ (ppm)	2.242±1.396	67.996±8.712
Water	$N-NO_3$ (ppm)	0.134±0.024	0.124±0.026
	$N-NH_4$ (ppm)	0.088±0.009	0.188±0.045
	$P-PO_4$ (ppm)	0.006±0.001	0.078±0.018
	Salinity (mg/l)	219.8±6.6	4650.0±332.1
	EC (mS)	457.8±13.5	8658.0±582.3

Table S3. *Onset dates for plants recorded in both sites (Elciola and Goara) and plots (Control and Removal) when 50% of* K. pentacarpos *individuals were at the principal growth stage of interest, i.e., blossoming (BBCH-5), flowering (BBCH-6), development of fruit (BBCH-7), ripening of fruit (BBCH-8), senescence (BBCH-9) according to the BBCH phenological stage. NA values indicate that no available plants on the day of monitoring were at the principal growth stage of interest.*

Stage	**Principal growth stage**	**Elciola Control**	**Elciola Removal**	**Goara Control**	**Goara Removal**
Blossoming	51	24 Jul	25 Jul	20 Jul	17 Jul
	55	11 Aug	4 Aug	13 Aug	12 Aug
	59	21 Aug	NA	21 Aug	NA
Flowering	61	12 Aug	NA	9 Aug	12 Aug
	65	NA	27 Jul	18 Aug	6 Aug
	69	21 Aug	31 Jul	17 Aug	19 Aug
Development of fruit	71	8 Sep	11 Jul	22 Aug	13 Aug
	75	6 Sep	14 Aug	31 Aug	21 Aug
	79	16 Sep	25 Aug	1 Sep	31 Aug
Ripening of fruit	81	NA	NA	NA	NA
	85	NA	NA	18 Aug	13 Aug
	89	18 Sep	8 Sep	16 Sep	13 Sep
Senescence	91	10 Sep	24 Aug	19 Sep	12 Oct
	95	6 Oct	19 Sep	12 Oct	12 Oct
	99	12 Oct	12 Oct	12 Oct	12 Oct

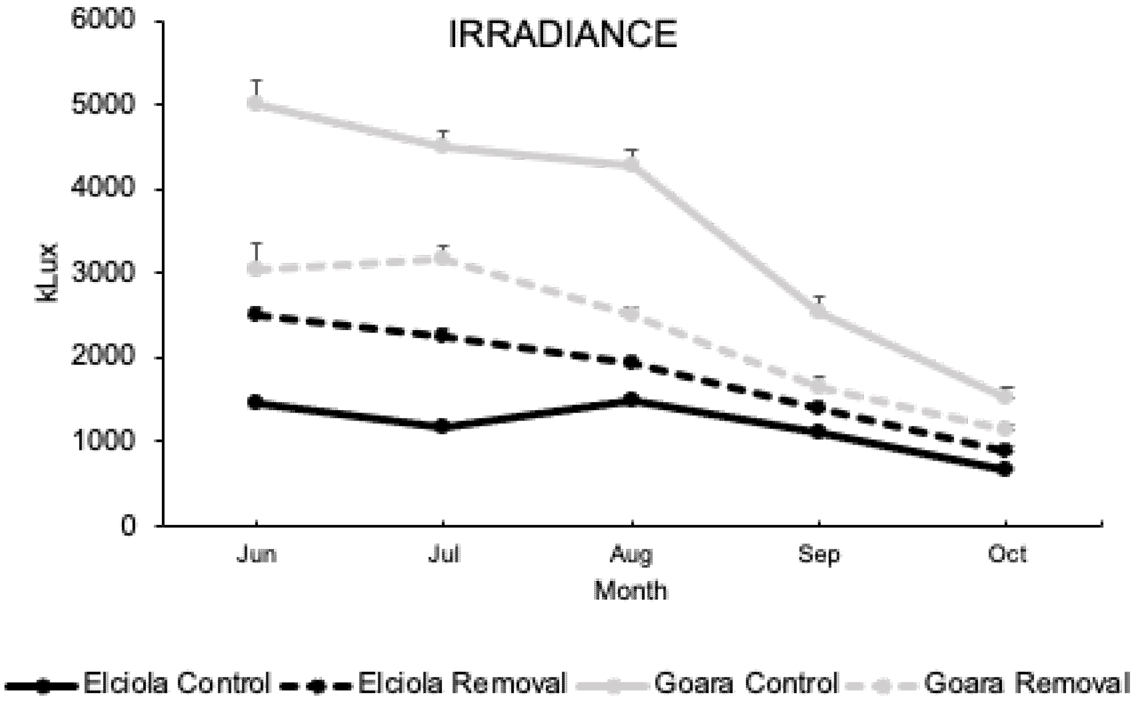

Figure S1. *Mean (± SE) irradiance recorded during the growing season in the sites and plots.*

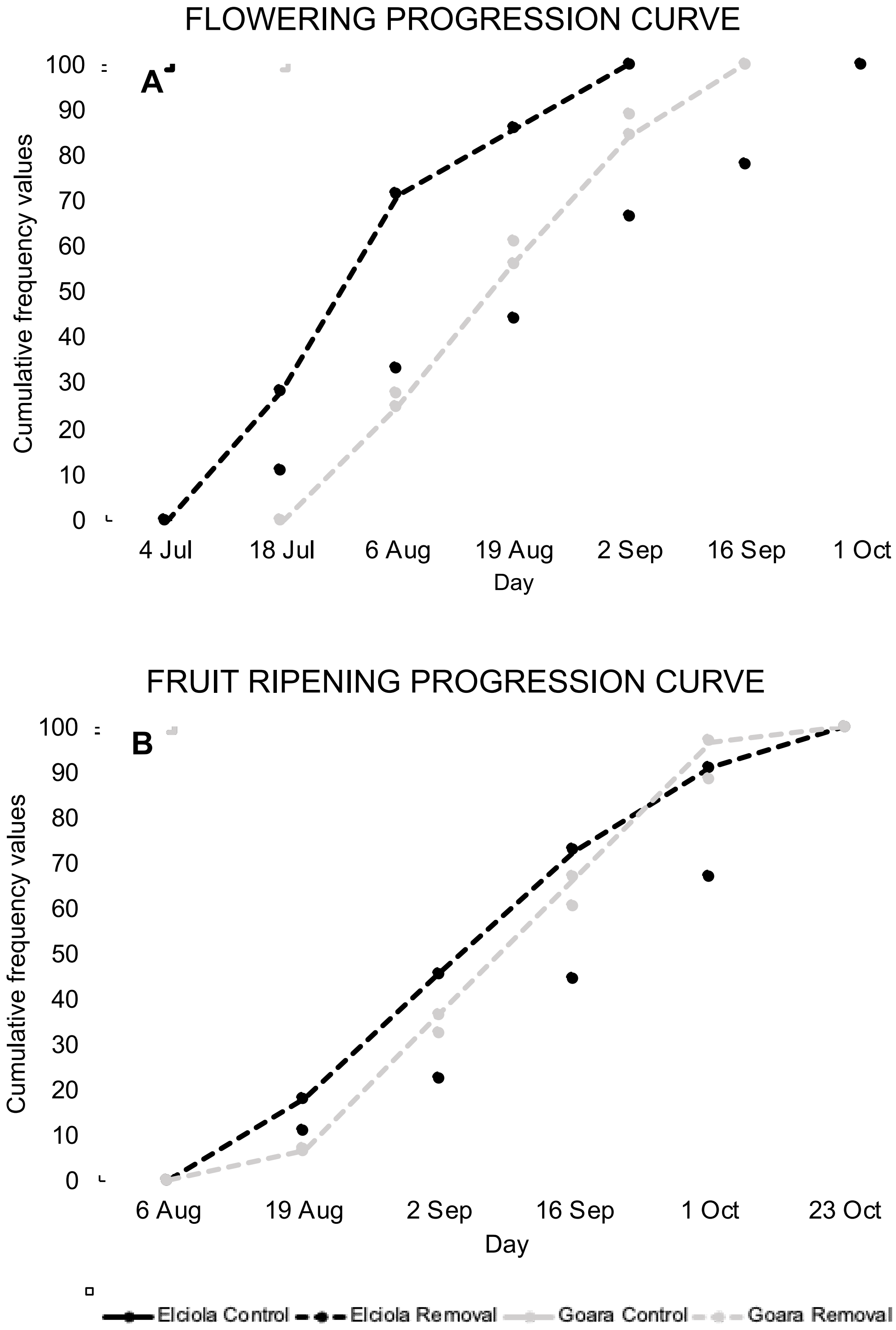

Figure S2. *Progression curves calculated by methods of Cumulative Stage Development on growth stages for BBCH-6 i.e. flowering (A) and BBCH-8 i.e. fruit ripening (B).*

Taxonomy and genetic debate

Kosteletzkya pentacarpos is a variable taxon. The target species jointly with *K. virginica* was published as species of *Hibiscus* in *Species Plantarum* (Linnaeus, 1753). Linnaeus stated that *Hibiscus virginicus* ([*K. virginica* (L.) C. Presl ex A. Gray) occurred in Virginia, USA (lectotype Clayton 567, LINN 875.35; Jarvis, 2007), and *Hibiscus pentacarpos* ([*K. pentacarpos* (L.) Ledeb.) was present in Venice, Italy (lectotype Zannichelli, Ist. Piant. T. 91. 1735; Alexander et al., 2012; Figure S3).

***Figure S3**. Lectotype of* Hibiscus pentacarpos*: [Icon.] "Ketmia palustris" in Zannichelli, Ist. Plant.; t 91. 1735.*

Historically, Cavanilles (1787) was the first botanist who considered *Hibiscus virginicus* to be a synonym of *H. pentacarpos*, which gave *H. pentacarpos* nomenclatural priority under the International Code of Botanical Nomenclature (McNeill et al., 2006; Art. 11.5). In 1835, Carl Borivoj Presl separated the genus *Kosteletzkya* from *Hibiscus*, giving honor to the Czech botanist Vincenz Franz Kosteletzky. Then, in 1849, Presl in Gray transferred *Hibiscus virginicus* to the genus *Kosteletzkya* (Alexander et al., 2012).

Until recently, the North American populations generally known as *K. virginica*, were formerly distinguished by the Eurasian *K. pentacarpos.* In particular, the American *K. virginica* occurred in the coastal wetlands of the Gulf of Mexico, on the east coast of the United States (USA), on the Bermuda Islands and on the southwestern coast of Cuba, whereas European populations of *K. pentacarpos* were restricted to the Mediterranean region, being found in the east coast of Spain, on the east coast of Italy, in France (Corsica), on the Black Sea coast (Georgia), on the southern and western coasts of the Caspian Sea (Iran and Azerbaijan) and in Russia (north coast of the Caspian Sea).

However, Blanchard (2008) turned the name into synonymy within *K. pentacarpos* because of the weak morphological differences between European and American populations; this finding has also been confirmed by other authors (Neubig et al., 2015; Iamonico & Peruzzi, 2014; Ferrer-Gallego & Laguna, 2015).

Given the common consideration of the North American and Eurasian populations, *K. pentacarpos* is the only species of the genus *Kosteletzkya* that has an ampiathlantic distribution, suggesting that the species may have been recently introduced (Blanchard, 2012) either through colonization of the North American species in the Mediterranean basin and shores of the Black and Caspian Seas or, on the contrary, from Atlantic Ocean to the east coast of North America, Cuba, and the Bermuda Islands from Eurasia. Considering the fragmented distribution range and the lower number of populations, it might be possible that the species is in its secondary range in Europe. However, to confirm this hypothesis is arduous, since historical information on plants in Europe are currently not known before the XVI century because of the lack of availability of the ancient herbaria.

One of the most interesting finding of the species in Italy is ascribable to *Istoria delle piante che nascono ne'lidi intorno a Venezia* (Zannichelli, 1735). Here, the author reported for the first time the occurrence of the species in the Venice lagoon, using the polinomy suggested by Linnaeus, *Ketmia palustris.* Zannichelli added also that the plant has never been noticed since then by other authors, with the exception of Plukenet: the British botanist in its *Phytographia* noted similarity with *Alcea indica*, which came from the “Indies” of Christopher Columbus i.e. America (Plukenet, 1691). In the area where the species has been firstly noticed, it is known that it has been cultivated in the *Hortus patavinus* (Padua, NE Italy) since the second half of the XVIII century, suggesting that the species might have been transferred from its natural environment to the botanical garden. *K. palustris* is also mentioned in the catalogue of *Horti Pisani* (Pisa, NW Italy) compiled by Tilli in 1723 as well as a specimen coming from Naples (SW Italy) by Napoleone Passerini dated back before XIX century is present in the herbaria. Lastly, a specimen is also present in the Erbario Campana (Ferrara, NE-Italy), without locality and suggested to be dated between 1736 and 1820. All of these findings may proof the introduction of the species in Europe from America by the end of the XVII century, reasonably through the main trade routes towards the Atlantic Ocean then the Mediterranean basin, where was later spread in the major Italian harbors, i.e., Venice and Naples. The same might have been introduced as ornamental plant thanks to its valuable beautiness, as happened also to the congeneric species *Hibiscus palustris*, previously cultivated in private house and then spread in natural environment (Farsetti, 1780). However, these suggestions have to be confirmed by solid genetic analysis. A preliminary genetic study (Posadas et al., 2019) addressing the native status of the species on Italian, Spanish and American populations showed that the European populations investigated must be considered a non-invasive alien species in Europe. However, this study has been simply presented as a poster in an international conference and need to be further developed and improved. New updated genetic analysis should be performed, with the inclusion of other European populations such as the one from Corsica.

Target species: *Bunias orientalis* L.

Invasion success of Bunias orientalis (Warty Cabbage) in grasslands: a mesocosm experiment on the role of hydrological stress and disturbance

Corli, A., Walter, J., Sheppard, C.S., 2021. Invasion success of *Bunias orientalis* (warty cabbage) in grasslands: a mesocosm experiment on the role of hydrological stress and disturbance. Frontiers in Ecology and Evolution (Published; https://doi.org/10.3389/fevo.2021.625587)

Abstract

Climate change is altering precipitation patterns, with higher frequency and magnitude of extreme events. Specifically, longer and more pronounced waterlogged conditions are predicted after rain spells as well as more frequent droughts, especially in Central Europe. Such hydrological changes can severely affect species performance and alter the function of ecosystems, as well as favor plant invasions. Competition with native communities may change depending on water stress. *Bunias orientalis* is an invasive plant that may benefit from disturbance or precipitation changes. We conducted a 3-year mesocosm experiment in a common garden to investigate how invasion success of *B. orientalis* in native German grassland communities is affected by varying hydrological conditions (from very dry to waterlogged). We measured the establishment and growth of *B. orientalis* in varying water table depths in bare soil (simulating disturbance) vs. in the community. Establishment and biomass of *B. orientalis* was generally highest under non-stress conditions. The species was also highly tolerant to dry conditions, but only when growing in bare soil. However, performance of *B. orientalis* was generally low, whereby interspecific competition in communities greatly limited invasion success. This might be due to the low competitive ability of the species in conditions of hydrological stress and the near-natural grassland communities with an extensive mowing regime used in our experiment. Our results suggest that invasion success of *B. orientalis* in grasslands will not increase if precipitation patterns change toward more extreme events. However, disturbance that creates bare soil patches might favor *B. orientalis* under drought conditions.

Keywords *Bunias orientalis* · common garden experiment · grassland · hydrological gradient · interspecific competition · plant invasion · precipitation changes

1. Introduction

Climatic change leads to changes in precipitation patterns as well as higher frequency and magnitude of extreme events, which are projected to increase further during the 21st century for many areas (IPCC, 2014). Dry regions in mid latitudes and subtropics are expected to receive less annual precipitation whereas moist mid-latitudinal regions and higher latitudes receive more. However, many uncertainties regarding possible changes and impacts of annual precipitation in the future remain (Orlowsky & Seneviratne, 2013).

In regions that overall receive more precipitation such as Central Europe (IPCC, 2014), hydrological change is predicted to severely affect soil moisture, causing longer and more pronounced waterlogged conditions after extreme rain spells and flooding. Nevertheless, as already observed in Europe in the last decade (Hanel et al., 2018), changes in precipitation patterns might also result more frequently in dry conditions, causing hot and dry summers. Notably, Southern Germany has been demonstrated to be a geographical center in terms of deficit volumes of precipitation and duration of drought (Laaha et al., 2017). Such changes in water availability affect plant growth and can change patterns of competition and facilitation between plants (Walter, 2018).

Grasslands are important biomes rich in biodiversity, accounting for nearly 25% of land surface on Earth (Gang et al., 2015); they offer key ecosystem services balancing the concentrations of global atmospheric greenhouse gases (O’Mara, 2012), protecting soil and water resources (Vogt et al., 2019) and providing livestock forage and therefore meat and milk for human consumption (O’Mara, 2012). Nevertheless, they are one of the most sensitive ecosystems to global climate change drivers (Seastedt & Pyšek, 2011), where anthropogenic influence accelerated their decline over the last decades (Czarniecka-Wiera et al., 2019). Indeed, because of abandonment and changing of traditional management toward intensification, European grasslands have experienced a decrease in species richness and changes in competitive regimes (Römermann et al., 2009), being also increasingly disturbed by frequent mowing or grazing (Stampfli et al., 2018).

Climate change and extreme events influence the structure and function of ecosystems worldwide, jointly with plant invasion (Vitousek et al., 1997; Parmesan & Yohe, 2003; Kelly & Goulden, 2008). By altering the dominant perennial plant cover, increasing resource availability and opening space for colonization, disturbance facilitates the arrival and dispersal of invasive species (Pierce et al., 2013; Herben et al., 2018), which are often characterized by competitive functional traits such as larger size, increased fecundity and faster germination than native species (Callaway & Ridenour, 2004). Invasive plants benefit disproportionately from disturbance (Smith & Knapp, 1999) and, notably in grasslands, they can act as drivers of change (Vitousek et al., 1997), shifting ecosystems to alternative stable states that are less diverse and dominated by alien species (Gibbons et al., 2017). However, some studies highlighted that their performance may not differ from dominant native species (Sheppard et al., 2012).

The warty cabbage, *Bunias orientalis* L. (Brassicaceae), benefits from excessive disturbance, copes very well with mowing due to rapid rosette regrowth and increased seedling recruitment (Woitke & Dietz, 2002) and forms tall dense stands that successfully compete with native grasses for sunlight, nutrients, and pollinators (Birnbaum, 2006). Over the last four decades the species has become highly invasive in various parts of Northern and Central Europe, including German grasslands

(Dietz et al., 1996, 1999; Woitke & Dietz, 2002). In this region, grasslands account for more than a quarter of farmed land, with more than half of all plant species found in the country depending on the habitat. However, in recent decades they have been under pressure, with a decline by 12% from over 5.3 million hectares reported in 1991 to 4.7 m ha nowadays (Umweltbundesamt, 2020).

In this study, we focus on how invasion success of *B. orientalis* in native grassland communities in Germany may change with varying hydrological conditions (ranging from very dry to waterlogged by adjusting water table depth) that are expected under climate change. We conducted a 3-year mesocosm experiment in a common garden addressing the following research questions: (i) Does the establishment and growth of *B. orientalis* differ among water table depths? (ii) Does the effect of water table depth depend on the presence and biomass of the native grassland community?

2. Materials and methods

2.1 Study species

Bunias orientalis is a tall (up to 1.2 m height) polycarpic biennial or perennial hemicryptophyte of high dispersal ability that may live up to 10 years or even longer; it grows as a semi-rosette with large leaves and numerous little flowers. The species is hermaphroditic and insect-pollinated, with a self-fertile breeding system (Dietz et al., 1999), but it can also reproduce vegetatively by root fragments (Bąba & Kompała-Bąba, 2008). *B. orientalis* has a high reproductive effort (from 15 up to 45% of reproductive biomass i.e., g dry weight of reproductive biomass per g aboveground biomass), high germination rates (up to 60%) and a high potential for vegetative growth following root fragmentation (Steinlein et al., 1996). Moreover, it has a persistent seed bank, with large seeds (mean seed mass 7 mg, Dietz et al., 1999) occurring in densities from 1,000 to 15,000 seeds/m2, able to be viable for more than 3 years (Steinlein et al., 1996).

The native range is Caucasus and southern Russia, where the species occurs in grasslands, sunny edges of forests, dappled shade and riverbanks. There, the species requires moist soils (Clapham et al., 1962). Multiple independent introductions in the 18th century brought *B. orientalis* to Europe where it rapidly became a dominant invasive species in many countries, including Germany, Czech Republic, Poland, Sweden, Norway, and elsewhere (Harvey et al., 2010). In other neighboring countries (France, Great Britain, Netherlands) the species is not yet considered invasive, although it occurs as an established neophyte. In the invaded range *B. orientalis* is highly versatile and easily establishes in a variety of semi-natural or disturbed habitats such as roadside verges, railways, fallow lands, floodplain meadows, pastures, ruderal sites, and even gardens (Birnbaum, 2006). In the invaded range the species is more tolerant to various soil conditions although it prefers open, nutrient rich clay or sand soils of a neutral pH and does not grow under shaded conditions. From these sites it can invade dry grasslands, especially neglected dry meadows (Birnbaum, 2006). Moderate disturbance caused by mechanical damage (e.g., mowing) or herbivore damage (e.g., slug grazing) promote the survival and establishment of the species (Bąba & Kompała-Bąba, 2008).

2.2 Experimental design

To study the effects of hydrological stress on the establishment and growth of the invasive plant *B. orientalis*, we set up a mesocosm experiment in a common garden (48°71′ N, 9°20′ E, 389 m a.s.l.) at the University of Hohenheim in Stuttgart, Germany. The climate is oceanic, subtype subcontinental, mild, with no dry season and warm summers. The long-term average annual temperature is 9.4◦C and annual precipitation is 718 mm (Agrarmeteorologie Baden-Württemberg, 2019). The experiment was conducted from April 2016 to November 2018 and included five hydrological treatments (chronic drought, dry, mesic, wet, waterlogged), applied to bare soil (simulating disturbance) and grassland community pots.

Naturally composed mature grassland communities were dug out in November 2015 from four typical hay meadows (Arrhenatherion elatioris), which all belonged to the same organic farm property in South-Western Germany (48°53′ N, 8°54′ E, 290-380 m a.s.l.). These dug-out communities (including soil, roots and the entire grassland community, with a soil depth of 15–20 cm) were placed in 20-L pots (28 cm × 35 cm ø), which were previously filled with a layer of sandy loam at the bottom. The communities thus had different species compositions (made up of mostly perennial grasses, forbs, and legumes), but provided a more natural environment to study invasion success. The communities contained on average 7.2 species at the start of the experiment in 2016, with the most common species being the grasses *Arrhenatherum elatius* (L.) P.Beauv. ex J. Presl & C. Presl., *Festuca pratensis* Huds., and *Poa pratensis* L. (Table S1). The bare soil pots were entirely filled with the same sandy loam used at the bottom of the grassland pots. All pots were then kept in the greenhouse until April 2016, when they were transferred to the common garden and placed inside large 260-L pools (Figure S1). Each pot was placed in a separate pool to avoid pseudo-replication (with the exception of one pot of bare soil, which shared a pool with one community pot). The pools also contained some other pots, which however are not part of the experiment described here. Seeds of *B. orientalis* were sown on 30 June 2016 in 50 pots, of which 40 were with communities (8 replicates per water table depth treatment), and 10 control pots without (bare soil, 2 replicates per treatment). Within the logistical limitations of our experiment, we allocated more replicates for the community treatments as we expected lower establishment success in the grassland. In each pot, 12 seeds were sown, with a total number of 600 seeds.

We aimed to mimic a realistic scenario with natural variation in precipitation, applying different soil moisture conditions on these near-natural grassland communities, ranging from waterlogged, over wet, mesic, dry to chronic-drought-exposed communities. We achieved the desired hydrological regimes by adjusting the water table depth in the pools to five levels (Figure S2). In the waterlogged treatment, the water table was kept at −5 cm below the soil surface, in the wet treatment at −15 cm and in the mesic treatment at −25 cm. The water table depth was kept constant by watering the pools twice per week and preventing higher water levels than desired with holes in the pools made at the respective height (Figure S2; see also Rai et al., 2018; Walter, 2020). In the dry treatment, pools contained no water (i.e., holes at the bottom) and pots received natural rainfall. Under chronic drought conditions, precipitation was additionally reduced by 30% with a partial rainout shelter, using curved, transparent PVC stripes mounted on a wooden construction that covered 30% of the area above the pools (Figures S1, S2).

The year 2016 was characterized by an exceptionally wet June (115 mm rainfall compared to the long-term 30-year average (1981–2010), mean of 87 mm), followed by an exceptionally dry period from July to September (only 117 mm rainfall compared to 212 mm in the long-term mean) (Agrarmeteorologie Baden- Württemberg, 2019). 2017 received almost 130 mm more rainfall than the long-term during the growing season (from April until November), with June and July being especially moist months (+ 67 mm). 2018 had a record-breaking dry and hot summer (+ 2.1◦ and 120 mm less precipitation than the long-term average, from April until November) (Agrarmeteorologie Baden- Württemberg, 2019). As expected, soil moisture differed among the applied hydrological treatments (see Walter, 2020 for details on average volumetric soil water content).

During the first year, germination of *B. orientalis* seedlings was monitored at weekly intervals until the end of October. In each year, number of *B. orientalis* individuals were counted in late May or beginning of June and at the end of October, after which all plant material (*B. orientalis* and the community) was cut 2 cm aboveground (mimicking extensive agricultural regimes). In each October, aboveground biomass of *B. orientalis* and the grassland communities were harvested, dried at 60◦ for 4 days and weighed.

2.3 Data analysis

Statistical analyses were performed with R 3.6.1 (R Core Team, 2019) using the lme4 R package (Bates et al., 2015). For those seeds that germinated during the first season (2016), we used a linear mixed effects model to test whether time of germination (i.e., week after sowing, up to week 14 referring to the week of harvest) was affected by water table depth (factor with 5 levels: chronic drought, dry, mesic, wet, waterlogged) and the presence of the community (2 levels: community, bare soil) and their interaction. Pot identity (factor with 50 levels) was included as a random effect. Marginal and conditional R2 was calculated using the MuMIn package (Barton, 2020).

For the analysis of number of individuals per pot over the 3 years, a generalized linear mixed model was employed with a Poisson distribution. The temporal (pseudo-)replicates of data-assessments were used as a random effect (5 levels: October 2016, May 2017, October 2017, June 2018, October 2018) as well as pot identity to account for repeated measures on the same pot. For (log-transformed) aboveground biomass, a linear mixed effects model was applied, using timing of harvest as a random effect (3 levels: October 2016, October 2017, October 2018). Note that in these models, due to singular fit issues, we were not able to account for pot identity. Water table depth and community as well as their interaction were used as fixed effects.

In a first step, we tested whether the interaction between water table depth and presence of the community was significant (tested using likelihood ratio tests with a chi-squared test statistic). If the interaction was significant, separate models for each community type were employed in a second step. For the pots with the community, we then additionally tested whether community biomass affected *B. orientalis* performance, adding this as a fixed effect to the above-mentioned models.

3. Results

3.1 Effect of water table depth and community on germination of Bunias orientalis

The percentage of germinated seeds of *Bunias orientalis* in 2016 was higher in bare soil (53% i.e., 64/120) compared to community pots (15% i.e., 72/480). The effect of water table depth on germination time was not significantly affected by the presence of the native grassland community (i.e., no significant interaction, Table 1).

However, seeds in bare soil pots germinated 3 weeks (on average) earlier than seeds in the community (Table 1, Figure 1). Time of germination was significantly affected by water table depth (Table 1), with an earlier germination in the wet treatment (mean germination time 5.95 ± 0.51 week after sowing, Figure 1) followed by the mesic and waterlogged treatments (mean 6.98 ± 0.52 and 7.20 ± 1.74 week, respectively). Notably, seeds in the dry (mean 8.18 ± 1.01 week) and chronic drought treatments (mean 8.78 ± 1.12 week) germinated on average 2–3 weeks later than seeds in the other treatments, irrespective of whether they were growing in bare soil pots or communities.

	Germination time	**No. individuals**	**Biomass**
Water table depth × Treatment	χ^2 (4 df) = 3.99; $p = 0.407$	χ^2 (4 df) = 5.09; $p = 0.278$	**χ^2 (4 df) = 17.13; $p = 0.002$**
Water table depth	**χ^2 (4 df) = 12.87; $p = 0.012$**	**χ^2 (4 df) = 34.87; $p < 0.001$**	**Community: χ^2 (4 df) = 18.79; $p < 0.001$** **Bare soil: χ^2 (4 df) = 8.84; $p = 0.060$**
Treatment	**χ^2 (1 df) = 18.11; $p < 0.001$**	**χ^2 (1 df) = 31.20; $p < 0.001$**	

Table 1. *Linear mixed effect models for germination time of Bunias orientalis in the first season (2016) and biomass over three years (2016-2018) and generalized linear mixed model for number of individuals over three years.*

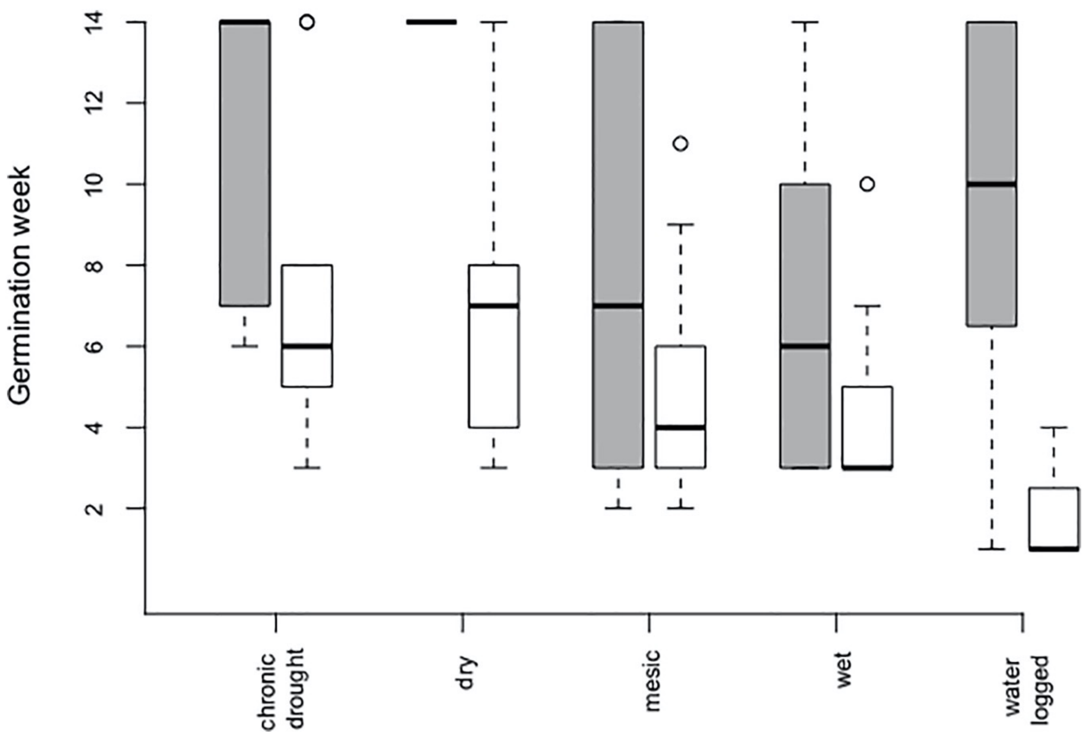

Figure 1. *Germination time in weeks after sowing of Bunias orientalis during the first season (2016) depending on water table depth treatment in the community (gray bars, n = 5, 3, 45, 34, and 7 individuals) and empty pots (white bars, n = 9, 14, 23, 22, and 3 individuals). Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers.*

Results from likelihood ratio tests for the effects of water table depth, treatment and their interaction are shown. (Marginally) significant values ($p < 0.1$) are indicated in bold characters. If the interaction was significant (as for biomass), the effect of water table depth was tested in separate models for community and bare soil.

3.2 Effect of water table depth and community on number of established individuals

The number of established individuals decreased over time: 136 seeds had germinated by October 2016, whereas 73 individuals (51 in bare soil, 22 within the community) were recorded in October 2017 and only 8 individuals (7 in bare soil pot, 1 within community) survived until October 2018 by the end of the experiment (Figure S3).

The effect of water table depth on the number of individuals did not depend on whether plants were growing in the community (Table 1, Figure 2 and Figure S3). Indeed, both in the community and in bare soil pots, the number of individuals significantly differed among water table depth (Table 1 and Figure 2). Most individuals established in mesic conditions and bare soil pots (mean 5.8 ± 1.6).

In communities, the number of individuals was also affected by community biomass [χ^2 (1 df) = 6.53; p = 0.011], which ranged between 0.29 and 130.48 g, with an average of 35.69 g/pot. The highest number of individuals that established per pot were observed in native grassland communities where community biomass was lower than the average (i.e., 10, 8, and 7 individuals/pot established respectively in community biomass of 17.57, 28, and 17.73 g), with a general trend of individuals having successfully established in communities with a biomass lower than 80 g (Figure S4).

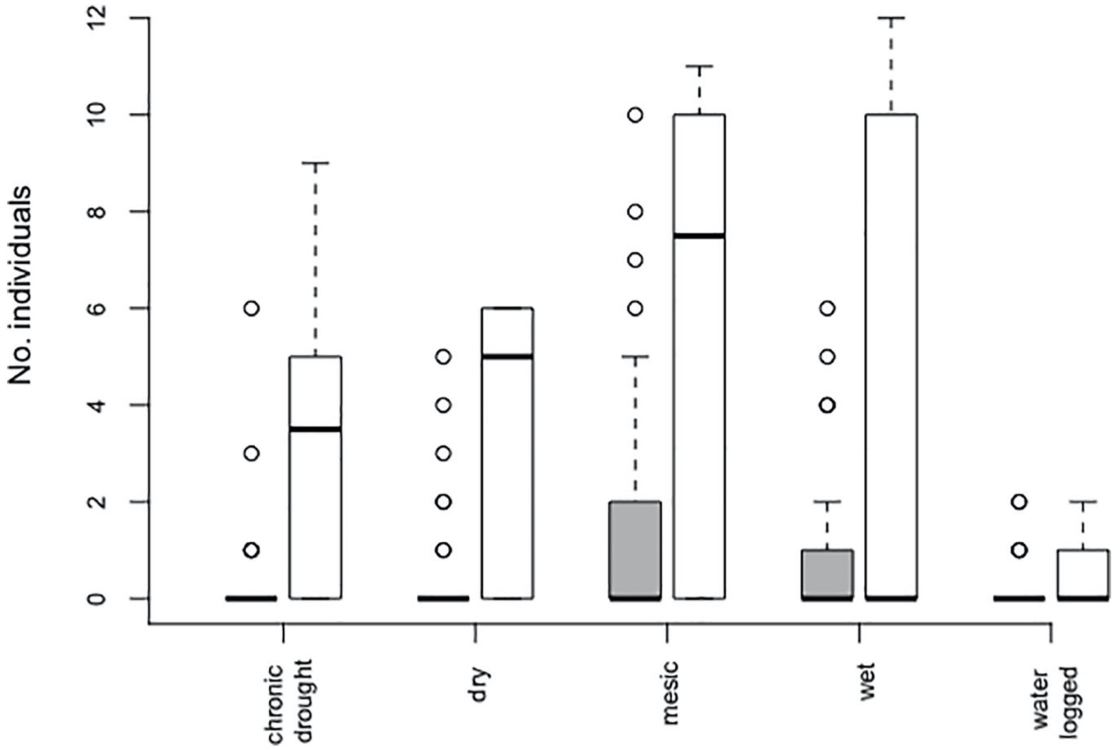

***Figure 2**. Number of Bunias orientalis individuals established per pot (from initially 12 sown seeds) over 3 years (2016–2018) depending on water table depth treatment in the community (gray bars, n = 40 each) and empty pots (white bars, n = 10 each). Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers.*

3.3 Effect of water table depth and community on plant biomass

The effect of water table depth on aboveground plant biomass significantly depended on whether plants were growing in the community (Table 1, Figure 3 and Figure S5). Both in the community and in bare soil pots plant biomass differed among water table depth (Table 1 and Figure 3). In communities, the species reported a significantly higher biomass in mesic (mean 0.051 ± 0.021 g) and wet conditions (0.047 ± 0.018 g), however community biomass did not affect *B. orientalis* biomass [χ^2 (1 df) = 0.031; p = 0.86]. Conversely, biomass of *B. orientalis* observed in bare soil was the highest in pots that received only natural rainfall, i.e., the dry treatment (1.571 ± 0.783 g, Figure 3), followed by the mesic treatment (mean 1.153 ± 0.737 g). We noted that *B. orientalis* never flowered during the experiment.

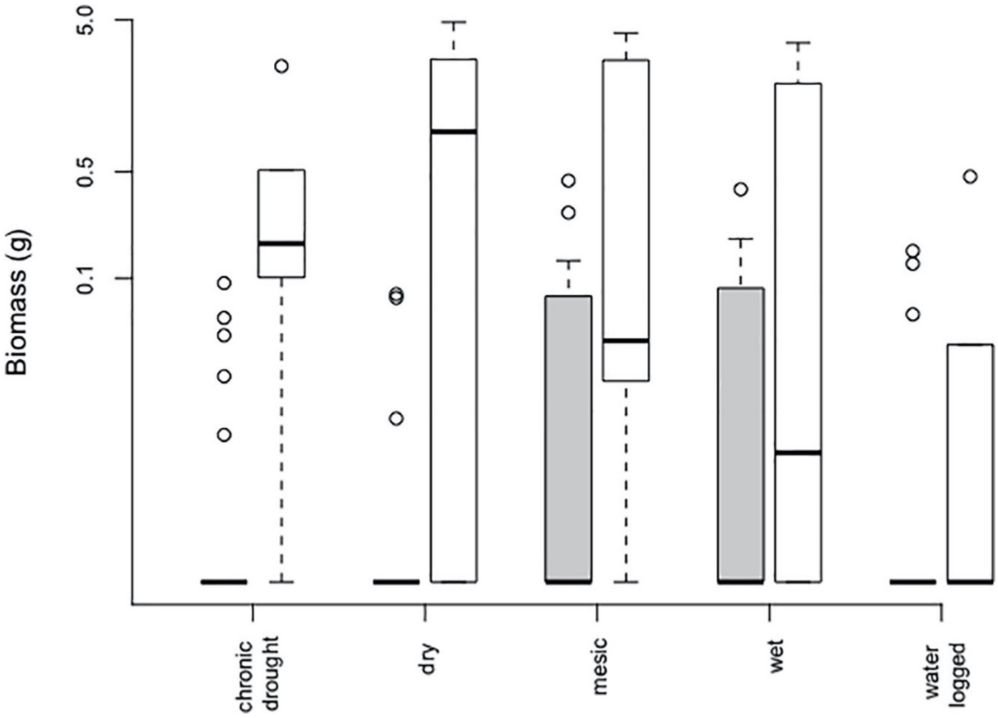

Figure 3. *Aboveground biomass of Bunias orientalis over 3 years (2016–2018) depending on water table depth treatment in the community (gray bars, n = 24 each) and empty pot (white bars, n = 6 each). Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. The y-axis is shown on a log-scale.*

4. Discussion

4.1 Establishment and growth of Bunias orientalis in bare soil and native grassland communities

In contrast to its previously demonstrated invasiveness (Steinlein et al., 1996; Dietz & Steinlein, 1998; Dietz et al., 1999; Woitke & Dietz, 2002; Birnbaum, 2006), the overall performance of *Bunias orientalis* in our experiment was rather low, with less than 23% of seeds having established after the first season and just 8 individuals surviving at the end of the experiment. Previous studies already reported a low germination rate (20%) in ruderal sites characterized by low intensity of disturbance (mowing) and soil deposition, whereas germination reached the highest rates (60.1%) in nitrogen rich, shady and moist soil (Steinlein et al., 1996). Probably competition by shading was too high in the still intact communities in 2016 (height of communities ranged between ca. 10–30 cm and cover between 80 and 100%) to allow for high germination rates. Moreover, the exceptionally dry period in mid-summer 2016 and the record-breaking dry and hot summer of 2018 could have impacted negatively on seed germination and early seedling establishment, a phase demonstrated to be the most critical for water stress and plant survival (Ahmad et al., 2009).

The low performance of *B. orientalis* was also reflected in the finding that plants never flowered during the experiment, although the species normally flowers in the second year or later (Bąba & Kompała-Bąba, 2008). However, plants that can grow either biennially or perennially may use different strategies depending on the conditions they grow in the reduced performance of plants may have allowed for a retention of accumulated resources for perennation (Miao & Bazzaz, 1990) and therefore selectively favored perennial plants (Steinlein et al., 1993).

In addition to the overall low performance, *B. orientalis* performance was clearly constrained by interspecific competition with the native grassland community. Other studies also showed higher growth of *B. orientalis* in the absence of competition, such as higher biomass (Koorem et al., 2018), higher cumulative leaf lengths of the species and higher seed numbers (Dietz et al., 1999). Reducing total cover of all other species, Kiełtyk (2014) demonstrated that *B. orientalis* cover in meadows increases as a consequence. Given that we used natural dug-out mature communities, competition was high because communities were already well established. Light competition from taller species might be a limiting factor in the establishment of the species (Dietz et al., 1996). Indeed, community biomass affected how many individuals established over the years, but notably it did not with regard to *B. orientalis* biomass, whereby either fewer individuals formed the classic large rosettes occupying the given area or more individuals remained smaller. To compensate for its low competitive ability compared with other ruderal forbs (Dietz et al., 1998; Birnbaum, 2006), *B. orientalis* has been shown to use other strategies to successfully invade: it requires moderate disturbance to establish, that facilitates invasion by reducing competition and changing resource conditions in favor of one species over another (Hager, 2004; Domènech & Vilà, 2008). For instance, formation of molehills which damages resident vegetation and creates open areas of bare soil, leading to less-compacted soil and vegetation with a lower-density rhizosphere, facilitates invasion of *B. orientalis* (Kiełtyk & Mirek, 2015). In contrast, mimicking standard management practices in extensively used semi-natural grasslands in our study, with mowing twice a year, did not favor the establishment, survival and potential invasiveness of the plants, maybe because the degree of disturbance was too low. However, simulating a high degree of disturbance with the bare soil pots greatly increased performance of *B. orientalis*.

4.2 Effect of hydrological stress on Bunias orientalis performance

The different soil moisture conditions that we created by adjusting water table depth, simulating wetness, temporary flooding and especially dryness strongly influenced the performance of the plants. Seed germination in the first season occurred earlier and the number of established individuals as well as biomass production over the following years was generally higher in the wet and mesic treatments. Indeed, even though B. orientalis exhibits a wide ecological tolerance, previous studies showed that the species establishes better in moist soils (Clapham et al., 1962; Steinlein et al., 1996).

Notably, *B. orientalis* showed greater performance and a wider ecological tolerance along the hydrological gradient in bare soil than in the community, with competition with the surrounding vegetation influencing the establishment and growth of the species. In particular, while establishment was highest in mesic and wet conditions regardless of the community, the presence of the community mostly limited plant growth to mesic and wet conditions. In contrast, plant biomass production in bare soil was not restricted to the non-stressful hydrological conditions, with the highest biomass found in dry condition, but moderate performance also observed in wetter treatments. This finding suggests that the smaller number of individuals on average established in dry treatments compensated for their number by producing more biomass. We note some limitation with the interpretation of the biomass results, however, because due to the limited number of pots in our experiment, we could not fully statistically account for non-independence among repeated measurements. Nevertheless, there are some likely explanations for our results. Reduced intraspecific competition might have favored faster growth rates in the dry treatments. In contrast, despite the lower aboveground biomass in dry communities, belowground competition for water might have become more severe, as found in other experimental studies (e.g., Walter, 2018). This may have limited plant growth of *B. orientalis* seedlings in interspecific competition under such resource stress, especially since seedlings usually have a smaller root system than established plants. The generally lower success of plants in waterlogged conditions (whereby differences between bare soil pots and community were less pronounced) may be attributed to anaerobic stress rather than increased competition, because special adaptations are necessary to cope with anoxia, as has been shown in other studies (Jung et al., 2009; Bartelheimer & Poschlod, 2016).

The results of our study have implications regarding possible changes in the dynamics between native and the invasive species in the future, based on the timing and frequency of precipitation patterns: the establishment of *B. orientalis* seedlings might turn into a successful invasion only if germination of seeds will occur in moderate precipitation conditions, followed by drought conditions coupled with disturbance. Therefore, rainy and wet springs and warmer summers might help the invasion success of this species, particularly if a disturbance creates patches of bare soil.

5. **Conclusion**

We conducted a 3-year mesocosm experiment in a common garden focusing on the invasion success of the warty cabbage *Bunias orientalis* in German native grassland communities. Thereby we compared the establishment and growth of plants in such communities with bare soil in varying hydrological conditions (from waterlogged to dry soil moisture), mimicking predicted changes in precipitation patterns under climate change. Establishment and biomass of *B. orientalis* differed among water table depth, with higher performance in wet and mesic conditions. However, despite its wide ecological tolerance in the absence of competing plants, invasion success of *B. orientalis* in the native grassland communities was generally low, which might be due to the low competitive ability of the species in conditions of hydrological stress and the fairly low degree of disturbance in our near-natural grasslands. Our results suggest that with changing of precipitation patterns toward more extreme events with frequent and pronounced waterlogged and drought conditions, the establishment of *B. orientalis* within grassland communities might be reduced. However, within patches of bare soil, this will not occur, where disturbance combined with drought will favor species establishment, survival and even invasiveness, a scenario which should be further tested in future studies.

Author Contributions JW and CS designed the study. AC and JW collected the data. AC and CS performed the analyses. AC wrote the first draft of the manuscript. All authors contributed substantially to the revisions.

Funding This work was supported by the German Research Foundation (DFG, WA 3442/2-1 and SH 924/1-1).

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Supplementary Materials

Table S1: *Species composition of the grassland communities at the beginning of the experiment (May 2016). Species richness ranged from 4 to 11 species (mean = 7.23). Note that species composition was only assessed in 17 out of the 40 grassland pots (randomly chosen). Total cover ranged between 80-100%.*

Species	**Family**	**Average cover (%)**
Achillea millefolium L.	Asteraceae	0.76
Arrhenatherum elatius (L.) P.Beauv. ex J. Presl & C. Presl.	Poaceae	17.12
Agrostis stolonifera L.	Poaceae	5.59
Bellis perennis L.	Asteraceae	0.82
Bromus erectus Huds.	Poaceae	1.18
Bromus hordeaceus L.	Poaceae	2.65
Centaurea jacea L.	Asteraceae	0.18
Cerastium holosteoides Fr.	Caryophyllaceae	0.18
Dactylis glomerata L.	Poaceae	7.94
Daucus carota L.	Apiaceae	0.18
Deschampsia caespitosa (L.) P. Beauv.	Poaceae	1.76
Festuca pratensis Huds.	Poaceae	14.70
Galium album Mill.	Rubiaceae	5.53
Geranium pyrenaicum Burm. f.	Geraniaceae	0.29
Glechoma hederacea L.	Lamiaceae	5.88
Heracleum sphondylium L.	Apiaceae	1.18
Holcus lanatus L.	Poaceae	2.94
Hypochaeris radicata L.	Asteraceae	0.59
Lolium multiflorum Lam.	Poaceae	0.29
Lolium perenne L.	Poaceae	4.82
Lotus corniculatus L.	Fabaceae	1.18
Luzula campestris DC.	Juncaceae	0.29
Lysimachia nummularia L.	Primulaceae	0.06
Medicago lupulina L.	Fabaceae	1.76
Ononis spinosa subsp. hircina (Jacq.) Gams	Fabaceae	0.18
Plantago lanceolata L.	Plantaginaceae	5.00
Poa pratensis L.	Poaceae	14.82
Ranunculus acris L.	Ranunculaceae	0.88
Ranunculus repens L.	Ranunculaceae	0.88
Salvia pratensis L.	Lamiaceae	1.29
Sanguisorba minor Scop.	Rosaceae	0.59
Taraxacum officinale (L.) Weber ex F. H. Wigg.	Asteraceae	1.29
Trifolium dubium Sibth.	Fabaceae	2.06
Trifolium pratense L.	Fabaceae	2.23
Trifolium repens L.	Fabaceae	5.06
Trisetum flavescens (L.) P.Beauv.	Poaceae	3.82
Triticum aestivum L.	Poaceae	0.29
Vicia L. spp.	Fabaceae	2.70

Figure S1. *Aerial view of the mesocosm experiment, showing the large 260-L pools with the smaller 20-L pots within. The white grids are the partial rainout shelters to reduce natural precipitation by 30% for the chronic drought treatment. Note that the experiment described here included 40 grassland community pots and 10 bare soil pots in 49 different pools (i.e. not all pools and pots in the picture were part of the experiment described here). Picture: V. Ferenc (June 2018).*

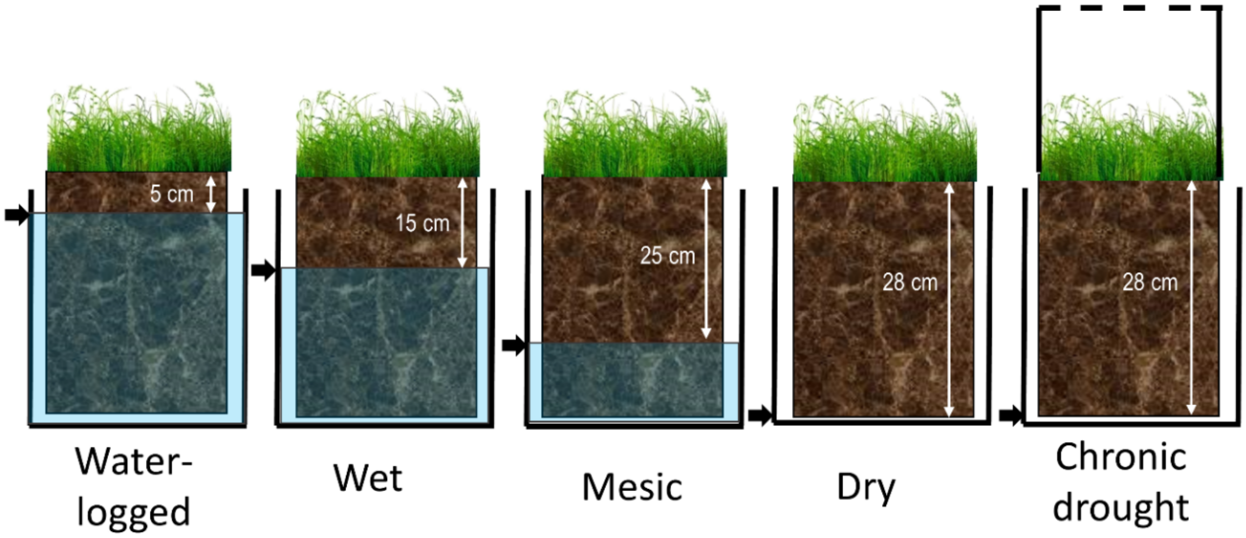

Figure S2. *Experimental design of the five hydrological treatments used in the mesocosm experiment: waterlogged, wet, mesic, dry and chronic drought conditions. Smaller 20-L pots were placed in large 260-L pools. The water table depth was adjusted by placing holes in the large pools at the depth indicated by the bold arrows on the left, with the white arrows indicating the water table depth in the respective treatments. The pools in the waterlogged, wet and mesic treatment were filled twice a week with water up to the respective depth. The dry treatment received only natural precipitation, while chronic drought additionally was covered by a partial rainout shelter reducing precipitation by 30%. The 20-L pots contained either a natural grassland community (with 8 replicates per water table depth treatment) or bare soil (2 replicates per treatment).*

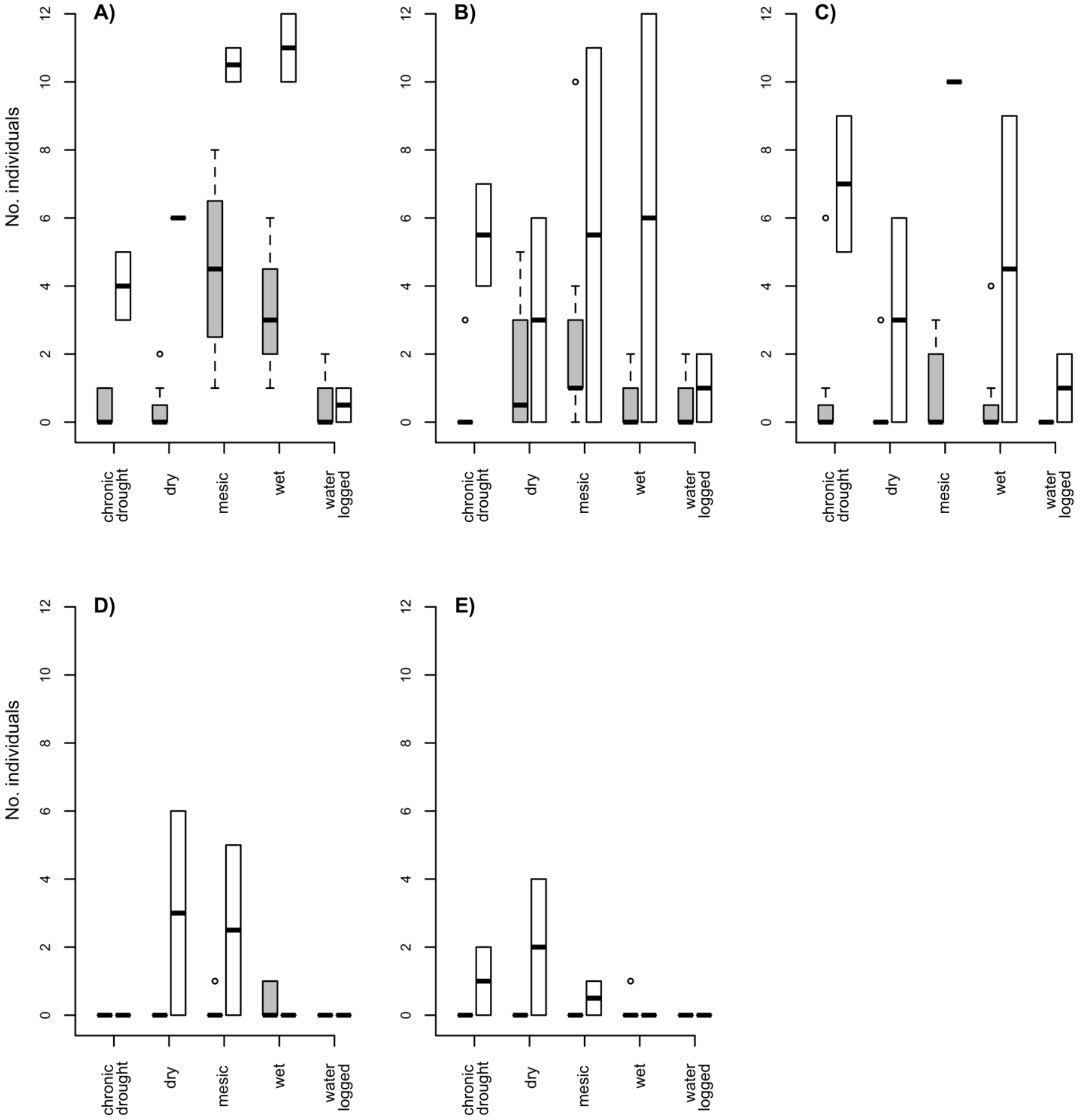

Figure S3. *Number of individuals established per pot (from initially 12 sown seeds) in A) October 2016, B) May 2017, C) October 2017, D) June 2018, and E) October 2018 depending on water table depth treatment in the community (grey bars) and empty pot (white bars). Boxplots show the median per group (solid line), crosses the mean, boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers.*

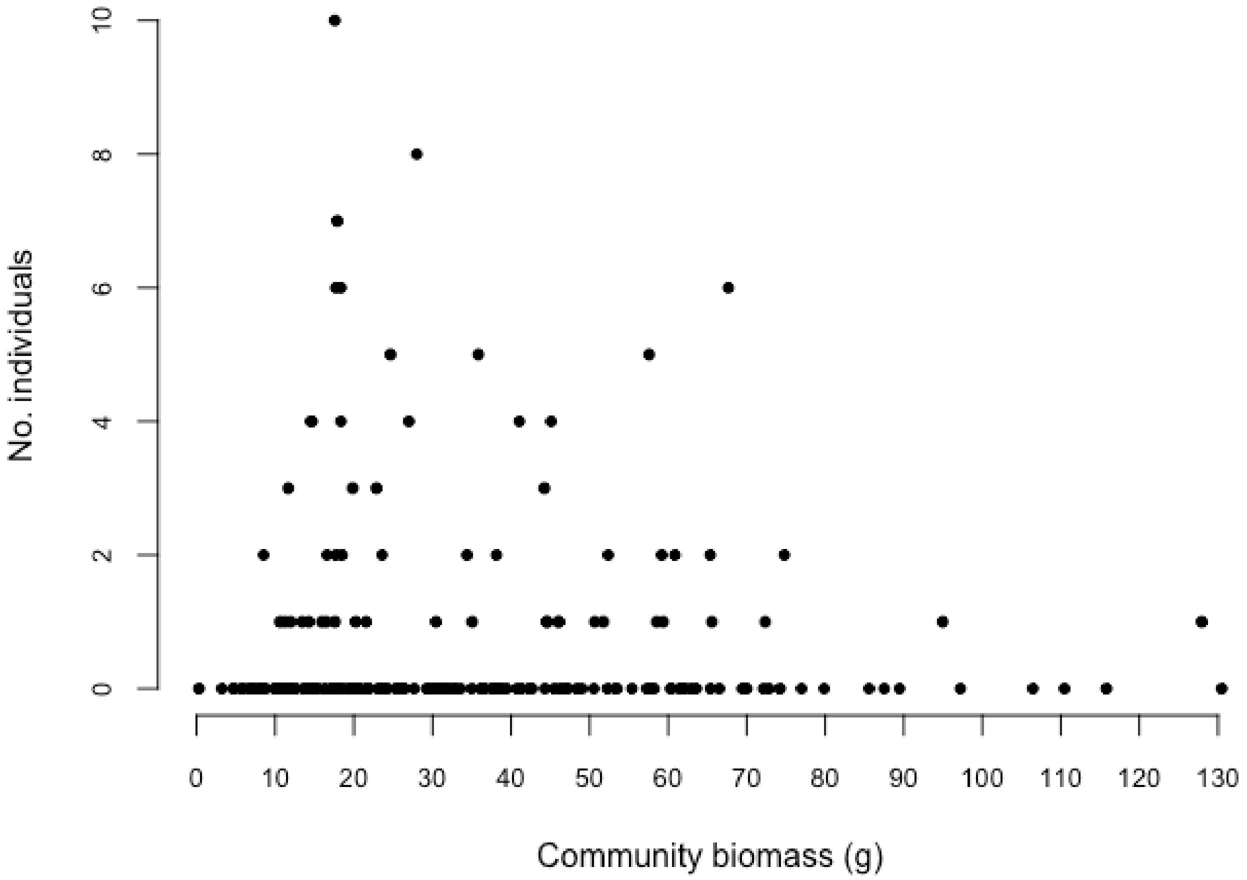

Figure S4. *Number of Bunias orientalis individuals established per pot (from initially 12 sown seeds) over three years (2016-2018, n = 117 as community biomass data was missing for 3 pots) depending on the native grassland community biomass.*

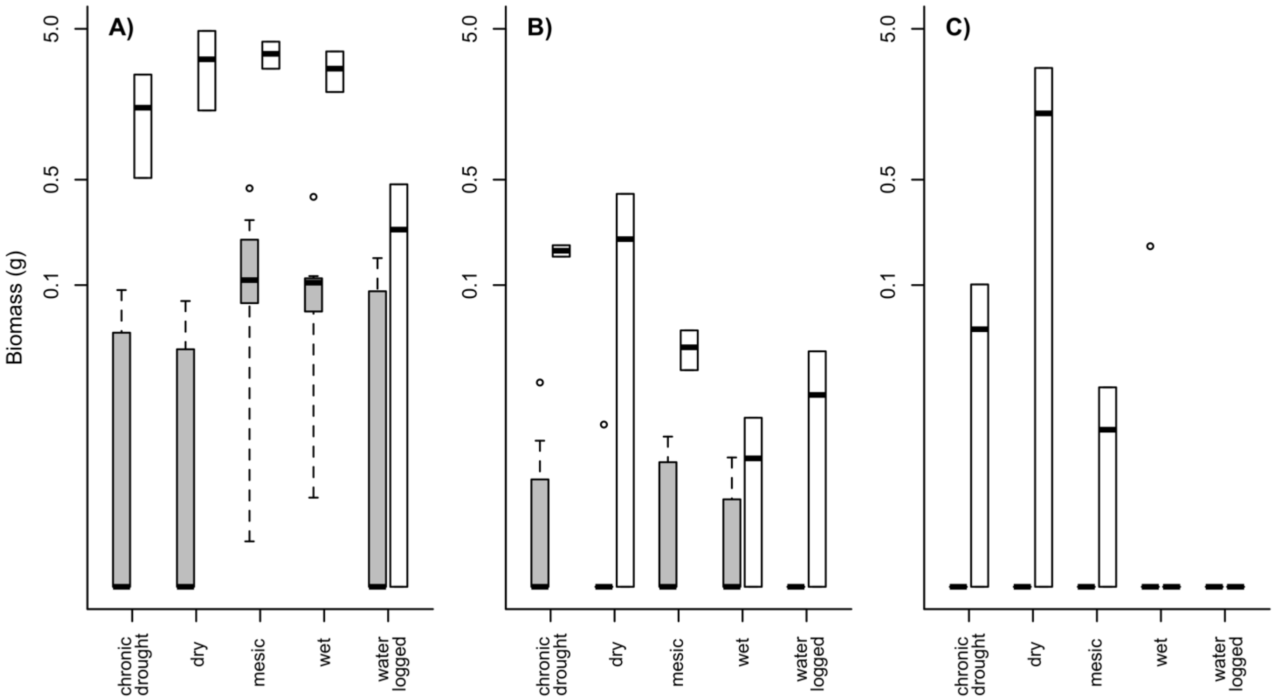

Figure S5. *Aboveground biomass per pot in A) October 2016, B) October 2017, and C) October 2018 depending on water table depth treatment in the community (grey bars) and empty pot (white bars). Boxplots show the median per group (solid line), boxes represent the 25th and 75th quantile, whiskers the normal data range and circles the outliers. The y-axis is shown on a log-scale.*

Conclusions

This work aimed at understanding the entity of interspecific interactions between species, focusing on the competition ability of a policy species vs its invasive potential competitor (i.e., *Marsilea quadrifolia* L. vs *Heteranthera reniformis* Ruiz & Pav) and competition ability of a policy species (*Kosteletzkya pentacarpos* (L.) Ledeb.) vs natural community, with the aim to individuate the ecological requirement of the species for their long-term survival and the development of a robust conservation plan. In addition, this work addressed the competitive ability of the invasive species *Bunias orientalis* L. vs native grassland community under different hydrological conditions.

Competitive interactions between species within plant communities strongly help in explaining coexistence, species diversity maintenance, ecosystems functioning and are important for understanding both plant conservation and biological invasions since they determine changes in community composition and abundance, especially in agroecosystems and human disturbed habitat, such in the case of the target species. However, the different experimental studies showed that the competition between plants is extremely vary and depends on several factors that may significantly affect the response of species basing on the resources for which species compete (i.e., light, water, nutrients) but also on the presence or absence of the natural vegetation or co-occurring species in environment where the species are adapted. In addition, the experimental studies showed that competition is largely species-specific dependent and changes with changing of the environment.

The study of the competition must then be addressed to understand the dynamics occurring among species, especially in disturbed and anthropized habitats such as wetlands and grasslands where invasive species are abundant and highly competitive but also several threatened species and diverse native communities still occur.

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Additional scientific output

Beside the beforementioned papers, during my PhD I published (and submitted) other scientific articles and chapters in international indexes journals. The following works deal with germination ecology, alpine environment, invasion biology, plant conservation.

Scientific papers

Plant regeneration above the species elevational leading edge: trade-off between seedling recruitment and adult performance

Mondoni, A., Orsenigo, S., Abeli, T., Rossi, G., Brancaleoni, L., **Corli, A.**, Iacumin, P., Tonin, R., Marchesini, R., Gerdol, R., 2020. Plant regeneration above the species elevational leading edge: trade-off between seedling recruitment and adult performance. Frontiers in Ecology and Evolution (Published; https://doi.org/10.3389/fevo.2020.572878)

Abstract

Mountains and their biota are highly threatened by climate change. An important strategy that alpine plants use to escape this change consists in seed dispersal and the ability of seeds to germinate and establish in new sites at higher elevation. Little is known about the environmental factors that can affect the regeneration of plants above the elevational limit of growth. We present the first field evidence of recruitment success and plant performance in consequence of upward shift from the alpine to the nival life zone. Seeds of four alpine grassland species were sown at the current elevational limit of growth (site A) and 200 m upward, in a nival environment (site N) located in the Italian Alps. At site N part of the seeds were subjected to experimental manipulation of temperature (using an Open Top Chamber, OTC) or soil (using soil from site A). Recruitment success, soil surface temperature and water potential were monitored for five consecutive years. At the end of the experiment, vegetative growth and foliar traits were measured on individuals from all treatments. Mean annual soil surface temperature and length of the growing season at site A were ca. 2◦C higher and ca. 44 days longer than at site N. Seedling emergence and seedling establishment generally were higher at N (with or without OTC) on local soil than at site A or at site N with soil originating from site A. Conversely, production was higher at site A and at site N with soil originating from site A. Recruitment success above the elevational leading edge was enhanced by coarser and nutrient-poor soil, which promoted seedling emergence and establishment but constrained plant growth. This trade-off between seedling recruitment and plant production underlines adaptive consequence and environmental filtering, both critical to forecast community assembly and responses of alpine species to climate warming.

Effects of residence time, auto-fertility and pollinator dependence on reproductive output and spread of alien and native Asteraceae

Corli, A., Sheppard, C.S., 2019. Effects of residence time, auto-fertility and pollinator dependence on reproductive output and spread of alien and native Asteraceae. Plants (Published; https://doi.org/10.3390/plants8040108)

Abstract

Alien plants benefit from auto-fertility to spread over areas where the lack of co-evolved mutualists would otherwise limit invasion success. However, the widespread generalists among mutualists and their large geographical ranges allow alien plants to be integrated into networks. The role of residence time also must be accounted for, as it takes time for a species to spread and adapt to a new area. We investigated how residence time, auto-fertility and pollinator dependence affect reproductive output and invasion success of Asteraceae in Germany. We conducted a multi-species common-garden experiment along an alien–native continuum including 42 species of natives, archaeophytes and neophytes (casual and established), subjecting plant individuals either to free access or exclusion of pollinators. Pollinator dependence does not play a crucial role in invasion success, with most Asteraceae being able to self-fertilize. Surprisingly, both established neophytes and natives showed higher abilities to self-fertilize, while archaeophytes and casual neophytes were more attractive to pollinators. In contrast to casual neophytes, the established neophytes' strategy was associated with a large reproductive output. Yet auto-fertility was not associated with range size, since archaeophytes reached the largest range sizes. Elucidating how breeding systems affect invasion success is crucial for predicting and managing invasions.

Richer, greener, and more thermophilous? – a first overview of global warming induced changes in the Italian alpine plant communities within the new GLORIA ITALIA NETWORK

Porro, F., Orsenigo, S., Abeli, T., Mondoni, A., **Corli, A.**, White, F.J., Lodetti, S., Tomaselli, M., Petraglia, A., Carbognani, M., Gualmini, M., Forte, T.G.W., Erschbamer, B., Nicklas, L., Carnicero, P., Mallaun, M., Unterluggauer, P., Stanisci, A., Giancola, C., di Martino, L., Barni, E., Oddi, L., Morra di Cella, U., Gentili, R., Dellavedova, R., Adorni, M., Pauli, H., Rossi, G., 2021. Richer, greener, and more thermophilous? – a first overview of global warming induced changes in the Italian alpine plant communities within the new GLORIA ITALIA NETWORK, Plant Biosystems (In press, https://doi.org/10.1080/11263504.2021.1992527).

Abstract

We announce the formation of the "GLORIA ITALIA NETWORK" and present an overview of the Italian alpine plant communities changes that have occurred in the last 20 years. This network will provide coordination between Italian GLORIA sites and enhance public awareness of changes in alpine plant diversity under climate change

Chapters

Corli, A., Orsenigo, S., 2021. Marsilea quadrifolia: from paddy field weed to threatened species. In: Reference Module in Earth Systems and Environmental Sciences, Elsevier. (Published; https://doi.org/10.1016/B978-0-12-821139-7.00045-3)

Abstract

In this contribution we illustrate a species on the verge of extinction, commonly known as flagship species of temporary wetland agro-ecosystems. *Marsilea quadrifolia* L., known also as European water clover, is a leptosporangiate pteridophyte typical of aquatic environments, considered a weed of paddy fields of southern Europe; it grows with a creeping rhizome either in submerged or in emerged condition, experiencing heterophylly. The life cycle is carried out by the alternation of generations and species reproduction occurs either with vegetative propagation or with sexual reproduction thanks to sporocarps. The species is ubiquitous in many soils at different levels of pH, preferring mesotrophic to eutrophic shallow waters; it can be found either in natural environments such as lakes and small rivers as well as in an agricultural context, such as paddy fields and ditches. *M. quadrifolia* is native to Eurasia and has a widespread distribution; it spreads also in America, where it is considered an alien species. For this reason, it is classified as “Least Concern” globally according to the IUCN criteria, however, since its distribution is scattered in Europe, it is assessed as “Vulnerable” and listed as threatened with extinction in most European countries. On the other hand, the species is cultivated in aquaria and has been used for centuries for its ethnobotanical and medicinal properties. Human activities, habitat loss and degradation, excessive water eutrophication, agricultural practices and the presence of alien species are the main threats for the species.

Many efforts have been applied to preserve this species, both through in situ reintroduction as well as ex situ techniques, with cultivation in botanical gardens, in vitro propagation as well as spore cryopreservation. However, despite the few successes of these efforts, it’s reappearance in paddy fields could represent a valid opportunity to combine nature conservation and sustainable agricultural practices, offering new possible income for farmers.

Corli, A., Orsenigo, S., Gerdol, R., Brancaleoni, L., Abeli, T., Rossi, G., 2020. Testing a threatened pteridophyte in paddy fields: the coexistence of rice production and Marsilea quadrifolia L. in Northern Italy.

Presented at the 115° Congress of the Italian Botanical Society helded Online, September 9th-11th 2020

Abstract

During the last century, many species of conservation interest, associated with agricultural environments experienced severe population declines, with agricultural intensification being the main responsible of their loss. The aquatic pteridophyte *Marsilea quadrifolia* L. was formerly considered as a weed in paddy fields of Southern Europe. However, with the introduction of new agronomic practices as mechanization, use of chemicals and simplified rotation, the species experienced a strong decline, being listed in the Annexes of Directive 92/43/EEC and Bern Convention and classified as “Endangered” in the Italian Red Lists.

Noteworthy, as a consequence of more sustainable rice cultivation practices, new spontaneous occurrences were recently recorded in some rice farms in Northern Italy (Po Valley), a region hosting more than 50% of the European rice production. For the mandatory improvement of the species conservation status, reintroduction has to be considered and cannot disregard the agricultural and semi-natural context where the species grows. However, in such an area of intensive agriculture, species conservation may be constrained by both landscape fragmentation and conflicts with farmers.

Aiming to stimulate the adoption of specific measures for the conservation of *M. quadrifolia* and set up a conservation plan, swards were cultivated in 2017 and 2018 in rice farms of the province of Pavia (N-Italy), to understand the effects of different farming management (organic, conventional, in transition from conventional to organic) with respect to *M. quadrifolia* presence/recovery. To evaluate the micro-environmental conditions that can affect species growth (sunny vs. shaded), rice was removed in 2018 to create an open area (1×1 m) around the swards. Plant biomass, ecophysiological (chlorophyll fluorescence and pigments concentration) and environmental data (temperature, irradiance, water and soil main chemical contents) have been evaluated.

Our study revealed that M. quadrifolia has the highest vegetative performance in organic farms, even though the species survived in conventional farms with reduced herbicides supply. Differences in water chemistry had limited effects on *M. quadrifolia* performance, because of its already demonstrated wide ecological amplitude. In addition, the shady conditions provided by rice canopy created micro-habitats suitable for *M. quadrifolia* growth, with open areas being quickly colonized by

exotic invasive species like *Heteranthera reniformis* Ruiz & Pav., *Ammannia coccinea* Rottb. and *Cyperus microiria* Steud.

The cultivation of the species in organic rice farms represents, then, an important opportunity for preserving this endangered species in areas of intensive agriculture. Considering that rice fields are worldwide recognized as surrogate habitats for wetland species and many rice farms in the crop district of Northwestern Italy are included in protected areas (e.g., the Special Protection Area “Garzaie della Lomellina”), a conservation plan should consider simultaneously the ecological requirements of the species and the needs of stakeholders (i.e., farmers, landowners, local policymakers). To this aim, ad hoc agro-environmental compensations or incentives from the EU Rural Development Plan (RDP) should support farmers willing to reintroduce and maintain *M. quadrifolia*, at least in organic rice farms, transforming potential conflicts between nature conservation and productivity into new income chances for farmers.

Corli, A., Orsenigo, S., Rossi, G., Gerdol, R., Brancaleoni, L., Abeli, T., 2020. Coexistence of intensive rice production and Marsilea quadrifolia in the organic rice farms of the Po Valley.

Presented at the Conference of Young Botanists, Genua, Italy, February 6th-7th 2020

Abstract

Agricultural activities are the main factor driving biodiversity loss, being responsible of 31% current species loss globally. Among these, rice cultivation caused considerable environmental impacts such as the contamination from massive herbicides use. However, the recent increase of organic rice farming with reduced use of herbicides and mechanical tillage, improved environmental conditions and suggested the potential reintroduction and survival of threatened species.

In this study, we tested the coexistence of intensive rice production and conservation of the aquatic fern *Marsilea quadrifolia* L. in the Province of Pavia, one of the most important areas for rice production in Italy.

This species was considered a major weed in paddy fields in the past, however because of modern agronomic techniques (e.g., herbicides) its occurrence drastically decreased, being listed as “Endangered” in Italian Red Lists and protected at EU level. Interestingly, recent occurrences were found in organic paddy fields, likely as a consequence of improved environmental conditions.

Swards of Marsilea have been transplanted in three different farming system (organic, in transition and conventional farms) in 2017 and 2018, aiming to understand to what extent they affect the performance of *Marsilea quadrifolia*. We noted that the species best performed in organic farms, reporting higher chlorophyll fluorescence but especially higher biomass, whereas plants died in conventional farms after the first year. These results will help us to identify the most suitable conditions that favor survival and spread of the species in rice fields for future conservation plan.

Posters

Corli, A., Sheppard, C.S., 2019. Alien-native species continuum approach: invasion dynamics among Asteraceae. Poster presented at the 114° Congress of the Italian Botanical Society (VI International Plant Science Conference), Padua, Italy, September 4th-6th 2019

Posadas IJ, Rossi G, **Corli A**, Weeks A, Saiz JCM, Aguilar JF, 2019. *¿Qué nos dicen los datos moleculares sobre la naturalidad de Kosteletzkya pentacarpos (Malvaceae) en el Mediterraneo?* Poster presented at the 9° Congreso de Biología de la Conservación de Plantas, Granada, Spain, July 9th-12th 2019

Acknowledgements

I would like to express my deep gratitude to my tutor Prof. Graziano Rossi who believed in me and gave me a constant academic and human support. I am grateful to all of my co-tutors: Prof. Renato Gerdol for his valuable help, Prof. Thomas Abeli for his precious suggestions, Dr. Simone Orsenigo for his useful critiques and assistance, Dr. Lisa Brancaleoni for her consistent advices and encouragement. Thanks also to Prof. Andrea Mondoni for his useful notes and scientific feedback regarding my work. Many thanks to Dr. Christine Sheppard who welcomed me in Germany twice, believing in my skills and giving me the opportunity to lead autonomously two projects; thanks also to Dr. Julia Walter for her scientific priceless help and grateful thanks to all of the German and international students and researchers I had the opportunity to meet and to share knowledge. Thanks to Dr. Roberta Marchesini for her valuable help and assistance in the laboratory, Mr. Fausto Molinari and Dr. Fabrizio Negrini who supported me in the field experiment, Mr. Mauro Menghini and Dr. Giovanni Nobili who kindly supported part of my research in the field.

Special thanks go to my friends and colleagues: Francesco, Adriano and Gloria from the Seed Bank; Francesco, Chiara and many other young researchers for valuable suggestions and advices, Dr. Paolo Cauzzi and Dr. Nicola Ardenghi for the priceless help they provided me; valuable thanks go also to the students that helped me in acquiring data, doing experiments, that taught me as well. Many thanks to the farmers, researchers, and botanists from all over the world with I contacted in these three years and that, many time unconsciously, have provided me with essential inputs to carry out my research.

Finally, I am deeply indebted to my Family for supporting me during my studies and personally, who always believed in me and in my progress, sometimes even more than me! I am grateful to my friends Alma, Luca, Ele, Ismael, Betta, Chiara, Davide and many others: you have been always there, ready to laugh and having fun, brightening my days.

Special thanks finally go to Tommi, who unexpectedly entered into my life and made wonderful things inside it.

Thank you all, sincerely.

With extreme gratitude and love,

Anna