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ARTICLE

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

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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

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 and Vidotto 2010; Hill et al. 1991). This led to a drastic decline of *Marsilea 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

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the EU Directive 92/43/EC "Habitats" and in the Annex I of Bern Convention, the primary legal instruments driving species protection in Europe (Online Resource 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 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; Online Resource 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 may 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 (Online Resource 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.

Materials and methods

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 (Online Resource 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 multiannual 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 (Online Resource 2). 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*.

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.

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 (Online Resource 3). 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 (13–20 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).

Results

Plant biomass, water chemistry and soil chemistry

Total biomass of *M. quadrifolia* was highest in organic farms both in 2017 and in 2018 (Fig. 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* $\Lambda = 0.010$). 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 (Fig. 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 (Online Resource 3). Conversely, soil chemistry did not differ significantly among farming systems ($F_{10,2} = 1.11$, $p < 0.56$; Wilk's $\Lambda = 0.023$; Online Resource 3).

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).

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.

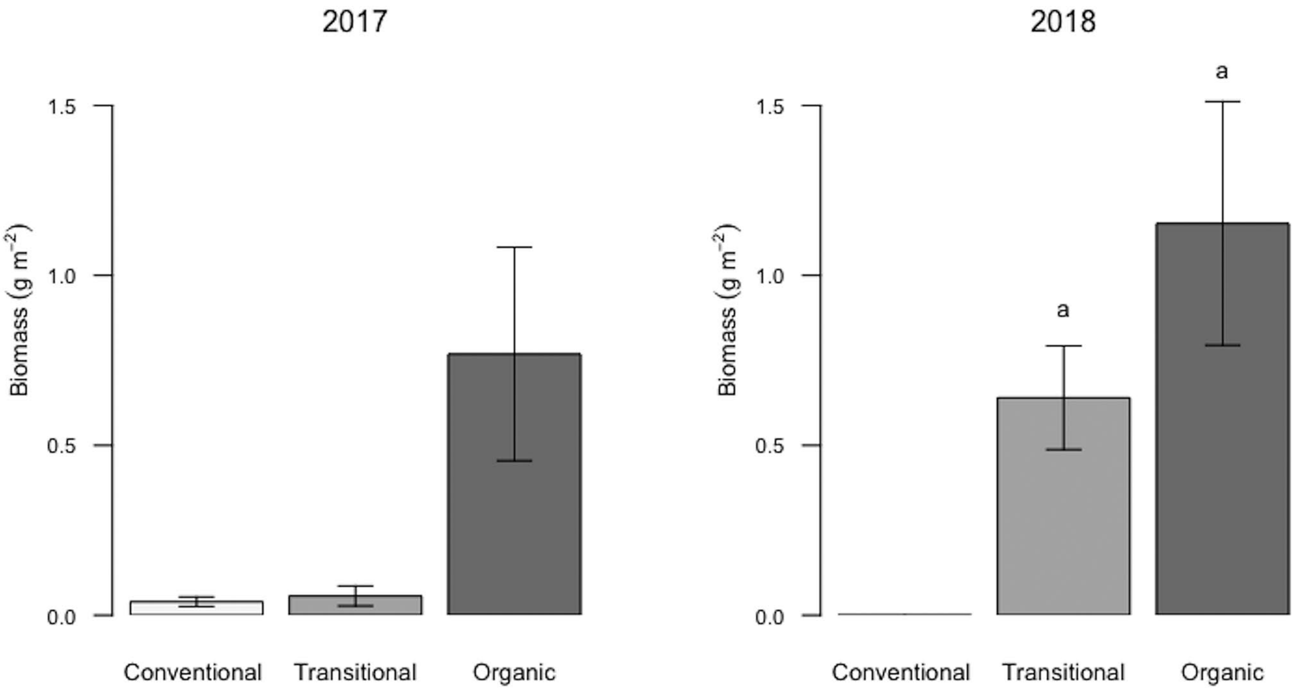
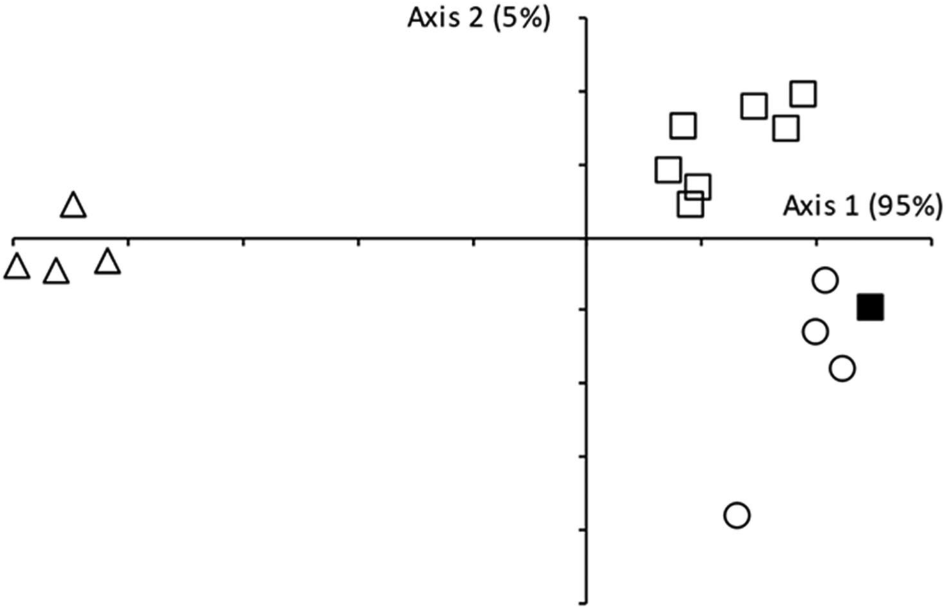


Fig. 1 Mean (+SE) total biomass of *M. quadrifolia* collected from different rice farming systems. Lowercase letters indicate significant ($p < 0.05$) differences between farming systems, according to post hoc Fisher's LSD tests

Fig. 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)



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

Table 1 Mean (+SE) values of chlorophyll fluorescence, concentrations and ratios of photosynthetic pigments in *M. quadrifolia* leaves from open areas and under rice canopy

	Open areas	Under rice canopy
Chlorophyll fluorescence	**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

Bold characters indicate significant differences in one-way ANOVAS ($p < 0.01$)

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 and 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.

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