



Mediterranean grassland succession as an indicator of changes in ecosystem biodiversity and functionality

José Antonio Molina¹ · Juan Pedro Martín-Sanz² · Inmaculada Valverde-Asenjo² · Abel Sánchez-Jiménez¹ · José Ramón Quintana²

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Abstract

The abandonment of agricultural lands triggers a secondary succession of plant species which implies important changes in soil quality. Annual Mediterranean grasslands are known to be persistent on abandoned agriculture lands in the western Mediterranean. We used plant taxonomic and functional approaches to determine the role of Mediterranean grasslands as an indicator of changes in ecosystem biodiversity and functionality. We tested the hypothesis that Mediterranean grasslands are a suitable model for monitoring biodiversity and soil fertility in a secondary succession. Soil and vegetation features on 21 permanent plots were monitored in 2016 and 2020. Numerical classifications based on floristic composition showed two different plant communities independently of the sampling year: early-stage grasslands in the first post-abandonment decade and late-stage grasslands after the first post-abandonment decade. Generalized linear model and redundancy analysis also revealed differences in growth forms, functional traits and soil functionality between communities. Late-stage grasslands was characterized by enriched bryophyte coverage and an impoverishment in hemicryptophytes and plant latex segregators growing on soils with a higher hydrolase enzyme activity and TOC content compared to early-stage grassland. Our results suggest that annual Mediterranean grasslands growing on siliceous soils denoting a mature-stage succession, and floristically characterized by the symbiont plant with Ascomycota, *Tuberaria guttata*, and a high bryophyte cover, are worthy of recognition for conservation.

Keywords Abandoned agrosystems · Annual dry grasslands · Secondary succession · Short-term · Soil biology

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✉ José Antonio Molina
jmabril@ucm.es

¹ Department of Biodiversity, Ecology and Evolution, Complutense University of Madrid, c/ José Antonio Novais 12, 28040 Madrid, Spain

² Department of Chemistry in Pharmaceutical Sciences, Complutense University of Madrid, plaza Ramón y Cajal s/n, 28040 Madrid, Spain

Introduction

Terrestrial ecosystems are subject to different human-induced drivers that alter the ecosystem services on which we depend, including the maintenance of biodiversity (Steffen et al. 2004). Climate change, N deposition, CO₂ concentration, biotic change and land-use change take place simultaneously and interact with each other (Doblas-Miranda et al. 2015). Mediterranean terrestrial ecosystems are in transition between arid and temperate ecosystems and show a high sensitivity to the effects of these drivers of ecosystem diversity (Sala et al. 2000). Rural land abandonment is an important syndrome of global change in European Mediterranean countries, and the Mediterranean Basin is one of the world's regions where land abandonment is most prevalent (MacDonald et al. 2000; Weissteiner et al. 2011). In the last 20 years, 2.95 million hectares of utilized agricultural area have been lost in Spain (MAPA 2020). Between 2015 and 2030 about 11% (more than 20 million hectares) of agricultural land in the EU will be at high potential risk of abandonment, with Spain among the most affected countries (Perpiña Castillo et al. 2018). Land abandonment in the Mediterranean has enhanced species richness and overall abundance (Pliening et al. 2014) and led to an improvement in ecosystem functionality through increased soil C and N, soil biological activity and plant diversity (Valverde-Asenjo et al. 2020). However, there is a lack of in-depth knowledge on the relationships between the changes in plant composition and diversity and the changes in soil functionality during this secondary succession.

Mediterranean grasslands are among the most diverse in the world (Faber-Langendoen and Josse 2010), and occupy about 1.3 million km² of the Earth's surface (Dixon et al. 2014). In the European Mediterranean basin, grasslands include a high diversity of vegetation types (Mucina et al. 2016); specifically, annual Mediterranean grasslands on acidic soils contain a high diversity of low-growing ephemeral herb and grass plants. This habitat reaches its highest abundance and diversity in the western Mediterranean area, and is considered to be vulnerable due to its severe qualitative decline over the last 50 years (Janssen et al. 2016). In the western Mediterranean, annual grasslands are an important component of plant succession even long after the land has been abandoned (Debussche et al. 1996; Valverde-Asenjo et al. 2020). Since dry grasslands are very suitable as a model system for biodiversity (Vrahnakis et al. 2013; Dengler et al. 2014), they can be used for long-term monitoring of changes in ecosystem biodiversity and functioning as occurs in the case of land abandonment. Despite their potential interest, there is a dearth of studies on secondary succession after abandonment in acidic soils and / in dry Mediterranean environments.

The main drivers of the secondary succession process in dry biomes are time since abandonment and soil properties (Carpenter et al. 1986; McAuliffe 1988; Agami et al. 1998; Dana and Mota 2006), and the principles of succession can be used to predict changes in biodiversity and ecosystem services over time (Prach and Walker 2011). Based on previous observations of secondary succession in abandoned vineyards in central Spain (Valverde-Asenjo et al. 2020), we hypothesize that there is a change in plant communities over time during the secondary succession after the abandonment of agricultural activity, from first-colonizing to late-successional species, in tandem with changes in soil microbiota activities and physicochemical properties. To test this hypothesis we studied the variability in the composition and biodiversity of annual grasslands and their relationships with the soil organic fraction and biological activity in a succession of abandoned vineyards, and their changes in the short term. This was done by examining the time of abandonment through

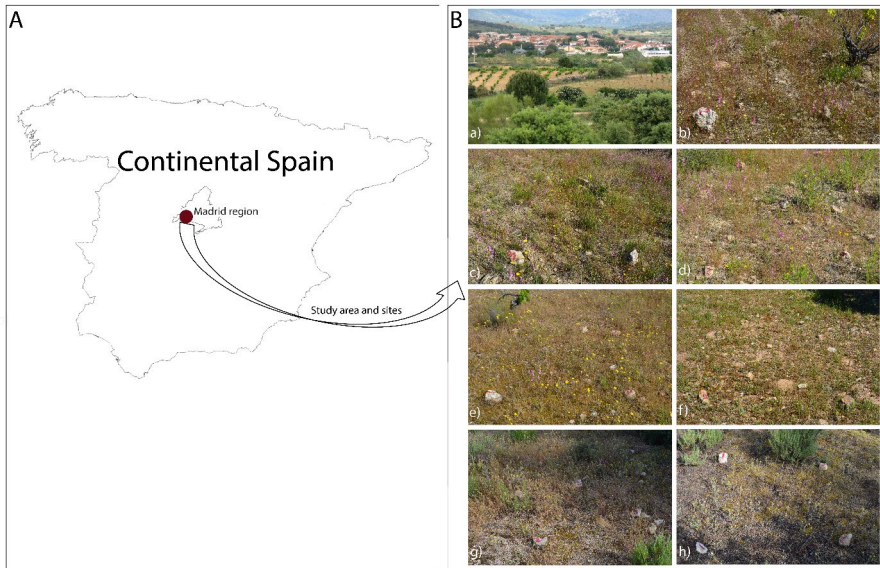


Fig. 1 A. Location of the study area. B. Examples of the study sites: (a) overall view of study area; (b) plot abandoned for five years in 2020; (c) plot abandoned for seven years in 2020; (d) plot abandoned for 11 years in 2020; (e) plot abandoned for 15 years in 2020; (f) plot abandoned for 29 years in 2020; (g) plot abandoned for 44 years in 2020; (h) control plot

a static chronosequence built with different times since abandonment (Valverde-Asenjo et al. 2020), and through a dynamic chronosequence by resampling permanent plots over a short-term period of five years (2016, 2020). Our results provide arguments to preserve and restore ecosystem function and biodiversity in abandoned agrosystems.

Methods

Study area

The study area is in a rural ecosystem in central Spain at about 700 m above sea level (Navas del Rey in the Madrid Region, central Iberian Peninsula, Fig. 1 A). The climate is typically Mediterranean, with mild winters and hot dry summers. Igneous rocks, mainly granites, are dominant in the area, and the predominant soils are Leptosols and Regosols according to the FAO classification (WRBI 2015), characterised by their coarse texture and good drainage (Consorcio Sierra Oeste 2009). The area lies within the Meso-Mediterranean bioclimatic belt and has a dry Mediterranean pluviseasonal-oceanic bioclimate (Rivas-Martínez et al. 2011). Biogeographically, the territory is in the West Iberian Mediterranean Province (Rivas-Martínez et al. 2017). The study area was used predominantly for vineyards for at least the last 100 years until recently, and the area occupied by vineyards has declined from 80 to 20% of the territory since the 1950s. There are several reasons for the abandonment of vineyards, the most important of which are the low soil productivity, the migration of the rural population towards the cities – mainly to the capital, Madrid – and the EU policy

promoting the grubbing-up of vines (Common Market Organization 2001, 2008). Since the vineyards belonged to small landowners the abandonment occurred randomly as they ceased their agricultural activity. The territory not dedicated to vineyards is occupied by natural vegetation (Vaquero Perea et al. 2020), in a plant landscape mainly constituted by patches of sclerophyllous oak (*Quercus rotundifolia*) woodlands, *Retama sphaerocarpa* tall shrublands, *Lavandula pedunculata* and *Thymus mastichina* low scrublands, and extensive grasslands mostly characterized by a high diversity of ephemeral plants. The study area is part of a typical traditional Mediterranean land-use system with few clearly-defined boundaries between farmlands and woodlands (Papanastasis 2007), and in which old fields form part of a dynamic equilibrium.

Sampling design

Our field research was based on the study of a chronosequence including six plots of abandoned vineyards (1, 3, 7, 11, 25, 40 years of abandonment in 2016), and one plot that had not been used as a vineyard in at least the last 60 years. In each plot, three random quadrats (1m²) within the areas covered by annual grasslands – the most abundant vegetation in all the study plots – were established as permanent plots (Fig. 1B). These kinds of permanent sites allow the observation of temporal vegetation dynamics (Chytrý et al. 2019; Bello et al. 2020). The procedure for choosing and identifying the time since the abandonment of each plot is explained in Valverde-Asenjo et al. (2020).

The field sampling took place in the second half of May when the grasslands are at the peak of their development. We studied vegetation attributes and soil properties in 2016 and 2020. These two years were climatically similar, so the interannual variability that is so characteristic of the Mediterranean climate is assumed to be buffered. The study of the chronosequence in both years will show if temporal patterns along the chronosequence are maintained in a short term such as five years and how each plot evolves throughout this time. These permanent plots are based on regular observation of temporal vegetation dynamics using sampling units with a fixed location in time, while the sampling approach remains consistent (de Bello et al. 2020).

The plant species in each quadrat were identified and their coverage was estimated as a percentage. The vegetation inventory included vascular plants, bryophytes, lichens and fungi. The percentage of bare soil and stones was also estimated. This information allows us to determine the between-plot floristic succession and the within-plot floristic dynamics. Each species was also assigned to its corresponding growth form. The following five growth forms were identified in our study to use in large-scale comparisons: bryophytes, chamaephytes, hemicryptophytes, thallophytes and therophytes. Based on the known plant physiological characteristics that can potentially be linked to biogeochemical cycles, eight plant functional traits were recognized in order to explain ecosystem functionality: Ascomycota-symbionts, cryptogams, hemiparasites, latex bearing plants, N-fixers, N-compounds (alkaloids+cyclopeptides) bearing plants, silica accumulators, sulphur accumulators and terpenoid accumulators. An undetermined plant functional trait was also taken into account. Coverages of plant growth forms and plant functional traits were determined as the number of quadrats in which they were found.

Soil was sampled at the same time as the vegetation was relevéd. Soil was collected within the contiguous 1×1 m quadrat outside the 1m² quadrat established for vegetation

sampling as a permanent site (Quintana et al. 2021). Soil samples were taken using two cores with a diameter of 5 cm from the top 0–5 cm in each quadrat.

Laboratory methods

Soil samples conditioned in plastic bags were taken to the laboratory and stored in a refrigerator at 4 °C until analysis. Fresh samples were sieved with a 2 mm sifter to perform all the analyses on the fine fraction. They were subdivided into two subsamples, one of which was air-dried for the physical-chemical analyses, while the other was used to determine the enzyme activity.

Soil nutrients were analysed by determining organic carbon (TOC), available phosphorus (AP) and available ammonium (NH₄-N). TOC was determined using the Walkley and Black (1934) wet oxidation procedure; AP was calculated with the Olsen and Sommers method (1982); and NH₄-N content was obtained by extraction in 2 M KCl and later measured by UV–visible spectrophotometry following the Keeney and Nelson (1982) method.

Nine enzyme activities were established related to the main macronutrient cycles (C, N, P and S). α -glucosidase (ALPHAGLU), β -glucosidase (BETAGLU), β -galactosidase (BETAGAL) and phenoloxidase (PHE) were studied for the C cycle; arylamidase (ARYLN), N-acetylglucosaminidase (NAG) and urease (URE) for the N cycle; phosphatase (PHOS) for the P cycle; and arylsulfatase (ARYLS) for the S cycle. All the activities were obtained following ISO 20,130 methods (ISO 2018) with the exception of phenoloxidase activity, which was obtained following DeForest (2009). All activity measurements were determined in a UV–visible spectrophotometer with a TECAN NANOQUANT INFI-NITE M200 PRO multi-well plate reader.

Statistical methods

We used both agglomerative and divisive classification methods for confirming different community types and diagnostic species. The floristic data sets corresponding to the 2016 and 2020 sampling years were subjected to an agglomerative classification method using the program JUICE 7.0 (Tichý 2002). The b-flexible linkage method ($b=0.25$) with Sørensen distance was chosen in order to evaluate the vegetation grouping, and determine whether it is maintained over the short term such as the five years covered by our work. Percentage cover values were square-root transformed with a view to reducing the importance of dominant species (Van der Maarel 1979). The crispness of classification was checked using the method in Botta-Dukát et al. (2005), which revealed the highest crispness at the level of two clusters for the 2016 and 2020 data sets, decreasing gradually as the number of clusters increased. A synoptic table was created with frequency values, average cover and diagnostic species. The clusters were standardized to an equal size (Tichý and Chytrý 2006) to calculate the diagnostic species values at the plant community level. We show only the diagnostic species with values of the phi coefficient (Φ) of association ≥ 0.10 for at least one cluster, and with a statistically significant affinity at the probability level < 0.01 according to Fisher's exact test. A divisive clustering classification was performed on the 2016 and 2020 floristic data sets using TWINSpan (Two-Way Indicator Species Analysis; Hill 1979). We chose the default cut levels since data were in the form of estimated percentage cover and we reduced the minimum group size to 4 because there were small datasets.

A generalized linear model with Poisson distribution and logistic link function was performed to assess the influence of the time since abandonment and/or the sampling year on plant growth forms and functional traits coverage, as the number of quadrats where they occur. For each plant growth form or plant functional trait, abundance was taken as the dependent variable while the sampling year (2016, 2020) and type of grassland (early- and late-abandoned grassland as shown by the cluster analysis) were considered as independent variables. The interaction between sampling year and type of grassland was tested in all cases, so a maximum model was developed including both sampling year and type of grassland variables and their interaction, followed by a backward stepwise selection of variables to obtain a final model with all its terms significant. If the interaction term was significant, it was split into two new models, one for each level of the variable type, to assess the different effect of the sampling year on the growth form/functional trait.

A linear regression model was performed to analyse soil physical-chemical and enzyme activity, taking the sampling year and the grassland type as independent variables and the value of the soil parameter in each quadrat as the dependent variable. Soil values were previously box-cox transformed to ensure normality and homoscedasticity. The variables were selected as described in the previous paragraph. All regression models were carried out in R (R Core Team 2022, version 4.1.3) under RStudio (RStudio Team 2022, version 2022.02.3 Build 492). The inclusion of the grassland variable as a random clustering factor in the models was previously ruled out as fittings were singular with a low ICC and a higher AIC than the models without the random component.

The relationships between plant growth forms and functional traits and soil enzyme activity and chemical features were also investigated through the multivariate redundancy analysis (RDA). Both functional traits and abundance of growth forms were standardized by the Hellinger method, while soil parameters were normalized and linearized by transformation with the box-cox method. The soil variables with the greatest impact on communities were selected by means of permutation tests following a forward stepwise procedure. The significance of each ordination component was also assessed through permutation tests. Only significant axes and soil variables were used to make the biplots. These analyses were done in R with the vegan package (Oksanen et al. 2019).

Results

Plant-community succession

The agglomerative classification performed on the floristic composition of the 21 permanent quadrats sampled in 2016 showed that they were arranged in two major groups (A, B) according to the crispness of the classification (Table 1). Both groups share a large number of high-frequency species. Some diagnostic species were also identified in each group. Group A included 9 relevés corresponding to the most recently abandoned vineyards (1, 3 and 7 years respectively). This early-stage grassland gathered a total of 56 species, with an average number of 20.6 species per sampling. Low grasses and herbs such as *Vulpia myuros*, *Bromus tectorum*, *Trifolium arvensis* and *Hypochoeris glabra* had higher frequencies and cover. In this group, only *Andryala integrifolia* acted as a diagnostic species, and with a moderate score. Group B clustered 12 relevés corresponding to the plots abandoned for the

Table 1 Synoptic table of the grasslands sampled in 2016 showing the frequency and average cover (upper number) of species in each of the two plant communities (columns) obtained in the classification analysis. Only species with a frequency of >30% in at least one group are included. Dark-grey, mid-grey and light-grey shaded values indicate diagnostic species with fidelity ($\Phi > 0.3$), ($\Phi > 0.2$) and ($\Phi > 0.1$), respectively.

Group	1	2
Number of plots	9	12
Number of species per Group	56	75
Average number of species	20.6	30
Simpson dissimilarity	0.4211	0.3521
Average Whittaker beta-diversity	1.7097	1.4931
<i>Andryala integrifolia</i>	78 ^{5.7}	
<i>Tuberaria guttata</i>		58 ^{34.3}
<i>Tortella nitidula</i>		75 ^{20.4}
<i>Andryala arenaria</i>		92 ^{4.3}
<i>Corynephorus fasciculatus</i>	33 ^{1.3}	92 ^{3.6}
<i>Psilurus incurvus</i>		67 ^{3.5}
<i>Vulpia myuros</i>	100 ^{13.3}	100 ^{11.9}
<i>Bromus tectorum</i>	100 ^{8.4}	83 ^{1.6}
<i>Trifolium arvense</i>	89 ^{18.1}	67 ^{4.9}
<i>Hypochoeris glabra</i>	89 ^{12.1}	83 ^{4.3}
<i>Rumex bucephalophorus</i>	89 ^{3.8}	100 ^{3.3}
<i>Logfia minima</i>	89 ^{1.6}	100 ^{2.3}
<i>Leontodon longirostris</i>	56 ^{5.6}	100 ^{5.1}
<i>Vulpia ciliata</i>	67 ^{2.7}	100 ^{5.7}
<i>Brassica barraelieri</i>	89 ^{6.1}	58 ^{3.9}
<i>Tolpis barbata</i>	78 ^{8.1}	92 ^{2.8}
<i>Hymenocarpus lotoides</i>	78 ^{6.9}	92 ^{6.7}
<i>Galium parisiense</i>	11 ¹	83 ^{1.7}
<i>Bryum sp.</i>	67 ^{18.5}	67 ^{19.5}
<i>Avena barbata</i>	67 ^{3.5}	50 ^{2.2}
<i>Triolium glomeratum</i>	67 ³	50 ^{2.3}
<i>Crepis capillaris</i>	33 ^{1.7}	58 ^{6.3}
<i>Silene colorata</i>	22 ^{2.5}	58 ^{1.9}
<i>Erodium cicutarium</i>	22 ^{1.5}	58 ^{1.4}
<i>Hymenocarpus cornicina</i>	11 ¹	58 ⁷
<i>Biserrula pelecinus</i>	11 ²	58 ^{2.3}
<i>Jasione montana</i>	56 ^{2.6}	33 ^{1.3}
<i>Sanguisorba verrucosa</i>	33 ²	50 ³
<i>Lathyrus angulatus</i>	44 ^{4.8}	42 ^{1.4}
<i>Ornithopus compressus</i>	44 ³	25 ^{2.3}
<i>Anthemis arvensis</i>	44 ^{1.8}	17 ¹
<i>Bellardia trixago</i>	44 ^{7.3}	25 ^{3.3}
<i>Vicia lathyroides</i>	11 ¹	42 ¹
<i>Trifolium cherleri</i>	33 ¹	33 ^{2.3}
<i>Taeniatherum caput-medusae</i>	33 ^{2.7}	17 ^{1.5}
<i>Carlina corymbosa</i>	22 ³	33 ^{6.3}
<i>Silene gallica</i>	11 ¹	33 ^{1.5}
<i>Ortegia hispanica</i>	22 ⁹	33 ^{3.5}
<i>Eryngium campestre</i>	22 ¹	25 ^{1.7}
<i>Spergula arvensis</i>	33 ¹	
<i>Coronilla dura</i>		58 ^{1.7}
<i>Cladonia foliacea</i>		50 ^{3.3}
<i>Geranium molle</i>		50 ^{1.3}
<i>Bromus rubens</i>		42 ^{1.6}
<i>Neatostema apulum</i>		42 ^{3.6}
<i>Plantago lagopus</i>		42 ^{1.4}
<i>Cladonia cervicornis</i>		42 ¹⁰

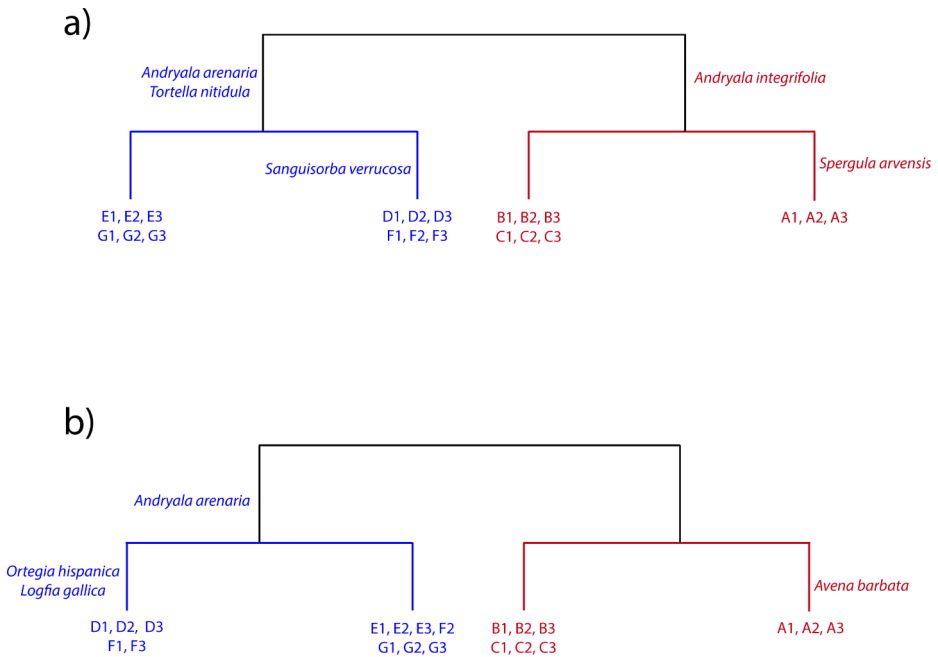


Fig. 2 Dendrogram obtained from a Twinspan classification analysis of 21 vegetation grassland samples relevéd in 2016 (a) and 2020 (b) from a chronosequence including six plots of abandoned vineyards and a plot that had not been used as a vineyard in at least the last 60 years

longest time (over a decade). This late-stage grassland encompassed a total of 75 species with an average of 30 species per sample. The Cistaceaea *Tuberaria guttata* and the moss *Tortella nitidula* were the diagnostic species with the highest phi scores. In this first year of sampling (2016), both the Whittaker beta-diversity and Simpson dissimilarity index showed higher scores in the early-stage than in the late-stage grassland (Table 1). Divisive classification supported the two main groups obtained by agglomerative hierarchical classification (Fig. 2a). One group included the most recently abandoned vineyards (1, 3 and 7 years post abandonment) and was floristically characterized by *Andryala integrifolia*, while the other group encompassed the vineyards abandoned for over a decade and had *Andryala arenaria* and *Tortella nitidula* as characteristic species.

The agglomerative classification performed on the 21 relevés sampled in 2020 revealed a similar pattern to five years earlier. Relevés were gathered in two major groups (A, B) according to the crispness of the classification (Table 2). Again, both groups shared a large number of species with a high frequency in each frequency group. Group A encompassed the earliest abandoned plots (now 5, 7 and 11 years post-abandonment respectively), including nine relevés with a total of 51 species and 23.8 as average number. These grasslands showed the highest frequency in therophytes such as *Vulpia myuros*, *Trifolium arvense*, *Rumex bucephalophorus*, *Tolpis barbata*, *Bromus tectorum*, *Andryala integrifolia* and *Trifolium glomeratum*. Group B gathered the longest abandoned plots, the same 12 plots as in 2016 (but now 15, 29 and 44 years post-abandonment, plus the control), and included a total of 66 species, with an average of 26 species per quadrat. Group B showed some diagnostic species such as *Tortella nitidula*, *Andryala arenaria* and *Tuberaria guttata* already identi-

Table 2 Synoptic table of the grasslands sampled in 2020 showing the frequency and average cover (upper number) of species in each of the two plant-communities (columns) obtained in the classification analysis. Only species with a frequency of >30% in at least one group are included. Dark-grey, mid-grey and light-grey shaded values indicate diagnostic species with fidelity ($\Phi > 0.3$), ($\Phi > 0.2$) and ($\Phi > 0.1$), respectively.

Group	1	2
Number of plots	9	12
Number of species per Group	51	66
Average number of species	23.89	26.08
Simpson dissimilarity	0.3257	0.3692
Average Whittaker beta-diversity	1.1349	1.5304
<i>Tortella nitidula</i>		100 ^{29.9}
<i>Andryala arenaria</i>		100 ^{8.7}
<i>Syntrichia ruralis</i>	11 ²	75 ^{3.7}
<i>Tuberaria guttata</i>		67 ^{11.3}
<i>Vulpia myuros</i>	100 ^{8.2}	100 ^{3.3}
<i>Trifolium arvense</i>	100 ^{4.6}	67 ^{9.4}
<i>Rumex bucephalophorus</i>	100 ^{3.8}	67 ^{1.5}
<i>Tolpis barbata</i>	100 ^{5.3}	75 ^{2.6}
<i>Hypochoeris glabra</i>	78 ^{5.1}	100 ^{2.3}
<i>Corynephorus fasciculatus</i>	67 ^{3.2}	92 ²
<i>Bryum sp.</i>	89 ^{6.5}	42 ^{8.8}
<i>Brassica barrelieri</i>	89 ¹⁴	42 ³
<i>Jasione montana</i>	89 ²	42 ^{1.2}
<i>Hymenocarpus lotoides</i>	78 ^{1.7}	83 ^{5.1}
<i>Vulpia ciliata</i>	78 ^{3.6}	67 ^{2.4}
<i>Logfia minima</i>	33 ^{1.7}	58 ^{1.6}
<i>Carlina corymbosa</i>	44 ^{11.3}	33 ^{4.8}
<i>Biserrula pelecinus</i>	33 ^{2.3}	42 ^{1.2}
<i>Bromus rubens</i>	22 ¹	42 ¹
<i>Crepis capillaris</i>	44 ^{2.8}	50 ^{2.5}
<i>Erodium cicutarium</i>	56 ^{1.2}	58 ^{1.3}
<i>Galium parisiense</i>	33 ¹	92 ^{1.3}
<i>Holcus setigulumis</i>	44 ³	17 ¹
<i>Hymenocarpus cornicina</i>	33 ^{2.3}	42 ^{2.6}
<i>Silene colorata</i>	78 ^{4.9}	58 ^{6.3}
<i>Taeniattherum caput-medusae</i>	33 ^{2.3}	8 ²
<i>Trifolium cherleri</i>	56 ^{2.8}	8 ²
<i>Trifolium glomeratum</i>	67 ^{2.2}	8 ²
<i>Vulpia ciliata</i>	78 ^{3.6}	67 ^{2.4}
<i>Vulpia membranacea</i>	56 ^{4.4}	8 ³
<i>Leontodon longirostris</i>	56 ^{8.6}	42 ^{2.6}
<i>Logfia gallica</i>	33 ^{1.7}	58 ^{1.6}
<i>Neatostema apulum</i>	11 ⁴	83 ^{1.6}
<i>Ornithopus compressus</i>	44 ^{1.5}	8 ¹
<i>Ortegia hispanica</i>	33 ^{9.3}	25 ^{8.3}
<i>Psilurus incurvus</i>	33 ^{2.7}	67 ^{1.8}
<i>Rumex angiocarpus</i>	33 ²	25 ²
<i>Sanguisorba verrucosa</i>	33 ¹	42 ²
<i>Andryala integrifolia</i>	78 ^{1.6}	
<i>Bromus tectorum</i>	67 ^{2.5}	
<i>Anthemis arvensis</i>	33 ^{1.3}	
<i>Avena barbata</i>	33 ²	
<i>Lactuca viminea</i>	33 ^{2.3}	
<i>Lavandula pedunculata</i>		58 ^{2.6}
<i>Polycarpon tetraphyllum</i>		50 ²
<i>Cladonia foliacea</i>		50 ^{2.5}
<i>Centranthus calcitrapa</i>		42 ^{1.2}
<i>Cladonia cervicornis</i>		42 ^{7.6}
<i>Bufonia tenuifolia</i>		33 ¹
<i>Lathyrus angulatus</i>	33 ¹	8 ¹²

fied in 2016 but whose frequency increased in 2020. The moss *Syntrichia ruralis* was also included this time as a diagnostic species with a high fidelity score. A comparison of the two years showed that the pattern of biodiversity indexes was reversed between 2016 and 2020, when both indexes had higher scores in mature grasslands than in grasslands growing on earlier-abandoned vineyards (Table 2). Divisive classification at the third division level confirmed the two main groups were the same as those obtained with agglomerative methods (Fig. 2b). One group with no characteristic species included the most recently abandoned vineyards (5, 7 and 11 years post-abandonment), while the other group gathered the rest of the plots (vineyards abandoned 15, 29 and 44 years earlier, and the control plot), and this method showed only the Compositae *Andryala arenaria* as a diagnostic species. Numerical classification thus revealed two main plant communities in 2016: early-stage grasslands in the first post-abandonment decade and late-stage grasslands after the first post-abandonment decade. This structure remains five years later in 2020 when the earlier-abandoned plots are still in or near the first decade of abandonment.

A Poisson regression to examine the evolution of the growth form cover through static and dynamic chronosequences showed the following most relevant patterns (Fig. 3). In three growth forms (bryophytes, hemicryptophytes and therophytes), their cover was influenced by both the grassland stage and the sampling year but with different pattern dynamics. Bryophyte cover increased significantly from early- to late-stage grasslands independently of the sampling year, and also from 2016 to 2020 (Fig. 3a). In contrast, hemicryptophyte cover significantly decreased along the chronosequence independently of the sampling year (Fig. 3b). Therophytes showed significant differences in cover between early-stage and late-stage grasslands depending on the sampling year (Fig. 3c). There was a more significant decrease in therophyte coverage in 2020 than in 2016, which was more notable in late-stage plots. In addition, chamaephyte cover only showed differences along the dynamic chronosequence (Fig. 3d) and thallophytes along the static chronosequence (Fig. 3e). Specifically, thallophyte cover increased from early-stage to late-stage grassland, whereas chamaephyte cover increased significantly from 2016 to 2020. Overall, the average cover of all growth forms increased along the chronosequence independently of the sampling year, with the exception of hemicryptophytes which showed the opposite tendency, and therophytes whose pattern (increasing or decreasing along the chronosequence) was dependent on the sampling year.

A Poisson regression to examine the evolution of plant functional trait cover showed the following (Fig. 4). Latex-accumulator plants decreased from 2016 to 2020, and with lower cover in late grasslands regardless of the sampling year (Fig. 4a). Terpenoid-accumulator plants increased in coverage throughout the sampling year but no differences were found between the grassland type (early or late) –Fig. 4b–. Cryptogam cover was always greater in late than in early grassland, but while early grassland had a significantly higher cover in 2016, late grassland did not show any significant differences between years (Fig. 4c). Coverage of N-fixers was significantly greater in 2016 than in 2020 for both early and late grassland, but the decrease in coverage in early grassland was much greater than the decrease in late grassland (Fig. 4d). In N-compounds accumulators there was a clear effect of interaction (Fig. 4e). In 2016 cover of this functional trait was higher in late grasslands than in early grasslands but no differences were found in 2020. While no significant differences were found in the coverage of silica-accumulator plants in early grassland between 2016 and 2020, their coverage in late plots decreased significantly from 2016 to 2020 (Fig. 4f). In

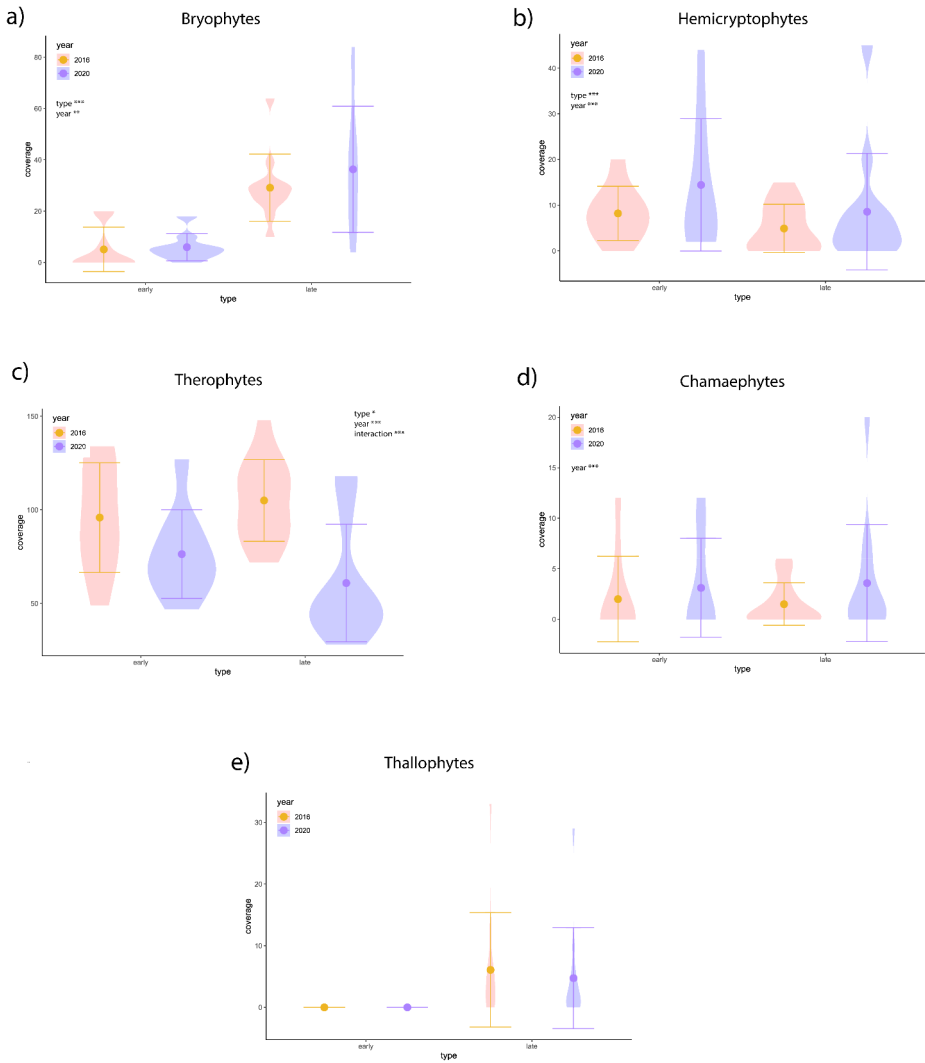


Fig. 3 Violin plots showing the cover distribution of growth forms corresponding to bryophytes (a), hemicryptophytes (b), therophytes (c), chamaephytes (d) and thallophytes (e) between early-stage and late-stage grassland for the 2016 (in red) and 2020 (in violet) sampling years. Inside each violin plot a 95% confidence interval for mean coverage is shown as a thin colour bar. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

contrast, sulphur-accumulator plants showed no significant cover differences in late grassland between 2016 and 2020, although their coverage increased significantly in early grassland in 2020 compared to 2016 (Fig. 4 g). Physiological traits such as symbiosis with fungi or hemiparasitism also revealed notable results. Hemiparasites coverage was higher in early grassland independently of the sampling year, and increased from 2016 to 2020 (Fig. 4 h). Ascosymbionts were absent in early grasslands, and their cover in late grasslands decreased in 2020 compared to 2016 (Fig. 4i).

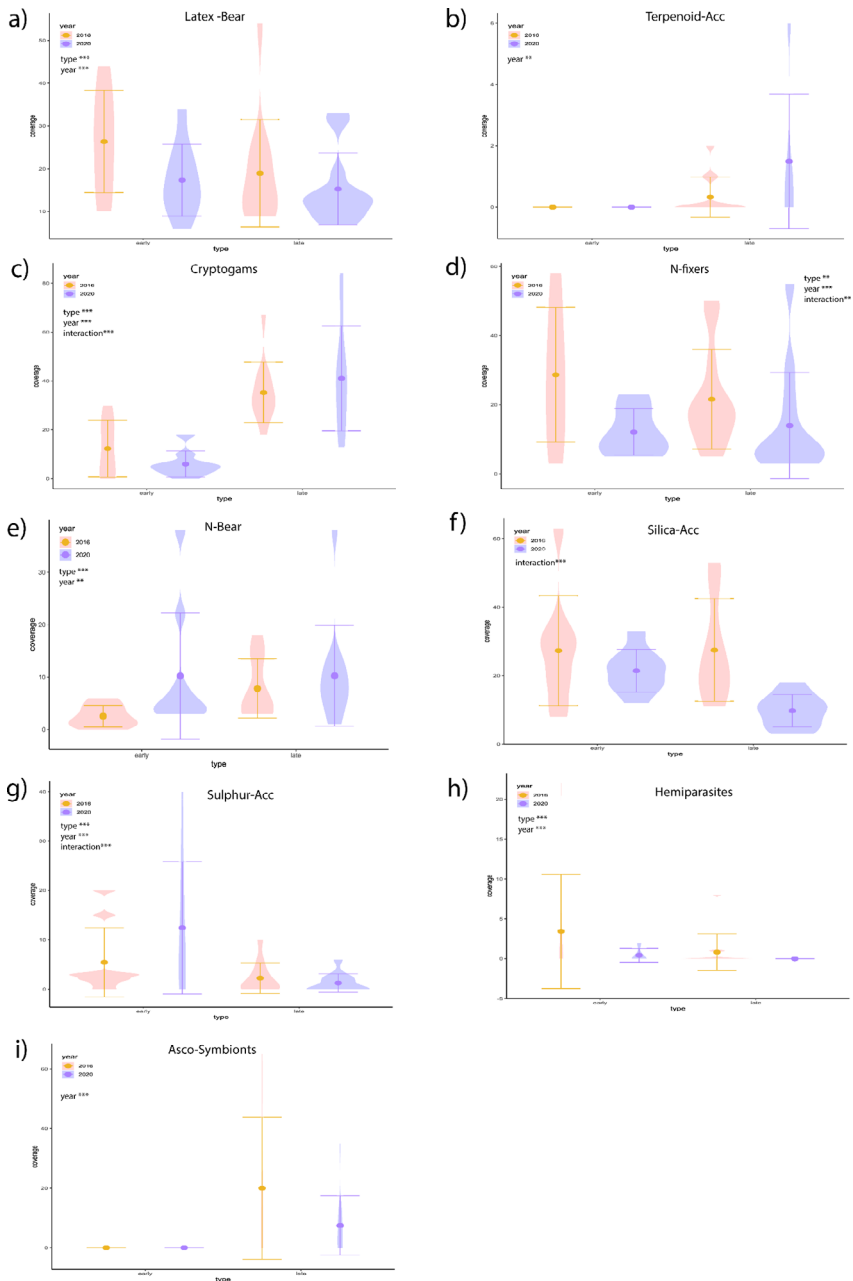


Fig. 4 Violin plots showing the cover distribution of functional traits corresponding to latex-secreters (a), terpenoid-accumulators (b), Cryptogams (c), N-fixers (d), N-containing compounds (e), silica-accumulators (f), sulphur-accumulators (g) Ascomycota-symbionts (h) and hemiparasites (i) between early-stage and late-stage grassland for the 2016 (in red) and 2020 (in violet) sampling years. Inside each violin plot a 95% confidence interval for mean coverage is shown as a thin colour bar. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The plant functional traits are abbreviated as follows: Latex-Bear=latex bearing plants, Silica-Acc=silica accumulators, Sulphur-Acc=sulphur accumulators, Terpenoid-Acc=terpenoid accumulators, Asco-Symbionts=Asco-Symbionts, N-Bear=N-bearing compounds (alkaloids + cyclopeptides)

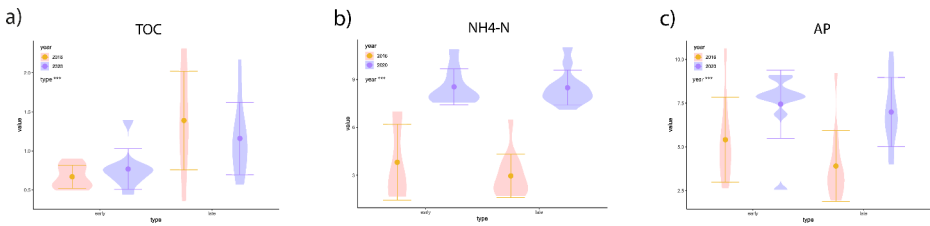


Fig. 5 Violin plots showing the soil TOC and nutrient contents between the early-stage and late-stage grassland for the 2016 (in red) and 2020 (in violet) sampling years. Inside each violin plot a 95% confidence interval for mean coverage is shown as a thin colour bar. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

Succession in soil properties and enzyme activities

A Poisson regression on the soil TOC, nutrient contents and enzyme activities revealed the following patterns. Soil TOC content was significantly higher in late-stage grasslands, and AP and $\text{NH}_4\text{-N}$ content showed a significant increase in 2020 (Fig. 5a-c). In regard to the enzyme activities related to C cycle, ALPHAGLU and BETAGLU activities increased significantly over the sampling years, and their activities were greater in plots located in late-stage grasslands regardless of the sampling year (Fig. 6a,b). BETAGAL activity only showed a significant increase from early to late grasslands (Fig. 6c) and PHE increased significantly throughout the sampling year (Fig. 6d). In regard to soil enzyme activities relating to the N cycle, ARYLN and NAG activities increased significantly throughout the sampling year, and their activity was greater in late-stage grassland whatever the year (Fig. 7a,b). URE activity showed a significant decrease throughout the sampling year (Fig. 7c). In terms of enzyme activities related to the P and S cycles, it is notable that both PHOS and ARYLS activities increased significantly in late-stage grasslands (Fig. 7d,e).

Ecological succession

The RDA calculated by stepwise selection to observe the distribution of the samples in relation to the plant growth forms and soil factors showed that the soil variables with most impact on communities were soil PHOS and PHE activity (Fig. 8). PHOS activity was closely related to Axis 1 and PHE activity with Axis 2. Partial correlation coefficients between growth forms and soil factors revealed that PHOS activity positively correlated with bryophyte and thallophyte cover, and negatively with therophyte and hemicryptophyte cover (Fig. 9). PHE activity was positively correlated with chamaephyte and hemicryptophyte cover and negatively with therophytes.

The RDA calculated by stepwise selection to observe the distribution of the samples in relation to the plant functional traits and soil factors showed that the soil variables with the greatest impact on communities were PHOS and URE activity and AP content; the first were closely linked to Axis 1 and the second to Axis 2 (Fig. 10). PHOS activity had a positive correlation with cryptogams, terpenoid accumulators, ascosymbionts and alkaloids, and a negative correlation with silica accumulators, sulphur accumulators and latex secretors (Fig. 11). URE activity was positively correlated with N-fixers and silica accumulators and negatively with cryptogams. Finally, soil AP content was positively correlated with N-fixers and negatively with cryptogams.

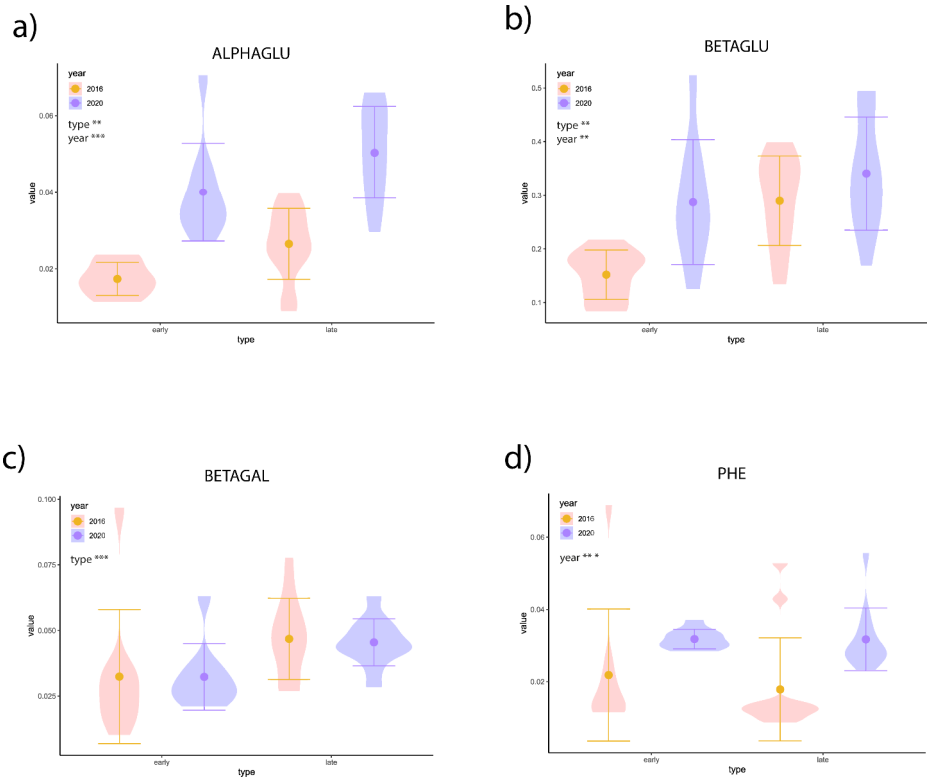


Fig. 6 Violin plots showing the soil enzyme activities related to C cycle between the early-stage and late-stage grassland for the 2016 (in red) and 2020 (in violet) sampling years. Inside each violin plot a 95% confidence interval for mean coverage is shown as a thin colour bar. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. The enzymes are abbreviated as follows: ALPHAGLU = α -glucosidase, BETAGLU = β -glucosidase, BETAGAL = β -galactosidase and PHE = phenoloxidase.

Discussion

Grassland floristic replacement

Plant succession focuses on spatial species replacement over time (Clements 1916), distinguishing pioneers from late successional species (Bazzaz 1979). In a classic plant succession scheme under a dry Mediterranean climate, annual grasslands play an important role in the initial stages before fields are finally encroached by woody vegetation encompassing low and tall shrubland, and with sclerophyllous *Quercus* forest acting as the climax (Rivas-Martínez et al. 2011). This model fits the pattern of abandoned Mediterranean vineyards. In the eastern Mediterranean there is rapid encroachment by woody vegetation accompanied by a decrease in annual plant species during the first decades of the succession (Ne'eman and Izhaki 1996); whereas in the western Mediterranean annual grasslands remain longer and with more cover after vineyards are abandoned, coupled with a slower encroachment of woody species (Houssard et al. 1980; Escarré et al. 1983; Valverde-Asenjo et al. 2020). Our results also clearly show that Mediterranean annual grasslands remain throughout the

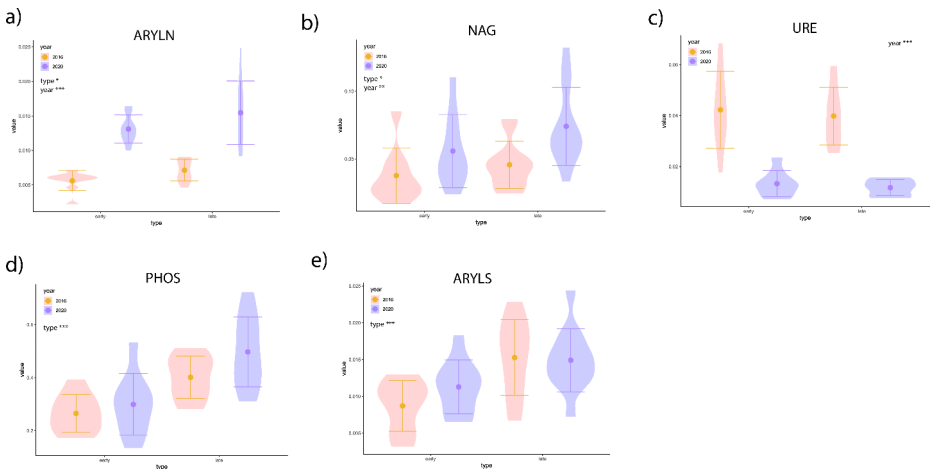


Fig. 7 Violin plots showing the soil enzyme activities related to N, S and P cycles (ARYLN, NAG, URE, PHO, ARYLS) between the early-stage and late-stage grassland for the 2016 (in red) and 2020 (in violet) sampling years. Inside each violin plot a 95% confidence interval for mean coverage is shown as a thin colour bar. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

succession and play an important role both as a pioneer and as late successional vegetation, albeit floristically differentiated based on the identification of frequent abundant characteristic and diagnostic plant species, all corresponding to Mediterranean annual low-growing ephemeral herb and grass vegetation (*Helianthemetea guttati*, *Helianthemetalia guttati*) (Mucina et al. 2016). Some ruderal plants were recognized in early-stage succession grasslands (*Brassica barrelieri*, *Andryala integrifolia*), whereas late-stage grasslands included symbionts with Ascomycota (*Tuberaria guttata*) and cryptogams. This floristic change along the static chronosequence was not related to clearly defined patterns in other biodiversity descriptors such as biodiversity indexes more closely linked to the sampling year (dynamic chronosequence).

It should be noted that both early- and late-stage grasslands can be included in the habitat type “Mediterranean to Atlantic open, dry, acid and neutral grassland” in the European Red List of Habitats that have been assessed as vulnerable (VU) based on a severe qualitative decline (Janssen et al. 2016), and within which no sub-habitats have been distinguished. Since the grassland stages identified can be related to the quality indicators recognized for this habitat type (e.g. medium disturbance regime, absence of nitrophilous species and absence of signs of advanced secondary succession such as encroachment of chamaephytes or shrub species), we here propose to distinguish mature-stage grasslands, which would warrant further assessment. This knowledge is useful from the point of view of biodiversity and conservation for policies regarding the management of old-field lands. It also should be noted that our study failed to pinpoint the key step at which the plant-community replacement (between early-stage and late-stage grassland) occurs. The history of use while the vineyard was active (amount of fertilizers and phytosanitary products, ploughing regime and intensity, among others) could determine the point when this change occurs (Jakovac et al. 2021). It would therefore be necessary to follow the chronosequence for more years in order to identify the threshold at which one community type changes to another.

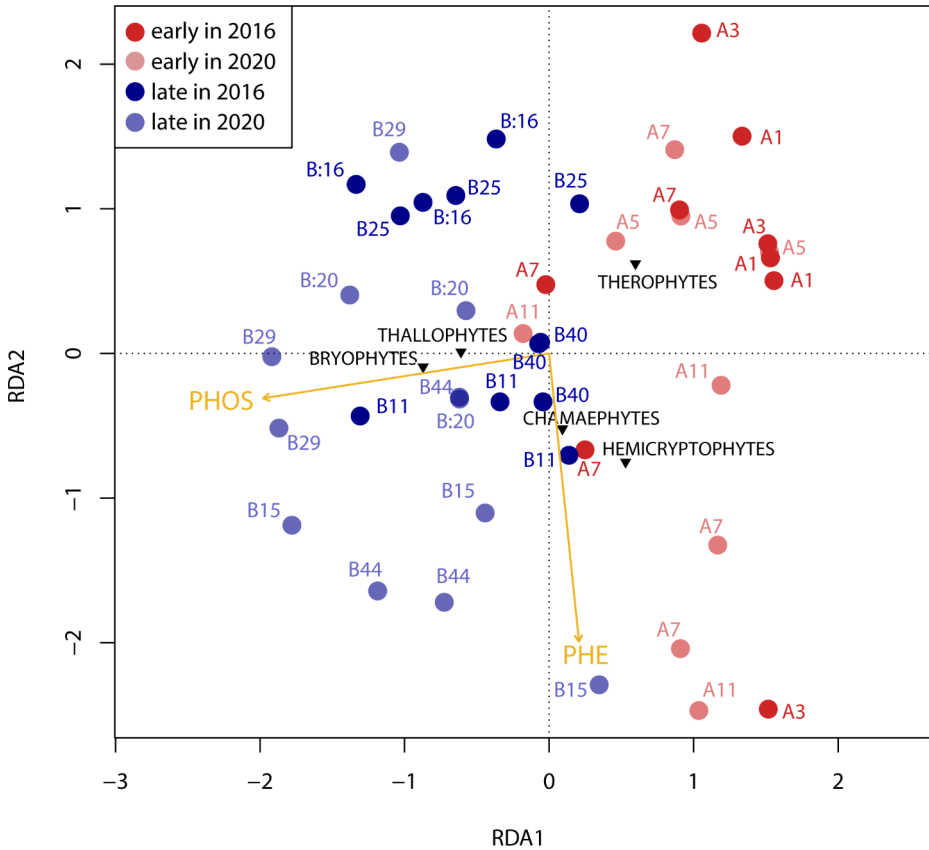


Fig. 8 Redundancy analysis triplot (RDA) showing the relationships between grassland types as circles; soil properties selected by the forward selection procedure as orange arrows; and growth forms as black triangles. The percentage of cumulative explained variance by the first two axes is 21.16. Different coloured circles depict grassland types classified as early-stage grassland (A) – in red – and late-stage grassland (B) – in blue – as detailed in Tables 1 and 2; colour intensity indicates sampling year: pale for 2016 and dark for 2020. The numbers after the group type (A or B) indicate the year after abandonment. Soil properties are abbreviated as follows: PHOS=phosphatase, PHE=phenoloxidase.

Secondary succession can also be monitored through changes in growth forms and functional traits (Tilman 1990; Robinson et al. 2015; Fan et al. 2019). Our work revealed a slow successional process where the main features were: (i) the increase in cover of all growth forms along the chronosequence, independently of the sampling year, with the exception of hemicryptophytes and therophytes; (ii) the appearance and increase along the chronosequence of certain functional traits such as Ascomycota symbionts and terpenoid accumulators. Bryophytes and lichens have been recognized as significant indicators of both early (Dettweiler-Robinson et al. 2013; Arróniz-Crespo et al. 2014) and late successional stages (García de León et al. 2016). Our results revealed that not only cryptogams, but also Ascomycota symbionts and terpenoid accumulators play an important role as indicators of late successional-stage grasslands in Mediterranean abandoned agrosystems. The fungi:bacteria ratio increases during secondary succession (Zhou et al. 2017a), which

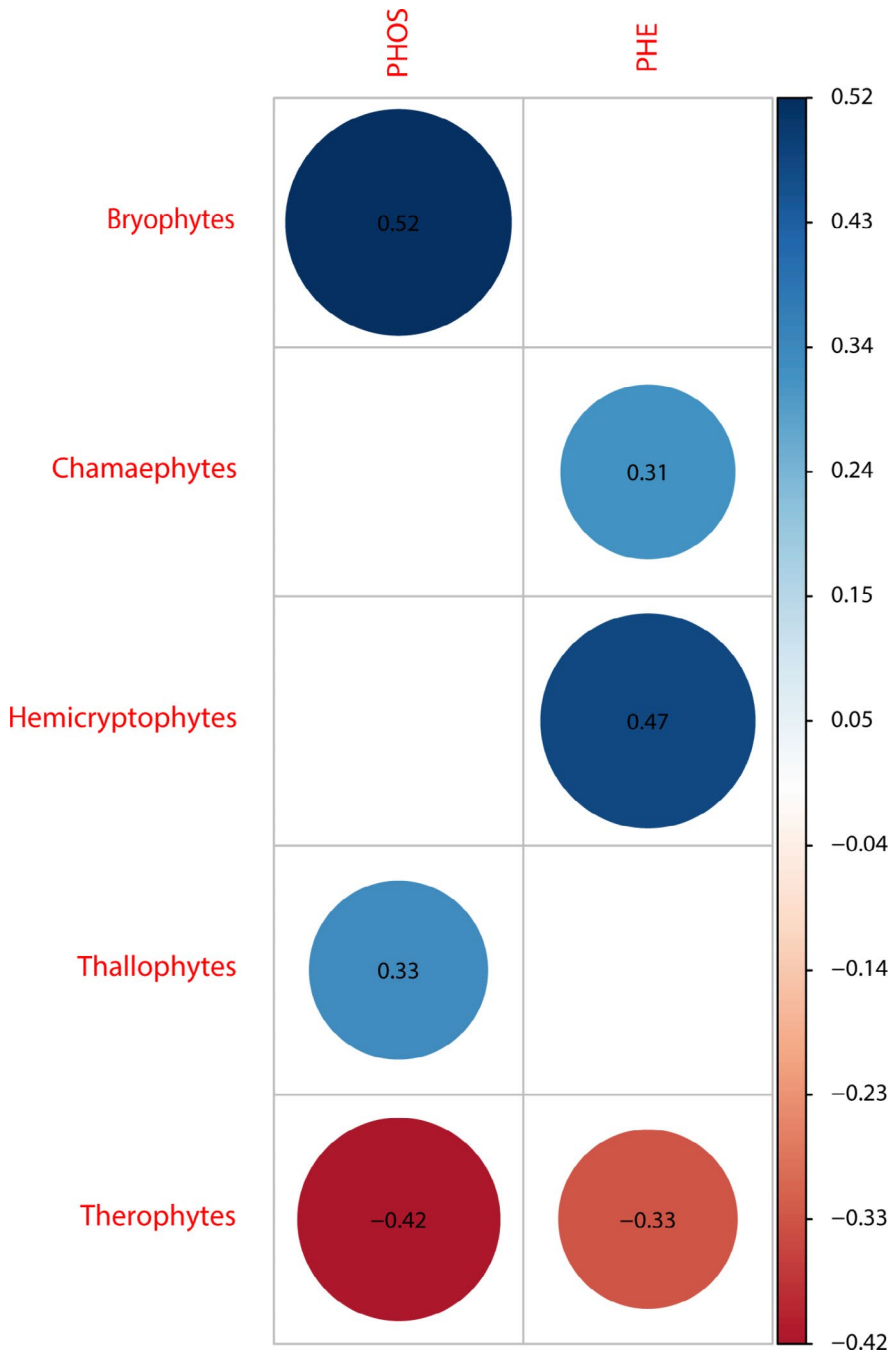


Fig. 9 Correlogram showing the significant correlations between each plant growth form and each soil variable. Positive correlations are displayed in blue and negative correlations in red. Colour intensity and circle size are proportional to the correlation coefficients

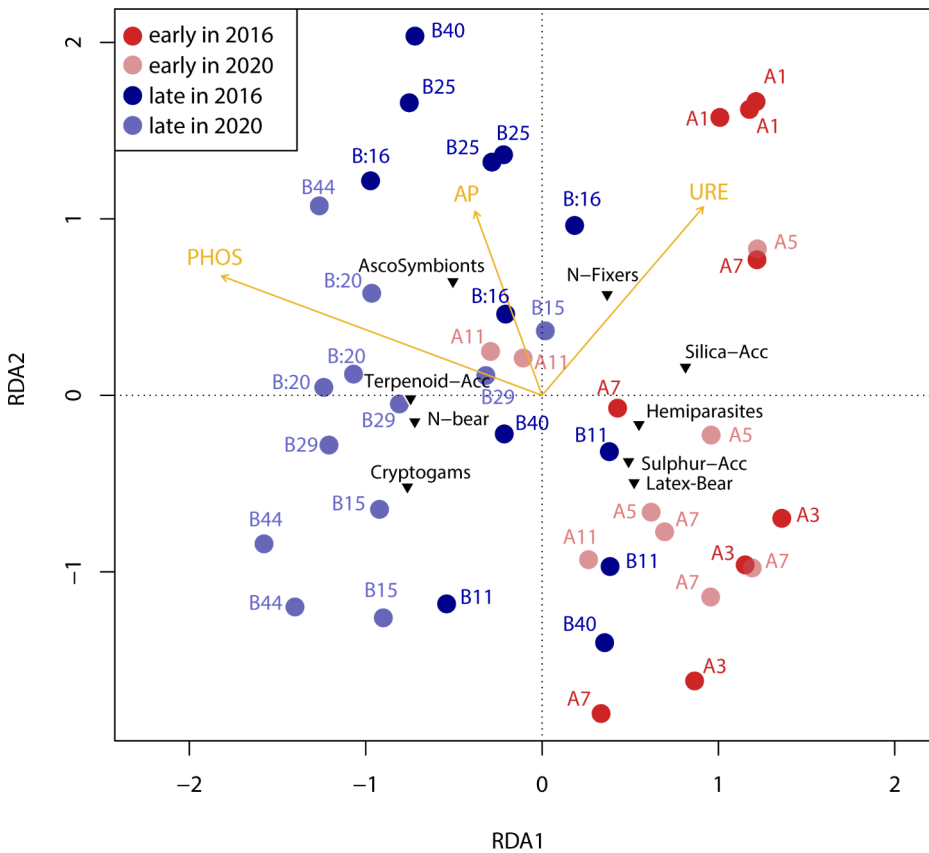
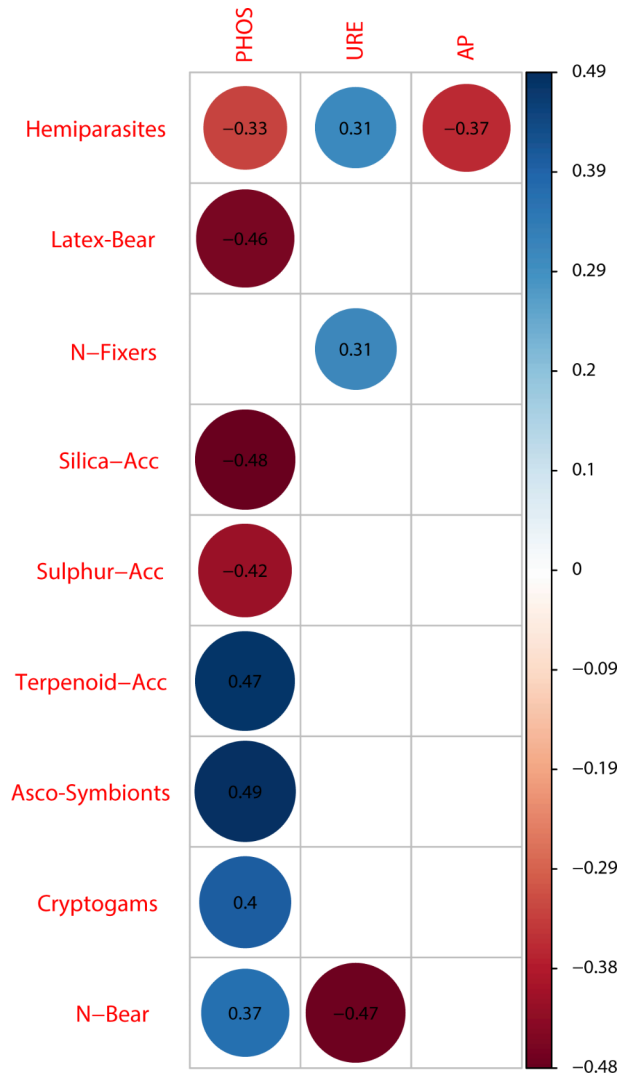


Fig. 10 Redundancy analysis triplot (RDA) showing the relationships between grassland types as circles, soil properties selected by the forward selection procedure as orange arrows, and functional traits as black triangles. The percentage of cumulative explained variance by the first two axes is 26.02. Different coloured circles depict grassland types classified as early-stage grassland (A) – in red – and late-stage grassland (B) – in blue – as detailed in Tables 1 and 2; colour intensity indicates sampling year: pale for 2016 and dark for 2020. The numbers after the group type (A or B) indicate the year after abandonment. Soil properties are abbreviated as follows: AP=available phosphorus, PHO=phosphatase, URE=urease.

helps explain both the presence of Ascomycota symbionts and the importance of both bryophytes and thallophytes – basically lichens – in late-stage grasslands. Rates of woody succession in old-field plant succession are highly correlated with both annual temperature and soil fertility, and decrease with latitude (Wright and Fridley 2010). A typical plant succession in a dry abandoned agricultural landscape includes a first-stage encroachment by dwarf shrubs after the early colonization of grasslands (Debusche 1996, Bonet and Pausas 2012). In our study this role corresponded largely to lavender (*Lavandula pedunculata*), which accounts for the importance of terpenoid accumulators (typically in Lamiaceae species) in late-stage succession. Cover of N-fixers, whose direction of change – according to our results – was dependent on the sampling year, could be used as an indicator of interannual climate variability since their abundance decreases in extreme drought (Quintana et al. 2021).

Fig. 11 Correlogram showing the significant correlations between each plant functional trait and soil variables with the greatest impact on communities. Positive correlations are displayed in blue and negative correlations in red. Colour intensity and the circle size are proportional to the correlation coefficients. The plant functional traits are abbreviated as follows: Latex-Bear=latex bearing plants, Silica-Acc=silica accumulators, Sulphur-Acc=sulphur accumulators, Terpenoid-Acc=terpenoid accumulators, Asco-Symbionts=Asco-Symbionts, N-Bear=N-bearing compounds (alkaloids+cyclopeptides). The soil variables are abbreviated as follows: PHOS=phosphatase, URE=urease, AP=available phosphorus



Ecological changes

There is growing interest in the fact that successional patterns in plant community changes are linked to corresponding changes in soil microbiota (Chang and Turner 2019). Land abandonment causes an increase in soil organic matter (Vesterdal et al. 2002; Zhu et al. 2010; Raiesi 2012a) and microbiota activity (García and Hernández 1997; Raiesi and Salek-Gilani 2018), and has been related to a decrease in organic N mineralization associated with soil organic matter variation (Zhang et al. 2012; Raiesi 2012b). Our results confirm that soil TOC content and enzyme activities related to labile C cycle and N, P and S cycles increased significantly in soils under late-stage grasslands in comparison with soils under early-stage grasslands in dry Mediterranean abandoned agrosystems. However, our study also revealed this improvement in functionality over time to be linked with a certain floristic occurrence

anean to Atlantic open, dry, acid and neutral late-stage grassland”. This new sub-habitat type is characterized by the occurrence of the symbiont with the Ascomycota plant *Tuberaria guttata* and a high cover of cryptogams combined with high hydrolytic enzyme activity and TOC content in the soil.

Author contributions All authors contributed to the conception and design of this study, led by JAM and JRQ. Field data were provided by JAM and JRQ. Laboratory analyses were carried out by JQR and JPMS. Statistical analyses were performed by ASJ and JPMS. The first draft of the manuscript was written by JAM and JQR and all authors commented on previous versions of the manuscript.

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Data Availability Associated data in a data repository.

Declarations

Conflict of interest The authors have no conflict or financial interest to report.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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