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REGULAR ARTICLE

Mediterranean forage legumes grown alone or in mixture with annual ryegrass: biomass production, N_2 fixation, and indices of intercrop efficiency

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Abstract

Aims To evaluate the productivity and N_2 fixation of a range of Mediterranean forage legume species as well as their ability to be grown in mixture with a forage grass, and to verify whether N transfer occurs from the legume to the non-legume component of the mixtures and, if so, to what extent this process is affected by legume species.

Methods Seven legume species (*Hedysarum coronarium* L., *Medicago scutellata* L., *Trifolium resupinatum* L., *Trifolium squarrosum* L., *Trigonella foenum-graecum* L., *Vicia sativa* L., *Vicia villosa* Roth) were grown alone or in mixture with annual ryegrass (*Lolium multiflorum* L.). Biomass and N yields and biological N_2 fixation (^{15}N dilution technique) were measured. N transfer from legume to the non-legume component was also assessed. The efficiency of the intercrops was evaluated using the land equivalent ratio (LER), aggressivity index, and competitive ratio.

Results Differences were observed among the monocropped legumes for biomass yield, N_2 fixation, and ability to utilize inorganic soil N. Moreover, the proportion of legume species to the total biomass yield of the intercrop varied from 30 % (*T. resupinatum*) to 69 % (*T. foenum-graecum*). All intercrops showed an advantage over monocrops in terms of biomass and N yields (LER and NLER values always >1). No N transfer occurred from legume to ryegrass in any of the mixtures.

Conclusions The large differences observed among the studied legumes must be taken into account when trying to develop cropping systems with more efficient N use. Moreover, as all legume–ryegrass intercrops used natural resources more efficiently than pure crops, intercropping is a relevant cropping strategy for sustainable agricultural systems in Mediterranean environments.

Keywords Intercrop · Fenugreek · Hairy vetch · Persian clover · Snail medick · Squarrosum clover · Sulla · ^{15}N-isotope techniques

Introduction

The ever-rising cost of fossil fuels necessary to synthesize agricultural chemicals on the one hand and widespread concern about environmental pollution and resources conservation on the other hand are driving a renewed interest in reintroducing legumes into agricultural systems. Including more legumes in cropping systems is generally suggested as an environmentally friendly way to achieve higher yields without increasing the use of nitrogen (N) fertilizers (Canfield et al. 2010), as legumes rely more on biological N_2 fixation to satisfy

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their N needs (Nyfeler et al. 2011). Most of the advantage of growing legumes is attributable to the increase in N availability for subsequent non-legume crops due to both N sparing during legume growth (usually attributed to the frugal use of soil N by legumes; Chalk 1998) and the release of N bound in the crop residues, most of which comes from N_2 fixation.

The amount of symbiotically fixed N_2 in legumes can vary from 0 to more than 450 kg N ha^{-1} $year^{-1}$ (Herridge et al. 2008; Peoples et al. 2008) depending on the legume species, the environment, and the method of technical management applied. However, despite the benefits in terms of increased N availability, the inclusion of legumes in cropping systems is not without potential drawbacks. For example, legumes are generally less efficient than non-N_2-fixing crops at recovering soil inorganic N during the growing season (Jensen and Hauggaard-Nielsen 2003); hence, the increase in soil mineral N availability that occurs both during the cycle of the legume crop and post-harvest can in some agroecosystems increase N leaching to the groundwater and/or losses in the atmosphere through denitrification or volatilization (Jensen and Hauggaard-Nielsen 2003). Intercropping annual legumes with grasses may contribute to a better use of N available in soil, thus reducing the environmental risks of its loss. Moreover, because mixtures generally use light, water, and nutrients more efficiently over time and space (Hauggaard-Nielsen and Jensen 2005; Corre-Hellou et al. 2006) compared to monocrops, growing mixtures may increase yield and yield stability; improve ecological sustainability by reducing the need for N fertilizer and the incidence of weeds, pests, and disease; and increase the yield of the subsequent crop (Stout et al. 1997; Anil et al. 1998). In forage systems, grass–legume mixtures can provide forage with more balanced nutritional traits than crop monocultures. Another point in favour of grass–legume intercropping is that N can be transferred from the legume to the associated non-fixing crop via decomposition of dead legume tissues or root nodules (Paynel et al. 2001), root exudation of N compounds (Fustec et al. 2010), or arbuscular mycorrhizal fungi linking the intercrop components (Frey and Schüepp 1992; He et al. 2003). However, data on N transfer from annual legumes to the companion grass in short-term intercrops are contradictory and range from no N transfer to up to 10–20 % of total N accumulated in the non-fixing companion (Høgh-Jensen 2006; Moyer-Henry et al. 2006).

The efficiency of a grass–legume mixture is affected by a range of environmental and agronomic factors (Ofori and Stern 1987). Benefits are more evident when mixtures are grown in poor soils or in low-input systems (Corre-Hellou et al. 2007). In any case, the best results are obtained when complementarity between the components is maximized and competition minimized (Willey 1979; Anil et al. 1998). Therefore, the success of an intercrop largely depends on the species to be grown together. Many studies have compared the efficiency of grass–legume mixtures with that of their respective monoculture systems, focusing on a single legume species, by varying certain agronomic practices (seeding ratio, plant arrangement, cutting management, etc.; e.g., Vasilakoglou and Dhima 2008; Giambalvo et al. 2011). However, few studies (e.g., Javanmard et al. 2009) have compared the productivity and efficiency of different combinations of grass–legume mixtures grown at the same time under the same environmental and agronomic conditions.

In the Mediterranean basin, a range of legume species are utilized or utilizable for forage production. However, they are very different from each other in terms of growth habit and rate, life cycle length, and regrowth ability following defoliation. Such morphological and biological differences suggest that their respective abilities to utilize available resources (light, water, and nutrients) and aptitudes for growth in mixtures with other crops may also differ. To plan and build more environmentally sustainable cropping systems, it is essential to have data on such differences. Unfortunately, however, to date there is very little information on these factors. To fill this gap, we performed an experiment aimed at evaluating the differences in productivity and N_2 fixation of seven Mediterranean forage legume species as well as their ability to be grown in mixture with a forage grass. Moreover, we tested whether N transfer occurs from the legume to the non-legume component of the mixtures and, if so, whether and to what extent this process is affected by legume species. In particular, the study included three legume species that are usually grown in Mediterranean forage systems for hay production (common vetch, fenugreek, and sulla; Ruisi et al. 2011) and four other legume species that are of potential interest but not yet widespread in the Mediterranean basin (hairy vetch, Persian clover, snail medick, and squarrosum clover).

Materials and methods

Experimental site

The experiment was carried out in the 2005–2006 and 2006–2007 growing seasons at the Pietranera experimental farm about 30 km north of Agrigento, Sicily, Italy (37°30′N, 13°31′E; 178 m a.s.l.). In both growing seasons, the soil was a Chromic Haploxerert (525 g kg^{-1} clay, 227 g kg^{-1} silt, and 248 g kg^{-1} sand; pH 8.2; 16.8 g kg^{-1} total C and 1.78 g kg^{-1} total N). The climate of the experimental site is semiarid Mediterranean with a mean annual rainfall of 581 mm, mostly in the autumn/winter (September–February; 76 %) and in the spring (March–May; 19 %). There is a dry period from May to September. The mean air temperatures are 15.9 °C in autumn, 9.7 °C in winter, and 16.5 °C in spring. The total rainfall at the site during the first growing season was 558 mm (Fig. 1), which was very close to the long-term average. Rainfall was well distributed over the growing season and allowed for satisfactory plant growth and yield for the trial environment. The mean temperature for the year was similar to the normal mean temperature. During the second growing season, the total rainfall was 645 mm, 20 % more than the long-term average. Rainfall occurred mainly between September and December (350 mm) and during spring, with a peak in March (150 mm). Weather data were collected from a weather station located within 200 m of the experimental site.

Experimental design and crop management

The experiment was set up in a randomised block design with four replications. Treatments consisted of seven Mediterranean forage legumes (Table 1) grown in pure stands and in mixture with annual ryegrass. Monocropped ryegrass was also included as a control treatment as either unfertilized or N-fertilized. In both experimental years, the growth and density of common vetch was dramatically reduced by root rot attacks; thus, data collected on this species were not included in the analysis and consequently are not reported in the present paper.

In both years the previous crop was wheat. Soil was ploughed in August and harrowed after the first autumn rainfalls; before harrowing, 69 kg P_2O_5 ha^{-1} was applied in all treatments. Crops were hand-sown in both seasons in the last 10 days of November using, for each species in pure stand, the seeding rate ordinarily adopted by farmers in the area (Table 1) and, for mixed stands, a 0.5:0.5 ratio. Species within intercrops were arranged in alternating rows. Plots were 4.0 × 2.6 m (13 rows, 0.2 m apart and 4 m long). Seeds were not inoculated with *Rhizobium* before planting because prolific nodulation occurs naturally at the experimental site. All plots were hand-weeded. In both years all plots were harvested by hand on the same day, when the latest legume species was at the full flowering stage (15 May in 2005–2006 and 22 May in 2006–2007). In no case did the differences in flowering time between the earliest and latest legume species exceed 7 days. All plants were cut at soil level.

The ^{15}N isotope dilution technique was used to estimate N fixation by the legume species. ^{15}N fertilizer ($[NH_4]_2SO_4$ with an isotopic composition of 10 atom%^{15}N) was uniformly applied, at a rate of 8 kg N ha^{-1}, in liquid form to a 2.88-m^2 microplot in the middle of each plot. The ^{15}N fertilizer was applied at crop emergence. In the N-fertilized ryegrass monocrop, microplots were labelled with 80 kg ha^{-1} of ^{15}N fertilizer with an isotopic enrichment of 1.57 atom%. In addition, the rest of the plot outside the ^{15}N-labelled area received a topdressing of ammonium sulphate in an amount equivalent to that in the microplots. After each application of labelled fertilizer all plots were irrigated (5 L m^{-2}) to prevent plant leaves from retaining the ^{15}N fertilizer and to facilitate a more uniform distribution of the added ^{15}N in the soil profile.

At crop harvesting, total fresh weight was determined and a sample of plant material taken from the centre of the microplots was hand-disaggregated into its botanical components (legume or ryegrass), dried at 60 °C for 36 h, weighed, ground to a fine powder (sieved using a 0.1-mm mesh size) in a fast-running mill, and analysed for total N and ^{15}N enrichment. The concentrations of total N and ^{15}N were determined using elemental analyser–isotope ratio mass spectrometry (Carlo Erba NA1500).

Calculations

Data on ^{15}N enrichment of biomass were used to calculate the percentage of legume N derived from

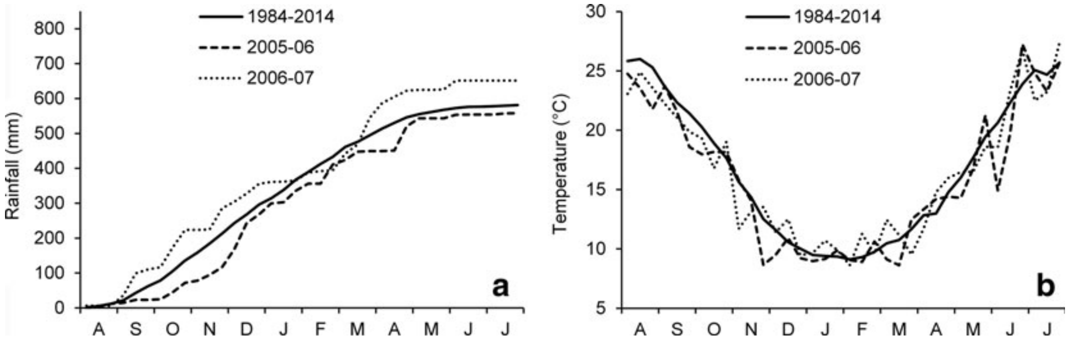

Fig. 1 Accumulated rainfall (**a**) and 10-day mean air temperature (**b**) at the experimental site during the two growing seasons (2005–2006 and 2006–2007); 30-year average 10-day temperatures and accumulated rainfall are also included

symbiotic N_2 fixation (%Ndfa) according to Fried and Middelboe (1977):

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom}\%^{15}\text{N}_{\text{Leg}}}{\text{atom}\%^{15}\text{N}_{\text{R}}}\right) \times 100,$$

where atom%^{15}N$_{\text{Leg}}$ represents the atom%^{15}N excess of legume tissue grown in mixture and pure stand and atom%^{15}N$_{\text{R}}$ represents the atom%^{15}N excess of unfertilized ryegrass tissue grown in pure stand. The ^{15}N-natural abundance of the atmosphere (0.3663%^{15}N) was used to calculate the atom%^{15}N excess of both crops at each cut.

The amount of N fixed (N_{fix}) by each legume species was estimated as follows:

$$N_{\text{fix}} = N_{\text{tot}} \times \frac{\%\text{Ndfa}}{100},$$

where N_{tot} represents the total N in the legume aboveground biomass. The percentage of N transferred (%N$_{\text{trans}}$) from each legume species to ryegrass was calculated according to Høgh-Jensen and Schjoerring (1997):

$$\%\text{N}_{\text{trans}} = \left(1 - \frac{\text{atom}\%^{15}\text{N}_{\text{R(Leg)}}}{\text{atom}\%^{15}\text{N}_{\text{R}}}\right) \times 100,$$

where atom% ^{15}N$_{\text{R(Leg)}}$ represents the atom% 15N excess of ryegrass tissue grown in mixture with legume. The recovery of the applied fertilizer N in ryegrass and legumes (%Ndff) was calculated as follows:

$$\%\text{Ndff} = \frac{N_{\text{tot}} \times \text{atom}\%^{15}\text{N}}{N_{\text{fert}} \times \text{atom}\%^{15}\text{N}_{\text{fert}}} \times 100,$$

where N_{fert} is the amount of N applied, N_{tot} is the amount of aboveground N yield from ryegrass or legume species grown in monoculture or in mixture, and atom%^{15}N and

Table 1 Species, cultivars, 1000-seed weights, and seeding rates used in the experiment

Species	Botanical name	Cultivar	Plant stature	Growth habit	Early vigour	1000-seed weight (g)	Seeding rate (n m^{-2})
Common vetch	*Vicia sativa* L.	Pietranera	Medium	Semi-prostrate, viney	High	61.3	500
Fenugreek	*Trigonella foenum-graecum* L.	Sicilian ecotype	Medium	Erect	High	15.6	800
Hairy vetch	*Vicia villosa* Roth	Capello	Medium	Semi-prostrate, viney	Medium	42.5	500
Persian clover	*Trifolium resupinatum* L.	Laser	Medium	Semi-erect	Low	1.6	1300
Snail medick	*Medicago scutellata* L.	Kelson	Short to medium	Semi-erect to erect	High	18.4	1300
Sulla	*Hedysarum coronarium* L.	S. Omero	Medium to high	Semi-erect to erect	Low	6.0	800
Squarrosum clover	*Trifolium squarrosum* L.	Tuscan ecotype	Medium	Erect	Low	5.2	1300
Annual ryegrass	*Lolium multiflorum* L.	Elunaria	Medium	Erect	Medium	3.2	1300

atom%^{15}N$_{fert}$ represent the atom%^{15}N excess both of crops in monoculture or in mixture and of fertilizer.

The intercrops were compared with the monocrops using the land equivalent ratio (LER), defined as the relative area of land growing monocrops that is required to produce the biomass yield achieved from intercropping (Willey 1979). Following De Wit and van Den Bergh (1965), LER values were estimated as follows:

$$\mathrm{LER} = \mathrm{LER_{Leg}} + \mathrm{LER_R} = \frac{Y_{\mathrm{Leg(R)}}}{Y_{\mathrm{Leg}}} + \frac{Y_{\mathrm{R(Leg)}}}{Y_{\mathrm{R}}},$$

where Y_{Leg} and Y_{R} are the biomass yield of each legume and ryegrass in pure stands, respectively; and $Y_{\mathrm{Leg(R)}}$ and $Y_{\mathrm{R(Leg)}}$ are the biomass of each legume intercropped with ryegrass and of ryegrass intercropped with each legume, respectively. Similarly, the N land equivalent ratio (NLER), defined as the relative area of land growing monocrops that is required to achieve the same N yield of the mixture, was estimated. LER and NLER values >1 indicate an advantage of intercropping over growing in pure stands in terms of the use of environmental resources; LER and NLER values <1 indicate that monocrops use resources more efficiently than intercrops.

The intensity of competition between species in mixtures was computed using the aggressivity index and competitive ratio. The aggressivity index (A_{Leg} for the legume species) was calculated according to McGilchrist and Trenbath (1971):

$$A_{\mathrm{Leg}} = \frac{Y_{\mathrm{Leg(R)}}}{Y_{\mathrm{Leg}}} - \frac{Y_{\mathrm{R(Leg)}}}{Y_{\mathrm{R}}}.$$

Positive values for A indicate that the legume species is dominant; negative values for A indicate that the legume species is being dominated; values for A not significantly different from 0 indicate that neither companion crop dominates the other. Competitive ratios ($\mathrm{CR_{Leg}}$ for the legume component and $\mathrm{CR_R}$ for ryegrass) were calculated according to Willey and Rao (1980):

$$\mathrm{CR_{Leg}} = \frac{\mathrm{LER_{Leg}}}{\mathrm{LER_R}} \quad \text{and} \quad \mathrm{CR_R} = \frac{\mathrm{LER_R}}{\mathrm{LER_{Leg}}}.$$

The higher the CR value, the greater the competitiveness of the species in the mixture.

Statistical analysis

All measured variables were tested using the Shapiro–Wilk test of normality. All variables corresponding to proportion were arcsine transformed before analysis to ensure a better fit with the Gaussian law distribution. Data from each season were analysed separately, and the homogeneity of variances was assessed using Bartlett's test before combined analysis was performed. A mixed model according to the experimental design was used on the combined 2-year data set, with year as a random factor. Treatment means were compared using Fisher's protected least significant difference test at the 5 % probability level. We performed regression analyses (using the GLM procedure) on the combined 2-year data set to determine if there were relationships between biomass yield and Ndfa and between both biomass yield and total N yield and N fixed. Cropping system (monocropping and intercropping) was included as a categorical variable in the analyses. SAS software (SAS 2008) was used for all statistical analyses.

Results

Plant growth, N yield, and N_2 fixation

Fertilization increased the biomass and N yields of monocropped ryegrass by 50 % compared to the unfertilized ryegrass, but it did not influence the biomass N content (Table 2). Snail medick was the most productive species, with a biomass yield significantly higher than that of the N-fertilized ryegrass, whereas hairy vetch had the lowest biomass yield, which was not different from that of the unfertilized ryegrass.

On average, the biomass of the intercrops was 6.5 % higher than that of the monocropped legumes. However, the advantage of intercropping legumes with ryegrass compared to monocropped legumes varied by species, being significant only for Persian clover, squarrosum clover, and sulla (+12.3 %, +15.8 %, and +9.7 %, respectively, of biomass yield). The proportion of a legume species to the total biomass yield of the intercrops varied greatly, ranging from 30 % (Persian clover) to 69 % (fenugreek). Intercropping resulted in a dramatic reduction in total N yield in comparison to the respective monocropped legumes, with great differences among legume species; these differences increased as the percentage

Table 2 Aboveground biomass and N yields, N content of annual ryegrass and of the legume species, N derived from the atmosphere (Ndfa, as a percentage of total N yield; and N fixed, as amount per unit area), ^{15}N-fertilizer recovery fraction (Ndff), and contribution of ryegrass to total ^{15}N recovered from the mixtures

Crop	Biomass yield		N yield		N content		Ndfa	N fixed	Ndff	Contribute of ryegrass on total Ndff
	(kg DM ha^{-1})		(kg N ha^{-1})		R (g kg^{-1} DM)	Leg	(%)	(kg N ha^{-1})	(%)	(%)
Monocrops										
Ryegrass (R)	6506		45		6.9	–	–	–	26.6	–
Ryegrass (R_{N80})	9736		67		7.0	–	–	–	33.3	–
Fenugreek (F)	9709		194		–	19.9	77	150	26.7	–
Hairy vetch (Hv)	7296		179		–	25.6	81	146	20.3	–
Persian clover (Pc)	7806		202		–	25.3	88	178	14.6	–
Snail medick (Sm)	10806		205		–	19.0	80	163	24.9	–
Sulla (S)	9923		196		–	19.7	85	165	18.4	–
Squarrosum clover (Sc)	9095		185		–	20.4	86	158	15.9	–
Mixtures										
F+R	9833	(69)[a]	169	(79)[a]	11.2	19.8	90	121	27.3	69
Hv+R	7962	(59)	151	(71)	14.1	23.7	90	97	28.9	78
Pc+R	8765	(30)	112	(52)	7.3	25.3	91	61	25.4	83
Sm+R	10226	(68)	155	(79)	10.2	17.8	86	106	28.4	64
S+R	10889	(45)	123	(61)	7.5	16.6	92	76	25.9	86
Sc+R	10531	(58)	147	(75)	8.4	18.4	90	102	26.7	76
P-value	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
[b]$LSD_{0.05}$	945.1	8.5	19.1	7.2	1.38	1.42	2.8	18.0	4.85	7.0

Data are means of 2 years ($n = 8$)

[a] Values in brackets indicate the percentage contribution of the legume species to the total biomass yield and the total N yield of the mixture

[b] $LSD_{0.05}$: Least Significant Differences at the 0.05 probability level

contribution of the legume species to the total biomass yield of the mixtures decreased. The greatest reduction was found in the Persian clover/ryegrass intercrop (−90 kg N ha^{-1} compared to monocropped Persian clover) and the smallest in the fenugreek/ryegrass intercrop (−26 kg N ha^{-1} compared to monocropped fenugreek). The proportion of the legume component to the total N yield of each intercrop was always more than 50 %, ranging from 52 % (Persian clover) to 79 % (fenugreek and snail medick). The N content (g kg^{-1} DM) of ryegrass grown in mixture was markedly higher than that of ryegrass grown in pure stand; in general, it increased as the proportion of ryegrass to the total biomass yield of the intercrops decreased. The N content of legumes in intercrops was similar to (fenugreek, Persian clover, and snail medick) or lower than (hairy vetch, squarrosum clover, and sulla) that of pure legume stands.

The average value of %Ndfa across all treatments was high (86 %). When legume species were grown in monoculture, values of %Ndfa ranged from 77 to 88 % (in fenugreek and Persian clover, respectively). The percentage of Ndfa was always significantly higher when legume species were grown in mixture compared to monocrops.

The total amount of N_2 fixed by legumes was always higher in pure stands than in mixtures. For this trait, differences among legumes were moderate when legumes were grown in pure stands (range = 146–168 kg N ha^{-1}) and great when legumes were grown in mixture with ryegrass (range = 61–121 kg N ha^{-1}). No significant differences were observed between intercropped and monocropped ryegrass for average atom% ^{15}N excess (data not shown), suggesting that no N transfer occurred from legume to ryegrass in any of the mixtures.

No relation was found between total biomass yield and %Ndfa in legumes grown in pure stands or mixed with ryegrass (Fig. 2a). However, close relationships were found between the amount of N_2 fixed in biomass and both the total biomass yield (Fig. 2b) and the total N yield (Fig. 2c). Moreover, we did not find significant differences in the regression equations obtained between legumes in pure stands and in mixture.

Great differences were found among legume species for %Ndff when legumes were grown in pure stands; the observed values for this trait ranged from 14.6 % (Persian clover) to 26.7 % (fenugreek). In addition, the %Ndff of the intercrops was on average 27.1 %; this value was markedly higher than the average value in the monocropped legumes (20.1 %) and similar to that of the unfertilized ryegrass (26.6 %). The contribution of the ryegrass to the total %Ndff of the mixtures was 76 % on average and decreased as the proportion of the legume to the total biomass of the intercrop increased ($r = 0.797$; $n = 48$).

Indices of intercrop efficiency

All intercrops showed an advantage over monocrops in terms of biomass and N yields. LER values ranged from 1.17 (snail medick + ryegrass) to 1.44 (sulla + ryegrass), and NLER values ranged from 1.31 (Persian clover + ryegrass) to 1.61 (hairy vetch + ryegrass; Fig. 3). The partial LER values of ryegrass were always higher than 0.50, ranging from 0.52 (intercropped with fenugreek) to 1.02 (intercropped with Persian clover); the partial LER values of legume species ranged from 0.31 (Persian clover) to 0.70 (squarrosum clover). The partial NLER values of ryegrass were always markedly higher than 0.50 (range = 0.71–1.01), whereas among the legumes, only Persian clover and sulla showed partial NLER values lower than 0.50.

The aggressivity index of the legume species calculated on the basis of biomass yield ranged from −1.31 (Persian clover) to 0.43 (fenugreek; Table 3); when calculated on the basis of N yield its values were equal to 0 (fenugreek, snail medick, and squarrosum clover) or negative (hairy vetch, Persian clover, and sulla). Competitive ratios of the legume species in terms of biomass yield were always greater than 1, except for Persian clover and sulla. When the competitive ratio was calculated based on N yield, it was less than or very close to 1 for all legumes. As expected, competitive ratios for ryegrass based on N yield were always greater than 1 but varied greatly depending on the intercropped legume species.

Discussion

In both years, total rainfall was similar to the long-term average for the trial environment and was adequately distributed; this was reflected in plant growth and biomass yields similar to those usually obtained for the forage species that are typically grown in the trial environment (Giambalvo et al. 2011; Saia et al. 2014).

Great differences were observed among the monocropped legumes in terms of biomass (range = 7.3 t ha^{-1} for hairy vetch to 10.8 t ha^{-1} for snail medick); on the whole, when biomass yield increased, quality (expressed as N content) decreased. Thus, differences in the biomass yields of monocropped legumes were not related to those in total N yield. Indeed, for the latter trait, differences among legume species grown in pure stands were generally moderate and lower than those observed for biomass yield.

The average value of %Ndfa across all legumes grown in pure stand was high (82 %) and higher than or similar to the values found in other studies in the Mediterranean region (Sulas et al. 2009; Giambalvo et al. 2012; Ruisi et al. 2012). Significant differences were found among legume species for this trait, ranging from 77 % (fenugreek) to 88 % (Persian clover). In agreement with the findings of Anglade et al. (2015), no relationship was found between %Ndfa and biomass yield. However, we found significant linear relationships between biomass yield and the total amount of N_2 fixed, as previously found by other authors (Peoples et al. 2001; Schipanski and Drinkwater 2012). It is intuitive that the greater the plant's growth, the greater its N needs, which it satisfies by increasing N_2 fixation. This suggests that the amount of N_2 fixed by a legume crop can be increased by acting on all factors that directly affect plant growth (water and nutrient availability, plant protection, etc.). Our average value across all legume species of 17.3 kg of N_2 fixed per ton of biomass is lower than that previously reported by Unkovich et al. (2010; 18.7 kg N_2/t of shoot dry matter), Herridge et al. (2008; 20 kg N_2/t of shoot dry matter), and Peoples and Baldock (2001; 25 kg N_2/t of shoot dry matter). Such discrepancies are probably due to several factors, including the species investigated, which differed in the cited experiments; variability in

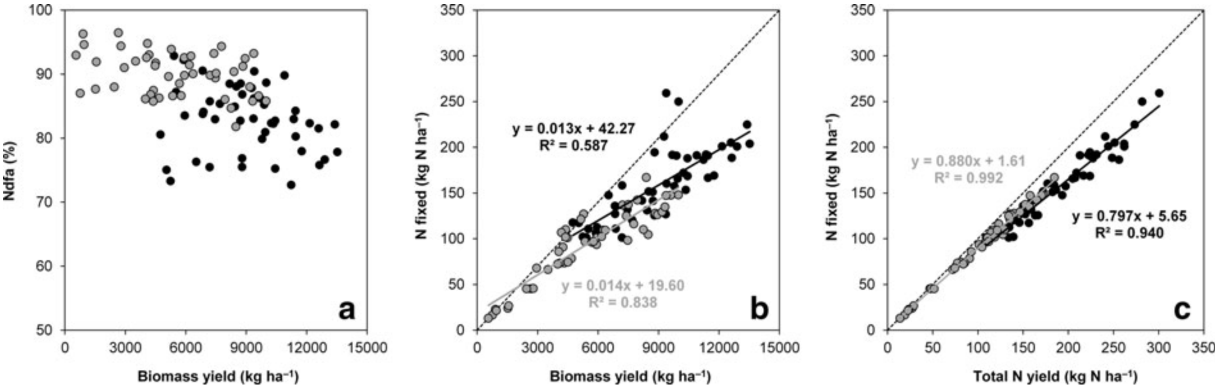

Fig. 2 The relationships between the fraction of N in biomass derived from atmosphere (Ndfa %) and the legume biomass yield (**a**), between the amount of N_2 fixed in biomass (N fixed) and the legume biomass yield (**b**), and between the N fixed and total N yield (**c**). Within each figure, symbols ($n = 48$; 6 legume species × 4 replications × 2 years), the regression line, and equations are displayed in grey for intercrops and in black for monocrops

climatic conditions, soil characteristics, and management practices; and finally the different methodologies used to estimate N_2 fixation. Unkovich et al. (2010) noted that the magnitude of this relationship is highly site specific. Furthermore, in line with Anglade et al. (2015), our data showed that the accuracy of the estimate of the amount of N_2 fixed by a forage legume can be markedly improved if the biomass yield is associated with its relative N content, as shown by the highly significant linear relationships between total N yield and the amount of N fixed.

The ^{15}N fertilizer recovery fraction (%Ndff) of monocropped ryegrass was about 30 %, which is similar to that observed in grass and cereal species grown in the same environment (Giambalvo et al. 2011; Ruisi et al. 2014, 2015). With the exception of fenugreek and snail medick, the legumes under study were less efficient than ryegrass at recovering N when grown in pure stand. Several authors have observed a greater ability of grasses than legumes to extract mineral N from the soil (Høgh-Jensen and Schjoerring 1997; Giambalvo et al. 2011). Munoz and Weaver (1999) explained this as the

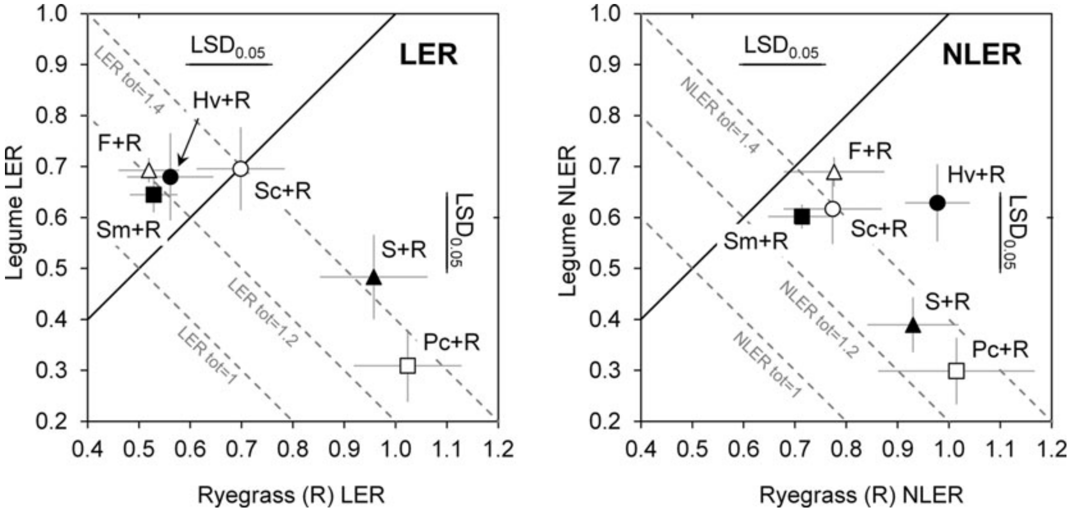

Fig. 3 The partial land equivalent ratio (LER) and partial nitrogen land equivalent ratio (NLER) of the legume species and ryegrass. *Dotted lines* indicate total LER and NLER values of 1.0, 1.2, and 1.4. The *solid line* indicates the theoretical condition of the balanced use of environmental resources by the legume species and ryegrass ($LER_{legume} = LER_{ryegrass}$; $NLER_{legume} = NLER_{ryegrass}$). All values are the mean ($n = 8$) ± SE (bars). *Vertical* and *horizontal bars* indicate LSD values at 5 % probability level for partial LER_{legume} or $NLER_{legume}$ and $LER_{ryegrass}$ or $NLER_{ryegrass}$, respectively. F, fenugreek; Hv, hairy vetch; Pc, Persian clover; S, sulla; Sc, squarrosum clover; Sm, snail medick; R, ryegrass

Table 3 Indices of aggressivity of the legume species (on biomass yield basis and on N yield basis) and indices of competitiveness of both the legume species and ryegrass (on biomass yield basis and on N yield basis) in the mixtures

Mixture	Aggressivity of the legume		Competitive Ratio			
	Biomass	N	Legume		Ryegrass	
			Biomass	N	Biomass	N
[a]F+R	0.43	0.05	1.44	1.02	0.76	1.16
Hv+R	0.32	−0.55	1.46	0.68	1.01	1.76
Pc+R	−1.31	−1.19	0.38	0.39	6.04	5.80
Sm+R	0.30	0.04	1.31	0.90	0.86	1.21
S+R	−0.84	−0.79	0.62	0.48	2.94	3.14
Sc+R	0.03	0.02	1.19	0.93	1.22	1.47
P-value	<.001	<.001	<.001	<.001	<.001	<.001
[b]$LSD_{0.05}$	0.427	0.586	0.447	0.278	1.25	1.486

Data are means of 2 years ($n = 8$)

[a] F, fenugreek; Hv, hairy vetch; Pc, Persian clover; S, sulla; Sc, squarrosum clover; Sm, snail medick; R, ryegrass

[b] $LSD_{0.05}$: Least Significant Differences at the 0.05 probability level

differential ability of the two crops (grass and legume) to deplete the mineral N supply of the soil and not as a result of differential access to N pools between the crops (due to a different weight or volume of the root system). In contrast, Morris et al. (1990) found that arrowleaf clover (*Trifolium vesiculosum* L.) in pure stand accumulated similar quantities of fertilizer N as ryegrass in pure stand. In the present experiment, the legume species with the highest %Ndff values were fenugreek and snail medick; these two species are characterised by a higher early crop growth rate, as we visually observed in this and in other experiments (unpublished data) and also by other authors (Nichols et al. 2007). Thus, it is likely that earlier growing species were more able than later growing species to catch soil mineral N derived from the mineralization of the organic matter during the fall and the early phase of the crop cycle. Such N was probably unavailable for later growing species because of the high leaching losses that probably occurred during the winter, as the soil received most of the total annual rainfall (about 70 %) in this season.

In our experiment, we observed a negative relationship between the %Ndfa and %Ndff of monocropped legumes. The higher %Ndfa observed in Persian clover, squarrosum clover, and sulla seemed to be mainly due to a low capacity to take up the mineral N from the soil rather than a need to satisfy a higher N demand.

On average, the legume–ryegrass mixtures showed advantages in terms of both total dry matter yield and total N yield over their components grown in pure stands (LER and NLER values always >1). This result is consistent with several findings that have shown a more efficient resource utilization of grass–legume intercrops than pure stands (Haynes 1980; Jørgensen et al. 1999; Sengul 2003). Several experiments have shown that a large part of such advantage is due to complementarity in the use of resources (especially N) when resources are limited (Bulson et al. 1997; Bedoussac and Justes 2010). In our experiment, N was surely a limiting factor since fertilization of ryegrass strongly increased its biomass, N uptake, and N recovery fraction. Therefore, when intercropped, the grass component could have access to a higher proportion of soil mineral N thanks to its greater interspecific competitive ability because of a more rapid and often deeper root development and higher N need, whereas the legume component should increase biological N fixation to meet its N demand in comparison with the sole cropping system.

The contribution of the ryegrass to the total biomass yield of the intercrop varied markedly among mixtures, highlighting different competitive behaviour among legumes, as suggested by all competition indices (A_{Leg} and CR). The earlier growing legumes (fenugreek, snail medick, and hairy vetch) showed a greater ability to compete against ryegrass for resources (e.g., soil N, as shown by values of %^{15}N recovered), so that, for such species, their contribution to the biomass of the intercrop was greater than 59 %.

The greater ability of the ryegrass to absorb N from soil in comparison with the legumes, as shown by the large contribution of ryegrass to total ^{15}N recovered (from 64 to 86 %), decreased the N available in the soil for the legumes and, as a consequence, increased their dependence on symbiotic N_2 fixation. In fact, the %Ndfa of intercropped legumes was markedly higher compared to the pure stands. Similar results were found by Vinther (2006) for white clover grown in pure stand and in mixture with perennial ryegrass and by Giambalvo et al. (2011) for berseem clover grown either alone or in mixture with annual ryegrass. However, the increased %Ndfa in the intercropped legumes was strongly counteracted by a reduction in total biomass and N accumulated by the legumes in mixture compared to pure stands, so that the largest amount of N fixed per hectare occurred in the monocropped legumes not the intercrops.

The mixtures had N recovery values markedly higher than the legume crops grown in pure stand. This highlights the fact that intercropping allows for a better exploitation of natural resources (e.g., N, as observed in this study) on the one hand and limits soil N losses (via leaching, volatilization, or denitrification) that can occur either in the same growing season as intercropping or in the subsequent one on the other. On the whole, the different legume species showed different abilities to compete with ryegrass for the exploitation of N available in soil (both native and from fertilizer). Fenugreek was the most competitive whereas sulla and Persian clover were the least competitive. These latter species also showed the greatest reductions in terms of biomass yield and N_2 fixed when grown in mixture with ryegrass relative to when they were grown as monocrops.

No N transfer from legumes to ryegrass occurred, as no significant differences were observed between intercropped and monocropped ryegrass for average atom% ^{15}N excess. Estimates reported in the literature for N transfer in legume–cereal intercrops show considerable variability (Chalk et al. 2014). As a general observation, significant N transfer from legumes to companion species during seasonal growth has been reported in pastoral systems (Dahlin and Stenberg 2010) and in perennial intercropping systems (Mårtensson et al. 1998), whereas little or no N transfer is often reported in annual grass–legume mixtures during concurrent growth (Izaurralde et al. 1992; Kurdali et al. 1996). It is probable that the mechanisms through which the N release from legume tissues and root zones and the N transfer to the associated non-legume crop take place (e.g., decomposition of plant tissues and root nodules) need time to become efficient. Høgh-Jensen and Schjoerring (1997) found that N transfer from white clover to an associated ryegrass increased with sward age, with the N transferred amounting to 3, 16, and 31 % of the N accumulated in the ryegrass in the first, second, and third production years, respectively. However, in some experiments, carried out mainly with warm-season species, remarkable amounts of N have been transferred from legumes to an associated non-legume companion in short-term intercropping systems (Fujita et al. 1992; Martin et al. 1991; Ofosu-Budu et al. 1995). This suggests that N transfer can be affected, beyond sward age, by environmental factors that favour N release accelerating decomposition processes of plant tissues and nodules (i.e., temperature and water). Furthermore, agronomic management (proximity and intimacy of the species in the mixture, cutting regime, pest protection strategy, fertilization, etc.) can also influence N transfer (Fujita et al. 1992). In our experiment, carried out in the autumn/spring period, environmental conditions and agronomic factors might not have favoured those processes that lead to the release and then transfer of N from legume to the associated ryegrass; this was true regardless of the legume species.

In conclusion, the legume species differed in their ability to utilize inorganic soil N and in their N_2 fixation capacity and biomass yield. Such differences, although displayed by using only one variety of each legume species, should be taken into account to develop cropping systems with more efficient N use. Legume–ryegrass intercrops used natural resources more efficiently than pure crop systems, which implies that intercrops are a valuable option for sustainable agricultural systems in Mediterranean environments. The advantages observed in this study depended on complementarity in the use of resources, as the %Ndfa of the intercropped legumes was markedly higher than that of the monocropped species, and this depended on the ability of the ryegrass to utilize soil mineral N and the ability of the legume to rely on N_2 fixation to satisfy its own N need. As N transfer from legumes to the companion grass was never observed, this mechanism apparently did not contribute to the success of the mixtures over the respective monocrops. Moreover, different legume species showed different aptitudes for growth in a mixture and different abilities to compete with ryegrass for the available resources. From a practical point of view, such information will be useful for farmers since it highlights how the success of an intercrop largely depends on the choice of the species grown together. Further research should aim to evaluate the genotypic diversity existing within each of the forage legumes studied for its ability to be grown in mixture with a forage grass.

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