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Screening suitable legumes for living mulches to support nitrogen dynamics and weed control in a durum wheat-forage sorghum crop sequence

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ABSTRACT

Relay intercropping of service legumes (living mulch) is a valuable agroecological practice to support nutrient availability, yield and non-chemical weed control in a crops sequence, if suitable legumes are chosen. This study tested the suitability of eight legume living mulches for relay intercropping in durum wheat (*Triticum durum* Desf.) evaluating their effects on the subsequent forage sorghum (*Sorghum bicolor* L. Moench) in a Mediterranean low-input cereal-based cropping system. Legumes include perennial (*Medicago sativa*, *Medicago lupulina*, *Trifolium repens*, *Hedysarum coronarium*), annual (*Trifolium incarnatum*, *Trifolium resupinatum*) and annual self-seeding (*Trifolium subterraneum*, *Medicago polymorpha*) species. A plot experiment repeating two times a wheat-sorghum crop sequence, was carried out in Central Italy to assess the effects of relay intercropping of legumes on agronomical and economic performance of the associated wheat and subsequent sorghum. The legumes were undersown in the already developed durum wheat in late winter. They were maintained after wheat harvest and used as green manure by biomass chopping and plowing in the following spring at sorghum sowing time. None of the intercropped legumes affected N uptake or grain protein content of the companion wheat with respect to the control (wheat sole crop). In the spring before sorghum sowing, N input from legume biomass residues was assessed and it sensibly changed according to legume species, ranging from 1.2 (*T. incarnatum*) to 182 kg ha^{-1} (*H. coronarium*). The legume treatments significantly affected the N uptake in the subsequent sorghum and it ranged from 20.2 to 172.6 kg ha^{-1}. Without the use of additional external nitrogen input, average sorghum dry biomass production preceded by *H. coronarium* (13.3 t ha^{-1}), *T. repens* (11.8 t ha^{-1}), *T. subterraneum* (11.0 t ha^{-1}) and *M. sativa* (9.3 t ha^{-1}) was in line with the productive level under conventional conditions. Biomass residues of *M. sativa* significantly reduced the total weed biomass in sorghum by 65% compared with the control. In sorghum, preceding legumes such as *T. resupinatum* and *T. incarnatum* promoted dicotyledonous weed growth. *Hedysarum coronarium* and *T. repens* were the best legumes for relay intercropping in the low-input wheat-sorghum crop sequence under Mediterranean conditions of this study according to agronomic and economic evaluation. These legumes were able to increase soil nitrogen content allowing to significantly reduce external nitrogen fertilization while optimizing sorghum production. When gross income is calculated at cropping system level, most legumes provide a positive economic balance.

1. Introduction

Nitrogen deficiency and weed competition are common concerns for crop production and are currently faced, in conventional cereal-based cropping systems, through the extensive use of inputs such as herbicides and synthetic nitrogen (N) fertilizers. Alongside the environmental issues related to the use of input intensive farming systems, their economic sustainability is increasingly questioned (Pimentel et al., 2005). Agricultural production is sensitive to variations in energy prices, either through direct energy consumption or through energy-related inputs. The cost per unit of agricultural product are increasing rapidly while prices for agricultural commodities do not increase at the same rate (Sands et al., 2011). Therefore, there is a growing demand for more sustainable agricultural systems, prioritizing production methods that are based on crop diversification and that exhibit a greater land use efficiency while conserving natural resources (Bedoussac et al., 2015; Li et al., 2020).

Legume living mulches (LMs) are often reported as a promising

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option for supporting crop diversification and reducing input reliance in conventional cereal-based cropping systems (Gaba et al., 2015). Living mulches are service crops (i.e., crops that are not directly used for productive purposes but for the provision of ecosystem services), often legumes, co-cultivated with the main crop and maintained as a living ground cover throughout the crop growing season (Hartwig and Ammon, 2002). Inclusion of a LM has been demonstrated to be an effective alternative to chemical weed control, and it supports the optimization of nutrient cycling and resource conservation with no or limited negative impact on crop productivity (Amossè et al., 2013b; Hiltbrunner et al., 2007; Leoni et al., 2022; Verret et al., 2017). Living mulches can be established at the same time as the main crop, i.e., contemporary intercropping, or through delayed sowing of the LM into a previously established cash crop, i.e., relay intercropping. The main advantage of relay intercropping is that it provides a competitive advantage to the main crop (Amossè et al., 2013a). Alternatively, it can be used in case the environmental conditions for LMs establishment are not met at the time of the main crop sowing (Lamichhane et al., 2023).

In addition to the positive effects that LMs can provide during the co-cultivation with the main crop, the most interesting attribute of LMs is their persistence and growth after the main crop is harvested, allowing to maintain the soil constantly covered until the subsequent crop. For example, in a Mediterranean cereal-based cropping system, winter cereals are typically harvested at the end of June, when drought impedes the sowing of a summer crop, while a LM is expected to persist and keep the soil covered until the following spring with positive effect on weed control, reduction of soil erosion and supports productivity of the subsequent crops. During this period, legumes can be utilized for forage production or as green manure before sowing the subsequent crop (Vrignon-Brenas et al., 2016a , Cougnon et al., 2022). This option allows nutrients, especially nitrogen (N), and allelochemical compounds released during legume residue degradation to improve the yield and weed control in the following crop, leading to a reduction in the use of external inputs such as fertilizers and herbicides (Amossè et al., 2013c);

Despite the numerous advantages that LMs can offer in a crop sequence, the impact of legumes LMs on the subsequent crop has received limited research attention. Most studies on LMs have primarily concentrated on their interactions with co-cultivated crops, often overlooking their effects within a crop sequence (Bergkvist et al., 2011; Amossè et al., 2013c; Mugi-Ngenga et al., 2022; Rouge et al., 2023). Indeed, the successful adoption of LMs depends on their capacity to provide benefits during entire crop sequence (Vrignon-Brenas et al., 2016b). Their success rate can be affected by several factors such as:

i) the ability of the living mulch to germinate and survive under the wheat canopy during the intercropping period,
ii) the capacity of legumes to establish a good coverage after the wheat harvest in relation to the local environmental condition,
iii) the production of a sufficient quantity and quality of biomass to support the production of the subsequent crop without the need for external inputs.

Therefore, the screening of legume species can be highly important in selecting suitable LMs for the Mediterranean environment (Garba et al., 2022a). This study focused on a typical crop sequence of Mediterranean cereal based cropping systems, such as durum wheat and forage sorghum, and aims to explore the effects of eight different legume living mulches used for relay intercropping with durum wheat on N dynamics, weed control and crop production of the subsequent forage sorghum. In the experiment we have included 4 perennial legumes such as *Medicago sativa*, *Medicago lupulina*, *Trifolium repens*, *Hedysarum coronarium* because they are expected to establish a dense coverage of living plants until the following spring and subsequently use as green manure before the subsequent cash crop (Bergkvist et al., 2011). However, in the southern regions of Europe, the summer season can be excessively dry, and this prevents a good establishment of perennial legumes after wheat harvest (Porqueddu et al., 2016). In this situation, annual self-seeding legumes can be a viable alternative to perennial legumes (Campiglia et al., 2001) and therefore *Trifolium subterraneum* and *Medicago polymorpha* were also evaluated in the experiment. Self-reseeding annual legumes, once they are established, can be managed to produce a sufficiently high amount of seeds to enable re-establishment in the following growing season without tillage (Bartholomew, 2014). Under Mediterranean conditions, the growth cycle of annual self-seeding legumes terminates in late spring, a few weeks before wheat harvest. After wheat harvest, biomass residues of these legumes are expected to form a dead mulch during the dry summer season and re-grow spontaneously from their seeds in autumn, to establish a cover crop until the subsequent spring (Ilnicki and Enache, 1992). Living mulches with annual forage legumes such as *Trifolium incarnatum* and *Trifolium resupinatum*, are not expected to regrow and they were included in the experiment to evaluate the effect of their dead mulch on the subsequent crop.

In Leoni et al. (2022) it was shown that legumes did not affect durum wheat grain yield and this was independent of the two cropping systems (high and low input system). In this paper we continued the research, and we focused on the low-input cropping system (Leoni et al., 2022) to test the legume's impact on durum wheat N uptake and grain protein content, an important parameter determining the quality of durum wheat flour for pasta production that is strongly dependent on N availability and N uptake by the wheat crop, and subsequently on nitrogen inputs to the following sorghum crop and the interaction this may have with the weed community. This research was carried out at crop sequence level and was performed only in the low-input cropping system in Pisa (Italy), contrary to the agronomic evaluation presented in Leoni et al. (2022) that compared the performance of wheat and the relay-intercropped legumes in a low and high input durum wheat system representing two different regions in the Mediterranean area. In the low-input system we expected to find differences among the tested legumes species in their capacity to provide nitrogen to durum wheat and to the following forage sorghum crop.

By following the effect of legumes until the harvest of the subsequent sorghum crop, an overall evaluation could be made based on trade-offs between the various phases in the crop sequence, to select legumes with the best overall performance. Finally, this study investigated the economic viability of legumes intercropped with cereals on wheat-sorghum crop sequence. We assumed that the legume species tested in this experiment would provide different services to the crops and affect the gross margin accordingly, allowing us to identify the most cost-effective ones for relay intercropping for a typical crop sequence in a Mediterranean low-input cropping system.

2. Material and methods

2.1. Site description

This experiment was carried out in Pisa in a rainfed area of the Centre for Agri-Environmental Research "Enrico Avanzi" of the University of Pisa (CiRAA, San Piero a Grado, Pisa, Italy, 43∘41′02.08′N, 10∘20′35.0′E). In order to replicate the trial over two consecutive crop seasons (2017–19, 2018–20) within a typical crop sequence for the Pisa plain area and to evaluate the effect of legumes on the following cash crop, experiments were set up in field A for 2017–19 (Crop Sequence Year 1, CSY1) and B for 2018–20 (Crop Sequence Year 2, CSY2) (Fig. 1). Before starting the experiment, the fields were cropped with soybean (Field A) and maize (Field B). However, in field A soybean failed due to a severe seeds predation. Soil types in field A and B were classified as silty-loam and a silt-clay respectively (Jahn et al., 2006). More in details, analyses of soil samples (0 – 0.30 m) collected in field A showed that the soil consists of 50.6% sand, 26.1% silt, and 23.3% clay, 1,77% SOM and pH 8.0. Analysis in Field B showed that the soil consists of 39.8% sand, 34.7% silt, and 25.5% clay and 1,18% SOM and pH 8.3. Soil chemical fertility consists of 1.20 and 1.23 total N g kg^{-1}, 10.5 and 7.0 available P

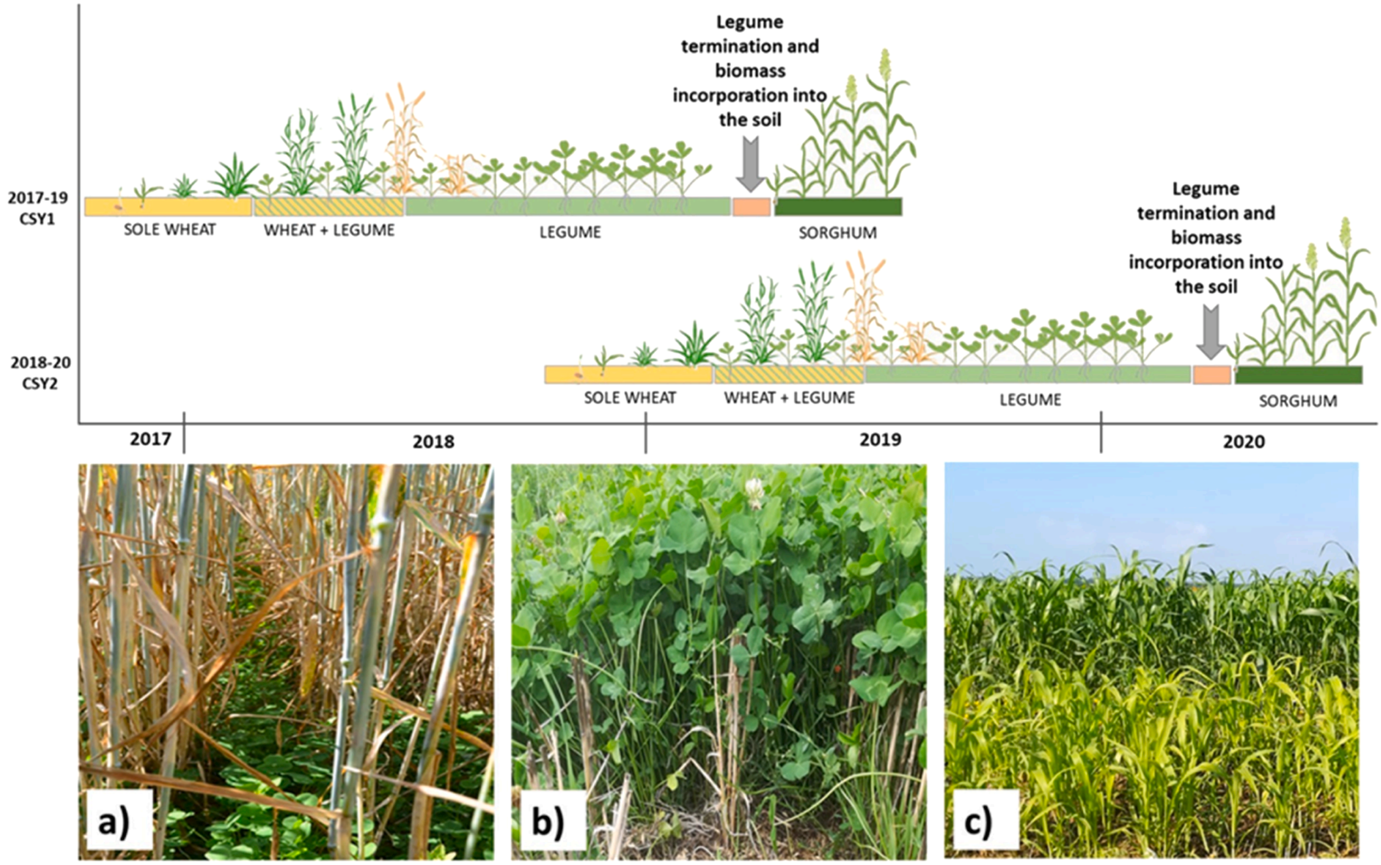

Fig. 1. Conceptual diagram of the experiment (Oct 2017 - Aug 2020). Relay intercropping of *T. repens* with durum wheat (a), *T. repens* after the cereal harvest (b) and effects of *T. repens* biomass residues on the subsequent summer crop (c) in contrast with the control.

mg kg^{-1} respectively for field A and B. Organic matter (SOM), total nitrogen (Total N) and available phosphorus (P) were respectively determined following the Walkley-Black method (FAO, 2019), Kjeldahl method (Bremner, 1960) and Olsen method (John and Pierzynski, 2009). The experimental site was subject to a Mediterranean climate, with mild winters, very warm summers and rainy autumns (Temperatures and monthly precipitations over the duration of the experiment, from November 2017 to August 2020, can be found in supp. Fig. SM1).

2.2. Experimental design and treatments

The trial was laid out in a randomised complete block design with four replications that was repeated twice, in adjacent fields. Each experiment consisted in a two-year crop sequence of durum wheat (*Triticum durum* Desf. cv. Minosse) - relay intercropped legume - spring crop (*Sorghum vulgare* cv. Sugar Graze) (Fig. 1). The durum wheat cv. Minosse is a commonly used wheat variety for pasta-making in Italy and it has been chosen for its lodging resistance. Sorghum cv Sugar Graze is used for forage production and has been chosen for its drought resistance since the experimental fields were not irrigated. In the Pisa plain, farmers are progressively abandoning the use of irrigated crops such as maize due to the lack of water during the summer and replacing them with more drought-resistant summer crops, such as sorghum for both grain and forage production. Eight legume species were undersown in durum wheat: i) four perennial legumes, *Medicago sativa* (cv. Gamma, 40 kg ha^{-1}), *Trifolium repens* (cv. Companion, 15 kg ha^{-1}), *Hedysarum coronarium* (cv. Carmen, 30 kg ha^{-1}), *Medicago lupulina* (cv. -, 40 kg ha^{-1}); ii) two annual legumes, *Trifolium incarnatum* (cv. Kardinal, 40 kg ha^{-1}) and *Trifolium resupinatum* (cv. Laser, 10 kg ha^{-1}); two annual self-seeding legumes, *Trifolium subterraneum* (cv. Mintaro, 35 kg ha^{-1}) and *Medicago polymorpha* (cv. Scimitar, 40 kg ha^{-1}). The legume species have been chosen to represent a diversity of morphological characteristics and growing cycle. Seeding rates were determined by contacting experts on pasture legumes and intercropping, and according to our experience. Seeding rate of legumes was adjusted according to the germinability test performed on each seed lot. A control treatment was implemented with wheat grown as the sole crop to evaluate the incidence of undersown legumes on wheat N uptake and grain protein content. In the control, after the wheat harvest, the soil was maintained uncultivated until the sorghum sowing (Fig. 1). Except for the legume presence, the control was managed as the other plots. The seed bed was prepared by ploughing at 25 cm depth and refining the soil with a rotary harrow. Plot size was 9 m^{-2} in CSY1, and 18 m^{-2} in CSY2. Durum wheat was sown with a mechanical plot seeder (5 Jens A. Schou Mek, 5 Øyjord 1971) on 22 November 2017 (CSY1) and 12 December 2018 (CSY2) in 18 cm spaced-rows at a rate of 350 viable seeds m^{-2}. Legumes were undersown with the same mechanical plot seeder in the wheat inter-rows spaces before the wheat elongation phase (BBCH 21–29) on 28 February 2018 (CSY1) and 18 February 2019 (CSY2). Durum wheat was mechanically harvested on 27 July 2018 (CSY1) and 10 July 2019 (CSY2), and straw was removed from the field. Legumes were maintained after the wheat harvest until the following spring on 14 April 2019 (CSY1) and 15 April 2020 (CSY2) (Fig. 1). Legume biomass was used as green manure by biomass chopping and ploughing in the following spring. The forage sorghum was sown at a rate of 150 viable seeds m^{-2} in 30 cm wide inter-rows following the legume plots (Fig. 1). Prior to the sowing of sorghum soil was refined with a rotary harrow. The plot management was based on the principle of low-input farming, with no application of fertilizers, herbicides and fungicides. Given the low productivity potential of this area, local farmers actually reduce as much as possible input costs in order to maximize the gross margin. In fact, the standard durum wheat production level in this area is on average only 2.6 t ha^{-1}.

2.3. Data collection

At the harvesting stage (BBCH GS 92), wheat aboveground biomass was cut at the base in two 54 × 50 cm quadrants (sub-replicates) per plot (replicates). Before processing, samples were weighed after oven-drying (40 °C in air oven). The durum wheat samples were further assessed for spike biomass and straw biomass. Threshing of wheat was carried out mechanically using Vignoli mod. Trident to obtain clean grains. Protein content was quantified using Infratec 1241 Grain Analyzer (FOSS). In order to evaluate the contribution of legumes living mulch on the wheat nitrogen uptake, total nitrogen content (N, %) was measured in the wheat straw following the Kjeldhal methodology (Bremner, 1960) and estimated in grains using a conversion factor from protein content of 5.81 (Fujihara et al., 2008). In the following spring, legumes were incorporated into the soil as green manure. Before termination, samples of 54 × 50 cm were hand harvested randomly in two points (sub-replicates) per plot (replicates) avoiding edges to determine the dry biomass (g m^{-2}) of legumes used in the experiment. At this sampling time, the total nitrogen content (N, %) was determined in the dry aboveground biomass (legume and weeds) following the Kjeldhal methodology (Bremner, 1960). For each sampling point, the total nitrogen content (N, %) was multiplied by accumulated dry biomass (kg ha^{-1}) to estimate the nitrogen inputs (N, kg ha^{-1}) from the biomass incorporation (legume and weeds). Sorghum and weed aboveground dry matter production (g m^{-2}) were measured in August with sorghum at booting stage (BBCH 45). Biomass samples of 60 × 50 cm (two rows and two inter-row spaces along 0.5 m) were hand harvested randomly in three points (sub-replicates) per plot (replicates) avoiding edges. Biomass of sorghum and weeds (splitting monocotyledonous and dicotyledonous species) was separated and weighed after oven-drying (40 °C in air oven). Total nitrogen content (N, %) in sorghum and weed biomass was determined following the same procedure used for legumes.

2.4. Nitrogen Use Efficiency (NUE)

The Nitrogen use efficiency was calculated according to EU Nitrogen Expert Panel (Oenema et al., 2015), as follows:

$$\mathrm{NUE}\ (\%) = [(\mathrm{Nup}_{S(trt)}) - (\mathrm{Nup}_{S(cnt)} + \mathrm{Nup}_{W(cnt)})]/(\mathrm{Nin}_{L(trt)} + \mathrm{Nin}_{W(trt)})$$

Where NUE (%) is the nitrogen use efficiency, $\mathrm{Nup}_{S(trt)}$ is the N uptake of sorghum preceded by legumes (N, kg ha^{-1}), $\mathrm{Nup}_{S(cnt)}$ and $\mathrm{Nup}_{W(cnt)}$ are respectively referred to the N uptake of sorghum and weeds in the control (sorghum without previous legume) and finally $\mathrm{Nin}_{L(trt)}$ and $\mathrm{Nin}_{W(trt)}$ are respectively referred to the N input from legume and weeds biomass residues (N, kg ha^{-1}). $[(\mathrm{Nup}_{S(trt)})-(\mathrm{Nup}_{S(cnt)}+\mathrm{Nup}_{W(cnt)})]$ represents the N uptake of sorghum, net of the N uptake in the control.

2.5. Economic assessment

Gross income analysis was used to determine the economic sustainability of relay intercropping of legumes with durum wheat adapting the approach applied by Antichi et al. (2022) and Vasileiadis et al. (2015). Gross income (GI) was calculated for wheat (GI_w) and sorghum (GI_s) separately. Cumulative Gross Income (GI_{w+s}) was then calculated to evaluate the effects of relay intercropped legumes at crop sequence level by summing gross income of wheat and sorghum. Gross income (GI) and cumulative gross income (GI_{w+s}) was calculated as follows:

$$GI_w\ (€\ ha^{-1}) = GPV_w - C_w;$$

$$GI_s\ (€\ ha^{-1}) = GPV_s - C_s;$$

$$GI_{w+s}\ (€\ ha^{-1}) = GI_w + GI_s;$$

Gross income is defined as the Gross Production Value (GPV, € ha^{-1}) minus the variable costs (C_w, € ha^{-1}) incurred in achieving that income. Notably, for the purpose of this study GI does not include the Common Agricultural Policy (CAP) payment. Gross Production Value (GPV) is the value of production at the point of sale (see supplementary material SM2). GPV was calculated as follows:

$$GPV_w\ (€\ ha^{-1}) = Y_w \cdot Q_w;$$

$$GPV_s\ (€\ ha^{-1}) = Y_s \cdot Q_s;$$

Where Y is the crop yield (t ha^{-1}) and Q is the quotation of that specific crop (€ ha^{-1}). In this experiment wheat and sorghum yields were experimentally evaluated. We assumed pasta production and fresh forage as destination of wheat grain and sorghum respectively. In the experimental site the price quotation refers to Bologna Stock Exchange for cereal grains (Italy) (Camera Di Commercio, Industria, Artigianato, Agricoltura Di Bologna, 2021) and to the Chamber of Commerce of Brescia for forage sorghum (Camera Di Commercio Industria Artigianato e Agricoltura di Brescia, 2021). According to the designated use of wheat and sorghum, the quotation (Q_w and Q_s) in July 2018 and 2019 was respectively 220 and 225 € ha^{-1} for durum wheat and 48 € t^{-1} in both of the seasons for forage sorghum (see supplementary material tab. SM2). The variable production costs (C_w, € ha^{-1}) used in the economic analysis included seed purchases and mechanical operations. Regarding the seed cost, durum wheat (cv. Minosse) was sourced at 600 € ha^{-1}, certified seeds treated with fungicide.

The price of legume seeds varied according to the species as follows: *Medicago sativa* (cv. Gamma): 2 € ha^{-1}; *Trifolium repens* (cv. Companion): 5 € ha^{-1}; *Hedysarum coronarium* (cv. Carmen): 6.6 € ha^{-1}; *Medicago lupulina* (cv. -): 7 € ha^{-1}; *Trifolium incarnatum* (cv. Kardinal): 3.7 € ha^{-1}; *Trifolium resupinatum* (cv. Laser): 4 € ha^{-1}; *Trifolium subterraneum* (cv. Mintaro): 7 € ha^{-1}, and *Medicago polymorpha* (cv. Scimitar): 7 € ha^{-1}. Forage sorghum (cv. Sugar graze 2) was provided by Padana semences at the cost of 2.4 € ha^{-1} (seeds treated with fungicides). The quotations for agriculture operations and services were obtained from Regional Agricultural Mechanic Entrepreneurs' Association Price List and include downtime, insurance, depreciation, labor, machinery servicing and maintenance. Details about quotation of agricultural operation used in this experiment are reported table SM3 of supplementary materials.

2.6. Statistical analysis

All response variables were analyzed with Linear Mixed Model (LMM) or Generalised Mixed Model models (GLMM), using the lme4 package for R (Bates et al., 2015). Explanatory variable including wheat N uptake, wheat grain protein content, N input (from legumes, weeds and total), sorghum biomass, N uptake (from sorghum and weeds) and NUE have been analysed by a LMM with legume species (9 levels: CNTR, HESCO, MEDSA, TRFRE, MEDLU, MEDPO, TRFSU, TRFIN, TRFRS) and experiment repetition, i.e. years (2 levels: CSY1 and CSY2) as fixed factors and sub-replicates nested into replicates (blocks) as random factor. In the subsequent sorghum, the total weed dry biomass was analysed separately for CSY1 and CSY2 using GLMM (with log link function for CSY2) assuming legume species, sorghum biomass and their interactions as fixed terms and sub-replications nested into blocks as a random term (model 7 and model 8 in Supplementary Material). Following the results of the analysis of variance, for significant explanatory variables, Sidak post-hoc test was performed to separate means ($p < 0.05$) using the R/'emmeans' package (Lenth et al., 2020). Normality and homogeneity of residuals variance have been studied respectively with Kolmogorov-Smirnoff and Levene test using R/'DHARMa' package (Hartig). To further investigate the interaction of sorghum biomass x previous legume species on weeds type in CSY2, the same models used for total weed biomass was ran for monocotyledonous and dicotyledonous weeds dry biomass and linear contrasts were employed to compare the trends (slopes) of each level of the interacting factors to zero. The resulting estimates of the differences in slopes were then tested for statistical significance ($p < 0.05$). These techniques were implemented using R/'emmeans' package (Lenth et al., 2020). The relationship between sorghum biomass production and N inputs from legumes and weed biomass in spring were analysed with a linear regression analysis for CSY1 and CSY2 (see model 12 in Supplementary Material). Statistical analyses of gross income for wheat (Gw), forage sorghum (Gs) and cumulative gross income (Gw+s) was performed using a Linear Mixed Model. The model was run with legume species and experiment repetition (CSY) as fixed effects and replication (blocks) as random effect. Data analysis was performed using R environment for statistical computing (R Core Team, 2020).

3. Results

3.1. Effect of legume living mulches on wheat grain protein content and wheat N uptake

The total wheat N uptake (biomass + grains) was significantly affected by the repetition of the experiment CSY ($p < 0.004$), slightly affected by the intercropped legumes ($p = 0.053$) but not by their interaction ($p = 0.508$) (Table 1). The mean effect of CSY1 and CSY2 show that the N uptake of wheat with *H. coronarium* (35.69 ± 5.95 kg N ha^{-1}) was significantly higher compared with *M. polymorpha* (24.65 ± 5.78 kg N ha^{-1}). However, compared with the control, legumes had no influence on the wheat N uptake whatever the legume species used in this experiment (Table 2). Wheat grain protein content was significantly affected by CSY ($p < 0.0004$) but not by legume species ($p = 0.74$) (Table 1). Grain protein content in CSY1 (15.4%) was significantly higher than CSY2 (12.7%) (Table 2). Relay intercropping of legumes did not have negative effects on grain yield of the co-cultivated durum wheat, whatever the legume species used in this experiment.

3.2. N input from legume biomass incorporation into the soil

Before legume termination and their biomass incorporation into the soil, N content (%) of legume dry biomass and their total N input (kg N ha^{-1}) were assessed. Legume nitrogen content ranged from 2.45% (*T. subterraneum* in CSY1) to 3.55% (*M. lupolina* in CSY1, see supp. Tab. SM4). Nitrogen input from legume biomass (kg N ha^{-1}) was significantly affected by legume species ($p < 0.0005$) and by the interaction between CSY and legume species ($p < 0.0001$) (Table 1). Nitrogen input from legume biomass residues varied considerably according to the legume species, seasonal conditions and their biomass production and it ranged from 1.22 to 182 kg N ha^{-1} (Fig. 2). During both CSY1 and CSY2, *H. coronarium* and *T. repens* (perennial) and *T. subterraneum* (annual self-seeding) had the highest nitrogen content and the nitrogen input from these species was respectively 182, 157 and 113 kg N ha^{-1} (Fig. 2). For other legume species, the different growth conditions in the field experienced in the two repetition of the experiment (CSY1 and CSY2) resulted in differences in nitrogen input related to the variation in biomass production of legumes among CSY1 and CSY2. Results on legume dry biomass production were detailed in Leoni et al. (2022). The nitrogen input of *M. sativa* was 43 and 91 kg N ha^{-1} respectively in CSY1 and CSY2, and N input of *M. lupulina* was 44 to 83 kg N ha^{-1} respectively (Fig. 2). Nitrogen inputs from *T. resupinatum* and *T. incarnatum* were respectively 74 kg N ha^{-1} and 149 kg N ha^{-1} during the CSY2 whereas it was only 1.97 kg N ha^{-1} and 1.22 kg N ha^{-1} in CSY1. (Fig. 2).

Table 1
Results of the analysis of variance for all studied variables: the degrees of freedom (d.f.), and associated probabilities of main effects and their interactions of dependent variables at different stages in the cropping system.

Stage of the crop sequence	Response variable	Source	d. f.	p-value
Wheat	N uptake	Leg. Species (Leg)	8	0.0531
		Exp. Repetition (CSY)	1	0.0041**
		Leg x CSY	8	0.5087 ^{ns}
	Grain protein content	Leg. Species (Leg)	8	0.7449 ^{ns}
		Exp. Repetition (CSY)	1	0.0004***
		Leg x CSY	8	0.4648 ^{ns}
Wheat-sorghum fallow period	N input (weeds)	Leg. Species (Leg)	8	< 0.0001***
		Exp. Repetition (CSY)	1	0.0363*
		Leg x CSY	8	< 0.001 * * *
	N input (legumes)	Leg. Species (Leg)	7	0.00059 * * *
		Exp. Repetition (CSY)	1	0.4449 ^{ns}
		Leg x CSY	7	< 0.001 * * *
	N input (total)	Leg. Species (Leg)	8	< 0.001 * * *
		Exp. Repetition (CSY)	1	0.4921 ^{ns}
		Leg x CSY	8	< 0.001 * * *
Forage sorghum	Biomass yield (sorghum)	Leg. Species (Leg)	8	< 0.001 * * *
		Exp. Repetition (CSY)	1	0.0016 * *
		Leg x CSY	8	< 0.001 * * *
	Biomass (weeds, CSY1)	Leg. Species (Leg)	8	0.0706 ^{ns}
		Sorghum biomass (S)	1	0.8369 ^{ns}
		Leg x S	8	0.0866 ^{ns}
	Biomass (weeds, CSY2) Link function: log	Leg. Species (Leg)	8	0.0044**
		Sorghum biomass (S)	1	0.2158 ^{ns}
		Leg x S	8	0.0105*
	N uptake (weeds)	Leg. Species (Leg)	8	0.8318 ^{ns}
		Exp. Repetition (CSY)	1	0.0815 ^{ns}
		Leg x CSY	8	0.4496 ^{ns}
	N uptake (sorghum)	Leg. Species (Leg)	8	< 0.001***
		Exp. Repetition (CSY)	1	0.6700
		Leg x CSY	8	0.0070**
Nitrogen Use Efficiency	NUE	Leg. Species (Leg)	7	< 0.001***
		Exp. Repetition (CSY)	1	0.2283 ^{ns}
		Leg x CSY	7	0.0054 * *

[*] indicates statistical significance at $p \leq 0.05$ level,* * at $p \leq 0.01$, * ** at $p \leq 0.001$. ns indicates $p > 0.05$.

Nitrogen content in weed dry biomass (%) ranged from 1.18 to 2.5% (see supplementary material SM4). Nitrogen input from weed biomass (kg N ha^{-1}) was significantly affected by legume species ($p < 0.0001$), CSY ($p = 0.03$) and their interaction ($p < 0.0001$) (Table 1). Nitrogen input from weed biomass was on average 30 and 14.6 kg N ha^{-1} respectively in CSY1 and CSY2 (Fig. 2). In CSY1, the N input from the weed biomass in plots with *M. polymorpha* (47.7 ± 5.90 kg N ha^{-1}) was significantly higher compared with *H. coronarium* (22.6 ± 3.0 kg N ha^{-1}), *M. sativa* (20.3 ± 3.4 kg N ha^{-1}), *T. resupinatum* (18.6 ± 7.4 kg N ha^{-1}) and *T. subterraneum* (25.0 ± 4.60 kg N ha^{-1}) (Fig. 2). However, compared with the control, presence of legumes had no influence on the N input deriving from the weed biomass whatever the legume species used in this experiment (Fig. 2). In CSY2 of the experiment the N input from weed biomass in the plots with *H. coronarium* (2.1 ± 2.0 kg N ha^{-1}), *M. sativa* (4.4 ± 1.9 kg N ha^{-1}), *T. repens* (1.9 ± 0.9 kg N ha^{-1}) and *T. subterraneum* (4.1 ± 1.7 kg N ha^{-1}) was lower compared with the

Table 2
Effect of relay intercropping of legumes on the wheat grain protein content and wheat N uptake. Values with the same letter are not significantly different at 0.05 confidence level. Data are pulled together across CSY1 and CSY2.

Code	Legume species	Wheat N uptake (kg N ha^{-1})		Grain protein content (%)	
		Mean	SE	Mean	SE
CNT	Wheat sole	33.22^{ab}	4.90	13.93^{a}	0.64
HESCO	*Hedysarum coronarium*	35.69^{b}	5.95	13.92^{a}	0.65
MEDLU	*Medicago lupulina*	27.38^{ab}	4.98	13.83^{a}	0.82
MEDSA	*Medicago sativa*	25.06^{ab}	3.46	13.75^{a}	0.48
TRFRE	*Trifolium repens*	28.79^{ab}	6.33	13.33^{a}	0.93
MEDPO	*Medicago polymorpha*	24.65^{a}	5.78	13.92^{a}	0.84
TRFSU	*Trifolium subterraneum*	30.60^{ab}	5.85	14.17^{a}	0.55
TRFIN	*Trifolium incarnatum*	29.63^{ab}	5.54	13.58^{a}	0.62
TRFRS	*Trifolium resupinatum*	29.86^{ab}	5.94	13.09^{a}	0.72

control (26.1 ± 1.3 kg N ha^{-1}) (Fig. 2). Considering the total legume and weed biomass per plot, the N input was significantly affected by legume species ($p < 0.0001$), and by the interaction between CSY and legume species ($p < 0.0001$) (Table 1). In both CSY1 and CSY2, *H. coronarium* (139 and 185 kg N ha^{-1} respectively in CSY1 and CSY2) and *T. subterraneum* (108 and 161 kg N ha^{-1} respectively in CSY1 and CSY2) had a higher N input compared with the control (Fig. 2). Total nitrogen input in plots with *M. polymorpha*, *M. sativa*, *T. incarnatum*, *T. repens* and *T. resupinatum* was higher compared with the control only during one repetition of the experiment.

3.3. Sorghum yield and weeds biomass

Overall, the linear regression analysis revealed a significant positive relationship between sorghum dry biomass production and the N input from legume biomass residues (data pooled among legume species) in both replications, CSY1 and CSY2. The slope of the regression line was significantly different from zero in CSY1: $y = 2.45 + 0.057\,x$ ($R^2 = 0.49$; $p < 0.0001$) and CSY2: $y = 8.18 + 0.034\,x$ ($R^2 = 0.21$; $p < 0.0001$). Analysis on the specific effects of each legume showed that the sorghum dry biomass was significantly affected by previous legume species ($p < 0.0001$), CSY ($p < 0.0016$) and their interaction ($p < 0.0001$) (Table 1). Sorghum dry biomass production in CSY1 (5.52 ± 0.63 t ha^{-1}) was lower than CSY2 (11,68 ± 0.98 t ha^{-1}) due to the lower sorghum emergence. Sorghum dry biomass production in control plots was 2.06 ± 0.18 t ha^{-1} and 5.91 ± 0.35 t ha^{-1} respectively in CSY1 and CSY2 (Fig. 3). In CSY1, sorghum preceded by *H. coronarium*, *T. subterraneum*, *M. polymorpha*, *T. repens* and *M. sativa* produced a higher dry biomass compared to the control (respectively +457%, +437%, +403%, +363% and +291%) (Fig. 3). Other legumes such as *M. lupulina*, *T. resupinatum* and *T. incarnatum* induced no effect on the sorghum biomass production (Fig. 3). In the CSY2, there was no difference in sorghum dry biomass production between the two treatments previously covered with *T. resupinatum* and *T. incarnatum* in comparison with the control (Fig. 3). Sorghum preceded by *H. coronarium* and *T. repens* produced a higher dry biomass than the control (respectively +291% and +272%) (Fig. 3). Moreover, *T. subterraneum*, *M. sativa*, *M. lupulina* and *T. resupinatum* treatments increased the sorghum biomass by 120%, 114%, 101% and 90% in comparison with the control (Fig. 3).

Perennial weeds such as *Convolvulus arvensis*, *Cynodon dactylon*, *Cyperus rotundus* and *Cirsium arvensis* were the dominant components in the weed community in the experimental field used during the first replication of the experiment (CSY1). In this case, during the sorghum cultivation the weed biomass was slightly affected by the previous legume species ($p = 0.070$) and by the interaction between legume species and sorghum biomass ($p = 0.086$) (Table 1). In the experimental field used in the second repetition 2018–20 (CSY2), the weed community composition was more diversified, and it was dominated by annual weeds. Among the annual weeds, *Chenopodium album*, *Amaranthus retroflexus*, *Datura stramonium* and *Lolium* spp. were the most representative species. In CSY2 the weed biomass was significantly affected by previous legume species ($p = 0.004$) and the interaction with the sorghum biomass ($p = 0.01$) (Table 1). In particular, sorghum preceded by *M. sativa* (46.36 ± 17.04 g m^{-2}) had a significantly lower weed dry biomass compared with the control (117.8 ± 33.9 g m^{-2}) and compared with other legumes such as *T. subterraneum* (99.2 ± 20.1 g m^{-2}), *T. incarnatum* (106.2 ± 24.25 g m^{-2}), and *T. repens* (139.4 ± 39.28 g m^{-2}) (Fig. 3). In CSY2, the effect of sorghum biomass on monocotyledonous and dicotyledonous weeds changed according to the previous legume species. Dicotyledonous weed dry biomass in sorghum preceded by *T. resupinatum* and *T. incarnatum* was positively correlated with sorghum dry biomass whereas dicotyledonous weed dry biomass was negatively correlated with sorghum biomass in the control (Table 3). Monocotyledonous weed dry biomass was negatively correlated with sorghum biomass in sorghum preceded by *M. lupulina* and *T. repens* (Table 3).

3.4. N uptake of the subsequent sorghum

The nitrogen content in the sorghum biomass was significantly affected by the previous legume species ($p = 0.0004$), CSY ($p < 0.0001$) and their interaction ($p = 0.023$) (Table 1). The nitrogen content in CSY1 was higher than CSY2 (1.10 vs 0.75%) (Table 4). In CSY1 nitrogen content of sorghum preceded by *T. repens* and *M. sativa* was significantly higher compared with the control (1.50 and 1.41 vs 0.87%) whereas in CSY2 only *H. coronarium* had a higher N content compared with the control (0.98 vs 0.58%) (Table 4).

The sorghum uptake (kg N ha^{-1}) was significantly affected by the previous legume species ($p < 0.0001$), and by the interaction between CSY and legume species ($p = 0.007$) (Table 1). In the control, the N uptake of sorghum was 20.1 ± 2.6 kg N ha^{-1} in CSY1 and 34.2 ± 1.6 kg N ha^{-1} in CSY2 (Fig. 4). During both repetition of the experiment, the N uptake of sorghum preceded by *H. coronarium* (88 and 173 kg N ha^{-1} respectively in CSY1 and CSY2) and *T. subterraneum* (98 and 102 kg N ha^{-1} respectively in CSY1 and CSY2) was significantly higher compared with the control (Fig. 4). The N uptake of sorghum preceded by *M. sativa* and *T. repens* was significantly higher compared with the control in CSY2 but not in CSY1. During the sorghum cultivation, the nitrogen content in the weed biomass (%) was significantly affected by the previous legume species ($p < 0.0001$), CSY ($p = 0.0002$) and their interaction ($p < 0.0001$) (Table 1). In CSY1 nitrogen content of weeds preceded by *M. sativa*, *M. polymorpha*, *H. coronarium* and *T. repens* was significantly higher compared with the control (respectively 1.52, 1.62, 1,65 and 1,80 vs 1.38%) (Table 4). In CSY2 legumes significantly affected the N content of weed biomass in comparison with the control except for *M. polymorpha*. In CSY2 weeds preceded by *M. sativa*, *H. coronarium* and *T. repens* had the highest biomass N content (1.83%, 1.83% and 1.84%) (Table 4). Despite the effect of legumes on the N content, no significant differences were observed in terms of weed N uptake (kg N ha^{-1}) in the subsequent sorghum crop during CSY1 whatever the legume species used in this experiment (Fig. 4).

3.5. Nitrogen Use Efficiency (NUE)

For sorghum, the nitrogen use efficiency (NUE %) was significantly affected by the previous legume species ($p < 0.0001$) and by the interaction between legume species and CSY ($p = 0.005$) (Table 1). During both replications of the experiment (CSY1 and CSY2), sorghum preceded by *T. repens*, *M. sativa* and *H. coronarium* showed a nitrogen use efficiency between 50% and 90% that is commonly considered as a desirable range of NUE (Fig. 5). The net nitrogen uptake of sorghum on plots previously covered by these legumes was on average 64%, 62% and 57% of the total nitrogen inputs from legume biomass and weed residues (Fig. 5). Sorghum preceded by *T. subterraneum*, *M. lupulina* and

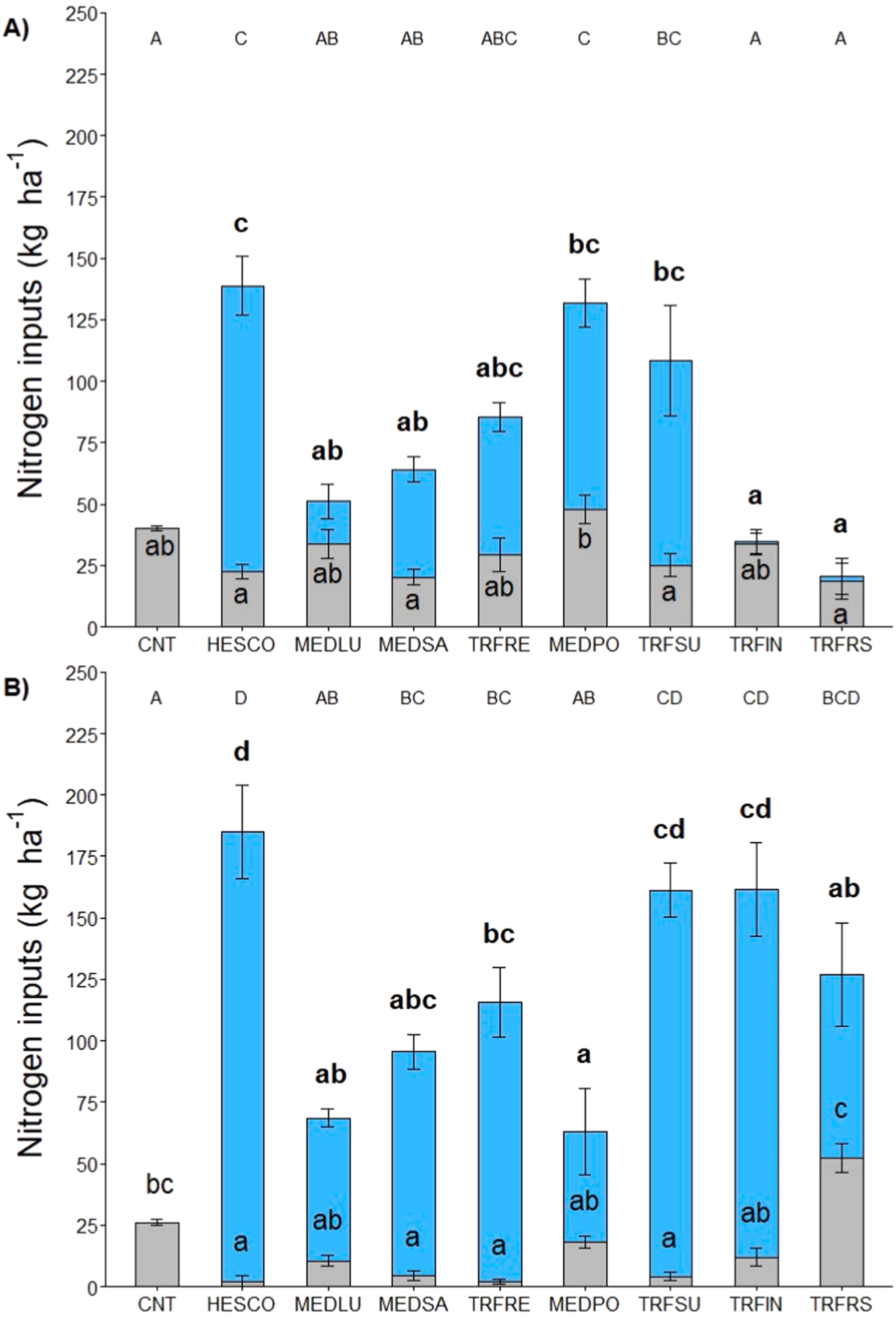

Fig. 2. Nitrogen inputs (N kg ha^{-1}) from the legume (blue bars) and weed biomass (grey bars) in the two replication of the experiment, CSY1 (A) and CSY2 (B). CNTR: Control plot (uncultivated after the sole wheat harvest); HESCO: *Hedysarum coronarium*; MEDSA: *Medicago sativa*; TRFRE: *Trifolium repens*; MEDLU: *Medicago lupulina*; MEDPO: *Medicago polymorpha*; TRFSU: *Trifolium subterraneum*; TRFIN: *Trifolium incarnatum*; TRFRS: *Trifolium resupinatum*. Values with the same letter are not significantly different at 0.05 confidence level. Data were analysed differently for legumes (bold letters) and weed biomass. Capital letters are referred to the total nitrogen input (legume and weed) Error bars represent standard error (SE).

M. polymorpha showed a desirable NUE only during one repetition of the experiment, whereas sorghum preceded by *T. incarnatum* and *T. resupinatum* showed very low nitrogen use efficiency (respectively 9% and 19.5%) (Fig. 5).

3.6. Economic viability of living mulches

The use of living mulches led to a higher total production cost that negatively affected the gross margin when only durum wheat yield and production costs are taken into consideration, despite the fact that grain yield was not affected by the presence of intercropped legumes. In the low-input management system, the total cost for grain production was 592 € ha^{-1} for the sole wheat treatment (see supplementary material SM2). The increased production cost for the relay intercropping system was due to the inter-seeding operation (116 € ha^{-1}) and the cost of legume seeds (variable according to market price). In particular, the cost

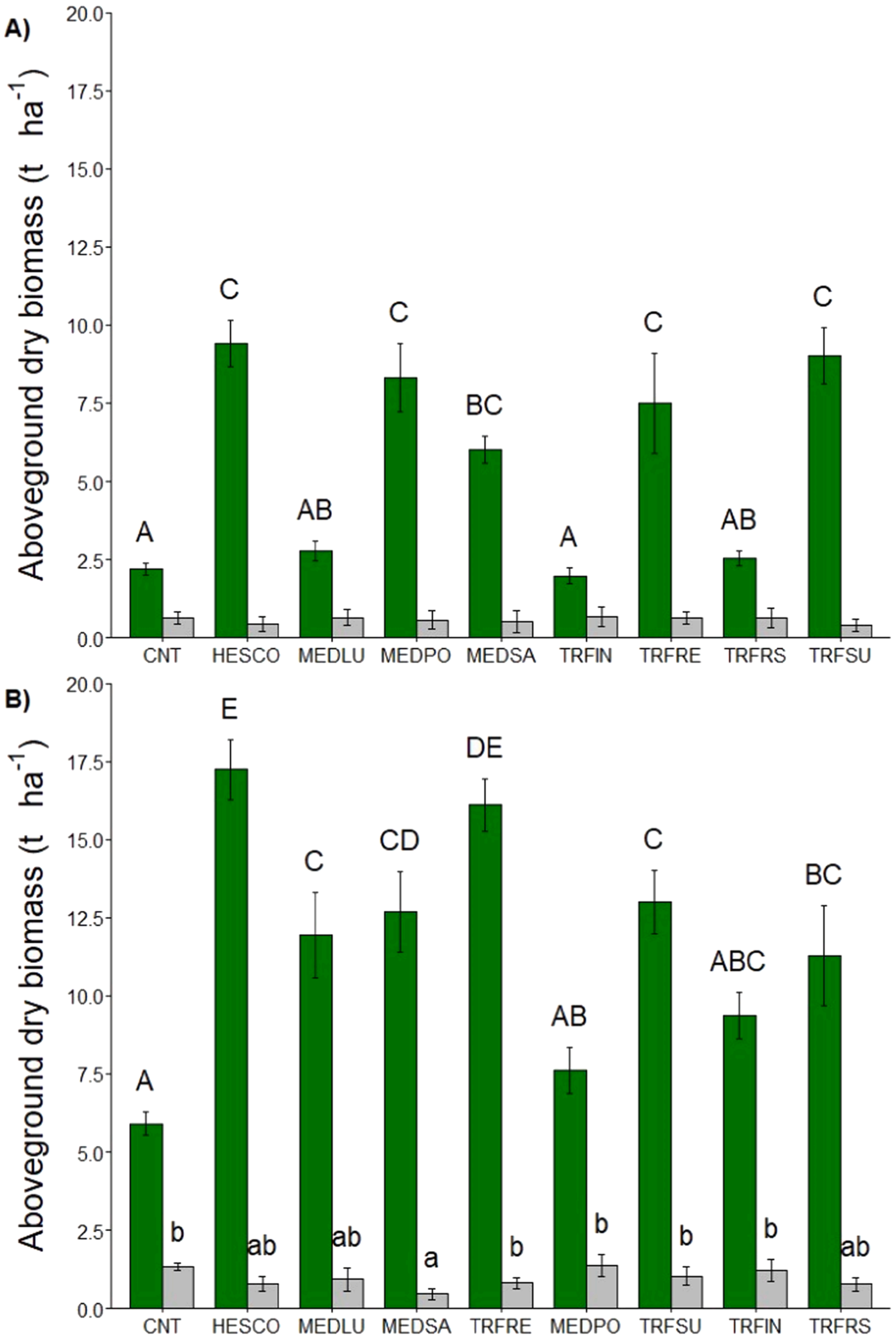

Fig. 3. Sorghum (green bars) and weed (grey bars) dry biomass at harvest time during CSY1 (A) and CSY2 (B). CNTR: Control plot (uncultivated after the sole wheat harvest); HESCO: *Hedysarum coronarium*; MEDSA: *Medicago sativa*; TRFRE: *Trifolium repens*; MEDLU: *Medicago lupulina*; MEDPO: *Medicago polymorpha*; TRFSU: *Trifolium subterraneum*; TRFIN: *Trifolium incarnatum*; TRFRS: *Trifolium resupinatum*. Values with the same letter are not significantly different at 0.05 confidence level. Error bars represent standard error (SE).

for inter-seeding changed according to the legume choice and, at the time of the experiment, it ranged from 156 € ha^{-1} for *T. resupinatum* to € ha^{-1} for *M. lupulina* and *M. polymorpha*. Therefore, the total production cost ranged from 748 € ha^{-1} to 988 € ha^{-1} (see supplementary material SM2). Relay intercropping impacted on the total production cost as follows: 20% for *T. resupinatum*, 24% for *M. sativa* and *T. repens*, 31% for *T. incarnatum*, 34% for *H. coronarium*, 37% for *T. subterraneum* and up to 40% for *M. lupulina* and *M. polymorpha*. During CSY1, the production level of wheat was extremely low due to unfavourable weather conditions (1.47 t ha^{-1}). For this reason, the cost exceeded the income for durum wheat and consequently the gross margin was negative even in the control treatment (−427 € ha^{-1}) (Table 5). Gross margin loss increased in the case of relay intercropping with legumes due to the higher production costs. Losses amounted to as much as 856, 893 and 826 € ha^{-1} for *M. lupulina*, *M. polymorpha* and *T. subterraneum* respectively (Table 5). In the second repetition of the experiment (CSY2), the

Table 3
Regression coefficients of sorghum dry biomass (g m^{-2}) preceded by different legume species on monocotyledonous, dicotyledonous and total weeds dry biomass (g m^{-2}).

Leg	Monocotyledonous		Dicotyledonous		Total weeds	
	Slope	SE	Slope	SE	Slope	SE
CNT	-0.00025 ^{ns}	0.00074	-0.00262*	0.00111	-0.00022 ^{ns}	0.00036
HESCO	+ 0.00063 ^{ns}	0.00065	+ 0.00050 ^{ns}	0.00069	+ 0.00053 ^{ns}	0.00040
MEDLU	-0.00141***	0.00035	-0.00030 ^{ns}	0.00056	-0.00116***	0.00026
MEDSA	-0.00065 ^{ns}	0.00044	+ 0.00032 ^{ns}	0.00059	-0.00042 ^{ns}	0.00030
TRFRE	-0.00139*	0.00069	-0.00123 ^{ns}	0.00096	-0.00134**	0.00047
MEDPO	-0.00135 ^{ns}	0.00079	+ 0.00014 ^{ns}	0.00099	-0.00096 ^{ns}	0.00057
TRFSU	+ 0.00006 ^{ns}	0.00059	-0.00036 ^{ns}	0.00055	-0.00015 ^{ns}	0.00038
TRFIN	-0.00093 ^{ns}	0.00074	+ 0.00188*	0.00095	-0.00034 ^{ns}	0.00049
TRFRS	+ 0.00012 ^{ns}	0.00038	+ 0.00155**	0.00056	+ 0.00034 ^{ns}	0.00028

CNTR: Control plot (uncultivated after the sole wheat harvest); *H. coronarium*; MEDSA: *M. sativa*; TRFRE: *T. repens*; MEDLU: *M. lupulina*; MEDPO: *M. polymorpha*; TRFSU: *T. subterraneum*; TRFIN: *T. incarnatum*; TRFRS: *T. resupinatum.* * indicates statistical significance at $p \leq 0.05$ level,* * at $p \leq 0.01$, * ** at $p \leq 0.001$.

Table 4
Nitrogen concentration (%) of forage-sorghum and its weeds during CSY1 and CSY2 experiment repetitions. Values with the same letter are not significantly different at 0.05 confidence level. Data are reported as mean ± standard error (SE).

Leg	CSY1				CSY2			
	N conc. sorghum (%)		N conc. weeds (%)		N conc. sorghum (%)		N conc. Weeds (%)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
CNT	0.87^{a}	0.09	1.38^{ab}	0.016	0.58^{a}	0.08	1.13^{a}	0.028
HESCO	0.98^{ab}	0.31	1.65^{d}	0.025	0.98^{b}	0.22	1.84^{d}	0.007
MEDLU	1.08^{ab}	0.31	1.44^{bc}	0.002	0.69^{ab}	0.14	1.51^{bc}	0.009
MEDSA	1.41^{bc}	0.09	1.52^{d}	0.072	0.89^{ab}	0.10	1.83^{d}	0.026
TRFRE	1.50^{c}	0.13	1.80^{e}	0.002	0.73^{ab}	0.07	1.83^{d}	0.015
MEDPO	1.11^{abc}	0.09	1.62^{c}	0.003	0.75^{ab}	0.07	1.13^{a}	0.019
TRFSU	1.08^{abc}	0.21	1.39^{b}	0.023	0.75^{ab}	0.15	1.59^{c}	0.058
TRFIN	0.91^{a}	0.03	1.38^{ab}	0.004	0.72^{ab}	0.16	1.50^{b}	0.014
TRFRS	0.99^{ab}	0.05	1.30^{a}	0.007	0.70^{ab}	0.12	1.46^{b}	0.086

CNTR: Control plot (sorghum sown after fallow period); HESCO: *H. coronarium*; MEDSA: *M. sativa*; TRFRE: *T. repens*; MEDLU: *M. lupulina*; MEDPO: *M. polymorpha*; TRFSU: *T. subterraneum*; TRFIN: *T. incarnatum*; TRFRS: *T. resupinatum.*

production level of durum wheat was higher than the average local production level (4.94 t ha^{-1}). For sole wheat, the gross margin was positive (682 € ha^{-1}) (Fig. 5). For the relay intercropping system, the gross margin was positive for all the intercropped legume species, however, the gross margins for *M. lupulina* (183 € ha^{-1}), *M. polymorpha* (291 € ha^{-1}) and *T. subterraneum* (386 € ha^{-1}) were significantly lower compared with the control (Table 5). In this relay intercropping system, the perennial and annual self-seeding legumes persisted in the field after the wheat harvest, until the sowing of the subsequent forage sorghum. In the second repetition also the annual legumes regrew from the seedbank. The production cost for sorghum was 661 € ha^{-1}. During the first repetition of the experiment, the production costs exceeded the income in the control, resulting in a negative gross margin (−413 € ha^{-1}) (Table 5). Legumes such as *H. coronarium*, *T. repens*, *T. subterraneum* and *M. sativa* supported the production level of forage sorghum resulting in a positive gross margin (respectively 433, 298, 229 and 85 € ha^{-1}) (Table 5). For sorghum preceded by *T. incarnatum*, *T. resupinatum*, *M. lupulina* and *M. polymorpha* the production increase provided by the legumes was not enough to cover the additional costs, causing a negative gross margin of – 234, – 126, – 87, – 35 € ha^{-1} respectively (Table 5). During the second repetition of the experiment, more suitable soil and weather conditions supported the beneficial effect of legumes on the subsequent sorghum. Forage sorghum preceded by *H. coronarium*, *T. repens*, *T. subterraneum* and *M. sativa* had a significantly higher biomass production compared to the control and the gross margin was positive (945, 845, 576 and 548 € ha^{-1} respectively) (Table 5). Gross margin for sorghum preceded by *M. lupulina*, *M. polymorpha* and *T. incarnatum* was positive but it was not significantly different from the control. The gross margin for the control was just below 0 (−42 € ha^{-1}) (Table 5). The overall economic assessment of durum wheat and forage sorghum showed that higher production costs of wheat due to the intercropping operation is balanced with the benefits provided by legumes in the subsequent forage sorghum for all legumes used in this experiment. In particular, *H. coronarium* and *T. repens* had the highest gross margin during the second repetition of the experiment (1381 and 1267 € ha^{-1} respectively) whereas these legumes guaranteed the lowest gross margin loss during the first repetition of the experiment, under unfavorable conditions (−274 and −324 € ha^{-1} respectively) (Table 5).

4. Discussion

In this experiment, environmental conditions varied between the two experimental repetitions (CSY1 and CSY2), particularly concerning the availability of water during the summer and early autumn seasons. In this stage precipitations are crucial for the establishment of legumes in the fallow winter period with a cascade effect on the subsequent cash crop yield benefits (Garba et al., 2022b). Specifically, cumulative precipitation from July to October was 88 mm in 2018 and 303 mm in 2019. These differing conditions had a significant impact on the behavior of legumes in the two experimental repetitions, especially affecting annual legumes like *T. incarnatum* and *T. resupinatum*. These conditions affected their growth and persistence in CSY1, resulting in minimal effects on the subsequent sorghum crop. For example, the nitrogen input from the biomass of these legumes was only 2 kg N ha^{-1} in the CSY1 but increased to 150 kg N ha^{-1} in the second year when better environmental condition occurred. Their differences were reflected in the subsequent forage sorghum production that was on average 2.25 t ha^{-1} in CSY1 and 10.33 in CSY2. Since *T. incarnatum* and *T. resupinatum* lack in specific characteristics for self-reseeding, the seeds they release on the soil surface during wheat harvest are more exposed to

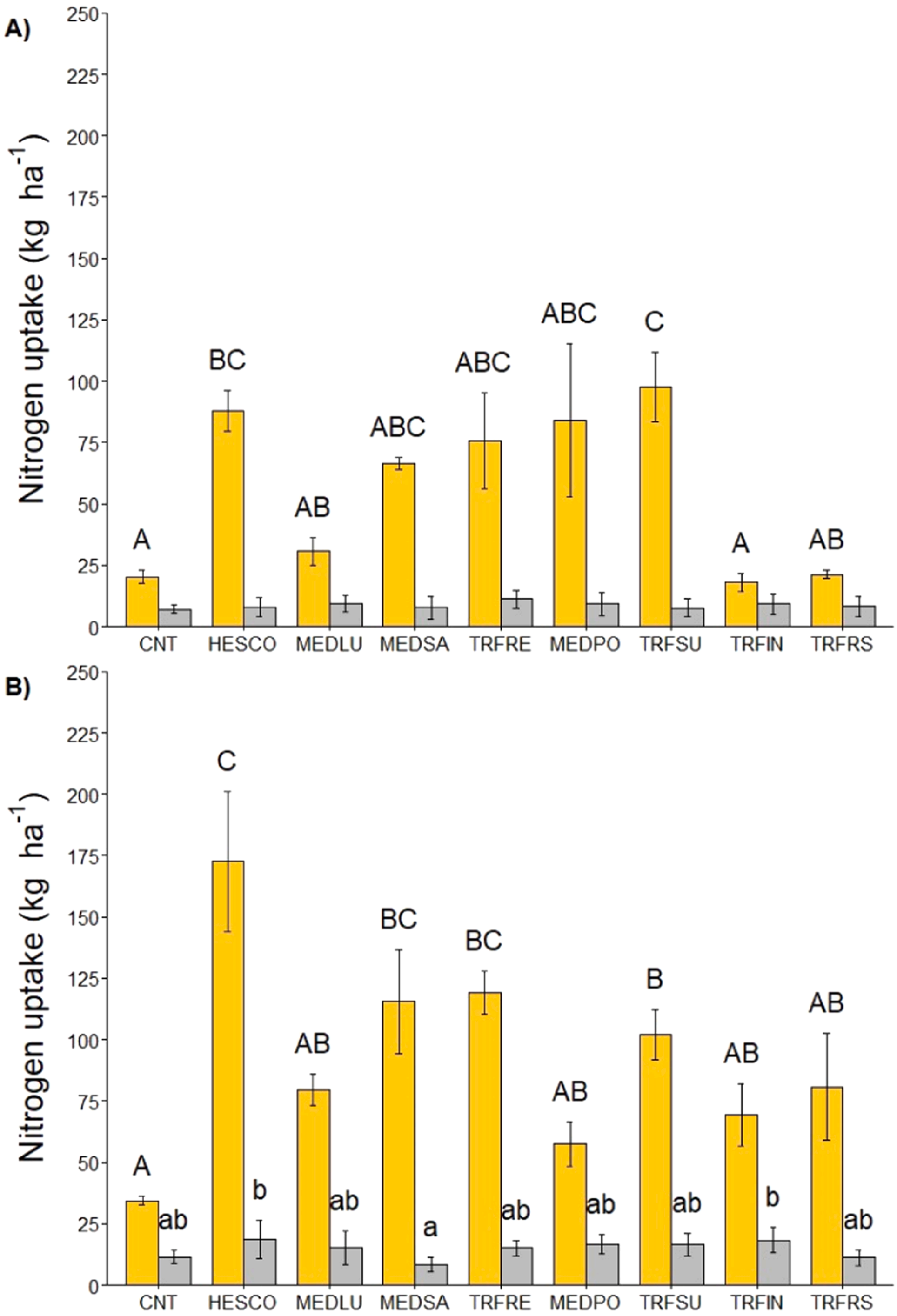

Fig. 4. Nitrogen uptake (N kg ha^{-1}) from sorghum (orange bars) and weed (grey bars) during the two repetition CSY1 (A) and CSY2 (B). CNTR: Control plot (uncultivated after the sole wheat harvest); HESCO: *Hedysarum coronarium*; MEDSA: *Medicago sativa*; TRFRE: *Trifolium repens*; MEDLU: *Medicago lupulina*; MEDPO: *Medicago polymorpha*; TRFSU: *Trifolium subterraneum*; TRFIN: *Trifolium incarnatum*; TRFRS: *Trifolium resupinatum*. Values with the same letter are not significantly different at 0.05 confidence level. Data were analysed differently for legumes (bold letters) and weed biomass. Capital letters are referred to the total nitrogen input (legume and weed) Error bars represent standard error (SE).

unfavorable environmental condition and this can compromise their vitality as probably was the case in CSY1.

In contrast to annual legumes, self-seeding legumes like *T. subterraneum* guaranteed a sufficiently high N input after their biomass incorporation and they were therefore able to support the biomass production of the subsequent sorghum crop in both CSY1 and CSY2, regardless of the differences in pedoclimatic conditions. This can be attributed to the ability of these species to bury their seeds directly into the soil in late spring and allowing a spontaneous regrowth in the subsequent autumn, which makes them less vulnerable to unfavorable environmental and climatic conditions and more resilient for this specific cropping system. Similarly, perennial legumes such as *H. coronarium*, *M. sativa*, and *T. repens* showed coherent results across both experimental repetitions. However, as already mentioned, in southern regions of Europe, the summer season can be excessively dry, preventing the successful establishment of perennial legumes after

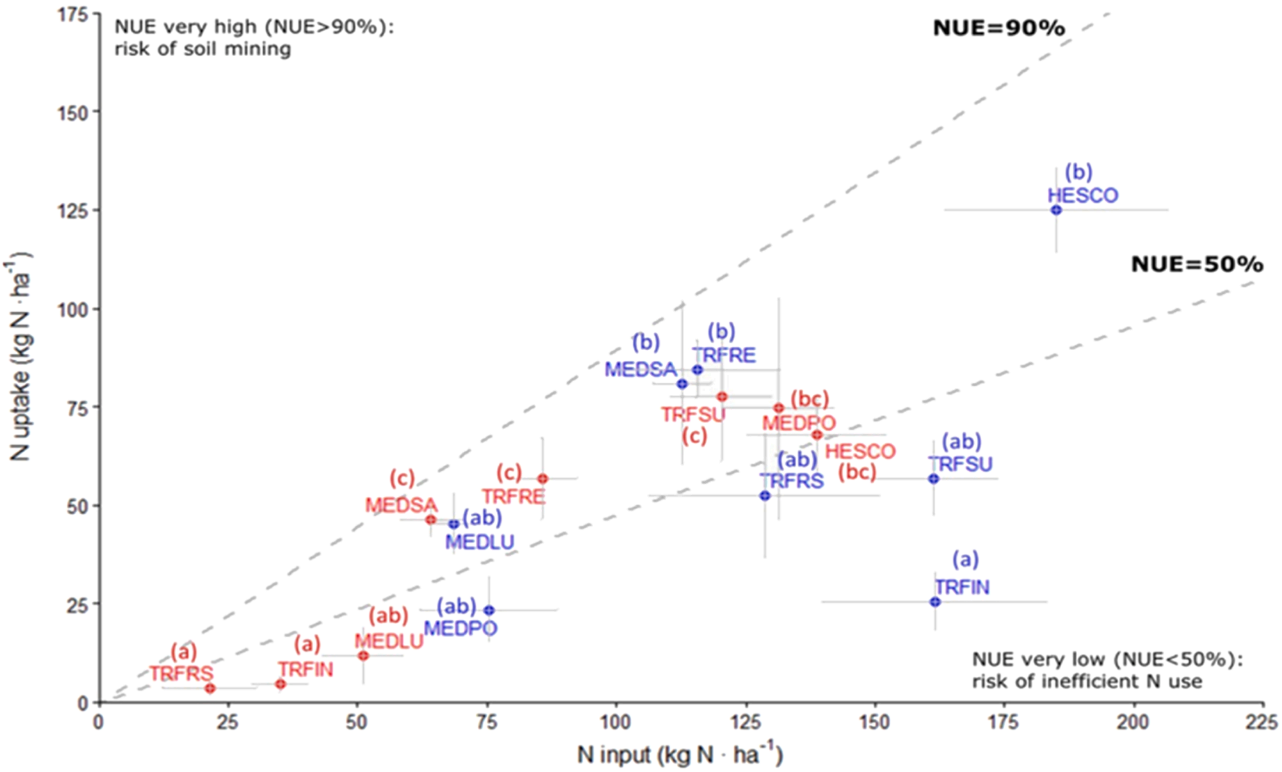

Fig. 5. The two-dimensional N input – N uptake diagram showing nitrogen use efficiency (NUE) for sorghum grown after incorporation of the biomass of varius legume species. The desirable range of NUE is between 50% and 90% (50% < NUE < 90%). For arable systems 50% and 90% were commonly used as lower and upper target values (50%) defining the desirable range of NUE (EU Nitrogen Expert Panel 2015). NUE (%) of sorghum in CSY1 (red) and CSY2 (blue). HESCO: *H. coronarium*; MEDSA: *M. sativa*; TRFRE: *T. repens*; MEDLU: *M. lupulina*; MEDPO: *M. polymorpha*; TRFSU: *T. subterraneum*; TRFIN: *T. incarnatum*; TRFRS: *T. resupinatum*. Values with the same color and letter are not significantly different at 0.05 confidence level. Error bars in horizontal (N input) and vertical (N uptake) directions represent standard error (SE).

Table 5

Gross Income (mean±SE) of durum wheat (GIw), forage sorghum (GIs) and cumulative gross margin (GIw+s) for CSY1 and CSY2. CNTR: Control plot (wheat sole stand crop); HESCO: *Hedysarum coronarium*; MEDSA: *Medicago sativa*; TRFRE: *Trifolium repens*; MEDLU: *Medicago lupulina*; MEDPO: *Medicago polymorpha*; TRFSU: *Trifolium subterraneum*; TRFIN: *Trifolium incarnatum*; TRFRS: *Trifolium resupinatum*. Different letters (a-e) indicate significant differences at the 0.05 level. GI do not include the Common Agricultural Policy (CAP) payment due to their variable rate in time and among countries.

Exp.	Leg	GI_W (€ ha^{-1})		GI_S (€ ha^{-1})		GI_{W+S} (€ ha^{-1})	
		Mean	SE	Mean	SE	Mean	SE
CSY1	CNT	-426.93^{d}	29.91	-413.86^{a}	22.58	-840.79^{a}	40.54
	HESCO	-707.31^{bc}	56.40	$+433.15^{e}$	77.86	-274.16^{c}	131.10
	MEDLU	-855.86^{a}	16.21	-87.04^{abc}	80.61	-942.91^{a}	94.57
	MEDSA	-630.29^{c}	42.27	$+85.65^{bcde}$	109.39	-928.21^{abc}	84.51
	TRFRE	-623.18^{c}	9.74	$+298.99^{de}$	106.32	-324.18^{bc}	114.44
	MEDPO	-893.01^{a}	11.76	-35.20^{bcd}	77.41	-928.21^{a}	84.51
	TRFSU	-834.77^{ab}	21.96	$+210.01^{cde}$	29.99	-624.75^{abc}	48.59
	TRFIN	-714.53^{bc}	20.38	-234.33^{ab}	36.19	-948.87^{a}	49.79
	TRFRS	-640.38^{c}	36.78	-126.71^{abc}	117.17	-767.09^{ab}	103.21
CSY2	CNT	$+681.94^{B}$	48.04	-42.07^{A}	30.28	$+647.57^{AB}$	82.74
	HESCO	$+433.08^{AB}$	56.20	$+945.21^{C}$	70.04	$+1381.46^{C}$	201.12
	MEDLU	$+291.64^{A}$	89.79	$+484.00^{ABC}$	160.39	$+752.18^{ABC}$	127.73
	MEDSA	$+433.45^{AB}$	39.17	$+548.35^{BC}$	179.40	$+981.93^{BC}$	167.89
	TRFRE	$+413.72^{AB}$	95.26	$+845.66^{C}$	40.02	$+1267.76^{BC}$	198.76
	MEDPO	$+172.47^{A}$	123.44	$+184.64^{AB}$	53.89	$+374.20^{A}$	90.77
	TRFSU	$+386.99^{AB}$	85.16	$+576.51^{BC}$	66.28	$+969.61^{BC}$	159.42
	TRFIN	$+404.83^{AB}$	78.69	$+259.96^{AB}$	73.23	$+662.36^{AB}$	113.02
	TRFRS	$+529.58^{AB}$	66.49	$+425.56^{ABC}$	241.72	$+940.53^{BC}$	144.97

wheat harvest (Porqueddu et al., 2016).

In low-input cropping systems, legumes represent a valuable internal source of nutrients thanks to their symbiotic N fixation capacity. Part of these nutrients can be made available to the contemporary cash crop (Corre-Hellou et al., 2006). Therefore, we investigated the effect of undersown legumes on N uptake and grain protein content in durum wheat. However, the results of our study showed that relay intercropping of legumes had no significant effect on wheat grain protein content but only on the subsequent cash crop. These results are consistent with previous studies on the effect of relay intercropped legumes on cereals that reported limited (Blackshaw et al., 2010a; Vrignon-Brenas et al., 2018) or even negative effects on grain protein content (Amossè et al., 2013a). In the case of simultaneous establishment of legume living mulch and wheat, i.e. contemporary intercropping, (Willey, 1979), the legume component is often developed enough to support additional N uptake of wheat (Rodriguez et al., 2001; Bedoussac et al., 2014; Pellegrini et al., 2021,). However, with contemporary intercropping the high biomass production of legumes can also compete with the wheat for space and limited resources or hinder the mechanical harvest operation with potential negative effects on grain production (Carof et al., 2007; Benincasa et al., 2012; Vrignon-Brenas et al., 2018). Delaying the sowing date of the legume living mulch (relay intercropping) is among the proposed solutions to limit the competition between the legume component and wheat in the intercropping system (Lamichhane et al., 2023). Albeit no immediate beneficial effect on durum wheat grain protein content was noticed, we strongly advocate the establishment of long-term studies able to measure the cumulative effect of repeated legume biomass incorporation also on the wheat crop in the successive crop rotation cycles.

Differently to what has been described for nitrogen, relay intercropping has been shown to increase the availability of other nutrients, such as phosphorus, immediately. A field experiment on relay intercropping of grain legumes, such as lentil, with durum wheat reported a phosphorus concentration increase in the relay intercropped wheat biomass compared with sole wheat (Koskey et al., 2022).

A good persistence of the legumes after the wheat harvest can determine a productive advantage to the subsequent crop avoiding the use of additional fertilizers (Bergkvist et al., 2011; Blackshaw et al., 2010b). Forage sorghum was then sown to evaluate the legacy effect of the biomass of each legume on the subsequent cash crop yield performance and weed community (Henry et al., 2010). Our results revealed that sorghum N uptake and biomass production were strongly correlated with the amount of N released from the decaying legume biomass. These observations are consistent with Bergkvist et al., 2011 who reported a significant increase of spring barley yield due to the residual effect of biomass of red clover and white clover undersown in the previous winter wheat. Results of our study showed that the production level of sorghum preceded by *H. coronarium*, *T. subterraneum*, *T. repens* and *M. sativa* had an up to five times higher biomass production compared with the control, ranging from 6.5 to 17.2 t ha^{-1} without any additional fertilizer and herbicide application. Notably, the forage sorghum preceded by these legumes had a comparable production level with the same sorghum type grown in conventionally managed systems in Italy (biomass production ranged from 13.5 to 15.4 t ha^{-1}) (Pannacci and Bartolini, 2016).

Although the production advantages of sorghum are mainly determined by the nitrogen input from legume and weed biomass in the previous spring, it is important to note that the N input alone does not fully explain the variability in the beneficial effects on sorghum productivity and nitrogen uptake. Indeed, also the quality of biomass from different legume species can modulate the positive effect on the subsequent sorghum crop (Hesterman et al., 1992). It is known that the mineralization and thus the availability of nutrients from legume biomass residues is highly variable, and depends on several factors, such as biomass composition, soil characteristics and climatic conditions, which determine differences in nitrogen use efficiency for the subsequent crop(s) (Fageria and Baligar, 2005). Therefore, there is a risk that timing and rate of mineralization of the relay intercropped legume cover crops is inadequate in satisfying the needs of the subsequent crop, leading to limited (Blackshaw et al., 2010b) or no advantage (Henry et al., 2010), at least in the immediately subsequent cash crop. On the long term the continuous accumulation of legume biomass and nitrogen may result in a more stable effect on both wheat and summer crops. In this study, the relationship between nitrogen input from legume biomass residues and the net N uptake of the subsequent summer crop showed that sorghum growing on plots previously covered by *H. coronarium*, *M. sativa*, *T. repens* had an optimal nitrogen use efficiency (50% < NUE < 90%). In this case the majority of nitrogen input from these legumes was immediately available for sorghum. For other legumes such as *T. incarnatum* and *T. subterraneum*, despite the high amount of nitrogen input (respectively 161.5 and 161.2 kg N ha^{-1}) in CSY2, the net uptake by the subsequent sorghum was significantly lower (respectively 25.5 and 56.6 kg N ha^{-1}) which corresponds to a NUE of 15% and 35%. We can therefore hypothesize that nitrogen input from *T. incarnatum* and *T. subterraneum* is only partially used by the subsequent sorghum and that these legumes may have a beneficial effect also on the following winter crop due to the slower release of nutrients. However, further studies such as the one by Angus et al. (2006) regarding the nitrogen release pathway following legume decomposition are needed. In order to optimize the use of nutrients released from legume residues, utilization of crop species efficient in absorption and utilization of N is an important strategy in improving NUE and maximizing the beneficial effect of legume residues (Fageria and Baligar, 2005). Differences in N uptake and utilization among crop species and cultivars for wheat, sorghum, corn, ryegrass, and soybean have been reported (Traore and Maranville, 1999). In particular, Pandey et al. (2001) reported that agronomic efficiency of N was higher in sorghum compared to pearl millet and corn.

Besides providing N, legume biomass residues can provide effective weed control in the subsequent cash crop and consequently improve NUE if managed properly (Fageria and Baligar, 2005). In our experiment weed control in sorghum preceded by intercropped legumes can be mainly attributed to increased competition of sorghum against weeds. Legumes that provided a high level of N input such as *H. coronarium*, *T. repens*, *T. subterraneum* and *M. sativa* significantly increased the sorghum biomass, and this supported the competition of sorghum for light, space, and soil resources on weeds. On the other hand, Hiltbrunner et al. (2007) reported that in case of low competition by the crop, weeds can take advantage from the nitrogen released by legume residues. In our study this happened with *T. incarnatum*, *M. polymorpha*, *T. resupinatum* and *M. lupulina*, species that slightly affected the sorghum biomass production while strongly promoting weed growth. In particular, *T. incarnatum* and *T. resupinatum* significantly increased the dry biomass of dicotyledonous weeds. Overall sorghum preceded by *M. sativa* showed the highest weed control capacity. Past studies on the residual effects of legumes biomass on weeds showed that *M. sativa* residues significantly decreased weed biomass after legume incorporation and that allelochemical compounds in the legume biomass can persist in the soil with relatively high concentrations up to one week after the biomass incorporation (Kruidhof et al., 2008; Carlsen et al., 2012). We speculate that the residual allelochemical effects of legumes can be involved in the weed control in the subsequent sorghum. However, it is important to address the question of whether these allelopathic compounds have any potential negative effects on the emergence and growth of the following crop. In our experiment there was no evidence showing any negative impact of legumes biomass residues on the establishment of sorghum. This could be attributed to the fact that the efficacy of allelochemicals is strongly linked to the seed size of the target plant, as larger seeds tend to exhibit greater resistance to the inhibitory effects of these compounds (Einhellig, 1994). Therefore, the utilization of a spring crop with a larger seed size, such as sorghum or maize, may facilitate a more selective inhibitory effect of allelochemicals on weeds, while minimizing any negative impacts on the subsequent crop.

The economic assessment conducted in our study reveals that relay intercropping reduced the profitability of the co-cultivated durum wheat due to the higher cost for inter-seeding but it maximized the gross income in a crop sequence if a suitable legume species was chosen. The expenses for seed purchases were the main cost item for intercropping and it varied according to the unit cost for seeds and the seed dosage of legumes. In general, the economic sustainability of living mulches is highly dependent on the local seed market for these legume species. The income gap due to intercropping expenses should be balanced through an increase in crop yield, or the provision of ecosystem services at crop rotation level i.e weed control or soil nutrient enrichment, in order to reduce the expenses for external inputs such as fertilizers and herbicides.

Indeed, the sowing of legumes is an investment from which we expect a return in the long term due to the cumulative effect of nitrogen rich legume biomass in the system, also the economic sustainability of relay intercropping should therefore be evaluated through the quantification of the ecosystem services provided by the legumes at crop sequence level. Ecosystem services provided by legume living mulches can limit the need for external inputs, reduce the total production cost and thus increase the cumulative gross income. A good persistence of the legumes after the wheat harvest determines a productive advantage to the subsequent crop without using additional fertilizer (Bergkvist et al., 2011; Blackshaw et al., 2010b). In the control, after the wheat harvest, no herbicide and fertilizer was used and only the biomass of spontaneous vegetation was incorporated into the soil before the sowing of subsequent sorghum. In this condition, the production costs of sorghum were higher compared to the income resulting in a negative gross income. Instead, the persistence of legumes after the wheat harvest and their biomass accumulation until the following spring supported productivity and economic income of the subsequent sorghum, as confirmed also in other studies (Bergkvist et al., 2011; Blackshaw et al., 2010b). The calculation of cumulative gross income (Table 5 and supplementary material SM3) reveals that the economic sustainability of relay intercropping can be achieved in a crop sequence only if suitable legumes are used. During the first repetition of the experiment (CSY1) unfavorable weather conditions reduced wheat and legume emergence with a negative impact on wheat yield and legume establishment. In the second growing season (CSY2), optimal growing conditions occurred, and grain yield was in line with the local production level. Despite the differences in climatic conditions during the two repetitions of the experiment, *H. coronarium* and *T. repens* had consistent performances and resulted as the best performing legumes from an economic point of view during both repetitions of the experiment. For these legumes, the costs of relay intercropping were balanced by the ecosystem services provided during the crop sequence. If on one side *H. coronarium* and *T. repens* had the highest gross margin during favorable growth conditions (respectively 1381 and 1267 € ha^{-1}), on the other side these legumes allowed to minimize economic losses during an unfavorable growing season (respectively −274 and −324 € ha^{-1}). The combined agro-economic evaluation of legumes for relay intercropping system in this study revealed that *H. coronarium* and *T. repens* can ensure, in the agro-environmental conditions of the experiment, both the highest cumulative margin and best agronomic performance resulting in the most suitable legume for the local environmental condition. However, the suitability of legumes can change according to the specific soil and climate conditions and therefore, the choice of living mulches at species and cultivar level need to be fine-tuned according to the local context. Another interesting option to be tested in the future is the use of mixtures between perennial and annual self-seeding legumes for stabilizing living mulch performance under different climate and pedoclimatic conditions.

The findings of this experiment emphasize the significance of choosing suitable species for this system. Perennial legumes and some of the self-seeding legumes, such as *T. subterraneum*, exhibit greater adaptability to fluctuating environmental conditions, making them more attractive to farmers interested in using living mulches. However, given the complexity of these cropping systems and the numerous variables that can influence their successful implementation, farmers should be supported through participatory and interactive approaches when selecting the components of the living mulch system (Leoni et al., 2023).

In this regard, a serious game and decision support systems (MERCI) has recently been developed respectively by Meunier et al. (2022) and Constantin et al., (2023) to support farmers' exploration of intercrops and cover crops by designing a wide-range of cereal and legumes for intercropping and cover cropping in given cropping system contexts and assessing the most relevant ecosystem services provided by intercrops, i.e., cereal and legume yields, cereal protein content, nitrogen supply to the following crop, impact on soil structure and weed, insect and disease control.

The current study presented relevant results on agronomic and economic performance of eight legume species used as living mulches with durum wheat assessing their ecosystem services provided at crop rotation level. These results can be used to support the design and implementation of locally adapted legumes for living mulches in Mediterranean cereal-based cropping systems through a participatory approach, like the one proposed by Meunier et al. (2022) and Constantin et al., (2023) . At the same time, there may be practical barriers for the uptake of cropping system diversification by farmers. These include commercial barriers due to the lack of a market for species that are suitable for use as living mulches, technical barriers due to the lack of adapted farm machinery, economical barriers to the cost of seeds of legume crops.

5. Conclusion

The current study highlights the importance of suitable legume selection for living mulches to support N uptake and weed control at crop sequence level. Legume living mulches did not affect N uptake and grain protein content in the co-cultivated wheat but according to the legume species used, a positive but variable effect on the subsequent cash crop was demonstrated. *Hedysarum coronarium*, *M. sativa, T. repens* and *T. subterraneum* were assessed as the most promising legumes for living mulching in low input cereal-based cropping systems because they were able to optimize the production of the next sorghum crop. The only legume that was able to reduce weed biomass in the subsequent sorghum crop was *M. sativa*. Although no significant effects on weed biomass was detected, the other legumes affected monocotyledonous and dicotyledonous weed species differently. From the economic point of view *H. coronarium* and *T. repens* maximized the cumulative gross income in the wheat-sorghum crop sequence under low-input management conditions. For these legumes, lower profitability of wheat due to the intercropping expenses are balanced by their beneficial effects on sorghum biomass production without the use of additional chemical inputs. For the local pedo-climatic conditions the combined agronomic and economic evaluation suggests that *H. coronarium* and *T. repens* are the most suitable species.

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CRediT authorship contribution statement

Leoni Federico: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lazzaro Mariateresa:** Supervision, Methodology, Investigation, Conceptualization. **Carlesi Stefano:** Supervision, Methodology, Investigation, Conceptualization. **Moonen Anna Camilla:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2023.109246.

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