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Nitrogen uptake in lentil cultivar mixtures is not predictable from pure stands performance but is correlated with agronomic parameters and experimental conditions

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Abstract

Background In the context of rising costs of raw materials and environmental degradation caused by livestock farming, the agri-food sector faces significant challenges in sourcing sustainable proteins. Grain legumes have emerged as cost-effective protein sources, with lower water footprint and GHG emissions compared to animal sources. However, their cultivation is threatened by strong yield fluctuations. Leveraging intra-specific diversity through cultivar mixtures in cropping systems can effectively buffer biotic and abiotic stresses, hence increasing yield stability. In this study, we investigate the effect of intra-specific diversity on lentil nitrogen uptake under pot (2020) and field conditions (2021). We hypothesize that cultivars with higher affinity for nitrogen fixation influence the other components of the mixture, and that nitrogen uptake dynamics are a possible driver in modulating cultivar mixture behaviour. We designed two-, three-, and four-cultivar mixtures with a trait-blind approach and compared them to sole cultivars.

Results and conclusions Our results show inconsistencies across the two experimental years, indicating that lentils may shift their nitrogen source from the atmosphere to the soil when grown in pots. Mixtures ^{15}N enrichment was not always consistent with pure stand performance, suggesting that cultivar mixtures may have an unpredictable cumulative effect on nitrogen uptake. Regarding correlations with agronomic parameters, we observed a significant correlation between nodules number and nitrogen concentration, regardless of experimental conditions. Finally, we found that ^{15}N excess emerged as a significant predictor for pure stands' yield, but the differences were diluted with the increase in diversity levels. The findings on ^{15}N enrichment responses, cultivar impacts, and complex mixture effects on soil microbiota underscore the need for further research.

Keywords Agroecology, Functional agrobiodiversity, Resource use complementarity, Grain legumes, Pulses, Isotope dilution, Cultivar mixtures design, Ecological services, Underlying mixture mechanisms

Background

The seek for sustainable sources of proteins is amongst the main challenges of the agri-food sector, as the livestock sector is confronted with the increasing costs of raw materials and environmental degradation [1, 2]. Grain legumes are a cost-effective protein source for humans: overall they contain around 25% of proteins and have a lower water footprint and GHG emission compared to equivalent proteins from animal sources [3, 4].

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Moreover, thanks to nitrogen fixation, pulses favour the growth of subsequent crops in the rotation and minimize the overall use of synthetic N fertilizers [5]. Nevertheless, grain legumes still represent a minor sector both in Italy (1.3% of total agricultural area) and Europe (1.5%) probably due to the lack of suitable genetic material and the subsequent unreliable agronomic performances, together with low profitability [1, 4, 6, 7].

Within grain legumes, lentil (*Lens culinaris* Medik) is a founder crop in the Italian and Mediterranean food culture [7], however, in Italy its production has been significantly decreasing until the beginning of the millennium [8]. Currently, only 2% of national consumption is domestically produced [9]. Boosting the consumption and the production of lentils in Italy would, therefore, benefit both the agroecosystem and the autonomy in protein production.

The experiment reported here is part of a larger inquiry on biodiversity-based practices, known for their positive impact on production and environment [10]. Leveraging crop diversity in agriculture may occur at different levels and scales, e.g., at the level of species diversity, the diversity of species in an agroecosystem, or at the level of genetic diversity, the diversity of cultivars within a given cropped species. Genetic diversity, or intra-specific diversity, can be increased through breeding for multi-genomic mixtures: i.e., a certain number of cultivars grown together on the same field [11]. The implementation of intra-specific diversity in cropping systems has the potential to positively affect ecosystems services and productivity, with minimal technical disadvantages [12]. There are evidences for mixtures' advantages in term of pathogens control [13–15] and increase of yield amount, stability and quality [16, 17].

The mechanisms underlying such effects have not been fully unveiled, but the intrinsic functioning can be related to well-known ecological theories: niche differentiation and functional complementarity [5, 18, 19].

In this study, the target service consists in the crop's ability to fix atmospheric nitrogen through the association with rhizobia: lentils with higher N fixation indeed are expected to contain more proteins, as well as to have higher yield and growth (thus requiring less inputs), finally to leave more nutrients in the soil for the subsequent crop [5, 20–24].

It has already been proven how crop variety represents a major determinant for nitrogen uptake [2, 5, 21, 25], but studies on the effect of cultivar mixtures on nitrogen uptake are lacking. Still, cultivar mixtures effects on soil microbial diversity were observed [26–28] and may be the underlying reason for certain mixtures behaviour that yet did not find an explanation.

The aim of this study is to investigate the effect of intra-specific diversity on lentil nitrogen uptake both under pot and field conditions. We expect that interactions between cultivars when grown in mixtures may influence nitrogen parameters, and that nitrogen parameters may correlate with the effects highlighted in Lorenzetti et al. study [29]: i.e., grain production, total biomass and nodules number. In their study indeed, Lorenzetti et al. [29] proposed that nitrogen dynamics might clarify the behaviour of lentil cultivar mixtures, urging further investigation in this direction. Consequently, we aim to explore nitrogen uptake as a potential catalyst in modulating the dynamics of cultivar mixtures, contributing to a more comprehensive understanding of intraspecific diversity for sustainable protein production. The uptake of ^{15}N was investigated to study lentil N acquisition at the peak of N fixation [30].

Materials and methods

Soil, plants and growing conditions

An experiment was conducted in 2020 and 2021 at San Piero a Grado (43.6628N, 10.3485E), ca. 9 km SW of Pisa, Central Italy.

San Piero a Grado is part of the Mediterranean zone, characterized by mild, relatively rainy winters and hot, dry summers. Annual precipitations reach 910 mm, ranging from 25 mm in the driest month (July) to 145 mm in the wettest (November), with an irregular pattern throughout the year (autumn being the rainiest season).

The soil was sandy, with a composition of 795 g/kg of sand, 129 g/kg of silt and 76 g/kg of clay. The soil had a pH (water)of 7.5, 2% Organic Matter, 1.4 g/kg total nitrogen, C/N ratio of 8.9, 29.1 mg/kg of P2O5 (Olsen method) and 91.5 mg/kg K (BaCl2).

In 2020 the experiment was conducted in pots placed outdoors, filled with the topsoil (0–0.3 m) taken from the experimental field. In 2021, the experiment was repeated in field conditions instead of pots, following an adaptive approach [31] since in 2020 nitrogen enrichment values were not in line with literature. We therefore allocated the experiment in a dedicated section of the plot from Lorenzetti et al. [29] trial, we carried out separate destructive samplings in designated areas corresponding to each experiment within the plot. This approach facilitated the examination of additional parameters within an experimental framework that aligned with the testing of our hypotheses.

In 2020 the experiment started on the 5th of May and finished on the 22nd of July. During that time, we registered a total precipitation of 276 mm; the average daily maximum temperature was 22.6 °C, while the average minimum temperature was 9 °C (Fig. 1).

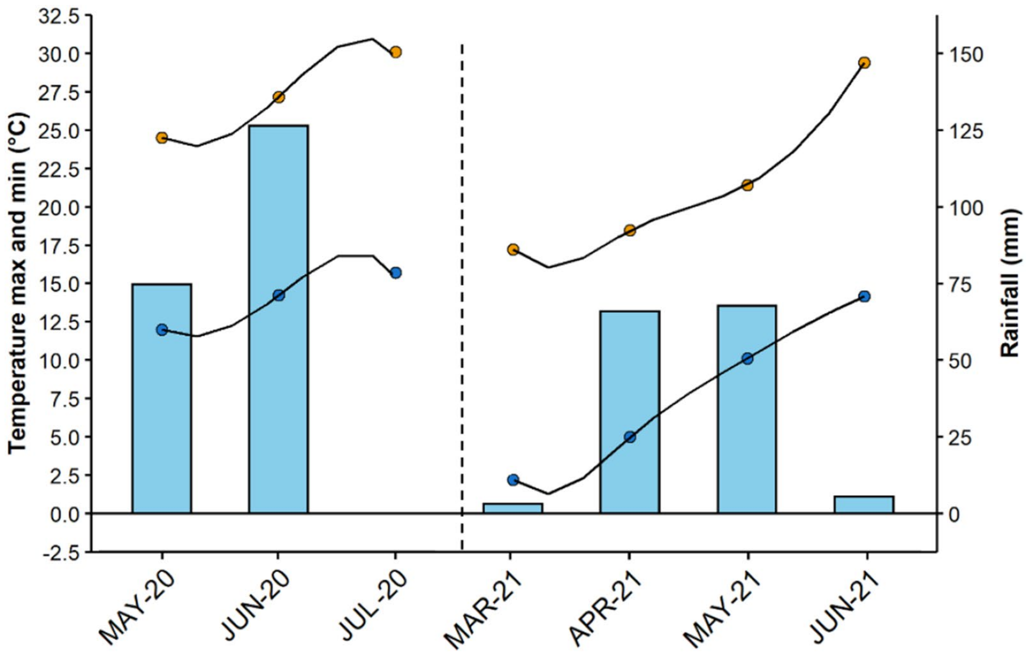

Fig. 1 Seasonal climate in San Piero a Grado during the experimental years, from May to July 2020 and from March to June 2021. On the left *y* axis, the maximum (top lines) and minimum (bottom lines) monthly average temperatures, on the right *y* axis the monthly average rainfall (bars)

In 2021 the trial was sown on the 25th of February and harvest took place on the 6th of July. During the study period we registered a total precipitation of 142 mm; the average daily maximum temperature was 21.8 °C, while the average minimum temperature was 8 °C (Fig. 1).

Cultivar mixtures design

The framework of this study is a wider inquiry into lentils' cultivar mixtures dynamics: the present work is a follow-up to a previous study [29] from which it derives methodology and experimental design.

Four commercial lentil cultivars were chosen to compose the mixtures. The Italian seed market experiences a lack of diverse genetic material, which is consequence of a lack of breeding programmes and dedicated research for grain legumes compared to cereals [6, 32]. Therefore, we had limited ground for designing cultivar mixtures and opted for a trait-blind approach [12]. The main criteria for cultivar choice were (i) availability on the market, (ii) seed colour and (iii) seed dimension: cultivars with smaller seeds (microsperm) are better appreciated on the market and are less subject to damage during harvest. Within microsperm type, we selected cultivars with different seed weights, as seed dimension is known to affect nitrogen uptake through the regulation of nodules number [33], and we opted for including the highest seed colour diversity, being colour genes possibly linked to nodulation genes, as it is the case in chickpea [34]. The material was provided by the partner farm in Tuscany (cv. *Robin* and cv. *Screziata*, respectively, with brown and dotted brown seeds) and by the Apulian branch of an agricultural consultancy firm (cv. *Turca* and cv. *Nera*, respectively, with dotted red and black coated seeds).

The four lentil cultivars were assembled in 16 treatments, representing all the possible combinations between cultivar pairs and triplets, plus the quartet and the sole cultivars (Table 1). A plot in each block was left unseeded as a control for checking weed development.

Experimental design

The trial was arranged in a randomized block design with four replicates each year.

In 2020 the trial was conducted in pots, to increase the control of environmental effects. During winter the pots were filled with topsoil from the field trial: pots' surface measured 30 × 30 cm, while the soil was filled up to 30 cm height. Seeds were hand seeded as 18 seeds per row, with a row spacing of 12 cm (3 rows per pot).

In 2021, a few weeks before sowing the soil was ploughed at 25 cm depth, then the seedbed was prepared with a rotary harrow at 10 cm depth. Mixtures and sole cultivars were established in 6 × 1.5 m plots by sowing 360 germinable lentil seeds m^{-2} with a row spacing of 18 cm (plot seeder from Wintersteiger, OYJORD model).

The seed proportion of each lentil cultivar in mixtures was 0.5 in the two-variety mixtures, 0.33 in the

Table 1 Increasing levels of diversity within lentils mixture combinations

Baseline: Pure stand cultivars	First level: 2 cultivar mixture	Second level: 3 cultivar mixture	Third level: 4 cultivar mixture
Robin (Ro)	Robin + Screziata (RoSc)	Robin + Screziata + Turca (RoScTu)	Robin + Screziata + Turca + Nera (RoScTuNe)
Screziata (Sc)	Robin + Turca (RoTu)	Robin + Screziata + Nera (RoScNe)	
Turca (Tu)	Robin + Nera (RoNe)	Robin + Turca + Nera (RoTuNe)	
Nera (Ne)	Screziata + Turca (ScTu)	Screziata + Turca + Nera (ScTuNe)	
	Screziata + Nera (ScNe)		
	Turca + Nera (TuNe)		

three-variety mixtures and 0.25 in the four-variety mixtures.

Isotope dilution method was identified as the most appropriate technique for assessing the subtle variations in nitrogen uptake across lentil cultivars [35]. Although the distribution of ^{15}N fertilizers may be irregular within the root system [36], the lentil cultivars under study exhibit minimal morphological variations, thereby negating the need to account for such an effect.

Before flowering stage (on the 22nd of May in 2020, on 21st of April in 2021) the pots, and an equivalent portion of plots (30 × 30 cm) were irrigated with 500 mL of water into which 0.47 g of ammonium sulphate salts containing 99 atom. percent ^{15}N had been diluted (corresponding to 11.6 kg ha^{-1} of N). In 2020, the same amount of ^{15}N enriched fertilizer was applied to one pot containing a non-fixing control (*Panicum miliaceum*) in each block. The solution was evenly distributed with a dedicated tool. The surface was kept clean from weeds during the cropping season to avoid the uptake of enriched N from species other than the target ones. No other fertilizers were applied. The choice of the growth stage for ^{15}N application was meant at obtaining a sufficient root mass for minimising the losses of fertiliser and at describing differences in N uptake when most likely occurring, i.e., at the peak of N fixation and plant N demand (from before flowering to pod filling stage) [30].

In 2020, the pots received drip irrigation until harvest. In 2021, irrigation was provided only when rain was lacking in the 3 weeks after sowing to ensure germination.

Sampling procedure and analysis

In 2020 sampling took place at harvest stage on the 22nd of July.

In 2021 sampling occurred at pod filling stage, before plants senescence, on the 6th of July.

In both years, all the 30 × 30 surface was harvested with a sickle. Lentil biomass was oven-dried at 40 °C until constant weight. Subsequently, biomass was finely ground and a subsample placed into tin capsules (~ 5 mg). Due to the impossibility to identify individual cultivars within the mixtures (no phenological differences), analysis refers to the whole mixture.

The sampling provided data on lentil total biomass (in 2021), N content (g m^{-2}), N concentration (%) and ^{15}N enrichment, expressed as atomic percent excess (^{15}N excess): i.e., the abundance of ^{15}N in a labelled sample minus the abundance of ^{15}N in nature (i.e., 0.366%, from Unkovich et al. [35]. ^{15}N excess is thus the expression of the nitrogen share derived from soil [35]. ^{15}N excess was measured using an Isoprime isotope ratio mass spectrometer (Isoprime Ltd.), connected to a Eurovector CN elemental analyser (Eurovector) that provided information on N content and concentration.

Data on lentil yield production (g m^{-2}) and nodules number come from the related study by Lorenzetti et al. [29].

Statistical analysis

Residual analyses were carried out utilizing the *DHARMa* R package [37] to determine the most suitable distribution for each variable. In instances where the variable distribution deviated from normality as per the Kolmogorov–Smirnov test, various link functions were assessed. If none of the tested link functions adequately fit the data, alternative distributions (primarily Gaussian and Poisson) were explored.

Concerning total nitrogen and 2021 ^{15}N excess, data were analyzed with a General Linear Model (GLM) with a Gamma distribution (with a logarithmic link function) to study the effect of treatment.

N concentration in 2020 and 2021, and ^{15}N excess in 2020 best fitted to a linear mixed model framework (gaussian distribution), thus a Restricted Maximum Likelihood (REML) procedure and Satterthwaite's method for the t-test were used to analyze the model performance. Treatment was set as a fixed term, while Block was set as random term.

Treatment means were compared with the Bonferroni post-hoc test using the R package *emmeans*, version 1.7.5 [38]. Treatments were divided in levels of diversity for the post-hoc test to identify significant differences within each level: the third level of diversity, consisting in the mixture of four cultivars, was not included in the post-hoc comprising of one single treatment. Orthogonal contrasts were built to compare the performance of each mixture with that of its components. This procedure allowed us to study the interaction effect between cultivars: when the contrast highlights better results for mixtures components compared to pure stands, it indicates a synergistic effect among lentil cultivars promoted by the mixture, otherwise it indicates an antagonistic effect. Contrasts were performed with *emmeans* package (Bonferroni based method). Orthogonal contrasts were built within the whole three levels of diversity: two, three and four cultivar mixtures.

Correlations of the results of the current study (N concentration, N pool, ^{15}N excess) and those of the joint study from Lorenzetti et al. [29] (yield, nodule number and biomass) were performed by comparing a baseline model with an improved model though ANOVA. The baseline model consisted of a linear mixed model with a dependent variable, an independent variable and block as a random term, while the improved model sees the addition of a second independent variable and the interaction terms between the two independent variables. We studied nitrogen content (g), nitrogen concentration (%) and yield as dependent variables, treatment, biomass, ^{15}N excess, nodules and nitrogen concentration as independent variables.

All data analyses were performed in R for Windows, version 4.0.3 [39]. Data visualization was done using the R packages ggplot2, version 3.3.5 [40]. When applicable, interactions were studied with the emtrends function in emmeans package. In addition to significant results (alpha < 0.05), results showing a p comprises between 0.05 and 0.1 were taken into account and discussed to fully exploit the potentials of the study and obtain suggestions for further research.

Results

Nitrogen concentration

The average N concentration (%) in lentil biomass in the pot experiment in 2020 and in the field experiment in 2021 was, respectively, 2.3% and 2.4%, with relatively low differences in spite of the different experimental conditions.

Treatment effect on nitrogen concentration in 2020 was not significant, but it fell within the $\alpha < 0.1$ threshold ($p = 0.08$, $\chi^2 = 22.05$) while it was significant in 2021 ($p = 0.04$, $\chi^2 = 24.9$).

In the pot experiment (2020) nitrogen concentration varied significantly among pure stands, where cv. *Nera* and cv. *Screziata* showed higher performances than cv. *Robin* and cv. *Turca* (on average + 16% ca.) (Fig. 2). Concerning the two-cultivar mixtures, the differences were diluted in accordance with the performances of the pure stands, with the exception of the TuNe mixture that, considering the $\alpha < 0.1$ threshold, showed a higher nitrogen concentration ($p = 0.069$) than the average of its components (TuNe = 2.49%, Ne = 2.38% and Tu = 2.06%). Finally, the mixtures of three cultivars show predictable differences, in relation to the increase in diversity: the

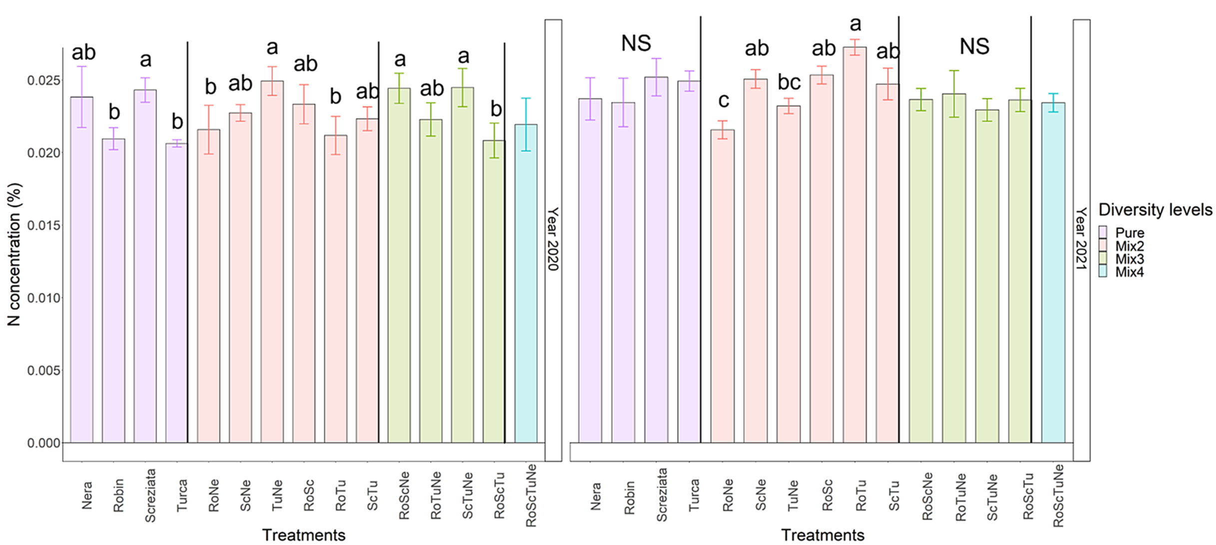

Fig. 2 Mean shoot N concentration (%) in lentil pure stands, two-, three- and four-cultivar mixtures in 2020 (on the left) and 2021 (on the right). Pure stands are represented by purple bars, two-cultivar mixtures by pink bars, three-cultivar mixtures by green bars and four cultivar mixtures by blue bars. Different letters indicate significant differences within diversity levels at $p \leq 0.05$ (ANOVA results, Bonferroni-based method). Error bars represent the standard errors of the means (SE). Ne = cv. Nera, Ro = cv. Robin, Sc = cv. Screziata, Tu = cv. Turca

combinations containing both high concentration cultivars also registered higher N%.

In the field experiment (2021) the main differences were registered among the mixtures of two cultivars (Fig. 2). In detail, the RoTu mixture's N concentration was significantly higher than that of the mixtures RoNe and TuNe. RoTu performance also tended to be higher ($p = 0.069$) than the average of its components (RoTu = 2.75%, Ro = 2.36% and Tu = 2.51%). The behaviour of RoTu mixture in field conditions was opposite than in pot conditions (2020), when it registered the lowest performance of the two cultivar mixtures; TuNe mixture instead, from holding the highest N concentration in pot conditions (2020), was among the poorest in field conditions (2021).

Nitrogen content-total (g m^{-2})

The analysis of nitrogen content (N pool size) (g N m^{-2}) resulted in a non-significant effect of Treatment in field condition in 2021 ($p = 0.5$, $\chi^2 = 13.86$).

Nitrogen content did not show significant differences among pure stands and mixtures of two cultivars, while among mixtures of three cultivars some differences emerged (Fig. 3): ScTuNe mixture's nitrogen content was significantly lower than that of RoTuNe mixture, being only one of the three components swapped (cv. *Screziata* with cv. *Robin*). ScTuNe mixture also shows a slight difference from the expectations according to its pure stand performances, but the difference was not significant as $p = 0.12$ (ScTuNe = 1.72 g N m^{-2}, Sc = 2.34 g N m^{-2}, Tu = 2.43 g N m^{-2}, Ne = 2.73 g N m^{-2}).

Enriched nitrogen share

The analysis of ^{15}N excess resulted in a non-significant effect of Treatment both in pot conditions in 2020 and in field conditions in 2021, even though in 2021 the effect significance fell within the $\alpha < 0.1$ threshold ($p = 0.07$, $\chi^2 = 22.34$) (Fig. 4).

Significant differences emerged among pure stands, two-cultivar mixtures and three-cultivar mixtures in 2021 (Fig. 5): cv. *Turca* was associated with a lower ^{15}N excess when grown alone, but the trend was not always confirmed in mixtures. Among two-cultivars mixtures the main difference concerns ScNe and RoNe mixtures,

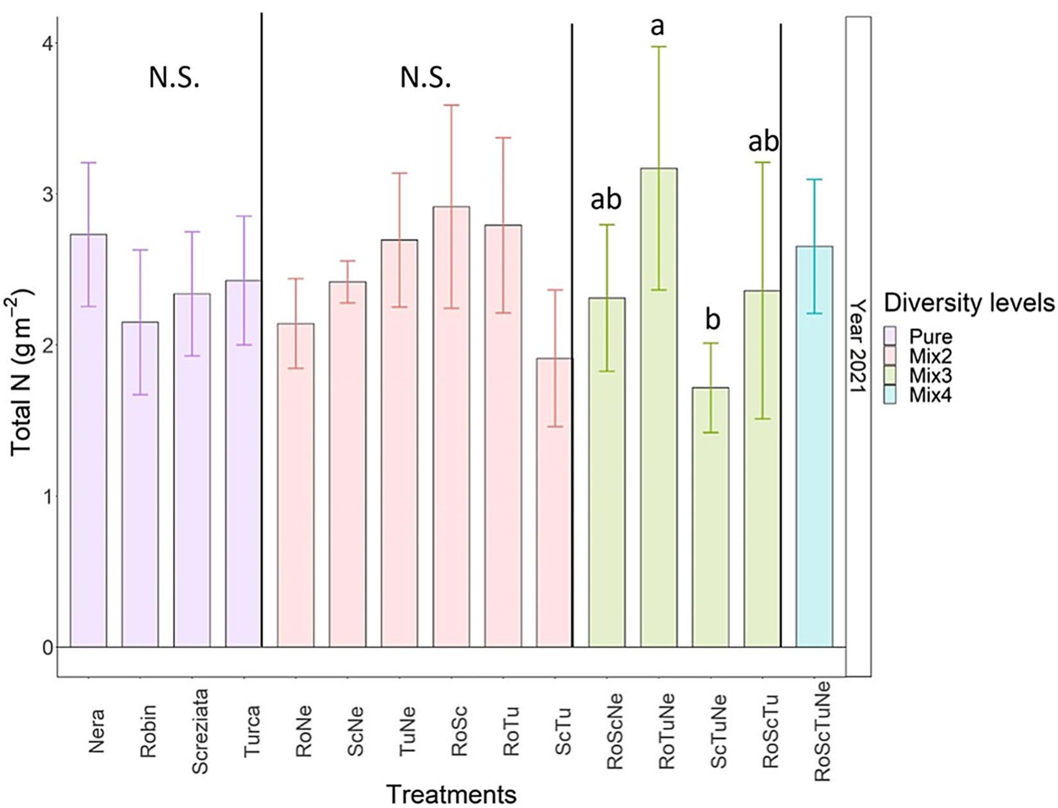

Fig. 3 Mean shoot N content (g m^{-2}) in lentil pure stands, two-, three- and four-cultivar mixtures in 2021. Pure stands are represented by purple bars, two-cultivar mixtures by pink bars, three-cultivar mixtures by green bars and the four cultivars mixture by blue bars. Different letters indicate significant differences within diversity levels at $p \leq 0.05$ (ANOVA results, Bonferroni-based method). Error bars represent the standard errors of the means (SE). Ne = cv. Nera, Ro = cv. Robin, Sc = cv. Screziata, Tu = cv. Turca

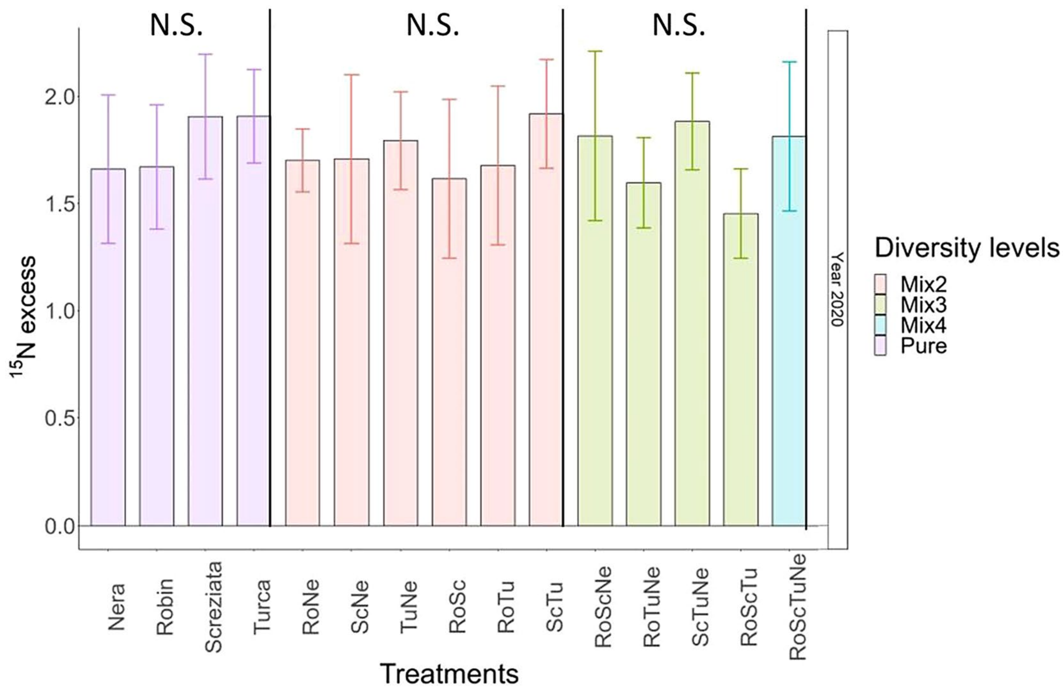

Fig. 4 Mean 15N excess in % (15N enrichment %–0.366%, i.e., natural abundance) in lentil pure stands, two-, three- and four-cultivar mixtures in 2020. Pure stands are represented by purple bars, two-cultivar mixtures by pink bars, three-cultivar mixtures by green bars and the four cultivars mixture by blue bars. Error bars represent the standard errors of the means (SE). Ne = cv. Nera, Ro = cv. Robin, Sc = cv. Screziata, Tu = cv. Turca

so not containing cv. *Turca*, while among three-cultivar mixtures it concerns RoScTu e ScTuNe mixtures, both containing cv. *Turca*.

Concerning contrasts between mixtures and their components, a significant interaction emerged: ScNe mixture showed a ^{15}N excess significantly lower than the average of its components (ScNe = 0.279%, Sc = 0.461% and Ne = 0.507%).

There ^{15}N excess was clearly lower in field conditions in 2021 (0.396%) than in pot conditions in 2020 (1.743%), thus with an average decrease of 77%.

Finally, a non-significative trend emerged between the treatments' typologies: with the increase of the diversity level, ^{15}N excess decreased (from pure stands to mixtures of four component). This trend suggests that there may be a negative correlation between the gradient of diversity and nitrogen uptake from the soil. The mean ^{15}N excess for pure stands was 0.427%, for mixtures of two cultivars was 0.394%, for mixtures of three cultivars 0.387% and finally 0.339% for the mixtures of four cultivars.

Orthogonal contrasts

The analysis of ^{15}N excess and N concentration revealed an emergent effect of some mixtures, meaning that the performance of the mixture was significantly different from the average of its components.

In pot conditions in 2020 the mixtures NeRo and NeTu showed a significantly lower yield than their components. While NeRo mixture did not result significant to contrasts with any other parameter, NeTu mixture's N concentration and nodules were significantly higher than those of its pure stands components.

In field conditions in 2021 RoTu mixtures showed a higher N concentration than its components, but a lower yield, while NeSc performed worse than its components concerning ^{15}N excess, while better concerning yield (Table 2).

Correlation analyses

The correlation analysis between data from the current study and the earlier from Lorenzetti et al. [29] produced significant results (Table 3).

Concerning yield in pot conditions (2020), the correlation analysis resulted in a strong effect of the additive model with treatment and N% as predictors. No interaction between fixed terms emerged, meaning that the effect of N concentration on yield is the same for each treatment, despite the different basal levels of yield.

Concerning N% in pot conditions (2020), the correlation analysis resulted in a significant effect of the additive model with treatment and biomass as predictors, while neither of the terms was significant in a baseline model. No interactions emerged.

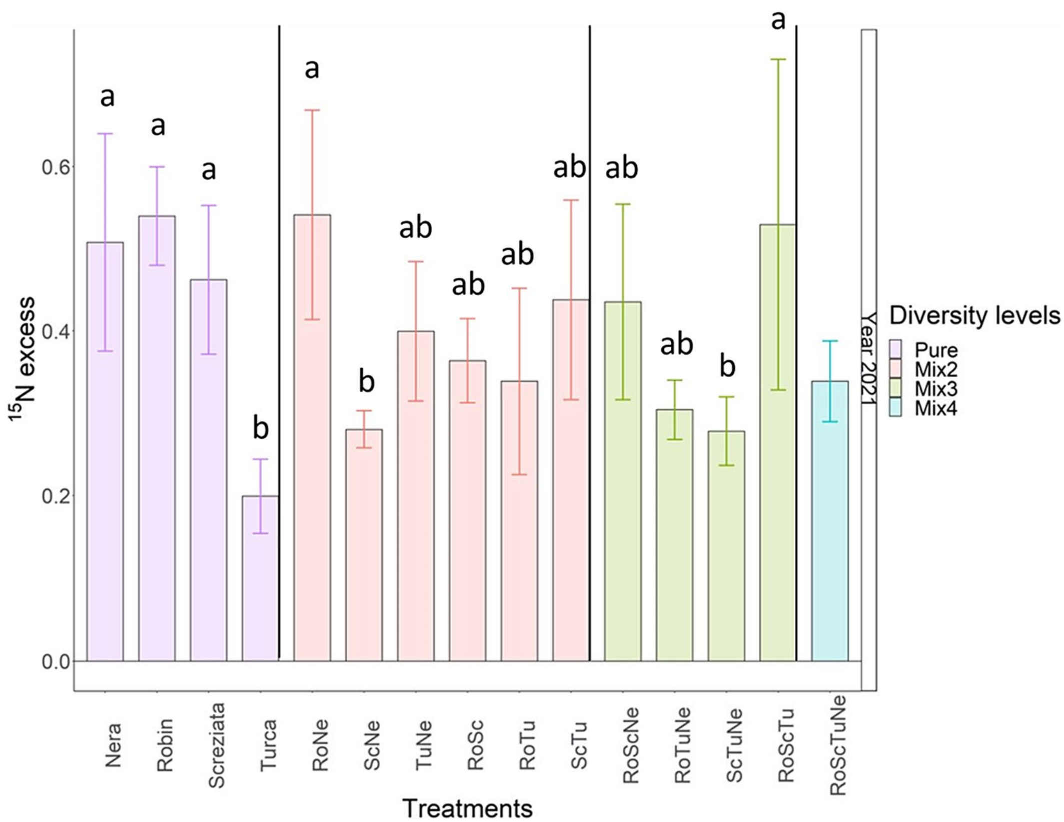

Fig. 5 Mean ^{15}N excess in % (^{15}N enrichment %–0.366%, i.e., natural abundance) in lentil pure stands, two-, three- and four-cultivar mixtures in 2021. Pure stands are represented by purple bars, two-cultivar mixtures by pink bars, three-cultivar mixtures by green bars and the four cultivars mixture by blue bars. Different letters indicate significant differences within diversity levels at $p \leq 0.05$ (ANOVA results, Bonferroni-based method). Error bars represent the standard errors of the means (SE). Ne = cv. Nera, Ro = cv. Robin, Sc = cv. Screziata, Tu = cv. Turca

Table 2 Significant results of orthogonal contrasts for all lentil mixtures in 2020 and 2021 concerning N concentration (%), ^{15}N excess, nodules and yield

		N%			**Enrichment**			**Nodules**			**Yield**		
	Contrasts												
20	NeRo versus ½ Ne + Ro										0.83	(0.07)	*
	NeTu versus ½ Ne + Tu	0.3%	(0.001)	+				1.18	(0.10)	+	0.64	(0.06)	***
21	NeSc versus ½ Ne + Sc				0.58	(0.16)	*				1.24	(0.16)	+
	NeTu versus ½ Ne + Tu							1.18	(0.10)	+			
	RoTu versus ½ Ro + Tu	0.3%	(0.001)	*							0.71	(0.09)	**

Nodules are expressed as the result of three experimental years (2019, 2020, 2021) according to Lorenzetti et al. [29] analysis

The values represent the ratio between the mixture performances and that of its components (S.E.) For enrichment, nodules and yield when the ratio is < 1 the mixture perform worse than its components, if it is > 1 the mixture performs better than its components. For *N*%, when the ratio in positive the mixture performs better than its components, when negative the mixture perform worse than its components. + indicates statistical significance at $p \leq 0.1$, * at $p \leq 0.05$ level, ** at $p \leq 0.01$, *** at $p \leq 0.001$ (Estimated Marginal Means post hoc test). Ne = cv. Nera, Ro cv. = Robin, Sc = cv. Screziata, Tu = cv. Turca

In addition, nodules were found a critical predictor for N concentration in pot conditions (2020).

Concerning N% in field conditions in 2021, correlations emerged with biomass and with ^{15}N excess (as predictors of two different baseline linear models). Finally, a separate analysis identified as significant the additive model with treatment and nodules as predictors, N% as dependent variable. Again, no interaction between predictors emerged.

Concerning nitrogen content (g) in field conditions in 2021, as previously shown, treatment cannot explain

Table 3 Result of the correlation analysis between the parameters observed in this study and the agronomic parameters of Lorenzetti et al. [29]

Year	Response variable	Predictors	Test statistics	*p*-value	Parameter coefficient
2020	Yield	Treatment +	χ^2 31.608	0.005	
		N%	χ^2 3.349	0.067	
2020	*N*%	Treatment +	χ^2 27.575	0.016	
		Biomass	χ^2 5.349	0.021	
2020	*N*%	Nodules	χ^2 2.827	0.093	$1.831e^{-04}$
2021	Yield	Treatment*	χ^2 5.238	0.982	
		^{15}N excess	χ^2 0.095	0.758	
		Treatment:^{15}N excess	χ^2 33.689	0.002	
2021	*N*%	Biomass	χ^2 2.994	0.084	$-9.101e^{-06}$
2021	*N*%	^{15}N excess	χ^2 7.457	0.006	− 0.0038
2021	*N*%	Treatment +	χ^2 36.888	0.0008	
		Nodules	χ^2 11.9	0.0006	
2021	N content (g)	Nodules	F 10.671	0.001	0.126

+ indicates additive models, * indicates interaction models

the differences observed, but nodules were found a critical predictor for N content.

The ^{15}N excess generally correlated positively with yield: for cv. *Robin* ($p = 0.002$), cv. *Turca* ($p = 0.03$) and the mixture of two cultivars RoNe ($p = 0.02$), but with a negative trend for cv. *Screziata* ($p = 0.08$). Apart from RoNe mixture, the effect is diluted in the mixtures, that do not show yield dependency from ^{15}N excess (Fig. 6).

Discussion

Nitrogen enrichment and concentration under contrasting experimental conditions

In this study, a clear difference between years emerged in terms of nitrogen enrichment: in 2020 the pot experiment resulted in a very high ^{15}N excess for lentils, in contrasts with the 2021 field experiment, with ^{15}N excess of lentils almost five times lower. Although the use of pots for nitrogen fixation estimates is widely validated from literature [5, 41], concerns on its reliability has arisen [42, 43]. One explanation could be differences in water availability, as rhizobia are sensitive to humidity [44, 45]. Pots indeed tend to lose moisture faster than in the field. In addition, in our case the high level of water table in the field may have played an important role in maintaining a constant moisture. From the other side the drier conditions in the pots may have contributed to preserve nitrogen fertilizer in closer proximity to the root system, compared to field conditions. In the field, the persistent moisture content could have led to increased nitrogen losses through diffusion, particularly when considering the sandy nature of the soil. Additionally, plants in field conditions have the ability to access nitrogen reservoirs beyond the limited area where enriched nitrogen is applied, as the root system is not constrained, thus diluting the enriched nitrogen share in the tissues. These findings raise questions regarding the practice of conducting pot experiments to study nitrogen fixation, as they reveal that the implications for biological processes may significantly differ from those observed under field conditions.

From the other hand, the mean ^{15}N excess of lentils in 2020 exceeded that of the non-fixing control (i.e., *Panicum miliaceum*), but this phenomenon was already observed in literature as the result of different N uptake patterns from different crops [18]. This observation seems to support the recent criticism regarding the use of a single non-fixing control species and one single enriched nitrogen application [18, 46, 47]. In our study, the objective was to compare cultivar performances, not to determine the exact quantity of biologically fixed nitrogen, therefore we opted to exclude the non-fixing control from the second-year replicate.

While the extent of the differences that we registered between the two methodologies was very high we could compare the trends between treatments within each experimental year.

As observed by Lorenzetti et al. [29], cultivar mixtures of commercial lentils behaved very similarly under high environmental stress conditions (i.e., the pot experiment in 2020), while they were expected to show they adaptation potential in such conditions. One explanation is that high genetic homogeneity in available cultivars may be a crucial obstacle for designing effective lentil mixtures with a trait-blind approach. To overcome this problem, use of local landraces may be considered as a source of trait variability to test a trait-based approach to cultivar mixture design.

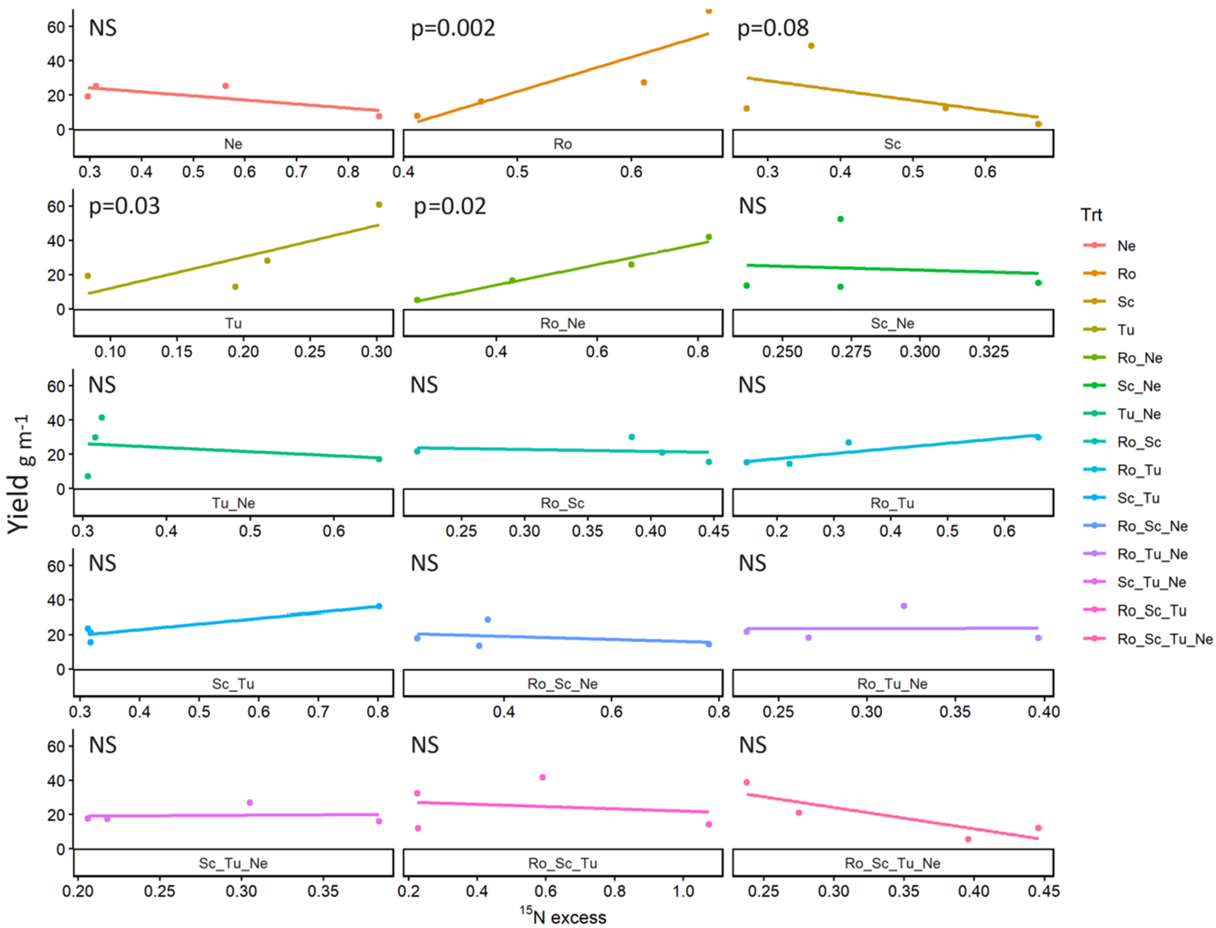

Fig. 6 Interaction between 15N excess (on the *x* axis) and treatment (each color corresponds to a treatment) as explanatory variables of lentil yield (expressed as g m^{-1} on the *y* axis) in 2021. The slope of the line indicates the presence of interaction: when the interaction is significant, *p* value is expressed, when non-significant N.S. Ne = cv. Nera, Ro = cv. Robin, Sc = cv. Screziata, Tu = cv. Turca

A second explanation may be deducted by the observation of nitrogen concentration, which, in spite of experimental differences, shows nearly equal values in the 2 years: 2.3% in 2020 and 2.4% in 2021. Considering the great differences in ^{15}N excess between pot and field conditions, this result suggests that lentils shifted their source of nitrogen from atmosphere to soil under stress conditions in the pots. Additionally, the correlation analysis revealed that N % in 2021 is better explained by ^{15}N excess rather than treatments, but the relation is not confirmed in 2020: this could be a further clue that in 2020 lentils relied to a larger degree on soil N for nitrogen accumulation at the flowering stage. This suggests a plasticity of the trait involved in the ability to fix N or to absorb N from the soil [21, 48], which is an interesting adaptation strategy from the crop perspective.

N enrichment and implications on microbiota

In 2020 we did not notice significant differences in nitrogen enrichment, but in 2021 the value of ^{15}N excess was significantly affected by treatments, meaning that the four lentil cultivars under study have a different strategy in nitrogen provision. Cv. *Turca*, in particular, seems not to rely as much on soil nitrogen, upon a similar N concentration and content than the other cultivars. The behaviour of the mixtures mainly reflects that of the pure stands, with the combinations containing cv. *Turca* resulting in a lower ^{15}N enrichment, but this trend was not always confirmed: the value dropped significantly from expectations for ScNe mixture and, even though not significant, for RoTu, while the enrichment was higher (although non-significant) for RoScTu and ScTuNe mixtures.

These results may be explained by cultivar influences at a rhizosphere level: indeed, Sawyer et al. [27] found out how switchgrass cultivars affected both fungal and bacterial communities. Similar studies were conducted by Yang et al. [28] with potato mixtures, and by Mauger et al. [26] that observed how wheat mixtures have a synergistic effect on microbiota richness, especially on fungi microbiota.

In turn, differences in microbiota competition, especially among mycorrhizal fungi, are proven to be related to nodulation and nitrogen fixation capacities [19, 24, 49–51], even within different cultivars of a given species [52]. The differences and the emergent phenomena concerning enriched nitrogen in our study may indicate that lentils cultivars affect the microbiota in the rhizosphere (considering N enrichment as a proxy for microbial behaviour) and that such effects may be modulated with the manipulation of within-species diversity. Apart from a couple of exceptions (RoScTu and RoNe), the increase of mixtures diversity was associated with a lower N enrichment (although not significant), suggesting a shift in soil N uptake when cultivars are cropped in mixtures.

On the base of our observation, we believe that more refined studies could be performed in seek of a clearer comprehension of nitrogen fixation dynamics in mixtures, e.g., designing mixtures on the base of rhizobia affinity and nitrogen fixation ability, as well as mixtures with higher levels of diversity and higher intra-specific heterogeneity. Indeed, it was reported that mixtures designed according to specific cultivar characteristics are associated with higher trait complementarity [17, 53, 54]. Similarly, it was proven that the higher the number of cultivars composing a mixture, the higher the benefits [12, 55], as also confirmed in Lorenzetti et al. study [29].

Orthogonal contrasts and correlation analysis

In our study nitrogen concentration and content was found significantly dependent on nodule number, both in 2020 and 2021. The result is not surprising per se, as literature asserts the association of nitrogen content and nodules [56, 57], but it is interesting considering the critical issues of the 2020 experiment. In 2020 the experiment from Lorenzetti et al.'s [29] earlier study was conducted in field, while the present study in pots, yet the correlation between nodules number and nitrogen concentration is confirmed. In 2020 the addition of treatment as a predictor for N% was not significant, in contrast to 2021, probably due to the more optimal conditions of field grown crops.

Nitrogen concentration also resulted as negatively dependent from total biomass in both experimental years, due to a dilution effect. It is interesting to note how total biomass did not represent a crucial observation in Lorenzetti et al. [29] study, but indeed its distribution is related to that of nitrogen concentration.

In 2020, we noted a significant dependence of yield on treatment and N%, while in 2021, the yield exhibited dependence on treatment and ^{15}N excess that in its turn was found influencing N% in the same year. This result may support the theory of yield dependency on nitrogen fixation that is not consistently confirmed in literature [58]. Indeed, the effect of ^{15}N excess on yield varied according to the treatments, but it was diluted with the increase of the diversity levels, probably as a result of a niche complementarity effect: each cultivar showed a different preference in the N source to sustain grain production, but when grown together the result is a lower dependency between N source and yield. The case of NeRo mixture, tough, question the hypothesis, suggesting that other mechanisms may take place besides niche complementarity.

Such mechanisms, not determined yet, may also be responsible for the regulation of interactions within mixtures components: concerning mixtures of two cultivars, indeed, several correspondences emerged between the interactions observed in this study and in the previous study of Lorenzetti et al. [29]. In 2020 NeTu mixture resulted significantly different from its pure stands concerning yield, nodules number and nitrogen concentration: while N% and nodules were higher than in pure stands, yield was lower. In 2021 RoTu showed a similar behaviour, with an improved N% performance compared to the pure stands, but a lower yield, while NeSc mixture, inversely, showed a higher yield and lower ^{15}N excess compared to its components.

The experimental design of the current study was conceptualized to test correlations between N uptake and production parameters, but not to fully investigate nitrogen dynamics. We recommend that future studies take into consideration different nitrogen availability levels to obtain a more comprehensive insight into the dynamics of lentil cultivar mixtures.

Conclusions and future perspectives

Results exhibit inconsistency across the two experimental years, implying that experiments on nitrogen cycling in pot conditions may not faithfully represents the target biological services. In particular ^{15}N enrichment resulted the most affected by the experimental setup, suggesting that lentils may shift their source of nitrogen from atmosphere to soil when cropped in pots.

In 2021 under field conditions ^{15}N enrichment was significantly affected by lentil cultivars, indicating distinct nitrogen provision strategies among the four cultivars studied. Mixtures enrichment though was not always in line with pure stand performance and it decreased with the increase of diversity levels (even though not significantly). Considering N enrichment as a proxy for microbial behaviour, the result suggests that cultivar mixtures may have an unpredictable cumulative effect on soil microbiota entailing a shift in the source of N uptake: the hypothesis should be tested with specific studies that take in consideration microbiota diversity and function. To a wider extent, these results may contribute to determining cultivar impact on protein production in different pedoclimatic zones.

Concerning correlations with agronomic parameters from Lorenzetti et al. [29], nodules number was found a critical predictor for N concentration, regardless for the experimental conditions. In addition, we noted that total biomass, although not showing significant trends in the Lorenzetti et al. study [29], significantly correlated with nitrogen-related parameters and may thus be used as a proxy to identify nitrogen efficient cultivars.

Finally, ^{15}N excess emerged as a significant predictor for pure stands' yield, probably due to cultivar preference for the N source to sustain grain production. However, these differences were diluted with the increase of diversity levels, revealing a lower dependency between N source and yield in cultivar mixtures (niche-complementarity effect). This result supports the use of lentil cultivar mixtures to minimize yield fluctuations linked to environmental variability. However, some exception emerged, suggesting that besides niche differentiation theories, other mechanisms may have intervened.

We recommend for future research to take in account different nitrogen availability levels, to obtain a more comprehensive insight into the dynamics of lentil cultivar mixtures. In addition, the results of this study may find stronger confirmation if maximising the genetic heterogeneity of mixture components, e.g., through the use of local landraces as a source of variability, and designing trait-based mixtures that specifically take into consideration cultivar rhizobia affinity and nitrogen fixation ability.

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Author contributions
Conceptualization, EL and PB; methodology, EL, SC, AM and PB; software, EL and SC; validation, EL, SC, AM and PB; formal analysis, EL and SC; investigation, EL and SC; resources, PB; data curation, EL and SC; writing—original draft preparation, EL; writing—review and editing, EL, SC, AM and PB; visualization, EL; supervision, AM, SC and PB; project administration, PB; funding acquisition, PB. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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