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Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our Editorial Policies and the Editorial Policy Checklist.

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
☐	☒	A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☐	☒	The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
☐	☒	A description of all covariates tested
☐	☒	A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
☐	☒	For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐	For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☐	☒	For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐	Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	Data collection was not performed using any softwares.
Data analysis	Data analysis was performed using R version 3.6.3 with the packages: reshape2, vegan, lme4, agricolae, lattice, MASS and permute and igraph. All code used to perform the analysis can be found at the Open Science Framework project: https://osf.io/qn3jc

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The data supporting the findings presented here are available from the corresponding authors on request and from the Open Science Framework Repository (https://doi.org/10.17605/OSF.IO/QN3JC). The sequencing data are available in the NCBI repository with the identifiers https://www.ncbi.nlm.nih.gov/sra/PRJNA1137244 and https://www.ncbi.nlm.nih.gov/sra/PRJNA1136844 for bacteria and fungi,

respectively. Source data files are also provided with this paper.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	It does not apply to this study.
Reporting on race, ethnicity, or other socially relevant groupings	It does not apply to this study.
Population characteristics	It does not apply to this study.
Recruitment	It does not apply to this study.
Ethics oversight	It does not apply to this study.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☒ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

Study description	The aim of this study was to evaluate if and how plant diversity impact carbon use efficiency (CUE) in an agricultural soil. To do so we sampled the TwinWin Plant Diversity experiment at the University of Helsinki (Finland). The TwinWin experiment was established in 2019, was designed to evaluate the impact of ecological intensification within agroecosystems by evaluating the potential benefits of undersowing diverse species mixtures to promote ecosystem functioning. Here, barley (Hordeum vulgare L. var. Harbinger) is planted as the main crop in monoculture and with increasing levels of undersown plant diversity (i.e. barley monoculture, barley plus 1, barley plus 2, barley plus 4 and barley plus 8 plant species). The undersown plant species were selected based on their functional root traits (i.e. nitrogen fixation capacity and rooting depth) and were: Trifolium repens L., T. hybridum L. (shallow rooting, nitrogen fixing), T. pratense L., Medicago sativa L. (deep rooting, nitrogen fixing), Lolium multiflorum Lam., Phleum pratense L. (shallow rooting, non-nitrogen fixing), Festuca arundinacea Schreb., Cichorium intybus L. (deep rooting, non-nitrogen fixing). We sampled the barley rhizosphere soil when barley is planted in monoculture and under increasing levels of plant diversity (barley plus one specie, barley plus four species and barley plus eight species). Barley is first sown in equal abundance in every plot in 12cm wide rows and a week later, the undersown species are sown in 10cm wide rows between the barley plants. The number of seeds from each undersown species at diversity level 1 (barley plus one; "D1") is calculated to reach a similar plant cover after discussion with farmers. In the higher diversity levels barley plus two ("D2"), barley plus four ("D4") and barley plus eight ("D8"), 1/2, 1/4 and 1/8 of this proportion is sown, respectively. Experimental plots were 4x10m and follow a randomized block design totaling 60 plots. Barley rhizosphere soil was collected during August 2020 on the barley monoculture, barley plus one, barley plus four and barley plus 8 treatments. For the D1 treatment, we sampled the rhizosphere soil from barley growing under the influence of four different plant species (L. multiflorum, F. arundinacea, T. hybridum and M. sativa).
Research sample	Our research sample are rhizosphere soils, which we defined as the soil attached to the roots of barley after excavating the barley plants and shaking them. This soil was sieved to 2 mm on site before being transported back to the lab 2 hours after sampling. Immediately upon arrival at the lab, one subsample of each soil sample was dried to constant mass at 65oC overnight to determine soil moisture content. The remaining soil was left in zip-log plastic bags at room temperature overnight. Different soil subsamples were then used for microbiological assays and soil organic matter analyses. The barley above ground plant biomass was dried in an oven for 48 hours at 105oC.
Sampling strategy	The sampling design was performed to obtain 24 samples from each treatment (barley monoculture, D1 treatment from four undersown species, D4 and D8) totaling 168 rhizosphere soil samples obtained from distinct barley plants. We based this on previous studies to determine the sample size needed to build co-occurence association networks for the distinct plant diversity treatments (Jacquiod et al., 2020 A core microbiota of the plant-earthworm interaction conserved across soils. Soil Biology and Biochemistry and Romdhane et al., 2020 Unraveling negative biotic interactions determining soil microbial community assembly and functioning. ISME Journal).
Data collection	Data collection was performed by Seraina L. Cappelli and Rashmi Shrestha. Random barley plants were selected for each

Data collection	experimental plot, excavated and shaken to remain only the rhizosphere soil (soil attached to the roots). This soil was sieved and transported to the lab within 2 hours of sampling for further processing.
Timing and spatial scale	The full experiment was performed within 15 days. Due to the huge number of samples and various assays performed here we randomly sampled 30 rhizosphere soil samples every day to ensure that soil samples would not wait different periods before being analyzed. The 18O-H2O-CUE assay was performed 48 hr after sampling and the soil was frozen at the end of incubation to wait for DNA extraction.
Data exclusions	One sample showed negative 18O-atom% excess, which result in a negative growth value and therefore were excluded from the analysis.
Reproducibility	To reduce stochasticity during soil DNA extraction, all extractions were performed in quadruplicates and pooled before quantification, sequencing and determination of 18O-DNA enrichment. The barley plants were chosen randomly in the plots. The authors did not attempt to replicate the entire experiment.
Randomization	Rhizosphere soil samples were sampled in a random order.
Blinding	Data acquisition and analysis was done blindly as every microcosms was assign a specific number and I did not know to which treatment that specific number corresponded during data acquisition and analysis.

Did the study involve field work? ☒ Yes ☐ No

Field work, collection and transport

Field conditions	The soil sampling was performed within 15 days, the temperature during the day ranged between 15-20 Celsius and it did not rain during the sampling period.
Location	TwinWin Experiment, University of Helsinki, Finland
Access & import/export	It does not apply to this study.
Disturbance	This is an agricultural experiment and soil samples are harvested with a regular frequency. No additional disturbancy was caused by this study.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☐	☒ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Plants

Seed stocks	The barley rhizosphere samples were collected from one of the most common barley varieties planted in Finland the Harbinger variety (see https://www.vikingmalt.com/wp-content/uploads/2017/11/Viking-Malt-Barley-News-2017.pdf). The seeds used within the TwinWin experiment were obtained with the local farmers.
Novel plant genotypes	It does not apply to this study.
Authentication	This is an agricultural experimental site. Therefore, this doesn't apply here.