Fertilizer regime and cultivar affect barley growth and rhizobiome composition

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A B S T R A C T

To combat climate change and environmental pollution, agriculture must reduce mineral fertilizer use and adopt sustainable, low-input, and organic practices. In such systems, plants depend on the soil microbiome for nutrient acquisition and growth. Thus, enhancing plant-microbiome interactions is vital for maintaining or even increasing production sustainably. Modern plant breeding has led to barley cultivars that yield high under substantial mineral fertilizer application. However, this selective breeding may have diminished traits essential for plant-microbiome interactions, making these cultivars less suitable for sustainable agriculture. In contrast, older cultivars might have preserved traits from their common wild ancestor, enabling them to prosper under low nutrient conditions. This study evaluated four modern elite barley cultivars and three older (pre-1980). Grown under various fertilizer regimes, we assessed their root microbiome using 16S rRNA amplicon sequencing. Our objectives were: i) to determine the impact of nutrient availability on plant nutrient uptake and biomass production, and ii) to understand how the time since domestication (domestication age), individual cultivar, and fertilizer regime influence the root microbiome. We found that without fertilizer, older cultivars had superior biomass production and higher leaf concentrations of nitrogen, potassium, sulfur, iron, zinc, and copper than modern cultivars. This indicates that older barley cultivars may have retained their wild ancestors’ capability to synergize with the soil microbiome, enhancing nutrient acquisition in low-input systems. Notably, the diversity of the rhizobiome was not significantly affected by domestication age but varied with individual cultivar and fertilizer treatment.

1. Introduction

Humans have been cultivating cereals like wheat, rice, maize, and barley for over 10,000 years (Meyer et al., 2012). Since the onset of the so-called “green revolution” in the mid-20th century, plant breeding has provided high-yielding crop cultivars that depend on high input of mineral fertilizers and pesticides (Pawar et al., 2008; Bresghello and Coelho, 2013). However, concerns about negative impacts on the environment and climate, as well as rising costs of fertilizers and pesticides, question this high input-high yield strategy. Hence, there is a growing demand on farmers to implement sustainable practices with lower chemical input (Gomiero et al., 2020). Still, organic fertilizers, as compost and green manure, must be mineralized by the soil microbiome prior to release of inorganic nutrients for plant uptake (Cassidy-Duffey et al., 2020). Consequently, crops supplied with only organic fertilizers rely on an efficient interaction with the soil microbiome (Kurzem et al., 2020; Miransari, 2011).

While breeding efforts have improved plant growth and yield, modern crops have lost a significant part of their genetic diversity (Condon et al., 2008; Olsen and Wendel, 2013). In this process, plant traits facilitating belowground plant-microbiome interactions have likely been lost unintentionally (Iannucci et al., 2017; Grando and Ceccharelli, 1995; Nerva et al., 2022). Hence, modern cultivars probably perform sub-optimally in organically fertilized systems, as they are ill-equipped genetically to interact with, and benefit from, the soil microbiome. Older cultivars, which have not undergone the same strong breeding selection for high-yield at high nutrient availability may have preserved traits of their wild ancestors that better facilitate interaction with the soil microbiome (Chen et al., 2021). Therefore, wild progenitors and older crop cultivars, pre-dating the green revolution, are promising candidates to possess traits suitable for agricultural systems with minimal chemical input (Swarup et al., 2021).

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Plants shape their root microbiome (rhizo-microbiome) through root morphology (Herms et al., 2022) and by releasing root exudates (Sasse et al., 2018), which promote colonization of the rhizosphere by beneficial microorganisms from the soil (Zhalnina et al., 2018). While root exudates stimulate the micro-organisms, these in turn benefit the plant by increasing nutrient availability, by hormone release, and by suppressing pathogens (Qu et al., 2020). As domestication has altered root morphology (Grando and Cecarelli, 1995; Isaac et al., 2021) and exudate profiles (Iannucci et al., 2017; Yue et al., 2023), it is likely that host control of, and interaction, with the rhizobiome have also changed.

Bulgarelli et al. (2015) found that the root microbiomes of wild and domesticated barley had similar richness and diversity but differed in composition. Similarly, Brisson et al. (2019) found that in maize, rhizosphere microbiomes of the wild progenitors and older domesticated hybrids were relatively similar, while both differed from modern cultivars. Mauger et al. (2021) found that root-endospheric prokaryotic and fungal communities of old and modern wheat cultivars differed significantly. They concluded that modern cultivars were more prone to colonization by pathogens in the root endosphere, indicating a reduced ability to shape the microbiome. Spor et al. (2020) and Kim et al. (2020) used co-occurrence network analysis in wheat and rice, respectively. They showed that modern cultivars tend to form simpler networks with fewer keystone taxa compared to their wild progenitors and older cultivars, pre-dating the green revolution.

Thus, several studies found significant differences between microbiomes associated with modern cultivars and their wild or older relatives. This suggests that plant breeding has altered the composition of the rhizosphere in modern crops. However, it is still largely unclear whether these changes have compromised modern cultivars’ ability to thrive in low-input agricultural systems.

Here, we investigated growth, biomass production, nutrient concentration, and rhizo-microbiome composition of seven spring barley (Hordeum vulgare L.) cultivars grown in a greenhouse at eight different fertilizer regimes, i.e., no fertilizer, organic fertilizer at six different levels, and mineral fertilizer. We selected seven commercially available cultivars (Langelandsbyg, Babushka, Salka, Irina KWS, Flair, Feedway, and RGT Planet). We divided these cultivars into two domestication age categories: old (pre-1980): Langelandsbyg, Babushka, and Salka modern: Irina KWS, Flair, Feedway, and RGT Planet. The four modern cultivars are commonly used in conventional agriculture today.

We anticipated cultivar and fertilizer regime to affect rhizo-microbiome composition. Moreover, we expected that cultivar, fertilizer regime, as well as microbiome composition would affect plant nutrient uptake and growth. These complex interactions were assessed by testing four specific hypotheses. As we expected older cultivars to have retained traits from their wild ancestors, enabling them to effectively recruit and sustain a beneficial rhizo-microbiome, we hypothesized that: i) Older cultivars will exhibit higher microbial rhizo-microbiome diversity and richness than the modern ones, and will thus facilitate a microbiome that more efficiently facilitates uptake of nutrients from organic fertilizer; ii) Increasing fertilization will generally result in a decrease in microbial richness and diversity, as high nutrient availability will favour a narrower selection of copiotrophic microorganisms. Further, the higher rhizo-microbiome diversity and richness will result in; iii) Older cultivars exhibit relatively better growth at low nutrient availability compared to modern, indicated by higher aboveground biomass and higher nutrient concentration. Finally, while fertilizer addition will enhance nutrient uptake and growth in all plants, iv) modern cultivars will benefit more from high fertilizer levels, as they have been specifically bred for high-input systems.

2. Materials and methods

2.1. Experimental design

2.1.1. Experimental overview

The experiment involved a complete two-factorial design with seven barley cultivars (three old and four modern) × eight fertilizer treatments, i.e., 56 different treatments, each in three replicates, i.e. in total 168 experimental units. We grew the plants in pots containing agricultural soil, either with no fertilizer, with a gradient of organic fertilizer, or with mineral fertilizer (0.2 g N kg dry soil⁻¹). After 35 days, we harvested, performed chemical analyses of plants and soil, and investigated the rhizo-microbiome (rhizosphere and rhizoplane combined) composition. We measured the shoot biomass, leaf nitrogen concentration, and soil parameters from all 168 experimental units. We measured other plant nutrients from 63 experimental units (fertilizer treatments: No fertilizer, Organic 4, and Mineral). We performed the molecular rhizo-microbiome analyses on 105 experimental units (fertilizer treatments: No fertilizer, Organic 2, Organic 4, Organic 6, and Mineral).

2.1.2. Barley cultivars

The three old cultivars included i) Babushka, a six-row barley originating from crosses between a Swiss (BRGC13154) and Indian (Lyallpur 3647) landrace and a modern cultivar from the 1960 (Nackta) (Karl-Josef Müller (www.cultivari.de)) via agroligica.dk; ii) Langelandsbyg (Langeland henceforth), a two-row Danish landrace dating back to the late 1800s (Skovgaard Gods (skovgaard.dn.dk)); iii) Salka, a Danish barley cultivar from 1970 (agroligica.dk). Further, we selected four modern cultivars popular amongst farmers in Denmark today: i) Irina KWS (KWS Lochow GMBH), ii) Feedway (Nordic Seed), iii) Flair (Sejet Planteforædling), and RGT Planet (Nordic Seed).

2.1.3. Soil

We collected a coarse sandy soil (0–25 cm, Orthic Haplohumod) in August 2019, from an organic field (S. Jutland, Denmark, 54° 54’ 2.68”, 9° 7’ 34.05”). For soil physical, chemical properties, and cropping history see Table S1. We air-dried, sieved (2 mm), and mixed the soil.

2.1.4. Fertilizers

For organic fertilizer, we harvested organic maize leaves (S. Jutland, Denmark, 55° 03’ 36.8”, 9° 08’ 07.2”, August 2019), oven-dried (70 °C), and pulverized them (Retch Ultra Centrifugal mill with titanium rotor, to avoid trace element contamination). The maize fertilizer contained 2.5 % nitrogen (Dumas combustion) and had a C:N ratio of 18:1. Table S2 shows concentration of other nutrients in the organic fertilizer (determined by ICP-OES). For mineral fertilizer, we used a standard NPK fertilizer (Yara ScanFarm NPK 14–3–15). Table S3 shows the nutrient composition of the mineral fertilizer.

2.1.5. Experimental setup

We homogenized the mineral NPK fertilizer and mixed it into the soil to a concentration of 0.2 g nitrogen kg dry soil⁻¹. We added organic fertilizer to the soil at six levels (Organic 1–6): 0.94, 2.34, 4.69, 9.37, 18.75, and 28.12 g per pot (0.04, 0.1, 0.2, 0.4, 0.8 and 1.2 g N kg dry soil⁻¹). We based levels on anticipated plant nitrogen requirements, i.e., four levels below, one level matching, and one level above the standard fertilization rate of 0.2 g N kg dry soil⁻¹. Hereby, assuming a mineral fertilizer equivalent of 4, and a nitrogen availability slightly lower than in organic household compost (López-Rayo et al., 2016). We mixed the organic fertilizer into the soil just before experimental start, to ensure that all mineralization occurred during the experiment.

The experimental units were 168 cylindrical plastic pots (Ø: 13 cm, height: 10 cm, vol.: 0.86 l). To each pot, we added a 2 cm bottom layer of vermiculite and then 580 g of either soil, soil mixed with mineral fertilizer or soil mixed with one of the six levels of organic fertilizer.

For each cultivar, we germinated seeds in vermiculite for six days. At
experimental start, we transferred five seedlings to each of the 168 pots. The plants grew in a greenhouse at a relative humidity of 65 % with 16 h daily light (275–280 μmol m$^{-2}$ s$^{-1}$) at 19 °C and 8 h in the dark at 15 °C. We watered the pots thrice a week with 150 ml of double deionized water and rearranged them during growth to minimize systematic errors.

2.2. Harvest and sample preparation

After 35 days, we destructively sampled all pots. We dipped shoots 20 times in a 0.1 % Tween 20 solution (Merck, NJ, USA) followed by 20 rinses with milli-Q water to remove surface trace element contamination (Schmidt et al., 2013). We then freeze-dried shoots for 48 h and recorded the dry biomass. We homogenized green, fully developed, freeze-dried leaves in a shaker, using zirconium balls (Intensive shaker of Fluid Management, SO-40a, The Netherlands), for nutrient analyses. We collected root/rhizosphere material (fresh roots with attached soil particles) for 16S amplicon sequencing and bulk soil samples for chemical analyses and stored them at –20 °C and 4 °C, respectively, until further processing.

2.3. Chemical analyses

2.3.1. Soil

We measured soil pH in a 0.01 M CaCl$_2$ solution at a 1:2.5 soil to solution ratio (Mikkelsen et al., 2020). To determine soil ammonium-and nitrate-N, we suspended 10 g soil (fresh weight) in 50 ml ddH$_2$O for 60 min on an orbital shaker (KSS01 digital, IKA-Labortechnik, Germany) at 200 rpm and filtered the suspension through 2.7 μm filters (Whatman™ Grade GF/D Glass Microfiber Filters, Cytiva, USA). Ammonium- and nitrate-N was measured by flow injection analysis (Foss FIAstar Analyzer 5000, Denmark). We determined total carbon and nitrogen content by Dumas combustion of 15 mg pulverized dry soil (Euro EA 3000 Elemental Analyzer, Eurovector SPA, Milan, Italy).

2.3.2. Leaf nutrient concentration

We measured leaf total carbon and nitrogen concentration using an elemental analyzer (EA) (PYRO Cube Elemental Analyzer, Elementar, Hanau, Germany) coupled to an isotope ratio mass spectrometer (IRMS) (Isoprime100, Elementar, Manchester, UK) (Novak et al., 2019). To determine leaf element concentrations, dried, homogenized samples were digested in 70 % HNO$_3$ and 30 % H$_2$O$_2$ at 240 °C and 200 bars for 15 min using a pressurized microwave digestion system (Ultrawave, Milestone Srl, Sorisole, Italy) (Hansen et al., 2013), after which we performed inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Agilent 5100, Agilent Technologies, USA). We evaluated the analytical accuracy by measuring certified reference material (apple leaf (NIST 1515), wheat flour (NIST 1567b)) for every ten samples throughout the run and used blanks to correct for background signals. We rejected element contents below a Limit of Detection <80 % of the reference value, defined as three times the standard deviation of seven method blanks. Hereby, we quantified the macronutrients: K, Ca, Mg, P, and S and the micronutrients: Fe, B, Mn, Fe, Zn, and Cu.

2.4. Statistics

We analysed data using R Statistical Software (R Core Team, 2021) and RStudio (Posit team, 2023). As each cultivar was associated with a specific domestication age (old or modern), we used nested linear models to investigate the impact of the categorical variables (fertilizer treatment, domestication age, and cultivar). Using this approach, we could explore the effects of both domestication age and cultivar in the same model. Response variables were log-transformed if needed, and we checked normality and variance homogeneity of residuals for all models using quintile-quantile and residual plots.

For aboveground biomass, the residuals for the linear model showed signs of heterogeneity across fertilizer treatments, even after transformation. Therefore, we instead fitted a linear mixed-effects regression model, using the lmer function in the lme4 package (Bates et al., 2015). We performed ANOVAs (Type III sum of squares) based on the models and Tukey HSD post hoc tests using emmeans (Lenth, 2023). We analysed the linear relationship between organic fertilizer addition (0–28 g pot$^{-1}$) and the soil parameters ammonium-N, nitrate-N, C:N ratio, and pH. We used the ggplot2 package (Wickham, 2016) to create graphs.

2.5. Root associated microbiome

2.5.1. DNA extraction and sequencing

We extracted root associated (rhizosphere and root) DNA collectively from 100 mg frozen root with attached soil particles from each pot in the treatments: No fertilizer, Organic 2, 4, and 6, and Mineral fertilizer, using the DNeasy® PowerSoil® Pro Kit (Qiagen, Hilden, Germany). We selected treatments for molecular analysis based on maximum difference in plant performance. We transferred the samples directly into the PowerBead Pro Tubes provided in the kit, and vortexed at maximum speed for 15 min using a Vortex-Genie® 2.0 (Scientific Industries, NY, USA). We purified DNA according to the instructions in the kit. To obtain bacterial and archaeal community profiles, we prepared amplicon sequencing libraries using a two-step PCR. We amplified the V3-V4 region of the 16S rRNA gene using primers Uni341F (5'-CCTAYGGGRBGCASCAG-3') and Uni806R (5'-GGAC-TACHVGGGTWTCTAAAT-3') (Yu et al., 2005; Sundberg et al., 2013; Caporaso et al., 2011) and purified the products from the first PCR, using the HighPrep clean-up (MagBio Genomics, Gaithersburg, MD, USA) in a 0.65 l (beads:PCR reaction) volumetric ratio. In a second PCR using PCRBIO HiFi (PCR Biosystems Ltd., London, UK), we added Illumina sequencing adapters and sample-specific dual indexes (IDT Integrated DNA Technologies, Coralville, IA, USA). Products from the second PCR were purified as described above and concentrations were normalized using SequaPrep Normalization Plate (96 Kit) (Thermo Fisher Scientific, Waltham, MA, USA). We pooled and concentrated the libraries using DNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, USA). The final 9pM library was sequenced on an Illumina MiSeq platform using Reagent Kit v3 (2 × 300 cycles; Illumina, San Diego, CA, USA), following manufacturer’s instructions.

2.5.2. Amplicon sequencing reads processing

We used the QIIME2 pipeline (v>2022.8.0; (Bolyen et al., 2019)) to predict Amplicon Sequence Variants (ASVs). We removed primers from the demultiplexed paired-end reads using cutadapt plugin. We used q2-dada2 plugin to filter and quality trim reads, remove chimeras, find ASVs and estimate their abundance. We trimmed forward and reverse sequence reads at positions 280 and 235, respectively, based on the quality scores. Taxonomic classification for the predicted ASVs was inferred using q2-feature-classifier plugin with pre-trained Naive Bayes classifier: “silva138 99% OTUs 4 full-length sequences” downloaded from https://docs.qiime2.org/2022.2/data-resources.

We analysed the resulting ASV table with R software, using a custom script with the following packages: Phyloseq (v1.44.0; (McMurdie and Holmes, 2013)) for pre-processing and beta-diversity analysis; decontam (v1.20.0; (Davis et al., 2018)) to identify and remove contamination; and Vegan (v2.6.4, (Oksanen et al., 2022)) for statistical analyses of beta-diversity. We removed ASVs assigned to chloroplasts and mitochondria, without genus-level annotations, and present in <1 % of the samples. Differences in sequencing depth was accounted for by rarefaction to 11,000 reads per sample.

We estimated alpha diversity with Chao1 and Shannon indices using the phyloseq estimate_richness function. For beta-diversity, we used canonical analysis of principle coordinates (CAP) (Anderson and Willis, 2003), and calculated the dissimilarities between the samples using the rarefied data with constraints on domestication age and fertilizer treatment. The dissimilarity matrix was tested by Permutational
Multivariate Analysis of Variance (PERMANOVA) using adonis2 function in the Vegan package. This was done over 10,000 permutations to calculate the statistical significance.

To identify genera that were differentially enriched or depleted across fertilizer treatments, as well as between individual cultivars within each fertilizer treatment, we estimated microbial differential abundance using DESeq2 (v1.40.1; (Love et al., 2014)). This was done through pairwise comparisons of samples applying a false discovery rate (FDR) cut-off of <0.05.

To identify species specific to a particular fertilizer treatment, we identified indicator species for each using the R package “indicspecies” multipatt() function with 9999 permutations and point biserial correlation coefficient (r.g) (Caceres and Legendre, 2009). We also assigned functions to these indicator species using PICRUSt2 (version 2.5.0; Douglas et al. (2020)) with reference to KEGG orthology (KO) gene families (Mao et al., 2005). The KO is structured as a hierarchy called Directed Acyclic Graph (DAG) where a term may have more than one parent and more than one child. It consists of four levels. The top level consists of five categories that include metabolism, genetic information processing, environmental information processing, cellular processes, and human diseases. The second level divides the five functional categories into finer sub-categories. The third level corresponds to more specific pathways, and the fourth level consists of the specific functions.

3. Results

3.1. Visual observations and aboveground biomass

At harvest, we observed distinct differences in biomass and plant health between the cultivars and fertilizer treatments (Fig. 1). Plants with no fertilizer, or low amounts of organic fertilizer, had a stiff upright appearance, general chlorosis, necrotic tissue, senescence of older leaves, and anthocyanosis of the stem. For most cultivars, the symptoms lessened with increasing fertilizer. However, Feedway and Salka had lower aboveground biomass and accelerated senescence at the highest organic fertilizer level(s). Not all plants survived until harvest, and all died at Organic level 5 after approximately two weeks (Table S4).

Individual cultivars, but also old versus modern plants, responded differently to the fertilizer treatments (Fig. 2A). There were significant interactions between fertilizer treatment and cultivar as well as domestication age. The results of the joint_tests can be found in Fig. S1, and relevant F and p-values are shown in Fig. 2A.

To investigate the significant interactions, we applied Tukey HSD
Fig. 2. (A) Shoot dry weight and (B) nitrogen concentration in three old and four modern barley cultivars after 35 days growth in a greenhouse. Plants received either no fertilizer, different levels of organic fertilizer (Organic 1, 2, 3, 4 and 6) or mineral fertilizer (Mineral). For each fertilizer amendment, cultivars are grouped by domestication age (old: white, modern: blue). The box plots display the interquartile range, with the median marked by a line and the minimum and maximum values indicated by whiskers. F- and p-values (only shown when significant) are from joint-test analyses on nested linear models testing fertilizer, domestication age, and cultivar (Fig. S2–3). Adjusted p-values (Tukey multiple comparison HSD test) are indicated by connected lines between bars (*: p < 0.1, **: p < 0.05, ***: p < 0.01). See Table S5 for p-values for all pairwise comparisons. The dashed horizontal line in Fig. 2B indicates the adequate foliar nitrogen concentration according to Kirkby (2023).
post-hoc tests. For each fertilizer treatment, we compared the aboveground biomass of the domestication age groups and the individual cultivars (Tables S5–6). The older cultivars produced significantly more aboveground biomass in the No fertilizer treatment \((p = 0.0125)\), while the modern cultivars tended to have higher biomass in the Mineral fertilizer treatment, though not significantly \((p = 0.0531)\) (Fig. 2A). The pairwise comparisons between all cultivars within each treatment, revealed that the old cultivar Langeland produced significantly more

![Fig. 3. Leaf concentration of selected (A) macronutrients (K, P, and S) and (B) micronutrients (Fe, Zn, and Cu) of three old and four modern barley cultivars after 35 days growth in a greenhouse pot experiment. For each fertilizer treatment, cultivars are grouped by domestication age (old: white, modern: blue). The box plots display the interquartile range, with the median marked by a line and the minimum and maximum values indicated by whiskers. Dots represent individual measurements, with colours indicating different cultivars. Fertilizer treatment significantly affected all nutrients, except Fe, \((p < 0.0001)\) in nested linear models.

Domestication age significantly affected K \((F = 10.489, p = 0.0025)\), S \((F = 9.564, p = 0.0037)\), Fe \((F = 9.693, p = 0.0035)\), Zn \((F = 10.084, p = 0.0029)\), and Cu \((F = 12.029, p = 0.0013)\). Further, we observed significant interactions between fertilizer treatment and domestication age for P \((F = 3.969, p = 0.0270)\) and Zn \((F = 3.593, p = 0.0370)\), as well as between domestication age and cultivar for P \((F = 2.946, p = 0.0238)\) and Zn \((F = 2.657, p = 0.0368)\). P-values from a Tukey HSD test comparing the means of old and modern cultivars within the same fertilizer treatment are shown in the figures: \(\ast: p < 0.1, \ast\ast: p < 0.05, \ast\ast\ast: p < 0.01, \ast\ast\ast\ast: p < 0.001\). See Table S8–S9 for p-values for all pairwise comparisons. Dashed horizontal lines indicate adequate foliar levels of the nutrient in question according to Kirkby (2013). (A) Macronutrients (% of dry matter). (B) Micronutrients (ug g\(^{-1}\) dry weight). Note that due to insufficient leaf biomass for some cultivars in some fertilizer treatments, we only had material enough to analyse the multi-element concentrations of leaf tissue on a subset of the fertilizer treatments, i.e. No fertilizer, Organic 4 and Mineral fertilizer.
aboveground biomass than Babushka \((p = 0.0126)\), Feedway \((p = 0.0236)\), and RGT Planet \((p = 0.0031)\) in the No fertilizer treatment.

We also compared the aboveground biomass of the individual cultivars across fertilizer treatments using a Tukey HSD post-hoc test (Table S7). All cultivars displayed more aboveground biomass in the Mineral fertilizer treatment, generally followed by the Organic 4 and 6 treatments, the No fertilizer treatment, and lastly the Organic 1–3 treatments. However, for Feedway, addition of organic fertilizer did not increase the aboveground biomass compared to the No fertilizer treatment.

### 3.2. Leaf nutrients

#### 3.2.1. Nitrogen

Fertilizer treatment and domestication age significantly affected leaf nitrogen concentration. In all cultivars, leaf nitrogen tended to increase with increasing organic fertilizer. The Organic 4 treatment was an exception, with a noticeably low nitrogen concentration. Mineral fertilizer provided the highest nitrogen concentration in all cultivars. Older cultivars tended to contain more nitrogen than modern, though only significantly in the No fertilizer treatment (Fig. 2B). Only plants in the No fertilizer and Organic 1 treatments were below the adequate foliar nitrogen concentration, defined by Kirkby (2023).

#### 3.2.2. Other macro- and micronutrients

Fertilizer treatment significantly affected all nutrients measured except Fe \((p < 0.0001, \text{Figs. S3-S12})\). Generally, leaf nutrient concentration was lowest in the No fertilizer treatment and highest in the Mineral fertilizer treatment. The macronutrients K, P, S (Fig. 3A) and the micronutrients Fe, Zn, and Cu (Fig. 3B) significantly differed between old and modern cultivars. The mineral fertilizer treatment displayed significantly higher levels of K \((p = 0.0048)\), P \((p = 0.0252)\), and Cu \((p = 0.0143)\) in old cultivars compared to modern. In all fertilizer treatments, macronutrient levels were generally sufficient, but the micronutrient Fe was consistently insufficient for all cultivars according to Kirkby (2023) (Fig. 3).

#### 3.3. Soil carbon, nitrogen, and pH

The soil C:N ratio at harvest correlated significantly negatively with organic fertilizer amendment (Fig. S13A). The C:N ratio in the Mineral fertilizer treatment and No fertilizer treatment were similar (Table S10). Organic fertilizer amendment correlated positively and significantly with plant available ammonium-N and nitrate-N (Fig. S13B). Soil pH correlated significantly positively with organic fertilizer amendment (Fig. S13C). The pH in the Mineral fertilizer treatment was approximately 5.3 (Table S10).

### 3.4. Rhizo-microbiome

The 16S rRNA sequencing resulted in 7705 ASVs, that we used for further analyses. Fertilizer treatment significantly affected the alpha diversity of the rhizo-microbiome (Fig. 4A). A Dunn’s test revealed that the treatments with the highest nutrients availability (Organic 6 and Mineral fertilizer) had the lowest alpha diversity (Fig. 4A). Neither domestication age, nor cultivar affected alpha diversity significantly.

Beta diversity analyses in terms of CAP showed a clear clustering based on fertilizer treatment (Fig. 4B). Permutational analysis of variance showed a significant effect of fertilizer treatment, cultivar, and the interaction between the two on shaping the rhizo-microbiome composition. Domestication age did not have a significant effect on the beta diversity (Fig. 4B).

The distribution of ASVs amongst fertilizer treatments showed that almost 13% (978 ASVs) occurred in all treatments. About 7% (506

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**Fig. 4.** Rhizo-microbiome alpha (A) and beta diversity (B) of three old and four modern barley cultivars grown for 35 days in a greenhouse pot experiment at different fertilizer amendments. Different colours refer to different fertilizer amendments. (A) Boxplots showing Chao1 richness and Shannon diversity; boxes not sharing any letters are significantly different (Dunn’s test). Kruskal-Wallis test on Shannon diversity index shows a significant effect of fertilizer \((p < 0.01)\). (B) Canonical Analysis of Principal Coordinates computed on Bray-Curtis dissimilarity matrix of the rhizo-microbiome across selected fertilizer treatments. The percentages on the axes represent the percentage of variance explained by each axis. Dot shapes represent domestication age. The table at the top right corner of the plot shows the results of PERMANOVA analyses in terms of effect size and p-values for domestication age (Age), fertilizer treatment (Fertilizer), cultivar and their interactions. The asterisks represent significant p-values whereas the dot represents a p-value <0.1.
ASVs) occurred exclusively in the No fertilizer and the Mineral fertilizer treatments, and about 6% (500 ASVs) occurred exclusively in the No fertilizer and the Organic 2 treatments. The largest number of unique ASVs occurred in the Organic 2 treatment (432) followed by the No fertilizer (375) and the Organic 6 (350) treatment (Fig. 5A).

We found distinct differences between relative abundance of different bacterial phyla in the different fertilizer treatments. Of the top ten most abundant phyla across all treatments, ASVs belonging to Bacteroidota were almost twice as abundant in the Organic 6 treatment as in the mineral treatment. ASVs belonging to Firmicutes were more abundant in organic treatments as compared to both Mineral and No fertilizer treatments. ASVs belonging to Actinobacteriota, Acidobacteriota, and Gemmatimonadota were less abundant in Organic 4 and Organic 6 treatments, than in the No fertilizer, Mineral, and Organic 2 treatments. ASVs belonging to Patescibacteria were most abundant in the Mineral treatment (almost five times more abundant than organic and No fertilizer treatments). ASVs belonging to Verrucomicrobiota and Myxococcota were least abundant in the Mineral treatment, and Chloroflexi ASVs were most abundant in the No fertilizer treatment (Fig. 5B).

Pairwise comparisons of genera significantly enriched or depleted in the rhizo-microbiome in particular fertilizer treatments showed that increased organic fertilizer resulted in more genera of the phyla Bacteroidota, Firmicutes, Verrucomicrobiota, and Myxococcota, and in fewer genera in Actinobacteria. The No fertilizer treatment contained most genera belonging to Chloroflexi. In case of Proteobacteria, no general pattern for enrichment/depletion could be observed across fertilizer treatments (Fig. 6).

When comparing significantly enriched or depleted genera in the rhizo-microbiome of individual cultivars within each fertilizer treatment we found no clear pattern (Fig. S14). However, compared to the other cultivars, Babushka had enriched genera of Proteobacteria and Bacteroidota in the mineral fertilizer treatment.

Based on the indicspecies analyses, we identified microbial species associated with a particular fertilizer treatment (indicator species). The Organic 6 treatment had the highest number of indicator species (185), followed by the No fertilizer treatment (150), the Organic 2 (143), and Organic 4 treatments (133). The Mineral fertilizer treatment had the lowest number of indicator species (93).

Functional annotation of these indicator species for each treatment based on unique KO annotation (as described in the methods section) showed that the Organic 6 and No fertilizer treatments had a broader range of functions (total of 52 and 51 respectively) as compared to the
Mineral fertilizer treatment which had the least number of different functions overall (total of 14). The KO level 2 categories with the largest difference amongst Organic 6 and Mineral fertilizer treatment include amino acid metabolism, lipid metabolism, and metabolism of cofactors and vitamins. In case of No fertilizer treatment, the most diverse functional category as compared to other fertilizer treatments was environmental information processing which mainly includes secretion, signalling, and transporter pathways (Fig. S15).

4. Discussion

4.1. Growth of the different cultivars

We hypothesized that older cultivars would perform better under low organic or no fertilizer treatments (Hypothesis iii), reasoning that these cultivars, having evolved under less intensive traditional agricultural practices, likely retained adaptations for thriving in less fertile soils. Supporting our hypothesis, older cultivars produced significantly more aboveground biomass without fertilizer (Fig. 2A). We also observed a tendency for modern cultivars to produce more aboveground biomass with mineral fertilizer application, aligning with Hypothesis iv. This reflects the breeding objectives of modern cultivars for high productivity under abundant nutrient supply (Lammerts van Bueren et al., 2011).

Typically, older cultivars allocate more resources to both root and shoot growth, unlike modern cultivars, which have been selectively bred to prioritize grain production, often at the expense of overall biomass (Bektas et al., 2016). However, in our experiment, only in one of the seven fertilizer treatments, older cultivars outperformed the modern, in terms of shoot biomass. This suggests that increased shoot biomass is not a universal trait for older cultivars under all fertilizer conditions but rather a specific adaptation to very low nutrient availability.

These results are supported by other studies, which have shown that older cultivars and landraces sustain growth under nutrient-limited and stressful conditions by maintaining high nutrient uptake (Pourazari et al., 2015; Zhu et al., 2022). This is likely achieved through an
enhanced allocation of resources to root biomass (McGrail and McNear, 2021). Additionally, as domestication have significantly affected the root exudate profiles of modern cultivars compared to older relatives (Iannucci et al., 2017), altered exudation profiles between our modern and old cultivars could also explain why the two groups reacted differently in systems with no fertilizer addition.

Contrasting to our results, Rajala et al. (2017) observed that modern cultivars generally outperformed older ones even at low nutrient levels in a study of 195 barley cultivars. In another study, Wacker et al. (2002) found that old and modern winter barley cultivars produced equal biomass without fertilizer, but modern cultivars showed a better response to mineral fertilizer application, consistent with our results.

In our experiment, the oldest cultivar, Langeland, was the main contributor to the higher aboveground biomass in old cultivars in the No fertilizer treatment. However, none of the older cultivars outperformed the modern in any of the organic fertilizer treatments. This suggests, in contrast to what we anticipated (Hypothesis i), that the older cultivars were not better at stimulating mineralization in the presence of organic fertilizer. It is noteworthy, however, that the modern cultivar Feedway did not thrive in any of the organic fertilizer treatments, even at the highest levels. We suggest that Feedway is genetically poorly equipped to grow under organic fertilizer conditions, which is in line with Hypothesis i. Thus, a major conclusion of this study is that although some of our older cultivars perform better under low nutrient and/or organic fertilizer conditions, performance is more dependent on specific choice of cultivars than on cultivar domestication age.

### 4.2. Plant nutrient uptake

Plant nutrient uptake is a key factor for plant growth. Hence, to make relevant comparisons of the different treatments, it was necessary that plants were short of essential nutrients in some of the fertilizer treatments. We succeeded in this approach. Just before harvest, all cultivars, regardless of domestication age, displayed poor growth and clear signs of nutrient deficiency in the lower fertilizer treatments (Fig. 1). The predominant deficiency symptoms were yellow leaves and purple stems, probably related to nitrogen and phosphorus, respectively (de Bang et al., 2021). These symptoms lessened with mineral fertilizer and, for most cultivars, at higher organic fertilizer levels.

However, the effect of organic fertilizer is complex. Besides the nutrients, the organic fertilizer also contains carbon, which stimulates microbiome activity and growth. This may lead to either nutrient mineralization or nutrient immobilization depending on the C/N ratio of the organic fertilizer (Probert et al., 2005). Further, increased microbiome activity may result in oxygen depletion (Siedt et al., 2023) and/or production of toxic metabolites (Duke and Dayan, 2011). Thus, the intricate balance between these factors will determine the net effect of the organic fertilizer. We saw that this balance tipped in disfavour of the plants in the Organic 5 treatment, where all died.

In the modern cultivar Feedway, increased organic fertilizer levels generally affected plants negatively, resulting in severely reduced growth. Whereas this is in line with our Hypothesis i, that modern cultivars cannot utilize organic fertilizer efficiently, the old cultivar Salka also reacted negatively to the highest level of organic fertilizer. The varied response to increased organic fertilizer illustrates the complex interplay between individual cultivar, microbiome, and fertilizer regime, all affecting plant performance (Pour-Aboughadareh et al., 2022; Fekadu et al., 2023).

Low levels of organic fertilizer decreased the growth and performance of all cultivars compared to the No fertilizer treatment. In contrast, higher levels of organic fertilizer boosted growth. This could be because our maize leaf fertilizer had a higher C:N ratio, compared to the soil (18:1 vs. 14:1, respectively). The microorganisms would likely try to balance their C:N ratio by acquiring readily available nitrogen already present in the soil (Sawada et al., 2015). This process would inadvertently reduce the pool of mineral nitrogen available for the plants and explain the decreased growth observed in the low organic treatments. Thus, low levels of organic fertilizer caused net immobilization due to its higher C:N ratio as compared to the soil. In contrast, addition of higher levels of organic fertilizer caused extensive microbial growth and activity, resulting in net mineralization (Hobbie and Hobbie, 2013). As we increased the level of organic fertilizer, we also increased the total amount of labile carbon and nitrogen available to the microbiome, stimulating microbial activity further and thus increasing nutrients available for plant uptake.

### 4.3. Plant nutrient concentration

Overall, older cultivars had significantly higher leaf nitrogen concentration than modern in the No fertilizer treatment (Fig. 2B). The combination of a significantly higher aboveground biomass and leaf nitrogen concentration suggests that on average, the older cultivars acquired nitrogen for growth at non-fertilized conditions more efficiently than modern cultivars, confirming our hypotheses. Similarly, Görny (2001) found that, as compared to modern cultivars, wild barley and old barley landraces, took up nitrogen more efficiently under stressful conditions, when nitrogen was limited. Foulkes et al. (1998) showed that nitrogen uptake and utilization in old winter wheat cultivars was higher than in modern, when no fertilizer was applied. However, addition of optimal levels of mineral fertilizer reversed this pattern, so modern cultivars outperformed older cultivars. These results, combined with ours, show that older cultivars have the potential to use nitrogen more efficiently than their modern relatives, but only when no fertilizer is applied, and nutrient availability therefore is limited.

Including results from more studies makes the picture more ambiguous. Bingham et al. (2012) found no differences in aboveground N content of 15 barley cultivars (released between 1931 and 2005) grown without fertilizer. However, with mineral fertilizer, modern cultivars accumulated significantly more N in the aerial parts compared to older. Furthermore, Abeledo et al. (2008) compared four Argentinian barley cultivars (released between 1944 and 1998) and found no differences in total N in the aboveground biomass, even at the low mineral fertilizer application rate. Muurinen et al. (2006) also found no differences in nitrogen uptake or overall nitrogen use efficiency between 17 Nordic barley cultivars of varying age (from 1902 to 1998), arguing that breeding has exhausted variations in the trait. Again, the results suggest that performance depends on specific choice of cultivars.

Besides nitrogen, we also found that older cultivars accumulated significantly more S, Fe, Zn, and Cu in the leaves than the modern cultivars, when no fertilizer was applied (Fig. 3). Especially deficiencies in Fe and Zn are prevalent in global food systems (Gregory et al., 2017), and our results suggest that certain older cultivars could potentially help alleviate this problem.

In accordance with our results, Schmidt et al. (2019) reported superior Mn, Zn, and Cu concentrations in leaves of a barley landrace adapted to marginal soils compared to modern elite relatives, resulting in improved photosynthesis and yields. Thus, the authors proposed that an interplay between increased root exudates and more efficient and specific root transporters were responsible for the increased uptake. We suspect that similar properties were enhancing the uptake of nutrients in the older cultivars in our experiment. However, as with the shoot biomass and leaf nitrogen concentration, the differences in uptake of other nutrients were most pronounced in treatments without fertilizer addition. This indicates that older cultivars did not significantly stimulate mineralization and thus nutrient acquisition in treatments with the addition of organic fertilizer compared to their modern relatives. We therefore propose that the increased nutrient accumulation in the leaves of older cultivars was not directly an effect of increased interaction with the rhizosphere microbiome, but rather an effect of root physiological characteristics.
4.4. Rhizo-microbiome

The microbial composition in the surrounding soil makes up the pool that defines which microorganisms are available for recruitment to the rhizo-microbiome (Hamonts et al., 2018; Park et al., 2023). From this pool, the actual composition of the rhizo-microbiome will be determined by factors as pH, soil organic matter amount and quality, and inorganic nutrients. Furthermore, plants actively regulate the composition of the rhizo-microbiome by releasing root exudates and through root morphology (Herms et al., 2022; Sasse et al., 2018). As both root exudate profiles and root morphology can differ significantly between cultivars (Iamucci et al., 2017; Iamucci et al., 2021), we expected that the rhizo-microbiomes would also differ between our cultivars. We found that there was a significant interaction between fertilizer regime and cultivar, indicating that the effect of fertilizer on the microbiome differed between cultivars. However, based on our analysis of significantly enriched and depleted genera between cultivars in specific fertilizer treatments we could not see a clear trend explaining this interaction (Fig. S14). Furthermore, based on the alpha diversity and CAP plot (Fig. 4), it was clear that the main driver for changes in microbial diversity and composition was the fertilizer treatment.

While it is not surprising that fertilizer regime and cultivar had a significant impact on the rhizo-microbiome, it was unexpected that domestication age had no effect, which conflicted with our Hypothesis i. In accordance with previous studies (Zhou et al., 2017; Xu et al., 2020), we saw that increasing fertilization, and thus nutrient availability, reduced microbial richness and diversity (Fig. 4A). Like Leff et al. (2015), we found dominance of fewer but fast-growing taxa in the rhizo-microbiome (Fig. 6). Also in line with Dang et al. (2022), organic fertilizer stimulated the growth of the copiotrophic bacterial phyla Bacteriodota and Firmicutes. These phyla both contain genera considered beneficial for plant growth, and therefore important components of the rhizo-microbiome (Jorquera et al., 2012). That organic fertilizer stimulated microbial activity, and therefore carbon mineralization, is also reflected in the reduced soil C:N ratio and increased levels of ammonium and nitrate-N at the higher fertilizer levels (Fig. S13).

Conversely, compared to the No fertilizer and Mineral fertilizer treatments, addition of organic fertilizer reduced the relative abundance of Actino- and Acidobacteriota. These phyla contain important plant growth-promoting genera but are usually considered slow growing oligotrophs, and decomposers in low carbon systems (Bao et al., 2021; Kielak et al., 2016; Brzeszcz et al., 2016). Furthermore, Acidobacteriota are sensitive to increases in pH and thrives in more acidic soils (Conradie and Jacobs, 2020). As we saw that organic fertilizer addition caused an increase in soil pH, the reduction in relative abundance of Acidobacteriota could in part be explained by increasing soil pH.

The relative abundance of Proteobacteria was unaffected by fertilizer treatment, displaying the same relative abundance in all treatments (Fig. 6). Proteobacteria are normally abundant and dominating in soil and rhizobiome (Qang et al., 2022). Other studies have also shown that this phylum remains abundant regardless of fertilizer regime (Ren et al., 2020).

The No fertilizer and Organic 6 treatments resulted in the highest number of rhizo-microbiome indicator species and the broadest functional range of the microbiome (Fig. S15). Furthermore, despite its lower alpha richness and diversity, the Organic 6 treatment exhibited higher microbial activity (Fig. S13) and plant growth (Fig. 2A) compared to the No fertilizer treatment and the other organic treatments. This suggests that addition of high amounts of organic fertilizer created a less diverse and more specific rhizo-microbiome but with high functional capabilities. This environment benefitted all cultivars except Feedway.

In the No fertilizer treatment, the older cultivars collectively had higher shoot biomass and concentration of leaf nutrients (Fig. 2 and Fig. 3). Contrary to the Organic 6 treatment, we observed high alpha richness and diversity in the No fertilizer treatment indicating a more diverse rhizo-microbiome. In line with the Organic 6 treatment however, we also found a significant number of indicator species and a broad functional range of the microbiome. These results indicate that collectively, the older cultivars were more adept at leveraging the diverse and functionally rich soil microbiome for their growth and nutrient uptake when nutrient availability was very low. We attribute this ability to a long history of adaptation to diverse and less controlled soil environments, enabling these cultivars to effectively utilize the broad range of microbial functions, particularly those related to nutrient cycling and environmental stress resilience.

In line with previous studies (Stradnick et al., 2013; Li et al., 2015), addition of mineral fertilizer resulted in less rich and diverse rhizo-microbiomes (Fig. 4), with the lowest amount of indicator species and functionalities (Fig. S15). Clearly, however, these reductions did not negatively affect the cultivars who all benefitted from the addition of mineral fertilizer resulting in higher growth and nutrient uptake (Figs. 2 and 3).

These findings suggest, that the older cultivars thrived relative to modern only in systems with very low nutrient availability, high alpha diversity, and high functional range of the rhizo-microbiome. In treatments where fertilizer was added (both organic and inorganic), all cultivars, regardless of domestication age, were heavily dependent upon the effect of the fertilizer type and amount on the microbiome.

4.5. Limitations and perspectives

This study sheds valuable light on how different barley cultivars respond to fertilization, yet there are limitations to consider. The foremost is the need for extended field trials to validate our laboratory findings in more realistic, natural conditions and with more replicates for robust results. In addition, a more in-depth analysis of soil parameters would enhance our understanding of cultivar-fertilizer interactions and their effects on the rhizo-microbiome.

Advancing to molecular techniques like metagenomics could also provide a detailed view of the rhizo-microbiome and its function, essential for understanding nutrient cycling and plant growth. Despite these limitations, this research lays a foundation for future studies, offering a stepping-stone towards improving agricultural practices and understanding barley-microbiome interactions in response to fertilizer regime.

5. Conclusion

We found that plant performance depended more on choice of cultivars, than on domestication age. Still, the old cultivar, Langeland, for example, performed better in the No fertilizer treatment, and old cultivars overall contained more nutrients, albeit, not in all cases significantly. Our results indicated that these differences are more likely rooted in plant physiological characteristics than in differences in the microbiome associated with domestication age. Furthermore, the results suggest that introducing older barley cultivars and landraces into farming and breeding programs is timely and highly relevant, as they show the potential to grow better and accumulate more vital macro- and micronutrients under very low nutrient availability, compared to their modern relatives.

CRediT authorship contribution statement

Nikolaj L. Kindtler: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. Sanea Sheik: Writing – review & editing, Writing – original draft, Visualization, Validation, Data curation. Emilie Krog: Writing – review & editing, Methodology, Data curation, Conceptualization. Lorrie Maccario: Writing – review & editing, Investigation, Data curation.
Mette Vestergård: Writing – reviewing & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. Rute R. da Fonseca: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. Flemming Ekelund: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Data curation, Conceptualization. Kristian H. Laursen: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Data curation, 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

The raw data used for this project is available at ERDA: https://sid. erda.dk/sharelink/Ra8sVkJ1. All R scripts for analysing the data and generating the figures can be found at: https://github.com/PlantMicrobiome/BetterBarley_16SAmplicon.

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Appendix A. Supplementary data

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References


