Soil microorganisms decrease barley biomass uniformly across contrasting nitrogen availability.

Victoria Munkager[a*], Andreas Altenburger[b], Anders Priemé[a], Toke Bang-Andreasen[ac], Regin Rønn[a], Mette Vestergård[d], Flemming Ekelund[a]

[a]Department of Biology, University of Copenhagen, Copenhagen, Denmark, Universitetsparken 15, 2100 Copenhagen Ø, Denmark

[b]The Arctic University Museum of Norway, UiT - The Arctic University of Norway, Lars Thørings veg 10, 9006 Tromsø, Norway

[c]Department of Environmental Science, Aarhus University, Frederiksborgvej 399, 4000 Roskilde, Denmark

[d]Department of Agroecology, Aarhus University, Forsøgsvej 1, 4200 Slagelse, Denmark

* Correspondence: victoria.munkager@bio.ku.dk, tel.: +45 27 12 16 33, address: Universitetsparken 15, Building 1, 2100 Copenhagen Ø, Denmark

Abstract

Aims Microorganisms play a dichotomous role in the soil nitrogen cycle through mineralization and immobilization. We aimed to understand how nitrogen availability modifies the effect of microorganisms on plant growth. We hypothesized that soil microorganisms would increase plant biomass following amendment with a substrate rich in organic nitrogen (net mineralization), be neutral when adding inorganic nitrogen, and decrease biomass when adding organic nitrogen-limited substrate (nitrogen competition).

Method Barley (*Hordeum vulgare* L., cv. Evergreen) was exposed to either i) limited, organically bound nitrogen, ii) organically bound nitrogen or iii) inorganic nitrogen. In these amendments, we assessed the difference in plant biomass and physiology between plants with or without soil microbiome addition.

Results The soil microbiome reduced shoot biomass equally (12%) across all nitrogen amendments. However, nitrogen availability did modulate the effect of the soil microbiome on plant physiological parameters associated with nitrogen deficiency.

Conclusions The results indicate that the net negative effect of complex microbiomes on shoot biomass is independent of nitrogen availability. Thus, microbiome addition was deleterious to biomass even in a nutrient-stress-free environment. We suggest that strategies for improving plant growth through manipulation of microorganisms should not exclusively focus on beneficial and pathogenic microorganisms, but also include minimizing plant metabolic costs of microbiome interactions.

Keywords Nitrogen competition · Nitrogen mineralization · Plant growth · Plant-microbiome interaction · Protists · Bacteria

Abbreviations:

C: Carbon

C/N ratio: Carbon/Nitrogen ratio

Fv/Fm: Maximum quantum efficiency of photosystem II

N: Nitrogen

n: number of observations

1. Introduction

Soil varies with respect to a myriad of different factors such as nutrient availability, moisture, and salinity that all influence the health and development of plants. Add the living component of the soil including bacteria, fungi and microeukaryotes, and the picture becomes even more complex and unpredictable as several known and unknown interactions occur simultaneously. Some bacteria influence the abundance of other bacteria [1] while others modulate fungal abundance [2]; e.g. strains of *Pseudomonas cepacia* suppress the growth of the pathogenic fungus *Fusarium moniliforme* consequently reducing plant disease [3]. In turn, protists graze on bacteria but with an inclination towards specific bacterial species over others, thereby also altering the bacterial community composition [4].

Much research has been dedicated to understanding which rhizosphere microorganisms can promote plant growth and which are pathogenic or deleterious. Apart from suppressing plant pathogens [5-9] and reducing plant susceptibility to pathogens [10], microorganisms can benefit plants as biofertilizers [11] or by abating abiotic stress [12]. Deleterious rhizobacteria, unlike pathogenic bacteria, do not parasitize the plant but still confer a negative effect such as delayed development or reduced growth of shoots or roots [2]. Despite recent progress, we are far from understanding the conditions that facilitate or hinder plant-microbiome interactions and yield a net effect on plant performance. Rubin, et al. [13] showed that bacteria isolated from the roots of blue grama (*Bouteloua gracilis)* increased shoot biomass when re-inoculated on blue grama, but decreased shoot biomass on maize (*Zea mays*). Additionally, the microbiome effect was stronger under well-watered conditions than under drought conditions. Thus, the effects of a microbiome on plants are not universal across plant species and environmental conditions [14]. Moreover, the net

effect of a particular microbiome on a particular plant is the result of combined positive, neutral, and negative effects [11].

Due to the complexity of plant-microbiome-soil interactions, it is paramount to adopt a reductionist approach to understand how particular abiotic factors affect plant-microbiome interactions before we can piece together the full picture. Previous studies comparing plants grown either with or without a soil microbiome have found contrasting results. Plant biomass was increased by compost microorganisms [15] and re-inoculated root endophytes [16] whereas other studies found the effect on plant biomass to be negative, positive or neutral depending on the origin of the microbial community [17-19].

Research has suggested that the negative effect of microorganisms on plants increased when microorganisms were extracted from rhizospheres of distantly related plants compared to more closely related plant species [17, 20]. This is in line with the theory that plants can recruit specific microorganisms, which confer benefits to the plant [5, 21-24] and that the composition and functioning of a microbiota shaped by plants differ between plant genotypes [25, 26]. Microorganisms that benefit one plant species can be deleterious to another [13]. Contrastingly, it has long been recognized, that continuous monocropping leads to build-up of specific pathogens [27] and that sterilization of low-yielding monocropped soils abated the negative effect [28-30]. On the other hand, disease suppression may develop when the same crop is cultivated continuously [6]. It is evident that plant-soil microbiome interactions do not always develop in favor of the plants and we still lack sufficient knowledge on how soil conditions mediate the plant-microbiome interactions in order to predict whether a particular soil microbiome will contribute a net benefit to plant growth.

Plants need nitrogen for several essential functions, yet most soils are nitrogen-limited [31, 32]. When nitrogen is scarce, plants often increase their root system to scavenge for nutrients [33] and increase root exudation [34]. Increased exudation rates alter the rhizosphere microbiome composition and functioning and it has been hypothesized that increased exudation incites rhizosphere microorganisms to mineralize nitrogen from organic matter by providing a labile source of carbon [35-37]. The microbial loop hypothesis thus suggests that this labile carbon source primes microbial activity and scavenging for N from soil organic sources. Subsequently, predation on the microbial biomass, notably by bacteriovorous protists, results in the excretion of inorganic N [4, 35, 37, 38]. Thus, microorganisms are capable of increasing plant available nitrogen, but microorganisms can also compete with plant roots for nitrogen with an uptake efficiency superior to plants [39, 40]. This way, microorganisms have a dichotomous function in the soil nitrogen cycle, as they can both mobilize and immobilize nitrogen. Therefore, in soils supplied with material with a high carbon/nitrogen (C/N) ratio, most nitrogen end up in the microorganisms (nitrogen immobilization), especially if the soil is already nitrogen-limited [40]. Oppositely, in carbon-limited situations, such as many agricultural soils, the microbiome excretes excess nitrogen during decomposition of organic matter as inorganic nitrogen [40, 41]. Additionally, the short life cycle of microorganisms and the predation of bacteria by protists and other microbivorous organisms release inorganic nitrogen to the soil where plants can access it [37, 38, 40].

Microbial responses to fertilizer addition are less well studied in the rhizosphere than in bulk soil [42]. To our knowledge, no study has focused on how the net effect, i.e. the combined positive and negative impact, of the rhizosphere microbiome on plant growth and survival is related to carbon and nitrogen availability and C/N ratio.

We aimed to address if nitrogen availability mediates the impact of microorganisms on plant nitrogen status. In sterilized microcosms depleted of carbon and nutrients, we performed a full factorial experiment, a) with or without microbiome amendment at b) three nutrient amendments; ground sawdust with high (ca. 10,000) C/N ratio, kidney bean with C/N ratio 11, and inorganic fertilizer without carbon (Table 1). We extracted our microbiome from a soil conditioned by other plant species than barley to mimic agricultural conditions, as barley is rarely grown in soil conditioned by barley due to crop rotation practices and catch crops. Because microbiomes may become more detrimental to plant growth if they have been conditioned by distantly related plant species [20], we extracted our soil microbiome from a soil with different grass species. Based on the C/N ratios and total nitrogen contents of the nutrient amendments, we made four main hypotheses.

Firstly, we hypothesized that microbial growth would increase from the sawdust treatment (high C, low N) to the inorganic fertilizer (low C, high N), and be highest in the bean treatment (high C, high N). We expected this gradient in microbial growth based on both nitrogen and carbon availability, where root exudation would play an important role in the supply of labile carbon to the rhizosphere microorganisms, especially in the inorganic fertilizer treatment.

Secondly, we hypothesized that the microbiome amendment would improve plant nitrogen status compared to plants without microbiome amendment in the bean treatment. Most of the nitrogen in the bean treatment was organically bound and thus largely unavailable to the plants unless mineralized by microorganisms. The bean substrate had a C/N ratio of 11 and was therefore expected to favor net nitrogen mineralization [43, 44].

Thirdly, we hypothesized that the microbiome amendment would have little effect on plant nitrogen status in microcosms with the inorganic fertilizer treatment. A surplus of nitrogen was added and immobilization of nitrogen would not affect plant nitrogen status due to low availability of carbon consequently limiting microbial growth.

Finally, we hypothesized that the microbiome amendment would negatively affect plant nitrogen status in the sawdust treatment compared to plants without microbiome amendment. The sawdust amendment contained organically bound nitrogen in a very low quantity which would limit growth for both microorganisms and plants creating a system where the soil microorganisms would scavenge any accessible nitrogen.

2. Materials and methods

2.1. Experimental design

We conducted the plant microcosm experiment in a climate chamber to investigate how sources of nutrients may alter the effect of soil microorganisms on plant growth and physiology. Plants were grown in microcosms treated with three nutrient sources varying in available nitrogen and carbon, either with a complex community of soil microorganisms or without.

We constructed the plant microcosms in 50-ml Falcon tubes modified to seal off the belowground compartment from exogenous microbial contamination in a time- and cost-efficient way (see Fig. 1 for details). As a tradeoff, the 50-ml microcosms provided limited rhizosphere space, which is known to inhibit root development and thus will not reflect field conditions perfectly [45]. The microcosms were filled with 75 g (50 ml) sand (quartz sand type: no. 2, particle size: 0.71-1.22 mm, Dansand A/S, Brædstrup, Denmark), ignited (550 °C, 6 hours) to remove all organic material and

autoclaved at 121˚C to create an inert substrate free of microorganisms, organic matter, and nutrients.

Location of Figure 1

2.1. Treatments

We used a full factorial experimental design, with three different nutrient amendments with or without soil microbiome addition; 15 microcosms were prepared for each of the six nutrient and microbiome treatment combinations; i.e. a total of 90 microcosms.

2.1.1. Nutrients

The three nutrient amendments (Table 1) consisted of i) 100 mg ground, dried, red kidney bean equivalent to 4.4 mg nitrogen (N) and 45.7 mg carbon (C) (C/N ratio: 11), ii) 100 mg ground sawdust, equivalent to 0.005 mg N and 50.7 mg C (C/N ratio: 10636), and iii) 10 ml of inorganic plant fertilizer (Substral Vita Plus, Substral, Skovlunde, Denmark) equivalent to a total of 4.62 mg N and 0 mg C (C/N ratio: 0). All nutrients were autoclaved (121 ˚C). The 100 mg of bean and sawdust was mixed with the sand substrate before transplanting the seedlings. For the inorganic fertilizer treatment, 5 ml (7 ml Substral per 1000 ml ddH_2O) was pipetted into the sand substrate before transplanting and another 5 ml was pipetted into the sand halfway through the experiment. The nutrient dose was split in two for the inorganic fertilizer because the nitrogen was readily available for uptake unlike the organically bound nitrogen in sawdust and bean.

2.1.2. Microbiome

For the microbial inoculum, we collected soil from the top 3 cm of a grass lawn dominated by annual meadow-grass (*Poa annua*) and common meadow-grass (*Poa pratensis*) (location: 55°42’6”N 12°33’32”E). We mixed 10 g of fresh soil with 1 l of sterile ddH_2O, followed by

189 shaking for 60 min on a rotary shaker. The mixture was allowed to settle for 40 min and the
190 supernatant was separated from debris and sediment by decantation. We used one ml of this
191 supernatant as inoculum for each of the microcosms destined to receive microbiome amendment
192 (see below). This means that each microcosm was inoculated with the number of microorganisms
193 that could be expected from approximately 10 mg of soil. This would provide enough
194 microorganisms to represent a diverse microbial community.
195

Table 1. Nitrogen, carbon and C/N ratio of nutrient amendments and hypothesized outcome of nutrient and microbiome treatments.

	C (mg C g^{-1})	N (mg N g^{-1})	C/N	Hypothesized effect of microbiome amendment on plant nitrogen status	Presumed plant-accessible N
Sawdust	50.7	0.005	10,000	Negative, due to N competition	Very low
Bean	45.7	4.4	11	Positive, due to mineralization of organic N	Low
Inorganic fertilizer	0	4.6	0	No effect, N already plant available	High

196

197 **2.2. Plants**

198 Spring barley grains (*Hordeum vulgare* L. cv. Evergreen, Nordic Seed Galten, Denmark) were
199 stored at 4˚C prior to use. Evergreen is a high-yielding (87.3 hkg/ha), mlo-resistant cultivar suited
200 for organic agriculture. We sterilized 200 undamaged grains within the weight range of 55±5 mg in
201 250 ml 1% $AgNO_3$ (w/w) for five minutes and rinsed in 30 ml sterile ddH_2O [46], five consecutive
202 times. Sterilized grains were germinated in the dark (15 °C, 48 hours) on Potato Dextrose Agar
203 plates (39 g L^{-1} ddH_2O, Sigma) with ten grains per plate. The sterilization procedure should result in
204 88% sterility [46], so we only used grains from non-contaminated plates. A single grain was placed
205 in each microcosm in the middle of the largest pipe. A volume of 1 ml of the microbial inoculum or
206 sterile ddH_2O was pipetted on the germinated grain for the microbiome and control treatment
207 respectively, before sealing the pipe with sterilized, water-repellent cotton wool. We changed the

208 position of the cotton wool around the growing seedling when needed, to keep the microcosms
209 sealed without hindering plant growth. Seedlings which never emerged above the plastic tube were
210 discarded (n=13, Table S1). Microcosms were checked daily and watered when needed with sterile
211 ddH_2O to maintain the water level at 15 ml per microcosm. Microcosms were placed in a climate
212 chamber at 23.5 ˚C on a 14/10 h day/night cycle. Photon flux was set to 228 µmol m^{-2} s^{-1}.

213

214 **2.3. Plant measurements**

215 The objective of our study was to assess whether nitrogen availability mediates the effect of
216 microorganisms on plants. We therefore focused on plant parameters often affected by nitrogen
217 availability: survival, biomass, root volume, chlorophyll content index, and nitrogen content [47-
218 54]. We also assessed shoot length and phenological stage, as microorganisms are known to alter
219 plant development [55]. Measuring the maximum quantum efficiency of photosystem II, allowed us
220 to test whether microorganisms altered the photosynthetic capacity of the plants.

221

222 Shoot length, measured from the ground to the tip of the longest leaf, was estimated semiweekly.
223 Each week, we measured chlorophyll content index thrice per plant on the healthiest looking area of
224 the leaf tissue using a chlorophyll meter (SPAD-502, Konica Minolta, Japan). Weekly, the
225 maximum quantum efficiency of photosystem II, measured as the ratio of variable fluorescence
226 over the maximum fluorescence value (Fv/Fm), was quantified on a chlorophyll fluorimeter (Handy
227 PEA, Hansatech Instruments, King´s Lynn, UK) after dark adapting the leaves for at least 35 min.
228 Chlorophyll content index and fluorescence were measured in accordance with the manuals of the
229 manufacturers.

230

231 Before harvesting, we assessed the phenological stage in accordance with the BBCH scale for
232 cereals [56].
233
234 Microcosms were destructively sampled under sterile conditions 30 days after transplanting. The
235 shoot was separated from the roots just above the remains of the grain to keep the root system
236 intact. The microcosms were limited in space and the root system was dense throughout the whole
237 Falcon tube. Therefore, all the sand substrate was sampled as rhizosphere soil. The sand substrate
238 was frozen at -20˚C for later quantification of bacteria, carbon, nitrogen, and pH. The root systems
239 were washed to remove sand particles and kept moist until the root volume was estimated by the
240 software WinRhizo (Regent Instruments, Quebec City, Canada) in accordance with the
241 manufacturer's manual. Dry weight was measured when sample weight was stabilized at 60˚C after
242 72 hours.
243
244 **2.4. Quantification of bacteria and protists**
245 The abundances of bacteria and protists were quantified to test whether our hypothesis that Bean
246 was the most optimal growth substrate and sawdust the least optimal. Furthermore, the
247 quantification of bacterial and protist abundances of the un-inoculated treatment was used to control
248 whether the modified Falcon tubes prevented microbial contamination.
249
250 We quantified the copy number of bacterial 16S rRNA gene in the rhizosphere (i.e. the sand
251 particles removed from the plant roots as described above) using quantitative PCR (qPCR). DNA
252 was isolated following the manufacturer's protocol using Powerlyzer™ Powersoil® DNA isolation
253 kit (MoBio, Carlsbad, California, USA) with the following exceptions: Our centrifuge needed extra
254 time to reach 10,000 x g, so 60 seconds was added to the recommended centrifuge time. Extracted

255 DNA samples were stored at -80˚C. qPCR was carried out on a real-time T100™ Thermal Cycler
256 (Bio-Rad, Copenhagen, Denmark) with technical duplicates of 20 µl containing 4 µl HOT FIREPol
257 "EvaGreen" qPCR Supermix (Solis Biodyne, Riia, Estonia), 1 µl bovine serum albumin (BSA) (20
258 mg mL^{-1}, Bioron, Römersberg, Germany), 1 µl DNA template (10 times diluted), 12.2 µl ddH_2O,
259 and 0.4 µl (10mM) of the forward and reverse primers [341F: 5′-CCTAYGGGRBGCASCAG -3′
260 and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′] [57]. Triplicates of sterile water were used as
261 negative control and a dilution series of genomic DNA from *Escherichia coli* K-12 (with seven
262 copies of 16S rRNA genes) was used as a standard [58, 59]. Thermal conditions were an initial
263 denaturation at 95°C for 12 min followed by 40 amplification cycles of 95°C for 15 secs, 56 ˚C for
264 30 secs, and 72°C for 30 secs, and finally 72°C for 3 min. No-template controls gave negligible
265 values.
266
267 The number of cultivable protists were estimated by a most probable number method, where we
268 prepared eight replicated 1:3 dilution series (based on 5 g rhizosphere material in 100 ml Neff's
269 amoebae saline [60]) in 96-well microtiter plates (Costar® 3598, Corning, Vordingborg, Denmark)
270 with 0.1 ml of 0.1 g L^{-1} tryptic soy broth (TSB) (#211825, Becton-Dickinson, Lyngby, Denmark)
271 [61]. The plates were stored in the dark at 15 °C, and the wells were examined for the presence of
272 flagellates, amoebae and ciliates after one and three weeks using an inverted microscope (200 x
273 magnification) (Olympus CK X31). The most probable numbers were calculated as in Briones and
274 Reichardt [62].
275
276 **2.5. pH, nitrogen and carbon**
277 pH was determined from rhizosphere substrate using a pH-meter. We measured the carbon and
278 nitrogen content in the sand and in the ground bean and sawdust. Total carbon and nitrogen were

279 measured in tin capsules of 300 mg pulverized sand, bean, or sawdust with TruSpec Carbon
280 Nitrogen Determinator (LECO, St. Joseph, Michigan, USA). Shoot and root nitrogen were
281 measured on 4 mg finely crushed samples with EuroVector Elemental Analyzer (EuroVector,
282 Pavia, Italy) using Dumas combustion samples weighed into tin capsules.
283
284 **2.6. Statistics**
285
286 All statistical analyses were run in R (version 3.3.1 3) Statistically significant differences were
287 based on a .05 significance level. Main effects and interactions were removed when $P < .05$ and .1,
288 respectively. All plots were generated using the R package "ggplot2" [63].
289
290 *2.6.1. Survival*
291 Due to perfect separation of the survival data, the differences in survival between plants inoculated
292 with or without microbiome amendment were analyzed with a Firth's Bias-Reduced logistic
293 regression (R package "logistf" [64]), followed by a likelihood-ratio test. The analyses were run
294 independently for each of the three nutrient treatments.
295
296 *2.6.2. Plant, microbial, and rhizosphere parameters*
297 To evaluate effects of nutrient and microbiome amendment on plants (shoot and roots) biomass,
298 root volume, phenological stage, nitrogen and carbon content (shoot and roots), the rhizosphere
299 substrate (total nitrogen and carbon, pH), and the rhizosphere microbiota (16S rRNA gene copy
300 numbers, and MPN of protists), we analyzed each variable individually with a two-way analysis of
301 variance with nutrient amendment and microbiome amendment as the factorial variables. Analyses
302 were run on a subset where replicates with dead plants were removed. Normality and

homoscedasticity were confirmed with QQ plots and scale-location plots, respectively. A likelihood-ratio test was run to test for significant *P*-values of an interaction and main effects. A Tukey's test on least-squares means (R package "emmeans" [65]) was used to identify significant differences between treatment combinations.

2.6.3. Chlorophyll content index, chlorophyll fluorescence, and shoot length

To analyze the effects of nutrient and microbiome amendment on chlorophyll content and chlorophyll fluorescence we used a three-way ANOVA between the three categorical variables nutrient treatment, microbiome treatment, and time. For shoot length, we ran an ANOVA on a linear mixed effect model with random effect of plant, and residual errors within plants with an exponentially decreasing correlation (R package "nlme" [66]). The model was reduced stepwise using Akaike Information Criterion (AIC). A Tukey's test on least-squares means (R package "emmeans" [65]) was used to identify significant differences between treatment combinations.

3. Results

3.1. Biotic rhizosphere characteristics at harvest in response to nutrients and microorganisms

3.1.1. Nutrient source and microbial abundance

In the microcosms inoculated with soil microorganisms, 16S rRNA gene copy numbers (Fig. 2) and protists numbers (Fig. 3) were highest in the bean treatment followed by the inorganic fertilizer treatment whereas the sawdust treatment had very low abundances of both bacteria and protists. In microcosms with microbiome amendment, the bean treatment had 50-fold more 16S rRNA gene copy numbers and more than 250-fold higher abundance of protists (MPN g^{-1} rhizosphere substrate) than the sawdust treatment, while in microcosms amended with inorganic fertilizer the abundance of bacteria and protists was approximately 10 times higher than in the sawdust treatment.

Location of Figure 2 and 3

3.1.2. Bacterial and protist abundance in microcosms not amended with microorganisms

In 19 out of the 39 replicates in the control treatment, we did not detect bacterial 16S rRNA genes or protists. The 20 remaining replicates had no protists, but contained some bacteria, yet in significantly lower quantity (60,675±11,103 16S rRNA gene copy numbers mg^{-1} rhizosphere substrate) than the microcosms inoculated with a microbiome. The difference between the microcosms with and without microbiome amendment was highest for the bean treatment. Here, microbiome amendment led to more than a 300-fold increase in 16S rRNA gene copy numbers compared to controls. For sawdust, where nitrogen was limited, microbiome amendment only led to a 3-fold increase in 16S rRNA gene copies per mg compared to controls. In microcosms with inorganic fertilizer, microbiome amendment increased 16S rRNA gene copy numbers 10-fold compared to those without microbiome amendment.

3.2. Abiotic rhizosphere characteristics at harvest in response to nutrients and microorganisms

Soil microbiome amendment caused a reduction in total nitrogen in microcosms amended with nitrogen-rich substrates (bean and inorganic fertilizer), although this was only statistically significant for the bean treatment (Table S2). Amendment with nitrogen-poor sawdust caused a nearly significant increase in rhizosphere nitrogen, in microbiome amended systems (P=.06, Table S2). Total rhizosphere carbon varied little between inoculated and non-inoculated systems regardless of nutrient treatment (Table S2). Soil microorganisms slightly raised pH in systems amended with bean substrate, but no difference was found in the microcosms amended with sawdust and inorganic fertilizer. Overall, pH ranged between 7.2 and 7.7 (Table S2).

3.3. Plant survival and development in response to nutrients and microorganisms

Location of Fig. 4 and 5

Replicates where plants did not emerge above the microcosm lid were eliminated from the experiment, so plants categorized as "dead" initially showed growth, subsequently followed by withering and death. In all microcosms without microbiome amendment, plants survived until harvest, whereas with microbiome, only plants with inorganic fertilizer had 100% survival (Fig. 4). With microbiome amendment, 5 out of 14 plants died before harvest when fertilized with bean. In microcosms with microbiome amendment and sawdust, 13 of 14 plants survived. The difference was not significant.

Plant aboveground biomass (dry weight) declined when inoculated with soil microbiome for all three nutrient treatments (Fig. 5A) whereas belowground dry weight (Fig. 5B) and root volume (Fig. S1) only declined with the application of inorganic fertilizer. The relative loss in aboveground biomass caused by soil microorganisms was very similar across nutrient treatments and ranged between 12.4-12.6%. The soil microbiome amendment increased shoot/root ratio in systems with inorganic fertilizer whereas they decreased shoot/root ratio with bean amendment (Fig. S2). In microcosms with sawdust, the microbiome amendment had no effect on the ratio (Fig. S1). By contrast, microbiome amendment had no effect on shoot length (except for inorganic fertilizer, day 19) and phenological stage regardless of nutrient amendment (Fig. S3 and Fig. S4, respectively).

3.4. Plant physiology in response to nutrients and microorganisms

The chlorophyll content index did not differ between plants amended with soil microbiome and those without for any of the nutrient treatments (Table 2). The nitrogen contents of shoots (Table 2)

was also unaffected by soil microbiome amendment in systems with sawdust and inorganic fertilizer. Similarly, we found no difference due to microorganisms in the maximum quantum efficiency of photosystem II (Fv/Fm) for plants fertilized with inorganic fertilizer (Table 2). Plants in the sawdust treatment were too small to measure Fv/Fm, so data is missing. Contrastingly, the bean treatment led to an increase in Fv/Fm by the second half of the growth period and a decrease in nitrogen content of shoots when amended with soil microbiome. We detected no differences in root nitrogen for any of the nutrient amendments in response to the microbiome amendment (Fig. S5).

As time progressed, the chlorophyll content index was stable for plants receiving inorganic fertilizer, but declined close to harvest for the two other nutrient amendments (Table 2). By the end of the experiment, Fv/Fm had declined for plants receiving bean or inorganic fertilizer (Table 2).

Table 2. Plant physiological response to microbiome and nutrient amendment.

		Chlorophyll content index (AU)			**Shoot nitrogen content mg N g^{-1} leaf dry weight**	
		13 DAT[a]	20 DAT[a]	27 DAT[a]		
Sawdust	Control	25.2±1.4 (13)	12.6±0.9 (13)	9.5±0.8 (13)	8.9±0.9 (13)	*P*=.29
	Inoculated	26.2±0.7 (13)	12.3±0.9 (13)	10.4±1.2 (13)	9.6±2.1 (13)	
Bean	Control	31.0±0.8 (14)	15.1±1.6 (14)	19.2±1.3 (14)	14.1±1.1 (14)	***P*=.03**
	Inoculated	25.9±1.1 (9)	15.6±1.7 (9)	18.3±2.5 (9)	12.3±1.9 (9)	
Inorganic fertilizer	Control	37.7±0.4 (12)	39.1±0.7 (12)	37.3±0.9 (12)	17.7±2.5 (13)	*P*=.23
	Inoculated	37.9±0.8 (14)	36.7±0.7 (14)	35.3±0.8 (14)	16.6±3.1 (13)	
		Inoculation: F(1,215)=0.5, *P*=.50			Inoculation x nutrient:	
		Nutrient: F(2,215)=624, *P*=<.0001***			F(2,69)=3.0, *P*=.055	
		DAT: F(2,215)=131, *P*=<.0001***				
		Nutrient x DAT: F(4,215)=34, *P*=<.0001***				

Chlorophyll fluorescence (Fv/Fm) (AU)

		8 DAT[a]		14 DAT[a]		21 DAT[a]	28 DAT[a]	
Sawdust	Control	0.82±0.003 (12)	[b]	0.81±0.003 (13)	[b]	0.76±0.008 (13)	0.78±0.025 (3)	[b]
	Inoculated	0.82±0.002 (13)		0.78±0.27 (13)		0.77±0.010 (12)	NA (0)	
Bean	Control	0.82±0.002 (13)	*P*=.67	0.81±0.004 (14)	*P*=.50	0.74±0.004 (14)	0.71±0.049 (3)	***P*=<.0001**
	Inoculated	0.82±0.002 (8)		0.81±0.004 (9)		0.77±0.007 (9)	0.78±0.016 (3)	
Inorganic fertilizer	Control	0.83±0.002 (11)	*P*=.98	0.82±0.001 (12)	*P*=.61	0.81±0.002 (12)	0.80±0.005 (12)	*P*=.30
	Inoculated	0.83±0.002 (14)		0.82±0.002 (14)		0.81±0.006 (14)	0.80±0.004 (14)	
Inoculation x nutrient x DAT[a]: F(3,160)=5.5, *P*=<.0001***								

[a] DAT= Days after transplanting

[b] For Fv/Fm no regression was done on sawdust treatment as plants were too small to acquire sufficiently replicated measurements

4. Discussion

A myriad of morphological, physiological, and molecular changes can co-occur in a plant when exposed to a rhizosphere microbiome. Our study illustrates that the presence of a complex soil microbiome reduced plant aboveground biomass by 12% regardless of the availability of nitrogen. Yet, the microbiome effects on plant survival, biomass allocation between shoot and roots, maximum photosynthetic capacity and nitrogen status were all modulated by rhizosphere nutrient availability. Thus, we suggest that for our specific plant-microbiome interaction, the negative net effect on shoot biomass cannot be explained solely by nitrogen competition between the plant and rhizosphere microorganisms despite evidence that the rhizosphere nutrient availability modified the interaction in other ways.

399 We grew barley plants in inert substrate deplete of carbon and nutrients. Our three nutrient
400 amendments were designed to test the microbial effect on plant growth under varying nitrogen
401 availability. Expectedly, in microcosms amended with a surplus of plant available nutrients
402 (inorganic fertilizer), plants produced more biomass, had a higher chlorophyll content index, and
403 more nitrogen than plants in systems amended with organically bound nitrogen (sawdust and bean),
404 which required enzymatic breakdown by microorganisms to release inorganic nitrogen. From a
405 microbial perspective, the most nitrogen-limited system, sawdust, was carbon-rich, but since it
406 contained little nitrogen (Table 1), microbial growth was minimal (Fig. 2 and 3). The most optimal
407 substrate for microorganisms was bean, providing both nitrogen and carbon. This manifested in 50-
408 and 250-fold higher abundances of bacteria and protists, respectively, than in sawdust. The
409 abundance of bacteria and protists in systems with inorganic fertilizer and microbiome amendments
410 was 10-fold higher than in sawdust. Thus, the inorganic fertilizer amendment was more optimal for
411 microorganisms than sawdust, but less optimal than bean. This was in line with our initial
412 assumption because whilst inorganic fertilizer contained a quantity of nitrogen similar to the bean
413 substrate, no carbon was provided. Consequently, the microorganisms relied on the plant to supply
414 carbon via root exudates [67, 68].
415
416 Generally, limitations to plant-available rhizosphere nitrogen manifest as reduced biomass,
417 chlorophyll content, and total nitrogen per leaf mass. These three symptoms co-occur and have been
418 shown under both field and laboratory conditions for various plant species [47-52]. A fourth
419 common symptom of nitrogen limitation is a reduction in the shoot/root ratio [54, 69]. In
420 microcosms with bean, amendment with the soil microbiome unexpectedly reduced shoot dry
421 weight, shoot nitrogen, and shoot/root ratio.
422

We measured chlorophyll content index on the healthiest looking part of the plants, which may explain why total nitrogen per shoot mass showed an adverse effect of soil microbiome amendment, while a change in chlorophyll content index was undetectable. Thus, it seems likely that the soil microorganisms exacerbated plant nitrogen limitation in systems amended with bean. This finding contrasts our first hypothesis as we expected the bean substrate to provide an opportunity for microorganisms to assist plant nitrogen uptake through enzymatic breakdown of the organic bean substrate. The substrate C/N ratio of 11, was well below the threshold of 20-25 generally reported as the optimal ratio for microbial consumption [43] and thus should have favored net nitrogen mineralization [44]. Similar to our study, Weidner, et al. [70] found that when only organically bound nitrogen was provided, microbial diversity, positively correlated to abundance, increased, which reduced soil NO_3^- and *Arabidopsis thaliana* root nitrogen content after four weeks. In the bean treatment, soil microbiome amendment decreased the average rhizosphere nitrogen by almost 70%. As we measured total nitrogen, microbial nitrogen is included in this average. In the bean systems, plants were smaller, shoot nitrogen was reduced by 13%, and root nitrogen was unaffected by soil microbiome amendment. Consequently, nitrogen must have been lost from the plant-microcosm systems in the bean-microbiome treatment. The growth substrate, sand, has high drainage and the microcosm were plastic tubes thereby preventing leaching, thus it seems likely that reduction of NO_3^- to gaseous nitrogen, mainly N_2, by denitrifying bacteria explains the loss of nitrogen in the bean-microbiome systems. The significant increase in rhizosphere pH in systems amended with bean and microbiome is another indication of denitrification as the process is known to raise pH [71]. In the short-term, microorganisms are superior to plants in the battle for nitrogen [39, 72], thus competition for mineralized nitrogen between plants and denitrifying bacteria would likely have favored the activity of denitrifying bacteria due to the presumed anaerobic conditions in parts of the rhizosphere. This could explain the symptoms of N-limitations in plants from the bean-

447 microbiome systems. Plants are especially sensitive to nitrogen limitation during the early stages
448 [70], which can affect seedling survival [53]. Additionally, roots exposed to prolonged oxygen
449 deficiency also lead to diminished survival [73]. Thus, the reduced survival of plants in systems
450 with bean and soil microbiome amendment could be explained by nitrogen limitation, oxygen
451 deficiency, or a combination of both.
452
453 When inorganic fertilizer was added, all plant-essential nutrients were provided in a form available
454 for plant uptake independently of microorganisms and in such a surplus, that plant-microorganism
455 competition for nitrogen would not be a limiting factor. By adding inorganic fertilizer, we could
456 explore which net effects on plant morphology and physiology were altered by soil microorganisms
457 independently of nitrogen competition. In contrast to systems amended with bean substrate, plant
458 survival and shoot nitrogen content was not affected by soil microorganisms. The shoot/root ratio
459 actually increased in response to soil microorganisms, thereby showing the opposite effect of what
460 would be expected from nitrogen limitation. In plants amended with soil microbiome and inorganic
461 fertilizer, we only found a single symptom of nitrogen limitation, i.e. reduced biomass. Biomass
462 reduction is not necessarily a sign of nitrogen deficiency as several other factors, e.g. reduced
463 photosynthetic capacity and an activated defense system [74] can also inhibit growth.
464 Consequently, the microorganisms do not appear to have caused plant nitrogen deficiency in the
465 inorganic fertilizer treatment.
466
467 The bean substrate induced nitrogen competition favoring microorganisms over plants whilst
468 systems with sawdust and inorganic fertilizer minimized the nitrogen competition because there
469 was no nitrogen to compete for in the first and plenty for all in the latter. Yet, despite these
470 contrasting conditions, which led to varying degrees of plant nitrogen limitation, soil

471 microorganisms caused a 12% shoot biomass reduction equally across the three nutrient
472 amendments. Besides the nitrogen limitation of the bean and sawdust treatments, the plants were
473 grown under otherwise optimal conditions without water, temperature, or light stress and we
474 observed no visible symptoms of pathogenic infections. Plants amended with inorganic fertilizer
475 were all healthy-looking plants. This strongly indicates that the deleterious net effect of
476 microbiomes on plant biomass in our microcosms was not significantly modified by nitrogen
477 availability. This is in accordance with a study that found no evidence that the positive effect of
478 microorganisms on plant biomass was due to improved macro- or micro-nutrient status [15], but is
479 in contrast to the hypothesis that under optimal, stress-free conditions, microorganisms have
480 minimal to no effect on plants [10].
481
482 We suggest that the reduction in plant biomass due to soil microorganisms could be linked to an
483 altered sink allocation of assimilated carbon by the plants. When plants are exposed to rhizosphere
484 microorganisms, several events can occur. Up to 40% of the carbon photosynthetically fixed by
485 plants become root exudates [67, 68] but the presence of microorganisms has been shown to
486 significantly increase the exudation rate [68] presumably because the microbial turnover of
487 exudates increases the concentration gradient of metabolites across the root-soil interface, which
488 results in greater exudates efflux [68]. Whether the increased carbon efflux imposed by microbial
489 exudate usage solely explains the reduced carbon allocation to plant biomass production may be
490 resolved by testing if addition of surplus labile carbon (e.g. glucose) to inoculated systems will
491 alleviate the biomass decrease. Further, microorganisms can penetrate the plant tissue e.g. through
492 wounds [75] and more carbon-containing compounds and nutrients are directed towards the
493 infected plant tissue area during a pathogen attack [67, 75]. Currently, it is not fully understood how
494 plants differentiate between pathogenic and non-pathogenic microorganisms that are closely related

[67]. If the differentiation between friend and foe is incomplete under some circumstances, perhaps some non-pathogenic microorganisms also activate the aforementioned reallocation of carbon away from building biomass.

Plants host gene regulation showed similarities between exposure to the commensal bacteria *Sphingomonas melonis* FR1 and the pathogenic bacteria *Pseudomonas syringae* DC3000 [76], leading the authors to suggest that at least some parts of the plant immune system were activated by the commensal bacteria as well. The plant immune system is highly complex, but some mechanisms involved are production of reactive oxygen species, nitrogen oxide, and antimicrobial metabolites as well as hormone signaling [67]. To our knowledge no study has quantified the energy and production costs of these mechanisms, but most likely increased exudation, activation of the immune system and potentially also a redirection of carbon towards microbial entry points due to the presence of microorganisms are not cost-free processes for plants. As our study showed no indication that the photosynthetic apparatus was altered by the soil microorganisms, we hypothesize that the deleterious effect on above-ground biomass could be caused by a redirection of resources from biomass accumulation towards interactions with rhizosphere microorganisms. Further research is needed to provide a mechanistic understanding of how non-pathogenic soil microorganisms can alter the resource allocation in plants leading to lowered plant growth. If rhizosphere microorganisms per default lower plant growth not solely because of pathogens, strategies for improving plant growth through manipulation of microorganisms do not exclusively mean identifying the beneficial and pathogenic microorganisms, but could also include minimizing the metabolic cost of interacting with microorganisms.

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521

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7. Figure legends

Fig. 1: Microcosms were built from Falcon tubes (50 ml, Greiner Bio-One cat. No. 227 261), where lids were modified with two holes, fitted with plastic pipes made from the broadest half of one 1 ml and one 100 µl pipette tip. The smallest pipe was sealed with a cotton swap and used for watering (blue arrow). The largest pipe was sealed with cotton wool around the plant stem (black arrow). The Falcon tubes were covered by tin foil to avoid sunlight as to mimic natural soil conditions. All microcosm components were autoclaved at 121 ˚C and all work was done under sterile conditions.

Fig. 2: Mean (±SE) bacterial 16S gene copies (mg^{-1} rhizosphere substrate) across nutrient and microbiome treatments (n=9-14).

Fig. 3: Mean (±SE) number of protists (MPN mg^{-1} rhizosphere substrate) across nutrient and microbiome treatments (n=9-14).

Fig. 4: Mean (±SE) survival percentage of spring barley (*Hordeum vulgare* L. cv. Evergreen) at harvest (30 days after transplanting) in response to nutrient treatment and microbial inoculation treatments (n=12-14). *P*-values are based on Firth's bias-reduced logistic regression at significance level <.05 comparing control vs. microbiome amendment separately for each nutrient amendment.

Fig. 5: Mean weight (±SE) in mg of A. shoot dry weight and B. root dry weight of spring barley (*Hordeum vulgare* L. cv. Evergreen) at harvest (30 days after transplanting) in response to nutrient and microbiome amendment (n=9-14). In the top left corner of each graph is given the results of the highest order of significant effects (interaction or main) from a two-way factorial ANOVA. For models with a significant interaction, the P-values are provided from a Tukey's test on least-squares means adjusted for multiple comparisons testing for significant differences between control and microbiome amendment within each nutrient amendment.

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