SoilCare
SOILCARE FOR PROFITABLE AND SUSTAINABLE CROP PRODUCTION IN EUROPE

Soil fungal diversity in long-term experiments focusing on soil improving cropping systems

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SoilCares Research

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SoilCare
SOILCARE FOR PROFITABLE AND SUSTAINABLE CROP PRODUCTION IN EUROPE

SoilCares Research

Soil Fungal Diversity in Long Term Experiments

Focusing on Soil Improving Cropping Systems

D.P. Di Lonardo, S.E. Hannula, A.J. Termorshuizen, P. van Erp

Title EU project:	SoilCare
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Short lay summary

The overall aim of SoilCare (https://www.SoilCare-project.eu/) is to assess the potential of soil-improving cropping systems and to identify and test site-specific soil-improving cropping systems that have positive impacts on profitability and sustainability in Europe. As part of SoilCare, this research report presents the results of a study that aims to develop a method to measure soil quality.

Current methods to measure soil quality are too expensive for farmers. A cheap method by which important chemical soil characteristics can be measured such as organic matter content, pH, nutrients and texture, is based on near- and mid-infrared (NIR/MIR) technology. Since it is known that NIR and MIR are quite sensitive especially for organic matter, we thought that fungal composition of soil samples could also be measured using NIR/MIR, since many fungi heavily depend on organic matter. The basic question for this research was, therefore, whether fungal composition, as determined using modern DNA techniques, is different for differently managed soils. This we studied in 12 existing long-term experiments (LTEs) in 4 countries (Denmark, Hungary, UK and Belgium) that aimed to design soil-improving cropping systems (SICS), such as minimum-tillage and including cover crops in the rotation.

All LTEs except one gave at least one significant effect of a soil-improving treatment on the fungal composition. Those LTEs that had been installed longer and had a more extensive experimental set-up clearly showed more significant effects than those that had been installed more recently. Anyhow, for all LTEs variation in results was rather large as well as variation between LTEs. This was especially clear for the number of fungal species that showed a significant effect. We concluded that a general parameter reflecting soil quality based on fungal composition is not yet possible. One reason for this could be that the work relies on a database for identification of DNA sequences, and that the number of species included in that database is still limited.

Although we did not manage to find overarching ecological patterns from the fungal dataset, this work provided valuable, novel, and appreciated information that could be used by the site managers of the LTEs that were involved in this study. For example, although pathogens are not suitable for predicting soil quality, the presence of certain pathogens can affect the crop choices in the near future.

Summary

The overall aim of SoilCare (https://www.SoilCare-project.eu/) is to assess the potential of soil-improving cropping systems and to identify and test site-specific soil-improving cropping systems that have positive impacts on profitability and sustainability in Europe. As part of SoilCare, this research report presents the results of a study that aims to develop a method to measure soil quality.

Maintaining or improving soil quality and soil health is crucial for crop production and can, in particular, contribute to remediating subtle forms of soil degradation. To quantify the health/quality status of a soil is not easy. Generally speaking, the better a method reflects soil health or quality, the more difficult and thus costly the method is. For example, methods to quantify soil structure such as bulk soil density and organic matter stability are too costly to be performed on a regular and field-specific basis. Hence, for farmers more affordable methods to assess soil health are urgently needed.

Since soil organisms depend on soil organic matter and other factors that are affected by soil structure, including soil moisture-related factors such as water holding capacity and aeration, it is likely that their communities reflect, at least partially, different statuses of soil quality. A great range of organisms occur in soil, including viruses, bacteria, oomycota, fungi, protozoa, nematodes and earthworms. Among these, the microbes (bacteria and fungi) have been studied in most detail. A technique which is now becoming mainstream, is to map biodiversity using metabarcoding. Fungi have important roles in soil functioning, including in agricultural soils: together with bacteria, they decompose organic residues, returning nutrients in plant available form, with their dense hyphal networks they contribute to soil structure (saprotrophs), a range of species suppress plant pathogenic fungi (mycoparasites) and nematodes and other soil animals (animal pathogens) and a considerable part of the fungal microflora lives in symbiosis with plant roots, i.e. the mycorrhizal fungi. In arable farming, arbuscular mycorrhizal fungi (AMF) live together in symbiosis with the roots of most agricultural crops (mainly except cabbage crops and sugar beets). In this symbiosis, the fungi deliver nutrients such as phosphorous and water to the plants, and the plants deliver photosynthates to the fungi. Furthermore, there are fungi that can have detrimental effects on plant growth (pathogens).

Metabarcoding is a technique for the evaluation of biodiversity from environmental DNA, in our case soils, that combines Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) in order to get an impression of the biodiversity of soil fungi. A standard marker (barcode) for fungal species detection is the nuclear ribosomal Internal Transcribed Spacer (ITS). The possibility of identifying fungi at species level using the ITS region gives the opportunity to divide them into the above-mentioned functional groups. Results of the metabarcoding are expressed in operational taxonomic units (OTUs). A specific OTU can be a known species with a full name or a sequence type of unknown identity. Usually, the clustering of OTUs is based on 97% similarity (i.e. two OTUs are always more than 3% different from each other in their targeted genetic code). The contribution that metabarcoding can give to soil quality assessment as well as management recommendations is quite new and still challenging.

The general hypothesis underlying this study is that the soil fungal community represents a fingerprint of soil quality. This is expected, since a major part of it represents saprotrophs (i.e. fungi living from (dead) organic material), which depend on the quantity and quality (composition) of soil organic matter. In other words, different types and quantities of soil organic matter are likely to contain different fungal communities.

SoilCares Research develops and provides techniques to quantify nutrient composition, pH, organic matter content and texture of soil at a given field. This is possible with a hand-held scanner which produces NIR-spectra or with a larger device that produces MIR-spectra of soil samples. In both cases, no wet chemistry is needed, thus making soil analysis considerably faster and cheaper compared to existing laboratory methods. It is expected that this technique is commercially viable, because the lower costs enable farmers to measure their abiotic soil condition on a regularly and frequently. The hypothesis is that the NIR/MIR-spectra also contain information on soil fungal community and hence on soil health status. So, if a reliable correlation can be made between the soil

fungal community and NIR/MIR-spectra, then SCR could provide additional, useful information with only marginal extra costs.

In SoilCare, participants from 4 countries brought in 16 existing long-term experiments (LTEs) that aimed to design soil-improving cropping systems (SICS). In this study, 12 LTEs were selected from countries that included a treatment on tillage (e.g. minimum tillage, no-tillage, reduced tillage) to be able to standardize the community measurements across countries. On the one hand, from the onset it was clear that this approach presented challenges because of great differences in set-up of these experiments, on the other hand costs could be strongly reduced because these field experiments were already existing. Moreover, because of the very nature of LTEs, long-term effects on especially soil structure and probably also soil communities cannot be seen if experiments would be started within the time frame of SoilCare, which had a duration of 5 years. Furthermore, none of the sites had been ever sampled for fungal community structure so this project also generated useful information to the site managers. One of the major drawbacks that appeared in the course of the project was that proper indicators for soil structure or soil quality were not available for most of the LTEs. Since in many of the experiments maximization of the yields was also not a target, and since soil organic matter density only changes very slowly, we had to rely on the assumption that any cropping system that had been applied within these experiments was by definition considered "good" for soil quality. For example, in a LTE where non-tillage was compared with tillage, it was assumed that non-tillage resulted in a "better" soil than the tilled soil. Irrespective of whether this is true or not, we also considered it reasonable to assume that if a certain SICS like reduced-tillage or compost amendment had been implemented for multiple years, that at least there had to be a measurable difference in soil fungal community in order to make the business case potentially viable.

In this study, we only analyzed the soil fungal community using metabarcoding, since insufficiently distinctive characteristics were found to relate the metabarcoding results to NIR- or MIR-spectra.

The metabarcoding results from 16 LTEs were analyzed in two ways:

(1) In one large dataset with the metabarcoding results of all LTEs, where the hypothesis was that any SICS tested would lead to some general signal representing soil improvement. The results of this dataset have been published (Hannula et al., 2021).
(2) Separate analysis of the metabarcoding results of each LTE individually. These results have not yet been published and are presented here.

The LTEs included 4 sites from Denmark (pedoclimatic zone Atlantic North, sandy to sandy loam soils), 4 from Hungary (Pannonian, sandy loamy soils), 2 from UK (Atlantic Central/North, heavy loamy soils) and 2 from Belgium (Atlantic Central, loamy soils) with various soil-improving treatments, such as no- or limited tillage, liming, and including cover crops (Table 2).

Between April and October 2016 one composite soil sample was taken from each selected field and analyzed for fungal composition. From the DNA metabarcoding data the following potentially interesting parameters were extracted: fungal richness (i.e. number of unique fungal OTUs detected in a sample), fungal community (i.e. the composition of all fungal OTUs detected together in the sample), fungal index (i.e. richness of beneficial fungal OTUs (i.e. AMF, mycoparasites and animal pathogens) – richness of potentially detrimental fungal OTUs (plant pathogens)), individual fungal taxa (individual taxa, identified as described above, were considered to be different between the treatments if they had a least a ^{2}log difference in their relative abundance (i.e. a 2× difference or larger), measure used often in gene-expression analysis to avoid false-positive differences). Data were collected for the following fungal functional groups: plant pathogens, arbuscular mycorrhiza fungi, mycoparasites, animal pathogens, and saprotrophs.

All LTEs except one gave at least one significant effect of soil-improving treatment on the fungal composition (Table 5). Those LTEs that has been installed longer and had a more extensive experimental set-up, clearly showed more significant effects than those that had been installed more recently. Anyhow, for all LTEs variation in results was rather large as well as variation between LTEs. This was especially clear for the number of fungal species that showed a significant effect.

For the global perspective on the effects of SICS on soil quality, we conclude that the current study has been carried out too early, i.e., with a UNITE database (used for identification of DNA sequences) that is currently too limited and DNA metabarcoding being too expensive and labor-intensive to collect representative number of samples seeing the large diversity detected here.

Although we did not manage to find overarching ecological patterns, this work provided valuable, novel, and appreciated information on individual LTEs that could be used by the individual farmers (UK, BEL) and site managers (HUN, DEN). For example, although pathogens are not suitable for predicting soil quality, the presence of certain pathogens can affect the crop choices in the near future.

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Abbreviations used

AMF	arbuscular mycorrhizal fungi
K	potassium
LTE	long-term experiment
MIR	mid-infrared
N	nitrogen
NGS	next-generation sequencing
NIR	near-infrared
NMDS	nonmetric multidimensional scaling
OTU	operational taxonomic unit
P	phosphorus
PCR	polymerase chain reaction
SCR	Soil Cares Research (participant 16 of the SOILCARE project)
SICS	soil-improving cropping systems

1. Introduction

The overall aim of SoilCare (https://www.SoilCare-project.eu/) is to assess the potential of soil-improving cropping systems and to identify and test site-specific soil-improving cropping systems that have positive impacts on profitability and sustainability in Europe. Specific objectives of SoilCare are:

- To review which cropping systems can be considered soil-improving, to identify current benefits and drawbacks, and to assess current and potential impact on soil quality and environment,
- To select and trial soil-improving cropping systems in 16 study sites across Europe, representing various pedo-climatic zones and socio-economic conditions following a multi-actor approach,
- To develop and apply an integrated and comprehensive methodology to assess benefits, drawbacks and limitations, profitability and sustainability of soil-improving cropping systems in the study sites, taking into account pedo-climatic, socio-economic and legislative conditions,
- To study barriers for adoption and to analyse how farmers can be encouraged through appropriate incentives to adopt suitable soil-improving cropping systems,
- To develop and apply a method to upscale study site results to European level, taking into account different pedo-climatic and socio-economic conditions in different parts of Europe, to come up with Europe-wide information on which soil-improving cropping systems would be most beneficial where in Europe,
- To develop an interactive tool for selection of soil-improving cropping systems throughout Europe,
- To analyze the effect of agricultural and environmental policies on adoption of cropping systems, and to support these policies in order to improve adoption,
- To disseminate key-information about soil-improving cropping systems including agronomic techniques to all stakeholders.

This research report presents the results of a study that aims to develop a method to measure soil quality. From the text of the grant proposal:

> 'SCR (participant 16) will carry out fungal biodiversity measurements and, together with collaborators, analyze them in the context of other measurements ongoing in the framework of SoilCare. SCR will carry out the soil sampling needed in the Study Sites for the analysis of fungal biodiversity, thus minimizing variation in soil sampling and optimizing the comparability between samples in the monitoring program (WP3, 4). With the analyses, SCR will investigate the links that exist between fungal biodiversity and soil health & crop production. SCR profits from SoilCare because of the magnitude of the study across Europe, which optimizes the ability to interpret the biodiversity data in a way that it is useful for farmers, and by extending its network.'

One of the major vehicles of SoilCare's work was to use existing sites of long term field experiments (LTEs) as study objects.

In this introduction, the general approach of using fungal soil biodiversity as a soil quality monitoring tool is explained (par. 1.1), including how this could be marketed (par. 1.2), followed by an overview of the general approach (par. 1.3).

1.1. Fungal soil biodiversity as a potential soil quality monitoring tool

Maintaining or improving soil quality and soil health is crucial for crop production and can, in particular, contribute to remediating subtle forms of soil degradation (Bünemann et al., 2018). The term cropping system refers to crop type, crop rotation, and the agronomic management techniques used on a particular field over a period of years (Nafziger, 2012). Such systems can be considered soil-improving if they result in a durable increased ability of the soil to fulfil its functions, including food and biomass production, buffering and filtering capacity, and provision of other ecosystem services. A

healthy soil is a soil that is sustainably managed, so that it continues to produce profitable crop yields within agriculture, whilst also reducing biodiversity loss. However, to quantify the health/quality status of a soil is not easy. Generally speaking, the better a method reflects soil health or quality, the more difficult and thus costly the method is. For example, methods to quantify soil structure such as bulk soil density and organic matter stability are too costly to be performed on a regular and field-specific basis.

Since soil organisms depend on soil organic matter and other factors that are affected by soil structure, including soil moisture-related factors such as water holding capacity and aeration, it is likely that their communities reflect, at least partially, different statuses of soil quality. A great range of organisms occur in soil, including viruses, bacteria, oomycota, fungi, protozoa, nematodes and earthworms. Among these, the microbes (bacteria and fungi) have been studied in most detail. A technique which is now becoming more and more mainstream, is to map biodiversity using metabarcoding. Fungi have important roles in soil functioning, including in agricultural soils: together with bacteria, they decompose organic residues, returning nutrients in plant available form, with their dense hyphal networks they contribute to soil structure (saprotrophs), a range of species suppress plant pathogenic fungi (mycoparasites) and nematodes and other soil animals (animal pathogens) and a considerable part of the fungal microflora lives in symbiosis with plant roots, i.e. the mycorrhizal fungi (Frąc et al., 2018). In arable farming, arbuscular mycorrhizal fungi (AMF) live together in symbiosis with the roots of most agricultural crops (mainly except cabbage crops and sugar beets). In this symbiosis, the fungi deliver nutrients such as phosphorous and water to the plants, and the plants deliver photosynthates to the fungi. Furthermore, there are fungi that can have detrimental effects on plant growth (pathogens).

Metabarcoding is a technique for the evaluation of biodiversity from environmental DNA, in our case soils, that combines Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) (Nguyen et al., 2016; Orgiazzi et al., 2015) in order to get an impression of the biodiversity of soil fungi. A standard marker (barcode) for fungal species detection is the nuclear ribosomal Internal Transcribed Spacer (ITS) (Schoch et al., 2012). The possibility of identifying fungi at species level using the ITS region gives the opportunity to divide them into functional groups (such as potential plant pathogens, beneficial mutualistic fungi, decomposers and for example animal pathogens), which represent ecologically meaningful categories. Results of the metabarcoding are expressed in operational taxonomic units (OTUs). A specific OTU can be a known species with a full name or a sequence type of unknown identity. Usually, the clustering of OTUs is based on 97% similarity (i.e. two OTUs are always more than 3% different from each other in their targeted genetic code). The contribution that metabarcoding can give to soil quality assessment as well as management recommendations is quite new and still challenging (Sun et al., 2016).

The general hypothesis underlying this study is that the soil fungal community represents a fingerprint of soil quality. This is expected, since a major part of it represents saprotrophs (i.e. fungi living from (dead) organic material), which depend on the quantity and quality (composition) of soil organic matter. In other words, different types and quantities of soil organic matter are likely to contain different fungal communities (Frąc et al., 2018; Hättenschwiler et al., 2005; van der Wal et al., 2013). Crop species is important as well, since it determines litter quality (Cleveland et al., 2014; Fanin and Bertrand, 2016) and can modulate fungal community through carbon released to the rhizosphere as root exudates. In addition, the mycorrhizal arbuscular soil community also depends on soil organic matter quality, next to the fertilization status (notably the P nutrition status) of the soil (Avio et al., 2013; de Vries et al., 2007; Lin et al., 2012; Brito et al., 2012; Jansa et al., 2003; Mathimaran et al., 2007). In that respect, the part of the fungal soil community that represents the plant pathogens, probably is the group of fungi that is least related to soil quality, since this likely depends more on the types of crop present now and in past rather than directly on soil-related factors (Raaijmakers et al., 2009).

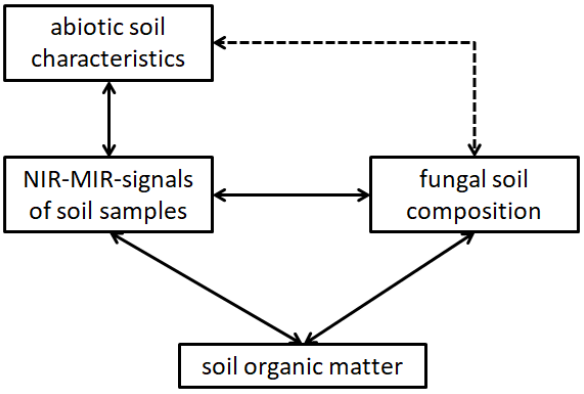

Figure 1. Scheme representing the relation between fungal soil composition, soil organic matter and NIR-/MIR-signals from soil and the already existing relation between NIR-/MIR-signals and abiotic soil characteristics.

This would be the first but essential step needed to arrive at the business case (par. 1.2), where NIR/MIR-spectra of soil samples would be used as proxy of the fungal soil community. NIR- and MIR-spectra are known to be especially sensitive to organic matter and so are many fungal species.

1.2. Business case

SCR develops and provides techniques to quantify nutrient composition, pH, organic matter content and texture of soil at a given field. This is possible with a hand-held scanner which produces NIR-spectra or with a larger device that produces MIR-spectra of soil samples. In both cases, no wet chemistry is needed, thus making soil analysis considerably faster and cheaper compared to existing laboratory methods. It is expected that this technique is commercially highly viable, because the lower costs enable farmers to measure their abiotic soil condition much more frequently.

As explained in par. 1.1, the hypothesis is that the NIR/MIR-spectra also contain information on soil fungal community and hence on soil quality/health. So, if a reliable correlation can be made between the soil fungal community and NIR/MIR-spectra, then SCR could provide additional, useful information with only marginal extra costs. The business case is quite interesting because methods to measure soil quality/health at field level at for farmers affordable costs are currently non-existing: typical costs of analysis on bulk soil density, organic matter stability, basal respiration or microbial activity are in the order of magnitude of € 75-150 or more per sample.

As with other NIR/MIR techniques, the bulk of costs are at the initiation of product development, more precisely, setting up of the calibration curve, where the relation between spectra and fungal soil community and soil health/quality is set up. In SoilCare, the target was to do the first steps to see whether setting up a calibration curve is possible at all. If so, then, based on the experience of SCR, the prospect is that for setting up a calibration curve, 10.000 and 1000 samples for NIR and MIR spectra would be required, respectively, requiring costs of fungal sequencing (€ 50 per sample) of € 500.000 and € 50.000 respectively for NIR and MIR (Table 1). These differences between NIR and MIR are to be expected: for NIR, large datasets are needed, but once they have been set up, they will be valid for large areas, while for MIR small datasets suffice, but they will be then only valid for relatively small areas. Additional costs will be the maintenance of the dataset, i.e. the metabarcoding of additional samples that are regarded as outliers. Overall it is reasonable to take into account that 5% of the samples will be detected as outlier. Adding the costs for this, and assuming a commercial price of € 75 per sample, the break-even point for NIR and MIR would be approx. 6.900 and 690 samples, respectively, for NIR- and MIR-spectra.

Table 1. Some key figures of the business case for NIR- and MIR-spectra to be used to quantify soil health/quality. For explanation, see text.

	NIR	MIR
initial # samples needed for calibration curve	10.000 samples	1000 samples
costs of fungal metabarcoding (€ 50 per sample)	€ 500.000	€ 50.000
dataset maintenance (% of commercial samples needing metabarcoding)	5%	5%
break-even point (# commercial soil samples needed at € 75 / sample)	6900 samples	690 samples

Here, we mainly focus on one necessary, intermediary step for this business case, i.e. characterization and interpretation of the fungal community using metabarcoding.

1.3. General approach

In SoilCare, participants from 4 countries brought in 16 existing long-term experiments (LTEs) that aimed to design soil-improving cropping systems (SICS). In this study, 12 LTEs were selected from countries that included a treatment on tillage (e.g. minimum tillage, no-tillage, reduced tillage) to be able to standardize the community measurements across countries. On the one hand, from the onset it was clear that this approach presented challenges because of great differences in set-up of these experiments, on the other hand costs could be strongly reduced because these field experiments were already existing. Moreover, because of the very nature of LTEs, long-term effects on especially soil structure and probably also soil communities cannot be seen if experiments would be started within the time frame of SoilCare, which had a duration of 5 yr. Furthermore, none of the sites had been ever sampled for fungal community structure so this project also generated new, useful, information to the site managers. One of the major drawbacks that appeared in the course of the project was that proper indicators for soil structure or soil quality were not available for most of the LTEs. Since in many of the experiments maximization of the yields was also not a target, and since soil organic matter densities only change very slowly, we had to rely on the assumption that any cropping system that had been applied within these experiments was by definition considered “good” for soil quality. For example, in a LTE where tillage was compared with non-tillage, it was assumed that non-tillage resulted in a “better” soil than the non-tilled soil. Irrespective of whether this is true or not, we also considered it reasonable to assume that if certain SICS like reduced-tillage or compost amendment had been carried an extended period of time (multiple years), that at least there had to be a measurable difference in soil fungal community in order to make the business case (par. 1.2) potentially viable.

In this study, we only analyzed the soil fungal community using metabarcoding, since insufficiently distinctive characteristics were found to relate the metabarcoding results to NIR- or MIR-spectra.

The metabarcoding results from 16 LTEs were analyzed in two ways:

(3) In one large dataset with the metabarcoding results of all LTEs, where the hypothesis was that any SICS tested would lead to some general signal representing soil improvement. The results of this dataset have been published (Hannula et al., 2021) and will be discussed shortly in Ch. 4.

(4) Separate analysis of the metabarcoding results of each LTE individually. These results have not yet been published and will be presented in Ch. 3.

2. Materials and Methods

Long-term experiments (LTEs). SoilCare uses a large number of Study Sites (16) spread throughout Europe, that cover all main pedo-climatic zones and various cropping systems. The project partners responsible for these Study Sites also have are included long-term experiment (LTE) fields, used to assess both benefits and drawbacks of soil-improving CS, as well as to assess how drawbacks can be minimized. SCR sampled several LTE fields located in four different European countries (i.e. Denmark, Hungary, UK and Belgium), covering different pedo-climatic conditions (Table 2). Summaries of the LTEs involved in this study are provided in Annex 1.

Table 2. Description of long-term experiments (LTEs).

country	experiment code	pedoclimatic zone and soil type	treatments
Denmark	DEN-1	Atlantic North, sandy	liming, P-fertilizer, fallowing
Denmark	DEN-2	Atlantic North, sandy loam	straw incorporation, cover crops
Denmark	DEN-3	Atlantic North, sandy loam	manure, fertilizer
Denmark	DEN-4	Atlantic North, sandy loam	tillage, cover cropping
Hungary	HUN-1	Pannonian, sandy loam	stalks/straw incorporation, manure, fertilizer
Hungary	HUN-2	Pannonian, sandy loam	tillage, N-fertilizer
Hungary	HUN-3	Pannonian, sandy loam	rotation, manure
Hungary	HUN-4	Pannonian, sandy loam	fertilizer, timing of fertilization
UK	UK-1	Atlantic Central/North, heavy loam	tillage
UK	UK-2	Atlantic Central/North, heavy clay loam	cover crops
Belgium	BEL-1	Atlantic Central, loamy	compost, tillage
Belgium	BEL-2	Atlantic Central, loamy	compost

Soil sampling, processing and measurements of physico-chemical properties. Soils were tested to assess several physico-chemical parameters by the Golden Standard Lab (GSL) of SoilCares Research. Between April and October 2016 from each field about 5 soil samples were taken using an auger of diam. 4 cm from 0-20 cm depth to make up one joint sample of about 1 kg. All samples were cooled during transportation. Within two days after collection, samples were dried at 40°C and sieved to remove roots and rocks. The soil was crushed. Tubes of 50mL were filled with roughly 30 g of soil and stored at -20°C until further processing (DNA extractions). Measured properties of soils included pH, organic carbon, total nitrogen, C:N ratio, total phosphorous, total potassium and texture (as clay content). A summary of the physico-chemical properties of all the soils is listed in Table 3.

DNA extractions and fungal community determination. DNA was extracted using modified Power Soil protocol (MoBIO laboratories). Fungal DNA was amplified using primers ITS4ngs and ITS3mix1-5 (Tedersoo et al., 2015, 2014) and purified using AMPure magnetic beads (Beckman Coulter). Dual tags were added to samples (Illumina dual indexing kits v1-3) using 7 cycles of PCR. PCR products were further purified using magnetic beads (AMPure). The DNA was quantified using Qubit fluorometer and equimolar pooled into a library of 255-285 samples. One mock community sample with 8 fungal strains was sequenced along the samples in each library. Sequencing was performed using Illumina MiSeq pair-end 2x300bp.

Table 3. Summary of physico-chemical properties of LTEs in this study. Median values are shown.

experiment code	pH-KCl	C-organic (g/kg)	N-total (g/kg)	K-total (g/kg)
DEN-1	5.0	13.7	0.85	7.63
DEN-2	6.0	14.0	1.32	12.3
DEN-3	5.9	11.1	0.95	11.9
DEN-4	5.8	21.1	1.85	13.1
HUN-1	7.0	12.4	1.40	17.1
HUN-2	7.4	13.3	1.52	17.6
HUN-3	7.3	13.0	1.48	17.9
HUN-4	6.5	8.2	0.95	16.1
UK-1	5.4	29.3	3.28	15.2
UK-2	6.9	26.3	3.23	16.0
BEL-1	6.6	16.8	1.71	16.3
BEL-2	6.1	11.8	1.24	15.2

The reads were assigned to samples based on tags at both ends. No mismatches were allowed. The reads were processed using PIPITs pipeline in Ubuntu (Gweon et al., 2015). In short, first paired end reads were joined and low quality and non-paired reads were filtered out. Then ITS region was extracted using ITSx (Bengtsson-Palme et al., 2013) and sequences were re-oriented. The resulting sequences were clustered using UPARSE (Edgar, 2013). Taxonomy was assigned using UNITE database (Kõljalg et al., 2013) and certain artefacts (such as chimeric sequences) were removed. Sequences from non-fungal origin were removed from the dataset.

Statistical analyses. We used samples from LTE fields from Belgium, Denmark, Germany, UK and Hungary to investigate relationships between the soil fungal community and soil chemical properties. Statistical analyses were performed in R using packages 'phyloseq', 'DESeq2' and 'vegan'. Proportion of a read from total reads was used to correct for differences in number of reads between samples. Relative abundance of a functional group or an OTU is calculated as a % from total OTUs in that sample to compensate for difference in read numbers between samples. Number of species is simply the sum of unique OTUs assigned to a certain functional group in a sample. Non-metric multidimensional scaling (NMDS) with Bray-Curtis transformation was used to explore and visualize complex species data (clustering of the samples, e.g. Fig. 1). Each dot is a sample and the closer dots are to each other the more similar the communities are. PERMANOVA ('adonis') with Bray-Curtis transformation was used to compare the community composition between treatments. PERMANOVA tells if the community structure of fungi is significantly affected by the treatment and how much of the variation is explained by the treatment (R^2 value). DESeq2 was used to investigate OTUs whose abundance was significantly and strongly (over ^{2}log) affected by treatments. ANOVA (with Tukey post-hoc) was used to compare differences in number of sequences and/or OTUs between treatments. Finally, Principal Component Analysis (PCA) was used to investigate the relevance of some selected chemical parameters in shaping the soil fungal community. In addition, Pearson's correlation coefficient was used to test for the relationships between functional groups and several soil chemical parameters.

The following potentially interesting parameters were extracted from the DNA metabarcoding data:

- Fungal richness: number of unique fungal OTUs detected in a sample;
- Fungal community: the composition of all fungal OTUs detected together in the sample;
- Fungal index: richness of beneficial fungal OTUs (AMF, mycoparasites and animal pathogens) – richness of potentially detrimental fungal OTUs (plant pathogens). Positive values indicate more beneficial fungi, negative values more potentially detrimental fungi;

- Individual fungal taxa: individual taxa, identified as described above, were considered to be different between the treatments if they had a least a ^{2}log difference in their relative abundance, measure used often in gene-expression analysis to avoid false positive differences.
- Functional groups:
  - plant pathogens.
  - arbuscular mycorrhiza fungi (AMF): a special group of fungi that lives in symbiosis with plant roots providing the plant nutrients in exchange for carbon. Occurs for nearly all crops except Brassicaceae.
  - mycoparasites: a group of fungi that parasitizes other fungi (e.g. *Trichoderma harzianum* and *Coniothyrium minitans*).
  - animal pathogens: a group of fungi that parasitizes various animals; agriculturally interesting because they may also parasitize plant parasitic nematodes.
  - saprotrophs: fungi colonizing dead organic material.

3. Results

The results are presented per LTE.

3.1. Denmark experiments

The partner from Denmark participated with 4 LTEs.

3.1.1. Denmark-1 (DEN-1; Liming, P fertilization and fallow in St. Jyndevad)

This experiment at St. Jyndevad involved the long-term effects of liming and fertilization with P. In 1996, one field was grown with barley and one field was left fallow. For more details on the experimental set-up, see Annex 1.

The fungal index, based on relative abundance, was calculated for all fungal OTUs, so including identified and non-identified OTUs. Liming had a significant negative effect on the fungal index (Fig. 2). Interestingly, when considering only the OTUs that could be identified, effects looked different (Fig. 3), with no effect of liming, except for the 8000 kg/ha lime in the fallow treatment, where fungal richness was higher. The fungal composition analyzed by NMDS revealed a clear gradient of liming (Fig. 4, 5). Especially there was a clear separation between no or low-liming (4000 kg/ha) and high levels of liming (8000 and 12000 kg/ha). The NMDS plot also shows a difference between barley and fallow (Fig. 5).

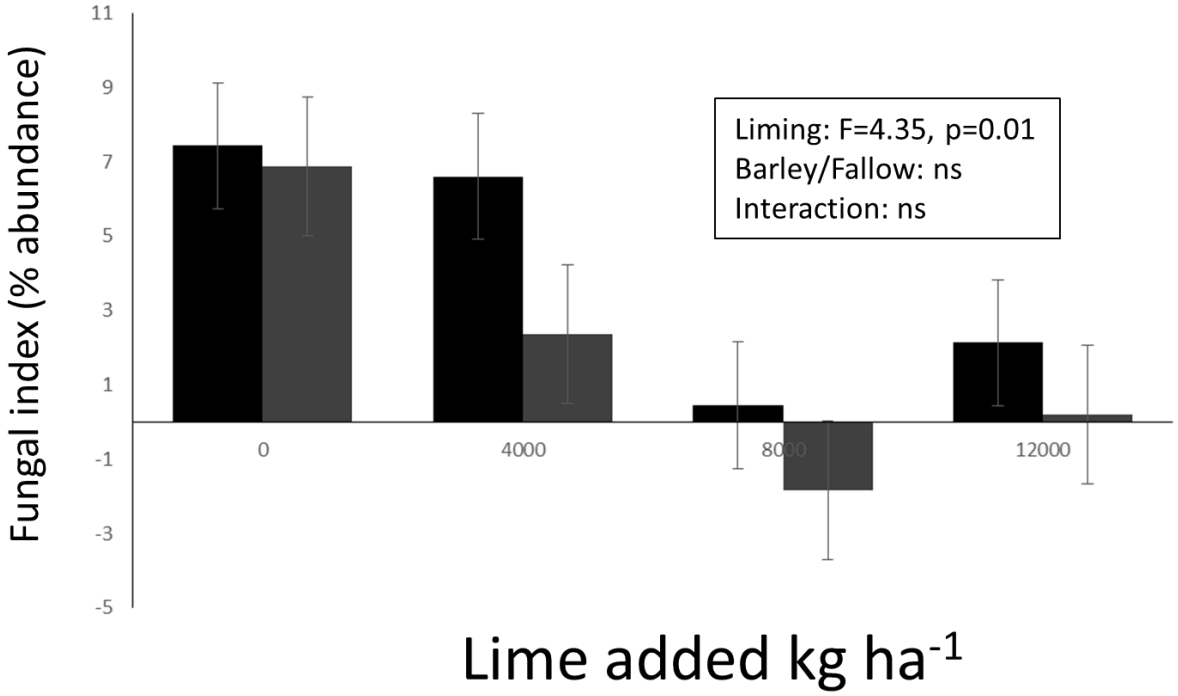

Figure 2. DEN-1. Fungal index based on abundance as function of liming, for barley (black/left bars) and fallow (grey/right bars).

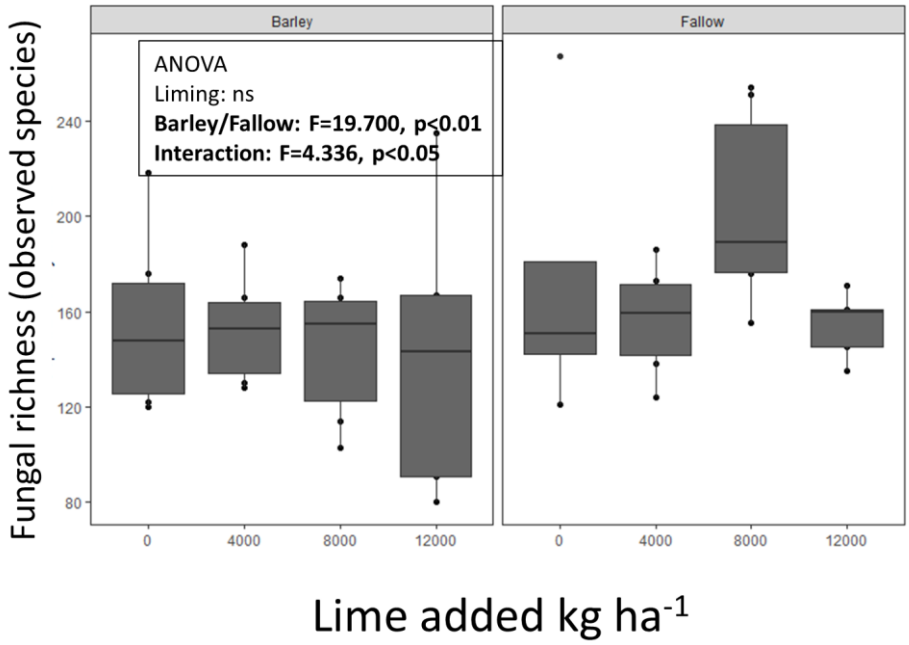

Figure 3. DEN-1. Fungal richness based on observed (identified) species as function of liming, for barley (left) and fallow (right).

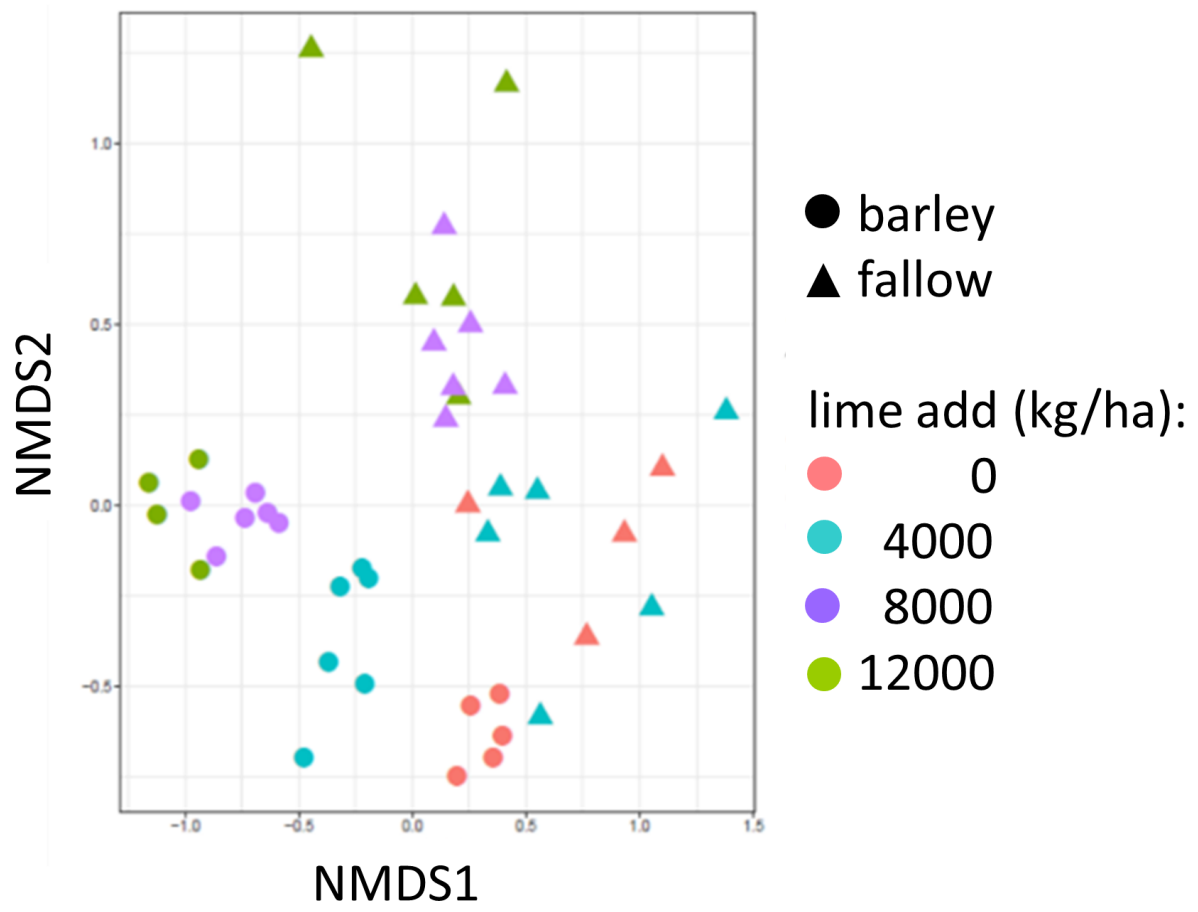

Figure 4. DEN-1. NMDS plot of fungal composition for the liming treatments and separated for the barley and fallow fields. Each point represents one sample. The more distant the points are, the more different they are in fungal composition.

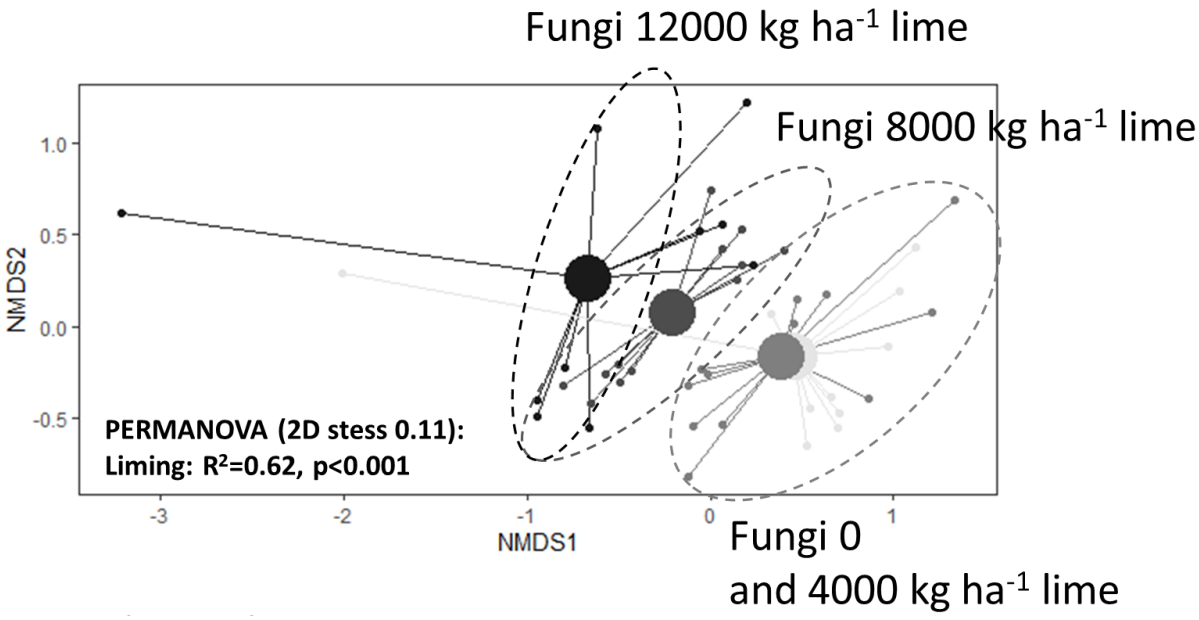

Figure 5. DEN-1. As Figure 4 but only showing the effect of liming. Each point represents one sample, and the four large points are the centroids of each treatment. The more distant the points are, the more different they are in fungal composition. Overlapping datapoints and centroids (such as here between 0 and 4000 kg ha^{-1} liming treatments) indicate no significant differences.

When analyzed separately, the functional fungal groups AMF (arbuscular mycorrhizal fungi), animal pathogens, mycoparasites, plant pathogens and saprotrophs were affected significantly (P<0.001) by liming as well as by cropping (barley vs. fallow) (shown for all fungi together in Fig. 4). However, P-fertilization did not affect the community structure in general nor any of the groups (data not shown). For AMF this was surprising, since it is well-known that P affects AMF negatively. The interaction of liming with P-fertilization was also in most cases significant (P<0.05), except for AMF, where there was only a tendency of significance (P=0.064). In any case, the interaction effects were always smaller than the main effects, and therefore we pay most attention to the main effects. Plant pathogens were more common in the 4000 kg/ha lime-barley and 8000 kg/ha lime-barley/fallow plots (Fig. 6, top). AMF showed no effect of liming in the barley treatment but was strongly increased by liming in the fallow treatment (Fig. 6, bottom).

At the individual taxon level, 17 taxa were affected by liming (Fig. 7), 4 negatively and 13 positively. Remarkably, although there was a clear positive effect of liming on AMF in the fallow treatment, no individual taxa was found to be affected by liming. Most of the taxa were at the level of fungal genus, e.g. *Penicillium* and *Umbelopsis* sp. The following species were affected by liming: positively: *Acremonium rutilum*, *Cladophialophora chaetospira*, and *Phallus impudicus*; and negatively: *Mortiellea longigemmata* and *Clonostachys candelabrum*. More fungal taxa were found to be affected by the contrast barley-fallow: 47 taxa, most of them being saprotrophs. (Fig. 8). P-fertilization did not show such effects.

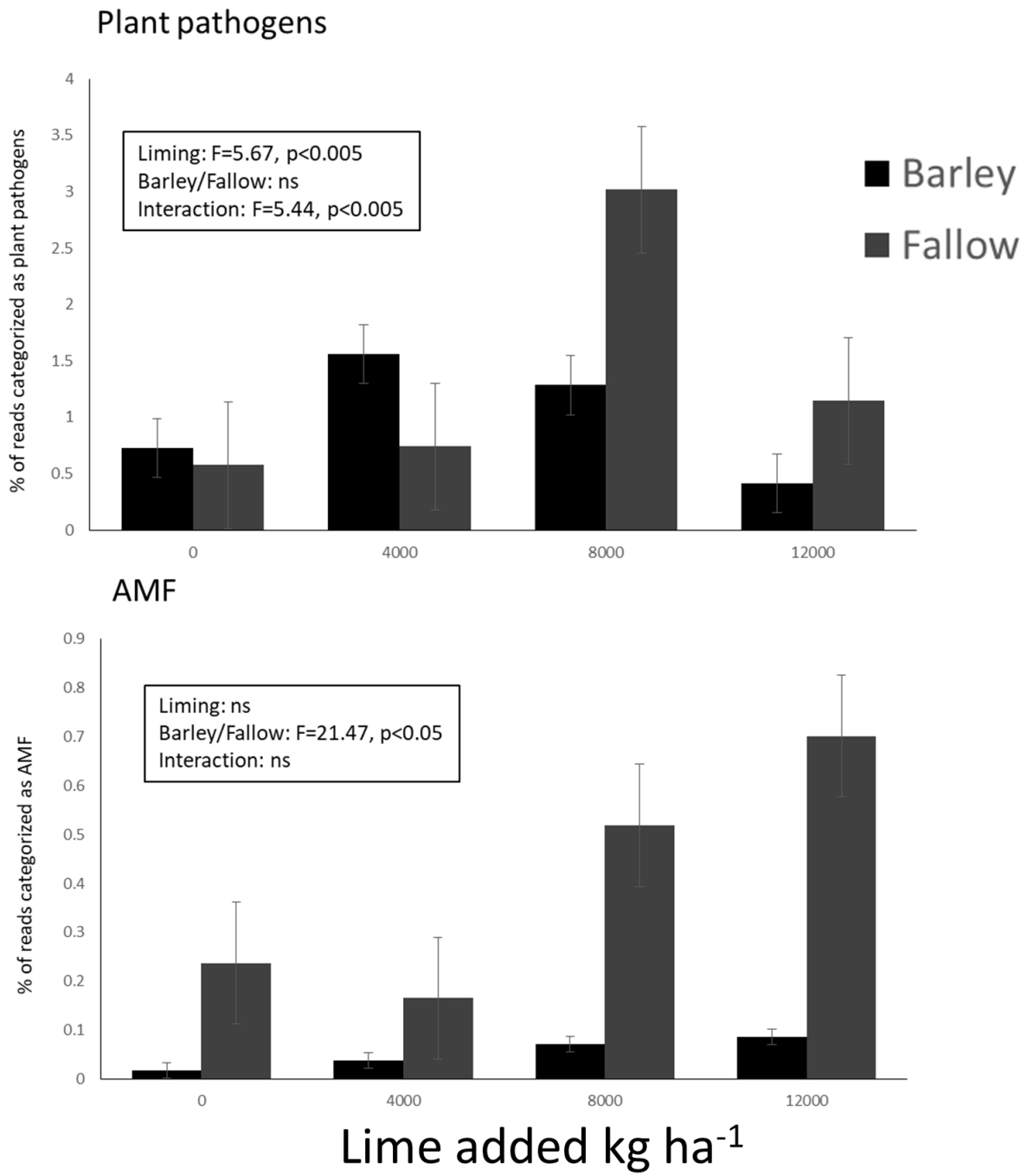

Figure 6. DEN-1. Relative abundance of plant pathogens (top) and arbuscular mycorrhiza fungi (AMF; bottom) as function of liming, for barley (black bars, left) and fallow (grey bars, right).

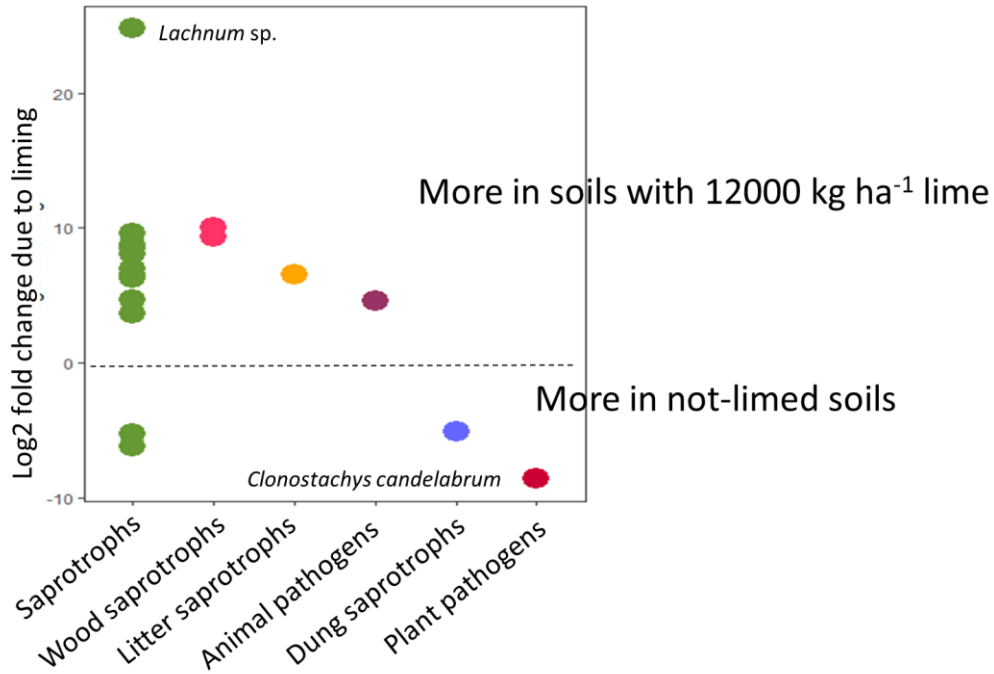

Figure 7. DEN-1. Fungal taxa affected by liming.

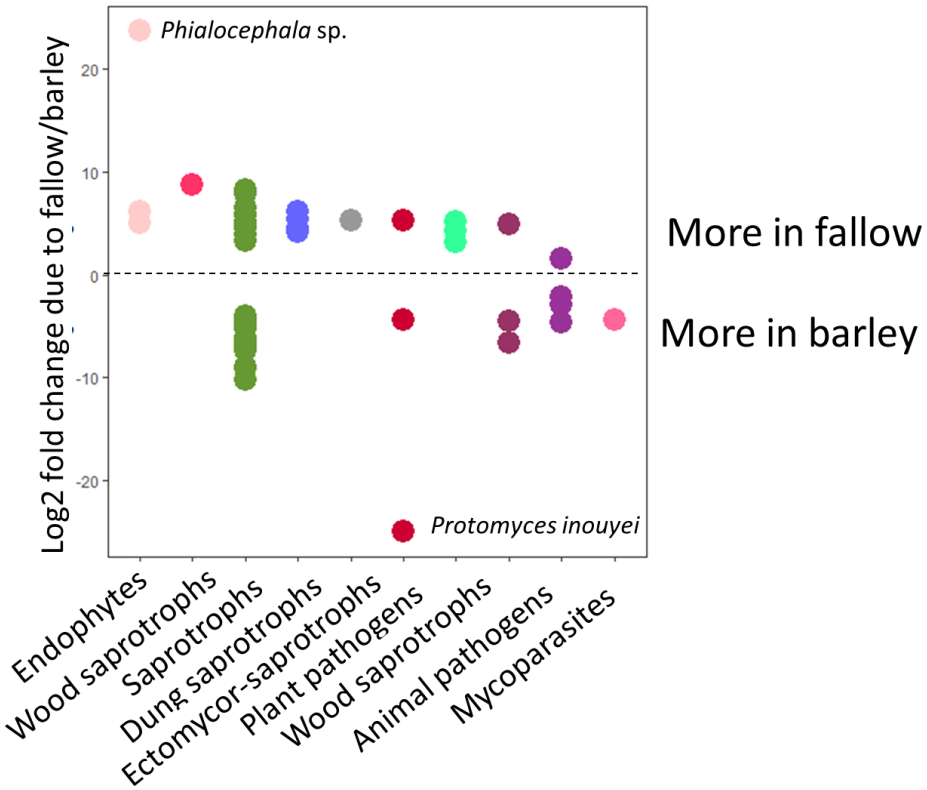

Figure 8. DEN-1. Fungal taxa showing difference between the barley and fallow treatment.

3.1.2. Denmark-2 (DEN-2; straw incorporation and cover crops in Askov)

This experiment at Askov involved the study of the effects of addition of straw at 0, 4, 8 and 12 tons/ha per year from 1980 onwards (with the exception of 5 years where no straw had been applied). The main straw plots were subdivided into six subplots in 2002 to allow for the subsidiary treatments: no catch crop, a catch crop of ryegrass, and a catch crop of ryegrass/white clover. For more details on the experimental set-up, see Annex 1. Only the 0 and 12 ton/ha treatments were included in this study.

Fungal richness and fungal index (indicating there are more beneficial fungi) increased in the straw-amended plots (Fig. 9). The effects were more prominent (but non-significant) in the plots with grass/clover as cover crop. Also the fungal composition was clearly different between the straw treatments (Fig. 10). Among the functional groups only effects were observed on plant and animal pathogens (Fig. 11). Straw amendment reduced the number of plant pathogens ($P<0.05$), with no effect of cover crop. Animal pathogens were also reduced by straw amendment, but only in the plots without cover crop. At species level, there were 27 fungal taxa that showed significant difference between the straw amendments; 6 taxa were more prominent in the plots without straw and the remainder was more common in the straw-amended plots (Fig. 12). Most of these species are saprotrophs; the fungal antagonist *Trichoderma* was found to be more common in the straw-amended plots. Though plant pathogens as a group declined due to straw amendment (Fig. 11, top), no individual species could be assigned to this effect. The cover cropping with grass/clover did not lead to significant changes in specific fungal taxa.

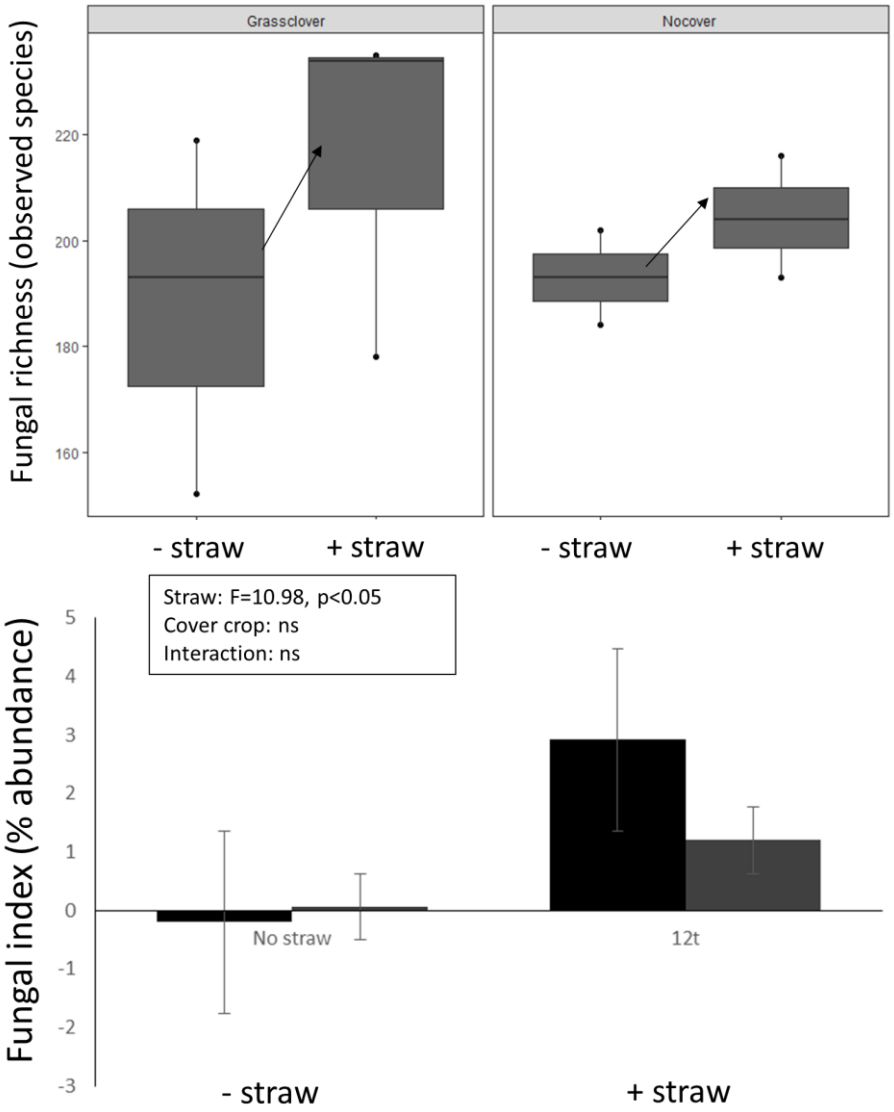

Figure 9. DEN-2. Fungal richness (top) and index (bottom) as function of straw addition (0 or 12 ton/ha/yr) and grass/clover as cover crop or without cover crop. Bottom plot: black bars: grass/clover; grey bars: no cover crop.

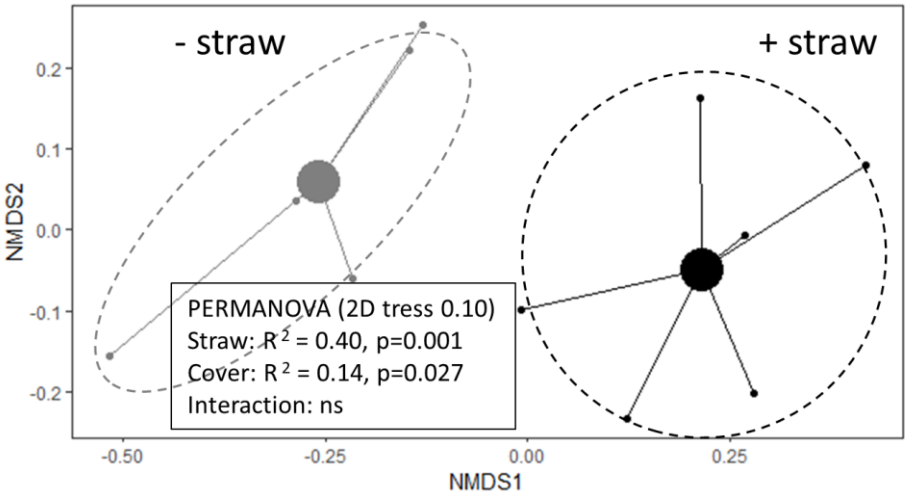

Figure 10. DEN-2. Fungal composition in NMDS plot with straw additions (-: 0 ton/ha/yr; +: 12 ton/ha/yr) Each point represents one sample, and the two large points are the centroids of each treatment. The more distant the points are, the more different they are in fungal composition.

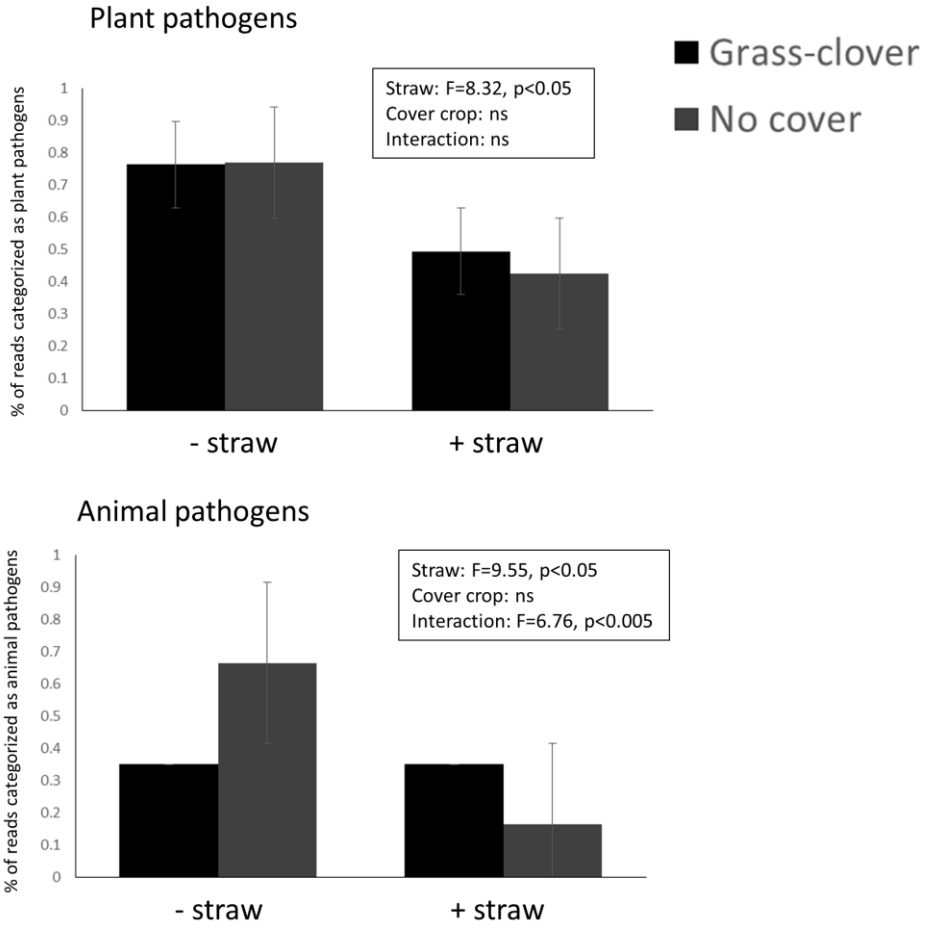

Figure 11. DEN-2. Relative abundance of plant pathogens (top) and animal pathogens (bottom) as function of straw addition (-: 0 ton/ha/yr; +: 12 ton/ha/yr) liming, for grass/clover as cover crop (black bars) and plots without cover crop (grey bars).

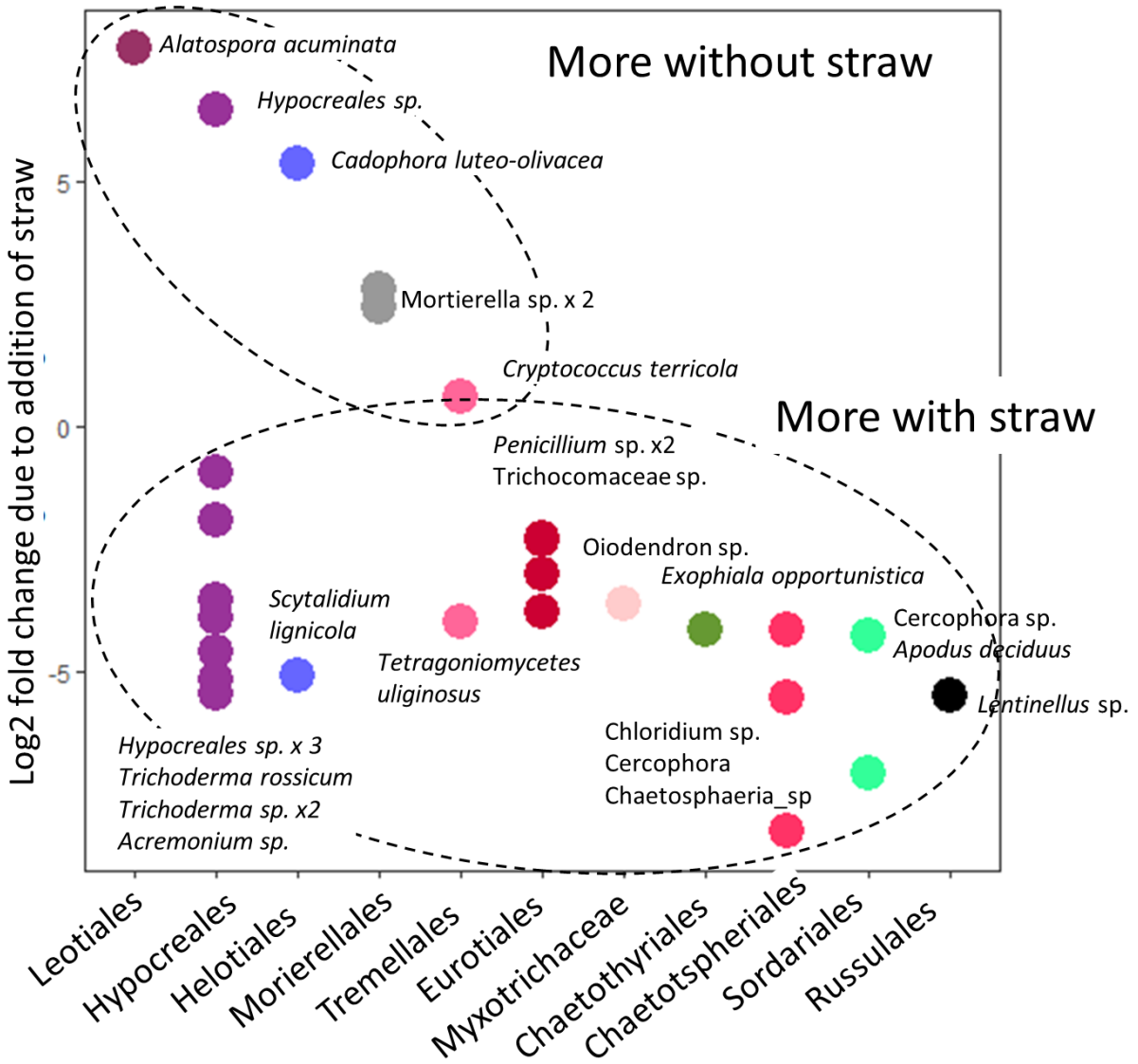

Figure 12. DEN-2. Fungal taxa showing difference between the plots with and without 12 ton/ha/yr straw amendment.

3.1.3. Denmark-3 (DEN-3; manure/fertilizer experiment in Askov)

This long-term experiment at Askov started in 1894 and mainly involves the long-term effects of organic fertilization with animal manure, inorganic fertilization (NPK) compared to no fertilization. For more details on the experimental set-up, see Annex 1.

Fungal richness was not affected by fertilization, but each treatment did result in a different fungal composition (Fig. 13), which was clearly reflected especially in the composition of plant pathogens and arbuscular mycorrhizal fungi (AMF) (Fig. 14). The abundance of plant pathogens was in decreasing order NPK > unfertilized > manure. Notably common were *Verticillium*, *Drechslera* and *Ilyonectria* in the NPK-fertilized treatment. Of these, *Verticillium* most likely refers to *V. albo-atrum* infected alfalfa (*Medicago sativa*). The abundance of AMF was in decreasing order unfertilized > NPK > manure, with in the unfertilized treatment a remarkably large group of unidentified taxa. The latter can be expected, since the best known habitats are well-fertilized, agricultural soils.

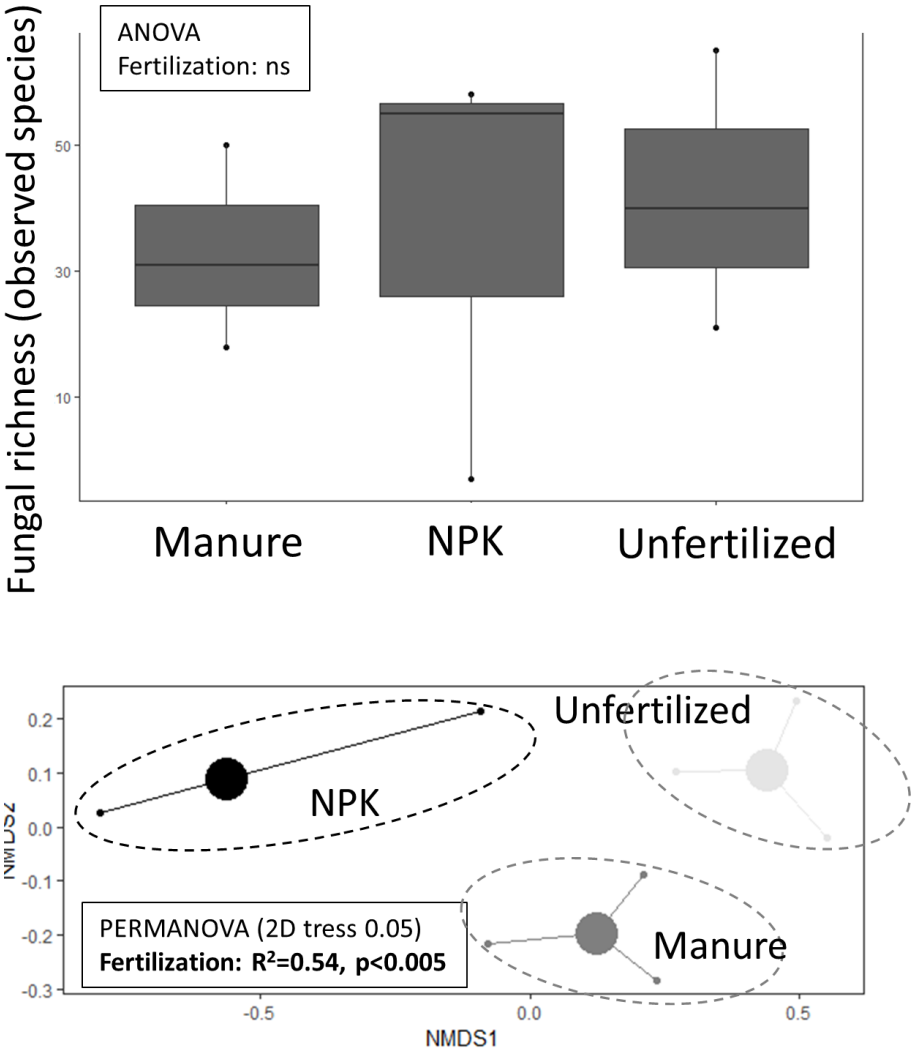

Figure 13. DEN-3. Fungal richness (top) and fungal composition (bottom) as function of fertilization (organic fertilization with animal manure, inorganic fertilization (NPK) and unfertilized. In the bottom figure, each small point represents one sample, and the three large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

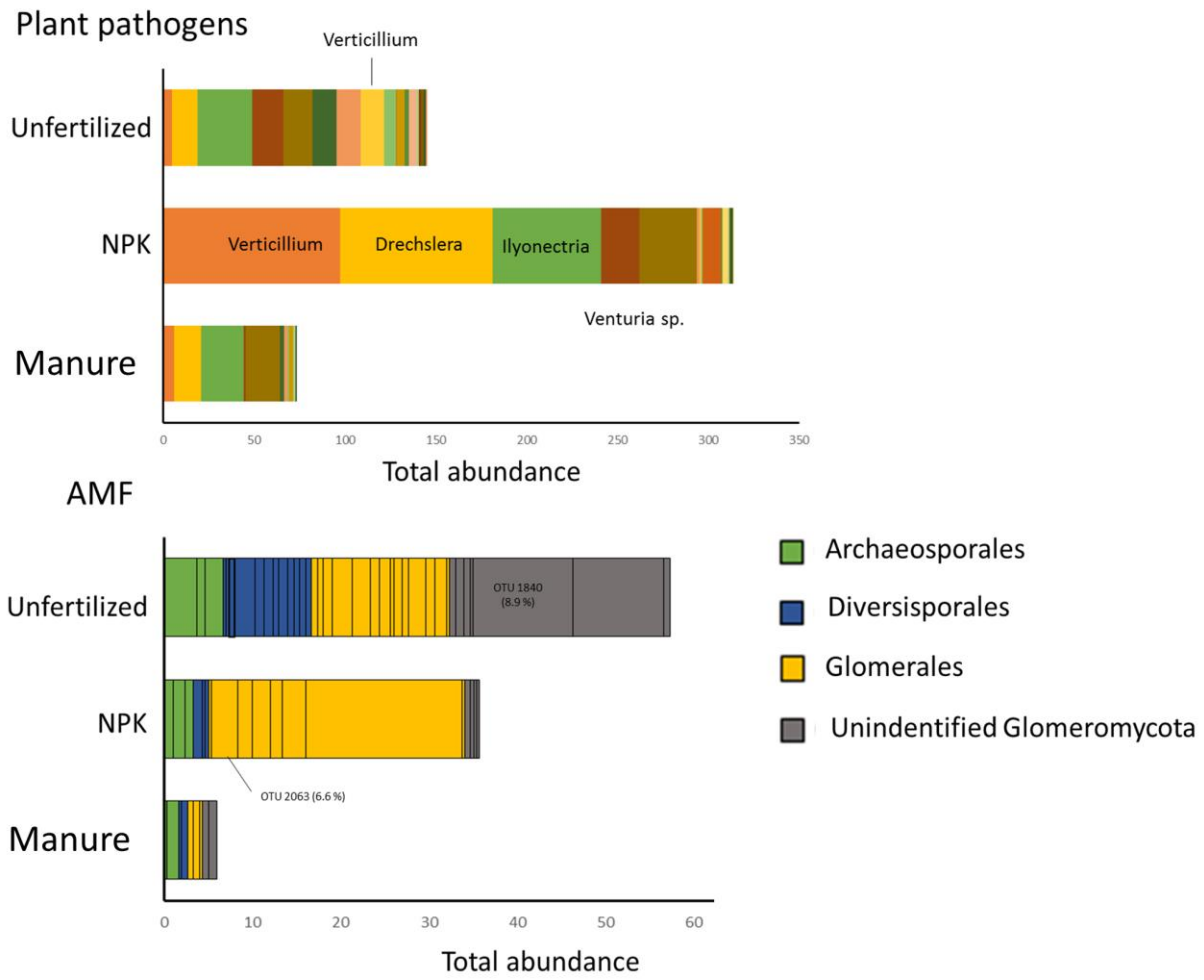

Figure 14. DEN-3. Difference in composition of fungal plant pathogens and arbuscular mycorrhizal fungi (AMF) between unfertilized treatments and plots treated with inorganic fertilizers (NPK) and organic fertilizers (manure).

3.1.4. Denmark-4 (DEN-4; type of tillaging and cover cropping)

This experiment at Foulum, started in 2002, involved the effects of different tillage methods: direct drilling, harrowing or conventional tillage and the presence of a cover crop. For more details on the experimental set-up, see Annex 1.

Fungal richness was not affected by tillage type or cover cropping (Fig. 15). However, fungal composition was significantly affected ($P<0.05$), though not so clear as in the other Danish experiments (par. 3.1.1-3.1.3: reflected in lower R^2 value here) (Fig. 16). Among the functional groups, only the saprotrophs showed some tendency for fewer saprotrophs in the conventional tillage treatment (Fig. 16). Only a few specific species showed significant effects, with *Pyrenopeziza* sp., *Tremellales* sp. and *Fusicladium* sp. being more common in the direct drill treatment, and *Cryptococcus podzolicus*, *Acremonium rutilum* and *Pyrenochaetopsis leptospora* more common in the conventional tillage treatment.

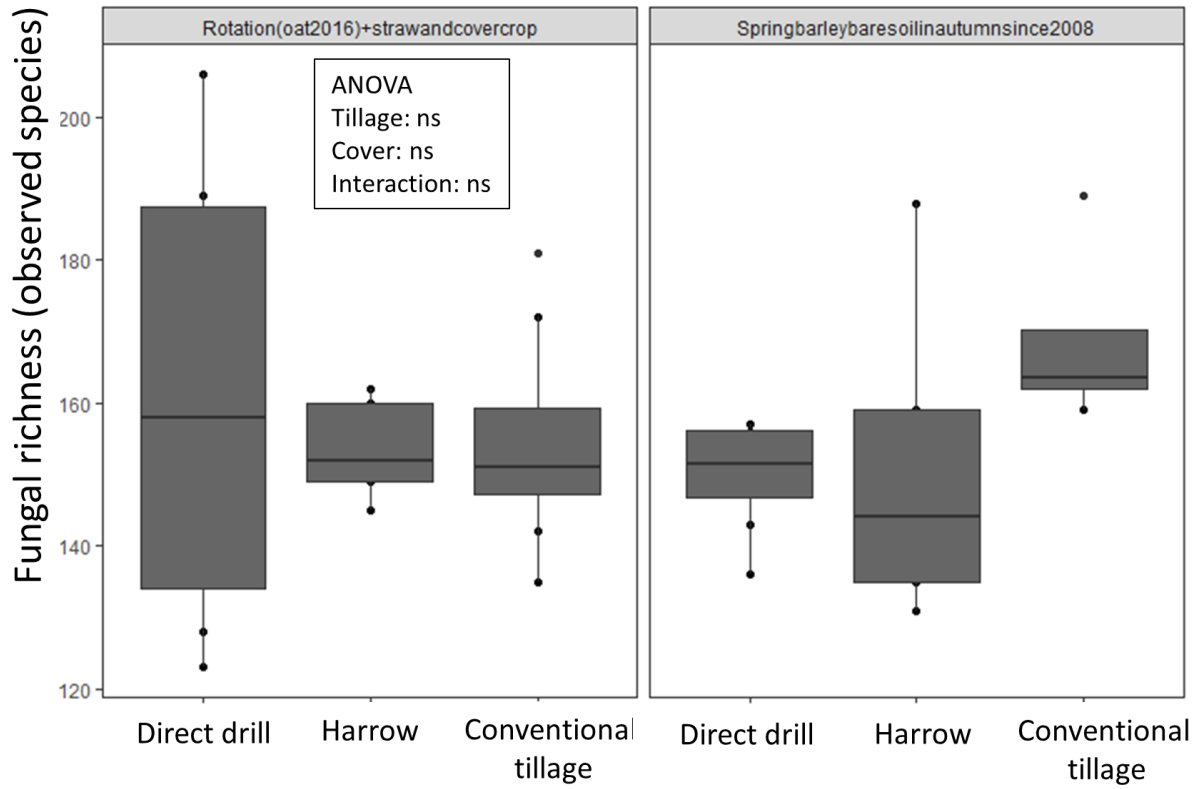

Figure 15. DEN-4. Fungal richness as function of tillage type.

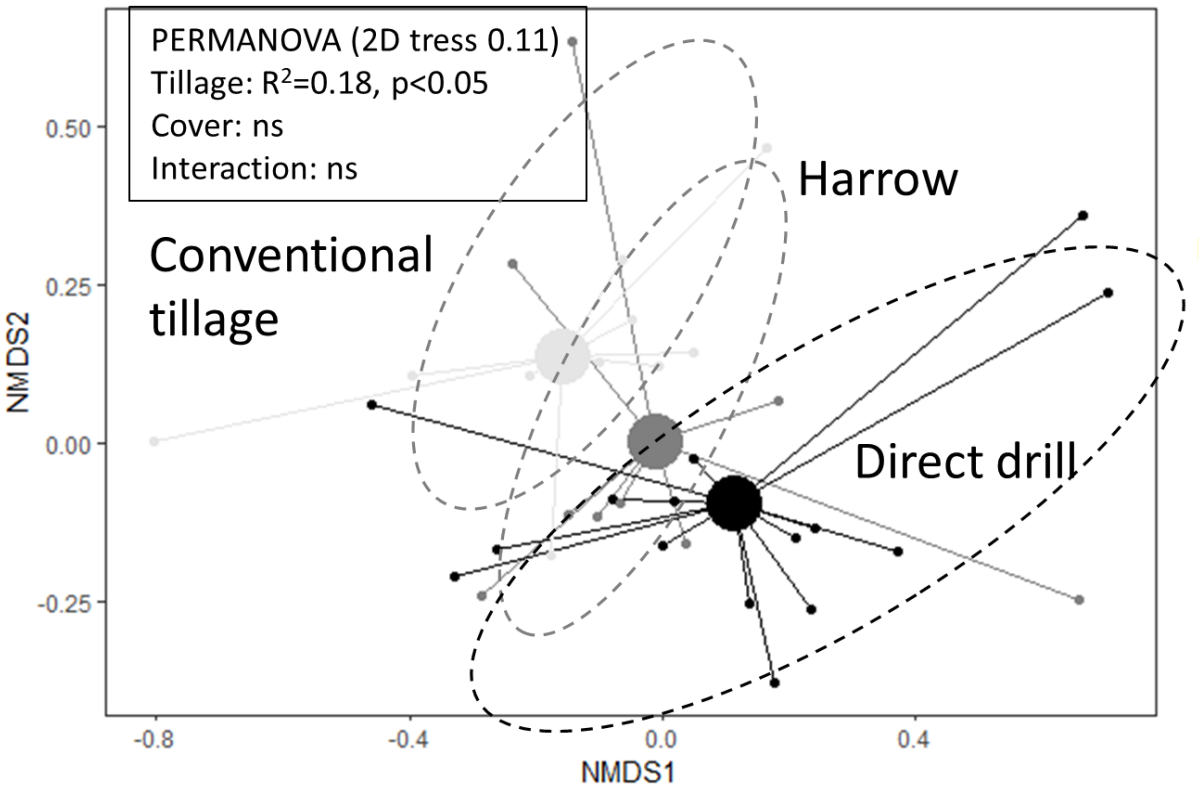

Figure 16. DEN-4. Fungal composition as function of type of tillage. Each small point represents one sample, and the three large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

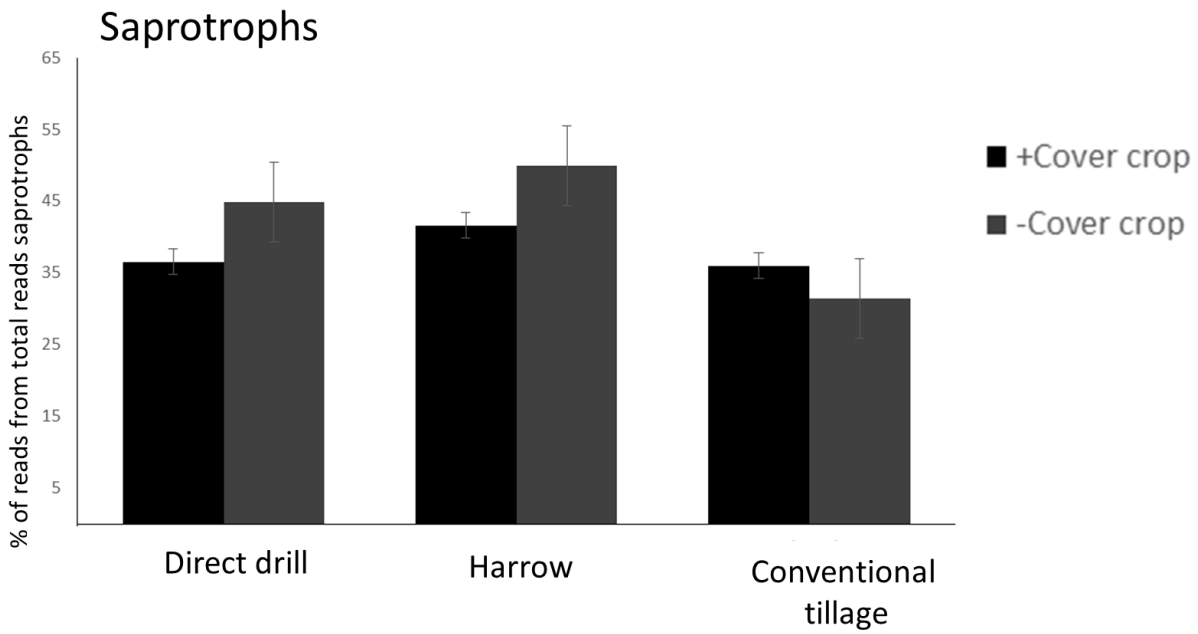

Figure 17. DEN-4. Abundance of fungal saprotrophs as function of tillage type and cover cropping (black (left): with cover crop; grey (right): without cover crop).

3.2. Hungary experiments

The partner from Hungary participated with 4 LTEs.

3.2.1. Hungary-1 (HUN-1; stalks and straw incorporation, manure and nitrogen fertilization)

This experiment with a rotation of maize, winter wheat and winter barley started in 1983 and studied the effects of organic amendment management, with 3 treatments: 1) organic manure, 2) stalk and straw incorporation and 3) no amendment (control), all at varying levels of nitrogen. For more details on the experimental set-up, see Annex 1.

Effects of the treatments on the fungal richness and the fungal index were weak, although the organic amendments did show a lower richness compared to not adding anything (Fig. 18). For stalk amendment, this reduction in fungal richness was most severe when excess nitrogen (N4) was added. Fungal index (number of beneficial fungi) was further significantly lowered by addition of manure. Nitrogen level only had a negative effect on richness but not on index. No difference in fungal composition could be detected between treatments, except for the number of species of arbuscular mycorrhizal fungi, with about 3-5× less species in the manure treatments irrespective of added N (Fig. 19).

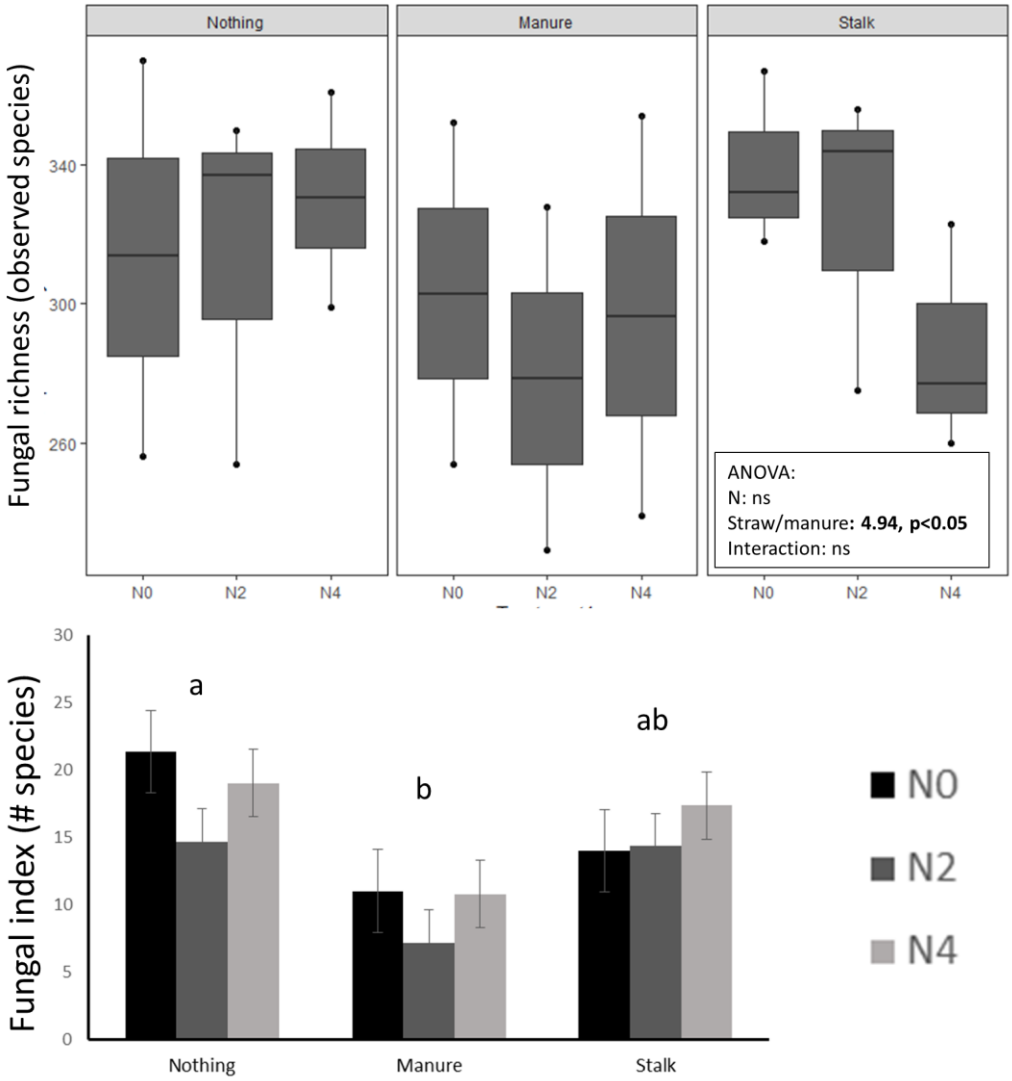

Figure 18. HUN-1. Effect of treatments on fungal richness (top) and fungal index (bottom). Nothing: no organic amendment; manure: manure amendment; stalk: stalk and straw amendment. N0, N2, N4: 0, 140 and 210 kg N/ha/yr, respectively.

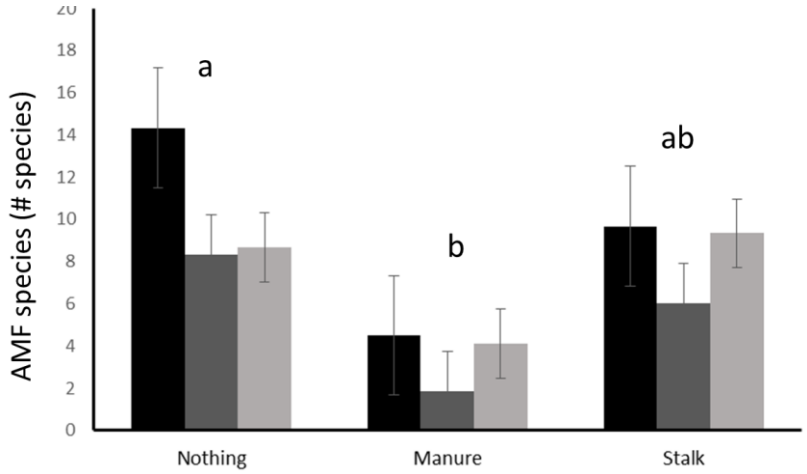

Figure 19. HUN-1. # of species of arbuscular mycorrhizal fungi (AMF) as function of organic amendment and N-application. Black, dark grey and light grey bars represent 0, 140 and 210 kg N/ha/yr, respectively.

3.2.2. Hungary-2 (HUN-2; tillage and nitrogen fertilization)

This experiment with a rotation of cereals started in 1972 and studied the effects of three different cultivations: deep winter ploughing (conventional tillage system), shallow winter disking (shallow tillage system) and disking just before drilling (minimum tillage system) at 5 N fertilizer levels. For more details on the experimental set-up, see Annex 1.

No effect of type of tillage was observed on fungal richness, on the other hand the fungal index was negatively affected by the intensity of tillage, in decreasing order minimum tillage > shallow disk > conventional tillage, as well as by N-fertilizer (Fig. 20). Effects of tillage and N-fertilizer on fungal composition were statistically significant but not large; the largest effect was on conventional tillage, which was somewhat separated from the two other tillage treatments (Fig. 21). The relative abundance of plant pathogens was significantly and positively affected by N-fertilizer (Fig. 22, top). This was not attributable to specific species, except for *Ustilago maydis* (the causal agent of corn smut disease), which was more common in the conventional tillage treatment in the presence of N-fertilizer (Fig. 22, bottom). About 18 species were affected by tillage, and 27 by fertilization (Fig. 23).

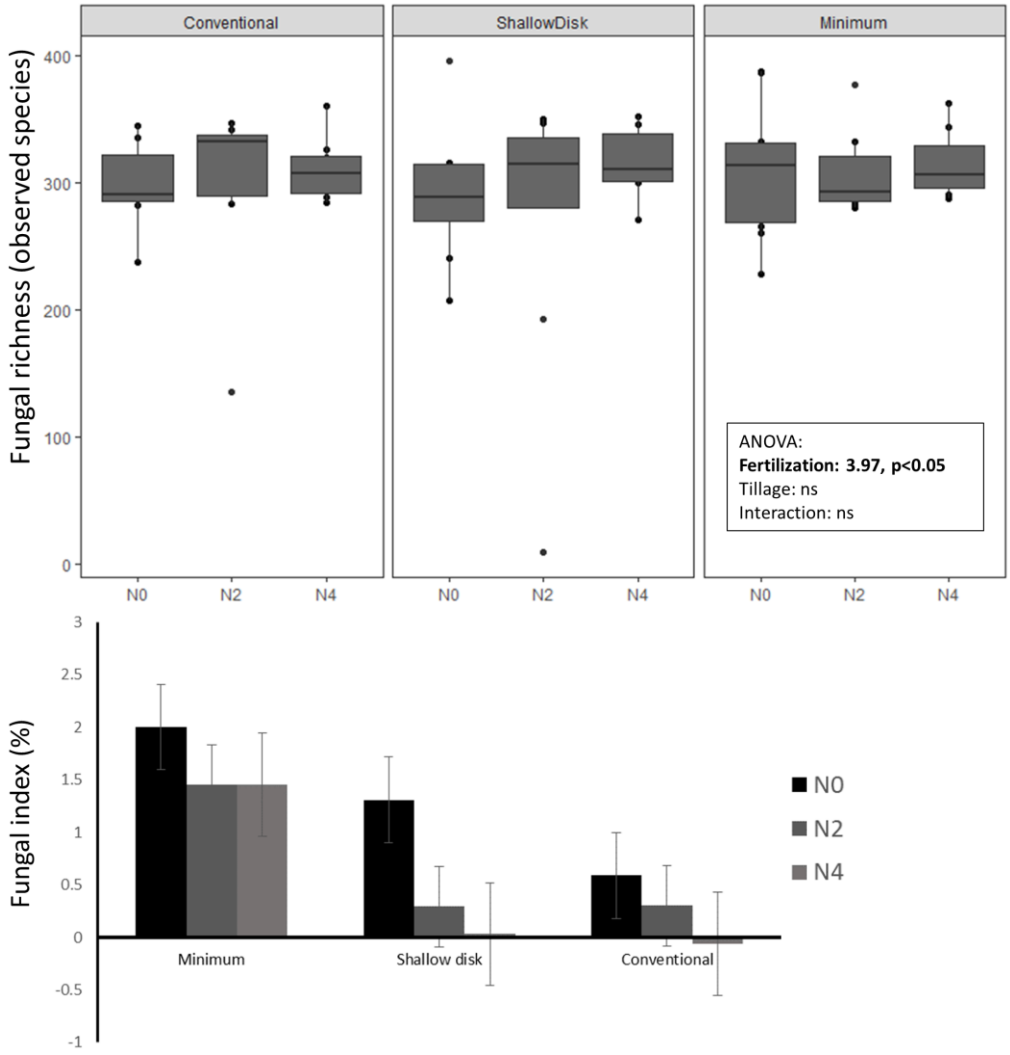

Figure 20. HUN-2. Effect of type of tillage and amount of N-fertilizer on fungal richness (top) and index (bottom). N0 (left), N2 (middle), N4 (right): 0, 120 and 240 kg N/ha/yr, respectively.

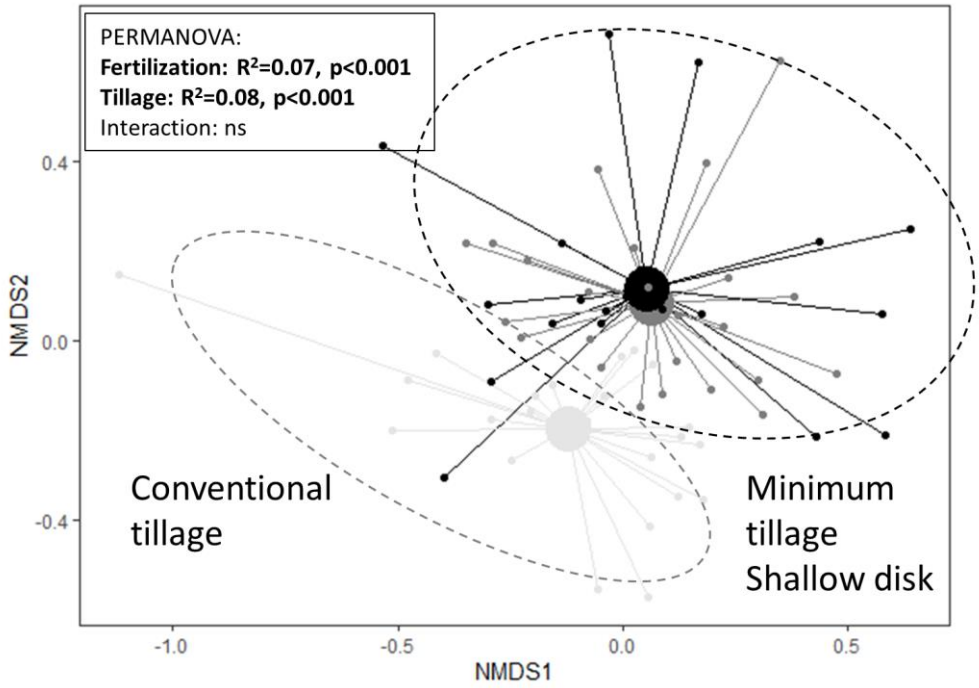

Figure 21. HUN-2. Fungal composition as function of type of tillage. Each small point represents one sample, and the three large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

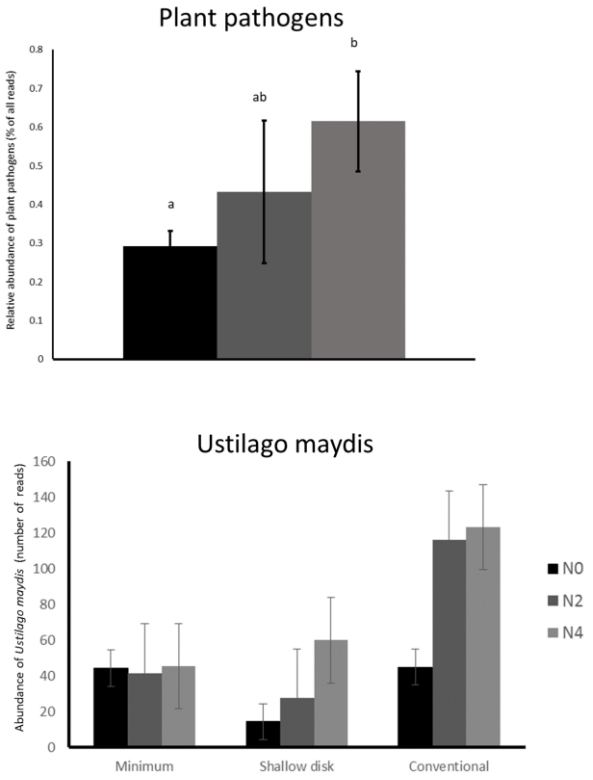

Figure 22. HUN-2. Effect of N-fertilizer on the relative abundance of plant pathogens and the effect of tillage and N-fertilizer on *Ustilago maydis*. Black bars/left: fertilization level N0; gray bars/middle: fertilization level N2; light gray bars/right: fertilization level N4.

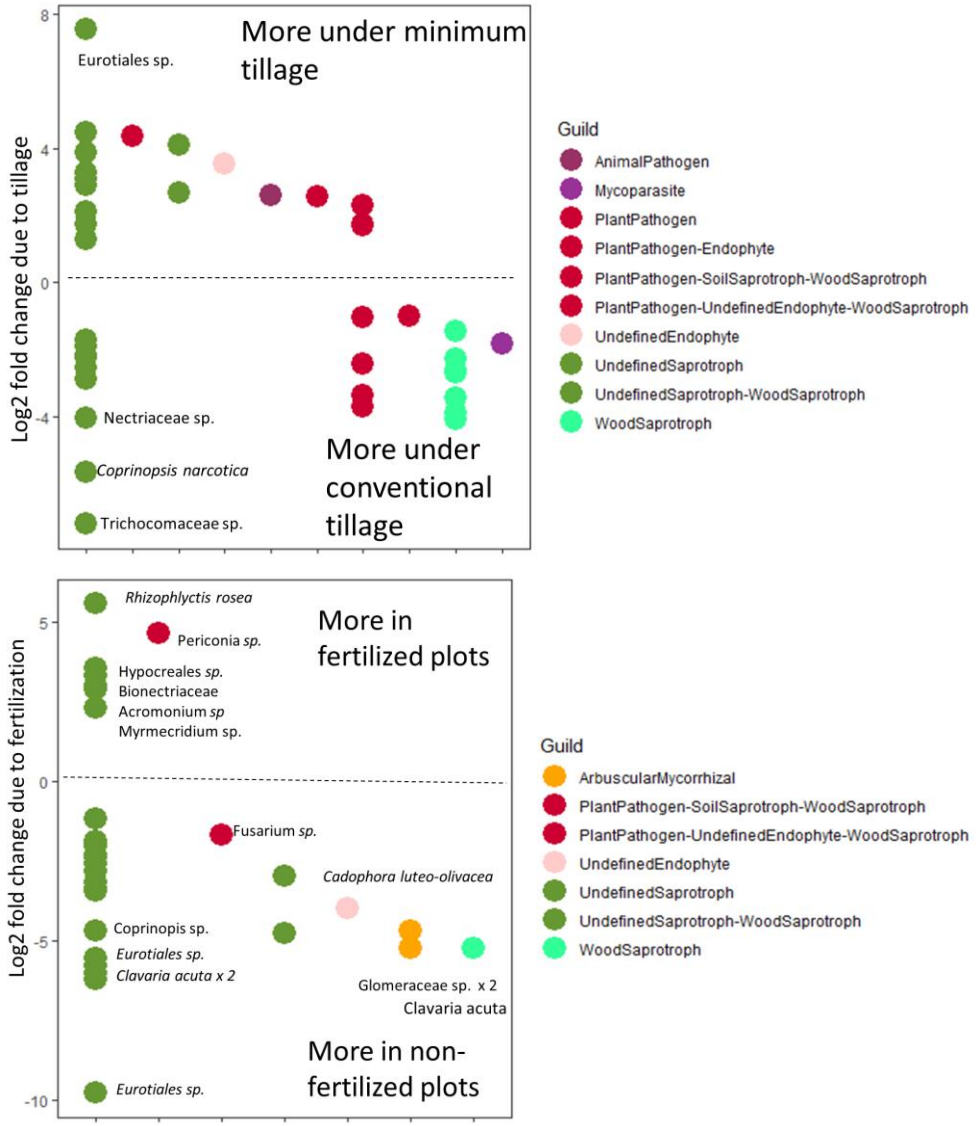

Figure 23. HUN-2. Fungal taxa significantly affected by type of tillage (top; contrast between conventional tillage on the one hand and shallow disk and minimum tillage on the other hand) and N-fertilizer (bottom; contrast: non-fertilized (N0) verses N-fertilized (N2 and N4).

3.2.3. Hungary-3 (HUN-3; rotation, current crop species and manure addition)

This experiment started in 1963 and studied the effects of two different rotations (rot1: winter wheat - alfalfa - alfalfa - winter wheat - maize; rot2: winter wheat - oats and vetch - winter wheat - sorghum - maize) and of fertilization (no fertilization (control), 520 kg NPK/ha /5yr, 2080 kg NPK/ha/5yr and 2080 kg NPK + 35 t farmyard manure/ha/5yr). The farmyard manure is applied before the maize in every fifth year of the rotation. The fertilizer rates applied annually are different according to the different needs of the different crops, but the total quantity is the same in each rotation during one rotation period. For more details on the experimental set-up, see Annex 1. Soil sampling took place for rotation 1 in winter wheat and for rotation 2 in maize.

Rotation had no effect on fungal richness (Fig. 24, top), but it had so on fungal index (Fig. 24, bottom), where rot1 was more than 2× higher in the relative abundance of beneficial fungi than rot2,

irrespective of the current crop. Fertilization had significant effects only on fungal community composition (resp. Figs. 24 and 25) while it did not affect fungal richness (Fig. 24, middle). Furthermore, the fertilizer regime had a stronger effect on fungal composition than the current standing crop (Fig. 25). Especially the unfertilized treatment separated off from the other fertilizer treatments having very different fungi in the soils. Fungal richness was affected by the current plant species and was higher when the current crop was winter wheat. The functional group of animal pathogens were more present in rot1, while mycoparasites were more present in rot2 (Fig. 26). The difference in animal pathogens was due to the (soil) insect parasite *Metarhizium* sp. The two rotations had 73 fungal taxa showing significant differences, with 16 taxa more common in rot1 and 57 taxa more common in rot2 (Fig. 27). In contrast, only 3 taxa showed treatment effects of fertilization; these 3 taxa, *Podospora* sp., *Thermomyces dupontii* and *T. lanuginosus*) were more common in NPK with manure compared to NPK only. The great majority of the taxa were saprotrophs (Fig. 27).

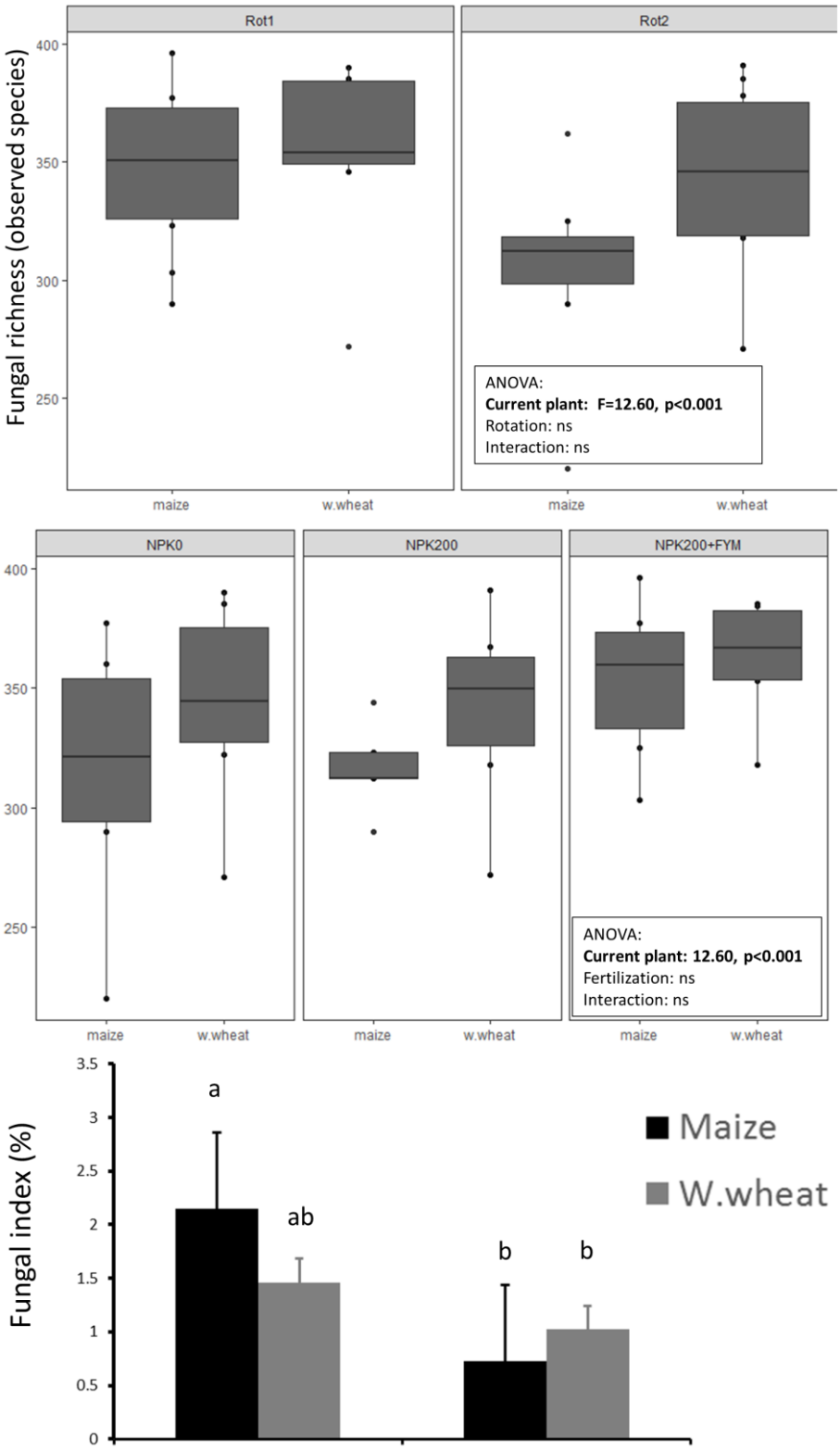

Figure 24. HUN-3. Effects of rotation (top; rot1, rot2, see for explanation the introduction of this section) and fertilization (bottom; idem) on fungal richness, and effects of rotation and crop species at the moment of sampling (maize or winter wheat) on fungal index (bottom).

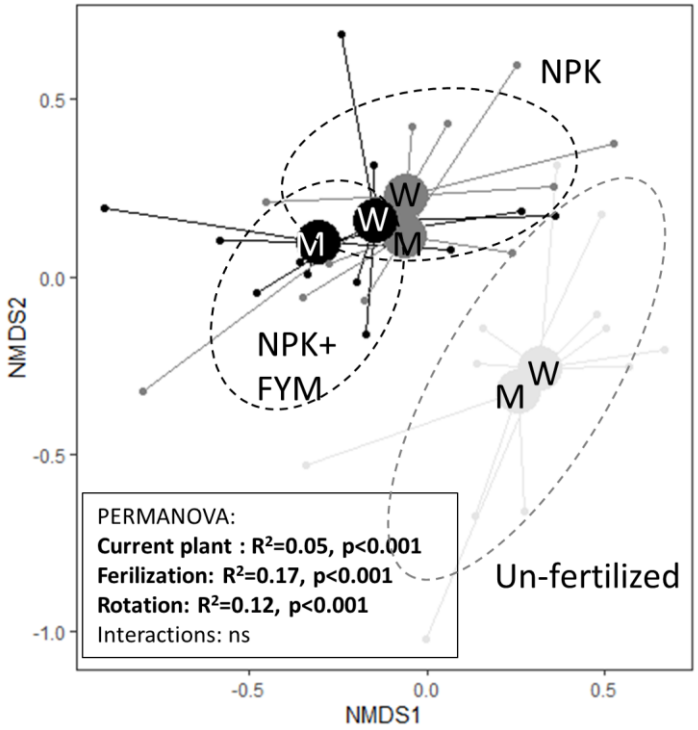

Figure 25. HUN-3. Fungal composition function of the treatments and last crop before sampling. M = maize, W = winter wheat. Each small point represents one sample, and the six large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

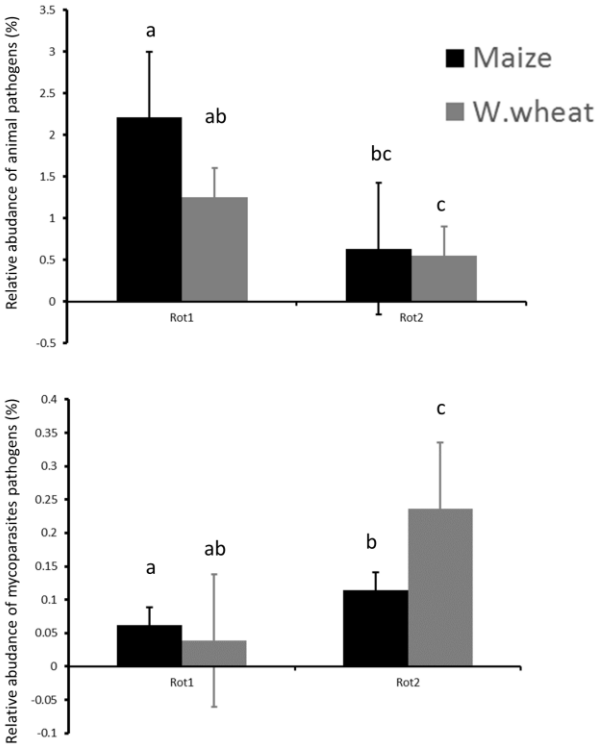

Figure 26. HUN-3. Effects of rotation (rot1 and rot2, for explanation see the introduction of this section) and current crop at sample taking (maize or winter wheat) on the relative abundance of animal (top) and fungal pathogens (mycoparasites; bottom).

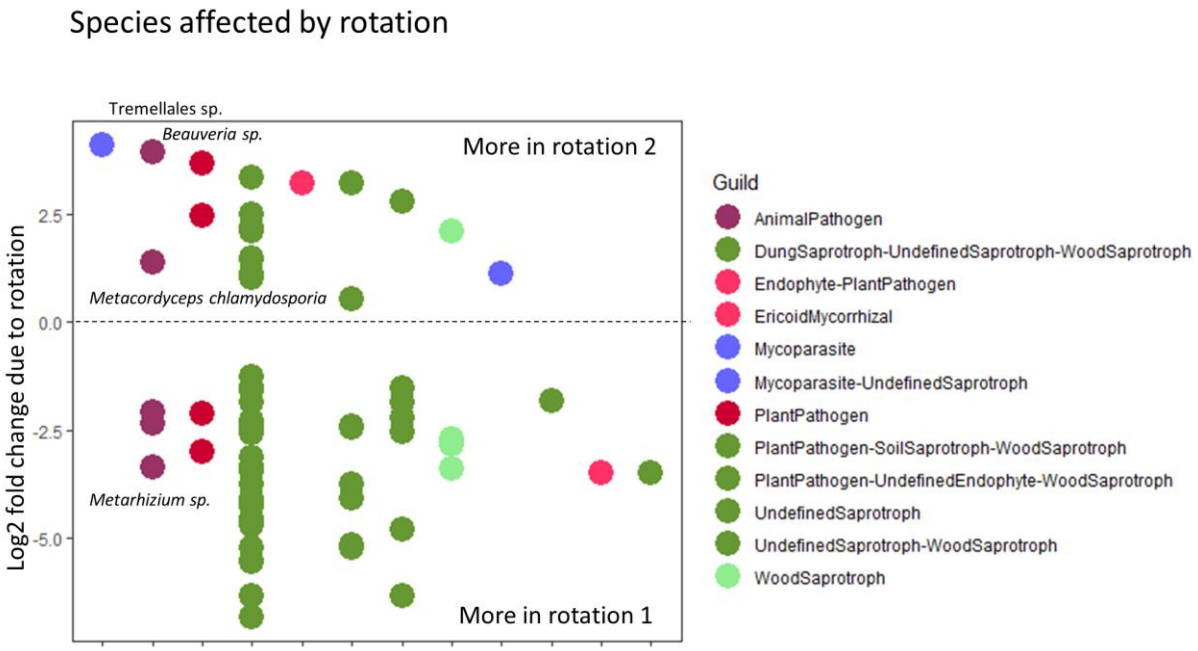

Figure 27. HUN-3. Fungal taxa specifically affected by rotation. For the rotation types, see the introduction of this section.

3.2.4. Hungary-4 (HUN-4; amount of mineral fertilizer and timing of fertilizer application)

This experiment with continuous maize started in 1969 and studied the effects of 4 increasing rates of mineral NPK fertilizers: 0, 300, 600 and 900 kg N:P_2O_5:K_2O/ha/yr (N:P_2O_5:K_2O = 1:1:1) and timing of N application: once in autumn, once in spring or twice in spring. For more details on the experimental set-up, see Annex 1.

Fungal richness was significantly higher ($P<0.05$) in the 900 kg NPK/ha/yr than in the unfertilized treatment, but was not affected by the timing of application (Fig. 28). More significant was the effect of fertilization on fungal composition (Fig. 29). About 17 taxa had been affected positively and the same amount negatively by fertilization (Fig. 30).

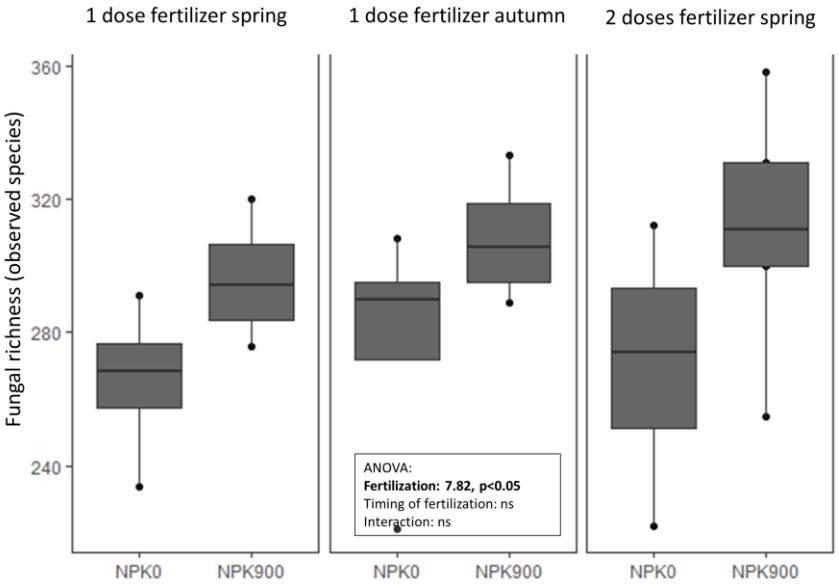

Figure 28. HUN-4. Effect of amount of fertilizer and timing of fertilizer on fungal richness.

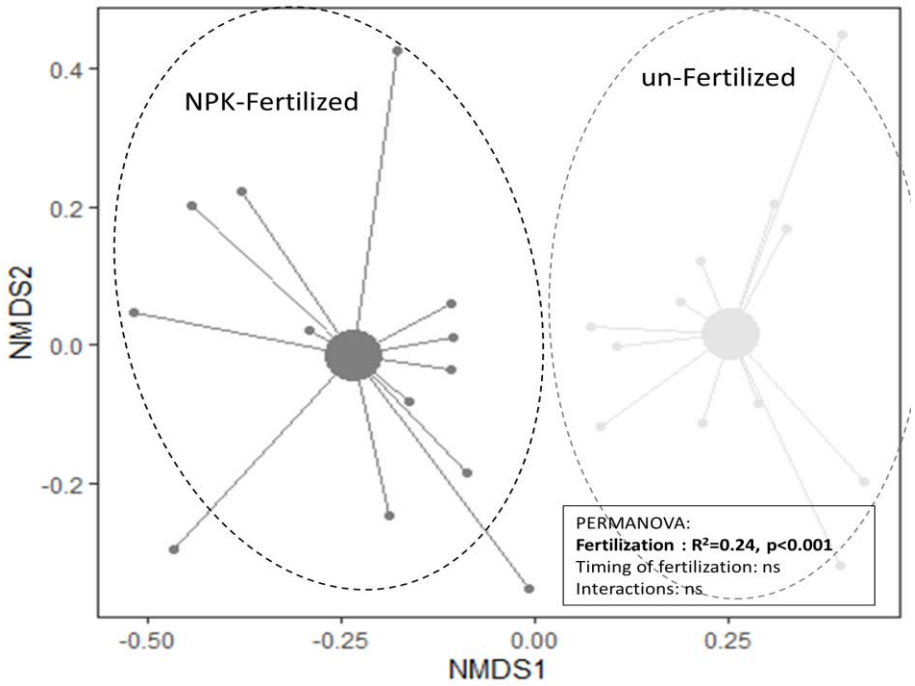

Figure 29. HUN-4.Effect of fertilization treatment on fungal composition. NPK-fertilized = 900 kg/ha/yr NPK. Each small point represents one sample, and the two large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

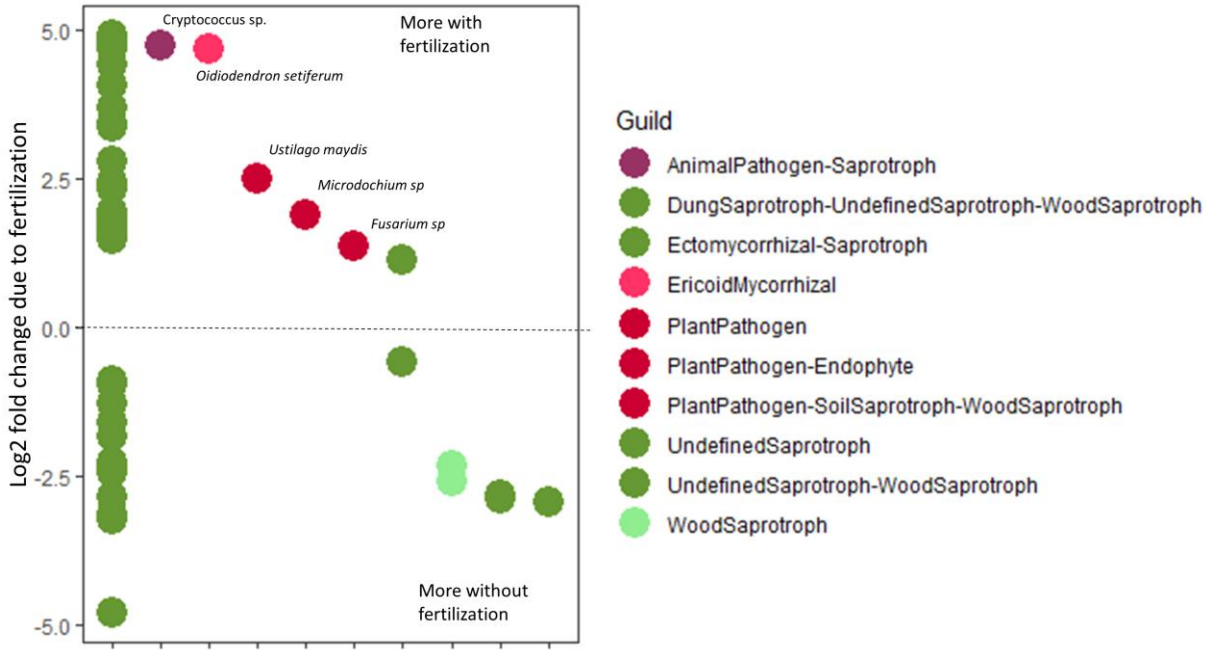

Figure 30. HUN-4. Fungal taxa specifically affected by fertilization.

3.3. United Kingdom experiments

The partner from the UK participated with 2 LTEs.

3.3.1. United Kingdom-1 (UK-1; tillage/no tillage)

The effect of tillage was studied at Loddington, Stonton and Normanton, where three farmers each split one field into a section receiving tillage and one receiving no-tillage. For more details on the experimental set-up, see Annex 1.

Effects of tillage on fungal richness was insignificant (Fig. 31), but on fungal index tillage had a significant ($P<0.05$) effect as well as on animal parasites, but no effect on arbuscular mycorrhizal fungi (AMF) (Fig. 32). Tillage also had no effect on overall fungal composition (Fig. 33). No-tillage significantly increased a species of *Exophilia* and *Apodus deciduous*, while species of *Trichoderma*, *Uncobasidium*, *Penicillium* and *Trichocomaceae* were more common in the fields receiving tillage.

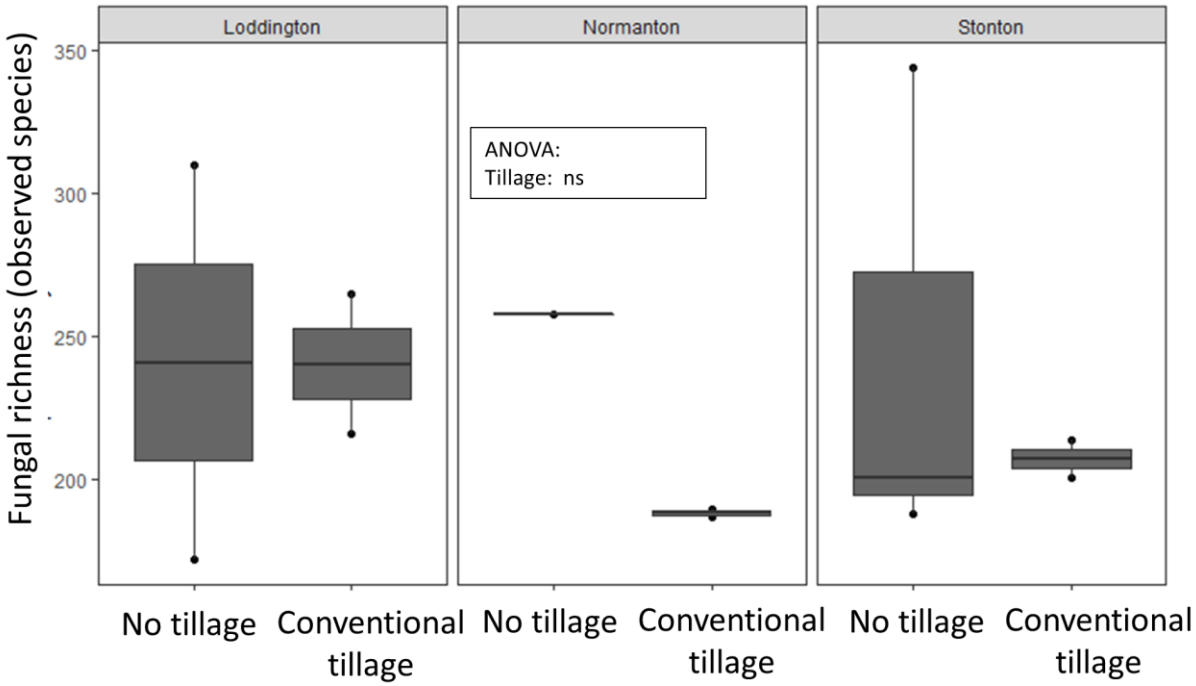

Figure 31. UK-1. Effect of tillage on fungal richness at three locations.

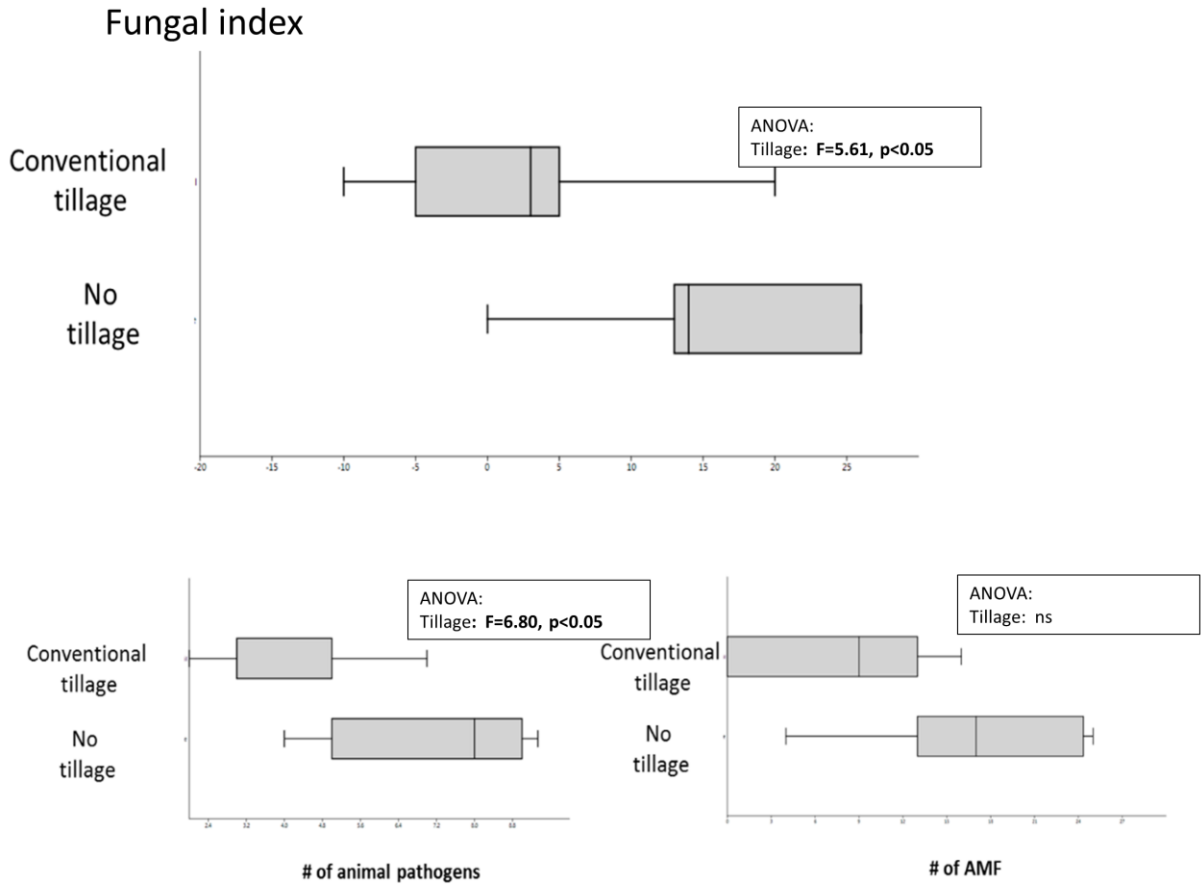

Figure 32. UK-1. Effect of tillage on the fungal index; top: all fungi; bottom-left: animal pathogens; bottom-right: arbuscular mycorrhizal fungi (AMF).

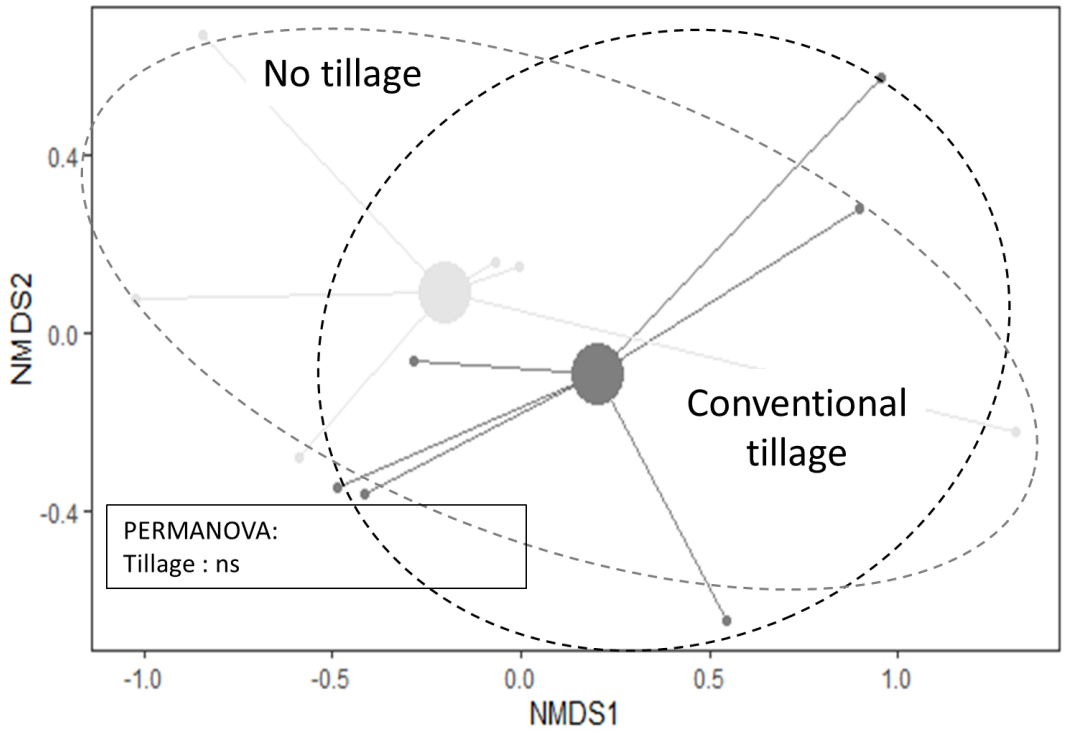

Figure 33. UK-1. Effect of tillage on fungal composition. Each small point represents one sample, and the two large points are the centroids per treatment. The more distant the points are, the more different they are in fungal composition.

3.3.2. United Kingdom-2 (UK-2; cover crops)

The effect of cover cropping was studied at Loddington, existing of oats and phacelia (mix1), oats, phacelia, rye and radish (mix2) or oats, phacelia, radish, buckwheat, and the legumes vetch, crimson clover and berseem clover (mix3). The control was left fallow in winter time. For more details on the experimental set-up, see Annex 1.

Cover crop did not have an effect on fungal richness (Fig. 34) or fungal composition (Fig. 35), but in the latter, mix3 seemed to have an effect. At OTU-level, the control (no cover crop) had more unique OTUs (88) than with cover crop (8) (Fig. 35). The treatments with cover crop also had quite some unique OTUs, for the three mixtures mix1, mix2 and mix3 59, 87 and 63, respectively (Fig. 36). Quite some species (6, 8 and 12 in mix1, mix2 and mix3, respectively, and 7 in the control) were significantly more abundant in one or more of the treatments (Table 4). Although not a big difference, it is noticeable that this number of species increased with the number of crop species in the cover crop mixture. The same picture appeared for the unique number of OTUs for the mixtures (Fig. 36, bottom-right).

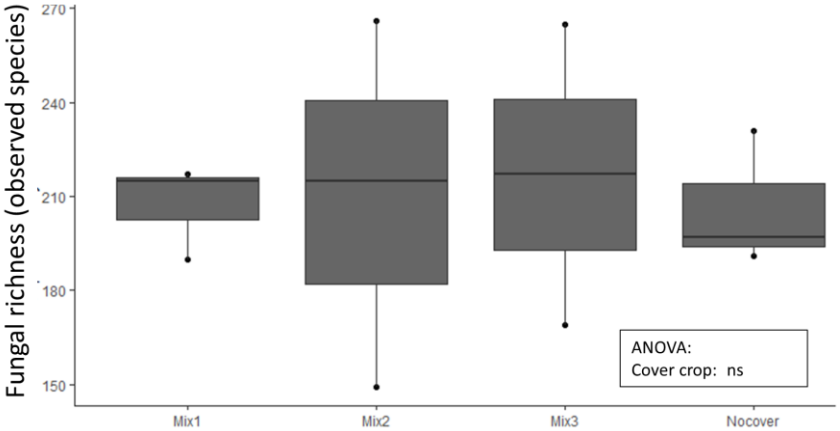

Figure 34. UK-2. Effect of cover crop on fungal richness; cover crop mixtures: mix 1: oats and phacelia; mix2: oats, phacelia, rye and radish; mix3: oats, phacelia, radish, buckwheat, and the legumes vetch, crimson clover and berseem clover; nocover: no cover crop (control).

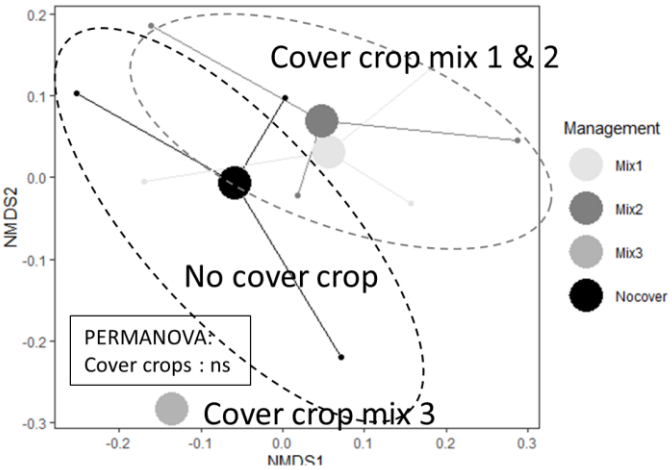

Figure 35. UK-2. Effect of cover crop on fungal composition; cover crop mixtures: mix 1: oats and phacelia; mix2: oats, phacelia, rye and radish; mix3: oats, phacelia, radish, buckwheat, and the legumes vetch, crimson clover and berseem clover; nocover: no cover crop (control).

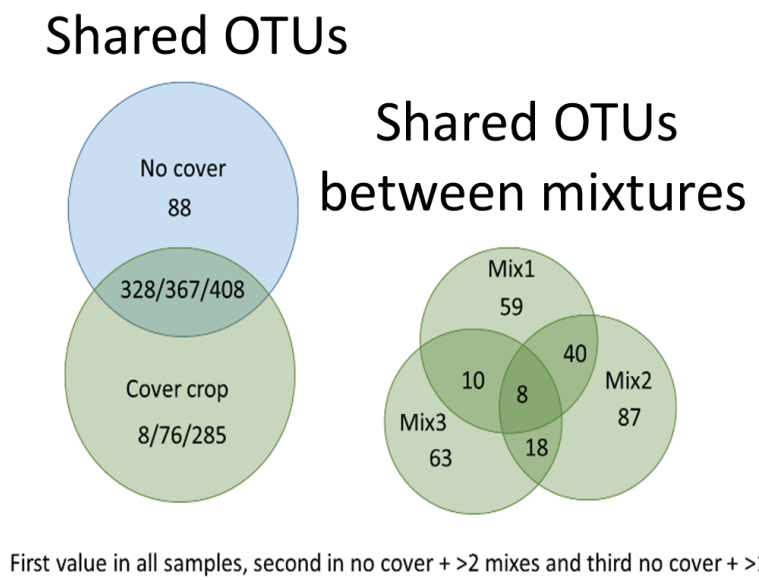

Figure 36. UK-2. Effect of cover crop on OTUs. Left: the three values mean, respectively: present in all samples / present in control (no cover) and 3 mixes / present in control (no cover) and 2 or 3 mixes; cover crop mixtures: mix 1: oats and phacelia; mix2: oats, phacelia, rye and radish; mix3: oats, phacelia, radish, buckwheat, and the legumes vetch, crimson clover and berseem clover; nocover: no cover crop (control).

Table 4. UK-2. Species specifically stimulated in the various treatments; cover crop mixtures: mix 1: oats and phacelia; mix2: oats, phacelia, rye and radish; mix3: oats, phacelia, radish, buckwheat, and the legumes vetch, crimson clover and berseem clover; nocover: no cover crop (control). * = presence in some samples; ** = presence in over half of the samples; *** = presence in all samples of the treatment.

Fungal taxon	host	mix 1	mix 2	mix 3	no cover
Ilyonectria sp.	unknown			**	
Verticillium sp.	unknown			*	*
Devriesia pseudoamerica	apple	*	*		
Blumeria graminis	Gramineae			**	
Clonostachys sp.	unknown		*	**	*
Colletotrichum sp.	Unknown	*	**	*	
Phaecytostroma ambigum	maize, sorghum			**	
Tilletiaria anomala	unknown				*
Ustilago trichohora	grasses			**	
Ilyonectria robusta	woody dicots	*		**	
Protomycetes inouyei	Compositae	*	**	*	**
Cylindrocarpon sp.	unknown		***		*
Ustilago maydis	maize		*	***	
Verticillium sp.	unknown	*	**	***	***
Ilyonectria sp.	unknown	***	***	***	***

3.4. Belgium experiments

The partner from Belgium participated with 2 LTEs.

3.4.1. Belgium-1 (BEL-1; compost and tillage regime)

This experiment involves the comparison of conventional tillage (25-30 cm soil depth) versus non-inversion tillage and started in 2001 (at Huldenberg) and in 2004 (at Lubbeek) with a rotation of winter wheat, winter barley, sugarbeet and potato. For more details on the experimental set-up, see Annex 1.

This experiment has not been replicated well and therefore here only the observed trends are presented shortly. Fungal richness was greater for the conventional tillage treatment (Fig. 37). Effects of tillage on composition seemed about as large as, or smaller than, the effect of the standing crop (maize vs. potatoes) (Fig. 38).

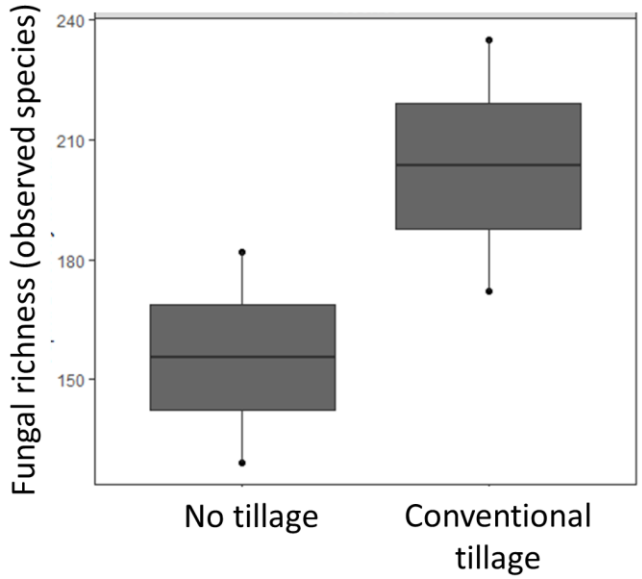

Figure 37. BEL-1. Effect of tillage on fungal richness.

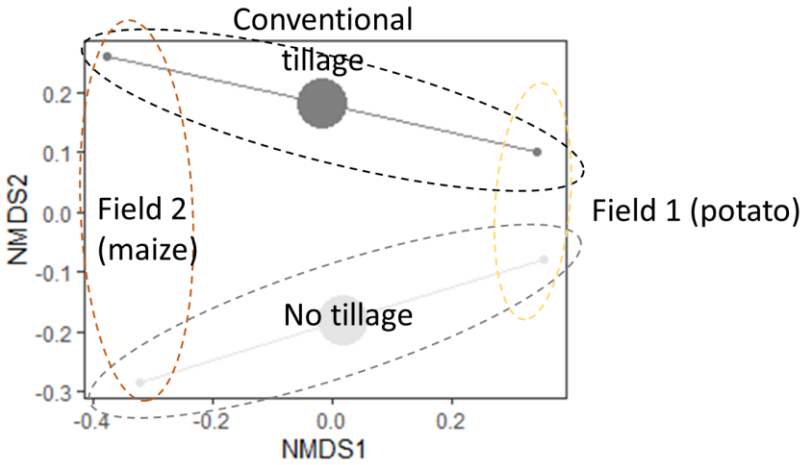

Figure 38. BEL-1. Effect of tillage and standing crop on fungal composition.

3.4.2. Belgium (BEL-2; long-term compost trial)

This experiment was set up in 1997 on a loamy soil in Flanders to study the effect of compost, with as treatments compost made of vegetable, fruit and garden waste (15, 30 or 45 tonnes/ha) applied one-, two- or three times a year. Controls included an amendment with mineral fertilizer, a non-fertilized control and a non-fertilized fallow. The rotation included sugar beet, potatoes, winter wheat, carrots and onions. For more details on the experimental set-up, see Annex 1.

Fungal richness was not affected by the treatments, although there seems to be a tendency towards a lower richness in the compost-amended treatments (Fig. 39). Fungal community composition was affected by the addition of compost, more than adding mineral fertilizers. Also the amount of compost added affected the community composition (Fig. 40). The relative number of arbuscular fungi was significantly lower in all treatments as compared to the non-fertilized plots (Fig. 41).

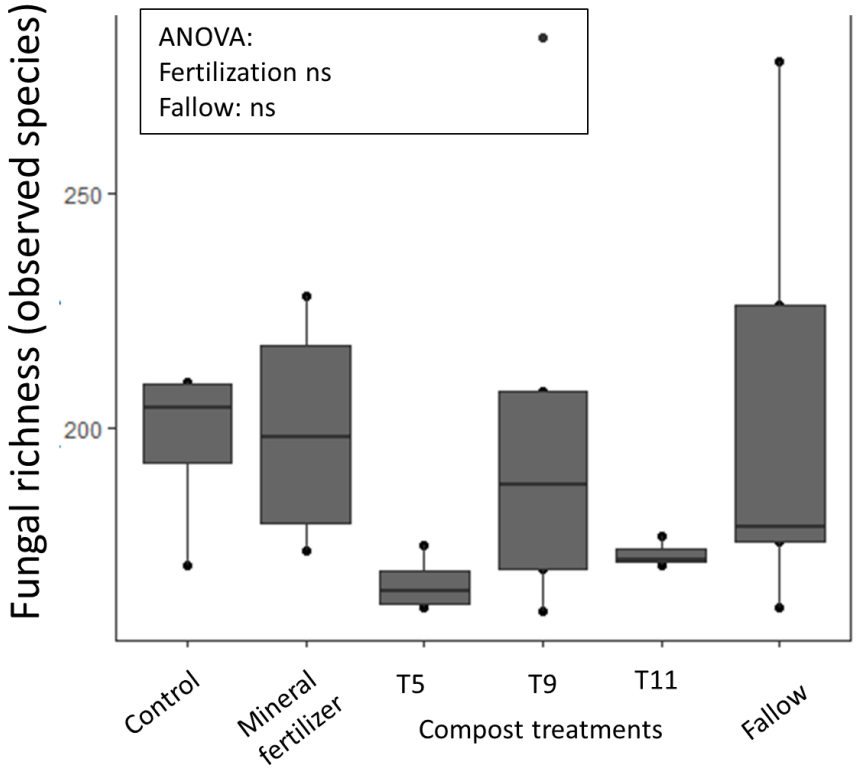

Figure 39. BEL-2. Effect of compost on fungal richness. Compost treatments: T5 = tri-annual compost amendment (45 tonnes/ha in total), T9 = annual compost amendment (15 tonnes/ha), T11 = annual compost amendment (45 tonnes/ha).

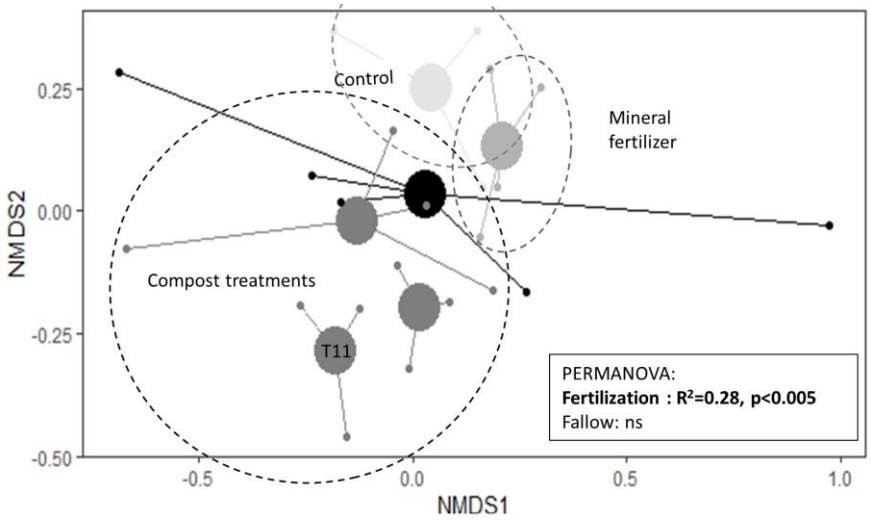

Figure 40. BEL-2. Effect of compost amendment on fungal composition.

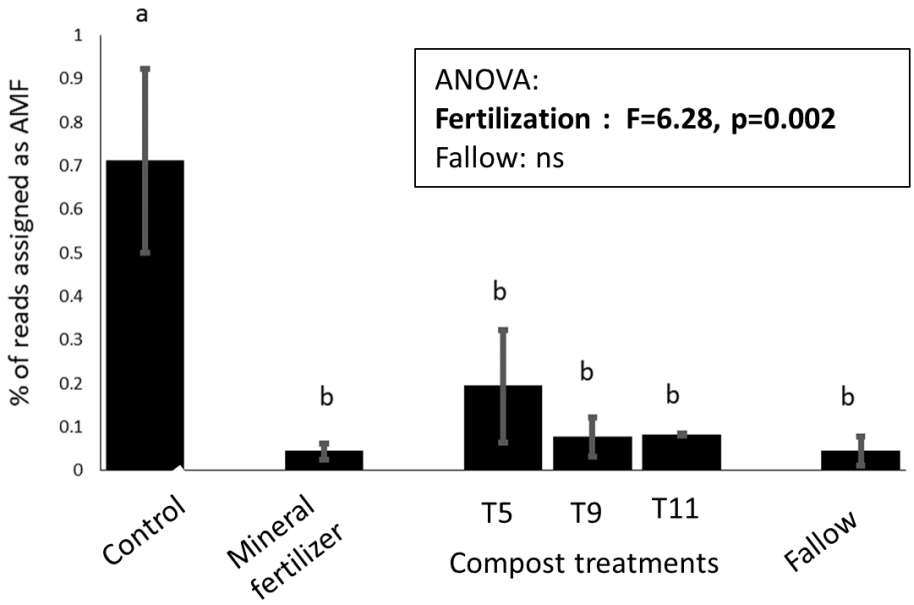

Figure 41. BEL-2. Effect of compost amendment on relative number of arbuscular mycorrhizal fungi (AMF).

3.5. Summing up

Table 5 provides a short summary of the significant effects found for the different LTEs. Clearly effects have been found, but they differ considerably for the different LTEs. For example, tillage had clear effects in HUN-1, but much less so in DEN-4 and BEL-1 (Table 5). Also fungal index and richness did not show similar effects. Perhaps more importantly, also specific taxa showing significant differences between treatments were relatively small, with only few functional groups showing significant effects. If all the methods of the various LTEs to improve soil quality were compared with the controls, no general picture appeared in (elements of) the fungal community that pointed in the same direction (Hannula et al., 2021).

Table 5. Short summary of significant effects found in par. 3.1-3.4. For more details, see respective paragraphs. * = P<0.001, ** = P<0.01, * = P<0.05.

		fungal				pathogens		sapro-trophs	AMF	myco-parasites
exp.	treatment	index	rich-ness	compo-sition	taxa (#)	plant	animal			
DEN-1	liming (L) fallowing (F) P-fert. (P)	L**		L***	L:17 F:47 P:0	L**			L*	
DEN-2	straw (S) cover crop (C)	S*	S*	S*** C*	S:27 C:0	S*	S*			
DEN-3	manure (M) artificial fertilizer (F)			M**	M:8 F:1					
DEN-4	tillage (T) cover crop (C)			T*	T:6 C:0					
HUN-1	stalks (S) nitrogen (N) manure (M)	N*	S*		S:0 N:0 M:1				M*	
HUN-2	tillage (T) nitrogen (N)	T* N*	N*	T*** N***	T:18 N:27	N**				
HUN-3	rotation (R) manure (M)	R*	R*	R*** M***	R:73 M:3		R*			R*
HUN-4	fertilization (F) timing of fert. (Ti)		F*	F***	F:34					
UK-1	tillage	*			6		*			
UK-2	cover crops				15					
BEL-1	compost (C) tillage (T)		T*		C:0 T:0					
BEL-2	compost				0					

Although the variation in results between LTEs is big, one striking result that can be seen from Table 5 is that relatively many significant effects in fungal community differences between treatments have been found for the Danish and Hungarian LTEs, with in total 29 significant effects for 8 LTEs (average 3.6 per LTE); while on the other hand the average was 0.75 per LTE for those from UK and Belgium. The number of fungal taxa showed the same tendency, with 33 and 5.3 species per LTE showing some significant effect for, respectively, Denmark/Hungary and UK/Belgium. In part this difference could be explained by the experimental set-up: UK-1, UK-2 and BEL-1 were LTEs managed by different farmers, and repetitions were done at the farmer's level only, while the Danish and Hungarian LTEs were performed by experimental stations on relatively small fields with relatively many repetitions. Also, the LTEs from Belgium and UK were installed more recently than those of Hungary and Denmark. Possibly, because of the large field sizes in UK-1, UK-2 and BEL-1, within-field and between-farmer variation explains the shortage of significant effects. If this is true, then it would be difficult to find useful parameters for soil quality based on fungal soil communities. The second LTE from Belgium, BEL-2, is in many respects exceptional, since this is a well-repeated experiment on relatively small fields, yet no significant effects were found. For this we have no explanation. In a previous study on this experiment, it appeared that effects of compost on bulk density were significant but not very large (i.e. a change from 1.46 g/cm^3 in the non-amended compost to 1.38-1.42 g/cm^3 in the various compost amendments in a period of 14 years; de Clercq et al., 2016).

However, even if we would deal only with the Danish and Hungarian LTEs, then still it is difficult to find a general pattern in the results with, for example, little effects in DEN-4 and HUN-1, strong effects of manure applications in DEN-3 and HUN-3, but no effects in HUN-1. Hence, it is difficult to generalize beyond the observation that most agricultural practices do affect soil fungal

community structure, and in some cases also the richness and number of beneficial fungi (measured as the index).

In spite of the many significant effects that have been found, the effects at the fungal taxon and functional group level were limited (Table 5) and we were further hampered with the low number of shared species across sites. It could be that still unknown taxa or even clusters of fungal taxa are affected by soil improving management styles and further work in characterization of soil fungi re needed.

3.6. Linking fungal community composition with soil chemistry

Although it is not the main goal to study the relation between soil chemistry and texture and the fungal soil community, knowledge on its relation contributes to the understanding of fungal soil community dynamics. Taking all the samples in this study together and correlating this with soil chemical data, it seems that soil texture, C/N ratio and organic matter content are important contributing factors explaining the fungal variation (Fig. 42).

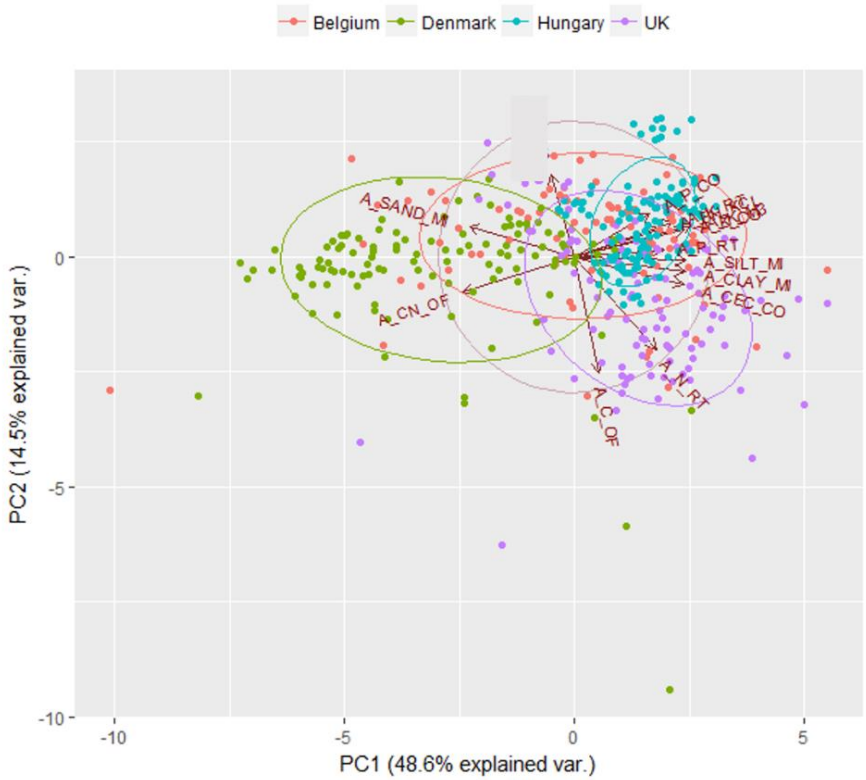

Figure 42. Principal component analysis of community structure of fungi correlated with soil chemical and texture data. A_SAND_M, A_SILT_M and A_CLAY_M: proportion of sand, silt and clay, respectively; A_CN_OF: C/N-ratio, A_C_OF: organic matter content, A_N_RT: total N.

4. Discussion and conclusions

This study aimed to set the first steps to use soil fungal community structure as estimate for soil quality. The first question here was, can a soil be judged in terms of soil quality/health based on fungal soil community data obtained by metabarcoding? The approach was to analyze soil samples of 12 long-term experiments (LTEs) that aimed to improve the quality of soil. The hypothesis was that LTEs would change the fungal community structure in a consistent, detectable manner. The subsequent step, to indirectly assess the fungal-based characterization of soil quality using NIR/MIR-spectra was not done here since our dataset was too small to carry this out, together with the high variability in fungi between sites and the insufficiently significant differences within LTEs (Table 5). With a "too small dataset" we primarily mean that the number of identified taxa and from this the number of taxa we could assign to certain functional groups were too limited. This problem will be discussed further below. In this section we discuss how the fungal soil community structure could become an estimate for soil quality, and how from this a business case can be made.

The dataset was analyzed in two ways: (1) all data of the different LTEs combined or (2) the data of each LTE separately. The first approach, all data of the different LTEs combined, was published recently (Hannula et al., 2021). The major conclusion from this study was, that there did not appear a consistent picture that the various soil improving cropping systems (SICS) were changing the fungal communities in a predictable way. The second approach is detailed in this report. In line with Hannula et al. (2021), the comparison of the different LTEs did not show a consistent pattern (Table 5). But, as presented in par. 3.5, also looking at the individual LTEs it is difficult to come up with specific fungal indicators that could predict the soil health status at that specific LTE. Although the fungal index based on relative abundances of beneficial and detrimental soil fungal groups and fungal richness indicating soil fungal diversity were thought to show clear differences between control soils and soil improving practices, these parameters only occasionally showed strong effects and moreover, only in three cases they had similar effects when experiments with similar treatments (such as tillage) were compared to each other (Table 5). More consistent were the effects of treatments on fungal community (Table 5). However, this measure is difficult to use as soil health parameter, since it is evaluated only relative to other treatments (in this case, the control treatment). The functional groups of fungi, which we expected to have the most interesting value and soil quality indicators had only occasionally some significance, and again the observed effects were not comparable between experiments (Table 5). Finally, also identified fungal taxa did not show strong effects (Table 5) that were, moreover, difficult to interpret. So, overall, the results of the fungal metabarcoding show that the most soil improving practices change the soil fungal community structure but that it is difficult to draw general patterns or predict the effects on larger scale from the data collected here. There could be various reasons for these results:

- Soils are superdiverse in fungal species. Using fungal soil community as indicator for soil quality requires ecological knowledge of these species. In total, 2101 OTUs were detected, and although to these for over 95% some taxonomic level could be assigned, only to 2% a species name and to 66% a genus name could be assigned. To make any ecological inference from OTU data, except perhaps for arbuscular mycorrhizal fungi, a species name is needed. The low number of species names recovered may be caused by the fact that probably a high proportion of fungi cannot be cultured, a trait that is still indispensable to attach names to OTUs. This may change in future when we will know more about ecological traits of unculturable fungi having certain OTUs. The continuously improving UNITE database is due to increase the number of cultured and described species that is included, and genomic information derived from uncultured organisms in soils will certainly provide more ecological traits of unculturable fungi in the near future (Pölme et al., 2021).
- The data from metabarcoding are relative, hence not quantitative. Relative quantities can be difficult to interpret, as an observed change of a certain functional group can be a true change, but the change can also be only apparent due to the change in quantity of another group. There are

ways around this, like measuring also ergosterol content or PLFAs. For some targeted species, qPCR is also possible, but this makes the whole assessment too costly.

- DNA-metabarcoding includes viable and unviable DNA (Emerson et al., 2017). Although this could be regarded as problematic, it could also be seen as an advantage, since by taking into account DNA from organisms that have recently died makes a sample less dependent of the time when it was taken.
- DNA-metabarcoding does not provide information about the status of activity of the fungi revealed (Emerson et al., 2017); it may be dormant or active. Like the previous point on viability, this also was not thought to be problematic, since by including active and dormant propagules the metabarcoding would give a less time-dependent picture of the fungi present.
- The assignment of species names to their ecological function can be difficult, because (1) many fungi can act differently under different circumstances (e.g. plant pathogens that may also behave like saprotrophs), (2) fungi appearing from metabarcoding as the same species may actually belong to different strains that vary strongly in ecology (e.g. strains of *Fusarium oxysporum* and *Rhizoctonia solani*) and (3) of many fungi their precise function is not yet known.
- In the analysis of all LTEs together (Hannula et al., 2021), differences may be due to the different set-ups of experiments, their duration, and the different soil types on which they have been carried out. These results together point out that due to immense diversity of soil fungi, many more sampling points and LTEs need to be sampled in order to predict the soil fungal communities

One general drawback of this study is that we had to rely on existing LTEs on which data on soil structure was lacking; so a direct link between the fungal soil community and parameters directly related to soil structure could not be made. However, even if these data were available, then still the large variation between treatments across LTEs would have been problematic (Hannula et al., 2021).

For the global perspective on the effects of SICS on soil quality, we conclude that the current study has been carried out too early, i.e., with a UNITE database that is too limited and DNA metabarcoding being too expensive and labor-intensive to collect a representative number of samples considering the large diversity detected here. Furthermore, more soil parameters relating to soil health should have been collected simultaneously with fungal community analysis to further learn about the link between soil fungi and soil health. A step forward would be to repeat the study first on smaller scale, with one LTE with treatments that already have led to clear differences in soil structure as determined by various quantitative parameters, and relate this to the fungal community based on various samples taken during the growing season and from various crops. Also novel methods to characterize organic matter, such as pyrolysis-gas chromatography/mass spectrometry (Derenne & Quenea, 2015) could contribute to the unravelling of the relation between fungal biodiversity and functioning and soil structure-related parameters. A second future perspective is to increase the number of sites studied and limit the investigation to few SICS whose effects on soil fungi can be then compared across sites. With more sites repeated across soil types, it would be possible to generalize what are the effects of specific soil management on soil fungal communities, soil health as well as crop yield. This larger dataset would also allow full comparison of links between soil chemistry measured via NIR/MIR and soil fungal community.

Although we did not manage to find overarching ecological patterns, this work provided valuable, novel, and appreciated information on individual LTEs that could be used by the individual farmers (UK, BEL) and site managers (HUN, DEN). For example, although pathogens are not suitable for predicting soil quality, the presence of certain pathogens can affect the crop choices in the near future.

5. References

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Annex 1. Description of LTEs

DEN-1: The St. Jyndevad Long-Term experiment on liming and superphosphate fertilization (St. Jyndevad-lime and P)

This experiment was initiated in 1942 at St. Jyndevad Experimental Station, Denmark (54º 54' N, 9º 07' E) at 16 m above sea level. The mean annual precipitation is 870 mm and mean annual temperature is 7.9 C. The landscape is flat, naturally well drained and soil has developed on melt water sand from the Weichselian glacial stage. The soil is a coarse sandy soil (Orthic Haplohumod, siliceous, mesic), containing less than 5% clay (Nielsen & Moberg, 1985). The treatments in the field experiment includes all combinations of four levels of liming and four levels of P fertilization in three replications within each field. The liming treatments applied every 4-7 years as limestone ($CaCO_3$) enriched with 6 to 10 % dolomite (calcium-magnesium carbonate (Ca-Mg$(CO_3)_2$)) were: No lime applied (L0), 4 ton/ha (L4), 8 ton/ha, (L8) and 12 ton/ha (L12). Target pH measured in 0.01 M CaCl2 in the three treatments receiving lime were 5.4, 6.2, and 6.7 respectively. The P treatments applied as triple superphosphate are 0 kg P/ha, 156 kg P /ha initially at the beginning of experiment, 15.6 kg P/ha yearly and single dose of 156 kg P/ha plus 15.6 kg P/ha yearly. The lime treatments started in 1942 while the P treatments started in 1944.

The field experiment is encompasses four different fields designated V1, V2, V3 and V4. The present use of V1 and V2 are arable. At field V3 treatments were omitted since 1996 when the field was set aside as permanent grassland. At field V4 treatments were omitted since 1964 when trees were planted.

The arable fields are ploughed yearly to 20 cm depth (occasionally a ploughing depth of up to 22 cm is reached) in spring prior to seed bed preparation and sowing. There are three blocks with 16 treatments in each field. Within each block, the lime treatments are laid out as main plot factors and the four P fertilizer treatments are split-plot factors within the lime treatments. Plot arrangement is systematic. For V1 and V2, the size of each treated plot was 11.25 m × 8 m, while the harvested plot was 7.6 m x 3.3 m. Other nutrients such as nitrogen (N), potassium (K) and magnesium (Mg) are applied at adequate levels for crop growth. During the first eight experimental years the following crop rotation were practiced: Swedish turnip, oat, lupine and rye (Dorph-Petersen, 1953), but spring barley (*Hordeum vulgare L.*) has been grown continuously for the past 35 years. Further details on the long-term experiment can be found in Rubæk (2008), Rubæk et al. (1998) and Azeez et al. (2020).

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DEN-2: The Askov Long-Term Straw experiment (Askov-Straw)

The experiment was established in 1980 on the Lermarken O1-field at Askov Experimental Station, Denmark (55°28'N, 09°07'E; elevation 63 m a.s.l.). Annual average precipitation, potential evapotranspiration and temperature are 862 mm, 543 mm and 7.7 °C, respectively (1961-1990

averages). The soil is a light sandy loam derived from Weichelian glacial deposits (Nielsen & Møberg, 1984) and classifies as Ultic Hapludalf (Soil Survey Staff, 2014) and as Aric Haplic Luvisol (IUSS Working Group WRB, 2015). The topsoil (0-20 cm) has 10% clay (<2 µm), 12% silt (2-20 µm), 43% fine sand (20-200 µm) and 35% coarse sand (200-2000 µm).Soil pH is maintained in the range 5.5-6.5 by addition of magnesium-enriched lime every four years.

Spring barley dressed with NPK mineral fertilizers has been grown every year since 1981, except for the years 2000, 2001, 2002, 2008 and 2013 when wheat replaced barley. Starting autumn 1980, different annual rates of barley straw (0 (only stubbles), 4, 8 and 12 t straw/ha) has been incorporated in separate plots after harvest of barley, except for the years 1999, 2000, 2001, 2002, 2008 and 2012 when straw was removed from all plots leaving only stubbles behind.

The main straw plots were subdivided into six subplots in 2002 to allow for the subsidiary treatments: no catch crop, a catch crop of ryegrass, and a catch crop of ryegrass/white clover. The catch crops were undersown in barley in spring. These treatments were combined with two ploughing times: October-November and February-March.

On 9 September 2016 (shortly after barley harvest and before any tillage operations) soil used in this experiment was sampled.

DEN-3: The Askov Long-Term Experiments on Animal Manure and Mineral Fertilizers (Askov-LTE)

The Askov Long-Term Experiments on Animal Manure and Mineral Fertilizers (Askov-LTE) was established in 1894 on the Lermarken site at Askov Experimental Station, Denmark (55º28'N, 09º07'E; elevation 63 m a.s.l.), the same site as DEN2. Annual average precipitation and potential evapotranspiration is 862 mm and 543 mm, respectively, and mean annual temperature is 7.7 ºC (1961-1990 averages). The Askov-LTE consists of four blocks termed B2-, B3-, B4- and B5-field and carries a four-course rotation of winter wheat (*Triticum aestivum*), silage maize (*Zea maize*), and spring barley (*Hordeum vulgare*) undersown with a grass-clover mixture (*Trifolium hybridum, Medicago sativa, Lotus corniculatus, Lolium perenne, Festuca pratensis, Phleum pratense*) that is used for cutting in the following production year.

The soil is a light sandy loam derived from Weichselian glacial deposits and classified as Typical Hapludalf according to the USDA Soil Taxonomy System. The topsoil (0-20 cm) has 10% clay (<2 µm), 12% silt (2-20 µm), 43% fine sand (20-200 µm) and 35% coarse sand (200-2000 µm). Soil pH is maintained in the range 5.5 – 6.5 by addition of magnesium-enriched lime every four years. Sulphur is applied annually at a rate of 12.5 kg S/ha.

The main treatments of the Askov-LTE are unfertilized plots (UNF) and plots amended with different levels (½, 1, 1½ and 2 times the standard rate for a given crop) of nitrogen (total-N), phosphorus (P) and potassium (K) in animal manure (AM) or in mineral fertilizers (NPK). Subsidiary treatments are mineral fertilizer N, P and K, added individually (1 N, 1 P and 1 K) or in combinations of two (1 NP, 1 NK and 1 PK). The nutrient levels were increased in 1923, 1949 and 1973 to comply with the general development in agriculture, but within each period, almost equivalent amounts of nutrients have been applied to corresponding NPK and AM treatments.

Since 1973, 1NPK and 1AM have corresponded to 100 kg total-N ha^{-1}, 20 kg P ha^{-1} and 90 kg K ha^{-1} (annual input across the rotation). Animal manure has been cattle slurry with an average of 5 % dry matter and 60-70 % of its total-N being ammoniacal-N. Nutrient level 1 typical corresponds to the addition of 25 t/ha of slurry (fresh weight). Mineral fertilizer N, P and K are given in calcium-ammonium-nitrate, triple-super-phosphate and potassium chloride, respectively. Not all treatments are present in all fields and the number of treatment replicates within each field differs.

Soil sampling for the present study took place in the B5-field on 9 September 2016 beneath the undersown grass-clover (following harvest of spring barley).

DEN-4 – The CENTS long-term field experiments on soil tillage and cover crops at Foulum

This experiment was established on a sandy loam soil at Foulum (56°30'N9°35'E) in Denmark in autumn 2002. The soil is developed on morainic deposit from the last glaciation and classified as a

Typic hapludalf according to the U.S. soil taxonomy and Mollic Luvisol according to the FAO system . The textural composition is 92 g kg^{-1} clay (<µ2 mm), 126 g kg^{-1} silt (2-20 µm) 444 g kg^{-1}fine sand (20-200 µm) and 307 g kg^{-1} coarse sand (200-2000 µm). The average organic carbon content was at the initiation of the experiment 18 g kg^{-1}. The normal annual temperature is 7.3 and the normal annual precipitation is 626 mm (1961-1990).

The experiment is randomized and replicated in four adjacent fields. Each field includes four crop rotations where four tillage regimes are carried out as main plots of a split plot arrangement within each crop rotation. Each tillage plot consists of two 3 m wide tillage bands of 72.2 m length. Different cover crops regimes are introduced in subplots within each tillage plot (Hansen et al., 2015; Abdollahi et al., 2013). For this study, we sampled soil in the crop rotation where mainly spring barley have been grown and where straw have been left in the plots every year. We sampled the treatments "direct drilling", "harrowing 8 to 10 cm depth" and "plowing" and we sampled two cover crop regimes: "without cover crop" (since 2008) and "with a cover crop" (fodder radish since 2007, perennial ryegrass before 2007).

For this study, on 9 September 2016 (shortly after harvest and before any tillage operations), soil was sampled

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HUN1-HUN4: **Description of experiments involved in Keszthely, Hungary**

The soil class is an Eutric Cambisol containing 41 % sand, 32 % silt, and 27 % clay. The available phosphorus content of this sandy loam soil is low (AL- P_2O_5: 60-80 mg kg^{-1}), the potassium content is medium (AL-K_2O: 140-160 $mgkg^{-1}$) and the pH-KCl value is 7,3. The topsoil is poor in lime, but the deeper layers of soil profile are calcaric.

The climate is semi-continental, the 100 year average annual precipitation is 683 mm, but the distribution is often uneven. The long-term annual mean temperature is 10.5 °C.

Monthly precipitation and mean temperature values in Keszthely, Hungary (1901-2000)

Months	Precipitation (mm)	Annual mean temperature (°C)
I.	34,5	-1,1
II.	35,0	1,4
III.	38,5	5,5
IV.	52,0	10,9
V.	69,0	15,8
VI.	79,0	19,0
VII.	76,0	20,9
VIII:	72,0	20,3
IX.	61,0	16,3
X.	56,0	10,6
XI.	61,0	5,2
XII.	49,0	1,3
Sum:	**683,0**	-
Average:	-	**10,5**

In the experiments crop residues are generally removed from the plots after harvest except the plots where the strow is applied as an extra organic amendment in accordance with the experimental treatments.

The generally applied mineral fertilizers are: nitrochalk (27% N), superphosphate (18% P_2O_5) and KCl (60% K_2O).

HUN-1: Organic amendments and Inorganic N Fertilization Experiment

The experiment was established in 1983 at Keszthely Experimental Station, Hungary (46°43'59.7"N, 17°13'47.9"E). The trial is a bifactorial experiment with a strip-plot design having three replications. In the experiment a three course cereal crop rotation is grown containing maize, winter wheat and winter barley in this order. The factors of the experiment are the complementary application of different forms of organic amendments and increasing rates of mineral N fertilization. The organic amendments have 3 different variants: no organic fertilizer application (control, straw is removed), farmyard manure (FYM) application (35 t/ha, in every third year before maize, straw is removed), straw/stalk (St) incorporation. After winter barley on the St plots an extra green manure (GM) crop, oilseed radish is grown (*Raphanus sativus* var. *Oleiformis*) as a 2nd crop. The N rates are 0-70-140-210-280 kg ha^{-1} in case of maize. P and K are uniformly applied as a rate of 100 kg ha^{-1} P_2O_5 and K_2O on each plot, even on the N control plots.

HUN-2: Soil Tillage Experiment

The experiment was established in 1972 at Keszthely Experimental Station, Hungary (46°44'04.2"N, 17°13'47.1"E).

The experiment has two factors; the main factor is tillage, with three different cultivations: deep winter ploughing (conventional tillage system), shallow winter disking (shallow tillage system) and disking just before drilling (minimum tillage system). The second factor is increasing rates of mineral N fertilization. N-rates in case of maize are 0-120-180-240-300 kg ha^{-1}. The trial was arranged in a split-plot design with four replications. P and K are uniformly applied as a rate of 100 kg ha^{-1} P_2O_5 and K_2O on each plot, except N control plots, where no P and K is applied.

HUN-3: Organic and inorganic fertilization in 5 course crop rotations

The experiment was established in 1963 at Keszthely Experimental Station, Hungary (46°44'05.2"N, 17°13'56.9"E). The bifactorial experiment has a strip-plot design, and four replications. In the experiment two 5 course crop rotations are grown including and excluding perennial legume. The factors of the experiment are the different crop composition of the rotations (winter wheat - alfalfa - alfalfa - winter wheat - maize, winter wheat - oats and vetch - winter wheat - sorghum - maize) as well as the increasing rates and forms of fertilizers. The study was conducted in the 1st wheat and maize sequences of the rotations.

The fertilizer treatments have four variants including no fertilization (control), 520 kg NPK ha^{-1} $5yr^{-1}$, 2080 kg NPK ha^{-1} $5yr^{-1}$ and 2080 kg NPK + 35 t farmyard manure ha^{-1} $5yr^{-1}$. The farmyard manure is applied before the maize in every fifth year of the rotations. The fertilizer rates applied annually are different according to the different needs of the different crops, but the total quantity is the same in each rotation during one rotation period.

HUN-4: Rates and timing of fertilization in continuous maize

The experiment was established in 1969 at Keszthely Experimental Station, Hungary (46°44'30.2"N, 17°14'23.5"E). The only crop has been growing in the experiment is maize. The experiment is bifactorial having a split plot design. The experimental factors are fertilization as well as timing of N application. Fertilization includes 4 increasing rates of mineral NPK fertilizers: 0, 300, 600 and 900 kg $N:P_2O_5:K_2O$ ha^{-1} yr^{-1} ($N:P_2O_5:K_2O$ = 1:1:1). Timing of N application has 3 different variants: once in autumn, once in spring, twice in spring (1/3 N is applied as top dressing after germination).

UK-1: Tillage experiments on farms

Four individual farms had left 2 of their fields un-tilled and with 2 neighboring fields conventional tillage was applied. The farms were located in Stonton (N 052° 32′ 55″, W 00° 54′ 57″), Loddington (N 052°36′53″ W 00°50′31″), Normanton (N 052° 38′ 38″ N, W 00° 37′ 19 ″)and Goadby (N 052° 35′ 0″, W 00° 53′ 0″; only 2 fields). Soils are predominantly a heavy clay loam, from the Denchworth series.

UK-2: Cover crop experiment

The experimental area was set up in September 2015 at the Allerton Project—a 300 hectare mixed arable and livestock Research, Demonstration and Education Farm (Game & Wildlife Conservation Trust), in Loddington, Leicestershire, UK (N 052°36′53″ W 00°50′31″; 186 m a.s.l). The Allerton Project historically had a wheat–rape rotation with a "break" spring crop. Soils are predominantly a heavy clay loam, from the Denchworth series: texture 45% clay, 35% silt, and 20% sand. Winter wheat was harvested across three fields (totaling an area of ~20 hectares) and three different cover crop mixes were established on day after, in large "field-scale plots" (covering an area of nearly 20 hectares), replicated across three fields in a randomized block design (Crotty & Stoate 2019). Each plot was 24 meters wide and up to 200 meters long, set out between the tramlines within each field. Each mixture was sown over an area of around a half hectare per field. During the cover crop period (September 2015–March 2016), 389 mm of rainfall was recorded, just over half the long-term annual average (664 mm [1961–1990, based on the Standard Average Annual Rainfall (SAAR) 1-km grid dataset from the Met Office]).

The three cover crop mixes were planted at the manufacturer's recommended seed rates for mixtures using a direct drill ("Eco M," Dale Drills), rolled with a segmented ridged roller (Cambridge rolled) to flatten land, with no fertilizer applied at this stage. Cover crop mixes were either C + P—oats (*Avena sativa*) and phacelia (*Phacelia tanacetifolia*) (EFA Greening mix); C + P + R—oats, rye (*Secale cereale*), phacelia, and radish (*Raphanus sativus*) (Soil Structure mix); C + P + R + L—oats, phacelia, radish, buckwheat (*Fagopyrum esculentum*) and the legumes, vetch (*Vicia sativa*), crimson clover (*Trifolium incarnatum*), and berseem clover (*T. alexandrinum*) (Biodiversity mix); or a "no cover" control that was left as bare stubble (there was no herbicide applied, so natural regeneration did occur). The cover crops were left undisturbed to grow over the autumn and winter before all cover crops were terminated on in April with 360 g/L glyphosate at 3.5 L/ha (Azural, Monsanto) prior to planting the spring oats. The soil samples were collected during the growth of the spring oats.

BEL-1: The long-term non-inversion tillage trials in Huldenberg and Lubeek (Belgium)

The two experimental fields in Huldenberg (50°48'N, 4°36'E) and Lubbeek (50°55'N, 4°48'E) are located in the Belgian Loess Belt (Table 1). For each of the experimental fields, the upper soil layer (0-30 cm) had a loam, silt loam or sandy loam soil texture (Luvisols). Climate in Belgium is temperate with mild rainy winters and cool humid summers. Long term (1981-2010) average yearly temperature and precipitation are 10.5 °C and 852 mm year-1.

The experimental fields are cultivated by professional farmers. To obtain realistic results, all management decisions (crop rotation, sowing date, fertilization, pesticides, herbicides, …) were taken by the farmers. The only restriction is that for each field all farming practices, apart from tillage, needed to be similar for both tillage treatments.

Two treatments are considered: conventional tillage (CT) and non-inversion tillage (NIVT). CT is carried out with a moldboard plough that inverts the soil and mixes the crop residues with the soil. The soil is loosened and weeds are buried. Tillage depth in the experimental plots is about 25 to 30 cm. When NIVT is applied, the soil is not inverted and crop residues are left at the surface. NIVT is mostly combined with harrowing for seedbed preparation and in some cases with subsoiling to loosen the soil below the plough layer.

The experimental field in Huldenberg was set up in 2001. Since 2001, one part of the field is conventionally tilled while the other part of the field has non-inversion tillage (split-plot design). NIVT is carried out with a V-shape at a 30 cm depth. Year after year, grain maize is cultivated.

The experimental field in Lubbeek was set up in 2004. Since 2004, one part of the field is conventionally tilled while in the other part of the field is non-inversion tillage is applied (split-plot design). NIVT is carried out at a 15 cm depth. On this experimental field, a common rotation of winter wheat, winter barley, sugar beet and potato is cultivated. In 2016, potatoes were grown in Lubbeek.

BEL-2: The Boutersem long-term compost trial (Belgium)

A long-term field trial with VFG (vegetable, fruit and garden waste) compost was set up by the Soil Service of Belgium in 1997 on a loamy soil in Flanders, Belgium. The site has a maritime temperate climate, with significant precipitation in all seasons (no dry season) and a warm summer (according to the Köppen climate classification: Cfb). The average temperature during the trial period is 11.0 °C and the average annual precipitation 760 mm.

The trial is set up as a randomized complete block design. The field is divided into 48 plots, each one having a surface of about 100 m². Twelve treatments were laid out in four replicates, including an unfertilized control treatment, a control treatment with only mineral fertilization, treatments with three-yearly applications of VFG-compost (15, 30 and 45 tons per hectare), with two-yearly applications of VFG compost (15, 30 and 45 tons per hectare), with yearly applications of VFG compost (15, 30 and 45 tons per hectare) and an unfertilized fallow plot. Starting from 1998, in treatments with respectively 45 tons VFG/ha 3- yearly and yearly, the plant cover (crop and weeds) was removed in part of the plots (5×5 m²), in order to study the mineralization of the applied compost. Since 1997, the following crop rotation was applied: sugar beet (1997), winter wheat (1998), potatoes (1999), carrots (2000), sugar beet (2001), winter wheat (2002), potatoes (2003), carrots (2004), winter wheat (2005), sugar beet (2006), winter wheat (2007), winter wheat (2008), potatoes (2009) and winter wheat (2010), sugar beet (2011), winter wheat (2012), carrot (2013), winter wheat (2014), onion (2015), winter wheat (2016).

The VFG compost was applied before the growing season (in spring for the root crops and just before sowing for the winter cereals).

Each year, at the end of the winter period (start of the growing season) a soil sample was taken in the ploughing layer (0–23 cm) of the field trial for the analysis of pH, carbon content (C%), phosphorous (P_2O_5), potassium (K_2O), magnesium (MgO), calcium (CaO) and sodium (Na_2O). Based on this analysis, a standard fertilization and liming recommendation was calculated using the BEMEX expert system of the Soil Service of Belgium. The basic fertilization (P, K, Mg, Ca, Na) and lime applications in the trial were always performed according to these recommendations. In the different treatments of the trial, soil samples were taken each year in the soil layers 0-30 cm, 30-60 cm and 60-90 cm for the analysis of mineral nitrogen (N) (3 layers), pH and carbon content (only top layer 0-30 cm). The samples were taken in the four replications and mixed per treatment for the analysis. This was done at the end of the winter period, in order to determine the available mineral nitrogen stock for the following crop and to calculate a nitrogen fertilization recommendation with the N-INDEX expert system.

The fertilization of the different trial treatments was applied as follows: treatment 1 received no fertilization (control); treatment 2 always received mineral fertilization according to the fertilization recommendation. Treatments 3 to 11 received no fertilization during the first trial years (1997–2002). However, starting from 2003, the expected amount of nutrients released by the applied compost during the growing season was supplemented each year with mineral fertilizer up to the recommended level.

The compost used in this trial was VFG compost, provided by Ecowerf, a professional company in the compost industry in Flanders. Prior to each compost application, a representative sample was taken and analyzed. Each year, the composition of the applied composts approached the average composition of VFG compost, the heavy metals were far below the legal standards and the composts contained very low amounts of stones and impurities and no viable seeds.