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# Study of arbuscular mycorrhizal fungal diversity in long-term field experiments

PhD Thesis

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## BACKGROUND AND OBJECTIVES

The mutualistic relationship between most plant species and arbuscular mycorrhizal (AM) fungi is the most widespread symbiosis in natural and agricultural vegetations (WANG and QUI 2006). Upon entering into the intercellular space between the cortical cells of the root tissue of the host plant and into the cells themselves (although not into the cytoplasm), arbuscular mycorrhizal fungi (AMF) form characteristic structures: arbuscules and sometimes vesicles. This association is beneficial for both partners: the fungi receive ready-made nutrients from the plant, and – in an exchange – more water and minerals are available for the plant due to the vast mycelium network of the fungi (SMITH and READ 1997). Mycorrhizal plants are more tolerant to stress caused by salts, drought (AUGÉ et al. 2008) and heavy metals (GILDON and TINKER 1983, LEYVAL et al. 1997, HILDEBRAND et al. 2007), and AM fungi directly or indirectly enhance the resistance of the plant partner against pathogens and pests (POZO and AZCÓN-AGUILAR 2007). This association plays an even more significant role in agricultural plants since AM fungi promote the development of plants under stress conditions, may reduce the risk of soil sickness, and the quantities of fertilizer may be diminished by improving the nutrient utilisation of the roots.

The development of AM, which constitute the most ancient and most widespread type of mycorrhiza, dates back to more than 400 million years, and AM fungi are likely to have played a role in the propagation of terrestrial plants as well. In spite of this, the number of AMF species is only 236, and they belong to twenty genera of ten families of four orders (*Archeosporales*, *Diversisporales*, *Glomerales*, *Paraglomerales*) of the class *Glomeromycetes* of the phylum *Glomeromycota* (SCHÜßLER et al. 2001) branching off as a sister group of *Ascomycota* and *Basidiomycota* according to the current taxonomic classification (KRÜGER et al. 2012).

Such unique experiments as the long-term field experiments initiated in the experimental area of the Agricultural Research Institute of the Hungarian Academy of Sciences, including a site in Martonvásár, provide excellent opportunities to study the long-term effects of various agrotechnical factors, including the cultivation and land use methods which are most frequently applied in Hungary, on the diversity of soil microorganisms. In several cases, intensive agricultural cultivation, which is characterised by the use of large quantities of fertilizers and chemicals, results in deteriorating soil conditions and other environmental problems, for example, reduced soil life, including reduced diversity of AMF. Several studies confirmed that agrotechnical procedures, such as soil cultivation (JANSA et al. 2002, 2003, ROLDÁN et al. 2007), fertilization (BHADALUNG et al. 2005, FRANKE-SNYDER 2001), and the use of pesticides (OEHL et al. 2004), influence the natural AM fungal communities of the soil.

In Hungary, data about the mycorrhization of 163 plant species have been published in the scientific literature so far (KOVÁCS 2008). With a few exceptions (LANDWEHR et al. 2002, FÜZY et al. 2008, KOVÁCS et al. 2007), these included status studies and investigations into ectomycorrhizal fungal communities, and were targeted to plant species of natural habitats. Despite the fact that most terrestrial plants, including crops, form arbuscular mycorrhiza, molecular studies into the changes in the AM fungal communities of corn, a crop playing a major role in the European agriculture, were primarily conducted in

tropical areas; in addition, insufficient information is available on the AMF communities of all cultivated plant species of Hungary (KOVÁCS 2008).

Studying the differences in the diversity of AM fungi living in natural and agricultural ecosystems is essential in order to have a more accurate understanding of the role and importance of arbuscular mycorrhizal fungi and to obtain information with a view to their use as inoculum.

On the basis of our results, we expected to answer the following question: how do various agrotechnical interventions change the incidence of AMF species, their proportions and the structure of AM fungal communities? Therefore, the objectives of our study include:

1. assessing the effect of plant density and
2. large quantities of inorganic fertilizer (400 kg $ha^{-1}$ NPK) and organic fertilization (7.5 t $ha^{-1}$ corn stalk) on the diversity of AM fungi in long-term corn monocultures, and
3. comparing the diversity of AM fungal communities of plants from long-term monoculture-based cultivation and various crop rotation systems (3 years of alfafa / 5 years of corn, 2 years of wheat / 2 years of corn, and corn / spring barley / peas / wheat [Norfolk type] crop rotation systems).

Our investigations were targeted to:

- determining the number of AM fungal spores in 1 g of the rhizosphere soils of plants,
- estimating mycorrhization percentages as indicators of the colonisation by AM fungi,
- identifying the mycorrhizal fungi actively colonising the roots of plants by molecular techniques, and
- revealing the phylogenetic relationships among the members of the AM fungal community.

## MATERIALS AND METHODS

*Sampling*

Plant samples came from long-term experiments set up by Béla Győrffy in the Agricultural Research Institute of the Hungarian Research Academy of Sciences in Martonvásár at the end of 1950s and the beginning of 1960s. The soil was classified as humus loam of the chernozem type with forest residues, slightly acidic in the ploughed layer, with poor supplies of available phosphorus and good supplies of potassium.

To assess the effect of the plant density on AM fungi, four replications of Norma SC hybrid maize roots were collected from 70 000 plant $ha^{-1}$ (normal, ND) and 100 000 plant $ha^{-1}$ (high, HD) maize monocultures at16 June 2008.

To assess the effect of fertilization on AM fungi, four replications of Norma SC hybrid maize roots were collected from non-treated control maize monoculture (NON), maize monoculture treated with 400 kg $ha^{-1}$ NPK mineral fertilizer (IF) and with 7.5 t $ha^{-1}$ corn stalk residues (OF). Samples were collected at 13 June (start of flowering), 3 July (start of seed filling), 7 August (start of biological ripening), and 20 October (immediately after harvesting) in 2008.

To compare maize monoculture with different rotation systems, four replication of Norma SC hybrid maize roots were collected from maize monoculture (CRM), 3 years of alfalfa / 5 years of corn rotation (CR3), 2 years of corn / 2 years of wheat rotation (CR5) and Mv Magvas hybrid winter wheat roots from maize / spring barley / peas / wheat rotation (Norfolk type, CR7). Samples were collected at the same times as written above.

*Extraction of AMF spores from soil and spore counts*

AMF spores were isolated from 5 g air-dried soil samples by wet sieving (GERDEMANN and NICOLSON 1963) followed by sucrose gradient centrifugation (IANSON and ALLEN 1986). Spores were counted under stereomicroscope at 100x magnification and spore abundance was expressed as the number of AMF spores per gram soil.

*Assessment of AMF root colonization*

Five root fragments (corresponding to 1.5 g fresh weight) from every each plant were collected as representative sample, washed with tap water and stained with ink and vinegar technique (VIERHEILIG et al. 1998). Percentage of root length colonization as a degree of symbiosis was calculated (without examination of fungal structures inside the root) in 4 replication under stereomicroscope at 100x magnification using the gridline intersect method (GIOVANNETTI and MOSSE 1980).

*DNA extraction from plant roots*

To assess the effect of the density of 70,000 plant $ha^{-1}$ and 100,000 plant $ha^{-1}$ on AM fungi, crude DNA were extracted from 5 different lateral root segments using the boiling procedure (DI BONITO et al.

1995). The extracted DNA templates (1x2x4x5=40) were stored at –20°C until subsequent PCR amplification. Among the samples collected in 2008 five different lateral root segments of each plant belonging to the sample times June and August were subjected to DNA extraction using the DNeasy®plant Mini Kit (Quiagen) following the manufacturer's instructions. The extracted DNA templates (to assess the effect of fertilization: 2x3x4x5=120; to compare maize monoculture with different rotation systems: 2x4x4x5=160) were stored at –20°C until subsequent PCR amplification.

*Nested-PCR, isolation of DNA fragments from agarose gel, ligation, transformation of E. coli with plasmids*

Amplification of a portion of AM fungal 18S rDNA by nested-PCR was performed using universal eukaryotic primers (AMV4.5F-AMV4.5R) in the first step and AMF specific PCR primers (AMV4.5NF-NR) in the second step (SAITO et al. 2004). Amplification products were detected and separated by agarose gel electrophoresis in 2% (w/v) agarose gel, stained with 0.1 µl $ml^{-1}$ ethidium bromide. PCR products of the expected size (~650 bp) were purified from agarose gel with GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Amersham Biosciences). PCR fragments were pooled according to RENKER et al. (2006) within each of the treatments and the same sampling time and cloned into pGEM®-T Easy Vector (3015 bp) with pGEM®-T Easy Vector System (Promega) following the manufacturer's instructions and then transformed into *E. coli* DH5α.

*Plasmid DNA isolation and DNA sequencing*

Plasmids from putitative positive clones were extracted with the Wizard® Plus SV Minipreps DNA Purification System Kit (Promega) following the manufacturer's instructions. Determination of the nucleotid sequence of recombinant plasmids was made by T7 primer starting from one of the cloning site.

*Sequence analysis*, *statistical analysis*

After editing the sequences, molecular operational taxonomical units (MOTUs) were distinguished on a similarity level of 97% using the Mothur software program. The representative sequences for each MOTU were blasted against MaarjAM *Glomeromycota* database (http://maarjam.botany.ut.ee/). References for phylogenetic analysis were chosen from the Virtual Taxa showing the highest similarities based on the blast search. MEGA 4.0 software was used to generate a neighbor-joining (NJ) consensus tree, assessing Kimura two-parameter model as distance method and 1000 replicates of non-parametric bootstrapping. Diversity and structure similarity indices were estimated also with Mothur program. Statistical analyses of mycorrhization data were performed using linear modeling.

## RESULTS

### The assessment of the effect of plant density on AMF

Colonisation rates (ND: 36.50 ± 4.12 %; HD: 37.50 ± 3.42 %) and number of AMF spores per gram of rhizosphere soils (ND: 2.75 ± 0.50; HD: 2.50 ± 0.58) of corn roots collected from normal density (ND; 70,000 plant $ha^{-1}$) and high density (HD; 100,000 plant $ha^{-1}$) parcels showed no significant differences.

Upon editing 33 *Glomeromycota* sequences during the molecular mapping of AM fungal communities, 9 MOTUs were distinguished on a similarity level of 97% using the Mothur software program. Phylogenetic analyses suggested that seven of these MOTUs (90.9% of the sequences) belong to the family *Glomeraceae* (former *Glomus* Group A), and the other two (9.1%) belong to the family *Claroideoglomeraceae* (former *Glomus* Group B). A total of seven MOTUs were detected in the normal density parcels, and a total of five in the high density parcels**.** In the stands with 70,000 plant $ha^{-1}$ density, the MOTU belonging to the genus *Septoglomus* (a part of the former *Glomus* Group Aa) constituted 61.11% of the AM fungal community, while the same MOTU had a share of only 20% in the stands with 100,000 plant $ha^{-1}$ density. In the latter, the MOTU representing the predominant AM fungal community (40% of the sequences) belongs to the *Glomus* Group Ad.

Based on the comparisons of the averaged non-parametric species richness estimators (ACE and Chao1) with the number of actually distinguished MOTUs, it may be concluded that a considerable part, i.e., 95.24% of the AM fungal communities of the 100,000 plant $ha^{-1}$ (HD) stands were mapped, while the same value was only 41.8% for the AM fungal communities of the 70,000 plant $ha^{-1}$ (ND) stands. The number of MOTUs distinguished and estimated by us was also higher in the 70,000 plant $ha^{-1}$ (ND) stands than in the 100,000 plant $ha^{-1}$ (HD) stands.

### The assessment of the effect of fertilization on AMF in corn monoculture

Evaluation of the level of mycorrhization of corn roots from monocultures receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer (IF) or 7.5 t $ha^{-1}$ corn stalk (OF), or from untreated control (NON) monoculture demonstrated that both the treatments ($p<0.01$) and the sampling times ($p<0.001$) had significant effects on root colonisation. During the vegetation period, increasing mycorrhizal colonisation was detected in both the control and fertilizer-treated stands reaching a peak value (IF: 47.50 ± 3.42 %; OF: 52.50 ± 1.29 %; NON: 47.50 ± 2.0 %) in August, i.e., during the biological maturation phase of corn. Compared to the rates measured in control plants at identical time points, plants receiving inorganic and organic nutrient supply exhibited significantly higher root colonisation rates in July ($p<0.01$) and in August ($p<0.001$), respectively. In all treatment, the number of AMF spores per gram of rhizosphere soils was 3-4 in June and increased to 12-15 spore $g^{-1}$ soil by August. Between August and October, fertilizer-treated plants showed reduced spore numbers, but those in control parcels showed further increases (19 spore $g^{-1}$ soil) at the sampling time point in October. Thus, in October, the number of AMF spores per gram of rhizosphere soils of fertilizer-treated plants was significantly lower ($p<0.001$) than that of the rhizosphere soils of control parcels.

In the framework of the molecular evaluation of the AM fungal communities, a total of 252 clones (42/treatment/time point) were analysed for their nucleotide sequence, and 179 (71%) belonged to *Glomeromycota*. Upon editing the sequences, 21 MOTUs were distinguished on a similarity level of 97% using the Mothur software program. Phylogenetic analyses suggested that 18 of these MOTUs (96.09% of the sequences) belong to the family *Glomeraceae* (former *Glomus* Group A), one (1.12%) to the family *Claroideoglomeraceae* (former *Glomus* Group B), one (0.56%) to the family *Archaeosporaceae* and one (2.23%) to the family *Paraglomeraceae*. 7 MOTUs were detected in the plants receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer, 14 MOTUs in those receiving 7.5 t $ha^{-1}$ corn stalk and another 14 MOTUs in the control plants. Members of the genera *Rhizophagus* and *Sclerocystis* (collectively, the former *Glomus* Group Ab) were present in the corn roots from both the control and the fertilizer-treated areas, and constituted 47.73%, 10.77% and 9.13% of the AM fungal communities of plants receiving inorganic fertilizer treatment, control treatment and recycled corn stalk residues, respectively. MOTUs belonging to the genera *Funneliformis* and *Septoglomus* (collectively, the former *Glomus* Group Aa) were not detectable in the roots of corn receiving inorganic fertilizer treatment, but constituted 51.69% of the AM fungal communities of the area treated by corn stalk residues. Their proportion in the control stands was also as high as 46.68%. The Theta indexes, which take into account the abundance of MOTUs as well, suggest that the greatest similarity (81.58%) in the composition of the AM fungal communities was detected between the treatment by 7.5 t $ha^{-1}$ corn stalk residues and the control treatment. On the other hand, the lowest level of similarity (1.52%) was detected between the AM fungal communities in the plants receiving control (NON) treatment and treatment by 400 kg $ha^{-1}$ inorganic NPK fertilizer. Based on the comparisons of the averaged non-parametric species richness estimators with the number of actually distinguished MOTUs, it may be concluded that – except for the sampling time point for the control plants in August – a considerable part, i.e., 81.6% of the AM fungal communities were mapped on average. In June and August, the unfertilized control corn monoculture had higher Shannon-Wiener diversity values (H':2.2-2.38) than the corn monoculture receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer (H':1.64-1.82). In June, the corn monoculture receiving 7.5 t $ha^{-1}$ corn stalk residues showed identical values (H': 2.2), but with the progress of the vegetation period in August, again lower AM fungal diversity (H': 1.87) was detected in comparison with the control plants. At the sampling time point in June, the detected MOTUs suggested a ranking of NON=OF>IF among the treatments, the estimated MOTUs suggested OF>NON>IF, and the Shannon-Wiener diversity indices suggested NON=OF>IF. In August, both the estimated MOTUs and the Shannon-Wiener diversity indices suggested a ranking of NON>OF>IF (despite the fact that only 42.79% of the AM fungal communities of the control plants were mapped by that time).

**Comparing maize monoculture with different rotation systems**

The lowest colonisation rates were detected in June (CRM: 23.75% - CR7: 41.00%) in the roots of flowering corn and in wheat in full maturation, and in October in the roots of corn and wheat from stubble-fields (CRM: 35.75% - CR7: 43.75%). At all sampling time points, the root colonisation rates of wheat plants from a Norfolk type (CR7) crop rotation system – achieving 61.25% and 60.50% in the wax

maturation and the blight of wheat, respectively – were significantly higher ($p<0.01$) than the root colonisation rates of corn plants from monocultures. In July, significantly lower ($p<0.01$) average colonisation rates were detected form the alfalfa-corn and wheat-corn crop rotation systems (43.88% and 41%, respectively) in comparison with the average root colonisation rates of corn plants from monocultures (51.25%). In July, the rhizosphere soils of corn plants from monocultures were characterised by an average of 10 spores per g of soil; at the same time, significantly lower ($p<0.01$) average AMF spore numbers (5.5 spores per g of soil) were detected in the wheat-corn crop rotation system. In October, all crop rotation systems exhibited significantly lower ($p<0.001$) spore numbers than the corn monocultures, which were characterised by an average of 24.5 AMF spores per gram of rhizosphere soil.

Molecular identification of the AM fungal communities of corn plants from corn monoculture (CRM) and from 3 years of alfalfa / 5 years of corn (CR3) and 2 years of wheat / 2 years of corn (CR5) crop rotation systems, and of wheat plants from corn / spring barley / peas / wheat (Norfolk type, CR7) crop rotation system were conducted twice, i.e., in June and in August. A total of 340 clones (42-44 clones/treatment/time point) were analysed for their nucleotide sequence, and 179 AMF sequences were subjected to further analyses. Upon editing the 179 *Glomeromycota* sequences, 18 MOTUs were distinguished using the Mothur software program. Phylogenetic analyses suggested that 12 of these MOTUs (91% of the sequences) belong to the family *Glomeraceae* (former *Glomus* Group A), three (4%) to the family *Claroideoglomeraceae* (former *Glomus* Group B), one (1%) to the family *Diversisporaceae* and two (4%) to the family *Paraglomeraceae*. 11 MOTUs were detected in corn monoculture, 9 MOTUs in the 3 years of alfalfa / 5 years of corn, 6 MOTUs in the 2 years of wheat / 2 years of corn and another 6 MOTUs in the corn / spring barley / peas / wheat (Norfolk type) crop rotation systems. The Theta indices suggest that the greatest similarity (66%) is between the AM fungal communities of corn plants from the 3 years of alfalfa / 5 years of corn crop rotation system and those of corn plants from the 2 years of wheat / 2 years of corn crop rotation system. On the other hand, the lowest level of similarity (1.23%) was detected between the AM fungal communities from corn monoculture and those from the 3 years of alfalfa / 5 years of corn crop rotation system. The detected and estimated number of MOTUs, as well as the diversity indices, showed dramatic reductions from the corn monoculture to the Norfolk type crop rotation system, especially at the sampling time point of August. In June, the highest AM fungal diversity indices were associated with the alfalfa – corn crop rotation system (H':1.63) but with the corn monoculture in August (H':1.25). The AM fungal diversity of the corn monoculture and crop rotation systems was also declining with the progress of the vegetation period. For the sampling time point in June, the detected MOTUs suggested a ranking of CR3>CRM>CR5=CR7 among the cultivation systems, the estimated MOTUs suggested CR3>CRM>CR5>CR7, and the Shannon-Wiener diversity indices suggested CR3>CRM=CR7>CR5. For the sampling timepoint in August, the detected MOTUs suggested a ranking of CRM>CR3=CR5>CR7, the estimated MOTUs suggested CRM>CR3=CR5>CR7, and the Shannon-Wiener diversity indices suggested CRM>CR5>CR3>CR7.

**New scientific results**

- For the first time, we have provided data for the arbuscular mycorrhizal fungal (AMF) communities of a cultivated area in the region of the Carpathian Basin, including Hungary.
- For the first time, we have assessed the effect of plant density on AMF community in corn monocultures. In the 70,000 plant $ha^{-1}$ (ND) corn monocultures – although 41.18% of the AMF community was mapped –, the number of detected and estimated molecular operational taxonomical units (MOTUs) was higher than in the 100,000 plant $ha^{-1}$ (HD) corn monoculture, where 95.25% of the AMF community was mapped.
- We have detected higher AMF diversity values and more MOTUs from corn monocultures than those detected so far in corn monocultures using molecular techniques.
- The predominant MOTU in the AMF community of corn monoculture characterised by normal plant density and low nutrient supply belongs to the genus *Septoglomus*. However, in corn monoculture receiving large quantities of inorganic nutrient supply and in crop rotation systems including leguminous flowering plants this MOTU was not detectable suggesting that the AM fungus representing it is sensitive to the increased soil nutrient levels, especially to soil nitrogen levels.
- Upon comparing the different cultivation systems, different MOTUs were identified as predominant members of the AMF communities. The detected and estimated number of MOTUs, as well as the diversity indices, showed dramatic reductions from the corn monoculture to the Norfolk type crop rotation system, especially at the sampling time point of August.

## CONCLUSIONS AND PROPOSITIONS

In order to efficiently utilise the beneficial effects of the interaction between AM fungi and plants in agricultural cultivation, it is vital to precisely explore the structure of the AMF communities characteristic for the ecosystems of Hungarian; the present work was intended as a contribution to this.

Average colonisation rates and average number of AMF spores per gram of rhizosphere soils of corn roots collected from 70,000 plant $ha^{-1}$ (ND) and 100,000 plant $ha^{-1}$ (HD) parcels showed no significant differences; one explanation may be that the sampling was conducted in the middle of June when the stand is not entirely closed yet. As obligate fungi, AM fungi have different needs in terms of the available quantity of plant carbohydrates (SAITO et al. 2004), therefore, the structure of the AMF communities involved in the root colonisation may have been changed and this may not necessarily be reflected in the root colonisation rates and spore numbers. Literature values for the colonisation rates and number of AMF spores per gram of rhizosphere soils from corn monocultures show considerable variations, which mostly reflect the differences in the climatic and cultivation conditions.

In the framework of the assessment of the effects of nutrient supply on AM fungi and the comparisons of the corn monocultures and crop rotation systems in 2008, root colonisation rates of the plants and AMF spore numbers in the rhizosphere soils were measured at four sampling time points: in June, July, August and October. The root colonisation rates and the AM fungal spore numbers also changed during the vegetation period. Ploughing type of soil cultivation reduces the inoculum potential of the AM fungi, which in turn induces a delay in the root colonisation and sporulation (KABÍR 2005), as also demonstrated by our results. In all cases, root colonisation started to increase in June and reached a peak in August, i.e., at the time of the biological maturation of corn – and the blight of winter wheat in the case a Norfolk type of crop rotation –, and showed a decline from August. In accordance with the delay in sporulation, AMF spore numbers in corn rhizosphere soils continued to increase up until October, in the control corn monoculture during the assessment of the effects of nutrient supply, and both in the corn monoculture and in the 3 years of alfalfa / 5 years of corn crop rotation system during the comparison of the monoculture and the crop rotation systems. BHADALUNG et al. (2005) described identical changes in AMF spore numbers of rhizosphere soils during the vegetation period in long-term (27-year) fertilization experiments in Thailand.

In the studied corn monocultures, both the organic (7.5 t $ha^{-1}$ corn stalk residues) and inorganic (400 kg $ha^{-1}$ NPK) nutrient supply induced the same reduction in the AMF spore numbers of rhizosphere soils, which became remarkable in October, after the harvest of corn. This is not surprising since plants with a better nutrient supply are less in need of the advantages offered by AM fungi (JOHNSON 1993); therefore, less stored plant carbohydrates may be available as the energy fuelling the sporulation of AM fungi. During the studies conducted in the above mentioned 27-year fertilization experiments, BHADALUNG et al. (2005) found that an increase in the rate of inorganic fertilizer from 0-0 to 180-180 $N\text{-}P_2O_5$ kg $ha^{-1}$ induced 70% reduction in the AMF spore numbers in the soil (47 to 14 spores $g^{-1}$ soil).

In terms of root colonisation rates, the overall ranking of corn monoculture was second to the crop rotation systems, i.e., to the Norfolk type crop rotation; on the other hand, it was undoubtedly the first in

terms of the AMF spore numbers of rhizosphere soils in October. The significantly higher root colonisation rates of the plants of the Norfolk type crop rotation system is likely to result from the effects of the previous plant (green peas); furthermore, the phenological phases of winter wheat are earlier than those of corn, thus colonisation may have occurred earlier, i.e., during early spring. However, the low spore production rates may reflect the effects of the plants themselves given that wheat – unlike corn – is considered as a facultative mycotrophic plant (PLENCHETTE et al. 2005). For example, even in the 2 years of wheat / 2 years of corn crop rotation system showing the lowest average spore production rates, only wheat is alternating with corn. In Hungary, wheat-corn rotation is highly widespread but appears to have a negative effect on the spore production of AM fungi.

In order to study the effects of cultivation and soil use methods on AM fungi in more detail, molecular techniques were used to identify the members of the AMF communities actively colonising the roots of plants from long-term experiments with monocultures and various crop rotation systems, and explored the phylogenetic relationship among them.

In the corn monoculture as compared with the crop rotations systems, in the 70.000 plant $ha^{-1}$ (ND) stands used for the studies into the effects of plant density, as well as in the control corn monoculture serving the assessment of the effects of nutrient supply, the predominant molecular operational taxonomic units (MOTUs) of the AM fungal communities of corn plants included MOTUs phylogenetically related *Glomus viscosum* BEG 126, a species of the *Septoglomus* genus. AMF species related to these MOTUs (*Septoglomus constrictum*) and AMF species belonging to the former *Glomus* Group Aa, i.e., to the current *Septoglomus* and *Funneliformis* genera, have been already detected as predominant members of the AMF communities of corn monocultures (BAINARD et al. 2012, OEHL et al. 2005).

However, during the assessment of the effects of plant density (70,000 and 100,000 plant $ha^{-1}$), the MOTU encompassing the majority (40%) of the sequences was found to belong to the *Glomus* Group Ad in the 100,000 plant $ha^{-1}$ (HD) corn monoculture. No known AMF species of this group have been described so far but their presence have been detected in both natural and anthropogenic ecosystems (HELGASON et al. 1998, SAITO et al. 2004, BALESTRINI et al. 2010). In the studied 100,000 plant $ha^{-1}$ (HD) corn monoculture, this *Glomus* Group Ad took over the predominant role from the *Septoglomus* genus, although it is suggested that members of this group have high carbohydrate demands (SAITO et. al. 2004). In the literature, no studies are available regarding the effect of plant density on AMF community, although on the basis of the species richness estimators and rarefaction curves, the number of sequences was not sufficient for an accurate determination of diversity; therefore, no conclusions were made.

The assessment of the effect of nutrient supply on AM fungi showed that the number of MOTUs detected in the parcels receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer was 50% less (7 MOTUs) than in the control parcels (14 MOTU) and those receiving 7.5 t $ha^{-1}$ corn stalk residues (14 MOTUs). In the latter two, the predominant MOTU belonged to the *Septoglomus* genus. Among the AMF species belonging to the genera *Septoglomus* and *Funneliformis* (former *Glomus* Species Group Aa), *F. mosseae* and *Septoglomus constrictum* can be characterised as species with a preference for organic nutrient supply (OEHL et al. 2004).

In addition, BHADALUNG et al. (2005) described *F. mosseae* as a species sensitive to high rates of N-$P_2O_5$ supply. Accordingly, both their predominance in the stands treated with 7.5 t $ha^{-1}$ corn stalk and their absence in those treated with 400 kg $ha^{-1}$ inorganic NPK fertilizer is plausible. In the plants receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer, the majority of the sequences were shared by 4 major abundant MOTUs belonging to the genera *Rhizophagus* and *Sclerocystis*, to the *Glomus* Species Group Ad, and – with the Glo4 phylotype – to the sensu lato *Glomus* genus. The predominance of the members of the genera *Rhizophagus* and *Sclerocystis* in the fertilizer-treated parcels is not surprising given that high nutrient supply, and especially high phosphorous levels in the soil are most frequently tolerated by the members of the *Rhizophagus* genus (JOHNSON 1993, MATHIMARAN et al. 2005); in our case, the AL-$P_2O_2$ content of the parcels receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer was, for example, three times higher (103.5 ppm) than in the control parcels (29.8 ppm), but those of the parcels receiving 7.5 t $ha^{-1}$ corn stalk was only twice as high (57.5 ppm). The Glo4 phylotype was characterised by ÖPIK et al. (2006) as a slightly ruderal taxon, and classified by SAITO et al. (2004) as having high demand for plant carbohydrates, which may explain their predominance in parcels receiving large quantities of inorganic nutrient supply and their absence in the control parcels. The way different AM fungi respond to fertilization is influenced by both direct and indirect effects. This may be related to the quantity and quality of plant carbohydrates available for the symbiotic partner (DOUDS and SCHENCK 1990), to the changes in root morphology caused by the available nitrogen levels of the soil (JOHNSON 1993), and to the changes occurring in the saprotrophic fungal communities as a result of recycling of corn stalk residues. The relevant literature suggests that large quantities of inorganic fertilizers may have a negative effect on the diversity of AM fungi (OEHL et al. 2005) and may also induce changes in the composition of the AM fungal communities (BHADALUNG et al. 2005). This is also confirmed by our results given that the AMF communities of plants from parcels receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer treatment are quite different from those of plants receiving no fertilizer treatment. Assumingly as a result of the proton release from the roots upon ammonium uptake (MARSCHNER and DELL 1994), inorganic nutrient supply reduces the pH of rhizosphere soils and this has an effect on the structure of the AM fungal communities (CLARK 1997, GIOVANNETTI 2000). Our soil data were not directly related to the rhizosphere but the pH in the soil of the parcels receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer was, for example, lower (pH 5.82) than that of the control parcels (pH 6.23) and those receiving 7.5 t $ha^{-1}$ corn stalk residues (pH 6.05). The AMF communities of the plants receiving 7.5 t $ha^{-1}$ corn stalk were 81.58% identical to those of the plants from control parcels; furthermore, AMF diversity was also the same in June, but dropped by August, while the 400 kg/h inorganic NPK treatment showed improving tendencies. This may be due to the fact that fertilizers and corn stalk have different nutrient exposure dynamics; therefore, both the ammonium uptake by the roots and the pH of the rhizosphere soils are different in the two cases and these differences may have been reduced during the vegetation period. Unlike us, HIJRI et al. (2006) detected lower (H': 0.8) AMF diversity from corn monoculture receiving 170-70 N-$P_2O_5$ kg $ha^{-1}$ fertilizer (using molecular techniques), which may result from the differences of the techniques and markers used or from the different soil conditions. On the contrary, OEHL et al. (2010) detected remarkably high diversity (H': 2.31) – with a total of 25 detected AMF species – from the soil of corn monoculture receiving 190-40 N-$P_2O_5$ kg $ha^{-1}$

fertilizer (cambisol); however, these results were based on morphological studies of AMF spores isolated from the soil.

In the framework of the identification of the AMF communities of plants from monoculture and crop rotation systems, a total of 11 MOTUs were distinguished in corn monoculture (CRM), and the predominant MOTU was again the one phylogenetically related to *Glomus viscosum* BEG 126, an AM fungus belonging to the *Septoglomus* genus. This phylotype was completely absent in those rotation systems that had leguminous flowering plants (CR3 and CR7) among the plants constituting it, and also disappeared as a result of the 400 kg $ha^{-1}$ inorganic NPK fertilizer treatment. Accordingly, it was concluded that the fungus representing this MOTU may have a key role in the nutrient supply of corn in corn monocultures with low nutrient supply. As nutrient levels increase, this role is likely to be diminished and ruderal, generalist AM species with high carbohydrate demand such as *Rhizophagus intraradices* or the AM fungus with the Glo4 phylotype may become more abundant. This is also confirmed by the finding that the MOTUs belonging to the Glo4 phylotype were predominant in the 3 years of alfalfa / 5 years of corn crop rotation system and in the corn monoculture receiving 400 kg $ha^{-1}$ inorganic NPK fertilizer. The same holds for the MOTUs exhibiting close phylogenetic relationship with *Rhizophagus intraradices* both in the corn / barley / green peas / wheat (Norfolk type) crop rotation system and in the corn monoculture receiving inorganic fertilization. In summary, our results indicate that crop rotation induced considerable changes in the composition of the AMF communities in comparison with the corn monoculture, and this is in accord with the relevant literature data (JOHNSON et al. 1991, HENDRIX et al. 1995, DANIELL et al. 2001). Furthermore, our results confirm the fact that only a few of the AM fungi actively colonising the roots of the plants are predominant even in ecosystems with high plant diversity indices (DEBELLIS and WIDDEN 2006, HUSBAND et al. 2002; STUKENBROCK and ROSENDAHL 2005). As a result of the changes in the structure of the AMF communities, the AMF diversity of the corn monoculture and crop rotation systems was also declining with the progress of the vegetation period, which is again in accord with the corresponding results of the literature (DANIELL et al. 2001, BAINARD et al. 2012). The competition between the AM fungi actively colonising the roots of the plants for new colonisation sites and for the photosynthetic products of the plant results in the predominance of one or two successful AM fungi, which in turn displace the others. Thereafter, the predominant root colonisers become almost unrivalled in the AMF community and other fungi may completely disappear. As indicated by our results, this process leads to reduced AMF diversity with the progress of the vegetation period of the host plant, most remarkably in the wheat of the Norfolk type crop rotation, which was in full blight already in the beginning of August. This phenomenon was referred to as overdominance by DUMBRELL et al. (2010); however, they found no connection between the ecosystems and the predominant AM fungi when the results of different authors were compared. The fact that different authors identified different AMF phylotypes as predominant members of the AMF communities was explained by the AM fungi locally adapting to the physical and chemical characteristics of the soil and by the key role of the plant community. Our results suggest that crop rotation in comparison with monoculture has no positive effect on AMF diversity, except for flowering corn in the 3 years of alfalfa / 5 years of corn crop rotation system. In summary, the Norfolk type crop rotation system

(with the upcoming wheat) and the wheat-corn crop rotation system were the least favourable in terms of AMF diversity. Accordingly, it was concluded that the structure of the AMF community is determined by the plant composition of the crop rotation system but sill rather indirectly, via its effects on the nutrient levels of the soil. Previous studies also indicated that plant composition has an effect on the structure of AM fungal communities, especially in soils with low nutrient levels (HELGASON et al. 2007, DUMBRELL et al. 2010). In such soils, some AM fungi may have a key role in the nutrient supply to the plants. The results of our comparisons of the AMF diversity of corn monoculture and crop rotation systems again differ from the corresponding literature data indicating positive results for crop rotation (HIJRI et al. 2006, OEHL et al. 2003, OEHL et al. 2009). This is likely the result of the fact that all monocultures and crop rotation systems studied by the different authors received some kind of fertilization.

By providing assistance to agricultural practices, the results of the present work may extend the existing knowledge for determining the composition of an efficient inoculum combination of AM fungi best adapted to these cultivation systems. Among the predominant MOTUs detected by us, several had corresponding phylotypes that are already extensively discussed in the literature (Glo4, Glo7) but no described AMF species could be assigned to them. Therefore, the molecular methods should be combined with spore morphology assessments in the future to enable the isolation of these AM fungi.

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