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Superior differentiation of arbuscular mycorrhizal fungal communities from till and no-till plots by morphological spore identification when compared to T-RFLP

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ABSTRACT

In our experiments we intended to document the impact of tillage on arbuscular mycorrhizal (AM) fungal communities under temperate climate conditions and intensive agriculture in a Luvisol of a fertile Loess area in Saxony (Germany). AM fungal community structure and diversity have been examined in a long-term field experiment in plots subjected to conditions of intensive agriculture with continuous 3-year rotation of winter wheat, winter wheat and sugar beet and managed by till and no-till treatments for thirteen years. AM fungal diversity was assessed by sequence analysis and terminal restriction fragment length polymorphism (T-RFLP) using the nested PCR-primers LR1/FLR2 and FLR3/FLR4 as well as by morphological spore identification. While both analyses resulted in essentially similar pictures of fungal community composition and of negative effects of tillage for AM fungal diversity, morphological spore identification allowed a considerable better differentiation of fungal taxa and a more sensitive detection of changes in community composition and diversity when compared to molecular methods.

1. Introduction

For a number of reasons tillage can be identified as one of the less sustainable practices in modern agriculture justifying current efforts to reduce this practice: Tillage is responsible for a large proportion of fuel consumption in agricultural management, and therefore was identified as a highly significant factor for the negative agricultural carbon footprint (Lal, 2004). This impact on the agricultural carbon footprint is further aggravated by the negative effect of tillage on soil organic matter (SOM), resulting in the liberation of considerable amounts of carbon from the soil and in a long-term reduction of soil fertility (Dawson and Smith, 2007; Hobbs et al., 2010). In addition, tillage is an important factor driving soil erosion, which has been identified as an important global threat to soils used for agriculture (Montgomery, 2007). Reduction of tillage, or complete abolishment, however, is connected to problems in plant mineral nutrition and to increases in weeds and pests (Cook, 2006). Problems in nutrient cycling may occur in particular in the first years after introduction of no-till, since the subsequent build-up of SOM establishes an additional sink for mineral nutrients. Problems with weeds and pests are due to the fact that reduced levels of field disturbance allow overwintering of a number of organisms usually eliminated during tillage. Arbuscular mycorrhizal (AM) fungi at least theoretically have the potential to counteract these problems in the establishment of no-till practices. AM fungi support plants in the acquisition of mineral nutrients under conditions of nutrient scarcity and they strengthen plant tolerance regarding a number of pests (Azcón-Aguilar and Barea, 1996; Harrier and Watson, 2004). Since no-till management has been described to have positive effects for AM fungi (Oehl et al., 2011), the re-establishment of semi-natural AM fungal communities under no-till conditions could be regarded as part of a natural healing process, initiated by the abolishment of tillage and resulting in the re-establishment of natural mechanisms regulating nutrient cycling and plant pathogen defence.

In our experiments we investigated whether such healing processes also occur under conditions of intensive agriculture. We studied this question on a highly productive loess stand in central Europe (Lüttewitz, Saxony) managed by the "Südzucker AG Division Agriculture/Commodity Markets" and subjected to continuous

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crop rotation comprising two subsequent years of winter wheat cultivation followed by sugar beet. Individual plots on this stand have been managed by conventional plowing or by no till cultivation since 1996 (Lübke-Al Hussein et al., 2008). Productivity of the stands (Koch et al., 2009), soil erosion and water infiltration, soil microbial activity and mesofauna (Lübke-Al Hussein et al., 2008) have been studied intensively, demonstrating clear positive effects of no-till management for soil stability, water infiltration and earthworm populations. Community structure of AM fungi was studied in samples obtained from these plots in May at the peak of the growing season and end of July at the end of the growing season. To our knowledge, this is the first study where comprehensively concomitantly morphological analyses of AM fungal spores and molecular analyses were applied in the same field soils, which allows us to compare and evaluate both these methods.

2. Materials and methods

2.1. Long-term field experiment

To answer the research questions, a long-term field experiment was chosen managed by the "Südzucker AG Division Agriculture/Commodity Markets" in Lüttewitz (Saxony, Germany) since 1996. The soil was a silty-loamy Haplic Luvisol which is typical for arable Loess areas in flat areas of temperate climates in Central Europe. Continuous crop rotation comprised two subsequent years of wheat cultivation (*Triticum aestivum*, 2009, 2010 and 2012, 2013) followed each by one year of sugar beet (*Beta vulgaris*, 2011 and foreseen for 2014) with *Sinapis alba* as a cover crop prior to sugar beet. Individual plots (each of about 100 × 600 m) have been managed by conventional plowing (to a depth of about 27 cm) or by zero-till cultivation since 1996 (Lübke-Al Hussein et al., 2008). Fertilization regime and plant protection measures (including fungicide applications) were largely similar for the two plots, with an occasional additional herbicide treatment for the zero-till plot during the time period of analyses. As expected, organic carbon contents in the top soils are slightly higher in zero-till than in conventional tillage (16.3 and 10.6 g C_{org} kg^{-1}, respectively), and also available phosphorus and potassium contents (using DL double lactate as extractant, see e.g. Oehl et al., 2005), appear to be slightly increased in zero-till soils compared to conventional tillage (170 and 131 mg P kg^{-1}, and 198 and 173 mg K kg^{-1}, respectively). According to Thiel (2010), however, both plots showed similar contents of available nitrogen. Especially the available P contents are quite high when compared to other studies on agricultural soils in Central Europe (e.g. Oehl et al., 2005, 2010), pointing at the intensive agricultural production at the study site. In addition, detailed analyses from 2000 to 2008 had demonstrated significantly higher levels of microbial biomass in the zero-till plot when compared to the conventional plot (519 μg organic carbon g^{-1} dry mass *versus* 275 μg organic carbon g^{-1} dry mass in the upper 10 cm of soil; Thiel, 2010). Similarly, the zero-till plot contained higher levels of organic carbon (1.29% *versus* 1.08%), although this difference had not been shown to be significant (Thiel, 2010).

2.2. Collection of soil and root samples

Samples for DNA-extraction were obtained in two years of subsequent wheat cultivation, in 2009 (end of July; root samples) and in May 2010 (root and soil samples). Samples for assessing the inoculum potential of soils were obtained in July 2012. Soil samples for extracting and morphological identification of spores were taken in September 2012. In 2009, 2010, samples were obtained from 10 randomly distributed locations within the till and no-till plots excluding 2 m wide strips at the edges of the plots; in 2012, five subplots with corresponding location with regard to the relief of the plots were defined for each of the two treatments. In each of these subplots, six samples were obtained from random locations and subsequently pooled resulting in five samples per treatment. All samples or subsamples were approximately 8 cm in diameter and 12 cm in depth and were taken from the surface.

2.3. Assessment of inoculum potential and extraction of spores

Trap cultures for the assessment of inoculum potential were prepared according to Oehl et al. (2005) on the day of soil sampling. Large pots (upper diameter: 22 cm, lower diameter: 19 cm, height: 25 cm) were filled with a mixture of equal volumes of sand and expanded clay. In an intermediate layer undisturbed soil pieces from individual soil samples (200 g/pot) were placed. One trap culture was prepared for each soil sample referring to a total of 10 trap cultures. After watering the pots, three germinated seeds of *Lolium perenne*, *Trifolium pratense* and *Plantago lanceolata* were placed in the upper substrate layer. Trap cultures were cultivated from July 4th until October 17th in an open greenhouse in Bad Lauchstaedt under ambient light and temperature regime and were watered regularly. Root systems were harvested from each pot and washed extensively with water. Five root samples were taken from random positions within the root systems from each pot. Roots were stained for the analysis of AM structures according to Vierheilig et al. (1998) using ink (Sheaffer, Middlesex, UK). Unless colonization rates were clearly below 1%, mycorrhizal colonization of roots was estimated using the "magnified intersection method" described in McGonigle et al. (1990) using a magnification of 200×.

For spore extraction and identification, the soil was sieved (5 mm mesh), air dried for two weeks and stored at room temperature. Spores were extracted from the soil samples by wet sieving and sucrose centrifugation (Sieverding, 1991; Oehl et al., 2005), transferred to Petri dishes and counted under the dissecting microscope. Then, they were mounted on slides in PVLG and PVLG + Melzer's reagent and identified consulting current identification manuals for AM fungi (e.g. Schenck and Pérez, 1990; Błaszkowski, 2012) and an institutional collection of all original AMF species descriptions and emendations.

2.4. DNA extraction, PCR and terminal restriction fragment length polymorphism (T-RFLP)

DNA from root systems was extracted using the DNeasy Plant Mini-Kit (Qiagen, Hilden, Germany) and DNA from soil was extracted using the FastDNA®Spin Kit for Soil (MP). DNA from the soil was further purified by agarose gel electrophoresis. The band containing the bulk DNA in these gels was excised and DNA was extracted using the Wizard® SV Gel and PCR-Clean Up System (Promega, Mannheim, Germany). Quality and quantity of isolated DNA was analyzed using the Nanodrop microphotospectrometer. T-RFLP for assessing fungal diversity was based on a respective analysis from Mummey and Rillig (2007) starting with a nested PCR for an approximately 400 bp fragment from the 28S rRNA DNA using the primer pairs LR1/FLR2 and FLR3/FLR4 (Gollotte et al., 2004). For detection in T-RFLP, primer FLR3 was labeled by 6-carboxyfluorescein (FAM). PCR amplification was done using the Taq PCR Mastermix from Qiagen (Hilden, Germany) in the case of the root samples and using the Phusion-Polymerase from Thermo Fisher Scientific (Waltham, USA) in the case of the soil samples with 30 cycles and an annealing temperature of 58 °C in both cases. Quality and quantity of PCR-products was checked by agarose gel electrophoresis. Samples with clear single bands of the expected size were excised and purified using the Wizard® SV Gel and PCR-Clean Up System (Promega, Mannheim, Germany); an aliquot of