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The effect of long-term fertilizers application on soil chemical properties, plant species composition and arbuscular mycorrhizal fungi of grasslands

(Vliv dlouhodobého hnojení na chemické vlastnosti půdy, rostlinné společenstvo a arbuskulární mykorhizní houby v travních společenstvech)

PhD Thesis

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CONTENT

1. GENERAL INTRODUCTION

With increasing usage of mineral fertilizers at the beginning of the last century, there has also been a higher demand for a more multidisciplinary and holistic contribution to the agronomic research and development (Conway, 1985; Schellberg *et al*., 1999). Agroecosystems provide high variety of valuable services such as production of food, fiber, fuel and many others. However human activities linked with more intensive land use have also serious negative consequences. From many of them the best known are chemical contamination of the soil, water and air, decrease of biodiversity, soil erosion, alteration of the most important biogeochemical cycles and others. Nonnegligible portion of these changes is caused by fertilization (Wood *et al*., 2000; Schröder *et al*., 2008).

Fertilization, liming and other agronomical techniques have been used as an instrument improving hay production for centuries (Hejcman *et al*., 2013; Kennedy *et al*., 2005). For a long period of time, these practices not only sustained and increased hay yields, but also had negative effects on grassland ecosystems (Schellberg *et al*., 1999; Egerton-Warburton *et al*., 2007). Among others, they caused considerable changes in plant species composition leading in most cases to the reduction of species richness (Silvertown *et al*., 2006; Kennedy *et al*., 2005) due to a lower capability of indigenous plants to compete with more productive pasture species (Schellberg *et al*., 1999). Furthermore these practices caused changes in soil and biomass chemical properties and shifts of soil microbial communities, including arbuscular mycorrhizal fungi (Hejcman *et al.*, 2010; Johnson *et al*., 2010).

Arbuscular mycorrhizal fungi establish association with plant roots mutually beneficial for both partners where plant hosts provide carbohydrates in exchange for mineral nutrients, water or other provision from the fungi (Allen, 1991; Jansa *et al.*, 2003; Parniske, 2008). They are very important components of the soil microbial community forming symbioses with more than two thirds of terrestrial plant species (Smith and Read, 2008; van der Heiden, 1998; Bonfante and Genre, 2010). In addition to the improvement of plant nutrient uptake, arbuscular mycorrhizal fungi can provide better drought tolerance or resistance against pathogens for the host (Newsham *et al*., 1995; Smith *et al*., 2010) and their hyphae form an underground network in the soil which helps to create stability of soil aggregates and reduce soil erosion (Gryndler *et al*., 2004; Chaudhary *et al*., 2009). It has been shown that more diverse arbuscular mycorrhizal fungi community significantly increases species richness and productivity of plant communities (Johnson, 1993; van der Heijden *et al*., 1998; Liu *et al*.,

2012). This can be explained by unique functional trait of individual arbuscular mycorrhizal fungus which can differently utilize particular component of resource (van der Heijden *et al*., 1998; Egerton-Warburton, 2007). Consequently, any changes altering community composition of arbuscular mycorrhizal fungi can change function of plant community (Johnson, 1993; Corkidi *et al*., 2002; Johnson *et al*., 2003). Arbuscular mycorrhizal fungi thus create a pivotal link between plants and soil and they are essential parts of grasslands (Grime *et al*., 1987; Gange *et al*., 1990; Sanders and Koide, 1994).

To overcome the problems related to eutrophication or intensification caused by long-term fertilization, professional knowledge of the effects of soil nutrient supply is necessary. For these proposes, in the middle of the 19th century (after the industrial production of fertilizers), the long-term experiments were set up to contribute the research at the level of botany, soil, microbiology, agriculture or landscape ecology. Long-term studies represent a perfect source providing knowledge on complex interactions between fertilizer input, plant species composition, soil chemical properties, productivity, forage quality and soil biota including mycorrhizal fungi (Schellberg *et al*., 1999; Liu *et al.*, 2012). Some effects are only noticeable after a longer period of time and long-term experiments can help to avoid the difficulties which can occur due to the seasonal variation in growing conditions. By recognition of all impacts and ecosystem functioning, grasslands or other agroecosystems protection would be more effective (Thurston *et al*., 1976; Liu *et al.*, 2012).

Requirements for sustainable agriculture are not only biological or technical, but also economic, political, social, and they are very dependent on the requirements of a society which is often interested only in exploitation and obtaining maximum crops. This attitude needs to be changed more toward nature protection and a long-term stabilization. Therefore, all agriculture activities such as fertilizing, row spacing, harvesting, crop species, pest control or rotations should be properly investigated and carefully planned for conservation of resources and energy, for public or environmental health (Altieri *et al.*, 1983).

2. AIMS AND HYPOTHESES OF THE THESIS

The aim of the dissertation thesis was to investigate the effect of fertilizer application on plant and mycorrhizal community and soil chemical properties in grassland ecosystems and to further examine how the different nutrient enrichment changes plant species composition and production, soil and biomass chemical properties, community of arbuscular mycorrhizal fungi, their presence, abundance and other parameters. This thesis was divided into three specific aims:

Specific aims:

2.1 THE STEINACH GRASSLAND EXPERIMENT: SOIL CHEMICAL PROPERTIES, SWARD HEIGHT AND PLANT SPECIES COMPOSITION IN THREE CUT ALLUVIAL MEADOW AFTER DECADES-LONG FERTILIZER APPLICATION

The aim of this experiment was to analyze the effect of nitrogen, phosphorus, potassium, and calcium application on biomass and soil chemical properties, compressed sward height, and plant species composition in Steinach Grassland Experiment, probably the oldest still-running grassland fertilizer experiment in Continental Europe.

H_1: Decades-long fertilization with nitrogen, phosphorus, and potassium will change soil chemical properties (in particular soil pH, total nitrogen, organic carbon and plant available concentration of phosphorus, potassium and calcium in the soil).
H_2: Nutrients addition (either alone or in combination) will affect productivity of the vegetation estimated using compressed sward height.
H_3: Enrichment with nitrogen, phosphorus, potassium, and calcium will change plant species composition of the sward.
H_4: Nitrogen enrichment will reduce species richness of vascular plants.

2.2 LONG-TERM AGRICULTURAL MANAGEMENT MAXIMIZING HAY PRODUCTION CAN SIGNIFICANTLY REDUCE BELOWGROUND CARBON STORAGE

The aim of this study was to investigate the responses of soil carbon and arbuscular mycorrhizal fungi to seventy years of application of lime and mineral fertilizer. The goal of this work was to predict responses of soil carbon stocks to grassland inputs. We examined the

abundance of arbuscular mycorrhizal fungi inside and outside plant roots and used molecular techniques to determine the abundance of a few individual arbuscular mycorrhizal fungal taxa.

H_1: Lime and mineral fertilizers (nitrogen and phosphorus) will reduce plant allocation of photosynthates to belowground structures including the arbuscular mycorrhizas.
H_2: Differences in lime and fertilizer input among treatments will affect long-term buildup of soil carbon stocks, co-incident with the effects on arbuscular mycorrhizal fungi.
H_3: Nitrogen enrichment will increase allocation to arbuscular mycorrhizal fungi.
H_4: Fertilization with nitrogen and phosphorus will decrease allocation to arbuscular mycorrhizal fungi, consequently reducing the soil carbon sequestration.

2.3 *ZEA MAYS* RESPONDS TO 70 YEARS OF DIFFERENTLY MANAGED SOILS

The aim of this experiment was to examine the responses of investigated plant *Zea mays* (height of plant, number of live and dead leaves, dry plant above-ground biomass, flowering occurrence), grown on soils from long-term experiment Rengen Grassland Experiment which differ in soil chemical parameters affected by long-term fertilization of nitrogen, phosphorus, and calcium.

H_1: Enrichment with nitrogen, phosphorus and calcium will have significant effects on soil chemical properties.
H_2: Soil chemical properties (pH, content of nutrients in the soil) will significantly affect morphologic and qualitative parameters of investigated plants.
H_3: Fertilization with more nutrients will result in higher height of plants.
H_4: Soil nutrient addition will increase dry plant above-ground biomass and number of leaves of the plant.

3. LITERATURE OVERVIEW

3. 1 INTRODUCTION

Raising supply of nutrients into ecosystems is now recognized as a one of the major factors causing global change (Vitousek *et al.*, 1997; Craine *et al*., 2007). In Europe, the most discussed nutrients in context of fertilization are nitrogen and phosphorus which can induce an eutrophication. At the same time, nitrogen and phosphorus are the most limiting nutrients in terrestrial ecosystems and adding even small amounts of these nutrients can affect ecosystem functioning. Long-term enrichment of these elements from human activities that exceeds normal concentration can change species community composition, productivity, nutrient retention (Vitousek *et al.*, 1997; Weiss, 1999), soil biogeochemical processes and decrease species richness (Bobbink *et al.*, 1998; Bowman *et al.*, 2006; Silvertown *et al*., 2006), high deposition of nitrogen can facilitate invasion of nitrophilic annual grasses (Brooks, 1999), which can increase fire frequency (D'Antonio and Vitousek, 1992). Changes in nutrient availabilities in the soil can further induce shifts in carbon allocation by plants and then influence arbuscular mycorrhizal fungal communities (Egerton-Warburton *et al.*, 2001, 2007; Liu *et al*., 2012).

Responses of plant and soil biota to fertilization can differ significantly depending on many factors. For instance, nitrogen enrichment can increase plant or microbial biomass when added into phosphorus-deficient grasslands while nitrogen application into phosphorus-rich grasslands often decreased it (Johnson *et al*., 2003; Wilson *et al*., 2009; Liu *et al*., 2012). This can be explained by functional equilibrium model. This conceptual model predicts that plants will preferentially allocate their photosynthates to structures acquiring the most limiting resources. In infertile soils plants will allocate more to roots and mycorrhizas and in nutrient rich soils plants will allocate more to shoots and leaves (Brower, 1983; Johnson *et al*., 2003). Increasing nitrogen concentration in phosphorus-limited soils makes phosphorus even more limiting and enhances the importance of acquiring phosphorus through arbuscular mycorrhizal symbioses. Plants are thus expected to increase allocation of photosynthates belowground to roots or arbuscular mycorrhizal fungi. In contrast, when nitrogen is added into phosphorus-rich soils, the limitation by any below-ground resource is likely to remove and plants are expected to shift allocation of photosynthates aboveground to shoots and away from arbuscular mycorrhizal fungi (Brower, 1983; Johnson, 2010).

3. 2 AGROECOSYSTEM

Agroecosystems are types of ecosystems influenced by human activity, such as changes into its species composition, nutrient nets or energy flows. Agroecosystem is defined by higher input of energy or fertilization which is necessary due to the reduction of basic regulating functions and loss of the ability to provide own soil fertility and pest control. This typically leads to eutrophication, simpler species composition and energy and nutrient flows than in natural ecosystems (van de Fliert and Braun, 1999; Peden, 1998). Agronomy in Western and Central Europe evolved from little scale farming with site specificities into high technology systems oriented to maximum yield during the 20th century. Yield is maintained by investing pricey external sources of doubtable future and with technologies that induce ecosystem degradation (Altieri *et al.*, 1983; Altiery, 1999). Farmers, who were used to apply sophisticated crop rotations, changed into monocultures or two-crop-systems and shifted to protein rich production for animals. The utilization of synthetic fertilizers and pesticides together with the usage of modern equipment enabled deeper tillage, improved soil management and economical usage of resources and resulted in high production and food security.

Function of internal regulation in agroecosystems is very dependent on the level of biodiversity which includes all species of animals, plants, and microorganisms existing and interacting within an ecosystem (McNeely *et al.*, 1990; Altieri, 1999). When the level of biodiversity is markedly decreased or lost due to the biological simplification, the economic and environmental expenses can be significant. Global maintenance of biodiversity is especially important to agriculturalists since agriculture covers about 25 - 30 % of world's land area and fundamentally affects biological diversity. It was estimated that the extent of global cropland increased from about 365 million ha in 1700 to approximately 1.5 billion ha today, mainly from forests and grasslands. Only very limited areas have remained without any agriculturally induced changes (Altieri and Nicholls, 2004).

The whole range of the domestic plants used in agriculture is originally from wild species which have been adapted by hybridization, domestication or selective breeding. Agroecosystems provide valuable genetic data about populations of adapted as well as wild relatives of pants for crop improvement (Altieri, 1999). It is desirable to maintain or increase individual functional biodiversity as a crucial instrument for sustainability of the production. According to diversity-productivity hypothesis, interspecific differences in plant usage of resources allow plant communities with higher diversity to process more limiting sources and

thus achieve greater productivity. Also nutrient leaching losses from ecosystems should be decreased by plant diversity because more diverse ecosystems have the ability of greater nutrient capture and immobilization (Tilman *et al*., 1996). The present-day trend of the agriculture in most places in the world is to promote simpler structure with considerably less cultivated plant species instead of naturally rich communities. In most cases, only about 12 species of grain crops, 23 vegetables crop species, about 35 fruit and nut crop species are used in the world's agriculture landscape (Fowler and Mooney, 1990; Altieri, 1999). Alarming fact is that no more than seventy plant species is grown on approximately 1440 million ha of currently cultivated land.

To comprehend any biological community it is important to understand interspecific competition - how the niches of particular species are divided because no two kinds could live together constantly if their niches were too similar (Altieri, 1999; Vandermeer, 2010). Plant operates within ecological niches, beyond the essential nutrient resource profile, there are also the environmental requirements of species: temperature, soil moisture, rainfall distribution, frosts, winds, relationships with insect, weeds, crops or other interactions (e.g. competition of species for the same resource). Furthermore, agro-niches exploit various species relationships where two or more species are able to offer different ways for success (one species is deep rooted, other shallow rooted and each plant receives water and nutrients from different resources). Therefore, good understanding and utilizing niche dynamics help to achieve favorable cropping outcomes (Wojtkowski, 2008).

Especially important is the diversity of microorganisms and other soil biota which have received substantially less attention in the past (van Bruggen and Semenov, 2000). These organisms highly contribute in soil health which helps to sustain the biodiversity and stabilize high levels of internal cycling of nutrients in agroecosystems. Its instability can be expressed like plant and animal disease or low resistant ability of the biological community to stress or to suppress plant pathogens or pathogens density in the soil. Soil organisms involve different taxonomic groups including fungi, nematodes, bacteria, protozoa, arthropods or earthworms and can be assessed as species and genetic diversity or structural and functional diversity. These organisms maintain soil homeostasis and the larger and more diverse the pool is, the faster ecosystem can return to stable stadium after exposure to disturbance or stress. Microbial communities are very unstable and fluctuate with changes of environment or stress factors (Begon *et al*., 1999; van Bruggen and Semenov, 2000).

We distinguish three kinds of stress factors:

- **Physical:** extreme temperatures, matric and osmotic potentials (rewetting and drying) or high pressure (e.g. by agriculture equipment)
- **Chemical:** soil reaction (soil pH), excess or deficiency of organic or inorganic nutrients, salinity, anoxia, and biocides (e.g. heavy metals, pesticides, radioactive hydrocarbons and pollutants)
- **Biological:** nutrient excess or shortage, unoriginal organisms with high ability of competition and uncontrolled growth of predators or pathogens

These factors usually operate together, not separately, and they can enhance or weak other stress factors. Any disturbance in agroecosystem may lead to a succession of organisms, to a degradation and then to an increase or decrease of biodiversity. A degree and duration of the succession depends on the extent and duration of the perturbation. Short-term disturbance will relatively fast lead to the initial condition but long-term or permanent stress may lead to a new ecological equilibrium (Begon *et al*., 1997; van Bruggen and Semenov, 2000).

Modern intensive agriculture has its yield limits. According to Wood *et al.* (2000), the overall capacity of ecosystems is declining in production of the goods and services on which we depend. Drastic changes in agroecosystems led to rapid degradation of the environmental ecosystems quality. High doses of fertilizers and pesticides leached into other ecosystems, into aquatic systems, where increased level of nitrogen and phosphorus changed a trophic state of rivers, to the ground water and consequently to the marine ecosystems (Schröder *et al.*, 2008).

3. 3 GRASSLAND

Grassland is one of the major landscape forms on the planet, which covers about 40 % of the world terrestrial surface. From this area, about half is currently used in agricultural production (Lal, 2007). Grasslands in forms of steppe, alpine and alluvial grasslands occurred naturally in Central Europe long before agriculture stared. It is presumed, that they first appeared in the early Neolithic, approximately 5500 before Christ, their area was small and fragmentary distributed in the forest (Hejcman *et al.*, 2013). Majority of grasslands was created as a result of a human activity during the evolution, but the participation of large herbivores on their creation was not as significant as originally thought (Vera, 2000). Grasslands were typically

maintained by regular defoliation - grazing or by haymaking and more productive soils usually rotated crops. During the 19th century, intensification of land use and increasing demand for crop production led to the decrease in pasture area and increase in mown lands (Buček, 2000). In the Czech Republic, after World War II, the huge areas of meadows were turned into the arable lands due to the foodstuff shortage, ploughed and reseeded by more productive species resulted in reduction of species richness (Hejcman *et al.*, 2013).

According to Pavlů *et al.* (2003), species richness of grasslands in Central Europe is usually in the range of 10 – 25 species per m^2, the highest species richness in grasslands (89 vascular plants per m^2) was found in Argentina (Wilson *et al.*, 2012). Generally, the most species-rich grasslands in Europe are dry grasslands with high soil pH (Karlík and Poschlod, 2009) despite the fact that Central Europe is dominated by acid soils (Chytrý *el al.*, 2003). This can be explain by evolutionary processes in Pleistocene (glacial times, 2588 million to 9500 before Christ) when calcareous soils in the dry continental climate prevailed and in the glacial refugia of temperate flora was mostly in mountains of southern Europe with prevailing limestone and dolomite (Pärtel, 2002; Hejcman *et al.*, 2013).

Grassland ecosystems perform a variety of ecological services including regulation of microclimate, recycling of nutrients, affecting local hydrological processes, detoxification noxious chemicals or suppression of undesirable organisms. Vegetative cover of grassland reduces soil losses through erosion, enhances infiltration and reduces water runoff during flooding (Perry, 1994; Altieri, 1999). The ecosystem functioning and sustainability is influenced by many factors including climate, geographic location, amount and distribution of annual precipitation, management practices, and particularly by the level of fertilization (Sala *et al.*, 1988; Conant *et al*., 2001). For sustainment all of the valuable grassland services, soil organic matter is crucial. Soil organic matter supplies plants with nutrients, increases cation exchange, soil aggregation and water holding capacity and reduces soil erosion (Schulten and Schnitzer, 1997; Daynes *et al*., 2013).

Grasslands naturally comprehend a lot of soil organic matter but large part of grasslands worldwide is overexploited and inadequately managed which leads to its losses. Unappropriate treatment, especially intensive cultivation of native grasslands, over grazing or poor pasture management can cause transfer of soil organic matter to the atmosphere in form of carbon dioxide (Kern and Hohnson, 1993; Abril and Bucher, 1999; Conant, 2011). However, losses of soil organic matter can be reversed and atmospheric carbon sequestered with appropriate management. Recent interest to decrease rising carbon dioxide concentration in the atmosphere has led to the practices optimizing grassland management for carbon

storage and improving soil organic matter (Conant and Paustian, 2002; Lal, 2008). Practices, such as elimination of bare fallowing, tillage, usage of cover crops or perennials in rotations can potentially sequester large amounts of atmospheric carbon. Soil carbon can be further increased by eliminating disturbances to the soil, irrigation, introduction of earthworms, intensive grazing management, sowing of favorable forage grasses and legumes and by increasing primary production. As forage production increases supplemental benefit may increase sequestration of atmospheric carbon (Conant *et al*., 2001; Lal, 2013).

Among variety of management techniques evolved to increase forage production for livestock which has the potential to increase soil organic matter, very effective treatment has so far been fertilization. According to numerous studies, the most efficient grassland fertilization which has been shown to be associated with increased soil carbon is long-term application of nitrogen (Conant *et al*., 2001; van Groenigen *et al*., 2006), proving that fertilization is potentially an important driver of soil carbon sequestration. Usage of fertilizers can often double or even triple crop yields. To sustain or increase carbon sequestration and biomass production soil needs to be maintained with balanced supply of nutrients and adequate amounts of micro- and macronutrients. This recognition has led to the development of soil testing laboratories and complex fertilizers studies (Gowariker *et al.*, 2009). The efficiency of particular fertilizer not only on the soil organic matter but also on yield response or other soil and biomass properties can be tested by adding various amounts of fertilizers to neighboring plots, and then measuring and comparing the crop yields and other parameters.

Especially important are long-term experiments because of their value or potential contribution to investigate the long-term influence of fertilization. Unfortunately, the existence of the majority of long-term experiments all over the world was terminated due to the acquisition costs for maintaining or because of the fact that their potential for further research had not been recognized. The oldest still running experiments are in the United Kingdom, Germany, Australia, the Czech Republic, Poland, the Netherlands, and Slovakia (Hejcman and Schellberg, 2008).

- Park Grass Experiment (England) was set up in Rothamsted in 1856 by Lawes and Gilbert. It is the oldest still running fertilizer experiment on permanent grassland, very well documented with more than 170 scientific papers published from this site (Crowley *et al.*, 2005; Silvertown *et al.*, 2006)
- Palace Leas hay meadow experiment (England) was established in 1896 in Cockle Park (Shiel and Batten, 1988)

- Grass Garden in Giant Mts. (the Czech Republic) was first described in 1786 by Haenke (Haenke *et al.*, 1791), probably established in the second half of the 16th century (Semelová *et al.*, 2008)
- Steinach Grassland Experiment (Germany) established on an alluvial *Alopecurus pratensis* meadow in 1933 (Diepolder *et al.*, 2005)
- Rengen Grassland Experiment (Germany) set up on low productive *Nardus stricta* grassland in 1941 (Schellberg *et al.*, 1999, Hejcman *et al.*, 2010)
- Wageningen Experiment (the Netherlands) established on an *Arrhenatherion* hay meadow in 1958 (Pierik *et al.*, 2011)
- Černíkovice Experiment (the Czech Republic) set up on alluvial *Alopecurus pratensis* meadow in 1965 (Honsová *et al.*, 2007; Hrevušová *et al.*, 2009)

Apart from long-term, there are also short-term experiments which can also provide valuable information but, they are not able to predict long-term effects. Residual influence of nutrient enrichment can differ largely depending on various types of ecosystems and nutrients used. There have been observed big differences between lower and higher altitudes depending on climatic and/or soil conditions. In most cases at lower altitudes with less extreme conditions, the effect of fertilizer amendment is shorter (Hejcman and Schellberg, 2008). These effects can last decades and changes in plant community in some cases can even be irreversible (Semelová *et al.*, 2008).

3. 4 FERTILIZATION AND NUTRIENTS

According to The Fertilizer encyclopedia, fertilization is an enrichment of soil by organic or chemical fertilizers (Gowariker *et al.*, 2009). Fertilizer might be named any natural or manufactured material, added to the soil, containing minimally 5 % of one or more of the three essential nutrients - N, P_2O_5, K_2O (FAO, 2000). In the broadest sense, they are products improving availability of nutrients, physical or chemical properties of the soil, and finally directly or indirectly affecting the quality, yield or growth of the plants (Vaněk *et al.*, 2007). Principles of plant mineral nutrition were discovered by Sprengel in 1826 and in 1840 popularized by Justus von Liebig (van der Ploeg *et al.*, 1999) who formulated the "Law of the minimum", stating that a development and a growth of plants are limited by an essential mineral which is in the relatively shortest supply. This discovery changed the way farmers

had managed the field for 12 000 years and persuaded them to maintenance of fertility and the soil health. Nutrients have been returned to arable fields by applying organic material, such as woodland litter, sods or waste from the settlement and cattle dung after the stubble was grazed (Hejcman and Schelberg, 2008).

The application of fertilizers has been known long before Sprengel's discovery and paddock manuring (Hejcman *et al.*, 2002), wood ash mixed with slurry or manure (Semelová *et al.*, 2008) were generally applied by farmers. Slurry was already used in the Alps in the middle age and it is known that Romans used lime, limestone or marl. There were also created different methods like shifting cultivation or cereal-legume crop rotation to improve agronomic production or soil health (Gowariker *et al.*, 2009). As for the modern fertilizers, the first superphosphate was manufactured by Lawes in Deptford in England in 1842 (Leigh, 2004) and the first industrially produced superphosphate in the Czech Republic was made in Ústí nad Labem in 1856 (Vaněk *et al.*, 2007). Since then, the chemical fertilizer industry has grown rapidly.

Mineral fertilizers are now universally used in agriculture and its compilation and content is carefully counted. About sixteen elements are essential for the growth of plants and these are derived from the surrounding air (e.g., carbon as carbon dioxide) and soil.

These elements are divided into:

a) **Macronutrients:** nitrogen, phosphorus, potassium, hydrogen and oxygen as H_2O, calcium, magnesium, sulphur
b) **Micronutrients:** iron, manganese, zinc, copper, boron, molybdenum, and chlorine

Other chemical elements can be also beneficial for some plants, but they are not essential to all of them (Vaněk *et al.*, 2007).

There are several ways how elements can enter the soil: by application of mineral or organic fertilizers, as an atmospheric deposition or they might be released by decomposing rock material which determines quality and quantity of the element. Clay and organic matter (also iron hydroxides) keep nutrients in a form available for plants, attached to these soil compounds (*adsorption complex*). The natural fertility of the soil is defined by its ability to retain a certain amount of nutrients (*storage* or *adsorption capacity*). Nutrients are attracted by the clay materials and the organic matter according to its charge (cations and anions) (FAO, 2000; Vaněk *et al.*, 2007).

Roots can only take up elements in dissolved form from *soil solution* (soil water containing nutrients in dissolved form) where they are released from adsorption complex. There is equilibrium in the soil between the nutrients released into the soil solution and the nutrients adsorbed on the soil particles. When the equilibrium is disturbed by nutrient uptake, the adsorption complex releases nutrient to establish a new equilibrium. This process is very important and the difference in adsorption strength of cations and anions influences how and when to apply fertilizers in order to avoid pollution by leaching and to receive the highest efficiency (Marschner, 1995; Vaněk *et al.*, 2007).

In uncropped soils, after some time nutrients can be released and accumulated in the soil solution (especially nitrogen or potassium) and washed out to the ground water which can caused eutrophication and other environmental problems (Marschner, 1995; Schellberg *et al*., 1999). Some nutrients (chlorides and sulphates of sodium, calcium, and magnesium) under semi-arid conditions can move with evaporation water to the surface and cause salt damage. Phosphate (PO_4^{3-}) is highly immobile and it is strongly held by the positively charged position of certain clay minerals and soils constituents like calcium, iron and aluminium. To the contrary, chlorine (Cl^-) and nitrate (NO_3^-) or sulphate (SO_4^{2-}) usually stay in the soil solution and move with soil water to the roots (*mass flow*) (FAO, 2000; Vaněk *et al.*, 2007).

Soil fertility and so plant growth is determined mostly by soil organic matter (including microbial biomass), soil structure and texture, content of nutrients, soil depth, storage capacity (adsorption capacity), soil reaction and absence of toxic elements. Soil contains mineral particles of various sizes, weathering products of the parent material, and organic matter, different amounts of water and air. The solid particles are classified by size in *gravel and stones* (more than 2 mm in diameter), *sand* (2.0 to 0.02 mm), *silt* (0.02 to 0.002 mm) and *clay* (less than 0.002 mm) (FAO, 2000).

Availability of nutrients in the soil is determined by soil reaction, pH unites indicates soil reaction - lower values are with excess concentration of hydrogen ions (H^+) at the adsorption complex and higher values indicate alkalinity (a predominance of calcium (Ca^{2+}) and/or sodium (Na^+) cations). Soil acidity is constantly increased with acidic rains from atmosphere, plant root exudates and cation uptake. The pH value of normal, productive soils ranges between 4 and 8. In the humid tropic soil pH tends to be rather acidic because of the leaching effect of heavy rainfall. In the dry subtropics, soil reaction may be higher than 7 (alkaline) due to the accumulation of alkaline elements such as calcium and sodium. Lower soil pH value causes increase availability of phosphorus because of a decreased concentration of $H_2PO_4^-$ and limits plant growth by toxic concentration of Al^{3+} and Mn^{2+}. Nitrogen uptake depends on

the supplied form, uptake of ammonium (NH_4^+) is decreased with lower pH value whereas uptake of nitrate (NO_3^-) is increased but the relationship is complicated because of side-effects such as cation balance on root function and metabolism (Marschner, 1995; Schellber *et al*., 1999).

Long-term fertilization mainly application of nitrogen fertilizers is known to develop soil acidification (Johnston *et al*., 1986), which is recognized as one of the main limits of agricultural production and provision of ecosystem services on a wide range of soils (Tilman, 1996; Scott *et al*., 2000; Egerton-Warburton *et al*., 2007). For those proposes, calcium in different forms (e.g., in carbonate form as a lime) is used to reverse soil acidification which has positive effect of precipitating the free aluminium and thus controlling its toxicity. A negative effect of liming reaching pH values to 7 can cause micronutrient deficiency (except molybdenum) in tropical soils. Whenever possible, lime and fertilizers (with macro- or micronutrients) should not be applied at the same time, but at certain intervals. In soils with high pH (alkaline soils), acid-forming fertilizers such as sulphate of ammonia, ammonium sulphate-nitrate, ammonium nitrate or urea should preferably be used in order to correct alkalinity. On saline/sodic soils gypsum is a useful soil amendment for the removal of sodium.

Accessibility of nutrients in the soil can also be influenced by soil organisms as an inherent part of grassland ecosystems. Their presence and activities are very beneficial because they decompose organic matter creating humus, aggregate soil practices that helps for better structure of the soil, they can protect roots from parasites and disease, hold nitrogen and other nutrients from leaching out, produce hormones for better plants growth and delegate pollutants to lead them into the soil (FAO, 2000; Vaněk *et al.*, 2007; Johnson *et al.*, 1999). One of the very important group of soil organisms are mycorrhizal fungi which help to provide nutrients which would otherwise be unavailable for the plant, especially low mobile phosphorus and zinc but also nitrogen or water (Jansa *et al.*, 2003; Parniske, 2008; Bonfante and Genre, 2010).

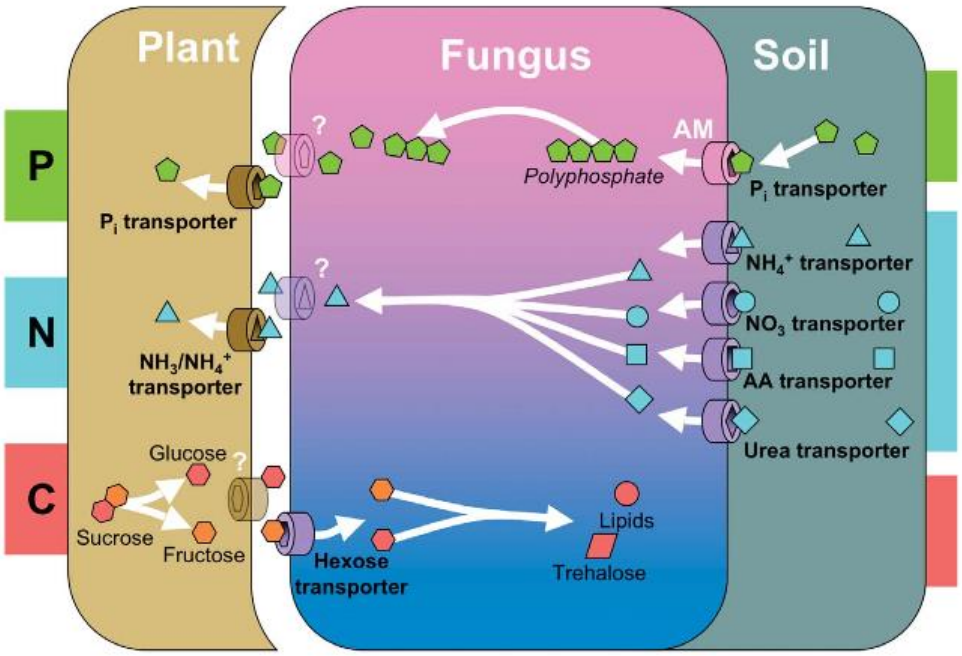

Figure 1 Schema summarizing the main nutrient exchange processes in mycorrhizal symbiosis (Bonfante and Genre, 2010).

3. 5 MYCORRHIZA

Roots of most plants including cultivated crop species, grasses or forest trees are infected with a specific type of soil organisms - mycorrhizal fungi. The term mycorrhiza comes from Greek words for fungus and root because mycelium network of these fungi form an association with plant roots and create a new organ – mycorrhiza extending thus a surface area of the roots (Smith and Reed, 2008; Bonfante and Genre, 2010). Mycorrhiza is ubiquitous, generally mutualistic relationship in which plant hosts receive soil resources (mineral nutrients and water) from the fungi, while fungal endophytes receive photosynthetically derived carbon compounds from the plant. Both of partners profit from the cooperation, mycorrhizal fungi can increase plant growth and improve nutrients uptake and in return, up to 20 % of plant-fixed carbon is converted to the fungus (Jansa *et al.*, 2003; Parniske, 2008). Among improving plant status or nutrition condition, mycorrhizal fungi can deliver variety of other benefits such as physically stabilization of soil through the fixing of soil particles by filamentous hyphal networks and production of glomalin (a soil protein and component of the hyphal walls). They can increase the resistance towards pathogens, improve water relations,

drought tolerance or other unfriendly conditions. There is even an intention to commercially use the ability of mycorrhizal fungi to positively affect plant production and nutrient status in cultured grown plants (Abbott and Robson, 1982; Smith and Smith, 2011; Gadkar and Rillig, 2006; Chaudhary *et al.*, 2009).

We can distinguish two main categories of mycorrhizal fungi: septate Ascomycota and Basidomycota or aseptate endphytes Glomeromycota. More frequent classification represents anatomical perspective and defines two groups according whether the fungus penetrates the cortical root cells of the plant and create cells inside: ectomycorrhiza, predominant on trees in temperate forests and endomycorrhiza which is further divided into ericoid, orchid and arbuscular mycorrhiza (Bonfante and Genre, 2010). Arbuscular mycorrhiza (AM) is the oldest, globally widespread and abundant type of mycorrhiza which was found in 95 % of mycorrhizal and 80 % of vascular plant families (Gryndler *et al.*, 2004; Smith and Reed, 2008). Even though this monophyletic group occurs in all soils on the Earth (AM fungi were found to occupy soils from alpine or boreal zones to tropical forests, grasslands or croplands), it currently contains only about 230 species known to science so far (Smith and Read, 2008; Helgason and Fitter, 2009; Bonfante and Genre, 2010).

AM fungi are considered to be asexual with hundreds of nuclei sharing the same cytoplasm where nuclei are steadily transferred with fast cytoplasmic streaming. Likewise, large asexual spores contain hundreds of nuclei making generic approaches very uneasy. Nutrient transport and symbiotic development occur in tree-shaped structures created by AM fungi within plant cells inside the root. These formations are called arbuscules coming from Latin "arbusculum" which means little tree or bush. Arbuscules are places of main nutrient exchange between plant and symbiotic fungi. All fungal branches in a plant cell are surrounded by a plant-derived periarbuscular membrane which is continuous with the plant plasma membrane and excludes the fungus from the cytoplasm of the plant. The apoplastic interface between the plant-derived periarbuscular membrane and the fungal plasma membrane is called the perarbsucular space. Because of the cell-wall synthesizing potential of both the fungal and the plant membrane, periarbuscular space comprises fungal and plant cell-wall material (Gryndler *et al*., 2004; Parniske, 2008; Bonfante and Genre, 2010).

AM fungi extend the radius of the plant root with their hyphae reaching about 100 meters of hyphae per m^3 of the soil. The AM fungal mycelium exists both outside of the host roots (extraradical mycelium) and within the root (intraradical mycelium). Extraradical hyphae of AM fungi are very often the largest single contributor (estimated 30 – 60 %) to microbial biomass in agricultural soil and structure the soil (Olsson *et al.*, 1999; Martinez and Johnson,

2010) and they also serve as an important pathway for translocation of plant assimilates to the soil.

Plants can survive without their fungal symbiont, but AM fungi have so far known to be unculturable without the presence of its host unable to accept carbohydrates other way then through plant root which makes them obligate biotrophs completely dependent on their green host. Evolutionary, AM fungi show successful ecological strategy where advantage coming from this hazardous association overcome the risk of losing saprotrophic skills.

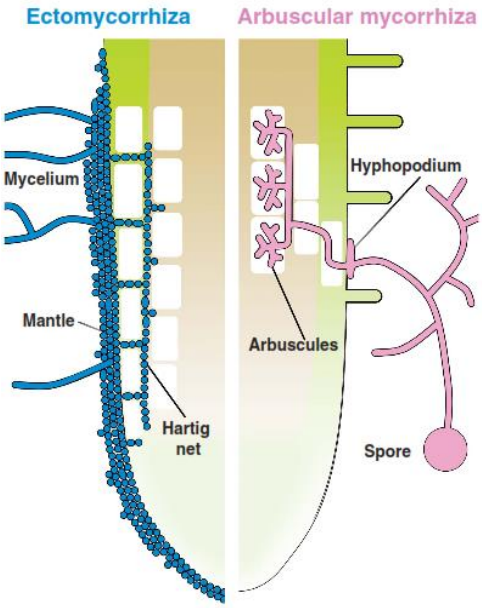

Figure 2 Schema of root coloniazation sturctures in ectomycorrhizal (blue) and arubuscular mycorrhizal (pink) interaction.
The ectomycorrhizal fungus surrounds the root tip with a thick mantle of closely appressed hyphae, whereas the Hartig net develops around epidermal cells (green). In the case of arbuscular mycorrhizas, the root tip is usually not colonized. Hyphae develop from a spore and produce a hyphodium on the root epidermis. Intraradical colonization proceeds both intra and intercellularly and culminates with the formation of arbuscules, little fungal trees, inside inner cortical cells (brown) (Bonfante and Genre, 2010).

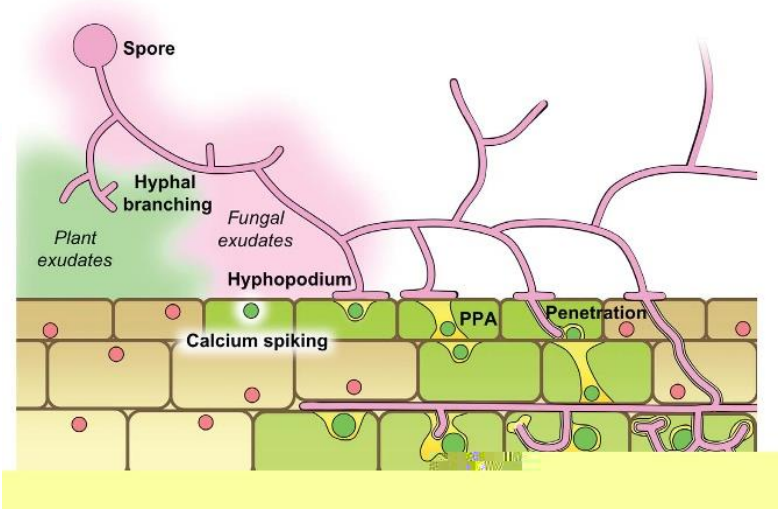

Figure 3 Schema of root colonization process by AM fungi.
The germination of a resting spore is followed by the production of a short explorative mycelium. The perception of plant exudates, released by the host root, induces recursive hyphal branching, increasing the probability of a direct contact between the symbionts. In the meantime, fungal exudates are perceived by the root, where they trigger calcium spiking through the activation of the common SYM pathway. Signal transduction leads to the activation of cellular and transcriptional responses (green cells and nuclei). The contact between the plant and fungus is followed by the adhesion of hyphopodium to the root surface. This triggers the assembly of a broad aggregation of cytoplasm (yellow), named the prepenetration apparatus (PPA) in the contacted epidermal cell and underlying outer cortical cell. Subsequent intracellular fungal colonization strictly follows the route of PPAs from the epidermis to the inner cortex. Here, intercellular hyphae can develop along the root axis. The PPA mechanism is then replicated in the contacted inner cortical cells, both before fungal entry and – on a smaller scale – branching. (Bonfante and Genre, 2010).

Mycorrhizal symbiosis is very intensively investigating topic in the world as well as in the Czech Republic although there had been some delays in our country comparing to others. Mycorrhiza was firstly observed on *Iris* by Nägeli in 1842 but the fungi colonizing the plant root had not been known until middle of 20th century when the first isolation of AM fungi was done by professor Barbara Mosse (Mosse, 1953). First work about the mycorrhiza in the Czech Republic was study from Krčmář about *Pyrola* in 1899 (Gryndler *et al*., 2004).

AM fungi are of a great importance both ecologically and physiologically to the most of agriculture ecosystems including grasslands. Many long-term experiments have shown that changing contents of nutrients, pH values or land-use can cause serious changes in plant species composition and chemical or biological properties of the soil (Hejcman *et al.*, 2007; Hrevušová *et al.*, 2009; Kull and Zobel, 1991; Kirkham *et al.*, 1996; Silvertown *et al.*, 2006). So far, more attention has been given to study this effect on plant communities, but impact has been observed within AM fungal communities as well (Liu *et al*., 2012). Enrichment with nutrients, mainly with nitrogen and phosphorus has been described to cause significant alteration to AM fungal community structure and this could further impact plant community function and contribute to global changes in grassland productivity (Egerton-Warburton *et al.*, 2007). Understanding mycorrhizal systems and their responses to different soil management, especially to long-term fertilizer application is very important for their utilization and comprehension (Kahiluoto *et al.*, 2000). Numerous of interactions among soil biota including mycorrhizal fungi and its effects illustrate a level of complexity that we are only beginning to understand (Antoninka *et al.*, 2009; Chaudhary *et al.*, 2009).

Therefore, experiments were established to generate a broader understanding of the drivers contributing these changes. Studies investigating this topic were carried out in many countries, e.g., Switzerland (Jansa *et al.*, 2002), USA (Martinez and Johnson, 2010) or China (Yongjun *et al.*, 2012). It has been shown that AM fungi are structured by plant species composition, soil properties or nutrient availabilities but responses of AM fungal communities can differ largely depending on many factors. Some studies reported negative correlation between AM fungi and plant species richness, some showed no relationship and others reported a positive correlation suggesting that the responses of AM fungi as well as responses of plant communities are not always similar to environmental variation (Antonika *et al*., 2009; Liu *et al*., 2012). Plant community structure can be further influenced by AM fungi because this nutritional symbioses influence the outcome of plant interactions with competitors, parasites or herbivores. Furthermore, AM fungi are known to influence the community composition of soil microbes, including populations of pathogenic soil bacteria and fungi

(Linderman, 2000; Antoninka *et al.*, 2009). Important driver shifting AM fungal community is fertilization. Application with nitrogen and phosphorus is known to reduce abundance of AM fungi which can be a result of direct responses to increased concentration of nitrogen and phosphorus in the soil or indirect responses to changed plant community. AM fungi as an obligate biotrophs should be reduced when the amount of root substrate is decreased (Liu *et al*., 2012).

Basic method for practical investigating of mycorrhiza is mycorrhizal bioassay which is according to Johnson *et al.* (1999) a proxy measure of mycorrhizal propagule densities. Plants in bioassay are grown in defined conditions using soil collected from the field, and the extent of mycorrhizal colonization is assessed after a predetermined period. The complex characteristics of mycorrhizal associations depend on root growth and architecture as well as fungal diversity in the soil and inside roots (Varma and Kharkawae, 2009). Abundance and community composition of AM fungi is evaluated by indentifying the asexual spores to genus or species. This approach dominated in mycorrhizal research for about fifty years until a development of molecular methods using to estimate community composition of AM fungi and allowing better insight into their mechanisms (Parniske, 2008; Bonfante and Genre, 2010).

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5. RESULTS OF THE THESIS

1. Hejcman M., **Sochorová L**., Pavlů V., Štrobach J., Diepolder M., Schellberg J. (2014): The Steinach Grassland Experiment: Soil chemical properties, sward height and plant species composition in three cut alluvial meadow after decades-long fertilizer application. *Agriculture, Ecosystems and Environment* (184): 76 – 87.

2. **Sochorová L.**, Jansa J., Verbruggen E., Hejcman M., Schellberg J., Kiers E.T., Johnson N.C. (2016): Long-term agricultural management maximizing hay production can significantly reduce belowground C storage. *Agriculture, Ecosystems and Environment* (220): 104 – 114.

3. **Zemková L.** (2016): *Zea mays* respons to 70 years of differently managed soils. *Scientia Agriculturae Bohemica* (Submitted paper).

Further are attached papers which have connection to the topic and were done with cooperation of Institute of Microbiology, Academy of Sciences of the Czech Republic:

4. Gryndler M., Beskid O., Hršelová H., Bukovská P., Hujslová M., Gryndlerová H., Konvalinková T., Schnepf A., **Sochorová L.**, Jansa J. (2015): Mutabilis in mutabili: Spatiotemporal dynamics of a truffle colony in soil. *Soil Biology & Biochemistry* (90): 62 – 70.

5. Gryndler M., Beskid O., Hujslová M., Konvalinková T., Bukovská P., **Sochorová L.**, Hršelová H., Jansa J. (2016): Soil receptivity for ectomycorrhizal fungi: *Tuber aestivum* is specifically stimulated by calcium carbonate and certain organic compounds. *Mycorrhiza* (Submitted paper).

5. 1 PAPER I.

The Steinach Grassland Experiment: Soil chemical properties, sward height and plant species composition in three cut alluvial meadow after decades-long fertilizer application

Hejcman M., Sochorová L., Pavlů V., Štrobach J., Diepolder M., Schellberg J.

2014, *Agriculture, Ecosystems and Environment* (184): 76 – 87

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The Steinach Grassland Experiment: Soil chemical properties, sward height and plant species composition in three cut alluvial meadow after decades-long fertilizer application

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ABSTRACT

The Steinach Grassland Experiment (SGE) is probably the oldest still-running grassland fertilizer experiment in Continental Europe. It was established on an alluvial *Alopecurus pratensis* meadow in southeast Germany in 1933. The aim of this study was to provide detailed information on this experiment concerning the effects of decades-long nitrogen, phosphorus, potassium, and calcium application (46 treatments in total) on (1) soil chemical properties, (2) sward height, (3) plant species composition, and (4) species richness of vascular plants derived from a field survey in spring 2008.

(1) A steep gradient of soil properties was recorded, namely plant-available (Mehlich III) P (14–161 mg kg^{-1}), K (82–1018 mg kg^{-1}) and Ca (532–3336 mg kg^{-1}) concentrations, C:N ratio (6.9–10.4) and pH (H_2O) (4.7–7.0).

(2) Compressed sward height in the third week of May 2008 ranged from 11 cm in the control to 47 cm in the limed plot with high N, P, and K application.

(3) Although fertilizer application altered the plant species composition, diversification of plant communities was not as high as in the case of fertilizer application on low productive soils in other experiments. This was caused by sufficient nutrients enabling the survival of species adapted to high nutrient availability, even in the unfertilized control. The species composition in productive alluvial grasslands is substantially less affected by fertilizer application than in low productive grasslands.

(4) Long-term fertilizer application negatively affected the species richness of vascular plants directly by soil acidification and indirectly by an increase in sward height. The extent to which N application negatively affected species richness was dependent on whether N was applied alone or in combination with other nutrients and whether N application acidified the soil.

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1. Introduction

In Europe, there are many still-running decades-long grassland fertilizer experiments, but results from these experiments have rarely been published in international scientific journals (see Hejcman and Schellberg (2009) for an overview of 10 experiments). To our knowledge, there are only six experiments that have been running for at least 40 years with well-published results, of which the Park grass experiment established on *Lolium perenne* grassland in 1856 (Crawley et al., 2005; Rothamsted Research, 2006; Silvertown et al., 2006) is the best known, followed by the Palace Leas Hay Meadow Plots Experiment established on *Agrostis capillaris* grassland in 1896 (Hopkins et al., 2011), both in the UK. Recently, an analysis of plant species richness pattern was published from the Wageningen Experiment established on an *Arrhenatherion* hay meadow in the Centre of the Netherlands in 1958 (Pierik et al., 2011). The next well-published experiment is the Rengen Grassland Experiment established on low productive *Nardus stricta* grassland in SW Germany in 1941 (Schellberg et al., 1999; Hejcman et al., 2010), the Černíkovice Experiment established on alluvial *Alopecurus pratensis* meadow in the Centre of the Czech Republic in 1965 (Honsová et al., 2007; Hrevušová et al., 2009) and the Vel'ká Lúka experiment established on an alluvial *A. pratensis* meadow in 1961 in the Centre of Slovakia (Vargová

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et al., 2012). Decades-long fertilizer experiments are an excellent source of knowledge on complex interactions between fertilizer regime, soil chemical properties, plant species composition, productivity and forage quality (Silvertown et al., 1994; Liu et al., 2010; Pavlů et al., 2012). Each experiment is unique and all experiments together cover a wide range of environmental conditions, such as climate and soil, management, including the fertilizer regime and the frequency of defoliation, as well as the resulting productivity and forage quality.

In Europe, eutrophication mainly through the application of mineral nitrogen (N) and phosphorus (P) fertilizers is considered to be mostly responsible for the loss of species-rich grasslands (Janssens et al., 1998; Wellstein et al., 2007; Klaus et al., 2011a, 2013). In studies performed on grasslands at the landscape scale, management practices combined with fertilizer application are often insufficiently known and hence, the effects of different fertilizer regimes on plant species composition can hardly be identified correctly. In addition, changes in fertilizer regimes during the last fifty years have frequently also been connected with changes in cutting date and frequency, hampering the study of the effect of fertilizer application on plant species composition (Hopkins and Wilkins, 2006). In contrast to this, long-term fertilizer experiments under controlled and constant management allow precise identification of the real effects of fertilizer application on plant species composition.

One of the oldest still-running grassland fertilizer experiments in Continental Europe is the Steinach Grassland Experiment (SGE) established on an alluvial *A. pratensis* meadow in SE Germany (Bavaria) in 1933 (Rieder, 1988, 1989; Diepolder et al., 2005). Although treatments are not well replicated as in all similar long-term experiments established before the 1940s, a unique and rewarding feature of this experiment is the large number of fertilizer treatments applied (46 altogether), which generate a wide gradient of soil chemical properties, plant species composition and herbage production. To our knowledge, a similar large number of fertilizer treatments as in the SGE is only present in the Park Grass Experiment (Silvertown et al., 2006).

In the period from 1985 to 2001, aboveground biomass production in the SGE ranged from 4.1 t ha^{-1} in a treatment with an application of 174 K ha^{-1} to 10.8 t ha^{-1} in a treatment with an application of dung water (66 m^3 ha^{-1}) supplemented with 44 kg P ha^{-1} in the form of Thomas slag, or in a treatment with mineral application of 120 kg N, 44 kg P, and 174 kg K ha^{-1} (Diepolder et al., 2005). Another peculiarity of the SGE is the combination of organic fertilizers (dung water–mixture of urine and technological water with nutrients leached from faeces) together with synthetic chemical fertilizers (Thomas slag and lime) in some treatments, which is rarely used in long-term experiments. Details of the experimental layout and a detailed analysis of the effects of long-term fertilizer treatments on the relationship between soil chemical properties, plant species composition and species richness have never been presented to the international scientific community from this unique experiment. After a field survey of all treatments performed in 2008, we concluded that it is necessary to present an inventory of the state of the experiment, explore major drivers and effects and then assess the value of its particular combinations of fertilizer treatments for further studies. We therefore suspected that the analysis of soil chemical properties and plant species composition would be most promising in this respect and that historical herbage yield data should be considered in subsequent analyses. In this initial report, we decided to focus on the following questions:

(1) What is the effect of decades-long N, P, and K application on soil chemical properties, in particular pH (H_2O), total N, organic C and concentrations of plant-available nutrients (P, K, Ca, Mg)?
(2) How do nutrients (either alone or in combination) affect productivity of the vegetation as estimated via compressed sward height?
(3) How do different fertilizer treatments affect plant species composition of the sward? We were especially interested in understanding the impact of nutrient application on the abundance of all species, not only dominant ones. As we had observed a complete replacement of species along nutrient gradients already in other long-term experiments on poorer soils (Hejcman et al., 2007, 2012; Semelová et al., 2008), we wanted to study whether N, P, and K application affects the plant species composition similarly on a naturally richer alluvial soil, which is well supplied with water.
(4) Which of the nutrients most effectively influence the species richness of vascular plants and how is species richness related to sward height and soil chemical properties? We hypothesised that species richness in well-fertilized treatments is significantly lower than in unfertilized treatments.

2. Material and methods

2.1. Study site

The long-term Steinach grassland experiment (SGE) was established in 1933 on an alluvial meadow near the village of Steinach (Straubing-Bogen, Bavaria, Germany, 48°57'N, 12°36'E). The altitude of the study site is 344 m a.s.l., the mean annual precipitation is 840 mm and the mean annual temperature is 7.3 °C (Steinach meteorological station). The soil type is a pseudogley and in 2008, the pH(H_2O) was 5.4 in the upper 10 cm layer in the control without any fertilizer application and liming. The depth of the underground water table varied between 1 and 2 m. According to phytosociological nomenclature (Botta-Dukát et al., 2005), the vegetation of the experimental meadow was classified as *Deschampsion cespitosae* (syn. *Alopecurion pratense*).

2.2. Experimental design

In 1933, five fertilizer treatments plus a control (group I to V, see Appendix A) were established without any replication, on plots 300 m^2 in size (Rieder, 1989). Subsequently, the experimental plots were divided and thus the number of fertilizer treatments increased. The first division was performed in 1954 (10 fertilizer treatments), the second in 1965 (20 treatments), and the last in 1986 (46 treatments). Some modifications to the fertilizer regime, particularly an increase in N application rates, were performed in several treatments in 1971. The history of the fertilizer regimes in each plot is given in Appendix A.

As a result of the reorganization of treatments, the SGE today represents three neighbouring strips (Fig. 1), which are regularly cut three times a year. Each strip is divided into 25 plots (75 monitoring plots in total). Standard individual plot area is now 18.75 m^2 (3.75 × 5 m) in 60 plots. The area of 15 additional plots is 12.5 m^2 (2.5 × 5 m).

Six control plots without any fertilizer and lime input (orange in Fig. 1) now exist, plus 69 fertilized plots. In these fertilized plots, 25 were limed in 1986 (blue) with 8 t ha^{-1} of dolomite lime (Diepolder et al., 2005), whereas 44 plots still remain without any direct liming (green). Treatments include limed plots with and without dung water, a combination of mineral P and dung water, liming only, $(NH_4)_2SO_4$, a combination of $Ca(NO_3)_2$, $(NH_4)_2SO_4$ and NH_4NO_3 (amount of applied N was 60, 50, and 50 kg N ha^{-1}) and a combination of mineral N, P, and K fertilizers. The fertilized plots without any direct liming include plots with dung water only, a combination of P and dung water and a combination of mineral N, P, and

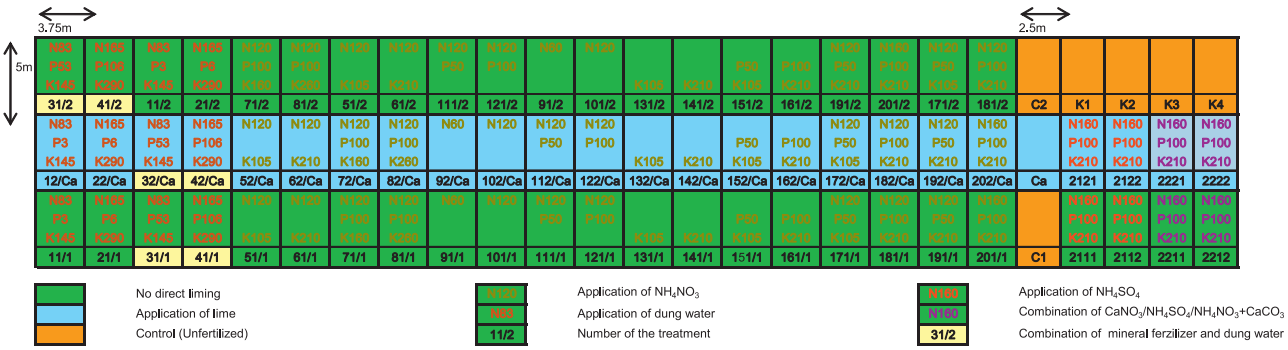

Fig. 1. Spatial arrangement of treatments in the Steinach Grassland Experiment. Numbers indicate the amount of annually applied N, P, and K (kg ha^{-1}). Black numbers and letters in the bottom of each plot are original marks of monitoring plots. Green indicates unlimed plots with application of N, P, and K fertilizers. Blue indicates plots directly limed in 1986 and with application of N, P, and K fertilizers. Orange indicates control plots without any fertilizer input. Brown letters and numbers indicate dung water application, green indicates mineral N, P, and K fertilizer application, red indicates the application of mineral N in the form of $(NH_4)_2SO_4$ and violet indicates the application of N as a combination of three different mineral fertilizers. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

K fertilizers. Some plots received $(NH_4)_2SO_4$, or a combination of $Ca(NO_3)_2$, $(NH_4)_2SO_4$, and NH_4NO_3.

Annual application rates of N were 60, 120, and 160 kg ha^{-1} in mineral fertilizer treatments and 116 and 232 kg N ha^{-1} in organic fertilizer treatments. Annual application rates of P were 22 and 44 kg ha^{-1} in mineral fertilizer treatments applied as Thomas slag and 4 and 8 kg ha^{-1} in organic fertilizer treatments. Thomas slag also supplied the soil with Ca at an annual rate of 107 and 214 kg Ca ha^{-1} in treatments with an annual application of 22 and 44 kg P ha^{-1}, respectively. Annual application rates of K were 87, 133, 174, and 216 kg ha^{-1} in mineral fertilizer treatments applied as a mixture of KCl and $MgSO_4$ and 232 and 465 kg ha^{-1} in organic fertilizer treatments. Mineral P and K fertilizers were applied in the autumn and all N fertilizers during the vegetation season. The amount of Ca and Mg applied in the form of dolomite lime (53% CaO and 25% MgO) in 1986 was 3032 and 1206 kg ha^{-1}, respectively, in limed treatments (Diepolder et al., 2005).

2.3. Soil chemical properties

In mid-April 2008, 10 separate soil samples per individual experimental plot (see Fig. 1 and Appendix A for list of all plots) were taken from a 0 to 10 cm layer and thoroughly mixed to obtain one representative soil sample per plot. The soil samples were air-dried, ground in a mortar, and sieved to 2 mm after removal of living roots. All analyses were performed at the Eko-Lab, Žamberk (http://www.ekolab.zamberk.cz), an accredited Czech national laboratory, using the Mehlich III extraction to predict plant-available Ca, K, Mg and P (Mehlich, 1984). The determination of organic C (C_{org}) was performed spectrophotometrically after oxidation in a mixture of $K_2Cr_2O_7$ solution with H_2SO_4, at 135 °C. The determination of total N (STN) was performed using a TruSpec f. Leco instrument, where the soil sample was combusted at 950 °C. Soil pH(H_2O) was measured in a suspension of 10 g dry soil mixed with 50 mL distilled H_2O.

2.4. Compressed sward height

Compressed sward height (CSH) measurements were performed with a rising plate meter (Castle, 1976) in the third week of May 2008. The mean CSH was calculated from 10 single measurements within the central part of each plot.

2.5. Plant species composition

In the third week of May 2008, the percentage cover of all vascular plant species was visually estimated in each plot. Based on description of vascular plants in the local flora (Rothmaler et al., 2000), all plant species within the study area were *a priori* classified according to their main traits. We recognized five functional groups: legumes, tall graminoids (average high ≥0.5 m), short graminoids (bellow 0.5 m), and similarly tall forbs and short forbs (see Appendix B). To obtain total cover of functional groups, the cover of individual species belonging to a particular group were totaled. To eliminate edge effects, relevés were taken only in the centre of each plot in an area of 14.6 m^2 (60 plots) and 9 m^2 (15 plots). As species richness can be affected by sampling area (Crawley et al., 2005; Hejcman et al., 2010), smaller plots (six control and nine fertilized plots) were omitted from the analyses. Species richness was determined by counting the number of species in each relevé.

2.6. Data analysis

We used simple linear regressions in STATISTICA 8.0 software (Statsoft, Tulsa, AZ, USA) to analyse the relationship between the amount of applied nutrients, soil chemical properties, CSH, species richness and cover of functional groups. We used this simple analytical approach because of the absence of proper replications of individual treatments in the SGE. However, such a large number of different fertilizer treatments created a considerably high gradient of soil chemical properties and sward heights. Thus, we analysed these gradients and applied a regression approach. Detrended Correspondence Analysis (DCA) and Redundancy Analysis (RDA) in the CANOCO 4.5 program (ter Braak and Šmilauer, 2002) were used to evaluate the plant species composition data based on the cover estimates described above. In addition, a Monte Carlo permutation test with 999 permutations was used to reveal whether the tested explanatory (environmental) variables (amount of applied nutrients, soil chemical properties and CSH) significantly affected the plant species composition data. The results of DCA and RDA analyses were visualised in the form of ordination diagrams constructed by the CanoDraw program. The percentage of plant species data variability explained by the environmental variables was used as a measure of their explanatory power.

3. Results

3.1. Soil chemical properties

Soil pH(H_2O) ranged from 4.7 in plot number 2112 ($N_{160}P_{44}K_{174}$–numbers indicate the amount of applied N, P, and K in kg ha^{-1}), with the application of $(NH_4)_2SO_4$ and in plot number 61/2 ($N_{120}P_0K_{174}$), to a value of pH 7 in the limed plot

Table 1
Soil chemical properties in a selection of the most contrasting plots. For plot abbreviations see Fig. 1. Plot C—mean values from all control plots without fertilizer input. The abbreviation "Ca" in plot numbers indicates lime application in 1986. Plots with applications of mineral P were indirectly limed by Thomas slag.

	Experimental plot									
	C	141/1	162/Ca	31/2	52/Ca	61/2	192/Ca	21/1	2112	2222/Ca
N (kg ha^{-1})	0	0		116	100	120	120	232	160	160
P (kg ha^{-1})	0	0	44	26			22	8	44	44
K (kg ha^{-1})	0	174	174	232	87	174	174	485	174	174
SN_{tot} (g kg^{-1})	3.6	3.6	2.6	2.9	3.1	3.3	2.5	3.9	5.0	3.2
SC_{org} (g kg^{-1})	29	34	19	20	26	26	19	33	44	23
C/N	8.2	9.6	7.4	6.9	8.2	7.9	7.4	8.5	8.7	7.2
pH(H_2O)	5.1	5.2	6.7	5.9	5.5	4.7	6.4	5.9	4.7	6.5
SK (mg kg^{-1})	135	869	214	173	135	287	170	1018	238	180
SP (mg kg^{-1})	26	24	161	36	14	18	53	35	103	73
SMg (mg kg^{-1})	201	218	386	164	321	164	360	222	190	388
SCa (mg kg^{-1})	1506	565	2150	1689	1377	532	2007	1155	1153	2474

N, P, and K–amount of annually applied N, P, and K. SN_{tot}—total nitrogen content in the soil; SP, SK, SCa, and SMg—plant-available concentrations of P, K, Ca, and Mg in the soil; SC_{org}—content of organic carbon in the soil.

number 122/Ca ($N_{120}P_{44}$) with the application of NH_4NO_3 (see Table 1 for the pH in other selected plots). The mean pH value was 5.7 in plots without direct liming in 1986 and 6.1 in plots with direct liming in 1986, with significant differences between them (one-way ANOVA; DF = 1; $F = 10.223$; $P = 0.002$). As expected, there was a significant positive effect of direct liming performed in 1986 on soil pH and on the concentration of plant-available Ca in the soil (Table 2, analyses 29 and 31).

The content of organic C ranged from 18.8 g kg^{-1} in the limed plot number 192/Ca ($N_{120}P_{22}K_{174}$) to 44.5 g kg^{-1} in plot number 2111 ($N_{160}P_{22}K_{174}$) receiving $(NH_4)_2SO_4$. There was a significant negative relationship between the pH value and the content of organic C in the soil (Table 2, analysis 40). From the whole data set, we did not identify any effect of N and P application on organic C in the soil (Table 2, analyses 2 and 11).

The content of total N in the soil ranged from 2.5 in plot number 192/Ca to 5.0 g kg^{-1} in plot number 2112 (Table 1). The amount of applied N, either organic or chemical-synthetic, had no effect on the content of total N in the soil (Table 2, analysis 1).

The C:N ratio of soil organic matter ranged from 6.9 in plot number 31/2 ($N_{116}P_{26}K_{232}$) with a combination of mineral P and dung water application, to 10.4 in the control plot K2 ($N_0P_0K_0$). The plant-available P concentration in the soil ranged from 14 mg kg^{-1} in limed plot number 52/Ca ($N_{120}K_{87}$) to 161 mg kg^{-1} in limed plot number 162/Ca ($P_{44}K_{174}$). There was a strong and significant positive effect of P application on the P concentration in the soil (Table 2, analysis 10).

The plant-available K concentration in the soil ranged from 82 mg kg^{-1} in limed plot number 122/Ca ($N_{120}P_{44}$) to 1018 mg kg^{-1} in plot number 21/1 ($N_{232}P_8K_{465}$) with dung water application. There was a strong and significant positive effect of K application on K concentration in the soil (Table 2, analysis 19).

The plant-available Ca concentration in the soil ranged from 532 mg kg^{-1} in plot number 61/2 ($N_{120}K_{174}$) to 3336 mg kg^{-1} in plot number 121/1 ($N_{120}P_{44}$).

3.2. Compressed sward height

The CSH ranged from 11 cm in the control plots ($N_0K_0P_0$) and in plot number 141/1 (K_{174}) to 47 cm in limed plots number 2222 and 2221 ($N_{160}P_{44}K_{174}$) with a combination of all synthetic chemical N fertilizers (Appendix B). The CSH was significantly and positively affected by N, P, and K application as well as by plant-available concentrations of P, K, and Ca in the soil (Table 2, analyses 3, 12, 21 and 13, 23, 33). In contrast, there was no effect of Ca application by liming in 1986 or of total N content in the soil on CSH (Table 2, analyses 32 and 4).

Table 2
Results of linear regression analyses of nutrient application on the content of nutrient and pH in the upper 10 cm soil layer and on compressed sward height.

Analysis	Ind. var.	Dep. var.	Equation	*R*	*P* value
1	N	SNtot	$y = 3232 - 0.3248x$	−0.04	0.750
2	N	SC_{org}	$y = 2.66 + 0.0003x$	0.03	0.811
3	N	CSH	$y = 19.0 + 0.0797x$	0.49	<0.001
4	SNtot	CSH	$y = 37.7 - 0.0033x$	−0.16	0.193
5	SNtot	TG	$y = 63.9 - 0.006x$	−0.11	0.365
6	SNtot	SG	$y = -14.2 + 0.0136x$	0.36	0.003
7	SNtot	L	$y = 25.2 - 0.0036x$	−0.12	0.354
8	SNtot	TF	$y = 9.8 + 0.0042x$	0.13	0.295
9	SNtot	SF	$y = 28.9 - 0.0014x$	−0.05	0.706
10	P	SP	$y = 19.0 + 0.7201x$	0.76	<0.001
11	P	SC_{org}	$y = 2.80 - 0.0017x$	−0.14	0.222
12	P	CSH	$y = 15.9 + 0.1716x$	0.79	<0.001
13	SP	CSH	$y = 20.0 + 0.1147x$	0.55	<0.001
14	SP	TG	$y = 29.9 + 0.2442x$	0.45	<0.001
15	SP	SG	$y = 39.5 - 0.1655x$	−0.43	<0.001
16	SP	L	$y = 15.4 - 0.0305x$	−0.09	0.447
17	SP	TF	$y = 29.9 + 0.2442x$	−0.53	<0.001
18	SP	SF	$y = 39.5 - 0.1655x$	−0.00	0.999
19	K	SK	$y = 146.5 + 0.8713x$	0.39	<0.001
20	K	SC_{org}	$y = 2.9 - 0.0016x$	−0.27	0.020
21	K	CSH	$y = 19.9 + 0.0502x$	0.45	<0.001
22	K	SR	$y = 29.2 - 0.0081x$	−0.22	0.056
23	SK	CSH	$y = 43.5 + 0.0051x$	0.11	0.377
24	SK	TG	$y = 33.3 - 0.015x$	0.04	0.723
25	SK	SG	$y = 8.3 + 0.202x$	−0.18	0.147
26	SK	L	$y = 15.4 - 0.0305x$	0.29	0.017
27	SK	TF	$y = 22.7 + 0.0021x$	0.03	0.807
28	SK	SF	$y = 29.5 - 0.0197x$	−0.30	0.012
29	Ca	SCa	$y = 1670 + 363x$	0.23	0.040
30	Ca	SC_{org}	$y = 2.8 - 0.2018x$	−0.18	0.127
31	Ca	pH	$y = 5.72 + 0.4709x$	0.35	0.002
32	Ca	CSH	$y = 23.4 + 4.4116x$	0.19	0.097
33	SCa	CSH	$y = 20.5 + 0.0036x$	0.27	0.026
34	SCa	TG	$y = 30.4 + 0.0077x$	0.22	0.072
35	SCa	SG	$y = 35.6 - 0.0033x$	−0.13	0.289
36	SCa	L	$y = 27.5 - 0.0075x$	−0.36	0.003
37	SCa	TF	$y = 37.0 - 0.0074x$	−0.35	0.004
38	SCa	SF	$y = 7.27 + 0.0092x$	0.47	<0.001
39	SCa	SR	$y = 31.2 - 0.0017x$	−0.25	0.029
40	pH	SC_{org}	$y = 5.52 - 0.4808x$	−0.57	<0.001
41	TG	SR	$y = 31.3 - 30.07x$	−0.39	<0.001

Ind. var.–independent variable; Dep. var.–dependent variable; Equation–regression equation; *r*–coefficient of determination; *P* value–corresponding probability value; N, P, and K–applied nutrients; Ca–amount of Ca applied by liming in 1986; SNtot–total nitrogen content in the soil; SP, SK, and SCa–plant-available concentrations of P, K and Ca in the soil; CSH–compressed sward height; SC_{org}–content of organic carbon in the soil; TG–tall graminoids; SG–short graminoids; L–legumes; TF–tall forbs; SF–short forbs; SR–species richness.

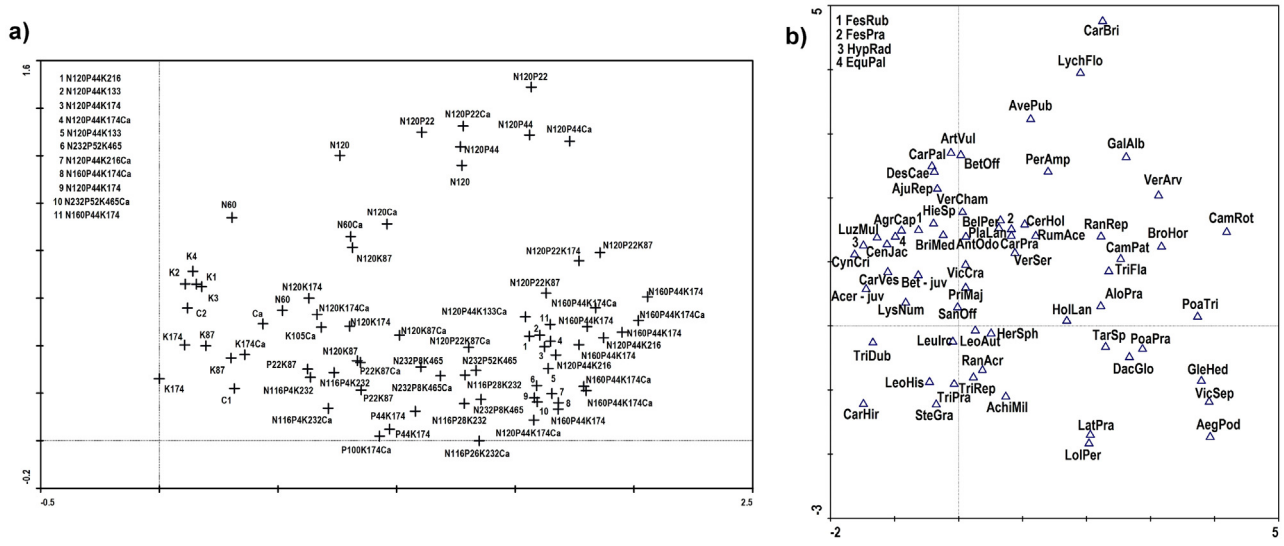

Fig. 2. Ordination diagrams showing the results of DCA analysis of plant species composition data: (a) the scatter plot of individual experimental plots and (b) the scatter plot of plant species. Letters and numbers in plot names indicate the amount of applied N, P, and K in kg ha^{-1} according to Appendix 1. Ca indicates lime application in 1986. Abbreviations of control plots without any N, P and K application are the same as in Fig. 1: Ca–control limed in 1986, K1–K4, C1 and C2. Plant species abbreviations: Acer–juv–*Acer* seedlings, AchiMil – *A. millefolium*, AegPod–*A. podagraria*, AgrCap–*A. capillaris*, AjuRep–*A. reptans*, AloPra–*A. pratensis*, AntOdo–*A. odoratum*, AvePub–*A. pubescent*, BelPer–*B. perennis*, BetOff–*B. officinalis*, Bet–juv–*Betula* seedlings, BroHor–*B. hordaceus*, CamPat–*C. patula*, CarBri–*C. brizoides*, CarHir–*C. hirta*, CarPal–*C. pallescens*, CarVes–*C. vesicaria*, CarPra–*C. pratensis*, CerHol–*C. holosteoides*, CynCri–*Cynosurus cristatus*, DacGlo–*D. glomerata*, DesCae–*D. caespitosa*, EquPal–*E. palustre*, FesPra–*F. pratensis*, FesRub–*F. rubra*, GalAlb–*G. album*, GleHed–*G. hederacea*, HerSph–*H. sphondylium*, HolLan–*Holcus lanatus*, HypRad–*H. radicata*, LatPra–*L. pratensis*, LeoHis–*L. hispidus*, LeuIrc–*L. ircutianum*, LolPer–*L. perenne*, LuzMul–*L. multiflora*, LychFlo–*L. flos-cuculi*, LysNum–*L. nummularia*, PerAmp–*P. amphibia*, PimMaj–*P. major*, PlaLan–*P. lanceolata*, PoaPra–*Poa pratensis*, PoaTri–*P. trivialis*, RanAcr–*R. acris*, RanRep–*R. repens*, RumAce–*R. acetosa*, SanOff–*Sanguisorba officinalis*, SteGra–*S. graminea*, TarSp–*T.* sp., TriDub–*Trifolium dubium*, TriPra–*Trifolium pretense*, TriRep–*T. repens*, TriFla–*T. flavescens*, UrtDio–*U. dioica*, VerArv–*V. arvensis*, VerCham–*V. chamaedrys*.

3.3. Plant species composition

Across all plots, 68 vascular plant species were recorded, i.e. 21 graminoids, 6 legumes, 38 other dicotyledonous and 3 seedlings of woody species (Appendix B). Calculated by DCA, the first axis and the first four canonical axes explained 29 and 50% of the variability in the plant species composition data, respectively. The length of the environmental gradient in DCA was 2.0. In the ordination diagram derived from this analysis, the plots without any N application together with their characteristic species grouped on the left side, whereas plots receiving high rates of N with their characteristic species were located together on the right side of the diagram (Fig. 2).

Calculated by RDA, the amount of applied nutrients, soil chemical properties, and CSH together explained 49.5% of the variability in the plant species composition data (Table 3, analysis 1). This analysis is visualised in an ordination diagram in Fig. 3, which displays the response of individual species to nutrient application, soil chemical properties and CSH.

The amount of applied N, P, K, and Ca together explained 43% of the variability in the plant species composition data (Table 3, analysis 2). The highest amount of variability from applied nutrients was explained by N (Table 3, analysis 3), followed by P (Table 3, analysis 4), and K (Table 3, analysis 5). Therefore, long-term N application most effectively diversified plant species composition in the SGE, either applied alone or in combination with other nutrients. The amount of applied Ca by direct liming in 1986 alone explained only a non-significant part of the variability in plant species composition (Table 3, analysis 6).

All soil chemical properties together explained 28.2% of the variability in plant species composition data (Table 3, analysis 7) and this is much less than the amount of fertilizer applied. The

Table 3
Results of RDA analyses of plant species composition data.

Analysis	Expl. var.	Covariables	% ax 1 (all)	F 1 (all)	P1 (all)
1	N, P, K, Ca, SNtot, SP, SK, SCa, CSH	–	33.5% (49.5%)	32.8 (8.1)	0.001 (0.001)
2	N, P, K, Ca	–	31.0% (43.0%)	32.0 (14.0)	0.001 (0.001)
3	N	P, K, Ca	19.6%	17.0	0.001
4	P	N, K, Ca	13.4%	10.8	0.001
5	K	N, P, Ca	12.5%	10.0	0.001
6	Ca	N, P, K	2.1%	1.5	0.091
7	SNtot, SP, SK, SCa	–	19.4% (28.2%)	16.8 (6.8)	0.001 (0.001)
8	SNtot	SP, SK, SCa	3.0%	2.1	0.023
9	SP	SNtot, SK, SCa	10.0%	7.8	0.001
10	SK	SNtot, SP, SCa	2.8%	2.0	0.035
11	SCa	SNtot, SP, SK	7.8%	5.90	0.001
12	CSH	–	26.7%	26.6	0.001
13	CSH	N, P, K, Ca	6.7%	4.9	0.001
14	CSH	SNtot, SP, SK, SCa	17.6%	17.7	0.001

Expl. var.—explanatory (environmental in Canoco terminology) variables; % ax 1 (all)—species variability explained by canonical axis 1 or by all constrained axes (measure of explanatory power of the environmental variables); F1 (all)–statistics for the test of particular analyses; P 1 (all)—corresponding probability value obtained by the Monte Carlo permutation test; N, P,K, and Ca—amount of applied nutrient; SNtot—total nitrogen content in the soil; SP, SK, and SCa–plant-available concentrations of P, K and Ca in the soil; CSH—compressed sward height.

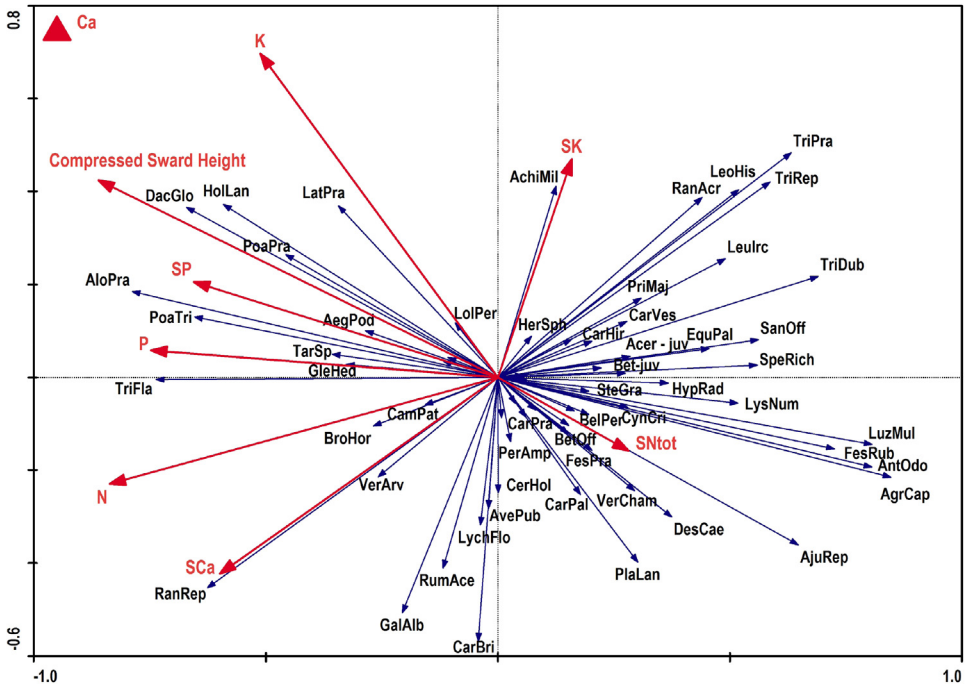

Fig. 3. Ordination diagram showing the result of RDA analysis of plant species composition data in relation to the amount of applied nutrients, soil chemical properties, and compressed sward height according to analysis a1 in Table 3. Environmental variables: N, P, K, and Ca—applied N, P, K, and Ca by fertilizers; SNtot—total nitrogen content in the soil; SP, SK, SCa—plant available concentrations of P, K and Ca in the soil. Abbreviations of plant species are given in Fig. 2.

highest amount of variability from soil chemical properties was explained by plant-available P (Table 3, analysis 9), followed by plant-available Ca (Table 3, analysis 11), total N (Table 3, analysis 8), and plant-available K in the soil (Table 3, analysis 10). Here, plant-available P rather than total N in the soil had the strongest impact on the explained variability in the data set.

Compressed sward height alone explained 26.7% of the variability in plant species composition data (Table 3, analysis 12). The explained variability by CSH strongly decreased after removal of the effect of applied nutrients (Table 3, analysis 13) and soil chemical properties (Table 3, analysis 14) from the analysis, thus indicating a relatively high effect of applied nutrients and soil chemical properties on sward height.

Calculated by RDA, soil chemical properties, the amount of applied N, P, K, and Ca, and CSH together explained 50% of the variability in plant functional group data ($F = 7.4$, $P = 0.001$, results are visualized in Fig. 4). A high cover of tall graminoids was associated with high values of CSH and high application rates of N, P, and K and high plant-available concentrations of P and K in the soil. Alternatively, a high cover of tall graminoids was connected with a low cover of legumes, short and tall forbs, short graminoids, and with a low plant species richness.

Calculated by linear regression, there was a positive significant effect of total soil N content on the cover of short graminoids, but there was no significant effect on the cover of tall graminoids, legumes, tall forbs, and short forbs (Table 2, analyses 5–9). Furthermore, we found a significant positive relationship between the concentration of plant-available P in the soil and the cover of tall graminoids (Table 2, analysis 14), but a negative correlation between P and short graminoids or tall forbs (Table 2, analyses 15 and 17). A significant effect of soil P on legumes and short forbs was not observed (Table 2, analyses 16 and 18). The response of functional groups on soil K was positive for legumes and short forbs and no effect of K was recorded on tall graminoids, short graminoids, and tall forbs (Table 2, analyses 24–28). The concentration of plant-available Ca caused a negative significant effect on legumes and tall

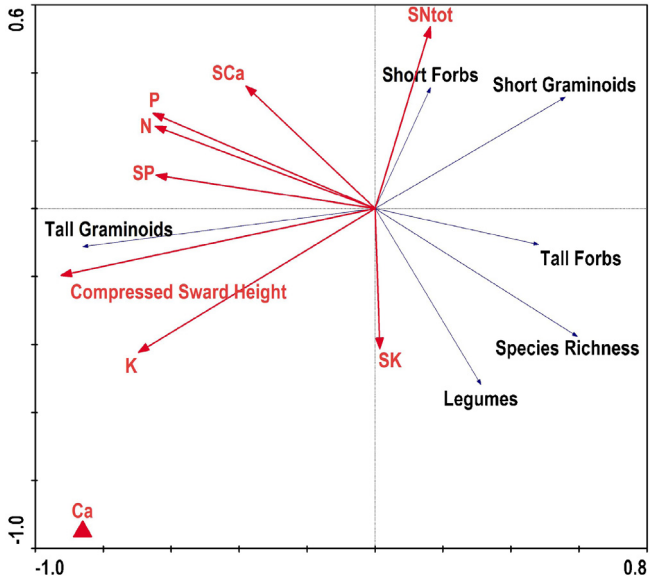

Fig. 4. Ordination diagram showing the result of RDA analysis of functional group data and species richness in relation to the amount of applied nutrients, soil chemical properties, and compressed sward height. Environmental variables: N, P, K, and Ca—applied N, P, K, and Ca by fertilizers; SNtot—total nitrogen content in the soil; SP, SK, SCa—plant-available concentrations of P, K and Ca in the soil.

forbs, a positive effect on short forbs, but no significant effect on tall and short graminoids (Table 2, analyses 34–38).

With respect to plant species composition, a highly positive response to combined N, P, and K application was found, with an increasing cover of especially tall grasses, namely *Holcus lanatus*, *A. pratensis*, and *Dactylis glomerata*. In contrast, the short grass *Anthoxanthum odoratum* and the tall grass *Festua rubra* responded negatively to N, P, and K application. We also observed that N application decreased the cover of legumes. Only the legumes

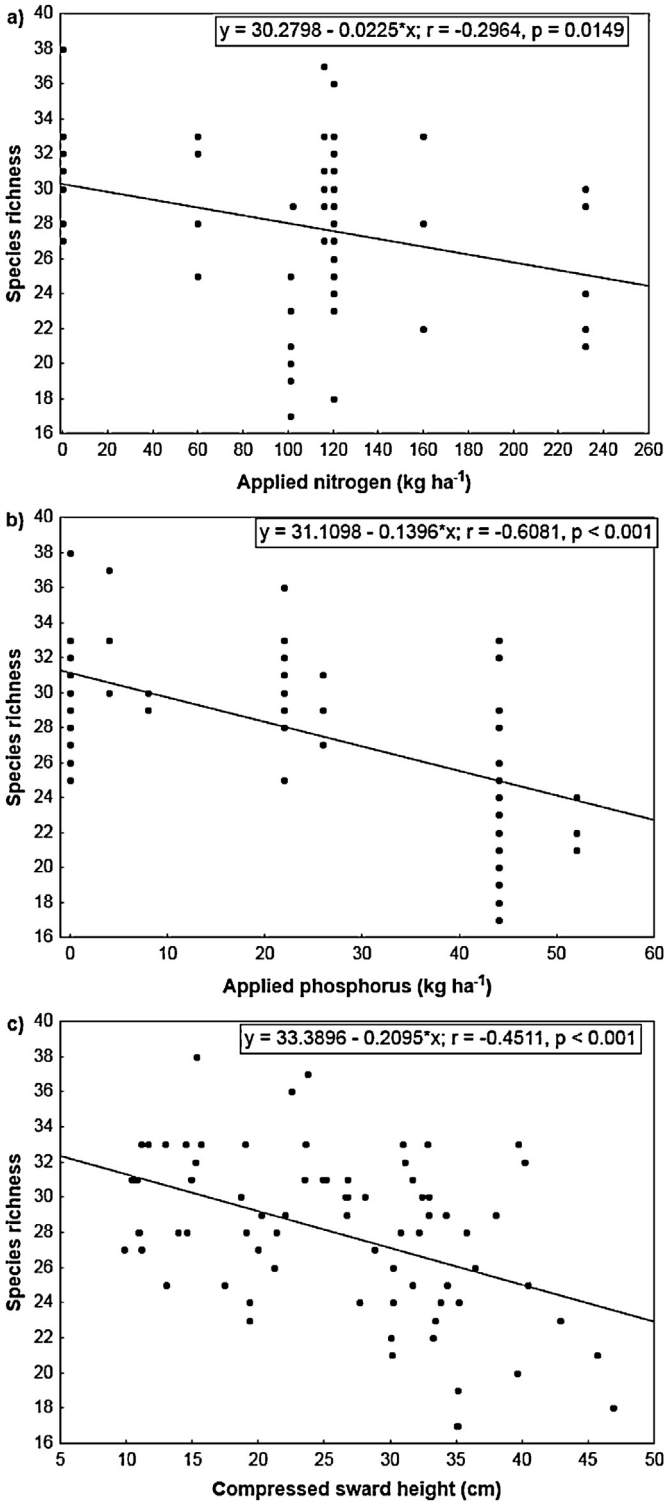

Fig. 5. Effect of (a) nitrogen and (b) phosphorus application and (c) compressed sward height on species richness (per 18.75 m^2 area) of vascular plants.

Lathyrus pratensis, *Trifolium pratensis*, and *Trifolium dubium* were able to survive moderate application of N. The highest cover of the herbs *Leontodon hispidus*, *Lysimachia nummularia*, *Plantago lanceolata*, *Ranunculus acris*, and *Sanguisorba officinalis* was recorded in non-fertilized control plots.

The species richness of vascular plants ranged from 17 to 38 species per plot (18.75 m^2) and its overall relation to applied nutrients, soil chemical properties and cover of individual species and functional groups is visible in the RDA ordination diagrams (Fig. 3 and Fig. 4). As depicted in Fig. 5, species richness was negatively affected by N and P application and by CSH. In addition, species richness decreased with the Ca concentration in the soil and with the cover of tall graminoids (Table 2, analyses 39 and 41). The total soil N content, soil pH and plant-available K in the soil did not affect species richness (data not shown).

4. Discussion

4.1. Soil chemical properties

The application of different fertilizers resulted in a wide gradient of soil pH that ranged from moderately acid to neutral. High soil acidification was already recorded in treatments with a long-term application of $(NH_4)_2SO_4$ (Silvertown et al., 2006; Clark et al., 2007; Hopkins et al., 2011). Soil acidification can substantially change the soil chemical properties and availability of many elements. Furthermore, the accumulation of soil organic matter in plots with the most acid soils has been recorded previously, indicating a negative effect of soil acidity on the mineralization of soil organic matter and on the release of mineral N. The accumulation of soil organic matter under low soil pH was also recorded in other long-term fertilizer experiments (Diepolder and Schröpel, 2003; Silvertown et al., 2006; Hejcman et al., 2010; Hopkins et al., 2011). In contrast, neutral soils in the SGE plots with Ca application and with the lowest content of organic matter indicate a positive effect of Ca application on soil microbial activity and the associated rate of N and C cycling. This conclusion is also supported by the fact that the C:N ratio in soil organic matter was lowest in neutral soils and highest in acid soils.

Although N fertilizers were applied for several decades, we did not observe any effect of N application on the total soil N content. Instead, the total N content was affected by soil pH rather than by N application. The fact that there was no effect of N application on the total soil N content supports results from other long-term grassland experiments (Silvertown et al., 2006; Hejcman et al., 2010; Hopkins et al., 2011). Probably, increased N uptake by harvested biomass was sufficient to remove the N applied by fertilizers.

In the SGE, the content of plant-available P and K in the soil was positively affected by P and K fertilizer application, as in other long-term experiments (Hejcman et al., 2009). We also found a strong relationship between plant-available P and K concentrations and N application, since these were highest in plots with P and K but without N application. This can be explained by a negative effect of N deficiency on plant growth and therefore, on herbage yield and a reduced uptake, leading to a higher accumulation of applied P and K in the soil (Hrevušová et al., 2009; Hejcman et al., 2010; Pavlů et al., 2011a). In addition, we measured a high concentration of plant-available P in all plots with an annual application of 44 kg P ha^{-1}, because P application probably exceeded P uptake by harvested biomass and thus P accumulated in the soil.

The same is true for plant-available Ca in the soil, which substantially increased with lime application and partly because of Thomas slag application (P fertilizer) because of the high amount of applied Ca exceeded the amount of Ca removed by harvested biomass and leaching.

4.2. Compressed sward height

It is well known that the height of a sward is highly correlated with the standing aboveground biomass and is thus a predictor of herbage yield (O'Donovan et al., 2002; Hakl et al., 2012; Pavlů et al., 2013). Therefore, an increase in sward height with N application even without any P and K application, indicates N limitation of biomass production at the experimental site. Nitrogen limitation

of biomass production in alluvial grasslands agrees with results of other authors from different regions in Europe (Beltman et al., 2007; Hrevušová et al., 2009; Loeb et al., 2009).

4.3. Plant species composition

Although N, P and K fertilizers have been applied in the SGE for several decades, there were relatively small differences in plant species composition across the full range of fertilizer treatment, compared to long-term experiments established on naturally low productive soils (Silvertown et al., 2006; Hejcman et al., 2007; Semelová et al., 2008). One possible explanation is that the plant species composition of the alluvial grassland has adapted to relatively high nutrient concentrations even without any fertilizer input. A high tolerance of plant species composition to nutrient input is a typical feature of alluvial grasslands, because a high amount of nutrients can be deposited during floods. Furthermore, high rates of N, P and K might be released through mineralization of soil organic matter or clay minerals (Beltman et al., 2007; Klaus et al., 2011b). A clear indication of nutrient deposit and mineralization in the SGE is given by the fact that the majority of species, including those with high nutrient requirements such as *A. pratensis*, were recorded even in the control plots of the SGE, whereas in other long-term experiments on low productive soils, unfertilized control and fertilized plots rarely shared the same species.

Apart from small differences in floristic composition, we found some fertilizer effects, as depicted in Fig. 3, i.e., a strong positive effect of N application on the cover of tall graminoids, but a negative effect on species richness as well as on the cover of legumes, short graminoids and forbs. This is consistent with results from several other long-term experiments on alluvial grasslands and indicates high N requirements of tall grasses and therefore their N limitation without any N application (Honsová et al., 2007; Rothamsted Research, 2006; Vargová et al., 2012). In addition, tall graminoids were partly limited in their expansion by obviously insufficient P supply in plots with N and K application only. The concentration of plant-available P in the soil was lowest from all plots and the cover of tall graminoids was not as high as in plots with simultaneous N, P, and K application. The lowest cover of tall graminoids in plots with K application was caused by a strong N and partly P limitation. In treatments that received K only, the concentration of plant-available N was probably below the tolerance limit for *A. pratensis*, *D. glomerata*, *P. pratensis*, and *P. trivialis.* It is well documented that these grasses generally require high N supply rates throughout the growing season (Prach, 2008; Čop et al., 2009; Liu et al., 2010; Klaus et al., 2011b; Pavlů et al., 2011b; Hejcman et al., 2012).

In contrast to other tall graminoids, *H. lanatus* responded positively to the application of $(NH_4)_2SO_4$. This is generally due to its relatively high N requirements connected with its tolerance to acid soils and aluminium toxicity (Kidd and Proctor, 2001; Rothamsted Research, 2006). In addition, this grass species has been dominant in plots with dung water application, where applied N is mainly in the form of NH_4^+. A high tolerance of *H. lanatus* to high soil acidity can be thus also connected to N nutrition, i.e. a preference for NH_4^+ rather than for NO_3^-. In contrast, *Poa trivialis* negatively responded to the application of $(NH_4)_2SO_4$, probably because of its lower tolerance to acid soil reaction. *A. pratensis* responded highly positively to N application under a wide range of soil pH, indicating its high N requirements, ability to take up NH_4^+ and NO_3^- and tolerance to acid soils. *Festuca rubra* was the only tall grass that exhibited a negative response to N application and its highest cover was recorded in the control. The low N requirements of *F. rubra* in comparison to other tall forage grasses were also recorded by other authors (Berendse et al., 1992; Gastal et al., 2010; Hejcman et al., 2012; Pavlů et al., 2012).

As well as the effects of N, P, and K application in the SGE, the dominance of some species might also have been influenced by the permanent three-cut system in this experiment. For instance, *A. pratensis* obviously benefits from a low cutting frequency and suffers from frequent cutting, in contrast to *T. repens* (Pavlů et al., 2011a, 2012).

The highest cover of the short graminoids, especially *A. odoratum* and *A. capillaris*, in plots with N and K application but without any P application, was caused by lower P requirements than that of tall grasses (Merunková and Chytrý, 2012; Pavlů et al., 2012). Because of insufficient P supply, rapidly growing tall grasses were probably not able to suppress short grasses with lower P requirements under N and K application. *Trisetum flavescens* positively responded to the application of $(NH_4)_2SO_4$, probably because of its tolerance to acid soil reaction (Pavlů et al., 2011b).

The low cover of legumes in plots with N application agrees with results from other long-term experiments (Velich, 1986; Pavlů et al., 2011a, 2012). The increased competitive ability of tall graminoids suppressed legumes under high N supply. Furthermore, a higher sensitivity of legumes to an increased mineral N concentration in the soil is known to reduce N_2 fixation and the competitivity of legumes (Neuberg et al., 2011). Only some legumes such as *L. pratensis*, *Trifolium repens, T. pratensis* and *T. dubium* survived moderate applications of N similar to in other long-term experiments (Hejcman et al., 2007; Honsová et al., 2007; Pavlů et al., 2011a).

Herbs, predominately short ones, possess a lower competitive ability under high application rates of N, P, and K fertilizers, mainly because of shading by tall grasses. The only forbs that exhibited a high competitive ability under N, P, and K application were *Glechoma hederacea* and *Achillea millefolium* (Honsová et al., 2007). *G. hederacea* is well known as a shade-tolerant species that can survive in dense canopies of tall vegetation (Daßler et al., 2008). *Ranunculus repens* positively responded to the application of $(NH_4)_2SO_4$, probably due to its high N requirements connected with a tolerance to an acid soil reaction.

Ajuga reptans was most commonly found in the control as well as in K, and NK treatments and *Leonthodon hispidus*, in plots with K application, whereas *L. nummularia* and *Veronica chamaedris* preferred plots without any fertilizer input. All these short species were probably suppressed by shading of tall graminoids in plots with N, P, and K application. Notably, *P. lanceolata* was the most common species in all plots, irrespective of fertilizer input. A great tolerance of this species to a wider range of nutrient availability in grassland soils has also been found in the Rengen grassland experiment (Hejcman et al., 2013).

4.4. Species richness of vascular plants

Species richness was negatively affected the most by simultaneous N, P, and K application, similar to in other experiments (Crawley et al., 2005; Honsová et al., 2007; Hejcman et al., 2010; Pierik et al., 2011; Wesche et al., 2012; Dufková and Libichová, 2013). Under non-limiting N, P, and K supply, tall swards developed in the SGE, with a dominance of several tall graminoids. This partly explains the negative effect of sward height on species richness also recorded in other experiments (Clark et al., 2007; Gough et al., 2012). Most importantly, there was almost no negative effect of N application in the form of NH_4NO_3 on species richness, since almost the same number of species was recorded in the control (29 species) as in plots with only N application (27 species under 60 and 120 kg N ha^{-1}). In contrast, we found a negative effect of $(NH_4)_2SO_4$ application on species richness, similar to observations by Crawley et al. (2005). In plots which received only N in the form of $(NH_4)_2SO_4$, the number of species per plot was only 18. We suggest that this effect on species richness was predominately due to soil acidification. In Europe, acid soils are generally

associated with a low species richness, because the majority of vascular plants in the regional species pool are adapted to neutral and alkaline soils. This is mainly due to evolutionary processes within the current flora that occurred on predominantly alkaline soils during the Pleistocene (Ewald, 2003; Merunková and Chytrý, 2012). This result indicates that not only the amount of applied N is important for species richness, but also the form of applied N and whether N is applied together with other limiting nutrients, in particular, P and K. A low plant species richness in acid soil conditions also directly explains the results of observational studies showing that a high atmospheric deposition of N compounds markedly decreases the species richness of European grasslands (Stevens et al., 2011). This decrease is evidently caused not only by indirect effects of N application via an increase in biomass production, competition for light and therefore by exclusion of less competitive species, but also directly via soil acidification, which creates a pH that lies outside the tolerance range of many species.

5. Conclusions

(1) Over decades of different fertilizer application, a steep gradient of soil chemical properties developed, namely in N, P, K, and Ca availability, the content of soil organic matter and pH.
(2) Soil chemical properties were responsible for the development of a steep gradient of aboveground biomass production expressed by sward height.
(3) Although long-term fertilizer application induced significant diversification of the plant species composition in the SGE on the alluvial meadow, this effect was not as high as that resulting from fertilizer application on naturally low productive soils of other long-term experiments discussed here. Clearly, a high nutrient availability enabled the survival of species that were well adapted to these conditions, even in the unfertilized control. We therefore conclude that the plant species composition in alluvial grasslands responds substantially less dramatically to N, P, and K application than in low productive grasslands. Alluvial grasslands dominated by *A. pratensis* are thus substantially less affected by fertilizer application than other low productive grasslands.
(4) Long-term fertilizer application negatively affected plant species richness directly by soil acidification ($(NH_4)_2SO_4$ particularly) and indirectly by an increase in aboveground biomass production, thus increasing competition for light, which supports the exclusion of low competitive species. The magnitude of the negative effects of N application on species richness was dependent on (i) whether N was applied alone or in combination with other nutrients, particularly P, K, and Ca, and (ii) whether the N application acidified the soil.

Acknowledgments

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Appendix A.

List of plots and fertilizer regimes applied since the start of the experiment in 1933. Numbers indicate the amount of annually applied N, P and K in kg ha^{-1}. The abbreviation "Ca" in plot numbers indicates lime application in 1986. Bold frames indicate plots exposed to the same fertilizer treatment. Since 1933, original groups of plots I to VI have been divided into subgroups and therefore, the amounts of either N, P, and K applied have been increased in most plots. Plots C_1, C_2 and C_{Ca} and K_1–K_4 have never received any fertilizer input.

Group	Plot number	Period 1	Period 2	Period 3	Period 4	Period 5
		1933–1953 (21 years)	1954–1964 (11 years)	1965–1970 (6 years)	1971–1986 (17 years)	1987–2008 (22 years)
I Dung water	11/1, 11/2 & 12/Ca	N116P4K232	N116P4K232	N116P4K232	N116P4K232	N116P4K232[1]
	21/1, 21/2 & 22/Ca	N116P4K232	N116P4K232	N232P8K465	N232P8K465	N232P8K465[1]
	31/1, 31/2 & 32/Ca	N116P4K232	N116P4K232	N116P26K232	N116P26K232	N116P26K232[1]
	41/1, 41/2 & 42/Ca	N116P4K232	N116P4K232	N232P52K465	N232P52K465	N232P52K465[1]
II	51/1, 51/2 & 52/Ca	N40K87	N40K87	N40K87	N120K87	N120K87[1]
N-	61/1, 61/2 & 62/Ca	N40K87	N40K87	N80K100	N120K174	N120K174[1]
K	71/1, 71/2 & 72/Ca	N40K87	N60P40K133	N80P44K133	N120P44K133	N120P44K133[1]
	81/1, 81/2 & 82/Ca	N40K87	N60P40K133	N80P44K216	N120P44K216	N120P44K216[1]
III	91/1, 91/2 & 92/Ca	N40	N40	N40	N60	N60[1]
N	101/1, 101/2 & 102/Ca	N40	N40	N80	N120	N120[1]
	111/1, 111/2 & 112/Ca	N40	N40P22	N40P22	N120P22	N120P22[1]
III	121/1, 121/2 & 122/Ca	N40	N40P22	N80P44	N120P44	N120P44[1]
IV	131/1, 131/2 & 132/Ca	P22K87	K87	K87	K87	K87[1]
	141/1, 141/2 & 142/Ca	P22K87	K87	K174	K174	K174
	151/1, 151/2 & 152/Ca	P22K87	P22K87	P22K87	P22K87	P22K87[1]
	161/1, 161/2 & 162/Ca	P22K87	P22K87	P44K174	P44K174	P44K174[1]
V	171/1, 171/2 & 172/Ca	N40P22K87	N40P22K87	N40P22K87	120N22P87 K	120N22P87K[1]
	181/1, 181/2 & 182/Ca	N40P22K87	N40P22K87	N80P44K174	120N44P174 K	120N44P174K[1]
V	191/1, 191/2 & 192/Ca	N40P22K87	N40P40K133	N120P22K174	N120P22K174	N120P22K174[1]
	201/1, 201/2 & 202/Ca	N40P22K87	N40P40K133	N120P44K174	N160P44K174	N160K44P 174[1]
VI	2111, 2112, 2121/Ca, 2122/Ca	–	–	–	–	N160P44K174[2]
	2211, 2212, 2221/Ca, 2222/Ca	–	–	–	–	N160P44K174[3]

[1] Application of NH_4NO_3.
[2] Application of NH_4SO_4.
[3] Combination of $CaNO_3$/NH_4SO_4/NH_4NO_3.

Appendix B.

Cover (in %) of bryophytes, functional groups of vascular species and vascular species, compressed sward height (cm), and species richness (per 9 m^2 in C, 2112, and 2222 plots and per 14.6 m^2 in all other plots) measured in May 2008. Species with a cover below 0.1% are not presented. C–mean values from all control plots without any fertilizer input. Many species were recorded only in several control plots, therefore, the mean species richness per control plot was 29.3 and the number of species recorded over all control plots was 64. The abbreviation "Ca" in plot numbers indicates lime application in 1986. Plots with an application of mineral P were indirectly limed by Thomas slag.

Species	Experimental plot									
	C	141/1	162/Ca	31/2	52/Ca	61/2	192/Ca	21/1	2112	2222/Ca
N (kg ha^{-1})	0	0		116	100	120	120	232	160	160
P (kg ha^{-1})	0	0	44	26			22	8	44	44
K (kg ha^{-1})	0	174	174	232	87	174	174	485	174	174
Bryophytes	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Compressed sward height	11.3	9.8	26.7	31.7	24.9	19	26.8	32.4	35.1	46.9
Species richness	29.3	27	29	31	31	33	31	30	19	18
Tall graminoids	15.6	5.2	49	64.2	37.2	17.2	55	75.2	96.1	52
Alopecurus pratensis	1.6	–	10	8	20	8	20	10	30	30
Avenula pubescent	0.1	–	–	0.1	–	–	–	–	–	–
Carex vesicaria	0.1	–	–	–	–	–	–	–	–	–
Deschampsia caespitosa	0.1	0.1	–	–	–	–	–	–	–	–
Dactylis glomerata	0.1	–	5	5	5	0.1	20	3	5	5
Festuca pratensis	0.8	0.1	6	0.1	4	0.1	3	12	1	–
F. rubra	10.2	3	–	–	0.1	3	–	–	–	–
Holcus lanatus	2.2	2	25	50	8	6	10	50	60	5
Poa pratensis	0.1	–	2	–	–	–	1	0.1	0.1	–
P. trivialis	0.3	–	1	1	0.1	–	1	0.1	–	12
Short Graminoids	55.8	31.2	5.2	25.2	12	64	24	20.2	18.2	40.1
Agrostis capillaris	22.5	6	–	–	2	15	–	–	–	–
Anthoxanthum odoratum	13.7	20	5	10	8	45	3	8	0.1	0.1
Briza media	0.1	–	–	–	–	–	–	–	–	–
Bromus hordaceus	0.1	–	0.1	0.1	–	–	1	0.1	0.1	–
Carex hirta	0.1	–	–	–	–	–	–	–	–	–
Carex pallescens	0.1	–	–	–	–	1	–	–	–	–
Carex brizoides	9	0.1	–	–	–	–	–	–	–	–
Cynosurus cristatus	0.6	–	–	–	–	–	–	–	–	–
Lolium perenne	0.1	–	0.1	0.1	–	–	–	0.1	–	–
Luzula multiflora	9.2	5	–	–	–	1	–	–	–	–
Trisetum flavescens	0.3	0.1	–	15	2	2	20	12	18	40
Legumes	28.5	31	35	13.2	7	1.2	2.2	11	0	2.2
Trifolium repens	12.2	10	20	0.1	4	0.1	0.1	8	–	0.1
T. pretense	4.7	1	12	3	2	1	0.1	2	–	0.1
T. dubium	10.3	20	2	0.1	–	–	–	–	–	–
Lathyrus pratensis	1.1	–	1	10	1	0.1	2	1	–	2
Vicia cracca	0.1	–	–	–	–	–	–	-	–	–
Vicia sepium	0.1	–	–	–	–	–	–	–	–	–
Tall Forbs	20.3	16.2	23.1	18	42.3	58.2	15	30.2	3.5	4
Aegopodium podagraria	0.1	–	–	–	–	–	1	–	–	–
millefolium	1.1	6	4	2	4	40	1	15	3	–
Artemisia vulgaris	0.1	–	–	–	–	–	–	–	–	–
Betonica officinalis	0.1	–	–	–	–	–	–	–	–	–
Centaurea jacea	0.1	–	–	–	–	–	–	–	–	–
Leucanthemum ircutianum	1.6	10	8	–	0.1	5	5	0.1	0.1	–
Galium album	0.1	–	–	2	4	–	–	0.1	–	1
Lychnis flos-cuculi	0.1	–	0.1	–	–	–	–	–	–	–
Pimpinella major	0.1	–	–	–	0.1	0.1	–	–	–	–
Persicaria amphibia	0.1	–	1	–	–	–	–	–	0.1	–
Ranunculus acris	7.3	0.1	7	2	0.1	0.1	1	1	0.1	–
Rumex acetosa	2.5	0.1	2	5	4	5	7	8	0.1	3
Sanquisorba officinalis	6.8	–	1	7	30	8	–	5	0.1	–
Silaum silaus	0.1	–	–	–	–	–	–	1	–	–
Urtica dioica	0.1	–	–	–	–	–	–	–	–	–
Short Forbs	25.9	17.5	8.4	9.6	20.5	16.4	34.6	13.5	6.2	43.2
Ajuga reptans	4.4	5	–	0.1	0.1	5	–	–	–	–
Alchemilla vulgaris	0.1	–	–	–	–	–	–	–	–	–
Bellis perennis	0.1	0.1	0.1	0.1	0.1	–	–	0.1	–	–
Campanula patula	0.1	–	–	–	–	–	0.1	–	–	–
Campanula rotundifolia	0.1	–	–	–	–	–	–	–	–	–
Cardamine pratensis	0.4	–	–	–	–	–	0.1	–	–	–
Cerastium holosteoides	0.6	0.1	0.1	0.1	0.1	0.1	1	0.1	0.1	0.1
Equisetum palustre	0.1	–	–	–	–	–	–	–	–	–
Glechoma hederacea	0.1	–	–	0.1	–	–	1	–	–	–
Heracleum sphondylium	0.1	–	–	–	2	4	–	0.1	–	–
Hieracium sp.	0.1	–	–	–	–	1	–	–	–	–
Hypochaeris radicata	0.1	–	–	–	–	0.1	–	–	–	–

Appendix B (*Continued*)

Species	Experimental plot									
	C	141/1	162/Ca	31/2	52/Ca	61/2	192/Ca	21/1	2112	2222/Ca
Leontodon autumnalis	0.1	–	–	–	1	0.1	–	–	–	–
L. hispidus	2	12	4	0.1	3	–	0.1	2	–	–
Lysimachia nummularia	3.8	–	–	2	0.1	0.1	0.1	0.1	–	–
P. lanceolata	2.4	0.1	1	4	10	2	2	7	0.1	1
Ranunculus repens	3.5	0.1	3	2	4	2	25	4	5	40
Stellaria graminea	0.1	–	–	0.1	–	–	–	–	–	–
Taraxacum sp.	0.1	–	0.1	1	0.1	2	4	0.1	1	2
Veronica chamaedrys	7.4	0.1	0.1	–	–	–	1	–	–	–
Veronica serpyllifolia	0.1	–	–	–	–	–	0.1	–	–	–
Veronica arvensis	0.1	–	–	–	–	–	0.1	–	–	0.1

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5. 2 PAPER II.

Long-term agricultural management maximizing hay production can significantly reduce belowground C storage

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Long-term agricultural management maximizing hay production can significantly reduce belowground C storage

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ABSTRACT

Liming and fertilization of grasslands have been used for centuries to sustain hay production. Besides improving hay yields, these practices induce compositional shifts in plant and soil microbial communities, including symbiotic arbuscular mycorrhizal (AM) fungi. However, in spite of increasing interest in soil carbon (C) sequestration to offset anthropogenic CO_2 emissions, little is known about the long-term effects of these agronomic interventions on soil C stocks. We examined how plants, AM fungi, and soil C respond to more than seven decades of annual applications of lime, mineral nitrogen (N), and mineral phosphorus (P) to test the hypotheses that (1) management practices increasing aboveground plant production decrease C allocation to roots, AM fungi and the soil; and (2) the relative availability of N and P predicts belowground C allocation in a consistent manner. Our study was conducted at the Rengen Grassland Experiment, established in 1941. Lime combined with N increased hay yields and promoted development of AM fungal hyphae in soil, while reducing relative C allocation to roots. Simultaneous enrichment of soil with lime, N, and P further boosted hay production, promoted grasses and suppressed other plant functional groups. This treatment also decreased soil organic C and strongly suppressed AM fungi in the soil, although the response to P varied among different AM fungal taxa. Our results indicate that agricultural management practices aimed at maximization of hay production may, in the long run, significantly (−20%) reduce belowground C storage. This is a great concern with respect to the intended use of grasslands as anthropogenic CO_2 sinks because the fertilization-induced decrease in soil C stocks can partly or fully negate the C sequestration potential of the grassland ecosystems as a whole.
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1. Introduction

Grasslands cover about 40% of the terrestrial surface of the planet and about half of this area is currently used in agricultural production (Lal, 2007). Because soils contain more organic carbon (C) than both the atmosphere and biota combined and because grassland soils generally have much larger C storage capacity than annually cropped soils, grasslands are extremely important for global soil C sequestration (Conant et al., 2001; Lal, 2013). Long-term productivity of grasslands is influenced by many factors including climate, geographic location, and management practices, and particularly by the level of fertilization (Sala et al., 1988; Conant et al., 2001). For sustainable hay production, soil organic matter is critical because it supplies plants with nutrients, helps to reduce soil erosion, and increases cation exchange and water holding capacity (Schulten and Schnitzer, 1997; Daynes et al., 2013). Consequently, management practices that restore and/or maintain high levels of soil organic matter are desirable. This in combination with recent interest in soil C sequestration, as a means to offset rising CO_2 levels in the atmosphere, has led to a keen interest in optimizing grassland management for C storage

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(Conant and Paustian, 2002; Lal, 2008). An earlier review of 115 studies from around the world demonstrated that grasslands fertilization, especially with nitrogen (N), tends to be associated with increased soil C (Conant et al., 2001; van Groenigen et al., 2006), suggesting that fertilization is potentially an important driver of soil C sequestration. However, the relationship is difficult to predict because it may critically depend on the type of fertilizer, and is likely to be nonlinear (Wander et al., 1994). One reason for this is that belowground C allocation by the plants and subsequent C transformations within soils likely depend on plant and microbial responses to relative, not absolute nutrient limitations (Cleveland and Liptzin, 2007; Kaiser et al., 2014).

The soils and roots of plants in natural and managed grasslands are abundantly colonized by arbuscular mycorrhizal (AM) fungi. These fungi, belonging to the phylum Glomeromycota, form symbiotic relationships with plants that are often mutually beneficial for both partners. Plant hosts supply carbohydrates in exchange for fungal provisioning of mineral nutrients, especially phosphorus (P) and zinc, some of which would otherwise be unavailable for plants (Allen, 1991; Jansa et al., 2003). Besides facilitating nutrient uptake, AM fungi can provide benefits to colonized plants, such as resistance against pathogens and drought tolerance (Newsham et al., 1995; Smith et al., 2010). High densities of AM fungal hyphae in soil form underground networks that contribute to creation and stability of soil aggregates and reduce soil losses through erosion (Chaudhary et al., 2009). Because many of these features are considered beneficial for the sustainability of agricultural production, there is a great interest in increasing mycorrhizal symbioses through agricultural management (Gianinazzi, 2010; Fitter et al., 2011).

Mycorrhizal fungi are key components of grassland C sequestration (Treseder and Allen, 2000; Zhu and Miller, 2003; Johnson et al., 2006; Wilson et al., 2009). Hyphae of AM fungi may comprise a large fraction (estimates go up to a half) of microbial biomass in grassland soil (Olsson et al., 1999), and therefore their responses to fertilization are highly relevant to soil C dynamics. Although AM fungi are expected to rapidly channel and immobilize a significant portion of plant-derived C through the soil, there is an on-going debate about the importance of this C highway in the buildup of stable soil organic matter and soil C sequestration (Staddon et al., 2003; Phillips et al., 2013; Averill et al., 2014; Balasooriya et al., 2014). Density of AM fungal hyphae in the soil was previously found to be positively correlated with the responses of soil organic C to long-term N enrichment and burning in a North American tall-grass prairie, while application of fungicides suppressed AM fungal hyphae and reduced soil organic carbon (Wilson et al., 2009). Responses of AM fungi to N fertilization have been shown to vary with availability of soil P. For example, N enrichment of P deficient grasslands often increases AM fungal biomass but N enrichment of P rich grasslands often decreases it (Johnson et al., 2003; Wilson et al., 2009; Liu et al., 2012).

The functional equilibrium model can help explain why N enrichment may either increase or decrease AM fungal biomass. This conceptual model states that plants should preferentially allocate photosynthates to structures that acquire the most limiting resources; plants growing in infertile soil should allocate more to roots and mycorrhizas and plants growing in nutrient rich soil should allocate more to shoots and leaves (Brouwer, 1983; Johnson et al., 2003). Increasing soil N availability in P-limited soils exacerbates P limitation and enhances the importance of symbiotically acquired P; thus plants are expected to increase allocation of photosynthates belowground to AM fungi. In contrast, N enrichment of P-rich soils is likely to remove limitation by any belowground resource and, in accordance with the functional equilibrium model, plants are expected to shift allocation of photosynthates aboveground to shoots and away from AM fungi (Johnson, 2010).

Changes in nutrient availabilities and consequent shifts in C allocation by plants can have large effects on AM fungal communities (Egerton-Warburton et al., 2001, 2007; Liu et al., 2012). Application of fertilizers systematically increases plant productivity, but also reduces plant diversity (Silvertown et al., 2006; Hejcman et al., 2007; Semelová et al., 2008). Generally, the most discussed nutrients in relation to the management of grasslands are N, P, and calcium (Ca), of which the last mentioned is most often provided in carbonate form (lime) not to primarily correct for plant nutrient deficiencies, but to reverse soil acidification, developing mainly due to application of N fertilizers (Johnston et al., 1986). Soil acidification is recognized as one of the main limits of agricultural production and provision of ecosystem services on a wide range of soils (e.g., Scott et al., 2000). The effect of liming on AM fungi is not easy to predict, in some cases the application of Ca promoted AM fungal colonization of roots (Johnson et al., 2005), whereas in others it had no effect (Kennedy et al., 2005). Recent unconstrained monitoring of AM fungal communities in a number of agricultural soils (including grasslands) in Switzerland pointed to a particular importance of soil pH in structuring indigenous AM fungal communities (Jansa et al., 2014), which provides some mechanistic explanation on how could induced soil pH shifts (due to liming of acidic soils) affect the indigenous AM fungi. The effects of fertilization on individual AM fungal taxa are highly context-dependent and are often confounded with the effects of soil disturbance and crop rotation (Jansa et al., 2006 and references therein). Individual taxa of AM fungi differ in their responses to fertilization; some species increase and others decrease in abundance in response to nutrient enrichment (Douds and Schenck, 1990; Johnson, 1993; Egerton-Warburton et al., 2001).

The aim of this study was to analyze the responses of soil C and AM fungi to 70 years of annual applications of lime and mineral fertilizers at one of the oldest agricultural experiments worldwide—the Rengen Grassland Experiment. Our goal was to test the ability of the functional equilibrium model to predict responses of soil C stocks to grassland inputs. We examined the abundance of AM fungi inside and outside plant roots and used molecular techniques to determine the abundance of a few individual AM fungal taxa. This allowed us to test the hypothesis that lime and fertilizers would reduce plant allocation of photosynthates to belowground structures including the mycorrhizas. We predicted that the differences of lime and fertilizer inputs among treatments would have consequences for the long-term buildup of soil C stocks, co-incident with the effects on AM fungi: N enrichment will increase allocation to AM fungi due to increasing relative demand for limiting P, while fertilization with both N and P will decrease allocation to AM fungi, consequently reducing the soil C sequestration.

2. Material and methods

2.1. Study site description and experimental design

This study was conducted at the Rengen Grassland Experiment established in 1941 by the University of Bonn in low productivity *Nardetum* grassland in the Eifel Mountains, Germany (50°13′N, 6°51′E). The site is 475 m above sea level, average annual precipitation is 811 mm and the mean annual temperature is 6.9 °C. Soil type is a pseudogley. The experimental plots (3 m × 5 m) received annual amendments of lime (in the form of $Ca(OH)_2$, obtained by combination of CaO with water) and fertilizers for more than seven decades, reaching levels considered sufficient for sustainable hay production in that area (Wendland et al., 2012). We

sampled four treatment combinations (average inputs in kg ha^{-1} yr^{-1}): Ca (715 Ca), CaN (100 N, 752 Ca), CaNP (100 N, 35 P, 936 Ca), and a control (unfertilized plots). The different amounts of Ca in the different treatments were due to the forms of applied fertilizers (such as Ca_3PO_4 in the CaNP treatment, which were not counterbalanced by reducing lime inputs in the same treatment as compared to the other limed treatments). The experimental plots are arranged in two blocks containing each five randomly arranged replicates of the Ca, CaN and CaNP treatments, totaling 30 experimental units with ten replicate plots per treatment. No differences in the underlying geology or soil properties were detected between the two blocks when comparing concentrations of available P, total N, organic C, mobile Ca in the soil and the soil pH (analyses not shown), justifying pooling the replicates across the two blocks. Since the original field experiment did not contain non-fertilized controls, ten control plots were designated as late as 1998 on adjacent grassland strip below one of the blocks. This grassland strip has never been fertilized or grazed, it has been subjected to 2 hay cuts since 1941, but for historical reasons the plots are not spatially randomized with the other treatments. However, no significant soil gradient within soil properties has been found in the experimental area either between the blocks (see above) or along the axis perpendicular to the block arrangement, except the soil pH, which showed a significant shift with the distance from the control plots, driven by the strong difference in soil pH between the controls and the other (limed) plots (see Table 1). These analyses justified inclusion of the control plots to the comparison with the other soil treatments even if they were not perfectly randomized with the other treatments in the experimental field. Further details of the site and the experimental design have been published previously (Schellberg et al., 1999; Hejcman et al., 2007).

2.2. Sampling procedure and plant analysis

Experimental plots were cut twice a year, first in early June and again in mid-October. Aboveground biomass for laboratory analyses was collected along a strip 1.25 m wide and 5 m long and weighted. Subsamples of 500 g (fresh weight) from each of the plots were dried at 60 °C for 48 h to determine the dry biomass (hay yield). The percentage of surface cover by individual vascular plant species was estimated visually in twenty plots (i.e., one experimental block and half of the control plots) on 20 June 2011 according to a regional plant species list (Rothmaler et al., 2000), considering three plant functional groups (grasses, herbs (i.e., dicots without legumes) and legumes). The nomenclature of plant species was based on Kubát et al. (2002). To eliminate edge effect, relevés were taken in the center of each plot, considering an area of 1.8 m × 3.2 m.

Table 1
Soil chemical properties (0–10 cm depth) of the Rengen Grassland Experiment. Soil data measured on samples collected in 2011 are shown. Mean values (n = 10) are shown of soil pH, available soil phosphorus (P), total soil nitrogen (N) and soil mobile calcium (Ca) concentrations. Different letters within a column indicate significant differences between treatment means according to the least significant difference post-hoc test following significant ANOVA (F- and p-values are shown in italics).

Treatment	pH	P (mg kg^{-1})	N (g kg^{-1})	Ca (mg kg^{-1})
control	5.32c	0.307b	3.79bc	70.5
Ca	6.96a	0.663b	4.11a	138.3
CaN	7.06ab	0.452b	4.03ab	121.9
CaNP	7.14b	9.947a	3.57c	103.7
F value	*211.34*	*92.98*	*6.26*	*2.40*
p-Value	*<0.001*	*<0.001*	*0.002*	*0.084*

Three types of belowground samples were collected: (1) composite soil + root samples, (2) root standing biomass, and (3) new root growth. All samples were transported to the Czech University of Life Sciences in Prague and frozen within 24 h of collection.

(1) On 20 October 2011, eight small diameter cores (2.5 cm diameter × 10 cm depth) were collected across the central area of each plot and mixed into one composite sample. Roots recovered from a portion of each composite sample were analyzed for AM fungal colonization and the remaining soil from the composite samples was analyzed for extraradical (soil-borne) AM fungal hyphae and other soil properties as described below.
(2) At the same time, one large (5 cm diameter) core was taken from the center of each plot to measure the standing root biomass. The deepest possible core was taken and the resulting core depths varied from 10 to 15 cm. Each core was weighed and its exact length was recorded. A subsample of each core was used to estimate gravimetric soil moisture by weighing the soil sample before and after drying at 70 °C for 72 h. The rest of the core was used to extract root biomass (see below). Each hole created through this sampling was immediately filled with granitic river sand.
(3) In mid June 2012, the area was re-sampled using the same corer and sampling depth as above (point 2) to estimate new root production during eight months of root growth.

Roots were removed from the large core samples by soaking them in soapy water for 5–10 min, and then rinsed with tap water through a 1 mm sieve. Roots were also carefully removed from the new growth (sand) cores by rinsing with tap water through a 1 mm sieve. Cleaned roots were weighed, dried at 80 °C for 48 h and weighed again. Standing root biomass in the topsoil (10 cm depth) per ha was calculated by using estimated root biomass (×2) and the soil core volume. Root-to-shoot biomass ratio was then calculated as the ratio of dry weight of roots (t ha^{-1}) and annual yield (t ha^{-1}, average value from years 2008 through 2012).

2.3. Determination of AM fungal colonization and extraradical hyphal length

The extent of root colonization by AM fungi was determined in the roots from the composite soil samples. Roots were soaked in 5% KOH at room temperature for two days, and stained using the ink and vinegar technique (Vierheilig et al., 1998). Stained roots were examined for mycorrhizal colonization using the magnified intersection method, scoring 100 intersections per sample (McGonigle et al., 1990) at a magnification of 200×. Then we calculated percentages of root length colonized by arbuscules, coils, vesicles, mycorrhizal hyphae, and the fraction of root length devoid of any mycorrhizal structures.

Composite soil samples were thawed and a 5 g subsample was used for determination of extraradical hyphal length density following the protocol described in Rillig et al. (1999), with minor modifications. In short, soil was suspended in a solution containing 112 ml of deionized water and 420 mg of $Na(PO_4)_6$, shaken for 30 s, allowed to settle for 30 min, and decanted into a 38 μm sieve. All material on the sieve was suspended in 200 ml of deionized water, shaken, and 3 ml was transferred onto a membrane filter with a pore size of 0.45 μm (GN-6 25 mm; Pall Life science, Mijdrecht, the Netherlands), placed in a vacuum manifold. Material on the filter was stained with 0.02% trypan blue in lactoglycerol for 5 min, washed with excess water and the filter was then mounted on microscopic slides. AM hyphae were distinguished from other hyphae according to descriptions in Mosse (1959): “dark-to

light-blue stained aseptate hyphae with characteristic unilateral angular projections". Intersects in 30 random grids were counted using a compound microscope at 200× magnification. Hyphal length was calculated per g soil dry weight by estimating soil water loss after drying in an oven at 80 °C for four days.

2.4. Real-time PCR

A subsample of each composite soil sample was dried at 80 °C for 48 h, homogenized, and sieved through a 2 mm sieve. The soil DNA was extracted from 500 mg (dry weight) samples using the Macherey-Nagel NucleoSpin Soil DNA kit (Macherey-Nagel GmBH & Co., Düren, Germany) following the standard protocol and stored at −20 °C until use. Abundances of six AM fungal taxa were estimated using quantitative real-time polymerase chain reaction (qPCR) with specific AM fungal primers and hydrolysis (TaqMan) probes, using an AB StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA.). We employed taxon-specific markers targeting the nuclear large ribosomal subunit (nLSU) genes (Wagg et al., 2011; Thonar et al., 2012) for *Cetraspora pellucida*, *Claroideoglomus claroideum*, *Funneliformis mosseae*, *Diversispora celata*, *Gigaspora margarita*, and *Rhizophagus irregularis*. Additionally, for detection of *Rhizophagus* sp., we also used a marker targeting the mitochondrial large ribosomal subunit (mtLSU) gene (Kiers et al., 2011; Couillerot et al., 2012). Twenty billion copies of an internal standard (linearized plasmid carrying a fragment of cassava mosaic virus, GenBank accession AJ427910) were added into each sample before extraction, and quantified after the extraction, to estimate the DNA extraction efficiency and DNA extract quality, namely absence of PCR inhibitors (Thonar et al., 2012; Janoušková et al., 2015). Each qPCR reaction mixture contained 2 μl template (DNA extracts diluted 1:10), 0.4 μl forward primer (25 μM), 0.4 μl reverse primer (25 μM), 0.1 μl hydrolysis probe (25 μM), 4 μl of 5× HOT FIREPol® PROBE qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia), and 13.1 μl PCR-grade water.

Cycling conditions were as follows: initial DNA denaturation and DNA polymerase activation at 95 °C for 15 min, then 50 cycles each with denaturation at 95 °C for 10 s, annealing conditions specified in Table S1 (Electronic supplement), and elongation at 72 °C for 10 s.

2.5. Soil properties

To measure soil pH, 30 g of dry soil was suspended in 30 ml deionized water for 20 min, centrifuged at 3044 g for 20 min and the supernatants (soil extracts) were filtered through a paper filter to remove floating particles. The pH was determined using a combination pH electrode G-P Combo w/RJ (Corning, USA). The concentration of mobile Ca^{2+} ions in the supernatants brought to pH 7.0 ± 0.2 (using 1% HCl or KOH) was measured using a Ca-selective electrode (type 20-35, Monokrystaly, Turnov, Czech Republic) and corrected for acidic error using the calibration of the electrode response with solutions of known Ca concentrations at different pH values. Soil organic C and total N concentrations were analyzed using a CN analyzer (Flash EA 2000, ThermoFisher Scientific, Waltham MA, USA). Soil available P was determined spectrophotometrically in aqueous soil extracts (3 g dry soil and 30 ml of water) after shaking for 25 h, subsequent centrifugation at 3044 g for 20 min and filtration through a 0.22 μm filter (CME, Carl Roth, Karlsruhe, Germany). This approach to measure P availability was chosen because of large differences in soil pH between the different soil application treatments. Soil pH can namely largely affect the estimates of P availability in soils when assessed by various extraction methods (Demaria et al., 2005).

2.6. Bulk density and soil carbon stocks

Bulk density was determined from the large soil cores (5 cm diameter). The entire core was weighted fresh, and a half of the core was then taken, weighted, dried at 70 °C for 3 days and weighted again. The ratio of fresh to dry weights of the soil subsample was used to calculate dry weight of the entire soil core of known dimensions. In order to determine the amount of organic C per ha, the volumetric organic C content of the topsoil was calculated by using gravimetric organic C concentrations measured on the composite soil samples (cores with 2.5 cm diameter, dried and sieved to pass 2 mm sieve) and the soil density assessed on the large soil cores, and scaling up to 1×10^6 l of soil (1 ha surface, 10 cm depth). Both gravimetric and volumetric C concentrations were used to compare the differently treated soils whereas the volumetric C concentration was then used to establish a relationship with the extraradical hyphal length density. This is because a gravimetric approach would artificially amplify the differences due to the well-established fact that more organic soils have a lower bulk density (Aggelides and Londra, 2000; Celik et al., 2004; Hati et al., 2007).

2.7. Statistical analyses

Prior to analysis, root colonization data (percentage values + 1) were square root-arcsine transformed (Zar, 1999). To evaluate differences between treatments, one-way ANOVA in STATISTICA 8.0 (StatSoft, 1995) and an additional post-hoc test using the least significant difference *F*-test ($p = 0.05$) was used. Control plots were considered as the fourth treatment in addition to the Ca, CaN, and CaNP for statistical comparisons, given the apparent absence of underlying soil gradients on the experimental grounds (see above). Redundancy analysis (RDA) and canonical correspondence analysis (CCA) in the CANOCO 4.5 program (ter Braak and Šmilauer, 2002) were used to examine the relationships among AM fungal variables, soil properties, and hay yields. Further, a Monte Carlo permutation test with 499 permutations was used to reveal if the tested explanatory environmental variables (soil C, available soil P, total soil N, available soil Ca, soil pH, and hay productivity at the plot level) had a significant impact on the AM

Table 2
Gravimetric and volumetric carbon (C) concentrations of the soils, soil bulk density, and the soil C/N ratios as affected by the different management treatments. Mean values ($n = 10$, ± standard errors) are shown. Different letters within a column indicate significant differences between treatment means according to the least significant difference post-hoc test following significant ANOVA (*F*- and *p*– values are shown in italics).

Treatment	Gravimetric C concentration ($g\,kg^{-1}$)	Soil bulk density ($kg\,l^{-1}$)	Volumetric C concentration ($g\,l^{-1}$)	Soil C/N ratio
Control	51.7 (±0.6) ab	0.97 (±0.02) b	50.3 (±0.9) b	13.6 (±0.09) a
Ca	55.5 (±1.9) a	1.00 (±0.03) ab	55.4 (±1.7) a	13.5 (±0.20) a
CaN	49.0 (±1.9) b	1.03 (±0.02) ab	50.5 (±1.8) ab	12.1 (±0.14) b
CaNP	41.5 (±1.9) c	1.06 (±0.02) a	44.1 (±2.3) c	11.6 (±0.23) c
F value	*12.7*	*2.96*	*6.84*	*33.97*
p-Value	*<0.001*	*0.045*	*<0.001*	*<0.001*

fungal community composition and abundance. Results of RDA and CCA were visualized in an ordination diagram constructed by the CanoDraw program. Simple linear regression was used to examine the relationships between the volumetric organic C content of the topsoil, density of AM fungal hyphae in soil and hay production.

3. Results

3.1. Soil properties

Long-term fertilization and liming caused large changes in soil chemical properties across the different management treatments (Tables 1 and 2). Plant available P in soil was an order of magnitude higher in P fertilized plots compared to the other treatments (Table 1). Long-term liming, with or without fertilizer additions, considerably increased soil pH. It increased with liming and fertilizer from 5.32 in control plots to 7.14 in CaNP plots (Table 1, Table S2). The fertilization had a profound effect on the total N and organic C concentrations in the soil, but neither of the practices had significantly changed mobile Ca levels. Soil bulk density varied between individual samples from 0.83 to 1.18 kg l^{-1}, and was significantly lower in the unfertilized control treatment as compared to the CaNP treatment (Table 2, Table S2).

3.2. Plant responses to lime and fertilizers

Hay yield was significantly increased by application of lime and the N and P fertilizers ($F = 118.8$, $p < 0.001$; Fig. 1a). The standing biomass of roots in October 2011 tended to have lower values in the CaN treatment as compared to the other treatments, yet the differences remained insignificant ($F = 2.06$, $p = 0.123$; Fig. 1b, Table S3). Root biomass in June 2012 showed similar pattern as in October 2011, with no significant differences between treatments ($F = 1.58$, $p = 0.21$), but the values were about 3 times lower than those in October 2011 (data not shown). Root-to-shoot biomass ratio was higher in the unfertilized control and in the limed treatments than in the CaN and CaNP treatments ($F = 13.8$, $p < 0.001$; Fig. 1c). Plant species richness decreased with the complexity of soil amendments, with significantly fewer species being found in the CaNP plots than the other treatments ($F = 11.1$, $p < 0.001$; Fig. 1d); relative cover of herbs tended to decrease ($F = 3.16$, $p = 0.0535$; Fig. 1e), grass cover increased ($F = 5.22$, $p = 0.011$; Fig. 1f), and cover of legumes decreased ($F = 5.53$,

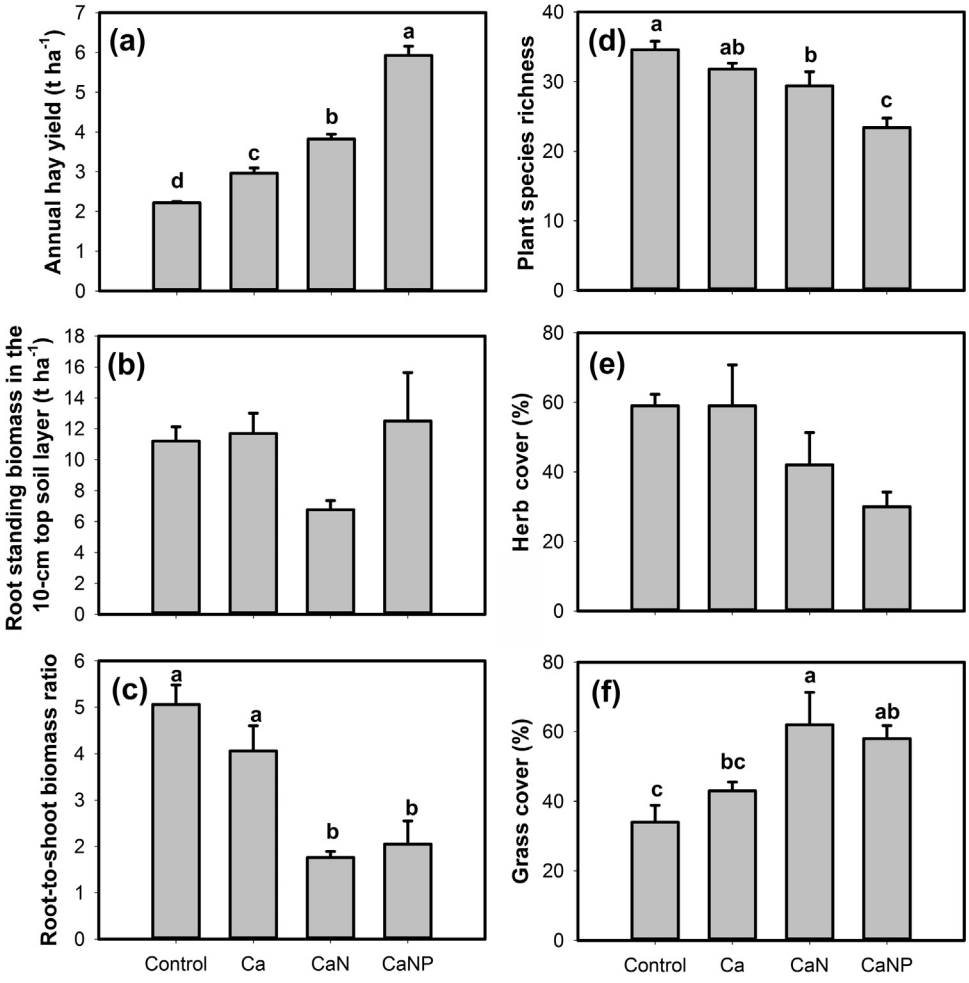

Fig. 1. Annual hay yield (a), root standing biomass (b), root-to-shoot biomass ratio (c), plant species richness (d), cover of herbs (e), and cover of grasses (f) as affected by the management treatments (Control—no amendments, Ca—lime added, CaN—lime and nitrogen added, CaNP—limed and nitrogen and phosphorus fertilizers added). Bars represent means +1 standard errors ($n = 10$ for a–c and $n = 5$ for d–f). Different letters indicate significant differences between treatment means (least significant difference F-test, $p < 0.05$). Biomass of roots has been estimated only for the topsoil (0–10 cm depth) in October 2011, and upscaled to 1 ha surface to compare with hay yields. Average yields of seasons 2008–2012 are shown.

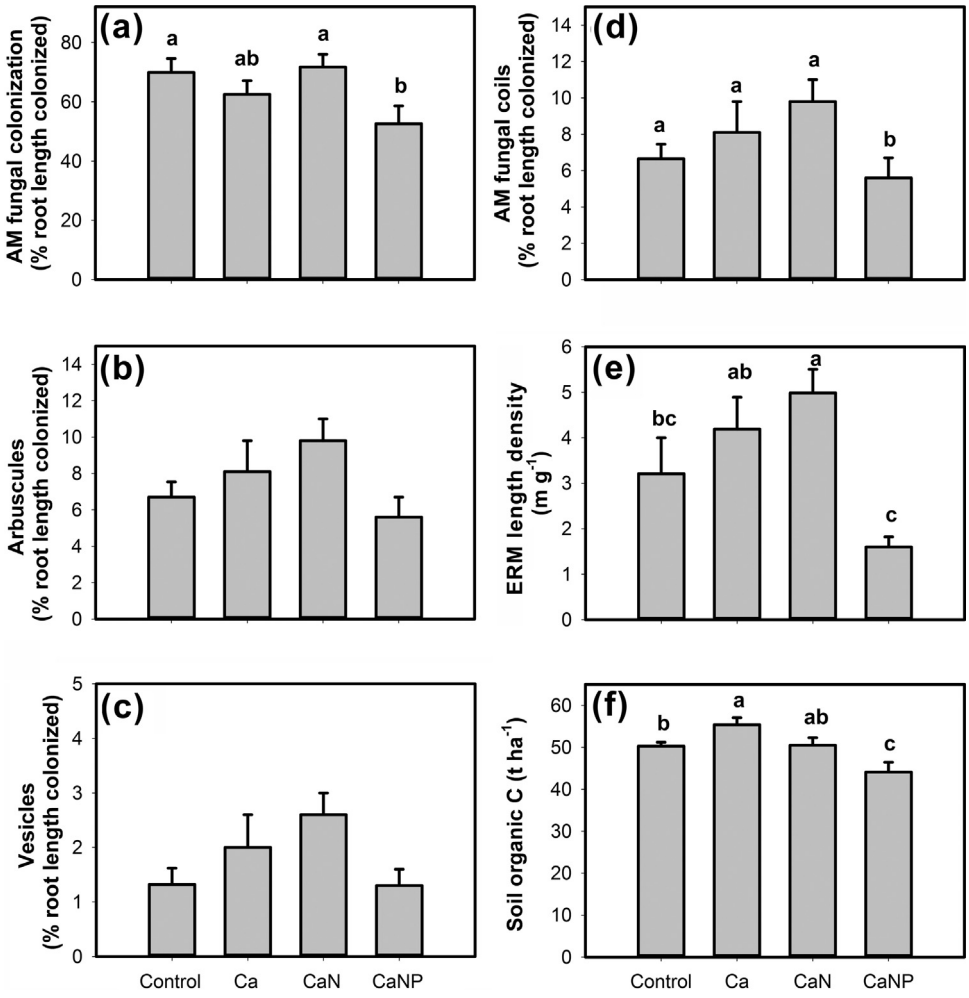

Fig. 2. Extent of root length colonized by any of the arbuscular mycorrhizal (AM) fungal structures (a), and the fraction of root length colonized specifically by arbuscules (b), vesicles (c), and AM fungal coils (d). The density of the extraradical mycelium (ERM) of AM fungi in the soil (e) and the concentration of organic carbon (C) in the topsoil (f) as affected by the management treatments. Bars represent means +1 standard errors ($n = 10$). Different letters indicate significant differences between treatment means (least significant difference F-test, $p < 0.05$). The percentage values were arcsin-square root transformed for the statistical comparisons so as not to violate ANOVA assumptions. Soil C stocks have been estimated only for the topsoil (0–10 cm depth) and upscaled to 1 ha surface.

$p = 0.015$, data not shown) with increasing complexity of the soil amendments.

3.3. Mycorrhizal responses to soil amendments

The percentage of root length colonized by any AM fungal structures varied significantly among the treatments ($F = 3.18$, $p = 0.036$; Fig. 2a) and was lower in the CaNP treatment than in the unfertilized control. The colonization of roots in the CaN treatment showed similarly high values as in the unfertilized control plots (Fig. 2a, Table S4). A similar picture was observed when comparing the impact of the management practices on the rates of root colonization by specific AM fungal structures (Fig. 2b–d), but only the coils (Fig. 2d) showed statistically significant ($F = 3.64$, $p = 0.022$) decrease in abundance in the CaNP as compared to the other treatments. The density of extraradical AM fungal hyphae in the soil was significantly higher in the CaN plots as compared to the control and the CaNP treatments, with the latter having the lowest hyphal density among all the management treatments ($F = 5.91$, $p = 0.002$; Fig. 2e). According to the CCA (Monte Carlo permutation test $p = 0.018$; Fig. S1), root colonization and density of AM fungal hyphae in the soil were negatively related to available soil P and hay yield and positively related to the N and C concentrations in the soil.

The qPCR profiling of individual AM fungal taxa abundance in soil yielded positive detection between 30% (*Claroideoglomus*) and nil (*Cetraspora*) of the analyzed samples (details not shown). There were either no effects of soil management on the abundance of the tested AM fungal taxa or the abundance in the limed and/or fertilized treatment was higher than in the control plots (Fig. S1). Additionally, there was an obvious difference in the response to P fertilizer between *Claroideoglomus*, whose abundance decreased in response to P fertilization as compared to N fertilization only, and *Diversispora*, which was not significantly affected by P fertilization (Fig. S2a and b). Multivariate analysis (Fig. 3) showed that the abundances of the tested AM fungal taxa were significantly affected by soil properties (Monte Carlo permutation test $p = 0.02$) and mostly driven by soil amendments, positively correlating with pH (*Rhizophagus, Claroideoglomus, Diversispora*), yields and/or soil P levels (*Funneliformis*).

3.4. Soil C stocks

The amount of soil organic C in the top 10 cm was significantly increased by liming without mineral fertilizer amendment, and significantly decreased by simultaneous addition of lime, N and P as compared to the unfertilized controls ($F = 12.7$, $p < 0.001$; Fig. 2f). There was also a strong decrease in the soil C/N ratio with

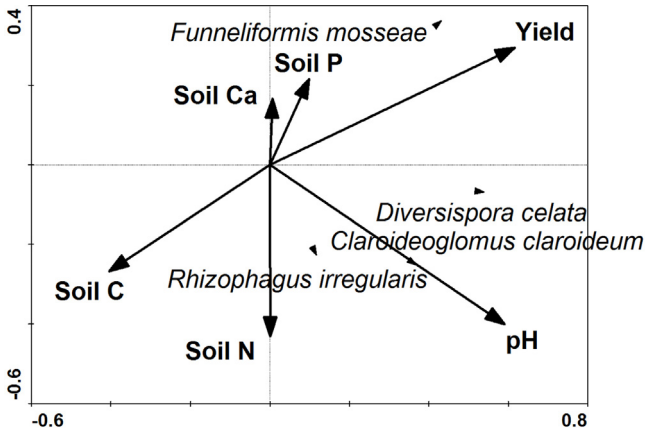

Fig. 3. Ordination diagram showing results of redundancy analysis of abundance of individual arbuscular mycorrhizal fungal taxa in soil in relation to soil chemical properties and hay yield. *Rhizophagus irregularis* abundance was assessed with quantitative PCR marker targeting mitochondrial large ribosomal subunit (LSU) gene whereas the other AM fungal taxa were assessed with markers for nuclear LSU genes. Environmental variables: Soil N and C—total nitrogen and organic carbon concentrations in the soil, respectively. Soil P and Ca—available phosphorus and mobile calcium concentrations in the soil, respectively. Yield—hay productivity per year (average of 2008–2012 seasons). First and second axes explain 62.8% and 25.1% of the mycorrhizal fungal community and environmental data relationship, respectively.

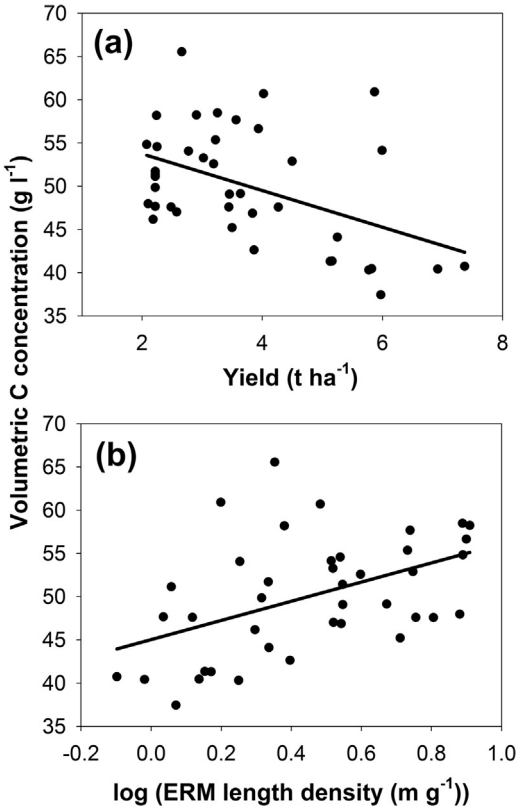

Fig. 4. Negative relationship between volumetric soil carbon (C) concentration and hay yields (a) and a positive relationship between the volumetric soil C concentration and the length density of extraradical mycelium (ERM, log transformed) of the arbuscular mycorrhizal fungi in the soil (b). Every dot represents a separate experimental plot. Both regression lines $p < 0.01$.

increasing complexity of amendments (Table 2). Soil C/N ratios were higher in the control and limed treatments as compared to the other treatments, with the values being still significantly higher in the CaN than in the CaNP treatments (Table 2). Volumetric organic C content of topsoil had a negative relationship with hay yields ($R^2 = 0.21$, $p = 0.003$) and a positive relationship with the density of AM fungal hyphae in the soil ($R^2 = 0.22$, $p = 0.002$; Fig. 4), whereas it showed no significant relationship with any of the AM fungal abundance estimates inside the roots (analyses not shown).

4. Discussion

Our results show that seven decades of yield-promoting measures, particularly N and P fertilization, dramatically reduced soil C stocks in a temperate grassland. This reduction in the top 10 cm is about 6 t C ha^{-1} when comparing the fully fertilized treatment with the unmanaged control. There is even a higher reduction of 11 t C ha^{-1} when the fully fertilized treatment is contrasted against the lime-only treated plots. This latter comparison represents a reduction of 20.4% of the C stocks in the topsoil of the limed-only plots as compared to CaNP treatment, which is more than half of the figure to be expected due to land use change from grassland to cropland (−36%, Poeplau et al., 2011). The C loss from the topsoil of the CaNP treatment over the last 70 years outweighed the C contained in the increased annual hay yield by 6- and 12-fold when compared to the control with no inputs and to the limed treatment, respectively. This means that increased hay yields over 6–12 subsequent years are just compensated by reduction of belowground C stocks within the top 10 cm. This comparison takes into account average hay yields over the last fifteen years, assuming 45% C content of hay. Due to lack of records, we are unable to draw a full C balance of the system over the entire 70 years duration of the experiment or for soil depths greater than 10 cm. Nevertheless, the magnitude of the observed effect calls for a very careful consideration of the design of grassland management practices that aim at both maximizing hay yield and enhancing belowground C sequestration. There is an urgent need to better understand the mechanisms that control the accrual of soil C stocks in agricultural systems and, based on this information, to design highly productive agrosystems with high C sequestration potential.

Photosynthesis is the source of the vast majority of C in grassland soils, and thus one would assume that the greater the productivity, the greater the C remaining in the system. There are, however, many complicating factors: the processes of decomposition and mineralization determine the ultimate fate of soil organic C; and the stoichiometry of C, N, and P within the system has a strong influence on the complex interactions involved in these processes. Organic C enters the soil as root or shoot debris (necromass), root exudates, AM fungal biomass (trophically completely dependent on host plants) or AM fungal necromass. Thereafter, organic C enters the belowground food web involving soil animals, fungi and prokaryotes. At every trophic step, a portion of organic C is lost as CO_2 and a part is stabilized in the soil over shorter or longer periods of time. As a result, realized soil C stocks depend on plant productivity (i.e., input quantity), biomass partitioning between roots and shoots, degree of association of plants with AM fungi, degradability of plant and/or AM fungal necromass (i.e., input quality), activity of soil microbes, and competition among plants, AM fungi, and soil saprobes for essential resources such as N and P, as well as fluctuations of environmental properties such as temperature and moisture (Cotrufo et al., 2013). Theoretically, plants and associated AM fungi could be seen as competitors of soil saprobes for available P and/or N thus suppressing their decomposition activity (Leifheit

et al., 2015). Alternatively, AM fungi and fine roots could prime soil saprotrophic life with fresh C from aboveground (Murphy et al., 2015), thus increasing activity of soil saprobes and eventually reducing the soil C stocks. Below, we will describe three non-mutually exclusive mechanisms that may explain the decline of soil C that was associated with P fertilization in our study: (1) allocation to roots and AM symbioses, (2) degradability of litter inputs, and (3) stoichiometric control of soil microbial activity.

4.1. Allocation to roots and AM symbioses

The functional equilibrium model predicts that plant allocation to roots will decrease when the plants are not limited by mineral nutrients (Brouwer, 1983; Nielsen et al., 2001; Hermans et al., 2006). Here we showed that the root-to-shoot biomass ratio significantly decreased in the CaN and CaNP treatments, corroborating the idea that plants allocate biomass to the organs required to gain access to the most limited resources. Enrichment with N or with N and P together increased relative allocation of biomass/C aboveground to shoots and reduced allocation to roots, indicating greater limitation of plants' growth by photosynthesis than by nutrients in those soil management treatments (Fig. 1). The functional equilibrium model applies to plant allocation to AM symbioses as well as roots (Johnson et al., 2003, 2013). It is interesting to note that in our study allocation to roots was reduced in both the CaN and CaNP treatments, while allocation to AM fungi (soil hyphae) was only reduced in the CaNP treatment (Fig. 2e). This strongly suggests that AM symbioses are critical for the P nutrition while roots are critical for the N nutrition of plants.

The opposite relationships of hay yield and AM hyphae with soil C suggest that plant allocation to shoots (hay) and away from AM symbioses may have a large (and possibly a causal, AM-mediated) impact on soil C storage (Fig. 4). The positive correlation between soil C and AM fungal hyphae could arise from increased C inputs via hyphal biomass and necromass as well as increased C storage through enhanced soil aggregation and hence protection against degradation (Zhu and Miller, 2003; Rillig, 2004; Leifheit et al., 2015), as well as from lower microbial activity in the unfertilized soil due to low P availability for the microbes, which could further be enhanced by direct competition for resources like available P between AM fungi and other soil biota (van Groenigen et al., 2006; Welc et al., 2010). Other studies have also reported a positive correlation between AM fungal abundance and soil C levels (Treseder and Allen, 2000; Johnson et al., 2006; Wilson et al., 2009). Future research is needed to elucidate the mechanisms generating these correlations. The degree to which AM fungi and their associated microbes increase soil C stocks through direct input of recalcitrant forms of C versus their influence on soil aggregate structure (and protection from decomposition) is currently unknown. It is important to recognize that in some systems, AM fungi might actually reduce soil C levels by stimulating (nutrient rich) organic matter decomposition by other soil microbes (Hodge et al., 2001; Cheng et al., 2012). This idea is also supported by the view that most C allocated to the AM fungi flushes through the ecosystem and is respired back to the atmosphere within few days (Johnson et al., 2002; Staddon et al., 2003; Lendenmann et al., 2011). Depending on the relative importance of either of these processes (increased C inputs and stabilization or stimulation of microbial decomposition), the effects of AM fungi on C stocks may be positive, negative or neutral (Verbruggen et al., 2013). From our correlative findings we can simply conclude that AM fungi thrive in more organic soil. Indeed, some common organic compounds (i.e., chitin or cellulose) may significantly affect the presence and abundance of AM fungi in soil (Gryndler et al., 2003; Joner and Jakobsen, 1995).

The community composition of plants and AM fungi was strongly influenced by the different fertilizer treatments. Apart from stimulating mycorrhizal fungi in general (Fig. S1), application of lime together with N increased the abundance of AM fungal taxa *Claroideoglomus* and *Diversispora* (Fig. S2). In contrast, P enrichment drove a sharp decrease of extraradical AM hyphae in the soil, although the specific response of the individual fungi to P application differed, with some taxa such as *D. celata*, not responding at all to addition of P (Fig. S2). This may suggest that plants either selectively down-regulate certain fungal taxa in response to high P, or that high P-levels directly inhibit some AM fungal taxa whereas others are indiferent to elevated P levels. This interpretation is supported by another fertilization experiment that shows the relative abundance of *Diversispora* spp. increases while that of *Glomus* spp. decreases with N and P fertilization (Liu et al., 2015). It should be noted that in our study the observed changes in the fungal community composition could only roughly explain changes in AM fungal hyphal density in soil because the method used to screen for the AM fungal taxa abundances misses many taxa for which the quantitative molecular markers are not yet available. This was the case of the control treatment that showed a complete absence of both *Diversispora* and *Claroideoglomus*, although other AM fungi were obviously present in great abundance (Fig. S2). Our measured abundances of certain AM taxa, although correct, do not signal lower activity of AM fungi in general, but only of the particular focal species (Jansa et al., 2014; Ohsowski et al., 2014). Finally, reduced mycorrhization in the CaNP treatment could have arisen because of selection of a plant community with reduced mycorrhizal dependency over the course of the experiment. The shift from a diverse herb-rich community to a less diverse grass-dominated community (Fig. 1) can also be expected to generate many changes that could generate strong feedbacks on soil C dynamics other than mycorrhizal abundance.

4.2. Degradability of litter inputs

Litter quality is likely to vary across the different fertilizer treatments and this may have a strong influence on decomposition. Previous research at the Rengen Grassland Experiment has shown that P concentration in harvested forage from the CaNP plots was about twice as high as in the other plots, with most of the other parameters remaining the same (Schellberg et al., 1999; Hejcman et al., 2010). Concentration of N in the harvested forage from the CaN plots was higher than in the CaNP plots, but only slightly and insignificantly elevated as compared to control and the limed-only plots (Hejcman et al., 2010). This resulted in much lower N/P ratio of the harvested forage in the CaNP treatment (4.8) than in the other three treatments (where the mean ranged between 13.6 and 17.7, Hejcman et al., 2010). The P-rich biomass in the CaNP treatment is likely to be degraded faster than if P in the biomass is low (Cornelissen and Thompson, 1997; Freschet et al., 2013), resulting in more C to be removed from the P-rich organic inputs than if the inputs are of low quality.

In addition to the direct influence of chemical fertilizers on forage quality, the indirect effect through altered plant community composition is also likely to be important. We observed the highest soil C storage in the treatments with the highest plant diversity, the unfertilized control and the limed plots (Fig. 1), in agreement with other observations from the Jena and Wageningen biodiversity experiments, where plant diversity correlated positively with belowground C storage (Steinbeiss et al., 2008; Cong et al., 2014). In our case this correlation could be explained either through production of more degradation-recalcitrant litter by the plants adapted to nutrient-limited environments (Pastor and Cohen, 1997), or through changes in the composition and/or activity of soil microbial communities (including the AM fungi) in response to the

differential management. Indeed, it has recently been demonstrated that manipulation of the soil microbial diversity could have dramatic consequences on multiple ecosystem functions including litter decomposition, nutrient cycling and C sequestration (Wagg et al., 2014).

4.3. Stoichiometric control of soil microbial activity

Decomposer microorganisms require a certain balance of essential nutrients and the most limiting nutrient may control the decomposition process (Sinsabaugh et al., 2013). The processes of mineralization and immobilization are well known to be sensitive to the C/N ratio of the substrate and surrounding environment; however, C/N/P ratios can also be very important (Elser et al., 2000). Many grassland systems, particularly those on highly weathered or carbonate rich soils are P limited and N enrichment of these systems may exacerbate P limitation and immobilize organic substrates. For instance, in a laboratory based decomposition study, Craine et al. (2007) found that increasing mineral N levels reduces litter decomposition, potentially leading to higher soil C accumulation. Also, van Groenigen et al. (2006) found a strong positive effect of N availability on build-up of the soil C reservoir. Looking at actual C stocks in grasslands, Fornara and Tilman (2012) and Fornara et al. (2013) report significantly higher C under only-N fertilization in studies lasting for 27 and 19 years, respectively. In the latter study, simultaneous addition of N and P entirely negated this effect. These observations were more recently confirmed by both laboratory and field studies, indicating that both C mineralization and gaseous N emissions are promoted by P additions to soil (He and Dijkstra, 2015; Fisk et al., 2015). This broadly agrees with our results: when P limitation is removed, soil C storage decreases. However, in our work the N-only addition did not significantly increase soil C compared to the controls, even after 70 years of fertilization (Fig. 2f). Yet it tended (not significantly) to decrease standing root biomass and showed a tendency to increase the abundance of AM fungal hyphae in soil, indicating the possible interconnections between AM fungal biomass/activity and the buildup of soil C stocks.

5. Concluding remarks

The Rengen Grassland Experiment provides an unprecedented opportunity to examine how long-term management influences plant and microbial communities and ecosystem processes such as C sequestration. Our study provides correlative evidence that AM symbioses contribute to soil C stocks and that this C pool may be diminished if AM fungal biomass is reduced in response to P fertilization. Future experimental work is needed to more thoroughly understand the mechanisms causing these correlations. Isotopes can be used to measure C pools and track C fluxes through plants, mycorrhizas and decomposer food-webs. Also, stoichiometric models should be tested to elucidate the relative importance of C/N/P ratios in soil C dynamics. Although we acknowledge a number of limitations of our study, such as lack of records on C stocks in deeper soil horizons and limited data on the total AM fungal biomass and community composition, our results convey a very clear message—that designing future agroecosystems to simultaneously maximize aboveground yields and maximize belowground C storage may be a difficult or even an impossible goal to achieve.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2015.12.026.

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5. 3 PAPER III.

Zea mays respons to 70 years of differently managed soils

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(Photo: Lenka Zemková)

Zea mays responds to 70 years of differently managed soils

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Abstract

This study has shown that seventy years of different fertilizer treatment at the Rengen Grassland Experiment (RGE) caused changes in soil chemical properties which have resulted in significantly various plant responses. The goal of this study was to examine the responses of *Zea mays* to the soils collected from the RGE and grown in greenhouse. In June 2012, soil from four fertilizer treatments (only limed, limed with nitrogen, limed with nitrogen and phosphorus, and unfertilized control without any nutrient input) was collected in RGE and used to plant germinated seedling of *Zea mays* for nine weeks. Once a week, height of plant, number of leaves was counted and dry above-ground biomass was weighted at the end of experiment.

We found that long-term fertilization and liming caused high variation in soil pH, plant available concentration of phosphorus and organic carbon in the soil but it did not significantly altered total content of nitrogen and plant available calcium in the soil. Also, height of plants varied markedly across the treatment with the highest values in limed soils enriched with nitrogen. The same trend was observed for number of leaves and dry above-ground biomass.

Key words: bioassay, *Zea mays*, nitrogen, phosphorus, calcium, long term grassland experiment

Introduction

Grassland as the cheapest and easiest source of feed for livestock is of a great interest for agronomists all over the world. To obtain sufficient yield production and to improve oligotrophic grasslands, mineral fertilizer application has been a common treatment (Schellberg *et al*., 1999; Sochorová *et al*., 2016). Nowadays nutrient supply is universally used and its content very carefully monitored. Farmers and agronomists are aware of an importance of plant nutrition and its direct connection with yield, quality and cost which has to be optimized not only because of financial outcome but also because of stabilization of grassland functions. With constantly increasing application of mineral fertilizers since the beginning of last century together with growing anthropogenic nutrients input to the ecosystem there has been an ongoing debate what are the consequences and how it will influence biotic communities (Vitousek *et al*., 1997; Craine *et al*., 2007). Numerous field studies worldwide have shown that improved soil fertility can alter soil chemical properties, plant species composition, it can increase plant productivity but also it can cause a loss of indigenous species, reductions of species diversity and alteration of soil microbial community (Tilman, 1996; Silverman *et al*., 2006; Egerton-Warburton *et al*., 2007). The reaction of grassland is not easy to predict as it can differ largely depending on many effects including the average temperature, amount and distribution of annual precipitation or previous management practices including level of fertilization (Sala *et al*., 1988; Conant *et al*., 2001).
Elements can enter plant or soil with applied mineral or organic fertilizers, they can be released by the process of rock material decomposition or enter as an atmospheric deposition. Even though the nutrients are present in the soil solution, the uptake of nutrients by plant can be influenced by various factors such as nutritional status of the plant, kind of species or phenological development of the plant (Duru *et al*., 1993). Profound effect on root uptake but also to plant growth has soil acidity which defines the availability and mobility of cations and anions in the soil (Silvertown *et al*., 2006; Clark *et al*., 2007; Hopkins *et al*., 2011). It is a very actual topic because soil pH is permanently increased with acidic rains from the atmosphere, plant root exudates and cation uptake. Lower soil pH is known to increase availability of phosphorus due to a decreased concentration of $H_2PO_4^-$ and to limit plant growth by toxic concentration of Al^{3+} and Mn^{2+}. Nitrogen uptake depends on the supplied form, uptake of ammonium (NH_4^+) is decreased with lower pH whereas uptake of nitrate (NO_3^-) is increased however interpreting results might be complicated because of side-effects such as cation balance on root function and metabolism (Marschner, 1995; Schellberg *et al*., 1999).

Also, the reactions of the plant can be very different. Plants planted on nutrients rich soil tend to transfer more photosynthate to above-ground biomass and less to below-ground because plant preferentially sends photosynthates where they are the most needed. There is an assumption that plants in fertilized soil will invest more to leaves and shoots and plants growing in nutrient poor soil will invest more to roots (Brouwer, 1983; Johnson *et al.*, 2003). Addition of nitrogen to phosphorus limited soil can further increase phosphorus limitation and force plants to allocate more photosynthates below-ground but enrichment with nitrogen to phosphorus rich soils should exacerbate plant growth above-ground to shoots and leaves (Johnson, 2010; Sochorová *et al*., 2016).

Uncertainty about the impact of increasing nutrient levels limits the ability to predict responses of grasslands to global changes in resource availability. To solve the problems coming from eutrophication of ecosystems, investigation and professional understanding is necessary. And thus, since the middle of nineteen century, long-term fertilization experiments have been set up to investigate the influence of nutrient amendments. Long-term experiments are especially valuable as they are able to provide information on comprehensive interactions between nutrient input, soil and biomass chemical properties, species composition, productivity and yield quality noticeable only after several decades of management (Schellberg *et al*., 1999; Liu *et al*., 2012). Understanding the underlying mechanisms causing plant responses to differently managed soils may facilitate predictions of ecosystem responses to anthropogenic eutrophication of terrestrial systems.

We studied the responses of *Zea mays* plants to the soils affected by long-term fertilizer treatment to test the following hypotheses: H_1: Enrichment with nitrogen, phosphorus and calcium will significantly affect soil chemical properties; H_2: Soil chemical properties (soil pH, content of nutrients in the soil) will have significant effects on morphologic and qualitative parameters of investigated plants; H_3: Soil amended with more nutrients will result in higher height of plants; H_4: Fertilization treatment will increase plant dry above-ground biomass and number of leaves of the plant.

Materials and methods

Study sites

Soil samples were taken from the long-term Rengen Grassland Experiment (RGE) set up in 1941 by University of Bonn in the Eifel mountains (Germany, 50°13' N, 6°51' E) at 475 m above sea level. A mean annual temperature at the study site is 6.9°C and an average annual precipitation is 811 mm (Rengen meteorological station). Before the establishment of the experiment, the area was extensively grazed, dominated by *Calluna vulgaris, Nardus stricta* and *Danthonia decumbens*. The soil type was classified as a pseudogley (Arens *et al*., 1958) indicated by temporarily wet conditions in the turf layer after rainfall in spring and winter and slow vertical rise of capillarity water in summer. Experimental plots were arranged in two blocks containing 5 complete randomized replicates with a total of forty experimental units. Each unit was 15 m^2 (3 m x 5 m). Experimental plots were cut twice a year in late June to early July and in mid-October. More details on RGE are given in Schellberg *et al*. (1999) and Hejcman *et al*. (2007).

Soil sampling and experiment set up

For purposes of this experiment plots with four treatment combinations were sampled: limed only, limed with nitrogen, limed with nitrogen and phosphorus, and unfertilized control without any fertilizer or lime input. The focus of this study was on experimental manipulations of nitrogen and phosphorus and so experimental plots with additional application of potassium were excluded, as potassium has only minor effects on plant communities (Chytrý *et al*., 2009). Soil samples were collected from the upper soil layer (0 – 10 cm) on 22 June 2012 from plots covering all the combinations, carefully homogenized and transported to the Czech University of Life Science in Prague and stored at -4°C within 24h of collection. Soil from one plot was used to fully fill each of six 0.3 L pots with soil. Each pot was planted with one germinated seedling of *Zea mays* for nine weeks. Once a week, height of all plants and number of dead and live leaves were counted, flowering noted and all pots were randomized. After determination of the experiment, each plant was cut, dried at 65°C for seven days and weighted.

Soil chemical properties

Soil pH was measured using 30 g of dry soil suspended in 30 ml of deionized water for 20 minutes, centrifuged for another 20 minutes and the soil extracts were filtered through a paper filter to remove floating particles. To determine the soil pH, a combination pH electrode G-P Combo w/RJ (Corning, USA) was used. The concentration of mobile Ca^{2+} ions was measured with a Ca-selective electrode in the supernatants which were brought to pH 7.0 ± 0.2 (using

1% HCl or KOH) and corrected for acidic error using the calibration of the electrode response with solutions of known calcium concentrations at different pH values. Organic carbon and total nitrogen concentrations in the soil were analyzed using a CN analyzer (Flash EA 2000, ThermoFisher Scientific, Waltham MA, USA). Available phosphorus in the soil was spectrophotometrically measured in aqueous soil extracts (3 g dry soil and 30 ml of water) vortexed for 25 hours, centrifuged for 20 minutes and filtered through a 0.22 μm filter. We chose approach of measuring phosphorus availability according to Demaria *et al*. (2005) because soil pH can differ largely between the different soil application treatments.

Statistical analyses

To evaluate differences in soil chemical properties and dry plant above-ground biomass between treatments, one-way ANOVA in STATISTICA 8.0 (StatSoft, 1995) and additional post-hoc test using the least significant differences F-test (P = 0.05) was used. Multiple ANOVA in STATISTICA 8.0 was used to examine the height of plants, flowering appearance and number of live and dead leaves each week over the whole duration of the experiment. Simple linear regression in Statgraphics 3.1 was used to analyze the relationships between the concentration of nutrients in the soil and its application.

Results

Soil chemical properties

The mean pH value was 5.3 in control plots without any liming or fertilization application and 7.1 in plots amended with lime (limed only, limed with nitrogen and limed with nitrogen and phosphorus) and with significant differences between them (Fig. 1, $F = 211.3$, $P < 0.001$). Soil pH ranged from a value of 4.9 in control plot to a value of 7.3 in plot with application of calcium, nitrogen, and phosphorus. Total content of nitrogen in the soil ranged from value of 3.6 g kg^{-1} in the plot fertilized with nitrogen, phosphorus and calcium to 4.1 g kg^{-1} in only limed plot (Fig. 2a, $F = 6.26$, $P = 0.002$). Plant available concentration of phosphorus in the soil ranged from 0.3 mg kg^{-1} in control plot to 9.9 mg kg^{-1} in fully fertilized plot (Fig. 2b, $F = 93$, $P < 0.001$). There was a strong and significant positive effect of phosphorus application on concentration of phosphorus in the soil ($F = 92.98$, $P < 0.001$, data not shown). There was no effect of amount of applied nitrogen on content of total content of nitrogen in

the soil. Plant available calcium concentration in the soil ranged from 70.5 mg kg^{-1} in control plot to 138.3 mg kg^{-1} in only limed plot (Fig. 2c, F = 2.4, P = 0.084). Value of soil carbon ranged from 3.7 % in the plot enriched with calcium, nitrogen, and phosphorus to value of 7 % in plot amended with calcium and nitrogen (Fig. 2d, F = 12.7, P < 0.001). There was found significant relationship between nitrogen and phosphorus application on organic carbon in the soil and no effect of the pH value on the content of organic carbon in the soil.

Plant responses to soil properties

Dry plant above-ground biomass was significantly altered by management treatment. The highest dry plant above-ground biomass was in soil enriched with lime and nitrogen, followed by limed only and fully fertilized soil and control plots (F = 10.86, P < 0.001, data not shown). Height of measured plants was also affected by fertilization treatment. The tallest plants were found in the soil amended with calcium and nitrogen, followed by only limed treatment. Surprisingly, the second lowest height was found in pots with fully fertilized soil with calcium, nitrogen, and phosphorus and the smallest plants grew in soil from control plots (Fig. 3a, Treatment: F = 37.55, P < 0,001; Date: F = 261.36, P < 0.001, Treatment*Date: F = 0.33, P = 0.99). Similarly, plants with highest number of leaves grew on soil enriched with nitrogen and calcium, followed by limed only, fully fertilized treatment and control pots without any fertilizer input (Fig. 3b, Treatment: F = 9.56, P < 0.001; Date: F = 259.37, P < 0.001; Treatment*Date: F = 0.76, P = 0.77). The same trend was observed for dead leaves with the highest number of dead leaves observed in soils amended with calcium and nitrogen but followed by fully fertilized soil, limed only and control treatment (Fig. 3c, Treatment: F = 5.58, P < 0.001; Date: F = 1280.95, P < 0.001; Treatment*Date: F = 1.13, P = 0.30). After five weeks of experimental duration some of plants started flowering (mostly in pots with soil from control treatment but the relationship was not significant).

Discussion

We found that seventy years of different fertilizer treatment caused changes in soil chemical properties which have resulted in significantly different plant responses. Long-term amendment with nutrients caused a wide gradient of soil pH that ranged from slightly acid to neutral. It is known that higher soil acidification is very often associated with long-term

nitrogen input to the soil. Consequent alterations of the soil pH might considerably change properties of the soil, availability of nutrients or the accumulation of soil organic matter (Silvertown *et al*., 2006; Clark *et al*., 2007; Hopkins *et al*., 2011). In the substrate form RGE soil organic carbon was not significantly affected by pH however the accumulation of organic matter under lower pH was observed in some long-term fertilizer experiments (Dieplolder and Schröpel, 2003; Silvertown *et al*., 2006; Hejcman *et al*., 2010). Also, application of nitrogen did not have any significant effects on total content of nitrogen in the soil even though it was applied for seven decades. This observation is in accordance with findings from other grassland studies running for a long period of time (Silvertown *et al*., 2006; Hejcman *et al*., 2010; Hopkins *et al*., 2011). This was probably caused by higher nitrogen uptake by harvested biomass than the amount of nitrogen added to soil by fertilizers and/or due to high mobility of nitrogen in the soil (Marschner, 1995; Vaněk *et al*., 2007). In contrast, phosphorus enrichment positively influenced plant available concentration of phosphorus in the soil which is in accordance with other experiments (Hejcman *et al*., 2009; Hrevušová *et al*., 2009). It is known that phosphorus is low mobile (Marschner, 1995; Vaněk *et al*., 2007) and stays in the soil even decades after the determination of fertilizer application (Hrevušová *et al*., 2009).

The lowest dry plant above-ground biomass on average was observed in control plots without any fertilizer input and was increased with nutrient addition. This is consistent with numerous of other experiments in grasslands (Beltman *et al*., 2007; Hrevušová *et al*., 2009; Loeb *et al*., 2009). Surprisingly, the highest dry plant above-ground biomass on average was found in the soils amended with nitrogen and calcium, not in fully fertilized treatment with phosphorus application as expected. This can be explained either by increased phosphorus limitation in fully fertilized soil or other possible explanation can be insufficient size of experimental pots (Poorter *et al*., 2012). A small container can imply reduction in the availability of water and nutrients for the plant and a small quantity of soil or other substrate. In addition to decreased nutrient availability, small pots may slow or block root growth. When taking into account that most plants have roots with more than 1 m in length very soon after their germination (Drew, 1975; Fusseder, 1987; Jackson *et al*., 1996) this can be very limiting condition affecting plant height, dry plant above-ground bimass or number of leaves. Also, large plants growing in small pots can have a big portion of so-called pot-bound roots and many other secondary consequences (Herold and McNeil, 1979; Poorter *et al*., 2012). Using of small containers can has its advantages such as higher number of experimental pots in growth chamber facilities, increased number of replication or more experiments accomplished. Another advantage can be that the usage of small containers does not exceed the capabilities of the transport to move

the weight of pots with the plant. Still, size of pots have received inadequate attention and consideration in the scientific literature and very often is not even mentioned in the materials and methods section of publication (Poorter *et al*., 2012).

We also found a strong relationship between treatment and height of plants. Again, plant height was the highest in pots with soil amended with nitrogen and calcium and second lowest in fully fertilized soil. Possible explanation can be the same as for the dry plant above-ground biomass. The same trend was observed in number of leaves with the highest number of leaves found in limed soil with nitrogen application, followed by limed only and fully fertilized treatment and the smallest number of leaves found in control plots without any fertilizer input. This is in accordance with functional equilibrium model which predicts that plants allocate photosythates to structures more limited by nutrients. This means that number of leaves should increase with fertilization (Brouwer, 1983; Johnson *et al.,* 2003). The exception in contrary to functional equilibrium model is the fully fertilized treatment which induced the same pattern in responses for number of live and dead leaves, height of the plant and dry plant above-ground biomass suggesting that phosphorus addition limits the plant growth. Also, after five weeks of experimental duration some of plants started flowering mostly in pots with control treatment even though not significantly. This is a common phenomenon, when plant does not have enough nutrients for growth it focuses on reproduction and allocates photosynthates to flowering and seeds (Marschner, 1995; Vaněk *et al*., 2007).

Our findings elucidate the different soil managements associated with increased fertilizer application and thus soil fertility can significantly affect availability and concentration of nutrients in the soil, height of plants, dry plant above-ground biomass, and number of leaves. Therefore, not only balanced application of amendments and also an appropriate size of pot need to be carefully planned before setting experiment.

Conclusions

We have observed that several decades long fertilization with lime, nitrogen, and phosphorus caused changes in soil chemical properties which have resulted in significantly various plant responses. The present study has illustrated that there was significantly different responses in height of plants, number of live and dead leaves and dry plant above-ground biomass among soils treated with different fertilizer option with the highest values surprisingly reaching in limed soils with nitrogen addition instead of expected fully fertilized soils. This can indicate

either increased phosphorus limitation in fully fertilized soils or more likely, it was caused by insufficient size of experimental pots due to the need of adequate number of replications and deficiency of experimental soil. Also, we found that long-term fertilization and liming caused high variation in soil pH, plant available concentration of phosphorus and organic carbon in the soil but it did not significantly altered total content of nitrogen and plant available calcium in the soil.

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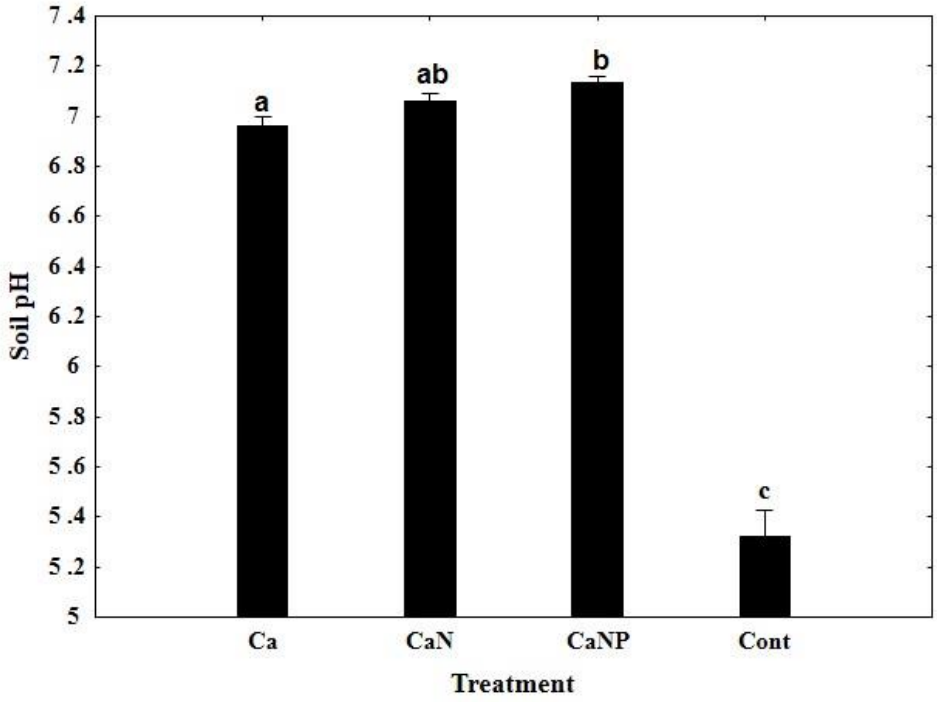
Figure legends:

Fig. 1. Soil pH as affected by the management treatments (Cont – no nutrients input, Ca – lime added, CaN – lime and nitrogen added, CaNP – lime, nitrogen and phosphorus added). Different letter letters indicate significant differences between treatment means (least significant difference F- test, $P < 0.05$).

Fig. 2. Total content of nitrogen in the soil (a), plant-available concentration of phosphorus in the soil (b), plant available concentration of calcium in the soil (c), soil carbon in the soil (d) as affected by the management treatments (Cont – no nutrients input, Ca – lime added, CaN – lime and nitrogen added, CaNP – lime, nitrogen and phosphorus added). Different letter letters indicate significant differences between treatment means (least significant difference F-test, $P < 0.05$).

Fig. 3. The extend of height plant (a), number of live leaves (b), and number of dead leaves of the plant (c). (Cont – no nutrients input, Ca – lime added, CaN – lime and nitrogen added, CaNP – lime, nitrogen and phosphorus added)



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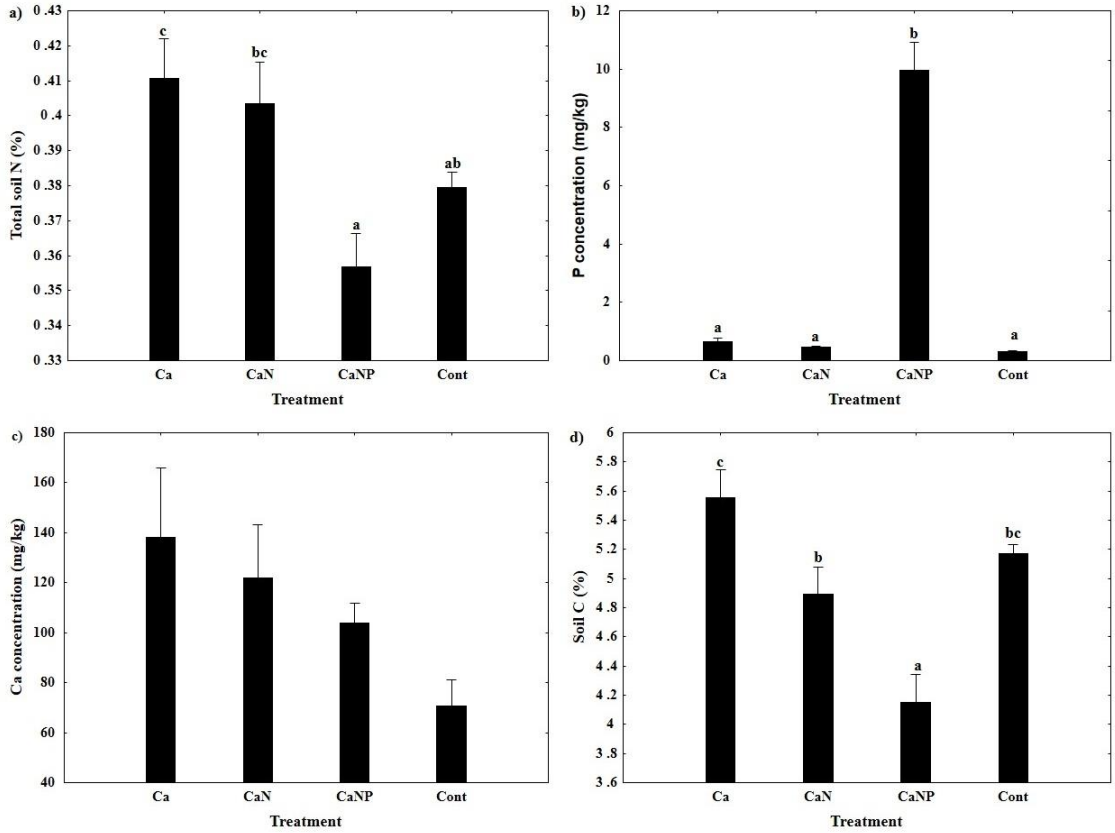

Fig. 2. Total content of nitrogen in the soil (a), plant-available concentration of phosphorus in the soil (b), plant available concentration of calcium in the soil (c), soil carbon in the soil (d) as affected by the management treatments (Cont – no nutrients input, Ca – lime added, CaN – lime and nitrogen added, CaNP – lime, nitrogen and phosphorus added). Different letter letters indicate significant differences between treatment means (least significant difference F-test, $P < 0.05$).

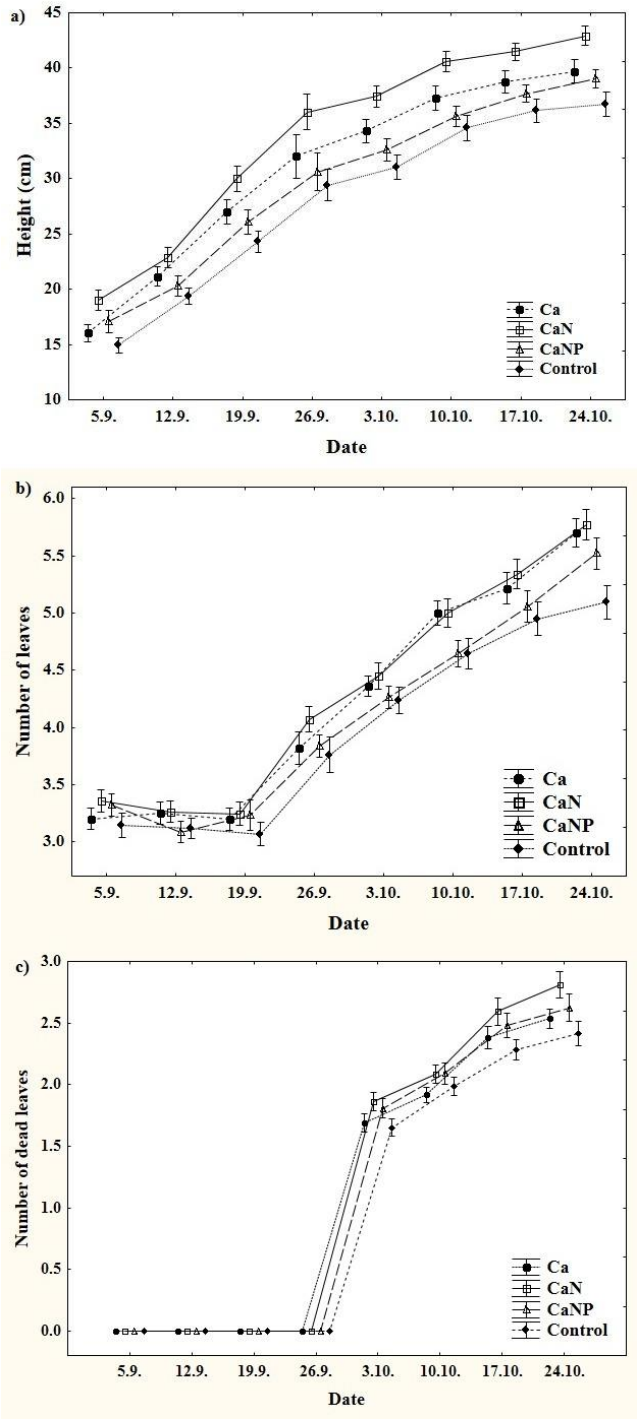

Fig. 3. The extend of height plant (a), number of live leaves (b), and number of dead leaves of the plant (c). (Cont – no nutrients input, Ca – lime added, CaN – lime and nitrogen added, CaNP – lime, nitrogen and phosphorus added)

5. 4 PAPER IV.

Mutabilis in mutabili: Spatiotemporal dynamics of a truffle colony in soil

Gryndler M., Beskid O., Hršelová H., Bukovská P., Hujslová M., Gryndlerová H., Konvalinková T., Schnepf A., Sochorová L., Jansa J.

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Mutabilis in *mutabili*: Spatiotemporal dynamics of a truffle colony in soil

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ABSTRACT

The functioning of ectomycorrhizal (ECM) symbioses is closely related to the development of the soil mycelial phase the ECM fungi. The properties and spatiotemporal dynamics of such mycelia in ecosystems is, however, poorly understood. Here we show, using a soil colony of summer truffle (*Tuber aestivum*) as a model, that an ECM mycelium may only grow and colonize newly-opened soil patches when soil temperatures rise above certain threshold, in this case +10 °C, provided other requirements such as sufficient soil moisture are fulfilled. Extension rates of truffle mycelium in the fields was recorded as >0.3 μm min^{-1}, several-fold greater than that predicted from laboratory cultures. Further, we demonstrated that there was a consistent spatial differentiation in mycelium growth patterns within the fungal colony on a decimeter scale, changing from "diffusion" type of growth at the colony margin to "colony-front" pattern further away from the colony margin. This change was clearly accompanied by shifting structure of soil microbial communities with *Terrimonas* sp. and another unidentified bacterium correlating with the "colony-front" mycelium growth pattern, and *Sphingomonas* sp. and *Lysobacter brunnescens* with the "diffusion" type of mycelium growth. Possible implications of the observed truffle colony differentiation are discussed for processes like fruit-body formation and dispersal of this ECM fungus. Our data indicate that the thallus of *T. aestivum* has to be considered as a principally variable ("*mutabilis*") being in space and time, whose behavior correlates with conditions in ever changing soil environment ("*in mutabili*").

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1. Introduction

Ectomycorrhizal (ECM) symbiosis is a natural phenomenon playing a pivotal role in mineral nutrition, water uptake and co-existence of many tree species, as well as in soil development and ecosystem carbon fluxes (Marx et al., 1991; Smith and Read, 2008). Through their mycelium, the ECM fungi directly interconnect both roots and soil environments. The soil mycelium, unlike the visible fruit bodies of ECM fungi, constitutes the major portion of the fungal thallus (Wallander et al., 2001).

The mycelium of a fungal organism in soil forms up a fungal colony, macroscopic and highly dynamic structure. The colony can grow, degenerate and replace itself in space and time. As the colony is composed of hyphae, the main parameter characterizing the colony behavior is the hyphal extension rate. This parameter varies largely between different fungal species and is a prerequisite for the colonization of new substrates. Hyphal extension rates between 8.7 and 38 μm per minute were reported from in vitro experiments for very fast saprotrophic fungi (*Aspergillus giganteus*, *Phycomyces blakesleeanus*, *Botrytis cinerea*, *Trichoderma viride*, *Mucor hiemalis* and *Neurospora crassa*) already by Trinci and Saunders (1977). *Rhizoctonia solani*, a fungus capable of establishing symbiosis with orchids, grows in vitro at a rate of 5.4–6.2 μm per minute (Bartnicki-Garcia et al., 2000). It is assumed that the growth rates of ECM fungi may be much lower though. Description of in vitro growth of ECM fungus *Pisolithus* sp. (Lagrange et al., 2001) allows calculation of a hyphal growth rate reaching 0.12–0.31 μm per minute. Another ECM fungus, *Amanita muscaria*, grew at a rate of

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0.40–0.55 μm per minute in a glucose-rich medium (Tarkka et al., 2006). Similar values ranging from 0.30 to 1.59 μm per minute were observed for three arbuscular mycorrhizal fungi grown in a soil-sand mixture by Thonar et al. (2011).

The above growth rates, which were mostly measured under axenic (in vitro) conditions, may not reflect the real values in nature. Moreover, the growth rate itself, though very important, does not exhaustively describe the behavior of the fungus under natural conditions. Other important parameters such as mycelium density, the growth pattern ("diffusion" vs. "colony front" growth type, see Section 2.6) or formation of mycelial strands and rhizomorphs remain largely unnoticed. Further, the behavior of the fungus is determined by complex environmental factors, such as (local) soil conditions and annual and diurnal cycles of temperature and humidity.

Studying the growth characteristics of ECM mycelium in-situ requires a robust quantitative approach (Sung et al., 1995). Previously, fungal-specific biochemical markers, such as ergosterol (Salmanowicz and Nylund, 1988) or signature fatty acids (Olsson, 1999) have been used to meet this goal. Later, DNA methods have been introduced with unprecedented specificity and capacity of delivering quantitative results. Real-time quantitative PCR (qPCR) can now be used for estimation of mycelium density of virtually any ECM fungus. If properly calibrated, qPCR can also be used to estimate mycelial mass (weight) per unit weight of soil (Raidl et al., 2005; Parladé et al., 2007). This way, the temporal dynamics, vertical distribution and interspecific competition of *Lactarius deliciosus* mycelium in soil have been approached (Parladé et al., 2007; Hortal et al., 2008). Using a qPCR approach, DeLaVarga et al. (2013) recently demonstrated strong seasonal dynamics of the mycelial growth of *Boletus edulis* and *Lactarius deliciosus*, with the mycelium development coincident with the precipitation intensity. Using competitive PCR as another quantitative PCR-based method, Guidot et al. (2003) observed that *Hebeloma cylindrosporum* can be completely eliminated from the host roots within a single year.

Some qPCR-based studies were already dedicated to the quantitation of the *Tuber* spp. mycelia. Suz et al. (2008) demonstrated correlation between the mycelium density of *Tuber melanosporum* and its fruit body productivity, whereas Gryndler et al. (2013a) showed relatively high *Tuber aestivum* densities in soil, as compared with the mycelial density of other ECM fungi.

All the above results, and many others, represent valuable contributions to the knowledge of ECM fungal biology and demonstrate the arsenal of methods to study the mycelium as a fungal structure of substantial ecosystem-wide importance. However, the studies cited above mostly treat the fungal mycelium only as a biomass dispersed in soil/ecosystem. This approach neglects the fungal organism as living, growing and dynamic entity. To effectively separate the growth and decay, combination of the above methods with hyphal ingrowth cores (mesh bags) is required. Although previously these approaches have been used, they were usually not analyzed in a spatially discrete manner. For example, Wallander et al. (2001) measured mycelial growth using the mesh bags filled with sand and inserted into the studied soil, but analyzed each bag as a whole. The analysis of the homogenized bag content was thus effectively disabling any spatially discrete observation of mycelial structures, rendering those analyses unsuitable for estimation of extension rate of the hyphae or hyphal growth pattern. Further, the growth of ECM hyphae in sand is unlikely to properly reflect their behavior in soil where they come from Hendricks et al. (2006), because of different physico-chemical properties. Our own preliminary observations indicated very different growth rates of *T. aestivum* as depends on the soil used for filling such hyphal traps.

Here we intended to answer four questions regarding the growth and dynamics of *T. aestivum* mycelium under natural soil conditions:

1) What is the extension rate of the growing hyphae?
2) How does the growth rate vary with changing soil conditions (temperature, humidity) around the year?
3) Is the growth rate and/or pattern spatially homogeneous within the fungal colony?
4) What is the identity of microorganisms that associate with the growing mycelium?

To maximize ecosystem relevancy of our results, the entire study was conducted under field conditions, using a field colony of the fungus as a model (Gryndler et al., 2013b). The extent of the colony was identified by so called "brûlé" zone, the area of suppressed vegetation above a truffle colony, and by molecular quantification of the summer truffle in the host (hornbeam) roots sampled within a fine spatial grid (Gryndler et al., 2014). To answer the above questions, we quantified hyphal density of the summer truffle in spatially discrete compartments of newly devised hyphal traps, which were replaced bimonthly throughout a period of 12 months. The traps were filled with local soil devoid of summer truffle DNA, and the truffle development quantified by a highly specific and sensitive qPCR approach (Gryndler et al., 2013a). Microbial communities were then profiled in selected samples by 454-sequencing.

2. Materials and methods

2.1. Truffle growth in vitro

Two isolates of summer truffle (*T. aestivum* Vittad.), ae2 and ae5 (maintained in the Laboratory of Fungal Biology, Institute of Microbiology ASCR, Prague, Czech Republic) were cultivated on the truffle medium PEX (pH 6.3, Pebeyre s. a., Cahors, France), solidified by Phytagel (Sigma P8169), in 90 mm plastic Petri dishes at 4 °C, 10 °C, 20 °C and 25 °C. Increments of the colony radius were measured in 5 replicates after 66 days of incubation and mycelial growth (hyphal extension) rates were expressed in terms of μm per min. Growth rates measured at 20 and 25 °C were considered as the maximum attainable values for the summer truffle and served as the basis for design of the soil traps described below.

2.2. Preparation of soil free of truffle DNA

An amount of 700 g of sieved (2 mm) soil from the truffle locality (Velká Chuchle, Czech Republic, details provided by Gryndler et al., 2013a) was autoclaved at 121 °C for 25 min, re-inoculated with 20 ml unsterile soil filtrate (10 g soil from the same locality shaken with 100 ml water for 1 h, then filtered through a fast paper filter) and incubated for 52 days at 18 °C, with gravimetric water content being kept at 22%. The absence of *T. aestivum* rDNA was then confirmed by the qPCR approach (Gryndler et al., 2013a).

The above soil sample was then used as inoculum to re-inoculate another 1400 g of the same soil autoclaved as above. The soil-inoculum mixture was incubated for 6 months at 18 °C, with its water content being kept at 22%. Following this treatment, the soil lacked any qPCR-detectable amount of *T. aestivum* DNA.

2.3. Soil mycelium traps

The traps were prepared from plastic cylindrical vessels (50 ml polypropylene centrifugation tubes, 28 mm internal diameter, 114 mm length), with their tops (openings) covered by 42 μm nylon

mesh (Silk & Progress, Brněnec, Czech Republic) to prevent entrance of roots and animals (Fig. 1). The bottom of each trap (40% of the volume) was filled with fine (2–4 mm) limestone grit. On the top of it, 41 g of the above (truffle DNA-free) wet soil was placed, physically divided into 5 layers by coarse (1 mm) nylon mesh discs, positioned every 5 mm from the top (Fig. 1). Each compartment between the discs contained approximately 4.9 g soil. The layer between the last disc and the grit zone (27 mm thickness) contained approx. 26.3 g soil. After filling, the traps were stored at −20 °C. One week before inserting them to soil, the traps were removed from the freezer and kept at 25 °C until use.

Eight traps were inserted into the field soil every 20 cm on a linear terrain transect (Fig. 2), 5 cm below surface, slightly tilted down (approx. 10°) to prevent excessive accumulation of water inside the traps (Fig. 1). The traps were first inserted into the soil on 6th March 2013 and then replaced every 2 months (6th May, 7th July, 9th September, 7th November 2013, and 7th January 2014) by a new set of traps (Fig. 2). Last set of traps was collected on 6th March 2014. Upon recovery of each generation of the traps, additional soil samples were always taken from the space between trap locations 1 and 2, 3 and 4, 5 and 6, and 7 and 8 to minimize the disturbance of the soil in proximity of the trap openings.

Soil temperature and humidity were continuously monitored at the experimental site at a depth of 5 cm once every 12 min over the whole duration of the trap incubation (Fig. S1, Supporting information). Soil humidity was derived from the electric conductivity, with the readings being calibrated for gravimetric soil water content and corrected for thermal dependence using the "ratio model" method (Ma et al., 2011). Air temperature at 2 m above ground and precipitation intensities were obtained from a meteorological station 2 km away from the site.

2.4. Sample processing, DNA extraction and qPCR

Once the traps were recovered from the field, they were immediately disassembled and the soil collected from the different layers. The most distant soil (>25 mm from the mesh) from all the 8 traps was mixed and processed as one composite sample F. The only exception was the sampling on 9th September when high rates of truffle growth were expected and fungal ingrowth into these distant compartments was anticipated. In this case, soil samples from compartments F were processed separately for each trap. This enabled us to evaluate the variation of the hyphal density in compartment F in high summer. Soil samples collected in between the traps were processed alongside the trap samples.

DNA was extracted from 250 mg (fresh weight) soil samples using Macherey-Nagel NucleoSpin Soil DNA kit (Macherey-Nagel, Düren, Germany), following manufacturer recommendations, and using extraction buffer SL1 and enhancer SLX. After the initial homogenization of the samples in the extraction buffer, 1.8×10^{10} gene copies of an internal DNA standard (linearized plasmid carrying fragment of cassava mosaic virus, GenBank accession AJ427910) were added into each sample to evaluate DNA extraction efficiency (Thonar et al., 2012).

The DNA extracts were further used as templates in the qPCR to quantify development of summer truffle according to Gryndler et al. (2013a). The PCR reaction mixture contained 2 μl template, 10 μl 2×AB Environmental master mix (Applied Biosystems), 0.4 μl forward primer (25 μM), 0.4 μl reverse primer (25 μM), 0.1 μl probe (25 μM), and 7.1 μl water. With Ta0 marker, the cycling conditions in the StepOnePlus instrument (Applied Biosystems) were: initial denaturation for 15 min at 95 °C, followed by 60 cycles of 10 s denaturation at 95 °C, annealing for 10 s at 60 °C, and synthesis for 10 s at 72 °C. Raw qPCR data were corrected for internal standard losses in each sample as in Thonar et al. (2012).

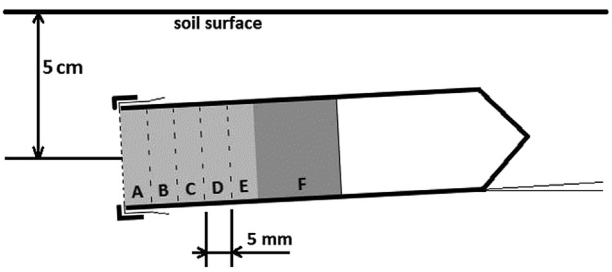

Fig. 1. The trap design and positioning beneath soil surface. Dashed lines indicate the positions of the meshes inside the trap. Layers A through E had an even thickness of 5 mm, whereas the layer F was much thicker and extended 25–42 mm from the 42 μm mesh (dashed line).

2.5. Estimation of hyphal growth rate

Extension rate of hyphae along the trap's longest axis was estimated by fitting the mathematical model of mycelial growth to the measured hyphal length densities inside the traps. These were obtained by conversion of the qPCR results to the hyphal biomass as described in the Supplementary information. The model was originally developed by Schnepf et al. (2008a) for arbuscular mycorrhizal fungi (see Supplementary methods for details). The values of the hyphal densities recorded outside the traps at the different times of the year were taken as the boundary conditions required by the model. Using the model, the parameters **b**, **d** and **v** describing the rates of branching, dying and longitudinal extension of mycelium, respectively, were calculated for different trap positions.

2.6. The 454-sequencing of soil microbiota

Following the quantification of truffle rDNA in the hyphal traps, prokaryotic and eukaryotic communities were analyzed in several soil samples by 454-sequencing of DNA amplicons. The samples were selected so as to represent the two types of hyphal in-growth pattern to the traps observed amongst the field samples, namely the "colony-front" and "diffusion" growth type of the truffle mycelium (see Fig. 2 for identity of the selected samples). The "diffusion" growth type of the mycelium is characterized by the density decreasing monotonously with increasing distance from the surrounding soil whereas "colony-front" type mycelial growth results in the highest hyphal density close to the growing front, i.e. at a distance from the surrounding soil. The obtained sequences have been deposited to the Sequence Read Archive (http://trace.ncbi.nlm.nih.gov/Traces/sra/) under study accession number SRP046077. The relationships between normalized abundances of different eukaryotic or bacterial operational taxonomic units (OTUs) and the truffle mycelium in the hyphal traps were analyzed using redundancy analysis (RDA) and significantly correlating taxa ($p \leq 0.05$) were visualized using t-values biplot with VanDobben circles delimiting the scores of OTUs (redundancy analysis with Monte Carlo permutation test/reduced rank regression, TerBraak and Looman, 1994). The similarity of microbial communities in groups of samples defined by different growth patterns of truffle mycelium growth were evaluated using the principal coordinate analysis (PCoA) at Euclidean distances of log-transformed data. RDA and PCoA were performed using the Canoco 4.5 software package (Biometris, Wageningen).

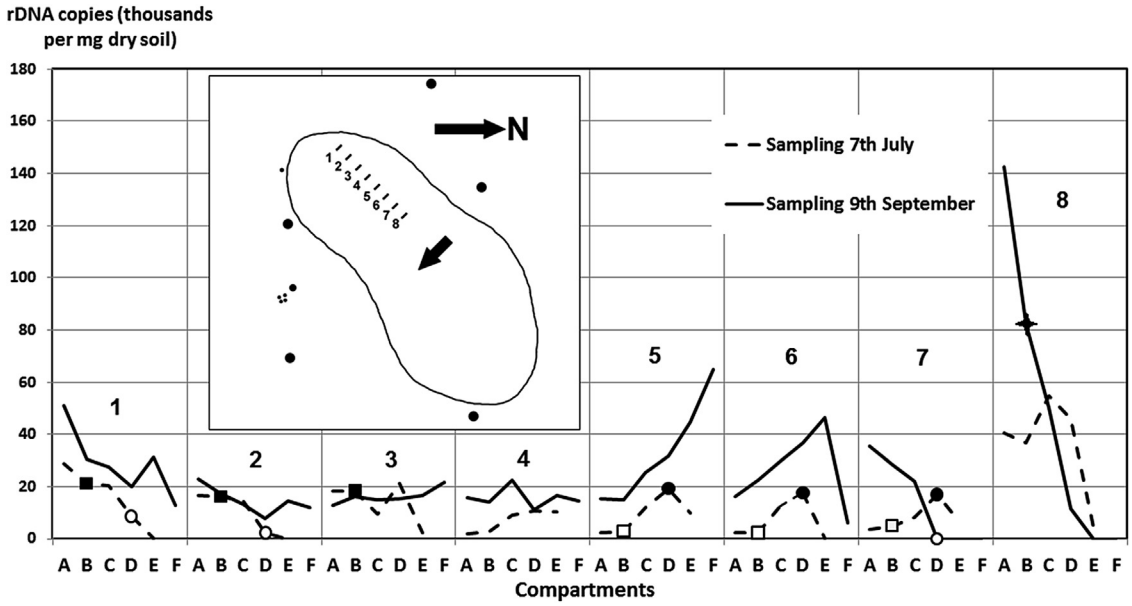

Fig. 2. Colonization by *Tuber aestivum* mycelium of the individual trap compartments as of 7th July and 9th September 2013. Samples highlighted with symbols (see also Fig. 5) were selected for metagenetic analyses of microbial communities. The locations of sampling points 1–8 relative to the brûlé area are shown in the small inserted figure, where the slope orientation is marked by a short arrow and orientation towards North by the long arrow. The length of the long arrow corresponds to 1 m distance. The small black points and circles indicate positions of host hornbeam trees.

3. Results

3.1. Truffle mycelium growth in vitro

The maximum growth rate of the isolate ae2 was recorded at 25 °C and reached 0.235 μm per minute (i.e., 20.7 mm per 61 days), the optimum temperature for the isolate ae5 was 20 °C with the value 0.042 μm per minute (Fig. 3). The growth rates of both isolates at 4 °C and 10 °C were negligible (Fig. 3).

3.2. Truffle mycelium growth in the fields: the rate and the seasonality

The concentration of the truffle rDNA in the soil outside the traps oscillated between 7.3 × 10^3 (January 2014) and 3.3 × 10^4

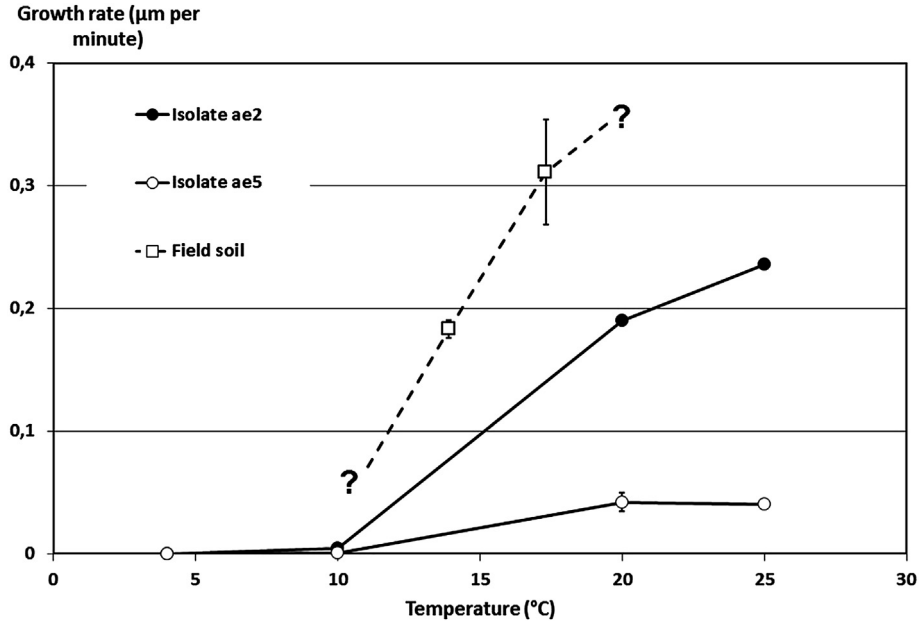

Fig. 3. Growth rate of *Tuber aestivum* mycelium in the field during late spring (samples collected in July) and summer (samples collected in September) as compared with the growth rates of two isolates of the same fungus in vitro at different cultivation temperatures. The estimates of field growth were derived from the mycelium growth model fitted on trap data and projected against the mean soil temperature recorded in the field during the sampled periods. The value calculated for summer period must be regarded as a lower limit rather than the actual growth rate. The question marks indicate that elongation rates of the hyphae in the field at corresponding temperatures could not be estimated on the basis of our data. Vertical bars indicate ±1 standard error of mean (n = 8 for soil mycelium, n = 5 for in vitro growth measurement).

(September 2013) copies per mg dry soil (Fig. 4). The values obtained in January and March 2014 samplings differed significantly ($p < 0.05$) and indicated a 213% increase in mycelial biomass density within the period of two months during late winter.

To characterize hyphal in-growth to the hyphal traps throughout the year (using truffle DNA concentration in the soil as the estimate of fungal/hyphal abundance), compartments A and E (Fig. 1) are the most illustrative as hyphal density in the compartment A reflects the density of growing hyphae in surrounding soil and hyphal density in the compartment E is a proxy of the hyphal extension rate. Furthermore, the compartments A and E usually included the highest recorded values in the traps, covering both the "diffusion" or "colony-front" types of growth (Fig. 2). The recorded hyphal densities in compartment A strongly increased from May to September 2013, reaching a maximum of 3.9×10^4 copies per mg soil. Thereafter, only fractions of the previous values were detected, with values close to 3×10^2 copies per mg soil recorded during winter (Fig. 4). Truffle hyphal densities in the compartment E showed even a greater temporal variation than in compartment A (Fig. 4). Values comparable to compartment A or surrounding soil were detected in July and September, whereas the values before and after this period reached much (3–4 orders of magnitude) lower values (Fig. 4). Although the absolute values of hyphal density were very low during the winter, there was a detectable increase of 189% between the samples collected in January and March, corresponding to the increase in mycelium density in the soil outside traps within the same period (Fig. 4).

The extension rate of the truffle hyphae as per the mathematical model for spring and summer samplings reached the values 0.183 ± 0.007 and 0.311 ± 0.043 μm per minute, respectively (Fig. 3). The summer extension rates were significantly higher than those recorded in spring so that the ratio of trap length and incubation time was small compared to hyphal growth rate. This resulted in all compartments of the traps being effectively colonized by the truffle during the high summer (2 month incubation), disabling estimation of the maximal depth of penetration of the hyphae to the traps. The growth rates calculated for the summer period must thus be regarded as the lower limit of this parameter, given the methodology constraints.

3.3. Spatial variation of T. aestivum mycelial growth

The densities of *T. aestivum* mycelium measured in the different traps showed considerable variability in growth rates and patterns (Fig. 2). Among the traps collected in July, sampling points 1 and 2 (close to the colony margin) showed a "diffusion" type of mycelial growth, the highest mycelial densities being detected in the compartments A and the lowest in compartments E (Fig. 2, broken lines). The growth pattern gradually changed from the sampling points 1 and 2 to points 5, 6 and 7 (deep within the truffle colony), where it resembled a so called "colony-front" growth type with very low mycelial densities in compartments A and B and high densities in the compartments D. The trap at sampling point 8 showed a unique developmental pattern with high density of hyphae in compartments A and B, and the density further increasing towards the compartments C and D (Fig. 2)

The truffle hyphal densities obtained on the same sampling points 2 months later (incubation July–September Fig. 2, solid lines) were generally higher and the pattern of growth slightly changing. The "diffusion" type of growth on sampling points 1 and 2 was still observable but elevated values of mycelial densities were noted in compartments E, falling down in the compartments F. Much higher densities were found in compartments E on sampling points 5 and 6 but not on sampling point 7 which now showed a typical "diffusion" growth pattern. Sampling points 5 and 6 differed greatly in the hyphal density in compartment F, which was very high at sampling point 5 and low at the sampling point 6. The trap at sampling point 8 showed very high mycelium densities in compartments A and B, but negligible density in the compartment E.

Mathematical modeling of the hyphal turnover and branching (Supplementary Figure S2) demonstrated very different behavior of the mycelium in different traps. In the traps incubated in the fields between July and September, the calculated hyphal extension rates gradually decreased from the colony margin towards its center.

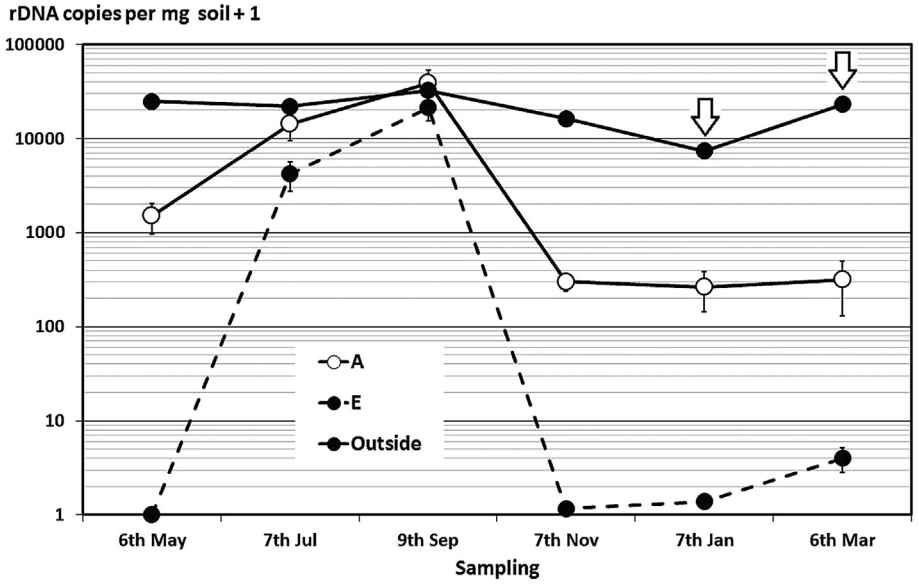

Fig. 4. Seasonal dynamics of *Tuber aestivum* mycelium at a field site expressed as rDNA copy number concentration. The data are shown for free soil (solid line, closed circles) and two trap compartments: A (solid line, open circles) and E (dashed line, closed circles). Vertical bars indicate ±1 standard error of mean. The arrows delimit period of a significant truffle growth (t-test, $p = 0.016$) during wintertime.

Branching rates were somewhat higher close to colony margin in early summer, but in late summer they showed higher values in the traps closer to the truffle colony center. Hyphal death rates showed elevated values in the very center and at the very margin of the truffle colony, with some inconsistencies between early and late summer samplings (Fig. S2).

3.4. Microbial communities in hyphal traps

The PCoA of the 454-sequencing data indicated great variation of the microbial communities between the groups and individual samples (Fig. 5). Prokaryotic communities in compartments B rich in mycelium (group 1) were clearly different from those in compartments D with low truffle mycelium concentration (group 2), with the samples belonging to either group holding together remarkably well (Fig. 5). This trend was less obvious for the eukaryotic microbial community. Composition of prokaryotic and eukaryotic communities in compartments B with low mycelial density (group 3) did not differ from the communities in compartments D (group 4) with high mycelial density. Interestingly, eukaryotic microflora present in the sample 8B extremely rich in truffle mycelium did not deviate from the other samples within group 1, whereas it differed markedly with respect to the prokaryotic profiles, resembling the group 4 instead. The score of the microbial community (both prokaryotic and eukaryotic) of the soil used for filling the traps was always far away from the scores of all other samples.

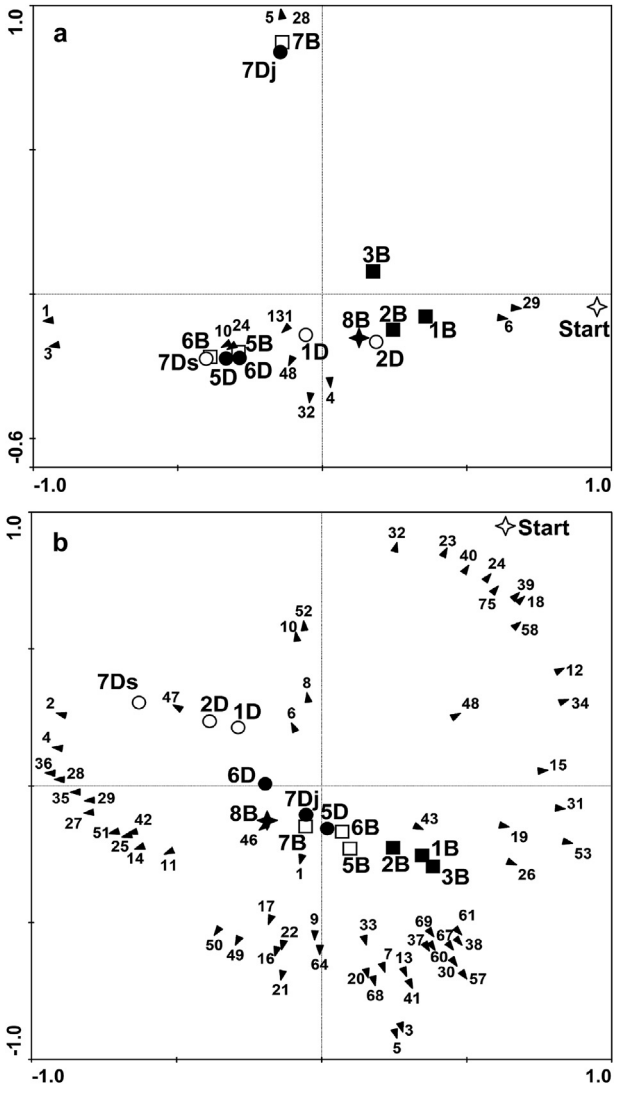

Fig. 5. Principal coordinate analysis biplot visualizing the consistency between the structure of eukaryotic (a) and prokaryotic (b) microbial communities and a-priori sample grouping highlighted in Fig. 2: compartments B with high concentration of *Tuber aestivum* mycelium (group 1, black squares), compartments D with low concentration of mycelium (group 2, white circles), compartments B with low concentration of mycelium (group 3, white squares), and compartments D with high concentration of mycelium (group 4, black circles). Position of the sample with extremely high concentration of truffle mycelium in the soil (trap 8, compartment B, late summer) is shown as a black asterisk. Position of the sample of soil used for filling the traps is labeled with "Start" (white asterisk). The numbers in the titles of sample scores indicate the trap locations in the field. Small numeric identifiers near triangular OTU scores correspond to OTU identifiers listed in the Supplementary file "OTU_Prokaryota_Eukaryota.xls". The characters "j" or "s" added to the titles of the sample 7D indicate that the sample was collected in July or September, respectively.

Abundant eukaryotic OTUs 2, 7, 9 and 66 were not included in the PCoA because they were not present in a majority of samples but may be still worth of interest. As seen in Supporting Table S1, the OTUs 2 and 9 corresponded to unidentified Thelephoraceae, which was abundant in trap locations 1, 2 and 3, where "diffusion" growth type and high mycelium concentration in compartments B was noted. OTUs 7 and 66 corresponded to *Sebacina incrustans* and were the most abundant sequences in B compartment of the trap collected from the location 8 with extremely high *T. aestivum* mycelial density.

The results of RDA (see Supporting Table S2 for details) proved that the prokaryotic community strongly interacted with the mycelial density in the traps and with the growth pattern of the mycelium ($p = 0.002$). First two canonical axes explained almost 55% of variability of species data. On the other hand, interaction of eukaryotic community with both of the truffle mycelium growth parameters was not significant ($p = 0.60$) and first two canonical axes explained only 13.3% of the variability. If the prokaryotic community data were treated by the reduced rank regression, 17 OTUs could be identified to positively correlate with the F_{sc} parameter describing the type of mycelium growth (see Supporting information for details, Fig. 6) whereas only 8 OTUs correlated negatively at $p \leq 0.05$. Of the most abundant prokaryotic OTUs, the positive correlation was noted for OTUs 5 and 3 (*Terrimonas* sp. and an unidentified bacterium, respectively), the negative correlation was detected for OTUs 2 and 4 (*Sphingomonas* sp. and *Lysobacter brunnescens*, respectively). The same statistical approach was used to analyze the prokaryotic taxa correlating with the truffle mycelium density. In this case, only 10 OTUs were detected to show positive and 6 OTUs a negative correlation (data not shown).

4. Discussion

4.1. Growth rate of the hyphae

The longitudinal extension rates of *T. aestivum* mycelium observed in the fields (0.183 and at least 0.311 μm per minute for the traps collected in July and September, respectively) are comparable with growth rates reported for other ECM fungi in laboratory cultures (Lagrange et al., 2001; Tarkka et al., 2006). However, these values are substantially higher than those recorded in the laboratory culture of the fastest isolate of *T. aestivum* ever cultivated

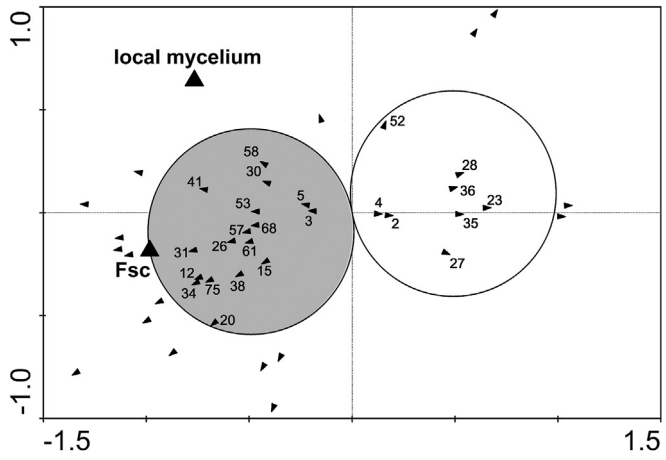

Fig. 6. Central section of t-values biplot with VanDobben circles delimiting the scores of prokaryotic OTUs significantly interacting with *Tuber aestivum* in the trap compartments highlighted in Fig. 2, at $p \leq 0.05$ (redundancy analysis/reduced rank regression). Local substrate colonization factor (F_{sc}, indicating the spatial pattern of trap colonization by truffle mycelium, see Supporting information for details) was used as the main environmental factor and mycelium density (local mycelium) as an additional environmental factor in this analysis. Gray circle encloses the scores of positively correlating OTUs, the empty circle contains the scores of negatively correlated ones. Small numeric identifiers near triangular OTU scores correspond to the OTUs (clusters) identified in the Supplementary file "OTU_Prokaryota_Eukaryota.xls".

in our lab. Interestingly, there is a recent report on expansion rates reaching 2–4 μm per minute of the macroscopic brûlé zone, delimiting the mycelial colony of *T. melanosporum*, which could be taken as a surrogate for longitudinal extension of the truffle mycelium (Liu et al., 2014). However, due to the nature of the studied system, these high rates might be confounded by fast spread of the host tree roots that are colonized de novo from the spores.

We originally thought that the hyphal growth observed in vitro would have the highest possible rate due to absence of environmental limitations. This resulted in underestimation of trap dimensions and/or excessive field incubation times and consequently hampered the estimation of the realized growth rate during the high summer, when the fungus colonized even the furthermost soil compartment within two months of growth. Consequently, the distance of colony front from the trap border could not be directly measured but only indirectly inferred from the mathematical model. Therefore, the values reported here of the mycelium growth rates in high summer shall be regarded as lower limits or conservative estimates of the actual growth rates. Regardless this limitation in summer data analysis, we can conclude that the growth rate observed in field soil was substantially higher than that under in vitro conditions (Fig. 3).

This unexpectedly fast extension of the field hyphae may have been caused by an unknown signal from the host plant or from members of the soil microbial community, i.e. contributors not tested under in vitro conditions. Accompanying soil microflora may strongly affect the growth of various ECM fungi. For example, some Actinobacteria are known to induce hyphal elongation of *A. muscaria* (Maier et al., 2004; Tarkka et al., 2006), and different isolates of the same prokaryotic species strongly differ in their effects on mycelial growth and gene expression in vitro. On the contrary, growth of *Lactarius rufus* was significantly decreased by bioactive compounds produced by *Paenibacillus* sp., with hyphal branching stimulated by the same (Aspray et al., 2013). Some bacteria are also reported to stimulate the formation of *Tuber* ectomycorrhizae (Dominguez et al., 2012), which may result in enhanced streaming of organic carbon to the soil and consequently stimulate the growth of soil mycelium of the truffles.

4.2. Seasonality

Our data demonstrate very strong seasonality of *T. aestivum* in a field ecosystem: explosive colonization of a newly opened soil patch was clearly restricted to a warm season, with average soil temperatures exceeding +10 °C. This information is rather unique within the published literature on ECM fungi, where generally all data is available either on a variation of hyphal abundance in unpatched soil or where hyphal density was observed in patches, but without explicit quantification of the different ECM species (Wallander et al., 2001; DeLaVarga et al., 2013). In unpatched soil, seasonal changes are much less dramatic due to pre-established hyphal networks of up to several hundred meters per g (see Fig. 4 and Supporting information for details). On the other hand, colonization of hyphal traps, especially of the compartments far from the root-excluding mesh, indicates that extension rate of the hyphae is severely restricted by a relatively narrow time window in the course of the year. This extreme seasonality in mycelium growth contrasts with much less prominent seasonality of truffle fructification, which occurs almost continuously throughout the year (Stobbe et al., 2013). Recently, annual fluctuations of hyphal length in soil were continuously monitored by Allen and Kitajima (2013) using a minirhizotron technology. There, the authors described a negative relationship between soil temperature and abundance of the hyphae in arid soil in California. This apparent contrast to our results is possibly explained by very different climatic conditions between the two locations, with the Dfb temperate climate in the Czech Republic (Köppen-Geiger classification, Peel et al., 2007, see Supporting Fig. S1 for details) as compared to arid climate in California. Taken together, the available evidence suggests primary limitation of ECM fungal growth by temperature, with certain levels of soil water availability being a must for their growth potential to realize (Allen and Kitajima, 2013; DeLaVarga et al., 2013)

4.3. Spatial differentiation of the mycelial colony

The variation of growth pattern of the mycelia ("diffusion" vs. "colony-front") throughout the brûlé space is probably the most striking and unexpected result of this study (Fig. 2). It seems that the hyphae at the colony margin preferentially grow longitudinally, resulting in a "diffusion" growth pattern, whereas the hyphae closer to the colony center branch more intensively, resulting in a "colony-front" pattern. This is particularly evident in the data from the high summer, i.e. in the traps harvested in September (Fig. S2). The type of growth at sampling point 8, which is somewhat cross to the big picture outlined above, is likely due to some other factors which might have caused unusually high death rate of the hyphae according to the mathematical model (Schnepf et al., 2008b). One possible explanation of the observed growth pattern is that fungal colony produces sparse exploratory structures (hyphal strands) which are branched only after a certain period of growth through the explored substrate (Agerer, 2001) and this period may vary in different parts of the colony. This hypothesis would be supported by the modeled branching rates from the late summer, but not by the data from early summer, suggesting high branching and death rates at the colony margin (Fig. S2). Interestingly, at some sampling points (e.g., 1 and 2), a change in growth pattern (branching replaced by longitudinal growth) could be observed between early and high summer. If colony front type of the growth is explained by increased mortality (death rate) of the older hyphae and reallocation of carbon and nutrients to newly formed hyphal strands (in accordance to direct observations

by Allen and Kitajima (2013)), increased death rates should be recorded close to the colony center. Yet the observed pattern (Fig. S2) was exactly the opposite (with a notable exception of sampling point 8 discussed above), requiring alternative explanation.

It is possible that the observed differences in the hyphal growth patterns are caused by intrinsic (as yet unknown) factors, related to age-specific differentiation or they are due to interaction with external (abiotic or biotic) cues. Intrinsic causes of any true differentiation would have to be genetically programmed. The fungal colony may either be composed of genetically heterogeneous portions (such as previously described from a colony of *Tricholoma matsutake*, Murata et al., 2005) or the colony is genetically homogeneous, but there is a systematic phenotype differentiation based on differential gene expression (Šťovíček et al., 2014). A good example of this scenario is the fructification, which, strictly speaking, represents a local deviation in gene expression (Lacourt et al., 2002). In this regard, it is interesting to note that in 2010, a single truffle ascocarp has been recovered in the proximity of the trap locations 6, 7 and 8 and other one or two ascocarps were probably collected at this very spot in 2013 by truffle hunters. This may support the hypothesis of the intrinsic programming of the *T. aestivum* colony differentiation. If this notion is confirmed, hyphal growth pattern could be used as an indication of preparedness of the mycelium to produce ascocarps – a hypothesis deserving further testing not only for curiosity reasons but also as a relatively simple bioindicator for the elusive truffle fruit body formation.

Among the external causes, colony differentiation may result from interactions with local soil microflora, which may affect the growth of ECM fungi in different ways (see Section 4.1). At the same time, the spread of soil microorganisms results in a kind of mosaic, with component dimensions ranging from micrometers to tens of meters (Gryndler et al., 2010; Kadowaki et al., 2014; Raynaud and Nunan, 2014). This should create a mosaic of diverse functional relations between organisms, including *T. aestivum*. Here we demonstrate that it is mainly the prokaryotic microflora in selected trap compartments that correlates with the pattern of truffle mycelial growth. A biological interaction between *T. aestivum* and other soil inhabitants is also suggested by high abundance of mycorrhizal fungus *Sebacina incrustans* in the trap showing the highest recorded *T. aestivum* mycelial densities (sampling point 8). Thus our results support the notion that important interactions between mycorrhizal fungi does not only occur in the mycorrhizosphere (Garbaye, 1994) but also in the "bulk" soil.

A question may appear whether the mosaic-like character of the distribution of microbial communities is determined by the non-homogeneous distribution of roots in the soil volume. This might hypothetically cause the observed non-homogeneity of the growth parameters of *T. aestivum* mycelium. Though we did not directly measure the density of roots in the proximity of our hyphal traps, we bimonthly sampled the soil between the traps to observe annual fluctuations of the density of *T. aestivum* mycelium, and picked the roots to remove them from samples. The fine roots were always found in samples, but no obvious pattern was noted in their distribution over the sampling transect. The density of fine roots formed under the dense host canopy thus seems to be high and rather homogeneous, resembling a continuous mat structure.

4.4. *T. aestivum* as a peculiar organism with specific growth requirements

Our study indicates that the thallus of summer struffle behaves in its natural environment in a substantially different way than when cultivated under the lab conditions. Mainly, the laboratory extension rates of *T. aestivum* heavily underestimate the rates observed in the field soil. The reason of this phenomenon is unknown and may present a challenge for further studies. The same may apply to other ECM fungi, though the relevant data are not yet available. It is possible that the mycelium extension rates of ECM fungi in their natural environments may be close to those of saprotrophic fungi and that the "growth potential" of these two fungal groups is similar and also limited by similar factors such as the rate of nutrient transport or the rates of synthetic processes involved in biomass building.

As the thallus of any soil fungus must colonize a very heterogeneous soil environment, it must exploit a whole range of phenotypic adaptations to achieve a good fit into the very local ecological niches – it is inconstant, variable ("*mutabilis*") within the varying soil environment ("*in mutabili*"). The colony thus differentiates – some parts behave differently than others, as also demonstrated in this study. We believe that differentiation of colonies of ECM fungi may be a prerequisite for initiation of such important events as is the fructification. However, this topic is currently *terra incognita* and needs more focused attention in the future. The research should pay particular attention to the zonality of soil fungal colonies, not only in terms of varying hyphal density but also in terms of spatial patterns of gene expression as a sign of colony differentiation. These efforts should always be accompanied by monitoring of abiotic and biotic properties of the soil and by manipulative experiments deciphering the biotic interactions between the fungus and other soil microbes.

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Appendix A. Supplementary information

Supplementary information related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.07.025.

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5. 5 PAPER V.

Soil receptivity for ectomycorrhizal fungi: *Tuber aestivum* is specifically stimulated by calcium carbonate and certain organic compounds

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Submitted to: *Mycorrhiza*

(Photo: Lenka Zemková)

Soil receptivity for ectomycorrhizal fungi: *Tuber aestivum* is specifically stimulated by calcium carbonate and certain organic compounds.

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Abstract
Mycelium of ectomycorrhizal fungi colonizes and interconnects two environments – biochemically and biologically rather stable roots and the surrounding soil which is extremely heterogeneous in terms of spatial arrangement and high diversity of compounds and microbial communities. Here we addressed which of the soil components would have the greatest influence on hyphal development of summer truffle (*Tuber aestivum*). We tested a range of inorganic and organic compounds and bacterial strains isolated from truffle hyphosphere, added to truffle grounds in small compartments accessible exclusively to the hyphae and not to host plant roots. Our results showed stimulation of truffle hyphal growth by lime powder addition, albeit in calcareous soil. Further, we compared the development of truffle hyphae through several different (unsterile) soils, and we also addressed hyphal response to a range of inorganic or organic compounds or bacterial cultures mixed with an inert carrier. We recorded significant stimulation of the hyphal growth by several organic compounds (gallic acid, cellulose and calcium formate), whereas no significant stimulation was observed due to any of the inorganic compounds or bacteria. These results indicate that the development of hyphae of *T. aestivum* in soil may well require complex and micro-heterogeneous environment, where specific organic compounds and calcium carbonate play particularly important roles.

Keywords
summer truffle, soil, gallic acid, cellulose, formate, heterogeneity, hyphal responses, quantitative real-time PCR (qPCR)

Introduction

The true truffles (including *Tuber aestivum*) are ectomycorrhizal fungi exploiting the nutrition provided by the host tree. In spite of their symbiotic life style, mycelia of true truffles have been observed not only as associated with true ectomycorrhizae but, at least in case of *T. aestivum*, they were also detected in the microbial film attached to the surface of decaying layers of suberinized cells of host roots beyond ectomycorrhizas (Gryndler et al. 2013b) as well as in the roots of many herbaceous plant species present at the natural locality of *T. aestivum* (Gryndler et al. 2014). The true truffles can significantly interact with the soil environment, which, in turn, may be important for the development of their mycelia and consequently possibly also for their fructification (Bencivenga and Granetti 1989; García-Montero et al. 2009a,b; Valverde-Asenjo et al. 2009). The complexity of these biological and ecological factors and their unknown interactions are likely the reasons for the variability of sporocarp yields in plantations and natural truffle grounds (Kües and Martin 2011).

The effects of organic compounds on mycelia of ectomycorrhizal fungi can be hypothetically very important because these fungi have considerable saprotrophic potential (Barry et al. 1993), though the use of this potential in the field is not satisfactorily known. Physical association of hyphae with organic particles in the soil may indicate a kind of utilization of such substrate or at least some of its degradation products, released by accompanying, typically saprotrophic microorganisms. The study of the effects of *T. aestivum* accompanying microbes is also justified by known stimulation of ectomycorrhizal fungi by saprotrophic helper bacteria (Dominguez et al. 2012).

Further, mineral nutrients such as phosphorus and nitrogen play very important roles in physiology of ectomycorrhizal symbiosis. These nutrients may be present in soil solution or can be liberated from decomposing organic matter (LeTacon et al. 2015). Then, mycelium of *T. aestivum* should explore the soil microsites enriched in organic or mineral nutrients to gain access to these compounds. In particular, available calcium seems to play a crucial role in the receptivity of the soil for true truffles (García-Montero et al. 2009a,b) and thus merits particular attention from both scientific and practical application perspectives.

Ectomycorrhizal fungi, as the inhabitants of the soil, are deeply embedded in complex interaction networks including both abiotic and biotic soil components. They should, in theory, respond to experimental manipulations of the availability of various forms of organic matter as well as of mineral nutrients. However, there have been little progress in understanding the possible effects of various soil amendments on the growth of truffle mycelium, in spite of the fact that molecular methods enable relatively simple experiments in this field. There are several basic unresolved questions directly related to the *Tuber* life strategy and its local ecophysiological function that can be straightforwardly answered via the analysis of the mycelial colonization of traps containing various bait materials. Therefore, within the present field study we aimed at answering the following questions:

1) Is the truffle mycelium development affected by organic matter such as tree litter, which is the most abundant source of soil organic matter at truffle grounds?

2) Is the content of main mineral nutrients (including calcium) important in this regard?

3) What is the role of microorganisms co-inhabiting the same soil volume as the truffle mycelium, in its development?

Materials and Methods

Trap fabrication

Two types of traps have been used in the study. The Type 1 traps contained autoclaved and microbially recolonized soil from the *T. aestivum* natural locality and were fabricated from 50-ml conical plastic centrifugation tubes and root-exclusion mesh exactly according to Gryndler et al. (2015). The volume of the soil (a mixture of soil samples 4, 5, 6 and 7, see below, autoclaved and recolonized by saprotrophs) in traps was divided into 5 mm thick compartments which were analyzed separately. The compartments are named A (the most proximal to the free soil/tube opening), B, C, D and E (the most distant from the free soil). The compartment A was separated from the free soil by 42 μm nylon mesh (Silk & Progress, Brněnec, Czech Republic) to prevent entrance of roots and animals.

The Type 2 traps were fabricated from 1.5 ml Eppendorf tubes with removed lids and covered by 42 μm nylon mesh. The internal space of these traps was not divided into compartments and was filled with various materials as indicated below.

Tested materials

Effects of calcium carbonate and pulverized hornbeam litter on *T. aestivum* mycelium were tested using the Type 1 traps. The litter was mixed with soil at a concentration of 1% (w/w). Calcium carbonate ($CaCO_3$) was used anhydrous, finely pulverized (particle size < 25 μm), at five different concentrations to reach 10, 30, 100, 1 000 and 10 000 mg of added Ca per kg soil. Soil without any additive was used in control traps. The soil samples (mixed with calcium carbonate or not) used for the trap filling were also subjected to measurement of water extractable and exchangeable calcium.. Briefly, the samples were suspended in deionized water (1:2, w:w) and saturation concentration of Ca^{2+} was measured using ion selective electrode in the supernatant after 12 h of incubation with shaking under room temperature at pH 7.0. Exchangeable Ca^{2+} was measured in 10 g soil extracted with 100 ml 1M KCl for 12 h upon shaking. The extracted Ca^{2+} was then measured using ion selective electrode, the measurement being corrected for contribution of K^+ as interfering ion in the extractant.

Three different sorts of materials were tested for their effects on growth of *T. aestivum* mycelium using the Type 2 traps: unsterile soils, chemical compounds (inorganic and organic), and bacterial cultures.

Seven different soil samples were tested in the Type 2 traps. The soils were the uppermost (0-10 cm) anthropogenic layers of three arable soils (samples 1-3) and four different samples (4-7) from the locality where *T. aestivum* was found, but not directly from the colony of this fungus (Gryndler et al. 2014, see supplementary information).

Soils were sieved through a 2 mm sieve to homogenize and to reach the same maximum particle size as the Perlite used in the other Type 2 traps. The aliquots produced from soils 4, 5, 6 and 7 (*T. aestivum* locality) were mixed. The soil samples were subjected to measurement of water extractable and exchangeable calcium as described above. The total carbonate content in the compound soil sample (mixture of samples 4-7, 2 mm fraction) was 16% (volumetric method using 10% HCl, expressed as $CaCO_3$).

The soil types, pH, water extractable Ca^{2+} and exchangeable Ca^{2+} levels are given in Table 1.

The absence of the mycelium of *T. aestivum* was tested in all 7 soil samples before they were used to fill the traps using PCR with species-specific primers Tu1sekvF/Tu2sekvR (Gryndler et al. 2011). Before the use, the soils were tested for their ability to admit the truffle mycelium in vitro as follows: the aliquots of 500 mg fine (0-2 mm) soil fraction were sterilized by gamma irradiation (25 kGy) and located excentrically to the surface of the solid PEX medium (25 ml volume) in 90 mm plastic Petri dishes inoculated with *Tuber aestivum* culture, isolate Tae2 (Fig 1). After 12 weeks of incubation at 25 °C, the cultures were visually inspected and the growth of mycelium through the soil sample was noted in all cases. No distortion of circular colonies or any obvious changes in aerial mycelium density were observed, which would be induced by the presence of the soils.

Chemical compounds mimicking the compounds that could potentially be met by the fungi in the soil (cellulose, chitin, laminarin, DNA, gum xanthan), sources of mineral nutrients (potassium phosphate, ammonium phosphate), components of root exudates of potential hosts (quinate, lactate, formate, acetate, oxalate, gallic acid) and potential organic soil amendants (pulverized hornbeam litter, cut cardboard) were used. Theophylline was included as a low-molecular heterocyclic source of organic nitrogen.

Quinate, lactate, formate and acetate were obtained by neutralization of respective 1% acids by 1% calcium hydroxide up to pH 6.3. Oxalate was precipitated from 1% solution by 1% calcium chloride and washed with water. The above calcium salts of organic acids were mixed with perlite at amount of 15 mg per trap. Theophylline and gallic acid were used at a rate of 10 mg per trap. Cellulose, DNA, gum xanthan and laminarin were provided at a rate of 50 mg per trap. Cardboard material was cut into 2 × 5 mm pieces and 1 cm^2 (50 mg), mixed with Perlite, was used per trap.

Ammonium phosphate solution (0.1%, pH 6.3) was imbibed into the perlite so that each trap received a dosis of 1.5 mg.

Bacterial cultures were isolated from soil in the same locality using solid cultivation medium A described in Gryndler and Hršelová (2012). Liquid cultivation medium B (ibidem) was imbibed into perlite in 100 ml Erlenmayer flasks, autoclaved, inoculated by 0.5 ml bacterial suspension in 0.1% $MgSO_4.7H_2O$ and incubated for 8 weeks at 24 °C. After the incubation period, the culture bearing perlite was filled into the traps.

Traps filled with pure perlite were established as controls.

Trap location, incubation and analysis

The locality of the study is covered with deciduous mixed forest with european hornbeam as a dominant tree species. The soil type is Rendzic Leptosol with Silurian limes as a bedrock. The Type 1 traps were immersed in soil from May 14th to July 16th, 2013. The Type 2 traps were buried on July 7th, 2013 and left to be colonize by the mycelium till August 12th, 2013. All traps were buried to the depth of 5 cm, almost horizontally, slightly tilted down to prevent accumulation of water in the traps. Exact positions of the traps within the *T. aestivum* soil colony are given in Supplementary information.

After the incubation period elapsed, the traps were recovered and stored at -20 °C thereafter. DNA was extracted from a 250 mg aliquot (Type 1 traps) or from 1 ml aliquot of

the fillings of the Type 2 traps using Macherey-Nagel NucleoSpin Soil DNA kit (Macherey-Nagel, Düren, Germany), following manufacturer recommendations, and using extraction buffer SL1 and enhancer SLX. After the initial homogenization of the samples in the extraction buffer, 1.8×10^{10} gene copies of an internal DNA standard (linearized plasmid carrying fragment of cassava mosaic virus, GenBank accession AJ427910) were added into each sample to evaluate DNA extraction efficiency (Thonar et al., 2012).

The DNA extracts were further used as templates in the quantitative real-time PCR (qPCR) to quantify development of summer truffle according to Gryndler et al. (2013b). The PCR reaction mixture contained 2 µl template, 10 µl 2×AB Environmental master mix (Applied Biosystems), 0.4 µl forward primer (25 µM), 0.4 µl reverse primer (25 µM), 0.1 µl probe (25 µM), and 7.1 µl water. With Ta0 marker, the cycling conditions in the StepOnePlus instrument (Applied Biosystems) were as follows: initial denaturation for 15 min at 95 °C, followed by 60 cycles of 10 s denaturation at 95 °C, annealing for 10 s at 60 °C, and synthesis for 10 s at 72 °C. Raw qPCR data were corrected for internal standard losses in each sample as in Thonar et al. (2012).

In the case of the Type 2 traps, the samples with extraction losses higher than 99% were excluded from further analyses. The total number of the Type 2 traps prepared per treatment varied between 5 and 10. The numbers of traps included in further analysis (after exclusion of traps with high DNA losses) is given in leftmost margin of the Fig. 4.

Data treatment

For observations of the effect of calcium carbonate amendment, the log-transformed concentrations of the *T. aestivum* rDNA were correlated with the concentration of calcium added to the soil using nonparametric Spearman´s order correlation. The correlation coefficient (r_s) was taken as significant at a level of p lower or equal to 0.05.

For observations of the effect of litter and for all data obtained using the Type 2 traps, rDNA copies in the treatments were compared with those found in control or in soil of *T. aestivum* locality by non-parametric Mann-Whitney test corrected for multiple comparison using false discovery rate method with δ=0.05 (Benjamini and Hochberg 1995).

The concentrations of rDNA copies have been expressed relative to the extracted material volume rather than to its weight, because the materials with very different specific weights were used in the study and the colonization of the volume is of main biological importance.

Results

Effect of calcium carbonate

Increasing amendment of soil with calcium carbonate increased the concentration of *T. aestivum* mycelium. This effect was manifested as significant positive correlation between the *T. aestivum* rDNA gene copy numbers and the concentration of calcium added to the trap soil (Fig. 2). This correlation was significant in compartments A and D. In treatments B, C and E, however, the same but insignificant trend has been noted.

In all treatments, the concentration of *T. aestivum* mycelium was highest in the compartment A and tended to decrease with increasing distance to free soil (trap opening).

Addition of $CaCO_3$ to the soil used for filling Type 1 traps had no detectable effect on water extractable calcium nor the exchangeable calcium in the sample immediately after mixing (data not shown).

Effect of litter

Pulverized litter of the European hornbeam did not show any significant effect on the concentration of *T. aestivum* mycelium in any of the compartments of the Type 1 traps (Fig. 3). Similarly as in the control, the traps filled with litter-amended soil showed monotonous decrease in mycelium density with increasing distance from the trap opening.

Type 2 traps

The most important result obtained using the Type 2 traps is the trend of increasing mycelial density (which was statistically significant for one soil sample) in traps containing soil samples, compared to the control traps filled with perlite. In some cases, the soil samples showed very large values of truffle mycelium concentrations, with the contrast to the controls exceeding 2 orders of magnitude (Fig. 4A).

Other significant effects were stimulations of *T. aestivum* mycelium in treatments amended by cellulose, gallic acid and calcium formate. All the three materials increased the mycelium density approximately by one order of magnitude with respect to the Perlite controls (Fig. 4A).

No significant effects of other organic and inorganic materials (including those rich in phosphorus or nitrogen) were noted. Similarly, bacterial cultures did show no significant, though some tendentious increase in truffle mycelium density.

When the overall effects of bacterial cultures, soils or organic materials were tested using pooled data per amendment class, highly significant effect were obtained for soils but not for the other two groups of materials (Fig 4B). The soil thus significantly stimulates the development of mycelium of *T. aestivum*.

During the analysis of the Type 2 traps we encountered problems connected with the efficiency of the DNA extraction, which obviously correlated with some kinds of trap amendments – e.g., all traps enriched with oxalate showed DNA extraction efficiency below 1% and thus were excluded from further analysis.

Discussion

Calcium carbonate

Our results correspond to the generally accepted opinion that *Tuber aestivum* requires high calcium availability in the soils (García-Montero et al. 2009a,b). At the same time, the concentrations of the added calcium carbonate were luxurious relative to the physiological needs of fungi and plants (Bücking and Heyser 2000). As we observed the simulation of the mycelium development even above these luxurious concentrations, we have to interpret the obtained data as an indication of quantitative physicochemical effects of calcium carbonate rather than a threshold response.

The truffle ground soil used in our work (samples 4-7) had the concentration of water extractable Ca^{2+} close to 3 mmol/kg, which corresponds approximately to 2% of exchangeable Ca^{2+}. The obtained value of exchangeable Ca^{2+} (153 mmol/kg) is close to lower limit of the range reported for Rendzic Leptosols in Czech Republic (Žigová et al., 2014). As the content of calcium in soil used in Type 1 traps in our experiment is well above the minimum reported for *T. aestivum*, we originally supposed that the stimulation of the mycelial growth by $CaCO_3$ amendment would not be observed.

Interestingly, the stimulation occurred even when the $CaCO_3$ addition did not cause the instant increase in extractable and exchangeable calcium in the soil, which would be detectable within a 12 h extraction period. This indicates that though finely pulverized, calcium carbonate crystals need significant time to release detectable amount of Ca^{2+} ions to soil solution. This could well have happened during the two months of incubation of the traps in the field, although we were not able to measure instantaneous increase of calcium availability in our short-term batch extraction.

On its natural localities, *T. aestivum* is reported to prefer soils with the concentration of exchangable calcium at least 140 meq/kg (70 mmol/kg, Chevalier and Frochot 1997) corresponding to 2800 mg exchangable calcium per kg soil. The exchangeable calcium in our rendzic leptosol (sampes 4-7) and both orthic luvisols (samples 1 and 3) is well above this limit. The only soil sample being below the limit is dystric cambisol (sample 2). Nevertheless, even this soil did not inhibit growth of truffle mycelium in vitro, which excludes the possibility that calcium stimulates the mycelium development via co-precipitation of possible harmful anions that might be potentially present in soil.

Our highest calcium amendment (10 g $CaCO_3$ per kg soil, ~40 metric tons per hectare, if 20 cm of the soil profile is taken into account) is much higher than doses of 5000 kg per hectare reported in García-Montero et al. (2009a). According to the cited work, such a high dosis of $CaCO_3$ may result in favorization of *Tuber melanosporum* over *T. aestivum*. This was deduced from the fact that high concentrations of "active" carbonate (i. e. of a finely dispersed $CaCO_3$) were present in brûlé areas with rich fructification of *T. melanosporum*, relative to ascomata producing soil colonies of *T. aestivum* and *Tuber mesentericum* which were generally poorer in carbonate (Garcia-Montero et al., 2008).

Our data on stimulation of mycelium of *T. aestivum* by high doses of $CaCO_3$ do not support the above notion. However, this does not mean that the stimulation of *T. melanosporum* by $CaCO_3$ could not be even more intensive than the stimulation of *T. aestivum*. Alternatively, the lower $CaCO_3$ content in *T. aestivum* brûlé areas might be explainable by higher mobilization of $CaCO_3$ (resulting in soil $CaCO_3$ desaturation) under the influence of *T. aestivum*, compared to the soil affected by *T. melanosporum*.

The observed densities of rDNA copies of *T. aestivum* in the Type 1 traps (compartment A) and Type 2 traps filled with soil roughly correspond to these found previously (Gryndler et al. 2013b, 2014, 2015). This fact seems to be trivial but it must be noted at this point that the Type 1 traps, which have been also used in the latter mentioned report, were filled with autoclaved and microbially recolonized soil, whereas the Type 2 traps in the present study were filled with unsterile soil. Then, it could be concluded that the drastic procedure of sterilization does not affect the receptivity of the soil for *T. aestivum* mycelium, at least when

it is allowed to be precolonized by soil microbes. The same can be said for the dimensions and shape of both trap types, which were very different indeed.

In general, the trap data are burdened with high variability, which is most probably due to the natural variation in the fungal colony growth parameters (Gryndler et al. 2015). In spite of this problem, we were able to detect the influence calcium carbonate amendment in the Type 1 traps and of several kinds of trap amendments in the Type 2 traps.

Hornbeam litter

The lack of significant effects of hornbeam litter on growth of *T. aestivum* mycelium indicates that truffle mycelium stimulation by leaf litter is either weaker than that of $CaCO_3$ or does not exist at all. This suggests that mycelium of this fungus does not directly interacts with the litter, which disagrees with hypotheses about the role of ectomycorrhizal fungi in litter decomposition (Lindahl and Tunlid 2015).

Soils

As it could be intuitively expected, the highest concentrations of *T. aestivum* mycelium were observed in the soils. In spite of large variation of experimental data, when all soil data were pooled, truffle mycelium concentration was significantly higher in the soil treatments than in the control. This indicates that *T. aestivum* mycelium depends on unknown physicochemical soil characteristics (e.g. micropores) which are not met in the inert environment of perlite, and that these characteristics can be found in all the soil samples studied. Unfortunately, no literature data are available that could compare the density of *T. aestivum* mycelium in different soils.

This unknown stimulus present in soils but absent from hitherto used cultivation media may explain relative unwillingness of *Tuber* spp. to grow in pure culture (Fontana 1971, Chevalier 1972).

A standardized approach producing mutually comparable data on mycelium concentrations would be necessary if the results produced by different authors are to be used for a meta-analysis of effects of soils factors on mycelium.. Nevertheless, we believe, such an analysis will be useful to optimize the conditions in the truffle commercial cultivation. Interestingly, the values of mycelium concentrations in different soils varied considerably, which does not correspond to equal growth during the preliminary in vitro test of soil receptivity with laboratory culture of *T. aestivum*. This fact may either mean that the tested fungal isolate maintained under laboratory conditions had lost its original sensitivity to soil properties or the wild mycelium of *T. aestivum* is affected by a vital activity of a microbial component of soil biota that was eliminated by sterilization in our in vitro assay.

Microbial cultures

Microbial cultures did not produce significant overall or individual effects on trap colonization by *T. aestivum* mycelium. This disagrees with a general view of mycorrhizal fungi including *Tuber* spp. as fungi dependent on accompanying saprotrophic organisms (Garbaye 1994, Sbrana et al. 2002, Napoli et al. 2008, Mello et al. 2010, Gryndler et al. 2013a). This may indicate that though saprotrophic microorganisms constitute a kind of living

environment for the fungus, their effects are much weaker than those of the abiotic soil components.

The problem that does not allow to make strong conclusions from the data on effects of microbial component of the soil is that specific microbes accompanying *T. aestivum* mycelium in soil are generally not culturable (Gryndler and Hršelová 2012, Gryndler et al. 2013a) which hampers their use as baits. Then, the possibility that the presence of a particular soil microorganism is indispensable from the normal development of the mycelium still remains open.

Organic materials

Three chemically distinct organic compounds that significantly stimulated the colonization of the traps were gallic acid, cellulose and calcium formate. It is difficult to speculate about possible coincidences with stimulatory effects of these compounds. They are not sources of nitrogen or phosphorus. Polyphenols (containing gallic acid units) present in root tissues of many woody plants probably play a role in regulation of colonization of root tissues by ectomycorrhizal fungi and can possibly also be utilized by some of the fungi as source of carbon (Tam and Griffiths 1993). We thus cannot exclude the possibility that the attraction of *T. aestivum* mycelium by gallic acid could be connected with its potential value as an organic nutrient which is not easily accessible to its competitors. This is supported by the fact that polyphenols (tannins) have been observed to support in vitro growth of *Tuber melanosporum* as a carbon source (Callot 1999, p. 136). Further, the tannins may decrease the exploitation of cellulose by microorganisms (Madritch et al. 2007). This, in turn, may provide an unused pool of available organic matter which may be exploited by truffle mycelium.

Enrichment of the soil with various substrates including cellulose induces substantial changes in the composition of soil saprotrophic microflora (Hanson et al. 2008). Cellulose, as rather easily available carbon source, can be exploited by a number of soil organisms and can sustain so called priming effect, i. e. the release of hitherto sequestered organic carbon which then becomes available for soil organisms (Blagodatskaya et al. 2014). *T. aestivum* then may be sensitive to products of cellulose decomposition as a marker of available complex nutrients. Nevertheless, weak utilization of cellulose as a substrate is also not excluded (Mamoun and Olivier 1991). It is noteworthy in connection with the above facts that some enzymes involved in degradation of cellulose are present in *Tuber* genomes (Kohler et al. 2015, Martin et al. 2010), which suggests the importance of cellulose degradation in nutrition of truffles.

Formate (formic acid) is present in root exudates of woody plant (Sandnes et al. 2005) and represents a compound that may significantly affect fungal metabolism. It acts at least as a regulatory molecule specifically affecting production of N_2O in fungi (Ma et al. 2008) but its role in attraction of fungal hyphae is unknown. It is notable at this point that calcium lactate, calcium acetate as well as calcium quinate did not induce the significant stimulation of truffle mycelium in spite of the fact that they are the sources of calcium. This further suports the idea that calcium alone is not sufficient factor for the development of *T. aestivum* mycelium and that other soil factors are indispensable.

Unfortunately, oxalate hampered the efficient extraction of DNA from trap samples. Oxalate thus seems to significantly interact with soil DNA. It is known to extract allophanic

minerals as well as Fe and Al salts from the soil, which results in a decrease of the DNA binding on surfaces (Saeki et al. 2008). As this compound can strongly affect the solubility of different complex compounds present in soil, it can hypothetically induce the formation of solutes hampering DNA extraction procedure.

Conclusions

Research strategy involving soil traps enriched with different bait materials can deliver very inspirative data on ecology of *T. aestivum*. Though inherently loaded by high variation, it proved to be sufficient in screening the effects of different materials potentially useful in stimulation of truffle mycelium growth in soil.

Our data prove that soil complex environment is important for the development of the fungus, as is high availability of calcium. Our data further suggest that accompanying soil microorganisms, though showing insignificant stimulatory trend, are much less important for the stimulation of *T. aestivum* hyphae than the complete soil.

The fungus responds selectively to different organic materials provided as baits. Yet the information is far from complete and more research in this direction is certainly warranted to unfold the complex web of interactions between truffles and their soil environment.

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Table 1. The pH and extractable calcium levels (±SE, 3 technical replicates) in the studied soils

Soil samples	Soil type	pH (water)	Water extractable Ca (mmol/kg)	Exchangeable Ca (mmol/kg)
1	orthic luvisol	6.6	0.95±0.06	93±7
2	eutric cambisol	7.1	2.17±0.05	57±3
3	orthic luvisol	6.7	0.72±0.08	89±6
4-7 (mixed)	rendzic leptosol	7.6	2.76±0.02	153±2

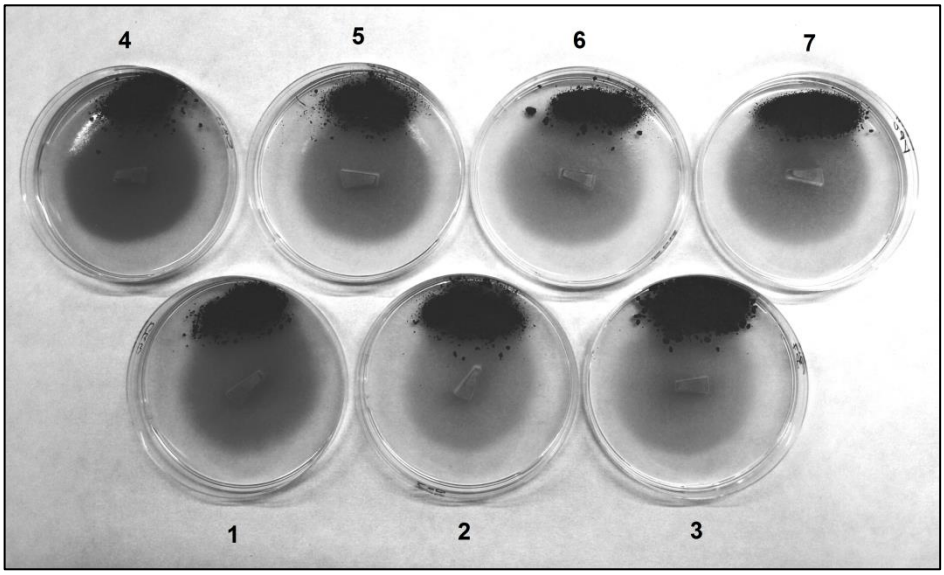


Fig. 1. In vitro cultures of *Tuber aestivum* isolate Tae2 after 12 weeks of cultivation together with gamma irradiated soil samples 1 - 7.

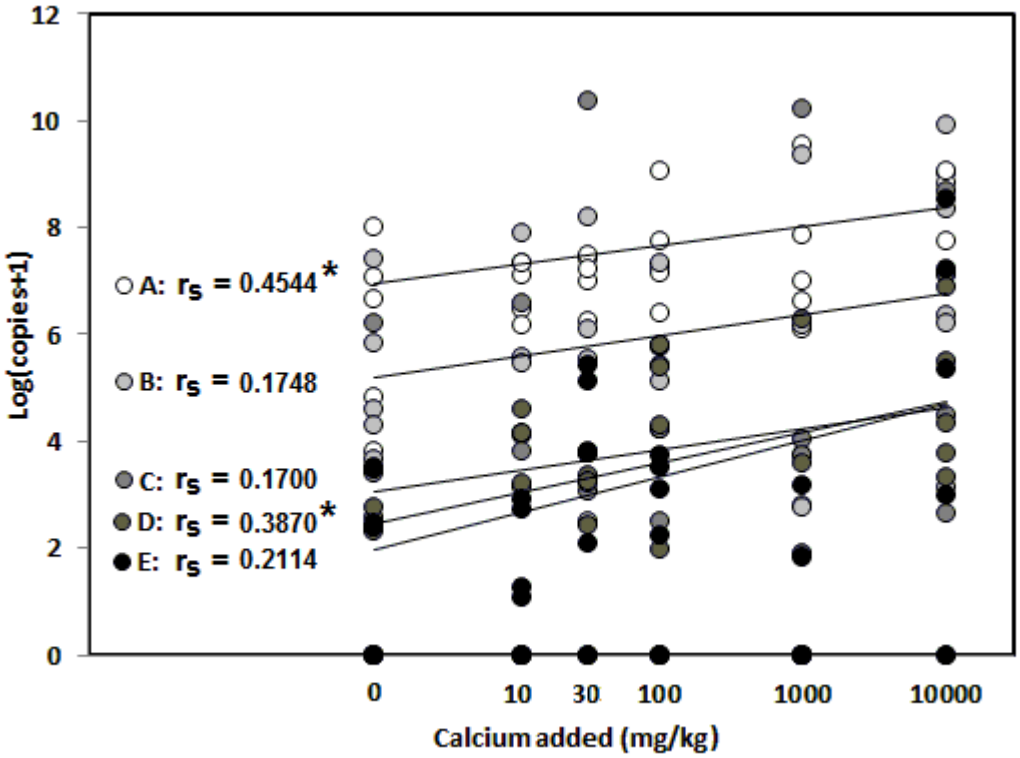


Fig. 2. Effect of calcium added in different amounts to the sterile soil filled in the Type 1 traps on the concentration of rDNA of *Tuber aestivum* per g soil. Different gray saturation levels represent the data from trap compartments A through E. Spearman´s coefficients of order correlation (r_s) followed by asterisks are significant at $p \leq 0.05$. The lines show loglinear trends of correlation. Number of replicates per calcium level and compartment is 5.

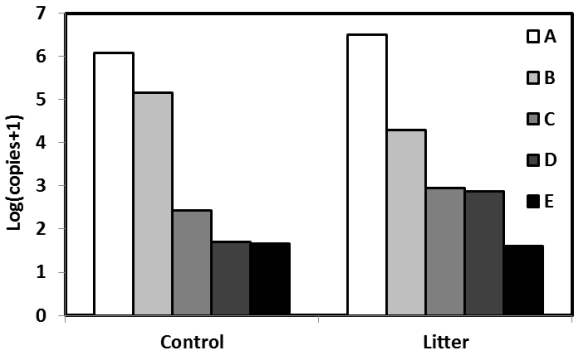


Fig. 3. Effect of pulverized hornbeam leaf litter amendment to the Type 1 traps on the concentration of rDNA of *Tuber aestivum* per g soil. Gray saturation levels present data from trap compartments A through E. No significant differences (Mann-Whitney test, $p \leq 0.05$) were observed between the respective pairs of trap compartments added or not with the litter. Number of replicates for control=5, number of replicates for amended treatment=8.

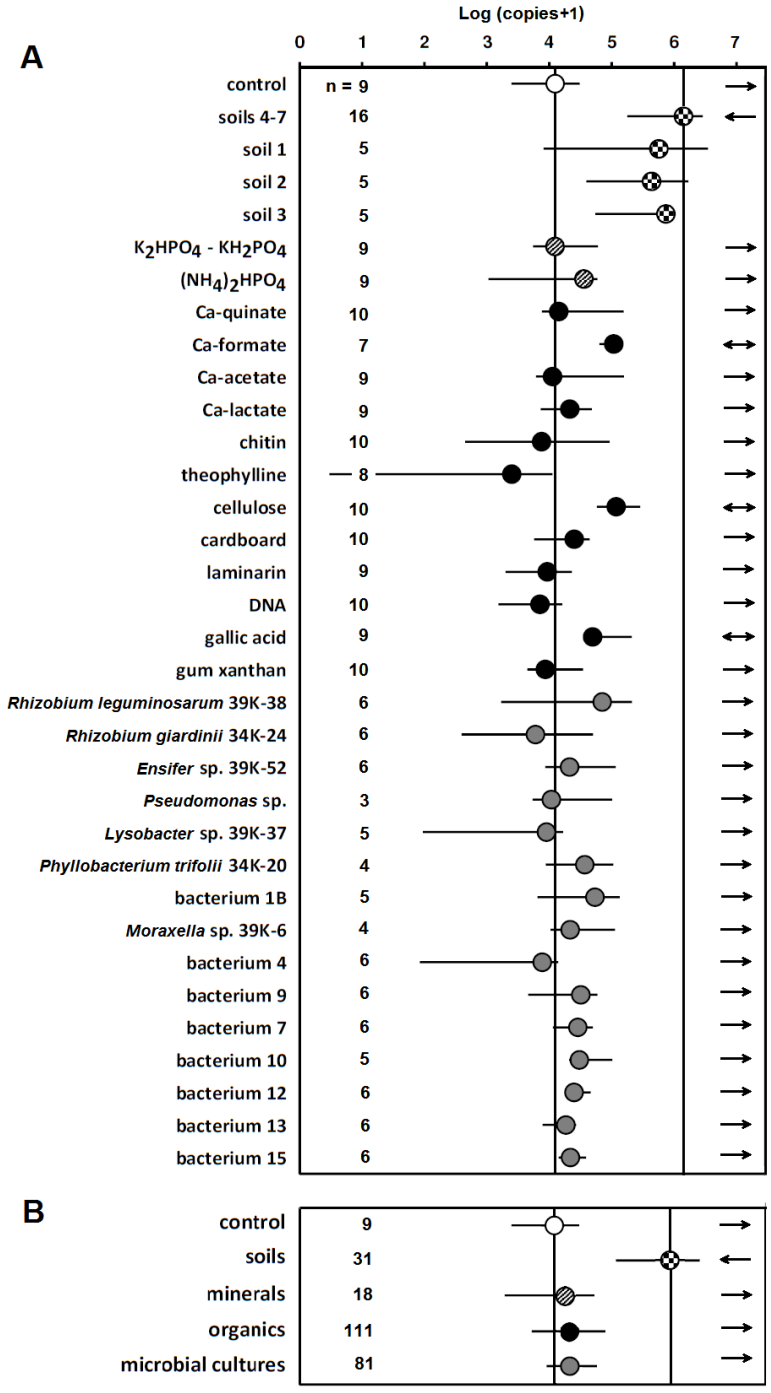
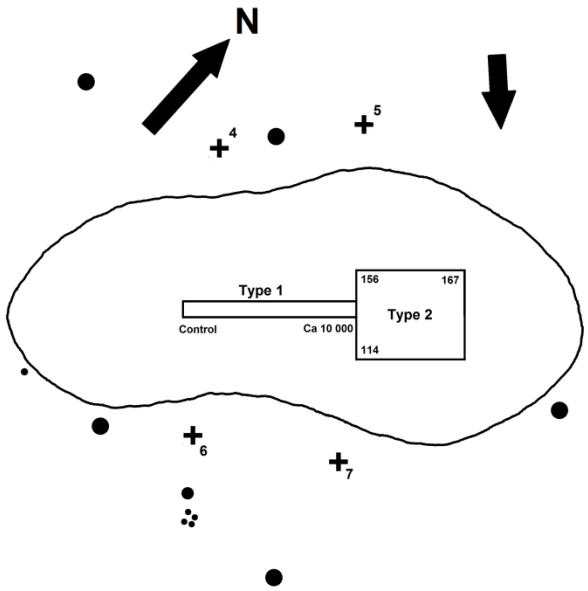


Fig 4. **A** - Effects of microbial cultures, soils, organic or well as mineral additives on the logtransformed concentration of rDNA of *Tuber aestivum* in the Type 2 traps, relative to the control treatment (pure perlite) and treatment with soil from natural locality of *T. aestivum* (vertical lines). n = number of replicates per treatment included into the statistical analysis. **B –** Overall effects of the microbial cultures, mineral nutrients or organic materials compared to pure perlite and soils (vertical lines). The left-peaking arrows indicate that the respective treatment median is significantly different from that obtained in the control treatment with perlite by Mann-Whitney test corrected for multiple comparison using false discovery rate method at δ=0.05. The right-peaking arrows show the significant difference of the respective treatment median from the median value obtained for soil from *T. aestivum* locality **(A)** or from the median value of all the soil treatments **(B),** respectively.

Arrangement of traps within the *Tuber aestivum* mycelial colony in the field



Arrangement of Type 1 traps:

Control (Ca0)	Ca10	Ca30	Ca100	Ca1000	Ca10000	Litter	Litter
Control (Ca0)	Ca10	Ca30	Ca100	Ca1000	Ca10000	Litter	Litter
Control (Ca0)	Ca10	Ca30	Ca100	Ca1000	Ca10000	Litter	Litter
Control (Ca0)	Ca30	Ca100	Ca1000	Ca10000	Litter	Litter	
Control (Ca0)	Ca10	Ca30	Ca100	Ca1000	Ca10000		

Arrangement of Type 2 traps:

156	73	106	186	166	318	116	216	136	251	196	312	266	236	97	282	256	408	241	261	278	300	390	167
417	226	267	360	80	176	246	330	274	294	86	107	227	306	137	117	270	288	87	402	187	197	354	336
126	366	98	348	295	146	283	428	378	157	342	391	247	384	177	217	420	331	337	396	372	415	147	108
319	206	409	242	403	118	262	228	148	138	198	207	74	81	188	139	426	119	367	343	75	158	427	208
307	325	392	88	218	257	252	99	313	168	120	416	140	324	289	237	89	349	301	149	127	355	361	189
379	178	190	284	268	199	338	362	271	326	128	159	275	397	229	179	421	111	296	253	314	404	308	410
209	344	302	385	320	109	90	332	219	373	150	411	263	309	210	243	132	220	285	100	351	180	380	297
290	356	350	101	76	303	238	279	169	414	248	368	429	170		291	82	258	386	230	430	374	151	412
387	315	363	130	181	121	191	160	200	221	91	141	231	327	110	345	321	201	398	276	129	211	394	161
244	333	357	83	212	310	92	152	286	123	272	339	292	102	182	352	381	122	192	171	213	264	405	131
142	322	103	304	172	254	298	232	277	202	334	422	222	369	375	245	364	328	431	399	316	77	413	143
418	370	112	162	193	259	183	340	124	346	249	388	239	269	323	153	395	203	93	358	406	341	425	400
78	94	204	432	423	214	144	280	173	223	299	104	84	163	233	133	376	419	293	389	113	287	371	335
224	365	382	234	134	154	184	359	240	273	305	311	255	194	401	265	174	383	215	235	205	185	135	225
114	164	424	377	329	175	95	317	407	125	353	250	281	347	260	79	115	155	145	165	105	96	85	195	393

The long arrow indicates the direction towards north and its length delimits distance of 1 meter. The short arrow indicates the slope orientation. The small and large dots indicate the positions of hornbeam trees, with the diameter indicating tree trunk size. The closed curved line represents the border of the brûlé area and numbered crosses represent the spots where the soil samples 4, 5, 6 and 7 for filling the traps were collected.

List of treatments and numbering of Type 2 traps:

Trap number	Trap content description	Trap number	Trap content description
(73-79)	(plaster + litter)	282-287	*Rhizobium leguminosarum* 39K-38
(80-85)	(plaster + soil 4)	288-293	*Rhizobium giardinii* 34K-24
86-96	K_2HPO_4 - KH_2PO_4	294-299	*Ensifer* sp. 39K-52
97-105	$(NH_4)_2HPO_4$	300-305	*Pseudomonas sp.*
106-115	Ca-quinate	306-310	*Lysobacter* sp. 39K-37
116-125	Ca-formate	312-316	*Phyllobacterium trifolii* 34K-20
126-135	Ca-acetate	318-323	bacterium 1B
136-145	Ca-lactate	324-329	*Moraxella* sp. 39K-6
(146-155)	(Ca-oxalate)	330-335	bacterium 1
156-165	chitin	336-341	bacterium 2
166-175	theophylline	342-347	bacterium 3
176-185	cellulose	348-353	bacterium 4
186-195	cardboard	354-359	bacterium 6
196-205	laminarin	360-365	bacterium 9
206-215	DNA	366-371	bacterium 7
216-225	gallic acid	372-377	bacterium 8
226-235	gum xanthan	378-383	bacterium 10
236-240	soil 1	384-389	bacterium 11
(241-245)	(soil 1 + plaster)	390-395	bacterium 12
246-250	soil 2	396-401	bacterium 13
(251-255)	(soil 2 + plaster)	402-407	bacterium 14
256-260	soil 3	408-413	bacterium 15
(261-265)	(soil 3 + plaster)	(414-419)	(plaster + 10 mM KH_2PO_4)
266-269	soil 4	(420-425)	(plaster)
270-273	soil 5	426-432	control
274-277	soil 6	311, 317	control
278-281	soil 7		

Treatments shown in brackets were not included in the analyses due to low DNA extraction efficiency.

6. CONCLUSIONS

This dissertation thesis is focused on investigating the impact of long-term fertilizer application on plant and mycorrhizal fungal community and soil parameters in grassland ecosystems and to further examine how the different nutrient amendment (nitrogen, phosphorus, calcium and potassium) changes plant species composition and production, soil and biomass chemical properties, presence, abundance and other parameters of mycorrhizal fungal community and their interaction.

5.1 We concluded that long-term fertilization in Steinach Grassland experiment caused steep gradient of soil chemical properties, namely nitrogen, phosphorus, potassium and calcium availability, soil organic matter and pH. Also, soil chemical properties were responsible for significant differences in above-ground biomass production expressed by sward height. Although decades of different fertilization induced significant changes in species composition on alluvial meadow, this effect was not as high as that resulting from other long-term experiments. Clearly, a high nutrient availability enabled the survival of species that were well adapted to these conditions, even in the unfertilized control. We therefore conclude that the plant species composition on alluvial grasslands responds substantially less dramatically to nitrogen, phosphorus and potassium enrichment then in low productive grasslands. Long-term fertilizer application negatively affected plant species richness directly by soil acidification and indirectly by an increase in above-ground biomass production which caused increasing competition for light and thus supported the exclusion of low competitive species. The magnitude of the negative effects of nitrogen application on species richness was dependent on whether nitrogen was applied alone or in combination with other nutrients or whether nitrogen application acidified the soil.

5.2 We found that seven decades of fertilization in Rengen Grassland Experiment caused large changes in soil chemical properties across the different management treatments, especially plant available phosphorus in the soil was an order of magnitude higher in phosphorus fertilized plots compared to the other treatments. Lime combined with nitrogen application increased hay yields and promoted development of arbuscular mycorrhizal fungal hyphae in the soil, while reducing relative carbon allocation to roots. Simultaneous enrichment of soil with lime, nitrogen, and phosphorus further increased hay production,

promoted grasses and suppressed other plant functional groups (herbs and legumes). This treatment also decreased soil organic carbon and strongly suppressed arbuscular mycorrhizal fungi in the soil, although the response to phosphorus varied among different arbuscular mycorrhizal fungal taxa. Our results indicate that agriculture management practices focused on maximization of hay production may, in long run, significantly reduce below-ground carbon storage. Long-term fertilization can thus induce changes decreasing soil carbon stocks and partly or fully negate the carbon sequestration potential of grassland ecosystem as anthropogenic carbon dioxide sink.

5.3 We observed that long-term nutrient enrichment caused significant changes in soil chemical properties which resulted in different plant responses. Several decades long fertilization induced a wide gradient of soil pH that ranged from slightly acid to neutral. Annual nitrogen application did not have any effects on total content of nitrogen in the soil as well as liming on concentration of calcium in the soil. In contrast, long-term fertilization with phosphorus had significant effect on plant available concentration of phosphorus in the soil. There were significantly different responses in height of plants, number of live and dead leaves and dry plant above-ground biomass among soils treated with different fertilizer amendment with the highest values surprisingly reaching in limed soils with nitrogen addition instead of expected fully fertilized soils. After five weeks of experimental duration some of plants started flowering mostly in pots with control treatment without any fertilization even though not significantly. Our findings elucidate the soil management associated with increased fertilizer application and thus soil fertility can significantly affect availability and concentration of nutrients in the soil, height of plants, dry plant above-ground biomass, and number of leaves.

7. SOUHRN (SUMMARY IN CZECH)

Tato disertační práce je zaměřena na zkoumání vlivu dlouhodobého hnojení na rostlinné společenstvo, půdní parametry a komunitu mykorhizních hub v travních ekosystémech a to konkrétně vliv jednotlivých živin (dusík, fosfor, vápník a draslík) na složení a produkci rostlinných společenstev, chemické vlastnosti půdy a biomasy, společenstvo mykorhizních hub, jejich výskytu, abundance a dalších parametrů a jejich vzájemné interakce.

5.1 Chemické vlastnosti půdy byly v pokusu Steinach Grassland Experiment průkazně ovlivněny dlouhodobých hnojením, především celkový obsah dusíku, dostupný obsah fosforu, draslíku, vápníku, pH a organické látky v půdě. Bylo zjištěno, že chemické vlastnosti půdy způsobily signifikantní rozdíly v produkci nadzemní biomasy mezi jednotlivými variantami vypočítanou pomocí stlačené výšky porostu. Ačkoliv dlouhodobé hnojení této aluviální louky významně ovlivnilo druhové složení rostlin, nebyly tyto změny natolik významné, jako tomu bylo u ostatních dlouhodobých pokusů. Přežití druhů i v nehnojených kontrolních plochách umožnila především vysoká dostupnost živin a dobrá adaptace rostlin na tyto podmínky. Dospěli jsme tedy k názoru, že rostlinné složení na aluviálních loukách nereaguje na dlouhodobé hnojení dusíkem, fosforem a draslíkem tak dramaticky jako na méně produktivních travních ekosystémech. Dále dlouhodobé hnojení snížilo druhovou rozmanitost rostlin, což bylo dáno přímo půdním okyselováním a nepřímo zvyšováním produkce nadzemní biomasy, která zároveň zvýšila konkurenci o světlo a tím podpořila vyloučení méně konkurenceschopných druhů. Intenzita, s jakou dlouhodobého hnojení dusíkem negativně ovlivnilo druhovou rozmanitost rostlin, záležela na tom, zda byl dusík aplikován samostatně či v kombinaci s dalšími živinami, nebo zda toto hnojení dusíkem způsobovalo okyselování půdy.

5.2 Z výsledků práce vyplynulo, že po sedmdesáti letech hnojení v pokusu Rengen Grassland Experiment byly patrné velké změny v chemických vlastnostech půdy v jednotlivých variantách obhospodařovaných různým způsobem, především dostupný fosfor v půdě se lišil o celý řád mezi variantami hnojenými a nehnojenými fosforem. Vápnění spolu s aplikací dusíku zvýšilo produkci nadzemní biomasy a podpořilo rozvoj hyf arbuskulárních mykorhizních hub v půdě, zatímco snížilo relativní alokaci uhlíku do kořenů. Současné obohacování půdy o vápník, dusík a fosfor dále zvýšilo produkci nadzemní biomasy,

podpořilo trávy a potlačilo ostatní rostlinné funkční skupiny (byliny a leguminózy). Hnojení dusíkem a vápníkem také způsobilo snížení obsahu organického uhlíku v půdě a silně potlačilo arbuskulární mykorhizní houby, ačkoliv reakce na fosfor se u jednotlivých taxonů arbuskulárních hub značně lišily. Zjištěné výsledky indikují, že způsoby zemědělského obhospodařování zaměřené na maximální produkci mohou v dlouhodobém měřítku způsobit změny, které snižují zásoby uhlíku v půdě a mohou tak částečně nebo zcela zamezit potenciálnímu ukládání uhlíky do travních ekosystémů, které slouží jako antropogenního uložiště oxidu uhelnatého.

5.3 Na základě toho experimentu bylo zjištěno, že dlouhodobé hnojení dusíkem, fosforem a vápníkem způsobilo signifikantní změny v chemických vlastnostech půdy, které měly za následek rozdílné růstové odpovědi rostlin. Několik desetiletí trvající hnojení vyvolalo rozpětí půdního pH, které dosahovalo hodnot od slabě kyselého po neutrální. Každoroční aplikace dusíku nijak neovlivnila celkový obsah dusíku v půdě stejně tak jako vápnění se neprojevilo v dostupném obsahu vápníku v půdě. Zcela opačný vliv mělo dlouhodobé hnojení fosforem, které ovlivnilo dostupnou koncentraci fosforu v půdě. Byly pozorovány průkazné rozdíly v růstových odpovědích rostlin pěstovaných v různě hnojených půdách (výška rostlin, počet živých a mrtvých listů a suchá nadzemní biomasa). Nejvyšších hodnot bylo překvapivě dosaženo ve vápněné variantě s přídavkem dusíku, ačkoliv bychom je očekávali v plně hnojené variantě s aplikací vápníku, dusíku a fosforu. Po pěti týdnech trvání pokusu některé rostliny vykvetly, především v kontrolních půdách bez jakéhokoliv přídavku živin, avšak neprůkazně v závislosti na variantě. Z výsledků našeho pokusu vyplývá, že způsob obhospodařování půdy spojený s vyššími dávkami dodaných živin a tím také zvýšenou úrodností půdy může závažně ovlivnit dostupnost a koncentraci živin v půdě, výšku rostlin, suchou nadzemní biomasu a počet listů na rostlině.

8. LIST OF PUBLICATIONS

Papers in scientific journals with impact factor:

Hejcman M., **Sochorová L.**, Pavlů V., Štrobach J., Diepolder M., Schellberg J. (2014): The Steinach Grassland Experiment: Soil chemical properties, sward height and plant species composition in three cut alluvial meadow after decades-long fertilizer application. *Agriculture, Ecosystems and Environment* (184): 76 – 87.

Sochorová L., Jansa J., Verbruggen E., Hejcman M., Schellberg J., Kiers E.T., Johnson N.C. (2016): Long-term agricultural management maximizing hay production can significantly reduce belowground C storage. *Agriculture, Ecosystems and Environment* (220): 104 – 114.

Gryndler M., Beskid O., Hršelová H., Bukovská P., Hujslová M., Gryndlerová H., Konvalinková T., Schnepf A., **Sochorová L.**, Jansa J. (2015): Mutabilis in mutabili: Spatiotemporal dynamics of a truffle colony in soil. *Soil Biology & Biochemistry* (90): 62 – 70.

Zemková L. (2016): *Zea mays* responds to 70 years of differently managed soils. *Scientia Agriculturae Bohemica* (Submitted paper).

Gryndler M., Beskid O., Hujslová M., Konvalinková T., Bukovská P., **Sochorová L.**, Hršelová H., Jansa J. (2016): Soil receptivity for ectomycorrhizal fungi: *Tuber aestivum* is specifically stimulated by calcium carbonate and certain organic compounds. *Mycorrhiza* (Submitted paper).

Papers in other scientific journals:

Sochorová L., Verbruggen E., Hejcman M., Schellberg J., Jansa J., Johnson N.C. (2013): Arbuscular mycorrhizal community responses to 70-year soil fertilization. *32nd New Phytologist Symposium*. Argentina. P95 – 96.

Sochorová L., Jansa J.,Verbruggen E., Hejcman M., Schellberg J., Johnson N.C. (2014): Long-term phosphorus inputs change structure of Arbuscular mycorrhizal fungi. *International Congress on Mycorrhizae*. Marrakesh, Morocco. P62.

Papers from scientific conferences in Czech:

Sochorová L., Verbruggen E., Jansa J., Johnson N.C., Hejcman M., Schellberg J. (2012): Vliv dlouhodobého hnojení P, Ca a N na kořeny a mykorhizu – Rengen Grassland Experiment. Sborník abstraktů z konference Kostelecké inspirování, *FŽP ČZU Praha*.

Sochorová L., Hejcman M., Pavlů V., Diepolder M. (2012): Plant species composition and soil chemical properties in alluvial meadow after 75 years of fertilizer application. Sborník abstraktů z konference Biodiverzita 2012, *FŽP ČZU Praha*.

Sochorová L., Verbruggen E., Hejcman M., Schellberg J., Jansa J., Johnson N.C. (2013): Arbuscular mycorrhizal community responses to 70-year soil fertilization. Sborník abstraktů z konference Kostelecké inspirování, *FŽP ČZU Praha*.

Sochorová L., Verbruggen E., Jansa J., Johnson N.C., Hejcman M., Schellberg J. (2013): The effect of long-term fertilization on roots and arbuscular mycorrhizal symbioses. Sborník abstraktů z konference Biodiverzita 2013, *FŽP ČZU Praha*.

Sochorová L., Jansa J., Verbruggen E. (2014): Arbuscular mycorrhizal community responses to 70 years of long term soil management. Sborník abstraktů z konference Biodiverzita 2014, *FŽP ČZU Praha*.