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Mycorrhizal symbiosis in wetlands – the effect of eutrophication on mycorrhizal fungi

Bachelor Thesis

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Annotation: This Bachelor Thesis represents the grant application for project dealing with effects of eutrophication on mycorrhizal fungi in wetland habitats.

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V Českých Budějovicích dne 3. ledna 2011

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Abstract

Wetlands are important and endangered habitats in recent time. One of the factors leading to their degradation is fertilization of neighboring fields and meadows resulting in eutrophication of water and soil. Further, eutrophication has impact on availability of nutrients, on plants and soil organisms and on their interactions and it also affects the biogeochemical cycles in wetland soils.

Little is known about the effect of eutrophication on arbuscular mycorrhizal fungi (AMF) and dark septate fungi (DSE). Therefore, in our project, we suggest to elucidate the effects of eutrophication on colonization intensity of AMF (and DSE) in roots and on biodiversity of AMF in soil and in roots of two wetland plants – *Ranunculus repens* and *Phalaris arundinacea.* In addition to sampling roots and soil directly from experimental field plots (field experiment on wet meadows with three fertilization treatments established five years ago), we will set up a mesocosm experiment with seedlings of *Phalaris arundinacea* and *Ranunculus repens*. We will simulate nutrients inputs and two water levels (low under soil surface and high just on the surface of soil). We will assess the effect of eutrophication and flooding on AMF presence and on colonization intensity of plant roots by AMF. Furthermore we will compare plants colonized by AMF (and DSE) and plants without any symbiosis using sterile soil without any symbiotic organisms and normal soil with living microorganisms. Moreover, seasonal dynamics of AMF colonizing the host plants' roots will be studied too.

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List of abbreviations

AM	Arbuscular mycorrhiza (arbuscular mycorrhizal symbiosis)
AMF	Arbuscular mycorrhizal fungi
C	Carbon
CBE	Chlorazol Black E
DSE/DSF	Dark septate endophytes/fungi
EMF	Ectomycorrhizal fungi
H	Hamr
INVAM	International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi
ITS	Internal transcribed spacer
LSCM	Laser scanning confocal microscopy
LSU	Large subunit
N	Nitrogen
P/Pi	Phosphorus/inorganic phosphorus
PA	*Phalaris arundinacea*
PCR	Polymerase Chain Reaction
RR	*Ranunculus repens*
SSU	Small subunit
TB	Trypan Blue
TBBR	Třeboň Basin Biosphere Reserve
T-RLFP	Terminal-Restriction Length Polymorphism
Z	Záblatské louky

CONTENTS

1. Review

1.1. Introduction

1.1.1. Wetlands

Wetlands are important habitats, with many crucial functions and of a great value for humankind. They can store water during floods and provide thus a natural protection. They take part in stabilization of global nutrient cycles (carbon, nitrogen and sulphur) and they improve water quality. Wetland habitats – when not negatively impacted - are crucial net sinks of carbon dioxide (Verhoeven et al., 2006; Mitsch and Gosselink, 2007).

From the ecological point of view wetlands represent a transition between terrestrial and water ecosystems. The environment of wetlands is quite variable, influenced by fluctuating water level and also by other factors like vegetation cover, nutrient supply, etc. (Mitsch and Gosselink, 2007). This makes them unique habitats. Wetlands are inhabited also by many endangered plant, animal, and fungal species (Primack et al., 2001).

There are soils typical for wetlands called hydric soils. In flooded conditions the diffusion of gases in soil is much lower and after O_2 depletion aerobic metabolism is often replaced with anaerobic one (utilizing alternative electron acceptors instead of oxygen such as NO_3^-, Fe^{3+}, SO_4^{2-}, *etc.*). Therefore wetland plants and other soil organisms have to be adapted to these conditions and they must be able to deal with stress such as anoxia, high salinity and water level fluctuations (Mitsch and Gosselink, 2007).

Eutrophication due to intensive conventional agricultural practices is one of the drivers causing degradation of wetlands all over the world. It results in a change of nutrients availability and soil biochemistry, which, in turn, leads to shift in vegetation and soil organisms. Then, wetlands may become carbon sources (Mitsch and Gosselink, 2007).

1.1.2. Mycorrhizal symbiosis

Mycorrhizal symbiosis is defined as a mutualistic association of fungi with roots of vascular plants, but it is also present in underground organs of the gametophytes of many bryophytes and pteridophytes (Gryndler, 2005; Smith and Read, 2008). We distinguish several types of mycorrhizal symbioses: ectomycorrhizal, endomycorrhizal (arbuscular, ericoid, orchid), and transitional types: ectendomycorrhizal, arbutoid and monotropoid mycorrhizal symbiosis. They differ in function, in typical fungal structures formed and in host plants' spectrum (Gryndler, 2005; Smith and Read, 2008). One fungal species can

form different types of mycorrhizal symbiosis and one plant species can also host different fungi (Smith and Read, 2008).

1.1.2.1. History and abundance of arbuscular mycorrhiza

Partnership of plants and arbuscular mycorrhizal fungi (AMF) is very likely the most widespread symbiosis on Earth (Hart and Klironomos, 2002; Wang and Qiu, 2006). It is estimated that about 90% of higher plant species are mycorrhizal and 80% of them associate with AMF (Trappe 1987. In: Aerts, 2002; Smith et al., 2003a; Smith and Read, 2008). In 2001, AMF were moved from the phylum *Zygomycota* into a new monophyletic phylum – *Glomeromycota* (Schüßler et al., 2001).

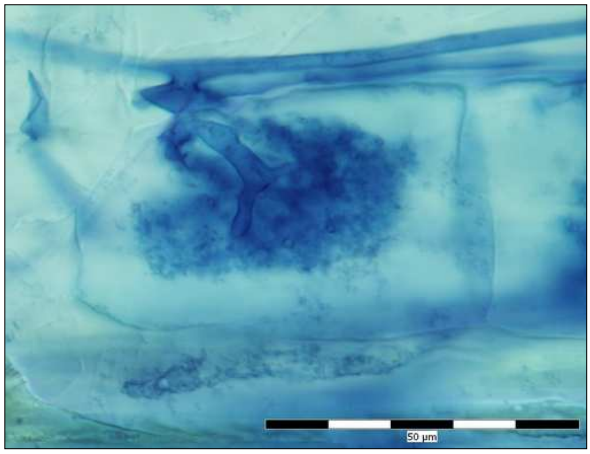
Mycorrhizal fungi co-evolved with their host plants (Schüßler et al., 2001; Brundrett, 2002; Smith et al. 2003a; Smith and Read, 2008). The long history of AMF symbiosis is based on molecular and fossil evidence and reaches back to the Devonian period (Remy et al., 1994). In that time first plants left water and invaded terrestrial ecosystems. Very likely AMF were essential for this transition (Hart and Klironomos, 2002). Among all other types of mycorrhizal symbioses, arbuscular mycorrhizal symbiosis (AM) occurs in all early-diverging lineages of plants and therefore represents the ancestral type of plant-fungal association (Wang and Qiu, 2006). The longevity and world-wide distribution of this type of mycorrhiza suggest that AMF are of a great importance for plant communities and whole ecosystems.

1.1.2.2. Basic features of arbuscular mycorrhizal symbiosis

AMF form a close relationship with their plant partners and AM is distinguishable by consistent structures formed (Smith and Read, 2008). These structures are: intra- and extra-radical mycelium, arbuscules (Pic. 1), hyphal coils and vesicles. Generally it is assumed that vesicles serve as storage. Arbuscules, on the other hand, are centers of metabolism and information exchange between the two symbiotic partners (Gryndler et al., 2005; Smith and Read, 2008).

The association is usually facultative for the plant but AMF are obligatorily biotrophic. Therefore they are completely dependent on the direct supply of organic carbon from their host (Brundrett, 2002; Jakobsen et al., 2002; Gryndler et al., 2005; Smith and Read, 2008). Without symbiosis they cannot complete their life cycle and it is impossible to cultivate them without the host plant (Fortin et al., 2005; Gryndler et al., 2005; Smith and Read, 2008).

The effects of AMF on host plants range from beneficial to antagonistic depending on identity of both partners, season and other environmental or physiological factors (Johnson et al., 1997; Smith and Read, 2008). Their main functions in this association are supposed to be to supply their host plants with water and nutrients (especially phosphorus), to enhance plant resistance to pathogens and protect against herbivores (Smith and Read, 2008). Thus AMF influence fitness and competitiveness of their partners. Plants, in turn, feed them with assimilates produced in the photosynthesis. Parasitic fungi, on the other hand, only use their host as a source of organic substances and the colonization leads to a disease of host plant (Smith and Read, 2008).



Pic. 1: Typical „tree-like“ intracellular arbuscule in the root of topinambur *Helianthus tuberosus*, stained with Trypan Blue. Photo (c) M. Vohník (http://mykorhizy.webpark.cz/)

Plants differ in reaction to AMF and the association between both partners (with its impact on ecosystem functioning) is a result of complex interactions among the AMF, the host plant, soil moisture, and nutrients levels and their stoichiometric ratios. Other important factors affecting symbiosis of plants and AMF are e.g. soil temperature, soil pH and light conditions (Smith and Read, 2008).

1.2. Wetland plants and mycorrhiza

Wetlands are often dominated by species from monocotyledonous families – Cyperaceae, Juncaceae, and Typhaceae (Mitsch and Gosselink, 2007). The *Carex* genus counts in wetlands for approximately 2000 species (Řepka, 2007) and wetlands in temperate zone represent habitats with highest diversity of sedges (Soukupová, 2002).

Members of these families have been long considered non-mycorrhizal (Brundrett, 1991. In: Smith et al. 2003a; Muthukumar et al., 2004; Smith and Read, 2008). According to Powell (1975) wetland plants rather increase their root length to enhance their P uptake than become mycorrhizal (Powell, 1975. In: Turner et al., 2000). However, many recent studies have confirmed that sedges, rushes, and cattails can be – under specific conditions - mycorrhizal (Turner and Friese, 1998; Cooke and Lefor, 1998; Miller et al., 1999; Turner et

al., 2000; Tang et al., 2001; Muthukumar et al., 2004; Gai et al., 2006; *etc.*). Therefore, it is more probable that mycorrhizal symbiosis plays a significant role also in wetland habitats.

1.2.1. Types of mycorrhizal fungi colonizing wetland plants

Plants in wetlands mostly associate with arbuscular mycorrhizal fungi (AMF) (Turner and Friese, 1998; Turner et al., 2000; Muthukumar et al., 2004; *etc.*). There are also several reports on ectomycorrhizal fungi (EMF) presence on wetland plants (Weishampel and Bedford, 2006; Muthukumar et al., 2004). Many wetland plants are also colonized by dark septate endophytes (or fungi) (DSE/DSF) (Jumpponen, 2001; Weishampel and Bedford, 2006; Dolinar and Gaberščik, 2010; Stevens et al., 2010). Their mycorrhizal status is not clear today and therefore they will be described in a separate chapter (1.3.).

1.2.2. Factors affecting the presence and diversity of AMF

Wetland plants can be mycorrhizal but the colonization of their roots can be seasonal (affected e.g. by plant phenology - Bohrer et al., 2004; Escudero and Mendoza, 2005; García and Mendoza, 2008) and/or controlled through the physico-chemical factors of the environment. These factors include: water table level and its fluctuations (Miller and Bever, 1999; Miller, 2000; Ray and Inouye, 2005; Ipsilantis and Sylvia, 2007; Dolinar and Gaberščik, 2010), nutrients availability and their additions (Cornwell et al., 2001; Tang et al., 2001; Aerts, 2002; Ipsilantis and Sylvia, 2007), content of soil organic matter (Gai et al., 2006; Gryndler et al., 2009) or host plant phylogeny (Miller et al.,1999; Lingfei et al., 2005; Gai et al., 2006). The abundance and importance of AMF vary also among different wetland types (Cornwell et al., 2001).

1.2.2.1. Host phylogeny and adaptations to non-mycotrophy

Plants need phosphorus and they take it primarily as inorganic phophate (Pi) from soil. Their growth in most ecosystems is limited by Pi availability but plants have evolved a range of adaptations (Smith et al., 2003b). They include symbiotic associations with fungi or formation of hairy root clusters, carnivory, root hemiparasitism, *etc.* (Cornelissen et al., 2003).

Diverse morphologic structures or/and metabolic mechanism that may represent and adaptation to non-mycotrophy can be found in wetland plants (Davies et al. 1973; Miller et al., 1999; Cornwell et al., 2001; Muthukumar et al., 2004; de Marins et al., 2009). These structures are described e.g. as “hair roots with bulbous swellings” (Miler et al., 1999) or

"swollen lateral roots" (Davies et al., 1973). Davies et al. (1973) observed these roots in many *Carex* species and suggest that they help in Pi absorption. Root clusters in Juncaceae and sand-binding roots in sedges and rushes are described on Mark Brundrett's website (http://mycorrhizas.info/nmplants.html#cap). These root clusters are capable of enhancing the availability of soil Pi (Smith et al., 2003b).

Miller et al. (1999) described three groups within the genus *Carex* that differ in the mycorrhizal status. Species are either: a) always mycorrhizal, b) always non-mycorrhizal, or c) variable in their mycorrhizal status across habitats, season, or other factors. Similar variation in mycorrhizal status was also described in Typhaceae (Cornwell et al., 2001).

1.2.2.2. Nutrients availability and fertilization

AMF take up nutrients from soil with their hyphae and transfer them through the extraradical mycelium into intraradical fungal structures. Nutrients are transported across symbiotic interfaces in the root cortex which include plasma membranes of both partners separated by an apoplastic interfacial compartment. Plants, in turn, supply AMF with products of photosynthesis. AMF get about 4-20% of the total fixed C (Smith and Read, 2008).

Soil nutrient availability and nutrient inputs have a large effect on AMF abundance and their role in the ecosystems. In terms of AMF, nitrogen (N) and phosphorus (P) are the most important elements. Turner et al. (2000) suggest that nutrient (especially P) availability may be the key to understand mycorrhizal colonization in wetland plant species.

As the cycles of N and P have changed through human activities (e.g. increased nutrient depositions via intensive agriculture), it might be expected that also the global abundance of AMF will be altered, together with their effects on the plant hosts, on the plant competitiveness and on ecosystem productivity (Egerton-Warbuton and Allen, 2000; Collins and Forster, 2009).

There are some general trends that might help us predict how the fungi will react to increased nutrient inputs. For instance, increased N inputs result in a relative shortage of P in ecosystems. Therefore AMF species should become more abundant and their role more crucial in the ecosystems (Aerts, 2002; Johnson et al., 2003). It is also known that P availability affects the AMF colonization and arbuscules formation. The effects, however, depends to a great extent on environmental factors and on identity of AMF and their host plant (Treseder and Allen, 2002; Smith and Read, 2008). However, as many studies show, it is difficult to summarize the effect of increased nutrients input on AMF because it is very

variable (Miller, 2000; Turner et al., 2000; Tang et al., 2001; Miller et al., 2002; Treseder and Allen, 2002; Johnson et al., 2003, *etc.*). The effect differs also among genera of AMF and among ecosystems (Treseder and Allen, 2002).

1.2.2.2.1. Phosphorus

AMF are especially efficient in the uptake of inorganic phosphorus (Jakobsen et al., 2002). It is known that plants colonized by AMF tend to have much higher capacity for inorganic P uptake and thus higher P concentrations in tissues than non-mycorrhizal species (Miller and Sharitz, 2000; Jakobsen et al., 2002; Jayachandran and Shetty, 2003; Smith and Read, 2008). Koide and Kabir (2000) confirmed that AMF can utilize organic forms of P, as well. They are capable of hydrolyzing organic P sources and then they transport the nutrients to their host plant.

One assumption would be that if there is enough P available in the soil, plants can take it themselves and AMF will not play such an important role. However, the situation is much more complex (Smith and Read, 2008).

There are different responses of AMF colonization in wetland plants on P availability and addition:

- No relationship (Miller, 2000)
- Low or absent colonization at high P availability (Wetzel and van der Valk, 1996; Lingfei et al., 2005; Smith and Read, 2008; controlled experiments by Tang et al., 2001; Ipsilantis and Sylvia, 2007)
- High levels of colonization in wetlands with low P availability (Turner et al., 2000).

1.2.2.2.2. Nitrogen

N addition has positive, negative or no effect on AMF colonization of plants (Aerts, 2002; Johnson et al., 2003; Mandyam and Jumpponen, 2008). Johnson et al. (2003) carried out an experiment in 5 semiarid grasslands in USA. They showed that N fertilization affects distribution of AM fungal structures in plant roots and alters species composition of AMF. N enrichment increased the amount of hyphae and arbuscules (Johnson et al., 2003). Mandyam and Jumpponen (2008) observed no significant effect of N amendments on abundance of AMF and DSE but it led to a shift in plant community (Mandayam and Jumpponen, 2008). Egerton-Warburton and Allen (2000) observed a shift in AMF community composition: species with larger spores (*Scutellospora*, *Gigaspora*) failed to

sporulate and were replaced by *Glomus* species. In the terms of the mutualistic – parasitic continuum, N fertilization may lead to selection for aggressive, less mutualistic fungi (Johnson, 1993).

1.2.2.2.3. Nitrogen and phosphorus together

Plant and fungal communities and relationships between them are governed not only by the response to N and P alone, but also by N and P limitation and the enrichment of soil by these nutrients together (Collins and Forster, 2009). Soil fertility mirrored in N:P ratio has been one of the best predictors of the effects of N enrichment on AMF distribution (Miller et al., 2002; Treseder and Allen, 2002; Johnson et al., 2003).

Fertilization with N and P together had no significant effect on root colonization by hyphae and vesicles (Treseder and Allen, 2002) or on total density of spores in soil (Johnson et al., 2003). On the other hand, an increase of vesicular colonization after N enrichment and its decrease after P enrichment were found by Johnson et al. (2003).

1.2.2.3. Water level and flooding

AMF are aerobic organisms and their life and survival is dependent on oxygen supply (Smith and Read, 2008). Therefore, AMF have been long considered to have a limited importance in wetland soils because the hydric soil is often anoxic (Keddy, 2000).

Water level, its fluctuations, and flooding belong to abiotic factors that affect the abundance of AMF and the extent of their colonization in host plant roots (Stevens and Peterson, 1996; Turner and Friese, 1998; Miller, 2000; Miller and Sharitz, 2000; Escudero and Mendoza, 2005; *etc.*). Changes in water level and water availability are also connected with redox-potential, amount of dissolved nutrients, or with plant community composition.

1.2.2.3.1. Adaptations of AMF

Organisms had to develop some adaptations to anoxia, increased salinity, and water fluctuations, to survive in wetland conditions (Mitsch and Gosselink, 2007). Miller and Bever (1999) described two mechanisms that could help AMF survive anoxic conditions when colonizing wetland plants. AMF either a) concentrate themselves near plant roots and acquire oxygen directly from the root or from the rhizosphere (Brown and Bledsoe, 1996. In: Miller and Bever, 1999), or b) differ in the tolerance to flooding and those AMF species that require less oxygen can be abundant in wetter habitats (Miller and Bever, 1999).

1.2.2.3.2. Effects observed in field and in mesocosms

Studies show different and sometimes conflicting patterns in how the water level affects AMF and DSE colonization. There are also big differences between field and mesocosm studies.

Field studies have shown that water-logging and soil moisture have either no significant (Bauer et al., 2003; Bohrer et al., 2004) or negative effect on AM fungal colonization levels in plants. Flooding leads to decrease in AM root colonization (Escudero and Mendoza, 2005; de Marins et al., 2009; Dolinar and Gaberščik, 2010) which is positively correlated with the length of non-flooded period (Ray and Inouye, 2006). Others observed higher colonization intensity in dry or intermediate regions (and the lowest in wet regions) along a hydrologic gradient (Stevens and Peterson, 1996; Miller, 2000; Jaychandran and Shetty, 2003).

Mesocosm studies showed higher colonization intensity of plants by AMF in wet treatments (Stevens and Peterson, 1996). Opposite results were observed by Miller and Sharitz (2000) and by Ipsilantis and Sylvia (2007). Plants in water-logged treatments were smaller and less colonized by AMF (Miller and Sharitz, 2000) and AM fungal colonization was almost eliminated by flooding (Ipsilantis and Sylvia, 2007).

Miller and Sharitz (2000) (in a mesocosm experiment) and Dolinar and Gaberščik (2010) (in field) observed that flooding leads to lower levels of AM fungal colonization in plants. However, once the symbiosis is established, flooding has no significant effect on it.

1.2.2.4. Seasonality effect

Seasonal variations in richness of AMF and their colonization intensity have been reported. Some of them show that fungal richness (Šmilauer, 2001) or AMF colonization intensity (Kabir et al., 1997; Bohrer et al., 2004; Likar et al., 2009) may correspond more to the phenology/development stage of the host plant than to environmental abiotic factors such as water level fluctuation or nutrients availability. Furthermore, Mandyam and Jumpponen (2008) suggest that the higher intensity of AMF structures observed late in the growing season may be linked to higher demands for P in plants.

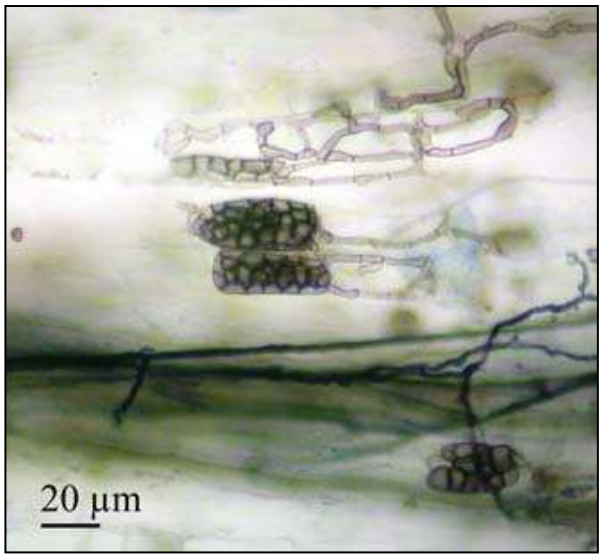
1.3. Dark septate endophytes/fungi (DSE/DSF)

Next to AMF, dark septate endophytes (DSE) or dark septate fungi (DSF) may play an important role in wetlands. Presence of these fungi has been reported also in wetland

plant species that often fail to form mycorrhizal association with AMF (Thormann et al., 1999; Muthukumar et al., 2004; Muthukumar et al., 2006; Weishampel and Bedford, 2006). DSE colonization appears to be most common in nutrient-limited ecosystems. These fungi might play a similar functional role for plants like AMF (Jumpponen, 2001; Muthukumar et al., 2004; Weishampel and Bedford, 2006).

1.3.1. Characteristic of DSE

DSE represent a various and very heterogenous group of ascomycetous fungi with a polyphyletic origin. They include also some fungi forming ectendomycorrhizas (Jumpponen, 2001). They can colonize roots intracellularly or intercellularly without causing obvious negative effects on plant hosts (Jumpponen and Trappe, 1998; Muthukumar et al., 2006). To the typical structures formed belong: appressorium, narrow septate hyphae (melanized or hyaline), coils or microsclerocia (Pic.2) (Weishampel and Bedford, 2006). Intraradical and extraradical hyphae do not differ morphologically (Muthukumar et al., 2006).



Pic. 2: Root of *Eurybia divaricata* colonized by DSE. Several hyphae ending in microsclerotia, which look like grape-like clusters. Retrieved from: http://botany.thismia.com/2010/02/28/dark-septate-endophytes/

DSE are rich in enzymes which allow them to break down dead organic matter and to utilize nutrients from it (Caldwell et al., 2000). They could therefore benefit their hosts in nutrient-limited ecosystems (Weishampel and Bedford, 2006).

1.3.2. Host spectrum of DSE

DSE can colonize a great variety of host plants (Jumpponen, 2001). They usually associate with herbs but they have been detected also in shrubs or trees (Muthukumar et al., 2006). They form frequently associations with monocots (Weishampel and Bedford, 2006; Kandalepas et al., 2010). Sometimes, DSE can co-occur with AMF in one individual (Lingfei et al., 2005; Weishampel and Bedford, 2006; Mandyam and Jumpponen, 2008; de Marins et al., 2009; Kandalepas et al., 2010). Usually one type of the plant-fungus

association is prevalent (Lingfei et al., 2005; Weishampel and Bedford, 2006; Kandalepas et al., 2010).

1.3.3. Effect of DSE on their hosts

Studies show a continuum in responses of the host plant to DSE colonization. Effects of DSE on the plants can be positive, neutral or negative (Jumpponen, 2001; Weishampel and Bedford, 2006). The effects are most likely governed by the identity of the fungus (taxon and also strain) and the environmental conditions (Jumpponen, 2001).

1.3.4. Are DSE mycorrhizal?

There is no clear consensus yet about the mycorrhizal status of DSE. They lack structures for nutrient exchange, such as arbuscules for AMF or Hartig net for EMF (Muthukumar et al., 2004). Therefore their role in nutrient acquisition and nutrition of the host plant is unclear.

On the other hand, under some experimental conditions they are capable to enhance host plant's growth and increase nutrient uptake (and P concentration in host's tissues) (Haselwandter and Read, 1982) and there was no injury or disease observed in the roots colonized by DSE (de Marins et al., 2009). Therefore they appear to be rather mutualistic than parasitic organisms (Haselwandter and Read, 1982; Jumpponen, 2001).

1.4. Methods of detection and quantification of AMF

AMF do not cause visible changes on the structure of roots (like for example EMF). Techniques that allow detection and quantification of AMF in their plant hosts are essential tools in mycorrhizal research (Gryndler et al., 2005).

1.4.1. Reviews and helpful sources

Gange et al. (1999) and Vierheilig et al. (2005) provided a comparison of (not only) staining methods with their possible pros and contras.

A nice overview of methods to study AMF is given in Gryndler et al. (2005). Very helpful are also websites of Mark Brundrett (http://mycorrhizas.info/index.html) and of International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM) (http://invam.caf.wvu.edu/methods/methodsindex.htm).

1.4.2. Description of methods

Application of a method depends on what we want to document. The colonization intensity and identification of AMF species is usually obtained by staining techniques in combination with microscopic observation (Vierheilig et al., 2005). It allows us to observe the fungal structures (such as arbuscules, vesicles and hyphae) that are used for morphological identification of AMF species (Brundrett, 2004).

These methods usually include clearing, staining and de-staining (Vierheilig et al., 2005). The most common used dyes are Chlorazol Black E (CBE), Trypan Blue (TB) and Acid Fuchsin (Gange et al., 1999), or ink as a simple, cheap and non-toxic alternative (Vierheilig et al., 1998).

Recently, molecular techniques for identification of symbiotic fungi have undergone a large progress and they have gained popularity. They allow us to detect the diversity of fungal community in soil or in roots (van Tuinen et al., 1994; Redecker, 2002; Redecker et al., 2003; Sýkorová et al., 2007).

In general, they are based on DNA isolation directly from roots (or from soil). After that PCR (Polymerase Chain Reaction) follows. Specific primers are needed for identification of fungi, e.g. primers targeting fragments from the SSU rRNA (small subunit of rRNA) (Filion et al., 2003), from LSU rRNA (large subunit of ribosomal RNA, primers FLR3 and FLR4) (Mummey and Rillig, 2007) or from internal transcribed spacers of ribosomal DNA (ITS) (Redecker et al., 1997). The PCR product is used for further analysis such as sequencing and T-RFLP (Terminal-Restriction Length Polymophism). With sequencing we identify the sequence of nucleotide bases of a certain DNA segment and we compare it with database, e.g. GenBank of NCBI using the method BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) (Altschul et al., 1997). The T-RFLP method uses one or more restriction enzymes that specifically cut the DNA strain. Products are separated in electrophoresis and then compared with known database profiles, e.g. with TRAMPR (FitzJohn and Dickie, 2007).

AM fungal rDNA is highly polymorphic, even in a single spore. This high variability makes the identification more difficult. Another drawback of molecular techniques is that it is very difficult to design primer specific only for Glomeromycota, excluding other fungi or even plants (Redecker et al., 2003).

We can also use other methods, such as immunochemical methods (Hahn et al., 1994; Wright and Upadhyaya, 1999; Rosier et al., 2008) or visualization using laser scanning confocal microscopy (LSCM) (Dickson and Kolesik, 1999; Vierheilig et al.,

2005). LSCM allows us to observe living AM fungal structures and can be used to measure AM symbiosis dynamics (Vierheilig et al., 2005).

1.5. Review summary

Despite the specific characteristics of wetland habitats, such as flooding resulting in oxygen shortage, AMF are able to colonize wetland plants. In the last decades researchers have paid attention to study of the effects of AMF on wetland plants and also to elucidation of factors that influence the presence, biodiversity of AMF and colonization intensity by AMF in plants. The results are often very conflicting, showing different patterns across plant species and experimental sites.

Among the most important factors governing the presence, biodiversity and colonization intensity of AMF in roots of wetland plants are: water level fluctuations, nutrient availability and host plant species.

High P availability or fertilization with P is usually connected with decreased colonization of plants by AMF (Wetzel and van der Valk, 1996; Tang et al., 2001; Smith and Read, 2008; *etc.*). Low P availability leads usually to increase in colonization of plants by AMF (Turner et al., 2000). However, some authors have reported no significant relationship between P availability and AMF colonization (e.g. Miller, 2000).

Responses of AMF to N enrichment are variable, causing no changes in AMF colonization (Mandyam and Jumpponen, 2008) or leading to shift in the community of AMF (Egerton-Warburton and Allen, 2000; Johnson et al., 2003) and even to selection for aggressive and less mutualistic fungi (Johnson, 1993).

Water level fluctuations usually result in oxygen shortage and in changes in soil biochemistry and redox-potential. Flooding usually leads to lower AMF colonization intensity (Stevens and Peterson, 1996; Turner and Friese, 1998; Escudero and Mendoza, 2005; *etc.*). A few studies show that flooding has no significant effect on mycorrhizal colonization if the symbiosis is already established in the roots (Miller and Sharitz, 2000; Dolinar and Gabeščik, 2010).

In wetlands, another group of fungi has been recently detected on plant roots – DSE. There is not much information about them and about their effect on host plants. However, some authors suggest that these fungi may have a similar role in host plant nutrition like AMF (Jumpponen, 2001; Weishampel and Bedford, 2006).

The relations between AMF/DSE and wetland plants are very complex and they are affected by many factors. Therefore further research is needed to shed light on the role of AMF and DSE also in wetlands. We should keep in mind that *"the study of plants without their mycorrhizas is the study of artefacts"* (motto of the International Bank for the Glomeromycota; http://www.kent.ac.uk/bio/beg/englishhomepage.htm).

2. Aims of the project

1) to assess the presence of AMF and DSE in two selected plant species in two wetland meadows
2) to assess if there is a seasonality pattern in colonization intensity of AMF and DSE
3) to evaluate the effect of soil eutrophication on the presence and colonization intensity of AMF and DSE in plants
4) to determine AMF and DSE diversity on the two studied wetland plants

3. Hypotheses

1) according to reviewed literature, AMF can play a significant role also in wetland habitats
2) the presence and colonization intensity of AMF and DSE in two selected plants is influenced by nutrient availability in soil and by water table level
3) fertilization (eutrophication) negatively affects the intensity of root colonization by AMF
4) seasonality (time period of sampling) does influence the root colonization intensity by AMF and DSE

4. Approach

4.1. Study sites

The two experimental sites are wet meadows situated in the Třeboň Basin Biosphere Reserve (TBBR), South Bohemia, Czech Republic. Záblatské Louky (Z) is a wetland meadow on peaty soils located in the inundation area of Záblatský fishpond. The water level is quite stable. However, the site is occasionally subjected to several-weeks-long shallow flooding or summer drought. The altitude is 426 m above sea level.

Hamr (H) is located near the village Hamr in the floodplain of a small river Nežárka on a silt-sand alluvial substrate. The water level is the same as in local drainage ditches connected with the river. Therefore, it is more variable than the water level in Z. The average water level, however, is lower in H. The altitude is 415 m above sea level.

From the phytocoenological data from 2007, Z is a sedge meadow dominated by *Carex vesicaria* and *Carex acuta*, and plant community in H is dominated by *Glyceria*

maxima and *Carex acuta*. *Carex acuta* and *Glyceria maxima* are listed as non-mycorrhizal species, *Carex vesicaria* as non-mycorrhizal or hosting AMF (Wang and Qiu, 2006).

In 2006, an experiment was established to observe effects of eutrophication on plant-soil interactions. A complete randomized block design was used in the study. Four blocks were established in each meadow in May with three plots (3.5 x 3.5 m) per block for each fertilization treatment: 1) no fertilizer addition (No), 2) low treatment – 65 kg NPK/ha/year (Low), and 3) 300 kg NPK/ha/year (High). The fertilizer was added in two half doses during growing season starting in 2007 (in mid-May and in mid-July). The plots in both sites are mown in early June.

4.2. Methods

4.2.1. Field measurements

The root samples will be taken from both experimental meadows (Záblatské Louky and Hamr) and all treatment plots. We have selected *Phalaris arundinacea* (*PA*) (Poaceae) and *Ranunculus repens* (*RR*) (Ranunculaceae) - both reported to be either non-mycorrhizal or to form AM association (for references, see Wang and Qiu, 2006). We will keep the samples in a freezer before further processing. We will sample in March, July and October.

The roots will be washed free of soil with tap water. Finer lateral roots will be selected and cleared in KOH (15-20 h), washed again with water and acidified with HCl. Then the roots will be boiled with the staining solution (containing either Chlorazol Black E or Trypan Blue dye). After that they will be de-stained for several days.

Microscope slides will be prepared and the AMF and DSE colonization intensity will be quantified under microscope.

To determine fungal diversity, DNA will be extracted from the roots of the two selected plants**.** The DNA will be amplified with PCR, followed by sequencing and T-RFLP. The method and use of primers will be first adjusted.

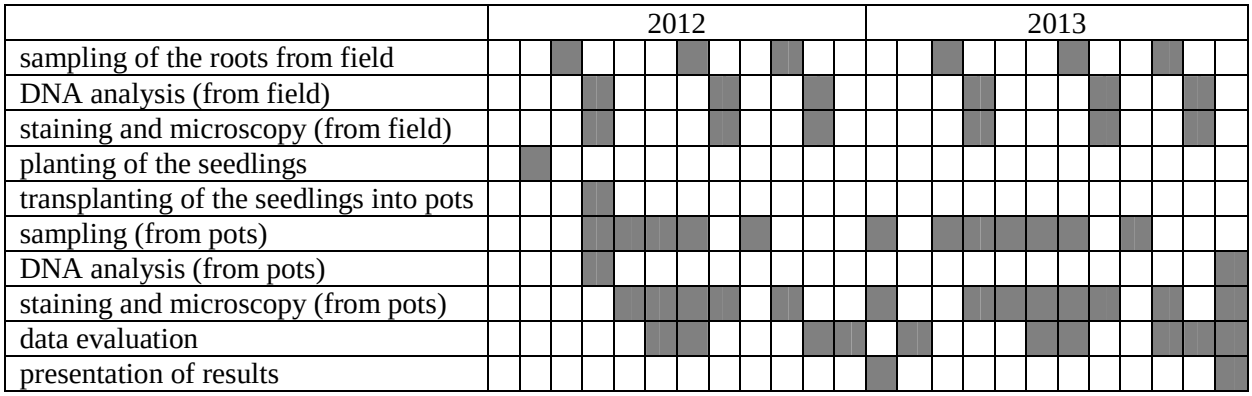
4.2.2. Mesocosm experiment

Seedling of *PA* and *RR* will be grown from seeds in the soil from the experimental site Z (mixed from several soil samples). When the seedlings will be big enough we will transplant them into experimental pots. We will assess the initial AMF biodiversity (molecular methods) and colonization intensity (staining and microscopy). The mesocosm

experiment will have following variants: fertilized with NPK (F+) x no fertilization (F-), flooded (W+) x un-flooded (W-), with microorganisms present in the soil (M+) x sterile (M-), and their combinations. For M- variants, soil will be sterilized with γ - radiation by Bioster, a.s. (http://www.bioster.cz/webpage.yhtml?id=1).

Roots will be sampled for staining and for colonization intensity measurements starting April 2012. During the growing season we will sample every 4 weeks from March till July, and two more samplings will be done in September and November. We will also analyze plant (biomass, P content in the biomass) and soil characteristics. The experiment will be set up for two years (2012 and 2013).

4.3. Time schedule



4.4. Finances

	2012 (thousands CZK)	2013 (thousands CZK)
Consumables	200	120
Overhead	30	18
Salary expenses	150	150
Travel expenses	20	25
Services	20	5
Total/year	420	318
Total (thousands CZK)	738	

Consumables: material for analyses – chemicals (Trypan Blue/Chlorazol Black E, lactic acid, kits for molecular analyses), laboratory equipment, equipment for field work, pots, seeds

Overhead: is set as 15% from all consumables

Salary expenses: salary of the part time employer and people who will help with sampling and analyses

Travel expenses: traveling to the study site and sample transport, travel expenses and fees for participation on conference

Services: γ - radiation (Bioser, a.s.) – sterilization of soil, expenses for company which will make pots and basins for mesocosm experiment, posters printing

5. Major impacts of the project

The major impacts of our project will include:

- New information about the effect of eutrophication on AMF biodiversity and on the level of AMF colonization in roots of *Phalaris arundinacea* and *Ranunculus repens*
- New information about the effect of AMF on the two selected wetland plants (on their biomass and P concentration in tissues) at different levels of water table and of nutrient inputs compared to plants without any mycorrhizal fungi
- New information about the seasonal dynamics of AMF colonization in roots of the two selected plants

The results of the project should increase our knowledge about functioning of wetland ecosystems under increased nutrient loadings. This information can be used for modeling of eutrophication effect on endangered wetland ecosystems as whole.

AMF are considered also in restoration activities. Therefore the project will have practical implication regarding wetland management and protection of rare plants dependent on mycorrhizal symbiosis.

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