University of South Bohemia in České Budějovice
Faculty of Science

# Amelioration of orchid germination in restored grasslands

Master thesis

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**Annotation:** Amelioration of orchid seed sown with fungal inoculum was tested in *in situ* experiment in White Carpathian Mountains. The thesis evaluated reintroduction potential of four meadow orchids (*Orchis mascula, Anacamptis pyramidalis, Platanthera bifolia* and *Gymnadenia conopsea*) to restored grasslands.

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My theses is part of the project of Grant Agency of the Czech Republic lead by Jana Jersáková. The experiment was designed by Jana Jersáková, Tamara Těšitelová and myself, I participated on field work together with Jana Jersáková, Tamara Těšitelová, Milan Kotilínek and several technicians. I did majority of laboratory work with guidance of Tamara Těšitelová, Hélène Vogt-Schilb, Milan Kotilínek and with help of technicians. The manuscript was written by me with help of my supervisor and my consultant.

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## Preface:

Orchids are well known for producing immense number of seeds at each reproductive event; this characteristic, however, does not ensure itself successful establishment and many orchid species are prone to extinction. This is caused by special demand of minute orchid seeds: for their successful establishment they need specialized symbiotic fungi as the seeds do not contain any reserves for early seedling growth. Mycorrhizal fungi are capable to supply the germinating seed with necessary resources through non-photosynthetic growth stadium.

Orchids experienced pronounced decline in their population densities and distribution in past decades, and the restoration efforts are needed, but the practical guidelines are missing. A question whether a restored habitat is suitable for regeneration of orchids from seeds is not easy to answer as we do not know distribution of mycorrhizal fungi. Most of orchid mycorrhizal fungi spend their whole life-cycle hidden below-ground. Expansion of molecular techniques in the last decades led to significant increase of studies oriented on soil ecology and symbioses between plants and other microorganisms. We are now, for example, able to determine mycorrhizal fungal strains without demanding cultivations. Furthermore, we can study interactions between orchids and mycorrhizal fungi and resolve species specificity of orchids.

Gained data can be used for practical conservation and restoration of orchid species and designing of protocols specific for individual species. This is a way for restoration of threatened orchid species in 21st century.

In this master thesis, I studied reintroduction of several threatened orchid species at restored grasslands via direct seeding with amendment of specific inocula. I hope that the result will improve restoration practice of orchid populations.

# Amelioration of orchid germination in restored grasslands

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## Abstract:

Restored grasslands on arable land are seldom colonized by orchids. Successful orchid germination is dependent on the presence of specific mycorrhizal fungi in soil and those are usually missing on restored sites. Here we tested whether sowing of orchid seeds with an addition of mycorrhizal fungus is a suitable technique for re-introduction of orchids into restored grasslands. In addition we asked whether the germination is enhanced by a type of inoculum and whether germination success depends on the time since restoration. The inocula of symbiotical fungi were based on the results of *in vitro* sowing experiment and they were subsequently used in *in situ* experiment established on nine grasslands restored from 1 to 20 years ago. Seeds of orchid species *Orchis mascula, Anacamptis pyramidalis, Platanthera bifolia* and *Gymnadenia conopsea* were enclosed in seed packets and incubated with different inocula types in soil for one year. It was found out that the time since grassland restoration had no effect on the orchid germination, all used fungal isolates survived and some had positive effect on orchid seed germination. The use of inoculated hay significantly affected size of protocorms of *Orchis mascula* and *Anacamptis pyramidalis*. Germination success of studied orchid species differed according to their breadth of fungus specificity. Our results imply that addition of seeds together with fungal inoculum may be a successful method for orchid restoration on grasslands.

## Introduction:

Human activities have been leading to habitat loss and fragmentation and inevitably also to decrease of biodiversity (Diaz et al., 2006). Despite those negative effects, the disturbed habitats represent also potential for restoration of natural communities after they are abandoned and not suitable for exploitation any more. There are two possibilities how to manage the disturbed areas: they can be left to spontaneous succession or actively restored to reach desirable vegetation type. Both attempts are suitable for revitalization of disturbed habitats and increase of their natural value (Prach & Hobbs, 2008). Decision whether to use spontaneous succession or restoration depends on target we want to achieve on a specific site. Even if spontaneous succession is a very cheap option, it cannot be applied on sites where we expect particular ecosystem services, or where target community type needs regular management, for example restoring grasslands for fodder production on former arable land (Valkó, 2016).

Restored grasslands experience significant change in management and lack of fertilization. High nutritional inputs have an important effect on succession of arable land to restored grasslands long after last fertilization. That brings changes not only to plant community (plant diversity, species composition, plant biomass and litter) but also to microbial community as they closely interact (Martınez-Garcıa et al., 2015). The fertilizers seems to have a negative effect on plant - fungi symbioses such are those of arbuscular fungi (Azcon et al., 2003), as well as saprophytic fungi (DeForest et al., 2004) and generally fungal biomass in the soil (Bittman et al., 2005). Prevalence of knowledge gained about mycorrhizal fungi is focused on arbuscular mycorrhiza. Arbuscular mycorrhizal community in restored ecosystems may be sorted similarly to plants on the base of immigration of propagules, biotical and abiotical environmental filters driving fungal succession (Jumpponen et al., 2012) on the other hand early successional stages were documented to be the most variable (Martınez-Garcıa et al., 2015) and seems to establish earlier than plants (García de León et al., 2016b).

Former arable land is low in C:N ratio with lack of ligninolytic fungi which is suitable condition for fungal opportunists (Van der Wal et al., 2006a; García de León et al., 2016a) and bacteria. In general, the early stage is characterized by prevalence of bacteria

over fungi in soil, with the latter increasing in dominance when the soil nutrients start to change to more natural stadium (Van der Wal et al., 2006b; Maharning et al., 2009). Recovery of the soil community is time demanding and can take up to decades. It was discovered that fungal biomass lowered by agriculture increases two years after farming termination (may be influenced by recovery of saprophytic fungi) with subsequent stagnation for at least three decades (Van der Wal et al., 2006b). Long-term spontaneous improvement is associated with increased soil C:N ratio, substrate lignification and heterogeneity, N immobilization and a decrease in soil pH which favors fungal growth (for summary see Maharning et al. 2009).

Once a restoration goal requires an active restoration, there are several ways how to establish a target community in a restored area. The cheapest and fastest technique is sowing seeds of desired plant community. For this method we need source of seeds that may be from already established community or special garden for seed production. The shortage of the method is that some species do not produce enough seeds or their seeds are not able to germinate on a restored site (Kiehl et al., 2010). Another method is transplanting seedlings or mature plants (Alvarez-Aquino et al., 2004). This technique is time and money demanding and is commonly used in forestry, but survival of transplanted orchid species is typically low (Batty et al., 2006a). It can be also used in translocation when source site for plant material with valuable species composition is planned to be damaged (Abeli & Dixon, 2016). If properly done, this technique may lead to successful restoration effect (Godefroid et al., 2011). In extreme cases, transplanting of whole blocks of plant communities is also possible, especially in cases when a donor community with valuable species composition is going to be destroyed (Aradottir, 2012; Mudrák et al., 2017).

Success of habitat restoration can be measured by different approaches belonging to the general ecosystem attributes: diversity, vegetation structure and ecological processes (Ruiz-Jaen & Aide, 2005). According to long term monitoring studies of restored plots of different time since restoration, it is clear that restoration success depends on habitat type, environmental conditions, composition of added species as well as plant species pool in surrounding habitats (Klimkowska et al., 2007; Kiehl et al., 2010), whereas rare species are reestablishing themselves with great difficulty (Pywell et al., 2002). This is particularly true for species with special requirements for particular pollinators or root/mycorrhizal symbiosis

which introduction to new restored area is difficult (Hudson et al., 2006; Swarts & Dixon, 2009) as they require not only suitable environmental conditions but also the organisms they interact with (McCormick & Jacquemyn, 2014). At the same time these species often need environment with low productivity and low competition (Press & Phoenix, 2005) and therefore some restored sites (e.g. post-mining sites) may be suitable for them. Orchids may serve as an example of such species, as they depend on occurrence and abundance of suitable mycorrhizal fungi and specific pollinators (Swarts & Dixon, 2009).

Orchids are threatened mainly by negative changes in their habitats due to intensive farming leading to high amount of fertilizers in soil (Brown & Mitchell, 2006; Swarts & Dixon, 2009) and expansion of highly competitive species (Janečková et al., 2006). Orchid populations are threatened not only by habitat degradation but also by habitat loss and fragmentation (Newman et al., 2013). Suitable patches for orchid growth in a habitat mosaic may be so remote that, despite airborne seeds, probability of colonization of a suitable patch is very low (Diez, 2007; Aewsakul et al., 2013; McCormick & Jacquemyn, 2014) and small populations are prone to extinction. Restored habitats therefore may represent additional sites in the matrix of suitable habitat mosaic and enhance viable metapopulation dynamics for the rare species. To be able to restore orchid populations in new habitats it is necessary to understand and take into account their complex relationships with other organisms (Wright et al., 2009).

One orchid plant produces thousands of dust-like seeds every year that are capable to travel thousands of kilometers (Arditti & Ghani, 2000) but most of the seeds still fall in the immediate vicinity of their mother plant (Jersáková & Malinová, 2007). For example, some experiments with seed sowing into unoccupied patches found germination and concluded that the orchids may be dispersal limited. Direct seeding can be then cheap and suitable restoration practice (Shefferson et al., 2008; De hert et al., 2013). This is, however, possible only when another conditions of orchid seed establishment are met and soil contains suitable mycorrhizal fungi as minute orchid seeds lack nutrient storage to support germination. Orchid seed with essential support from mycorrhizal symbiont is capable to develop into non-photosynthetic below-ground seedling called protocorm, which is fully mycoheterotrophic, i.e. obtaining all nutrients including carbon from a mycorrhizal fungus (Rasmussen, 1995). First leaves occur after few months to several years, and the orchid starts

photosynthesis and change to partially mycoheterotrophic (Gebauer et al., 2016). Some orchid species stay fully mycoheterotrophic for their whole life and do not produce any chlorophyll (Bidartondo, 2005).

Orchid restoration therefore needs to take into account biotic and abiotic characteristics of the environment (Zobel et al., 1998). It was found out that the results of seed sowing differ at the small scale more than at the landscape one (McCormick et al. 2018), which means that heterogeneity of the soil increases the probability of recruitment limitation and is leading to unsuccessful introduction (De hert et al., 2013). There are several factors playing an important role: soil micronutrients, pH, moisture content and suitable mycorrhizal fungi (Rasmussen, 1995; Diez, 2007). Several studies point out that not the presence of mycorrhizal fungi itself but their abundance is crucial for long-term support of orchid growth (McCormick et al., 2018; Jacquemyn et al., 2012). Low abundance of mycorrhizal fungi leads to seed dormancy and mortality (McCormick et al., 2018).

Restoration attempts of orchid species has been performed in different kind of habitats across most continents and used diverse techniques such as (i) direct seeding, (ii) planting of cultivated juveniles and (iii) transplantation of adults with soil bulk from natural populations.

(i) Direct seeding is the easiest technique which can lead to orchid reintroduction in case of dispersal limitation (Těšitelová et al., 2012; De hert et al., 2013). It is convenient to mix orchid seeds before sowing with substrates such as fine sand, sugar or forest soil (Huber et al., 2002; Wright et al., 2009). Disadvantage of this technique is demand for large quantity of seeds and subsequent wasting of significant part of them (Zettler & Piskin, 2011). Several successful restoration attempts, however were done so far (Huber, 2002; Wright et al., 2007). Improvement of seed germination is also possible to achieve by means of adding inoculum of suitable mycorrhizal fungus (Huber, 2002; Hollick, 2004; McCormick et al., 2012) by watering (Hollick, 2004; Wright et al., 2007), disturbing the soil (Wright et al., 2007) and addition of organic matter (Wright et al., 2007) and surface litter (Batty et al., 2001). Inocula with mycorrhizal fungi are mostly made out of natural organic material such as millets (Mursidawati, 2004; Wright et al., 2007), wood-chips (McCormick et al., 2012), rice hull and bran (Harris et al., 1993) or oatmeal agar (OMA) medium (Batty et al., 2006b). Evaluation of direct seeding is complicated especially during germination process, therefore

Rassmussen and Whigham (1993) invented technique for sowing seeds in seed packets which were repeatedly used to find suitable microhabitats for seed sowing with sufficient abundance of mycorrhizal fungi (Swarts, 2007; McCormick et al., 2012).

(ii) Planting juvenile orchid plants in nature is convenient because it can lead to avoidance of high mortality during germination phase and easy monitoring (Batty et al., 2001). Orchids can be propagated vegetatively e.g. by axillary bud multiplication (Martin, 2003) or via seeding. Seeds can be sown *ex vitro* in suitable substrate (Aewsakul et al., 2013) or in sterile condition *in vitro* asymbiotically (Ponert et al., 2011) or symbiotically (Aggarwal & Zettler, 2010). Seeds sown *ex vitro* can be propagated by sowing next to a nurse plant with well-developed mycorrhizal symbiosis and later transferred as clumps or "cake slice" to a pot with new substrate (Wright et al., 2009). Seeds sown asymbiotically have access to all nutrients from solid media (Yam & Arditti, 2009), on the other hand media for symbiotic sowing contain source of starch e. g. rolled oats which have to be broken down by suitable mycorrhizal fungus (Hadley, 1982). Germinated seeds and vegetative seedlings are transferred as they grow to single containers and placed in light. They are lately replanted to substrate and placed in a greenhouse to harden and adapt to natural conditions (Ponert et al., 2011). Symbiotically grown seedlings contain also mycorrhizal fungus in their roots thus their reintroduction can be easier (Reiter et al., 2016), the disadvantage of the method, however, is large investment into preparation of juvenile plants that needs complicated methodology which is available and tested only for a few species so far (Reiter et al., 2016).

(iii) Transfer of turfs containing target orchids is possible when a source locality is going to be destroyed (Wright et al., 2007; 2009). Advantage of this technique is that plants are already established and soil quality and soil organisms are transferred as well so that biotic conditions for plants are suitable. Disadvantage is that transport of soil blogs is expensive and can be done on short distance only and environmental conditions (apart of soil quality) must be optimal for the transplant success (Box, 1999).

From the above mentioned follows that direct seeding is the most promising and cheapest method for orchid introduction during restoration of habitats. Importance of

appropriate selection of site for orchid reintroduction remains to be very important (Wright et al., 2009). Seed sowing does not need long laboratory preparation and is not dependent on destruction of an existing locality (Wright et al., 2009). The main aim of this master thesis was to test this method as a technique for introduction of terrestrial orchids into grasslands restored on former arable land.

The study was performed in the Protected Landscape Area and UNESCO Biosphere Reserve in White Carpathian Mountains in the Czech Republic (Central Europe). The landscape is composed of well-preserved mosaic of forest, arable land, fens and grassland areas (Otýpková et al. 2011). Especially semi-natural grasslands with scatted trees traditionally managed for hay are valuable for their extraordinarily high diversity of vascular plants including orchids, the diversity is counted as the highest in the Czech Republic and Central Europe (Klimeš, 2008) and at a small scale as one of the highest diversity in the world (Merunková et al., 2012; Chytrý et al., 2015).

White Carpathian's grasslands went through complicated time in the past. In the last century large area of grasslands was turned into arable fields and about a third of the total historical grasslands was abandoned or degraded by intensive   use. Intensification of agriculture dropped since the Velvet Revolution in 1989 and approximately 7 000 hectares of arable land have been "re-grassed" (Jongepierová et al., 2007; Prach et al., 2013). Restoration of species rich grasslands was performed by leaving localities to spontaneous succession or actively managed by sowing commercial seed mixtures (containing several species of common grasses and legumes) and since 1998 a regional seed mixture of local origin (with a mix of grasses, legumes and other plants) was used (Jongepierová et al., 2007; Kiehl et al., 2010; Prach et al., 2014). On 500 hectares of restored grasslands almost all target species (98%), which were sown or established spontaneously 1 - 12 years after restoration (Prach et al., 2013). Orchids were not among sown species and their spontaneous establishment is still extremely rare although natural orchid rich meadows are abundant in the surroundings. First orchid plants were recorded eight years after restoration with others coming roughly after 15 years. Recorded species were: *Platanthera chlorantha, P. bifolia, Orchis ustulata, O. militaris, Anacamptis pyramidalis* and *Gymnadenia conopsea* (Fajmon, Jongepierová per com.). It is apparent that full restoration of the species rich grasslands can probably take decades or more, and orchids as a sign of meadow health and improved

interspecific relations reestablish as one of the last species (Jongepierová 2008; Prach et al., 2014).

In this study, we hypothesized that addition of a fungal symbiont inoculum would improve seed germination of several orchid species in restored grasslands. To test this hypothesis several combinations of mycorrhizal fungi inocula were introduced into soil with seeds of four meadow orchid species in nine restored grasslands of different time since restoration. Beside this main aim, we asked following questions:

(1) Does the abundance of mycorrhizal fungi have any impact on orchid seed germination?

(2) Is it possible to improve orchid seed germination independently of the time since grassland restoration?

(3) Do the orchid species differ in germination success due to differences in fungal specificity?

**Materials and Methods:**

- Sample collection for fungal isolation

For inoculation experiments, we isolated fungi forming pelotons from several orchid species from roots of adults or protocorms obtained by seed baiting technique (Rasmussen & Whigham, 1993) at several localities in White Carpathians area in 2014 - 2015 (Fig. S1). Three roots per plant and seed packets were carefully dig out to minimize damage to the orchid individuals. The roots were carried in plastic bags and stored in a fridge at 5°C until processing within a few days.

Subsequently the roots were cleaned with a brush in distilled water. The roots of an individual plant were divided into two parts: one part of roots was sterilized with SAVO 1:5 for 30 s, second was not sterilized. All roots were then washed 3x in distilled water. Roots were sliced with a razor blade and inspected under light microscope for presence of mycorrhizal structures. Protocorms were treated similarly to orchid roots (Swarts & Dixon, 2017). Whenever vital pelotons appeared in the sections, they were released by a laboratory needle and washed in four drops of autoclaved distilled water using automatic pipette as in Kohout et al. (2013). Finally they were transferred on a plate with (Melin, Marx, Norkrans) MMN media (Marx & Bryan, 1975) modified per 1 liter: 1 g of glucose, 0,3 g malt extract-powder, $KH_2PO_4$ 1 g, $MgSO_4$ 0,01 g, $ZnSO_4$ x 7 $H_2O$ 0,0057 g, $CuSO_4$ x 5$H_2O$ 0,0013 g and no thiamin HCl with addition of 50 mg $l^{-1}$ of Novobiocin antibiotic to suppress bacterial growth. The plates were sealed with Parafilm and placed at 22°C till the first hyphae appeared. Subsequently the fungi were transferred on new MMN plates without antibiotics. Those were kept for another few days till the diameter of the colony was bigger than 2 cm and then kept in a fridge at 5°C.

- Fungal identification

Isolated fungi were identified using molecular techniques. The DNA was isolated using NaOH technique: ca. 0,5 $cm^2$ block of agar with mycelium was put in an Eppendorf tube and homogenized in 40 ul 0,5 M NaOH and centrifuged for 2 min at 13 800 rpm. Supernatant was then transferred in a new Eppendorf tube and diluted with 1 : 10 of solution

100 mM Tris-HCL pH 8,3 and  stored at -20 °C. PCR was performed with universal primer pair ITS1/ ITS4 (White et al., 1990) and the PCR products were sequenced and analyzed as described in "Molecular evaluation of protocorms" below.

- Orchid seed collection

Seeds were collected from 2-3 populations per orchid species in White Carpathians. Ripe seed capsules were harvested in summer 2015 (*in vitro* experiment) and 2016 (*in situ* experiment) and kept at room temperature till the first experiment. Seeds collected in 2016 were from at least two different sites pooled per species. *Gymnadenia conopsea (L.) R. Br. (Gc)* seeds were collected from Zahrady pod Hajem and Certoryje, *Platanthera bifolia* (L.) Rich. (Pb) seeds from Zahrady pod Hajem and Drahy, *Orchis mascula* (L.) L. (Om) seeds from Certoryje, Zahrady pod Hajem and Letoviska, *Anacamptis pyramidalis* (L.) Rich. (Ap) seeds from Zahrady pod Hajem and Certoryje. Seed viability (percentage of seeds with viable embryo) was 57 % per *Gc*, 60 % per *Pb*, 56 % per *Om* and 39 % per *Ap*.

- In vitro experiment

Symbiotic *In vitro* germination is a convenient way how to make a backward control of orchid species-fungus compatibility for germination named as physiological specificity (Zettler et al., 2003). Thus, we (1) verified the compatibility of obtained isolates during *in vitro* germination and (2) according to the results we selected fungi for the *in situ* sowing experiment. In the *in vitro* experiment, each of the five fungal isolates (Tab. S1) was sown with seeds of the four focal orchid species (full factorial design) in February 2016.

Dried seeds were surface sterilized in plastic syringes with a semipermeable plug made out of netting. Needle 1,2 x 40 (18 G 1 1/2″, Luer-lock; Dispomedicor Zrt., Hungary) was used for the surface sterilization firstly for 5 minutes in 70% ethanol, washed three times in distilled water, 5 - 10 minutes (till lightening of testa) in $Ca(OCl)_2$ (with 5% of OCl, replaced ones during the time) and finally washed three times with autoclaved distilled water. The seeds were resuspended in a small amount of sterile distilled water  for sowing , subsequently the syringe needle was replaced by a new one of 1,8 x 40 (15 G 1 1/2″; Luer-

lock; Dispomedicor Zrt., Hungary) without netting. Four 2 x 2 cm pieces of autoclaved filter paper for seed sowing (one per one species of orchid) were placed in every Petri dish with OMA medium (Clemets et al., 1985). Before every injection on filter paper, the syringe was shaken to keep seeds resuspended in the fluid to obtain constant amount of seeds. In total four species were injected in one Petri dish. A small piece of an inoculum, i.e. mycorrhizal fungus grown on MMN medium (Marx & Bryan, 1975), was placed in the middle of every Petri dish except control asymbiotic treatment. Five dishes per fungus were made and sealed with Parafilm and kept in dark for 3 months at 23°C (Ponert et al. 2011).

Tab. 1: Orchid – fungal isolate compatibility in symbiotic in vitro germination experiment. The numbers denote replicates of Petri dishes with protocorm presence for species: *Orchis mascula* (*Om*), P*latanthera bifolia* (*Pb*), *Anacamptis pyramidalis* (*Ap*) and *Gymnadenia conopsea* (*Gc*).

| Fungal isolate | Fungal isolate identity | Isolate origin | *Ap* | *Gc* | *Om* | *Pb* | No. of replicates (no contamination) |
|---|---|---|---|---|---|---|---|
| **CER1N** | Ceratobasidium | *Gc*, protocorm | 0 | 5 | 0 | 0 | 5 |
| **CER2N** | Ceratobasidium | *Ap,* protocorm | 0 | 5 | 0 | 0 | 5 |
| **TUL1N** | Tulasnella | *Ap,* protocorm | 4 | 4 | 0 | 0 | 4 |
| **TUL3N** | Tulasnella | *Pb*, protocorm | 0 | 3 | 0 | 3 | 4 |
| **TUL4N** | Tulasnella | *Om*, root | 0 | 5 | 3 | 0 | 5 |

Only uncontaminated Petri dishes were evaluated (Tab. 1). Over one hundred seeds of each species was categorized as (1) ungerminated, (2) germinated but non-mycorrhizal, and (3) protocorms. We have chosen suitable orchid-fungus pairs for *in situ* germination on the base of the protocorms occurrence/ absence (Tab. 1).

- *In situ* experiment

The *in situ* experiment was designed based on the specificity detected in *in vitro* germination experiment (Tab. 1) with the seeds collected in summer 2016. It was established at nine sites of restored meadows (RV1, RV4, RH6, RH8, R11, R12, R20, R23 and R29 (Tab. 2; Figure 1), which were re-grassed with regional seed mixture (Jongepierová et al., 2007) and where broadleaved dry grasslands with dominant *Bromus erectus* Huds.. Soil characteristics were measured at the end of May 2016. Soil samples were collected from a

plot 20 x 20 m with 25 soil cores taken in a regular grid by 1 m and polled to make one mixed soil sample per each site. The soil samples were air dried and subsequently analyzed in the Analytical laboratory of the Institute of Botany ASCR in Trebon (Czech Republic). Soil characteristics were rather similar at all sites except for site R23 which had higher pH and lower amount of available phosphorus (Tab. 2).

In October 2016, 7 x 10 m plot was established at each site. In each plot, there were 70 holes made by a soil core (diameter 15 cm and 15 cm deep) organized in a regular grid with 1 m distance between holes. Within the grid, five treatments (Tab. 3) were organized in a complete randomized block (Fig. S2). The same design was applied to all experimental sites.

Tab. 2: Description of sites used in the *in situ* experiment (* values from natural meadows come from Vogt-Shilb et al. (unpublished)).

| Site | Year of restora-tion | coordinates | soil reaction pH H2O | availab-le cal-cium (mg/kg) | available phosphorus (mg/kg) | total nitrogen (%) | organic matter (%) | clay (%) | silt (%) | sand (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| R11 | 2012 | N 48.86602, E 17.44502 | 6,34 | 3488 | 33,2 | 0,299 | 7,59 | 23,4 | 50,7 | 25,9 |
| R12 | 2015 | N 48.85141, E 17.47953 | 6,75 | 3085 | 22,4 | 0,248 | 6,56 | 27,9 | 59,6 | 12,5 |
| R20 | 2012 | N 48.87078, E 17.39771 | 6,62 | 4560 | 21,2 | 0,405 | 10,41 | 21,5 | 56,5 | 22,0 |
| R23 | 2009 | N 48.89903, E 17.50266 | 7,30 | 7038 | 2 | 0,277 | 8,32 | 29,1 | 61,6 | 9,2 |
| R29 | 2004 | N 48.86997, E 17.40262 | 5,42 | 3901 | 29,6 | 0,407 | 10,86 | 27,0 | 52,5 | 20,5 |
| RH6 | 2003 | N 48.85089, E 17.47359 | 6,46 | 3929 | 33,0 | 0,312 | 9,24 | 30,7 | 58,3 | 11,0 |
| RH8 | 2007 | N 48.85162, E 17.47289 | 6,20 | 3538 | 38,8 | 0,341 | 9,56 | 26,4 | 57,2 | 16,4 |
| RV1 | 2001 | N 48.86520, E 17.43892 | 6,48 | 4731 | 38,2 | 0,453 | 12,69 | 21,8 | 66,6 | 11,7 |
| RV4 | 2008 | N 48.85941, E 17.44585 | 5,78 | 2992 | 33,7 | 0,278 | 8,12 | 23,7 | 46,0 | 30,2 |
| mean value for restored grasslands | | | 6,37 | 4140 | 28,0 | 0,336 | 9,26 | 25,7 | 56,6 | 17,7 |
| mean value for natural meadows* | | | 6,23 | 5094 | <2 | 0,487 | 14,19 | 17,6 | 64,2 | 18,2 |

Selected orchid species were in *in situ* experiment combined with fungal inocula as follows: *Om* - TUL4N; *Ap* - CER2N, TUL1N; *Pb* - CER1N, TUL3N; *Gc* - TUL1N, TUL3N, TUL4N, CER1N, CER2N which consequently led to a combination of two orchid species within one microsite (i.e. hole). Despite absence of symbiotic germination *in vitro*, *AP* and *PB* were tentatively sown also with CER isolates, because both species often form at adulthood mycorrhiza with Ceratobasidiaceae in nature and CER2 was even isolated from AP protocorms. Design of *in situ* experiment consisted from treatments with addition of fungus inoculum: (1) inoculated hay, further referred to as "hay" treatment, (2) sterile hay with agar plugs, further referred to as "agar" treatment, and control treatments: (3) disturbed soil, (4) not disturbed soil, (5) sterile hay without inoculum (for details see Tab. 3). Every microsite contained two replicates of seed packets per each orchid species (i.e. two seed packets per species and microsite).

Tab. 3: Treatments description and the number of replicates per plot. Five fungal isolates were used for inoculation treatments.

| treatment | control | Number of replicates per plot | Description of treatment |
|---|---|---|---|
| disturbed soil | yes | 7 | soil excavated, mixed and returned with seed packets |
| not disturbed soil | yes | 6 | soil cut with a knife and seed packets pushed down |
| only sterile hay | yes | 7 | soil excavated, mixed with sterile hay and returned with seed packets |
| inoculated hay "hay" | no | 5x for each of 5 fungal isolates | soil excavated, mixed with inoculated hay (15g of wet hay (» 5g dry hay)) and returned with seed packets |
| Sterile hay with agar plugs "agar" | no | 5x/ fungal species 5x 5= 25 | soil excavated, agar plugs - 1/5th from two inoculated 90mm agar plates mixed with sterile hay (15g of wet hay (» 5g dry hay)) and soil and returned with seed packets |

We have prepared two types of inocula used as treatments "hay" and "agar".

(1) Preparation of sterilized hay and fungal agar cultures

Sterilized hay was prepared with 100 g of cut hay placed in bottles with added water and left soaked for one hour. Subsequently redundant water was disposed of and the bottles with hay were autoclaved. Fungal inocula were grown on OMA medium for one month.

(2) Preparation of fungal inoculated hay

Cut hay was inoculated with one selected fungal strain at one time. About 15 agar plugs - 1cm² were cut of the fungal cultures grown on ½ MNM and added in the bottles with sterile hay. We made in total five bottles per fungus and kept them at 23°C. Fungus identity was checked after one month using molecular identification and left to grow from July/ August to September 2016.

Seed packets used in *in situ* experiment contained 350-400 seeds, that were placed in packets constructed from 42 μm nylon mesh (Silk and Progress Ltd, Brněnec, Czech Republic) and enclosed in 35 mm plastic slide mounts as in Rassmussen & Whigham, (1993). Number of seed packets was in total 3240, (per species: *Om* - 540 seed packets, *Pb* - 720 seed packets, *Ap* - 720 seed packets and *Gc* - 1260 seed packets).

- *In situ* experiment - evaluation

After one year (in October 2017) in the soil the seed packets were retrieved and stored at 5°C until examination of seed development. Every packet was rinsed with water and seeds were examined under a stereoscopic microscope. They were categorized as 0 - ungerminated, 1 - imbibed seed with broken testa, 2 - round protocorm with first trichomes, 3 - enlarged pear-shaped protocorm with leaf primordium (Ramsay et al., 1986). Embryos in categories 2 & 3 were recorded as protocorms and further processed. Three largest protocorms were photographed and their length was measured in ImageJ (http:// rsbweb.nih.gov/ij), afterwards they were stored for molecular analysis at -20°C. Results from *in situ* germination experiment were for statistical evaluation divided to individual orchid species because of the differing set of mycorrhizal fungi. We have evaluated number of protocorms per microsite, size of the biggest protocorm per microsite as well as abundance of orchid mycorrhizal fungi. Mycorrhizal fungi in selected protocorms were identified by molecular methods.

- *In situ* experiment - molecular identification

Although the seeds were sown with inoculum, the seeds could germinate with other fungi naturally occurring at the sites; thus, we had to verify identity of mycorrhizal fungi in protocorms. We have selected protocorms from up to 3 microsites per site, orchid species and treatment, if available. If protocorms occurred in both replicate seed packets within a microsite, protocorms from only one packet were used for molecular identification. For identification of mycorrhizal fungi in *Gymnadenia conopsea* only larger protocorms were chosen. DNA was isolated by CTAB technique (Doyle & Doyle, 1987) and stored at -20°C.

To identify isolates without costly sequencing, we developed specific primers to detect the inoculated fungi in protocorms and in the soil samples. We were successful in designing specific primers for TUL1N, TUL3N and TUL4N (Tab. 4), unfortunately designing primers specific to CER1N and CER2N failed. Specific primers to *Tulasnella* isolates were used for amplification of protocorms cultivated with respective inoculum. In case of a successful amplification and a clear band on a gel, a few samples were selected for sequencing and compared with the original sequences of inoculated fungal isolates. Protocorms from microsites inoculated with *Ceratobasidium* isolates were amplified with a primer pair ITS1F (Gardes & Bruns, 1993)/ LR21 Cer (Tedersoo et al., 2011) and sequenced. Samples with unsuccessful amplification with specific primers and samples from control treatments were amplified with less specific primer set ITS1 OFa,b/ ITS4 OF specific to orchid mycorrhizal basidiomycetes (Taylor & McCormick, 2008), ITS1/ ITS4 Tul (Taylor & McCormick, 2008) specific to some Tulasnellaceae, or fungal universal primer pair ITS1 F/ ITS4 (Gardes & Bruns, 1993; White et al., 1990). PCR reactions were made in 5 ul or 10 ul. Regular 10 ul volume contained 5 ul of Plain PP Master Mix (Top-Bio, Prague, Czech Republic), 0.6 ul of each primer (5 pmol/ul) and 2.8 ul of sterile ddH$_2$O. Amplification cycle consisted from initial denaturation at 95°C for 4 min, subsequently 40 cycles starting with denaturation at 95°C for 30 s, annealing temperature of specific primer set (Tab. 4), ITS 1OFa,b/ ITS4 OF at 55°C for 30 s; ITS 1/ ITS 4Tul at 52°C for 30 s; ITS 1F/ ITS 4 at 54°C for 30 s and elongation at 72°C for 30 s for unspecific primers, for specific see Tab. 4; followed by final elongation at 72°C for 10 min on XP Thermal Cycler (Bioer Technology). The PCR products were visualized on 1.5 % agarose gel in 1 x TBE buffer. When successful

amplification occurred, samples with weak bands were purified with Exo-Ap (Fast Alkaline Phosphatase) for 15 min at 37°C and for 15 min at 85°C (Affymetrix, Santa Clara, CA USA). Subsequently, all samples amplified with general primers were subjected to Sanger sequencing in commercial company SEQme s.r.o..

Tab. 4: Primers specific to Tulasnella operational taxonomic units (OTUs) positioned in internal transcribed spacer (ITS). Primers specific to Ceratobasidiaceae are positioned in small and large subunit of nuclear ribosomal DNA.

| fun-gus | primer | primer sequence | num-ber of cycles | annealing tempera-ture | elon-ga-tion |
|---|---|---|---|---|---|
| TUL1 N | Tul1-fw373 | GACTGTTGCGAGGCTGAAGC | 40 | 59°C | 72°C = 15s |
| | Tul1rev | GGTGTAAACGTCAGAGGCTGTC | | | |
| TUL3 N | Tul3 fw68 | GGTTAAACCCGTCGCTCTG | 40 | 55 °C | 72°C = 7s |
| | Tul3rev1 68 | AGTTTATACAACTGGTGTTAGA-CTC | | | |
| TUL4 N | Tul4F | AGCACTCATTGGGGTGCTAG | 40 | 64°C | 72°C = 15s |
| | Tul4rev | CGCCGAGTGGTAACCATTGA | | | |
| CER | ITS 1F | CTTGGTCATTTAGAGGAAGTAA | 40 | 57°C | 72°C = 30s |
| | LR21 Cer300 | CGACTCGTTGAGAGCACAA | | | |

The putative taxonomic identity of the sequences was searched via Blast search in NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences were grouped into operational taxonomic units (OTUs) based on 97% similarity threshold (viewed as the most general and realistic threshold for fungal species surrogates, Hughes et al. 2009) and compared with original sequences of fungi used as inocula (TUL1N, TUL3N, TUL4N, CER1N, CER2N).

Problematic samples had to be cloned. They were amplified by high performance polymerase Phusion High-fidelity DNA polymerase (NEB, USA) and Takara Ex Taq polymerase (Takara, Japan). PCR conditions with Phusion High-fidelity DNA polymerase were as follows: Amplification of samples with primer pair for TUL1N was modified to annealing temperature 62°C and elongation at 72°C for 20 s. Amplification of samples with

primer pair for TUL3N was modified to annealing temperature 54°C and elongation at 72°C for 20 s. Amplification of samples with primer pair ITS1F/ LR21 Cer was modified to annealing temperature 55°C/ 57°C/ 59°C and elongation at 72°C for 40 s. PCR amplification with polymerase Takara Ex Taq and specific primer sets was proceed as above only with some changes: Amplification of samples with primer pair for TUL4N was modified to annealing temperature 54°C and elongation at 72°C for 45 s. Amplification of samples with primer pair ITS 1OFa,b/ ITS4 OF was modified to annealing temperature 54°C/ 56°C and elongation at 72°C for 45 s. Amplification of samples with primer pair ITS 1F/ ITS 4 with modified elongation at 72°C for 45 s.

PCR products were separated on TBE agarose gel (range from 1.5 % to 3 % depending on size of PCR products, 90 V) and visualized by UV. 1.5 % TAE Low melting agarose gel (NuSieve GTG Agarose, Lonza) was used for separation of unspecific PCR products. Under UV, fragments of expected size were excised from the gel and transferred into Eppendorf tube. Subsequently it was incubated at 65°C for 10 min, 5 ul and the thawed gel was mixed with 14 ul of PCR water and 1 ul of p-GEM (Promega). Suspension was incubated at 65°C for 10 min in heat-block. 10 ul of suspension was subsequently mixed with 10 ul of 2x ligation buffer and 1 ul of ligase (Promega). Ligation was done at 16°C overnight. Transformation was performed as follows: 20 ul of ligation mix was added to 100 ul of thawed competent cells DH5alfa and mixed carefully.The suspension was kept on ice for 30 minutes and after that the mixture were heated at 42°C for 45 s in water bath. The tube was transferred quickly on ice and incubated for 5 min. Than 500 ul of SOC media was added to suspension and incubated in a horizontal shaker at 37°C, 200 rpm for 45 min. 200 ul of bacterial suspension was plated on LB (Sigma) plates (supplemented with ampicillin 100 mg/ml, 50 ul X -gal 20 mg/ml in dimethylformamid and 10 ul IPTG. 200 mg/ml and incubated at 37°C ON. Tranformants were selected at the base of blue white screening. 7white colonies from each transformation were transfered into 20 ul of PCR water using sterile toothpicks and denaturated in heat-block at 94°C for 5 minutes. 1 ul of suspension was used as a template for PCR. PCR conditions were  the same as for PPmaster mix (45 s, 40 cycles). We used SP6/T7 as primers. 2 ul of PCR products were separated on 1.5%TBE agarose gel, 90V. 3 positives colonies were subsequently sequenced for insert determination. We generally used T7 as a sequencing primers.

- *In situ* experiment - molecular evaluation of soil samples

Examination of protocorms was complemented with analysis of soil collected in the immediate vicinity to the seed packets during their retrieval in October 2017. Soil samples were collected and evaluated for treatments TUL1N and TUL3N of three middle-aged sites R23, RH8 and RV4. The soil samples were separately homogenized, sieved and desiccated in a dryer at 80°C. Subsequently DNA was isolated from 0,25 g of dry weight soil using the DNeasy PowerSoil Kit (QIAGEN, Germany) according to manufacturer's instructions and stored at -20°C. Soil samples were examined by quantitative PCR separately for TUL1N and TUL3N fungi with two setups for each primer pair with control samples.

Initially, soil samples were amplified to adjust temperature and length of PCR cycle on XP Thermal Cycler (Bioer Technology). Next, the concentration of DNA was measured by samples with added dye on the Qubit fluorometr (Invitrogen, Massachusetts, USA) with Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific, Oregon, USA) in volume 2 ul for adjusting standard curve. Reaction of quantitative PCR was performed with an ABI Step One Real-Time PCR Instrument (Applied Biosystems, California, USA), 20 ul reaction mixture contained: 1,2 ul of each primer (5 pmol/ul), 10 ul of FastStart Universal SYBR Green Master (Merck KGaA, Germany), 0,4 ul Bovine serum albumin (BSA) (Fermentas, Italy), 0,5 ul dimethylsulfoxide (DMSO) and 3,7 ul of $H_2O$ and 3 ul of DNA. Amplification was started with initial denaturation for 10 min at 95 °C followed by 40 cycles consisting of 30 s of denaturation at 95°C, 30 s of annealing at 59°C for TUL1N and 55°C for TUL3N, terminated by elongation for 15 s at 72°C for TUL1N and 15 s at 69°C for TUL3N. Every analysis was enclosed by melting curve analysis with measurement of fluorescence. Each sample was amplified in duplicates with added control samples without template DNA. Standard curve was made out of pure isolate *Tulasnella* sp. (TUL1N and TUL3N, in Tab. S1) amplified with PCR (specific primers of TUL1N/ TUL3N), purified with DNeasy PowerClean Pro Cleanup Kit (QIAGEN, Germany) and diluted to range ($79,3 \cdot 10^{-5}$ - $79,3 \cdot 10^{-9}$ ng/ul of target DNA) for TUL1N and in range ($22,2 \cdot 10^{-1}$ - $22,2 \cdot 10^{-5}$ ng/ul of target DNA) for TUL3N. The DNA quantity measured in samples was converted to copies of gene/ g dry weight and copies of gene/ ng DNA.

- Statistical evaluation

Statistical analyses were made in R (R Core Development Team 2009) using package nlme (Pinhiero et al. 2009). Orchid species were tested separately because of the difference in applied treatments. We tested effect of treatment and age (time since restoration) and their interaction on protocorm presence at a microsite using linear mixed-effect model with site as a random factor. Treatment without disturbance was selected as the control treatment. Same model was applied to test differences in the number of protocorms and size of the largest protocorm (logarithmic transformation of both) between treatments in microsites with germination. Treatments with too low number of microsites with germination were excluded from this analysis.

Presence of mycorrhizal fungi was also evaluated in soil in the immediate vicinity to the seed packets by quantitative PCR (qPCR). Abundance of mycorrhizal fungi was tested only for TUL1N and TUL3N fungi which were analyzed separately. We tested effect of treatment (hay and agar) and presence of protocorms within the microsite (categories 2 & 3) on abundance of mycorrhizal fungi (copies of gene per g of dry soil – gDRY, logarithmic transformation) using linear mixed-effect model with site as a random factor.

## Results:

- Results of *in situ* sowing

Seed germination was recorded for all species (*Gc*, *Pb*, *Ap*, *Om*) and all studied sites. Inocula of all fungal isolates (TUL1N, TUL3N, TUL4N, CER1N and CER2N) survived and induced germination in both inoculation treatments (hay and agar treatments).

*Orchis mascula*

*Orchis mascula w*as planted with mycorrhizal fungus TUL4N and control treatments (Tab. 3). Protocorms occurred at all sites of the experiment, with up to 5 microsites with protocorms per site and treatment (Tab. S2). Protocorms were found in 48 microsites (318 protocorms in total) with 47 microsites (317 protocorms) in TUL4N treatments and one microsite (1 protocorm) in a control treatment (disturbed soil) (Fig. 1).

Mycorrhizal fungus was isolated from 39 protocorms and in all cases identified as TUL4N. Among the identified protocorms was also the single protocorm from the control treatment which also germinated with TUL4N.

Time since restoration had not significant effect on germination (lme, $p>0.05$) as well as interaction between time since restoration and treatment ($p>0.05$) but treatment had significant effect on the presence of protocorms (lme, $F_{4, 252} = 46,6$; $p< 0.001$). Treatments TUL4 agar (lme, $t_{252} = 7,3$; $p< 0.001$) and hay (lme, $t_{252} = 10,0$; $p< 0.001$) had significantly higher amount of  microsites with protocorms than control (*no disturbance*). Number of protocorms was not affected by inoculation treatment (hay x agar), time since restoration or their interaction. Size of the largest protocorm was influenced only by treatment (lme, $F_{1, 37} = 5.96$, $p = 0.0195$), with protocorms in hay treatment being significantly bigger than in agar one (lme, $t_{37} = 2,4$; $p < 0.05$).

*Anacamptis pyramidalis*

*Anacamptis pyramidalis* was planted with CER2N and TUL1N inocula and control treatments (Tab. 3). Protocorms occurred at all sites of the experiment, with up to 4 microsites with protocorms per site and treatment (Tab. S3). Protocorms were found in 36 microsites (122 protocorms) in total with 32 microsites (106 protocorms) in TUL1N treatment, 1 microsite (4 protocorms) in CER2N treatment and 3 microsites (12 protocorms) in control treatments (Fig. 1).

Mycorrhizal fungus was identified in 33 out of 34 isolated protocorms (one protocorm per microsite for TUL1N, one per seed packets for CER2N and control treatments). 27 successfully isolated protocorms from TUL1N treatments were from 100 % identified as TUL1N. From two protocorms from two seed packets within one microsite of the CER2N treatment only one germinated with CER2N fungus; the other germinated with TUL1N naturally presented on the site RH6. Similarly, 4 protocorms from three microsites of control treatments associated with TUL1N.

Time since restoration of the sites did not have a significant effect on germination (lme, p>0.05) as well as interaction between time since restoration and treatment (lme, p>0.05) but treatment had significant effect on the presence of protocorms (lme, $F_{6, 343}$ = 19.9, p< 0.001). Treatments TUL1 agar (lme, $t_{343}$ = 6.1, p < 0,001) and hay (lme, $t_{343}$ = 6.9, p < 0,001) had significantly higher amount of microsites with protocorms than control (*no disturbance*). Treatments hay and agar of TUL1N differed in size of protocorms, which were significantly larger in hay treatment (lme, $t_{22}$ = 2.3, p = 0,0308).

*Platanthera bifolia*

*Platanthera bifolia* was planted with CER1N and TUL3N inocula and control treatments (Tab. 3). Protocorms occurred at only 6 out of 9 sites, they were not present at: R11, R23 and RV1. There were maximally 2 microsites with protocorms per site and treatment (Tab. S4). Protocorms were found in 22 microsites (81 protocorms) in total with 14 microsites (32 protocorms) in TUL3N treatments, 3 microsites (11 protocorms) in CER1N treatments and 5 microsites (38 protocorms) in control treatments (Fig. 1).

Mycorrhizal fungus was identified in 21 out of 22 isolated protocorms. 13 protocorms from TUL3N treatments were in 92 % of cases identified as the inoculated fungal strain TUL3N. Three protocorms from CER1N treatments did not germinate with the inoculated fungus. Fungi from these protocorms as well as 5 protocorms from control treatments were identified as other fungi from Ceratobasidiaceae and Tulasnellaceae.

Time since restoration of the sites had no significant effect on germination (lme, p>0.05) as well as interaction between time since restoration and treatment (lme, p>0.05) but treatment had significant effect on the presence of protocorms (lme, $F_{6, 335}$ = 3.59, p=0.0018). Treatments TUL3 agar (lme, $t_{341}$ = 2.2, p = 0,0255) and TUL3 hay (lme, $t_{341}$ = 3.2, p = 0,0015) had significantly higher amount of microsites with protocorms than control treatment (*no disturbance*). However, the treatments did not differ neither in the number of protocorms (lme, $F_{6, 10}$ = 0.48, p > 0.05) nor size (lme, $F_{6, 10}$ = 2.14, p>0.05).

*Gymnadenia conopsea*

*Gymnadenia conopsea* was planted with TUL1N, TUL3N, TUL4N, CER1N, CER2N inocula and control treatments (Tab. 3). Protocorms occurred at all sites of the experiment, with up to 5 microsites with protocorms per site and treatment (Tab. S5). Protocorms were found in 122 microsites (806 protocorms) in total (details from germination in Tab. 5), (Fig. 1).

Identification was successful in 84 out of 99 protocorms, which makes 85 %. The fungi which were not assigned to inoculated fungal isolates and also fungi from protocorms from 20 microsites in control treatments were assigned to 14 naturally present fungal OTUs from Tulasnellaceae and Ceratobasidiaceae.

Tab. 5: Germination of seeds *Gymnadenia conopsea* and their evaluation.

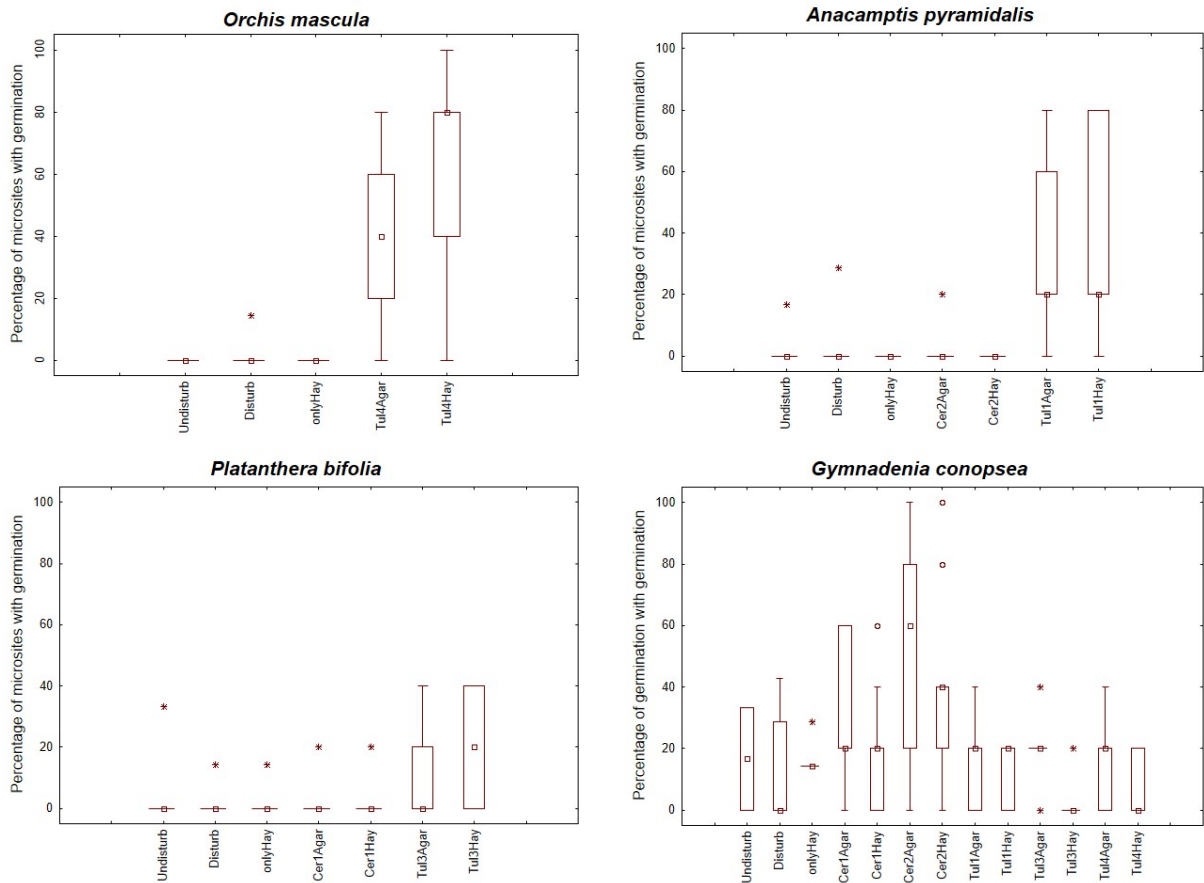| Treatment | Number of microsites/ protocorms | Number of isolated protocorms | Percentage of isolated protocorms with added fungal isolate |
|---|---|---|---|
| TUL1N | 13/ 65 | 8 | 50 % |
| TUL3N | 11/ 90 | 4 | 50 % |
| TUL4N | 10/ 42 | 8 | 63 % |
| CER1N | 22/ 180 | 12 | 58 % |
| CER2N | 39/ 264 | 32 | 78 % |
| control | 27/ 165 | 20 | — |



Fig. 1: Percentage of microsites with germination in different treatments in in situ germination experiment. Control treatments are: *Undisturb* - not disturbed soil, *Disturb* - disturbed soil and *onlyHay* - only sterile hay, treatments with added inocula CER1N (CER1Agar and CER1Hay), CER2N (CER2Agar and CER2Hay), TUL1N (TUL1Agar and TUL1Hay), TUL3N (TUL3Agar and TUL3Hay) and TUL4N (TUL4Agar and

Time since restoration of the sites did not have a significant effect on germination (lme, p>0.05) as well as interaction between time since restoration and treatment (lme, p>0.05) but treatment had significant effect on the presence of protocorms (lme, $F_{12, 593}$ = 5.0; p< 0.001). Treatments CER2N agar (lme, $t_{605}$ = 4.0, p < 0,001) and CER2N hay (lme, $t_{605}$ = 2.6, p = 0,0107) had significantly higher amount of microsites with protocorms than control (*no disturbance*). Also size of protocorms was significantly affected by applied treatments (lme, $F_{12, 101}$ = 2.07, p=0.0253) with protocorms from treatments TUL1N hay (lme, $t_{101}$ = -2.4, p = 0,0163) and TUL4N hay (lme, $t_{101}$ = -2.0, p = 0,0490) being significantly smaller than protocorms in control (*no disturbance*).

- Quantification of mycorrhizal fungi in soil

Soil samples coming from TUL1N and TUL3N treatment microsites were used for quantification of inoculated fungi by qPCR and were processed separately.

We have processed 35 samples from TUL1N treatments within two runs with control sample to ensure comparability. qPCR detecting TUL1N fungus had R2 values for standard curves > 0.98 with the slope -3.65 for both runs giving the estimated amplification efficiency 88 %. Microsites with TUL1N treatments contained seed packets of *Anacamptis pyramidalis* and *Gymnadenia conopsea.* Neither presence of protocorms of an orchid species associated with TUL1N nor agar vs hay inoculation significantly (lme, p>0.05) influenced abundance (copies of gene per gram of dry soil) of TUL1N fungus.

We have processed 34 samples from TUL3N treatments within two runs with control sample to ensure comparability. qPCR detecting TUL3N fungus has R2 values for standard curves > 0.99 with the slope between -3.85 and -3.92 giving the estimated amplification efficiency between 82 and 80 %, respectively. Microsites with TUL3N treatment contained seed packets of *Platanthera bifolia* and *Gymnadenia conopsea.* Neither treatments nor microsites with or without germination differed in the abundance of TUL3N per copies of gene per gram of dry soil (gDRY).

**Discussion:**

- Comparison of amelioration treatments and survival of added fungal isolates

Results of orchid seed sowing experiment in restored meadows in White Carpathians supported our hypothesis that addition of fungal symbiotic inocula improves orchid germination. The inoculated fungi were capable to survive in restored meadows at least one year and had a significant impact on the presence of protocorms.

There are several ways how to ameliorate seed sowing with addition of fungal inocula. Fungal inoculum is often added on complex organic substrate. McCormick et al. (2012)  used wood chips in their study on forest orchids. Addition of wood positively affected growth of saprotrophic fungi forming mycorrhiza with their studied orchids (Rasmussen & Whigham, 1998). We have chosen hay as an organic substrate for inoculated fungi despite we do not know about any study that would use hay as substrate for inoculation. Hay was selected as the most natural material available in grasslands and possible substrate for saprotrophic fungi growing there. Fungal growth on sterile hay was, however, time demanding and the identity of the growing fungus had to be verified because of occurrence of contaminant fungi.

As the other method of inoculation, we used inoculated OMA medium mixed with sterile hay at seed packet burial  ("agar" method). Inoculated OMA medium was used before by Hollick (2004) and Batty et al. (2006b) in form of "cubes made out of rolled oats". Quay et al. (1995) used potato dextrose agar (PDA). From our experience, usage of solid medium was convenient because of the fungi fast growth, uncomplicated handling and absence of contamination.

The success of orchid germination is also affected by the amount of added inoculum, although we tried to add comparable amount for both hay and agar treatments, we do not have any data to prove it. *Ex vitro* studies show positive as well as negative impact of quantity of added inoculum to the seeds. Mursidawati (2004) faced in her study problems with vigorous growth of added inocula. That was probably cause by unsuitable inoculated material (millets) and also high quantity of inoculum. Positive effect of added inoculum was described by Batty et al. (2006b), they achieved the best germination results when inoculated with the highest tested volume 300 g of inocula per microsite.

Despite the fact that the amount of inoculum added during seed sowing in the two types of inoculation (inoculated hay vs inoculated agar with sterile hay) was different, the type of inoculation did not have significant effect on the number of protocorms of any orchid species. The only difference we found was that protocorms of *Orchis mascula* and *Anacamptis pyramidalis* were bigger in microsites inoculated with hay. Other control treatments, such as organic material addition or disturbance did not significantly increase protocorm presence of any of the studied orchid species compared to undisturbed control. These results are dissimilar from positive effect of addition of organic matter and soil disturbance to direct seeding described by Wright et al. (2007).

We confirmed survival of fungal inocula by quantifying its abundance and indirectly by evaluating seed germination on inoculated microsites. Survival of inoculated fungi was compared between species and it was confirmed that all fungal isolates survived although they did not induce germination of all studied orchid species *in situ.*

- Mycorrhizal specificity in vitro and in situ

Fungal isolates for *in situ* experiment were initially chosen on the base of results of *in vitro* sowing experiment to select efficient mycorrhizal symbionts. In our study, seed germination was induced by fungi isolated from both protocorms (TUL1N, TUL3N, CER1N and CER2N) and mature orchid (TUL4N). Although orchid seeds of all four species were inoculated *in vitro* with all five fungal isolates, germination occurred only at some combinations (Tab. 1). Seeds of *Anacamptis pyramidalis* germinated *in vitro* only with TUL1N although also fungal isolate CER2N was isolated from *Anacamptis pyramidalis* protocorm. Isolates used in *in vitro* experiment induced seed germination also in *in situ* experiment. Although generalist Gymnadenia conopsea germinated *in vitro* with all fungal isolates (the largest protocorms were achieved when inoculated with Ceratobasidiaceae (data not shown)), *in situ* was germination significantly induced only by CER2N fungal isolate. Results for Gymnadenia conopsea are similar to general view that fungal isolates inducing germination of some orchids under laboratory conditions in *in vitro* experiments are often incorrectly mistaken as mycorrhizal fungi inducing germination also *in situ* (Batty et al., 2001). It would be more accurate to describe the fungi as "potentially specific" because they

can be unsuccessful in establishment of symbiosis in natural condition (Masuhara & Katsuya, 1994).

It was found out that co-occuring orchid species growing in vicinity tend to germinate with different fungi (Waterman et al. 2011; Jacquemyn et al., 2012; Těšitelová et al., 2013) to avoid competition. We have examined 12 microsites where at least two protocorms from different orchid species occurred, 5 microsites did not share the same fungus contrary to 7 examined microsites where protocorms shared the same fungus. This is the first study to our knowledge where two orchid species were inoculated *in situ* with one fungal isolate. Because of the fact that single fungal isolate was shared by two orchid species (four orchid species in *in vitro* experiment) the coexistence of orchids is still questioned.

In two species, *Orchis mascula* and *Anacamptis pyramidalis*, we detected marked effect of inoculated fungi on the protocorm presence. Protocorms of these orchids associated only with inoculated fungi. In the rare cases of germination in control treatments, the same fungi likely naturally occurring at the sites were detected. *Orchis mascula* has high fungus specificity and is known for germination with only one fungal isolate in Europe (Jacquemyn et al., 2010; 2012; Vogt-Shilb et al., unpublished). Although *Anacamptis pyramidalis* germinated in our study with only two fungal strains, it is known to forms relationship with higher number of mycorrhizal fungi (Pellegrino et al., 2014; Çiğ et al., 2018). These two orchid species colonize restored sites only sporadically because their symbiotic fungi occur at restored sites only rarely (Vogt-Shilb et al., unpublished).

That is in contrast with *Platanthera bifolia* and *Gymnadenia conopsea.* These species have low fungal specificity (*Pb* - Esposito et al., 2016; *Gc* - Jacquemyn et al., 2012; Těšitelová et al., 2013), and germinate with naturally occurring opportunistic fungi, which likely represent more stable and long-lasting support (McCormick et al., 2006; 2018). That can cause overestimation of germination success in our experiment, when the protocorms are only counted and not molecularly identified. There were 3 OTUs found in protocorms of *Platanthera* and 14 OTUs found in protocorms of *Gymnadenia* which were not added to the sites but occurred naturally. Especially ability to germinate with opportunistic fungi present at yearly stages of restored sites makes these orchids good colonizers (Stark et l., 2009; Vogt-Shilb et al., unpublished). Sucháček (2015) states in his study that *Gymnadenia*

*conopsea* germinated at restored meadows in White Carpathian Mountains independently on time since restoration.

- Abundance of inoculated fungi

Abundance of inoculated mycorrhizal fungi was evaluated only for fungal isolates: TUL1N and TUL3N because of problems with design of specific primers or their low effectivity in quantitative PCR. We have compared application of hay versus agar inocula on fungal abundance as well as seed germination and did not find any effect on abundance of fungi which is in contrast with research of McCormick et al. (2009, 2012, 2016, 2018). Abundance of inoculated fungi was determined in place of buried inoculum and both microsites with and without protocorm presence were sampled. Inoculated fungi were detected in all the microsites that confirms fungal presence exceeding seed germination as described by McCormick et al. (2016). McCormick et al. (2018) in their study pointed out that it is important to sample soil from vicinity to the orchid otherwise there will not be any relationship detected. They pointed out that soil is heterogenous and abundance of mycorrhizal fungi differs at a small scale. However Hollick (2004) detected ones growth of inoculated fungus up to 50 cm from the outset in the first growing season and another inoculated fungus was still detected even after three years. These findings seldom are positive start for further long term studies.

However, the absence of any relationship between protocorm presence and inoculum abundance could be caused by low number of sampled microsites (only 3 per treatment and site). Fungal abundance largely varied: $6.61*10^3$ - $7.08*10^5$ copies of gene/ g dry soil for TUL1 and $1.81*10^5$ - $2.74*10^8$ copies of gene/ g dry soil for TUL3 and the number of replicates was likely too low for such variable results. We suggest that fungal abundance could have been measured also from the vicinity of naturally growing orchids. Suitable orchid of our interest would be the most fungal specific one - *Orchis mascula*. However, primers amplifying fungal isolate TUL4N were not effective enough for amplification with qPCR. Since fungal abundance was in our study measured only after first season we lack long term data to observe interactions of added fungal inocula with the soil community as well as ontogenetic development of sown orchid seeds.

- Influence of abiotic factors

Abiotic soil properties of restored grasslands differed in several edaphic factors from natural meadows (Tab. 2). However, after addition of fungal inoculum (in *Anacamptis pyramidali*s and *Orchis mascula*) or even without (*Gymnadenia conopsea*), orchids established at all studied sites, regardless edaphic conditions at the sites. In congruence, Sucháček (2015) observed frequent germination of little specific *Gymnadenia conopsea* and *Neottia ovata* on restored meadows, while only sporadical germination was found in other species. Thus, the fungus availability seems to be the main limiting factor for establishment of specific orchid species in restored grasslands.

The edaphic conditions likely influence the fungal community composition, potentially leading to absence of mycorrhizal fungi for orchids. Average values of natural and restored grasslands differed significantly in amount of available phosphorus. Level of phosphorus was 14 times larger in restored  than in natural grasslands as a residue of fertilization which may require up to 50 years to decrease to pre-fertilization levels (Fagan et al. 2008). High values of labile phosphate has also negative effect on saprophytic fungi (Van der Wal et al. 2006b), P-rich soils are favored by Ascomycota with reduced representation of Basidiomycota (Lauber et al., 2008). When comparing natural and restored meadows Ceratobasidiaceae family was more frequent on P-rich restored meadows compare to natural meadows where Sebacinales were abundant (Vogt-Shilb et al., unpublished).

Follow-up dissimilar factor distinguishing restored and natural meadows was total nitrogen and mainly organic matter which was in natural grassland almost over half of the amount larger (Tab. 2) and likely plays crucial role for slow-growing saprotrophic Basidiomycetes (De Boer et al., 2005). Although amount of nitrites in soil was not documented in our experiment, their inhibition effect on orchid germination was repeatedly shown in *in vitro* experiments (Ponert et al. 2013). Available phosphorus and organic matter seems to be among most important factors having effect on mycorrhizal fungi and indirectly also on orchids themselves (Vogt-Shilb et al., unpublished).

## Conclusions:

Restored plant communities often lack rare species such as orchids. It is not well documented what is limiting factor of their occurrence in these ecosystems, and why orchids particularly are not present or their reintroduction is slow. Nevertheless it is apparent that mycorrhizal fungi play a crucial role in orchid reintroduction. The fungal diversity is the lowest in the first years after habitat restoration and it may take decades to restore fungal community similar to natural sites. The process of orchid reintroduction at restored sites can be fastened by sowing orchid seeds complemented with addition of suitable mycorrhizal fungus. This technique seems to enhance protocorm formation compared to direct sowing without any fungal supplements. It is especially valuable for restoration of terrestrial orchids with high fungal specificity on localities with unknown occurrence of suitable mycorrhizal fungi.

Nevertheless further research is still needed to confirm long-term survival of fungi and orchids in new areas and establishment of a permanent colony. This research was focused only on the initial phase of orchid and fungi introduction to restored grasslands. In future work we suggest to focus on a long term research with orchid seeds sown directly to localities and regular quantification of mycorrhizal symbiont in the immediate vicinity of the seeds.

**References:**

Abeli T. & Dixon K. (2016): Translocation ecology: the role of ecological sciences in plant translocation. Plant Ecology editorial: 1–3.

Aewsakul N., Maneesorn D., Serivichyaswat P., Taluengjit A. & Nont Achaiy Apoom S. (2013): *Ex vitro* symbiotic seed germination of *Spathoglottis plicata* Blume on common orchid cultivation substrates. Scientia Horticulturae 160: 238–242.

Aggarwal S. & Zettler L. W. (2010): Reintroduction of an endangered terrestrial orchid, *Dactylorhiza hatagirea* (D. Don) Soo, assisted by symbiotic seed gemination - First report from the Indian subcontinent. Nature Science 8: 139–145.

Alvarez-Aquino C., Williams-Linera G. & Newton A. C. (2004): Experimental native tree seedling establishment for the restoration of a Mexican cloud forest. Restoration Ecology 12: 412–418.

Aradottir A. L. (2012): Turf transplants for restoration of alpine vegetation: does size matter? Journal of Applied Ecology 49: 439–446.

Arditti J. & Ghani A. K. A. (2000): Tansley review No. 110: numerical and physical properties of orchid seeds and their biological implications. New Phytologist 145: 367–421.

Azcon R., Ambrosano E. & Charest C. (2003): Nutrient acquisition in mycorrhizal lettuce plants under different phosphorus and nitrogen concentration. Plant Science 165: 1137–1145.

Batty A. L., Brundrett M. C., Dixon K. W. & Sivasithamparam K. (2006a): New methods to improve symbiotic propagation of temperate terrestrial orchids from axenic culture to soil. Australian Journal of Botany 54: 367–374.

Batty A. L., Brundrett M. C., Dixon K. W. & Sivasithamparam K. (2006b): *In situ* seed germination and propagation of terrestrial orchid seedlings for establishment at field sites. Australian Journal of Botany 54: 375-381.

Batty A. L., Dixon K. W., Brundrett M. & Sivasithamparam K. (2001): Constraints to symbiotic germination of terrestrial orchid seed in a mediterranean bushland. New Phytologist 152: 511- 520.

Bidartondo M. I. (2005): Tansley Review: The evolutionary ecology of mycoheterotrophy. New Phytologist 167: 335–352.

Bittman S., Forge T. A. & Kowalenko C. G. (2005): Responses of the bacterial and fungal biomass in a grassland soil to multi-year application of dairy manure slurry and fertilizer. Soil Biology & Biochemistry 37: 613–623.

Box J. (1999): Habitat translocation and mineral workings. Mineral Planning 79: 11–14.

Brown, J. & Mitchell, N. (2006): Partnerships and protected landscapes: New conservation strategies that engage communities. Paper presented at the 2005 George Wright Society Conference on Parks, Protected Areas, and Cultural Sites: Hancock, Michigan.

Chytrý M., Dražil T., Hájek M., Kalníková V., Preislerová Z., Šibík J., Ujházy K., Axmanová I., Bernátová D., Blanár D., Dančák M., Dřevojan P., Fajmon K., Galvánek D., Hájková P., Herben T., Hrivnák R., Janeček Š., Janišová M., Jiráská Š., Kliment J., Kochjarová J., Lepš J., Leskovjanská A., Merunková K., Mládek J., Slezák M., Šeffer J., Šefferová V., Škodová I., Uhlířová J., Ujházyová M. & Vymazalová M. (2015): The most species-rich plant communities in the Czech Republic and Slovakia (with new world records). Preslia 87: 217–278.

Çiğ A., Demirer D. E. & Isler S. (2018): *In vitro* symbiotic germination potentials of some *Anacamptis*, *Dactylorhiza*, *Orchis* and *Ophrys* terrestrial orchid species. Applied Ecology and Environmental Research 16(4): 5141-5155.

Clemets M. A., Muir H. & Cribb P. J. (1985): A preliminary report on the symbiotic germination of European terrestrial orchids. Kew Bulletin 41: 437-45.

De Boer W., Folman L. B., Summerbell R. C. & Boddy L. (2005): Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiology Reviews 29: 795–811.

DeForest J. L., Zak D. R., Pregitzer K. S. & Burton A. J. (2004): Atmospheric nitrate deposition, microbial community composition, and enzyme activity in northern hardwood forests. Soil Science Society of America Journal 68: 132–138.

De hert K., Jacquemyn H., Provoost S. & Honnay O. (2013): Absence of recruitment limitation in restored dune slacks suggests that manual seed introduction can be a successful practice for restoring orchid populations. Restoration Ecology 21: 159–162.

Dıaz S., Fargione J., Chapin F. S. &Tilman D. (2006): Biodiversity loss threatens human well-being. PLoS Biology 4: e277.

Diez J. M. (2007): Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. Journal of Ecology 95: 159-170.

Doyle J.J. & Doyle J. L. (1987): A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19: 11–5.

Esposito F., Jacquemyn H., Waud M. & Tyteca D. (2016): Mycorrhizal fungal diversity and community composition in two closely related *Platanthera* (*Orchidacea*e) species. PLoS ONE 11: 1–14.

Fagan K. C., Pywell R. F., Bullock J. M. & Marrs R. H. (2008): Do restored calcareous grasslands on former arable fields resemble ancient targets? The effect of time, methods and environment on outcomes. Journal of Applied Ecology 45(4): 1293-1303.

García de León D., Moora M., Öpik M., Jairus T., Neuenkamp L., Vasar M., Bueno C. G., Gerz M., Davison J. & Zobel M. (2016a): Dispersal of arbuscular mycorrhizal fungi and plants during succession. Acta Oecologica 77: 128–135.

García de León D., Moora M., Öpik M., Neuenkamp L. & Gerz M. (2016b): Symbiont dynamics during ecosystem succession: co-occurring plant and arbuscular mycorrhizal fungal communities. FEMS Microbiology Ecology 92: fiw 097.

Gardes M. & Bruns T. D. (1993): ITS primers with enhanced specificity for Basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology 2: 113–118.

Gebauer G., Preiss K. & Gebauer A. C. (2016): Partial mycoheterotrophy is more widespread among orchids than previously assumed. New Phytologist 211: 11–15.

Godefroid S., Piazza C., Rossi G., Buord S., Stevens A-D., Aguraiuja R., Cowell C., Weekley C. W., Vogg G., Iriondo J. M., Johnson I., Dixon B., Gordon D., Magnanon S., Valentin B., Bjureke K., Koopman R., Vicens M., Virevaire M. & Vanderborght T. (2011): How successful are plant species reintroductions? Biological Conservation 144: 672-682.

Hadley G. (1982): Orchid Mycorrhiza. In: Arditti J. (ed). Orchid Biology Review and Perspective Vol. 2. Cornell University Press, Ithaca New York. pp. 85-100.

Harris A. R., Schisler D. A. & Neate S.M. (1993): Culture of *Rhizoctonia solani* and binucleate R*hizoctonia* spp. on organic substrates for inoculation of seedlings in containers. Soil Biology & Biochemistry 25: 337-341.

Herrera H., Valadares R., Contreras D., Bashan Y. & Arriagada C. (2017): Mycorrhizal compatibility and symbiotic seed germination of orchids from the coastal range and Andes in south Central Chile. Mycorrhiza 27: 175–188.

Hollick P. S. (2004): Mycorrhizal specificity in the endemic Western Australian terrestrial orchids (subtribe *Diurideae*): implications for conservation. PhD Thesis, Murdoch University, Perth.

Huber A. G. (2002): Mountain lady's slipper (*Cypripedium montanum*): Establishment from seeds in forest openings. Native Plants Journal 3: 151-154.

Hudson P. J., Dobson A. P. & Lafferty K.D. (2006): Is a healthy ecosystem one that is rich in parasites? Trends in Ecology & Evolution 21: 381–385.

Hughes, K. W., Petersen R. H. & Lickey E. B. (2009): Using heterozygosity to estimate a percentage DNA sequence similarity for environmental species' delimitation across basidiomycete fungi. New Phytologist 182: 795–798.

Janečková P., Wotavová K., Jersáková J. & Kindlmann P. (2006): Relative effects of management and environmental conditions on performance and survival of populations of a terrestrial orchid, *Dactylorhiza majalis*. Biological Conservation 129: 40–49.

Jacquemyn H., Brys R., Lievens B. and Wiegand T. (2012): Spatial variation in below-ground seed germination and divergent mycorrhizal associations correlate with spatial segregation of three co-occurring orchid species. Journal of Ecology 100: 1328–1337.

Jacquemyn H., Honnay O., Cammue B. P. A., Brys R. & Lievens B. (2010): Low specificity and nested subset structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*. Molecular Ecology 19: 4086–4095.

Jersáková J. & Malinová T. (2007): Spatial aspects of seed dispersal and seedling recruitment in orchids. New Phytologist 176: 237-241.

Jongepierová I., Mitchley J. & Tzanopoulos J. (2007): A field experiment to recreate species rich hay meadows using regional seed mixtures. Biological Conservation 139: 297–305.

Jongepierová I. (ed.) (2008): Louky Bílých Karpat. Grasslands of the White Carpathian Mountains. ČSOP Bílé Karpaty, Veselí nad Moravou, Czech Republic.

Jumpponen A., Brown S. P., Trappe J. M., Cazares E. & Strommer R. (2012): Twenty years of research on fungal–plant interactions on Lyman Glacier forefront – lessons learned and questions yet unanswered. Fungal Ecology 5: 430–442.

Kiehl K., Kirmer A., Donath T. W., Rasran L. & Holzel N. (2010): Species introduction in restoration projects – evaluation of different techniques for the establishment of semi-natural grasslands in Central and Northwestern Europe. Basic and Applied Ecology 11: 285–299.

Klimeš L. (2008): Druhové bohatství luk. In: Jongepierová I. (ed.), Louky Bílých Karpat, ZO ČSOP Bílé Karpaty, Veselí nad Moravou, Czech Republic. pp. 89–94.

Klimkowska, A., van Diggelen, R., Bakker, J. P. & Grootjans, A. O. (2007): Wet meadow restoration in Western Europe: A quantitative assessment of the effectiveness of several techniques. Biological Conservation 140: 318–328.

Kohout P., Těšitelová T., Roy M., Vohnik M. & Jersáková J. (2013): A diverse fungal community associated with *Pseudorchis albida* (*Orchidaceae*) roots. Fungal Ecology 6: 50–64.

Lauber C. L., Strickland M. S., Bradford M. A. & Fierer N. (2008): The influence of soil properties on the structure of bacterial and fungal communities across land-use types. Soil Biology & Biochemistry 40: 2407–2415.

Maharning A. R., Mills A. A. & Adl S. M. (2009): Soil community changes during secondary succession to naturalized grasslands. Applied Soil Ecology 41: 137–147.

Martin K. P. (2003): Clonal propagation, encapsulation and reintroduction of *Ipsea malabarica* (Reichb. f.) JD Hook., an endangered orchid. In Vitro Cellular & Developmental Biology - Plant 39(3): 322–326.

Martınez-Garcıa L. B., Richardson S. J., Tylianakis J. M., Peltzer D. A. & Dickie I.A. (2015): Host identity is a dominant driver of mycorrhizal fungal community composition during ecosystem development. New Phytologist 205: 1565–1576.

Marx D. H. & Bryan W. C. (1975): Growth and ectomycorrhizal development of Loblolly pine seedlings in fumigated soil infested with the fungal symbiont *Pisolithus tinctorius*. Forest Science 21: 245–254.

Masuhara G. & Katsuya K. (1994): *In situ* and *in vitro* specificity between *Rhizoctonia* spp. & *Spiranthes sinensis* (Persoon.) Ames. var. *amoena* (M. Beiberstein) Hara (*Orchidaceae*). New Phytologist 127: 711–718.

McCormick M. K. & Jacquemyn H. (2014): What constrains the distribution of orchid populations? New Phytologist 202: 392–400.

McCormick M. K., Taylor D. L., Juhaszova K., Burnett R. K., Whigham D. F., & O'Neill J. P. (2012): Limitations on orchid recruitment: Not a simple picture. Molecular Ecology 21: 1511–1523.

McCormick M. K., Taylor D. L., Whigham D. F. & Burnett R. K. Jr. (2016): Germination patterns in three terrestrial orchids relate to abundance of mycorrhizal fungi. Journal of Ecology 103: 744–754.

McCormick M. K., Whigham D. F. & Canchani-Viruet A. (2018): Mycorrhizal fungi affect orchid distribution and population dynamics. New Phytologist 219: 1207–1215.

McCormick M. K.,Whigham D. F., O'Neill J. P., Becker J.J., Werner S., Rasmussen H. N., Bruns T. D. & Taylor D. L. (2009): Abundance and distribution of *Corallorhiza odontorhiza* reflects variations in climate and ectomycorrhizae. Ecological Monographs 79: 619–635.

Merunková K., Preislerová Z. & Chytrý M. (2012): White Carpathian grasslands: can local ecological factors explain their extraordinary species richness? Preslia 84: 311–325.

Michalcová D., Chytrý M., Pechanec V., Hájek O., Jongepier J.W., Danihelka J., Grulich V., Šumberová K., Preislerová Z., Ghisla A., Bacaro G. & Zelený D. (2014): High plant diversity of grasslands in a landscape context: A comparison of contrasting regions in central Europe. Folia Geobotanica 49: 117–135.

Mudrák O., Fajmon K., Jongepierová I. & Doležal J. (2017): Restoring species-rich meadow by means of turf transplantation: Long-term colonization of ex-arable land. Applied Vegetation Science 20: 62–73.

Mursidawati S. (2004): Mycorrhizal association, propagation and conservation of the myco-heterotrophic orchid *Rhizantella gardneri*. Thesis at University of Wester Australia, Perth, WA, Australia.

Newman B. J., Ladd P., Brundrett M. & Dixon K. W. (2013): Effects of habitat fragmentation on plant reproductive success and population viability at the landscape and habitat scale. Biological Conservation 159: 16–23.

Otýpková Z., Chytrý M., Tichý L., Pechanec V., Jongepier J. W. & Hájek O. (2011): Floristic diversity patterns in the White Carpathians biosphere reserve, Czech Republic. Biologia 66 (2): 266–274.

Pellegrino G., Luca A. & Bellusci F. (2014): Relationships between orchid and fungal biodiversity: Mycorrhizal preferences in mediterranean orchids. PlantBiosystems 150: 180-189.

Pinhiero J., Bates D., DebRoy S., Sarkar D. & R Core team (2009): Nlme: linear and nonlinear mixed effects models R package version 31–96.

Ponert J., Figura T., Vosolsobě S., Lipavská H., Vohník M. & Jersáková J. (2013): Asymbiotic germination of mature seeds and protocorm development of *Pseudorchis albida* (*Orchidaceae*) are inhibited by nitrates even at extremely low concentrations. Botany 91(10): 662-670.

Ponert J., Vosolsobě S., Kmecová K. & Lipavská H. (2011): European orchid cultivation - from seed to mature plant. European Journal of Environmental Sciences 1(2): 95–107.

Prach K. & Hobbs R. J. (2008): Spontaneous succession versus technical reclamation in the restoration of disturbed sites. Restoration Ecology 16: 363-366.

Prach K., Jongepierová I. & Řehounková K. (2013): Large-scale restoration of dry grasslands on ex-arable land using a regional seed mixture: establishment of target species. Restoration Ecology 21: 33–39.

Prach K., Jongepierová I., Řehounková K. & Fajmon K. (2014): Restoration of grasslands on ex-arable land using regional and commercial seed mixtures and spontaneous succession: successional trajectories and changes in species richness. Agriculture, Ecosystems & Environment 182: 131–136.

Prach K., Fajmon K., Jongepierová I. & Řehounková K. (2015): Landscape context in colonization of restored dry grasslands by target species. Applied Vegetation Science 18: 181–189.

Press M. C. & Phoenix G. K. (2005): Impacts of parasitic plants on natural communities. New Phytologist 166: 737– 751.

Pywell R. F., Bullock J. M., Hopkins A., Walker K. J., Sparks T. H., Burke M. J. W. & Peel S., (2002): Restoration of species-rich grassland on arable land: assessing the limiting processes using a multi-site experiment. Journal of Applied Ecology 39: 294–309.

Quay L., McComb J. A. & Dixon K. W. (1995): Methods for *ex vitro* germination of Australian terrestrial orchids. HortScience 30: 1445–1446.

Ramsay R. R., Sivasithamparam K. & Dixon K. W. (1986): Patterns of infection and endophytes associated with Western Australian orchids. Lindleyana 1: 203–214.

Rasmussen H.N. (1995): Terrestrial Orchids: From Seed to Mycotrophic Plant. Cambridge University Press, Cambridge, UK.

Rasmussen H.N. & Whigham D.F. (1993): Seed ecology of dust seeds *in situ*: a new study technique and its application in terrestrial orchids. American Journal of Botany 80: 1374–1378.

R Development Core Team (2009): R: a language and environment for statistical computing R Foundation for Statistical Computing Vienna Austria. http://wwwR-projectorg.

Reiter N., Whitfield J., Pollard G., Bedggood W., Argall M., Dixon K., Davis B. & Swarts N. (2016): Orchid re-introductions: an evaluation of success and ecological considerations using key comparative studies from Australia. Plant Ecology 217: 81–95.

Ruiz-Jaen M. C. & Aide T. M. (2005): Restoration success: how is it being measured? Restoration Ecology 13: 569–577.

Shefferson R. P., Kull T. & Tali K. (2008): Mycorrhizal interactions of orchids colonizing Estonian mine tailing hills. American Journal of Botany 95: 156–164.

Stark C., Babik W. & Durka W. (2009): Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. Mycological Research 113: 952–959.

Sucháček P. (2015): Liší se klíčivost vybraných druhů vstavačovitých rostlin na přirozených a obnovených loukách? Č. Budějovice, diplomová práce (Ing.). Jihočeská univerzita v Českých Budějovicích. Zemědělská fakulta.

Swarts N. 2007. Integrated conservation of the rare and endangered terrestrial orchid *Caladenia huegelii* H.G. Reichb. Ph.D. Thesis, University of Western Australia, Perth.

Swarts, N. D. & Dixon K. W. (2009): Terrestrial orchid conservation in the age of extinction. Annals of Botany 104: 543–556.

Swarts N. D. & Dixon K. W. (2017): Conservation methods for terrestrial orchids. Plantation, FL, USA: J. Ross Publishing.

Taylor D. L. & McCormick M. K. (2008): Internal transcribed spacer primers and sequences for improved characterization of basidiomycetous orchid mycorrhizas. New Phytologist 177: 1020-1033.

Tedersoo L., Bahram M., Jairus T., Bechiem E., Chinoya S., Mpumba R., Leal M., Randriaanjohany E., Razafimandimbison S. & Sadam A. (2011): Spatial structure and the effects of host and soil environments on communities of ectomycorrhizal fungi in wooded savannas and rain forests of Continental Africa and Madagascar. Molecular Ecology 20: 3071–3080.

Těšitelová T., Jersáková J., Roy M., Kubátová B., Těšitel J., Urfus T., Trávníček P. & Suda J. (2013): Ploidy specific symbiotic interactions: divergence of mycorrhizal fungi between cytotypes of the *Gymnadenia conopsea* group (*Orchidaceae)*. New Phytologist 199: 1022–1033.

Těšitelová T., Těšitel J., Jersáková J., Říhová G. & Selosse M-A. (2012): Symbiotic germination capability of four *Epipactis* species (*Orchidaceae*) is broader than expected from adult ecology. American Journal of Botany 99: 1020–1032.

Valkó O., Deák B., Török P., Kelemen A., Miglécz T., Tóth K. & Tóthmérész B. (2016): Abandonment of croplands: problem or chance for grassland restoration? Case studies from Hungary. Ecosystem Health and Sustainability 2(2): e01208.

Van der Wal A.,Van Veen J. A., Pijl A. S., Summerbell R. C. & De Boer W. (2006a): Constraints on development of fungal biomass and decomposition processes during restoration of arable sandy soil. Soil Biology & Biochemistry 38: 2890–2902.

Van der Wal A., Van Veen J. A., Smant W., Boschker H. T. S., Bloem J., Kardol P., Van der Putten W.H. & De Boer, W. (2006b): Fungal biomass development in a chronosequence of land abandonment. Soil Biology & Biochemistry 38: 51–60.

Waterman R. J., Bidartondo M. I., Stofberg J., Combs J. K., Gebauer G., Savolainen V., Barraclough T. G. & Pauw A. (2011): The effects of above and belowground mutualisms on orchid speciation and coexistence. American Naturalist 177: E54–E68.

White T. J., Bruns T. D., Lee S. & Taylor J. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A.& Gelfand D. H. (eds). PCR Protocols: A Guide to Methods and Applications. Academic Press: London, pp. 315 – 322.

Wright M., French G., Cross R., Cousens R., Andrusiak S. & McLean C. B. (2007): Site amelioration for direct seeding of *Caladenia tentaculata* improves seedling recruitment and survival in natural habitat. Lankesteriana 7: 430– 432.

Wright M. M., Cross R., Dixon K., Huynh T., Lawrie A., Nesbitt L., Pritchard A., Swarts N. & Thomson R. (2009): Propagation and introduction of *Caladenia*. Australian Journal of Botany 57: 373–387.

Yam T. W. & Arditti J. (2009): History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnology Reports 3: 1–56.

Zettler L. W. & Piskin K. A. (2011): Mycorrhizal fungi from protocorms, seedlings and mature plants of the eastern prairie fringed orchid, *Platanthera leucophaea* (Nutt.) Lindley: A comprehensive list to augment conservation. American Midland Naturalist 166: 29–39.

Zettler L.W., Sharma J. & Rasmussen F. N. (2003): Mycorrhizal diversity. In: Dixon K. W., Kell S. P., Barrett R. L. & Cribb P. J.(eds). Orchid conservation. Natural History Publications: Kota Kinabalu, Sabah, Malaysia. pp.185-226.

Zobel M., Van der Maarel E. & Dupré C. (1998): Species pool: the concept, its determination and significance for  community restoration. Applied Vegetation Science 1: 55-66.

**Supplements:**

Tab. S1: Mycorrhizal fungi used in in vitro sowing, (Om - Orchis mascula, Ap - Anacamptis pyramidalis, Pb - Platanthera bifolia, Gc - Gymnadenia conopsea)

| OTU (species origin) | Site of origin | Isolation source |
|---|---|---|
| TUL1N (*Ap,Gc*) | restored meadow near Hruba Vrbka | *Ap*, protocorm |
| TUL3N (*Pb, Gc*) | Zahrady pod Hajem | *Pb*, protocorm |
| TUL4N (*Om*) | Certoryje | *Om*, root |
| CER1N (*Gc*) | restored meadow near Hruba Vrbka | *Gc*, protocorm |
| CER2N (*Ap*) | Certoryje | *Ap*, protocorm |
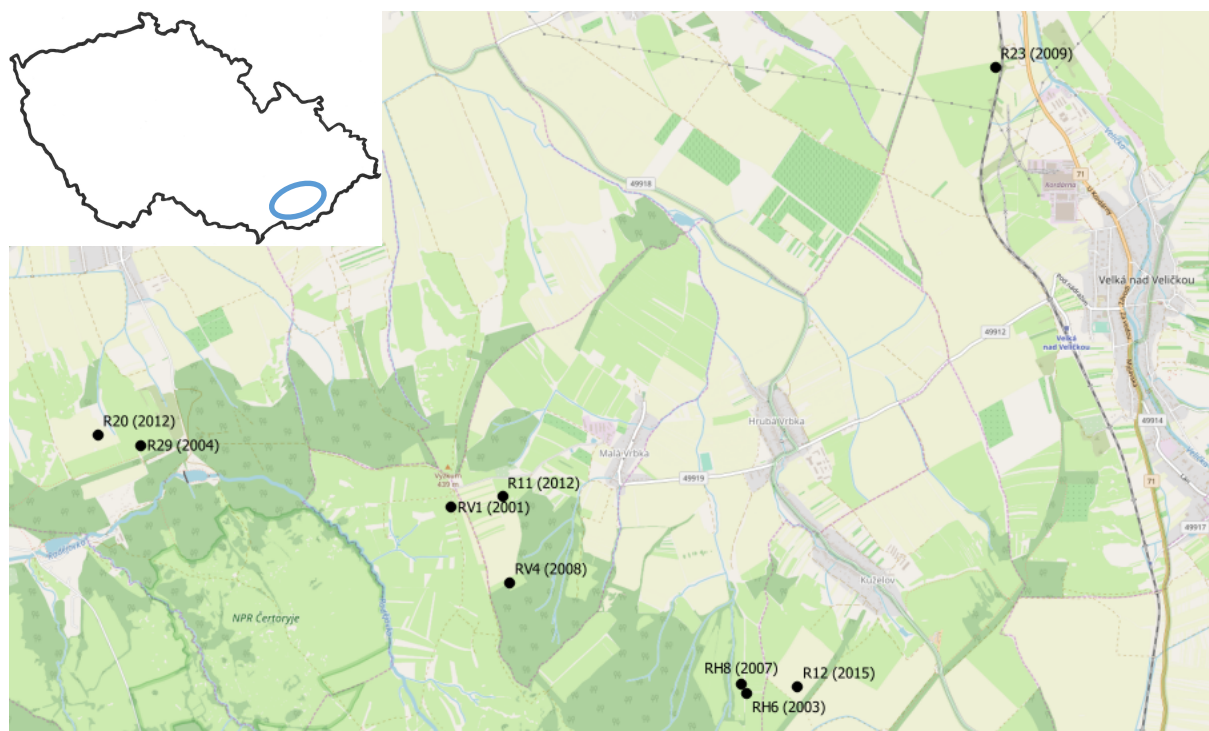


Fig. S1: *In situ* experiment was established on nine restored grasslands differing in year of restoration.

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Fig. S2: Complete randomized block with individual treatments. Same design was applied to all sites.

Tab. S2: *Orchis mascula*

| *Orchis mascula* count of microsites/ frames/ protocorms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R11 | R12 | R20 | R23 | R29 | RH6 | RH8 | RV1 | RV4 |
| Disturb | | | | | | | 1/ 1/ 1 | | |
| no dis-turb | | | | | | | | | |
| onlyHay | | | | | | | | | |
| Tul4a-gar | 4/ 6/ 13 | 1/ 1/ 2 | 3/ 5/ 54 | 2/ 3/ 14 | 4/ 7/ 25 | 2/ 2/ 3 | | 1/ 1/ 6 | 3/ 5/ 13 |
| Tul4Hay | 3/ 6/ 27 | 4/ 5/ 22 | | 4/ 5/ 7 | | 2/ 2/ 5 | 5/ 6/ 24 | 4/ 6/ 28 | 5/ 8/ 75 |

Tab. S3: *Anacamptis pyramidalis*

| *Anacamptis pyramidalis* count of microsites/ frames/ protocorms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R11 | R12 | R20 | R23 | R29 | RH6 | RH8 | RV1 | RV4 |
| Cer2a-gar | | | | | | 1/ 2/ 4 | | | |
| Cer2hay | | | | | | | | | |
| Disturb | | | | | | 2/ 3/ 10 | | | |
| no dis-turb | | | | | | 1/ 1/ 2 | | | |
| onlyHay | | | | | | | | | |
| Tul1agar | 1/ 2/ 6 | 1/ 1/ 1 | 1/ 2/ 2 | 3/ 3/ 4 | | 1/ 2/ 2 | 4/ 5/ 21 | 3/ 6/ 12 | 1/ 2/ 4 |
| Tul1hay | | | 4/ 4/ 10 | 4/ 7/ 12 | 1/ 2/ 4 | 2/ 3/ 5 | 1/ 1/ 3 | 4/ 5/ 16 | 1/ 2/ 4 |

Tab. S4: *Platanthera bifolia*

| *Platanthera bifolia* count of microsites/ frames/ protocorms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R11 | R12 | R20 | R23 | R29 | RH6 | RH8 | RV1 | RV4 |
| Cer1A-gar | | | 1/ 1/ 3 | | | | | | |
| Cer1Hay | | | 1/ 1/ 7 | | 1/ 1/ 1 | | | | |
| Disturb | | | | | | | | | 1/ 1/ 1 |
| no dis-turb | | | | | | | | | 2/ 2/ 24 |
| onlyHay | | | 1/ 2/ 12 | | | | 1/ 1/ 1 | | |
| Tul3agar | | | 2/ 2/ 4 | | | 1/ 1/ 2 | 1/ 1/ 2 | | 2/ 3/ 7 |
| Tul3Hay | | 1/ 1/ 1 | 1/ 2/ 3 | | | 2/ 2/ 4 | 2/ 4/ 7 | | 2/ 2/ 2 |

Tab. S5: *Gymnadenia conopsea*

| *Gymnadenia conopsea* count of microsites/ frames/ protocorms | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | R11 | R12 | R20 | R23 | R29 | RH6 | RH8 | RV1 | RV4 |
| Cer1Agar | 1/ 1/ 1 | 1/ 1/ 1 | 3/ 5/ 57 | | 1/ 1/ 2 | 3/ 3/ 6 | | 1/ 2/ 54 | 3/ 3/ 9 |
| Cer1Hay | 1/ 1/ 1 | 1/ 2/ 3 | 2/ 3/ 11 | | 1/ 1/ 2 | | | 1/ 2/ 20 | 3/ 4/ 13 |
| Cer2agar | | 4/ 6/ 33 | 3/ 4/ 19 | | 4/ 5/ 43 | 2/ 3/ 33 | 3/ 4/ 11 | 1/ 2/ 8 | 5/ 6/ 23 |
| Cer2hay | 1/ 1/ 2 | 2/ 3/ 8 | 1/ 1/ 2 | 2/ 2/ 3 | | 2/ 3/ 5 | 4/ 6/ 28 | | 5/ 6/ 46 |
| Disturb | | | 3/ 3/ 3 | | | | 2/ 2/ 2 | | 2/ 2/ 5 |
| no disturb | | 1/ 1/ 1 | | | 2/ 2/ 4 | 1/ 2/ 27 | 2/ 2/ 13 | 2/ 2/ 6 | 2/ 3/ 44 |
| onlyHay | 1/ 2/ 27 | 1/ 1/ 2 | 1/ 2/ 18 | 1/ 1/ 1 | 2/ 2/ 6 | 1/ 1/ 1 | 1/ 1/ 1 | 1/ 1/ 2 | 1/ 1/ 2 |
| Tul1agar | 1/ 1/ 3 | | 1/ 1/ 2 | | 1/ 2/ 9 | | 2/ 4/ 39 | 1/ 1/ 1 | 2/ 2/ 4 |
| Tul1hay | | 1/ 1/ 1 | | 1/ 1/ 2 | 1/ 1/ 1 | | 1/ 1/ 1 | 1/ 1/ 2 | |
| Tul3agar | 1/ 1/ 2 | 1/ 2 /5 | 2/ 4/ 44 | 1/ 1/ 1 | 1/ 2/ 7 | 1/ 1/ 1 | 1/ 2/ 4 | | 1/ 1/ 2 |
| Tul3Hay | | | 1/ 1/ 21 | | | | | | 1/ 1/ 3 |
| Tul4agar | 1/ 2/ 8 | | 1/ 1/ 14 | | 1/ 2/ 2 | | 1/ 2/ 8 | | 2/ 2/ 2 |
| Tul4Hay | | | | 1/ 2/ 4 | 1/ 2/ 2 | | 1/ 1/ 1 | | 1/ 1/ 1 |