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**Original Paper**

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# Influence of the late harvest period on reproductive spore quality of Burgundy truffles (*Tuber aestivum* Vittad.)

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The work presents new findings about the influence of the late harvest period on reproductive spore quality of Burgundy truffles (*Tuber aestivum* Vittad.) assessed by microscopic method and by inoculation experiments with hornbeam *Carpinus betulus* L. seedlings. Carpophore samples from one locality were collected in two terms in each month from November to January. Reproductive quality of spores was assessed by microscopic method. The presence of mycorrhiza on the roots was assessed by morphometric analysis. Quality of spores in winter decreased rapidly. Percentage of plants with *Tuber aestivum* mycorrhiza in the tested variants ranged from 19.72 % (early November) to 2.98 % (end of January). In variants with fruiting bodies collected in November observed level mycorrhized roots were statistically highly significant different from variants inoculated by carpophores from January and December. Lower percentage of *Tuber aestivum* mycorrhiza on root system of hornbeam seedlings in late periods of carpophores collection significantly influenced length of root system, while the length of stems was not statistically significant.

**Keywords:** Burgundy truffle, hornbeam, reproduction quality, mycorrhization

## 1. Introduction

Burgundy truffle (*Tuber aestivum* Vittad., syn. *Tuber uncinatum* Chat.) is indigenous to nearly all European countries. The natural geographical distribution of *T. aestivum* in Europe ranges from North Africa to Sweden and from Ireland to Russia (Chevalier and Frochot, 1997; Wedén et al., 2001).

Burgundy truffle is characterized by its relative resistance to climate fluctuations, changes of hydrological soil conditions and low temperatures (Streiblová et al., 2012). Southern natural habitatsof the species with a warm climate reach higher altitudes (Morocco, ~1,600 m above sea level (asl), northern habitats are often located near sea level (Sweden, <50 m asl).

Annual mean temperature varies here from 6.8 to 11.5 °C, which is typical for Mediterranean to temperate climates. Of particular importance are the mean temperatures of the coldest and warmest months, which may account for a stop in truffle production due to frost or a decrease of production due to heat-induced drought. The mean value for the coldest month generally ranges >0 °C, whereas significantly lower air temperatures can easily be buffered by snow cover, with the corresponding belowground values describing less distinct depressions (Stobbe et al., 2012).

Annual precipitation totals range from ~400 to 1,500 mm, making rainfall alone a very variable factor. The annual distribution of rain seems to be a much more crucial point for truffle growth (Büntgen et al. 2011, 2012). Strong ecological relationships exist between Tuber spp., host plants and soil type (Lulli et al., 1999). Soils must be permeable, well-aerated and well-drained, without stagnant water and also poor, in terms of N, P and Fe, but with a good content of Ca, K and S (Granetti, 1994; Bencivenga, 1998; Pacioni and Comandini, 1999). The ground of utmost truffle habitats is calcareous with pH levels >7 (Chevalier and Frochot, 1997) however, there are sites with pH >5.9 and the absence of active carbonate (Gogan et al. 2012).

*Tuber* spp. relies on a symbiotic association to form ectomycorrhiza with certain host plants and to complete their life cycle with the development of spore-bearing fruit bodies – the truffles (Mello et al., 2006).

Available data on structure of natural plant communities with occurrence of *T. aestivum* are oriented especially on host tree species determination and abundance of other plant species in range of these trees (Chevalier and Frochot; 1997 Wedén et al., 2004; Hilszczańska et al., 2008). Among numerous suitable host trees are oak species, a frequent symbiotic association to *Quercus robur* L. has been observed for temperate habitats. In Mediterranean habitats, this relationship shifts toward a preference for *Quercus cerris* L. Similarly important and widely distributed hosts are *Corylus avellana* L. and *Fagus sylvatica* L. (Stobbe et al., 2013).

The fruiting season usually has two maxima in the temperate habitats, although finds occur throughout the entire year (Stobbe et al., 2012). An earlier fruiting season

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is observed in warmer regions and at lower altitudes (from May to July in Greece), and a shift toward winter occurs in northern habitats and at higher altitudes (August to November in Sweden) (Diamandis and Perlerou, 2008; Wedén et al. 2004a, b).

Unlike other truffle species, Burgundy truffle overcomes the winter in a state of vegetative hibernation (Streiblová et al., 2012).

According to previous results, in genetic diversity of European *Tuber aestivum* populations is evident that this species is quite variable. Genetic variations of *Tuber aestivum* suggests the sexual reproduction mode with a presumed chromosome number 5 or 2n = 10 (Streiblová et al., 2010). In the Czech Republic, three different genotypes of this fungus were found, and it seems that its natural populations may have some unique properties (Streiblová et al., 2012).

The knowledge of spatial distribution and genetic diversity of the most relevant truffle taxa can provide a tool for planning in situ conservation programs. It is possible to identify a reserve by selecting the territory capturing the maximum of diversity within the least number of grid cells (Rebelo and Sigfried, 1992). Nonetheless, the assessment of *Tuber taxa* spatial distribution is difficult given that the underground ascomata can be localized only using specifically trained dogs (Gajos and Hilszczańska, 2013).

The goal of this study was to determine effect of the late harvest period on reproductive quality of Burgundy truffle (*Tuber aestivum* Vittad.) spores.

## 2. Material and methods

Inoculation experiments to study mycorrhization with spores from carpophores collected in late period of fructification were carried out with seedlings of hornbeam *Carpinus betulus*.

### 2.1 Seedlings of hornbeam

Hornbeam seeds were naturally stratified. Before sowing, the seeds were soaked in 80% ethanol. Then seeds were washed in tap water and sown on peat substrate with pH-adjusted to 7.6 by ground limestone. Hornbeam seeds in the number of 100 seeds were sown on in each variant. As growing containers were used plastic containers 600 × 360 × 160 mm.

### 2.2 Truffle carpophores

Truffle carpophores were collected from natural locality by trained dog in period from November 2008 to January 2009 always in two terms: 1st and 3rd decade (in article identified as early and late corresponding month – variants). At the end of October were ripe truffles collected in locality with aim do not mix carpophores ripened in autumn and conserved in soil with experimental ones. Climatological characteristics natural truffieres were identified by Miklós et al. (2002). In experiment were used only truffles with typical aroma. Macroscopic morphological properties (size and weight) of carpophores and degree of spore ripening (evaluated by microscope OPTIKA B-350 and camera MOTICAM 2000) were used for analysing the quality of inoculums. The presence of spores and spore maturation level was assessed according to the microscopic visual criteria: ascus – with presence or without ascospores, pigmentation of ascospores – complete or partial and development of the spore ornamentation - complete or incomplete by modified method according to Zeppa et al. (2004), mature spores = percentage of fully developed spores in 50 asci.

### 2.3 Preparation of inoculum

Inoculums were prepared from fresh carpophores by grinding and then they were stored in refrigerator with temperature up to -18 °C. Hornbeam seedlings were inoculated in April 2012. Inoculum in amount 2.0 $g.l^{-1}$ of substrate was applied in each variant. Inoculum was applied in mixture with inert carrier in rows between the plants at a depth of about 2 cm, and then was covered by substrate. The seedlings were grown in a greenhouse under standard system of treatment.

### 2.4 Evaluation of mycorrhiza

Level of mycorrhization on root system of inoculated plants was assessed 7 months after inoculation under microscope using a set of morpho-anatomical characters listed by Agerer (1987 to 2006) by counting of *Tuber* mycorrhized root tips. Plants were gently removed from the plastic containers. After 60 minutes soaking the root systems in water, the substrate remains were removed under tap water. Morphological identification of mycorrhiza on the roots divided plants into two groups (with and without mycorrhiza). On plants of both groups were analysed following characteristics: the length of the whole plant, stem length and root length. Data were analysed by one-way ANOVA following by LSD post-hoc test in STATISTICA CZ software.

## 3. Results and discussion

In the Slovak Republic, truffles were mentioned and traditionally collected in natural habitats as early as in the 16th century. Their consumption, gastronomic knowledge and market increased continuously, culminating in the beginning of the 20th Century under the Austro-Hungarian Empire. In the last decades, however, their occurrence in the nature declined and consequently they were classified as rare or extinct (Gažo et al., 2005).

In period from 2005 to 2007, more than 30 natural localities of *T. aestivum* were identified in Western



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**Table 1** Selected parametres of truffles carpophores collected in period from November 2008 till January 2009

| Variant | Average weight (g) | Min (g) | Max (g) | Coefficient of variation (%) | Fully developed spores (% fds)* |
|---|---|---|---|---|---|
| Early November | 10.30 | 1.20 | 19.40 | 34.08 | II. (58 %) |
| Late November | 8.70 | 2.05 | 13.75 | 22.42 | II. (53 %) |
| Early December | 7.65 | 2.10 | 12.40 | 22.58 | III. (24 %) |
| Late December | 5.80 | 0.75 | 6.15 | 15.50 | III. (26 %) |
| Early January | 4.55 | 1.40 | 5.30 | 14.45 | III. (19 %) |
| Late January | 3.80 | 0.90 | 4.80 | 17.11 | III. (17 %) |

*Degree of maturation: I. – Fully developed spores more than 75 % II. – Fully developed spores 50 – 75 %, III. – Fully developed spores less than 50 % IV. – Asci missing or empty (no spores)

Slovakia. Most sites were found in forest communities consisting of trees, shrubs and ground vegetation except four sites where the influence of the shield canopy shrubs and ground vegetation almost did not occured (Gažo et al., 2005; Miko et al., 2008a).

Natural sites hosting summer truffles are found in warm climate zone to the temperate climate in the subregion slightly dry with mild winters and average annual temperature of 7–8 °C, the average temperature in January from -2 to -3 °C, in July from 17 to 18 °C. The average annual rainfall is in range between 550 to 650 millimeters in the month of July 60–80 mm. Average numbers of days with snow cover is up to 40.

The ground of utmost truffle habitats in Western Slovakia is calcareous with pH ($H_20$) levels 6.36 till 7.47. In addition to pH was assessed eight selected agro chemical parameters total limestone, organic matter – humus, inorganic nitrogen, assimilable phosphorus, exchangeable potassium, sodium, exchangeable magnesium characterizing the soil conditions of natural habitats (Miko et al., 2008b). Soil and climatic requirements of *T. aestivum* can be also met in some productive agricultural areas in Slovak territory. In addition to the distinct general advantages mentioned above, this fungus is the most suitable of all truffles for commercial cultivation (Chevalier, 2001; Belloli et al., 2001) and the only species with fruit-bodies ripening advantageously from late May through all autumn. In some years, when vegetation is stopped by freezing weather, fruiting bodies can also be found during the winter months. Their reproduction quality is problematic.

Influence of truffle fructification period on reproduction quality of ascospores was studied by analysis quantity of mycorrhiza in artificially inoculated hornbeam seedlings.

Mild winter 2008/2009 allow us to obtain suitable fruiting bodies in the period from November to January. Variability of truffle carpophores weight found in the study area ranged from 14:45% (early January) to 34.08% in early November (Table 1). Fruit body with the lowest weight was found at the end of December (weight 0.75 g). Maximum weight fruit-body (19.40 g) was collected at the beginning of November.

To find methodological procedure for assessment maturity of spores, fruit-bodies samples were collected during whole fructification period from May till January. Reproductive quality of spores increases in spring period (Figure 1). The highest quality is reached in summer and early autumn period (Figure 2). Quality of spores in winter decreased rapidly (Figure 3).

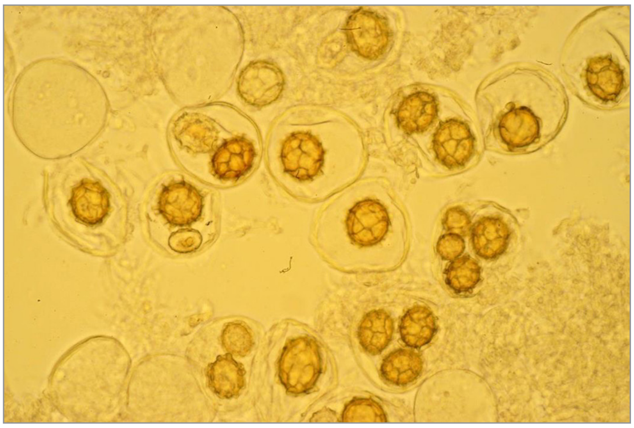

**Figure 1** Fructification period (May – July)

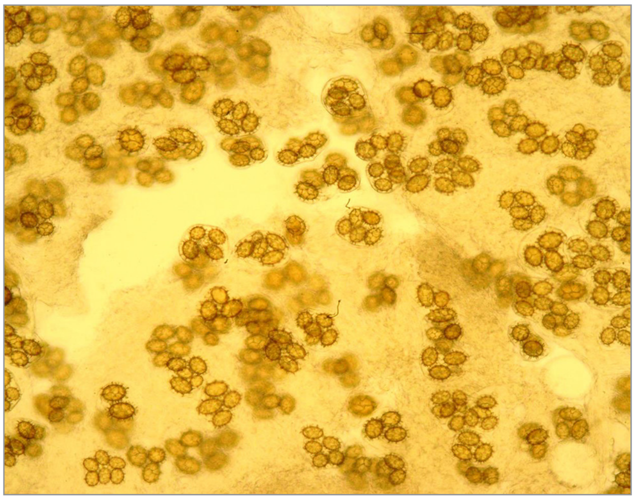

**Figure 2** Fructification period (August – October)

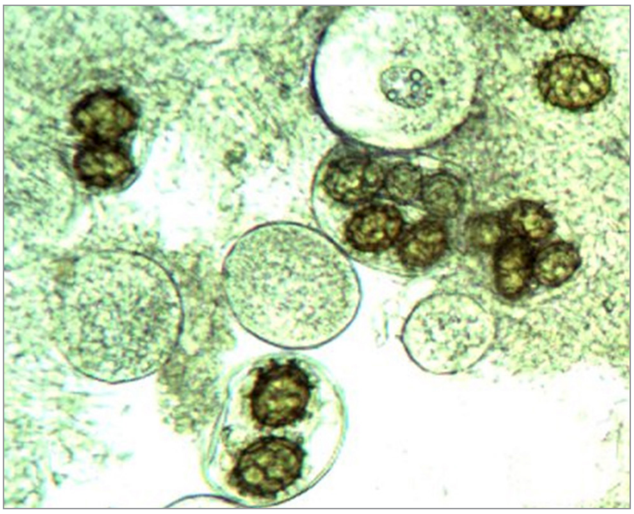

**Figure 3** Fructification period (November – January)

Hornbeam, as experimental material for inoculations with *T. aestivum* was prefered as the most common host species in natural truffle localities in the study area. Slow growth in the first years after sowing, allows realizing inoculation experiments in pots with natural development of the root system, without limitation by volume of cultivation containers which can cause malformations in shape of the root system (Miko and Gažo, 2007).

This woody plant is very tenacious and adaptable species of forest trees with high regeneration ability after clear cuts. In Slovak forests hornbeam and hazel tree species are probably capable support long term keeping of truffle mycorrhiza in natural habitats where the wood is harvested. Inoculation experiments with hornbeam were evaluated in autumn 2012. Percentage of plants with *T. aestivum* mycorrhiza in the tested variants ranged from 19.72 % (early November) to 2.98 % (end of January). In variants with fruiting bodies collected in November observed level mycorrhized roots were statistically highly significant different from variants inoculated by carpophores from January and December (Table 2).

Lower percentage of *T. aestivum* mycorrhiza on root system of hornbeam seedlings in late periods of carpophores collection significantly influenced length of root system (Table 4), the length of stems was not statistically significant (Table 3). Mycorrhized seedlings had shortened root system. Control variant (without mycorrhiza) was characterized by a statistically significant longest root system compared to mycorrhized variants (Table 3).

The morphology of the root system of mycorrhized seedlings compared to control variant was significantly different (Figure 4). Effect of root length non mycorrhized seedlings compared to mycorrhized was reflected in

**Table 2** Statistical significance between level of mycorrhization (in %) of hornbeam seedlings inoculated by spores collected from November till January

| Variant | F-test | Significance | Mean | Homogenous groups | |
|---|---|---|---|---|---|
| | | | | α = 0.05 | α = 0.01 |
| **Late January** | 56.83 | 0.000 | 2.98 | a | a |
| **Early January** | | | 4.85 | a b | a |
| **Late December** | | | 6.98 | b | a |
| **Early December** | | | 6.99 | b | a |
| **Late November** | | | 19.23 | c | b |
| **Early November** | | | 19.72 | c | b |

**Table 3** Statistical significance between stem lengths (in cm) of hornbeam seedlings inoculated by spores collected from November till January

| Variant | F-test | Significance | Mean | Homogenous groups | |
|---|---|---|---|---|---|
| | | | | α = 0.05 | α = 0.01 |
| **Late December** | 1.76 | 0.107 | 6.99 | a | a |
| **Late January** | | | 7.33 | a b | a |
| **Early January** | | | 7.49 | a b | a |
| **Control** | | | 7.54 | b | a |
| **Late November** | | | 7.62 | b | a |
| **Early December** | | | 7.64 | b | a |
| **Early November** | | | 7.83 | b | a |



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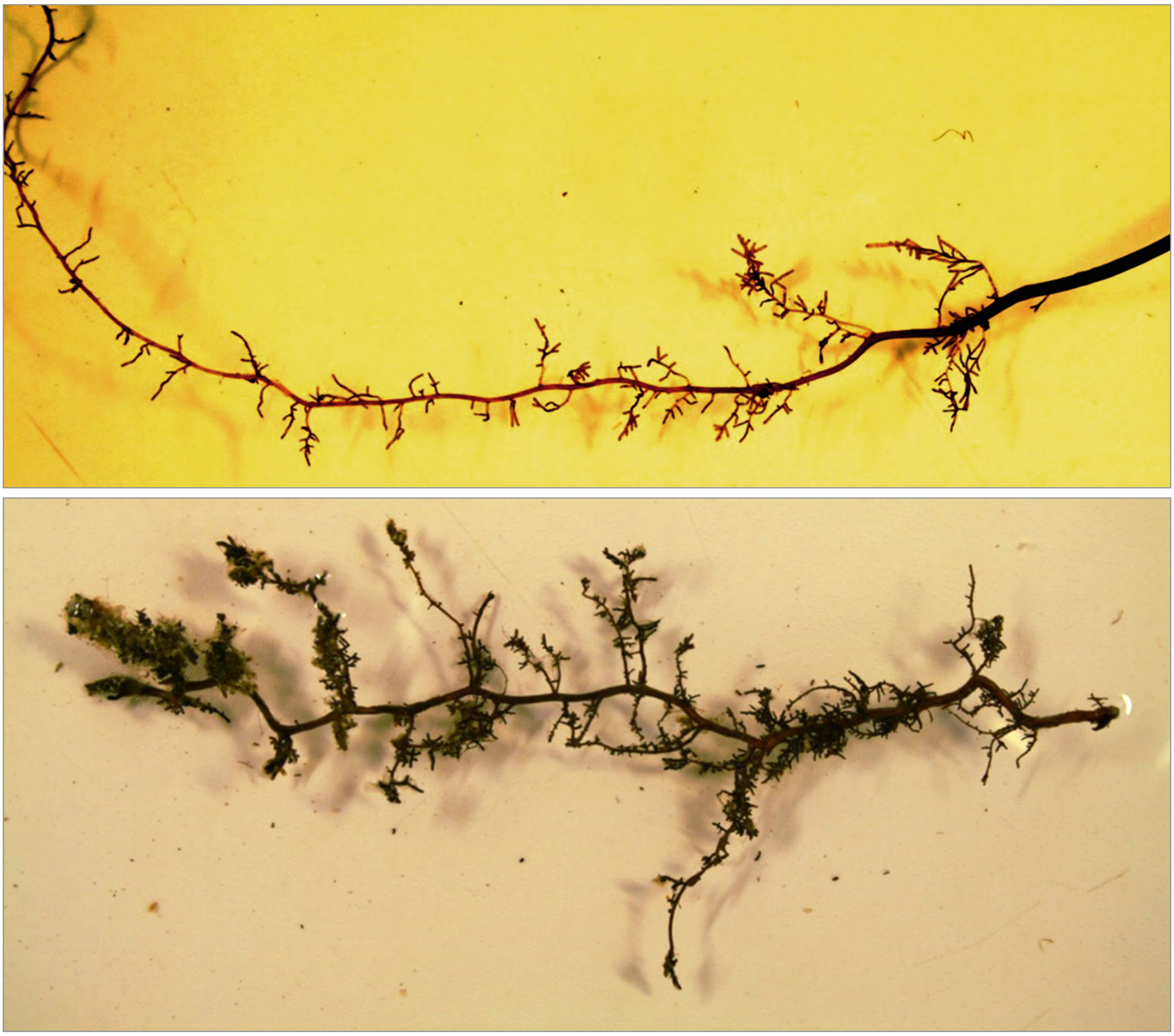

**Figure 4** Roots of hornbeam with lower percentage of mycorrhiza (above) and with higher percentage (below)

**Table 4** Statistical significance between root systems (in cm) lengths of hornbeam seedlings inoculated by spores collected from November till January

| Variant | F-test | Significance | Mean | Homogenous groups | |
|---|---|---|---|---|---|
| | | | | α = 0.05 | α = 0.01 |
| **Late November** | 9.596 | 0.000 | 8.47 | a | a |
| **Late December** | | | 8.68 | a b | a |
| **Late January** | | | 8.96 | a b | a b |
| **Early January** | | | 9.20 | a b | a b |
| **Early December** | | | 9.54 | a b | a b |
| **Early November** | | | 9.66 | b | a b |
| **Control** | | | 11.41 | c | b |



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**Table 5** Statistical significance between total plant lengths (in cm) of hornbeam seedlings inoculated by spores collected from November till January

| Variant | F-test | Significance | Mean | Homogenous groups | |
|---|---|---|---|---|---|
| | | | | α = 0.05 | α = 0.01 |
| **Late December** | 8.70 | 0.000 | 15.67 | a | a |
| **Late November** | | | 16.09 | a | a |
| **Late January** | | | 16.29 | a b | a b |
| **Early January** | | | 16.69 | a b | a b |
| **Early December** | | | 17.18 | a b | a b |
| **Early November** | | | 17.49 | b | a b |
| **Control** | | | 18.95 | c | b |

the total length of the seedlings where variant without mycorrhiza was significantly different from mycorrhized ones (Table 5).

## 4. Conclusions

The presence of empty asci and immature spores were in relation to a lower variability of fruiting bodies weight. In the inoculum experiments, this fact led to a significant decrease in the proportion of plants with mycorrhiza with increasing period of winter, to level at 2.97 % at the end of January. Statistical analysis revealed significant effect of mycorrhiza to change the length of the root system. The root system with present mycorrhiza is shortened, and increased its branch in particular in the subsurface of the growing medium.

Variants with different periods of ripened fruiting bodies had not significant effect on stem length of hornbeam seedlings with mycorrhiza on root system. The absence of similar findings in the literature does not allow comparing them, but they are a motivation for further study of symbiotic systems ectomycorhizal mushrooms.

Slovakia is characterized by varied geological structure and the relatively low proportion of soils on limestone bedrock. Group of black truffles with special requirements for complex environmental factors makes this group of fungi in our country particularly vulnerable. Probability distribution of spores by truffle eating forest animals into suitable environmental conditions for their reproduction is limited. A stock of germinating spores in natural areas is largely dependent on the amount of fruiting bodies bearing ripe spores, which are not removed from the natural habitats. Truffles are included into National Programme on Conservation Genetic Resources for Food and Agriculture in the Slovak Republic since 2005. Program includes inventory research, study of inoculation techniques and production inoculated seedlings for establishment of ex-situ agroforestry plantations. According our experiments we can summarized that protection of gene pool of *T. aestivum* in the Slovak Republic have to be carried out in complex approach with respect to natural truffle habitats.

The aim of the truffle research is to achieve a state in the future that in our country will in agro forestry plantations grown truffles with domestic origin. It requires comprehensive protection of natural habitats as a unique and irreplaceable repository of genetic resources.

## 5. Acknowledgements

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