First results from experimental truffle orchards established in Hungary in the framework of INRA-ELTE cooperation

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Abstract: In the framework of INRA-ELTE scientific cooperation 11 experimental truffle orchards were established between 2004 and 2006 in the Carpatho-Pannonian region. Seedlings with controlled quality were produced by ROBIN PÉPINIÈRES (France). The seedlings of four host plant species were inoculated with spores of *Tuber aestivum* biotypes from several European regions. Morphological and molecular examinations were carried out on the mycorrhiza of randomly selected seedlings from 3-year-old plots. The work focused on the determination of mycorrhizal frequency, the description of host plant phenology, the definition of contaminant mycorrhizal types, and their identification based on their whole ITS sequences. The results of the above mycorrhizal research were evaluated in relation to the data of previous studies on plant physiology, pedology, meteorology, and phytoindication in order to find answers to the following questions: To what extent does the efficiency of mycorrhization of different biotypes depend on the ecological features of Central European habitats? How is the host plant mycorrhization influenced by local biotic and abiotic factors? What ecological factors tend to promote early stage mycorrhiza substitution? A comparison of the truffle orchards with the best mycorrhization and the most productive natural truffières revealed a similarity in the ecological factors. The comparative evaluation of databases for natural habitats and truffle orchards could promote the more rapid development of truffle cultivation in the Carpatho-Pannonian region.

Zusammenfassung: Im Rahmen der wissenschaftlichen INRA-ELTE Kooperation wurden zwischen 2004 und 2006 11 experimentelle Trüffelkulturen im Karpaten-Pannonischen Gebiet angelegt. Setzlinge mit kontrollierter Qualität wurden von ROBIN PÉPINIÈRES (Frankreich) hergestellt. Die Setzlinge von vier Wirtsarten wurden mit Sporen von *Tuber aestivum* Biotypen aus einigen europäischen Regionen inokuliert. Morphologische und molekulare Untersuchungen der Mykorrhizen stichprobenartig ausgewählter Setzlinge von dreijährigen Untersuchungsflächen wurden durchgeführt. Der Schwerpunkt lag dabei auf Mykorrhizafrequenz, Beschreibung der Phänologie der Wirte, Erkennung kontaminierender Mykorrhizen und deren Identifizierung basierend auf der kompletten ITS-Sequenz. Die Ergebnisse dieser Mykorrhizastudien wurden früher erhobenen pflanzenphysiologischen, boden-

biologischen, meteorologischen und pflanzensoziologischen Daten gegenübergestellt, um folgende Fragen beantworten zu können: In welchem Ausmaß hängt die Effizienz der Mykorrhizierung durch unterschiedliche Biotypen von ökologischen Faktoren mitteleuropäischer Habitate ab? Welche ökologischen Faktoren fördern tendenziell Mykorrhizasubstitution im Frühstadium? Ein Vergleich der Trüffelkulturen mit der besten Mykorrhizierung mit den produktivsten natürlichen Trüffelplätzen zeigte eine Ähnlichkeit in den ökologischen Faktoren auf. Die vergleichende Auswertung von Datenbanken für natürliche Habitate und Trüffelkulturen könnte die raschere Entwicklung der Trüffelkultur in der Karpaten-Pannonischen Region fördern.

The majority of the truffles collected in the Carpatho-Pannonian region are summer (burgundy) truffles. According to LEHOTA & KOMÁROMI (2007) the annual quantity collected amounts to 6-7 tons, but in good years the total may be twice as much. On low-lying areas large numbers of truffles are produced even in summer, while in hilly habitats larger yields are given in autumn. Natural habitats are rapidly being destroyed by over-collection, due to the lack of any legal regulation, so there is already a strong need to satisfy demand in the region through the rapid development of the cultivation of burgundy truffles in truffle orchards.

The first opportunity for the establishment of truffle orchards in Hungary came after 1989. Initial efforts used technologies described in Hungarian translations of French papers (BRATEK & BENE 2003) in order to cultivate both burgundy truffles (*Tuber aestivum*) and black périgord truffles (*Tuber melanosporum*), despite the fact that the latter species is not indigenous. In order to save on the costs of producing mycorrhized saplings, several orchard owners attempted to use older methods, such as planting acorns on truffle patches or dipping the acorns in clay containing a spore suspension. This latter method was employed to establish the largest (15-hectare) truffle orchard in Hungary (GÓGÁN CSORBAINÉ & al. 2008). Many often unsatisfactory methods were also used to determine where to establish orchards and how to construct the irrigation system. In the early years (1990s) other orchard owners chose to import certified saplings from France. Mycorrhized saplings are now produced by several companies in Hungary, who, on request, will use local truffles to mycorrhize plant material from forests in the neighbourhood of the truffle orchard. Even in the greenhouse this solution may lead to a higher level of mycorrhization (MAR & al. 2008), while the host trees exhibit more successful acclimatisation after planting out. The conditions required for intensive truffle cultivation are now available in Hungary. Nevertheless, various questions have arisen in relation to the cultivation technologies routinely used in Western Europe, since the soil and climatic conditions in Central Europe are considerably different. A further question is how saplings mycorrhized using the patented INRA technique will behave in orchards in the Carpatho-Pannonian region. In order to clarify this question, scientific cooperation was set up between INRA and Eötvös University, Budapest (ELTE), in the framework of which a network of experimental truffle orchards was established, beginning in 2003, with the involvement of members of the Hungarian Truffle Growers Association. The present paper reports on the first analyses carried out on these orchards.

Recent molecular analyses indicate that summer truffle (*Tuber aestivum*) and burgundy truffle (*Tuber uncinatum*) can be regarded as a single biological species (PAOLOCCI & al. 2004, WEDÉN & al. 2005). The summer (burgundy) truffle is one of the most widespread truffle species in Europe, and is a ubiquitous and competitive species. The species generally prefers basic soils, rich in organic matter, with a high C/N ratio, which are of heavier texture than those favoured by *Tuber melanosporum*.

It tolerates climates ranging from moderately to very wet, and from oceanic (though more rarely) to semic-continental or continental.

Material and methods

Between 2004 and 2006 11 experimental truffle orchards were set up following a preliminary analysis covering more than 30 habitats. The certified saplings were supplied by the French company Robin, where the mycorrhizing technique patented by INRA is applied. 12 summer truffle biotypes were used to inoculate four host plants of French origin (*Pinus nigra*, *Quercus* spp., *Carpinus betulus*, *Coryllus avellana*). These were, in 2004: "France" (France); in 2005: "Hongrie" (Hungary), "Bouchet", "Verne" (France), "Piemont Calvo", "Ascoli Pisceno" (Italy), "Suéde" (Sweden), "Jaillifer" (France); in 2006: "Roumanie" (Romania), "Italie" (Italy), "Suéde" (Sweden), "Bouchet" (France). The young trees were planted with tree and row spacings of 2-3 × 4 m, in rows with approximately east-west orientation. After plantation the orchard was fully mapped using GPS coordinates. The map, showing each sapling with its individual identification code and all other major objects on the site (bushes, trees, lakes, fences), was prepared using the ArcView GIS 3.3 program package. In the first three years orchard maintenance did not involve liming, regular irrigation or mulching in any of the orchards.

In order to determine the stratification of the soils, 2-metre soil profiles were sampled from each orchard and mean samples were subjected to soil analysis at the Research Institute for Soil Science and Agricultural Chemistry of the Hungarian Academy of Sciences.

A full-scale coenological survey was made of the herbaceous vegetation of the orchards in order to determine its phytoindicative value (BORHIDI 1993).

Mycorrhiza samples were taken from the NE sector of the root zone of 3-year-old plants, from at least five trees in each variant, but whenever possible from 8-12. The degree of mycorrhization was determined using the methods of G. CHEVALIER (pers. comm.) and FICHER & COLINAS (1996). The statistical evaluation of the mycorrhization data was carried out as a function of orchard location, host plant and fungus biotype (giving 50 variants) using the (Compare meanst) One-Way ANOVA program of the SPSS Statistics 17.0 program package; significance was tested with LSD. Contaminating mycorrhiza types were identified on the basis of their whole nrITS sequences. Phenological analysis included measurements of plant height and tree base diameter, and the data were analysed using linear regression.

A rapid and technically simple way to measure plant vitality is to determine photosynthetic activity on the basis of chlorophyll fluorescence. In the present work correlations were measured between the plant phenological data, the mycorrhization parameters and the vitality recorded by fluorescence induction. Data collected from three sun leaves (indicative of plant status) were bulked for each test plant to characterise the trees.

Table 1. Summarised results of mycorrhizal investigations in the experimental plantations established during the INRA-ELTE cooperation.

Host plant species	Fungal species	Samples No.	Mycorrhizal frequency %				contamination
			Mean	St. Dev.	min	max	%
Carpinus betulus	*Tuber uncinatum*	190	61,8	27,4	0,0	98,0	3,6
Corylus avellana	*Tuber uncinatum*	127	43,2	28,5	0,0	88,3	5,7
Pinus nigra austriaca	*Tuber uncinatum*	135	49,7	31,1	0,0	100,0	16,2
Quercus spp.	*Tuber uncinatum*	221	36,7	29,1	0,0	94,9	14,4

Results and discussion

Preliminary mycorrhiza analysis on samples taken by G. CHEVALIER in August 2006 confirmed the preservation of the summer truffle mycorrhiza in all the orchards. Detailed mycorrhiza analysis on over 600 saplings, starting in 2007, made it clear that on average all the tree species were able to maintain the level of mycorrhization detected during the certification process, while for *Carpinus betulus* an increase was observed (Table 1). All in all, it would thus seem that saplings mycorrhized using the INRA technology were successful over a 3-year term in planting locations in Hungary despite the great heterogeneity of the orchards, with soils ranging from sandy to heavy clay and with climates ranging from continental to sub-mediterranean.

The analysis of the 12 summer truffle biotypes in 11 experimental orchards, independently of tree species and orchard, produced the following top ranking for the degree of mycorrhization: "Italie" (72.56%), "Verne" (63.82%), "Ascoli Pisceno" (58.51%), "Suéde" (54.13%) and "France" (50.51%), with deviations of around 20% for all the biotypes. In the case of individual host plants, the "Italie" and "Suéde" biotypes proved to be best for *Carpinus betulus* (over 70%), while "Bouchet", "Hongrie" and "Piemont" were the worst (below 50%). The "Italie" biotype also gave the best mycorrhization value for *Corylus* and *Pinus nigra* (over 60%), while the poorest biotypes for these hosts were "Suéde" and "Hongrie", with values of 30.1% or less, and "Piemont Calo" and "Bouchet", both below 38%. Considering the *Quercus* species together, the best results were found for "Bouchet" (55.2%) and the worst for "France". It should be noted that the "France" biotype gave extremely poor results in wetter habitats.

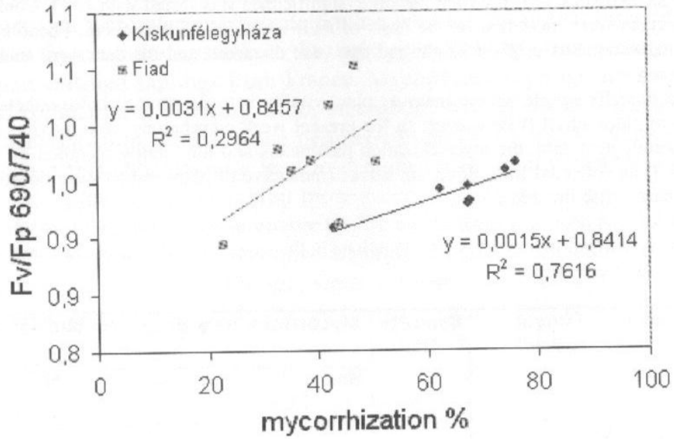

Fig. 1. Fv/Fp values recorded as an indicator of vitality at 690/735 nm on *Quercus pubescens* trees with roots free of contaminating fungi and with various degrees of *Tuber uncinatum* (France, Bouchet) mycorrhization. Depending on the growing site, the value of the vitality parameter rose in close correlation with an increase in the mycorrhization percentage.

Differences between biotypes in their soil, climate and ecological requirements are known (WEDÉN & al. 2004). So it is important to compare the mycorrhization of the biotypes with as few variables as possible. When ANOVA was carried out for different biotypes on the same host plant in the same orchard, the ratio of significant deviations was 32%. This figure was somewhat higher, 34%, if the only variable was the host plant, while it was much higher, 46% when the orchard location was the only variable. When ANOVA was carried out for all three variables (orchard location, host plant, biotype) the ratio of significantly different comparisons/pairings was 49%. This suggests that the degree of mycorrhization depends to the greatest extent on the ecological conditions in the orchards, followed by the host plant, while the least decisive factor was the summer truffle biotype.

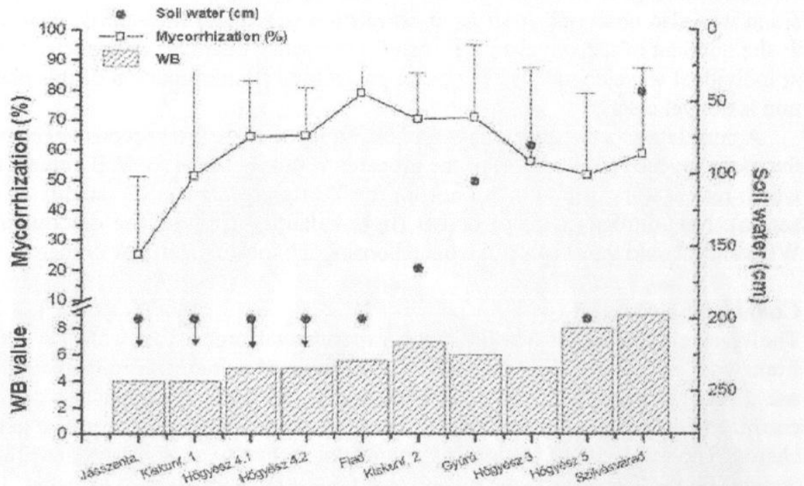

Fig. 2. Orchards ranked in increasing order of water supplies (taking into consideration the groundwater level and the maximum WB values estimated on the basis of the herbaceous vegetation). Mean + s. d. *Tuber aestivum* mycorrhization is given for *Carpinus betulus*.

Mycorrhizas involving undesirable, contaminating fungi were observed in all the orchards. One negative example, involving a high rate of mycorrhiza substitution, was the orchard in Jásszentandrás, where a relatively old (approx. 10 year-old) stand of pedunculated oak (*Quercus robur*), a small plantation of fir trees (*Abies*) and a planted avenue of poplars (*Populus*) can be found in the close vicinity of the truffle orchard. The level of contamination was high (37%) and alien fungi could be observed on the roots of approx. 78% of the saplings examined. The contaminating mycorrhizas were all typified morphologically, and for some the whole ITS sequence was identified. The most frequently occurring contaminating fungi were *Suillus* species (*S. granulatus*, *S. collinitus*), as well as *Tuber maculatum*, *Tomentella* spec., *Scleroderma bovista* and *S. areolatum*.

It is often found that mycorrhiza formation has a positive effect on the growth of the host plant. When data from all the orchards were analysed together, a significant positive correlation (r = 0.36675, p = 0.00749) was observed between plant height and degree of total mycorrhization for *Pinus nigra*, while for *Quercus pubescens*, in addition to a correlation between these parameters (r = 0.3928, p = 0.04714), there was also a significant correlation between the stem-base diameter and the degree of mycorrhization (r = 0.45127, p = 0.02067). The analysis of phenological data only gives a true picture if it is carried out for each orchard separately, since the ecological factors in each orchard may differ considerably. One example of this is the fact that the combined analysis indicated no correlation whatsoever between total mycorrhization and stem-base diameter for *Carpinus betulus*, while a strong positive correlation was found for these parameters in the Gyúró orchard (r = 0.34978, p = 0.0394).

The results of fluorescence induction measurements indicated a positive correlation between the mean actual quantum efficiency of plant leaves and both the growth data and the degree of mycorrhization of the roots. Dependence on the taxa composition was also observed. A stronger correlation was found for vitality, characterised as the quotient of the wavelengths, than for the actual quantum efficiencies measured at individual wavelengths, but the exact physiological interpretation of this phenomenon is not yet clear.

A correlation between the phytoindication value of the herbaceous vegetation and the mean mycorrhization level of the orchards was only found for WB values (Fig. 2), which reflect water supplies, but not for the TB (temperature), RB (acidity or soil reaction), NB (nitrogen supply) or LB (light balance) indexes. The determination of WB values could thus be useful when choosing the locations of new orchards.

Conclusions

The research carried out so far in the experimental orchard network set up in the framework of INRA-ELTE cooperation has produced numerous results that will be of use in the cultivation of truffles in Hungary. It has become clear that saplings mycorrhized using the INRA technology can be successfully employed in Hungarian orchards. The influence of certain environmental factors (e.g., soil water supplies, soil texture) on the *Tuber aestivum* mycorrhiza frequency has also been detected. The differences between mycorrhization levels are influenced to the greatest extent by orchard conditions (soil and climatic factors) and by the presence of contaminating sources, though the role of the host plant and biotype may also be important. Certain contaminating species were typical of a number of orchards, and were particularly frequent on *Pinus nigra*. A method involving phytoindication has been successfully developed to improve the selection of locations for new orchards. It will be possible to provide a better clarification of the ecological requirements of summer truffle biotypes if these results are compared and jointly analysed with those of experimental orchards established using the INRA technology in other European countries.

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