THESIS OF DOCTORAL DISSERTATION

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Strain comparative examinations and practical developments in the King Oyster Mushroom [*Pleurotus eryngii* (DC.:Fr.) Quél.] cultivation

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INTRODUCTION

The white mushroom [*Agaricus bisporus* (J.E. Lange)], oyster mushroom [*Pleurotus ostreatus* (Jacq.) P. Kumm.] and shiitake [*Lentinula edodes* (Berk.) Pegler] species represent only a small segment of cultivable mushroom species. In my opinion it is very important to get acquainted with other cultivable mushroom species and possibilities of their cultivation, improvement of cultivation technology and adaptation of these technologies to the Hungarian conditions. In the course of my work I performed experiments with the king oyster mushroom [*Pleurotus eryngii* (DC.:Fr.) Quél.], a species with good perspectives and very good taste. In addition, this mushroom has other advantageous features, such as relatively long shelf-life and low rate of sporulation, higher market price, etc.

In its natural habitat this species shows facultative biotrophic behaviour and appears most commonly with certain species of the *Apiaceae* family. It weakly parasites the root and stem of the plants, but later on it occurs as a saprophytic white-rot fungus. Nevertheless, during intensive cultivation this species does not require the above mentioned plants as raw material. Phylogenetic relationships of the *P. eryngii* species and populations of the *P. eryngii* species complex are still under debate.

Nowadays the king oyster mushroom gets more and more popularity amongst the growers and consumers all over the world. Though the species conquers new markets, it is still not cultivated on industrial scale in Hungary. With my work the main aim was to get new results that can be used for basic research and in cultivation practice, as well. The main goals of my research were to call the Hungarian growers' attention to the species and to compile a manual for them.

On the basis of the content I split the dissertation into three parts. In the first part I would like to get a picture about the phylogenetic relationship of my own isolates by means of molecular biology tools. In addition to that I would like to verify the presence (or absence) of dsRNA mycovirus species. In the second part I examined the cultivation possibilities of the species in order to help Hungarian growers. Part three contains the results of investigations that aimed the improvement of nutritional value; at the same time I wanted to get acquainted with the relationship between the mineral element content of the substrate and the fruiting bodies, together with the role of casing in mineral uptake and water transport.

AIMS

- To get a picture about the taxonomic relationships of my own *P. eryngii* isolates; furthermore, I searched for the identification possibilities on varietas level by the analysis of certain regions of two genes.
- To ascertain whether dsRNA molecules, thus mycoviruses were present in the isolates.
- To get knowledge about the *in vitro* vegetative growing features and cultivation attributions of my strains.
- To establish the adaption of the species to the Hungarian circumstances and get a picture about how to enhance the biological efficiency by cultivation.

- To get knowledge about possibilities that might help to improve nutritional value of the cultivated mushroom by changing the composition of substrate and its supplementation with mineral elements.
- To unravel the impact of casing soil on the quantity of yield and its role in the mineral uptake and water transport.
- To assemble a cultivation manual for Hungarian growers on the basis of my own results and literature data, in order to promote the cultivation of the species in Hungary.

INTRODUCTION

Habitat and 'host plants'

The *Pleurotus* species associated with members of the *Apiaceae* family can be found on the Northern hemisphere between the 30° and 50° latitudes (ZERVAKIS & BALIS, 1996). These species mainly occur in the subtropics region of the Mediterranean Sea, but are present also in Central Europe, Russia, Ukraine, Central Asia and Iran. The species can be found on steppes, arid meadows and even in mountain regions. The *P. eryngii* species complex is a unique group within the genus, because its members grow as facultative biotrophs on numerous species of the *Apiaceae* (*Umbelliferae*) family (including *Eryngium campestre, E. maritimum, E. alpinum, E. moroccanum, E. planum, Ferula communis, F. sinkiangensis, Laserpitium latifolium, L. siler, Elaeoselinum asclepium, Thapsia garganica, Cachrys ferulacea*) and certain species of the *Asteraceae* (*Compositae*) family (ZERVAKIS & BALIS, 1996, ZADRAZIL, 1974, LEWINSOHN et al., 2002, ZERVAKIS et al., 2001a,b; RODRIGUEZ ESTRADA, 2008).

Taxonomy

Since there are a few taxonomic groups with uncertain labelling that are connected to the *P. eryngii* (De Candolle ex Fries) Quelet *sensu lato* taxa, the concept of 'species complex' was introduced (ZERVAKIS et al., 2001a; BAO et al., 2004; RODRIGUEZ ESTRADA, 2008). The *P. eryngii* species complex includes the following varieties and species: var. *eryngii* (DC.: Fr) Quel.; var. *ferulae* Lanzi /syn.: *P. fuscus* var. *ferulae*/; var. *elaeoselini* Venturella et al.; var. *nebrodensis* (Inzenga) Sacc.; var. *tingitanus* Lewinsohn et al.; var. *tuoliensis* C. J. Mou; *P. hadamardii* Constantin; *P. fossulatus* (Cooke Sacc.) (CANDUSSO & BASSO, 1995; VENTURELLA, 2000; ZERVAKIS et al., 2001a,b; LEWINSOHN et al., 2002; KAWAI et al., 2008; RODRIGUEZ ESTRADA, 2008; RODRIGUEZ ESTRADA et al., 2010).

Molecular biology experiments

The classical characteristics (mostly micro- and macromorphological features), traditionally used for identification of a given taxa, can be modified and masked by the environment, thus the identification might by seriously hampered. Taxonomists often solve this problem with investigation of nucleic acids. The most popular and most frequently used region in fungal taxonomy is the internal transcribed spacer (ITS) region, which is part of the ribosomal gene cluster. In the *P. eryngii* this locus and a partial β-tubulin sequence was investigated in order to map the phylogenetic relationship amongst members of the *P. eryngii* species complex. Unfortunately, neither the ITS nor the β-tubulin region showed enough variability for differentiation of varieties (RO et al., 2007; RODRIGUEZ ESTRADA, 2008; RODRIGUEZ ESTRADA & ROYSE, 2008; RODRIGUEZ ESTRADA et al., 2010).

Certain regions of the translation elongation factor (EF1α) and the gene coding for the second largest subunit of the RNA polymerase II (*rpb2*) are thought to be

adequate for molecular phylogenetic examinations and identification on varietas level (LIU et al., 1999; ROGER et al., 1999; MATHENY et al., 2002; MATHENY, 2005; RO et al., 2007; RODRIGUEZ ESTRADA, 2008; RODRIGUEZ ESTRADA et al., 2010). The EF1α is a protein with binding function and necessary for ribosomal protein synthesis in eukaryotic cells. MARONGIU et al. (2005) reported that the gene of EF1α (*tef1*) bears nucleotide substitutions, which might be useful for differentiation of the *eryngii* and *ferulae* varieties.

The *rpb2* gene has twelve strongly conserved domains that might serve as appropriate primer binding regions for PCR. The *rpb2* and other genomic regions were used for identification of *Cortinarius* and *Inocybe* species (FROSLEV et al., 2005; MATHENY, 2005). In my experiments I planned to investigate the polymorphism of these two loci (*tef1* and *rpb2*) in our own *P. eryngii* isolates.

Mycoviruses

Majority of the mycoviruses bear dsRNA as genetic material (GHABRIAL, 1998; GHABRIAL & SUZUKI, 2009) and mostly spread via hyphal anastomosis, because the extracellular route for infection is not present. Mycoviruses may cause serious crop losses for mushroom growers, thus numerous methods (EM, electrophoresis, ELISA, etc.) were developed for their investigation. A very sensitive and specific immunoblot method for detection of mycoviruses was reported by GEÖSEL et al. in 2008. In my experiments I used this latter method to find out whether the phenotypic differences between our isolates were caused by mycoviruses or not.

Cultivation

The first cultivation experiments in Hungary were started in the 1950s (KALMÁR, 1960; SZILI & VÉSSEY, 1980; SZILI, 1994). Nowadays cultivation methods can

be grouped in a couple of ways; on the basis of location (indoor, outdoor, semi-indoor), packaging of raw materials (bag, block, bottle, crate), heat treatment of raw materials (sterilization, dry heat treatment, wet heat treatment), casing (cultivation without and with casing) (RODRIGUEZ ESTRADA & ROYSE, 2005; RODRIGUEZ ESTRADA, 2008). Though numerous cultivation techniques could be developed by the combination of these methods, in my opinion, the wet heat treatment technology could be adapted the best to the Hungarian conditions. As raw material, lignocellulose raw materials (secondary forestry and agricultural products) might be subjects to count on, but enrichment is necessary in order to achieve the optimum biological efficiency (BE, %).

Enrichment and accumulation of metals

Certain mushroom species are able to accumulate mineral elements in their vegetative mycelia and fruiting bodies. As long as a given element is essential for human nutrition, growers can improve the nutritional value of mushrooms by supplementation of raw materials with the necessary elements.

MATERIALS AND METHODS

Collection

Fruiting bodies were collected from grassy plains of Novaj, Eger Felnémet-Pásztorvölgy, Bogács, Tószeg, Kecskemét and Heves. Pure cultures of isolates were made by plectenchyma inoculation or spore printing. The strains were maintained on wooden sticks, perlite mixture and in liquid nitrogen.

Investigation of phylogenetic relationships by RAPD method

For isolation of fungal DNA, I used a modified, optimized version of a protocol published by SHURE et al. (1983). For RAPD investigations the OpA and OpB primer series (Operon Technologies) were applied. Selection of primers was performed on the basis of RAPD fingerprints. The presence or absence of bands was visually scored and binary coded. Binary matrices were set up for each primer and distant matrices were created by the PHYLTOOLS software, using the Nei-Li coefficient. The neighbor-joining tree was built by the NEIGHBOR software, part of the PHYLIP software package (FELSENSTEIN, 1995; NEI & LI, 1979).

In-depth sequence analysis of *tefl*α and *rpb2* loci

In order to differentiate varieties and find polymorphisms, I performed a detailed sequence analysis on the *tefl*α and *rpb2* genes. Both regions were amplified by PCR, and then the amplicons were cleaned and sequenced. Assembly of the sequences was performed by the ClustalX software and nucleotide discrepancies were visualized by the BoxShade program. The sequences were blasted against the NCBI GenBank. I used the *rpb2* sequence data to find possibilities for differentiation among the strains with PCR-RFLP.

Search for dsRNA mycoviruses

In order to extract the nucleic acid from sixteen *P. eryngii* strains, I used a modified protocol of LUKÁCS (1994) originally developed for nucleic acid extraction from plants. Nucleic acids were separated on 5% non-denaturing PAGE gel. Blotting was performed by a Semi-Dry (Biorad) instrument and a Zeta Probe (Biorad) membrane. The membrane was incubated together with dsRNA specific J2 monoclonal antibodies, then with 'Goat Anti Mouse IgG (H+L)' (Jackson Immunoresearch, USA) secondary antibodies conjugated with alcalic phosphatase. Development of the immunoblot was done by BCIP-NBT solution.

Growing tests with vegetative mycelia

In these experiments I investigated the *in vitro* growth rate of fifteen strains on agar plates at different temperatures (5-35 °C, in 5 °C steps) and pH values (pH=4-9, in 0.5 steps); in light and darkness; under aerobic and anaerobic atmosphere and with different osmotic concentrations (NaCl: 1%, 2%, 3%, 4%, 5%; glucose: 5%, 7,5%, 10%, 12,5%, 15%, 17,5%).

Strain comparative cultivation examinations

Spawn of sixteen strains was used to inoculate sterile substrate with the following composition (calculated for dry material): beech sawdust 65%, wheat bran 17%, beech chips 9%, gypsum 3.5% and soy-based growth supplement (Promycel 480) 5.5%. Water content of the substrate was 60%. The substrate was spawned and incubated at 25 °C then the bags were placed into a cold room (10 °C), cased by 3 cm thick layer of peat and covered with thin foil. The foil was removed when the primordia appeared. In the fruiting period the temperature was raised to 15-20 °C, CO_2 content decreased to 800 ppm and the relative water content was held between 90-95%. The substrate surface, floor and walls were sprayed by water every day.

At the end of the cultivation I calculated the following parameters (for 100 kg substrate): average yield, number of fruiting bodies, weight of fruiting bodies, harvest periods, biological efficiency (BE = weight of fresh mushroom (WFM) divided by the weight of dry substrate (WDS) × 100) and productivity (P = weight of fresh mushroom (WFM) divided by the wet weight of substrate (WWS) × 100) (STAMETS, 2000; ANDRADE et al., 2007). I have assembled documentation with photos and text about each developmental stage of the strains that contains the most important parameters.

I have calculated the dry material and nitrogen content of the substrates before and after the experiments and tried to answer the following questions: a) Is there any correlation between the decrease of substrate dry weight and the yield? b) Is there any correlation between the decrease of substrate wet weight and the yield? c) Is there any correlation between the total nitrogen content of the substrate and the yield? In order to answer the questions I performed Pearson correlation analysis with the SPSS 15 software.

Cultivation experiments with casing

With these experiments my aim was to find out what is the role of casing in yield and quality, on sterile substrate. Growing experiments were done without casing, and with different casing soils and mixtures used in variable thickness.

The spawned and supplemented lignocellulose substrate blocks were covered by peat-based casing mixture, lime powder and 1:1 ratio of mixture of peat casing soil and lime powder. Control substrate blocks were not covered by casing soil. Thickness of casing soil was 1, 2 and 3 cm. The yield and wave of production were recorded.

Investigation of element enrichment in fruiting bodies

Three microelements, which are essential in human nourishment, zinc (ZnCl), manganese ($MnCl_2$) and selenium (Na_2SeO_3) were used for enrichment of the lignocellulose substrate mixture in quantities of 50, 150, 300 and 600 ppm. Fruiting bodies were harvested in the first production wave of the PES strain. The fruiting bodies were chopped, desiccated and grinded then destructed by nitric acid. Quantitation of elements was performed by an ICP-MS (Perkin Elmer Elan DRC II.) instrument. Degree of metal enrichment was measured in the fruiting bodies of the species, in correlation with the amount of elements used for supplementation of the substrate. EMF ('factor of element mobilization') values

(EMF = element content of the supplemented mushroom / element content of the control mushroom) and EMF* values (EMF* = element content of the mushroom in a given experiment / element content of the mushroom in the previous experiment) were determined in order to describe the enrichment or accumulation with numerical values (RÁCZ et al., 1996; RÁCZ & OLDAL, 2000).

The impact of substrate quality on the nutritional values of the mushroom

The species is able to develop fruiting bodies on the surface of the substrate, so it can be grown without casing. Nevertheless, it is clear that casing has a positive effect on the yield, but the exact mechanism is not known yet. Some say the casing has two important positive effects: it hampers desiccation and attenuates environmental extremities in the growing house. Although fruiting bodies are able to develop on the surface of the substrate, the role of casing can not be narrowed to only these explanations above. In my opinion the casing soil plays role in the mineral uptake and water transport, as well.

In my investigations I had the following basic idea: if the mineral content of fruiting bodies produced with casing soil is higher than that of the control fruiting bodies (without casing), then the casing soil has a role in the uptake of minerals. Since this procedure is connected to water transport, it might confirm the role of casing soil in the water transport. In addition, both the growers and customers would appreciate mushrooms with higher nutritional value.

In these experiments I used the PES strain and the mineral content was investigated in fruiting bodies collected from three different substrates: 1. sterile straw chaff substrate; 2. mixture of sawdust and woodchips 37.6%, straw 11.28%, cooked rye 48.9%, gypsum 2.25%, without casing; 3. the same substrate as Nr. 2, but it was covered by peat casing soil in 3 cm thickness after spawning. Minerals were investigated as described above, and I determined the ash and protein content of the fruiting bodies.

Registration of causative agents and competitor organisms

In the course of the work I isolated those organisms (bacteria, mycoparasite and competitor moulds) that I met during my experiments. These organisms were isolated from the substrate and the fruiting bodies and majority of them was identified on genus level.

RESULTS

Results of isolation activity

I managed to isolate twelve *P. eryngii* strains from Hungary and three more strains were collected from strain collections from the Netherlands, Malaysia and Northern Italy. The strains could be well maintained with cryopreservation and on perlite mixture, but the wooden sticks are inadequate, since majority of the strains could not grow well on them.

Results of the RAPD experiments

In course of the RAPD reactions six primers were chosen (OpA 05, OpA 07, OpA 10, OpA 13, OpA 18, OpB 10) that resulted in the amplification of relatively well detectable and evaluable fragments and numeroues strains could be distinguished on the basis of the patterns. Most of the strains could be differentiated with the OpA05 and OpA13 decamers, but I found high degree of similarity between the Ple-1V/Ple-2V and Ple-3V/Ple-4V isolates, respectively. The Ple-1V and Ple-2V strains were isolated from the same place, but the Ple-3V and Ple-4V strains originated from different areas. Another interesting result was that the OpA05 did not distinguish the Ple-5V and Ple-6V strains but the OpA13 did, though the two strains were collected from the same field in two consecutive years. Certain primers resulted in patterns that were unique for the strains, so these could be used for characterization of a given strain. These results might be useful for spawn makers and breeders for strain protection.

The neighbor-joining tree (*Figure 1.*) demonstrates well the phylogenetic relationship amongst the cultivable *P. eryngii* strains of mostly Hungarian origin. The strains can be grouped into two big groups: some of them shows closer relation to the Malaysian strain, whereas others to the Western European strains. It is remarkable that we presumably managed to isolate the same strain from the same field in two consecutive years. Another interesting fact that two strains, isolated from Kecskemét and Demjén, show close genetic relationship, though the area where these strains were isolated from are 140 km far from each other.

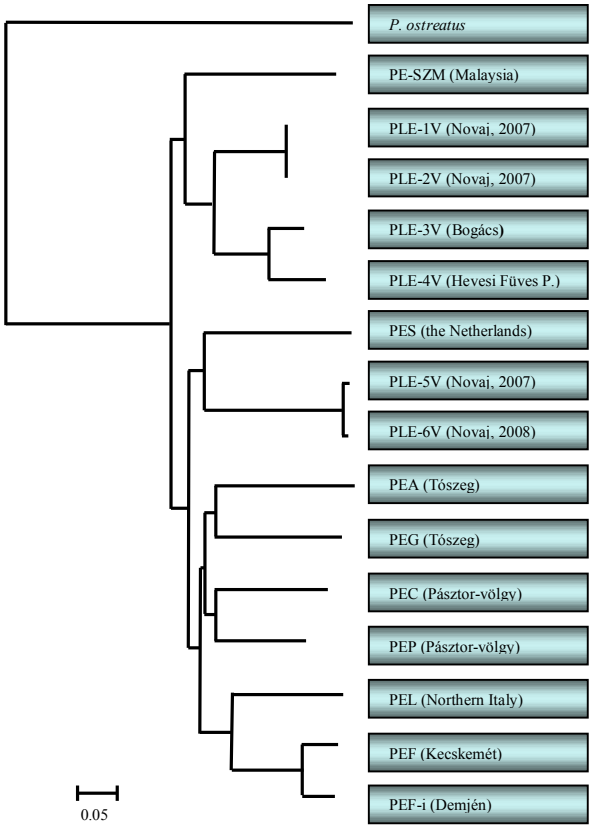

Figure 1. The dendrogram made by the Neighbor-Joining program (NEI & LI, 1979) from the results of RAPD analysis. The scale bar represents the genetic distance.

Sequence analysis of the *tef1α* and rpb2 regions

I found 100% identity in the analyzed nucleotide sequence of the *tef1α* gene, but in the *rpb2* region some nucleotide substitutions were detected. I did not find any information in the literature about what degree of polymorphism and what sort of sequence data could be used for differentiation of isolates at varietas level. Nevertheless, my results clearly show that some polymorphism was present in the *rpb2* locus of my isolates.

I blasted both sequences against the GenBank database and got unexpected results. The archived sequence data in the NCBI database are not sufficient for identification-comparison of the *P. eryngii* isolates. This result shows that molecular work with this species is still not easy.

On the basis of the nucleotide polymorphisms in the rpb2 locus, I performed restriction analysis with two restriction enzymes (BsmAI and TspDTI) and managed to divide my isolates into two groups. Results of the RFLP experiment are shown on *Figure 2* and *Figure 3*.

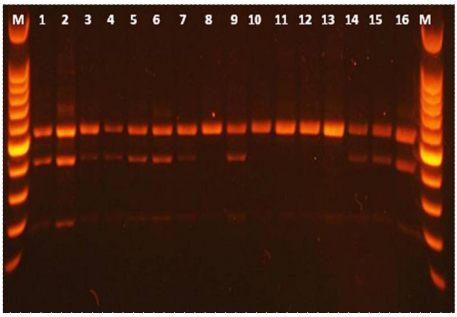

Figure 2. Restriction analysis with the BsmAI enzyme.

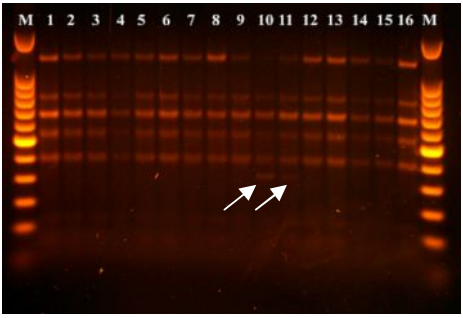

Figure 3. Restriction analysis with the TspDTI enzyme.

(M: size marker, 1. PEP; 2. PEC; 3. PES; 4. PEL; 5. PEF; 6. PEA; 7. PE-SZM; 8. PEG; 9. PEF-i; 10. PLE-1V; 11. PLE-2V; 12. PLE-3V; 13. Ple-4V; 14. PLE-5V; 15. PLE-6V; 16. PEK).
(M: 1500, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp)

Results of the dsRNA investigations

By the immunoblot method I used, no presence of dsRNA, hence no mycoviruses were detected, though the experiment was repeated three times. This result suggests that none of my strains were infected with dsRNA mycoviruses; therefore, there are other reasons in the background responsible for the significant differences in the yield and fruiting body quality.

Growing tests with vegetative mycelia

The strains showed very similar growing characteristics on lower and higher temperatures, as well. In contrast to that, remarkable differences were found in the growth rate on optimum or close to optimum temperatures among the strains. On 25 °C, the optimum temperature for the species, the daily growth rate was between 2.35-12 mm, depending on the strain. The PEC and PEFi strains showed most intensive growth rate, whereas the PEP strain had the slowest rate. On 20-25 °C certain king oyster mushroom strains (e.g. PEC, PEFi, PEA) showed similar growth rate to the K 357 (*P. ostreatus*) control strain.

The species has a wide optimum with respect to pH, but two pH optimum values were found in general: one is the acidic optimum at pH=4.5, the other one is the alkaline optimum in the pH=7.5-8.5 range. The two optimum values presumably ensure better adaptation and survival capabilities to the species in the nature. In contrast to the wide pH range toleration I found differences in the growth rate *in vitro* that developed especially in the extreme pH=4-4.5-5 range.

In the light/dark experiments I found that incubation in dark improved the growth rate of the vegetative mycelia of each strain. This result confirmed that light is not required in the vegetative phase of cultivation (spawning) and the use of black bags might be advantageous in this period.

When I investigated the tolerance of anaerobic conditions I found that the strains tolerated very well even a one week period under anaerobic atmosphere without oxygen (their viability and growth rate did not decrease), though fungi are aerobic, facultative anaerobic organisms. The good tolerance of anaerobic conditions allows successful spawning of compact substrates with low level of oxygen.

Increasing amounts of NaCl resulted in slower growth rate. I found a remarkable inhibition at 4% concentration and the growth stopped completely at 5% concentration. The glucose showed significant inhibition at 15% concentration and the growth stopped completely at 17.5% concentration.

Results of comparative strain examinations

The highest average yield was produced by the Ple-4V (41.5 kg/100 kg) and Ple-5V (39.5 kg/100 kg) strains. The lowest yield was produced by the PEL (9 kg/100 kg) and PEG (11 kg/100 kg) strains. The average yield of the species, calculated for 100 kg substrate, was 27.53 kg. The average number of fruiting bodies was 1488 pcs, the average weight of the *P. eryngii* fruiting bodies was 19.95 g. Yield values are shown on *Figure 4*.

In accordance with the results reported above, very high biological efficiency was found at the Ple-4V (156.18%) and Ple-5V (140.03%) strains. The lowest biological efficiency was found at the PEL (28.52%) and PEG (37.82%) strains. As an average of the strains, the biological efficiency of the species was 98.41%, whereas the productivity was 44.36%, calculated with the weight values of the used, depleted substrates.

Photo documentation was made about sixteen strains in each cultivation phase and I also recorded those data that I found to be the most important in terms of cultivation. In point of quality and quantity I think two strains are good for industrial production: PES and PEF.

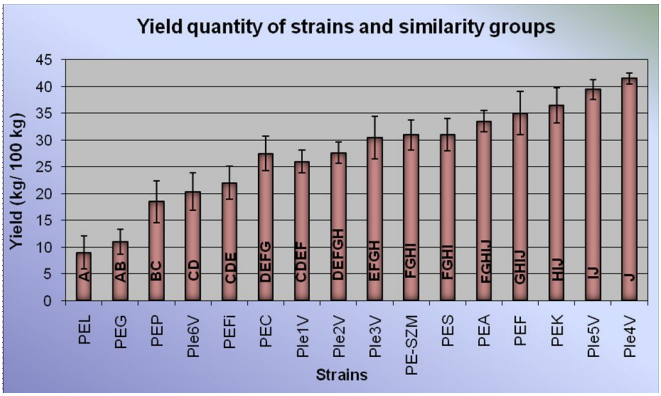

Figure 4. Yield quantity of strains (kg) calculated for 100 kg substrate and level of similarity and differences between strains. (The more identical characters mean higher similarity, while less identical characters refer to higher dissimilarity).

When correlation between the loss of the substrate's dry weight and yield quantity was investigated, the Pearson correlation coefficient showed that there was a higher than average positive correlation (r=0.542) between the loss of weight and the total yield. The correlation was realized on 5% significance level, so it is significant (α=3%). The loss of weight shows linear correlation with the yield quantity.

When the loss in the weight of the fresh substrate and yield quantity were investigated by means of the Pearson correlation coefficient, one could find that there was a higher than average positive correlation (r=0.655) between the phenomena. It was realized on less than 1% significance level (α=0.6%).

Significant correlation was found between the yield quantity and the nitrogen content, but it was negative and realized on a lower than average (r= -0.593) value and at least on 5% significance level (= -1.5%). On the whole it was clear that the nitrogen content of the depleted substrate was higher in case of those strains that produced lower yield. In contrast to that, the nitrogen content of the depleted substrate was lower in case of those strains that produced higher yield.

Results of experiments with casing soil

I found higher yield on those blocks that were covered with casing soil in different thickness than on the blocks without casing (*Table 1.*). The casing had obviously positive impact on the yield, though the species is able to produce fruiting bodies on lignocellulose substrate. The highest yield on the blocks covered with 100% ground limestone was found when the casing was 1 cm thick. The highest yield on the blocks covered with 'traditional casing soil' was found if the casing was 3 cm thick. At the same time, this latter casing resulted in the highest yield among each experiment. When the yield was measured, I found a very high level of standard deviation, when 50% casing soil and 50% ground limestone was mixed. The reason was unknown.

Table 1. Yield on blocks (kg) without casing and with various casing in different thickness, calculated for 100 kg substrate.

Thickness of casing (cm)	***100% 'traditional casing soil'***	***50% groundlimestone and 50% casing soil***	***100% ground limestone****	***Without casing***
1 cm	25.03	**30.10**	**30.15**	**22.70**
2 cm	25.48	23.88	27.80	
3 cm	**31.05**	25.90	**-**	

*Ground limestone = average size of particles was 1 mm;
'Traditonal' casing= 90% peat and 10% ground limestone.

The cropping period was 34 days in total. The harvesting started on the 14th day after casing contrary to the white button mushroom, which develops the first fruiting bodies on the blocks three weeks after casing. In most of the cases 80-90% of the yield was harvested on the first and second waves, so it is strongly advised to wait for these two waves. Between the first and second waves there was a 5-8 days long period, in which I could collect only a few 'intermediate' fruiting bodies. When the blocks were covered by paper, the mycelium developed 2-3 days earlier than on the non-covered blocks. Based on my experiments I would not recommend the ruffling unambiguously.

Results of experiments on microelement enrichment

When I measured the zinc, the control fruiting bodies contained 25 mg zinc /kg dry substrate. The highest amount (40.03 mg/kg dry substrate) was found in a fruiting body that was developed on a substrate supplemented with 300 ppm zinc. Additional results are shown in *Table 2.* 300 ppm zinc did not have any significant influence on the yield, but 600 ppm remarkably decreased it (to 18 kg/100 kg).

Table 2. Data of zinc supplementation experiments.

	Amount of Zn	***EMF***	***EMF****
Control	25.00	-	-
Zn 50	30.50	1.220	-
Zn 150	34.60	1.384	1.134
Zn 300	40.03	1.601	1.157
Zn 600	29.50	1.180	0.737

In the untreated control fruiting bodies I measured 4.13 mg/kg dry substrate manganese. The highest level of manganese was found after treatment with 300 ppm (7.3 mg/kg dry substrate). When the yield was measured, the highest amount was found after treatment with 50 ppm manganese, what might be interpreted as a positive impact on the manganese peroxidase enzyme. Treatment with more manganese did not have additional positive effects; the average yield was 25 kg/100 kg. More results are shown in *Table 3.*

Table 3. Data of manganese supplementation experiments.

	Amount of Mn	***EMF***	***EMF****
Control	4.13	-	-
Mn 50	5.30	1.283	-
Mn 150	7.07	1.711	1.333
Mn 300	7.30	1.768	1.033
Mn 600	6.70	1.622	0.917

When I evaluated the results of selenium supplementation, three aspects were taken into consideration. In biological aspect, when the substrate was supplemented with 600 ppm selenium, the species (PES strain) was able to accumulate significant amount of metal (520.03 mg/kg dry substrate) in contrast to the control (0.15 mg/kg dry substrate) (*Table 4.*). In cultivation only 300 ppm supplementation could be advised, because it did not have serious impact on the yield. In contrast to that, 600 ppm supplementation decreased the yield remarkably. When we evaluated the selenium content from the consumers' side, it became clear that supplementation even with the lowest concentration (50 ppm) of selenium resulted in a 13.8× amount of the recommended dietary allowance (RDA_{Se}= 55 μg) in 100 g raw mushroom, if we count with 100% absorption rate. This result showed that supplementation by selenium might lead to human toxicity problems. Nevertheless, selenium enriched mushrooms might be sold as functional products or could be used as food supplements.

Table 4. Data of selenium supplementation experiments.

	Amount of Se	***EMF***	***EMF****
Control	0.15	-	-
Se 50	76.03	506.889	-
Se 150	99.30	662	1.306
Se 300	230.20	1534.667	2.318
Se 600	520.03	3466.889	2.259

The impact of substrate on the mineral element content

When mineral element content of fruiting bodies developed on lignocellulose blocks with and without casing was compared, it was found that 19 out of 22 elements (Al, As, Ba, Ca, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Na, P, Se, Sr, Ti, V, Zn) were present in a higher amount in the fruiting bodies developed on blocks with casing. It showed that casing contributed to the accumulation of elements in the fruiting bodies, thus casing soil plays an important role in the mineral uptake of the mushroom. This finding led me to the conclusion that casing soil has significant effect on the water transport, as well. These results completed our knowledge about the role of casing soil and showed that its role is more than only decrease of desiccation and environmental effects.

Another observation was that fruiting bodies developed on lignocellulose substrate have higher protein content (*Figure 5.*) and fruiting bodies developed on straw substrate have higher ash content (*Figure 6.*).

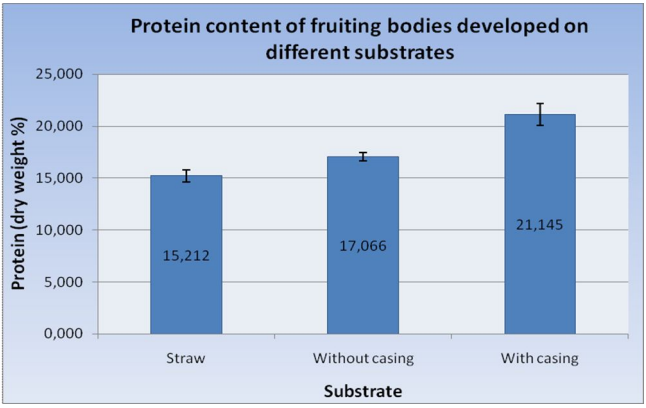

Figure 5. Protein content of fruiting bodies developed on different substrates.

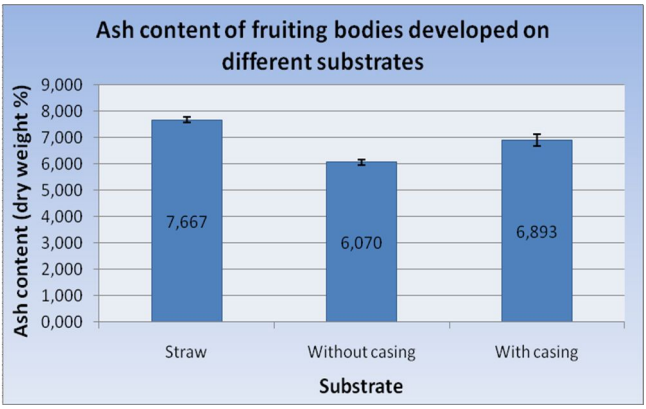

Figure 6. Ash content of fruiting bodies developed on different substrates.

Causative agents in cultivation

In the course of my experiments I found the following groups of causative agents: *Pseudomonas* species (brown blotch disease), mycoparasite *Trichoderma* and *Penicillium* species, *Dactylium* sp. (cobweb agent, known from cultivation of white button mushroom). *Aspergillus*, *Zygomycota* and other imperfect species were isolated from the substrate, but I could not identify these isolates on species level by means of traditional tools.

Various *Diptera* larvae are known to chew the substrate and the fruiting bodies. Important vectors might be the imagines (*Phoridae*, *Sciaridae* etc.). Certain mite species can be found on the substrate and the fruiting bodies.

The most frequent, presumably physiological disorders were the following: abnormally shaped fruiting bodies, brown stripes and/or grooves on the stipe. Fruiting bodies with large and thick stipe or with small cap are also often found, presumably due to the environmental conditions (climate, water transport, substrate), but these features might be characteristic for a given strain.

NEW OR NOVEL SCIENTIFIC RESULTS

On the basis of my experiments the new scientific results are the following:

1. A neighbor-joining tree was constructed on the basis of the molecular biology experiments that represent phylogenetic relationships of the investigated twelve Hungarian and three foreign *P. eryngii* isolates.
2. At first time in Hungary I performed sequence analysis on the *tef1*α and *rpb2* loci of the Hungarian isolates in order to find polymorphism and a method for distinguishment of varieties.
3. On the basis of the *rpb2* sequence data I developed a simple method for differentiation of strains by PCR-RFLP.
4. By the means of a special version of immunoblot I found out that dsRNA species, mycoviruses were not present in the investigated 16 strains, though these are frequently found in fungi. Hence, differences in yield and quality among strains can not be explained by the presence of dsRNA mycoviruses.
5. For fifteen strains I described the growth rate on various temperatures, pH, in light and dark, under aerobic and anaerobic atmosphere and among different osmotic conditions.
6. I performed comparative cultivation experiments with sixteen strains and recorded the important details, so I got a comprehensive picture about the cultivation characteristics of the strains. of the species.
7. I performed experiments with casing soils and found that the casing significantly improves yield. In addition, thickness and composition of the casing soil might be another tool for improvement of biological efficiency.

I found that nutritional value of fruiting bodies depends on the composition of lignocellulose substrate raw material and the presence/absence of casing soil.

8. I found that the role of the casing soil is not only to avoid desiccation and decrease climatic extremities of the growing house, but to help mineral uptake and water transport, as well.
9. I investigated the accumulation of three elements in the PES strain and found that zinc and manganese were not accumulated in significant amount, but selenium enrichment - depending on the concentration of selenium supplement - might be very remarkable. Therefore, this method could be applied for enrichment of fruiting bodies with selenium, which element has severe biological effect. Nevertheless, human toxicity effect must be taken into consideration when speaking about selenium accumulation.
10. Based on my own results and the literature I compiled a manual of cultivation to help Hungarian growers.

SUMMARY

In my dissertation I performed experiments with the king oyster mushroom (*Pleurotus eryngii*) species that is known about its good taste. Thirteen strains were collected from Hungary and three strains of foreign origin were obtained from Hungarian culture collections. Phylogenetic relationship of the isolates was investigated by means of RAPD-PCR and a neighbor-joining tree was built on the basis of the results. Sequence analysis of the tef1 and rpb2 loci of the isolates was done and I found a small-scale polymorphism among the strains. When the sequences were blasted against database sequences it turned out that identification of varieties with this method is very uncertain in this way. I managed to sort the

strains into two groups on the basis of single nucleotide polymorphisms in the rpb2 locus.

By the means of the immunoblot method I used no traces of dsRNA molecules, which are the signs of mycovirus infection, were found.

In the in vitro experiments I got a picture about the ecological claims of the strains and the species itself, and it might provide valuable information for cultivation, too. Comparative cultivation experiments were done with sixteen strains and I got a lot of information about the characteristics of the various strains.

My experiments with casing soils showed that thickness and composition of them might be important part in improvement of biological efficiency. I found that presence/absence of the casing soil has impact on the protein and mineral content of the fruiting bodies and on the mineral uptake and water transport.

Supplementation of raw material with zinc, manganese and selenium in different concentrations showed that the PES strain of the species was not able to accumulate the zinc and manganese, but accumulated such a large quantity of selenium, what might lead to human toxicity problems.

CONCLUSIONS

Taxonomic debates about the species are subject to be closed by comprehensive biological, speciation and coevolution research works. In the future it is necessary to screen the wild strains and it is advised to start breeding activities. It is necessary to protect the new hybrids and industrial strains and molecular biology methods are essential in this process.

If agricultural and forestry secondary products are used for cultivation, nitrogen content, heat treatment and casing of the lignocellulose substrate are of key

importance. In my opinion the wet heat-treated substrate has reason for existence in Hungary, because most of the substrate manufacturers have this technology.

It is necessary to determine the exact cultivation technology, or define even strain or variety specific technology in the future. Improvement of synchronized development and decrease of clustering (as a typical character of the species) might be additional task for the future. It is important to set up well defined requirements for quality. The species should be advertised in Hungary and it is important to estimate consumers' preferences.

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Research laboratories

Laboratory experiments were performed at: Laboratory for Strain Research, Laboratory of Molecular Biology and Spawn Plant of the Quality Champignons Ltd.; Department of Food Chemistry and Biochemistry of the Eszterházy Károly College; Department of Microbiology and Food Technology of the Eszterházy Károly College; Department of Plant Physiology and Plant Biochemistry, Corvinus University of Budapest.

Cultivation experiments were performed at: green house of the Faculty of Horticultural Sciences, Corvinus University of Budapest; Centre for Species Investigation, Quality Champignons Ltd.

PUBLICATIONS RELATED TO DISSERTATION TOPIC

Article published in journal with impact factor

J. SZARVAS, A. GEÖSEL, K. PÁL, Z. NAÁR, J. GYŐRFI (2011): Comparative studies of the cultivable king oyster mushroom [*Pleurotus eryngii* (DC.: Fr.) Quél.] isolates by RAPD-PCR method, *Acta Alimentaria, 40 (Suppl.),* 214-221.

Article published in English in peer-reviewed journal

J. SZARVAS, Z. NAÁR, A. GEÖSEL, J. GYŐRFI (2010): *In vitro* investigation of King Oyster Mushroom [*Pleurotus eryngii* (DC.: Fr.) Quél.] strains in vegetative growing phases. *International Journal of Horticultural Science, 16 (2):* 47-53.

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