DOCTORAL (PhD) THESIS

Amelioration of the characteristics of cultivated *Pleurotus ostreatus* hybrids and the production of new hybrids with the application of wilding phyla

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The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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1. Abstract of the research task and its scientific antecedents

The efficiency of oyster mushroom cultivation depends in a large measure on the species, the physiological and morphological properties of the cultivated living creature – just like the efficiency of every branch of cultivation that deals with living objects (agriculture, horticulture, etc.)

With the diffusion of oyster mushroom cultivation, both on the part of cultivators and customers, new demands are coming forward:

- The cultivated mushroom should be a good cropper.
- It should be resistant to diseases.
- Its breeding season should be short.
- A pellucid appearance is desirable – the colour is not inconsequential either, customers prefer a darker prime colour, while the canning industry favour lighter colours.
- It should disperse spores at a reduced number and preferably in the maturation period.

It is a market advantage if the environmental demands of the oyster mushroom species can be cheaply satisfied with simple equipment, and if it can be cultivated throughout the year independent of seasonal changes.

There is no single species of oyster mushroom, which can satisfy all the demands at once. Thus, there is no other choice, than broadening the variety of breeds, so that the cultivator could choose the breed most suitable for growing conditions and market demand.

In the world, the traditional methods of the selective breeding of plants and mushrooms have been supplemented and at the same time accelerated with the application of the methods of molecular biology in the past years. On the other hand, in Hungary, the methods of selective breeding of the cultivated large mushrooms are still using the classical methods, and domestic selective breeding and breed identification are still applying the methods of the 1980s. This does not mean that the traditional methods of selective breeding and breed separation are not applicable in the creation of new hybrids, or possibly to separate breeds with discreet morphological markers. Moreover, the case of the present domestic practice regarding mushrooms still shows that the methods of traditional selective breeding can be more efficiently applied. However, I believe a better solution would be to apply the newer methods of molecular genetics with the traditional methods. In my external research facility (Quality Champignons Ltd. Spawn Plant and Spawn Research Laboratory), we were able to develop a new molecular biology laboratory as a result of the company's expansion of the past few years. Although molecular biological work does not constitute a task undertaken in this

dissertation – first in the country, I started nucleic acid based breed separation examinations regarding mushroom production.

Mushrooms, particularly cultivated 'large mushrooms' react with extreme sensitivity to changes in their environmental conditions. This can be easily observed in the morphological diversity of the fruiting bodies of a given phylum, if they grow under different environmental conditions. Consequently, certain environmental and ecological influences, characteristic of the given breed, allow the development of extensive morphological varieties. The disadvantage in the course of mushroom production and selective breeding is: if the given breed is grown on differently composed substrates, in premises with different climates, with different production technologies, it may yield multifarious morphological markers. Because of the changes in environmental factors and the characteristics of a given breed, the morphological spectrum may range from deformed fruiting bodies to fine fruiting bodies that carry the characteristics of the breed. All this concludes that breed-separation based on the traditional morphological markers does not yield reliable results in every case, especially in the case of mushroom breeds.

In Hungary, there are only a few research facilities concerned with the research of mushroom breeds and the selective breeding of new breeds. Although mushroom production research (composting, selective breeding, application of new agro-technological methods, etc.) is starting to progress, it still lags significantly behind when compared to research related to biotechnology, the selective breeding of plants and the separation of plant breeds.

The central topic of my dissertation was the selective breeding of oyster mushrooms with classic technologies. However, since I started the doctoral school my research facility has undergone important developments, so I have tried to capitalize on the advantages offered by these developments. Naturally, this new opportunity has not made it possible to treat all the different details of breed separation relating to mushrooms, but I could lay the foundation of the molecular based separation of mushroom breeds. Hence, beside the central topic of my dissertation, that is the selective breeding of oyster mushrooms, I endeavoured to apply molecular biology based on the separation of oyster mushroom breeds. This is the first study of its kind in the country. Unfortunately, these types of mycological experiments are still new compared to biotechnological examinations of plants, so those methods are less elaborate in mycological work. Further molecular biological possibilities were partly limited because the equipment available at the research facility primarily allows of PCR (needs words spelled out first if it is an acronym) based work. I hope that my examinations on molecular biological

based mushroom breed separation will constitute good groundwork for further domestic mycological research.

My objectives were the following:

- To produce oyster mushroom hybrids with favourable cultural characteristics, applicable in large-scale production, by using wild oyster mushroom phyla and hybrids currently available at the market.
- I thought it was expedient to continue the work effectively done by the notable oyster mushroom selective breeder, Pál Gyurkó and his research team in the 1980s. The hybrids they produced can be taken as an excellent basis for producing newer hybrid phyla. In my selective breeding work I used the 'Gyurkó - hybrids,' and I tried to create new phyla applying the methods used during their development. In the selective breeding I employed the newly collected wild *P. ostreatus* phyla as well.
- As the central topic of my dissertation, besides classic selective breeding of oyster mushrooms, I endeavoured to separate oyster mushroom breeds with molecular biological, PCR based methods.

2. The methods of the examinations

2.1. Production of new hybrids by crossbreeding

Phyla used in crossbreeding

Today it would be difficult to market the propagation material of parent phyla used in the selective breeding of HK35 as an individual breed. At the same time, these phyla provide an excellent opportunity, complemented with newer, wild *P. ostreatus* phyla, to produce hybrids differing from HK35 but competitive against them.

That is why we used the culture of the following phyla, as the selective breeding parent material:

- Gyurkó hybrids: H7, G24, HK35-strains from different sources, HK44;
- Wild ostreatus phyla: from PO1 to 25, from OL1 to 7, *P.ostreatus var. florida*.

With the parent phyla we performed the following crossbreeding:

	Po 1-25	P.o. var. florida	H7	HK35	G24	HK44	OL1-7
Po 1-25	**0**	**X**	**X**	**0**	**X**	**X**	**0**
P.o. var. florida	**X**	**0**	**0**	**0**	**0**	**0**	**X**
H7	**X**	**0**	**0**	**0**	**0**	**X**	**X**
HK35	**0**	**0**	**0**	**0**	**X**	**0**	**0**
G24	**X**	**0**	**0**	**X**	**0**	**0**	**X**
HK44	**X**	**0**	**0**	**0**	**0**	**0**	**X**
OL1-7	**0**	**X**	**X**	**0**	**X**	**X**	**0**

(X= crossbred phyla; 0= phyla that were not crossbred)

In the production of new hybrids we applied two procedures, the monosporic and the multisporic methods.

Production of dicarionta cultures with a monosporic procedure.

The individual steps in the monosporic procedure are as follows:

- Production of spore suspensions
- Casting agar discs
- Inoculation of spores
- Germination
- Extracting germinating spores
- Microscopic examination of the culture
- Grafting the monosporic cultures from different sources and observing compatibility
- Sustaining dicarionta cultures

Production of dicarionta cultures with a multisporic procedure.

The individual steps in the procedure are as follows:

- Production of spore suspensions
- Mixing the spore suspensions
- Grafting on agar discs
- Germination and observing the heterogenic culture
- Isolation from the sectors and clarification of the isolates

- Verification of channel formation
- Sustaining dicarionta cultures

Comparison of the monosporic and the multisporic procedures

We consider the monosporic procedure to be more labour-intensive than the multisporic one because of the extraction of germinating spores. When monosproc cultures are composed and the spores are not related to each other – something we cannot be sure of in advance – then only a quarter of the grafting will yield dicarionta cultures. In this case there is unnecessary work involved. Otherwise, it is worth grafting the monocariontas in every possible combination, once we have isolated them. On the other hand, the monosporic procedure is advantageous, because it is certain that artificially grafted cultures will yield a dicarionta culture from the fusion of two unrelated monosporic cultures. A further advantage of the procedure is that the resulting culture is microbiologically pure since the grafting is preceded by cleansing it in several steps.

In the case of the multisporic procedure, according to our experiences, we can produce numerous dicarionta cultures more rapidly, but we do not have any evidence confirming that the resulting cultures do not stem from the fusion of the hyphae of two related germinated spores, but that they are descended from the fusion of two unrelated ones. Although exact experiments prove that between the unrelated ones affinity is great, but this only makes the fusion of unrelated ones likely, without proving it. We can only be sure if the parent cultures have differing individual characteristics, which are definitely distinguishable, and if these characteristics appear jointly in the progeny. This circumstance can be regarded as proof of crossbreeding.

In the case of the multisporic procedure, we can use the spores of a fruiting body, or those of two or more unrelated fruiting bodies.

We usually use the spores of a single fruiting body when we want to renew or rejuvenate an ageing, but worthy culture. Among the spore progeny of a single fruiting body we can find deviation, so we will choose the culture that best resembles the earlier phyla.

The diversity of the spore progeny of one fruiting body is surpassed if we use two or more different fruiting bodies, and the mixture of their spores, to create multisporic cultures, which result in greater diversity and variegation. Usually we follow this procedure to create completely new cultures. It is expedient to choose the fruiting body giving the spores after careful consideration.

2.2. Differentiation of mushroom breeds with molecular biological methods

We produce hundreds of hybrids annually with the above-mentioned methods. Initially we select these hybrids in experiments performed in small plots. Small plot experiments were carried out in glass containers. In laboratory autoclaves we sterilized the previously wetted and ground straw in glass containers. The stock produced this way was grafted with spawns. We covered the small glass containers with perforated foil or paper. The cross weaving was carried out in clean, acclimatized circumstances, used for spawn production, and the unit started to produce in a cool room. We ensured the necessary environmental circumstances for the formation of fruiting bodies with simple technologies: we ensured humidification with a household appliance and ventilation with small fans.

The objective of the examinations was to determine which phyla show identical or better results compared to the HK35 and HK44 control phyla. Experiments carried out in small plots or glass containers are not suitable to calculate productivity average, but they are highly applicable in the examination of the colour or morphology of the fruiting bodies, or for the time necessary for primordial development. Accordingly, we applied selection on these characteristics. The experiments in small plots lasted five to six months including repetitions, and finally we separated five to eight phyla for further growing experiments.

In the course of the examination of the RADP patterns of the examined oyster mushroom phyla we found differing marker bands using the following primers:

- OpA 11
- OpA 15
- OpA 17
- OpA 01
- OpA 4391
- OpA 20

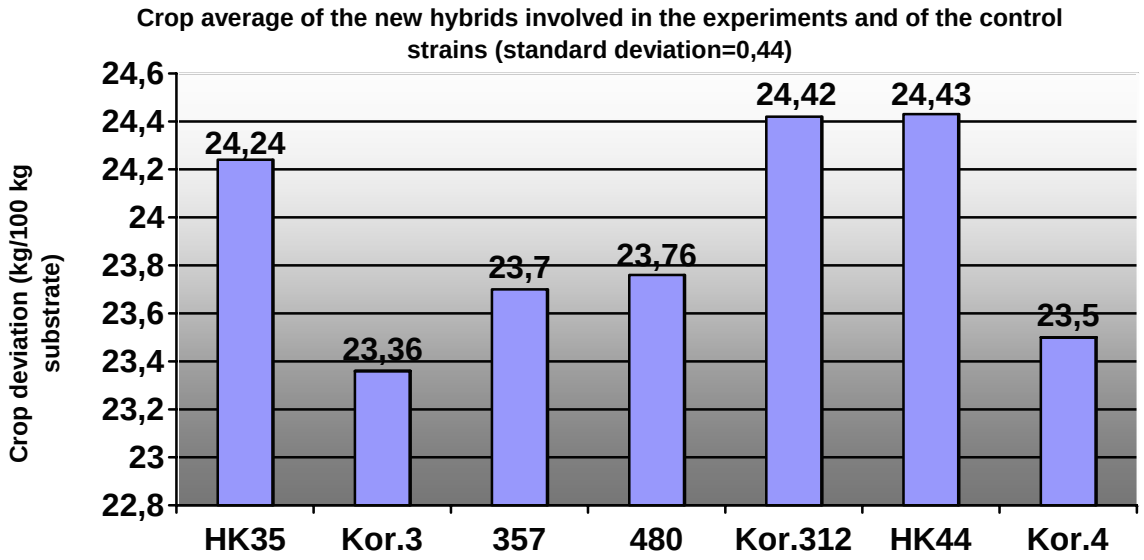
Among the phyla chosen for the purpose of RAPD analysis, were not only parent phyla, but also phyla originating from the propagation material of competitor firms. This is important because we can differentiate between the Korona 312 hybrid from the breeds of competitors with the help of the DNA method. We can also examine the deviation from or the relationship between the parent and related phyla applied in selective breeding.

3. Findings and their evaluation

3.1. Findings of crossing, characterising the new hybrids

The following diagrams show the results of some attempts to cultivate mushrooms. Production data come from experiments set on substrate produced with large-scale industrial technology. These experiments were repeated three times. Basic material enrichment was not done; ground straw was heat-treated with xerotherm technology. Results of cultivating experiments in two waves were accurately recorded. Besides the weight of the crop, the colour of the fruit body, the intensity of its development and its thickness were recorded.

Diagram 1.



This diagram shows that strains Korona 3 and 4 fall behind HK strains as for crop average. These strains can develop large number of fruit bodies. However, their thickness and weight fall behind that of the control groups'. For both strains the characteristics of *P.ostreatus var.florida* is dominant, which can be observed in the following two diagrams, where Korona 4 shows high productivity even in warm cultivating conditions.

Korona 312, however, yielded crops in similar quantity and quality as the two members of HK family, putting behind strains 357 and 480 of Korona Strain Research Laboratory.

We consider this the greatest result of our research.

Diagram 2.

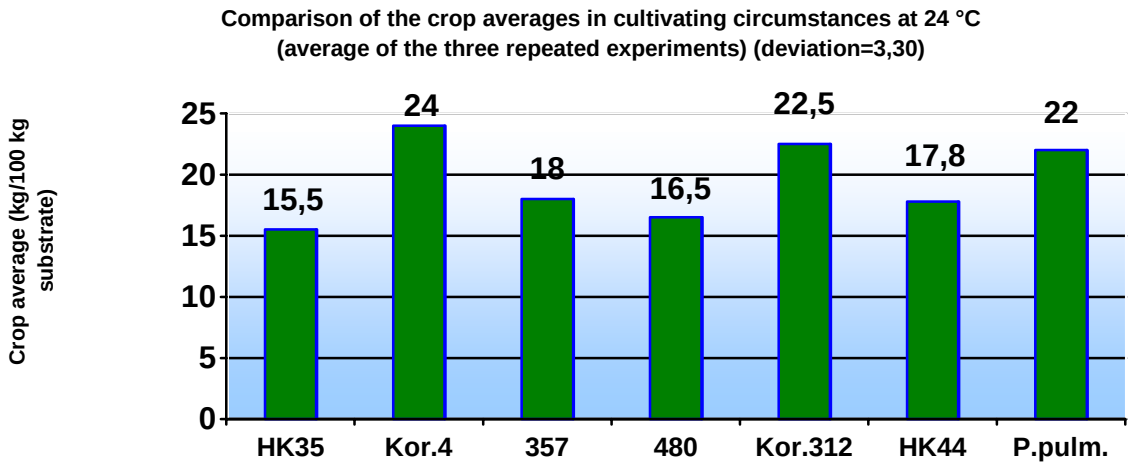
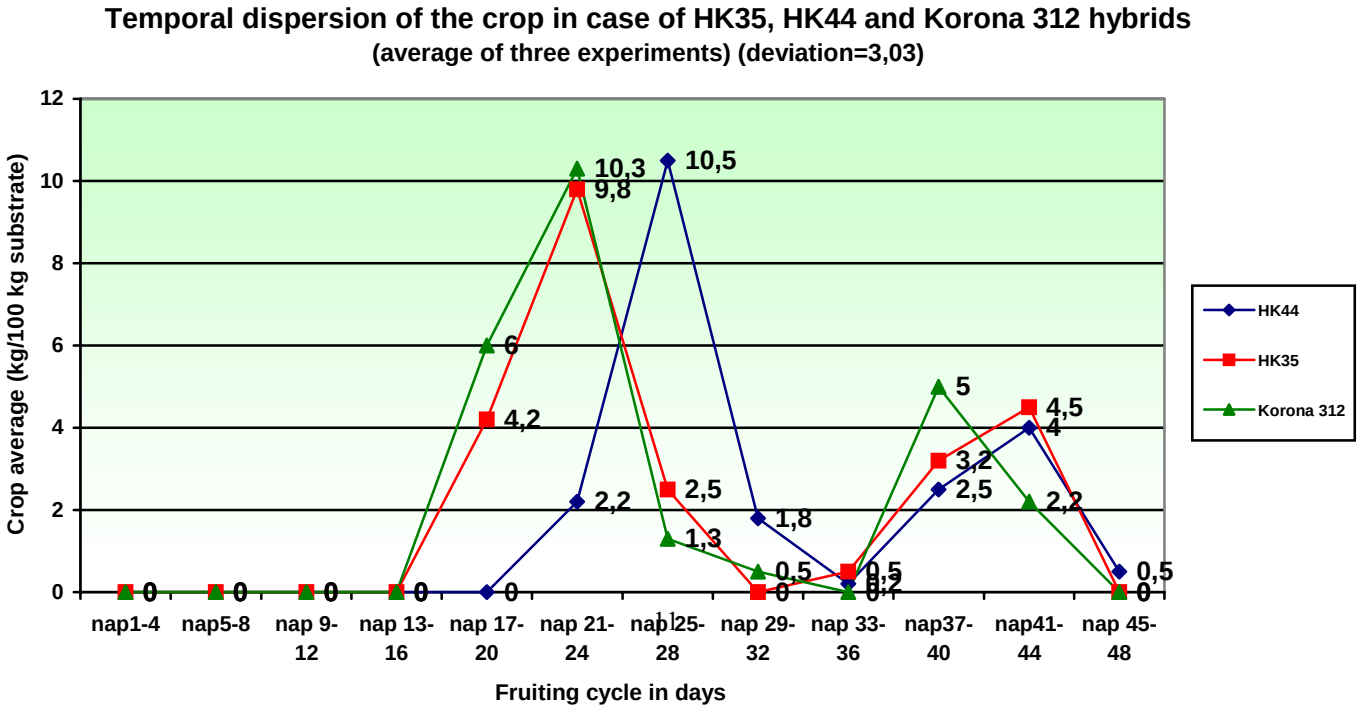


Diagram 2 shows the average results of cultivating experiments carried out in warmth, with 24 degrees Celsius as average daily temperature. *Pleurotus pulmonarius* wild strain was involved as a control, as this strain can bear these circumstances; in certain countries this strain is cultivated in large-scale plants. It can be seen that Korona 4 and 312 have outstanding results, which make these two strains suitable for summer cultivation in a large-scale plant.

Diagram 3 shows that the performance of the new Korona 312 hybrid does not fall behind strains HK 35 control strain even at the time of primordial forming. Cultivators expect that combining should be fast and intense so that they can avoid infections with a greater probability. Strains capable for basic material combining start to develop fruit body by the end of the third week, so on the fourth week after implementation the first wave can be harvested.

Diagram 3.



3.2. Summary on findings of molecular biological research

As a result of our molecular biological research it can be stated that we could separate Korona 312 hybrid produced by combination and examined in cultivating experiments from oyster mushroom hybrids produced by other domestic producers with primers having been planned on RAPD-PCR differentiating bands. We found differentiating bands during the use of OpA11, OpA111 decamers in RAPD-PCR, but we could not differentiate between strains with our own primers tested as a result of cloning, sequencing or primer planning work. In the case of OpA01 the aim was not to let the primer differentiate between different oyster mushroom hybrids so that it could be used in multiplex PCR coming up as an outcome of the work. We managed to produce it as a universal pair of primer, namely, the planned amount of amplicon returned in all the hybrids within the desired measurement province.

We succeeded in isolating two differentiating strains during the use of decamer OpA17. With primers designed for sequence data of one of these lines oyster mushroom strains included in the research could be divided into groups. In the case of the following strains and hybrids, the presence of amplicons could be observed: *P.ostreatus var.florida*, G24 and Korona 12 which were bred by our research team. These prove that characteristics of *P.ostreatus var.florida* dominate in hybrid Korona 12 since *P.ostreatus var.florida* was one of the confining strains.

We designed another primer from the other differentiating lines of OpA17 with the help of which strains and hybrids could also be divided into two groups. PCR product was observable in the followings: *P.ostreatus var.florida*, Korona 312, G24, Po12, Hk44.

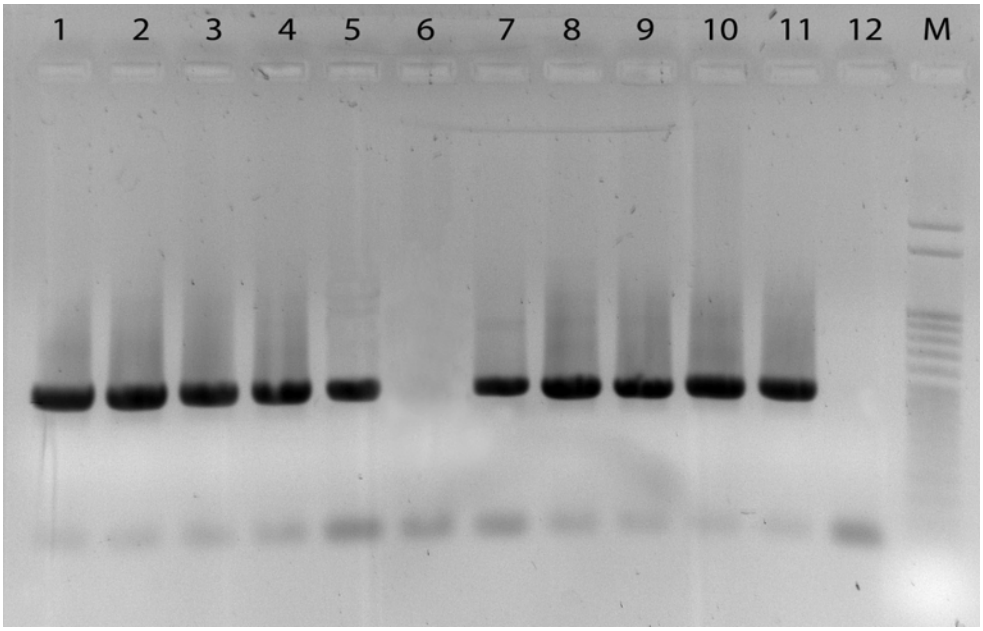
With designed primers set with OpA4391 decamer originating from RAPD-PCR, the presence of amplicon could be observed in six species, so the oyster mushroom strains included in the research were also divided into two groups. The product was observable by using the following templates: 1-0-30, Bu-La, GHK35, K357-1, *P.ostreatus var.florida* and Korona 312.

Using OpA 20 random primer we could gain that in the case of Korona 312, which is our own improvement, there was no amplicon at around 800 bp, in contrast with other strains.
The order of applying the samples was the following:

- 1-0-30, the breed of a French spawn producing plant (1)
- Bu-La, a common breed of mushroom spawn laboratories in Romania (2)

- GHK-35, the original Gyurkó hybrid (3)
- 357-1, the breed of Korona Plant, practically a HK selection (4)
- *P.ostreatus var.florida*, a wild phylum (5)
- Korona 312, a new hybrid (6)
- G24, Gyurkó hybrid (7)
- Po12, a European wild phylum (8)
- HK44, Gyurkó hybrid, not available in retail (9)
- OL1, Italian wild *P. ostreatus* (10)
- OL7, Italian wild *P. ostreatus* (11)

The sequencing data and designed primer:



Testing primers designed for RAPD-analysis differentiating lines by OpA 20 primer in PCR-reaction

Korona 312 strain does not give band, in contradiction to other hybrids. With this primer, it is possible to separate new and older strains and selective breeding materials.

Naturally, for us it would have been better if we had got a differentiating band in the case of Korona 312 and not in the case of the other strains. About 40 random primers were tested in RAPD-PCR without achieving the above mentioned result. However, I think the lack of amplicon with a self-designed primer is a similarly good result in strain-differentiating

research. Accordingly, as a result of PCR set with its own primers, in the case of all hybrids there was amplicon presence, with the exception of Korona 312 new hybrid. With this primer pair, we succeeded in separating this new oyster mushroom hybrid, which has good results in cultivating experiments proved by myself, from the other hybrids. This is one of the most significant achievements of this molecular biological work.

4. Summary of the new scientific achievements

During my improvements, I used the hybrids bred by Pál Gyurkó and I attempted to produce new strains by methods used in improving Gyurko hybrids. I involved several newly collected wild *P. ostreatus* strains, as well.

I did hundreds of combinations per year involving seven parental strains. New strains, selected from the new hybrids, were tested in laboratory and in cultivating conditions and they were selected again. Two to three lines per year were involved in large-scale cultivation and several litres propagation material (spawn) was sold inside and outside Korona company-group. With the methods mentioned in Chapter 3, we produced hundreds of hybrids per year. At the beginning, they were selected in small plot experiments. The small plot cultivation was done in glass containers on sterile base. The aim of the research was to find out which strains show similar or better results than HK35 and HK 44 control strains. Small plot, glass container cultivation is not suitable to calculate average crop. However, it can be excellently used to investigate primordial forming time, fruit body colour and its morphology. These small plot investigations took five to six months including repetitions. Eventually, we kept 5-8 strains for further experiments.

After we had selected strains showing promising characteristics from small plot cultivation, we inculcated them into 10-litre substrate and grew them on it, already in large-scale circumstances.

In comparative experiments not only the morphological features of the strain can be analysed but it is also possible to get the average crop results compared to the control strains. Of course, it is early to draw conclusions at this time, but after three repetitions the data can serve as the basis for the ultimate selection. From the examined five to eight strains only one or two as a maximum were selected, so that we can apply them in producing propagation material in large-scale production and sell it to consumers.

Before this, however, we involved the new strains in cultivation on a mushroom farm, which belongs to our company. This farm often allows experimentation with mushrooms and

produces exotic mushroom strains, as well. Here, we investigated the sensitivity and tolerance of the new strains to environmental influences.

By this testing method, we made several new hybrids, results of my scientific work, capable to be sold in market. My first hybrids were born by combining HK 44 with H7, and HK35 with G24. Korona 3 lines and Korona 4 strains, made in this way, started to produce and were later sold in market beginning in the year 2000.

In further years, we combined a strain, which was produced by combining *P.ostreatus var.florida* with a domestic Po5 strain, with an OL1 strain.

From among the strains, gained that way, one strain passed the selection procedure, Korona 312 hybrid. Its crop results definitely differentiate it from the control groups. The hybrid, simultaneously tested on productivity, was examined with molecular biological methods so that we can investigate the possibility of PCR differentiation of the strain.

Three great achievements of my scientific work, which have been going on since 1999, can be summarized as follows:

First, I carried out a breeding research, which lasted for eight years and the result of which was several hybrids, propagated in a plant and sold in market, including a new oyster mushroom hybrid called Korona 312. This hybrid proved during the experiments that it can be the competitor and the possible successor of HK35 hybrid, taking crop quantity and quality into account.

Second, I worked out a breeding protocol in our research laboratory, applying classical methods, bringing them to perfection, which protocol has not been recorded in such details so far.

Third, we worked out a strain-maintenance technology that could be used in maintaining xilophag mushrooms growing especially intensively and so, it is sure to maintain these mushrooms for a long time in a cost-saving way. We applied and brought traditional maintaining technologies to perfection, but gained new experience in practising criopreservation technology, as well.

I believe that applying DNA technologies, breeding will become faster and more effective, thus enabling Hungarian researchers to emerge into the international forefront again.

In my opinion, it is critical to carry on research in connection with molecular marker developments, because in the future, with the help of these units it will be possible to copyright and protect these hybrids. As far as I am concerned, legislators in Brussels are working on the legal background of regulations and strain-protection. Until coming into force,

there is a lot to do, so that we are not forced out of the international market and we are not limited in our activities due to our unprotected genetic basic materials.

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