Science and Cultivation of Edible Fungi, Van Griensven (ed.)
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Development of a sporeless strain of oyster mushroom *Pleurotus ostreatus*

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ABSTRACT: One of the major problems in the cultivation of oyster mushrooms *(Pleurotus ostreatus)* is the enormous production of spores by the fruitbodies, amounting up to several hundreds of millions of spores per gram of fruitbody tissue per day. Many workers in the production of these mushrooms develop an allergy with symptoms similar to an "extrinsic allergic alveolitis (EEA)".

For the development of a sporeless strain of P. *ostreatus* we used strain ATCC 58937. This non-commercial strain is completely sporeless. We have recovered both nuclear types of strain ATCC 58937 as monokaryons (hereafter referred to as nhl 1 and nh42) by protoplasting the mycelium.

Our studies indicate that sporelessness in strain ATCC 58937 is linked to a limited number of loci, present in both nuclear types. The exact number of loci remains to be determined. This is in contrast with previous reports which state that sporelessness is a dominant trait present only in nuclear type nh42. Our studies also show that sporelessness is not linked to the unfavorable appearance of the fruitbodies.

This information provides a good basis for the development of a sporeless strain of *P. ostreatus.* To accelerate our efforts in developing this strain we are currently working on the development of molecular markers linked to sporeless trait. As shown in our preliminary map one of these markers is linked to one of the loci involved in sporelessness.

1 INTRODUCTION

Next to the white button mushroom, the oyster mushoom *(Pleurotus ostreatus)* is the second largest crop of edible mushrooms. In 1997 annual production in the EU amounted up to 32050 tonnes, with Italy being the largest producer (19000 tonnes)(Bio Markets Research, 1999).

One of the main problems in growing *P. ostreatus* is the occurrence of respiratory problems among the people who are harvesting and handling oyster mushrooms. The symptoms of this disease are identical to the "mushroom workers lung". A more general term that is also used to identify a number of other respiratory diseases is extrinsic allergic alveolitis. Several authors have reported that the occurrence disease is associated with exposure of people to spores of *Pleurotus* species (Hausen *et al.,* 1974, Horner *et al,* 1988, Olsen, 1987). Cox *et al.* (1988) have shown under laboratory conditions that the development of an extrinsic allergic alveolitis is provoked by the inhalation of spores.

A sporeless strain would not only benefit the health of the mushroom workers, it would also reduce the risks of viral infections on the mushroom farms, In *P. ostreatus* viral infections have been reported in association with deviant fruitbody morphology (minicauliflower and indented margin, Poppe, 1987) and lower yields (Rinker *et al.,* 1993, Go *et al,* 1992a and b). Van Zaayen (1976) has shown that basidiospores are the vehicles by which viruses in *Agaricus bisporus* crops are spread and that these spores can germinate even after decades when kept at 4 °C.

Spore production by *Pleurotus* strains is immense. Traditional and present strains produce

between 200 and 660 million spores per gramme tissue per 24 hrs. Spore production by "poorly sporulating" strains is ten times lower (Sonnenberg *et al.,* 1996) but still high enough to yield spore concentrations that are well above the threshold values for the induction of symptoms of extrinsic allergic alveolitis.

A sporeless strain of P. *ostreatus* has been described by Eger (1976). This strain was obtained by mating two single spore isolates derived from the same spore print obtained from a fruitbody of P. *ostreatus* isolate "F" (Block *et al.,* 1958). We describe the use of this sporeless strain for the development of a commercial sporeless strain.

2 MATERIALS AND METHODS

2.1 *Strains and manipulations*

Single spore isolates (SSI's) were obtained from the normal sporulating *P. ostreatus* strain Somycel 3015 by germinating basidiospores on MMP medium (1% maltextract, 0.5% mycological peptone, 10 mM MOPS [3-(N-morpholino)propanesulfonic acid] pH 7.0). Monokaryons (protoclones) from the non-sporulating *P. ostreatus* strain ATCC 58937 were obtained by protoplasting vegetative mycelium as described by Sonnenberg *et al.* (1988). The monokaryotic nature of the SSI's and the protoclones was identified by microscopic evaluation of the presence of clamps. Strains were maintained at 4°C on slant tubes of wheat extract agar (64 g of wheat boiled for 2 h in tap water, filtered to remove the grains, and sterilized together with 40 g of agar).

Two monokaryons were mated by placing small pieces of mycelium at 2-3 cm from each other on MMP medium and incubating them at 24°C. After one week mycelium was sampled from both colonies (most) distal from the junction zone and subcultured on MMP. The periphery of the outgrowing colonies was examined for the presence of clamp connections. Since the migration of mitochondria in matings is considerably slower than nuclear migration, these procedures generally lead to the isolation of two strain types identical in nuclear content but different in mitochondrial genotype (Fisher & Wolfrath, 1997). In our experiments we have used the matings that have inherited the mitochondrial genotype from the ATCC strain.

Mating types were determined using tester strains that were kindly provided by Prof. Pisabarro and Prof. Ramirez from Universidad Publica de Navarra, Pamplona, Spain.

2.2 *Fructification trails*

Fruitbodies were obtained from small scale cultures. In plastic screw cap pots (0 105 mm, height 120 mm) 175 g of wet wheat straw was sterilized twice for 2 h at 121 °C on 2 subsequent days. An agar dish (MMP) completely colonized with *Pleurotus* mycelium was sliced and mixed with the substrate. Pots were capped loosely and cultures were incubated in the dark for 8 days in growing rooms at 25°C and relative humidity of 95%. Fructification was induced by removing the caps from the pots, lowering the temperature to 20°C and illuminating the cultures daily for 12 h. Relative humidity was kept at 95%. Pots were watered daily to prevent the straw substrate from drying. Spore production of fruit bodies was determined by phase contrast microscopy using 400 x magnification.

2.3 *Molecular biological techniques*

DNA was extracted using a modification of the method of Kim *et al.* (1990). In short, 50-100 mg lyophilized mycelium was homogenized in a lysis buffer (100 mM Tris/HCl pH 8.0, 10 mM EDTA, 2% (w/v) SDS, 0.2 mg/ml proteinase K), followed by cetyltrimethyl-ammonium bromide (CTAB) extraction of carbohydrates in 1.4 M NaCl, leaving nucleic acids in the supernatant. After chloroform-isoamylalcohol extraction of protein, carbohydrates were precipitated with ammoniumacetate and RNA was digested with RNase A. After a second chloroform-isoamylalcohol extraction, DNA was precipitated with isopropanol and washed with 70% (v/v) ethanol

Standard DNA manipulations of cloned DNA were carried out essentially as described (Sambrook et al., 1989). *E. coli* DHSoc (GIBCO BRL Life Technology, Gaithersburg MD.) was used for plasmid transformation and propagation. Restriction enzymes and other enzymes used for

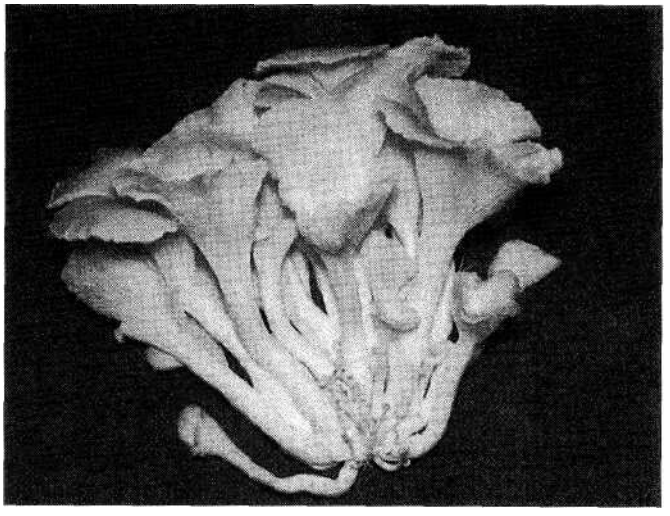

Figure 1. Morphology of fruitbodies *ofPleurotus ostreatus* strain 58937.

DNA manipulations were purchased from GIBCO BRL Life Technology (Gaithersburg MD.) and used according to the supplier's instructions. Probes were labeled with digoxigenin by using the Dig DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). Hybridization was carried out overnight at 65 °C in a standard hybridization buffer (5 x SSC, 0.1% lauroylsarcosine, 0.02% sodium dodecylsulfate and 1% Dig-blocking reagent). Detection of hybrids was carried out according to the conditions recommended in the Dig Chemiluminescent Detection Kit (Boehringer Mannheim).

3 RESULTS AND DISCUSSION

3.1 *The inheritance of the "sporeless" trait*

The characteristics of the sporeless strain ATCC 58937 described by Eger (1976) differ from those of commercial lines (Fig. 1). The fruit bodies are trumpet shaped with the stipe attached to the centre of the head. This is considered as a "poor" quality by mushroom producers and consumers hence the strain can not be used for commercial production.

For the introduction of the "sporeless" trait *(sp)* into a commercial line, we need to know the genetic determinants for this trait and whether the undesired characters are linked to the sporelessness. In her study on the genetic background of *sp* in strain ATCC 58937, Leal-Lara (1978) recovered its nuclear constituents designated nh42 and nhl 1 (nh standing for neohaplont). She mated both with monokaryotic single spore isolates of the stock "F" from which nh42 and nhl 1 were originally derived. From these experiments she concluded that the genetic determinant for *sp'* is located in the genome of nh42, that the nhl 1 is free of *sp'* and that, therefore, the trait is dominant. We have recovered both nuclear types from ATCC 58937 strain by protoplasting technique and the growth characteristics of the two types of colonies obtained match with those described by Leal-Lara (1978). The use of RFLP markers and a cross between the two types confirmed that we had isolated both nuclear types that were designated, as originally, nh42 and nhl 1, respectively.

To study the inheritance of *sp',* nh42 and nhl 1 were mated with compatible single spore isolates (SSI's) of the sporulating commercial line Somycel 3015 (Fig. 2). Previous test matings have shown that all factors of the mating types present in the ATCC strain and in the commer cial line are different thus permitting all possible combinations between the two strains. Both crosses (cross 1 and 2 in Fig. 2) developed fruit bodies with normal levels of spore production. This indicates that the genetic determinants for *sp'* are not dominant to corresponding alleles in

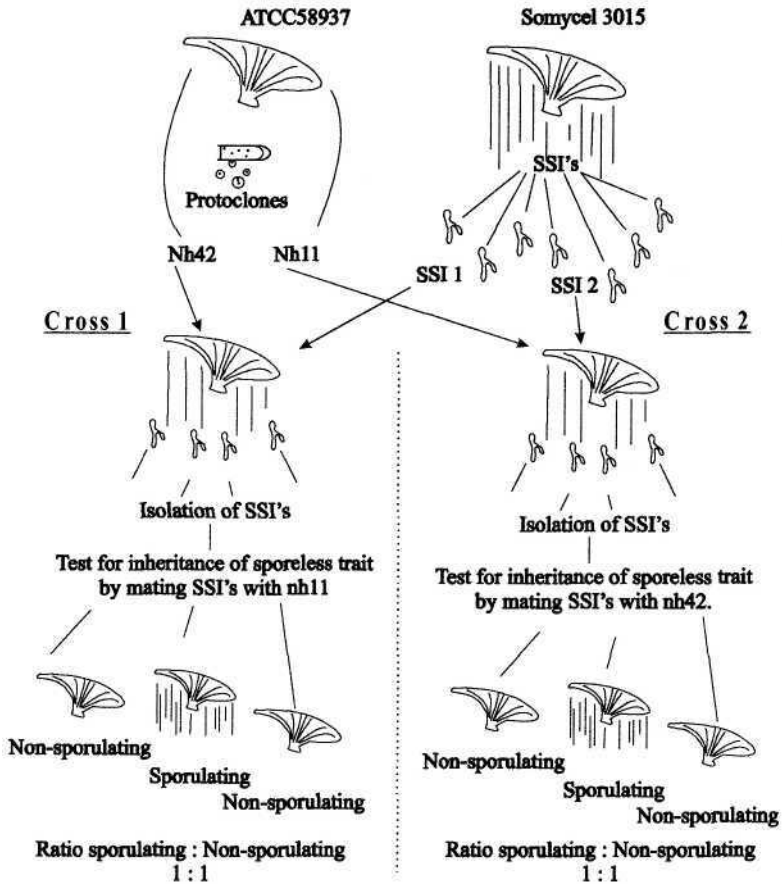

Figure 2. Schematic representation of the method to study inheritance of the sporeless trait in *Pleurotus ostreatus.*

the commercial line. According to Leal-Lara, nh42 exhibits its sporeless trait when crossed with nhll. If SSI's from cross 1 have inherited the necessary genetic determinant of nh42 for *sp"* , then a cross with nhl 1 should produce sporeless fruit bodies. To test this, 108 SSIs of cross 1 were mated with nhll. Fruit bodies obtained were either normally sporulating (51) or not sporulating (53). No fruit bodies were observed with a reduced spore production. This result shows a clear 1:1 segregation with respect to *sp'* and sp^+. The same test was performed on cross 2 (nhl 1 x SSI-2) by mating 120 SSIs with nh42. Fruiting tests showed again a segregation in normal sporulating (54) and not sporulating (67) fruit bodies closely to a 1:1 ratio. Again, no fruit bodies with a reduced spore production were observed. The segregation ratio's in both crosses suggest that in each nuclear type of the ATCC strain one factor is responsible for the *sp'* phenotype.

Based on these observations one would expect that matings between SSI's of both crosses that have inherited the appropriate factor for the *sp'* phenotype would produce sporeless fruit bodies. To test this hypothesis, seven sp" SSI's derived from cross 1 were mated with five *sp'* SSI's derived from cross 2. Fruiting tests revealed, however, that not all crosses produced-sporeless fruitbodies (Table !.)• The SSI's derived from cross 2 (nhl 1 x SSI 2), for example can be subdivided into three categories when mated with the *sp'* SSI's from cross 1. SSI's F, J, K and L produce sporeless fruit bodies in all matings, SSI's G and I produced normal sporulating

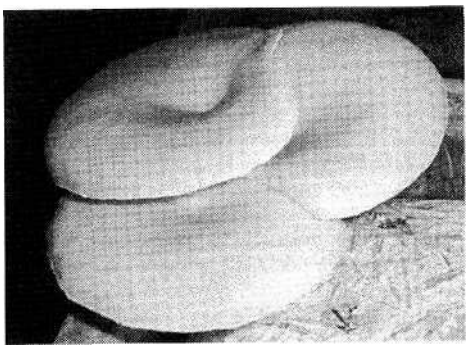

Figure 3. Morphology of a'sporeless strain derived from ATCC 58937 and Somycel 3015.

Table 1. Determination of sporelessness in matings between offspring of Cross 1 and Cross 2.

	SSI s derived from Cross 1				
SSI's derived from Cross 2	SSI A	SSI B	SSI C	SSI D	SSI E
SSIF	n.t.	-		-	
SSIG	+	+	+	+	+
SSIH	-		+	+	
SSI I	n.t.	+	+	+	+
SSIJ	n.t.	-		-	
SSIK	-	-		-	
SSIL	n.t.	-		-	

n.t.; not tested.

fruitbodies and SSI H produces sporulating and non sporulating fruitbodies, depending on the SSI's used from Cross 2. These results indicate that more than one factor in each nuclear type of the ATCC strain is involved in the *sp'* phenotype. Obviously, a factor in the offspring of both crosses is segregating only in a 1:1 ratio when they are crossed with a proper genetic background, i.e. the intact genome of nhl 1 or nh42. Both nhl 1 and nh42 are derived from stock "F". They might, therefore, be homoallelic for several loci, including some of those involved in the *sp'* phenotype. These loci are then masked resulting in a less complicated segregation. In crosses between SSI descendants of cross 1 and 2, these loci might be revealed again, resulting in a more complicated result. The limited variation shown in Table 1, however, indicated that not many independently segregating loci are involved.

The segregation analysis also indicates that the undesired fruit body morphology of the ATCC strain was not completely linked to the **sp** trait. Although most sporeless fruit bodies had a bad quality, some showed a normal morphology (Fig. 3). (The fruit bodies of the mating between SSI C and SSI L had the best quality but their yield was low and the time needed to produce fruit bodies was relatively long.) In order to trace genomic loci that are linked to the **sp** trait, genetic markers were generated by PCR. Primers were designed based on previously published sequences (Larraya *et* a/., 1999). These primers gave multiple bands when used in different combinations. By segregation analysis of these bands in the offspring of cross 1, a limited map was produced revealing at least 10 linkage groups (LOD= 3.0; JoinMap version 2.0; Stam & Van Ooyen, 1995). Although far from saturated, this map showed a clear linkage of genetic markers to the 1:1 segregation of the sp" / sp^{+} trait in cross 1. This indicates that at least for the 1:1 segregations of the **sp** trait in cross 1 and cross 2 tightly linked genetic markers can be found. An extension of matings between the **sp"** SSIs of both crosses and a more saturated map might reveal the additional loci that are linked to the **sp"** trait. Since repetitive sequences are very efficient in mapping loci (Sonnenberg, this volume), we are currently using strategies to isolate these sequences from the genome of P. *ostreatus*. Cross hybridization, for example,

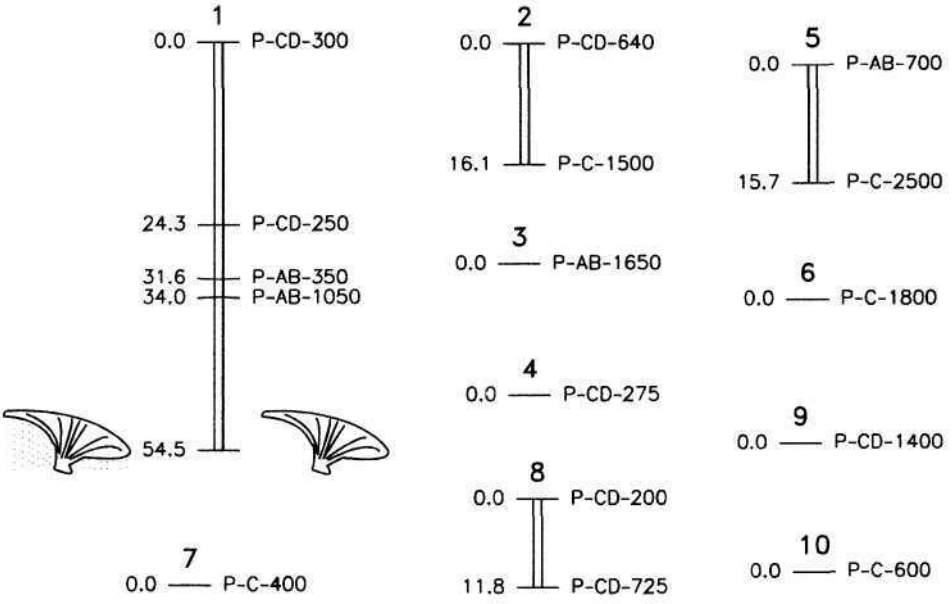

Figure 4. Preliminary genomic map of cross 1.

shows that human telomeric sequences are hybridizing to each *P. ostreatus* chromosome and give multiple bands on Southern blots. If the *P. ostreatus* telomere sequences are indeed identical to their human equivalent, a very useful marker is available for each chromosome end.

ACKNOWLEDGEMENTS

This research was funded by the Dutch Ministry for Agriculture (LNV). The authors would like to thank ms. in 't Zandt-Linders and mr. F. Hermans for skilled technical assistance.

REFERENCES

Bio Markets Research, Courvoisier,(1999) *mDer Champignon* 4: 192.
Block, S.S., Tsao, G. & Han, L., 1958, Production of mushrooms from sawdust. *J. Agric. and Food Chem. 6:* 923-927.
Cox, A., Folgering, H.T.M. & Van Griensven L.J.L.D. 1988. Extrinsic allergic alveolitis caused by spores of the oyster mushroom *Pleurotus ostreatus. Eur. Respir.J* 1:466-468.
Eger, G, Eden, G. & Wissig, E., 1976, *Pleurotus ostreatus,* breeding potential of a new cultivated mushroom. *Theoret. Appl. Genetics* 47: 155-163.
Fisher, M. & Wolfrath H., 1997, Mitochondrial DNA in Mon-Mon and Di-Mon pairings of Pleurotus ostreatus. *Botanica Acta* 110(2): 172-176.
Go, S.J., Cha D.Y. & Wessels J.G.H. 1992b, Symptoms of virus infected oyster

Figure 5. Southern hybridization of genomic DNA of *Pleurotus ostreatus* with DIG-labeled human telomeric sequences. From left to right: monokaryotic strains SSI 1 and nh42, dikaryotic strain Cross 1.

mushrooms, *Pleurotusflorida. Korean Mycol.* 20: 229-233
Go, S.J., Park, Y-N., Shin G.C. & Wessels, J.G.H. 1992a, Molecular analysis of double-stranded RNA in abnormal growing oyster mushrooms, *Pleurotusflorida* and *Pleurotus ostreatus* due to virus infection. *Korean Mycol.* 20: 234-239.
Hausen. B.M., Schulz, K.H. & Noster, U. 1974, Allergic disease caused by the spores of an edible fungus *Pleurotusflorida. Mushroom Sci.* 9: 219-225.
Homer, W.E., Ibanez, M.D., Liengswangwong, V., Salvaggio, J.E. & Lehrer,S.B. 1988, Characterization of allergens from spores of the Oyster mushroom *Pleurotus ostreatus. J. Allergy Clin. Immunol.* 82: 978-986.
Kim, W.K., Mauthe, W., Hausner, G. & Klassen, G.R., 1990, Isolation of high molecular weight DNA and double stranded RNAs from fungi. *Can. J. Bot.* 68: 1898-1902.
Larraya, L.M., Perez, G., Penas, M.M., Baars, J.J.P., Mikosch, T.M., Pisabarro, A.G. & Ramirez, L. 1999, Molecualr karyotype of the white rot fungus *Pleurotus ostreatus. Appl. Environm. Microbiol.* 65(8): 3413-3417.
Leal-Lara, H. 1978, Is sporelessness in *Pleurotus ostreatus* an infectious agent? *Mushroom Sci.* 10: 145-154.
Olsen, J.A. 1987, *Pleurotus* spores as allergens. *Mushroom J.* 172: 115-117.
Poppe, J., Samyn, G., Welvaert, W. & Meulewaeter, F., 1987, Mycoviroses and their control in Belgian mushroom farms, especially *onAgaricus. Med. Fac. Landbouww. Rijksuniv. Gent,* 52(3a): 1005-1013.
Rinker, D.L., Stobbs, L.W. & Aim, G., 1993, Effect of virus disease on oyster mushroom production. *Micol. Neotrop. Apl. 6:* 73-79.
Sambrook, J.. Fritsch, E.F & Maniatis. T., 1989. *Molecular cloning: a laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Sonnenberg, A.S.M., Wessels, J.G. & Van Griensven, L.J.L.D. 1988 An efficient protoplasting/regeneration system for *Agaricus bisporus* and *Agaricus bitorquis. Curr. Microbiol.* 17: 285-291.
Sonnenberg, A.S.M., van Loon, P.C.C. & Van Griensven L.J.L.D., 1996, The number of spores spread by *Pleurotus* spp. in the air. *De Champignoncultuur* 40(7): 269-272.
Stam, P. 1993, Construction of integrated genetic linkage maps by means of a new computer package: JoinMap. *Plant Journal* 3: 739-744.
Stam, P. & Van Ooijen, J.W. 1995, JoinMap™ version 2.0: Software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen.
Van Ooijen, J.W. 1994, DrawMap: A computer program for drawing genetic linkage maps,/. *Hered* 85: 66.
Van Zaayen , A., 1976, Diseases and pests of cultivated mushrooms: Virus disease. *Mushroom Growers J.,* 102: 1-3.