STRAIN IMPROVEMENT OF EDIBLE FUNGI WITH *PLEUROTUS ERYNGII* NEOHAPLONTS

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ABSTRACT

Pleurotus eryngii is an edible fungus with increasing interest for the local market as a gourmet product, due to its nice sensory characteristics and its thick and fleshy stipe. *Lentinula edodes*, on the other hand, is a fungus with interesting therapeutic properties whilst *Pleurotus* is widely cultivated in Mexico. A breeding program was undertaken in this study to develop improved strains combining *L. edodes* and *P. eryngii* characteristics. Eleven hybrids, obtained by pairing *P. eryngii* and *L. edodes* neohaplonts were grown on substrates suitable either for *P. eryngii* or *L. edodes*. Six hybrids preserved *Lentinula* phenotype and 3 of them, showed high biological efficiencies (119-153%). Additionally, 28 hybrids were obtained from Di-Mon matings by pairing different *Pleurotus* spp. dikaryons with *P. eryngii* neohaplonts, 19 hybrids were selected for fruiting on *P. eryngii* substrate; 16 hybrids showed biological efficiencies higher than 100%. Hybrids showed a wide variety of morphologies, *i.e.* different pile sizes, colors and shapes were observed though most of them with large, thick and fleshy stipe, resulting hence in suitable strains for a commercial exploitation.

Keywords: *Lentinula*, *Pleurotus*, inter-genera hybrids, neohaplonts, genetic improvement.

INTRODUCTION

Production of edible mushrooms has gained increased interest throughout the world, a multimillion dollar business has been developed producing a high quality food, rich in proteins, fiber, vitamins and minerals. World production of oyster mushrooms (*Pleurotus* spp.) is placed second following bottom mushroom (*Agaricus*). *Pleurotus eryngii* is a specialty mushroom showing a fleshy thick stipe, it is tasty with nice flavor and easy to combine with various types of foods. It is not produced commercially in Mexico nowadays but it is available as an imported "gourmet" mushroom with prices far superior to the bottom mushroom. Therefore, procedures for cultivation of *Pleurotus eryngii* and availability of improved strains are important factors for introducing this fungus into the local market.

Development of hybrid strains of *P. eryngii* by combining characters with commercial *Pleurotus ostreatus* strains and with strains of different genera, *i.e. Lentinula edodes* could yield strains retaining the tasty flavor, nice fleshy, thick stipe and long shelf life of *P. eryngii*. Hopefully, new interesting characteristics could arise, *i.e.* larger caps, new colors, flavors and taste, herewith strains showing a wider spectrum of characteristics would then become available for cultivation.

MATERIALS AND METHODS

Strains. Following strains were used for production of hybrids by mating neohaplonts and dikaryotic strains: A commercial *P. eryngii* dikaryon [1], 11 neohaplonts (designated from PeC9 to PeC45) recovered by dedikaryotization from the commercial *P. eryngii* dikaryon [2], 4 *L. edodes* dikaryons (L9, L10, L18, L21) [3], a dikaryotic *Pleurotus djamor* strain [4], 8 *Pleurotus* spp. dikaryons (Asp14, CP50, CP253, HK3539, IE200, IE201, P401 and *Pleurotus* sp. PB) and 6 neohaplonts (designated from L10-1S to L21-2S) recovered by dedikaryorization of *L. edodes* strains [2]. All strains are stored in the fungal collection of the Department of Food Science and Biotechnology at the Faculty of Chemistry (University of México). The strains were propagated in malt extract agar (MEA) (1.5% malt extract and 2% agar); cultures on MEA plates (Petri dishes) were stored at 2 to 4°C [3].

Hybrids from Matings of *P. eryngii* Neohaplonts with *L. edodes* Neohaplonts. Agar cubes (2 mm) full with growing mycelia were cut from the edge of growing cultures of selected *P. eryngii* and *L. edodes* neohaplonts. They were placed side by side on MEA plates and incubated at 24ºC. Developing colonies were inspected under the microscope during the following 7 days and those showing clamp connections were reseeded on MEA plates for further evaluation.

Hybrids from Di-mon Matings of *P. eryngii* Neohaplonts with *Pleurotus* spp. Dikaryons. Four agar cubes (2 mm) full with growing mycelia were cut from the edge of a growing culture of a selected *P. eryngii* neohaplont and symmetrically distributed on the surface of a MEA plate and incubated at 24ºC until development of 1 cm (∅) colonies. At this stage, a 2 mm agar cube was cut from the edge of a growing culture of a selected dikaryon and placed on the periphery of the growing neohaplont culture. Plates were again incubated at 24ºC and inspected under the microscope every day until appearance of clamp connections on the side opposite to the point of inoculation of the dikaryotic culture. The newly emerging dikaryotic strain (hybrid) was recovered on MEA plates. Mycelium growth of all resulting hybrids as well as of their respective parental dikaryons and neohaplonts was evaluated by placing 8 mm (∅) inocula cut from the edge of a growing culture and placed on MEA, with 3 replicates per strain. Colony diameters were measured after 3, 6 and 9 days incubation and when significant differences were established by variance analysis, strains were classified according to Duncan test.

Fruiting of Hybrid Strains. Two types of substrates were used for fruiting of hybrids, one is recommended for fruiting of *P. eryngii* and the second one for *L. edodes* (Table 1).

Table 1: Substrates for fruiting of *P. eryngii* and *L. edodes* hybrids and parental strains

Components	Substrates (% fresh weight)	
	L. edodes	*P. eryngii*
Sawdust	50.0	20.0
Cottonseed waste	36.0	60.0
Millet	6.0	-----
Sorghum (milled)	6.0	-----
Wheat bran	-----	16.0
Ammonium sulfate	0.5	-----
Citric acid	0.5	-----
Benlate	1.0	-----
Calcium carbonate	-----	3.0
Calcium sulfate	-----	1.0

Both substrates were used for fruiting hybrids of *P. eryngii* with *L. edodes* since each genus requires different substrates for good mycelium growth and fruiting. Hybrids of *Pleurotus eryngii* neohaplonts with *Pleurotus* spp. dikaryons were fruited only on *P. eryngii* substrate. Sawdust, cottonseed waste and millet were soaked in water for 24 h; after draining excess water, ingredients were thoroughly mixed according to formulation and water content of substrate was adjusted to 60%. *Lentinula* substrate (1.5 Kg) was filled into 25 x 35 cm polypropylene bags and *Pleurotus* substrate (1 Kg) was filled into 17 x 45 cm polypropylene bags. Sterilization and inoculation of substrates as well as preparation of spawn was according to Ramírez *et al.* [3]. After incubation for 9 weeks, *Lentinula* substrates were transferred into the fruiting room and polypropylene bags were completely detached in order to expose the whole surface of the substrate to the environment. After incubation for 9 weeks, *P. eryngii* substrates were transferred to the fruiting room, polypropylene bags were folded down in order to leave only the upper surface of the substrate exposed to the environment. Conditions in the fruiting room throughout the experiment were 70 to 80% air humidity, 20 to 23°C air temperature and 700 to 900 ppm CO_2. Fruit bodies developing on the substrates were harvested before pileus edge turned up completely. Harvesting period was 12 weeks for *P. eryngii* substrates and 8 weeks for *L. edodes* substrates. Weight of fresh fruit bodies was registered for each substrate bag and biological efficiency was determined as BE = g fresh fruit bodies/100 g dry substrate.

Statistical Analysis. Variance analysis of biological efficiencies of hybrids and parental strains were performed to evaluate significant differences and Duncan multiple range test was used to identify the highest producing strains (SPSS ver. 17 for Windows was used for both tests).

RESULTS

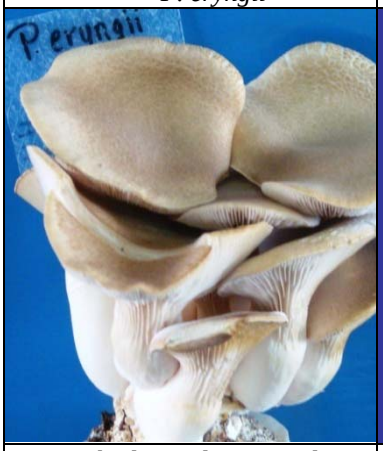
Hybrids from Matings of *P. eryngii* Neohaplonts with *L. edodes* Neohaplonts. Eleven hybrids obtained by mating *L. edodes* and *P. eryngii* neohaplonts were fruited on both types of substrates shown in Table 1 and biological efficiencies of the six hybrids presenting *L. edodes* morphology are shown in Table 2.

Table 2: Biological efficiencies of hybrids with *L. edodes* morphology

Strains	Biological efficiency (g fresh fruit bodies / 100 g dry substrate)							
	L. edodes substrate				*P. eryngii* substrate			
	$\bar{x}$	±	σ	Duncan test	$\bar{x}$	±	σ	Duncan test
L10	33.82	±	9.22	A				
PeC40 / L18-2S	50.63	±	16.25	B	44.17	±	8.04	a
L21	62.51	±	4.21	BC				
L18	67.67	±	7.53	C				
L9	84.13	±	7.84	D				
PeC40 / L18-1S	91.32	±	12.88	D	55.25	±	7.06	b
PeC40 / L10-1S	119.44	±	15.27	E	53.68	±	3.18	ab
PeC40/ L10-4S2	123.64	±	6.98	E	61.43	±	8.45	bc
PeC40 / L10-4S	123.64	±	6.98	E	53.68	±	3.18	ab
PeC40/ L21-2S	153.38	±	9.32	F	68.60	±	11.88	c

Different letters indicate significant differences in the same substrate

All the hybrids produced with *P. eryngii* neohaplont PEC40 produced fruit bodies with *Lentinula* morphology whereas hybrids derived from the other 5 neohaplonts belonging to mating type II paired with *L. edodes* neohaplont L21-3S resulted in fruit bodies with *P. eryngii* morphology. Biological efficiencies of hybrids producing fruit bodies with *Lentinula* morphology after 12 weeks of cropping period are shown on Table 2. Strain PeC40/ L21-2S produced the highest biological efficiency, 153%, though other 3 hybrids yielded high BE, 119-124%, in all cases better BE values than control *L. edodes* strains, whose BE ranged from 34 to 84%. Fig. 1 shows the fruit bodies produced by control *P. eryngii* dikaryon and two *L. edodes* strains as well as 4 different hybrids fruiting with *Lentinula* morphology.

Parental Strains		
P. eryngii	L10	L21
		
Fruit bodies: large. Pileus: brown grayish with regular edges. Stipe: white colored, long and thick	Fruit bodies: large. Pileus: dark brown, thin, slightly convex with scales on edges. Stipe: long and thin	Fruit bodies: medium. Pileus: dark brown, thick, without scales. Stipe: thin and very short

Hybrid Strains			
PeC40/L21-2S	PeC40/ L10-4S	PeC40/L10-4S2	PeC40/ L10-1S
			
Fruit bodies: medium, large and very large. Pileus: dark brown, thick, with few scales. Stipe: beige, long and medium sized, with scales	Fruit bodies: medium, large and very large. Pileus: dark brown, thick, with abundant scales. Stipe: beige with brown spots, long and thick, with scales	Fruit bodies: small, medium and large. Pileus: light brown, thick, with abundant scales. Stipe: thick medium sized, whitish with scales	Fruit bodies: small, medium, large and very large. Pileus: light brown, thick, with few scales. Stipe: light brown, long and thin, with few scales

Figure 1: Parental strains and highest producing hybrids with *L. edodes* morphologies

Biological efficiencies of the 5 hybrids producing fruit bodies with *P. eryngii* morphology are shown on Table 3. Hybrids cultivated on *P. eryngii* substrate had lower

biological efficiencies (13 to 67%) than the parental *P. eryngii* dikaryon (151%). Noticeably, 2 hybrids, PeC20/L21-3S and PeC29/L21-3S, produced higher yields on *P. eryngii* substrate and other 2 hybrids, PeC12/L21-3S and PeC45/L21-3S, conversely produced higher BE on *L. edodes* substrate, the last hybrid exceeding 100% BE. This suggests that nutrient requirements were differentially inherited by each group of strains, the first one similar to *P. eryngii* and the second one to *L. edodes.* The Figure 2 shows the fruit bodies produced by 3 different hybrids fruiting with *P. eryngii* morphology.

Table 3: Biological efficiencies of hybrids with *P. eryngii* morphology

Strains	Biological efficiency (g fresh fruit bodies / 100 g dry substrate)					
	P. eryngii substrate			*L. edodes* substrate		
	$\bar{x} \pm \sigma$		Duncan test	$\bar{x} \pm \sigma$		Duncan test
PeC20/L21-3S	43.61 ±	7.02	B	14.12 ±	1.94	a
PeC29/L21-3S	61.25 ±	8.63	CD	21.42 ±	5.94	ab
PeC12/L21-3S	13.16 ±	1.91	A	33.47 ±	3.61	b
PeC27/L21-3S	67.44 ±	10.96	D	56.38 ±	8.27	c
PeC45/L21-3S	56.13 ±	6.00	C	111.79 ±	17.55	d
P. eryngii	151.42 ±	11.49	E			

Different letters indicate significant differences in the same substrate.

PeC45/ L21-3S	PeC27/L21-3S	PeC12/L21-3S
		
Fruit bodies: large and very large. Pileus: light brown, thick, with undulated edges. Stipe: white, long and thick	Fruit bodies: small and medium. Pileus: white, with undulated edges. Stipe: white, medium, thin to thick	Fruit bodies: small, medium and large. Pileus: light brown, with regular edges. Stipe: white, medium, thin to thick

Figure 2: Hybrids between *P. eryngii* and *L. edodes* with *P. eryngii* morphologies on *L. edodes* substrate

Hybrids from Di-mon Matings of *P. eryngii* Neohaplonts with *Pleurotus* spp. Dikaryons. Mycelium growth rate of the 28 hybrids obtained by this procedure was measured to identify those showing similar growth to the parental strains, and those showing either faster or slower growth than the parental strains. For fruiting experiments, the following 19 hybrids were selected from these 3 groups:

hybrids with faster mycelium growth than parental strains:

PeC9/CP50 PeC20 / IE200 PeC38 / P401

PeC12 / P401 PeC35 / *P. djamor* PeC38 / ASP14
PeC12 / *P. djamor* PeC35 / HK3539 PeC44 / *P. djamor*
PeC20 / *P. djamor* PeC38 / *P. djamor* PeC44 / P401
hybrids with similar mycelium growth than parental strains
PeC11 /CP50 PeC35 /P401
PeC35/ *Pleurotus* sp. PB PeC44 / CP253
hybrids with slower mycelium growth than parental strains
PeC12 / CP253 PeC20 / ASP14 PeC20 / IE201

Biological efficiencies of the 19 hybrids produced by di-mon matings are shown in Table 4. Strain PeC11/CP50 showed up as the highest producing hybrid with an amazing 323% biological efficiency, however, 3 other hybrids produced also very high yields, *i.e.* PeC9/CP50 (271%), PeC12/P401 (207%) and PeC35/*Pleurotus* sp. PB (177%), in all cases, BE were significantly higher than BE of the parental *P. eryngii* dikaryon (151%). Remarkably, 12 more hybrids produced biological efficiencies higher than 100%; among them, 3 hybrids derived from *P. djamor* dikaryon with neohaplonts PeC12, PeC20 and PeC35 had significant higher biological efficiencies than the parental *P. djamor* dikaryon.

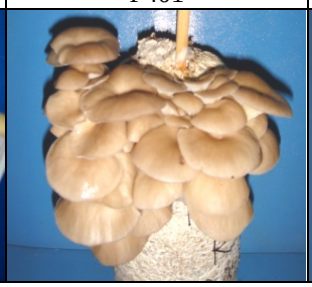
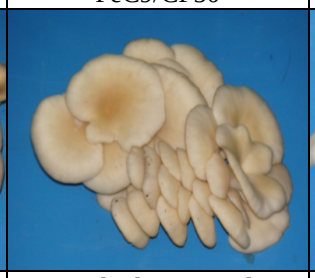
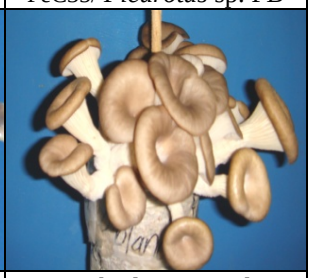
Parental Strains			
P. eryngii	CP50	P401	*Pleurotus* sp. PB
			
Hybrid Strains			
PeC11/ CP50	PeC9/CP50	PeC12/P401	PeC35/ *Pleurotus* sp. PB
			
Fruit bodies: small, medium and large. Pileus: light brown, with regular edges. Stipe: white, medium, thin, trumpet shaped	Fruit bodies: medium and large. Pileus: beige with regular edges. Stipe: white, medium, thin, trumpet shaped	Fruit bodies: large. Pileus: light brown with undulated edges. Stipe: white, medium, long and thin	Fruit bodies: medium and large. Pileus: light brown with regular edges. Stipe: white, medium, long and thin to thick, trumpet shaped

Figure 3: Parental strains and highest producing hybrids from di-mon matings with *Pleurotus* morphologies

Results from Table 4 also allow the identification of PeC35 as a neohaplont generating highly producing dikaryons when mated with different dikaryotic partners. These results also establish that most hybrids are higher yielding than the respective parental dikaryotic strains

employed for di-mon matings, *i.e.* IE201, CP253, IE200, CP50 and P401. Morphologies of fruit bodies produced by these hybrids are very interesting since wide varying characteristics were found among them, *i.e.* medium sized and long stipes of variable thickness and pileus of diverse color, shape and texture. Fruit bodies from 3 different hybrids are shown in Figure 3.

Table 4: Biological efficiencies of hybrids from di-mon matings of *P. eryngii* neohaplonts with *Pleurotus* spp. dikaryons

Strain	Biological efficiency (g fresh fruit bodies / 100 g dry substrate) $\bar{x}$ ± σ			Duncan test
IE201	26.58	±	3.98	a
CP253	35.05	±	2.98	a
IE200	40.67	±	8.73	a
CP50	43.47	±	9.74	a
PeC20/IE201	75.91	±	11.82	b
PeC44/*P. djamor*	78.27	±	3.84	b
Pleurotus sp. PB	78.66	±	17.56	b
P401	85.70	±	8.86	bc
P. djamor	92.11	±	8.75	bcd
PeC38/ *P. djamor*	99.54	±	2.15	bcde
PeC12/CP253	104.90	±	14.50	cdef
PeC44/P401	107.64	±	3.64	cdef
PeC35/P401	108.03	±	3.95	cdef
PeC20/IE200	108.44	±	12.80	cdef
PeC44/CP253	111.38	±	11.22	cdef
PeC20/ASP14	113.27	±	14.01	def
PeC38/ASP14	114.54	±	2.49	def
PeC38/P401	114.85	±	5.80	def
PeC12/*P. djamor*	119.83	±	21.84	ef
PeC20/*P. djamor*	122.87	±	10.52	ef
PeC35/*P. djamor*	127.86	±	9.81	f
P. eryngii	151.42	±	11.49	g
PeC35/HK3539	156.40	±	6.67	gh
PeC35/*Pleurotus* sp. PB	177.20	±	42.92	h
PeC12/P401	207.02	±	34.98	i
PeC9/CP50	271.39	±	43.84	j
PeC11/CP50	323.02	±	29.46	k

Different letters indicate significant differences.

DISCUSSION

Development of improved strains for commercial cultivation of edible fungi has been undertaken seriously by the bottom mushroom industry; *Agaricus* spawn producers perform this task continuously investing important resources. However, other cultivated fungi have not received this attention and thus offering strains with new characteristics for the industry has not occurred. This task is not such complicated as for *Agaricus* at least with fungi like *Pleurotus* spp. and *L.edodes*, both with heterothallic tetrapolar sexuality. For such fungi, dedikaryorization has been proposed as an effective procedure for recovery of the monokaryotic components of selected dikaryons making feasible a directed improvement of fungal strains by combination of monokaryotic cultures containing desirable characteristics [5].

In previous works, hybrids from 2 different genera, *i.e. P. ostreatus* and *L. edodes* have been produced by pairing neohaplonts from these 2 fungi [3, 6]. Hybrids yielding higher

biological efficiencies than the parental strains were then reported in accordance to the results presented in the present study where *P. eryngii* strains were used for the first time. Moreover, hybrids were now obtained yielding fruit bodies either with *Lentinula* or *P. eryngii* morphology contrasting to the previous report by Ramírez *et al* [3] where only hybrids producing fruit bodies with *P. ostreatus* morphology were recovered. Thus, in this study, hybrids with *Lentinula* morphology were obtained for the first time; 5 of these 6 hybrids showed higher BE than the parental *L. edodes* strains and 3 of them produced BE in the range of 119-153% (Table 2), becoming suitable for commercial cultivations. Furthermore, such strains exhibited interesting morphologies (Fig. 1), *i.e.* firmer pileus with fleshy texture as well as larger and thicker stipes. These newly acquired characters were possibly inherited from *P eryngii* whereas the higher yields on *Lentinula* substrate were probably received from parental *L. edodes* strains. On the other hand, although all hybrids with *P. eryngii* morphology showed lower yields compared to their parental *P. eryngii* dikaryon, hybrid PeC45/L21-3S remarkably produced higher BE on *Lentinula* substrate (111%) than on *P. eryngii* substrate, showing up also as an attractive strain for commercial cultivation and this fact again suggests that differential inheritance of nutrient requirements is present in these hybrids. Productivity of hybrids was also markedly increased in those obtained by di-mon matings of *P. eryngii* neohaplonts with *Pleurotus* spp. dikaryons (Table 4). Biological efficiency of 3 hybrids ranged from 323 to 200% while *P. eryngii* yielded 151% BE and 13 more hybrids produced BE higher than 100%.

Strikingly, a large diversity of morphologies are produced when different neohaplonts, derived from the same parental strain are paired with different partners as shown on Fig. 1, 2, 3, in some cases it was observed even in pairing with neohaplonts arising from the same parental strain, *i.e. P. eryngii* neohaplont PEC40 (Table 2). Furthermore, strains consistently showed variations in pileus (size, colors and shapes), and stipe (length, thickness and texture). Even though, this may be a desirable achievement in this study, such phenotypic variations should not be expected since all neohaplonts are supposedly carrying the same genetic information. However, this may be true in regards to the mating-type genes but obviously it is not true regarding the genetic information related to fruit body formation. This observation suggests that during dedikaryorization, some factors involved in the process of fruit body formation are separated into the neohaplonts in an irregular pattern, dissimilar as mating types do, thus arising a large variation in phenotypes. Such an observation is supported by the presence among neohaplonts of strains with abnormal mating types, *i.e.* unable to mate at all or with irregular mating pattern, varying mycelium morphologies and sometimes, the incapability of recovered compatible neohaplonts to reconstruct the original parental strain, when mated.

Finally, hybrids obtained in this study by mating neohaplonts consistently resulted in strains producing higher yields, regularly yielding more than 100% BE, in comparison with the traditional approach of strain improvement by matings of meiotic progenies. Following such an approach, Valencia del Toro and Leal Lara [7] obtained improved *Pleurotus* strains yielding 40-73% BE, while Galván [8] obtained improved *Lentinula* strains yielding 13-73% BE. Meiotic products show a large variation in genotype, making more difficult to find out combinations of monospore cultures giving higher yields. Such a variation was observed by Gharehaghaji et al. [9]; they germinated basidiospores from 5 *P. ostreatus* dikaryons recovering 17 monokaryons, which were paired to produce 27 hybrids. However, only primary mating characters were observed, *i.e.* morphological interaction in contact zones of mycelium, increased growth rate, change of colony morphology and presence of clamp connections to identify dikaryons, but no fruiting experiments were performed to evaluate productivity of the generated hybrids, a crucial step for assessing success of any breeding program since optimization of industrial mushroom production depends on improving the culture process and breeding new strains with higher yields and productivities.

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