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Monokaryotization by protoplasting heterothallic species of edible mushrooms

Jiong Zhao and Shu-Ting Chang*

After regeneration of protoplasts of seven heterothallic species of edible mushrooms, two types of monokaryons were recovered, being identified by the absence of clamp connections. In regenerated colonies, monokaryons grew more slowly than dikaryons and so could be distinguished from them. In all species, the yield of monokaryons in the regenerated colonies was over 45%. The mating types and auxotrophic markers were the same in the protoplasted monokaryons and parental monokaryons. In comparison with other monokaryotization methods, protoplasting is rapid, simple and effective; its applications in breeding of edible mushrooms are discussed.

Key words: Breeding, edible mushroom, monokaryotization, protoplast.

Monokaryotization allows the recovery of the two monokaryotic components from a dikaryon. Up to now there are three available methods: mechanical treatment; chemical treatment; and mycelium starvation. With mechanical treatment (Leal-Lara & Eger-Hummel 1982), only a few percent of cells survive and only one nuclear type is recovered. With chemical treatment (Miles & Raper 1956; Takemaru 1964a, b), there is a degree of toxicity and none of the chemicals has a general monokaryotizing effect. Leal-Lara & Eger-Hummel (1982) used medium containing glycine with low phosphate and magnesium ions to obtain monokaryotic strains. This technique was successful in all 14 strains of the five species tested. Although it is generally effective, it requires strict culture conditions and many steps. None of these methods appears therefore to be very satisfactory.

Protoplasting has been a part of mycological research for at least 30 years. The cell wall is a barrier which restricts some physiological and biochemical research, and cell-wall-free protoplasts may provide a better experimental system. Protoplasting was used for the isolation of homokaryons in homothallic *Agaricus bisporus* (Castle *et al.* 1987; Sonnenberg *et al.* 1988). In heterothallic species, monokaryotization by protoplasting has only been reported in a few strains and there are no reports on the distribution of different monokaryons in regenerated colonies (Wessels *et al.* 1976; Yoo *et al.* 1987). Therefore, in this report, seven heterokaryotic species from four genera were chosen for monokaryotization by protoplasting technique. Two types of monokaryons were obtained in each species, although the ratios of the two types differed. In heterothallic species of edible mushrooms, monokaryotization is only used for breeding sporeless strains (Leal-Lara & Eger-Hummel 1982). In this report, other potential applications are suggested and discussed and these aspects may be very useful for the breeding of edible mushrooms.

Materials and Methods

Strains and Media

The strains used in this study, which were all supplied by Prof. S. T. Chang of The Chinese University of Hong Kong, are listed in Table 1. The media used were: MYG [10.0 g malt extract, 4.0 g glucose, 4.0 g yeast extract, 14.0 g bacto-agar (Difco) and distilled water to 1l]; MYGM (MYG medium with 0.6 M mannitol); MYGMS [MYGM medium with 5% (w/v) soluble starch (BDH)]; CM [2.0 g peptone (Oxoid), 2.0 g yeast extract (Biolife), 20.0 g glucose, 0.005 g thiamine-HCl, 1.0 g K_2HPO_4, 0.46 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 14.0 g Bacto-agar and distilled water to 1l]; CMM (CM medium with 0.6 M mannitol); CMMS [CMM medium with 5% (w/v) soluble starch (BDH)]; PDY [24.0 g potato dextrose extract (Difco), 2.0 g yeast extract and distilled water to 1l]; and MM (2.0 g asparagine, 20.0 g glucose, 0.12 g thiamine-HCl, 1.0 g K_2HPO_4, 0.46 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$. 14.0 g Bacto-agar and distilled water to 1l].

Protoplast Production and Regeneration

Strains were cultivated on MYG solid medium at different temperatures for 1 to 2 weeks according to the strain requirement

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Table 1. Strains used in the protoplasting experiments.

Strain	Characters
Lentinus edodes	
3421 + 1315*	Dikaryon
54	Dikaryon, wild strain
3421	Monokaryon
Pleurotus sajor-caju	
21 + 36	Dikaryon
21	Monokaryon, ade⁻†
36	Monokaryon
P. florida	
67 + 11	Dikaryon
67	Monokaryon, ade⁻
11	Monokaryon
Coprinus cinereus	
5104 + 5026	Dikaryon
5104	Monokaryon, his⁻
5026	Monokaryon, ade⁻
Lentinus tigrinus	
1	Dikaryon, wild strain
Flammulina velutipes	
1	Dikaryon, wild strain

* Two monokaryons (e.g. 3421 and 1315) cross to become a dikaryon (3421 + 1315).
† Auxotrophic marker.

(Table 2). The mycelia were then transferred into PDY liquid medium and cultured for 2 to 6 days (Table 2). The cultures were harvested by filtration through a nickel sieve and washed twice with sterilized 0.6 M mannitol solution. The harvested mycelia were dried on absorbent paper and then suspended in filter-sterilized Lywallzyme solution (20 mg/ml in 0.6 M mannitol solution; Guangdong Institute of Microbiology, China) (Chang *et al.* 1985). Digestion of cell walls was allowed to proceed for 2 to 4 h (30°C, 100 rev/min) (Table 2). Hyphal fragments were removed by filtration through a column of cotton wool packed up to the 0.5 ml mark of a 5-ml syringe. Protoplasts were collected from the effluent by centrifugation (1400 × *g*, 10 min). Pellets were washed twice with 0.6 M mannitol, and then suspended in the same osmotic stabilizer. Protoplast yield was determined with a haemacytometer. For regeneration, 0.1 ml of protoplast suspension (about 10^4/ml) was plated on MYG medium containing 0.6 M mannitol as an osmotic stabilizer. The plates were incubated at different temperatures for 1 to 2 weeks (Table 2).

Identification of Mating Types in Protoplasted Monokaryons
Monokaryons were identified by the absence of clamp connections, and the mating types of protoplasted monokaryons were determined by a mating test. This test was conducted by crossing a protoplasted monokaryon with either of the parental monokaryons. In *Lentinus tigrinus* 1 and *Flammulina velutipes* 1, one protoplasted monokaryon was selected as the tester strain because of the lack of parental monokaryons. In the mating test, two different inoculated blocks were placed about 5 mm apart. After 6 to 12 days, hyphae from the contact zone were microscopically checked for the appearance of clamp connections.

Results

Protoplast Release and Regeneration
Using the procedures described, the yields of protoplasts were satisfactory (Table 3). The highest yield was in *Coprinus cinereus* (4.4 × 10^6 protoplasts/mg lywallzyme). In some difficult lysis strains, e.g. *Lentinus edodes* and *F. velutipes*, the yields were also good. The highest regeneration rate (9.6%), was in *P. florida* and the lowest (0.96%) in *L. edodes* (3421 + 1315).

Formation of Protoplasted Monokaryons
Protoplasts of *L. edodes* 54 were regenerated on MYGM medium. After culturing for seven days, 76 colonies appeared and 73 were dikaryons. In the following days, however, new colonies continued to appear and all were monokaryons (Figure 1). The same phenomenon was also observed in other strains. The first colonies appearing were larger and most of them were dikaryons. The colonies which appeared late were smaller and most of them were monokaryons. For further observation, colonies were transferred onto MYG medium. The morphology and size of the mono- and di-karyon colonies were compared; average diameters of protoplasted dikaryons and monokaryons were significantly different ($\alpha = 0.05$) (Table 4).

Table 2. Conditions for protoplast production and regeneration.

Strain	Cultivation		Digestion time (h)	Regeneration	
	Time (days)	Temperature (°C)		Time (days)	Temperature (°C)
P. florida	4	30	2	3	30
L. tigrinus	1	35	4	2	35
C. cinereus	3	30	2	4	30
P. sajor-caju	4	30	2	4	30
F. velutipes	3	25	4	5	25
L. edodes	6	25	4	7	25

Table 3. Monokaryotization by protoplasting heterothallic species of edible mushrooms.

Strain	Protoplast yield/mg enzyme	Regeneration rate* (%)	Monokaryon ratio† (%)	Monokaryon mating type A_xB_y:A_yB_x†	χ^2‡
P. florida (67 + 11)	1.0×10^6	9.6	46.0 (244:532)	1.13:1 (39:44)	0.30
L. tigrinus (1)	7.5×10^5	1.1	70.1 (162:231)	2.17:1 (13:6)	2.58
P. sajor-caju (21 + 36)	3.1×10^6	2.4	66.4 (194:292)	2.75:1 (55:20)	16.3
F. velutipes (1)	2.8×10^5	0.91	59.2 (116:195)	5.0:1 (35:7)	18.7
L. edodes (3421 + 1315)	4.0×10^5	0.62	60.3 (234:388)	1.93:1 (29:15)	4.45
L. edodes (54)	8.0×10^5	1.6	60.2 (110:183)	5.92:1 (71:12)	41.9
C. cinereus (5104 + 5026)	4.0×10^6	8.5	47.9 (192:400)	12.8:1 (64:5)	50.4
Control: *C.cinereus* (5104 + 5026)§	1.3×10^6	3.43	60.4 (128:212)	3.0:1 (48:16)	16.0

* Mean values from triplicate plates.
† Actual experimental data given in parentheses.
‡ χ^2 for 5% significance is 3.84 and for 1% is 6.63.
§ Regenerated in MYGMS medium.

Monokaryotization by Protoplasting different Edible Mushrooms
Dikaryotic strains from six species in four genera were tested for monokaryotization by protoplasting (Table 3). The yields of monokaryons in regenerated colonies were over 45%. Among these strains, the yield in *L. tigrinus* 1 (70%) was the highest. The lowest yield (46%) was in *Pleurotus florida* (67 + 11). In all tested strains, two mating types were recovered from each dikaryotic strain, but the ratios of the two mating types varied were not 1:1 in most cases.

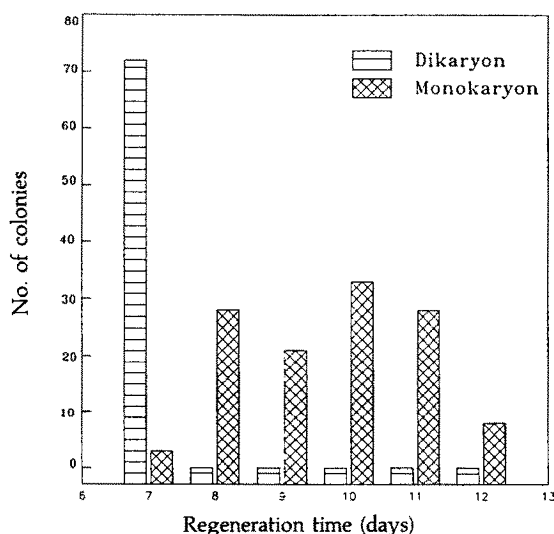

Figure 1. Difference in the regeneration time of dikaryons and monokaryons of *L. edodes* 54.

Comparison of Parental Monokaryons and Protoplasted Monokaryons
To demonstrate any differences between parental monokaryons and protoplasted monokaryons, colony diameter, mating type and auxotrophic markers were compared in *P. florida* (Table 5) (*P. florida* 67 was an auxotroph requiring adenine). Average colony diameter did not show any significant differences (at the $\alpha = 0.05$ level). To determine the mating types of protoplasted monokaryons, pairing

Table 4. Size of protoplasted monokaryon and dikaryon mycelium.

Strain	Cultivation time (days)	Dikaryon* (mm)	Monokaryon* (mm)
P. sajor-caju (21 + 36)	10	24.2 ± 0.9	14.1 ± 2.3
C. cinereus (5104 + 5026)	10	70.3 ± 4.2	36.9 ± 3.8
P. florida (67 + 11)	10	44.2 ± 2.8	23.5 ± 2.0
L. edodes (54)	10	44.7 ± 2.0	24.1 ± 4.2
L. edodes (3421 + 1315)	15	47.4 ± 1.6	29.0 ± 4.7
F. velutipes (1)	10	52.8 ± 2.1	6.7 ± 1.6

* All values are mean (±standard error) colony sizes of 10 samples randomly chosen from the regenerated colonies.

Table 5. Comparison of different characteristics of parental monokaryons and protoplasted monokaryons.

Character	*P. florida* 67		*P. florida* 11	
	Parental	Protoplasted	Parental	Protoplasted
Colony size (mm) (10 days in MYG)*	22.0 ± 1.2	20.0 ± 1.6	26.0 ± 1.2	26.8 ± 1.1
Mating type	A_1B_1	A_1B_1	A_2B_2	A_2B_2
MM medium	No growth	No growth	NT	NT
MM medium with adenine	Growth	Growth	NT	NT

* Means ± standard errors, based on 10 samples.
NT—Not tested.

reactions were carried out by using parental *P. florida* 67 (mating type A_1B_1) and *P. florida* 11 (mating type A_2B_2) as tester strains. The results implied that a protoplasted monokaryon maintains the same mating type as its respective parent and no third mating type was found. Protoplasted monokaryon *P. florida* 67 was checked for the auxotrophic marker. It did not grow on minimal medium but flourished on the supplemented minimal medium with 0.05 mg/ml adenine, showing that the protoplasted monokaryon maintained the same auxotrophic marker as its parental monokaryon.

Comparison of Regeneration Rate of Parental Monokaryons
The ratio of the two mating types of protoplasted monokaryons was expected to be 1:1. In fact, only *P. florida* (67 + 11) and *L. tigrinus* 1 showed this ratio (based on χ^2 test results). To understand why an unbalanced distribution of two mating types occurred in most strains, protoplasting of each parental monokaryon of *C. cinereus* (5104 + 5026), which gave the highest χ^2 value, was carried out. Their regeneration rates in different media, which have been reported suitable for the regeneration of protoplast (Kiguchi & Yanagi 1985), are shown in Table 6. The regeneration rates of *C. cinereus* 5104 in MYGM and MYGMS medium were 0.12% and 0.43%, respectively. The corresponding rates for its partner, 5026, were 2.42% and 0.83%, respectively. In all tested media, the regeneration rates of 5026 were significantly higher than those of 5104. These results might explain the uneven ratios of the two kinds of mating types in protoplasted monokaryons. After protoplasting of the dikaryon (5104 + 5026), 69 monokaryons were randomly selected for the mating reaction and, of these, 64 were determined as *C. cinereus* 5026 and only five as 5104. When another regeneration medium (MYGMS) was used for protoplasting the dikaryon (5104 + 5026) the χ^2 value was considerably less than that in MYGM medium (Table 3). The different regeneration rates may therefore be the major reason for the uneven ratio in the protoplasted monokaryons.

Table 6. Comparison of regeneration rate of parental monokaryons in different regeneration media.

Strain	Regeneration medium	Regeneration rate (%)
C.cinereus 5026		
	MYGM	2.42
	CMM	6.86
	MYGMS	0.83
	CMMS	0.0086
C. cinereus 5104		
	MYGM	0.12
	CMM	0.0046
	MYGMS	0.43
	CMMS	0.0044

Discussion

The isolation of protoplasts depends on three major factors: physiological state of the cells; lytic enzyme; and osmotic stabilizer (Peberdy 1989). The Lywallzyme used as lytic enzyme in this study is derived from *Trichoderma longibrachiatum* and is very effective in the lysis of several edible mushrooms. As an osmotic stabilizer, mannitol was generally suitable for the regeneration of protoplasts. The most difficult factor to control is the physiological condition of the mycelium although young or fast-growing mycelia tended to release a good yield of protoplasts. Following our protoplasting system, the protoplast yields are generally high (10^5 to 10^6/mg Lywallzyme) and the regeneration rates are also satisfactory (0.96% to 9.6%). Even if the plating density of protoplasts was 10^6, no colonies were found in MYG medium; this result confirmed the effectivity of the filtration system.

The time required for regeneration of parental dikaryons and monokaryons is clearly different, as shown in Figure 1. Initially, most of the regenerated colonies which appeared were dikaryons. It is suggested that the time of regeneration is a key factor in the development of the regenerated

colonies, and this time is mostly correlated with growth rates of the colonies. This phenomenon explains why some researchers fail to obtain any monokaryons (Magae *et al.* 1985). In order to avoid the mixed growth of dikaryons and monokaryons, the larger colonies which appear earlier should be removed to leave space for the growth of monokaryons. If regenerated colonies are isolated early, most or all of them will be dikaryons.

Mating type is one of the most important genetic characteristics in edible mushrooms. Because these protoplasted siblings are not the products of meiosis, genetic recombination will not appear in the mating types. Both mating types were recovered in all tested strains and no other mating types appeared. In addition, other characteristics, such as auxotrophic markers, mycelial growth rate and mycelial morphology, were compared in the parental and protoplasted monokaryons of *P. florida*. After protoplast release and regeneration, a protoplasted monokaryon still maintains the characters of its parental monokaryon. Although no differences were found between protoplasted monokaryons and parental monokaryons, the ratios of the two types of monokaryons of each strain were not always 1:1. Theoretically, if two mating types are isolated from a dikaryon, they should be evenly distributed. The unbalanced ratio is probably due to the different protoplast regeneration rate of individual parental monokaryons. In most cases, the less common of the two kinds of regenerated monokaryons grew slower than the more common partner. Care and patience are required if both types of monokaryons are to be isolated successfully. It may also be worth varying the medium since medium type can also affect the regeneration rates of two compatible monokaryons.

Electron microscopic and biochemical studies have shown that the structures in the fungal hyphal wall are different from the tip to the more distal region (Burnett 1979). The structure of the hyphae may influence the yield of protoplasts and the nuclear number contained in one protoplast. We observed the distribution of nuclei in protoplasts of *L. tigrinus* 1 by phase-contrast microscopy. The ratios of mononucleate, binucleate and anucleate protoplasts were 30%, 10% and 60%, respectively, and no significant differences were found at different times (data not shown). The number of nuclei in one protoplast is thought to be correlated with the distance between two compatible nuclei and the site of protoplast release.

Protoplasting may be an easy way to obtain monokaryotic siblings. Compared with the conventional single-spore-isolate method, protoplasting has several advantages (Figure 2). The conventional method requires many stages (formation of fruiting body, sporulation and germination) but the new technique, requires only one (the release and regeneration of protoplasts of mycelium) and is therefore simpler and quicker. If the mating components of the parental dikaryons are AxBx + AyBy, four mating types (AxBx, AyBy, AxBy and AyBx) can be obtained by the fruiting method and these are all meiosis products in which the genetic traits are diluted. In protoplasting, no meiosis occurs, only two parental mating types (AxBx and AyBy) appear and the genetic traits can be easily conserved. Since protoplasting is comparatively simple and effective, it will be a very useful tool in the breeding of edible mushrooms. For example, although sporeless strains of *Pleurotus* are very valuable in avoiding allergic problems, these strains are usually poor in quality and, since they are sporeless, it is difficult to obtain monokaryons from them for crossing with other monokaryons which may carry good traits. Wild strains may possess many good traits, such as resistance to disease and temperature tolerance but, some of them do not form a fruiting body under standard cultivation conditions. Protoplasting could be used to produce monokaryons from all these strains in a short time.

Another potential application of this technique is in the identification of protoplast fusants. Using protoplast fusion it is now possible to obtain interspecific and intergeneric hybrids of edible mushrooms. One principal advantage of

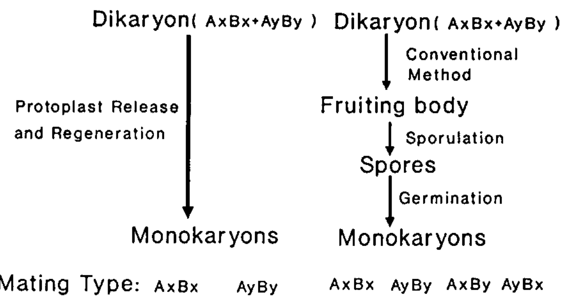

Figure 2. Comparison of the conventional method and the protoplasting technique.

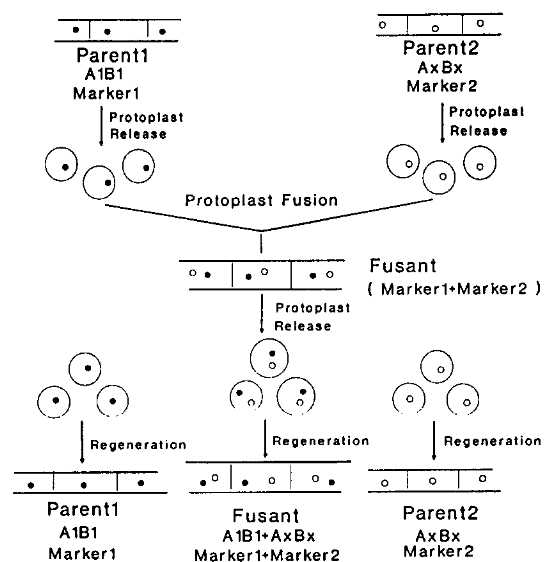

Figure 3. The identification of heterokaryotic fusants by the protoplasting technique.

this technique is that it can be used to set up crosses not possible by conventional hyphal fusion (Peberdy 1987). Identification of the protoplast fusants, which is the most difficult step in protoplast fusion, has been by: (a) observation of clamp connection; (b) isoenzyme analysis; (c) fruiting trials; and (d) basidiospore analysis. However, there are some imperfections in each of these methods. In all reports on fusion products, clamp connections have only been found in some interspecific fusion products and at a very low rate (Toyomasu & Mori 1989). Isoenzyme analysis depends on the establishment of biochemical loci (Stasz *et al.* 1988) but this is time consuming and the result can often be influenced by environmental and physiological conditions. The formation of a fruiting body is thought to be the best evidence for fusion products, but the possibility of monokaryotic fruiting cannot be excluded. Monokaryotization by protoplasting may provide a potential way to identify protoplast fusants. As shown in Figure 3, protoplasts are released from potential heterokaryotic fusants, with parents 1 (monokaryon, mating type A1B1, marker 1) and 2 (monokaryon, mating type AxBx, marker 2). After the regeneration of protoplasts, three kinds of colonies can be found, parent 1, parent 2 and protoplasted fusants. These colonies can be readily identified by mating tests and marker analysis etc. Kirimura *et al.* (1989) used this method to isolate both parents from suspected heterokaryotic fusants of *Aspergillus niger* and *Trichoderma viride*.

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