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An efficient protoplast isolation and regeneration system in *Coprinus comatus*

Linhui Li[1]*, Qingyu Yin[1], Xiaohong Liu[1] and Hong Yang[2]

[1]Key Laboratory of Southwest China Wildlife Resources Conservation, China West Normal University, Nanchong City 637009, P. R. China.

[2]Department of Biochemistry, Xichang College, Xichang City 615022, P. R. China.

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An efficient protocol for protoplast isolation and regeneration was first established in *Coprinus Comatus*. The highest yield of protoplasts was up to 8.9 × 10^6 cells/ml in digestion solution containing 2.0% lywallzyme and 0.6 M KCl after incubation of four-day-old mycelia at 30°C for 3 h; among which, about 1.4% protoplasts could be regenerated into mycelia after 4 - 6 days of incubation at 25°C in CYM medium with 0.6 M mannitol as osmotic stabilizer. The results are beneficial for breeding new cultivars by the methods such as protoplast fusion, mutagenesis as well as transformation. Moreover, the stepwise procedure for protoplast liberation and regeneration could be referred in other species.

Key words: *Coprinus comatus*, mycelia, lywallzyme, protoplast, liberation and regeneration.

INTRODUCTION

Wild *Coprinus comatus* (*C. comatus*) belonging to edible fungi is widely distributed in the world, and in P. R. China, it has been cultured in force in many provinces and regions (Huang, 1997), and the biological efficiency is over 100%. *C. comatus* has very important medical and edible values, for example, it can bring down blood sugar and blood fat, improve immunity and inhibit tumor growth (Liu and Zhang, 2003), so it was referred by FAO and WHO, to one of the 16 species rare mushrooms because it is characterized by nature, nutrition and health. We can search many literatures about the screening of culture media and comparing among strains (Tang et al., 2006; Mi, 2007; Li et al., 2008; Mi and Wang, 2008), but at present, its quality is descreasing and yield is falling, owing to the disease called “Black cap”. So, it is very nessary and significant to breed resistant strains against the pathogen resulting in the disease. Whereas conventional breeding methods including spore cross and mutagenesis are difficult to be used for breeding resistant strains in *C. comatu*, because the pure spores are hardly harvested by spore emission owing to the fruit body autolysis when it grows up into maturation. A feasible solution for screening resistant strains is to regenerate protoplasts from mycelia of *C. comatus*, and the basal work is to establish an efficient system suitable for liberation and regeneration of protoplasts. At present, the protoplasts from many species including some bacteria, yeasts, fungi and plants have successfully been regenerated (Morinaga et al., 1985; Yanagi et al., 1985; Sonnenberg et al., 1988), and many excellent strains have also been selected out in edible mushroom breeding (Gold et al., 1983; Kiguchi and Yanagi, 1985; Mukherjee and Sengupta, 1986; Kim et al., 2000; Sun et al., 2001; Dhitaphichit and Pornsuriya, 2005; Wang et al., 2005; Zhu et al., 2008; Chen et al., 2009). However, there are no reports on isolation and regeneration of protoplasts in *C. comatus* to date.

In the present study, an efficient protoplast isolation and regeneration system has been established in *C. comatus*, and the system is beneficial for screening the elite resistant strains against the pathogen resulting in “Black cap”.

MATERIALS AND METHODS

Strain and mycelial culture

The strain *C. comatus* was conserved in PDA medium (Table 1) in our labarary. A block of spawn was inoculated into 30 ml CYM broth (Table 1) in 500 ml Erlenmeyer flasks and incubated at 25°C to

*Corresponding author. E-mail: lilinhui6890@126.com.

Table 1. Media and their compositions.

Media	Compositions (in 1000 ml water)
PDA	200 g potato, 20 g sucrose and 15 g agar
CYM	20 g glucose, 2 g peptone, 1 g yeast extract, 0.5 g $MgSO_4$, 0.46 g KH_2PO_4 1 g K_2HPO_4, 20 g agar(only used in solid medium)
MYG	5 g maltose, 5 g yeast extract, 10 g glucose, 30 mg VB_1, 20 g agar

Table 2. The designed factors affecting protoplast release in *C. Comatus.*

Factors	Ladders or componentsbnb
Lywallzyme concentration (%)	1.5, 2.0, 2.5, 3.0
Osmotic stabilizer (0.6M)	$MgSO_4.7H_2O$, KCl, sucrose, mannitol
[a]pH of enzymatic solution	5.0, 5.5, 6.0, 6.5
Enzymatic digestion time (h)	2, 3, 4, 5
Mycelia age (d)	3, 4, 5, 6, 7
Enzymatic digestion temperature (°C)	25, 30, 35

[a]pH buffer was prepared by citrate-disodium hydrogen phosphate.

Table 3. The designed factors affecting protoplast regeneration in *C. Comatus.*

Factors	Ladder or components
Osmotic stabilizer	KCl, mannitol, sucrose, $MgSO_4.7H_2O$
Media	CYM, MYG, PDA
[a]Protoplast (h)	2.0, 2.5, 3.0, 3.5, 4.0
[b]Protoplast (d)	2 , 3 , 4 , 5 , 6

[a]Protoplasts from different incubation time; [b]Protoplasts from different mycelia age.

proliferate mycelia.

Protoplast liberation and purification

The basic procedure of protoplast liberation of *C. comatus* was conducted as follows:

(1) Mycelia collection and pretreatment. The proliferated mycelia were picked out with inoculation loop, and then rinsed thrice by osmotic stabilizer.
(2) Enzyme digestion of mycelia. 0.1 g mycelia were suspended in 1ml enzymatic mixture for digestion. The mixture containing lywallzyme (Guangdong Institute of Microbiology, P. R. China) and osmotic stabilizer was first prepared in pH buffer, and then sterilized by 0.22 m of pore membrane.
(3) Counting protoplast and stopping the reaction. The number of protoplast liberated was counted once every hour with globulimeter. When the highest concentration took place, the reaction was stopped.
(4) Removal of undigested mycelia fragment. The residual mycelia fragment in digesting solution was removed with a column prepared by filling a 2 - 3 mm thickness of cotton wool into 5 ml syringe and compressing gently.
(5) Protoplast purification. The protoplasts were purified by washing twice in the osmotic stabilizer the same with that used in enzymatic digestion, then the pellets were collected *via* centrifugation at 4°C and 4000 rmp for 10 min.

To optimize the conditions involved in protoplast release, in this experiment, six factors were considered (Table 2). All the digestion reaction was conducted under a gentle vibration of 50 rpm.

Protoplast regeneration and phenotypic observation

The basic procedure of protoplast regeneration of *C. comatus* was conducted as follows:

(1) Dilution of protoplast. The obtained protoplasts were first centrifugalized to remove the osmotic stabilizer, and then, the pellets were diluted with 0.6 M mannitol or sterile water to about 10^5 cells/ml. Here, the sterile water was used to eliminate the regeneration errors derived from the mycelia fragments in pellets.
(2) Plating protoplasts. 0.1ml diluted protoplasts were plated into Petri dishes (9 cm in diameter) containing 25 ml regeneration medium and incubated at 25°C for 4 - 6 days. The regeneration rate was calculated according to the following formula:

Regeneration rate (%) = (A-B) / plated protoplast number × 100%
A: colony number regenerated from protoplasts diluted with 0.6M mannitol.
B: colony number regenerated from protoplasts diluted with sterile water.
To screen the optimum regeneration condition, the following factors were designed to compare their effects on regeneration rate (Table 3). Moreover, The process from protoplasts to visible colonies were observed by microscope or naked eye, and partial visions were taken photographs.

RESULTS

Protoplast isolation

The protoplast concentration obtained under different factors was different, with a range from 2.6×10^6 - 8.9×10^6 protoplasts/ml. All the considered factors could affect protoplast release (Table 4), among of them, osmotic stabilizer seemed to have more significant effect than

Table 4. Effects of different factors on protoplast liberation.

Factors		Yield of liberated protoplast (×10[6] protoplasts/ml)	Factors		Yield of liberated protoplast (×10[6] protoplasts/ml)
[a] Lywallzyme concentration (%)	1.5	5.8	[d] Digestion time (h)	2	5.8
	2.0	8.1		3	8.1
	2.5	5.6		4	6.4
	3.0	3.6		5	6.2
[b] Osmotic stabilizers (0.6M)	$MgSO_4.7H_2O$	6.4		3	5.0
	KCl	8.9	[e] Mycelia age (d)	4	8.0
	Sucrose	2.6		5	6.3
	Mannitol	7. 8		6	6. 2
[c] Buffer pH	5.0	6.2	[f] Digestion temperature (°C)	7	4.8
	5.5	8.8		25	6.0
	6.0	7.7		30	8.0
	6.5	6.3		35	4.8

[a] The same condition was to incubate 6-d-old mycelia at 30°C for 3 h in the mixture of pH5.5 containing 0.6M KCl as osmotic stabilizer; [b] the same condition was to incubate 6-d-old mycelia at 30°C for 3 h in the mixture of pH5.5 containing 2% lywallzyme; [c] the same condition was to incubate 6-d-old mycelia at 30°C for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer; [d] the same condition was to incubate 6-d-old mycelia at 30°C in the mixture of pH5.5 containing 2% lywallzyme and 0.6M KCl as osmotic stabilizer; [e] the same conditions was to incubate 3 - 7 days of mycelia at 30°C for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer; [f] the same conditions was to incubate 6-d-old mycelia for 3 h in the mixture of pH 5.5 containing 2% lywallzyme and 0.6 M KCl as osmotic stabilizer.

others, the best one was 0.6M KCl, with a highest yileds of 8.9×10^6 protoplasts/ml; the worst was 0.6 M sucrose, with a lowest yileds of 2.6×10^6 protoplasts/ml. As for the others factors, there was no significant difference in protoplast release. From the data in Table 4, it was concluded that the optimal condition for protoplast liberations was to incubate 4 day old mycelia at 30°C for 3 h in the mixture of pH 5.5 containing 2.0% lywallzyme and 0.6 M KCl as osmotic stabilizer.

Protoplast regeneration

Six factors were designed for optimizing regeneration conditions, and the obtained data were shown in Table 5. Four osmotic stabilizers were used in medium to regenerate protoplasts, and 0.6M mannitol presented the highest regeneration rate (1.40%), followed by 0.6 M $MgSO_4.7H_2O$ (1.05%), while, both 0.6 M sucrose and 0.6 M KCl provided zero of regeneration rate, which suggested that sucrose and KCl were not adapt to act as osmotic stabilizer in regenerating protoplasts of *C. comatus*. Three media were employed in protoplast regeneration; as a result, CYM medium showed the highest regeneration rate 1.42%, but the difference between CYM and MYG was not significant. PDA medium displayed 0.51% of regeneration rate, much less than the others. The results revealed that the two media CYM and MYG were preferable to PDA in regenerating protoplasts of *C. comatus*. The protoplasts from different digestion time and mycelia age were also applied for evaluating the regeneration rate, the results showed no significant differences among the protoplasts from different digestion time, but, the regeneration rate from the protoplasts of different mycelia age seemed to have significant differences, among of them, 5-day-old mycelia showed the best regeneration rate 1.41%, whereas the 2-day-old mycelia showed the lowest regeneration rate 0.92%.

From the data in Table 5, it was concluded that the optimum regeneration conditions was to regenerate protoplasts isolated from 3 h of incubation of 5-day-old mycelia into mycelia in CYM medium supplemented 0.6 M mannitol as osmotic stabilizer.

Observation of protoplast regeneration process

Figure 1 showed the regeneration process from protoplasts to visible colonies in regeneration medium CYM. The isolated protoplasts (Figure 1a) were transferred onto regeneration media; they were first enlarged and then developed into a small yeast-like bud (Figure 1b). When more than one yeast-like buds occurred continuously from one mother protoplast, a lot of budding cells with different size was observed (Figure 1c), otherwise, when the new yeast-like buds appeared from one mother protoplast in a synchronous manner, the similar sizes of budding cells could be observed (Figure 1d). Every budding cells also began to produce new yeast-like buds

Table 5. Effects of different factors on protoplast regeneration.

Factors		Regeneration rate
[a]Osmotic stabilizer (0.6M)	KCl	0
	Mannitol	1.40
	Sucrose	0
	$MgSO_4.7H_2O$	1.05
[b]Media	CYM	1.42
	MYG	1.36
	PDA	0.51
[c]Protoplast quality (related to the incubation time) (h)	2.0	1.24
	2.5	1.33
	3.0	1.38
	3.5	1.30
	4.0	1.21
[d]Protoplast quality (related to the mycelia age) (d)	2	0.92
	3	1.21
	4	1.31
	5	1.41
	6	1.30

[a]The same condition was that protoplasts isolated from 3 hours of incubation of 4-day-old mycelia regenerated in CYM medium; [b]The same condition was that protoplasts isolated from 3 h of incubation of 4-day-old mycelia regenerated in medium supplemented 0.6M mannitol as osmotic stabilizer; [c]The same condition was that protoplasts isolated from 4-day-old mycelia regenerated in CYM medium supplemented 0.6M mannitol as osmotic stabilizer; [d]The same condition was that protoplasts isolated from 3 h of incubation regenerated in CYM medium supplemented 0.6M mannitol as osmotic stabilizer.

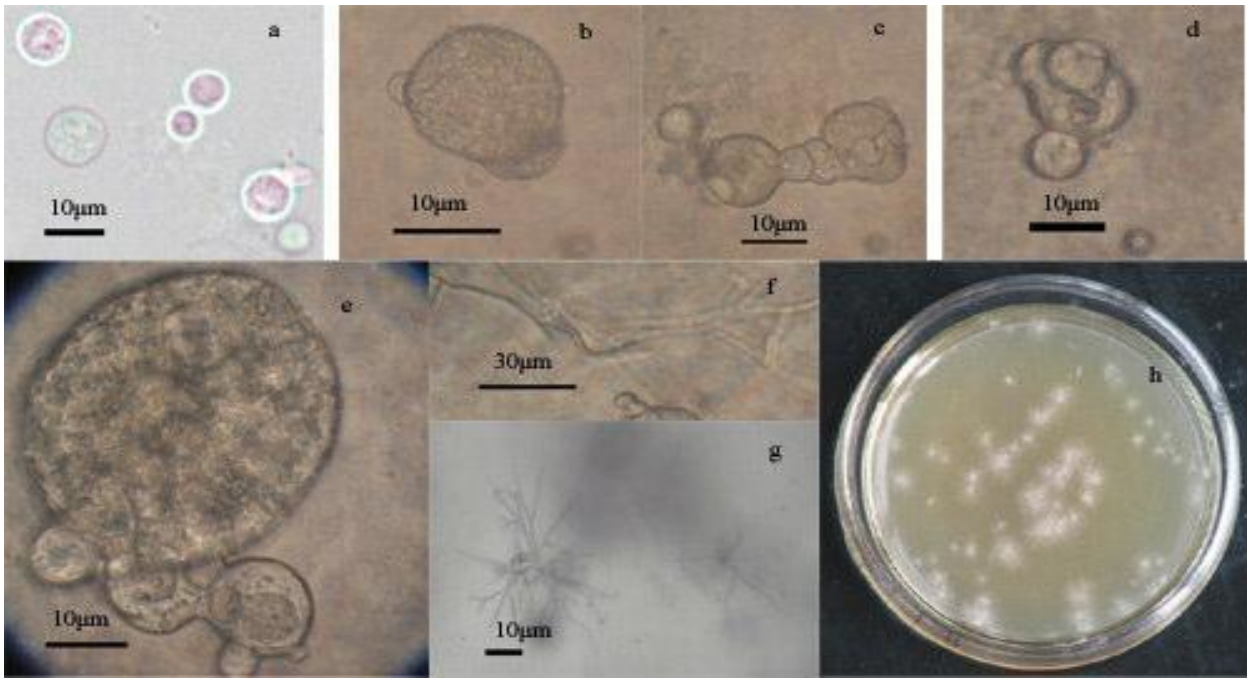

Figure 1. Regeneration process of protoplast of *Coprinus comatus.*
a: the isolated protoplasts; b: the enlarged protoplast; c: asynchronous protoplast budding; d: synchronous protoplast budding; e: new yeast-like buds continuously came out; f and g: the regenerated mycelia; h: the visible colonies.

as they grew up to a certain size (Figure 1e), meanwhile, the mother protoplasts could continue to budding, thus resulted in a continuous sizes of budding cells derived from one mother protoplast. When thesebudding cells regenerated to form cell walls, the mycelia could be observed (Figures 1f and g), and eventually, the propagated mycelia in regeneration medium formed visible colonies with different sizes (Figure 1h). The result was similar to the previous reports in other protoplast regeneration (Lau et al., 1985; Morinaga et al., 1985; Yanagi et al., 1985; Mukherjee and Sengupta, 1986).

DISCUSSION

C. comatus belonging to edible fungi has very important medical and edible values (Liu and Zhang, 2003) . But at present, its quality and yield are falling, duo to the disease called “Black cap”. So, it is very nessary and urgent to breed resistant strains against the pathogen resulting in the disease. However, conventional breeding methods including spore cross and mutagenesis can not efficiently be used to breeding resistant strains because of the autolysis of fruit body of *C. comatus* during maturation. An alterative method is to regenerate *C. comatus* from protoplasts, and the primary work is to establish an efficient system suitable for isolation and regeneration of protoplasts. At present, many species have successfully been regenerated from protoplasts (Morinaga et al., 1985; Yanagi et al., 1985; Sonnenberg et al., 1988), however, there are no reports in *C. comatus* to this day. To obtained an efficient protocol suitable for protoplast isolation and regeneration in *C. comatus*, in our studies, several factors affecting experimatal results were designed and evaluated, of which the four main factors including lytic enzyme concentrations, mycelia age, osmotic stabilizers and regeneration media were intensively discussed.

Lytic enzyme concentration is one of the most important factors affecting protoplast release. If too low, mycelia can not be digested sufficiently; too high, protoplast membranes will be injured in spite of high yields, which will lead to a low regeneration frequency (Kitamoto et al., 1988; Zhang and Li, 1993; Rao and Prakash, 1995; Naseema et al., 2008). Lywallzyme, a lytic enzyme produced by GuangDong Institute of Microbiology, P. R. China, has successfully been used to liberate the protoplasts of more than ten *basidiomycetes*, the previous used concentrations ranged from 1.5 - 2.5% (Sun et al., 2001; Wang et al., 2004; Li et al., 2006). The same lytic enzyme was also applied in our study, and four concentration values including 1.5, 2.0, 2.5 and 3.0% were designed to obtain the optimum concentration in *C. comatus*, the results showed that 2.0% is the best, with high up to 8.1×10^6 protoplasts in 1 ml digestion solution (Table 4).

Mycelia age can affect protoplast isolation and its regeneration, although both juvenile and older mycelia of *basidiomycete* could be used for protoplast isolation and regeneration, the protoplasts from elder mycelia can be regenerated into mycelia more rapidly and easily, this cause is probably that elder mycelia have more intact nucleus and organelles (Santiago et al., 1982a, 1982b; Peng et al., 1993; Wang et al., 2005). Nevertheless, too old mycelia are not preferred, because their protoplasts were not easy to be isolated by lytic anzyme, in addition, the obtained protoplasts are hardly regenerated into mycelia duo to bad quality. Thus, in our experiments, to make sure the optimum mycelia age, five mycelia possessing different ages (3, 4, 5, 6 and 7d) were evaluated on the protoplast liberation, the results showed that 4-day-old mycelia have the highest protoplast concentration (8.0×10^6 protoplasts in 1 ml digestion solution) (Table 4). Another five mycelia (2, 3, 4, 5 and 6d) were investigated on protoplast regeneration, and 5-day-old mycelia presented the highest regeneration frequency (1.41%) (Table 5).

The osmotic stabilizers, used for rinsing mycelia, diluting lytic enzyme and preparing regeneration media, is also an important factor influencing protoplast liberation and regeneration. As for protoplast liberation, an osmotic stabilizer is only suitable for a few species owing to the differences of cell wall compositions in different species (Cheng and Bélanger, 2000). For example, mannitol was the best for *Agrocybe aegerita* (Zhang et al., 2004), while, MgSO4 is the best one for *Sclerotium rolfsii* (Morinaga et al., 1985; Fari a et al., 2004). To ascertain the optimum osmotic stabilizer used to liberate protoplasts of *C. comatus*, in our experiments, four including sucrose, mannitol, $MgSO4.7H_2O$ and KC1 were selected from many available osmotic stabilizers and analyzed on protoplast liberation, as a result, 0.6 M KCl provided the highest yield (8.9×10^6 protoplasts per 1 ml digestion solution) (Table 4).

Regarding protoplast regeneration, the optimum osmotic stabilizers supplemented into regeneration media were also different in light of different species. For instance, sucrose was the best one in *A. bisporus* and *A. bitorquis* (Yanagi et al., 1985; Sonnenberg et al., 1988), while in *P. brevicompactum*, 0.8M KCl was much better then others (Varavallo et al., 2004).

In this present experiments, four osmotic stabilizers were respectively added into CYM medium (Table 5), as a reult, the CYM medium containing 0.6 M manitol was proved the best, with 1.4% of regeneration percentage, followed by the CYM appended with 0.6 M $mgSO_4.7H_2O$; whearas, the two CYM supplemented with 0.6 M KCl or 0.6 M sucrose could not regenerate protoplasts into mceylia at all. Mannitol, as osmotic stabilizer, has also been used for protoplast regeneration in plant and other fungi, according to speculation, it was the direct precursor of cell wall synthesis or the indirect one by metabolism and transformation, and thus, could speed up the cell wall synthesis and make protoplast regenerate more easily

(Lau et al., 1985; Peng et al., 1993; Kim et al., 2000; Chitnis and Deshpande, 2002; Balasubramanian et al., 2003).

Besides osmotic stabilizers, media can also influence significantly protoplast regeneration, for example, in the studies by Fari a et al (2004) on protoplast regeneration of *Sclerotium rolfsii* ATCC 201126, the MYG medium supplemented 0.6M sucrose (26%) or 0.7M NaCl (17%) displayed the highest regeneration rate, when CYM media with 0.6M sucrose was used as regeneration culture media, the regeneration rate decreased almost twofold, moreover, the protoplasts on MR medium could not be differentiated into mycelia. Thus, in our study, three media supplemented with the same osmotic stabilizer (0.6M mannitol) were used for protoplast regeneration; as a result, medium CYM was proved the best one, with 1.4% of regeneration percentage (Table 5).

Although the regeneration percentage reported here was not high in *C. comatus*, even much lower than those reported in other species within same genus (Morinaga et al., 1985; Yanagi et al., 1985), the value could sufficiently meet our demands in most studies, such as protoplast fusion, mutagenesis and even transformation. Certainly, we could also increase the regeneration rate by improving regeneration media and protoplast quality.

In summary, an efficient protocol for protoplast isolation and regeneration was first established in *C. comatus*, the highest protoplast yield reached 8.9×10^6 protoplasts / ml in digestion solution, among which, about 1.4% could be regenerated into mycelia. The results are beneficial for breeding new cultivars by these methods such as protoplast fusion, mutagenesis as well as transformation, moreover, the stepwise procedure for protoplast liberation and regeneration contributes to the related studies in other species. Of course, the resistant strains against the pathogen resulting in “Black cap” could be obtained by protoplast techneques.

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