Formation of interspecies fusants of *Agaricus bisporus* and *Agaricus bitorquis* mushroom by protoplast fusion

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Abstract Interspecies fusants are formed between *Agaricus bisporus* and *Agaricus bitorquis* by protoplast fusion technique. Protoplasts were isolated and regenerated by using Novozyme 234 lytic enzyme. Twenty slow growing isolates were separated from the protoplast regenerated colonies, which were assumed as homokaryons (putative homokaryons). These twenty isolates were subjected to growth rate, colony morphology and spawn run studies for screening of true homokaryons. Antifungal markers were developed for selection of fusants.

Keywords *Agaricus bisporus* · Homokaryons · Antifungal markers · Protoplast fusion · Fusants

Introduction

Agaricus bisporus (button mushroom) is one of the most popular species on the international mushroom market and has been undergoing a rapid increase in market share in recent years¹. The production and culture of new species of mushrooms is increasing. The breeding of new strains has significantly improved, allowing the use of strains with high yield and resistance to diseases, increasing productivity and diminishing the use of chemicals for pest control². Strain improvement of *A. bisporus* thus far has been based on conventional breeding methods³. Strain selection alone based on single spores, multispores or tissue culture may give improvement in the short term but it is unlikely to be as effective as methods involving controlled crossing. However, breeding of improved strains by conventional methods is a labour intensive and time-consuming procedure⁴. Protoplast fusion has been used as a method to create mushroom hybrids, especially when using conventional methods cannot achieve this result⁵. The technique has therefore opened up an important new area of study, which has both fundamental and applied importance⁶. In this study we have employed protoplast fusion technique for developing interspecies fusants of *Agaricus bisporus* and *A. bitorquis*.

Six cultures of mushroom were procured from IMTECH Chandigarh, IARI New Delhi and Department of Biological Sciences, R.D. University, Jabalpur, India. Protoplasts were isolated and regenerated for obtaining homokaryons of these six parental strains. A modification of procedure of Crowhurst *et al.*⁷ was used for preparation of the protoplasts. Protoplasts were stored at 4°C in STC buffer in refrigerator for further use. The estimated number of protoplasts formed per mL of osmotic stabilizer (0.8 M sorbitol, 50 mM CaCl₂, 50 mM TrisHCl, pH 7.5) was determined by using

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a haemocytometer⁵. Protoplasts were regenerated on MMP medium (1% Malt extract, 0.5% Mycological peptone and 1% Agar, pH 7.5) osmotically supported by 0.6 M sucrose. Protoplasts stored in STC buffer were diluted in 0.6 M sucrose to a concentration of 200–400 protoplasts/mL. One ml of the protoplast suspension was plated per petridish and incubated at 24±2°C in dark for 20 d. Similar studies were done by Sonnenberg *et al.*⁸ in which they have described protoplasting/regeneration system used for routine isolation of homokaryons of *A. bisporus*. Castle *et al.*⁹ obtained protoplasts from the heterokaryotic strains of *Agaricus brunne-scens*, and cultures from regenerated protoplasts were tested for homokaryosis. Khush *et al.*¹⁰ isolated homokaryons of *Agaricus bisporus* by screening slowly growing protoplast regenerates. Jin and Xu¹¹ made a study between fluffy and appressed types of *Agaricus bisporus*.

The protoplast-regenerated colonies were screened after 20 d for their growth characteristics. Only slow growing colonies were separated from protoplast regenerated colonies¹⁰. These slow growing isolates were assumed as homokaryons (putative homokaryons) and were subjected to growth rate (mm/d), colony morphology and spawn run studies on non-composted substrate for further confirmation

of their homokaryotic status¹². Slow growing isolates (SSI) were numbered from H–1 to H–20 (Table-1).

The isolates with a growth rate less than 1.4 mm/d failed in spawn running process. The eleven isolates i.e., H – 1, H – 3, H – 6, H – 7, H – 8, H – 10, H – 13, H – 15, H – 16, H – 17 and H – 20 never completed spawn running process even after 40 d. Isolate H – 1 of *A. bisporus* MTCC showed the least growth rate of 0.55 mm/d. The isolates of *A. bisporus* showed either appressed and powdery mycelial morphology or appressed with concentric rings type of morphology. The isolates of *A. bitorquis* exhibited dense mated type of mycelial growth but with very slow growth rate. The remaining nine isolates successfully completed spawn running and completely colonized the non-composted substrate. These nine isolates showed strandy, fluffier and good aerial growth, with or without sectoring. Such types of characteristics are exhibited by parental heterokaryotic strains. Therefore these nine isolates were excluded from further studies. Slow growing isolates were not recovered from *A. bisporus* FGCC M 02 and *A. bitorquis* MTCC 1794.

Resistance to fungicides was checked against Benomyl (Benlate 50%), Bavistin, Ketocanazole, Clotrimazole, Flucanazole and Griseofulvin by the method of Sobral *et al.*¹³.

Table 1 Mycelial growth rate, colony morphology and source of slow growing isolates of *A. bisporus* and *A. bitorquis*.

Isolate No.	Fungal Strain	Growth rate on MEA (mm/d)	Colony morphology	Spawn run in non-composted substrate in 0.5 kg bag
H – 1	<i>A. bisporus</i> MTCC 1792	0.55	Appressed and powdery	-
H – 2	<i>A. bisporus</i> MTCC 1792	1.56	Dense mycelia with no concentric rings	+
H – 3	<i>A. bisporus</i> MTCC 1792	1.20	Appressed with concentric rings	-
H – 4	<i>A. bisporus</i> MTCC 1792	2.31	Strandy with few fluffy sectors	+
H – 5	<i>A. bisporus</i> FGCC M 01	2.36	Strandy with no obvious sectors	+
H – 6	<i>A. bisporus</i> FGCC M 01	0.87	Appressed and powdery	-
H – 7	<i>A. bisporus</i> FGCC M 01	1.24	Appressed with concentric rings	-
H – 8	<i>A. bisporus</i> FGCC M 01	0.89	Appressed and powdery	-
H – 9	<i>A. bisporus</i> FGCC M 01	2.23	Strandy with few obvious sectors	+
H – 10	<i>A. bisporus</i> ITCC 1924	1.30	Appressed with concentric rings	-
H – 11	<i>A. bisporus</i> ITCC 1924	1.92	Dense mycelia with no concentric rings	+
H – 12	<i>A. bisporus</i> ITCC 1924	2.18	Strandy with aerial growth	+
H – 13	<i>A. bisporus</i> ITCC 1924	1.29	Appressed and powdery	-
H – 14	<i>A. bisporus</i> ITCC 1924	2.16	Strandy with aerial growth	+
H – 15	<i>A. bitorquis</i> ITCC 3619	0.92	Dense mated type of mycelium	-
H – 16	<i>A. bitorquis</i> ITCC 3619	0.82	Dense mated type of mycelium	-
H – 17	<i>A. bitorquis</i> ITCC 3619	0.89	Dense mated type of mycelium	-
H – 18	<i>A. bitorquis</i> ITCC 3619	2.22	Strandy and fluffier	+
H – 19	<i>A. bitorquis</i> ITCC 3619	1.92	Strandy and fluffier	+
H – 20	<i>A. bitorquis</i> ITCC 3619	0.82	Dense mated type of mycelium	-

Sign “+” indicate spawn run completed and sign “-” indicate spawn run not completed.

Table 2 Minimum concentration ($\mu\text{g/mL}$) of Antifungal agents required to develop a marker by inhibiting the growth of *A. bisporus* and *A. bitorquis*.

Homokaryotic strains	Antifungal agents ($\mu\text{g/mL}$)					
	Benomyl	Bavistin	Fluconazole	Griseofulvin	Ketoconazole	Clotrimazole
<i>A. bisporus</i> MTCC	32	45	220	220	190	30
<i>A. bisporus</i> ITCC	22	15	60	230	8	10
<i>A. bisporus</i> FGCC M 01	20	15	80	210	6	6
<i>A. bitorquis</i> ITCC	26	30	90	210	20	15

Table 3 Protoplast Regeneration frequency of *A. bisporus* ITCC H – 13, *A. bitorquis* ITCC H – 15 and their fusants

Fungal Strain	Protoplast yield/100 μL	No. of colonies appeared on regeneration medium	Regeneration frequency
<i>A. bisporus</i> ITCC H – 13	56	38	67.8%
<i>A. bitorquis</i> ITCC H – 15	50	31	62.0%
Fusants/Hybrids	55	09	16.4%

Fig. 1 Protoplast fusion of *Agaricus bisporus* ITCC H – 13 and *Agaricus bitorquis* ITCC H – 15

Antifungals were dissolved in DMSO at the concentration of 10 mg mL^{-1} (stock solution) and filter sterilized by using sartorius filter ($0.45 \mu\text{m}$) and stored in refrigerator. Mycelial discs were inoculated in the centre of Petridishes containing the culture medium with the antifungal agent at various concentrations. Three replicates were used per treatment.

An initial step critical in the development of an efficient protoplast fusion system for any organism is the availability of selectable markers, which allows positive selection of hybrids/fusants. Out of the six antifungals tested griseofulvin and clotrimazole were used as markers for the development of fusants, by protoplast fusion between isolate H – 13 of *A. bisporus* ITCC and isolate H – 15 of *A. bitorquis* ITCC. Growth of *A. bisporus* ITCC was inhibited at $230 \mu\text{g/mL}$ while that of *A. bitorquis* ITCC was inhibited at a concentration of $210 \mu\text{g/mL}$ of griseofulvin. *A. bisporus* ITCC

was unable to grow in presence of $10 \mu\text{g/mL}$ of clotrimazole while *A. bitorquis* ITCC was found to grow even at a concentration of $15 \mu\text{g/mL}$ of clotrimazole. Other homokaryons were not selected as there was close difference in their sensitivities to the tested antifungals, and the contrasting antifungal markers were not found for them (Table-2).

All the eleven homokaryons exhibited same level of sensitivity as was observed for their parental strains. Based on growth characteristics and marker development studies the homokaryotic isolate, H – 13 of *A. bisporus* ITCC and isolate H – 15 of *A. bitorquis* ITCC were selected for fusant development through protoplast fusion. Polyethylene mediated protoplast fusion was performed following the method of Sandhu and Venkateswerlu¹⁴. Regeneration frequency of fused protoplasts of homokaryons of *A. bisporus* and *A. bitorquis* was determined (Table-3) by using following formula:

$$\text{Regeneration frequency} = \frac{\text{No. of colonies appeared on regeneration medium}}{\text{Protoplast yield}} \times 100$$

Protoplast fusion started after 15 minutes of incubation with CaCl_2 and PEG 6000. The protoplasts of isolate H – 13 and H – 15 came closure and after 20 min of incubation they were more tightly compressed and finally looked like dumb-bell shaped after 45 min and fusion was completed (Fig. 1).

The MMP medium was supplemented with $210 \mu\text{g/mL}$ griseofulvin and $10 \mu\text{g/mL}$ clotrimazole for exclusively obtaining fusants, only. The regeneration frequency of homokaryotic strains was calculated and estimated between 60–70% (Table-3). In case of fusants this frequency was observed to be 16.4%, which is quit low as has been expected. Only 9 colonies were observed on regeneration medium after protoplast fusion.

Similar studies have been done by Dhitaphichit and Pornsuriya⁵, in which they fused two species of oyster mushroom *Pleurotus ostreatus* and *P. djamor* protoplast fusion. They detected 412 regenerated colonies but only two of them were selected as fusants by possessing clamp connections on their mycelia. The fusants were proved to be “hybrids” of *P. ostreatus* and *P. djamor*. Dahlberg¹⁵ produced and patented interspecies strain of *A. bisporus* and *A. bitorquis* by protoplast fusion of their homokaryons.

Further studies are required to be done to confirm the hybrid status of the fusants formed in this experimentation. The fusants will be subjected to small-scale fruiting trials to assess their productivity. The fusants are improved strains and possess new genetic traits, which could be more valuable than the available hybrids of *A. bisporus* and *A. bitorquis*.

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