J. Mycopathol. Res. 44(1) : 117-124 (2006)
© Indian Mycological Society, Department of Botany, University of Calcutta, Kolkata 700 019, India

Changes in nucleic acid content during fruitbody development in *Volvariella volvacea*

ADITI MUKHOPADHYAY, RATULA MUKHERJEE, MAITREYEE BANERJEE, SNIGDHA MAITRA, A. K. MANNA AND N. SAMAJPATI

Department of Botany, University of Calcutta, Kolkata 700 019, West Bengal

The present investigation dealt with some biochemical and genetical aspects of basidiocarp development of *Volvariella volvacea* (Bull. : Fr.) Sing. The tissue cultures were prepared from the basidiocarp and used in the present study. The experimental findings revealed that the combination of Novozyme 234 + cellulase + chitinase + pectinase enzymes was the best for the release of protoplasts from 4 days old mycelia of *V. volvacea* under sodium chloride osmotic stabilizer and phosphate buffer (pH 6.0). The regeneration of protoplast was also best in phosphate buffer (pH 6.0). In another experiment UV-mutants were produced and several asp⁻ and adn⁻ mutants were developed. In the progeny analysis it was found that 36 out of 38 asp⁻ mutant monosporous cultures and 45 out of 51 adn⁻ monosporous cultures retained their parent mutant phenotype. The comparative yield data of the parent and protoplast fusant hybrid strains revealed that the hybrid strain was superior than that of the parent strain. Moreover, the hybrid strain could produce fruit bodies throughout the twelve months of the year whereas the parent strain failed to produce yield during the months of October to January. The data on the protein content of the parent and the hybrid strains revealed that the amount of protein in button stage was maximum, 30.82% and 32.26% respectively. The protein content was more in hybrid strain than that of parent strain. The gel electrophoresis of protein of all the stages studied revealed that there was a new band in the mature stage of the basidoiocarp. The DNA-AP-PCR studies revealed that the genotype analysis of the parent and protoplast-fusant hybrid strains exhibited different DNA fingerprinting.

Key Words : *Volvariella volvacea*, protein, protoplast fusion, DNA

INTRODUCTION

In 1970, the critical energy crisis engulfed the world. During this time the scientists worked extensively on the recycling of organic wastes in order to combat this crisis and also to control the environmental pollution. Besides, this malnutrition is also creating a serious problem among the population of developing and underdeveloped countries. The low protein content of the food is one of the most important factors of malnutrition in India. In order to combat this protein maluntrition, new and cheap sources of protein are to be identified and these sources should be acceptable to the Indian population. Under the circumstances, cultivation of edible mushrooms utilising organic wastes will be an ideal proposal to combat the acute protein maluntrition and also to recycle efficiently the organic wastes for counteracting the problem of environmental pollution.

In West Bengal, Sur and Samajpati (1977) described the method of cultivation of *Volvariella diplasia* under agroclimatic conditions of West Bengal and reported about the role of different environmental and nutritional factors on the mycelial growth of *Volvariella diplasia.* They also reported about the role of temperature and pH on the yield of *Volvariella diplasia.*

Zhao and Chang (1997) studied the interspecific hybridization between *Volvariella volvacea* Vv 50

and *Volvariella bombycina* Vb 10 by protoplast fusion and subsequent polymerase chain reaction and pulsed-field gel electrophoresis of the fusant mycelial culture. Chiu and Moore (1999) reported the segregation of genotypically diverse progeny from self-fertilised haploids of *Volvariella volvacea.* Groot *et al.* (1998) reported the biochemical and molecular aspects of growth and fruiting in *Agaricus bisporus.* Cai *et al.* (1998) reported the beta-glucosidase components of the cellulolytic system of *Volvariella volvacea.* Chang and Miles (1989) furnished an excellent review about the physiology, biochemistry and molecular biology of the development of basidiocarp of edible fungi. No comprehensive information on the biochemical and genetical aspects of the development of basidiocarp of *Volvariella volvacea* from India has yet been reported.

In the present investigation an attempt has, therefore, been made to study some of the biochemical and genetical aspects of the basidiocarp development in *Volvariella volvacea.*

MATERIALS AND METHODS

Collection and preparation of tissue culture

Basidiocarps of *Volvariella volvacea* were collected from Nadia district, West Bengal, India which is situated between 22°45′N and 24°07′N latitude and 88°15′N and 88°50′W longitude and at an altitude of about 200 m above mean sea level. All the basidiocarps were collected during rainy season when these fungi grew abundantly in paddy straw heaps of the village farmers.

A small protion of the tissue from the juncture of pileus and stipe of basidiocarps, which was washed with 1% $HgCl_2$ previously, was removed aseptically from the freshly exposed surface and transferred to potato dextrose-agar medium slants supplemented with streptomycin sulphate (25 mg/l). These inoculated slants were incubated at 35°C (± 0.1°C) for 7 days in complete darkness for mycelial growth. The slants were subcultured repeatedly in the same medium at regular intervals.

All the tubes and flasks were maintained at 35°C in incubator. After 1-2 days pure mycelium started to grow on both the slants and the broth media. This pure culture was maintained throughout the period of study by subculturing at an interval of approximately a week and all the future experiments were carried out with this pure culture.

In order to study the physiological and biochemical aspects of the test fungus, screening test of medium was performed. A few number of liquid media were tried to determine the optimal mycelial growth of the fungus. However, the best medium was found to be glucose-asparagine (Lilly and Barnett, 1951) medium as a basal synthetic medium.

Protoplast Culture

The vegetative mycelium of *Volvariella volvacea* was grown in a liquid medium (pH 6.8) containing (w/v) glucose 20 g ; yeast extract 2 g and soytone 1.5 g at 32.°C in a rotary shaker (200 cycles per minute) for 4 days.

After four days the mycelia were harvested and washed three times with distilled water and then thrice with osmotic stabilizer solution (0.6 M NaCl in 0.01 M phosphate buffer ; pH 6.0). A Novozyme 234 soultion (5 mg/ml) was prepared in osmotic stabilizer solution and the enzyme solution was sterilized with the help of filter. A quantity of freshly harvested 4-days old mycelium (equivalent to about 20 mg dry wt.) previously washed with water and osmotic stabilizer, was incubated in 20 ml culture tubes with 3 ml of Novozyme 234 solution for 18 h at 32°C.

The released protoplasts were separated from the undigested and fragmented mycelia by filtering through G-2 sintered funnel and then centrifuged at 2000 g for 20 minutes. The sedimented protoplasts were suspended in osmotic stabilized solution and filtered through G-3 sintered funnel. The filtrate was again centrifuged at 2000 g for 20 minutes. The protoplast pellet was suspended in osmotic stablized solution and checked carefully under microscope to ensure the absence of any mycelial fragments. The number of protoplasts was counted with a hemacytometer.

The regeneration medium was prepared by adding glucose 10 g (w/v) and yeast extract 500 mg (w/v)

per liter in osmotic stabilizer solution and supplemented with agar 20 g (w/v). An aliquot of 0.1 ml of protoplast suspension containing about 60-70 protoplast was spread out on regeneration medium in Petriplate and incubated for 72 h at 32°C. Regeneration frequency was measured as the ratio of the number of colonies developed to the number of protoplasts added per plate of diluted protoplasts preparation.

The auxotrophs were produced by treating with UV-irradiation. An aliquot of 10 ml protoplast suspension (1.2×10^6) protoplasts / ml) was spread in a petridish (90 mm diameter) and irradiated with UV-irradiation for 15 minutes at 32°C in the dark. The UV-irradiation was provided with 15 W Philips germicidal lamp emitting at 254 nm at a dose rate of 1J / m^2 per second. After the exposure period the irradiated protoplasts were kept for 30 minutes in the dark to allow the full expression of the damage induced by UV-irradiation. The irradiated protoplasts were then diluted with osmotic stabilizer solution and plated for regeneration on solid medium. Simultaneously unirradiated controls were also prepared.

All the regenerated colonies were transferred to modified complete medium (CM) of Raper and Miles (1958). The MM contains (g/l) dextrose 20; K_2HPO_4 1; $MgSO_4$; $7H_2O$ 0.5; KH_2PO_4 0.46; agar (Difco) 15. The MM was suplemented with different amino acids (25 µg/ml), nucleic acid bases (20 µg/ml) or vitamins (1 µg/ml) to find out the nature of the auxotrophs.

For fusion of protoplasts, 1 ml of protoplasts suspension (about 10^7/ml) from two auxotroph strains (adenine requiring and aspartate requiring) were mixed

Maintenance

A polythene sheet was placed over the bed to cover but care was taken so that the sheet does not touch the bed. This was done to help the temperture to rise in the bed for the growth of the mycelium and also to keep the humidity high. Watering was done as and when required.

Cropping

Between 10-12 days after spawning, the pinheads or small white fruit-bodies were found to appear. At this stage the polythene sheet was removed for good ventilation. Within another 2 days the crops were ready for harvesting. The cropping continued for 2-4 days. The next flush was found to come after 8-10 days of gap period and the cropping continued for about 30 days.

Isolation of DNA and its analysis

Mycelial cultures of the material *Volvariella volvacea* were grown on PD broth medium prepared in 250 ml Erlenmayer flasks. After inoculation of the flasks with agar blocks from pure mycelial growth of *Volvariella volvacea* on PDA slants, the flasks were incubated at 30°C in complete darkness for 7-10 days. After the proper incubation period the mycelial mat was aseptically harvested from the flask, washed repeatedly with double distilled sterile water and stored at -40°C for future use.

The mushroom was cultivated as described previously and when the fruit body started to appear they were collected at three different maturation stages, namely, the initial, the button and the fully grown. All these samples were collected wearing a pair of gloves and with a sharp sterile scissors. These samples were washed with double distilled sterile water and then stored at -40°C for future use.

All the glass goods were also washed thoroughly first with detergent and then rinsed with double distilled sterile water and lastly made oven dry prior to use.

The extraction buffer for fungal DNA was prepared by taking 15% sucrose, 50 mM Tris-HCI (pH 8.0), 50 mM Na_2EDTA and 250 mM NaCl (buffer 1). After preparation of the solution on double distilled water the buffer was made sterile at 121°C for 15 minutes. After sterilization it was stored at 4°C. The second extraction buffer was prepared by mixing 10 mM Tris-HCl (pH 8.0) and 1 mM Na_2EDTA (buffer 2). The solution was made in double distilled water and autoclaved at 121°C for 15 minutes and stored

at 4°C. Sodium dodecyl sulphate 20% (w/v), was prepared in double distilled water and made sterile at 121°C for 15 minutes. This solution was stored at room temperature. Two different solutions of 5 M ammonium acetate and 3 M sodium acetate were separately prepared and after autoclaving at 121°C for 15 minutes stored at 4°C. Aqueous solution (70%) for dehydrated alcohol was also freshly prepared.

A stock solution of Rnase A was prepared as 1 mg in 1 ml sterile double distillted water. To make the working standard 100 µl of this stock solution was diluted to 1 ml. The stock solution of proteinase K was prepared by dissolving 2 mg in 1 ml sterile double distilled water. 100 µl of this stock solution was diluted to 1 ml with sterile double distilled water which served as the working standard. Phenol used in the isolation was first liquefied and made double distilled by an all glass distillation set. This double distilled liquid phenol was washed twice with 1 M Tris-HCl solution of pH 8.0 in a magnetic stirrer for 1-1½ h. After completion of the stirring the buffer was poured off and the phenol was kept immersed in 0.1 M Tris-HCl of pH 8.0 with 1-2 drops of 8-hydroxyquinoline.

Isolation of DNA from the sample

About 10 g of frozen mycelium was ground in liquid nitrogen using a mortar and pestle. The powder was readily suspended in about 100 ml of extraction buffer 1. The homogenated material was centrifuged at 5000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended gently in 100 ml of buffer and 2.5 ml of 20% SDS was also thoroughly mixed with it by gentle tapping. The total mixture was heated at 70°C for 5 minutes in water bath. After this incubation, 50 ml of 5 M ammonium acetate was added to the mixture and mixed with gentle tapping. Again the mixture was incubated at –20°C for 30 minutes. The mixture was then centrifuged at 10,000 rpm for 10 minutes at 4°C. This time the supernatant was decanted and the pellet was discarded. 2.5 times of the supernatant volume of chilled ethanol was then added to it and mixed gently but thoroughly. The solution was then kept at –20°C for overnight. Next day, pellets were observed at the base of the tube. The tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant alcohol was poured off and the pellet was washed twice with aqueous solution (70%) of ethanol. In the next step the pellet was made dry and dissolved in required amount of solution 2 and was stored at 4°C.

To purify the DNA component from the adhering protein and RNA components and following procedure was followed.

First of all RNase A was added at the rate of 100 µg/ml to the DNA suspension as prepared above and incubated at 37°C for 1 h in water bath. After the above incubation, proteinase K was added to the suspension at the rate of 200 µg/ml and incubated again at 37°C for 2 h in a water bath. Double distilled water and buffer saturated phenol was then added to this DNA suspension in equal volume and mixed thoroughly but gently at room temperature for 10 minutes. In the next step, the tubes were centrifuged at 10,000 rpm for 10 minutes at 4°C. The top layer of the centrifuged product was taken out by a micropipette and a mixture of chloroform and isoamyl alcohol (24 : 1) and phenol in 1:1 (v/v) was added to that layer and gently mixed for 10 minutes at room temperature. Again the mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C. The top layer was extracted similarly as described in the previous step and mixed with the mixture of chloroform and isoamyl alcohol (24 : 1). Again centrifugation was done at 10,000 rpm for 10 minutes at 4°C and the top layer was micropipetted out. 1-2% (v/v) 3 M sodium acetate was added to this top layer and kept at –20°C for 30 minutes. 2.5 times of chilled ethanol was added to it and the total solution was kept at –20°C for overnight. In the next step centrifugation was done at 13,000 rpm for 10 minutes at 4°C. The alcohol was poured off and the pellet was washed twice with freshly prepared aqueous solution (70%) of ethanol and finally dissolved in required amount of sterile double distilled water and kept at 4°C. Lastly to check the intactness of the DNA, the solution of DNA was run on an 0.8% agarose gel in a horizontal gel system.

RAPD analysis of the isolated DNA

Individual genomic DNA isolated from each of the four different growth stages of the material used as the template in the PCR amplification. The isolated

DNA was denatured for 5 minutes at 94°C and immediately cooled in ice for 3 minutes. After this 2 ng of the denatured DNA was added to a reaction tube containing 24 µl of the following mixture : 10X taq buffer containing 20 mM Tris-HCl (pH 8.4), 10 mM (KCl, 3 mM $MgCl_2$ and 0.2 mg/ml gelatin; 0.1 µM primer 1, primer 2 and primer 3 (Genei Laboratory) : 60 µM each of dATP, dCTP, dGTP and dTTP and 15 units of taq DNA polymerase. A drop of mineral oil was added to the 25 µl reaction mixture to minimize evaporation from the reaction tubes. The reaction tubes containing the mixtures were placed in a DNA Thermal Cycler of Perkin Elmer. The DNA amplification was performed with an initial cycle of denaturation (2 min. at 94°C); followed by 35 cycles of denaturation (2 min. at 94°C), annealing (2 min. at 40°C) and extension (1.30 min. at 72°C). Lastly, a final incubation was done at 72°C for 10 minutes.

After amplification, each reaction mixture was mixed well with 100 µl of chloroform and the aqueous phase was separated from the mineral oil-chloroform phase.

To assay the amplification products the method of agarose gel electrophoresis was used. A 1.5% agarose gel containing 89 mM Tris (pH 8.0), 89 mM H_2BO_3, 2 mM EDTA and 1.34 mM ethidium bromide was used for the assay. 2 µl of a loading and tracking dye (30% glycerol and 0.25% bromophenol blue in distilled water) were added to the amplification reaction mixture and 10 µl of the amplified sample was loaded onto the gel. Electrophoresis was conducted at a constant 80 V. After the electrophoresis the amplified products were viewed and photographed using a transilluminator.

RESULTS AND DISCUSSION

In the present investigation, attempts have been made to study some of the biochemical and genetical aspects of the basidiocarp development in the *Volvariella volvacea.*

Comparative account of the basidiocarp of the parent and protoplast hybrid strains

The parent and protoplast-hybrid strains of *Volvariella volvacea* were cultivated on paddy straw following the usual method of cultivation as described in Materials and Methods section.

The data revealed that the basidiocarps were almost similar except creamy white coloration of the pileus, less sturdy pileus, whitish gills, in the protoplast-hybrid strain.

The cultural characteristics also revealed that in protoplast-hybrid strain pure creamy white mycelial growth with light brown chlamydospores were obtained and the size of the chlamydospores were larger than that of the parent strain. The cultivation was done under natural conditions throughout the year. The data revealed that the maximum yield of basidiocarp was obtained in the month of May in both the strains. The parent strain could produce basidiocarp during the period of March to October of the year when the temperature varied from 33.4°C to 31°C. But the protoplast-hybrid strain could produce basidiocarps during the entire period, i.e. January to December with range of temperature between 25.1° — 36.1°C. But the yield was less in the months of January, February, November and December.

Protein determination

The protein content of the vegetative mycelium, button stage, egg stage and matured stage of the basidiocarps produced by the parent and protoplast fusant hybrid strains were determined following the standard methods.

The experimental data obtained are given in Table 1 and Fig. 1.

Table 1 : Total protein content (average of three replications) of the parental and protoplast-fusant hybrid strains of *Volvariella volvacea.*

Stages of development	Protein content (%)	
	Parental	Protoplast-fusant hybrid strains
Vegetative mycelium (15 days old)	25.20 ± 3.26	28.26 ± 3.40
Fruit body initiation (button stage)	30.82 ± 5.42	32.26 ± 5.62
Fruit body egg stage	24.26 ± 3.75	27.10 ± 4.12
Fruit body mature stage	22.10 ± 4.32	23.82 ± 3.84

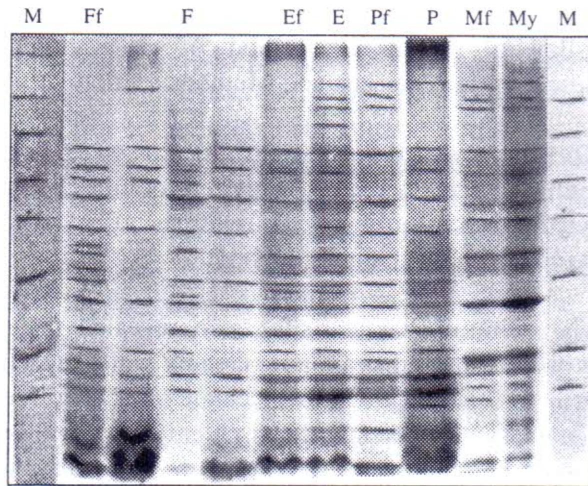

Fig. 1 : The protein bands of mycelium (My, Mf), basidiocarp pinhead (P, Pf), egg (E, Ef) and nature fructifrcation (F, Ff) stages of both parent and protoplast hybridized strain of *V. volvacea* by SDS-PAGE.

The data revealed the in both the parent and protoplast fusant hybrid strains the maximum protein content was found in button stage which was 30.82% and 32.26% respectively. This was followed by vegetative mycelium, egg stage and mature stage where it was 25.20% and 28.26%, 24.26% and 27.10% and 22.10% and 23.82% respectively.

The data further revealed that in all stages, the protein content was more in protoplast fusant hybrid strains than that of the parent ones.

From the Fig. 1, It is evident that there are about 11 distinct deeply stained bands accompanying with other fine faint very lightly stained bands in the mycelia of *V. volvacea*. In the button and egg stages there are marked differences in the number of bands and there was definitely one new protein band found in both cases. In the mature stage there are six prominent bands and a few very lightly stained bands only. There are three distinct bands of 16.5 K Da, 18 K Da and 26 K Da at the pinhead stage which were not present at the mycelial stage and one 16 K Da band was found in the present at the mycelial stage and one 16 K Da band was found in the mycelial stage. But this band (16 K Da) was absent in the basidiocarp stage.

DNA-Arbitrary-primed polymerase chain reaction (PCR)

Chiu *et al.* (1998, 1999) used the PCR technique with arbitrary primers for typing strains of fungi. After the extraction of genomic DNA of *V. volvacea*, the concentration and purity of DNA were measured by the spectrophotometric absorbance and the ratio greater than 1.8 of OD_{260} : OD_{280} was taken for PCR study.

Three arbitary primers were used. These were

1. 10 mer (5′–AGCCAGCGAA-3′)
2. 10 mer (5′–AATCGGGCTG-3′)
3. 10 mer (5′–TGCTCTGCCC-3′)

the genotype analysis by AP-PCR using above mentioned three primers were found to have identical DNA fingerprints of mycelium and fruit body of *V. volvacea* (Fig. 2).

But the genotypic analysis of the parent mycelia and protoplast fusant hybrid mycelia by AP-PCR using primer (5'–CGCCAGGGTTTTCCCAGTCA CGAC-3') exhibited different fingerprints (Fig. 3).

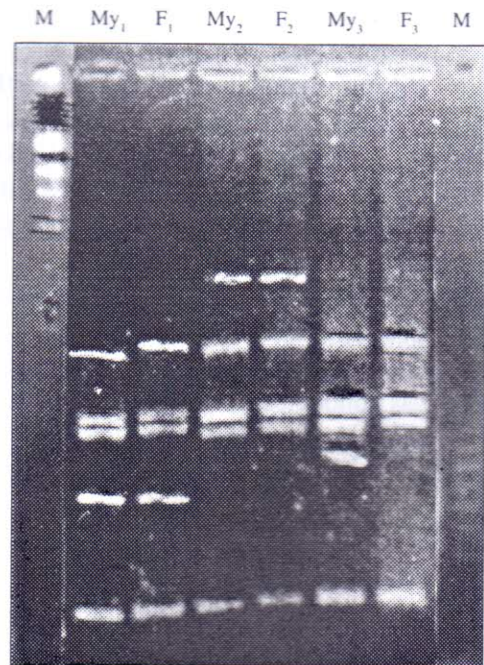

Fig. 2 : DNA fingerprint of the mycelium (My1, My2, My3) and Fructifucation (F1, F2 & F3) stages of *V. volvacea* using primer 1 (5′AGCCAGCGAA3′) primer 2 (5′AATCGGGATG3′) and primer 3 (5′TGCTCTGCC C3′) respectively.

PCR analysis of DNA obtained from mycelial and fruit body stages, amplified bands appeared between

1000 to 100 bp region. For the primer 1 one band apeared between 600-500 bp region which was absent in other two primers. In case of primer 2 one amplified band appeared having more than 1000 bp MW. which was absent in case of amplification products using 1 and 3 primers.

The data on the mycelial growth and yield of protein by the mycelia of *V. volvacea* in the basal liquid medium and the optimal liquid medium (formulated on the basis of experimental results) revealed that up to 12 days of incubation of growth and yield of protein increase gradually from day 1 and the growth and yield of protein are more in optimal liquid medium than that of the basal liquid medium. Similar observations are reported by several scientists earlier.

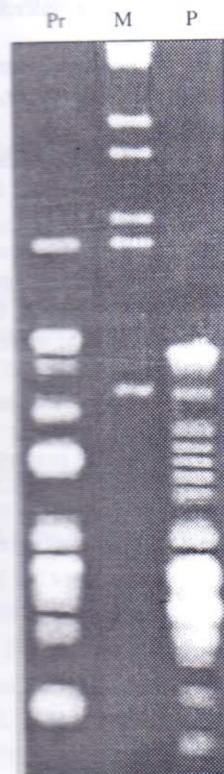

Fig. 3 : DNA fingerprints of fusant protoplast mycelia (Pr) and parent mycelia (P) of *V. volvacea* revealing differences.

The data on the release of protoplasts and their regeneration revealed that Novozyme 234+ cellulase+ chitinase and pectinase mixture enzyme solution was the best for regeneration purposes. The data also revealed that four days incubation was best for the release of protoplasts. Sodium chloride as osmotic stablizer and phosphate buffer (pH 6.0) were the best for release of protoplasts. The regeneration percentage of protoplasts was very low (6.36%) in *V. volvacea*. Similar observations were reported by Mukherjee and Sengupta (1986) on *V. volvacea*, Mukherjee and Sengupta (1992) and Das and Mukherjee (1995, 1996) on *Termitomyces clypeatus, Pleurotus sajor-caju, P. florida and P. ostreatus* respectively. The data also revealed that about 94.34% (average) monosporous isolates of Asp^- mutant protoplast strains and about 91.97% (average) monosporous isolates of $Aden^-$ mutant protoplast strains of *V. volvacea* retained their parental phenotype characters. Similar observatios were reported by Chang and Li (1991) on *V. volvacea* and Wang *et al.* (1991) on *Agaricus bisporus.*

It is known that *V. volvacea* is a primary homothallic species, i.e. self-fertile without any crossing. Chang and Yau (1971) first observed that there is a great variation among the monosporous isolates of *V. volvacea*. In order to find out the genetics of sexuality in this fungus the release of protoplasts and their regeneration were done by Santiago (1982 a, b), and Stille (1984).

The mushroom, *V. volvacea* has a haploid-diploid-haploid lifecycle. The majority of the basidiospores are uninucleate and several of them produce self fertile mycelia (Chang and Yau, 1971; Li and Chang, 1982; Chiu and Moore, 1999). There is evidence of conventional Mendelian segreggation as found in the present investigation and also reported earlier by Chang and Yau (1971), Chang (1978), Chang and Li (1991) and Royse *et al.* (1993). In *V. volvacea* it was reported that 50% of the progeny are self-fertile (Chiu and Moore, 1999).

In the present investigation it was observed that there is a difference in genomic AP-PCR in the parent and protoplast fusant hybrid strains. This clearly justified the differences in the yield behaviour of the two strains. But how the differences in DNA was achieved is not clearly understood from the present data of investigation. Chiu and Moore (1999) reported that similar differences in certain DNA bands occur in the

protoplast fusant and parental strain of *Volvariella bombycina* and *V. volvacea.*

REFERENCES

Cai, Y. J., Bushwell, J. A. and Chang. S. T. 1998. Beta glucosidase components of the cellulolytic system of the edible straw mushroom, *Volvariella volvacea. Enzyme–Microb. Technol.* **22**(2) : 122-129.

Chang, S. T. 1978. *Volvariella volvacea — The Biology and Cultivation of Edible Mushrooms.* Ed. S. T. Chang and W. A. Hayes. Academic Press. Ine. New York, pp 573-603.

Chang, S. T. and Li, S. 1991. Genetical studies on the sexuality pattern of *Volvariella volvacea. Sci. & Cult. Edible Fungi.* pp 119-122.

Chang, S. T. and Miles, P. G. 1989. Mushroom formation : Effects of environmental, nutritional and chemical factors. In *Edible Mushrooms and Their Cultivation.* S. T. Chang and P. G. Miles, CRC Press. pp 115-131.

Chang, S. T. and Yau, C. K. 1971. *Volvariella volvacea* and its life history. *Amer. J. Bot.* **58**(6) : 365-371.

Chiu, S. W. and Moore, D. 1999. Segregation of genotypically diverse progeny from self-fertilised haploids of the Chinese straw mushroom, *Volvariella volvacea. Mycol. Res.* **103**(10) : 1335-1345.

Das, N. and Mukherjee, M. 1995. Conditions for isolation of regenerating protoplasts from *Pleurotus sajor-caju. J. Basic Microbiol.* **35**(3) : 157-161.

Das, N. and Mukherjee, M. 1996. Preparation and regeneration of mycelial protoplasts of *Pleurotus florida* and *P. Ostreatus. Folia Microbiol.* **41** : 208-210.

Groot, P. W. J-de., Vissec, J., Griensven, J. D-van. and Scoop, P. J. 1998. Biochemical and molecular aspects of growth and fruiting of the edible mushroom *Agricus bisporus. Mycol. Res.* **102**(11) : 1297-1308.

Li, G. S. F. and Chang, S. T. 1982. The nucluc acid content of some edible mushrooms. *Eur. J. Appl. Microbiol. Biotechnol.* **15** : 237-240.

Lilly, V. G. and Brarnett. H. L. 1951. *Physiology of the Fungi* Mc. Graw Hill Book Co. London. 427.

Mukherjee, M. and Sengupta, S. 1986, Mutagenesis of protoplasts and regeneration of mycelium in the mushroom *Volvariella volvaeca. Appl. Environ. Microbiol.* **52**(6) : 1412-1414.

Mukherjee, M. and Sengupta, S. 1992, Induced mutation of mycelial protoplasts of mushroom, *Termitomyces Clypeatus* for obtaining auxotrophie mutants. *Ind. J. Exp. Biol.* **30** : 1206-1207.

Raper, J. R. and Miles, P. G. 1958. The genetics of *Schizophyllum commune. Genetics.* **43** : 530-546.

Royse, D. J., Bunyard, B. A. and Nicholson, M. S. 1993. Molecular genetic analysis of diversity in population of edible mushrooms. In *Mushroom Biology and Mushroom Products,* Ed. by S. T. Chang, J. A. Buswell and S. W. Chiu, The chinese University Press. pp. 49-54.

Santiago, C. M. 1982a. Protoplast isolation in the common tropical mushrooms using microbial enzyme. *Kali. Phil. J. Biol.* **11** : 365-371.

Santiago, C. M. 1982b. Production of *Volvariella* protoplasts by use of *Trichoderma* enzyme. *Mush. Newsletter. Tro.* **3** : 3-6.

Stille, B. 1984. Release of protoplasts from *Volvariella bombycina* (Schaeff. ex. Fr.) Singer, a method developed. *Mush. Newsletter. Trop.* **4** : 15.

Sur, C. K., and Samajpati, N. 1977. Cultivation of paddy straw mushroom in West Bengal, *Ind. Agric.* **21** : 145-148.

Wang, Z. S., Liao, J. H. and Wang, H. C. 1991. Protoplast fusion and fusant variation in *agricus bisporus. Scr. & Cult. Edible Fungi* 9-12.

Zhao, J. and Chang, S. T. 1997. Interspecific hybridization between *Volvariella volvarcea and Volvariella bombycina* by PEG-induced protoplast fusion. *World J. Microbiol. Biotechnol.* **13**(2) : 145-151.

(Accepted for publication December 12 2005)