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Isolation of monospores of *P.ostreatus* and *P.djamor* for interspecific hybridization and effect of temperature on dikaryotics

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Oyster mushroom (*Pleurotus spp*.) commonly called as Dhingri in India, is one among the commercially cultivated mushrooms throughout the world and stands second in world production. However, cultivated mushrooms face problems like loss of genetic diversity and strain degeneration. Hence, there is an increased demand for the development of new and improved strains with high production and better growth attributes. The present study was conducted to improve the strain of *Pleurotus* species by interspecific hybridization through crossing single spore cultures. *Pleurotus djamo*r is an early yielding pink coloured oyster mushroom having leathery texture and low biological efficiency (BE). It was crossed with the high yielding, *Pleurotus ostreatus*, which requires comparatively more days for primordial initiation. The experiment was conducted during 2021-2022 to find out the monokaryotic isolates of *P. ostreatus* (PO)and *P. djamor* (oyster pink or OP) for its dikaryotization with the help of rapidity in the growth performance. Seventeen monokaryotic cultures of *P. ostreatus* and twenty monokaryotic cultures of *P. djamor* were isolated by spore print, serial dilution and hyphal tip fragmentation techniques. Seventeen and twenty monokaryotic isolates of *P. ostreatus*, and *P. djamor* respectively were grown on Malt Extract Agar media (MEA) for testing their rapidity in radial growth. After the selection of monokaryotic isolates, interspecific hybridization was done between the monokaryotic and the dikaryotic culture was again tested in malt extract agar media under various temperature (24^0 C – 32^0 C) to checktheir growth performance. In MEA media, the highest AUMGC monokaryotic isolates viz.PO5M, PO6M PO7M for *P.*ostreatus ,OP1M,OP10M,OP11M,OP15M,OP16M,OP19M and OP 20 M for*P. djamor*(oyster pink/OP) was finally selected for hybridization. Among the sixty-two dikaryotic cultures tested at 24^0 C – 32^0 C .it was found that 30^0 C was best for all isolates but from sixty-two isolates, thirty-three dikaryotic isolates were able to grow at 32^0 C, which may be indicating the differences in tolerance of high temperatures.

Keywords: Dikaryotization, Malt extract agar, Monokaryotic, *Pleurotus djamor, Pleurotus ostreatus*

INTRODUCTION

High production and productivity, along with good quality produce are always the principal goals for agriculturally important crops including mushrooms. Mushrooms have been consumed by humans for their nutritional and therapeutic values for centuries throughout the world. Among edible mushrooms, *Pleurotus* species stand second in world production with 19 per cent contribution to total mushroom production (Sharma *et al*. 2017). Different species of *Pleurotus* grow well in wide temperature limits and thus is suited for year around cultivation in various regions of tropical country like India. Owing to their favourable organoleptic, nutraceuticals and therapeutic properties, its popularity is increasing worldwide, particularly in Asia and Europe. In addition to this, they have high biological efficiency and significant levels of proteins (Kalmis*et al*. 2008), while low in fats, calories, sodium, carbohydrates, and cholesterol (Gupta *et al*. 2011). The cultivated mushrooms face the problems like loss of genetic diversity and strain degeneration (Wang *et al*. 2012). Under the above scenario, there is a need for development of improved strain in *Pleurotus* spp. to ensure both cultivator and consumer preferences. There is another problem faced by the mushroom growers that is high temperature between 27 -35^0 C, during the day (Hernandez and Salmons,2008). Therefore, the selection for tolerance to high temperature is essential to obtain optimum yield and quality. *P. ostreatus* required 21.4 days for pinhead initiation in paddy

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straw substrate (Khaliq *et al*. 2013) with BE of 66.33 per cent (Sharma and Sharma, 2014). Pinheads emerged within 16 days in pink coloured *P. djamor,* with BE of 43.05 per cent in paddy straw (Satpal *et al.* 2017; Jose, 2018). These species of *Pleurotus* could be exploited to aggregate the beneficial growth attributes, thus improving the strain with high BE and shorter duration. Genetically improved strains not only increase the quality of cultivated mushrooms, but also reduce the cost of cultivation. They can also increase farmers' revenue in short term (Avin *etal.* 2012) Now-a-days for strain improvement in mushrooms, several modified breeding techniques such as selection and hybridization by the process of protoplast fusion, use of chemical mutagenesis, use of resistance markers, have been employed with new findings of high yielding, more nutritious, disease resistance and more biological efficiency. Among the various methods used for strain improvement, hybridization method has been proved to be the best and most sustainable for bringing genetic recombination and developing somatic hybrids. Therefore, the present study aimed at investigations of monokaryotic isolates and hybridization between the selected monokaryotic isolates of among the given species of *Pleurotus* and the newly formed strains were evaluated under different temperature to observe the mycelial growth of the dikaryotic isolates.

METHODS AND MATERIALS

Place of experimentation

The laboratory experiments were done in research Laboratory, Department of Plant Pathology, Uttar Banga Krishi Vishwavidyalaya, Pundibari, Coochbehar.

Media

The culture media were sterilized in an autoclave at 121^0 c for 15 minutes at 15 p.s.i. In this course of investigation, Malt extract agar (MEA) were used.

Mushroom species used and their maintenance

Two *Pleurotus* species namely *P.ostreatus* and *P.djamor* were collected from Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya.. From the tip of hyphal segment of mycelium was taken and transferred to MEA slants to get the pure cultures of these two species.

Isolation of monokaryotic culture of P.ostreatus and P.djamor

Isolation of monokaryotic culture is one of the most important steps to develop homokaryons before aiming hybridization for strain improvement. Various techniques such as Spore prints, serial dilution was followed to get the monousporus culture of these two species.

Spore print method

This method (Peterson and Ridley,1996) was followed for single spore isolation of *P.ostreatus* and *P.djamor* (oyster pink) (Fig.1 A,B)..

Serial Dilution method

The serial dilution method demonstrated by Bahukandi and Sharma (2002) was followed to get the monosporous cultures of the three species(Fig.1 C).Spore suspension was prepared from the spore print obtained by placing fresh mature sporocarp on a sterilized Petri plate. It was further diluted up to 10^{-4}, such that the spore concentration was as low as four to five spores when observed in Petri plate under low power microscope (100X magnification). The diluted spore suspension (1 mL) was pipetted out to the centre of sterile petri plate. A thin layer of two per cent plain agar was then poured into the Petri plate and was uniformly mixed. The spores were marked under low power microscope and were allowed to germinate for two days. The marked germinated spores were then picked by a sterile inoculation needle and were grown in potato dextrose agar (PDA) media at 28^0 C.Single spore cultures obtained were observed for their mycelial appearance and radial growth rate. Among the cultures, the fast growing, dense single spore cultures were selected from each strain.

Growth performance of Homokaryotic mycelium of P.ostreatus and P.djamor

Monosporous cultures were tested in 90 mm sterilised petri plates containing 15 ml of malt extract agar (MEA) media, where they were

inoculated aseptically with mycelial discs of 5 mm size taken from the margins of actively growing single spore isolates and incubated at 28 ^{0}C to determine the best suitable and fastest growth of homokaryotic isolates of those two species.Malt extract agar was chosen because, according to earlier research by Stanley and Nyenke (2011).Each isolate was replicated three times. Data pertaining to radial growth of the mycelium were recorded at three days of interval after inoculation. Isolates of these two-speciesorigin showing higher growth rate (AUMGC) was selected for hybridization.

Area Under Mycelial Growth Curve (AUMGC):

AUMGC calculated by:

$$\text{AUMGC} = \sum_{i=0}^{n} [\{(X(i+1) + Xi)/2\}(T(i+1) - Ti)]$$

in which X_i = colony diameter, expressed in mm at the i^{th} observation, t_i = time (days after inoculation) at the i^{th} observation, and n = total number of observations (Mueller *et al.* 2002).

Inter-specific Hybridization

The single spores isolates having higher growth potential was first inoculate separately in petri-plates containing malt extract agar media. Crossing between two homokaryotic isolates *P.ostreatus* and *P.djamor* (Table1)was performed by taking mycelial discs (five mm) of ten-day old single spore cultures were placed two cm apart in a nine cm diameter Petri plate containing malt extract agar media and was incubated at 25^0 C for seven days(Fig. 2 A, B).Based on the prominent interaction or after the contact of two colonies a small amount of inoculum was taking by chopping from the meetings point of two different isolates and dikayrotization was confirmed by presence of clamp connections(Fig. 2 C,D).Then, a culture disc of five mm size was cut off from the confluence region on the Petri plate and sub-cultured to MEA plate. In this way sixty-two of PO x OP dikaryotic isolates were picked from region. The heterokaryotic mycelia thus produced were screened; fast growing and thick stranded cultures were selected. These hybrids were cultured for three generations to see whether there is any segregation of characters or not.The third-generation hybrids were taken for further qualitative and quantitative studies.

Effect of temperature on interspecific strains of P. ostreatus and P.djamor

For studies on variability, sixty-two dikaryotic isolates were incubated at four different temperature viz.24 ^{0} C,28^0 C.30^0 C and 32^0 C (Fig.3A-D) Petri plates containing 15 ml of sterilized MEA medium were inoculated at the centre with five mm cork borer from 10 days old actively growing mycelium under aseptic conditions. Three replications for each treatment were maintained and growth performance was recorded separately. Further selection was made based on growth performance of dikaryotic isolates.

Statistical analysis

The laboratory trials were conducted following completely Randomized design. The replicated data generated from different experiments were analysed statistically using software JMP 8 and the ANOVA determined the probability for significant variation among the treatments.

RESULTS AND DISCUSSION

Selection of homokaryotic strains of P.ostreatus and P.djamor

Isolation of monousorus culture is the first step of hybridization in mushroom. Twenty monosporus strains of *P. djamor (*oyster pink/OP) designated as OP 1M to OP 20 M and seventy strains of *P.ostreatus* designated as PO 1M to PO 17M were isolated following the method described earlier (Fig. 3 E). The strains were grown on malt extract agar (MEA) media considered as homokaryons which evaluated for the variation in their growth rate in MEA.

The AUMGC (Area under mycelium growth curve) was calculated by following the formula.The highest AUMGC was recorded by PO 7M (208) and the second highest was PO 5M with a reading of 207 AUMGC and followed by PO 6M, recoded 200.3 AUMGC. So comparing the overall

Table 1. Parentage and interspecific hybrids of *Pleurotus*

Parentage	Hybrids
PO 5M x OP 1M	PO x OP1
PO 5M x OP 10M	PO x OP 2, PO x OP 3, PO x OP 4
PO 5M x OP 11M	PO x OP 5, PO x OP 6, PO x OP 7, PO x OP 8
PO 5M x OP 15M	PO x OP 9, PO x OP 10, PO x OP 11
PO 5M x OP 16M	PO x OP 12, PO x OP 13, PO x OP 14
PO 5M x OP 19M	PO x OP 15, PO x OP 16, PO x OP 17
PO 5M x OP 20 M	PO x OP 18
PO 6M x OP 1M	PO x OP 19, PO x OP 20, PO x OP 21,
PO 6M x OP 10M	PO x OP 22, PO x OP 23, PO x OP 24
PO 6M x OP 11M	PO x OP 25, PO x OP 26, PO x OP 27
PO 6M x OP 15M	PO x OP 28, PO x OP 29, PO x OP 30, PO x OP 31
PO 6M x OP 16M	PO x OP 32,
PO 6M x OP 19M	PO x OP 33, PO x OP 34, PO x OP 35, PO x OP 36
PO 6M x OP 20 M	PO x OP 37, PO x OP 38, PO x OP 39, PO x OP 40
PO 7M x OP 1M	PO x OP 41
PO 7M x OP 10M	PO x OP 42, PO x OP 43, PO x OP 44, PO x OP 45
PO 7M x OP 11M	PO x OP 46, PO x OP 47, PO x OP 48, PO x OP 49
PO 7M x OP 15M	PO x OP 50, PO x OP 51, PO x OP 52, PO x OP 53, PO x OP54
PO 7M x OP 16M	PO x OP 55, PO x OP 56, PO x OP 57
PO 7M x OP 19M	PO x OP 58, PO x OP 59,
PO 7M x OP 20 M	PO x OP 60, PO x OP 61, PO x OP 62

PO:*P.ostreatus,* OP: *P.djamor/oyster pink*

growth performance of the homokaryons of *P.ostreatus* ,there highest growing strains namely PO5M, PO6M, PO7M were selected as parents to be used in interspecific hybridizations(Fig. 4)Regarding the growth performance of homokaryons of *P.djamor* ,it was observed that highest AUMGC was recorded by OP 11M with reading of 238.33 AUMGC. The second highest AUMGC was observed in OP 10M (216.67) followed by OP 20M (211), OP 1M (173.33), OP15M (174.67), OP16M (169.33) and OP 19M (153 AUMGC). So from the overall growth performance of homokaryons of *P.djamor*,strains namely OP 1M ,OP 10M ,OP11M, OP15M, OP16M, OP19M and OP20M from the above experiment were selected as a parents to be used in hybridization(Fig .5).

Medium for hybridization

The results mentioned above made it clearly apparent that MEA were the ideal media for *P. ostreatus* and *P. djamor* monokaryotic strains.It was also observed that MEA, which has the best average growth rates for both *Pleurotus* species, is the ideal medium for interspecific hybridization between monokaryotic strains of *Pleurotus ostreatus* and *Pleurotus djamor.*This result supports the earlier findings of Hernandez and Salmones (2008).

Fig. 1 (A-C):(A) spore discharge (B) spore print (C) isolation of spore though serial dilution

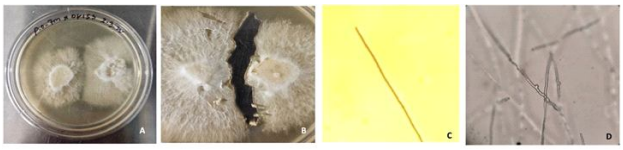

Fig. 2 (A-D):(A) Somatic hybridization of *Pleurotus* (B) Isolation of hybrids from meeting points (C) Hyphal tip without clamp (D) Presence of clamp connection,

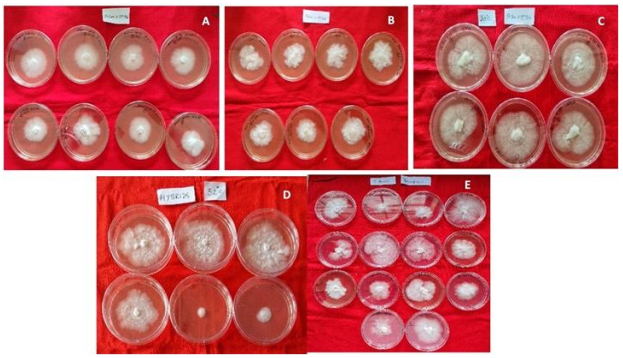

Fig.3 (A-D): Dikaryotic isolates at (A) 24^{0}C (B) 28^{0}C (C) 30^{0}C (D) 32 ^{0}C; (E) Monousporos culture

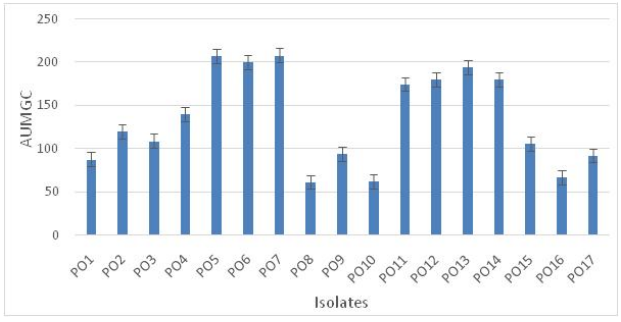

Fig.4: Growth rate (AUMGC) of *P.ostreatus*monosporous culture in MEA med

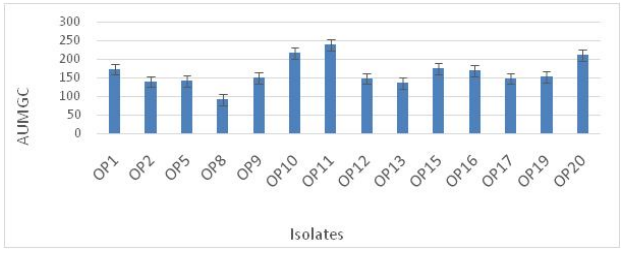

Fig.5: Growth rate (AUMGC) of *P. djamor*monosporous culture in MEA media

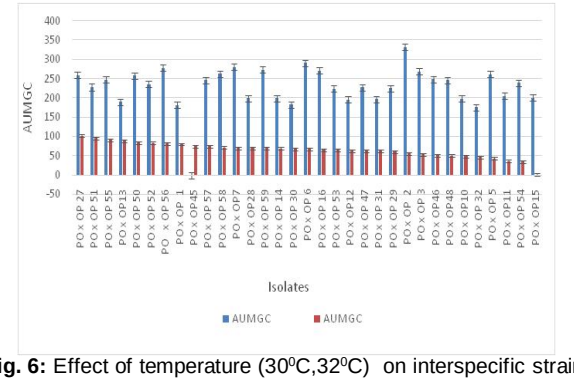

Fig. 6: Effect of temperature (30^{0}C,32^{0}C) on interspecific strains.

Effect of temperature on radial growth of interspecific strains

Temperature is a very important environmental factor for mycelium growth. This experiment was conducted to study the effect of range of temperature (24 -32^{0}C) on the radial growth of sixty-two dikaryotic isolates. From the ANOVA table it reveals that significant impact of treatment, isolates and their interaction on the observed data variances. In present study, mycelium growth was significantly higher at 30 ^{0}C irrespective isolates (Fig.6). This finding is in accordance with Vilas *et al*. (2020) who observed in their experiment that the mycelium growth rate of eleven *Pleurotus* isolates were significantly more at 30^{0}C.But in our experiment showed that among thesixty-two isolates tested in 32^{0}C temperature, thirty-three dikaryotic isolates showed mycelium growth, between them isolates PO x OP 27 was recorded with highest AUMGC at 32^{0}C as well as the mean irrespective of temperature, which may be indicating marked differences in tolerance of the isolates to high temperature. Temperature sensitivity is an index of *Pleurotus* spp. to revealed greater genetic differences among the genus *Pleurotus*.

CONCLUSION

In conclusion, variations in growth rate were observed in monokaryotic isolates in MEA medium and they are selected for dikaryotization of *P.ostreatus* and *P.djamor*. Dikaryotic mycelium performed best at temperature 30^{0}C for the fastest mycelium growth, however, some dikaryotic mycelium can tolerate temperature at

32^{0}C which can be grown on high temperature areas.

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DECLARATIONS

Conflict of interest: Authors declare no conflict of interest.

REFERENCES

Avin, F. A., Bhassu, S., Shin, T. Y., and Sabaratnam, V. 2012. Molecular classification and phylogenetic relationships of selected edible basidiomycetes species. *Mol. Biol*. Rep. **39**: 7355– 7364.

Bahukhandi, D., Sharma, R. K. 2002. Interspecific hybridization between Pleurotus species. *Ind. Phytopathol*. **55**: 61-66

Gupta, B., Reddy, B. N., Kotasthane, A. S. 2011. Molecular characterization and mating type analysis of oyster mushroom (*Pleurotus spp*.) using single basidiospores for strain improvement. *World J. Microbiol. Biotechnol*. **27**: 1-9

Hernandez, R. G.,Salmones, D. 2008. Obtaining and characterizing *Pleurotusostreatus* strains for commercial cultivation under warm environmental conditions. *Scientia Hort*. 118:106-110

Jose, A. 2018. Evaluation of biochemical and anticancerous activities of mushrooms. M. Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, pp-136.

Kalmis,E., Nuri,N., Hasan, Y., Faith.K.2008.Feasibility of using olive mill effluent (OME) as a wetting agent during the cultivation of oyster mushroom *Pleurotusostreatus*,on wheat straw.*Bioresour.Technol*.99:164-169

Khaliq, N., Khan, N. A.,Javed, N.,Gondal, A. S. 2013. Production efficiency of different strains of *Pleurotus ostreatus* using various cellulosic agro-wastes. *E Sci J. Plant Pathol.***2:** 37-43

Mueller, D. S., Dorrance, A. E.,Derksen, R. C.,Ozkan, E.,Kurle, J. E., Grau, C. R.,Gaska, J. M., Hartman, G. L., Bradley, C. A., Pedersen, W. L. 2002. Efficacy of fungicides on *Sclerotinia sclerotiorum* and their potential for control of Sclerotinia stem rot on soybean. *Plant Dis*. **86**:26-31

Petersen, R.H., Ridley, G.S. 1996. A New Zealand Pleurotus with Multiple Species Sexual Compatibility. *Mycologia*. **88:** 198-207

Sardar, H., Ali, M.A., Ayyub, C.M., Ahmad, R. 2015**.** Effects of different culture media, temperature and pH levels on the growth of wild and exotic *Pleurotus* species. *Pak. J. Phytopathol.***27:** 139-145

Satpal, S., Gopal, S.,Siddarth, R., Ankit, K., Bhanu, P., Priyanka, B., Ravikumar, P. 2017. Effect of different substrates on the growth and yield of oyster Mushrooms (*Pleurotus djamo*r). Int. J. Agric. Sci.**9**: 3721-3723

Sharma, R., Sharma, B. M. 2014. Strain improvement in *Pleurotus ostreatus* using UV light and ethyl methyl sulphonate as mutagens. *Afr. J. Microbiol. Res*. **8:** 432-436

Sharma, V. P.,Annepu, S. K., Gautam, Y., Singh, M., Kamal, S. 2017. Status of mushroom production in India. *Mushroom Res.***26:** 111-120

Stanley, H.O.,Nyenke, C.U. 2011. Cultural studies on mycelia of *Pleurotuspulmonarius* (oyster mushroom) in selected culture media. *International Journal of Sciences and Nature*. **2:** 183- 185

Vilas, P.M., Jadhav, A.C.,Dhavale, M.C.,Hasabnis, S.N., Gaikwad, A.P., Jadhav, P.R.,Ajit, P.S. 2020. Effect of cultural variability on mycelial growth of eleven mushroom isolates of *Pleurotus* spp. *J. Pharmacogn. Phytochem***.9**: 881-888

Wang, S., Yin, Y., Liu, Y., Xu, F. 2012. Evaluation of genetic diversity among Chinese *Pleurotus eryngii* cultivars by combined RAPD/ISSR marker. *Curr. Microbiol.***65**: 424–431