PHYLOGENY AND PHYSIOLOGY OF *PHELLORINIA* SPP.: A DELICACY OF INDIAN DESERT

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ABSTRACT

Phellorinia species is most commonly observed wild edible mushroom in the desert and have been very well reported from Rajasthan states. Forays have been made to collect, identify, catalogue and molecularly characterize this important mushroom of the desert region of Rajasthan state during August and September month of the year 2010 and 13. In total 85 specimens of the mushroom were collected from 12 different sites of Jodhpur, Jaisalmer districts. The Longitude and latitude of the sites ranged between N 26-27º, EO 71-72º and MSL 105-295m. The dominant vegetation at different sites was Leptadenia pyrotechnica, Calligonum polygonoides, Haloxylon recurvum, Lasirus sindicus and other desert grasses. All the samples were initially screened on the basis of colony characteristics and growth rate (3-7mm per day), which formed 29 groups. On the basis of DNA analysis using 10 random primers, the isolates have been categorized into 10 different groups. Nucleotide sequence comparisons of ITS 5.8S rDNA against NCBI databases showed 90 per cent identity with Phellorinia herculea. However, maximum likelyhood analysis of sequences using Mega 6 and Mr. Bayes software suggested the presence of at least three species of Phellorinia amongst the collections. Physiological studies have been made in order to domesticate the mushroom and results indicated, as malt extract medium to be the best while pH of 7.5 and temperature of 40°C are the optimal for the mycelial growth the mushroom. Preferred carbon and nitrogen sources for the growth of the mushroom were found to be dextrose and nitrates. Some success is achieved in domestication of this species.

Keywords: Wild mushroom, Desert, *Phellorinia*, Physiology, Molecular characterization

1. INTRODUCTION

Phellorinia is a genus of fungi in the Phelloriniaceae family of the Agaricales order. The genus is considered to be monotypic with the type species *Phellorinia herculeana* (Pers.) Kreisel [1], described by English naturalist Miles Joseph Berkeley in 1843 [2] as *P. inquinans*. The species *Phellorinia herculeana* is well distributed all over the world and is known by various species names. In India, the people of Rajasthan and adjoining areas of Sindh province in Pakistan are very much familiar with the Desert mushroom, *Podaxis pistillaris* and *Phellorinia herculeana*, commonly known as KHUMBHI and the false shaggy mane in English [3]. Local people collect this mushroom during rainy season and sell in market (Fig.1). It is most commonly observed in the desert and mountain (Thar and Kohistan) areas as well as in plains, meadows, coniferous forest etc. during and after rainy season of summer and rarely in winter too. It is very much relished by the local people, hence is hunted and eaten by the rural folk, since centuries. The mushroom is known for its nutritional and medicinal values amongst the rural masses.

Fig.1. Sale of *Phellorinia* in local market

The local Hakims (village doctors) of Sindh province of Pakistan use both the desert mushrooms in different ways for treatment of different food deficiencies and illness. Some times these are used with butter for bandage of broken bones [4, 5]. In countries like India, Afghanistan and Saudi Arabia, they are used as food [6]. It was often used by Aborigines to darken the white hair in the whiskers of old men, for body painting and as a fly repellent in Australia (Australian National Botanic Gardens: Fungi Web Site) [3]. Panwar and Purohit [7] reported its antimicrobial activities against *Pseudomonas aeruginosa* and *Proteus mirabilis*.

Limited information is available on molecular characterization, variability analysis and germplasm biodiversity of *Phellorinia* species throughout the world. Further, the

researchers have not attempted the physiological studies as well as artificial cultivation trials on this mushroom. In order to estimate the biodiversity and to standardize the artificial cultivation techniques of this mushroom, the studies have been taken up.

2. MATERIAL AND METHODS

Fungal Material

Forays have been made to collect, identify, catalogue and molecularly characterize this important mushroom of the desert region of Rajasthan state during August and September month of the year 2010 and 13. In total 85 specimens of the mushroom were collected from 12 different sites of Jodhpur, Jaisalmer districts of Rajasthan (India) (Fig.2). All the mushroom specimens were brought to the laboratory and cultured on malt extract agar medium (Malt extract 20g; Agar 20g). The tissue from the junction of pileus and stipe was used for the culturing (Fig.3).

Fig.2 Natural occurrence of *Phellorinia* in desert of Rajastahan

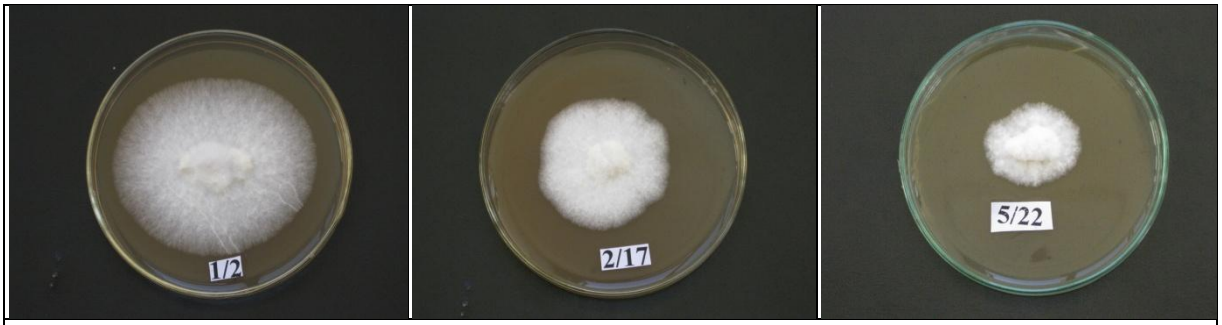


Fig.3. Pure culture of *Phellorinia*

DNA Extraction

Total genomic DNA was extracted from the mycelial cultures of 29 *Phallorinia* were raised in 150 ml conical flasks containing 50 ml of liquid culture medium (Malt extract- 10g L^{-1}; Glucose- 5g L^{-1}) for 10 days at 35±1°C under darkness and stationary culture conditions. Total DNA was extracted from 100 mg dried and liquid nitrogen ground mycelium by DNeasy plant mint kit (method of manufacturer Qiagen Ltd) and eluted in 100 µl of elusion buffer (Qiagen). The protocols suggested by the manufacturer were followed. The quantity of the DNA was determined by DNA flurometar (DyNA quant 200, Amarsham Biosciences).

RAPD Analysis

Multilocus genotyping by RAPD was performed using ten decamer arbitrary primers supplied by Operon Technologies namely, OPA-02, OPA-03, OPA- 08, OPA- 10, OPA- 13, OPA- 17, OPA- 19, OPA- 20, OPB-12, and OPH-11. Amplification was performed in a total reaction mixture of 25µl, each reaction mixture containing: decamer primer, 2 µl (50 pmol µl-1); dNTP mix, 2 µl (2 mM each from MBI, Fermentas); MgCl2, 1 µl (25 mM, MBI, Fermentas); Taq DNA polymerase, 1µl (5U µl-1, Sigma); 10x PCR buffer, 2.5 µl (100 mM, Tris-HCl, pH-8.3, 15 mM MgCl2, 250 mM KCl) and 16.5 µl of dH2O. To this 4µl of genomic DNA (approx 40-60 ng) was added. RAPD-PCR amplification were performed in a Master cycler (M J Research, USA) with lid heating option at 110 °C with initial denaturation step of 94 °C for 3 min followed by 36 amplification cycles of 94 °C for 40 sec, 50 °C for 40sec and 72 °C for 2 min and final elongation at 72 °C for 10 min. PCR amplification products were electrophoretically separated on 1.6% agarose gel (Sigma) prepared in 1x TAE. The ethidium bromide stained gels were visualized and photographed.

Amplification and Sequencing of ITS 5.8S rDNA Region

The polymerase chain reaction (PCR) was carried out in 50 µl reaction mixture containing: Taq DNA polymerase (1 Uμl^{-1}) (Sigma Chem.), 5 µl 10 x PCR buffer (10 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$. Sigma Chem.), 160 µM each of dATP, dCTP, dGTP and dTTP (MBI Fermentas), 50 pM each ITS-1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (5'-TCC TCC GCT TAT TGA TAT GC-3') primer, 2 µl of 5% glycerol, 40 ng of genomic DNA, and distilled water. Amplification of rDNA ITS region was performed in a master cycler, 34 cycles of 1 min denaturation at 95^0C, 30 s annealing at 50^0C, 80s elongation at 72^0C and ending by a 10 min final elongation step at 72^0C, with lid heating

option at 110^0C. The PCR-amplified ITS region containing ITS-1, 5.8S rDNA and ITS-2 was got sequenced from Delhi University.

Data Analysis

Nucleotide sequence comparisons were performed using Blast network and compared with database of the National Centre for Biotechnology Information (NCBI), USA data bases for molecular identification of showed 90 percent identity with *Phellorinia herculea.* The sequence data obtained from ITS-4 reverse primer were reversed and complimented using Gene doc software and clubbed with sequence data of ITS-1 to obtained complete sequences of amplified ITS product and nucleotide sequences were compared with data base of the National Centre for Biotechnology Information (NCBI) by using BLAST search. Species were identified using lowest e value (0.0) and maximum identity. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [8]. The tree with the highest log likelihood (-1169.7879) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 676 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [9]. The sequence data was also subjected to Baysian analysis to calculate the posterior predictive distribution to do predictive inference, i.e., to predict the distribution of a new, unobserved data point using Mr Bayes software using Markov Chain Monte Carlo (MCMC) Methods [10].

The RAPD gel photographs were scored for presence and absence of scorable bands with the assumption of positional homology. To establish the genetic relationship among the isolates, similarity coefficients were calculated between isolates and dendrogarm drawn using UPGMA Algorithm (Unweighted Pair Group Method using Arithmetic Averages) of the NTSYS-pc, Version 2.02h programme [11, 12].

Physiological Requirements

On the basis of phylogenetic analysis three strains were selected for further physiological studies. For physiological studies, the optimum temperature and pH for its growth was standardized on malt extract medium. Varying temperature range of 20° to 45 °C at an interval of 5°C was used while five ranges of pH from 5 to 9 were used during the study. To identify the best medium for growth of the test fungi, a total of eleven solid (Potato Dextrose, Malt extract, Asthana and Hauker's, Brown's, Czapak Dox, Dextrose Nitrate, Elliot's, Malt Extract Peptone Dextrose, Malt Rose Bengal Streptocyclin, Walkman's , Sabauraud's, and Glucose Peptone) and ten liquid media (Asthana & Hawkers, Browns , Czapekdox, Dextrose nitrate, Elliot's, Walksman, Sabourauds, Glucose peptone, Joffers and Malt extract peptone dextrose) were tested during the study. The growth of the test fungus strains on solid as well as liquid media was studied for a period of 15 days. Further, the preferred carbon and nitrogen sources by the three selected strains were studied by equating the carbon and nitrogen quantity in the medium. Also the trace elements (copper, iron, zinc and manganese) requirement by the test strains was studied using four different concentrations i.e. 0.025, 0.05, 0.075 and 0.1 per cent. Cupric sulphate, Ferric chloride, Manganese chloride and Zinc sulphate were used to supplement the media for Copper, Iron, Manganese and Zinc, respectively. The Czapek's dox medium was used in the study as the basal medium.

3. RESULTS AND DISCUSSION

Phylogenetic Analysis

Forays was made to collect, identify, catalogue and molecularly characterize *Phellorinia* mushroom's biodiversity of the desert region of Rajasthan state of India during August and September month of the year 2010 and 13. In total 85 specimens of the mushroom were collected from 12 different sites of Jodhpur and Jaisalmer districts. The Longitude and latitude of the sites ranged between N 26-27º, EO 71-72º and MSL 105-295m. The dominant vegetation at different sites was *Leptadenia pyrotechnica*, *Calligonum polygonoides*, *Haloxylon recurvum*, *Lasirus sindicus* and other desert grasses.

All the samples were initially screened on the basis of colony characteristics and growth rate (3-7mm per day), which formed 29 groups. PCR amplification of ITS region of

5.8S rRNA gene yielded ITS fragment between 800-900 bp lengths for all the isolates of *Phellorinia.* No inter or intra-species ITS length diversity could be detected (Fig. 4).

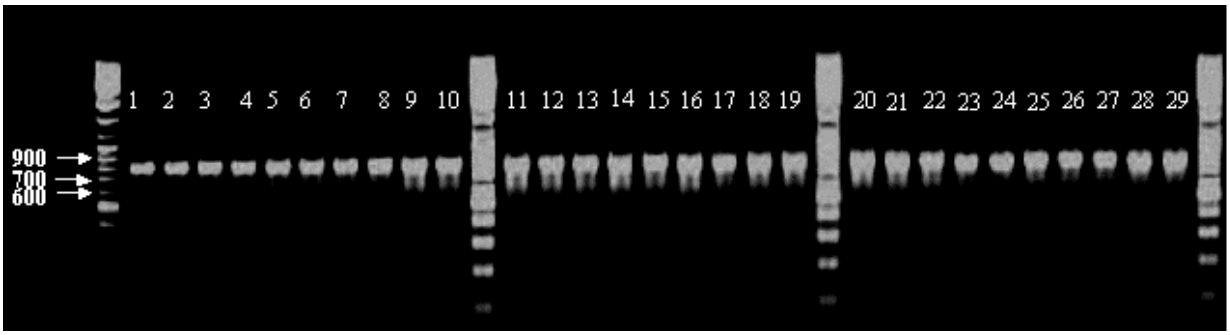


Fig.4. ITS profile of 29 *Phellorinia* isolates

All the representative sample of the groups was then genotyped using ten arbitrary decamer primers, which grouped them into 10 genetically different clades (Fig.5). The most diverse strains were found to be strain-5 and 10, which showed only 30% similarity with all other strains. The strains 1 and 4 also showed quite high diversity from the other strains. The biggest clade comprised twenty specimens showing 100% similarity within them. One representative from each group was taken for ITS 5.8S DNA region sequencing. In total 10 samples were sequenced and all the sequences were compared with NCBI databases through BLAST-N and phylogenetic analysis was done using fast minimum evolution method of NCBI, Maximum likelyhood method using Mega 6 and confirmed by Baysian statistics using Mr. Bayes software.

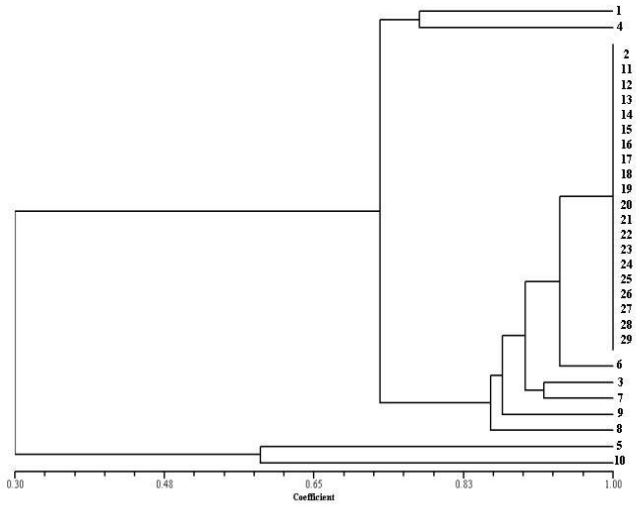


Fig.5 Genetic diversity in the collected *Phellorinia* specimens using ten RAPD primers

Although the BLAST analysis showed the maximum identities (90%) with *Phellorinia herculea* strain RA-1b along with an expect value of 9e-173, but during phylogenetic analysis using fast minimum evolution method, the most diverse specimen sample-9 did not come in the operational taxonomic unit of *Phellorinia herculea* but it came as the internal nodes with the *Phellorinia* species (Fig.6). Further, all the ten sequences were subjected to phylogenetic analysis using Mega 6, which grouped the sequences in to 3 three major clades (Fig.7) suggesting at least three different species of *Phellorinia* exist amongst the collected specimens. The confirmation of the results of maximum likelyhood analysis using Mega 6 software was done through baysian analysis, which confirmed the results. For further studies on physiological requirement of the test fungi, three representative strains, one from each clade, were selected.

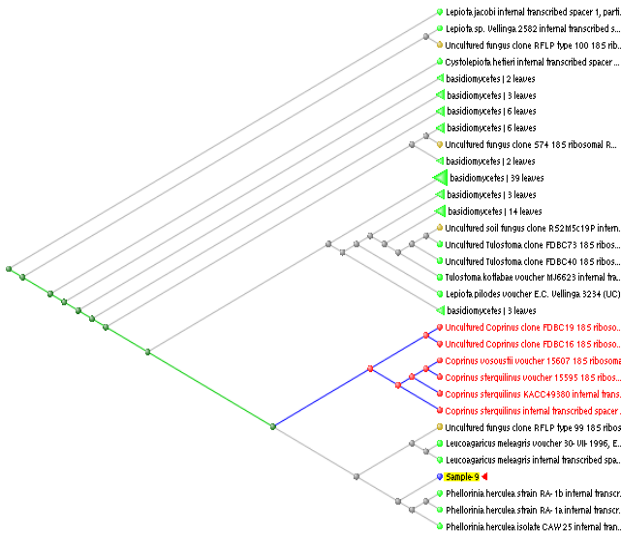


Fig.6 Phylogenetic analysis 5.8S rDNA sequence of sample-9 using fast minimum evolution method

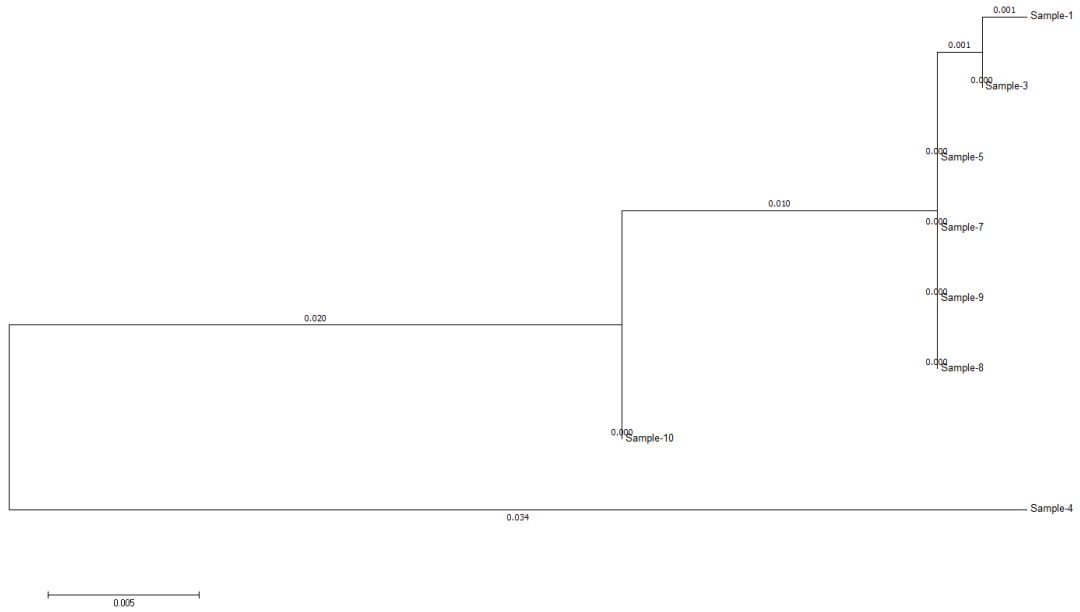


Fig 7. Molecular Phylogenetic analysis by Maximum Likelihood method with 1000 bootstrap comparisons. The tree with the highest log likelihood (-1169.7879) is shown. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 676 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [9].

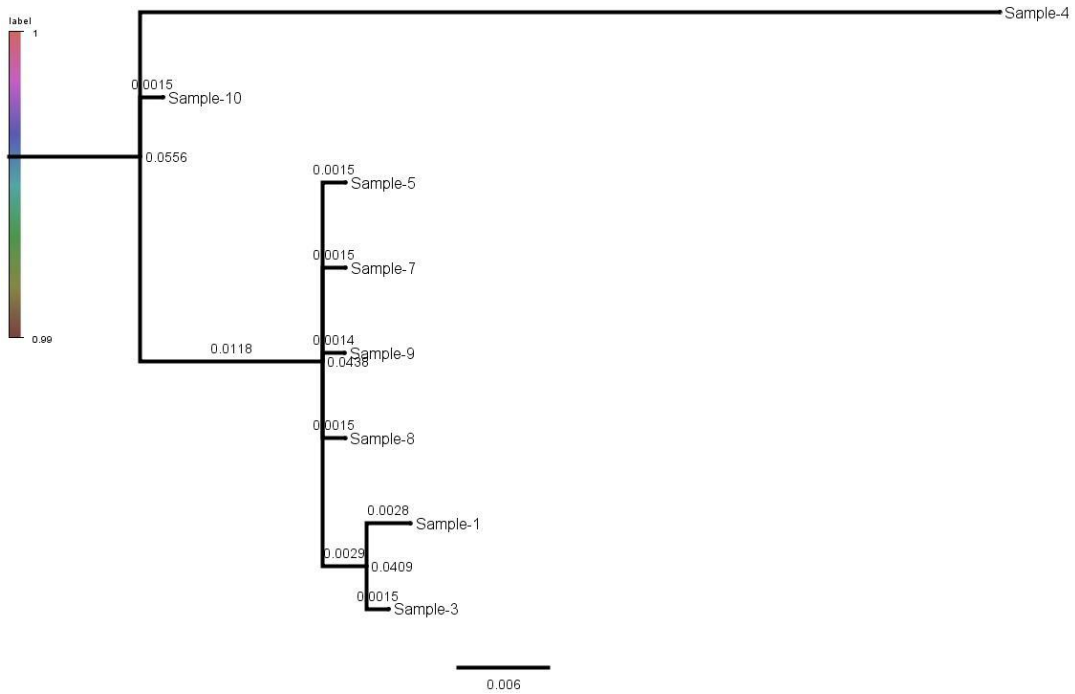


Fig 8. Phylogram generated by Mr Bayes based on Baysian analysis on the basis of posterior predictive distribution by MCMC methods. The Phylogram validated maximum likelihood analysis by Mega 6 software [Credible sets of trees (237 trees sampled): 50 % credible set contains 50 trees; 90 % credible set contains 94 trees; 95 % credible set contains 100 trees; 99 % credible set contains 106 trees] [10].

Description of the fruit bodies is given in Table 1 and microscopic features are presented in Fig. 8. The length of the carpophore varied between 5.0 to 13 cm. Cap diameter varied between 2.5-7.0 cm and was filled with spore mass. Fresh fruitbody weight varied between 11.40-80.54g. The fruit bodies were observed to have about 70 per cent moisture content. The average spore diameter was 6 µm. The mycelium was septate and the diameter of mycelium was found to be 1.87-2.55 µm (Fig 8).

Table 1. Morphological features of three *Phellorinia* isolates

S.No.	Feature	Sample number		
		1/2	**2/17**	**5/23**
1	Fruit body length (cm)	7.5-13.3	7.0- 11.0	5.0-12.5
2	Stipe length (cm)	3.1-8.0	4.5 -7.5	3-7.7.0
3	Cap diameter (cm)	3.0-7.0	2.8-5.1	2.5-6.0
4	Stipe diameter (mm)	12-17	13-26	17-24
4	Fresh fruit body weight (g)	11.40-55.73	13.57-49.04	16.01-80.54
5	Moisture (%)	57.42	69.50	70.69
6	*Spore size	6.1µm	5.80 µm	5.90 µm

		(4.95-7.73 µm)	(4.365-7.20µm)	(4.95-7..10µm)
7	Mycelium dia.	1.87µm	1.87µm	2.55µm
8	Intersepta distance	42.99µm	25.33µm	30.83µm

*=Observation based on 100 spore measurements

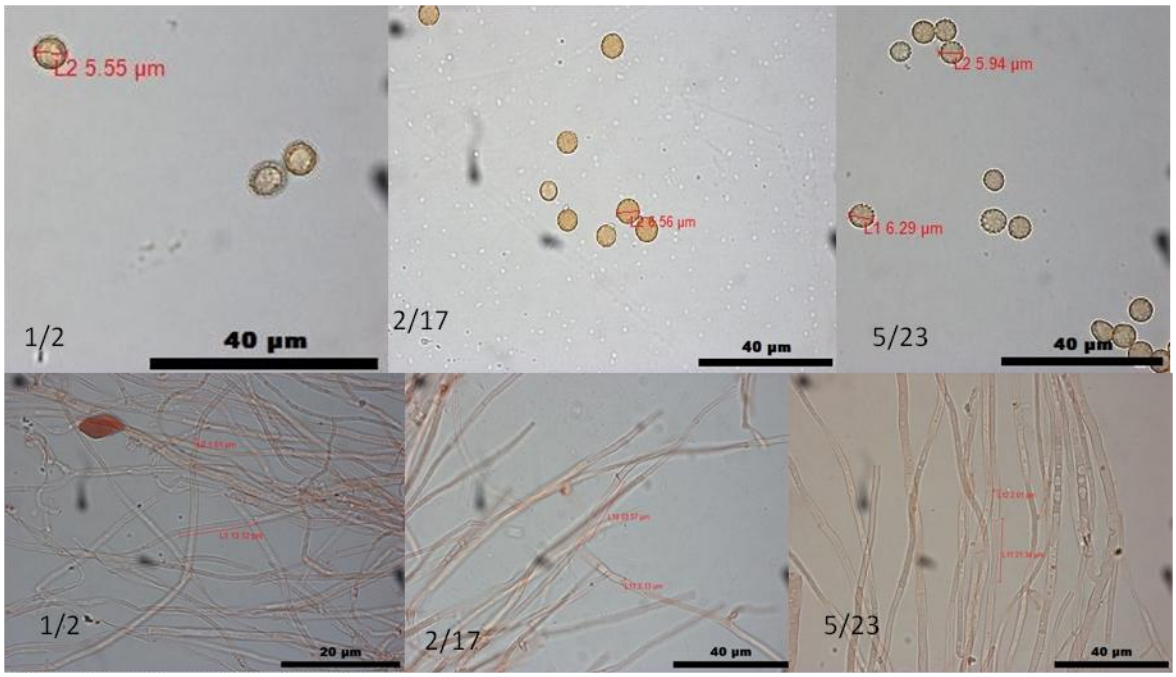
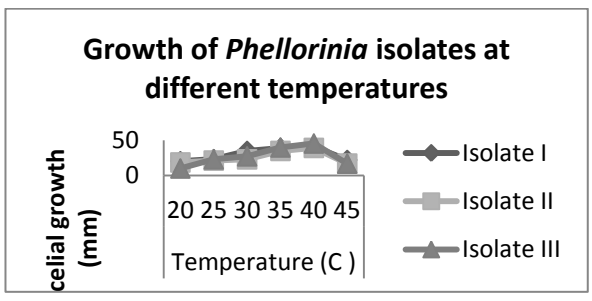


Fig.8. Morphological features of *Phellorinia* isolates

Physiological Requirements

The temperature preferences of all the *Phellorinia* strains under investigation were found to be 40°C (Fig.9). The optimum pH for all the test strains was observed to be 7.5. Although in strain 1/2 the growth declined sharply after pH 7.5 while in other strains it remained constant up to pH 8.5 (Fig.9). The results indicated that the fungus requires high temperature and slight alkaline pH, which is normally found in the deserts of Rajasthan (India). One earlier study on soil conditions *Phellorinia* mushroom natural sites revealed that it grows in coarse, well-aerated sandy soil with poor nutrients and high pH [13].



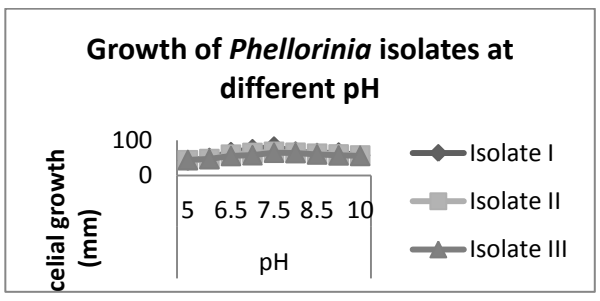


Fig.9. Effect of different temperature and pH on the mycelia growth of *Phellorinia* isolates

For further physiological studies, ten liquid and ten solid media were evaluated to identify a basal medium. For the media preference the strains differed from each other. Strain 1/2 preferred Elliott's medium while strains 2/17 showed best growth in Czapek's dox medium and 5/22 grew best on malt dextrose peptone medium (Fig.10). To perform the physiological studies a synthetic medium was required and all the test strains showed good growth on Czapek's dox medium hence used for all physiological studies.

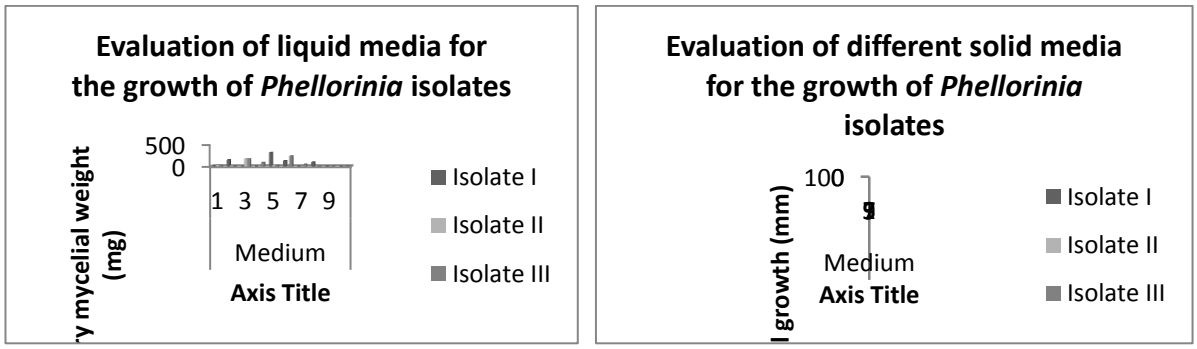


Fig. 10. Evaluation of different liquid and solid media for *Phellorinia* isolates
1- Asthana & Hawkers; 2 - Browns; 3 - Czapek's dox; 4 - Dextrose nitrate; 5 - Elliot's; 6 - Malt extract peptone dextrose; 7 - Walksman; 8 - Sabouraud's; 9 - Glucose peptone; 10- Joffer's medium

To study the physiological requirement, carbon and nitrogen source preference of the test fungi was studied. Also the trace element i.e. Copper, iron, manganese and zinc requirement of *Phellorinia* strains under study were studied. The results indicated that all the three strains differed in their carbon source preference. The strain 1/2 showed the maximum growth on sugar alcohols while strain 2/17 grew the maximum on sucrose as well as the sugar alcohols. The maximum growth amongst all the three strains were recorded in the strain 5/22 and the most preferred carbon source by the strain was found to be dextrose. The strain 5/22 was found to be the most potent strain and could grow on almost all the carbon sources (Fig11).

The results on the nitrogen source utilization also showed that the strains are different in their physiological requirement (Fig.12). The strain 1/2 and 2/17 showed the maximum growth on calcium nitrate while the strain 5/22 grew the maximum on ammonium sulfate. The difference in the physiological requirement of the test fungi also indicates that the specimens collected may belong to different species.

The trace element required by the test fungal strains were tested revealed that 1/2 showed very low growth on copper supplemented media whereas the other two strains showed relatively better growth on the copper supplemented media. All the test strains showed equal preference for the iron concentration and the maximum growth of the test strains was observed to be on 0.05% concentration of iron. All test strains have showed same growth rate on all the manganese concentrations tested. However, different strains showed different growth rates on manganese concentrations. The maximum growth of all the strains has been recorded on the 0.05% concentration of Zinc. However, maximum growth rate was recorded in strain 5/22 in all the treatments.

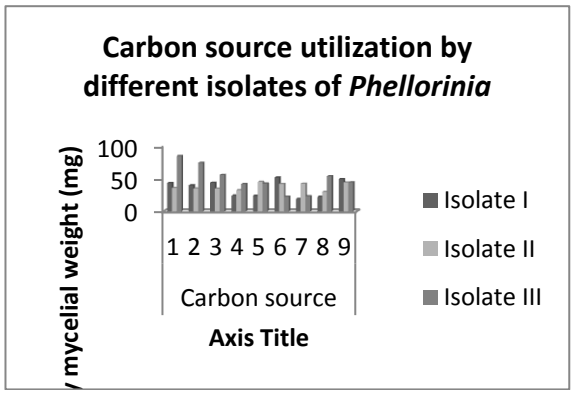


Fig. 11.Carbon source utilization by different *Phellorinia* isolates: 1-Dextrose; 2-Lactose; 3-Maltose; 4- Starch; 5-Sucrose; 6-Sorbitol; 7-Citric acid; 8-Cellulose; 9-Manitol

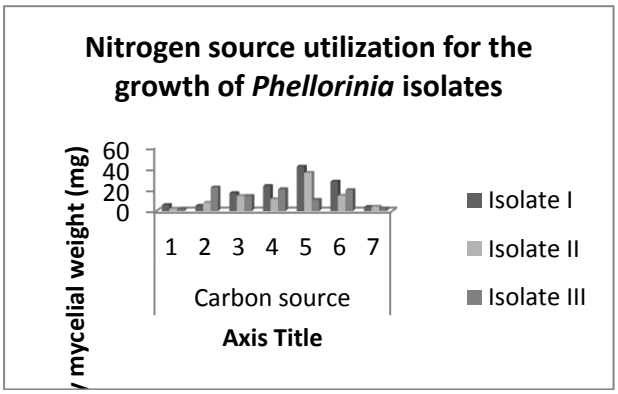


Fig.12. Nitrogen source utilization by different *Phellorinia* isolates: 1.Urea; 2**.** Ammonium sulfate;3. Ammonium chloride; 4. Sodium Nitrite;5.Calcium nitrate;6. Sodium nitrate; 7. Control

Cultivation

Cultivation of *Phellorinia* (1/2) was tried on wheat straw, paddy straw, alone and in combination (Fig.13). After soaking, 2 kg substrate was filled in PP bags and autoclaved at 1.55kg/cm^2 for 1 h. The bags were inoculated with 3 per cent spawn prepared on wheat grains. These bags were incubated at 35°C. Spawn run completed in 15 days and primordial developed on surface of the bags after 24 days. Casing did not helped in better fructification.

Fig.13. Cultivation of *Phellorinia*: a. In conical flasks, b. In pots, bags and trays, c. Developing primordial in bag cultivation

Not much study has been carried out on the *Phellorinia* mushroom since the mushroom is growing in dry and hot climate of deserts. However, a few studies have been carried out on the other desert mushroom *Podaxis pistallaris*. *P. pistillaris* has been reported from a number of countries including: Afghanistan [14], Africa [15], Argentina [16], Australia [17], Brazil [18], Congo [19], Iran [14], Israel [20, 21], South Africa [22] and USA [23]. In Sindh, preliminary experiments have been conducted on the artificial cultivation of Desert mushroom, *Podaxis pistillaris*, at Sindh Agriculture University Tandojam [4, 5, 24]. They reported that it can be easily grown as that of other cultivated mushrooms with a little difference. Only the need is to select sandy to sandy loam soil in the surrounding of thick grove of trees and or gardens, or ordinary shed, because direct sun rays are dangerous for this mushroom too. Ditches, caves, hut, hovels, cottage, sheds or shelters, bee-hive shaped huts, thatched or matted roofs as well as cellars, garages, kitchens, bathrooms or any other extra rooms of a house or so, can be used as a shed for mushroom cultivation. Cultivation of the fruiting bodies has also been reported by Jandaik and Kapoor [25], Arora [26] and Phutela *et al*. [27].

Looking at the economic importance of this mushroom for the desert areas of India, present study is an attempt to catalogue the biodiversity of this important mushroom of the

Indian deserts. At least three species of *Phellorinia* are existing in Rajasthan Desert of India. However, more detailed studies are required for exact identification of these species. At the same time the attempts have also been made to identify the physiological requirements of the mushroom so that this mushroom could be domesticated. The present studies revealed the initiation of primordia but the carpophores never developed into full grown fruit bodies. Most of the carpophores were found to occur in the vicinity of selective trees under the natural conditions, so some sort of mycorrhizhal association with local shrubs cannot be ruled out.

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