An insight on mycoflora associated with rice grain discolouration

ABSTRACT

Grain discolouration, a complex disease of rice is a new enemy to rice crops around the world, and it is becoming increasingly important due to the qualitative as well as quantitative loss of harvested crop. The present study was carried out on the ten different variety of rice (MEX-73, NLR-33892(Parthiva), MEX-48, MEX-61, PR-126, PR-128, PR-129, PR-130, Pusa-basmati -7 and Pusa basmati-1121). The incidence was calculated in one metre square quadrants randomly marked in the plot, where the highest incidence was observed in PR-126(36.23%) and the least was Pusa basmati-1121(19.77%). Several seed borne fungi were detected using blotter paper method. The Mycoflora observed were *Curvularia* sp*., Fusarium* sp*., Bipolaris oryzae, Sarocladium* sp*., Aspergillus* sp, *Penicillium* sp. The maximum frequency was observed for *Aspergillus* (25.73%) followed by P*enicillum* (24.53%), and the least observed was *Bipolaris oryze*(1.73%). The identity of the Aspergillus was confirmed using Inter transcribed Spacer primers (ITS1 & ITS4).

Keywords: Rice, Grain discolouration, Mycoflora, Aspergillus, rice crops, Aspergillus, crop growth

1. INTRODUCTION

Rice (Oryza sativa) is an important member of *Poaceae*, has been a major staple food for over half of the global population through many centuries. Asia produces most of the world's rice, with China leading (206.6 million metric tons), followed by India (135 million metric tons). India is the world's second largest rice producer, producing 135 million metric tons [1]. However, with the ever-growing population, which is speculated to reach 8 billion by 2025, the demand in rice may also see a 40% surge to fulfil rising food demand by 2030 [2, 3]. This crop faces many biotic and abiotic challenges, as it is grown in a variety of biotic conditions, which makes proper crop growth difficult [4].

Diseases can reduce crop output and quality at various growth stages and across different kinds. Diseases such as blast (*Magnaporthe grisea*), sheath blight (*Rhizoctonia solani*), brown spot (*Bipolaris oryzae*), bacterial blight (*Xanthomonas oryzae*), and tungro (*Rice Tungro Virus*) significantly reduce productivity, whereas Bakanae (*Fusarium moniliforme*), sheath rot (*Sarocladium oryzae*), false smut (*Ustilaginoidae virens*), early seedling blight (complex disease), and grain discoloration (complex disease) have a considerable impact on production, resulting in qualitative

and quantitative losses. When a fungus encounters seeds, it causes physical, physiological, and biochemical changes that reduce the seeds nutritional value [5].

Among the increasing minor diseases, grain discolouration (GD), also known as dirty panicle disease, is emerging as a threat in rice growing areas around the world. This complex disease can reduce the rice crop's output potential up to 6%. Disease occurrence and severity are significantly influenced by agricultural practices and climatic conditions (6). In India, GD is a serious problem in early and medium duration rice varieties grown in wet seasons with high relative humidity and warm temperatures throughout the growth and post-flowering stages (7). Many fungal pathogens, including *Alternaria alternata, Aspergillus flavus, Bipolaris oryzae, Chaetomium oryzae, Curvularia lunata, Fusarium moniliforme, Sarocladium oryzae,* and *Trichoderma* sp.., have been linked to rice GD [8]. *Fusarium moniliorme* and *Curvularia lunata* are two significant pathogens in charge of the discoloration of grains [9].

In the infected grains varying discoloration can be caused by varying pathogens, *viz., Curvularia geniculata* for eye-shaped spots, *Fusarium* sp. (*Fusarium oxysporum*, *Fusarium moniliformae*) for pink discoloration, and *Sarocladium oryzae* for light brown. *Alternaria alternata* (ashy grey discoloration) and *Helminthosporium oryzae* (black discoloration with dark brown patches) primarily affect the seed coat and endosperm area. Therefore, symptoms vary according to the microorganism and the severity of the infection. Overall, symptoms of this illness include brown or black patches on grains, hollow lightweight panicles, blackish-brown stripes on grains, and diseased panicles with unfilled grains. Grain discolouration affects grain morphology, including grain size and shape. Symptoms of this disease include rusty, water-soaked lesions on the palea, brown immature lighter grains on the panicles, grain discoloration, glume discoloration, and grain rot [10].

2. MATERIAL AND METHODS

2.1 Disease Incidence: **The content described under the section 2.1 seems inadequate. The information on the experimental set up, season and year of experiment conducted in required.**

Different rice cultivars were screened in 2022 during Kharif season at the agrifarm of LPU, Jalandhar using Randomised Block Design in three replicates. The disease incidence and sample collection were done using quadrant method (1 msq) from each replication of ten different varieties (MEX-73, NLR-33892(Parthiva), MEX-48, MEX-61, PR-126, PR-128, PR-129, PR-130, Pusa-basmati -7 and Pusa basmati-1121). The samples were collected in brown paper bag and were brought to the plant pathology laboratory and were separated based on healthy and discoloured grains with the help of visual observation method. The disease incidence was evaluated by using following formula:

$$\text{Disease Incidence (\%)} = \frac{\text{number of infected panicles/1mt}^2}{\text{Total number of grains/1mt}^2} \times 100 \quad [3]$$

Based on their response to the disease in the field, the genotypes were categorized into Highly Resistant (HR), Resistant (R), Moderately Resistant (MR), Susceptible (S) and Highly Susceptible (HS) groups using 0-9 Rating scale given by Venkatanagappa et al., [11].

Table 1: Disease rating scale (0-9) for grain discoloration

Score	Grains discoloured (%)	Response
0	No incidence	Immune
1	> 1	Highly Resistant (HR)

3	1-5	Resistant (R)
5	6-25	Moderately Resistant (MR)
7	26-50	Susceptible (S)
9	51-100	Highly Susceptible (HS)

2.2 Exploration of mycoflora based on blotter test on stored grains:

To explore the prevalent mycoflora associated with the rice GD, the stored discoloured grains of various rice varieties collected from the rice fields were screened using Blotter test method/ moist chamber technique. The sterile Petri plates (12 cm) were lined with double layer of moist sterile 12 cm blotter paper discs. Later, seventy-five discoloured grains from each variety, were surface sterilised with 1% sodium hypochlorite, followed by three washes in distilled water. Twenty- five sterilized seeds were transferred to the moist chamber in three replicates. The plates were incubated at 25±2°C and the humidity of the plates were maintained by spraying sterilised distilled water regularly. The frequency of mycoflora occurrence was estimated using the following formula:

$$\text{Frequency of mycoflora observed \%} = \frac{\text{Number of infected grains with a mycoflora}}{\text{Total number of grains}} \times 100$$

2.3 Morpho analysis and Molecular Characterization:

The fungal growth from the infected seeds were subjected to microscopy and the fungi were identified based on their conidia and conidiophores. The most prevalent fungi observed i.e., *Aspergillus* sp. was maintained on slants made using potato dextrose agar (PDA). DNA was extracted using CTAB method and amplification was done using the Internal Transcribed Spacer (ITS) markers *viz.,* ITS 1 & ITS 4. The amplicon was further sequenced and the sequence obtained was subjected to NCBI BLASTn to confirm the identity. The phylogenetic tree was constructed using MEGA X software using Neighbour joining method at 500 Bootstrap value.

2.4 Statistical Analysis: Data recorded was analysed using IBM SPSS Statistics 27.0 and Tuckey's test was performed with 0.05 p value [12].

3. RESULTS AND DISCUSSION

3.1 Disease incidence and Disease response

The disease incidence of rice GD of ten different varieties where observed as MEX-73 with 24.14%, NLR-33892(Parthiva) with 27.20%, MEX-48 with 23.77%, MEX-61 with 25.30%, PR-126 with 21.43%,PR-128 with 28.10%, PR-129 with 29.50%,PR-130with 36.23%, Pusa-basmati -7 with 31.03% and Pusa basmati-1121 with 19.77% was recorded. The variation in incidence was seen from variety to variety, wherein, the highest incidence was seen on PR-130 with the 36.23% and the lowest record of the disease incidence was seen on Pusa basmati 1121 with 19.77%. Remaining varieties showed incidence equivalent to each other. The disease response for ten varieties ranged from moderately resistant (MR) (MEX-73, MEX-48, MEX-61, PR-126,Pusa basmati-1121) to Susceptible(S) (NLR-33892(Parthiva), PR-128, PR-129, PR-130).

Table 2: Disease incidence homogeneous subset and response

S.N.	Variety	Disease Incidence*	Response
1.	MEX-73	24.14^{abc}	Moderately Resistant (MR)
2.	NLR-33892(Parthiva)	27.20^{abcd}	Susceptible (S)
3.	MEX-48	23.77^{abc}	Moderately Resistant (MR)
4.	MEX-61	25.30^{abc}	Moderately Resistant (MR)
5.	PR-126	21.43^{ab}	Moderately Resistant (MR)
6.	PR-128	28.10^{abcd}	Susceptible (S)
7.	PR-129	29.50^{bcd}	Susceptible (S)
8.	PR-130	36.23^{d}	Susceptible (S)
9.	Pusa basmati 7	31.03^{cd}	Susceptible (S)
10.	Pusa basmati 1121	19.77^{a}	Moderately Resistant (MR)

*values followed by same letter do not vary significantly; Tukeys hsd^{ab} test; p-0.05; CV- 11.55% & SE(d): 2.51

Our results are in corroboration with other workers, Baite et al., [13] screened thirty-seven rice genotypes where highest incidence was recorded in DRR-44 with 92.40% incidence and lowest incidence i.e., 25.00% was recorded in three genotypes (Dhala Heera, ratna & Khitish), Gouda et al., [14] screened thirty-eight where highest incidence was recorded in Jyoti with 46.50% and lowest incidence was recorded in BR-2655 with 4.75%. Raghu et al., 2020 screened twenty rice genotypes where highest incidence was recorded in Swarna Sub - I with 38.25% and lowest incidence was recorded in CR dhan 401/Reeta with 8.33%.

3.2 Exploration of mycoflora based on blotter test on stored grains:

Different diversities were obtained through the blotter paper method (fig. 1) when the discolored grains were observed on the regular interval period of time. Major pathogen such as *Alternaria* sp.*, Curvularia* sp.*, Fusarium* sp.*, Penicillium* sp.*, Bipolaris oryzae, Aspergillus* sp.*, Sarocladium oryzae and Chaetomium* sp. were observed. The frequency of mycoflora, varied with each variety *viz.,* for MEX-73, *Aspergillus* sp. (28.0%) was recorded highest and *Sarocladium oryzae* (2.67%) was the lowest, whereas in NLR-33892 (Parthiva) *Fusarium* sp. (21.33%) was recorded highest and *Alternaria* sp. (2.67%) and *Bipolaris oryzae* (2.67%) reported lowest. In MEX-48 *Penicillium* sp. (24.00%) and *Aspergillus* sp. (24.00%) recorded highest and *Alternaria* sp. (1.33%) was seen least. MEX -61 had *Penicillium* sp. (26.67%) as highest and *Bipolaris oryzae* (5.33%) at the lowest, whereas in PR-126 *Penicillium* sp. (25.33%) recorded highest and *Alternaria* sp. (2.67%) followed by *Sarocladium oryzae* (2.67%) at the least. In PR-128, *Penicillium* sp. (28.00%) was highest and *Bipolaris oryzae* (1.33%) was lowest, whereas in PR-129 *Aspergillus* sp. (30.67%) was highest and *Bipolaris oryzae* (1.33%) was the least, In PR-130, *Aspergillus* sp. (28.00%) was recorded highest and *Alternaria* sp. (5.33%) was the lowest followed by *Sarocladium oryzae* (5.33%), whereas in Pusa basmati 7 *Penicillium* sp. (30.67%) was highest and *Alternaria* sp. (10.67%) was the lowest. Pusa basmati 1121 had highest frequency of *Aspergillus* sp. (32.00%) whereas, *Chaetomium* sp. (4.00%) had the lowest. Overall, *Aspergillus* sp had the maximum

frequency with average of 25.73% followed by *Penicillium* sp. (24.53%) and the lowest was Bipolaris oryzae having an average frequency of 1.73% (Fig. 2; Table 3).

Figure 1: Blotter Test to assess the mycoflora associated with rice grain discolouration

Table 3: per cent contribution obtained through blotter paper method

Variety	*Alternaria* sp.	*Curvularia* sp.	*Fusarium* sp.	*Penicillium* sp.	*Bipolaris* sp.	*Aspergillus* sp.	*Sarocladium* sp.	*Cheatomium* sp.
MEX-73	6.67	14.67	16.00	24.00	0.00	28.00	2.67	8.00
NLR-33892(Parthiva)	2.67	20.00	22.67	21.33	2.67	20.00	6.67	4.00
MEX-48	1.33	17.33	21.33	24.00	5.33	24.00	6.67	0.00
MEX-61	0.00	20.00	22.67	26.67	5.33	25.33	0.00	0.00
PR-126	2.67	20.00	18.67	25.33	0.00	24.00	2.67	6.67
PR-128	4.00	16.00	21.33	28.00	1.33	24.00	2.67	2.67
PR-129	2.67	18.67	14.67	21.33	1.33	30.67	5.33	1.33
PR-130	5.33	16.00	18.67	25.33	1.33	28.00	5.33	0.00
Pusa basmati 7	10.67	21.33	16.00	30.67	0.00	21.33	0.00	0.00
Pusa basmati 1121	0.00	18.67	16.00	18.64	0.00	32.00	10.67	4.00
Average	3.60	18.27	18.80	24.53	1.73	25.73	4.27	2.67

The value stands average for three replicates

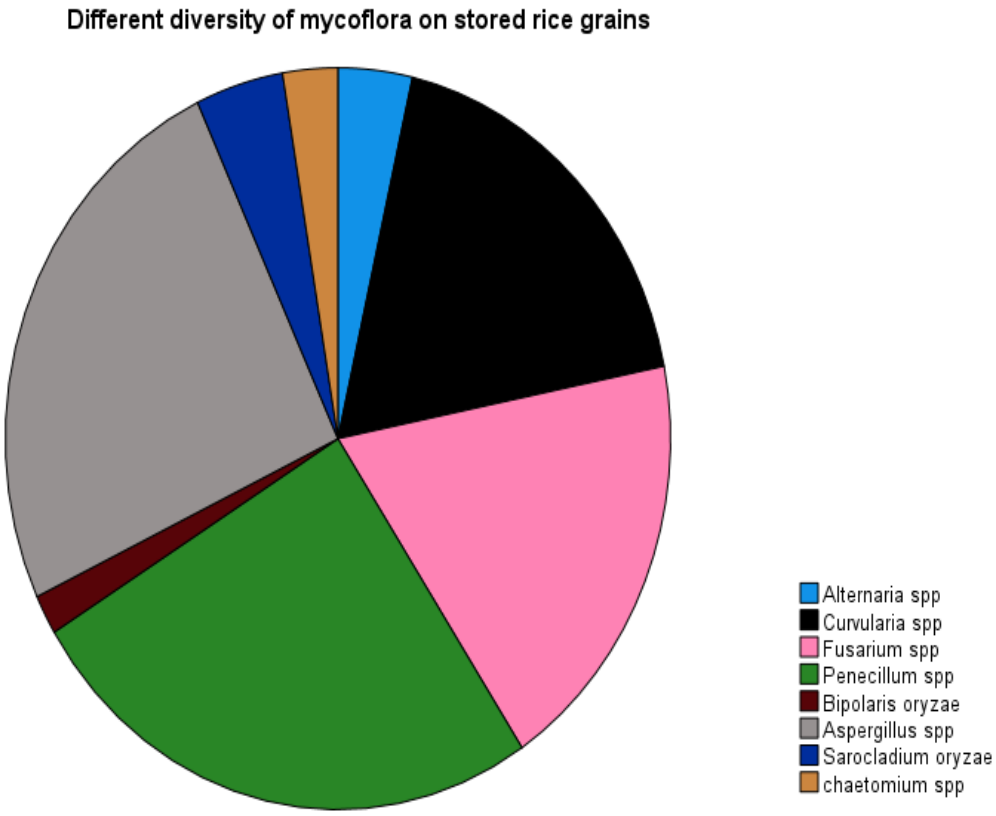

Figure 2: Diversity of different mycoflora observed on ten rice varieties using SPSS

Our results are in corroboration with other workers. Rusool, [15] observed eight fungi *viz., Pyricularia oryzae, Dreschlera oryzae, Curvularia lunata, Alternaria alternata, Fusarium moniliforme, Nigrospora oryzae* and *Sarocladium oryzae* where *Bipolaris oryae* had the highest frequency(49.95%) and *Pyricularia oryzae* to be the lowest (7.99%). Similar work was done by other workers and reported presence of several fungi. Kumar and kumar, [16] were they had observed Twelve fungi; *Alternaria alternata, Fusarium- oxysporum, F. verticillioides, Aspergillus niger, A. flavus, A. ochraceus, Nigrospora Oryzae, Penicillium sp., Bipolaris tetramera, Curvularia lunata, Curvularia pallescens* and *Bipolaris oryzae*, were found to be associated with different types of rice grain discolorations ,Uma and Wesely, [17] observed five pathogenic fungi, namely *Aspergillus flavus, A. niger, Penicillium citrinum, Alternaria padwickii* and *Rhizopus oryzae* which were associated with rice grain discoloration.

Many researchers have found higher *Aspergillus* contamination in the rice seed mycoflora especially *A. flavus*. However, in our study we found *A. quadralineatus* instead (Reddy et al., [18]; Reddy et al., [19]). The higher incidence of Aspergillus sp. can be due to the fact that rice crop is exposed to higher rain and humidity and Aspergillus sp. being a saprophyte, easily grow and contaminate the rice seeds especially during storage.

2.5 Morphoanalysis:

Major pathogen such as *Alternaria* sp., *Curvularia* sp., *Fusarium* sp., *Penicillium* sp., *Bipolaris oryzae, Aspergillus* sp., *Sarocladium oryzae and Chaetomium* sp. (Fig. 3) were observed on the basis of following features:

Table 4 : Morphometric analysis of major pathogen

S.N.	Pathogen	Conidia
1.	*Alternaria* sp.	muriform,5-6 transverse,2-3 longitudinally
2.	*Curvularia* sp.	Two central cells, transversally 3- septate
3.	*Fusarium* sp.	macro conidia, 1-3 septate microconidia, 0-1 septa
4.	*Bipolaris oryzae*	obclavate, having 5-11 pseudo-septation
5.	*Sarocladium oryzae*	Conidia were hyaline, aseptate and oblong
6.	*Penicillium*	globose, hyaline aseptate
7.	Aspergillus	globose or ovoid, hyaline aseptate, catenulate

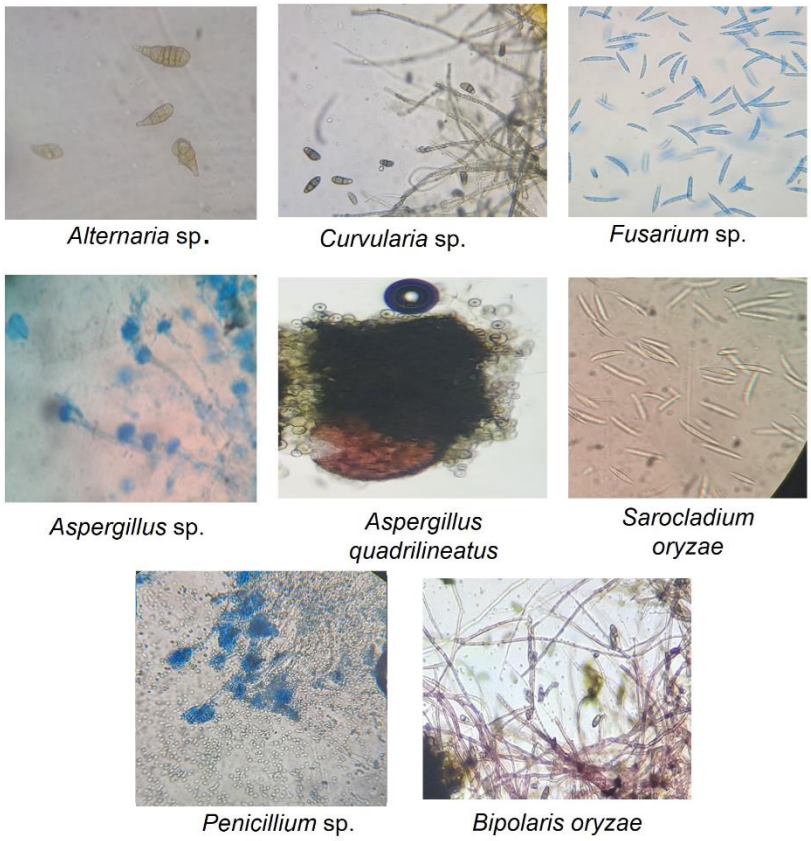

Figure 3: Various fungi observed associated with rice grain discolouration

3.4 Molecular Characterization & Phylogeny:

The amplicon generated was approximately 600 bp in size and the sequence obtained after Sanger-sequencing was of 501 bp.When subjected to NCBI BLASTn the fungus was confirmed to be *Aspergillus quadrilineatus*. A phylogenetic

tree (Fig. 4) was constructed and our test isolate was found to be closest with *Aspergillus quadrilineatus* strain KU20018.62 (accession no-MT487836) with 100 % similarity.

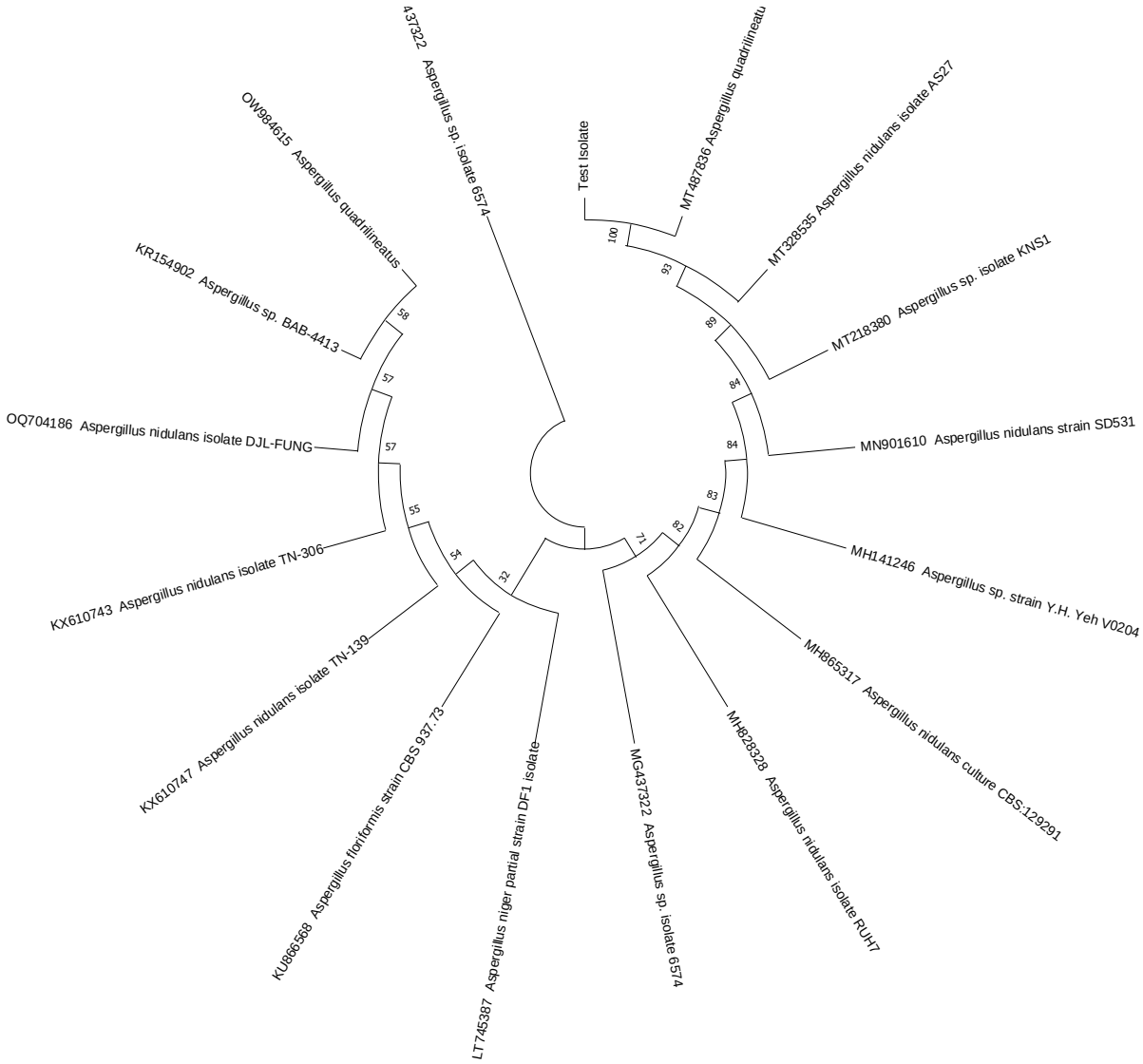

Figure 4. Phylogenetic tree method of the test isolate based on the ITS region, constructed using neighbour Joining method

Several workers have detected various fungi associated with rice seed and confirmed it using ITS primers. Persaud et al [20] found *Curvularia lunata* to be predominantly associated with the rice grain discolouration and based on the analysis of the ITS region sequence of the rDNA, they confirmed the identity of *C. lunata* as the sequenced showed 100% match with already published strains of this species. Similarly, Yuvarani [21] confirmed the identity of *C. lunata*

using ITS 1&4 primers. Mohamed and Gomaa [22] amplified the ITS region and found *Fusarium graminearum*, *F. verticilliodies* and *Bipolaris oryzae* from four rice varieties.

5. CONCLUSION

Rice grain discoloration which is a complex disease causing the qualitative and quantitative loss were different mycoflora are associated, different levels of incidence were observed within different rice varieties were PR-130 which was observed to have the highest disease incidence with 36.23% being susceptible to grain discoloration whereas Pusa basmati-1141 was observed to have the least incidence with 19.77% being moderately susceptible to grain discolouration. *Aspergillus* sp. was the most commonly found fungi associated with stored rice discoloured grains with average frequency of 25.73% whereas *Bipolaris oryzae* was the least with 1.73%. Molecular identity was confirmed using ITS primer and the species of *Aspergillus* was found to be *A. quadrilineatus*.

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AUTHORS' CONTRIBUTIONS

Aakash Gupta- designed the study, performed the statistical analysis and wrote the first draft of the manuscript.
Ajay S. Chavan- wrote protocol & reviewing
Malini Ray & Sneha Choudhary- Review, editing & final draft of manuscript

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