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Emergence of *Nigrospora* Leaf Blight as Major Biotic Stress in South Eastern Coastal Region of India

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ABSTRACT

Background: *Nigrospora* is one of the diversified pathogens with wide host range. Endophytic nature of *Nigrospora* oryzae has been reported previously. Daily variations in the climate led to the emergence of virulent infections from previously expressed weak ones. *Nigrospora oryzae* infestation comes as a major biotic stress under this present investigation.

Methods: A study was conducted to examine the variability of *Nigrospora oryzae* by collecting isolates from various locations in the south coastal region of Odisha. The level of virulence was assessed and analyzed between 2021 and 2023 to anticipate the emergence and progression of disease. The pathogen was identified using molecular techniques through ITS-r DNA sequencing method.

Result: Disease incidence was observed as a critical blast-like appearance, but greater in size, distinguishing it from blast. In the experiment, incomplete filling of grains was highest in MTU-7029 (39.73 plant^{-1}) followed by Niranjana and Gobindobhog; disease incidence was recorded to be highest in Niranjana (60.03%) followed by MTU-7029 and Barsha; and grain yield plant^{-1} was observed to be highly affected in Gobindobhog (14.58 g plant^{-1} followed by MTU-7029 and Niranjana. Molecular confirmation was established by ITS-rDNA sequencing, resulted sequence NO 9 (528 bp)- OP592217 and NO 15 (519 bp)- OP592218, exactly matches gene bank accession numbers- MH748173 (India) and ON514037 (India) of *Nigrospora* database, with 99% accuracy.

Key words: Biotic stress, Disease incidence, *Nigrospora*, Rice.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the major cereal crops of India and belongs to the family *Poaceae*. As per the latest statistics rice production turned to 523.9 million tonnes in the year 2023-24, which implied nearly 0.8% expansion from the 2022-23, consecutively FAO forecast it continues with a peak with an expansion of 0.9 percent with an annual production of approximately, 535.1 million tonnes (FAO STAT 2022, 2024). According to several estimates, the output of rice must grow by more than 40% by 2030 to keep up with the rising demand for it. Diverse abiotic stresses like soil salinity was also considered for yield reduction (Rani and Sharma, 2017). Though the southeastern coastal belt of Odisha is encircled with such favorable environmental conditions for rice cultivation every year heavy yield losses are occurring due to various biotic stresses (Pattanayk and Das, 2022). *Nigrospora oryzae* of rice was discovered in a paddy field in Fuyang, Zhejiang province, with symptoms similar to rice blast. Typically, it resulted in incomplete grain filling, which frequently caused a loss of 5 to 25% of grain weight (Liu *et al*., 2021; Pattanayak and Das, 2022).

Biotic and abiotic stresses significantly affect final crop yield (Bobode *et al*., 2021). The occurrence of *Nigrospora Oryzae* was found on the debris of various living and dead species and is considered a strong parasite that produces grain spots in rice, sorghum and maize. Very recently a leaf spot disease on Yam was reported by Xinyu *et al*. (2023) and on *Chrysanthemum morifolium* reported by Sha *et al*. (2023), on red elephant grass reported by Han *et al*. (2020). Depending on the host and environment, it has endophytic and parasitic characteristics. *Khuskia oryzae H. Huds* is a teleomorph of *Nigrospora Oryzae*. The monotopic genus *Khuskia* is found throughout the world, especially in tropical regions and is a member of the family *Trichosphaeriaceae* of the order *Trichosphaeriales*. The cosmopolitan nature of diverse species range of *Nigrospora* makes the pathogen more adaptive with the change in climataic conditions. Many *Nigrospora* species, including *N. osmanthi*, *N. camelliaesinensis*, *N. lacticolonia* and *N. vesicularis*, have overlapping morphologies, which causes uncertainty in the taxonomic classification of this genus.

The prolonged survey among different districts of south-eastern Odisha reflects that *Nigrospora* leaf blight

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turned to be a major biotic stress for rice cultivation. A severe disease incidence of 45-60% with a critical blast-like appearance makes the disease indistinguishable by visual observation from blast diseases. The present investigation was conducted to find out the emergence of the pathogen and disease severity.

MATERIALS AND METHODS

Estimation of disease was calculated by analysing disease time and disease pattern assessment in field condition. Random "Diamond" sampling pattern used to collect the sample data where different parameters like growth stage, date of sowing-transplanting, fertilizer application, soil type, texture, pH and various epidemiological parameters considered. The pathogen was isolated from twenty symptomatic plant samples which was collected from infected rice plant parts of various varieties from 4 different districts of Odisha namely Bhadrak (21.0574°N, 86.4963°E), Cuttak (20.4625°N, 85.8830°E), Gajapati (19.1912°N, 84.1857°E) and Ganjam (19.5860°N, 84.6897°E). Isolation of the pathogen was done on potato dextrose agar media. The cultured plate was maintained at 25±2°C for 24-48 hours.

The pathogen as subjected to two different types of pathogenicity tests, as follows-

Detached leaf assay, where the fresh and non-infected paddy leaf was artificially wounded and inoculated by placing a single mycelial disc on the wounded surface. Isolated culture was maintained under 25±2°C. The observation was taken from a 24-hour interval basis up to 144 hours (Liu *et al*., 2021).

In-vitro pot inoculation technique was used through spraying of spore suspension. In this investigation 3 quantitative traits like number of unfilled grains, disease incidence (%) and grain yield plant^{-1} (g) were considered to check the effectivity in 10 rice genotypes (CO 51, CR1017, MTU1140, MTU7029, Black Rice, Barsha, Gobindabhog, Pratiksha, Niranjana and Rajendra Masuri).

Cultural variability study was conducted under different nutritional media like potato dextrose agar, V8 agar, czapekdox agar, pikovaskay agar, different dextrose percentage and under different pH. Growth biology of different dextrose limit (5 gm, 10 gm, 20 gm and 25 gm) and different pH level (4.5, 5.5, 6.5 and 8) were tested. All the observations were taken from 24 hours interval basis upto 144 hours.

The CTAB DNA extraction procedure verified the molecular identification of isolate *Nigrospora oryzae*. It was found that Mucorales' ITS region served as a suitable barcoding marker. Primer pairs ITS-1 and ITS-4 to amplify the ribosomal DNA's internal transcribed spacers from the *Nigrospora oryzae* isolate. Amplification was carried out BIO-RAD MY cycler TM thermal Cycler (BIO-RAD, USA). Almost 528 bp product was extracted by sci Genom pvt limited and submitted through NCBI.

Phylogenetic analysis was done by comparative analysis of ITS region by neighbour joining method (among the closely related taxa of different countries derived from NCBI *Nigrospora* database by MEGA version 6.

Seed mortality test was performed by placing different rice germplasm on the periphery of dextrose agar plate and at the center mycelia disc placed. The observation was taken from 24 hours interval basis upto 144 hours by estimating seed mortality and seed infection level criteria.

Statistical analysis was performed in SPSS and OPSTAT software.

RESULTS AND DISCUSSION

From October to December 2021-2023 leaf blight of *Oryza sativa* caused by *Nigrospora oryzae* was observed for the first time at Ranadevi farm of Centurion University of Technology and Management, Paralakhemundi (20.2919°N, 85.8419°E) region of Odisha, India. Survey results revealed with 50-60% disease infestation level. Symptoms primarily appeared on any susceptible part like leaf, panicle *etc.* It created a brownish eye-shaped structure on the leaf surface and which is comparatively larger than the blast symptom (Fig 1), sometimes mixes up with blast and formed a disease consortium. Symptomatic expression of *Nigrospora oryzae* was measured on *Chrysanthemum morifolium* by Shah *et al*. (2023) and on wild rice by Liu *et al*. (2021).

Twenty fungal isolates were obtained from diseased sections of different rice varieties. Cultural characterization analyzed on the PDA media showed cottony white mycelia at the initial stage which turned to brownish to deep black at maturity (Fig 2). Cultural characterization under different nutritional media is represented in Fig 2.

Fig 1: *Nigrospora* leaf blight symptoms on rice leaf.

The hyphae were smooth and had a color ranging from hyaline to pale brown. They were branched and septate, with a diameter of 2-7.5 µm. Conidia were solitary, with a globose or subglobose shape and had a black, shiny and smooth appearance. They were aseptate and measured 11.5-15.5 × 10-15 µm. The conidia of *Nigrospora* were deeply pigmented and featured germ slits. Similar findings were observed through the morpho cultural description of (Abass *et al*., 2014 and Li *et al*., 2018).

Symptomatological expression comes after 7-8 days of post-inoculation. Virulency was estimated by the rapid expression of the symptoms and by observing the necrotic area of the lesion. The experimental trial was repeated thrice and every time same symptomatological appearance and virulency were noticed (Fig 3).

NO-9 and NO-15 isolates were identified as the most virulent among the tested samples. The fungal culture was preserved using PDA (Fig 2A). Morphological studies were based on morphometric characterization of the present isolates NO-9 and NO-15 (Fig 2C, D) with a comparative study with *Nigrospora oryzae.* The representative isolates NO-9, NO-15 and herbarium were deposited to the National Fungal Culture Collection (NFCCI) and the Ajrekar Mycological Herbarium (AMH) at the Agharkar Research Institute (ARI) in Pune and gained accession numbers NFCCI NO - 5477, NFCCI NO-5478 and AMH - 10510.

The pathogen has the ability to devastate rice crops, causing severe yield losses and threatening food security for millions of people worldwide. Typically, it showed incomplete grain filling, which frequently caused a loss of 5 to 25% of grain weight. Presenting results that uncover comparable findings from concurrent studies of Zhai *et al*. (2013), Chen *et al*. (2018), Liu *et al*. (2021), Obisesan and Ojo (2023).

In vitro pot inoculation technique conducted with 10 different rice genotypes resulted with susceptibility and superiority detection among the 15 quantitative traits (Fig 4). Disease incidence (DI) and grain yield per plant (GYP) showed variation, while other characters remain static. Comparative assay under treated and non-treated situations (Table 1 and 2) revealed that Niranjana showed the highest disease incidence (60.03%) and Black Rice showed the lowest disease incidence (29.90%) in the treated plot. On the other hand, Barsha showed the highest GYP value (22.00 g) and Gobindabhog showed the lowest GYP value (14.58 g) in the treated plot. Correspondingly, in

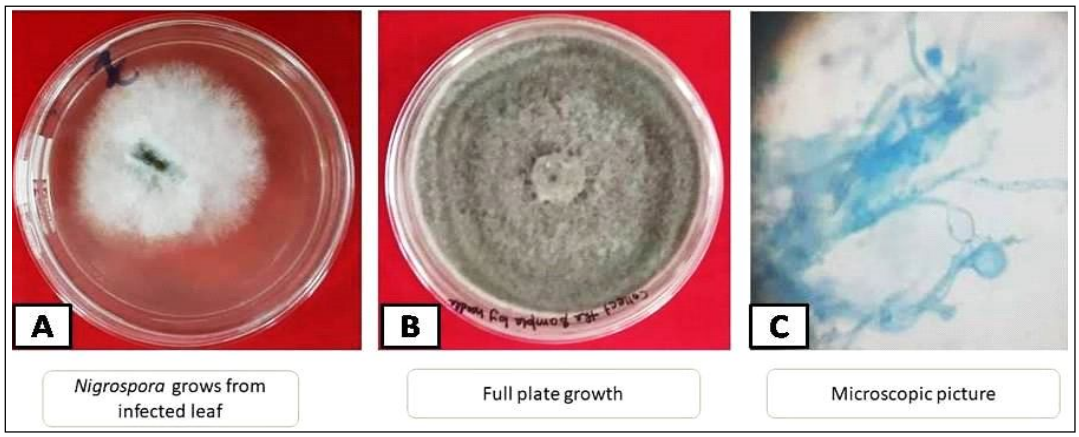


Fig 2: A *Nigrospora*grows from infected leaf sample. B-Pure culture of N. oryzae on PDA media. C- Microscopic observation *Nigrospora* mycelia.

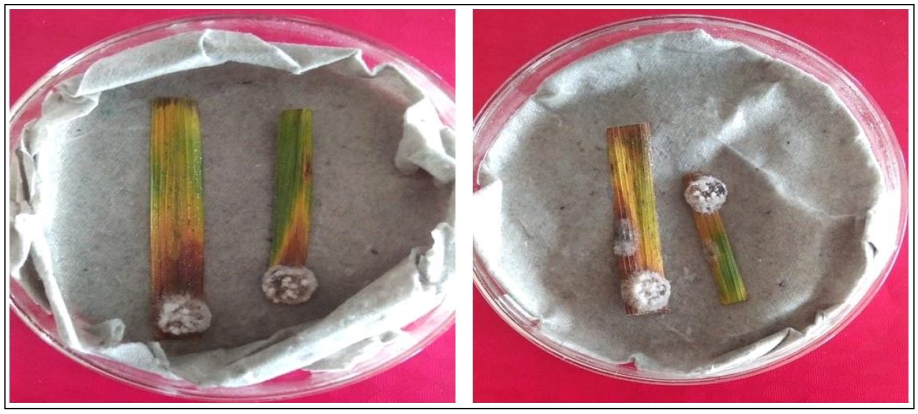


Fig 3: Pathogenicity test conducted by single mycelial disc inoculation technique, with two different isolates: NO-9 and NO-15 of *Nigrospora oryze.*

the non-treated plot, Gobindabhog showed the lowest disease incidence (4.53%) and Niranjana showed the highest disease incidence (8.47%), with addition to that CR1017 showed the highest GYP value (30.72g) and MTU1140 showed the lowest GYP value (23.53 g).

The PCR products separated in 1.5% TAE Agarose gel revealed on 550- 600 bp products (Fig 5). Isolated DNA, sequenced through Sanger dideoxy technique and resulted 528 bp and 519 bp sequences were deposited in Gene bank which assigned with the accession number OP592217 and OP592218. Molecular identification (ITS-rDNA) followed by blast search of NO 9 (528 bp) and NO 15 (519 bp) sequences are exactly matches with MH748173 (India) and ON514037 (India) with 99% accuracy with NCBI *Nigrospora* data base. Phylogenetic analysis was done by plotting the isolates through radial phylogeny and maximum likely hood parsimony test. The result reflects that OP592218 and EU918714 (Chaina) are conspecific on the same branch node. Similarly, OP592217 and MH748173 (India) were in another branch node with the highest similarity and closest. (Fig 6 and 7). The results having the parallel findings of Zhao *et al*. (2014), Liu *et al*. (2016), Han *et al*. (2019), Liu *et al*. (2021) and Wang *et al*. (2022).

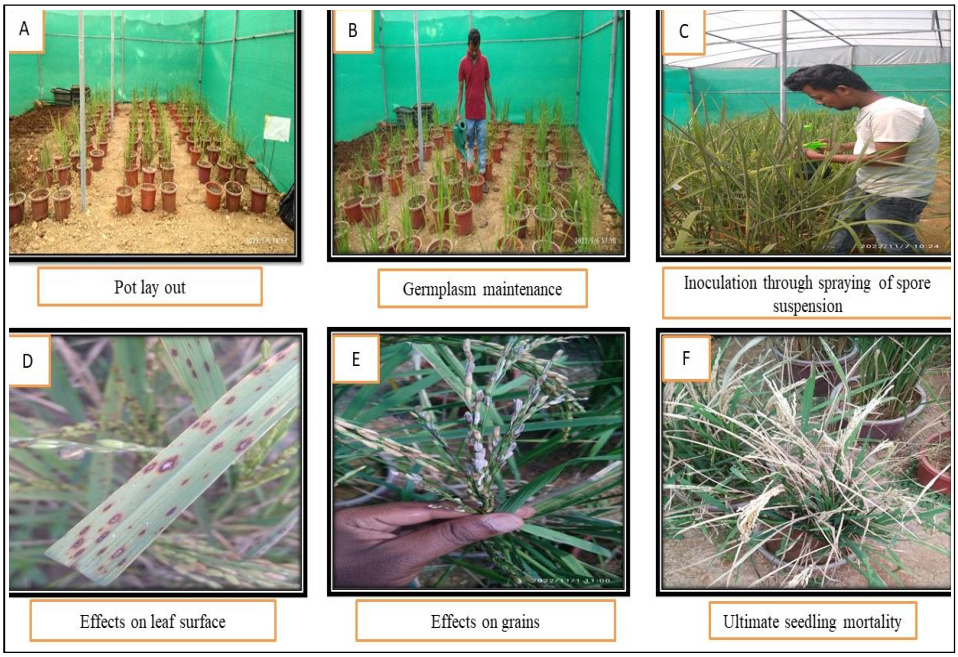


Fig 4: *In vitro* pot inoculation technique through spraying of spore suspension in different rice genotypes.

Table 1: Mean performance of 10 rice genotypes for quantitative traits under *Nigrospora* condition in non- treated plot.

Name of genotypes	Number of unfilled grains (NUFG)	Disease incidence (DI)	Grain yield per plant (GYP)
CO - 51	20.54	5.26	24.61
CR - 1017	21.61	5.77	30.72
MTU - 1140	22.23	6.24	23.53
MTU - 7029	35.98	8.25	25.71
Barsha	24.25	6.11	25.76
Pratiksha	24.97	5.17	25.08
Gobindabhog	26.72	4.53	27.52
Black rice	24.35	5.00	24.20
Rajendra Mahsuri	23.90	7.39	25.77
Niranjana	26.18	8.47	25.57
Mean	25.08	6.22	25.85
SE. (d)	0.89	0.21	0.93
SE. (m)	5.20	4.10	4.30
CV	0.06	0.15	0.64
CD @ 5%	0.20	0.40	1.90

Table 2: Mean performance of 10 rice genotypes for quantitative traits under *Nigrospora* condition in treated plot.

Name of genotypes	Number of unfilled grains (NUFG)	Disease incidence (DI)	Grain yield per plant (GYP)
CO - 51	27.40	34.14	18.02
CR - 1017	31.77	30.80	17.64
MTU - 1140	31.70	32.86	21.19
MTU - 7029	39.73	53.49	15.82
Barsha	33.03	43.70	22.00
Pratiksha	33.27	36.07	16.65
Gobindabhog	36.73	40.26	14.58
Black rice	32.93	29.90	21.36
Rajendra Mahsuri	31.07	40.23	19.50
Niranjana	36.90	60.03	16.43
Mean	33.45	40.15	18.32
SE. (d)	1.18	1.38	0.72
SE.(m)	4.20	4.20	4.80
CV	0.81	0.97	0.51
CD @ 5%	2.50	3.00	1.60

Table 3: Cultural characteristics of *Nigrospora* under different nutritional media.

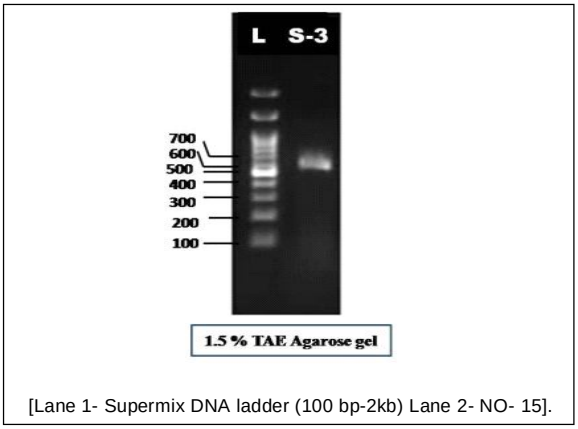
Media	Character
Potato dextrose agar	Whitish topale whitish, at maturity turned to brownish-blackish cottony structure
V8 agar	Produced ring like constriction region at the centre of the media
Pikovaskay's agar	Blackish zonation starts from the centre of media.
Czapek dox agar	Brownish pigment alteration starts from the from the centre of the media

Table 4: Cultural growth of *Nigrospora* under different nutritional media.

Name of the media	24 hour's growth	48 hour's growth	72 hour's growth	96 hour's growth	120 hour's growth
P.D.A	1.47	3.63	5.93	7.03	8.73
V8	1.43	3.07	5.50	6.70	8.27
Pikovskaya's	1.37	2.90	4.77	6.17	7.40
Czapekdox	0.57	0.97	2.10	3.07	4.33
C.D @5%	0.20	0.30	0.72	0.57	0.59
SE (m)	0.06	0.09	0.22	0.17	0.18
SE (d)	0.09	0.13	0.31	0.25	0.25
C.V.	8.61	5.99	8.23	5.23	4.29

Growth biology study of *Nigrospora* was studied under four different media which presented in Table 3. The highest radial growth of *Nigrospora* was recorded on PDA (8.73 cm), making it the most favorable medium for the fungus. V8 agar medium showed the next best performance, with a mycelial radial growth of 8.23 cm, followed by Pikovskaya's agar medium with a growth of 7.4 cm. Czapek's agar medium displayed the least mycelial radial growth of 4.3 cm (see Fig 8. Detailed result is depicted in Table 4.

Cultural variability study was conducted under the different dextrose concentrations. *Nigrospora* demonstrated the highest growth at 20 g dextrose concentration (8.9 cm) followed by 25 g and 10 g dextrose concentration, while the lowest growth was observed at 5 g dextrose concentration (3.9 cm) (Fig 9). Detailed result is depicted in Table 5.



[Lane 1- Supermix DNA ladder (100 bp-2kb) Lane 2- NO- 15].

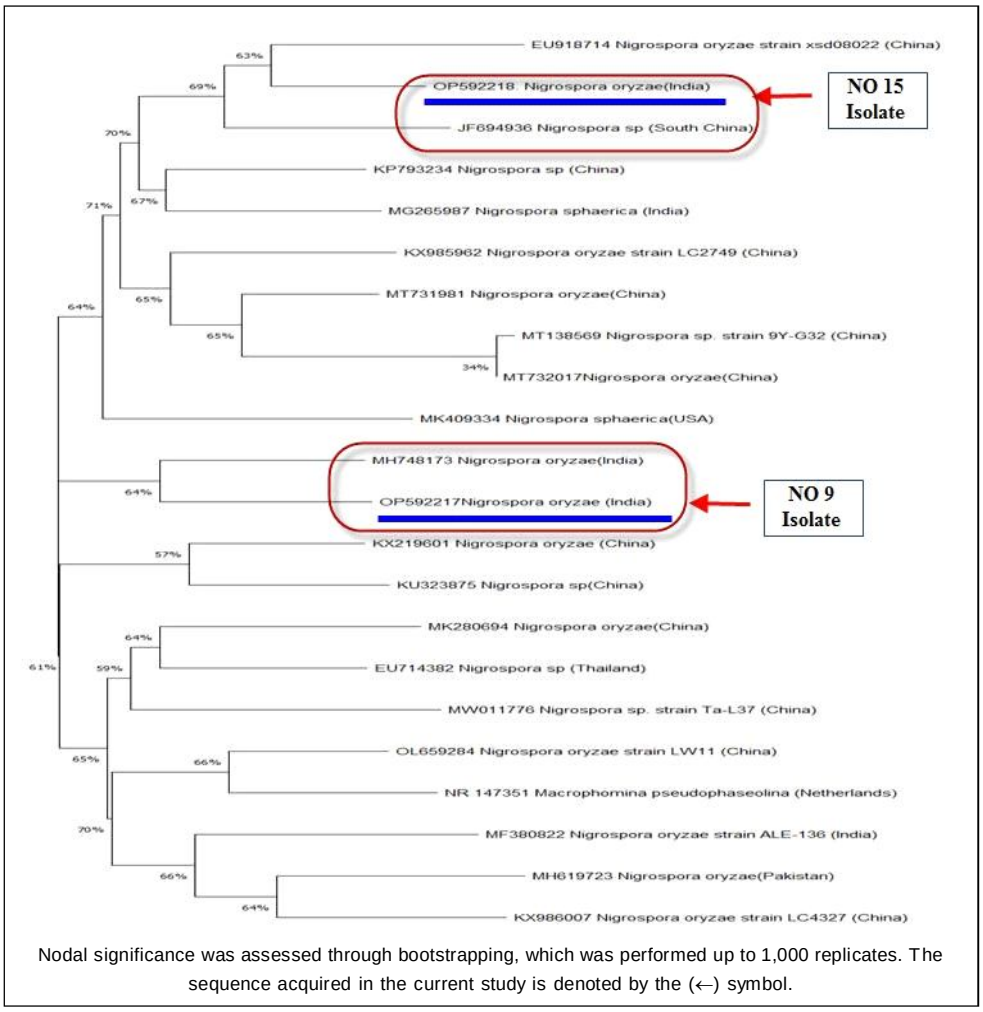
Fig 5: ITS PCR product of 528 bp DNAof *Nigrospora oryzae* isolate.

The seed mortality percentage of Pratiksha and Niranjana variety of rice was found to be nearly 60%. With addition to that Niranjana, Pratiksha and MTU 7029 were the most susceptible variety against *Nigrospora* found under this research (Fig 10). Detailed result is depicted in Table 6.

Growth biology of *Nigrospora* under different pH range was illustrated through Table 7 where 8.9 cm mycelia growth were found after 120 hours, occurred at pH 6.5. Additionally, pH 7.5 (7.63 cm) also supported favourable growth conditions, while the lowest growth was observed at pH 4.5 (4 cm). However, deviating from

Table 5: Cultural growth of Nigrospora under different dextrose concentration.

Dextrose amount/litter	24 hour's growth	48 hour's growth	72 hour's growth	96 hour's growth	120 hour's growth
5 g	0.40	0.90	2.10	2.70	3.90
10 g	0.90	1.67	2.40	3.10	4.60
20 g	1.50	3.63	5.90	8.00	8.90
25 g	1.30	2.83	4.90	6.20	7.70
C.D.@ 5%	0.18	0.29	0.60	0.68	0.44
SE (m)	0.05	0.09	0.18	0.20	0.13
SE (d)	0.08	0.13	0.26	0.29	0.19
C.V. %	8.98	6.79	8.23	7.06	3.62



Nodal significance was assessed through bootstrapping, which was performed up to 1,000 replicates. The sequence acquired in the current study is denoted by the (←) symbol.

Fig 6: Molecular phylogenetic analysis of *Nigrospora* oryzae, by neighbor-joining tree using the maximum composite likelihood method.

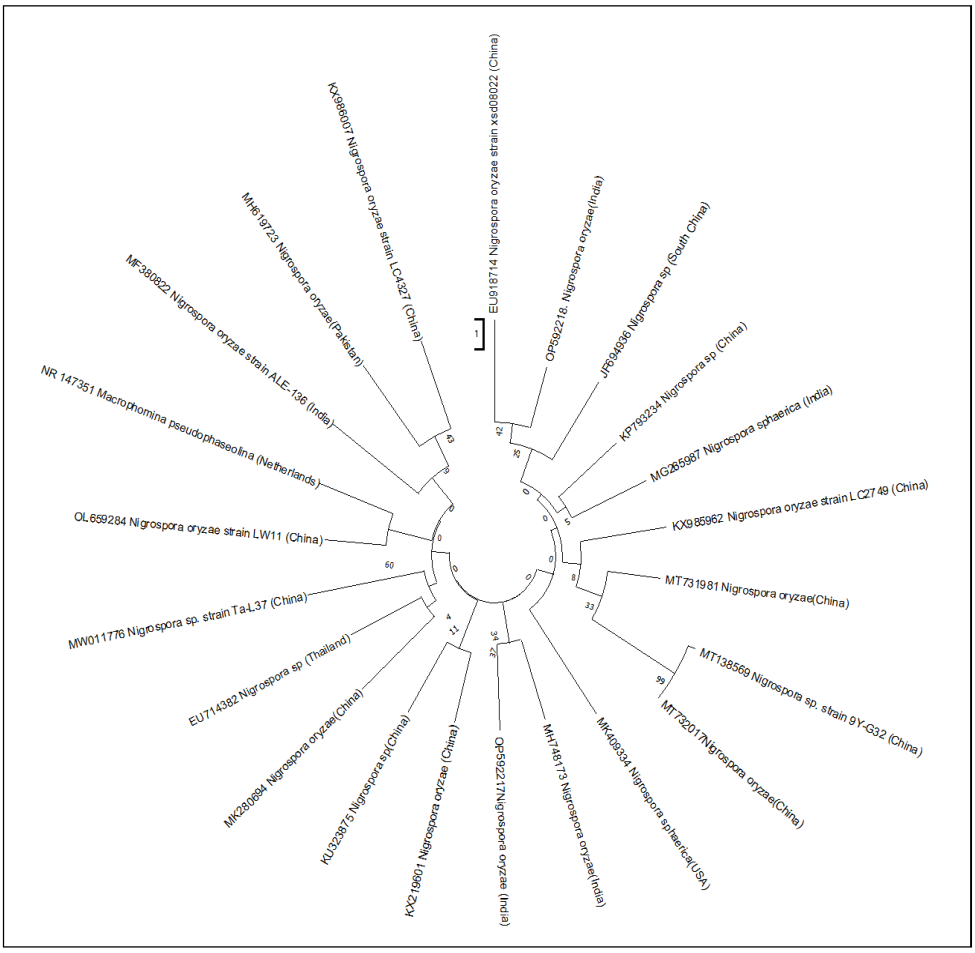


Fig 7: Radial cladogram indicates *Nigrospora oryzae* and it's closest taxa.

Fig 8: Effect of *Nigrospora oryze* on different varieties of rice germplasm.

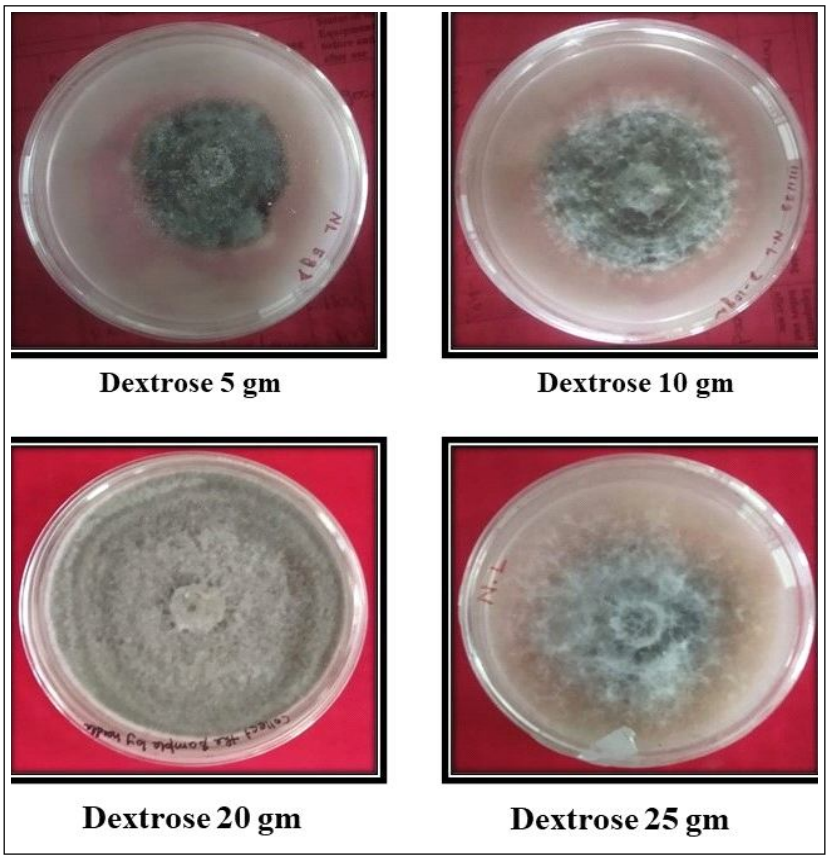


Fig 9: cultural growth of *Nigrospora* under the different dextrose concentration.

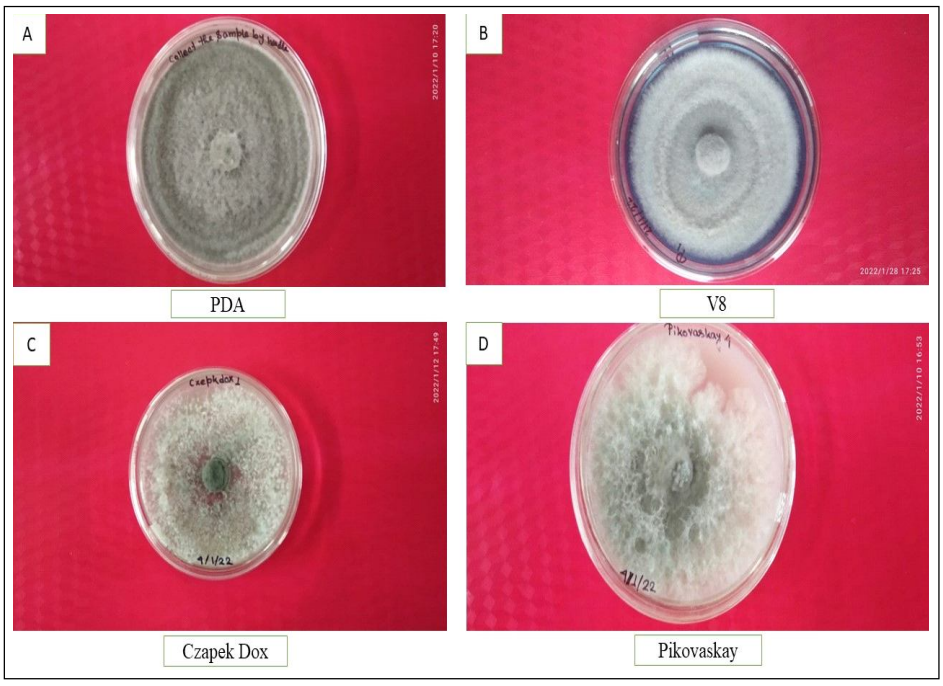


Fig 10: Cultural growth of *N. oryzae* under different nutritional media.

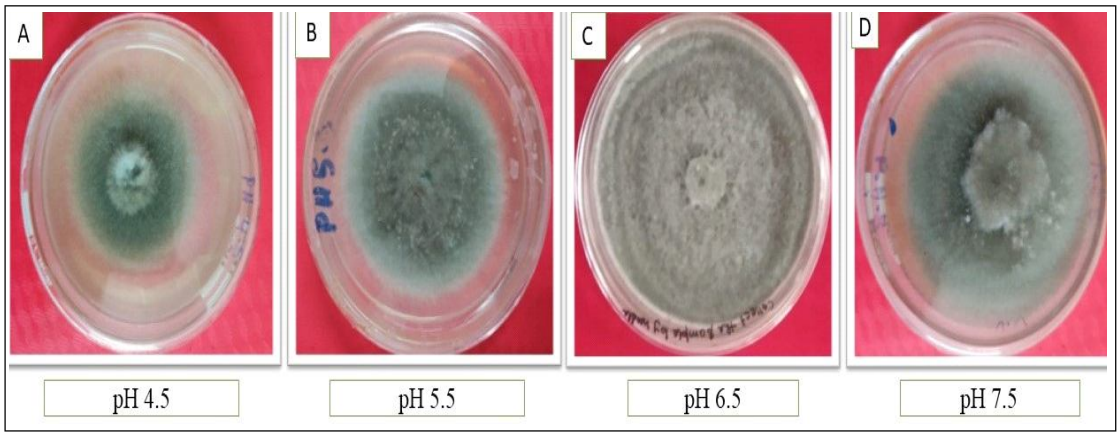


Fig 11: Cultural growth of *N. oryzae* under different pH concentration.

Table 6: Seed mortality percentage of different varieties of paddy through *Nigrospora oryzae* infestation.

DAI	Variety									
	Gobindabhog	CR51	CR1017	MTU7029	MTU1140	Barsha	Pratiksha	Niranjana	Rajendra	Black rice
1st	0	0	0	0	0	0	0	0	0	0
2nd	0	0	0	0	0	0	0	0	0	0
3rd	0	10	0	10	0	10	10	20	0	0
4th	10	10	10	20	10	10	20	20	10	0
5th	10	20	20	40	30	30	40	50	20	10
6th	20	30	40	50	50	40	60	60	40	20

Table 7: Cultural variability study under different pH.

pH concentration	Growth of *Nigrospora* (cm)
4.5	4.00
5.5	5.23
6.5	8.90
7.5	7.63
C.V	2.00
SE (m)	0.08
C.D @ 5%	0.24

pH 6.5, either by lowering or increasing the pH level, led to reduced growth for *Nigrospora*. Remarkably acidic or alkaline pH levels were found to be unsuitable for the pathogen's growth and sporulation (Fig 11). These results are consistent with the research of Tyagi and Paudel (2014), both indicating that an optimal pH level for fungus growth and sporulation is 6.0. Moreover, an elevation in pH levels demonstrated a hindering impact on both growth and sporulation.

CONCLUSION

The emergence of *Nigrospora oryzae* is a complex issue that demands a multi-dimensional approach. Previously, the appearance of these mild endophytes was reported in several other crops also but the pathogenic emergence on rice as leaf blight is the first time observed from southeastern coastal region of Odisha. Disease progression is accelerated by an ideal range of temperatures (25-30°C) coupling with high moisture levels (80-90%). Increasing disease severity reversely proportional to the grain yield, whereas other quantitative characters remains static from our present investigation we found Niranjana and MTU 7029 are the most susceptible variety against *Nigrospora* biotic stress and Black Rice and CR1017 are the most resistant variety.

Conflict of interest

The authors solemnly affirm that they have no conflicts of interest. They are solely associated with the affiliations stated in their contribution and have no ties to any other academic or research institutions. Furthermore, they declare that they hold no financial or non-financial (personal) interests with any other organization or individual. The content of this research paper is entirely original and any material derived from other sources has been properly cited in the references of this manuscript.

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