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Cultural and Morphological Characterization of *Pyricularia grisea* Causing Blast Disease of Rice

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ABSTRACT

Rice (*Oryza sativa* L.) is the world's most important crop and a primary source of food for more than half of the global population. In India, rice is a staple food for more than 65 per cent population. However, the crop is subjected to attack by many diseases among which rice blast caused by the fungus *Pyricularia oryzae* is one of the most economically important diseases. The pathogen was isolated from the rice field of Uttar Banga Krishi Viswavidyalaya Farm, purified, and characterized based on morphological and cultural characters. Highest mycelial growth of 28.50 mm and 71.33 mm were observed in oat meal agar (OMA) medium at 2 and 6 days after inoculation (DAI) respectively, whereas growth of 47.77 mm was observed in potato dextrose agar (PDA) medium at 4 DAI. The mycelial growth was highest at 30°C at 2 (27.00 mm) and 6 (72.17 mm) DAI whereas at 4 DAI, maximum growth of 43.50 mm was recorded at 25°C. The best nitrogen and carbon sources for the pathogen *in vitro* were found to be potassium nitrate and glucose, respectively whereas the best pH for the growth of the pathogen was found to be 6. The conidia in infected leaf sample were three-celled with an average size of 22.42 × 8.59 µm. The conidia on rice grain were predominantly two celled with an average size of 16.45 × 7.46 µm. Conidial production was not observed in any of the media tested including PDA, OMA. However, on rice grain, the fungus produced conidia at 55-60 days.

Keywords

Rice, *Pyricularia*, OMA, 30°C, Potassium nitrate, glucose, pH 6

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Introduction

Rice (*Oryza sativa* L.) is the world's most important crop and a primary source of food for more than half of the world's population. More than 90% of the world's rice is grown and consumed in Asia where 60% of the earth's people live (Kole, 2006). It is the main staple food in Asia and the Pacific region, providing almost 39 % calories (Yaduraju,

2013). Rice grain contains on an average 7% protein, 62-65 % starch, 0.7% fat & 1.3% fibre and rice is a main source of vitamin B1 (thiamin), B2 (riboflavin), B3 (niacin) & B5 (pantothenic acid). Rice occupies a pivotal place in Indian agriculture and is the staple food for more than 65 per cent of the Indian population, accounting for more than 43 per cent of total food grain production and 55 per cent of cereals production in the country.

Rice crop is subjected to attack by 50 diseases including 6 bacterial, 21 fungal, 4 nematodes, 12 viral and 7 miscellaneous diseases and disorders (Hollier *et al.*, 1993; Webster and Gunnell, 1992; Jabeen *et al.*, 2012). Among the fungal diseases, rice blast is the most important and the most destructive disease of rice, which is caused by the ascomycetous fungus *Pyricularia oryzae* (Teleomorph *Magnaporthe oryzae* Couch) formerly known as *Pyricularia grisea* (Cooke) Sacc.] (Couch and Kohn 2002).

Commonwealth mycological institute (CMI) description of the culture: Cultures greyish in colour, conidiophores single or in fascicles, simple or rarely branched, show sympodial growth. Conidia formed singly at the tip of the conidiophore at points arising sympodially and in succession, pyriform to obclavate, narrowed towards tip, rounded at the base, three celled, rarely one or two celled, hyaline to pale olive, $19-23 \times 7-9 \mu\text{m}$, with a distinct protruding basal hilum. Chlamydospores often produced in culture, thick-walled, 5-12 μm diameter. Fungus produces sexual fruiting bodies called perithecia within 21 days. Perithecia are flask-shaped that carry asci containing ascospores, the products of meiosis. Ascospores are arranged as unordered octads or as larger populations of randomly selected ascospores (Nicholas J. Talbot, 2003). Blast fungal isolates produced ring like, circular, irregular colonies with rough and smooth margins on oat meal agar media having buff colour, greyish black to black colour (Srivastava *et al.*, 2014). Culturing of different isolates of *Pyricularia oryzae* was studied by Priya Vanaraj *et al.* (2013) and reported that colonies of *P. oryzae* appeared as white on oat meal, rice polish and malt extract agar, grey on potato dextrose agar and whitish grey on rice agar. Ravindramalviya (2014) used four culture media for the study of mycelial growth of *P. grisea* under *in vitro*. Among them PDA media supported maximum

mycelial growth followed by Richard's Agar medium after 168 hours of incubation.

Materials and Methods

The laboratory work was done in Research Laboratory, Department of Plant Pathology, Uttar Banga Krishi Viswavidyalaya, Pundibari, Coochbehar.

Isolation of the pathogen (*Pyricularia grisea*)

The diseased leaves were collected from the experimental field in which susceptible rice variety Swarnamashuri was planted. 25-30 number of leaves from different places of a heavily blast infected field was taken at 70 days after transplanting. The necrotic patches of diseased leaves were cut into small pieces. These pieces were surface sterilized by dipping in mercuric chloride solution (1:1000) for one minute and were washed by sterilized water for several times. The cut pieces were inoculated in sterilized Petri dish containing potato dextrose agar medium (Riker and Riker, 1936) amended with streptomycin sulphate under aseptic condition and kept in BOD incubator at $25 \pm 1^{\circ}\text{C}$ for development of fungal growth. The fungus cultures were also maintained in culture tube to avoid contamination.

Purification of the pathogen (*Pyricularia grisea*)

Fungus isolation techniques as mentioned above has been used and it was transferred on sterilized PDA petri plates and incubated. The marginal mycelial growth that developed after 2-3 days was picked-up aseptically, transferred in PDA test tubes or PDA petri plates and incubated for getting pure culture of the pathogen. After getting the pure culture in the tubes or plates, the culture from the full grown plate or tube was transferred in another

fresh PDA tube or PDA plate. This is called sub culturing. In this way sub culturing was done at an interval of 15 days and preserved at low temperature ($5\pm 1^{\circ}\text{C}$) in refrigerator.

Cultural studies of the pathogen

The experiment was carried out in complete randomized design with three replications. Pure culture was maintained in Potato Dextrose Agar Medium (PDA) [Riker and Riker, 1936] and 7 days old culture was used for this experiment. Mycelial growth and sporulation of *Pyricularia grisea* was observed in different media [Potato dextrose agar (Peeled potato – 200g, Dextrose – 20g, Agar agar – 20g, Chloramphenicol – 0.05g, Distilled water – 1000 ml), Oat meal agar (Oat meal – 60 g, Agar agar – 20 g, Chloramphenicol – 0.05 g, Distilled water – 1000 ml), Malt extract agar (Malt extract – 30 g, Peptone – 6g, Agar agar – 15 g, Chloramphenicol – 0.05g, Distilled water – 1000 ml), Rice polish agar (Rice polish – 20g, Agar agar – 20 g, Chloramphenicol – 0.05g, Distilled water – 1000 ml), White rice agar (White rice extract – 20 g, Agar agar – 20g, Chloramphenicol – 0.05g, Distilled water – 1000 ml)], at different temperature levels (10°C , 15°C , 20°C , 25°C , 30°C , 35°C), on different carbon sources (Glucose, Dextrose, Maltose, Sucrose, Fructose), on different nitrogen sources (Potassium nitrate, Sodium nitrate, Ammonium nitrate, Calcium nitrate) and at different Hydrogen ion concentration (pH) levels (pH-5, pH-6, pH-7, pH-8, pH-9, pH-10). The data were observed at 2 days after inoculation, 4 days after inoculation & 6 days after inoculation.

Area Under Growth Progress Curve (AUGPC) was calculated following the formula:

$$\text{AUGPC} = \sum_{i=1}^{n-1} \left[\frac{(X_{i+1}) + X_i}{2} (T_{i+1} - T_i) \right]$$

Where, X_i is the growth of the pathogen on i th

date, T_i is the day on which observation was recorded and n is the number of scoring days.

Spore (conidia) morphology

For sporulation of the fungus, rice grain was used. First, 30 g rice grain was sterilized in conical flask in an autoclave at 15lb psi (121.6°C) for 20 minutes. Then 7 days old mycelium of the pathogen was inoculated in the sterilized rice grain and it was kept in a BOD at $25\pm 1^{\circ}\text{C}$. After 55-60 days spore was formed. This spore suspension was again inoculated in fresh rice leaves following detached leaf technique. Those leaves developed similar type of blast lesion and those lesions were moist chambered in BOD at $25\pm 1^{\circ}\text{C}$ for 3 days. The conidia both from leaf sample and from rice grain were observed under a microscope. 50 spores both from rice grain and leaf sample were measured (length and width) in μm with the help of motic image analyzing software and also septation pattern was observed from both leaf sample and rice grain.

Statistical analysis

The laboratory trials were conducted following Completely Randomized Design. The replicated data generated from different experiments were analyzed statistically using statistical package of INDOSTAT and the ANOVA determined the probability for significant variation among the treatments.

Results and Discussion

Isolation of the pathogen

The pathogen was isolated in Potato Dextrose Agar (PDA) media and purified. The pathogen produced white mycelial growth at first but later on it turned into black in colour. Sporulation was not observed in PDA even after 2-3 months of culture maintenance. PDA

was also used for isolation of *Pyricularia grisea* by Motlagh and Javadzadeh, (2010) and Priya Vanaraj *et al* (2013).

Cultural characterization of the pathogen

Growth of the pathogen in different media

Highest growth of 28.50 mm and 71.33 mm was recorded in Oat meal agar (OMA) medium at 2 and 6 days after inoculation, respectively whereas at 4 days after inoculation highest growth was achieved in Potato dextrose agar (PDA) medium (47.77 mm). The second highest growth at 2 and 6 days after inoculation of 28.33 mm and 71 mm respectively was recorded in PDA whereas second highest growth of 45.53 mm at 4 days after inoculation was recorded by OMA. Growth in PDA and OMA was statistically *at par*. Highest Area Under Growth Progress Curve (AUGPC) of 147.10 was recorded by PDA which is immediately followed by OMA (145.37) whereas the lowest AUGPC of 91.53 was recorded by White Rice Agar (WRA) medium (Table 1). So, PDA and OMA were found to be the suitable media for *Pyricularia grisea*. This result is in agreement with Ravindra malviya (2014) who studied that PDA medium supported maximum mycelial growth of *P. grisea* after 168 hr of incubation. This result is in accordance with the findings of Mahdieh (2013) who reported that PDA culture medium could provide the best medium for *P. oryzae* vegetative growth, regardless of light condition.

Growth of the pathogen in different temperatures

Highest growth of 27 mm and 72.17 mm was recorded at 30°C at 2 and 6 days after inoculation, respectively whereas at 4 days after inoculation highest growth was achieved in 25°C (43.50 mm). The second highest

growth at 2 and 6 days after inoculation of 25.33 mm and 70.67 mm respectively was recorded in 25°C whereas second highest growth of 42.17 mm at 4 days after inoculation was recorded in 30°C. Growth in 25°C and 30°C was statistically *at par*. Highest Area Under Growth Progress Curve (AUGPC) of 141.33 was recorded at 30°C which is immediately followed by 25°C (139.50) whereas the lowest AUGPC of 47.37 was recorded at 35°C. At 35°C, the growth was black in colour. In this temperature, huge amount of conidiophores were produced. This may be due to the fact that fungus produces conidia in stress condition. So, 25°C and 30°C were found to be the suitable media for *Pyricularia grisea* (Table 2). This result is in conformity with Awoderu *et al.* (1991), Okeke *et al.* (1992) and Arunkumar and Singh (1995) who reported that optimum temperature for the mycelial growth of *P. grisea* is 25 to 30°C. This result also confirms the findings of Okeke *et al.* (1992) who noted that the growth of *P. grisea* was optimum at 28°C, moderate at 23°C and minimum at 15°C and growth was inhibited at a temperature of 37°C.

Growth of the pathogen in different carbon sources

Highest growth in all the three days of observation was obtained by glucose (33.60 mm, 55 mm and 78.30 mm at 2, 4 and 6 DAI, respectively) whereas the second highest growth in all the three days was achieved by dextrose (32.59 mm, 52.47 mm and 72.60 mm at 2, 4 and 6 DAI, respectively). The AUGPC was also recorded highest in glucose (166.90) and second highest was in dextrose (157.65). The lowest AUGPC of 101.38 was recorded by sucrose (Table 3). This may be due to the fact that glucose is the simplest form of carbon source and the fungus finds it very easy to utilize this simple source of carbon for its good growth and thus the fungus growth is highest in this source of carbon. This result is

in conformity with the findings of Otsuka *et al.* (1957) who reported that Sucrose, glucose, maltose, fructose, lactose and xylose were the most suitable carbon sources for all the 47 isolates of *Pyricularia grisea* they tested. Similar type of results were also found by Otani (1953) who reported that carbon compounds like maltose, sucrose, glucose, inulin and mannitol as well as organic acids such as succinic acid were the best carbon sources whereas, lactose and galactose were not suitable for *Pyricularia grisea*.

Growth of the pathogen in different nitrogen sources

Highest growth of 78.33 mm was recorded in potassium nitrate at 6 days after inoculation. This is followed by the growth in ammonium nitrate where 73.10 mm growth was recorded at 6 DAI. Growth progress was also highest in potassium nitrate which is indicated by an AUGPC of 159.87. Second highest AUGPC of 152.97 was recorded in potassium nitrate whereas the lowest AUGPC of 103.77 was recorded by calcium nitrate. So, potassium nitrate was considered to be the best nitrogen source for the growth of the fungus (Table 4). This result is in conformity with Otani (1953) who found that among the nitrogenous compounds, KNO₃, NaNO₃, glycine, L-alanine, aspartic acid and asparagine markedly accelerated the growth of the isolates of *P. grisea* that he was using. Apparao (1956) also got similar type of results where he found that asparagine, peptone, NaNO₃ and KNO₃ supported good growth of *P. grisea*. He told that nitrate could be utilized by the pathogen and obtained the growth. One of the reasons for good growth in Nitrate nitrogen sources may be that nitrate may be the easiest form of nitrogen that can be utilized by the pathogen and thus all the forms of nitrate used here supported good growth of *Pyricularia grisea*. Our result is in partial agreement with the results of Vikram Pal

(2014) who studied that maximum mycelial growth of *P. grisea* was reported in barium nitrate followed by Ammonium nitrate. But he noted that minimum mycelial growth was found in Sodium nitrate and Potassium nitrate over control. None of nitrogen sources induced the sporulation of *P. grisea*.

Growth of the pathogen in different pH levels

Best growth was recorded in pH 6 which is indicated by highest growth of 29.67 mm, 47.02 mm and 72.83 mm at 2, 4 and 6 DAI, respectively. Second best growth was achieved at pH 7 as second highest growth was recorded in this pH. The growth in both these pH are statistically *at par*. Highest AUGPC of 149.52 was recorded at pH 6 which is closely followed by pH 7 with an AUGPC of 147.18 whereas the lowest AUGPC of 99.70 was recorded in pH 10. At pH 9 and pH 10 drastic decrease in growth was recorded (Table 5). So, it can be said that very high alkaline condition is not preferred by the fungus. The best growth of the fungus can be achieved at light acidic to neutral condition. This result is in agreement with the findings of Arun Kumar and Singh (1995) who observed differential response of *P. grisea* isolates from rice, finger millet and pearl millet to pH. They found that, pH 6.5 was best for the growth of rice and pearl millet isolates and pH 7.0 for finger millet isolates. Similar finding was observed by Mijan Hossain (2000) who studied that mycelial growth of *P. grisea* increased with increase in pH from 3.5 to 6.5. The pathogen showed maximum mycelial growth at pH 6.5.

Morphological characterization of the pathogen

In all the tested media only mycelial growth was noticed. No conidia were produced in those media.

Table.1 Growth of *Pyricularia grisea* in different media

Media	Growth (mm)			AUGPC
	2 DAI	4 DAI	6 DAI	
PDA	28.33	47.77	71.00	147.10
OMA	28.50	45.53	71.33	145.37
RPA	14.17	26.00	59.33	99.50
WRA	15.17	29.03	47.33	91.53
MEA	19.00	32.17	44.67	95.83
SEM±	2.3203	2.8353	2.0385	3.0176
CD (5%)	7.3111	5.7830	6.4235	9.5087
CV (%)	19.106	8.805	6.012	4.511

Table.2 Growth of *Pyricularia grisea* in different temperatures

Temperature (°C)	Growth (mm)			AUGPC
	2 DAI	4 DAI	6 DAI	
10	11.07	18.00	24.17	53.23
15	16.00	32.67	46.00	94.67
20	22.00	39.47	54.67	116.13
25	25.33	43.50	70.67	139.50
30	27.00	42.17	72.17	141.33
35	9.83	15.67	21.87	47.37
SEM±	2.6701	1.5934	1.4679	2.4837
CD (5%)	8.2274	4.9099	4.5231	7.6530
CV (%)	24.946	8.649	5.269	4.358

Table.3 Growth of *Pyricularia grisea* in different carbon sources

Carbon Sources	Growth (mm)			AUGPC
	2 DAI	4 DAI	6 DAI	
Glucose	33.60	55.00	78.30	166.90
Dextrose	32.59	52.47	72.60	157.65
Maltose	30.37	47.53	68.82	146.72
Sucrose	15.75	30.07	55.57	101.38
Fructose	19.83	30.80	65.50	116.17
SEM±	0.8022	1.0510	1.8904	2.5958
CD (5%)	2.5279	3.3119	5.9568	8.1796
CV (%)	5.258	4.217	4.804	3.264

Table.4 Growth of *Pyricularia grisea* in different nitrogen sources

Nitrogen Sources	Growth (mm)			AUGPC
	2 DAI	4 DAI	6 DAI	
Potassium nitrate	34.10	47.43	78.33	159.87
Sodium nitrate	26.33	46.00	72.53	144.86
Ammonium nitrate	30.07	49.80	73.10	152.97
Calcium nitrate	23.27	30.23	50.27	103.77
SEM ±	1.8016	1.1908	2.1123	3.6124
CD (5%)	5.8752	3.8835	6.8885	11.7806
CV (%)	10.972	4.756	5.336	4.458

Table.5 Growth of *Pyricularia grisea* in different pH levels

pH levels	Growth(mm)			AUGPC
	2DAI	4DAI	6DAI	
5	27.33	42.50	64.17	134.00
6	29.67	47.02	72.83	149.52
7	29.20	46.98	71.00	147.18
8	26.67	41.77	64.50	132.93
9	22.90	35.13	56.93	114.97
10	18.43	31.80	49.47	99.70
SEM ±	1.4404	2.5022	2.6996	3.9231
CD (5%)	4.4382	7.7100	8.3183	12.0883
CV (%)	9.707	10.605	7.404	5.238

The conidia of the pathogen were produced only in sterilized rice grain after 55-60 days. Length and breadth of the conidia of blast pathogen (*Pyricularia grisea*) was measured from both rice grain and from lesion developed by inoculation of that culture in the rice leaves. The size of the conidia was much higher from leaf sample than in the rice grain. The size of conidia measured about 17.96 - 26.64 µm × 7.36 - 9.22 µm (average 22.42 × 8.59 µm) and 12.06 - 19.95 µm × 5.38 - 9.06 µm (average 16.45 × 7.46 µm) from leaf sample and rice grain, respectively. This result is in conformity with Tochinai and Shimamura (1932) who classified 39 isolates into nine forms on the basis of cultural characteristics. On steamed rice straw, the conidia of the isolates belonging to four forms were short, the mean value ranged from 19.3

to 22.8 µm. The conidia of other five forms were long, the mean value ranged from 26.8 to 29.9 µm. Mijan Hossain (2000) also observed that mycelium in cultures was first hyaline in colour, then changed to olivaceous, 1–5.2 µm in width, septate and branched. The spore measurements were 15 – 22 µm × 4 – 7 µm (Average, 17.4 µm × 5.2 µm). Mostly 2 celled conidia were found in rice grain and 3 celled conidia were found in infected leaf sample.

In conclusion, these results demonstrated that no conidia were produced in all the media tested. Only in sterilized rice grain the conidia were produced after 55-60 days. The size of the conidia was much higher from leaf sample than in the rice grain. Mostly 2 celled conidia were found in rice grain and 3 celled conidia

were found in infected leaf sample. The best growth was achieved in PDA and OMA medium, 25°C to 30°C is the best temperature for growth of the fungus but for conidiophore production 35°C is best. Glucose is the best carbon sources for the fungus. Addition of potassium nitrate into PDA medium enhanced the growth of the fungus. Slightly acidic to neutral condition (pH 6 - pH 7) is the ideal condition for the fungus.

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