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RESEARCH ARTICLE

SCREENING, ISOLATION AND MOLECULAR IDENTIFICATION OF RICE PATHOGEN MAGNAPORTHE ORYZAE.

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Abstract

Rice, a major food crop and feeds more than half of the world's population, mainly in developing countries including Asia and Africa. *Magnaporthe oryzae*, a Blast pathogen attacks rice at any stage of its life cycle. Blast infected leaf samples were harvested from Lonavla susceptible Paddy field and then plant pathogens were screened and isolated to axenic form. Morphological identification of blast fungus showed grayish colony with circular smooth margin and concentric ring on potato dextrose agar. Spore Induction was performed using stem bits. Pathogenicity assay was performed in plastic pots and using detached leaf samples from susceptible paddy variety. Further desired blast pathogen i.e. Magnaporthe oryzae was identified with ITS region through Sanger sequencing.

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Introduction:-

Rice is important stapale food crop as it serves more than half of the world's population. India is one of the largest producer of rice accounting yearly 20% of the world's population. Rice plant growth is affected by as much as 36 different pathogens. They include bacteria, fungi, viruses and nematodes etc. *Magnaporthe oryzae* is the causal organism of the blast disease. Among that, Rice blast caused by fungus *Magnaporthe oryzae* is most distasterous disease which occur to rice and has reported yield losses up to 50% in Asia, Africa and some parts of America majorly rice growing regions of the world. Rice blast causes world's net production decline as 8% per year. (Wilson, 2009). Various strategies are being deployed to control disease on field. Magnaporthe oryzae is heterothallic fungus as antheridium and ascogonium are produced in two different thalli. *M. Oryzae* belong to class ascomycetae, members of this class are found in variety of habitats. Chief distinguishing characteristic of this class is formation of sac like structure called ascus. Ascospores are formed inside ascus. Mycelium stand have septa. Septa have minute holes or pores. From these pores protoplasm, nuclei move from cell to cell. *Magnaporthe* genus has been classified in separate family known as Magnaporthaceae.(Agrios, 2000). This rice pathogen attacks and infect rice plant at all developmental stages and causes blast symptoms on leaf, collar, neck and panicle if it attacks at stage of harvest. (J.W. Taylor, 2000)

In pathogenic fungi, morphological characteristics properties are used for the identification of the pathogen. Now a days, molecular identification is prefered commonly for two purposes mainly, first is precise and reliable identification of the disease causative organism so that preventive measures could be utilised correctly and second is time required for identification is very short as compared to the morphological identification so disease could be controlled at early stages to avoid further economic losses.

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Blast field samples were collected and screening was performed to isolate pure axenic culture of blast fungus. Objective of this study was screening and isolation of the causative organism of blast field isolate.

Materials and Method:-

Collection of infected leaves:

Infected leaf samples of Chimansal variety (Fig 1) were obtained from paddy fields from Agricultural Research Station, Lonavla, Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharashtra.

Infected leaf samples were strored in dry and cool place for overnight.

Screening and isolation of Plant pathogen:

Site of lesion was carefully cut and treatment of 0.1 % mercuric chloride was given as per the protocol prescribed. (Patel, 1989) Then, surface sterilised infected lesions were placed on potato dextrose agar plate. (Fig 2)

Steps followed were as follows:

1) Arrange four sterile petri dishes in a row near the flame.
2) Fill 0.1% Mercuric chloride solution in the first dish.
3) Pour asceptically sterile water in rest of dishes.
4) Select infected host tissues from advancing margin of lesion, cut into small pieces and place in 0.1% mercuric chloride solution for 5 minutes.
5) With sterile forcep, place sample into water for 5 minutes each for washing.
6) Repeat washing in water for 3 times.
7) Now place pre-treated infected cut tissue on potato dextrose agar plate.
8) Incubate inoculated Potato Dextrose Agar plates at 28ºC for 5-7 days. (Fig 3)
9) Subculture isolated culture further(Fig 4 and Fig 5)

Spore induction on stem bits:

Stem bits from rice (21 day old crop) were collected from field and cut into small pieces of 1cm in length. 10-15 pieces were placed in 50ml Erlenmeyer flask with filter paper at bottom and sterilized at 121ºC at 15lbs pressure for 20 minutes. Each flask was inoculated with 8mm disc of mycelia of 10 day old culture of isolated pure culture from PDA plate. (Vanaraj P, 2013) Then after 15 days, stem piece was taken in a test tube containing 2ml 0.01% tween 20 solution. Test tube was shaken well to dislodge the spores and then spore morphology was assessed using Lacto-phenol cotton blue stain under light microscope. (Fig 6 and Fig 7)

Pathogenicity test:

Pathogenicity test was performed on Paddy plants as well as detached leaves of Paddy Chimanasal variety. Standard procedure was modified from a procedure described by (Jia, 2003). For all experiments, plants were grown in controlled conditions at 24ºC-30ºC with sufficient day light and high humidity was maintained. (Laxman Singh Rajput, 2017)

Plants were inoculated in plastic pot and then leaves were surface sterilized with 0.1% $HgCl_2$ solution. Leaves were pierced slightly with sterile needle and 2ml spore suspension was inoculated with sufficient spore density. Then Plants were sealed with plastic bag as high humidity was required for fungal penetration into host plant. Spore inoculated plants were kept in fluorescent light for 24 hours and then shifted to controlled moisture and temperature chamber. Plants were maintained at 24ºC-30ºC and light and dark cycle of 16 hr and 8 hr respectively. Disease symptoms were monitored daily and plants were maintained till symptoms were visual.

Pathogenicity assay was also performed in detached leaves. 10 cm Paddy leaves were surface sterilized with 0.1% $HgCl_2$ solution. Then sterilized leaves were kept on sterile 15 cm diameter petri plates with moistened filter paper in it. Leaves were pierced slightly with sterile needle and then 2ml spore suspension was added to it. Plates were kept in temperature controlled cabinet until symptoms appear. Then symptoms were observed. (Fig 7 and 8)

Molecular identification of Blast pathogen:

Genomic DNA was isolated by CTAB DNA extraction method. (Doyle JJ, 1990)

The **ITS region of rDNA** was successfully amplified using fungal universal primers ITS4 & ITS5. The sequencing PCR was set up with ABI-Big Dye® Terminator v3.1 Cycle Sequencing Kit. The raw sequence obtained from ABI

3100 automated DNA sequencer was manually edited for inconsistency. The sequence data was aligned with publicly available sequences at NCBI & analyzed for confirmation of organism. Molecular identification was done at Agharkar Research Institute, Pune.

Result and Discussion:-

Isolation of fungus in pure axenic form was obtained. Morphological identification of blast fungus showed grayish colony with circular smooth margin and concentric ring pattern on potato dextrose agar. According to Koch's postulate pathogenic organism was found to be associated with disease at all stages of crop and was able to cause disease if re-inoculated with same host. So investigation was done by performing pathogenicity test of identified field strain on to the same variety of rice from where organism was isolated. Hence the Koch's postulate was proved. Spore induction was repeatedly attempted with different experiments but use of stem bits for sporulation induction was successful. Stem bits from 20 day germinated rice plant were used and good sporulation was achieved as described. (Vanaraj P. et al 2014). The conidiophores of the isolate was found to be slender, straight, bearing clusters of conidia which were typically of pyriform or obclavate and 2-3 septate. (Getachew et al., 2014). Tri-septate conidiation pattern were observed upon staining with Lactophenol cotton blue. (Fig 6) Characteristic feature of pyriform shaped conidial pattern confirms ***Pyricularia grisea*** as a asexual anamorph of ***Magnaporthe isolate***.

Different inoculation methods had been used for pathogenicity test. In Filter paper method (W. Takahashi, 2009), filter paper was kept on detached leaves and spore suspension was added, we had used filter paper below the leaf so as to keep leaves moist for 5-7 days. Results showed that isolated, identified fungus strain was ***Magnaporthe oryzae ck1***.

Molecular identification and confirmation of the field isolate was done by DNA extraction, PCR amplification with ITS pcr primers followed by Sanger sequencing. Molecular analysis of ITS rDNA sequencing was performed. Results were analyzed using Bio Edit Version 7.0.5.3. (Fig 10). Novel ITS r DNA region was uploaded in NCBI and Accession number for reference as MH553171.1.

Pictorial Diagrams:

Fig 1: Blast Infected Leaves of Chimansal Rice variety

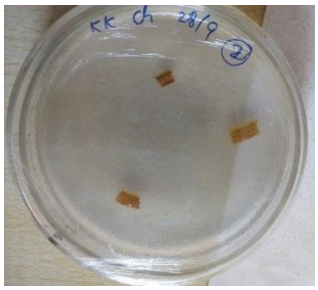

Fig 2: Excised Blast lesions on Potato Dextrose Agar plate

Fig 3: Day 5 representation of Inoculated Potato Dextrose Agar plate

Fig 4: Fungal Axenic culture of Blast pathogen

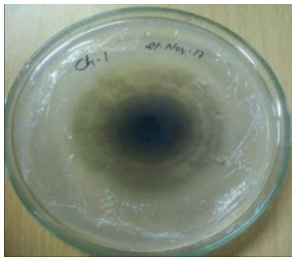

Fig 5: 10 day Culture plate of Blast fungus.

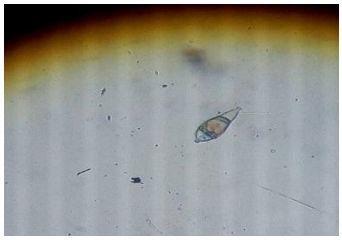

Fig 6: Single Conidia viewed under Light Microscope

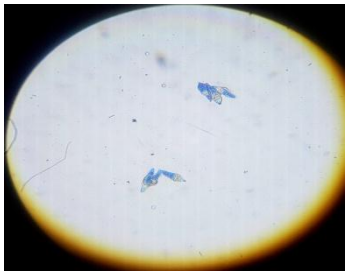

Fig 7: pyriform shaped sporulation pattern

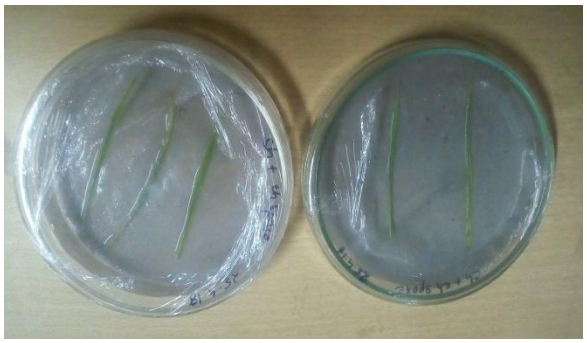

Fig 8: Spore inoculated 8-10 cm detached leaves from Chimanasal Variety

Fig 9: Blast infection observed on spore inoculated 8-10 cm detached leaves from Chimanasal Variety after 7-9 days.

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LOCUS       MH553171.1 Magnaporthe oryzae isolate ck1 small subunit ribosomal
RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal
RNA gene, complete sequence; and internal transcribed spacer 2, partial
sequence       483 bp
ORIGIN
        1 CGTAACAAGG TCTCCGTTGG TGAACCAGCG GAGGGATCAT TACTGAGTTG AAAAACTCCA
       61 ACCCCTGTGA ACATAACCTC TGTCGTTGCT TCGGCGGGCA CGCCCGCCGG AGGTTCAAAA
      121 CTCTTATTTT TTCCAGTATC TCTGAGCCTG AAAGACAAAT AATCAAAACT TTCAACAACG
      181 GATCTCTTGG TTCTGGCATC GATGAAGAAC GCAGCGAAAT GCGATAAGTA ATGTGAATTG
      241 CAGAATTCAG TGAATCATCG AATCTTTGAA CGCACATTGC GCCCGCCGGT ATTCCGGCGG
      301 GCATGCCTGT TCGAGCGTCA TTTCAACCCT CAAGCCTCGG CTTGGTGTTG GGGCGCCCGG
      361 GCCCTCCGCG GCCCGGGGCC CCCAAGTTCA TCGGCGGGCT CGTCGGTACA CTGAGCGCAG
      421 TAAAACGCGG TAAAACGCGA ACCTCGTTCG GATCGTCCCG GCGTGCTCCA GCCGCTAAAC
      481 CCC
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Fig 10: FASTA sequence view in BioEdit Version 7.0.5.3 (Hall, 1999)

Conclusion:-

Rice blast pathogen ***Magnaporthe oryzae ck1*** had been reported to be highly destructive and having huge potential to cause huge losses. Virulent field Blast pathogen was isolated from elliptical Blast lesions on leaf. Phytopathogen was studied in laboratory under controlled conditions. Axenic culture was obtained on potato dextrose agar. Colony morphology and other laboratory parameters were studied. Grayish Radial mycelial with concentric ring pattern of colony growth was obtained and identification using ITS region molecular marker was performed. Sequencing data has been uploaded in NCBI with Accession number MH553171.1.

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