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Detection of rice blast pathogen- Expedition from Petri plate to PCR tube - a mini review

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ABSTRACT

Rice blast caused by *Magnaporthe oryzae* (anamorph: *Pyricularia oryzae*) is a serious disease of rice threatening its production worldwide. The pathogen has high genomic plasticity, wide host range and rapid rate of evolution resulting in fungicidal resistance and breakdown of resistant varieties. Though integrated disease management modules have been adopted for the management of the pathogen, the disease continues to be a challenge to plant pathologists. The pathogen affects all the above-ground parts of the plant including seeds. Seeds are implicated as important overwintering source of inoculum of the pathogen and may serve as an important source of primary inoculum during the rice season. Further, the spread of pathogen to previously un- invaded areas has been found associated with the movement of the infected seeds. Therefore, use of pathogen-free healthy seeds should be an integral part of the rice blast management module, for quarantine purposes. Further, with the appearance of wheat blast as a global threat to wheat production and invasion of rice blast pathogen to previously uninvaded areas, accurate detection of *M. oryzae* strains is of paramount importance. Conventionally used seed testing assays are time-consuming, labor-intensive and have low sensitivity and specificity. Advances in the detection strategies of rice blast pathogen from blotter test to high throughput real time PCR is remarkable. But still, commercial application of these technologies is lacking. The review focuses on the advances in detection strategies of *M. oryaze* and their utility as commercial protocols.

Key words: Rice, blast, detection, PCR, *Magnaporthe oryzae*

Rice (*Oryza sativa* L.) is the second most important cereal crop of the world. It is a staple food for more than half of the world's population and approximately 90% of global rice production is contributed by Asian continent alone. Rice crop is affected by approximately 70 diseases (Zhang *et al*., 2009). However, major diseases affecting rice crop are blast, brown spot, bacterial leaf blight and leaf streak, sheath blight, sheath rot, bakanae, stem rot, tungro virus, false smut and post-harvest diseases (Sharma and Bambewale, 2008).

'Blast' caused by the heterothallic ascomycete *Magnaporthe* species complex (MSC) (Hebert) Barr. (Anamorph: *Pyricularia grisea* (Cooke) Sacc.) is one of the most devastating diseases of rice and can cause severe losses in rice-growing areas with congenial environmental conditions for disease development. The fungus is currently reported to be present in at least 85 countries. Strains of fungus can infect more than 50 graminaceous species including domesticated members of poaceae such as barley, rye, pearl millet, turf grasses, wheat (Igarashi *et al*., 2004) and ornamental plants belonging to family Marantaceae (Pappas and Paplomatas, 1998). Wheat blast caused by *M. oryzae Triticum* subpopulation, first reported from Brazil in 1985 (Igarashi *et al*., 1986), is another devastating pathotype of the pathogen. In 2016 wheat blast was reported from Bangladesh (Callaway, 2016) threatening wheat production in India, Pakistan and China and thereby wheat supply in south Asia.

The taxonomy and nomenclature of this fungus have been uncertain since the early years of the study of the pathogen. There is controversy regarding the nomenclature of the forms of the pathogen that attack

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different hosts. Saccardo (1880) established the genus *Pyricularia* based on *Tricothecium griseum* (cooke) on crab grass (*Digitaria sanguinalis*) from north America with *Pyricularia grisea* (cooke) Saccardo as the type species of the genus. The name *Pyricularia* refers to the pyriform shape of the conidia. Cavara (1891) first described the fungus causing blast disease of rice as *Magnaporthe oryzae* whereas the other morphologically indistinguishable forms on non-rice hosts such as ragi (*Eleusine coracana*), goose grass (*E. indica*), weeping love grass (*Eragrostis curvula*) and crab grass have been referred to as *M. grisea*. Recently, populations of *Magnaporthe* have been classified based on phylogenetic species concept (PSC), multilocus sequence typing (Choi *et al*., 2013) and infectivity on the host and grouped into subpopulations. Further, Gladieux *et al*., 2018, suggested that *M. oryzae* has multiple divergent lineages with host specificity to one host, these lineages have incomplete lineage sorting and genetic exchange between them has resulted in host shift or host expansion. Recent nomenclature of *Magnaporthe* sp is given in Table 1.

Table 1. Recent nomenclature of *Magnaporthe* sp. infecting members of poaceae

Host	*Magnaporthe* sp.	Subpopulation
Crab grass (*Digitaria sanguinalis*)	*M. grisea*	-
Rice (*Oryza sativa*)	*M. oryzae*	*Oryzae*
Wheat (*Triticum aestivum*)	*M. oryzae*	*Triticum*
Turf Grass (*Lolium partensse*)	*M. oryzae*	*Lolium*
Foxtail Millet (*Setaria italica*)	*M. oryzae*	*Setaria*
Fingermillet (*Eleusine coracana*)	*M. oryzae*	*Eleusine*

Seed transmission of the pathogen and its significance

M. oryzae infects most above-ground parts of the rice plant, but neck blast and panicle blast are the most damaging phases of the disease (Devi and Sharma, 2010) and have been shown to significantly reduce grain weight and milling quality (Surek and Beser, 1997). The fungus produces lesions on leaves and different parts of the panicle and grains of the rice plant (Fig. 1). The most destructive infection is panicle and neck blast, characterised by the infection of the base of the panicle. Stem infection causes blackening and shrinkage of the nodal region, penetrating into the tissues. The ears hang down from the stalk and sometimes they may break away. When the fungus colonizes the panicle node and adjacent tissues, the flow of the photosynthates to the developing grains can be inhibited, resulting in light grains or empty panicles.

Infected panicles produce infected seeds. Furthermore, seeds, crop residues and secondary hosts have all been reported as possible sources of *M. oryzae* primary inoculum (Lee, 1994; Teng, 1994). Seed transmission of the pathogen has been reported from Japan (Kuribayashi, 1928) and other parts of the world (Chung and Lee, 1983; Manandhar *et al*., 1998, Afouda *et al*., 2010, Dossou and Silue, 2018). The spread of wheat blast from South America to Asia is also suspected to be with infected seeds (Ceresini *et al*., 2018). It has been reported that fungus can withstand winter in rice seeds (Mew *et al*., 1988). In the tropics, where airborne inoculum is present throughout the year, overwintering of *M. oryzae* is not significant in the disease cycle (Ou, 1985). But in temperate regions overwintering of the pathogen in infected rice seed, rice straw, and rice residue have been implicated as important sources of primary inoculum for rice blast (Manandhar *et al*., 1998;). Infected seeds can also be the primary source of inoculum when seeds are densely sown in seedling boxes for mechanical transplanting, as has been practiced in Japan (Honda and Nemato, 1985).

Fig. 1. Symptoms of rice blast on above ground plant parts: A) On leaves, B) Neck blast symptoms

Manandhar *et al*. (1998) reported that there is a logistic relationship between the infection of neck blast symptom appearance and seed infection by *M. oryzae,* also diseased secondary branches of panicles produced high proportion of infected seeds. They also reported a positive correlation between plant infection in fields and seed infection by *M. oryzae.* In addition,

symptomless panicles and secondary panicles could also yield infected seeds, even in absence of foliar blast symptoms. Seeds from plants infected with higher neck infection have higher percentage of seed infection (Puri *et al*., 2007). These findings are important for seed certification programs which are based on field inspection.

Mycelium and conidia of *M. oryzae* are reported from glumes and caryopsis, thus enabling survival in unfavourable weather conditions. The fungus was found on rachilla, pedicel, palea, lemma and pericarp layers but not in embryo. Many investigators have suggested that *M. oryzae* can move systemically within a seedling from an infected seed (Sesma and Osbourn, 2004) but the systemic movement has not been demonstrated experimentally (Rampant *et al*., 2013). Sporulation of *M. oryzae* on shallow-planted infected rice seed has been observed on seed at or near the soil surface and may be an important source of primary inoculum that could initiate rice blast epidemics.

Lee (1994) reported infected rice seed and rice residue as potential sources of primary inoculum in Arkansas. Manandhar *et al*., (1998) reported that sporulation can occur on all parts of the seed, especially if the seed is not viable and preferential sporulation at embryonic ends in case of viable seeds. Long *et al*. (2001) reported that the use of a marked strain (a sul mutant) of *M. oryzae* confirmed the non-systemic aerial transmission of conidia from artificially infected grain inoculum to rice seedlings and showed that infected seed could initiate rice blast. Furthermore, the marked strain was recovered throughout the season from leaf, collar, and neck infections under field conditions in 1996 and 1997, further emphasizing the importance of the artificially infected grain as a source of primary inoculum. Guerber and TeBeest (2006) reported that planting infected seeds in the greenhouse and the field resulted in seedling infection. Planting naturally infected seed may result in disease development (i) from seedlings grown from infected seed planted beneath the soil surface, (ii) from seedlings grown from germinating seed left on the soil surface, (iii) from seed coats, or (iv) from non-germinated seed left on the soil surface after planting.

Furthermore, Rampant *et al.* (2013) proposed two scenarios for the development of rice blast epidemics in the field. (i) systemic colonization of plant from the infected seeds via systemic development of the pathogen through the vascular system (Guerber and TeBeest, 2006; Marcel *et al*., 2010). Though they found blast sporulation on symptomless seedlings, they could not found fungus in the main culm of the seedlings, further they concluded that the hemi-biotrophic nature of the pathogen could not support the systemic invasion by the pathogen, (ii) production of inoculum from dead seeds or seedlings and transportation of inoculum to healthy seedlings in field conditions. Transportation of inoculum to healthy seedlings via irrigation water is a possible mechanism of transmission, which requires growth of fungus under water. Interestingly they found that contaminated seeds placed within water resulted in diseased seedlings in opposition to previous studies in which blast infection was never detected in waterlogged conditions (Manandhar *et al.*, 1998). Irrespective of the mechanism of the transmission of blast pathogen from infected seeds to plant, it is evident that infected rice seeds serve as important inoculum source for initiation of epidemics. Thus, detection of blast pathogen should be pre-requisite for efficient and economical blast management module.

Conventional methods for detection of seed borne infection

Guidelines for comparative seed health testing (i.e. organization, design, procedures and analysis of results) initially issued by ISTA (International Seed Health Testing Association) were replaced by ISTA handbook of method validation for seed testing (Sheppard and Cockerell, 2008). Most commonly used seed assays for rice blast are, agar plate test, freezer blotter test (Neergaard, 1970) and blotter test (Fig. 2). In 1985 blotter test was recommended in the international rules for seed testing as a testing method for infection of *M. oryzae* in rice seed samples (Agarwal and Kharlukhi, 1985). Khan *et al*. (1988) compared blotter test, agar plate (ulster) test and freezer blotter test for identification of seed borne pathogens of rice. They found that blotter test yielded quantitatively more fungi than agar plate test and technique was comparatively more suitable for studying general micro-flora on rice seeds. Limonard (1968) reported that antagonism between different fungi is common problem during seed health testing by agar plate method. Deep freezing method

was found suitable for detection of deeply seated pathogens like *Fusarium* sp., *Trichoconis padwickii* and *Curvualria* sp. (Khan *et al*., 1988). Pande *et al*., (1994) evaluated different conventional methods for detecting seed borne infection of *M. grisea* in *Eleusine coracana* and found that the standard blotter test, freezer blotter test and agar plate test give similar infection count but sporulation of the pathogen was more on freezer blotter test.

Importance of seeds borne inoculum in epidemiology and disease cycle of pathogen (Narayanasamy, 2011):

(i) Quantitative estimation of pathogen
(ii) Assessment of management strategies
(iii) Seed certification
(iv) Assessment of germplasm for disease resistance
(v) Identification of new pathogen strains
(vi) Study the taxonomic and evolutionary relationships of plant pathogens
(vii) Study the intricacies of interactions between plants and pathogens to have an insight into the phenomenon of pathogenesis and gene functions

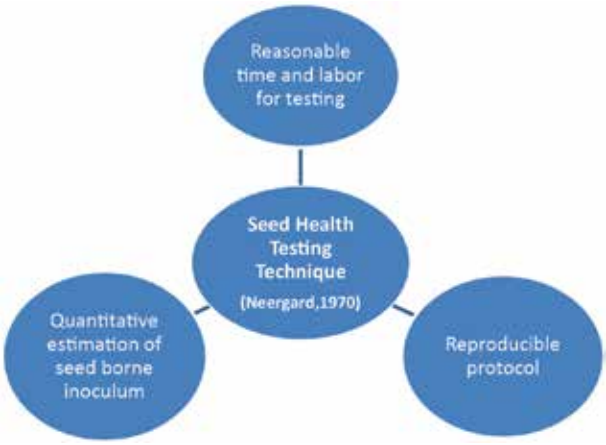

Fig. 2: Characteristics of seed health testing technique for detection protocols

Development of PCR assay specific to *Magnaporthe* spp.

Polymerase chain reaction (PCR) is *in vitro*, primer directed, enzymatic reaction capable of exponential amplification of DNA (Erlich *et al*., 1988; Saiki *et al*., 1988). The technique has various applications in different branches of biological sciences. Rasmussen and Wulff (1991) were first to apply PCR approach for the detection of a bacterial pathogen in diseased plants. Since then, the approach is widely used as viable alternative for detection and diagnosis of pathogens due to its advantages over conventional methods. PCR based detection assays are specific, sensitive, efficient, rapid, versatile (Henson and French, 1993) and relatively economical. These methods are ideal for detection of pathogen *de novo,* because they do not require isolation of pathogen in pure culture, thus saving time and resources (Walcott, 2003). Furthermore, parallel to serological methods PCR based assays have both narrow and broad specificities (Henson and French, 1993) and are relatively sensitive.

To develop a successful PCR test for a species or strain it is essential to identify DNA sequences that are unique or characteristic for species or strains under consideration. Among the factors affecting specificity of the primer pair, primer length, annealing temperature, Mg^{+2} concentration and formation of secondary structures are most important. Also, oligonucleotide primers of 18-30 bp in length, G+C content 50%, and without complementary 3' ends or inverted repeats are considered ideal for detection procedure (Henson and French, 1993). The products of PCR can be used for three different purposes: (i) as a target for hybridization, (ii) for direct sequencing of the DNA to determine strain variations and (iii) as a specific probe (Narayanasamy, 2011).

Despite potential applications, PCR-based seed assays have not been widely adopted for seed testing. Reasons for this are presence of secondary compounds and inhibitors in seeds which affect PCR results and errors in quantification of exact amount of pathogen. PCR based assays are unable to estimate the viability of the pathogen, as DNA from non-viable propagules may be amplified and give an impression of contamination (Henson and French, 1993). Since theoretically PCR is capable of amplifying a single DNA molecule from reaction mixture, possibility of contamination of commercial reagents with target sequences resulting in false positive is never ruled out (Saksena *et al*., 1991; Chou *et al*., 1992; Rochelle *et al*., 1992).Additionally, formation of non-specific products due to mis-priming and formation of artifacts in form of primer dimers add more complexities to the probability of correct interpretation. Thus, for wider

acceptance of PCR based assays it is necessary to address these problems. Solution of these problems is suggested by using hot start PCR, using primer with high annealing temperature for increasing specificity and precautions should be taken to avoid contamination of reagents and working area with target sequences (Henson and French, 1993).

PCR based detection assays have been proposed for *Magnaporthe* spp. using primers derived from different regions of genome. Harmon *et al*. (2003) reported development of PCR based assay for *Magnaporthe oryzae* from ryegrass (*Lolium perenne* L). Primers were designed to amplify a 687-bp fragment of the Pot2 transposon that is found in multiple copies in the genome of the pathogen. The protocol amplified amounts of purified DNA up to 5 pg and consistently and specifically detected *M. oryzae* in single diseased leaf blades as well as in field samples of infected perennial ryegrass. The total time required for detection was approximately 4 to 8 h. Karthikeyan and Gnanamanickam (2005) used the same primer pair to detect infection of blast from leaves of *Setaria italica.*

Chadha and Gopalakrishnan (2006), reported development of PCR based assay for detection of *Magnaporthe oryzae* from rice seeds. They developed mif23 gene based primer for detection of seed borne infection, and they found the assay to be highly specific. Sensitivity of the assay was 20pg and it could detect infection level of 0.2% after enrichment of infested seeds. Also, they did not find amplification directly from infected seeds and discussed due to low level of infection level in the seeds, enrichment is essential step in the procedure. Balodi and Kumar (2015) reported PCR based detection assay using PWL*2* gene. The sensitivity of the assay was found to be 50 pg and direct detection of the pathogen from seeds was reported. PWL2 gene belong to PWL gene family of the rice blast fungus and is ubiquitous in the genome of the fungus infecting wide range of the host (Kang *et al*., 1995). It was observed that with PWL2 gene specific primer pathogen could be identified from *Setaria italica* and *Eleusine coracana*. Thus assay was found to be very specific and durable in wide host range of the pathogen.

Real time PCR based detection

Real-time PCR is a variation of conventional PCR, which allows quantification of DNA or RNA in a PCR reaction mixture. With the help of sequence specific primers relative numbers of copies of a particular DNA or RNA sequence can be estimated by estimating C_t(threshold cycle) value for the sample. If a particular sequence (DNA or RNA) is abundant in the sample, amplification is observed in earlier cycles (low C_t value); if the sequence is scarce, amplification is observed in later cycles(high C_t value). Real-time PCR is technically advanced and provides following advantages over conventional PCR: (1) it allows real time monitoring of PCR reaction, (2) it enables measurement of amplicon after each cycle, (3) it increases range of detection of target sequence hence increases sensitivity of the assay, (4) since estimates are done in real time it does not require post amplification steps of gel electrophoresis thus saving time and resources (In-vitrogen).

With real-time PCR, DNA amplification is coupled with the production of a fluorescent signal that increases proportionally with the numbers of amplicons produced (Kurian *et al.*, 1999; Cockerill and Smith, 2002). The fluorescent signal is monitored on a computer in real-time and provides an indirect visual representation of DNA amplification. Detection of amplified DNA can be accomplished by staining with SYBR Green I (Molecular Probes Inc., Eugene Ore.) that binds double-stranded DNA indiscriminately or with the use of specific reporter probes like TaqMan (Applied Biosystems, Foster City, Calif.) (Taylor *et al.*, 2002). TaqMan probes are synthesized with reporter and quencher molecules at the 5' and 3' ends, respectively. Theses probe work on the principle of fluorescent resonance energy transfer (FRET), such that when reporter and quencher dye are proximal to each other there is no fluorescens and when they separate reporter dye fluoresces. The change in fluorescens is detected by photosensors and change in intensity is directly proportional to DNA amplification. Other detection systems including molecular beacon probes are also employed for real-time PCR, these also work on the same principles as TaqMan probe (Cockerill and Smith, 2002).

Real-time PCR offers key advantages that make it more acceptable than conventional PCR for routine seed testing *viz.* rapid cycling and real time analysis of PCR products makes it user friendly and well suited for swift analysis, reduction in cross contamination

Table 2. Sensitivity and specificity of the detection assays reported for *Magnaporthe oryzae* from rice

Method of detection	Sensitivity	Specificity	Reference(s)
Conventional methods (includes Blotter test, Freezer blotter test, Agar plate test)	NA	NA	ISTA
Polymerase Chain Reaction based methods	20-50 pg of genomic DNA of pathogen	Specific to targeted pathogen and no amplification was seen with other tested pathogens	Chadha and Gopalakrishnan (2006), Balodi and Kumar (2015)
Real Time Polymerase Chain Reaction based methods (QPCR)	6.9×10^{-5} ng of genomic DNA of pathogen	Specific to targeted pathogen and no amplification was seen with other tested pathogens	Sun *et al.* (2015)
Serological methods	60 ng fungal protein/ml	Specific to targeted pathogen and no reaction was reported on ELISA with other tested pathogens	Lee *et al.* (1998)
qLAMP	10 conidia	*M. oryzae* from turf grass	Villari *et al.* (2017)
LAMP	10-99 pg	*M. oryzae* from rice	Ortega *et al.* (2018)

due to closed system, reaction can be multiplexed with multiple probes and primers (Wittwer *et al.*, 2001). Real-time PCR can allow quantification of template DNA which may be of use in determining levels of seed infection. Though cost involved in real time PCR based detection is key constraint in its adoption as a routine technique in seed testing laboratories, but advantages are more than economics involved.

Detection assay based on real time PCR is reported by Zhao *et al.* (2012) for root infection of Poa and Festuca turf, caused by *M. poae*. They found a sensitivity of 3.88 pg of genomic DNA of pathogen and reported infection detected with real time PCR corresponded to visual assessment of disease. Further, Sun *et al.* (2015) using relative qPCR, SYBR Green 1 based assay using rice actin gene as house keeping gene, found sensitivity of the assay to be 6.9×10^{-5} ng of genomic DNA of pathogen. The results of the experiment showed potential of using real-time PCR for detection of the blast pathogen.

In another approach Villari *et al.* (2017) reported pathogen specific quantitative loop mediated isothermal amplification (qLAMP) coupled with a spore trap sytem for rapid detection and quantification of air borne inoculum of *M. oryzae* perennial ryegrass pathotype. They found that the system could detect 10 conidia, 12 days before symptom development. The technology has potential as a decision support system for fungicide application. Ortega *et al.* (2018) reported LAMP assay for detection of *M. oryzae* using calmodulin sequence with a sensitivity of 10-99 pg/ sample. Both are rapid testing methods and require less than 1 hour to complete the procedure. The sensitivity and specificity of various detection assays for *M.oryzae* from rice is given in Table 2.

Conclusion and future prospects

M. oryzae is a notorious pathogen and its management requires integrated approaches. Rapidly developing populations of the pathogen coupled with host expansion are threat to production of cereal crops. In addition, spread of the pathogen to previously uninvaded areas with infected seeds on the one hand increases the threat of bio-invasion on the other use of infected seeds may reduce production in existing areas. Thus, rapid and sensitive detection of the pathogen is needed for effective prevention of spread of pathogen with infected seeds. ISTA has recommended use of blotter test, freezer blotter test and agar plate test for detection of pathogen. These procedures lack required sensitivity, specificity and are time consuming. Further, rapid detection of the pathogen is needed for quarantine purposes. PCR and qPCR based detection procedures can be alternatives for existing problems. Recently many, detection assays are reported based on these technologies but commercial products are still having a long way to go. Detection of plant pathogens in general requires attention from plant pathologists. With the availability of high throughput technologies, visual identification based on symptomology requires upgradation.

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