BIO-DETECTIVE: A CUTTING-EDGE APPROACH FOR UNVEILING *Magnaporthe oryzae* pathotype *Triticum* (MoT) IN WHEAT SEEDS

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Abstract. To choose healthy seeds from a seed lot, an experiment was done to determine whether and how much wheat seeds had the blast pathogen. Total 145 wheat seed samples were gathered from three upazilas in Bangladesh's Meherpur district in the winter of 2019-Meherpur Sadar, Mujibnagar, and Gangni. Seeds were checked for any seed infection using a stereo-binocular microscope while incubated in moist blotter paper at room temperature (25°C±1). *Magnaporthe oryzae* pathotype *Triticum* (MoT) growth was confirmed by pathological analysis for any growth that was visible under stereo-binocular. 35 samples out of 145 were infected with MoT, with severity ranging from 2-16%. A PCR was conducted using the primers *MoT3* and Pot2a on fungal isolates from seeds with varying amounts of MoT infection (0, 5, 10, and 20%). Monomorphic bands of 350 bp and 361 bp were obtained by agarose (1.5%) gel electrophoresis. This proved the seed infection was caused by *Magnaporthe oryzae* pathotype *Triticum*, as seen in the moist blotter. *MoT3* and Pot2a primer were used to analyze 12 samples, finding 10 infected wheat samples, 1 seemingly healthy wheat seed sample, and 1 infected rice blast sample. According to the findings, 6 samples-including the one that was sampled and appeared to be in good health-clearly amplified bands of 350 bp by *MoT3* primer, and seven samples amplified bands of 361 bp by Pot2a primer. This study's findings show that the moist blotter technique effectively detects MoT presence in wheat seeds, offering farmers a tool to determine seed suitability for planting.

Key words: molecular; pathogen detection; pathological; seed borne disease; wheat blast.

INTRODUCTION

Most of the human population relies on wheat as a staple food, making it the most significant cereal crop. It is a popular winter crop that is grown all over the world under a variety of agro-ecological conditions and cropping strategies. Wheat is the food crop that offers the greatest amount of nutrition worldwide. The large range of food products made from wheat account for its popularity, which helps to explain why it has spread to areas where it has not traditionally been grown [25]. The grass family *Poaceae*, tribe *Triticeae*, includes the genus *Triticum*, which is the source of wheat [6]. Since Linnaeus' original naming in 1753, numerous modifications have been made to the taxonomic description of *Triticum* and its associated taxa. The genus *Aegilops*, which is closely related to wheat, has been combined with *Triticum*, as Bowden proposed [2] and now generally accepted. Diploid, tetraploid, and hexaploid species can be found in the genus *Triticum*. Therefore, polyploidy has played an important role in the evolution of wheat [17]. Following rice, wheat is the second-most significant cereal crop in Bangladesh, accounting for 7% of all food output. Its acreage is in increase as shown in wheat production statistics of Bangladesh. According to Rosegrant and Agcaoili the demand for wheat in the developing countries is expected to increase by 60% by 2050 [20]. However, *Magnaporthe oryzae* pathotype *Triticum*'s outbreak of wheat blast in Bangladesh in 2016 presented a terrifying threat to the country's wheat farming. The disease caused heavy loss of wheat production in eight districts of South-Southwestern part of the country [1, 4, 7]. Because wheat blast epidemic created a devastating loss to wheat production as blast infection in the spike caused total bleaching of wheat grains. No yield could be harvested, making a loss of 14% wheat grain production in the country [18]. If wheat cannot be grown for the devastating blast disease problem, can we imagine the setback in food production situation of Bangladesh and thus the food security? The wheat blast is now confined to the South and South-West region of the country [14]. An unguarded situation will, without any doubt, help spread this menace to major wheat growing northern part of the country. Therefore, we need to plan a strategy to limit the spread of the disease. The pathogen *Magnaporthe oryzae* pathotype *Triticum* is seed-borne but its seed transmission is not established [18]. Under Bangladesh conditions, the disease is mainly manifested as head infection during pre-heading. Symptoms on the leaves and leaf sheaths are seldom seen under natural field conditions [18]. Numerous MoT spores have been found in seeds of recently harvested infected wheat spikelets. However, with time, MoT levels in stored seeds decreased. It is unclear in this instance how much the seed-borne inoculum contributes to the infection of the wheat plant's emerging head. Under the correct environmental circumstances, the explosive hazard known as blast can reduce the output of sensitive cultivars by up to 100% [12]. The fungus's ability to impact grain yield and quality has important economic implications. MoT is seed-borne, according to numerous studies [10, 22]. In Bangladesh, this issue has not been addressed. However, it is crucial since seeds with MoT infection may induce head infection if sown. Thus, MoT must be removed from the seeds before they are chosen for sowing. Therefore, it is necessary to create a cheap and simple method for determining whether MoT is present in seeds. This was the purpose of the current inquiry, which had the following goals: using pathological and molecular assays, to identify the presence of the blast pathogen in seeds from a wheat seed lot in order to choose healthy seeds.

MATERIAL AND METHODS

Sample collection and preparation

Farmers in Meherpur's Meherpur Sadar, Gangni, and Mujibnagar Upazila provided the seeds of various wheat varieties, which were then gathered (Fig. 1). At Bangladesh Agricultural University in Mymensingh, the samples were kept in the laboratory conditions. The temperature was held at 40^0C during the storage period.

Analysis of seed samples for MoT through pathological assay

Following ISTA guidelines for seed testing, the Blotter incubation method was employed to identify the seed-borne pathogens connected to the seeds in seed samples [15]. In this technique, a plastic Petri dish of 9 cm in diameter was filled with 25 seeds after two layers of blotting paper had been soaked in sterilized water. For each sample, eight duplicates with 200 seeds each were created (Fig. 2).

For seven days, the seeds in the Petri dishes were kept in the incubation chamber at a temperature of 25°C and exposed to 12-hour cycles of near ultraviolet light and darkness. To keep the blotting paper moist, periodic watering was done. Data on germination were compiled and expressed as a percentage seven days after incubation in blotting papers.

To count the number of colonies of MoT, each incubated seed was examined under a stereomicroscope at 16X and 25X magnifications. By monitoring the growth characteristics of the incubated seeds on blotter paper, the majority of the linked infections were found. The findings were displayed as percent incidence for each pathogen. Data were collected by observing colony of MoT under stereomicroscope and by observing conidia and mycelia through preparing slides.

Analysis of seed samples for MoT through molecular assay

DNA extraction from fungal isolates: Genomic DNA extraction from fungal isolates utilized a modified mini-prep method from IRRI. Hygiene was upheld via autoclaving all glassware, micropipette tips, PCR tubes, distilled water, reagents and buffer solutions. Seeds crushed, mixed with 800 µL extraction buffer, incubated at 65°C for 20 mins. Added 800 µL chloroform mixture (24:1), tubes inverted 3 mins, then centrifuged 8 mins at 11000 rpm. From a 1.5 mL tube, drawn an aqueous part of 500 µL. Added 1000 µL cold ethanol (100%), tubes centrifuged 12 mins at 13200 rpm. Added 1000 µL cold ethanol (70%), tubes centrifuged 3 mins at 13200 rpm. Pellet air-dried on beach (~30-45 mins). Pellet resuspended in 100 µL TE buffer, dissolved in 65°C water bath for 1 hr. DNA stock stored at 4°C.

PCR and gel electrophoresis: The PCR mixture containing DNA was prepared with a volume of 10 µL per reaction according to the procedure outlined by Pieck *et al*. [19]. The mixture was then placed within PCR tubes and subjected to thermal cycling in a DNA thermal cycler. For individual PCR reactions, a combination of components was utilized. This included Green taq polymerase (3.5 µL), dNTPs (0.5 µL), DNA Polymerase (0.25 µL), and Primers (1 µL each for forward and reverse, as specified in table 1). Additionally, sterilized ddH2O (3.75 µL) was added. With this 9 µL PCR cocktail 1 µL genomic DNA was added and finally each PCR tube received 10 µL of the corresponding template DNA sample. The PCR amplification process involved initial denaturation at

Figure 1. Samples of 250-500g wheat seeds were collected through DD, DAE Meherpur

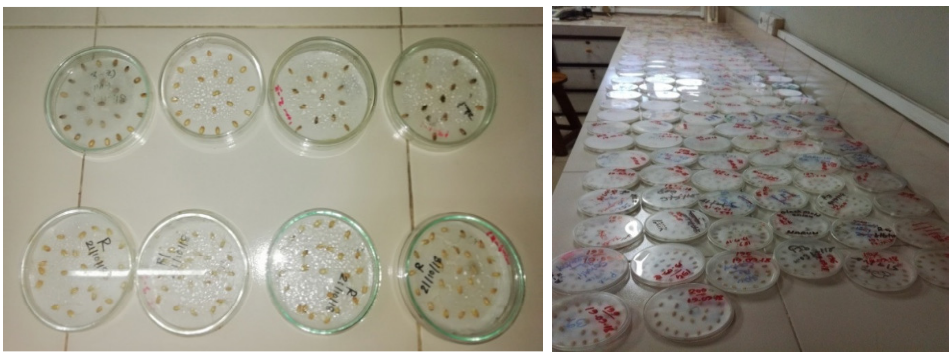

Figure 2. Placement of seeds in blotter

Table 1. The sequence and size of the selected specific primer used for MoT through molecular assay

Primer Name	Sequence		Annealing temp.(°C)	Target species
*MoT*3	Forward	GTCGTCATCAACGTGACCAG	62	*Magnaporthe oryzae* pathotype *Triticum*
	Reverse	ACTTGACCCAAGCCTCGAAT		
Pot2a	Forward	GCAATTTCATGCAACCGAAA	62	*Magnaporthe oryzae* pathotype *Triticum*
	Reverse	CGTACGCCAACCAGATTGAA		

94 °C for 90 seconds, followed by 30 cycles consisting of denaturation at 94 °C for 30 seconds, annealing at 62 °C for 30 seconds, extension at 72 °C for 60 seconds, and a final extension step at 72 °C for 2 minutes. The amplified PCR products were subsequently subjected to analysis on 1.5% agarose gels, which were then stained using ethidium bromide. Loading approximately 2 µL of each PCR product into the respective wells, a DNA size marker such as a 100 bp DNA ladder was employed for determining the sizes of the DNA fragments. Following staining, the gel was gently removed from the staining tray and positioned on a high-performance ultraviolet light box (UV-trans-illuminator) within a GEL Doc system for visualizing the DNA bands.

Data analysis

In order to statistically analyze the data on the different characters using analysis of variance (ANOVA) technique to find out the variations resulting from experimental treatments by using the software MSTAT-C.

RESULTS

Wheat blast symptoms on seed

Reddish-brown to dark-gray margins on seed surface were observed. Infected seeds appeared shriveled and shrunk. Size looked small and shapes were deformed (Fig. 3a and 3b).

Figure 3. Healthy(a) and infected(b) wheat seeds

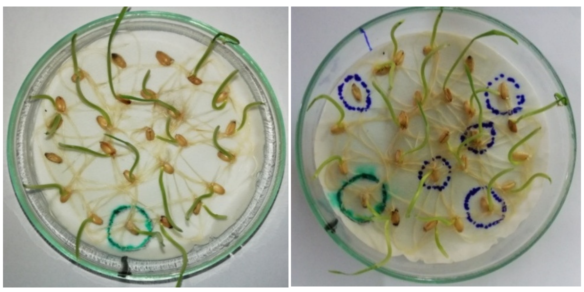

Figure 4. Seed germination and infection by wheat blast pathogen *Magnaporthe oryzae* pathotype *Triticum*

Observation of seed infection by MoT under microscope

Wheat seeds incubated in moist blotter when observed under the stereo-binocular microscope revealed the presence of MoT. Seed germination was not affected by the infection of MoT. Both germinated or dead seeds yielded MoT (Fig. 4).

On the seed surface *Magnaporthe oryzae Triticum* appeared as short conidiophore bearing conidia at and near the tip. At the tip, 3-4 conidia appeared in the whorls (Fig. 5a). Slides prepared out of the structures

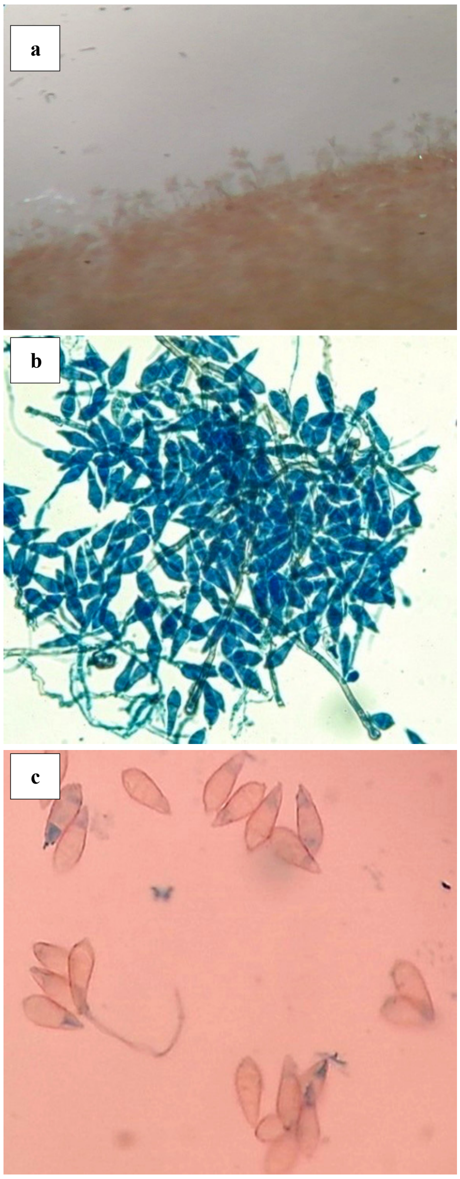

Figure 5. a) Conidia on conidiophores of MoT on the surface of wheat seed as seen under stereo-binocular microscope in 40X. b) Pyriform 3-celled hyaline conidia of MoT as seen under a compound microscope in 40X view c) Pyriform 3-celled hyaline conidia 100X view.

picked up from seed surface revealed innumerable pyriform conidia of *Magnaporthe oryzae* pathotype *Triticum* under the compound microscope (Fig. 5b). The number varied from 8×10^6 to 10×10^6 per mL suspension. Conidia were hyaline, pyriform, and 3-celled. The size of the conidia as measured was approximately 5-6.25 µm × 17.5-20 µm (Fig. 5c).

Seed-borne infection of MoT

The table 2 presents data on seed samples collected from different areas within Mujibnagar, Gangni, and Meherpur Sadar upazilas. At Mujibnagar upazila, the number of seed samples was 33 and there was no MoT found, where germination percentage was 90.8%. At Gangni upazila, the number of seed samples was 23 and there was no MoT found, where germination percentage was 90.26%. From two blocks of Meherpur Sadar 89 seed samples were tested. In Block 1, 10800 seeds representing 54 samples were tested, seed germination was 92.84%. No seed was found to show any infection of *Magnaporthe.* In Block 2, seed germination varied from 85 to 98% in 35 samples. Each of 35 samples yielded MoT. Seed infection varied from 2-21%. Seeds carrying 21% infection of MoT had 97% germination. Seeds with 2% infection of MoT had seed germination of 93-96%. Other fungi detected in seeds were *Bipolaris sorokiniana, Alternaria tenuis, Fusarium moniliforme, F. oxysporum, Aspergillus niger,* and *A. flavus*.

Molecular detection of MoT in wheat seeds

Six of the 12 examined samples had a band of 350 bp after PCR with the primer *MoT3*. Samples no. 1 ,2, 5, 8, 10 and 11 produced band (Fig. 6).

Sample no. 12 did not produce band which represented rice.Marker Pot2a when run in PCR produced band for samples no. 1, 2, 3, 5, 7, 8, 10 (Fig. 7).

Correlation between seed infection and seed germination

Seed infection by MoT ranged 2-21% while germination of the seeds ranged 85-98%. The regression equation indicates if seed infection is 21%, the seed germination is 90.6%. Conversely with 2% seed infection, the germination is 94.7% (Fig. 8). The relation is non-significant (r = 0.248) depicting no effect of *Magnaporthe* infection of seed on germination. When these two variables were plotted a straight-line equation of y=-0.231x +95.18 was obtained.

Table 2. Seed testing results including infection rates in different upazilas

Upazila	Seed samples	MoT found	Germination percentage
Mujibnagar	33	No	90.80%
Gangni	23	No	90.26%
Meherpur Sadar	89		
Block 1	54	No	92.84%
Block 2	35	Yes	Varied (85-98%)

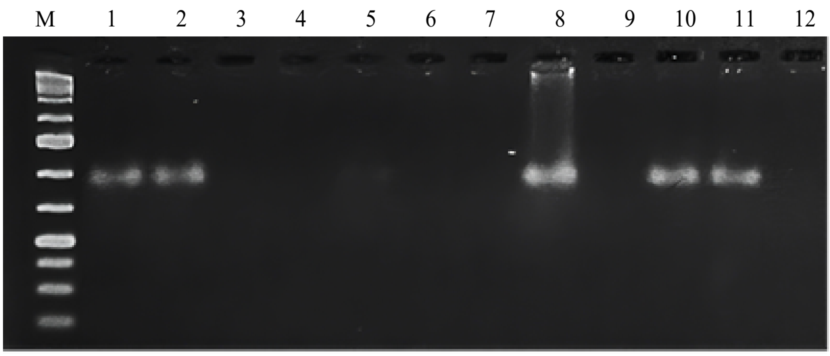

Figure 6. Ten (sl. 1-10) seed samples with wheat blast infection were electrophoresed on agar (1.5%) gel along with one apparently good-looking wheat seed (sl. 11) and one blast infected rice (sl. 12). The DNA was amplified with primer *MoT3*. Lane M is a 100 bp DNA ladder, while Lanes (1–12) contain the PCR results from the 12 seed samples. Six samples of wheat seeds included an amplicon with a size of 350 bp.

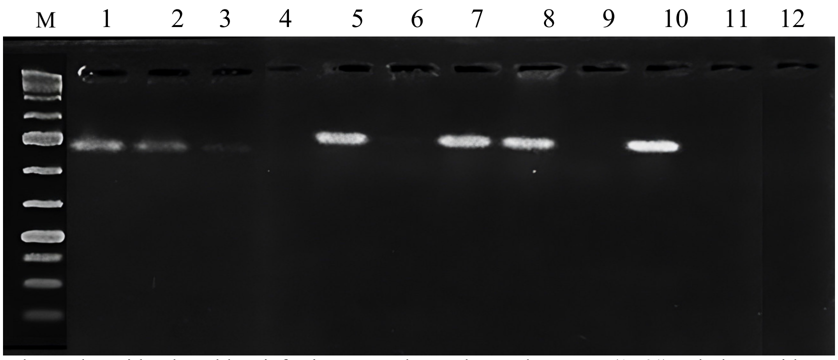

Figure 7. Ten (sl.1-10) seed samples with wheat blast infection were electrophoresed on agar (1.5%) gel along with one apparently good-looking wheat seed (sl. 11) and one blast infected rice (sl. 12). The DNA was amplified with primer Pot2a. Lane M is a 100 bp DNA ladder, while Lanes (1–12) contain the PCR results from the 12 seed samples. Seven samples of wheat seeds included an amplicon with a size of 361 bp.

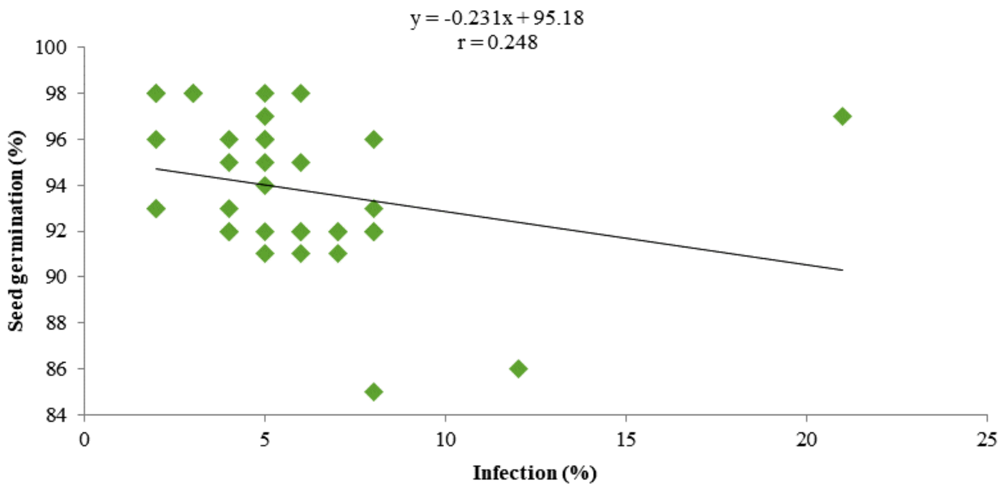

Figure 8. Effect of level of seed infection by *Magnaporthe oryzae* pathotype *Triticum* on the germination of wheat seeds

DISCUSSION

The control of wheat blast is crucial for the long-term productivity of wheat. Many studies have hypothesized that MoT is carried by seeds [9, 23, 24], hence treating seeds before sowing should provide effective protection against blast pathogen for wheat crops. To ensure that MoT isn't present in seed batches meant for sowing, though, it's crucial to conduct this inspection. The samples of seeds taken from various parts of the Meherpur district have several unusual characteristics, it supports to Goulart *et al.* [10], grains from blast-infected spikes of particularly sensitive cultivars are frequently small, withered, and deformed. When compared to healthy seeds, the visually estimated contaminated grain can be easily identified, with the infected seeds being small, malformed, and low in weight. Additionally consistent with earlier research are these results [16]. Consistent with our findings, previous research has documented that when wheat plants are afflicted with the wheat blast pathogen during the grain filling phase, the resulting kernels exhibit deformity, small size, wrinkles, and poor weight content. The current study's detection of MoT in seeds suggests that seeds are the main source of inoculum, which is consistent with Greer and Webster's [11] assertion. This result supports the hypothesis of Goulart *et al.* [9] that pathogens spread from contaminated wheat rachis to harvested seeds. Contaminated seeds were thought to be a significant factor in MoT long-distance dispersion, which is consistent with Goulart and Paiva's [8] assessment. Blast is transmitted in new wheat growing regions as a result of fungal contamination of wheat seeds. This research supports the hypothesis put forth by Urashima *et al.* [24] that seeds drive seed transmission and blast occurrence in newly established wheat-growing regions. According to Urashima *et al.* [23], seeds taken from healthy-looking and diseased spikes of specific cultivars may have similar levels of infection. This is supported by the fact that one of the seed samples utilized for PCR analysis (no. 11) using *MoT3* primer appeared healthy yet produced a band of typical 350 bp size. 110 of the 145 seed samples analyzed showed no signs of MoT. The remaining 35 seed samples had 2-21% infected seeds, nevertheless. This outcome is consistent with Goulart's [10] findings, according to which some seed samples contained MoT infection while others did not. The results of the current study are similar to those of Ceresini *et al.* [3], who discovered that primer Pot2a and primer *MoT3* produced bands for part of the samples they analyzed, which were 361 bp and 350 bp in size respectively. Lots of seeds with less than 20% seed infection showed that they were taken from blast prone fields and that the seed-borne inoculum MoT had infected the wheat seeds that had been grown on the mother plant. The sowing of these seeds is therefore not advised. Conidia were measured to be roughly 5-6.25 µm × 17.5-20 µm in size, which is consistent with Henry and Andersen's [13] estimation of MoT conidia (8-9 µm × 20-26 µm). The results of the present investigation indicate that seeds, whether they appear healthy or diseased, may harbor the wheat blast pathogen. As seeds are the primary sources of inoculum to new areas [16], this phenomenon is independent of whether or not the seeds were collected from blast-affected fields. In new wheat-growing locations, blast incidence is brought on by fungal contamination of wheat seeds [5, 24]. Contaminated seeds were thought to play a significant role in MoT long distance dispersion. Toledo [21] asserts that the primary inoculum of MoT can be eliminated with an efficient seed treatment. For this reason, it should be required that seed lots coming from blast-affected fields be treated with fungicides. It proves that wheat blast pathogen MoT is seed-borne. Wheat seeds in Bangladesh from any source of collection should be checked for the presence of MoT and treated with appropriate protective agent before sowing. Opinions are growing to know if the pathogen is seed borne as to design management strategy.

Experiments were conducted to find out about seed as a source of infection of wheat blast disease in the field. To select healthy seeds, studies were conducted to determine the extent of wheat seed exposure to the blast pathogen and identify the proportion of seeds that were actually infected.

In conclusion, the results of this study indicate that moist blotter technique is suitable for detecting the presence of MoT in seeds to suggest the suitability of a seed lot for sowing by the farmers. In addition, among the seed samples tested, a significant number showed no signs of MoT infection, and these seeds exhibited high germination rates. This demonstrates that the majority of seeds from that particular disease prone area are healthy and viable for planting. The study reinforces the necessity of treating seeds from blast-affected areas with appropriate fungicides to prevent the spread of wheat blast disease. Effective seed treatment could eliminate the primary inoculum, ensuring healthier crop production and reducing the risk of disease spread to new areas.

The limitation of the method is that, while effective, the moist blotter technique is labor-intensive, time-consuming, and requires skilled personnel for accurate microscopic identification of MoT. Additionally, it may not detect very low levels of infection, necessitating the use of molecular techniques like PCR for more sensitive detection.

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