Suitable methods for isolation, culture, storage and identification of wheat blast fungus *Magnaporthe oryzae Triticum* pathotype

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Abstract

Wheat blast disease caused by a South American lineage of *Magnaporthe oryzae Triticum* (*MoT*) pathotype has emerged as a serious threat to wheat production in Bangladesh since its first emergence in 2016. Efficient and suitable methods for isolation, storage, inoculum production and molecular characterization of the pathogen can help in achieving the target of sustainable management of the disease in a relatively short period of time. In this study, we aimed to develop suitable methods for isolation, storage and morphological characterization and molecular identification of *MoT* isolates collected from the blast-infected wheat fields in Bangladesh. This process included modification of existing protocols that were available for a related fungal pathogen *M. oryzae* or de novo method development and validation. We developed suitable methods for isolation of *MoT* from field-infected plant samples using modified monoconidial isolation technique and produced abundant conidia from a single mycelial plate for in vivo pathogenicity assay in a reproducible manner. Cultural and morphological characterization of the isolates revealed that all Bangladeshi *MoT* isolates are of a single clonal lineage with similar cultural and morphological characters. Molecular detection of isolates with *M. oryzae*-specific primers Pot1 and Pot2 and *MoT*-specific primers MoT3F and MoT3R produced bands with the expected size from all wheat-infecting isolates. We also successfully established a PCR-based detection system based on a commercially available detection kit for field-infected leaf and seed samples by detecting Pot2- and MoT3-specific bands. Additionally, the simple method we developed in our study for producing abundant conidia in a very short period of time will be very helpful in studying biology of the wheat blast fungus. This method was also proven to be more user-friendly and cost-effective than previously available methods. Successful characterization of *MoT* isolates at morphological and molecular levels coupled with detection of the pathogen in infected field and seed lots should be useful for efficient surveillance and management of the fearsome wheat blast disease.

Keywords: Wheat blast, Monoconidial isolation, Conidia production, Diagnostic method, Surveillance, MoT3 primer

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Background

Blast disease caused by *Magnaporthe oryzae* (Hebert) Barr (anamorph *Pyricularia oryzae*) is the economically most important fungal disease of rice, wheat, barley, millet, oat and many other plants of Poaceae family (Valent et al. 2019). The pathotype that infects wheat is known as *M. oryzae Triticum* (*MoT*) and was first reported in Paraná state of Brazil in 1985 (Igarashi et al. 1986). Immediately after that the disease spread fast and was found all over the wheat-growing regions in Brazil (Urashima et al. 1993) and neighbouring countries including Bolivia, Argentina and Paraguay within a few years (Barea and Toledo 1996; Viedma 2005). Wheat blast appeared for the first time in Bangladesh in February 2016 and caused up to 100% yield losses in eight districts of Southwestern part of Bangladesh (Islam et al. 2016). Out of a total 101,660 ha of cultivated wheat in those eight districts, an estimated 15% were affected by wheat blast. The disease severity and associated yield loss varied widely among these different districts (Islam et al. 2016). By collecting blast-infected plant samples and analysing with a field pathogenomics approach, we found that wheat blast in Bangladesh was caused by a South American lineage of *M. oryzae* (Islam et al. 2016; Islam 2018). The pathogen spread to 12 new districts in the following years with varying level of devastation (Surovy et al. 2020). Although India has border with five severely blast-affected districts of Bangladesh, no scientific report has been published on the occurrence of wheat blast in India (Islam et al. 2019). Prior to this, a detailed study on suitable methods of isolation, production of abundant conidia for artificial inoculation (in field and laboratory) assay, morphological and molecular characterization of the wheat blast isolates in Bangladesh has not been performed.

Accurate and reliable morphological and molecular characterization of the causal agents is a pre-requisite for surveillance and control of fungal plant diseases. An effective isolation and storage technique for fungal pathogens can speed up their pathological and molecular characterization and further study on the biology, ecology and epidemiology of pathogens. The most common problem in isolating *MoT* from a field sample is associated with contamination by fast-growing bacteria and other necrotrophic fungi. Although Jia (2009) reported a single spore isolation technique for obtaining a pure culture of *M. oryzae* isolate from rice and other grasses by dilution method, detailed description of single spore isolation technique of *MoT* isolates from field-infected wheat samples is scant. Farman et al. (2017) has described a single spore isolation method by rubbing the *MoT* conidia with a glass pestle and spreading on the culture plate. However, this technique is effective when the infected plant parts contain only a single type of organism. In the case of severely contaminated field samples in a tropical country like Bangladesh, both

aforementioned techniques were found either less effective or ineffective because of the prevalence of many other organisms that grow faster than *MoT* isolates. Besides isolation method, storage of *MoT* isolates is equally important for further study of this pathogen without losing its viability and virulence. The most common technique used for the storage of *M. oryzae* isolates is dry filter paper method (Jia 2009). Although the method is laborious and time-consuming, it is suitable for long-term storage of *M. oryzae* without losing its virulence. As *MoT*, the causal agent of wheat blast disease in Bangladesh is a new disastrous pathogen, suitable methods for isolation and storage of the pathogen from field-infected samples are essential to facilitate further study leading to the management of this fearsome plant disease.

The *MoT* isolates were reported exhibiting cultural variabilities on different media (Castroagudin et al. 2016; Perelló et al. 2017), and the characteristic features commonly include concentric ring, abundant white aerial mycelia, dark centre with gray sporulation (Castroagudin et al. 2016). Conidia are pyriform, hyaline with two septation, 25–30 μm long and 8–10 μm wide that arise from simple, short, erect conidiophore. A single conidiophore bears a cluster of conidia at the tip (Castroagudin et al. 2016). The airborne conidia are considered the means of disease dispersal from one plant to another and overwinter in seed and crop debris as a source of inoculum for the next infection (Goulart and de Paiva 2000). Under 18–25 °C and wetness for long hours, a climate condition conducive for wheat blast disease (Kohli et al. 2011), conidia germinate and form appressoria on hydrophobic wheat leaf surfaces, which generate very high turgor pressure to break down the host surface to penetrate and colonize the plant tissues (de Jong et al. 1997; Dixon et al. 1999). The rate of conidia production by a pathogen is directly related to disease severity (Berbegal et al. 2007; Akagi et al. 2015). Therefore, it is important to obtain a sufficient number of conidia in the laboratory for artificial inoculation and molecular biological study. Several factors influence the rate of conidia production of *M oryzae* isolates in laboratory conditions. Among them, the influence of light duration and media composition are well documented (Lee et al. 2006; Perelló et al. 2017). Nevertheless, the effect of incubation period on *MoT* isolates needs further investigation to maximize conidia production.

The blast pathogen *M. oryzae* is considered highly variable and is composed of a large number of physiological races or pathotypes possessing different host specificity. Recent studies showed that the *Triticum* pathotype is closely related to *Lolium* pathotype from ryegrass *(Lolium perenne* L.) that caused severe infection in wheat at the head stage in Kentuky, USA in 2011 (Farman et al. 2017). Apart from the *Lolium* pathotype,

Oryza pathotype is genetically distinct from the *Triticum* pathotype and generally do not infect wheat (Urashima et al. 1993). As very little or no cultural and morphological differences are observed among the pathotypes of *M. oryzae* (Castroagudin et al. 2016), molecular characterization along with morphological characterization is important for precise identification of the pathogen at pathotype level. Polymerase chain reaction (PCR)-based diagnostic assay is a common molecular technique used to detect or identify a number of plant pathogens (Chiocchetti et al. 1999; Barros et al. 2001; Larsen et al. 2002; Harmon et al. 2003; Pieck et al. 2017). This method is very reliable and rapid even in the absence of diagnostic morphological feature in symptomatic tissues (Pieck et al. 2017). With the advent of species-specific primers and diagnostic kits, it is now possible to identify the causal agent of a disease from infected plant samples within a very short period of time (Harmon et al. 2003). Molecular characterization of *MoT* isolates using conserved Internal Transcribed Spacer (ITS) region is not useful in detecting the fungi at the pathotype level (Pieck et al. 2017). In this study, pot2-transposon specific primers were used to amplify Pot2 region that is thought to be present in isolates of *M. oryzae* and *M. grisea* from different hosts (Harmon et al. 2003). A recently developed primer by Pieck et al. (2017) named MoT3 was also used, which is unique for identification of isolates of *M. oryzae Triticum* pathotype when used under stringent conditions (Gupta et al. 2019).

Wheat blast has emerged as new threat to wheat production in Bangladesh and there is no comprehensive literature about the isolation, storage, morphological characterization and molecular identification of this deadly fungus. Therefore, the objectives of the study are to develop suitable methods to (i) isolate, culture and store wheat blast fungus from naturally infected plants and seeds; (ii) produce large amount of virulent conidia from *MoT* isolates; (iii) characterize morphological features of *MoT* isolates; and (iv) identify *MoT* isolates at the molecular level from a pure culture or infected plant parts.

Results

Isolation and purification of *MoT* isolates

We established a suitable method for isolation of *MoT* by picking up a single conidium following the method described by Jia (2009) with some modifications. At first, infected leaves, panicles or seeds were washed with running tap water by gentle rubbing and then incubated on wet filter papers in a Petri dish to induce sporulation. Infected plant materials were placed on moist filter paper with the support of pipette tips to allow the sample not to come in direct contact with moist surface of filter paper placed inside the Petri dish followed by covering

with a lid, and incubated for 48 h. A part of infected plant samples was examined under a light microscope to check for sporulation of *MoT*. A sterilized dissecting needle was dipped into agar medium so that the needle tip would contain an agar layer. A few *MoT* conidia were then allowed to the needle tip by touching sample surface placed on the stage of a stereo microscope (Fig. 1). Conidia on the needle tip were subsequently transferred onto 2% PDA medium in a Petri dish and spread with a glass rod. Plates were incubated for 2 days at 25 °C. Under a stereo microscope, a single germinated conidium was marked with a needle by making a stretch around it and then agar block containing the germinated conidium was cut and transferred to another PDA plate for mycelial growth (Fig. 1). The *MoT* isolates were further purified and named by repeated culture from the growing mycelial tips.

Storage of *MoT* isolates

Development of an easy storage method for a fungal isolate is essential for its further study. Cryopreservation in liquid nitrogen is one of the most common techniques used for long-term storage of fungal isolates. Another storage method is dry filter paper method, which has also been used for storing plant pathogenic fungal isolates including *M. oryzae* (Fong et al. 2000; Jia et al. 2014). Here, we presented a detailed method for preservation of *MoT* isolates in dry filter paper. Briefly, Whatman filter paper (Cat. 1001 090) was cut into 1–2 cm pieces which were sterilized and placed on PDA medium in a Petri dish followed by placing a small block of *MoT* mycelial plug at the center of the plate. The plates were incubated at 25 °C until white mycelia covered the filter paper pieces. The filter paper pieces with mycelia were picked up using forcep and placed in a beaker containing silica gel. Then the filter paper pieces were desiccated using a desiccator and transferred into eppendorf tubes containing silica gel for storage at − 20 °C (Fig. 2). All these experimental procedures were carried out under aseptic conditions. To test the viability and virulence of the preserved culture, *MoT* isolates were tested for its regeneration capability on a regular interval (every three-month).

Effects of different media on growth characteristics of *MoT* isolates

A representative group of 15 isolates collected during 2016–2018 were used to study growth characteristics of the wheat blast pathogen in Bangladesh as influenced by different semi-synthetic media. The isolates showed distinctly different morphological features on different media but little difference on the same culture medium. Colonies were round in shape for all isolates in culture media (Additional file 1: Table S1). On PDA, there were
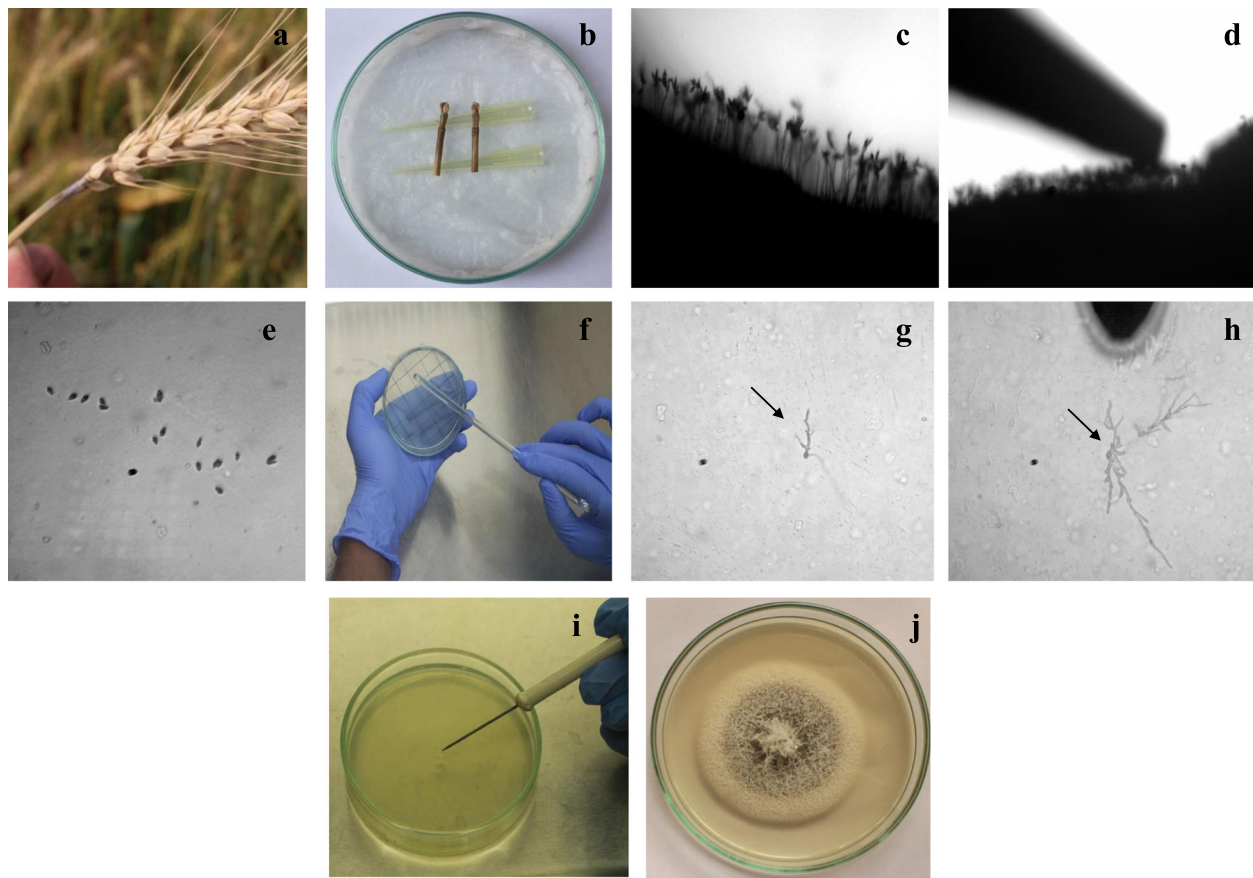
Fig. 1 Steps for obtaining single-spore isolates of *MoT*. **a** Collection of infected wheat spike. **b** Incubation of infected wheat neck for sporulation. **c** Microscopic view of sporulation of blast conidia after overnight incubation. **d** Touching conidia with a dissecting needle tip. **e** Transferring conidia on needle tip to PDA medium. **f** Spreading conidia in plate by a round-end glass rod. **g** Germination of a single conidium. **h** A single germinated conidia pointed by a needle (black U-shaped) under microscope. **i** Placing the single germinated conidia on PDA plate. **j** Purified isolate from a single conidium. Arrow heads indicate germinated conidia under microscope (g and h)

abundant white aerial mycelia on the colony surface. Dark centers and regular margins and sometimes concentric circles reaching up to 4.0–5.9 cm in diameter were usually observed by viewing from the reverse side of the colony within 7 days (Fig. 3 and Additional file 1: Table S1). On OMA, there were only sparse grey aerial mycelia on the colony surface, and dark black center and gray margin reaching up to 4.3–5.7 cm in diameter on the reverse side of the colony were observed. On V8 juice agar, the colony was 4.1–5.4 cm in diameter, with dispersed gray aerial mycelia. On CMA media, isolates showed diffused growth measuring 4.1–5.3 cm in diameter with a small black center and rough margin (Fig. 3).
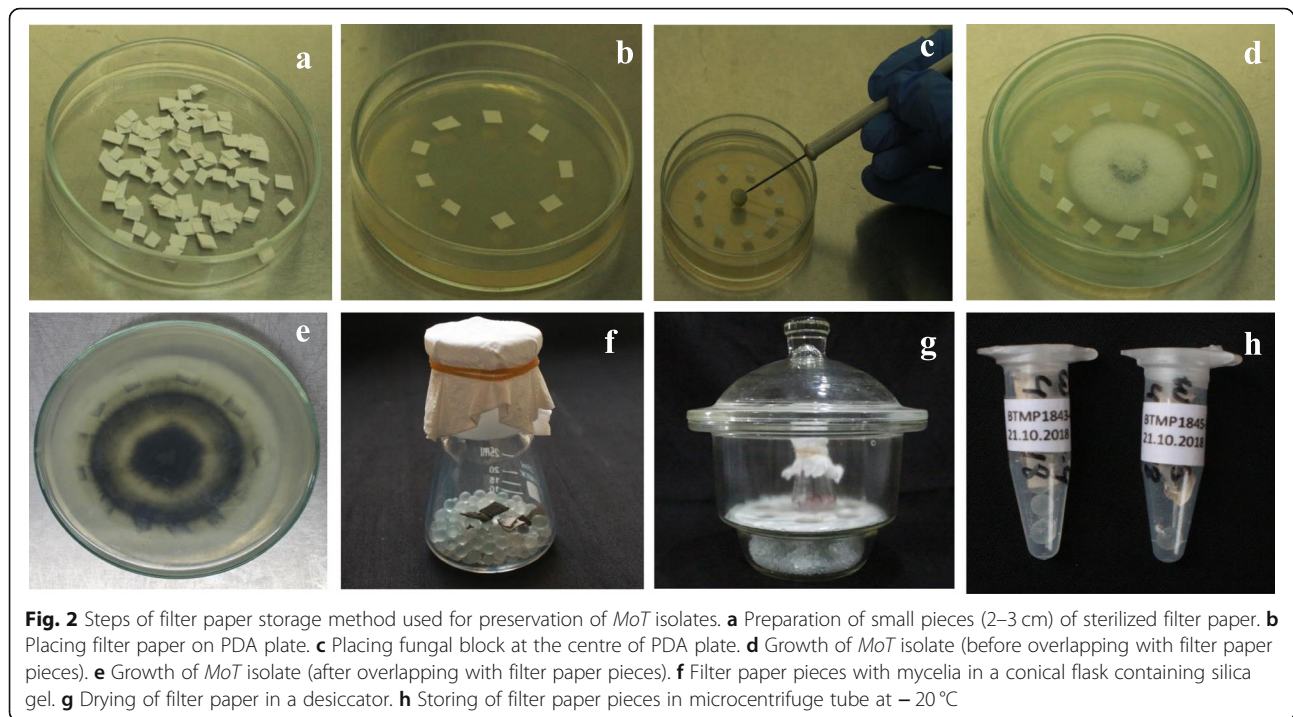
Effects of temperature on mycelial growth of *MoT* isolates
To determine the optimum temperature for mycelial growth of *MoT* isolates, six different temperatures, ranging from 15 to 38 °C were tested (Additional file 2: Figure S1). The results showed that mycelial growth of *MoT* isolates was significantly influenced by

temperature. Maximum radial growth of mycelia of *MoT* isolate BTJP4–1 was recorded at 30 °C (5.9 cm in diameter), followed by 25 °C (5.8 cm), 20 °C (4.8 cm), 15 °C (2.8 cm) and 35 °C (1.0 cm) after 7 days of incubation (Additional file 2: Figure S1). However, no mycelial growth was observed when *MoT* isolates were incubated at 38 °C. Although mycelial growth was restricted at 15 °C and 35 °C, a normal mycelial growth resumed when the culture plates of the isolates were transferred to 25 °C. However, no mycelial growth was observed when the culture plates were transferred from 38 °C to 25 °C after 7 days of incubation (data not shown) indicating non-survival of the *MoT* at 38 °C.
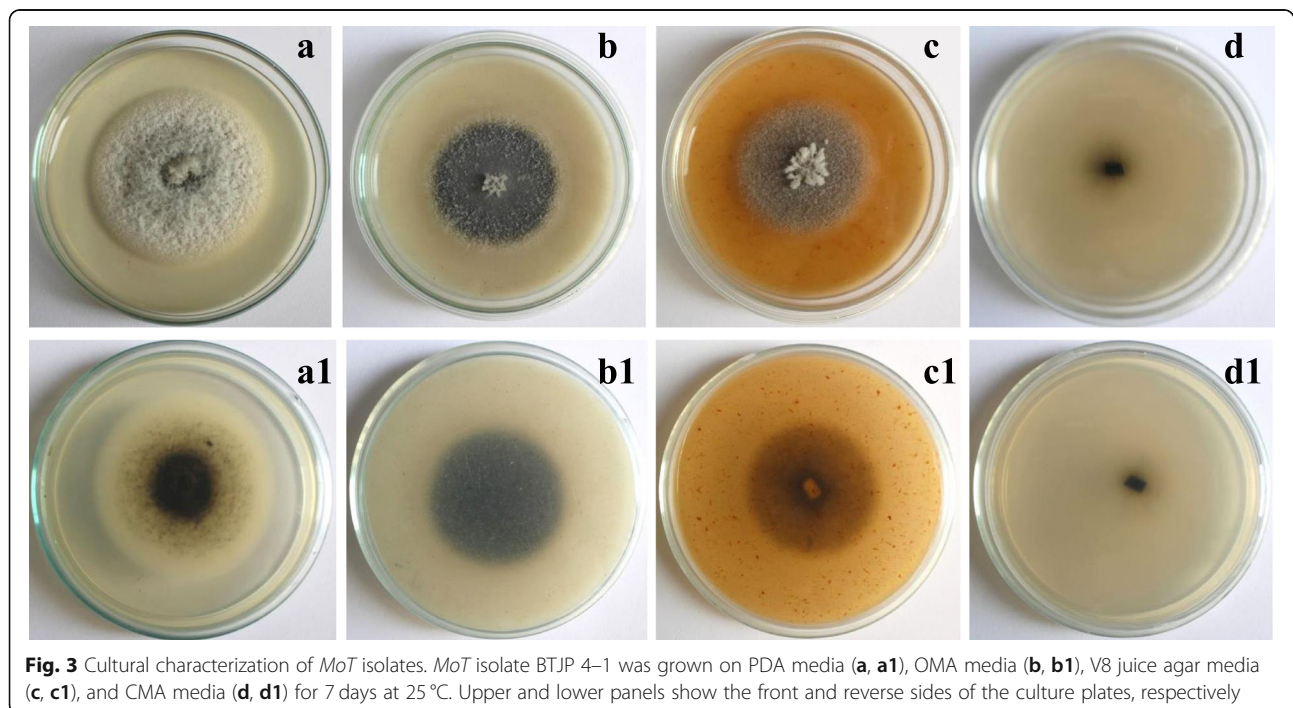
Morphological characterization of *MoT* isolates
To characterize the Bangladeshi *MoT* isolates, we conducted classical morphological analysis of the virulent isolates by light microscopy. Mycelia of all isolates were smooth, hyaline, branched, and frequently septate hyphae of 1.5–2 μm in diameter on PDA. Conidiophores

Fig. 2 Steps of filter paper storage method used for preservation of *MoT* isolates. **a** Preparation of small pieces (2–3 cm) of sterilized filter paper. **b** Placing filter paper on PDA plate. **c** Placing fungal block at the centre of PDA plate. **d** Growth of *MoT* isolate (before overlapping with filter paper pieces). **e** Growth of *MoT* isolate (after overlapping with filter paper pieces). **f** Filter paper pieces with mycelia in a conical flask containing silica gel. **g** Drying of filter paper in a desiccator. **h** Storing of filter paper pieces in microcentrifuge tube at − 20 °C

were solitary, erect and straight or curved, mostly unbranched, slightly swollen at the base, pale brown, smooth, 50–140 μm in length and 2.1–2.8 μm in width with 1–2 septa (Fig. 4c). Single conidiogenous cell produced 3–5 conidia arranged sympodially forming a cluster at the active apical tip (Fig. 4b, d). No significant

differences were observed in size and shape of the conidia among the isolates. Conidia were typically pyriform with a rounded base, acute and narrowed apex, pale brown in color and smooth, 2-septate, 3-celled within the range of 22.76–28.56 μm in length and 7.85–9.21 μm in width (Fig. 4a).
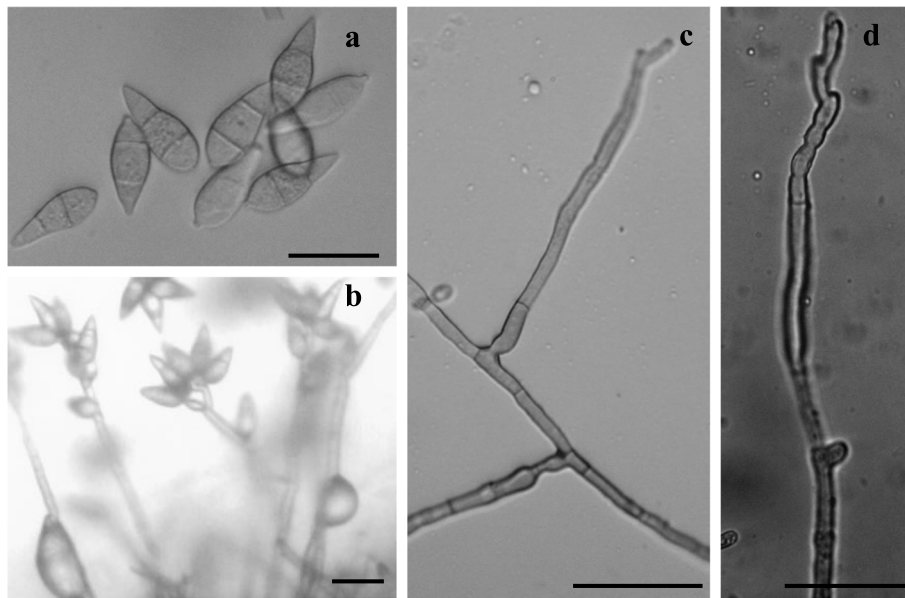

Fig. 3 Cultural characterization of *MoT* isolates. *MoT* isolate BTJP 4–1 was grown on PDA media (**a**, **a1**), OMA media (**b**, **b1**), V8 juice agar media (**c**, **c1**), and CMA media (**d**, **d1**) for 7 days at 25 °C. Upper and lower panels show the front and reverse sides of the culture plates, respectively

Fig. 4 Morphology of conidia and conidiophore of *MoT* isolates. BTJP4–1 was grown on PDA for 7 days and mycelia were then washed off and incubated for 24 h at 25 °C to induce conidia. Conidia and conidiophore morphology were observed under microscope at 40× magnification and photographs were taken with a camera attached to microscope. **a** Conidia. **b** Arrangement of conidia in conidiophore. **c** Hypha with conidiophore. **d** Conidiophore. Scale bar = 20 μm

Sporulation rate of the isolates in different media was tested. Conidia production rate among the isolates varied depending on the ingredients of media but not isolates. Isolates produced the highest number of conidia on OMA as well as on PDA ($5.5–5.6 \times 10^5$ conidia/mL). On V8 media, conidia production was lower ($5.6–7.4 \times 10^4$ conidia/mL) than that of OMA and PDA. Conidia production on CMA was the lowest, ranging from 5.4×10^3 to 6.2×10^3 conidia/mL (Additional file 1: Table S1).

Effect of incubation time on conidia production of *MoT* isolates

The influence of several factors such as media composition, temperature, and pH of the medium on conidia production of *M. oryzae* isolates has been reported (Landraud et al. 2013; Perelló et al. 2017). However, the effect of incubation time on the conidia production rate of *MoT* has not been studied yet. To test the effect of incubation time on conidia production, we used a *MoT* isolate BTJP4–1 to induce conidiation at varying incubation periods. Mycelia were removed from the PDA plate at the 4th to 9th days of incubation, and then a significant increase in conidia production was observed with incubation time. The average conidia production rate on day 4 of incubation was 4×10^5 conidia/mL, which increased to 6.6×10^5 conidia/mL on 8th day (Fig. 5). After 8 days of incubation, there was a slightly decreasing trend in the conidia production rate. These data clearly indicate that the incubation period significantly influence the rate of conidia production of *MoT*.

Development of a suitable method for conidia production of *MoT* isolates

Sufficient amount of inoculum is a prerequisite for disease development assay in the laboratory and field conditions. We developed a suitable method for repeated conidia production from a single culture of *MoT* isolates. The *MoT* isolate, BTJP4–1 was grown on PDA medium at 25 °C for 6 days. After a good radial growth, the fungal colony was flooded with 5 mL of sterilized water, and aerial mycelia were then washed off by gentle rubbing with a paint brush. Plates were incubated at 25 °C for 24 h in a laminar air flow cabinet by keeping the lids loosely closed to allow entry of air to the plates. In the first harvest, $5–6 \times 10^5$ conidia/mL were obtained from a single plate. After that, plates were again kept at the same condition for 1 day to induce conidia for a second harvest. A successive third harvest was done using the above procedure. In the second harvest, $2–6.2 \times 10^6$ conidia/mL were obtained from the same plate and $4–7.6 \times 10^6$ conidia/mL from the third harvest (Fig. 6a, b). In our study, we obtained ten times higher number of conidia in the second and third harvest compared to the first harvest (Fig. 6b). All
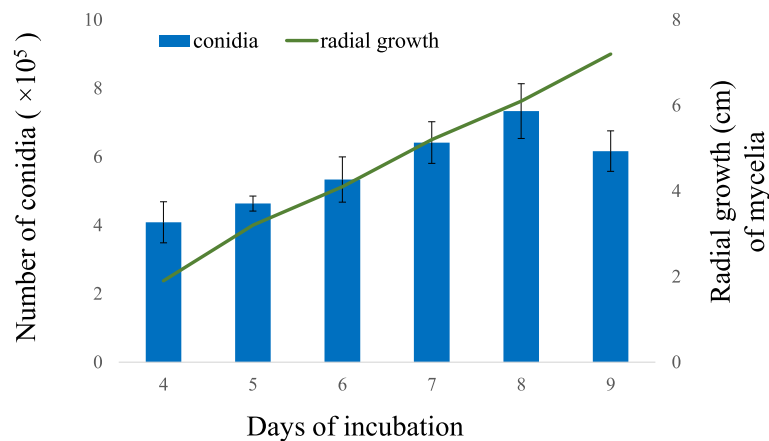
Fig. 5 Effect of incubation period on conidia production of *MoT* isolates. The *MoT* isolate BTJP4–1 was cultured on PDA plate at 25 ℃ for different incubation periods. Conidia were harvested from the plate and counted using a haemocytometer. Error bars indicate standard deviation from at least three independent replicates

conidia from the first, second and third harvests could produce characteristic blast symptoms on wheat seedlings (Fig. 6c). Our study for the first time reported the successive conidia production of *MoT* isolates from a single culture plate.

Molecular identification of *MoT* isolates

PoT, a specific primer pair for *M. oryzae* (Harmon et al. 2003), and MoT3, a recently developed primer pair for the detection of *MoT* (Pieck et al. 2017) were used for molecular identification of *MoT* isolates. Pot2 and
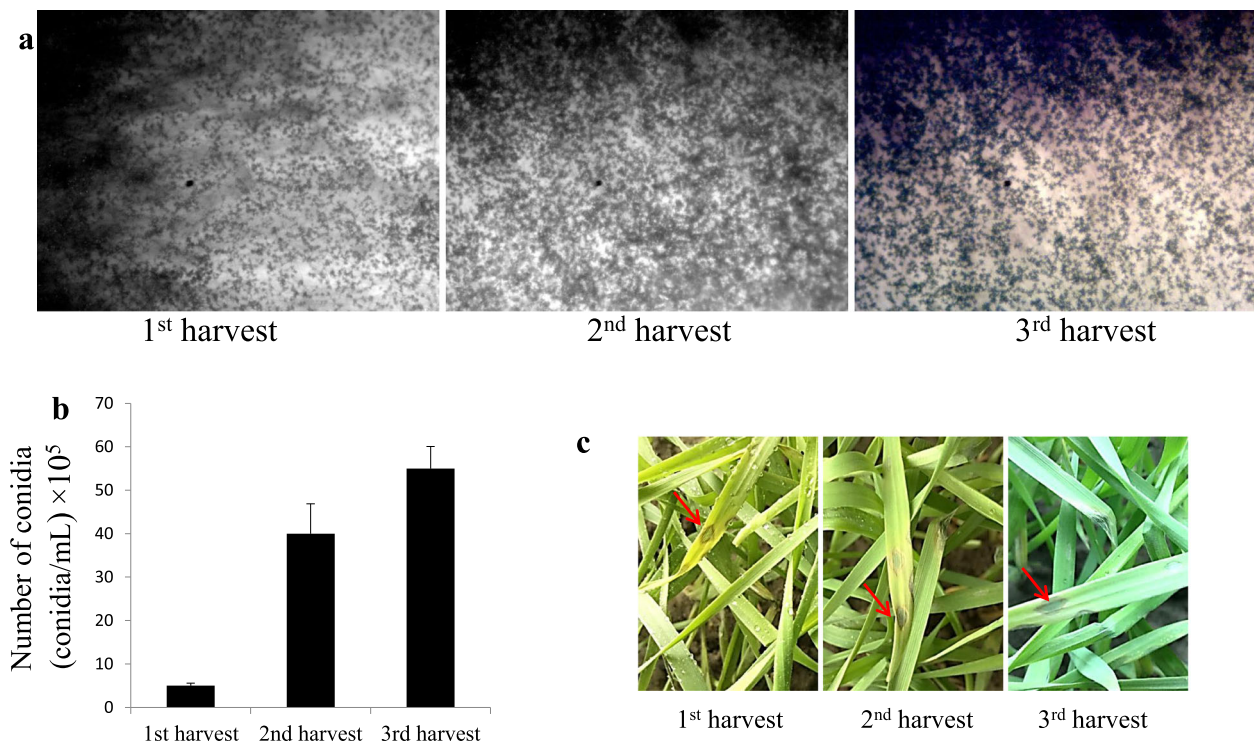

Fig. 6 Repeated conidia production of *MoT* isolates. **a** Microscopic view of conidia production of the BTJP 4–1 isolate on PDA plate in first harvest (left), second harvest (middle) and third harvest (right). **b** Graphical presentation of conidia production by *MoT* isolates at different harvesting frequency. Error bars indicate standard deviation from at least three independent replicates. **c** Blast symptom development on wheat leaves by spraying conidia collected from first harvest (left), second harvest (middle) and third harvest (right). Arrow heads indicate the eye-shaped blast symptom development on wheat leaves

MoT3 were tested against DNA extracted from six wheat and two rice *M. oryzae* isolates. One *Alternaria* sp. and one *Fusarium* sp. isolates were used as negative controls. All the *M. oryzae* isolates from wheat and rice showed a 687-bp band when amplified with Pot2, and a 361-bp band when amplified with MoT3 (Fig. 7a, b). However, the diagnostic Pot2- and MoT3-specific bands were not produced by *Alternaria* sp. and *Fusarium* sp. Meanwhile, all fungal isolates amplified a part of ITS region using primers ITS1 and ITS2 (Fig. 7c). Although MoT3 also amplified MoT3-specific band in rice isolates of *M. oryzae*, the intensity of the band was very low providing an option to discriminate rice and wheat isolates.

Detection of *MoT* isolates from infected wheat leaf and seed samples

Harmon et al. (2003) successfully detected *M. oryzae* isolates from infected perennial ryegrass in less than 8 h by using a commercially available kit. To establish the protocol for detection of *MoT* isolates from infected wheat plants, genomic DNA was extracted following the protocol described earlier (Harmon et al. 2003). Primers Pot2 and MoT3 were used to amplify the diagnostic bands, and the target bands of 687 bp and 361 bp were respectively amplified from 0.1 g of wheat leaf sample

either naturally infected or artificially inoculated with *MoT* isolates (Fig. 8a, b). Besides infecting leaves, *MoT* can also infect and survive in seeds as it is a seed-borne pathogen and disseminates from one place to another by seeds (Goulart and de Paiva 2000; Maciel et al. 2014). Seeds were collected from wheat blast-infected fields in the year 2016–2017 and incubated overnight to induce sporulation. Genomic DNA was extracted from infected seeds in both years followed by PCR amplification, and the results showed that both Pot2 and MoT3 primers produced their diagnostic bands in blast-infected wheat seed samples (Fig. 8a, b).

Discussion

Wheat blast has emerged as a new threat to wheat production in Bangladesh. Although the disease appeared for the first time in 2016 causing much devastation, it continued damaging wheat in subsequent years but in a smaller scale (Islam et al. 2019). As the pathogen *MoT* can survive in seeds and crop residues and disease severity is highly dependent on prevailing conducive weather, it is likely that the disease will affect wheat crop in coming years if an appropriate control measure can't be developed. To mitigate the loss from the disease, it is imperative to generate basic information of the
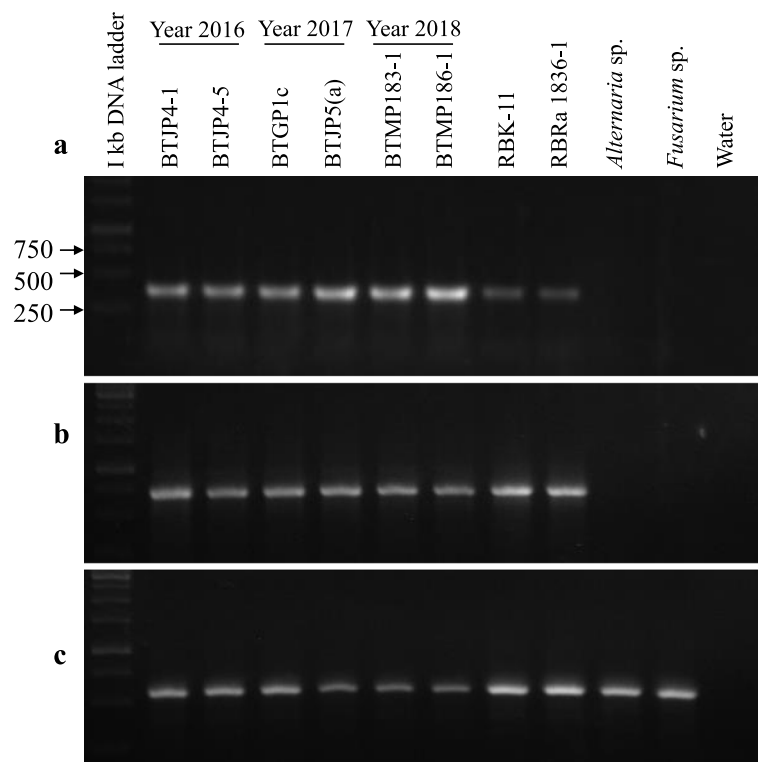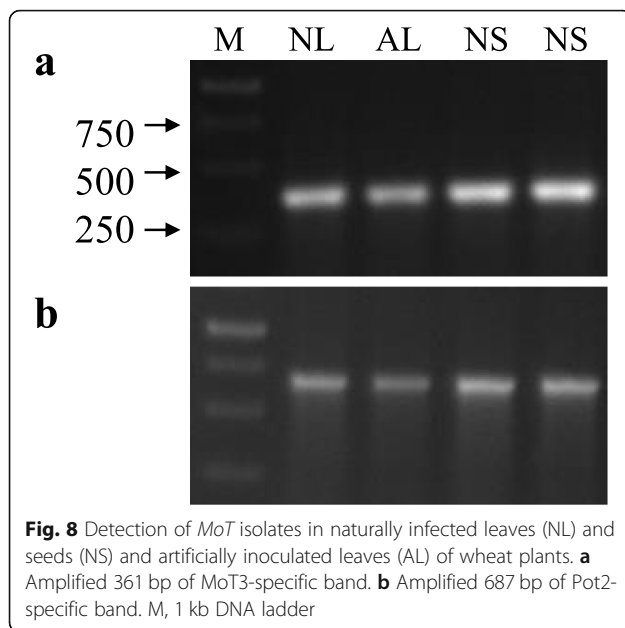

Fig. 7 PCR-based assay for the presence of MoT3 and Pot2 in 6 *Magnaporthe oryzae* isolates from wheat (lanes 2–7), 2 *M. oryzae* isolates from rice (lanes 8–9), with *Alternaria* sp. (lane 10) and *Fusarium* sp. (lane 11) as negative controls. **a** Amplified 361 bp of MoT3-specific band. **b** Amplified 687 bp of Pot2-specific band. **c** ITS1 and ITS2 primers were used as control to amplify partial ITS region

Fig. 8 Detection of *MoT* isolates in naturally infected leaves (NL) and seeds (NS) and artificially inoculated leaves (AL) of wheat plants. **a** Amplified 361 bp of MoT3-specific band. **b** Amplified 687 bp of Pot2-specific band. M, 1 kb DNA ladder

pathogen, its infection biology and epidemiology in Bangladesh environmental conditions. Information generated by the current study significantly improved our knowledge on new suitable and more efficient methods ranging from isolation of *MoT* pathogen to the investigation of its pathogenesis on wheat plant. These suitable methods should be instrumental for investigating complex biology and biochemical aspect of the pathogen in the laboratory as well as testing preventative and remedial measure of wheat blast in field conditions.

Our study for the first time described the morphological features of *MoT* isolates collected from blast-infected fields of different districts in Bangladesh. Morphological and cultural characteristics of 15 *MoT* isolates varied depending on the culture medium. Under the experimental conditions, the *MoT* isolates showed distinct but consistent cultural and morphological characters on different media. Only minor differences were observed among the isolates grown on PDA media. The growth of *MoT* isolates was faster on PDA compared to that on OMA or V8 medium. OMA and V8 juice agar media were reported as suitable media for the blast fungus (Trevathan 1982; Koley and Mahapatra 2015), however, we found that PDA and OMA were the best media for culturing Bangladeshi isolates of wheat blast fungus. Thus, future studies requiring abundant conidia, hyphal mass or both will have additional option for culture medium. The PDA medium is derived from organic source, which has simple formulation but more nutrient content that was found to favor the radial growth of mycelia of different fungal species (Saha et al. 2008; Koley and Mahapatra 2015). Many researchers used OMA medium for sporulation. Generally, OMA media is

prepared by using 50 g of rolled oat in 500 mL water followed by boiling at 70 °C for 1 h and then filtered through cheese cloth (Cruz et al. 2015). In our study, we used 40 g/L of broken oats and autoclaved the media for direct use. This medium supported significant sporulation similar to PDA medium. Our modified OMA medium preparation protocol reduced some steps compared with the previously described one making it more user-friendly and less time-consuming. There was no significant difference in the size of *MoT* conidia among the isolates tested. Furthermore, the size of conidia we isolated in Bangladesh was very similar with that from infected wheat fields of Brazil and Argentina (Castroagudin et al. 2016; Perelló et al. 2017). All of our isolates showed almost similar cultural and morphological characters indicating that the isolates probably originated from a single clade. Genome sequencing of the isolates in 2016 and 2017 (https://doi.org/10.6084/m9.figshare.5236381.v1) also revealed that they belong to the same clonal type.

Perelló et al. (2017) reported that the growth of *M. oryzae Triticum* fungus was favored by temperatures ranging from 25 to 30 °C in laboratory conditions. It has also been reported that the minimum and maximum temperatures required for the infection of wheat by *MoT* were 10 °C and 32 °C, respectively (Cardoso et al. 2008). Results obtained from our experiments proved that the preferred temperatures for *MoT* isolates from Bangladesh ranged from 20 to 30 °C for faster growth (Additional file 2: Figure S1). Interestingly, the *MoT* isolates did not survive or grow at 38 °C. During the last five decades, the average winter temperature of Bangladesh has shown an increasing trend and generally ranged between 18 and 21 °C (Miah et al. 2014). In the year 2016, prevailing high winter temperature together with long hours of leaf wetness from rainfall and high humidity during heading stage of wheat resulted in the blast epidemic in eight districts of Bangladesh (Islam et al. 2019).

Development of a method for efficient conidia isolation is important for obtaining pure culture from field samples. Field samples are always contaminated with various fast-growing necrotrophic fungi, which makes the isolation of *MoT* very complicated. Moreover, fetching infected field samples to the lab requires long distance travel, thus increasing the chance of contamination (Fei et al. 2019). Despite that the appropriate surface sterilization measure has been taken, various mold fungi, *Alternaria* spp., *Fusarium* spp., *Curvularia* spp. were always found growing along with the *MoT* isolates. Growth of *MoT* isolate is slower compared to other fungi, making it difficult to obtain pure culture of *MoT* from a field sample by utilizing the serial dilution method developed by Jia (2009) or single spore isolation method. In this study, we presented a modified and improved method to isolate *MoT* fungus

from a contaminated field sample. Contamination can be avoided by touching the specific conidia with a needle under microscope as shown in our result section. We used very simple instruments such as light microscope, needle and glass rod to isolate conidia from an infected sample. The success rate of our method can be very high so long as conidia are picked up by the needle tip to avoid other contaminants.

One of the hallmarks of our findings is the repeated conidia production from a single culture plate. We established a very suitable method for repeated conidia production from a single PDA culture plate, which is very helpful for Koch's postulation assay and also reduce the time and cost of the experiment. We obtained 10 times more conidia in the second and third harvest compared to the first (Fig. 6b). In the second and third harvest, radial growth of mycelia increased compared to the first, which may explain the increasing trends of conidia production by successive second and third harvests. The increasing trend of conidia production was also observed with incubation period as a result of increasing radial growth (Fig. 5). Conidia production from the plate incubated for 8 days is almost twice as much as that incubated for 4 days. However, conidia production rate declined at the 9th day despite the increased radial growth of mycelia (Fig. 5). After 9 days of incubation, water content in the media significantly decreased, which could be a reason for the decreasing trend in conidia production. Relative humidity (RH) of the culture environment is an important factor that induces conidia production on synthetic medium. Awoderu et al. (1991) showed the effect of relative humidity (RH) on conidia production and the production was higher at RH 92.9% and lower at RH 56.8%. During the time of mycelia removal and conidia harvest, water was used to wash off mycelia and conidia, and meanwhile increased the relative humidity of the plate, which may have acted as a positive factor for the increased conidia production. Further study is needed to understand the underlying molecular mechanism of asexual sporulation in *MoT*.

Another important finding of our research is the development of a suitable method for detection of *MoT* isolates from naturally infected wheat leaves, spikes and seeds. Although PCR-based rapid detection method of blast fungus in infected ryegrass using a commercial kit has been reported (Harmon et al. 2003), our study for the first time developed a suitable and rapid method for the precise detection of *MoT* isolates from the infected field and seed samples. This method is also suitable for detection of *MoT* isolates from other alternate or collateral hosts (data not shown). Pot2 primers specific for all *M. oryzae* isolates and MoT3 primers specific only for *MoT* isolates (Harmon et al. 2003; Pieck et al. 2017) were used in our detection method. We employed this

PCR protocol to detect Pot2- and MoT3-specific bands from symptomatic leaf tissues of wheat and other hosts with genomic DNA extracted using Extract-N-Amp Plant PCR kit as template. The protocol was found to be reliable and sensitive even when as low as 0.1 g of symptomatic leaf tissues was used. Genomic DNA extraction from infected seeds were carried out after 24 h of incubation to induce mycelial or conidial growth and produced good results. Since *M. oryzae* is a species complex and disease symptom of wheat blast is always confused with that caused by Fusarium head blight, it is therefore important to diagnose the disease accurately to apply suitable control measure timely.

Interestingly, our previous results using *M. oryzae* isolates from rice and wheat blast showed that MoT3 primers were not specific for *MoT* isolates under certain conditions (Gupta et al. 2019). This result was contradictory with that obtained by Pieck et al. (2017). MoT3 primers derived from *M. oryzae* gene MGG_02337 corresponding WB12 sequence. All *M. oryzae* isolates of rice and wheat produced WB12 amplicon in PCR analysis. We repeated this experiment with other isolates of rice blast from Bangladesh and obtained the same results. Although the PCR yielded fade band in rice blast isolates compared to that in wheat blast isolates, intensity of the band could be increased when annealing temperature was adjusted from 62 to 57 °C (data not shown). However, our results suggest that the MoT3 assay could be used for specific detection of *MoT* based on the difference of band intensity until a more reliable method is developed. It is assumed that wheat blast disease was inadvertently introduced into Bangladesh through seeds from outside of the country and favorable weather conditions supported the outbreak of the disease in South-western part of the country (Islam et al. 2016; Kamoun et al. 2019). Our results demonstrate a suitable diagnostic protocol for early detection of wheat blast pathogens from infected tissues, which can be utilized for monitoring disease incidence in wheat field and seed lots to prevent widespread dispersal of the pathogen.

Conclusions
This study for the first time developed suitable methods for the isolation, storage and characterization of *MoT* fungus in Bangladesh. The results of this study would be helpful for surveillance and further pathological and molecular studies of the wheat blast pathogen in Bangladesh, and also for the development of a sustainable management strategy for this cereal killer on the run.

Methods
Collection, isolation, purification and storage of *MoT* isolates
Blast-infected wheat seeds and spikes were collected from wheat fields in Bangladesh during 2016–2018.

Isolation of fungal strains was done by monoconidial isolation procedure. A detailed procedure of this isolation technique is described in the result section. Isolates were stored at − 20 °C in dry filter papers. A total of 150 isolates were obtained from the infected wheat seeds and spikes.

Growth media and morphological and cultural characterization of *MoT* isolates

Isolates were cultured on PDA (potato dextrose agar), CMA (corn meal agar), V8 juice agar and OMA (oat meal agar) medium. PDA and CMA were prepared according to manufacturers' instructions. For making 1 L of V8 or OMA medium, 15% V8 juice and 40 g of broken oat were used respectively, with 15 g agar plus 1 L of water and autoclaved. All media were amended with 0.05 g/L of phenoxymethylpenicillin (Sanofi Bangladesh Ltd.). Five isolates from each year were selected randomly for cultural characterization. Isolates were grown for 7 days in each medium at 25 °C in continuous dark conditions and colony diameter and cultural features of each isolate on PDA, CMA, V8 and OMA medium were examined. Each experiment was replicated three times and the assay was conducted twice.

Conidia production by *MoT* isolates

A detailed procedure of *MoT* conidia production technique is described in the result section. Conidial and mycelial suspension (5 mL) was filtered through two layers of cheesecloth. The volume of conidial suspension was adjusted to 1 mL by centrifugation and the concentration of conidia was measured with a haemocytometer and expressed as the number of conidia/mL. The shape and size of conidia were measured under a light microscope (Carl Zeiss Microscopy, Germany) and photographs were taken by a camera connected with the microscope (AxioCam ERc 5 s, Germany). At least, 50 conidia of each of the isolates were measured. All isolates were tested for their ability of conidia production on PDA, OMA, V8 and CMA media.

Effect of temperature on mycelial growth of *MoT* isolates

Effect of temperature on mycelial growth of *MoT* isolate was evaluated in vitro at various temperatures (15, 20, 25, 30 and 35 °C) on PDA medium. For all experiments, mycelial agar disks (6 mm in diameter) were placed at the center of the plate (80 mm in diameter). The growth was calculated by taking the average of two perpendicular measurements of diameter of each colony.

Artificial inoculation of *MoT* isolates on wheat seedlings

Isolate BTJP4–1 was used for artificial inoculation of wheat cv. Prodip. Twenty seeds were planted in each of 20-cm-diameter plastic pots filled with NPK fertilizer-amended soil. Ten days after emergence, the seedlings were thinned to 15 plants per pot and kept under natural conditions (temperature ranged from 25 to 30 °C) until inoculation. Plants were watered daily from the top. Inoculation assays were done as previously described (Islam et al. 2016). Plants were examined for lesion development at 7 days after inoculation. Artificially inoculated leaf samples were then used for molecular identification of pathogen from infected plant parts.

DNA extraction and amplification

Genomic DNA was extracted from *MoT* and other fungal isolates following the modified CTAB method (Zhang et al. 2010). The isolates were grown on filter paper on PDA medium for 10 days at 25 °C, and then the mycelia were harvested from the filter paper by scrapping. The harvested mycelia were ground in a mortar and pastel after adding 600 μL of extraction buffer (33 mM CTAB; 0.1 M Tris-HCl, pH 8.0; 7.8 mM EDTA; 0.7 M NaCl). Mycelial suspension was collected in an eppendorf tube and incubated at 65 °C for 30 min in a heating block with occasional shaking. Purification of DNA with phenol:chloroform:isoamyl alcohol (25: 24: 1) followed by precipitation with 700 μL of cold isopropanol were conducted. DNA was then washed with 70% ethanol, dried under vacuum and re-suspended in TE buffer (10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA) containing 10 μg/mL of RNase A and incubated at 37 °C for 30 min and stored at − 20 °C. Quantification of DNA was performed in a nano-drop spectrophotometer and diluted with sterile distilled water as needed.

DNA from symptomatic wheat leaves and seeds was extracted with the Extract-N-Amp™ Plant PCR kit (XNA-P2, Sigma Chemical Co., St. Louis, MO) and Extract-N-Amp™ Seed PCR kit (XNAS2-1KT, Sigma Chemical Co., St. Louis, MO) respectively, following the recommended protocol described by manufacturer. Approximately 0.1 g of infected leaf blades were placed in 1.5-mL eppendorf tubes and sufficient extract solution (provided in the kit) was added to cover the plant material (100 μL). After incubation at 95 °C for 10 min, an equal volume of dilution solution (provided in the kit) was added, and the sample was vortexed and then placed on ice. To extract genomic DNA from infected seeds, the seeds were ground and incubated in a mixture of extraction solution and seed preparation solution at 55 °C for 1 h. The mixtures were incubated at 95 °C for 3 min to stop the extraction. An equal volume of neutralization solution B was added and slightly vortexed. An aliquot of the extract was then added directly to the optimized PCR mix supplied.

PCR primers pfh2a (5-CGTCACACGTTCTTCAACC-3) and pfh2b (5-CGTTTCACGCTTCTCCG-3) were used to amplify a 687-bp region of the Pot2 transposon

(Harmon et al. 2003), and MoT3F (5-GTCGTCATCA ACGTGACCAG-3) and MoT3R (5-ACTTGACCCA AGCCTCGAAT-3) primers were used to amplify a 361-bp of DNA segment (Pieck et al. 2017). For purified genomic DNA from fungal isolates, the PCR was performed in a 50-μL reaction mixture which contained 0.5 μL of DNA *Taq* polymerase (2.5 U), 5 μL of 10× polymerase buffer, 3 μL of 25 mM MgCl$_2$, 1 μL of 10 mM dNTP, 2 μL of 20 pmol/μL of each primer, and 1 μL of the template (extracted genomic DNA at 50 ng/μL). For genomic DNA isolated from the symptomatic plant samples, the PCR was performed in a 20-μL reaction mixture that consisted of 4 μL of the diluted sample extract, 10 μL of 2× PCR mix (the Extract-N-Amp PCR ReadyMix in kit), and 1 μL of 20 pmol/μL of each primer. The PCR reaction for amplification of Pot2 was carried out in a thermal cycler (Applied biosystems, Thermo Fisher Scientific, USA) following the temperature conditions as described by Harmon et al. (2003). MoT3-specific gene sequence was amplified using the thermal cycler described by Pieck et al. (2017). The amplification products were subjected to electrophoresis in a 1% agarose gel and stained for 10 min in an ethidium bromide solution (10 μg/mL). Gel images were obtained with a digital imaging system (Alpha Imager MINI, Protein Simple, Santa Clara, CA).

Data analysis

The SPSS version 16 and Microsoft Office Excel 2010 program package were used for statistical analysis. The experimental design was completely randomized consisting of three replications for each treatment and all experiments were repeated twice. Means comparison of the treatments was performed by LSD test ($P \leq 0.05$).

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s42483-020-00070-x.

Additional file 1: Table S1. Cultural morphology, colony size and conidia production rate of 15 *MoT* iolates on PDA, OMA, V8 and CMA media.

Additional file 2: Figure S1. Effect of temperature on mycelial growth of *MoT* isolates. *MoT* isolate BTJP 4–1 was grown on PDA media and incubated at various temperatures for 7 days. Radial growth of the isolate at different temperatures was recorded by taking the average of two perpendicular measurements.

Abbreviations

CMA: Corn meal agar; CTAB: Cetyltrimethylammonium bromide; EDTA: Ethylenediaminetetraacetic acid; ITS: Internal transcribed spacer; *MoT*: Magnaporthe oryzae Triticum; OMA: Oat meal agar; PCR: Polymerase chain reaction; PDA: Potato dextrose agar

Authors' contributions
MTI conceived and designed the work, supervised the project, and wrote the manuscript; DRG, MZS and NUM conducted research, analyzed the data, and wrote the manuscript; MC, SKP, MSH, PB, MSM, KR, and RY collected and analyzed the data, and wrote the manuscript; MR interpreted the data, wrote and edited the manuscript; The authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

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