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ORIGINAL ARTICLE

Tracing seed to seedling transmission of the wheat blast pathogen *Magnaporthe oryzae* pathotype *Triticum*

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Abstract

Wheat blast caused by *Magnaporthe oryzae* pathotype *Triticum* (MoT), initially restricted to South America, is a global threat for wheat after spreading to Asia in 2016 by the introduction of contaminated seeds, raising the question about transmission of the pathogen from seeds to seedlings, a process so far not well understood. We therefore studied the relationship between seed infection and disease symptoms on seedlings and adult plants. To accomplish this objective, we inoculated spikes of wheat cv. Apogee with a transgenic isolate (MoT-DsRed, with the addition of being resistant to hygromycin). We identified MoT-DsRed in experiments using hygromycin resistance for selection or by observation of DsRed fluorescence. The seeds from infected plants looked either apparently healthy or shrivelled. To evaluate the transmission, two experimental designs were chosen (blotter test and greenhouse) and MoT-DsRed was recovered from both. This revealed that MoT is able to colonize wheat seedlings from infected seeds under the ground. The favourable conditions of temperature and humidity allowed a high recovery rate of MoT from wheat shoots when grown in artificial media. Around 42 days after germination of infected seeds, MoT-DsRed could not be reisolated, indicating that fungal progression, at this time point, did not proceed systemically/endophytically. We hypothesize that spike infection might occur via spore dispersal from infected leaves rather than within the plant. Because MoT-DsRed was not only successfully reisolated from seed coats and germinating seeds with symptoms, but also from apparently healthy seeds, urgent attention is needed to minimize the risks of inadvertent dispersal of inoculum.

KEYWORDS
Magnaporthe oryzae, reisolation, seed transmission, seedborne disease, wheat blast

1 | INTRODUCTION

Blast of wheat (*Triticum aestivum*), caused by the fungal phytopathogen *Magnaporthe oryzae* pathotype *Triticum* (MoT) (syn. *Pyricularia oryzae* pathotype *Triticum*), is a major disease that causes large reductions in yield and seed quality in many South American countries (Goulart et al., 2007; Kohli et al., 2011). This recent disease of wheat originated in 1985 in Brazil and was later reported from other countries of South America including Argentina, Bolivia, and Paraguay (Igarashi et al., 1986; Perelló et al., 2015; Prabhu et al., 1992). During early 2016, the disease occurred in Bangladesh (South Asia) (Malaker et al., 2016). Recently, in Zambia, wheat blast symptoms were observed for the first time

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on wheat grown in experimental plots and five farmers' fields in Mpika district of Muchinga Province during the 2017–2018 rainy season (Tembo et al., 2020).

Wheat blast affects all aerial parts of the plant, with symptoms appearing on leaves, stems, spikes, and seeds (Cruz & Valent, 2017). On leaves, initial symptoms are grey-green and water-soaked lesions with dark green borders that later become light tan with necrotic borders. After complete expansion, necrotic lesions are typically eye shaped with grey centres. The most significant symptom of wheat blast in the field is the premature bleaching of spikelets (Urashima, 2010). In severe cases, the entire head is damaged. The pathogen infects the rachis and develops dark brown discolouration at infection sites with or without dark brown mycelial growth. Spikes may be completely or partially bleached. Parts of spikes located above the infection site may dry quickly and thus leave spikelets empty if the infection takes place before the stage of grain filling. In the case of late spike infection, grains produced are small and shrivelled with black discolouration, and mostly remain sterile (Malaker et al., 2016; Urashima, 2010). The disease is among the most damaging diseases of wheat due to its multiple modes of survival (seed, secondary hosts, and crop), its fast spread and damage to spikes, causing losses ranging from 10% to 100%, and the lack of resistance in commonly grown wheat varieties. Cruz et al. (2016) determined that the translocation of the 2NS gene was associated with significant reductions (50.4%–72.3%) in the expression of the disease in spring and winter wheat under growth chamber conditions. In the same way, the lack of resistance was reported by Cruppe et al. (2020). From the screening of more than 780 accessions of elite spring and winter wheat cultivars, only eight non-2NS accessions were identified with moderate levels of resistance to wheat head blast. This is equivalent to only 1% of the evaluated genotypes offering some level of resistance, highlighting the rare occurrence of inherent protection to this devastating disease.

The disease may become an epidemic and devastate wheat crops within a week under the most conducive temperatures of 18–30 °C and a relative humidity of >80% during ear emergence or grain filling (CABI, 2019). Wet years, warm temperatures, and high humidity were found associated with wheat blast epidemics (Kohli et al., 2011).

Studies that evaluate the transmission of MoT by wheat seeds are still scarce (Gomes et al., 2018). In Brazil, Goulart et al. (1990) and Goulart and Paiva (1990) evidenced the lowest (0%) and the highest (47.3%) percentage of fungal transmission from wheat seeds to seedlings with 2% and 21% of incidence, respectively. Data from the literature suggests that long-distance dispersal of the pathogen occurs through infected wheat seeds (Urashima, 2010) and has an important role in terms of the long-distance dispersal consequences (Singh, 2017). Generally, fungal pathogens may be externally or internally seedborne, extra- or intra-embryonal, or associated with the seeds as contaminants (Singh & Mathur, 2004).

Wheat blast is categorized as a devastating disease on wheat, as it not only affects the development and growth of plants, but also infected apparently healthy seed plays an important role in the dispersion of this disease into new areas. More precise information is therefore needed about the development, spread, and transmission of MoT. This research was initiated to generate basic information on our understanding of seed to seedling transmission of MoT on wheat plants grown under artificial media and soil conditions. This objective was accomplished by quantifying the progress of the disease in wheat seedlings with and without symptoms, evaluating seed germination, seedling emergence, visual disease scoring, and monitoring pathogen recovery from vegetative plant tissues during a period of 42 days after sowing.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates and cultivation

The wild-type isolates PY15W and PY34W belong to an Argentinian collection of isolates of MoT taken from infected wheat plants in Argentina and maintained on dry filter paper at −80 °C at CIDEFI (Centro de Investigaciones de Fitopatología), belonging to the FCAyF UNLP (Facultad de Ciencias Agrarias y Forestales de la Universidad Nacional de La Plata). Isolates were chosen according to preexisting data showing contrasting degrees of symptoms on wheat plants of 14 different commercial Argentinian wheat cultivars tested at seedling and heading stage under greenhouse conditions (data not shown). Cultivation conditions for fungal isolates were as described by Martinez et al. (2019).

2.2 | *Agrobacterium*-mediated transformation of *M. oryzae*

Fungal transformation of MoT isolates PY15W and PY34W was conducted by using *Agrobacterium tumefaciens*-mediated transformation (Rho et al., 2001). In order to follow the infection process, isolates PY15W and PY34W were transformed with a construct carrying a gene conferring resistance to the antibiotic hygromycin (Hyg^R) and a gene encoding the fluorophore DsRed. Successfully transformed mutants were selected on complete medium (CM) agar containing 500 ppm Hyg (Bohnert et al., 2018); cefotaxime and streptomycin were supplied to the medium to prevent bacterial growth. Expression of the transgenes in mutants was validated via Hyg^R and DsRed fluorescence. Transformants were designated PY15-DsRed and PY34-DsRed. More detailed information about the transformation has been described previously (Bohnert et al., 2018; Odenbach et al., 2007). DsRed fluorescence was used as an optical marker for visualization of the infection process and followed using confocal laser scanning microscopy with a Leica TCS SP8 spectral confocal microscope (excitation 561 nm, emission monitored at 580–620 nm). In addition, a lambda scan was recorded to distinguish DsRed fluorescence from autofluorescence of, for example, dead fungal hyphae.

2.3 | Wheat plant cultivation and inoculation with MoT

The wheat cultivar Apogee was received from Eckhard Koch, JKI, and maintained at RWTH Aachen University. Apogee is a cultivar with a very short life cycle, flowering after 25 days, which makes it suitable to study diseases on spikes under laboratory conditions (Li et al., 2017; Wunderle et al., 2012). Wheat cv. Apogee was grown in pots in standard soil (type ED 73, Balster Einheitserdewerk GmbH) under controlled conditions at 22 ± 2 °C with a 16/8 hr light/dark cycle (210 µmol $\cdot m^{-2} \cdot s^{-1}$) and a relative humidity of about 65%, as described by Delventhal et al. (2014). At heading stage (GS 59; Zadoks et al., 1974), spikes were inoculated with a spore suspension of isolates PY15-DsRed or PY34-DsRed following a previously published protocol (Delventhal et al., 2014). For spore generation, the fungal isolates were grown on oatmeal agar (20 g/L agar, 2 g/L yeast extract, 10 g/L starch, 30 g/L oat flakes) at 25 °C under a 16/8 hr light/dark cycle under fluorescent tubes with black light (310–360 nm) for 7–10 days. Conidia were collected from these plates by rinsing with distilled water and using a spatula, filtering through gauze and diluting 1:1 (vol/vol) with gelatin/Tween solution (0.2% wt/vol gelatin, 0.1% wt/vol Tween-20). The spore concentration was adjusted to 250,000 spores/ml. Spikes were inoculated by spraying with the spore solution, and subsequently spikes were covered with a plastic bag for 24 hr to maintain a water-saturated atmosphere. Thereafter, plastic bags were removed and plants were incubated under the conditions specified above. Harvest of spikes took place 55 days after inoculation.

2.4 | Effect of MoT on spikes, weight and wheat seed germination, and quantification of the fungal incidence of harvested seeds

The spikes were evaluated for disease symptoms 21 days postinoculation, according to Martinez et al. (2019). To measure severity, the number of spikelets with symptoms was considered over the total number of spikelets in each spike evaluated. The experimental design consisted of two repetitions of six spikes for each isolate, plus the respective controls. A total of 12 spikes were used for each isolate. Moreover, the weight and the percentage of germinated seeds of wheat cv. Apogee harvested from spikes, previously inoculated with isolates PY15-DsRed, PY34-DsRed, PY15W, or PY34W were evaluated. The germination assay was performed under a blotter test (ISTA, 1999), at 26 ± 2 °C, with cool white fluorescent light plus black light (310–360 nm) with alternating cycles of 16/8 hr light/dark, and under controlled conditions at 22 ± 2 °C, 16 hr of light and a relative humidity of 65% with standard soil in plastic pots.

Collected seeds were visually inspected and grouped into three categories according to their appearance (shrivelled, shrivelled + discoloured, and apparently healthy). To determine the frequency of seeds in each category infected with the fungus, seeds were placed on CM agar supplemented with 500 ppm Hyg for the two transformed isolates, and for the wild type, isolates were placed on CM agar without Hyg. The incidence of each category was calculated for each MoT isolate tested. Incidence of the fungus on coats was included.

2.5 | Seed to seedling transmission of MoT on wheat plants by blotter test method under artificial media

Seeds derived from Apogee plants infected with MoT isolates PY15-DsRed or PY34-DsRed and classified according to visual inspection mentioned above, were used for testing the transmission from seed to seedlings. The test was carried out in between blotter paper (ISTA, 1999) using the blotter test method (Agarwal, 1994). Four blotters of 20 seeds for each category and isolate were evaluated. The assay was done in duplicate.

The rolls were placed upright inside a polyethylene bag to avoid drying during incubation. The bags were placed at 26 ± 2 °C and cool white fluorescent light plus near-UV light with alternating cycles of 16/8 hr light/dark for 21 days (until the third leaf was fully expanded). Seedlings were sampled and removed carefully from the rolled paper every 3 days, and this process was repeated for 3 weeks.

2.6 | Seed to seedling transmission of MoT on wheat plants in soil

Apogee seeds infected with MoT isolates PY15-DsRed or PY34-DsRed were grown in pots with standard soil at 65% humidity, 22 ± 2 °C, and a 16 hr light period for 42 days (until heading stage). The design consisted in six pots with five plants per isolate of MoT, where each pot was a replicate. For this test, seeds were randomized without classifying them into categories. Seedlings were sampled and removed carefully from the pots every 7 days and this process was repeated for 6 weeks. Collected seedlings were placed in individually labelled small Ziploc bags and carried to the laboratory for the fungal recovery analysis.

2.7 | Visualization of MoT on seeds and seedlings

During both the rolled paper towel assay and in soil, sampled wheat plants were pulled, washed, disinfected by immersion in 70% ethanol for 30 s, 5% sodium hypochlorite for 1 min, and subsequently washed three times with sterile distilled water (Chávez & Kohli, 2015), and then dissected in sterile conditions. Randomized plant parts (roots, coleoptiles, basal stem, middle stem, upper stem, leaves, immature inflorescence, flowers, and seeds, if present) were plated on CM agar plates supplemented with 500 ppm Hyg and incubated at 22 ± 2 °C and darkness for 7 days. Colonies developed from the vegetative tissues were directly analysed with a stereomicroscope. MoT colonies

recovered from each specimen were then further evaluated by fluorescence microscopy and interrogated for DsRed fluorescence. Colonies fulfilling this criterion were classified as successful reisolation of PY15-DsRed or PY34-DsRed, and numbers were used to calculate percentage of fungal recovery and transmission rate.

2.8 | Statistical analysis

The data were analysed using the InfoStat 2018 statistical analysis program. Data were statistically analysed by analysis of variance (ANOVA) and Tukey's test ($\alpha = 0.05$).

3 | RESULTS

3.1 | Generation of wheat seeds artificially infected with MoT

Apogee plants were inoculated with both transformants at heading stage (GS 59) and spikes were harvested 55 days after inoculation. Plants treated in the same way but without spores served as control. The untreated control (UTC) did not present symptoms, and no cross contamination was observed. This experiment revealed that cv. Apogee was similarly susceptible to both transformants. However, there were significant differences in disease severity caused by isolates PY15-DsRed and PY34-DsRed (68% and 84%, respectively; Figure 1a). Moreover, the isolate PY15-DsRed induced elliptical lesions with reddish-brown to dark grey margins in glumes (Figure 1b) whereas PY34-DsRed induced a partial or total bleaching of the spikes (Figure 1c).

To assess the effect of MoT infection on wheat seeds, the weight of seeds per spike and the germination of the seeds were evaluated. After harvest, spikelets were separated from the spike, grains were removed, cleaned from coats, and weighed. The average weight of grain per spike on plants from the UTC was 0.9 g (Figure S2). The weight of grain per spike from plants was reduced by the four isolates tested—wild types and mutants—but only statistically significant differences were found with the isolate PY34-DsRed, causing a reduction of 38% (Figure S2). In the case of germination, as shown in Figure S3, seeds harvested from UTC plants had germination rates of 85% and 74% as determined in blotter tests and by direct sowing into soil, respectively. Inoculation with wild type or transgenic MoT isolates reduced the germination rate in all cases. However, only the infection with PY34-DsRed led to a significant reduction, of 39% and 40% compared to the UTC in blotter tests and pot assays, respectively (Table S1).

3.2 | Verification of MoT transmission from seeds to plants grown on artificial media

Next, we wanted to evaluate the frequency of seeds infected by the different isolates of MoT. Seeds were visually inspected and grouped into three categories (shrivelled, shrivelled + discoloured, and apparently healthy). Remarkably, fungal colonies grew out from seeds of all categories. However, significantly higher rates of incidence were recorded for shrivelled grains from plants infected with the isolates PY15-DsRed (47%), PY34-DsRed (36%), and PY15W (57%) compared to seeds derived from plants infected with isolate PY34W (25%). In contrast, seeds from plants infected with isolate PY34W showed higher rates of "apparently healthy" seeds that gave rise to

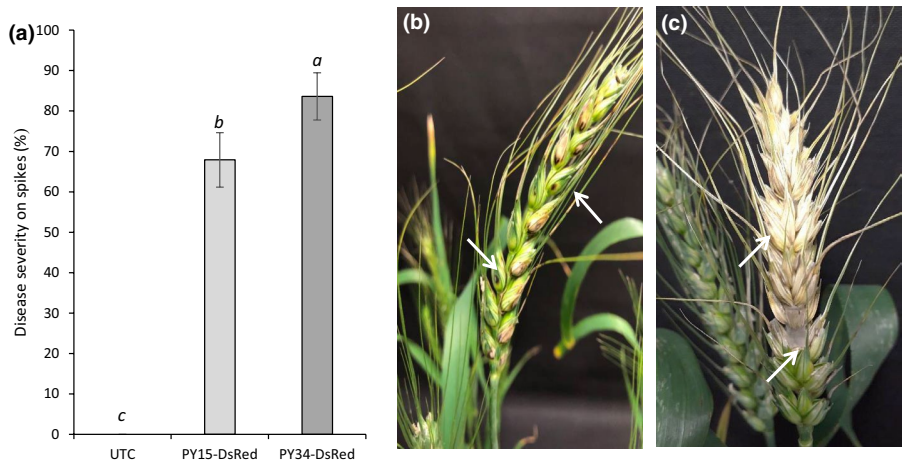

FIGURE 1 Spike infection caused by isolates PY15-DsRed and PY34-DsRed of *Magnaporthe oryzae* pathotype *Triticum* (MoT) on wheat cv. Apogee. An inoculation of MoT spores was carried out at a concentration of 250,000 spores/ml at the heading stage in two experiments of six spikes each; 21 days later an evaluation of severity (number of infected spikelets/total spikelets) (a) and type of symptoms were performed. PY15-DsRed showed necrotic spots on glumes (b) and PY34-DsRed caused total or partial bleaching of spikes with mycelium (c). The pictures were taken 21 days after inoculation. Different letters in (a) indicate significant differences between the columns at $p < 0.05$

fungal colonies (75%) compared with those coming from plants infected with isolates PY15-DsRed, PY34-DsRed, and PY15W (11%, 18%, and 18%, respectively). On coats, the infection rate was 70%–81% for isolates PY15-DsRed, PY34-DsRed, and PY15W, and 14% for isolate PY34W (Figure 2).

For evaluating the fungal transmission rate for the three categories of seeds, the rolled paper towel method was applied, which allowed the recovery of fungi from seedlings over a time span of 21 days. During the first 3 days of observation, abundant mycelia and spores were observed on coats and grains, indicating a high infection rate of the seed samples (Figure 3a,b). Analysing the progress of the infection, the symptoms appeared as brown lesions on the outer coleoptile tissue and roots of the seedlings (Figure 3c,d). At the 9th day after germination, spots on enlarged shoots and leaf sheaths were visually evident (Figure 3e). Moreover, after 12–21 days of development, some seedlings were observed to have spots on leaves and wilt symptoms caused by MoT (Figure 3f). The identity of isolates PY15-DsRed and PY34-DsRed was confirmed by fluorescence microscopy monitoring the DsRed fluorescence (Figure 4). Isolate PY34-DsRed was analysed for disease symptoms on the first leaf from seedlings using different techniques: stereoscope microscopy (Figure 4a), bright field microscopy (Figure 4b), and epifluorescence microscopy (Figure 4c,d). The growth on Hyg-containing agar plates and the confirmation of DsRed fluorescence confirmed the successful reisolation of MoT, not only from seeds, but also from seedlings derived from MoT-infected seeds.

Plant material used for the reisolation was always from the most recent (youngest) tissue which means that, for example, at 3 days after sowing the emerging coleoptile was examined, and at 15–16 days after sowing new leaves from tillers were sampled (Figure S4). The highest frequency for reisolation of MoT (isolates PY15-DsRed and PY34-DsRed) was found at 3 days (emerging coleoptile) for all batches of seeds, with 28%, 32%, and 12% for shrivelled, shrivelled + discoloured, and apparently healthy, respectively (Figure 5). A similar tendency for the transmission rate (incidences of fungal reisolation) was determined for all three batches of seeds during the evaluation time, but at different relative levels. Thus, at days 3–12, the recovery of MoT decreased to a minimum level, and then from days 15 to 21 it started to increase

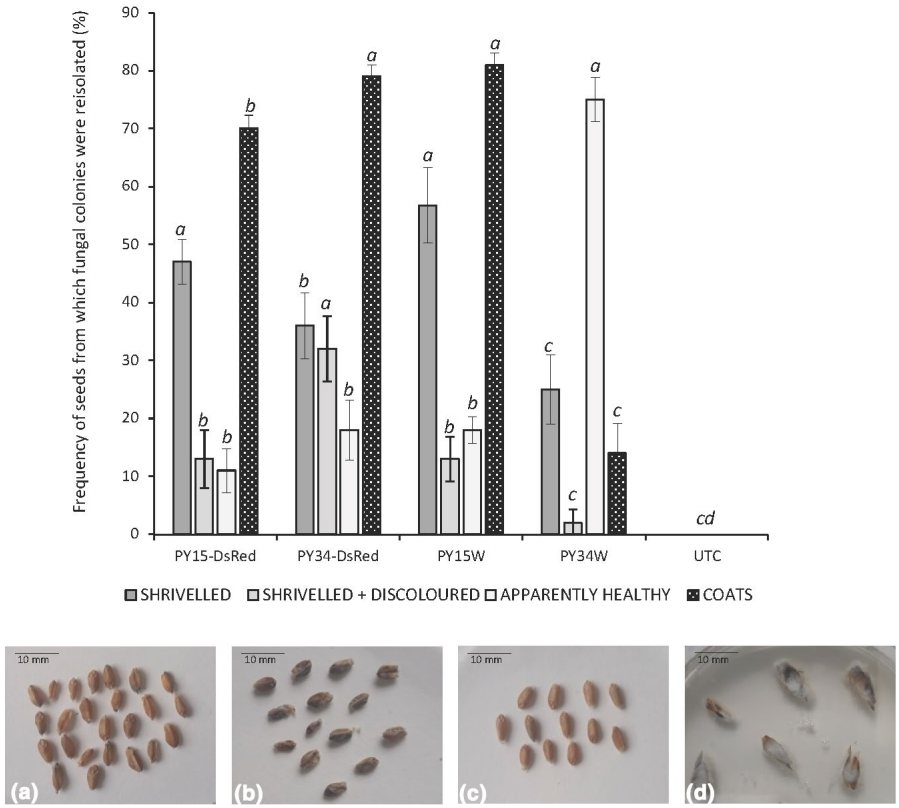

FIGURE 2 Determination of frequency of seeds infected with *Magnaporthe oryzae* pathotype *Triticum*. The seeds harvested from wheat cv. Apogee spikes, previously inoculated with the isolates PY15W, PY34W, PY15-DsRed, and PY34-DsRed, were grouped according to their visual appearance: shrivelled (a), shrivelled + discoloured (b), and apparently healthy (c). The seeds and coats (d) were disinfected and placed on complete medium plates supplemented with 500 ppm hygromycin and the addition of cefotaxime and streptomycin to prevent bacterial growth. Fungal colonies were examined by stereoscope microscopy. Data for percentage incidence was given following the formula of Mathur and Kongsdal (2003), as percentage of the fungal occurrence for each sample based on the quantification of the number of colonies developed from the seeds or coats compared to the total number of the sample. The bars are an average of four replicates with 25 seeds each, which were tested for each isolate and seed category. Different letters indicate significant differences between seeds categories at $p < 0.05$

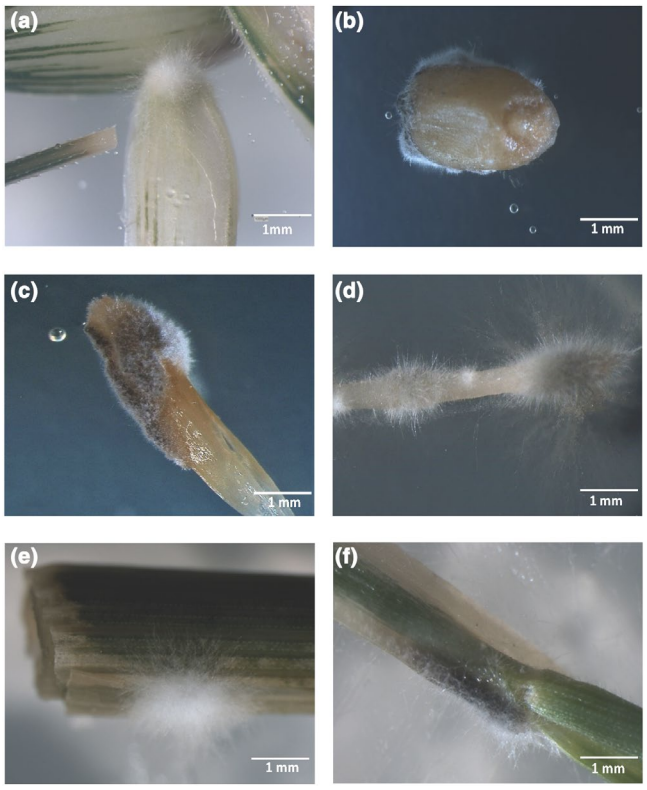

FIGURE 3 Reisolation of *Magnaporthe oryzae* pathotype *Triticum* from wheat seeds and seedlings derived from spikes of cv. Apogee infected with isolate PY15-DsRed. Seeds and vegetative tissues of seedlings were superficially disinfected, placed on complete medium agar plates supplemented with 500 ppm hygromycin and cultivated at 22 ± 2 °C in the dark to observe the development of the pathogen. Cefotaxime and streptomycin were added to prevent bacterial growth. Mycelia developed from coats (a), seeds (b), coleoptiles (c), roots (d), and piece of stems (e), observed by stereoscope microcopy. Typical blast lesions were found on 12-day-old seedlings (f)

again. These results point to a transmission of MoT from infected seeds to the youngest leaves of wheat plants over a time span of 21 days.

3.3 | Verification of MoT transmission from seeds to plants grown on soil

Next, an independent experiment was performed to verify the transmission of MoT from seeds to above ground plant organs for soil grown plants. In this case, we used a randomized mix of different categories of wheat seeds. The appearance of disease symptoms was visually monitored at different growth stages of wheat plants growing in pots with soil during 42 days of observations. During the whole period of inspection, no disease symptoms were detected on any of the plants grown in pots. To test whether the fungus developed without symptoms within the plants, vegetative tissues—germinating seeds, coleoptiles, stems, sheaths, and primary leaves—were collected and incubated on oatmeal agar medium containing Hyg. At 3 days after sowing, fungal structures (mycelia and spores) were successfully reisolated from 85% and 97% of plant samples (germinating seeds with coleoptiles) derived from seeds infected with PY15-DsRed and PY34-DsRed, respectively (Figure 6). The reisolation rate dropped to 3%–6% for plant samples harvested 14 days after sowing, corresponding to leaf material derived from main stems and tillers (Figure S4). Importantly, at 21 and 28 days after sowing, fungal material was again successfully recovered from plant samples. As described above, the identity of fungal material grown on agar plates was corroborated by fluorescence microscopy. From day 35 until day 42 of observation, no fungal material could be recovered. Both fungal isolates (PY15-DsRed and PY34-DsRed) showed the same pattern in the reisolation assay. MoT could not be recovered from stems, leaves, glumes, rachis, or immature grains (milk stage) of samples collected during the late stages (after 35 days) of disease monitoring.

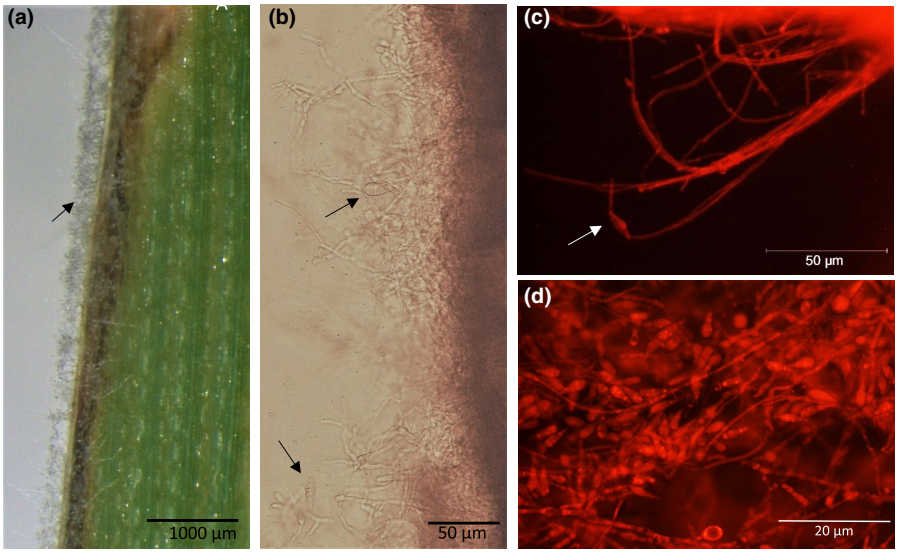

FIGURE 4 Visualization of *Magnaporthe oryzae* pathotype *Triticum* transmission on the first infected leaf of wheat cv. Apogee seedling. Typical wheat blast necrotic lesions caused by PY34-DsRed were observed by stereoscope microscopy (a). Associated with necrotic lesions, mycelia and spores (arrows) of the fungus were recorded. The same region was inspected using a higher magnification in bright field microscopy (b) and epifluorescent microscopy (c, d)

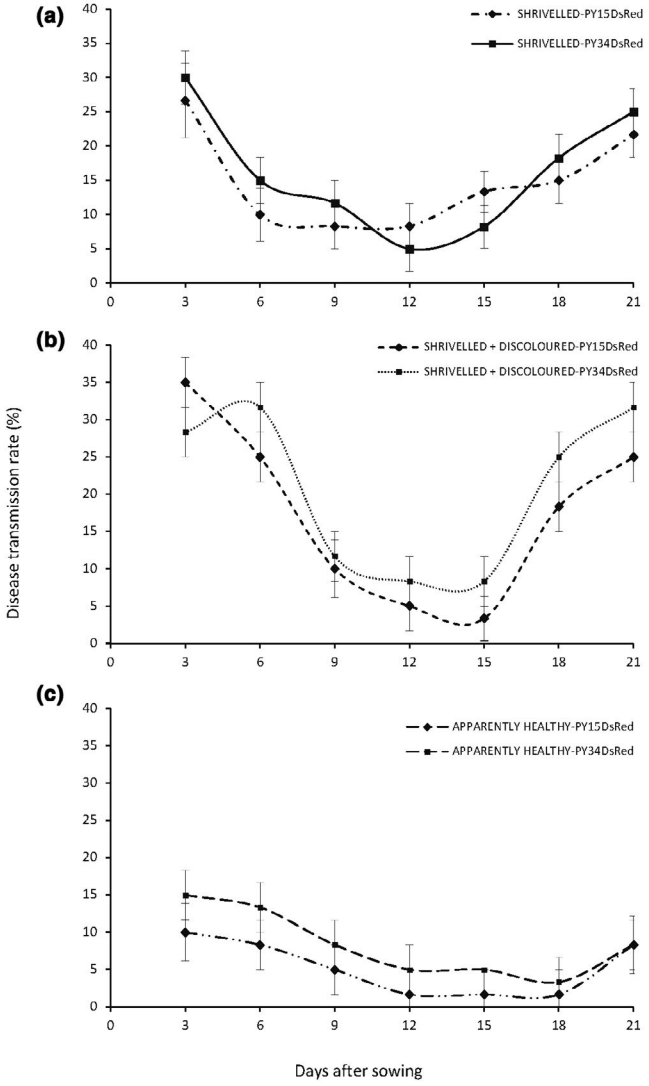

FIGURE 5 Recovery of *Magnaporthe oryzae* pathotype *Triticum* from plant parts derived from diseased seeds up to 21 days after sowing under laboratory conditions. The seeds classified as (a) shrivelled, (b) shrivelled + discoloured, and (c) apparently healthy, were superficially disinfected and sown on paper towels following the blotter test method (Agarwal, 1994) and incubated in a growth chamber. The paper layers were moistened with sterile distilled water until saturation. Four blotters of 20 seeds for each category and isolate were evaluated. The assay was done in duplicate. Every 3 days the seeds and samples of vegetative tissues were taken, cultivated on complete medium agar supplemented with hygromycin, and cefotaxime and streptomycin to prevent bacterial growth, and the number of reisolated colonies was recorded. The curves show the average transmission rate (incidence of fungal reisolation) for each seed category and isolate. Mean values and standard deviation are depicted

4 | DISCUSSION

Contaminated seeds are a prime source for primary inoculum and a critical step in plant pathogen ecology and disease epidemiology that leads to long-term inoculum survival, long distance dissemination, and the introduction of diseases into new areas. There are numerous examples in the literature of the inadvertent spread of plant diseases as a result of the importation of seeds infected or contaminated with pathogens (Bebber et al., 2014). A recent example is the occurrence of wheat blast in Bangladesh and the confirmation of infected seeds as the source of primary inoculum (Islam et al., 2016).

This study was conducted to substantiate the few reports of seed to seedling transmission for the pathogen MoT. For our studies we used the dwarf wheat cultivar Apogee, which flowers after a few weeks under laboratory conditions, making it very suitable for investigation of spike diseases. The infection of spikes from cv. Apogee by Argentinian MoT isolates was recently reported (Portz et al., 2020).

Accordingly, the results obtained here demonstrated statistically significant differences among the weights of infected seeds per spike of plants inoculated with isolate PY34-DsRed compared to the controls, where the average weight of grains per spike was 0.6 g for those inoculated with PY34-DsRed compared to 0.9 g presented by the UTC. Similar loss of grain weight was previously demonstrated by Martinez et al. (2019), where the relative thousand-grain weight of the four cultivars used in the experiment was significantly affected by the infection of eight isolates of MoT tested when compared with the controls. Moreover, our results demonstrated that the MoT infection reduced seed germination by 40% in artificial media under blotter test conditions and 39% for plants grown in soil. This result agrees with results by other authors; for example, Urashima et al. (2009) found a reduction in wheat seed germination in Brazil by MoT of 38%. Also, Perelló et al. (2017) reported that MoT decreased germination by 1%–65.9%, indicating that the effect on germination depended on the variety of wheat and the isolate used. Additionally, seedlings that developed from infected seeds showed poor plant development, weak appearance and yellowish colour, and reduced size and height. This was a characteristic observed in some evaluated plants in the present study (data not shown).

References about seed to seedling transmission are scarce for MoT and limited to experiences in Brazil (Gomes et al., 2018; Goulart et al., 1995; Urashima et al., 2009). More recently, Cruz and Valent (2017) pointed out that MoT can be transmitted from spike to seed, and from infected seeds to seedlings. In the frame of this study, we further substantiated these observations, and even expanded our current knowledge by showing that seeds collected from apparently healthy-looking wheat spikes also have the potential to cause a similar degree of infection to the ones with symptoms; that is, seeds collected from diseased and apparently healthy-looking wheat spikes may have a similar potential for infection. Key to these findings was our experimental approach of using transgenic isolates of the pathogen that were able to grow on selective medium containing the antibiotic hygromycin. In this way, we tested sample tissues from wheat plants and determined precisely whether or not they were infected with MoT, thus enabling us to corroborate and facilitate an accurate observation of MoT transmission. An additional advantage of our experimental design was the generation of seeds derived from artificially infected wheat spikes (inoculated with transgenic MoT isolates) instead of using seeds artificially inoculated with the pathogen by simply mixing a suspension of conidia with mature seeds.

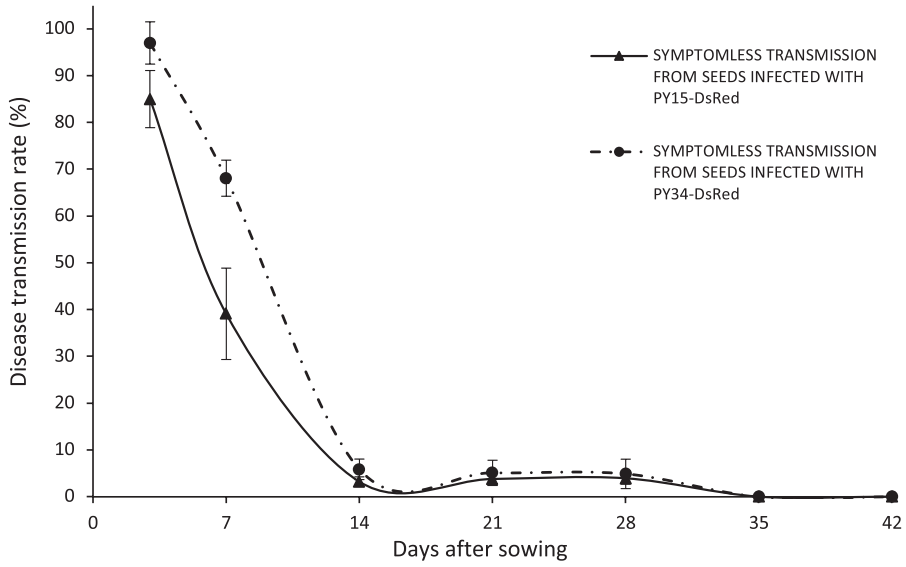

FIGURE 6 Recovery of *Magnaporthe oryzae* pathotype *Triticum* (MoT) from plant parts derived from diseased seeds up to 42 days after sowing for plants grown in soil. Wheat cv. Apogee seeds infected with PY15-DsRed and PY34-DsRed were sown in pots with standard soil and maintained in a phytotron for 42 days in a randomized complete block design with six pots with five plants per isolated of MoT, where each pot was a replicate. Seedlings were sampled and removed carefully every 7 days, cultivated on complete medium agar supplemented with hygromycin, cefotaxime, and streptomycin at 22 ± 2 °C in the dark and then the number of colonies developed for each evaluation time was calculated. The curves shown are the average transmission rate for each isolate, obtained after reisolation of the fungus. Average values and standard deviation are given. A negative control within the same experiment demonstrated that the reisolation is not based on external MoT contamination during handling

Using the experimental set-up described above, we documented here that MoT can be recovered from diseased and apparently healthy tissues, as evidenced by reisolation of mycelia and conidia. This result is concordant with previous observations by Urashima et al. (2009). In our transmission laboratory assays with Apogee plants, it was demonstrated that after seedling emergence, the pathogen can colonize newly developed tissues such as coleoptile, roots, stem, and primary leaves. Because MoT was found predominantly in infested seed coats and on the seed surface, we speculate that the transmission to hypocotyl/leaves started during the process of shoot differentiation under the ground. This was also shown for rice by Faivre-Rampant et al. (2013), who showed that spores of *M. oryzae* produced on contaminated seeds infect emerging seedlings and colonize the newly formed primary leaf and secondary roots.

Interestingly, our results indicated quantitative differences in the MoT transmission rate between the two assays performed, that is, plants grown using a blotter test (artificial medium) versus plants grown in soil (8%–28%, and no more than 5%, respectively). This is probably due to the faster spread of propagules on artificial media under the most favourable conditions of temperature and humidity at the time of observation (21 days). Analysing the progress of the transmission from seed to seedling, the initial recovery of the fungus was highest (31%) in the case of infected seedlings developed from shrivelled + discoloured grains, which also reached the highest final value of recovery (28%) compared with the other two grain categories. For seedlings derived from shrivelled grains, the initial and final recovery level from the samples of vegetative tissues was 28% and 23%, respectively. Importantly, seedlings grown from apparently healthy grains also carried the fungus, with an initial transmission rate at day 3 of 12% and at day 21 of 8%, which was the lowest value at this time point across all three grain categories.

Kohli et al. (2011) reported an increase in disease severity (>85%) at 25 °C with 40 hr of wetting. In addition, Danelli et al. (2019) established that the increase in MoT spores is due to the mean relative humidity, the mean daily temperature, and precipitation of less than 5 mm/day, managing to predict that the highest number of spores occurred in hours in which the temperature was between 15 and 35 °C and relative humidity >93%. Moreover, Kovaleski et al. (2020) established equations which identified that the highest production of conidia occurred between temperatures of 24 and 27 °C in experiments carried out in pots. As the greenhouse temperature and relative humidity were lower than those in laboratory conditions, we could speculate that, among other possibilities, the lack of optimum conditions is associated with the lower sporulation capability of the pathogen, and thus a lower rate of transmission under greenhouse conditions. The lack of production and dispersion of secondary inoculum due to the lack of wind and rain helping to disperse spores and mycelia from the leaves to the spikes, or among different leaves of the plants, could also be associated with the lack of recovery from the plant organs after 42 days.

The lack of these optimal conditions under phytotron conditions for plants grown in soil might explain, at least in part, the symptomless or cryptic type of fungal infection. Moreover, as happens with other pathogens (Malcolm et al., 2013), MoT could have a broad

repertoire of ecological interactions with its wheat host, which may include the capacity to infect and colonize without causing visible damage until the appearance of symptoms. Précigout et al. (2020) explained that many hemibiotrophic fungi such as MoT could, during latency, be inside the host and thus avoid or delay the damaging phase. Furthermore, this type of fungus has two phases, one symptomless where it lives within plant tissues, and the other necrotrophic. In this sense, many fungi are able to establish symptomless associations with their hosts. Nonetheless they cannot be seen as classical endophytes, rather these types of microbes are able to switch from an endophytic to a pathogenic lifestyle, depending on environmental conditions (Stergiopoulos & Gordon, 2014). In agreement, other studies have pointed out that, as a seedborne pathogen, MoT can be isolated even from symptomless seeds (Cruz & Valent, 2017). Similarly, Manandhar et al. (1998) reported sporulation of the fungus in symptomless rice seedlings, suggesting that latent infection can occur at temperatures too high or too low for lesions to develop. The latent infection and endophytic behaviour of MoT is not yet fully understood, and additional work with histological examinations is needed.

The results presented here provide a first clue for understanding the effect of environmental factors on the first stages of development of wheat blast epidemics and illustrate the imminent risk for dissemination of the disease from apparently healthy material under field conditions. Thus, there is a need to increase public awareness on aspects related to seed health and to develop suitable management for improving seed quality control systems.

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AUTHOR CONTRIBUTIONS

S.I.M. and A.P. performed most of the experiments, interpreted results, and drafted the manuscript. A.W. and S.B. helped with the fungal transformation assay and interpretation of results. U.S. and A.P. designed experiments and finalized the manuscript. All coauthors read and approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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