A Response to Gupta et al. (2019) Regarding the MoT3 Wheat Blast Diagnostic Assay

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ABSTRACT

This is a response to a recent Letter to the Editor of *Phytopathology*, in which Gupta et al. (2019) caution against the indiscriminate use of the MoT3 diagnostic assay that distinguishes isolates of *Magnaporthe oryzae* in the *Triticum* lineage from those that do not cause aggressive wheat blast. We confirm that the assay does reliably distinguish between wheat and rice isolates from Bangladesh and worldwide, as described in the

original paper by Pieck et al. (2017). We have been unable to reproduce the equally intense amplification of WB12 and WB12-like sequences reported in Figure 1 of the Letter. Other data presented by Gupta et al. (2019) support the specificity of the MoT3 assay. Therefore, cautions beyond those always associated with accurate reproduction of diagnostic assays are unwarranted.

LETTER TO THE EDITOR – RESPONSE

Our goal in developing the polymerase chain reaction (PCR)-based MoT3 diagnostic assay was to differentiate the aggressive *Triticum* isolates of *Magnaporthe oryzae*, currently restricted to South America and Bangladesh, from all other *M. oryzae* isolates, not just from rice pathogens (Pieck et al. 2017). The challenge was to find sequences that differentiate *Triticum* pathogens from extremely closely related isolates from *Lolium* species (Gladieux et al. 2018). This distinction gained practical importance when a native U.S. *Lolium* isolate (WBKY11-15) was recovered from a blasted wheat head in Kentucky in 2011 (Farman et al. 2017). Isolation of this native *Lolium* strain from wheat alerted us to the ability of some *Lolium* isolates to infect wheat in the field, although *Lolium* strains are much less aggressive pathogens on wheat than *Triticum* strains. The MoT3 primers amplify a sequence, WB12, which was perfectly correlated with 113 *Triticum* lineage strains among a total of 285 *M. oryzae* strains from 11 host-preference groups (Pieck et al. 2017). What appeared to be exceptions were not; for example, 2 out of 115 isolates tested from wheat in the field

were members of the *Lolium* lineage, according to phylogenetic analyses.

Prior to the publication of Pieck et al. (2017), the MoT3-based PCR assay was thoroughly tested in multiple laboratories. Subsequently, the U.S. Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) Center for Plant Health Science and Technology (CPHST), Beltsville Laboratory (the only U.S. Federal Plant Pathology accredited laboratory that conducts operational diagnostics for regulated plant pathogens), has validated the assay for confirmatory diagnostic testing of wheat blast (Bienapfl et al. 2018). In addition, another lab in Bangladesh has successfully implemented the MoT3 assay and obtained the predicted results using *M. oryzae* isolated from rice, wheat, and other grasses in Bangladesh (M. Muzahid-E-Rahman, P. Kumar Malaker, and N. Chandra Deb Barma, Bangladesh Wheat and Maize Research Institute, *personal communication*). In these additional labs, the MoT3 diagnostic assay performed exactly as predicted by Pieck et al. (2017).

We agree with Gupta et al. (2019) who “caution against the indiscriminant use” of a diagnostic assay to identify a plant pathogen; that standard should be applied to all DNA-based diagnostic assays for any plant pathogen. Implicit in publishing a diagnostic assay that includes all the details for reagents, reaction conditions, technology platform, etc., is that those conditions must be followed to obtain the reported results. That is a fundamental understanding to those who work in the field of diagnostics. The danger of failing to follow PCR assay specifications is that it commonly leads to inaccurate results. It is important to note that the concerns Gupta et al. (2019) raise about the MoT3 assay were based on two experiments in which the explicit instructions in Pieck et al. (2017) were not followed. These include altered primer concentrations, longer extension times, changes to the buffer composition, and the use of a polymerase with enhanced sensitivity and yield. After the authors obtained unexpected amplifications from rice blast isolates, they did not test if the published Pieck et al. (2017) protocol produced the same, unexpected results.

Rather than simply discounting the Gupta et al. (2019) findings as a consequence of not following a published protocol, three different laboratories performed additional experiments to test the assay's robustness to different protocols (USDA-ARS, Ft. Detrick, MD;

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Kansas State University; and the University of Kentucky, where MoT3 assays had never been performed previously). When the Pieck et al. (2017) protocol was followed, no unexpected amplifications were obtained with previously tested strains. We tested nine additional rice isolates, including two from India (O-249 and O-250), two from Nepal (O-260 and O-261), two from Brazil (O-282 and O-283), and three from Uruguay (U75, UB107, and U198), and all tested negative using the MoT3 primers (data not shown, all strains described in Yasuhara-Bell et al. 2018). Importantly, no unexpected amplifications were detected using the same reagents and conditions reported by Gupta et al. (2019), while parallel assays for the single-copy *MPG1* gene control produced abundant amplification in all lanes. On the other hand, using the Gupta et al. (2019) cycling parameters with ExTaq polymerase and buffers (Takara Bio, Mountain View, CA) did produce unexpected amplification in two samples (the annual ryegrass pathogen, ATCC64557; and BR32, a wheat blast isolate that is MoT3-negative but had yielded an amplicon in the Gupta et al. [2019] study). Critically, however, the nontarget, WB12-like bands were extremely faint (~50- to 100-fold less intense than genuine WB12 bands on the same gel, as estimated by densitometric scanning, and were barely visible until the gel image was

inverted to show black bands on a gray background). This is in striking contrast to Figure 1 of Gupta et al. (2019) where the WB12 and WB12-like bands had equal intensities. Sequencing of a representative ExTaq amplicon from isolate ATCC64557 confirmed that it originated from the nontarget WB12-like sequence reported by Gupta et al. (2019) (Farman lab, University of Kentucky).

We screened a set of control isolates using the MoT3 primers supplied to us by Gupta et al. (2019), but again were unable to obtain any unexpected amplifications. Finally, we obtained three of the Bangladeshi rice isolates used by Gupta et al. (2019) and tested them along with the Japanese rice isolate, INA168, which was used as a control in their study. Our predicted assay specificity was maintained with these strains as well, using both the Pieck et al. (2017) protocol (Fig. 1A) and the Gupta et al. (2019) protocol (Fig. 1B). In our labs, no rice isolate samples have ever resulted in an amplicon using the MoT3 PCR primers and protocol reported by Pieck et al. (2017); or the MoT3 loop-mediated isothermal amplification (LAMP) primers and protocol in Yasuhara-Bell et al. (2018). Based on our follow-up experiments, we cannot explain the equally intense amplicons shown for wheat and rice pathogens in Figure 1 of the Gupta et al. (2019) Letter.

Additional data presented in the Letter to the Editor of *Phytopathology* by Gupta et al. (2019) seem to provide insight into the discrepancy. When the PCR thermocycler was replaced with a new one, and an annealing temperature of 62°C was employed, they obtained the expected MoT3 specificity (their Supplementary Fig. S1A). However, inappropriate amplification of WB12-like sequences from rice isolates was obtained when the annealing temperature was lowered to 57°C (their Supplementary Fig. S1B). We tested the Gupta et al. (2019) protocol with an annealing temperature of 55°C and were able to amplify extremely faint bands from all the rice isolates, as well as from BR32. The intensities were such that the bands were not visible looking at the gel on the transilluminator; however, they were clearly visible when imaged. Thus, it appears that once they employed a validated primer stock and utilized a newer (and, presumably, more accurate) thermocycler, their results (shown in their Supplementary Fig. S1A) fully validated the MoT3 assay. Therefore, we are disappointed that these results were not included in the main manuscript and mentioned more prominently.

Gupta et al. (2019) conclude that the WB12 sequence is not present in all wheat-infecting isolates. However, as previously mentioned, our goal was to differentiate highly aggressive isolates in the *Triticum* lineage from isolates in the *Lolium* lineage, some of which show low levels of pathogenicity on wheat. Their bioinformatic results identifying WB12 and WB12-like sequences in 67 *M. oryzae* genomes actually support the specificity of the MoT3 assay. They identified one true exception, a strain that we had not tested before. Wheat isolate BR32 is in the *Triticum* lineage (Gladieux et al. 2018), but its genome sequence contains a WB12-like paralog of the MoT3 sequence and it is negative in our MoT3 assay (positive in Gupta et al. [2019]). However, the other mentioned exception, strain P28 (same as strain P-0028 in their Supplementary Table S1) is a *Lolium* lineage strain that was isolated on *Bromus* species growing in a heavily blasted wheat field in Paraguay in 2014 (Gladieux et al. 2018). The fact that P28/P-0028 has the WB12-like sequence and not the WB12 sequence fits expectations. The other named exception in Supplementary Table S1 (Gupta et al. (2019) was WBKY11 (i.e., WBKY11-15 described in Farman et al. 2017). Although this strain was isolated from a wheat head, it is from the *Lolium* lineage and it possesses only the WB12-like sequence. Therefore, the failure to amplify MoT3 is expected. The second *Bromus* isolate from Paraguay, P29 (same as P-0029, Gladieux et al. [2018]), is correctly labeled as belonging to the *Triticum* lineage in their Supplementary Table S1, and it contains the WB12 sequence. These strains illustrate that host of isolation is not always a reliable predictor of that strain's primary host, nor its genetic lineage. It is expected to find some

Fig. 1. Polymerase chain reaction (PCR) assays demonstrating the accuracy of the MoT3 assay. **A**, Use of the MoT3 primers results in a single amplicon of the predicted size (361 bp) from isolate T25, whereas the Pot2a primers produce an amplicon (389 bp) from all *Magnaporthe oryzae* isolates tested. T25 is an isolate of *M. oryzae* collected from wheat in Brazil. *M. oryzae* isolates RB3a, RB9c, and RB11a were collected from infected rice in Bangladesh and INA168 was collected from infected rice in Japan. The assay was performed at Kansas State University. Assay conditions and primer sequences (Pot2a and MoT3) have been previously described (Pieck et al. 2017). **B**, Use of the MoT3 primers results in a single amplicon of the predicted size (361 bp) from isolates BdKUS and Br80, whereas the MPG1 primers produce an amplicon (438 bp) from all *M. oryzae* isolates tested. BdKUS and Br80 are isolates of *M. oryzae* collected from wheat in Bangladesh and Brazil, respectively. *M. oryzae* isolate Guy11 was collected from rice in French Guyana. The assay was performed at the University of Kentucky using the assay conditions described by Gupta et al. (2019). MPG1 primer sequences have been previously described (Farman et al. 2017).

cross-infections under disease-conducive conditions. Therefore, out of analyzed genomes mentioned in Gupta et al. (2019), only 1 strain out of 67 (65 accounting for duplications) is a true exception—that being BR32.

ADDITIONAL TECHNICAL POINTS

The results presented in Figure 1B of Gupta et al. (2019) are directly contradicted by the results presented in their Supplementary Figure S1A. According to the data presented in Supplementary Figure S1A, this MoT3-based PCR assay performed as predicted by Pieck et al. (2017). The only difference presented between these two experiments is the use of a new thermocycler of the same model used for the experiment in Figure 1B. The authors highlight the faint band produced in the reaction with the rice strain RB9c using the UK primer set in the 62°C annealing temperature experiment (Supplementary Fig. S1A), but they do not mention the stronger band produced in their water control reaction using the UK primer set in the 57°C annealing temperature experiment (Supplementary Fig. S1B).

Accurate interpretation of a diagnostic assay requires that proper controls are included in the assay. The PCR assay represented by the gel in Figure 1A from the Bangladesh laboratory of Gupta et al. (2019) failed to include a known positive control, a known negative control, or a no-template control thus compromising the conclusion that the MoT3 assay produces an amplicon for all blast isolates collected from infected rice in Bangladesh. The isolates they labeled as controls had not been previously tested and therefore cannot serve as controls.

Gupta et al. (2019) alerted us to the exceptional *Triticum* lineage strain BR32 that has a WB12-like paralog of the MoT3 (WB12) sequence. BR32 is an early strain isolated from wheat in Brazil in 1991, and probably illustrates gene flow during the early days of wheat blast when we know at least some strains were highly fertile sexually (Cruz and Valent 2017). All of our recent isolates from the *Triticum* lineage have tested positive in the MoT3 assay.

Testing by Yasuhara-Bell et al. (2018), subsequent to the publication of Pieck et al. (2017), identified two isolates (FPH2015-44 and BR35) that are exceptions to MoT3 specificity. FPH2015-44, a U.S. Japanese forest grass isolate (Farinas et al. 2017); and BR35, isolated from *Brachiaria* in Brazil (Inoue et al. 2017), both have the WB12 MoT3 allele. Both strains appear to be hybrids between *Stenotaphrum* and *Brachiaria* pathogens (M. Farman, unpublished data). Importantly, Inoue et al. (2017) identified a *Brachiaria* pathogen as a likely contributor of the Type B PWT3 allele present in wheat blast isolate BR48. Thus, it is conceivable that wheat blast isolates and *Brachiaria* pathogens share recent ancestry. These two hybrid strains are the only non-wheat blast isolates that have yielded MoT3 amplicons out of 421 isolates tested in our laboratories.

SUMMARY

We stand by the MoT3 assay as a valuable tool for differentiating strains in the dangerous *Triticum* pathotype from strains in all other *M. oryzae* lineages worldwide. Our attempts to reproduce

inappropriate amplification of the WB12-like sequences in non-*Triticum* isolates have resulted in extremely faint bands in a few instances, but not the equally bright WB12 and WB12-like amplicon bands described in Figure 1 of Gupta et al. (2019). Additionally, the low rate of exceptions associated with the MoT3 assay are acceptable and not uncommon for useful diagnostic assays. All diagnostic assays, no matter how robust, can be compromised by poor reagents, omission of proper controls, faulty equipment, lack of adherence to published protocols, or human error.

We encourage and will support further improvement of the MoT3-based assay and the development of additional markers for wheat blast diagnostic testing. However, we have full faith and confidence in the current protocol as described by Pieck et al. (2017). It is our position that Gupta et al. (2019) unnecessarily erode confidence in the published MoT3-based assay. We will be happy to continue to work with them to resolve the differences.

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