scientific reports

Check for updates

OPEN

Amplification-free detection of Ascochyta blight in chickpea using a simple molecular beacon assay

Kazbek Dyussembayev[1,2], Victor Akpe[1], Talgat Yechshzhanov[3], Matthew J. Cheesman[4,5], Tak H. Kim[1,6] & Ian E. Cock[1,7]✉

Ascochyta blight is a major biotic stress that limits chickpea production globally. Fungicide application remains one of the effective control measures for the endemic spread. Due to the serious threat that synthetic fungicides pose to crop quality, early diagnosis of the pathogen is imperative. Whilst there have previously been several conventional lab-based diagnostic methods developed for early detection of Ascochyta rabiei, they require long assay times, specialised equipment and facilities, and trained personnel to process the samples. To overcome this challenge, a rapid amplification-free detection assay using a molecular beacon probe was developed. The method consists of a simple assembly assay that accurately detects pathogen within 30 min. The developed assay is species-specific and has a similar sensitivity level as conventional amplification-based methods. Although it is still a lab-based technique, considering the simplicity of the assay, it has a great potential to be developed further as a reliable in-field diagnostic device for early detection and quantification of fungal pathogen spores.

Ascochyta blight (AB) arises from infection by the spore-based necrotrophic fungus *Ascochyta rabiei*. AB is a threat to global production of chickpea and has the potential to cause substantial crop losses. The fungal pathogen mostly spreads in cool, wet weather conditions and affects all parts of plants[1]. AB is generally managed by intensive fungicide application, crop rotation, seed treatment and the use of AB-resistant cultivars[2]. Of concern, overexposure of infected chickpea plants to fungicides may cause long-term damage to the ecosystem. Due to the absence of highly resistant chickpea cultivars, fungicides are commonly used to achieve an acceptable level of disease control[3]. This generally includes a fungicide spray regime based on weather forecast (prior to rain events), as well as a mandatory spray before sowing. The major problem in using chemicals to control or manage this pathogen is the potential for isolates to lose sensitivity[4]. Together with the impacts of climate change, the overuse of fungicide is proposed to have driven selective adaptation and improved the evolution potential of the pathogen[5]. Most recently, a highly aggressive adapted haplotype was observed in increased frequencies within the Australian clonal *A. rabiei* population, which is able to overcome resistance within the widely adopted resistant host cultivars[6].

Fungicides are currently widely used as a preventative measure to assist in generating profitable chickpea production, as well as to eradicate AB infections[4]. There is a need to develop fast, accurate and reliable disease diagnosis to avoid the overuse of chemicals that may compromise the crop quality and environmental security[7,8]. Ideally, these detection methods should not only be sensitive and specific, but they should also be rapid and simple enough to facilitate in-field analysis. Early pathogen detection will enable agriculturalists to monitor the health quality of their crops and respond rapidly with effective management strategies[9,10]. Whilst several current molecular diagnostic methods offer relatively sensitive and specific approaches to plant pathogen detection, they are limited by complicated sample preparation methods, time-consuming assays, and the use of costly/sophisticated equipment and reagents[11,12]. These constraints have limited the uptake of these methods by primary producers, who instead continue to use indiscriminate fungicide management approaches.

Molecular beacons (MBs) are single-stranded oligonucleotide fluorescent probes that were originally developed by Tyagi and Kramer[13] for the detection of specific nucleic acids homogeneous solutions. MBs are most commonly used as a qPCR probe. They are designed with loop and stem structural moieties flanked by a fluorophore molecule and a quencher system at the 5' and 3' positions, respectively. The detection sequence is

[1]School of Environment and Science, Griffith University, Nathan, QLD 4111, Australia. [2]L.N. Gumilyov Eurasian National University, Astana 010000, Kazakhstan. [3]Pedagogical Institute, Astana International University, Astana 010000, Kazakhstan. [4]Menzies Institute, Griffith University, Nathan, QLD 4111, Australia. [5]School of Pharmacy and Medical Sciences, Griffith University, Gold Coast, QLD 4222, Australia. [6]Queensland Micro and Nanotechnology Centre, Griffith University, Nathan, QLD 4111, Australia. [7]Centre for Planetary Health and Food Security, Griffith University, Nathan, QLD 4111, Australia. ✉email: I.Cock@griffith.edu.au

designed to be complementary to the target gene. Once the probe assays are developed, the detection method using MBs is relatively quick and simple. When a target DNA sequence is hybridized with the loop portion of the MB probe, the fluorophore generates a fluorescence signal whilst the quencher is located far from the fluorophore and cannot prevent the fluorescence signal (Fig. 1A). Conversely, a mismatch between the MB probe and the target sequence facilitates steric proximity and signal quenching.

MBs have been utilized in other ways, such as in applications for real-time monitoring of PCR amplification, mutational analysis, clinical diagnosis, genotyping, allele discrimination, and pathogen detection[14]. With regard to their usage in disease diagnostics, they can be coupled to PCR detection techniques[15], loop-mediated isothermal amplification (LAMP)[16], various other biosensors[17], and may even be coupled with mobile phone integration as a portable screen detection platform[18]. To date, MB probes have generally been used as part of the complex diagnostic assays mentioned above. However, MBs can generate a fluorescence signal upon positive responses, yet they have never been used without additional involvement of amplification steps or biosensing platforms. Herein, we describe the development of a novel, simple-to-use, amplification-free assay for the detection of AB disease in chickpea samples. The assay time post DNA extraction is less than 30 min, which is a substantial improvement on current methodologies. The assay involves a simple hybridization process based on a temperature change and relies on MB's fluorescence emission upon a positive response (Fig. 1B).

Materials and methods

Plant samples and genomic DNA extraction

Twenty-four AB-infected chickpea samples (PBA Striker cultivar) were collected on 7/10/2021 from chickpea fields within Merredin, Western Australia. Five uninfected plant samples were grown under a controlled environment in a growth room (Griffith University, Australia) and used as non-infected controls. The cultivation and collection of all plant material complied with the IUCN policy on Research Involving Species at Risk of Extinction, and the Convention on Trade in Endangered Species of Wild Fauna and Flora. Genomic DNA was extracted from freshly cut leaves of each plant using the DNeasy® Plant Mini Kit (QIAGEN, Germany) and the purity of DNA determined by a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA). DNA integrity was determined using agarose (0.8%) gel electrophoresis following by staining with RedSafe™ Nucleic Acid Staining Solution (Scientifix Pty Ltd, Australia) and visualized under an UV transilluminator (Bio-Rad, Australia). The DNA was quantified using an Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific, USA).

Probe design

Synthetic target DNA (T_ST47_g9244), mismatched synthetic target (containing a single nucleotide mismatch; TM_ST47_g9244) and the MB probe (MB_ST47_g9244) (Table 1) were designed prior to purchase from Integrated DNA Technologies (Coralville, IA, USA). The target oligonucleotides were designed from one of the 53 predicted unique glycoside hydrolase genes (ST47_g9244, genome accession JYNV00000000.1) of *A. rabiei*[19]. The loop portion of the MB probe was designed by adding four extra nucleotides to the 3' end of the ST47_g9244 forward primer sequence. Furthermore, six nucleotides were added as complementary arm sequences to both ends of the MB probe (Fig. 2). A 6-FAM (carboxyfluorescein) fluorophore and a Dabcyl (dimethylaminoazobenzenesulfonic acid) quencher were added to the 5' and 3' ends of the arm sequences, respectively.

Assay conditions and fluorescence signal detection

The assay used a simple protocol to detect AB. Firstly, 1 ng of target DNA and 10 ng of MB probe were dissolved in 200 µL of 100 mM Tris-HCl (pH 8) (Sigma-Aldrich Pty Ltd, Australia) containing 1 mM $MgCl_2$ (Sigma-Aldrich Pty Ltd, Australia) in a 1.5 mL microcentrifuge tube. The mixture was subjected to a denaturation step at 95 °C for 3 min to denature the MB's arm sequences as well as the target dsDNA, and then incubated for 10 min on a thermomixer (Eppendorf, Germany) (300 rpm) at 63 °C (annealing temperature) to allow hybridisation of target ssDNA with MB. The reaction mixture was subsequently incubated at room temperature (25 ± 2 °C) for 10 min to stabilise the hybridisation process. The mixture was transferred into black 96-well immune-plates (Thermo Fisher Scientific, USA) for fluorescence intensity measurements.

Fluorescence (FL) intensity of emitted light was measured using a single emission wavelength at 515 nm on a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, USA) at room temperature (25 ± 2 °C). The excitation and emission wavelength for the assembled assay were taken at the MB's reported excitation and emission at 495 and 520 nm respectively[20]. The same reaction mixtures in the absence of target and MB probe were used as non-template and non-probe controls for the MB probe respectively in order to assess the presence of nucleic acid contamination. All reactions and measurements were carried out in technical triplicates and the results are reported as means ± standard deviation (SD).

Assay specificity and sensitivity

To determine the specificity of the MB_ST47_g9244 probe, a synthetic target sequence containing a single nucleotide mismatch (TM_ST47_g9244) was tested. For the sensitivity test, the target T_ST47_g9244 DNA was serially diluted across ten final quantities: 1 ng, 0.5 ng, 100 pg, 50 pg, 10 pg, 5 pg, 1 pg, 0.5 pg, 100 fg, 50 fg. The DNA template was diluted with Ultra-Pure H_2O (Thermo Fisher Scientific, USA). The assay conditions were as previously described. All reactions were conducted in technical triplicates.

For determination of the copy numbers of a template, the following formula was used:

$$Number\ of\ copies = (amount\,(ng) \times 6.022 \times 10^{23})/(length\,(bp) \times 1 \times 10^{9} \times 650)$$

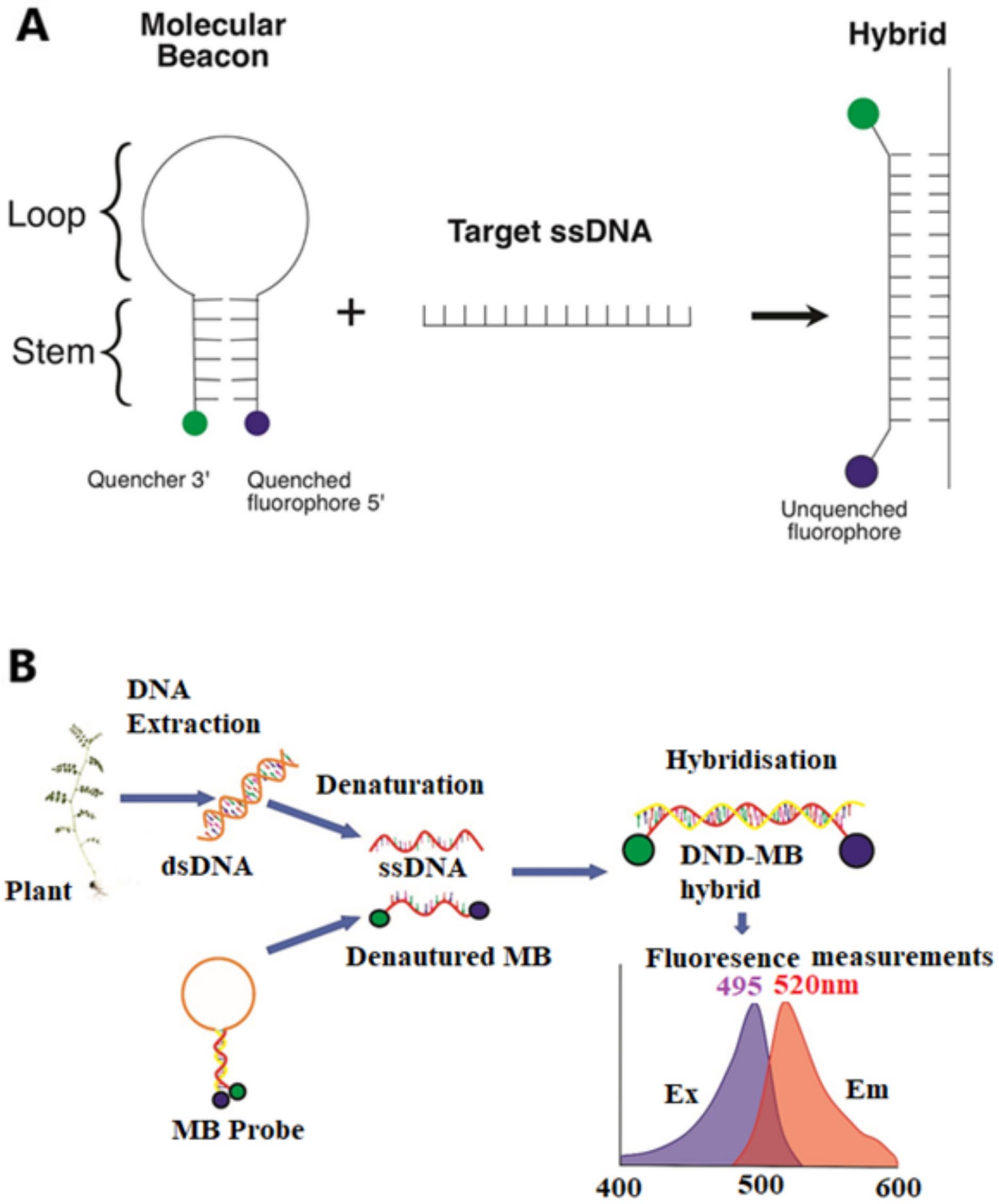

Fig. 1. (**A**) Working principle of molecular beacon. When a target sequence is hybridized within the loop portion of the MB probe, the ordered helical structure changes to a random coil configuration, demarcating the fluorophore from the quencher and allowing the fluorophore to fluoresce. Conversely, if there is no loop formation, hybridization of complementary arm sequences at the stem portion of the MB probe keeps the fluorophore and the quencher close to each other. Thus, the steric proximity of the quencher will block the fluorescence signal, even when the fluorophore is excited. (**B**) Schematic illustration of the MB assay. dsDNA was extracted from AB-infected chickpea plant samples and both the dsDNA and MB probe were subsequently denatured. The mixture was incubated at annealing temperature for hybridization and then at room temperature for stabilization of the hybridization process. Once the hybrid was formed, the fluorophore fluoresced as it was located far from the quencher and the fluorescence intensity was recorded.

Oligonucleotides	Sequences (5' → 3')
T_ST47_g9244	GAC GCC AGT GAC GTT GAC CTC GAA
TM_ST47_g9244	GAC GCC AGT GAC ATT GAC CTC GAA
MB_ST47_g9244	/56-FAM/AG CGC GTT CGA GGT CAA CGT CAC TGG CGT CCG CGC T/3Dab/
ST47_g9244 Fw	TTCGAGGTCAACGTCACTGG
ST47_g9244 Rv	CACACCTGCCTTCGATGAGT

Table 1. Oligonucleotide sequences used in this study.

For this calculation, the total sequence length (40,917,385 bp) of a reference *A. rabiei* genome[21] was used.

qPCR validation

A SYBR® Premix Ex TaqTM II (TIi RNaseH Plus) kit was used to evaluate target gene expression on a Bio-Rad CFX96 quantitative PCR detection system (Bio-Rad, Australia). PCR was carried out in a total volume of 20 µL, which contained 10 µL of 2x SYBR® Premix Ex TaqTM II (TIi RNaseH Plus), 0.6 µM of each primer, 7.8 µL of nuclease-free water and 1 µL of DNA template. The amplification included the ST47_g9244 forward and reverse primers from Table 1 and information on their design can be found in Supplementary information; ST 1. The cycling conditions included 15 s at 95 °C (initial denaturation), followed by 38 cycles of 95 °C for 15 s and 64 °C for 15 s. A melt curve analysis was undertaken for each 0.5 °C temperature change (every 10 s) across the 65–95 °C temperature gradient. The reactions were performed with technical triplicates and the two closest data points taken for analysis. A non-template control was added for each primer combination to assess potential contamination by gDNA or primer dimers.

Results and discussion

Assay functionality and optimization

To optimise the assay performance, the assay buffers (base solutions), as well as the template and MB probe quantities, were first evaluated.

The following parameters were tested:

(I) Various quantities of DNA template (10-fold: 100 ng to 10 fg) were tested at a constant quantity of MB (1 ng) in the presence of H_2O (Fig. 3A);
(II) The same dilutions of DNA template were tested at constant MB quantity in the presence of 100 mM Tris-HCl containing 1 mM $MgCl_2$ (Fig. 3A);
(III) Different quantities of MB (10-fold: 100 ng to 10 fg) were tested with a constant DNA template quantity (1 ng) in the presence of H_2O (Fig. 3B); and.
(IV) The same dilutions of MB at constant template DNA were tested in the presence of Tris-HCl containing $MgCl_2$ (Fig. 3B).

When Tyagi and Kramer[13] first developed the MB probe, it was dissolved in 100 mM Tris-HCl (100 mM, pH 8.0) containing 1 mM $MgCl_2$ to hybridize the stem portion. According to this study, the presence of magnesium ions stabilises the stem hybrid of the MB probe with short arm sequences (five to eight bp) in temperatures up to 56 °C. Therefore, Tris-HCl (100 mM, pH 8.0) was chosen in this study as a primary mixture solution and H_2O was also tested as a positive control under the same assay conditions. The 100 mM Tris-HCl buffer containing 1 mM $MgCl_2$ was deemed optimal for the MB probe for target hybridization as it generated a substantially higher FL intensity compared with H_2O. When a standard quantity of MB (1 ng) was tested in Tris-HCl buffer, DNA template quantities > 1 ng provided the highest FL intensity of around 162 relative fluorescence units (RFU). In contrast, dilutions containing < 1 ng showed substantially lower signals. Notably, adding 1 ng of MB probe (experiment IV) produced similar results (~ 162 RFU). However, at quantities of 10 and 100 ng MB, assay performance was substantially improved (~ 183 RFU). The stock of the MB probe contained fluorophore, quencher and additional arm sequences that may add mass to the loop portion of the probe, while the target DNA stock contains only the synthetic DNA fragments that are complementary to the loop. Therefore, these optimal quantities of the target (1 ng) and the MB probe (10 ng) were used in subsequent experiments. It should be noted that the cost of the MB is a limiting factor for the development of an inexpensive diagnostic method, with 40 nM of the probe costing around 900 AUD. Ideally, cheaper fluorophores and quenchers should be incorporated into the MB and tested in future optimisation studies. Additionally, the MB-based assay requires further optimisation that would facilitate the use of lower concentrations of the probe.

Further assay development steps involved optimization of the incubation time and annealing temperature for the hybridization of MB probe with the target DNA fragment. Six time-points between 5 and 30 min were tested, with an interval of 5 min between each time point (Fig. 4A). Interestingly, a 5 min incubation generated a lower signal (174.68 RFU), while 10 to 30 min of incubation time generated approximately 183 RFU. Thus, a 10 min incubation at the annealing temperature, coupled with a further 10 min incubation at room temperature, was found to be sufficient to allow hybridization between the MB probe and DNA target. This means that a total of 30 min is required to complete the assay, which is substantially faster than other detection methods currently used to quantify copies of genomic DNA. Further evaluations were also undertaken to optimise the annealing temperature of the assay. The highest optimal fluorescence intensity was observed when the assay incorporated

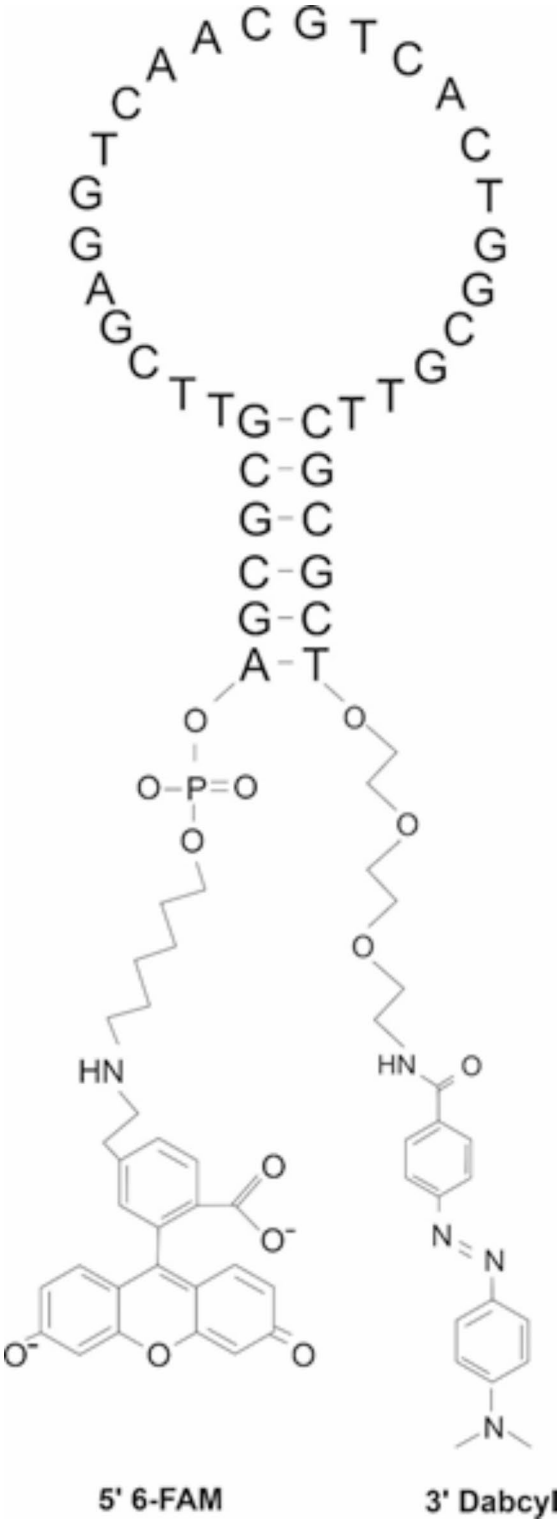

Fig. 2. Structure of MB probe. The oligonucleotide circle represents the loop portion of the probe and consists of 24 nucleotides that are complementary and specific to the target gene sequence. The stem portion is a hybrid of six base pairs complementary arm sequences connected to the loop portion from one side and to the fluorophore-quencher from the other side. A 6-FAM (carboxyfluorescein) fluorophore and a Dabcyl (dimethylaminoazobenzenesulfonic acid) quencher are attached to the 5' and 3' ends of the arm sequences, respectively.

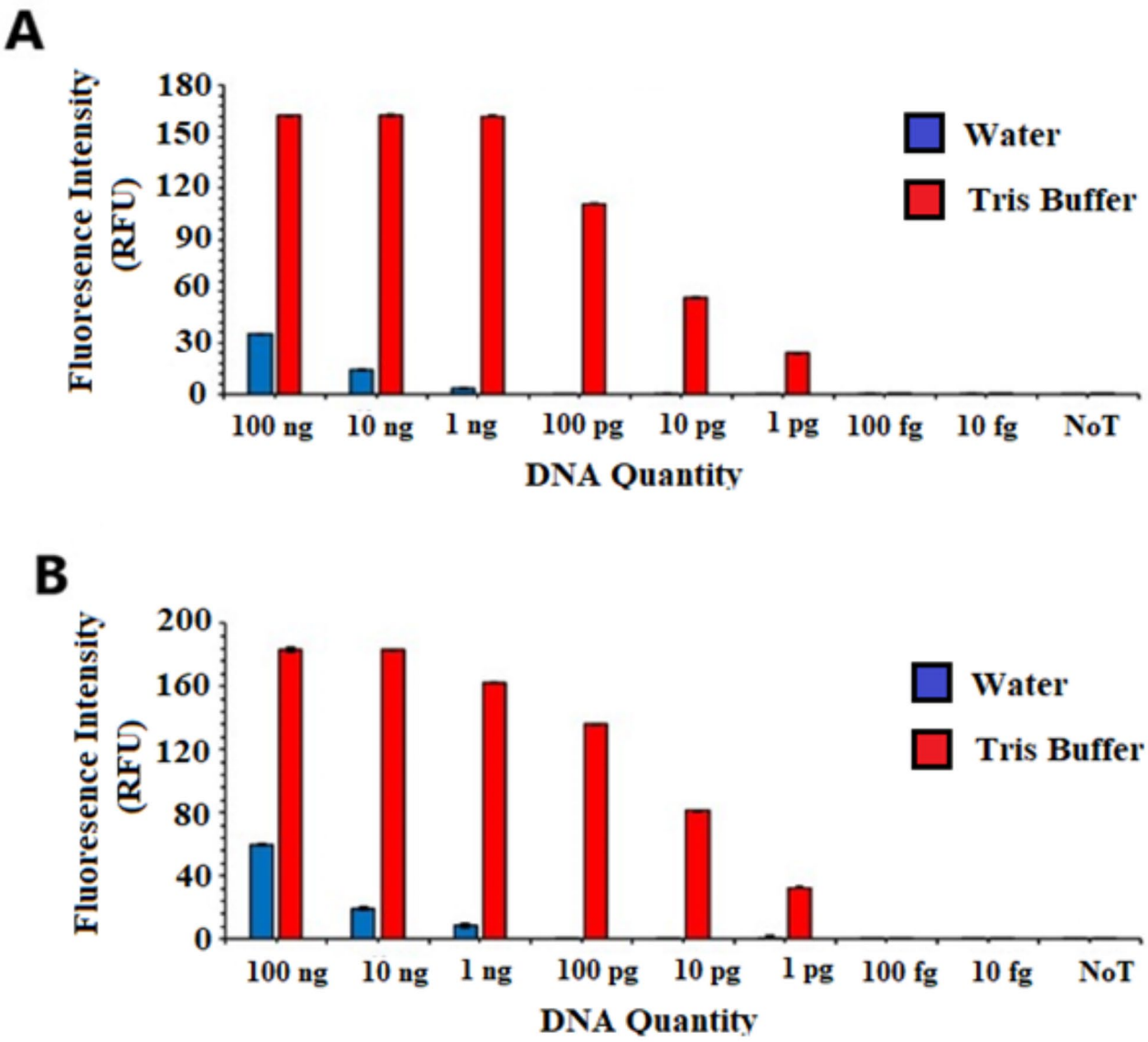

Fig. 3. Optimization of a mixture solution and DNA template/probe quantities: (**A**) Quantity effects of DNA template at constant MB quantity (1 ng) in the presence of H_2O and Tris-HCl + $MgCl_2$; (**B**) Quantitative effects of MB at constant DNA mass (1 ng) in the presence of H_2O and Tris-HCl + $MgCl_2$. NoT stands for non-template control. Fluorescence intensity was measured in relative fluorescence units (RFU). Values are the mean ± SD of technical triplicates.

an annealing temperature of 63 °C (Fig. 4B), which could be predicted according to a high CG content of the target DNA sequence.

Assay specificity and sensitivity

As discussed earlier, the loop portion sequence of the MB_ST47_g9244 probe has been tested previously for specificity in silico as well as in vitro via PCR and hybridisation testing against closely related species from the National Centre for Biotechnology Information database by our group[22]. A synthetic target sequence with a nucleotide mismatch (TM_ST47_g9244) was designed to verify the specificity of the developed probe in the current study. The MB probe detected the target, although it did not hybridize with the TM_ST47_g9244 (non-target) sequence, confirming that the assay is species-specific. The assay could discriminate between target DNA sequences within genomes obtained from other species by as little as a single nucleotide mismatch. This substantially decreases the likelihood of producing false positives using this assay.

Our findings with the sensitivity tests revealed that the MB probe was able to detect as little as 0.5 pg of synthetic target DNA. This is equivalent to 11.3 genome copies. The sensitivity level is lower compared to other diagnostic techniques that have been used for different plant pathogens. However, it should be noted that the detection limit of 0.5 pg of DNA obtained in the MB assay is similar to the limit of detection reported for the

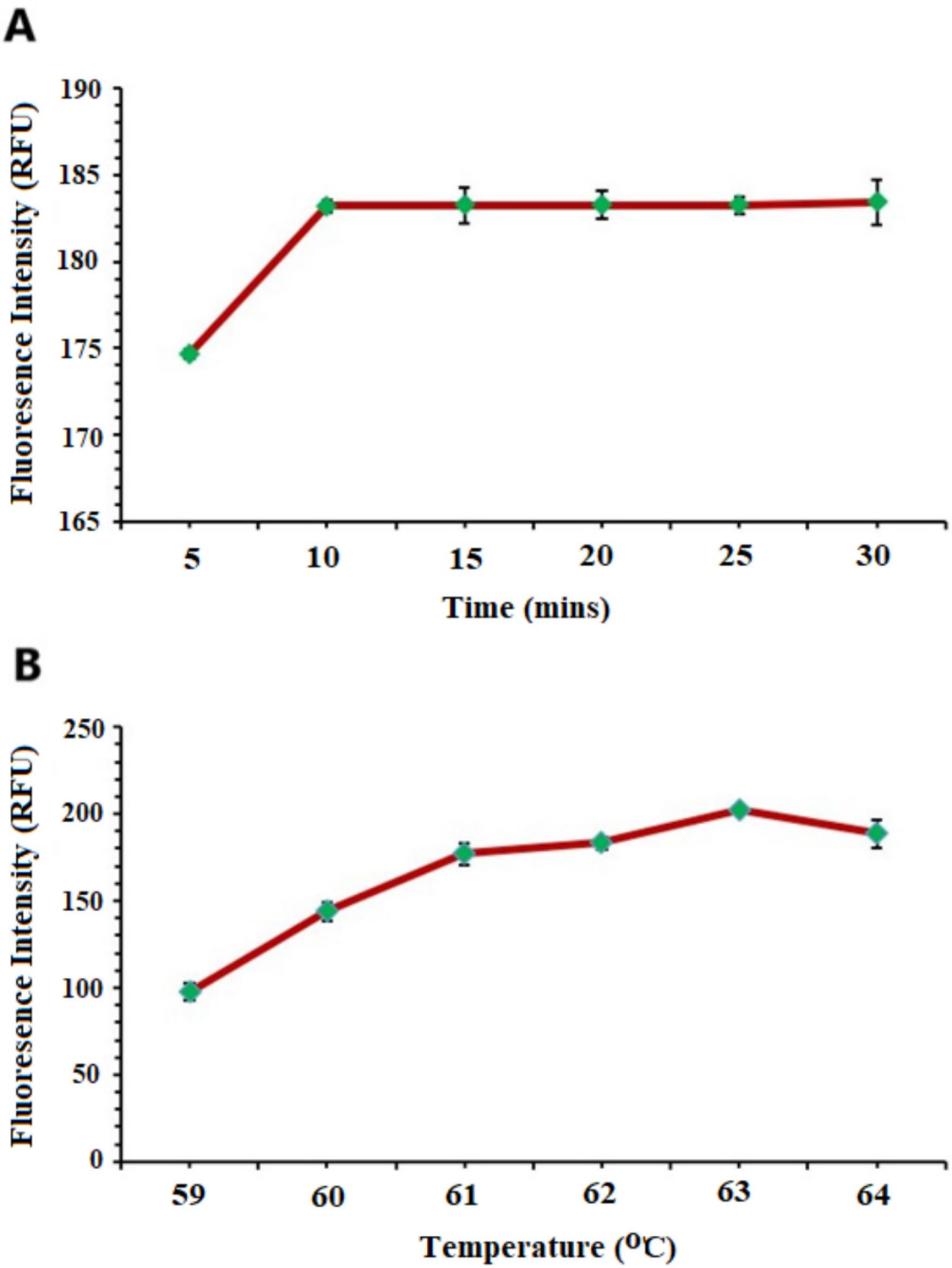

Fig. 4. Optimization of (**A**) incubation time and (**B**) annealing temperature for target-MB hybridization process. Fluorescence intensity was measured in relative fluorescence units (RFU). Values are the mean ± SD of technical triplicates.

qPCR method developed by Baite, et al.[23] for the detection of *A. rabiei*. Importantly, the sensitivity for this method as well as the assay in the present study is sufficient for the early diagnosis of the AB disease before symptoms are visible (Fig. 5). Whilst there is no study conducted using molecular beacon probes without amplification, there are many examples of various amplification-free biosensor assays based on only hybridization between probe and single-stranded target DNA. Moreover, most of those assays have higher sensitivity than MB assays, or any

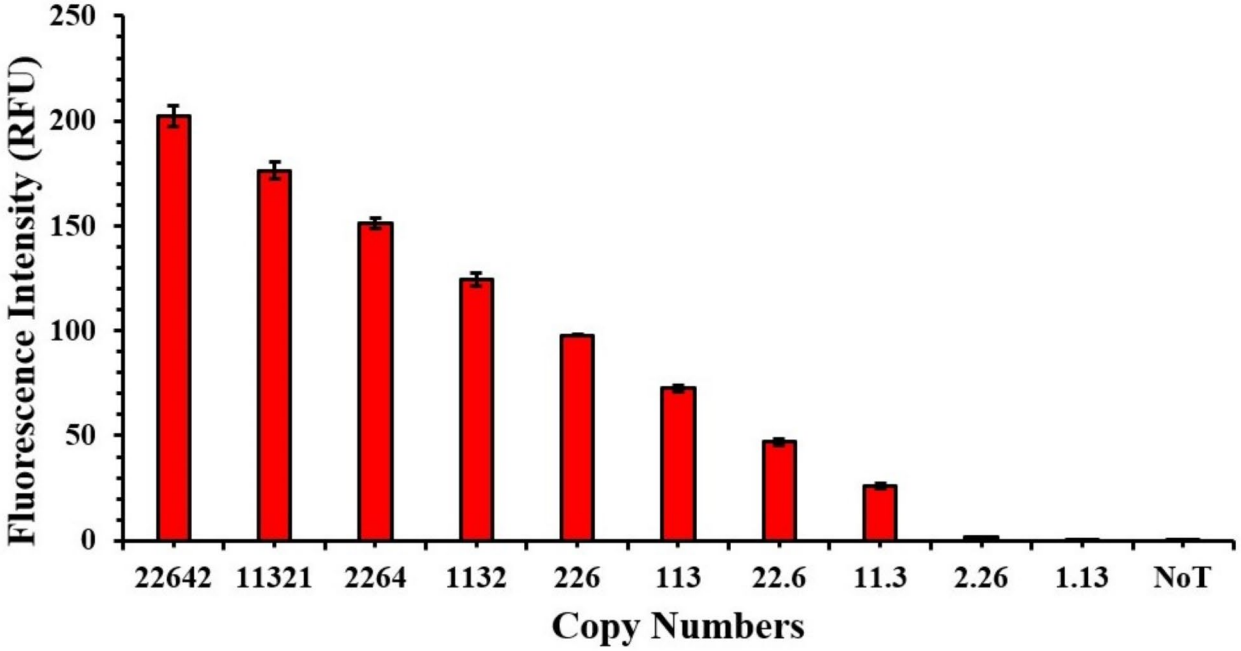

Fig. 5. Assay sensitivity. The limit of detection of the MB assay is 11.3 genome copies. NoT = non-template control. Fluorescence intensity was measured in relative fluorescence units (RFU). Values are presented as the mean ± SD of technical triplicates.

conventional amplification-based assays. A review paper written by Chen et al.[24] provides an overview of highly sensitive nucleic acid-based amplification-free biosensor assays.

Assay validation on field samples

The optimised MB assay was tested on chickpea gDNA extracted from chickpea plants collected from fields of Western Australia to verify its performance using field-derived samples. Twenty-four plant samples that were verified to be infected by the target pathogen *A. rabiei* were collected and used in this study, with five additional chickpea plants grown in a controlled environment acting as uninfected, negative control plants. Amplification of the gDNA was performed using a method currently classed as the standard method for pathogen detection and quantification (qPCR) and compared with the results obtained by MB analysis. Initially, to validate the results, calibration curves were generated for both methods for the detection and quantification of DNA concentrations and genome copy numbers (Fig. 6). The standard curves for *A. rabiei* were generated by plotting the genome copy numbers against the FL intensity values (Fig. 6A) and the Ct values determined by qPCR (Fig. 6B). Both MB assay and qPCR standard curves demonstrated good linearity across the concentration range evaluated, yielding high correlation coefficients of $R^2 = 0.999$ and $R^2 = 0.997$, respectively. Notably, the detection limit of qPCR assays targeting the same glycoside hydrolase gene of *A. rabiei* as the MB assay was 100 fg of pathogenic DNA (2.26 genome copies), which is five times more sensitive than the MB assay. Considering the MB probe was designed from the sequence of ST47_g9244 forward primer, this indicates that amplification-based method can generate a greater dynamic range of detection compared to hybridization alone.

A comparative study was undertaken by calculating genome copies that could be detected using both of the tested methods. Both the MB and qPCR assays generated similar outcomes for all 24 infected samples (Fig. 7). The majority of the naturally infected plant samples were at an early stage of infection, except for T3 L3, T3 L5, T3 L6b, T4 L4 and T4 L6c. These plants had already been significantly impacted by the pathogen and the disease symptoms were clearly visible on their leaves and stems at the time of collection. Neither method detected any signs of the disease on the five plant samples from the growth room, indicating that they were not susceptible to the generation of false positive readings.

The outcomes of this study confirm that the MB-based assay is a reliable, robust and simple detection method for the early identification and quantification of this significant fungal disease of chickpea. Moreover, the use of 96-well plate assay method permits the testing of a large number of field samples within a short period of time. However, the assay is quite expensive due to the cost of the MB probe, and thus further optimisation of the assay is necessary in order to develop a less costly MB. Apart from the probe cost, there are few challenges that are needed to be further addressed for successful in-field application of the MB assay. In current disease management practices, the timing of first fungicide application depends on visual assessment of initial symptoms of *A. rabiei*. However, visual identification of the fungus by first leaf lesions requires strong epidemiology knowledge within the field. Moreover, it is difficult to differentiate different fungal species with similar symptoms that can co-exist in the same field, and they may have different levels of resistance to certain fungicide. As such, a robust on-site diagnostic device could provide a reliable detection of pathogens. For that, an effective sample-collection

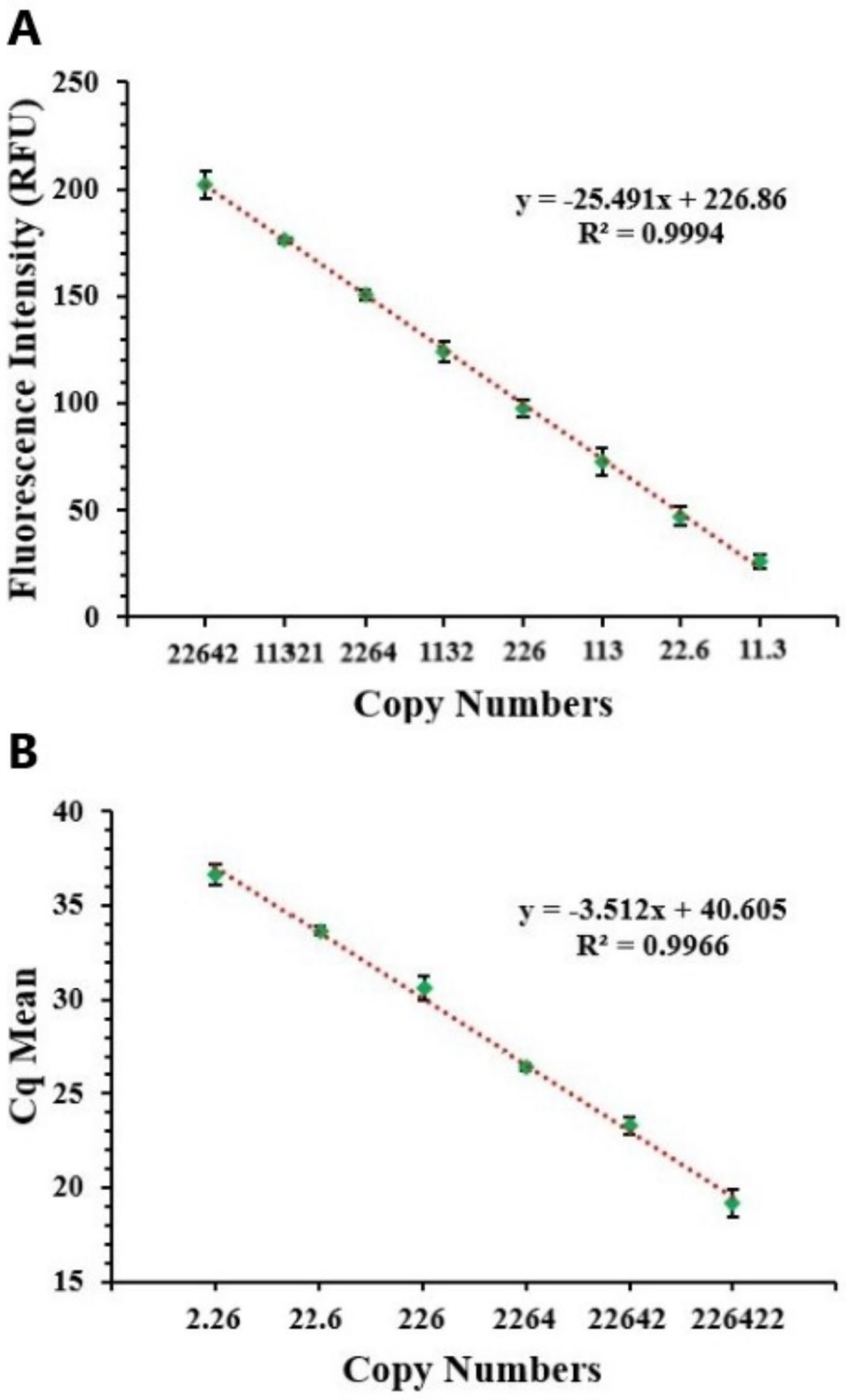

Fig. 6. Standard curves: (**A**) MB assay standard curve generated from fluorescence signals produced by hybridization of eight different quantities of *A. rabiei* gDNA (1 ng – 0.5 pg) with the MB probe; (**B**) qPCR standard curve generated from the amplification of 10-fold quantities of *A. rabiei* gDNA (10 ng – 100 fg) with ST47_g9244 primer set. The detected DNA quantities were calculated to genome copy numbers. Both curves revealed good linear relationships between the copy numbers and FL intensity value (MB assay)/Cq value (qPCR). Values are the mean ± SD of technical triplicates.

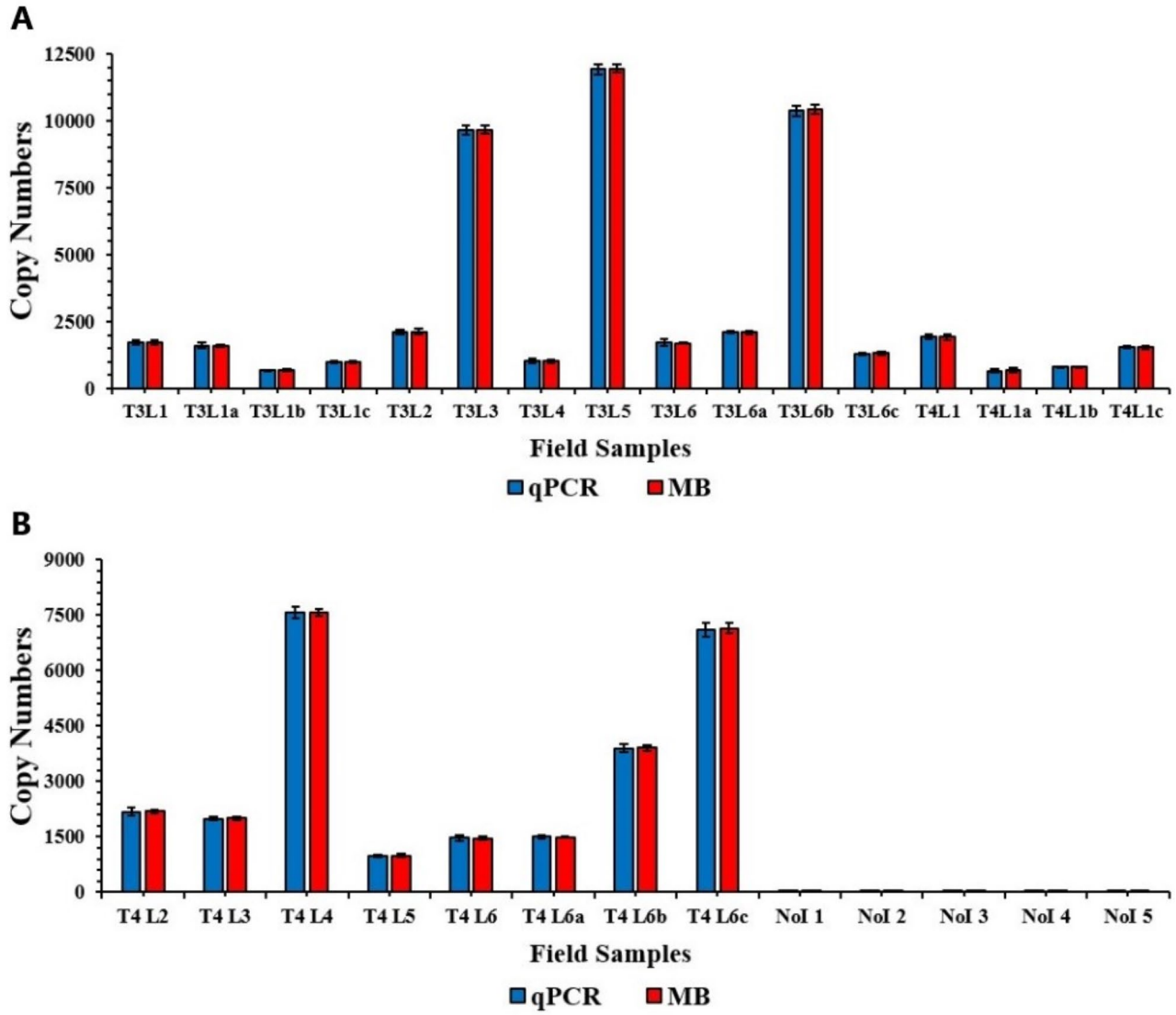

Fig. 7. Assay performance on field plant samples and validation of the results with an established method. For detection of AB disease, twenty-four infected plant samples (PBA Striker cultivar) were collected from chickpea fields within Merredin, Western Australia and five non-infected control samples (NoI 1–5) were grown under controlled environment. The disease detection was performed via the MB assay developed in this study and qPCR and the results were compared. The detected copy numbers using both methods were similar. Plant samples T3 L3, T3 L5, T3 L6b, T4 L4 and T4 L6c had already been significantly impacted by the *A. rabiei* pathogen, whereas the disease was in its early stages of development in other plant samples. The detected copy number values are presented as means ± SD of technical triplicates.

strategy is needed to be designed that could represent an entire field. Indeed, this could be the solution for informed disease management.

This study was an in-principal examination to determine whether MB assays are as useful as other existing lab-based diagnostic assays. Future studies could be undertaken to develop the assay into an in-field assay kit. For that to be achieved, equipment and reagents needed for DNA extraction and fluorescence signal detection should be optimized. As was described in the methods section, an expensive commercial kit was used for gDNA extraction from plant leaves. In order to reduce the cost of the whole assay kit, as well as to keep equipment and chemicals for on-site DNA extraction at minimum, future studies should focus on possibility of performing the MB assay using crude extracts crushed by a hand-held homogeniser from field samples. Although the basic mechanism of developed assay is simple, it still requires some equipment and materials to perform the detection. A mini thermoblock, Tris-buffer and MB probe are needed for both DNA denaturation and hybridization. Most importantly, a portable fluorescence detector could be used for fluorescence measurements, along with easy-to-understand instructions. In addition, a smaller hand-held version of the microplate reader with a temperature control up to 95 °C (for target DNA denaturation) would allow to use only one piece of equipment for the detection performance excluding the mini thermoblock. This would allow the in-field user to purchase the

equipment as a one-off expense, then obtain the consumables as required. Future studies to develop and optimise this method are required before this assay is applicable to in-field testing.

Significant improvements in the disease management of *A. rabiei* would occur through the availability of a quick, accurate and cost-effective diagnosis and quantification of the pathogen. In turn, this would allow reductions in the use of targeted fungicides (and decreased agricultural costs). Conventional diagnostic techniques are time consuming, require specialised technical personnel and access to sophisticated equipment, all of which precludes these conventional assays from in-field use. Although several amplification-based assays have been developed in the past for the detection of *A. rabiei*[25–28], there are problems associated with their specificity and complexity[10]. In addition, most of these methods cannot provide real-time detection, rendering them less suitable for on-field testing and for early warning systems. As such, the development of an advanced diagnostic device that can detect the disease during the early stages of infection and before an epidemic occurs is essential. The MB detection method described in the present study describes the design and use of an improved method for AB detection. Assay optimisation efforts are underway in our laboratory in order to reduce costs and further simplify the assay for use in the field.

Conclusion

The MB assay developed in this study was both sensitive and selective. Despite its lower sensitivity compared to a number of optical and electrochemical biosensor technologies, its relative simplicity and rapid assay time are desirable features for in-field applications. Several aspects require further optimisation to improve its reliability, expediency, and accuracy. The assay's limitation is the high cost of the MB probe which requires further study in order to find a solution to the expense of the probe. Despite this, our MB assay is substantially simpler to use and more expedient than any of the existing diagnostic assays used for the detection and quantification of AB disease of chickpea. Ongoing testing of the MB assay will also focus on its selectivity for a broader panel of other chickpea fungal pathogens that may be present in the field alongside AB. Finally, the sample preparation should be simplified by using hand-held homogenisers for on-site DNA extractions. Together, these endeavours would help facilitate an increase in the use of the MB assay as an in-field diagnostic tool for early detection of AB in chickpea.

Data availability

Any additional data can be obtained from the corresponding author upon reasonable request.

Received: 4 May 2023; Accepted: 26 September 2024
Published online: 11 October 2024

References

1. Deokar, A. et al. QTL sequencing strategy to map genomic regions associated with resistance to ascochyta blight in chickpea. *Plant Biotech. J.* **17**, 275–288. https://doi.org/10.1111/pbi.12964 (2019).
2. Garg, V. et al. Integrated transcriptome, small RNA and degradome sequencing approaches provide insights into Ascochyta blight resistance in chickpea. *Plant Biotech. J.* **17**, 914–931. https://doi.org/10.1111/pbi.13026 (2019).
3. Davidson, J. A. & Kimber, R. B. E. Integrated disease management of ascochyta blight in pulse crops. *Eur. J. Plant Path.* **119**, 99–110. https://doi.org/10.1007/s10658-007-9132-x (2007).
4. Singh, R. et al. Ascochyta rabiei: A threat to global chickpea production. *Mol. Plant Path.* **23**, 1241–1261. https://doi.org/10.1111/mpp.13235 (2022).
5. Donoso, A. & Valenzuela, S. In-field molecular diagnosis of plant pathogens: Recent trends and future perspectives. *Plant Path.* **67**, 1451–1461. https://doi.org/10.1111/ppa.12859 (2018).
6. Bar, I. et al. Current population structure and pathogenicity patterns of Ascochyta rabiei in Australia. *Mic. Gen.* **7**, 000627. https://doi.org/10.1099/mgen.0.000627 (2021).
7. Miller, S. A. et al. Plant disease diagnostic capabilities and networks. *Ann. Rev. Phys.* **47**, 15–38. https://doi.org/10.1146/annurev-phyto-080508-081743 (2009).
8. Martinelli, F. et al. Advanced methods of plant disease detection. A review. *Agron. Sustain. Dev.* **35**, 1–25. https://doi.org/10.1007/s13593-014-0246-1 (2015).
9. Ali, Q. et al. Research advances and applications of biosensing technology for the diagnosis of pathogens in sustainable agriculture. *Env. Sci. Pol. Res.* **28**, 9002–9019. https://doi.org/10.1007/s11356-021-12419-6 (2021).
10. Dyussembayev, K. et al. Biosensor technologies for early detection and quantification of plant pathogens. *Front. Chem.* **9**, 636245. https://doi.org/10.3389/fchem.2021.636245 (2021).
11. Li, Z. et al. Agricultural nanodiagnostics for plant diseases: Recent advances and challenges. *Nan. Adv.* **2**, 3083–3094. https://doi.org/10.1039/C9NA00724E (2020).
12. Chaudhary, V. A paradigm of internet-of-nano-things inspired intelligent plant pathogen-diagnostic biosensors. *ECS Sens. Plus* **1**, 031401. https://doi.org/10.1149/2754-2726/ac92ed (2022).
13. Tyagi, S. & Kramer, F. R. Molecular beacons: Probes that fluoresce upon hybridization. *Nat. Biotech.* **14**, 303–308. https://doi.org/10.1038/nbt0396-303 (1996).
14. Huang, K. & Marti, A. A. Recent trends in molecular beacon design and applications. *Anal. Bio. Chem.* **402**, 3091–3102. https://doi.org/10.1007/s00216-011-5570-6 (2012).
15. Tolba, S. R. *et al.* Real-time assays for detection of Phytophthora spp. and identification of an avr3a gene variant. *J. Plant Dis. Prot.***125**, 331–337. https://doi.org/10.1007/s41348-018-0150-z (2018).
16. Sherrill-Mix, S. et al. Detection of SARS-CoV-2 RNA using RT-LAMP and molecular beacons. *Gen. Biol.* **22**, 1–17. https://doi.org/10.1186/s13059-021-02387-y (2021).
17. Saisuk, W. et al. A molecular beacon biosensor for viral RNA detection based on HyCaSD strategy. *Anal. Chim. Acta* **1221**, 340134. https://doi.org/10.1016/j.aca.2022.340134 (2022).
18. Kerr, E. et al. Amplification-free electrochemiluminescence molecular beacon-based microRNA sensing using a mobile phone for detection. *Sens. Act. B Chem.* **330**, 129261. https://doi.org/10.1016/j.snb.2020.129261 (2021).
19. Verma, S. *et al.* Draft genome sequencing and secretome analysis of fungal phytopathogen Ascochyta rabiei provides insight into the necrotrophic effector repertoire. *Sci. Rep.* **6**, 24638. https://doi.org/10.1038/srep24638 (2016).

20. Cooksey, C. J. Quirks of dye nomenclature. 9. Fluorescein. *Biotech Hist.* **92**, 506–512. https://doi.org/10.1080/10520295.2017.1359751 (2017).
21. Shah, R. M. *et al*. Reference genome assembly for Australian Ascochyta rabiei isolate ArME14. *G3 Gen. Gen. Gen.* **10**, 2131–2140. https://doi.org/10.1534/g3.120.401265 (2020).
22. Dyussembayev, K. *et al*. Electrochemical platform assay for Ascochyta blight detection in chickpea. *Manuscript in preparation.*
23. Baite, M. S. *et al*. Development of a sequence-characterized amplified region marker for detection of Ascochyta rabiei causing Ascochyta blight in chickpea. *Fol. Mic.* **65**, 103–108. https://doi.org/10.1007/s12223-019-00711-5 (2019).
24. Chen, Y. et al. Nucleic acid amplification free biosensors for pathogen detection. *Biosens. Bioelect.* **153**, 112049. https://doi.org/10.1016/j.bios.2020.112049 (2020).
25. Phan, H. *et al*. A rapid and sensitive polymerase chain reaction (PCR) assay for detection of Ascochyta rabiei, the cause of ascochyta blight of chickpea. *Aus. Plant Path.* **31,** 31–39. https://doi.org/10.1071/AP01056 (2002).
26. Bayraktar, H. *et al*. Determination of Ascochyta blight disease in chickpea using real-time PCR. *J. Plant Dis. Prot.* **123**, 109–117. https://doi.org/10.1007/s41348-016-0017-0 (2016).
27. Chen, X. *et al*. Development of a loop-mediated isothermal amplification method for the rapid diagnosis of Ascochyta rabiei L. in chickpeas. *Sci. Rep.* **6**, 25688. https://doi.org/10.1038/srep25688 (2016).
28. Valetti, L. et al. Early detection of Ascochyta blight (Ascochyta rabiei) of chickpea by traditional PCR. *Crop Prot.* **143**, 105463. https://doi.org/10.1016/j.cropro.2020.105463 (2021).

Acknowledgements

The authors acknowledge the Kazakhstan Presidential Bolashak Scholarship Program for providing the PhD scholarship to KD for his research. The authors also acknowledge Prof Rebecca Ford group (Griffith University, Australia) for providing the field chickpea plant samples.

Author contributions

K.D. conducted experiments and wrote the main manuscript text. V.A. was involved in data analysis. T.Y. prepared Figs. 1 and 2 and the graphical abstract. M.J.C., T.H.K. and I.E.C. edited the manuscript. I.E.C. supervised the research and corrected the manuscript. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-74564-5.

Correspondence and requests for materials should be addressed to I.E.C.