


Cite this: *RSC Adv.*, 2023, 13, 1587

# Fluorescent detection of brown spot of tobacco caused by *Alternaria alternata* based on lambda exonuclease-induced DNAzyme amplification

Kai Lian,<sup>†a</sup> Guangyan Chen,<sup>†a</sup> Xiaoqiang Wang,<sup>b</sup> Wenna Zhang,<sup>c</sup> Xihao Hu,<sup>d</sup> Hui Wang,<sup>b</sup> Yusen Li,<sup>a</sup> Dongmei Xi<sup>a</sup> and Ying Wang<sup>id</sup> <sup>\*a</sup>

A rapid, simple, and sensitive fluorescent detection method for brown spot of tobacco is established by lambda exonuclease-induced  $Mg^{2+}$ -dependent DNAzyme amplification. It contains hybridization of the *Alternaria alternata* genome and HP1, digestion of the 5'-phosphorylated strand of the hybrid dsDNA by lambda exonuclease, acquisition of complete  $Mg^{2+}$ -dependent DNAzyme, cleavage of the substrate modified with FAM and BHQ-1, and fluorescent detection. The proposed assay exhibits good sensitivity ( $10\text{ pg L}^{-1}$ ), selectivity and reproducibility. The method does not require pure DNA and expensive instruments, and can be performed within 2.5 hours. To the best of our knowledge, this is the first report of fluorescent detection of *Alternaria alternata* and its tobacco field samples. This method can be applied to the rapid and sensitive detection of *Alternaria alternata* in tobacco and its seedlings, and is particularly important for the green prevention and control of tobacco brown spot disease.

Received 6th September 2022  
Accepted 27th December 2022

DOI: 10.1039/d2ra05616j

rsc.li/rsc-advances

## 1. Introduction

Brown spot is a serious tobacco disease caused by *Alternaria alternata* (*A. alternata*), and occurs mainly at the mature stage of tobacco leaf.<sup>1</sup> Since it was first reported in the United States in 1892, the disease has repeatedly caused huge economic losses to the tobacco industry around the world.<sup>2–4</sup> It mainly harms leaf, stem, pedicel, and capsule, and greatly reduces the economic value of tobacco.<sup>5</sup> Lots of methods including plate culture method, serological method, PCR method, Mass spectrometry,<sup>6</sup> high performance liquid chromatography,<sup>7</sup> and surface-enhanced Raman spectroscopy have been reported for detection of *A. alternata*.<sup>8,9</sup> However, these detection methods are tedious, time-consuming, costly, and require precise, advanced equipment and professional detection personnel, so they are not suitable for on-site detection.<sup>10</sup> At present, immunocolloidal gold dipstick method is commonly used for qualitative detection in field detection, and it is difficult to detect the whole population of a pathogen because of the restriction of the antigen antibody reaction method.<sup>11,12</sup> For example, a colloidal gold test strip can be used to detect brown spot disease caused

by *Alternaria longus* in tobacco, and the detection limit is  $20\text{ ng mL}^{-1}$ , but it is not suitable for detecting brown spot disease caused by other species of *Alternaria alternata*.<sup>13</sup> Therefore, it is very important to establish an economical, accurate and rapid detection method for prevention and control of brown spot of tobacco.

Deoxyribozyme amplifies signals through specific recognition and shear cycling, which is very attractive for efficient and rapid detection of pathogens.<sup>14–16</sup> Deoxyribozymes are single-stranded DNA molecule with catalytic function obtained by *in vitro* screening technology.<sup>17,18</sup> They are folded into specific spatial structure to catalyze specific reactions, such as RNA cutting,<sup>19,20</sup> DNA/RNA linking,<sup>18,21</sup> DNA phosphorylation,<sup>22</sup> etc. Compared with traditional protein enzymes, deoxyribozymes have good chemical stability and are hardly affected by pH and temperature. They have small molecular weights and are easy to be synthesized and modified.<sup>23</sup> Lambda exonuclease is a 5'-3' exonuclease, which catalyze the removal of nucleotides stepwise at the 5'-phosphorylated strands of double-stranded DNA, and shows low activity to non-phosphorylated substrates, such as cleaved DNA, single-stranded DNA and double-stranded DNA. Thus, lambda exonuclease provides an alternative methods for strategies related to exonuclease-based cascade amplification.

In this study, a novel fluorescent method is established for *A. alternata* detection by lambda exonuclease and  $Mg^{2+}$ -dependent DNAzyme. A species-specific sequence of *A. alternata* is hybridized to HP1, and 5'-phosphorylated strand of HP1 in the hybrid strand are degraded stepwise by lambda exonuclease, releasing partial sequence of  $Mg^{2+}$ -dependent DNAzyme. This product is hybridized to P1 bearing another part of the  $Mg^{2+}$ -

<sup>a</sup>College of Life Science, Linyi University, Linyi 276005, China. E-mail: m13854869219@163.com

<sup>b</sup>Plant Protection Research Center, Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao 266101, China

<sup>c</sup>Shandong (Linyi) Institute of Modern Agriculture, Zhejiang University, Linyi 276000, China

<sup>d</sup>Shandong Tobacco Company Qingdao Branch, Qingdao 266101, China

<sup>†</sup> These authors contributed equally.



dependent DNAzyme sequence to assemble the complete  $Mg^{2+}$ -dependent DNAzyme. In the presence of  $Mg^{2+}$ , the enzyme recognizes and cleaves the ribonucleotide of its substrate MB. FAM and BHQ-1 modified at both ends of MB are separated at room temperature, and the fluorescence signal appears.

## 2. Materials and methods

### 2.1. Chemicals

Lambda exonuclease is acquired from Thermo Fisher Scientific Ltd (Shanghai, China). 20 bp DNA ladder is purchased from TaKaRa Bio Ltd (Dalian, China). Rapid DNA Extraction Detection Kit (KG203), InRcute IncRNA First-Strand cDNA Kit (KR202), and RNA Easy Fast Plant Tissue RNA Rapid Extraction Kit (DP452) are acquired from Tiangen Biotech Co., Ltd (Beijing, China). Ultrapure water (electrical resistance above 18.2 MΩ) used in the experiment is acquired from an ULUP-IV system (Shanghai, China). Other reagents used in this experiment are analytically pure grade and have not been further purified.

Internal transcribed spacer (ITS) of the species of *A. alternata* were highly conservative. In order to improve the sensitivity and selectivity of the detection method, ITS sequences of *A. alternata* genome were acquired from NCBI, and aligned by DNAMAN software (version 7, LynnonBiosoft Co., Ltd). The GenBank IDs of these *A. alternata* ITS were MG601454.1, KY550233.1, NR\_136120.1, KC492447.1, MN654955.1, EU520171.1, MT704965.1, OW986459.1, ON796496.1-ON796488.1, ON745551.1, ON740895.1, ON738701.1, ON738588.1. A high conserved segment (Target, Table 1) was found in the ITS region of *A. alternata* genome, which originally used as primer in PCR.<sup>24</sup> So, we designed the fluorescent detection strategy to target this segment of the *Alternaria alternata* genome. Strains of *A. alternata*, *Rastonia solanacearum*, *Phytophthora nicotianae*, and tobacco samples carrying *A. alternata*, *cucumber mosaic virus* (CMV), *tobacco mosaic virus* (TMV), 4 positive tobacco samples and 26 field tobacco samples, were kindly provided from a laboratory of Plant Protection Research Center in Tobacco Research Institute of Chinese Academy of Agricultural Sciences. Oligonucleotides were synthesized by Sangon Biotech (Shanghai) Co., Ltd and listed in Table 1.

### 2.2. Apparatus

Fluorescence was monitored by a F4600 spectrofluorimeter (Hitachi, Tokyo, Japan). Range of emission spectra was from 500 to 650 nm and the excitation and emission wavelengths were 494 nm. The excitation and emission slit widths were set at 5 nm. NanoDrop 2000 (Thermo, New York, USA) was used for

concentration measurements of genomes in the experiment. Isothermal reaction was performed in a heating block (Thermo, New York, USA). Polyacrylamide gel electrophoresis (PAGE) is performed in double board sandwich type vertical electrophoresis apparatus (DYCZ-30C, Liuyi Co., Ltd, Beijing, China). Gel pictures were captured by a WD-9413A imaging system (Liuyi Co., Ltd, Beijing, China).

### 2.3. Genome extraction

Tobacco plants infected with *A. alternata* and healthy tobacco plants were put into grinding bags respectively, and then buffer solution (10 mM Tris-Cl, 1 mM EDTA pH 8.0) with twice the quality of tobacco leaf was added for grinding. The suspensions were heated in boiled water for 5 min, and cooled down in ice-water, and vortexed for 1 min. The above steps were repeated one more time. The genomic DNA was released from the cell wall of *A. alternata*. The result suspension was centrifuged, and the genome of *A. alternata* in the supernatant was used as the target, and its concentration was quantified by this method. Rapid DNA Extraction Detection Kit (KG203) was used to extract and purify genomic DNA of *Alternaria alternata*, *Phytophthora nicotianae*, and *Ralstonia solanacearum* from their liquid medium. These pure genomes were used for sensitivity, selectivity, and reproducibility tests in this method. Total RNAs of tobacco infected by TMV or CMV were purified by RNA Easy Fast Plant Tissue RNA Rapid Extraction Kit (DP452). cDNAs of TMV and CMV were obtained by InRcute IncRNA First-Strand cDNA Kit (KR202). Reverse transcription of TMV and CMV were done with 5× reverse transcription supermix (2 μL), primer (10 μM, 2 μL), and total plant RNA (6 μL). The reaction was performed at 42 °C for 30 min, followed by suppression of reverse transcriptase activity at 85 °C for 5 min.

### 2.4. Procedures for *A. alternata* detection

Genomic DNA of *A. alternata* was hybridized to HP1 in 1× reaction buffer of lambda exonuclease. In the presence of lambda exonuclease, HP1/*A. alternata* genome hybrid was degraded stepwise from the 5-phosphorylated end of HP1 until it becomes a single-stranded DNA. It was reacted in 50 μL 1× reaction buffer (67 mM Glycine-KOH 2.5 mM  $MgCl_2$  0.01% (v/v) Triton X-100 (pH 9.4 @ 25 °C)). Hybrid product and lambda exonuclease (1U) were added into the reaction solution. It was performed at 37 °C, and then incubated at 75 °C for 10 min to inactivation of lambda exonuclease. P1 was then introduced in the solutions, and incubated for a period time at room temperature. At last, 30 μL of 1 μM MB, 10 μL of 20 mM  $Mg^{2+}$

Table 1 Sequence of oligonucleotides designed in the present study

Name	Base sequence (5'-3')
Target	TGCAATCAGCGTCAGTAACAAAT
HP1	pATTTGTTACTGACGCTGATTGCACAGACCTCCACCCTGCTCAGCGATGGTGGAGGTCTG
P1	CAGACCTCTTTTCACCCATGTTAGACCT
MB	FAM-TGACGTAAGGTCTATAGGAGCAGTACGTCA-BHQ-1
A-F	AGGCCGGCTG CCAATTAC



and 160  $\mu\text{L}$  of buffer solution (25 mM HEPES, 100 mM NaCl, pH 7.0) were introduced into the solution. The solution was incubated at room temperature for 40 min, and the fluorescence intensity was then measured by the F4600 spectrofluorimeter. To improve hybridization efficiency, samples to be hybridized were mixed and incubated at 95  $^{\circ}\text{C}$  for 1 min to eliminate their secondary structures, followed by incubation at room temperature for a period of time for subsequent reactions. DNA that need to be hybridized went through this procedure in the experiment.

The results of the hybridization and digestion were analyzed by PAGE(8%). It was carried out in  $1\times$  TBE (50 mM Tris-boric acid, 1 mM EDTA). Each lane was loaded with 5  $\mu\text{L}$  of sample, and electrophoresed at 120 V for 120 min. The PAGE gel is colored by silver stain. Gel picture was scanned by the WD-9413A imaging system.

### 2.5. PCR method

PCR systems included Taq PCR Master Mix (25  $\mu\text{L}$ ), Target (10  $\mu\text{M}$ , 2  $\mu\text{L}$ ), A-F (10  $\mu\text{M}$ , 2  $\mu\text{L}$ ), DNA template (2  $\mu\text{L}$ ), and  $\text{H}_2\text{O}$  (19  $\mu\text{L}$ ). PCR condition was divided into three steps, the first step was a one minute incubation at 94  $^{\circ}\text{C}$ , and the second step was 35 thermal cycles, including denaturation at 94  $^{\circ}\text{C}$  for 30 s, annealing at 55  $^{\circ}\text{C}$  for 30 s, and extension at 72  $^{\circ}\text{C}$  for 30 s. The last step was an extension at 72  $^{\circ}\text{C}$  for 5 min. PCR products were then analyzed by 1% agarose gel electrophoresis.

## 3. Results and discussion

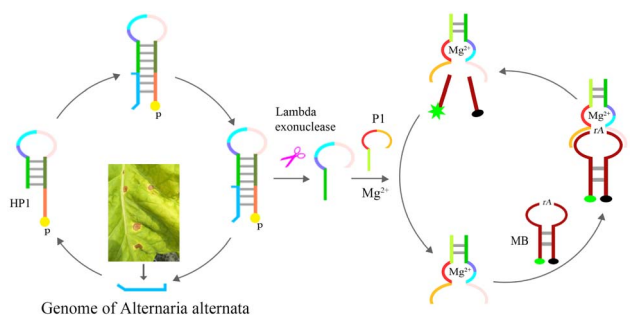
### 3.1. Principle of the assay

The method is shown in Scheme 1, and it involves three steps, (1) hybridization and exonuclease reaction, (2) formation of complete  $\text{Mg}^{2+}$ -dependent DNAzyme, (3) cleavage of molecular beaconing (MB) and fluorescent detection. HP1 is designed with three regions, (a) the *trans*-complementary sequence of part ITS

gene of *A. alternata*, position 1st–23rd of 5'-terminal of HP1 (orange in Scheme 1), (b) the stem part of HP1, position 24th–35th (grass green) and 48th–59th (light green), (c) part sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme, position 36th–47th of HP1 (light pink, sky blue and purple). The 5'-terminal hydroxyl group of HP 1 is attached to a phosphate group. P1 contains a *trans*-complementary sequence (1st–8th, fluorescent green) of HP1 (52nd–59th), a short interval sequence (9th–12th, fluorescent green), and another part sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme (13th–28th, Indian red and yellow). The ribonucleotide-containing molecular beacon (MB) is a fluorescent-labeled oligonucleotide consisting of a loop region, a stem region, a FAM and a BHQ-1 group, which is the substrate of  $\text{Mg}^{2+}$ -dependent DNAzyme. First, genome of *A. alternata* (deep sea blue) is extracted in tobacco leaves with brown spots, and hybridized with nucleotides of 1st–23rd of 5'-terminal of HP1. Lambda exonuclease can efficiently degrade the 5'-phosphorylated strand of dsDNA hybrid until it becomes a single DNA strand, that is, 1–35 nucleotides of hybrid HP1 is removed at 37  $^{\circ}\text{C}$ . Partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme (36th–59th of HP1) is released from the stem-loop structure of HP1. Genome of *A. alternata* goes free, and starts a new round of hybridization with HP1, and cleavage process in the presence of lambda exonuclease. Second, P1 is then introduced to the solution, and hybridized with the partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme to obtain the complete  $\text{Mg}^{2+}$ -dependent DNAzyme, which is hybridized and nicked MB at rA site in the presence of  $\text{Mg}^{2+}$ . As a result of this scission of the substrate, the distance between FAM and BHQ is enough to generate great fluorescence intensity, thus providing the optical readout signal for sensing of *A. alternata*. The  $\text{Mg}^{2+}$ -dependent DNAzyme continuously proceeds hybridization-cleavage step of MBs, and the fluorescence signal increases significantly. However, if there is no genome of *A. alternata*, 5'-protrude terminal of HP1 cannot be digested by lambda exonuclease, partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme is still caged in the duplex structure of HP1. No intact  $\text{Mg}^{2+}$ -dependent DNAzyme is assembled, and the ribonucleotide-containing MB is not be cleaved, and fluorescence can almost not be monitored.

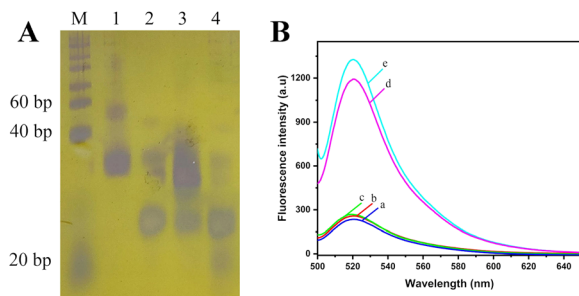
### 3.2. Feasibility of fluorescent detection of *A. alternata*

The genome of *A. alternata* contains millions of nucleotides, and the hybridization between genome of *A. alternata* and HP1 cannot be determined by PAGE. In order to better reflect the effect of hybridization and enzyme digestion, genomic DNA of *A. alternata* was replaced by Target sequence. Target is a 23 nucleotide DNA sequence, which is a high conserved sequence found in ITS region of *A. Alternata* genome (Table 1). 20 bp DNA lander consists of 13 double-stranded DNA fragments from 20 bp–200 bp, and is used as a measure of molecular weight of double-stranded DNA in PAGE. Since HP1, Target, and HP1/Target hybrid are short single-stranded DNA or partial double-stranded DNA in the assay, the lander could only be used as a reference, and not an accurately indication of the size of these DNAs. The effect of hybridization and enzyme digestion in this assay was verified by 8% PAGE (Fig. 1A). HP1 was in lane 1, and



**Scheme 1** Schematic illustration of lambda exonuclease-induced DNAzyme amplification for fluorescent detection of *A. alternata*. Genome of *A. alternata* is extracted in tobacco leaves with brown spots, and hybridized with 5'-terminal of HP1, which is degraded by lambda exonuclease. Partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme is released from HP1, and then hybridized with P1 to obtain the complete  $\text{Mg}^{2+}$ -dependent DNAzyme, which continuously cleaves ribonucleotide-containing molecular beacon (MB) substrate in the presence of  $\text{Mg}^{2+}$ . FAM moves far away from BHQ, greater fluorescence intensity was detected.





**Fig. 1** (A) Analysis of HP1/Target hybridization and lambda exonuclease digestion by 8% PAGE. M, 20 bp DNA ladder, lane 1, HP1, lane 2, Target, lane 3, HP1 + Target, lane 4, HP1 + Target + lambda exonuclease. (B) Fluorescence emission spectra of solution containing only MB (a), genome DNA of healthy tobacco plant (b), and in the absence of lambda exonuclease (c), total DNA of tobacco plant infected by *A. alternata* (d), and genome DNA of *A. alternata* (e). The concentrations of HP1, P1, MB, and lambda exonuclease are 1.0  $\mu\text{M}$ , 1.2  $\mu\text{M}$ , 1  $\mu\text{M}$ , and 1 U, respectively.

Target was in lane 2. The 5'-terminal of HP1 and Target were hybridized and formed HP1/Target hybrid (lane 3). Lambda exonuclease catalyzed the degradation of the double-stranded portion of the HP1/Target hybrid. HP1 was degraded from the 5'-phosphorylated end until it becomes a single strand, that is, the 1st to 35th nucleotides of HP1 were successively degraded to single nucleotides. The product was a DNA sequence consisting of 24 nucleotides (positions 36–59 of HP1), bearing a partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme. Target (23 nucleotides) was released from HP1/Target hybrid. Since the nucleotide number of the partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme and Target is similar, their electrophoretic bands were overlapped (lane 4). The results show HP1 could hybridize to Target, and 1–35 nt sequence of HP1 was indeed digested by lambda exonuclease.

In order to verify the feasibility of this method for quantitative detection of *A. alternata*, a series of samples were detected by this method, and the fluorescence emission spectra of each solution were obtained. As shown in Fig. 1B, no obvious fluorescence signal was detected in the solution containing only MB (curve a). The distance between FAM and BHQ was close, and the fluorescence was quenched. Similar fluorescence signals were detected in the solutions with genomic DNA of healthy tobacco plants (curve b) or reaction system without lambda exonuclease (curve c). In the presence of total DNA of tobacco plant infected by *A. alternata* (curve d) or genome of *A. alternata* (curve e), significant fluorescence signals near 520 nm were detected in the solutions. The results indicated that the proposed method could effectively monitor *A. alternata*, and was used for the detection of *A. alternata* in tobacco plants.

### 3.3. Optimizing the conditions for maximizing the fluorescence intensity

In this experiment, the fluorescence intensity depends on the reaction conditions of lambda exonuclease-induced DNAzyme amplification, such as digestion time by lambda exonuclease,

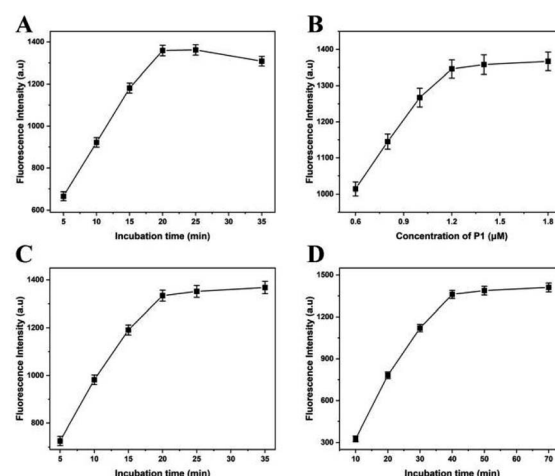
concentration of P1, hybrid time of *A. alternata* genome and HP1, and the part sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme and P1. In order to obtain the best performance for quantitative detection of *A. alternata*, these experiment conditions were optimized respectively.

Lambda exonuclease catalyzes the progressive removal of nucleotides from the 5'-terminal of double-stranded DNA. The optimal substrate for this enzyme is 5'-phosphorylated double-stranded DNA, while the degradation rate of non-phosphorylated substrates is relatively low. To reduce the cost and reaction time, digestion time of lambda exonuclease was optimized, and the concentration of *A. alternata* genome was 0.1  $\text{ng L}^{-1}$ . As shown in Fig. 2A, the fluorescence intensity increased with the reaction time from 5 to 20 min and dropped slightly beyond 20 min, indicating the completion of digestion reaction. Excessively long reaction time may lead to excessive digestion of HP1 in hybrid of *A. alternata* genome/HP1, thereby reducing the fluorescence intensity in the solution.<sup>25</sup> Therefore, reaction time of 20 min was the best condition, and applied to the following experiment.

The concentration of P1 was increased from 0.6 to 1.8  $\mu\text{M}$ , and the fluorescence intensity increased correspondingly. When the concentration of P1 was lower than 1.2  $\mu\text{M}$ , the fluorescence intensity increased rapidly with the increase of P1 concentration.

When the concentration of P1 was greater than 1.2  $\mu\text{M}$ , the fluorescence intensity increased slowly with the increase of P1 concentration (Fig. 2B). Therefore, P1 concentration of 1.2  $\mu\text{M}$  was the optimal concentration, and used for the following experiments.

In order to improve the hybridization efficiency and shorten the hybridization time, genome of *A. alternata* and HP1 were mixed and subjected to 95  $^{\circ}\text{C}$  for 1 min to remove secondary structures, then slowly reduced to room temperature. The



**Fig. 2** Optimization of digestion time of lambda exonuclease (A), concentration of P1 (B), hybridization time of HP1 and genome of *A. alternata* (C), and formation time of intact  $\text{Mg}^{2+}$ -dependent DNAzyme (D). Concentration of *A. alternata* genome was 0.1  $\text{ng L}^{-1}$  in the above experiments. Error bars in the figures represent the standard deviations of three experiments, and the same below.





mixture was incubated at room temperature for 5–35 min. The results showed that the fluorescence intensity reached a plateau when the incubation time was more than 20 min (Fig. 2C). Therefore, 20 min of incubation time was optimal and used for the following experiments.

The *A. alternata* genome/HP1 hybrid was digested with lambda exonuclease to obtain the partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme, which was then mixed with P1 containing another partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme. After the similar heating, cooling and incubation processes, the complete  $\text{Mg}^{2+}$ -dependent DNAzyme was acquired. As shown in Fig. 2D, fluorescence intensity increased along with the incubation time from 10 to 70 min. When the reaction time was 10 min, fluorescence signals could hardly be distinguished from the background. However, the fluorescence intensity was hardly no longer elevated once the reaction time was above 40 min. Therefore, the optimal time to form complete  $\text{Mg}^{2+}$ -dependent DNAzyme was 40 min, which was used for the subsequent experiments.

### 3.4. Analytical performance of the fluorescence detection method

In order to evaluate the sensitivity of the assay, the fluorescence intensity of the solution was detected by this method when the genome concentration of *A. alternata* was in a range of 0–0.2  $\text{ng L}^{-1}$ . Greater amounts of *A. alternata* genome were used in

the assay, and more *A. alternata* genome/HP1 hybrids were acquired. Lambda exonuclease digested the genome/HP1 hybrids, and greater amounts of partial sequence of  $\text{Mg}^{2+}$ -dependent DNAzyme were obtained. The products were hybridized to more P1s, assembling more complete  $\text{Mg}^{2+}$ -dependent DNAzyme. More MBs were nicked by  $\text{Mg}^{2+}$ -dependent DNAzyme in the presence of  $\text{Mg}^{2+}$ , causing a significant increase of fluorescence intensity. As shown in Fig. 3A, a dramatic increase of fluorescence intensity was acquired as the increase of *A. alternata* genome concentration, and the increase rate of fluorescence intensity decreased as the concentration of the genome was more than 0.1  $\text{ng L}^{-1}$ . The fluorescence intensity was linearly correlated with the logarithm of *A. alternata* genome in the concentration range of 10  $\text{pg L}^{-1}$  to 0.1  $\text{ng L}^{-1}$ . The linear regression equation was  $F = -638.7 + 1040 \lg(c)$ , and the correction coefficient was 0.992 (Fig. 3B).  $F$  and  $c$  represent the fluorescence intensity and concentration of the genome of *A. alternata*, respectively. Based on the sum of blank response and three times standard deviation, the detection limit of the method was calculated to be 10  $\text{pg L}^{-1}$ . The results show that this strategy has considerable sensitivity for detecting *A. alternata*. Moreover, the sensitivity of the assay was also compared with that of other methods for *A. alternata* detection (Table 2).

The reproducibility of the assay was detected with 0.1  $\text{ng L}^{-1}$  *A. alternata* genome. The intra-assay reproducibility was performed for three times, and the relative standard deviation value of three times was 2.2%. The inter-assay reproducibility test was performed for 3 consecutive days using the same amount DNA of *A. alternata* every time, and the relative standard deviation value was 3.8%. The results showed that the strategy had satisfied reproducibility.

### 3.5. Specificity of the method

*Rastonia solanacearum*, *Phytophthora nicotianae*, CMV and TMV are common pathogens in tobacco. Since co-infection of these pathogens with *A. alternata* is very common in tobacco fields, they are selected as the control for the specific test of this strategy. Since tobacco and pathogens contain a large amount of DNA and RNA, the specificity requirement of the method for detecting *A. alternata* is very high. The results show that the fluorescence intensities of the solutions bearing genomes of TMV (1), CMV (2), *Rastonia solanacearum* (3),

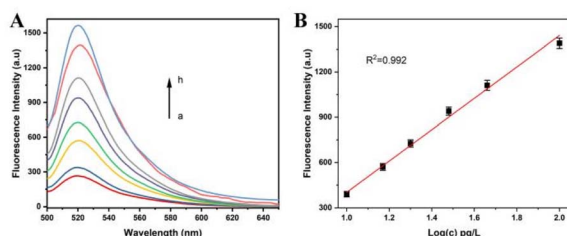


Fig. 3 (A) Fluorescence intensity of the solutions at different genome concentrations of *A. alternata*. Arrow from a to h represents the genome concentrations of 0  $\text{pg L}^{-1}$ , 10  $\text{pg L}^{-1}$ , 15  $\text{pg L}^{-1}$ , 20  $\text{pg L}^{-1}$ , 30  $\text{pg L}^{-1}$ , 50  $\text{pg L}^{-1}$ , 0.1  $\text{ng L}^{-1}$ , and 0.2  $\text{ng L}^{-1}$ . (B) Linear correlation between fluorescence intensity and logarithm of *A. alternata* genome concentration from 10  $\text{pg L}^{-1}$  to 0.1  $\text{ng L}^{-1}$ . Pg means picogram, and ng means nanogram.

Table 2 Comparison of detection limit of different method for *A. alternata* detection

Analytical method	Detection limit	Reference
Surface-enhanced Raman spectroscopy based silver nanodots array method	$1.0 \times 10^3 \text{ cfu mL}^{-1}$	8
Colloidal gold test strips based method	20 $\text{ng mL}^{-1}$	26
Real-time fluorescence PCR based method	1.9 $\text{pg } \mu\text{L}^{-1}$	27
Loop-mediated isothermal amplification based method	3 $\text{pg } \mu\text{L}^{-1}$	28
Chemiluminescence enzyme immunoassay based method	0.068 $\text{ng mL}^{-1}$	29
PCR based method	$1.0 \times 10^2 \text{ cfu mL}^{-1}$	30
Time-resolved fluoroimmunoassay based method	0.78 $\text{ng mL}^{-1}$	31
This method	10 $\text{pg L}^{-1}$	



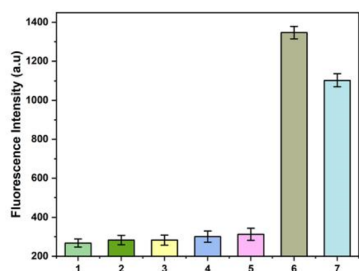


Fig. 4 Selectivity of the proposed method. Total genome of tobacco plant infected by TMV (1), CMV (2), *A. alternata* (7). Genome of *Rastonia solanacearum* (3), genome of *Phytophthora nicotianae* (4), genome of healthy tobacco plant (5), and genome of *A. alternata* (6). Concentration of *A. alternata* genome was  $0.1 \text{ ng L}^{-1}$  in the experiments.

Table 3 Real samples detection

Sample	PCR		Fluorescent	
	Positive	Negative	Positive	Negative
4	4	0	4	0
26	11	15	11	15

*Phytophthora nicotianae* (4) were almost the same as that of the healthy tobacco plant (5). The fluorescence intensity were very low. However, the fluorescence intensity was high in the solution containing *A. alternata* genome (6) or total DNA of tobacco infected by *A. alternata* (7). The results show that the method had good selectivity in monitoring of *A. alternata* (Fig. 4).

### 3.6. Application of this method in tobacco field

PCR is the most common method for detecting target DNA. To verify the application of this strategy in field samples, 4 positive samples and 26 field samples were tested by this method and PCR. The detection results of both methods show that all positive samples and 11 field samples were infected by *A. alternata*. The results of the two methods were completely consistent, indicating that the method could be used for the detection of *A. alternata* in tobacco field (Table 3).

## 4. Conclusions

In this study, a lambda exonuclease-induced DNzyme signal amplification strategy for fluorescent detection of *A. alternata* is established. The proposed assay exhibits good sensitivity ( $10 \text{ pg L}^{-1}$ ), selectivity and reproducibility. To the best of our knowledge, this is the first report of fluorimetric detection of *A. alternata* and its tobacco field samples. The method does not require pure DNA and expensive instruments, and can be performed within 2.5 hours. This method can be applied to the rapid and sensitive detection of *A. alternata* in tobacco and its seedlings, and is particularly important for the green prevention and control of tobacco brown spot disease.

## Author contributions

Kai Lian and Guangyan Chen, data curation, writing original draft. Wenna Zhang and Hui Wang, formal analysis, methodology, Yusen Li, conceptualization and methodology, Xiaoqiang Wang and Xihao Hu, writing – review & editing. Dongmei Xi, funding acquisition and writing – review & editing. Ying Wang: funding acquisition, supervision, validation, writing, review and editing.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

This work was financially supported by ShanDong Students' Platform for innovation and entrepreneurship training program (S202210452017), Key Research and development project of Linyi City (2021030), Commissioned research projects of Linyi University (HX210035, HX210046), the Open Project Foundation of Shandong (Linyi) Institute of Modern Agriculture, Zhejiang University (ZDNY-2021-FWLY02012). Major science and technology projects of China Tobacco Corporation (110202101054(LS-14), 2022370200270186).

## References

- 1 J. Staveland and L. Slana, *Phytopathology*, 1971, **61**, 73–78.
- 2 J. LaMondia, *Plant Dis.*, 2001, **85**, 230.
- 3 N. Tadakazu, U. Tamio and F. Hiroshi, *Tetrahedron Lett.*, 1982, **23**, 4469–4472.
- 4 A. Tanaka, H. Shiotani, M. Yamamoto and T. Tsuge, *Mol. Plant-Microbe Interact.*, 1999, **12**, 691–702.
- 5 Y. J. Jia, D. D. Cheng, W. B. Wang, H. Y. Gao, A. X. Liu, X. M. Li and Q. W. Meng, *Physiol. Plant.*, 2010, **138**, 164–175.
- 6 R. Deshidi, S. Devari, M. Kushwaha, A. P. Gupta, R. Sharma, R. Chib, I. A. Khan, S. Jaglan and B. A. Shah, *ChemistrySelect*, 2017, **2**, 362–368.
- 7 Z. L. Xiao, Y. L. Wang, Y. D. Shen, Z. L. Xu, J. X. Dong, H. Wang, C. Situ, F. Wang, J. Y. Yang and H. T. Lei, *Food Anal. Methods*, 2018, **11**, 635–645.
- 8 T. Pan, D. Sun, H. Pu, Q. Wei, W. Xiao and Q. Wang, *J. Food Eng.*, 2017, **215**, 147–155.
- 9 A. Crespo Sempere, N. Estiarte, S. Marín, V. Sanchis and A. J. Ramos, *Int. J. Food Microbiol.*, 2013, **165**, 214–220.
- 10 O. Laczka, E. Baldrich, F. X. Munoz and F. D. Campo, *Anal. Chem.*, 2008, **80**, 7239–7247.
- 11 S. Nara, V. Tripathi, H. Singh and T. G. Shrivastav, *Anal. Chim. Acta*, 2010, **682**, 66–71.
- 12 Y. D. Wu, M. J. Xu, Q. Q. Wang, C. X. Zhou, M. Wang, X. Q. Zhu and D. H. Zhou, *Vet. Parasitol.*, 2017, **243**, 199–203.
- 13 L. H. Yan Ji, X. Cai, C. Xu, Z. Fan, M. Shi, G. Tang, H. Ding and D. Yan, *Tob. Sci. Technol.*, 2022, **55**, 10–15.
- 14 Y. Wang, J. Liu and H. Zhou, *Sensors*, 2019, **19**, 1298.
- 15 Y. Wang, B. Li, J. Liu and H. Zhou, *Anal. Bioanal. Chem.*, 2019, **411**, 2915–2924.



- 16 J. Riu and B. Giussani, *TrAC, Trends Anal. Chem.*, 2020, **126**, 115863.
- 17 S. C. B. Gopinath, *Anal. Bioanal. Chem.*, 2007, **387**, 171–182.
- 18 R. R. Breaker and G. F. Joyce, *Chem. Biol.*, 1994, **1**, 223–229.
- 19 A. Sreedhara, Y. Li and R. R. Breaker, *J. Am. Chem. Soc.*, 2004, **126**, 3454–3460.
- 20 D. R. Semlow and S. K. Silverman, *J. Mol. Evol.*, 2005, **61**, 207–215.
- 21 K. Schlosser, J. Gu, L. Sule and Y. Li, *Nucleic Acids Res.*, 2008, **36**, 1472–1481.
- 22 A. J. Camden, S. M. Walsh, S. H. Suk and S. K. Silverman, *Biochemistry*, 2016, **55**, 2671–2676.
- 23 K. S. Scott, *Org. Biomol. Chem.*, 2004, **19**, 2701–2706.
- 24 X. Zhongyu, W. Xiaoqiang, S. Hong, S. Guangjun, L. Bin, C. Zhiyan, Z. Yongfeng, R. Mao, C. Haitao and Z. Shuai, *Tob. Sci. Technol.*, 2021, **54**, 11–16.
- 25 L. Liu, J. Lei, F. Gao and H. Ju, *Talanta*, 2013, **115**, 819–822.
- 26 J. Yuan, H. liwei, C. xiangjie, X. chengyue, F. ziyang, S. mowen, T. gangling, D. huimin and Y. ding, *Tob. Sci. Technol.*, 2022, **55**, 10–15.
- 27 F. J. Wang, X. L. Zhang, M. A. Hai-Bo, X. Z. Feng, J. L. Feng and P. Hua, *Food Sci.*, 2013, **34**, 170–173.
- 28 Z. Xinyue, X. Guojie, T. Huaqi, L. Yanpeng and L. Chunsheng, *J. AOAC Int.*, 2017, 99–103.
- 29 C. Y. Yao, Z. L. Xu, H. Wang, F. Zhu, L. Luo, J. Y. Yang, Y. M. Sun, H. T. Lei, Y. X. Tian and Y. D. Shen, *Food Chem.*, 2019, **283**, 359–366.
- 30 M. Pavon, I. Gonzalez, N. Pegels, R. Martín and T. García, *Food Control*, 2010, **21**, 1745–1756.
- 31 Y. Ji, L. Hu, W. Xiong, Y. Wang, F. Yang, M. Shi, H. Zhang, J. Shao, C. Lu, D. Fang, H. Deng, Z. Bian, G. Tang, S. Liu, Z. Fan and S. Liu, *J. Appl. Microbiol.*, 2022, **132**, 1250–1259.

