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ORIGINAL PAPER

Establishment and Application of a Multiplex PCR Assay for Detection of *Sclerotium rolfsii*, *Lasiodiplodia theobromae*, and *Fusarium oxysporum* in Peanut

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Abstract

Southern blight, stem rot, and root rot are serious soil-borne fungal diseases of peanut, which are caused by *Sclerotium rolfsii*, *Lasiodiplodia theobromae*, and *Fusarium oxysporum*, respectively. These diseases are difficult to be diagnosed in early stage of infection, causing the optimal treatment period was often missed. Therefore, establishing a rapid detection system is of great significance for early prevention of peanut soil-borne fungal diseases. Here, we have invented a multiplex PCR detection system to detect fungal pathogens of peanut southern blight, stem rot, and root rot at the same time. The quarantine fungal pathogen primer pairs were amplified to the specific number of base pairs in each of the following fungal pathogens: 1005-bp (*F. oxysporum*), 238-bp (*L. theobromae*), and 638-bp (*S. rolfsii*). The detection limit for the single and multiplex PCR primer sets was 1 ng of template DNA under in vitro conditions. Amplification of fungi of non-target species yielded no non-specific products. The validation showed that the multiplex PCR could effectively detect single and mixed infections in field samples. Overview, this study proved that this mPCR assay was a rapid, reliable, and simple tool for the simultaneous detection of three important peanut soil-borne diseases, which facilitated prompt treatment and prevention of peanut root diseases.

Keywords *Sclerotium rolfsii* · *Lasiodiplodia theobromae* · *Fusarium oxysporum* · Multiplex PCR · Detection

Introduction

Peanut is a vital oil crop and cash crop in the world [1], and China is the largest peanut producer [2]. In China, Shandong Province ranked second in peanut planting area in 2019, with a planting area of 650,000 hectares. Peanut soil-borne fungal diseases are one of the disastrous factors limiting peanut yield, which seriously threaten the quality of peanut and the safety of agricultural products [3]. The main causes of the serious soil-borne disease of peanut were the peanut continuous cropping barrier and the lack of disease resistant varieties [4].

At present, southern blight, stem rot, and root rot are three typical soil-borne fungal diseases of peanut [5], which have caused huge economic losses to the development of peanut-related industries. Peanut southern blight, caused by *Sclerotium rolfsii*, is a critical fungal disease in peanut worldwide [6–8]. Peanut stem rot is a vital soil-borne disease [9], which is caused by *Lasiodiplodia theobromae* that is a necrotic phytopathogen with a wide host range and widespread distribution [10]. Additionally, peanut root rot is caused by *Fusarium oxysporum*. The incidence rate of peanut root rot has greatly increased and already become a devastating disease [11–13]. These three diseases mainly damage the root and base of the stem, which were often difficult to diagnose in the early stage, so the best prevention period was easily missed. Therefore, establishment of rapid detection system of these three diseases is significant for protection of peanut cultivation.

Although individual PCR assay method that detects only single pathogen at a time is effective, PCR technology

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is time-consuming and expensive when applied to a large number of pathogen species leading to a variety of diseases [14, 15]. However, the emergence of multiplex PCR technology provides an effective method, which adds multiple pairs of primers in a reaction system at the same time and amplifies a large number of target fragments while using multiple DNA templates [16]. In recent years, based on the advantage of faster speed, higher sensitivity, and stronger specificity, multiplex PCR has been widely used [17–20].

In addition, fungal parasitism on seeds is an important factor in the spread of plant diseases. Thus, it is very important to establish a system to quickly detect and distinguish these three diseases. However, a multiplex PCR rapid detection system for detecting the main rhizome mycoses of peanuts has not been established. The contributions of this study were as follows: (1) designed the specific primers to detecting *S. rolfsii* and *L. theobromae* and used the universal primers for detection of *Fusarium* spp. and (2) established a multiplex PCR rapid detection system for southern blight, stem rot, and root rot of peanuts, which provided technical support for the early and rapid diagnosis of the three diseases, and this system also could be used for the rapid detection of diseased plants and seeds of peanut.

Table 1 List of fungal collections

Species	Code	Host
S. rolfsii	BJ-2	Peanut
L. theobromae	JF4-9	Peanut
F. oxysporum	FL-Y4-5	Peanut
R. solani	RS-1	Potato
P. myriotylum	PM-Y2	Tomato
R. cerealis	RC-3	Wheat
F. graminearum	HF310	Peanut
S. sclerotiorum	ST-2	Canola
F. pseudograminearum	XY-29	Wheat
P. parasitica var. *nicotianae* Tucker	YM-2	Tobacco

Table 3 The reaction system of single PCR

Component	Volume (μL)
10×GC buffer	2.5
dNTPs (2.5 mM)	2
Taq DNA polymerase (5 U/μL)	0.5
Primer F (20 μM)	0.5
Primer R (20 μM)	0.5
Template DNA (50–100 ng)	1
ddH_2O	18
Total volume	25

Materials and Methods

Tested Strains

The numbers and sources of all isolates are listed in Table 1. *Sclerotium rolfsii, L. theobromae*, and *F. oxysporum* were used as target strains for multiplex PCR detection. The specificity of primers was verified by *Rhizoctonia cerealis*, *F. graminearum*, *F. pseudograminearum*, *R. solani*, *Sclerotinia sclerotiorum*, *Phytophthora parasitica* var. *nicotianae* Tucker, and *Pythium myriotylum*. The strains were obtained from plant pathology laboratory of Shandong Agricultural University. These fungi were incubated in Petri dishes containing potato dextrose agar (PDA) medium for 3–5 days at 28 °C [21].

Primer Design

Specific primers were designed using Primer 5 software based on the specific gene beta-tubulin gene (GenBank accession no. MN078927.1) of *S. rolfsii*, the cytb-specific gene (GenBank accession no. MH880818.1) of *L. theobromae*, and the universal primer of *F. oxysporum* [22, 23]. As shown in Table 2, all primers were synthesized by Biochemical and Biological Engineering (Shanghai) Co., Ltd.

Table 2 Primers used in the multiplex PCR assay

Species	Primers	Sequence (5′–3′)	Product (bp)
S. rolfsii	BJ-bt-F3	CCGAAGGTGTCTGACACTGTTG	238
	BJ-bt-R4	TGCGCAAGTCCGAGTTAAGTTG	
L. theobromae	JF-cb-F1	GCAATGCATTACAATCCTAGTG	638
	JF-cb-R4	GGATTTGCCATAACATAATTCTCTG	
F. oxysporum	F1	ATTACTCCAGCATCCTTGC	1005
	F2	TTTACAACTCCCAAACCCC	

PCR Amplification

The single PCR system is shown in Table 3, and the multiplex PCR mixture system is shown in Table 4. Single PCR conditions: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 40 s, annealing at 63 °C for 40 s, extension at 72 °C for 1 min, followed by 30 cycles, and final extension at 72 °C for 10 min [24]. Similar conditions were used for multiplex PCR, except for the annealing temperature.

Optimization of the Annealing Temperature for Multiplex PCR

The temperature gradient PCR procedure was designed between 58.6 and 63.4 °C, and temperatures were 58.6 °C, 59.2 °C, 59.9 °C, 60.6 °C, 61.3 °C, 62 °C, 62.8 °C, and 63.4 °C [25]. After the reactions, 5 µL of each product was used for 1% agarose gel electrophoresis (200 V, constant for 20 min), after ethidium bromide staining and then observed under UV light. All tests were repeated thrice.

Multiple PCR Primer-Specific Amplification

The mixed DNA of *S. rolfsii*, *L. theobromae*, and *F. oxysporum* was used as the positive control and ddH_2O was used as the negative control. The single DNA of *S. rolfsii*, *L. theobromae*, and *F. oxysporum* and the genomic DNA of *R. cerealis*, *R. solani*, *S. sclerotiorum*, *P. nicotianae*, and *P. fulvum* were used as templates for triple PCR detection at the determined multiplex PCR annealing temperature to test the specificity of triple PCR primers [26]. All experiments were repeated thrice.

Table 4 The reaction system of multiplex PCR

Component	Volume (µL)
10 × GC buffer	2.5
dNTPs (2.5 mM)	2
Taq DNA polymerase (5U/µL)	0.5
Primer BJ-bt-F3 (20 µM)	0.4
Primer BJ-bt-R4 (20 µM)	0.4
Primer JF-cb-F1 (20 µM)	0.4
Primer JF-cb-R4 (20 µM)	0.4
Primer F1 (20 µM)	0.4
Primer F2 (20 µM)	0.4
Template DNA (50–100 ng)	1
ddH_2O	16.6
Total volume	25

Sensitivity Analysis of Single and Multiplex PCR

First, dilute the initial template concentration of DAN of *S. rolfsii*, *L. theobromae*, and *F. oxysporum* to 10 ng/µL and then serially dilute tenfold (10 ng to 10 fg) with distilled water [27]. In addition, the initial template concentration of the mixed DAN of the three pathogenic fungus genomes was diluted to 10 ng/µL and serially diluted 10 times to evaluate the sensitivity of mPCR detection. Single multiplex and multiplex PCR were performed under optimized conditions [28]. All experiments were repeated thrice.

Multiple PCR Vaccination Testing

Sclerotium rolfsii, L. theobromae, and *F. oxysporum* were cultured in an incubator at 25 °C for 2 days. Then, five fungal culture blocks were taken out from those and transplanted into the wheat kernel medium, incubated at a constant temperature at 25 °C for 4 days, and shaken four times a day. Fungi used for inoculation experiment were cultured with wheat grains. Two wheat grains with different fungi were placed at the base of the peanut stems after the seedling emergence and cultivated in a biochemical incubator at 25 °C and a humidity of 80%. Drying-weighing method is used for soil moisture measurement [29]. The disease incidence of plants at 12 h, 16 h, 20 h, 24 h, and 48 h was observed and recorded. Total genomic DNA of the rhizome was extracted by the improved CTAB method [30], which was used as a template for PCR detection. The established triple PCR system was used for detection of pathogenic fungi in peanut.

Detection of Pathogenic Fungi in Peanut

One hundred and two peanut plant samples were collected from peanut fields in Linyi, Tai'an, and Jinan in Shandong Province in August 2020; 216 peanut seed samples from peanut fields in Linyi, Tai'an, and Jinan in Shandong Province. The total DNA of rhizomes and seeds was extracted and detected by the established triple PCR system.

Results

Optimization of the Annealing Temperature for mPCR

As shown in Fig. 1, all genes were amplified between 58.6 and 63.4 °C, with the highest amplification at 60.6 °C.

Therefore, 54.9 °C was selected as the optimal annealing temperature.

Sensitivity of the Single and Multiplex PCR

Genomic DNA was sequentially diluted for the sensitivity assay of single and multiplex PCR. A single PCR amplification sensitivity test detected 1-ng, 10-pg, and 10-pg DNA for *S. rolfsii, L. theobromae*, and *F. oxysporum*, respectively

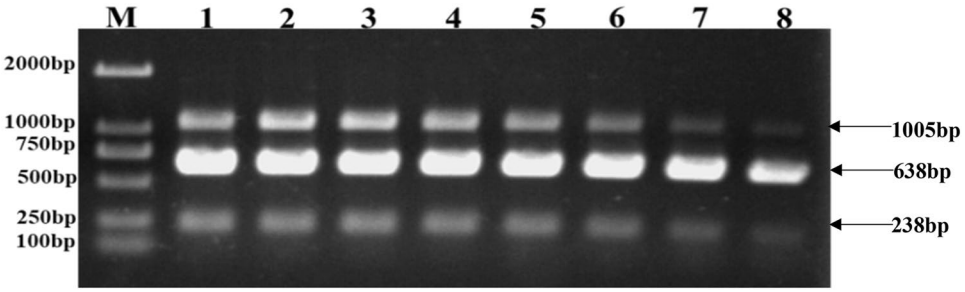

Fig. 1 Optimization of the annealing temperature for multiplex PCR. Lane M: DL2000 marker. Lanes 1–8: samples amplified at annealing temperatures of 58.6 °C, 59.2 °C, 59.9 °C, 60.6 °C, 61.3 °C, 62 °C, 62.8 °C, and 63.4 °C, respectively

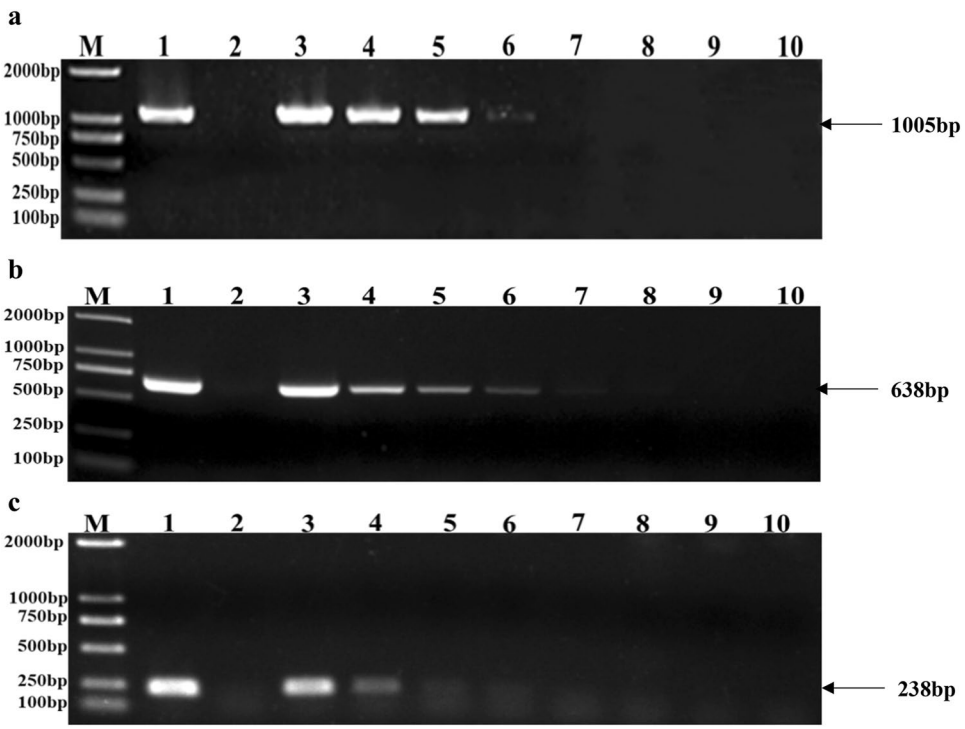

Fig. 2 Sensitivity of the single PCR assay for *F. oxysporum* (**a**), *L. theobromae* (**b**), and *S. rolfsii* (**c**). Lane M: DL2000 marker. Lane 1: positive control; Lane 2: negative control; Lane 3: 10 ng; Lane 4: 1 ng; Lane 5: 100 pg; Lane 6: 10 pg; Lane 7: 1 pg; Lane 8: 100 fg; and Lane 9: 10 fg

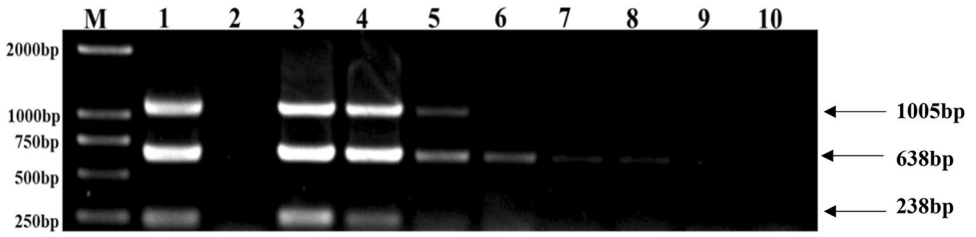

Fig. 3 Sensitivity of the multiplex PCR assay. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: negative control; Lane 3: 10 ng/μL; Lane 4: 1 ng/μL; Lane 5: 100 pg/μL; Lane 6: 10 pg/μL; Lane 7: 1 pg/μL; Lane 8: 100 fg/μL; and Lane 9: 10 fg/μL

(Fig. 2), while the mPCR detected 1 ng, 10 pg, and 100 pg for *S. rolfsii*, *L. theobromae*, and *F. oxysporum*, respectively (Fig. 3). In addition, for the detection sensitivity of stem rot and southern blight, the results of mPCR were similar to that of the single PCR. However, as for root rot, the detection sensitivity of mPCR was decreased compared with that of the single PCR.

mPCR Primer Specificity Amplification

The mPCR assay yielded fragments of the expected size using *S. rolfsii, L. theobromae*, and *F. oxysporum* as genomic DNA with specific primers, while no products were obtained with non-target species (Fig. 4), thereby validating the specificity of the mPCR assay.

Evaluation of Multiple PCR in Vaccination Testing

The three fungi were inoculated in a biochemical incubator with 25 °C (soil temperature 23.9 °C) and 80% humidity (soil humidity 53.6%) and collected inoculated plants and control (uninoculated) plant. The DNA of the rhizome was extracted for multiplex PCR detection. After inoculation for 20 h, 24 h, and 48 h, the southern blight fungus, stalk rot fungus, and root rot fungus were detected, respectively (Fig. 5). Moreover, plants exhibited no obvious symptoms, indicating that the system could detect these three pathogens in plants that have not yet shown disease symptoms and could be used for early detection of peanut diseases in the field.

Application of mPCR Detection of Peanut

For the collected 102 peanut samples, DNA was extracted from the rhizomes and subjected to mPCR detection. As shown in Fig. 6, a total of 24 plants with peanut southern blight, 15 plants with peanut root rot, and 6 plants with peanut stem rot were detected. Among them, 4 peanut plants were co-infected with stem rot fungus (*L. theobromae*) and southern blight fungus (*S. rolfsii*), which proved that this multiplex PCR system could be used to detect the plants with lesions caused by these three diseases (Table 5).

For 216 peanut seeds collected in Shandong Province, mPCR detection was performed after extracting the seed DNA. As shown in Fig. 7, a total of 71 peanut seeds carrying southern blight, 16 peanut seeds carrying root rot, and 12 peanut seeds carrying stem rot pathogen were detected. Among them, 3 seeds had the phenomenon of co-infection, 1 seed was detected with southern blight fungus and stem rot fungus at the same time and 2 seeds were detected by southern blight fungus and root rot fungus at the same time (Table 6). This result indicated that the established multiplex PCR system can be used to detect seeds and then effective control measures can be taken to reduce the damage of seed-borne diseases and minimize the losses from diseases caused by seeds with fungus.

Discussion

In general, staining, culture, and biochemical identification are major traditional methods to detect pathogenic fungi, which are time-consuming and possess low sensitivity [31, 32]. With the rapid development of molecular technology, molecular detection technology based on PCR technology is increasingly valued and favored [32–35]. Among them, a single PCR is suitable for detecting a single disease [36]. However, the peanut soil-borne diseases often mixing occurs, thus the single PCR is usually difficult to screen a large number of peanut samples for the presence of pathogens. Although the detection sensitivity of multiplex PCR is slightly lower or close to that of single PCR [37], multiplex PCR can simultaneously amplify multiple fragments and the characteristics of simplicity and convenience in practice and relatively low cost [38–41], which satisfies the needs of rapid detection of peanut root diseases.

In addition, it was the first time to detect the peanut soil-borne diseases by multiplex PCR. In this study, the multiple PCR assays established for the detection of pathogenic fungi of peanut stem rot, root rot, and southern blight with

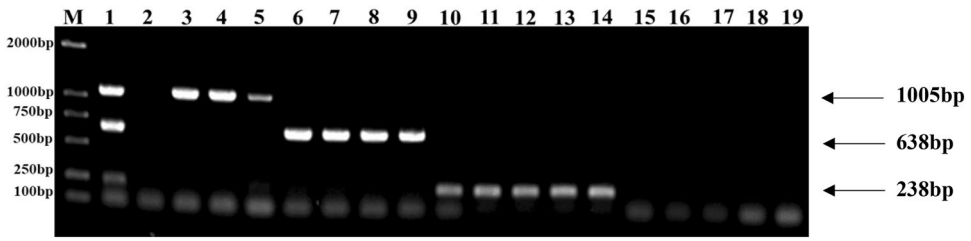

Fig. 4 Specificity of multiplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: negative control; Lane 3–5: *F. oxysporum*; Lane 6–9: *L. theobromae*; Lane 10–14: *S. rolfsii*; Lane 15: *R. solani*; Lane 16: *R. cerealis*; Lane 17: *S. sclerotiorum*; Lane 18: *P. parasitica* var. *nicotianae* Tucker; and Lane 19: *P. myriotylum*

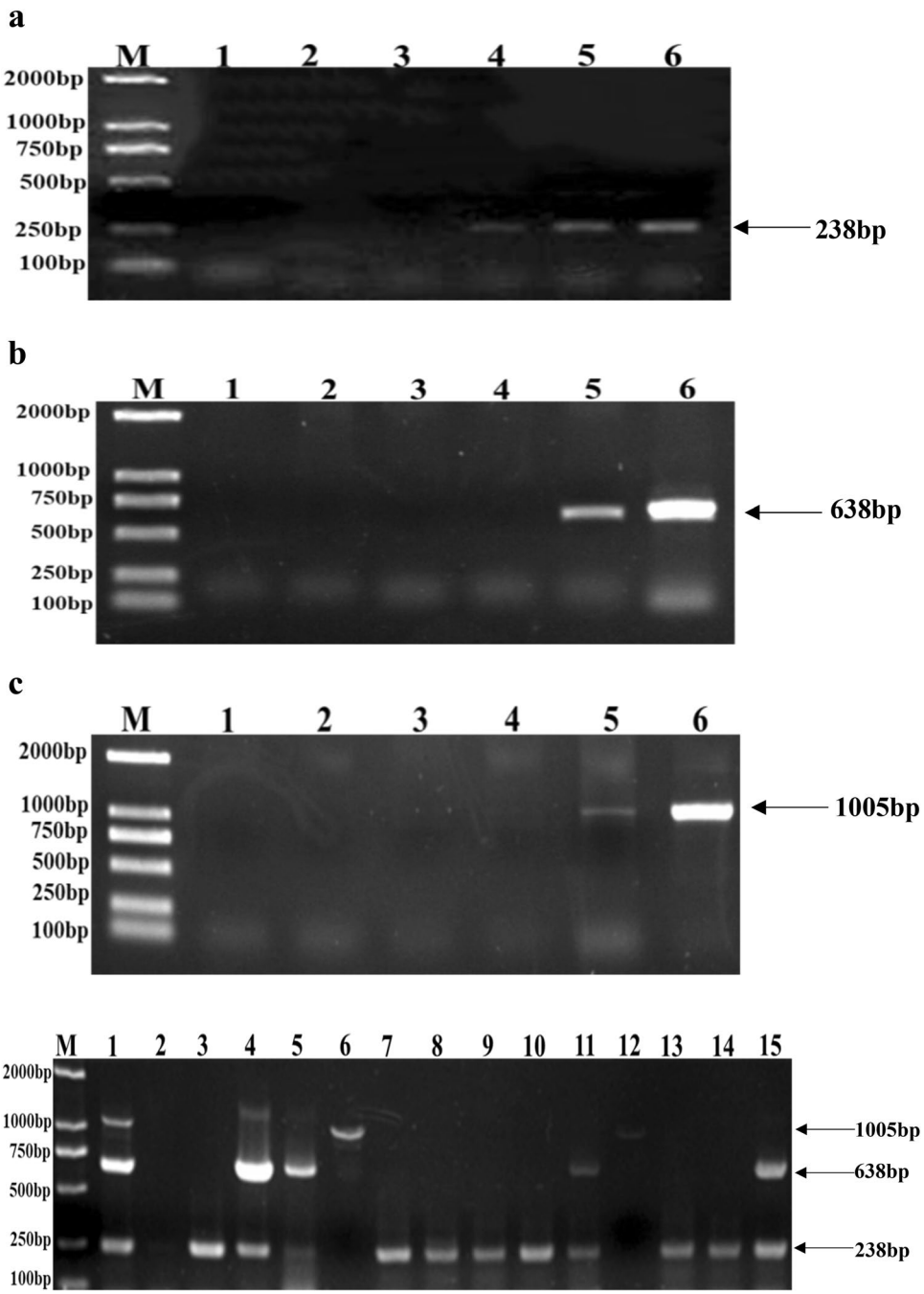

Fig. 5 Test results at different times of inoculation. Lane M: DL2000 marker; Lane 1: negative control; Lane 2: 12 h; Lane 3: 16 h; Lane 4: 20 h; Lane 5: 24 h; and Lane 6: 48 h

Fig. 6 Multiplex PCR detection of peanut root and stem diseases in some areas of Shandong Province. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: negative control; and Lane 3–15: field peanut samples

Table 5 Multiplex PCR detection of peanut root and stem diseases in eastern part of the mountain

Pathogen	*S. rolfsii*	*F. oxysporum*	*L. theobromae*	*S. rolfsii* and *L. theobromae*
Number of positive detections	24	15	6	4
Positive detection rate (%)	23.529	14.706	5.882	3.922

10 pg/L, 100 pg/L, and 1 ng/L, respectively (Fig. 3). Similar findings have been reported before [42–44]. Compared with the single PCR sensitivity test results (Fig. 2), the sensitivity results of stem rot pathogen and southern blight pathogen were the same and the sensitivity of root rot fungus decreased from 10 to 100 pg/μL (Fig. 3). Presumably,

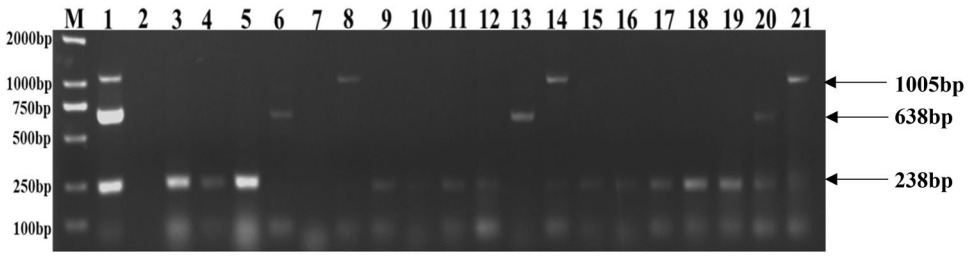

Fig. 7 Partial electrophoresis image of peanut seeds detected by multiplex PCR in some areas of Shandong Province. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: negative control; and Lane 3–21: peanut seed samples

Table 6 Multiplex PCR detection of peanut seeds in some areas of Shandong Province

Pathogen	*S. rolfsii*	*F. oxysporum*	*L. theobromae*	*S. rolfsii* and *L. theobromae*	*S. rolfsii* and *F. oxysporum*
Number of positive detections	71	16	12	1	2
Positive detection rate (%)	32.870	7.407	5.555	0.463	0.926

the decreased sensitivity is due to competition between the three pairs for *Taq* DNA enzymes and dNTPs, decreasing the number of amplification products [20, 45], another reason may be that the three pairs of primers interact with each other so that the binding of the primers to the DNA template is not tight and does not occur sufficiently [14, 46].

According to the relevant research, the phenomenon of mixed occurrence of diseases on the same plant is common [47, 48]. At present, a variety of diseases have been detected in strawberries, grape, and mango [49–51]. Since the stem rot, root rot, and southern blight disease of peanut can infect the seeds and make the seeds carry bacteria, the established multiplex PCR system is able to detect and verify not only the field peanut plant samples, but also the peanut seed samples. It has been reported that, in the seeds of rapeseed (*Brassica campestris* L.) [52] and sunflower (*Solanum tuberosum* L.) [53], multiplex PCR has been successfully used in detecting multiple seed-borne pathogens. Our results showed that three pathogens could be detected in peanut and peanut seeds with a relative high positive detection rate. The relative high rate of positive detection of peanut plants may be the collection of peanut plant samples with more typical diseases (Table 5) [54–56]. The positive detection rate of peanut seeds was not higher than that of plants (Table 6), may be due to the low amount of seed epidermis with fungi.

The results of the testing of field samples showed that it was found that there was a phenomenon of co-infection of peanut southern blight and peanut stem rot on peanut plants. There are co-infection of peanut southern blight fungus and peanut stem rot on peanut seeds, as well as peanut southern blight fungus and peanut root rot fungus in practice, indicating that these three diseases are indeed mixed. Studies have shown that this co-infection occurs not only in peanuts but has also been found in sugarcane, watermelon, and wheat. [20, 57, 58]. In this study, the peanut mPCR system enables pathogen detection of co-infected peanut soil-borne diseases, which contributed to effectively controlling peanut diseases.

In conclusion, the mPCR system established in our work for the detection of peanut southern blight, stem rot, and root rot is simple, rapid, and sensitive. The system has important practical value for the control of peanut root and stem diseases to ensure the safety of peanut crops.

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Author Contributions AXL, XL, and JW conceived and designed research. XL, JW, XLH, and MQL conducted experiments. XYS and CH analyzed data. JW and CH wrote the manuscript. All authors read and approved the manuscript.

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

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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