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Development of rapid DNA extraction and PCR amplification methods for fungi and parasitic plants

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Abstract

The main culprits of catastrophic agricultural losses or crop mortality are phytopathogenic fungi and parasitic plants. To manage the plant pathogens, a simple and rapid disease diagnosis is needed. The aim of the experiment was to develop simple and rapid genomic DNA extraction and PCR amplification methods for fungi and parasitic plants. For the DNA extraction, mycelia from 16 fungi species and stems from two parasitic plants species were incubated in a lysis buffer and were homogenised using a sterilised wooden stick. The homogenates were incubated at 95°C temperature for 10 min. Crude extracts served as a template for the PCR amplification containing *UKOD* polymerase. The application of lysis buffer, mechanical and heat disruption resulted in a fast DNA extraction from fungi and parasitic plants. DNA amplification time is reduced when using Master Mix containing *UKOD* polymerase.
The presented results confirm that these simple and rapid DNA extraction and PCR amplification methods are applicable to diverse fungi species and parasitic plants.

Keywords: lysis buffer, pathogenic fungi, mechanical and heat disruption, parasitic plants, PCR amplification.

Introduction

Phytopathogenic fungi and parasitic plants are the leading cause of devastating agricultural losses or death in crops. Phytopathogenic fungi damage plants by killing their cells and causing biotic stress (El-Baky et al., 2021), while parasitic plants penetrate their host tissues and form vascular connections to redirect the host's nutrients via the haustoria in their stems or roots (Těšitel, 2016). To characterise the phytopathogens, besides morphological characterisation, one of the reliable and trending methods is molecular identification (López et al., 2009). In general, the molecular identification process begins with DNA extraction followed by polymerase chain reaction (PCR) amplification; then gel visualisation for positive desired bands is used before sending the products for sequencing. Lastly, the identity of sequences is verified using the National Center for Biotechnology Information (NCBI) GenBank.

The development of DNA extraction and PCR amplification methods have been the topic of research since the invention of PCR in 1983 by K. B. Mullis (1990). Most of the time, DNA extraction involves the use of liquid nitrogen to break the cell wall or tissues (Umesha et al., 2016). However, the cost of liquid nitrogen is a limitation for studies involving a large number of samples. To facilitate the DNA extraction process, besides chemicals such as phenol, chloroform, and isoamyl alcohol (Aamir et al., 2015), microwave energy (Umesha et al., 2016) has been used. Using these chemicals and microwave energy during the extraction process can cause adverse effects to health (Renshaw et al., 2015; Sudharshan, 2016). Moreover, some laboratories in developing countries are lacking facilities to dispose these harmful chemicals. In addition, to perform DNA extraction, glass was used (Aamir et al., 2015). A frequent surface contact of samples with a pestle and a mortar, glass and a spatula increases the risk of contamination (Zhang et al., 2010). Mechanical and thermal disruption using a water bath have been applied in DNA extraction, and the methods are effective, easy, and rapid to extract DNA (Englen, Kelley, 2008; Zhang et al., 2010).

In the amplification process, the replication process of the DNA template relies on DNA polymerases (Bębenek, Ziuzia-Graczyk, 2018). These polymerases are extracted from several microorganisms such as *Pyrococcus furiosus* (*Pfu* polymerase), *P. woesei* (*Pwo* polymerase), *Thermococcus fumicolans* (*Tfu* polymerase), *T. litoralis* (*Wind* or *Tli* polymerase, or *Vent* polymerase), *T. kodakarensis* (*KOD* polymerase), *Thermotoga maritima*

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(*Tma* polymerase), *T. eapolitana* (*Tne* polymerase), *Thermus aquaticus* (*Taq* polymerase), *T. flavus* (*Tfl* polymerase), and *T. thermophilus* (*Tth* polymerase) (Drouin et al., 2007; Špibida et al., 2017). Many commercial DNA polymerases are available in the market, and their influences on the specificity, efficiency, and fidelity of PCR were reviewed comprehensively by Drouin et al. (2007). When working with the PCR, researchers face the problem of non-specific amplification, where there are primers annealing to non-complementary DNA sequences or forming dimers prior to PCR cycling (Lorenz, 2012; Mubarak et al., 2020). To address the problem of non-specific amplification, the modified hot start KOD DNA polymerase (*UKOD* polymerase) (Toyobo, Japan) has been used. *UKOD* polymerase has the advantages of inhibiting the DNA polymerase and 3' to 5' exonuclease activities at ambient temperature using two premixed monoclonal antibodies (Drouin et al., 2007). According to Toyobo's manufacturer, the *UKOD* polymerase is resistant to inhibition and is largely unaffected by the unpurified crude sample. Moreover, the addition of an enhancer and the genetic modification of *UKOD* polymerase enable the fast PCR.

The nature of PCR gives rise to a plethora of application developed independently. One particular application is the use in plant disease management (Balodi et al., 2017). PCR is specific, sensitive, efficient, rapid, versatile, and relatively economical (Henson, French, 1993; Balodi et al., 2017). PCR saves time and costs, because pathogen isolation in pure culture is not necessary (Balodi et al., 2017). In plant disease management, the PCR has been applied in fungal and parasitic plant diagnosis (Annamalai et al., 1995; Delavault et al., 1996). Fungi, viz. *Puccinia thaliae*, *Athelia rolfsii*, *Talaromyces siamensis*, *Neoscytalidium dimidiatum*, *Nigrospora sphaerica*, *Nigrospora aurantiaca*, *Colletotrichum siamense*, *Lasiodiplodia theobromae*, *Neopestalotiopsis cubana*, *Epicoccum sorghinum*, *Fusarium oxysporum*, *Diaporthe ueckerae*, *Hypoxylon hypomiltum, Fusarium proliferatum*, *Daldinia eschscholtzii*, and *Macrophomina phaseolina*, and parasitic plants, viz. *Cuscuta australis* and *Cassytha filiformis*, are the common pathogens that have been reported to cause plant disease (Rattan, 2005; Li et al., 2015; Ghuffar et al., 2018; Lee et al., 2019; Elahi et al., 2021; López-Cardona et al., 2021; Khoo et al., 2022 a; b; c; d; e; f; g; h; i; j; k; l). To identify and detect the pathogens, the PCR is used to target the specific regions in pathogens' genomic DNA (Lau, Botella, 2017).

The aim of this study was to develop rapid DNA extraction and PCR amplification methods for fungi and parasitic plants. To our knowledge, a rapid plant disease diagnosis enables the prompt action to be taken to manage the disease. In view of the aforementioned concern, a simple and rapid genomic DNA extraction using a lysis buffer, heat, and mechanical disruption to extract the genomic DNA of fungi and parasitic plants rapidly in the form of crude DNA extracts was presented. This method is low-cost, presents a low contamination risk and is free from the use of harmful reagents. Besides, no research has used *UKOD* polymerase in the PCR amplification of crude extracts of fungi and parasitic plants in plant disease diagnosis. In this study, unpurified crude DNA extracts as a template in the PCR Master Mix containing *UKOD* polymerase were used. The PCR amplification is fast, and the results are unaffected by the unpurified crude sample.

Material and methods

In this study, fungi *Puccinia thaliae*, *Athelia rolfsii*, *Talaromyces siamensis*, *Neoscytalidium dimidiatum*, *Nigrospora sphaerica*, *Nigrospora aurantiaca*, *Colletotrichum siamense*, *Lasiodiplodia theobromae*, *Neopestalotiopsis cubana*, *Epicoccum sorghinum*, *Fusarium oxysporum*, *Diaporthe ueckerae*, *Hypoxylon hypomiltum*, *Fusarium proliferatum*, *Daldinia eschscholtzii*, and *Macrophomina phaseolina*, and parasitic plants *Cuscuta australis* and *Cassytha filiformis*, which have been reported to cause a serious growth retardation and in some cases the death of hosts (Li et al., 2015), were used (Table). They were obtained from the Genetics Laboratory of the Faculty of Science and Natural Resources, Universiti Malaysia Sabah.

At least 0.1 g of mycelium from the 10-day-old culture and stems of parasitic plants were transferred into a 1.5-ml microcentrifuge tube containing 300 µL of a lysis solution made of Tris-HCl (0.1 M), pH 9.5, sodium chloride (NaCl) (1 M), and ethylenediaminetetraacetic acid (EDTA) (0.01 M), pH 8. The mixture was homogenised using a sterilised wooden stick prior to incubation in a water bath at 95°C temperature for 10 min. The concentration and purity of unpurified crude extract (approximately total volume of 300 µL) containing genomic DNA were measured using a spectrophotometer Multiskan SkyHigh Microplate (Thermo Scientific, USA) before being kept at −20°C temperature until further use.

Polymerase chain reaction (PCR) amplifications were performed in 20 µL of the volume consisting of 10 µL KOD One PCR Master Mix containing *UKOD* polymerase, nuclease free water (added up to 20 µL), 0.6 µL of each primer (10 µM), and unpurified crude extract (20 ng μL^{-1}). To amplify parasitic plant *C. australis* or fungi other than *P. thaliae*, primers ITS1 (5'-AGAGTTTGATCCTGGCTCAG-3') and ITS4 (5'-CCGTCAATTCCTTTGAGTTT-3') were used. For the PCR amplification of fungi *P. thaliae* (20 ng μL^{-1}), primers Rust28SF (5'-TTTTAAGACCTCAAATCAGGTG-3') and LR5 (5'-ATCCTGAGGGAAACTTC-3') were used, while for the PCR amplification of parasitic plant *C. filiformis* (20 ng μL^{-1}), primers MatK-3FKim (5'-CGTAC AGTACTTTTGTGTTTACGAG-3') and MatK-1RKim (5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3') were used. For each reaction, annealing temperatures were 55°C for the ITS region and MatK gene, and 57°C for the 28S ribosomal RNA gene. Cycling parameters were 94°C for 10 s, 55 or 57°C (depending on the primers used) for 5 s, and 72°C for 2 s, for 30 cycles, and the initial denaturation of 94°C for 3 min and a final extension step of 72°C for 5 min.

PCR products were resolved by electrophoresis via 2% (w/v) agarose gels in the 1X TAE buffer (40 mM Tris-

Table. Details of fungi and parasitic plants in the NCBI database used in the study

No.	Fungi	Host	Substrate, locality	Accession No.	Nucleotide length bp
1.	*Puccinia thaliae*	*Canna indica*	leaf, Malaysia	OK462969	901
2.	*Athelia rolfsii*	*Basella rubra*	soil, Malaysia	OK465460	625
3.	*Talaromyces siamensis*	*Hibiscus sabdariffa*	leaf, Malaysia	OK667950	464
4.	*Neoscytalidium dimidiatum*	*Selenicereus megalanthus*	stem, Malaysia	OK458559	535
5.	*Nigrospora sphaerica*	*Selenicereus megalanthus*	stem, Malaysia	OK448496	525
6.	*Nigrospora aurantiaca*	*Pandanus amaryllifolius*	leaf, Malaysia	OK469308	539
7.	*Colletotrichum siamense*	*Crinum asiaticum*	leaf, Malaysia	OK458683	568
8.	*Lasiodiplodia theobromae*	*Aloe vera*	leaf, Malaysia	OK209451	503
9.	*Neopestalotiopsis cubana*	*Ixora chinensis*	leaf, Malaysia	OM320626	545
10.	*Epicoccum sorghinum*	*Basella alba*	leaf, Malaysia	OM501130	507
11.	*Fusarium oxysporum*	*Basella rubra*	leaf, Malaysia	OK469301	507
12.	*Diaporthe ueckerae*	*Pachira aquatica*	leaf, Malaysia	OL306312	566
13.	*Hypoxylon hypomiltum*	*Cymbopogon* sp.	leaf, Malaysia	ON054207	576
14.	*Fusarium proliferatum*	*Basella rubra*	leaf, Malaysia	OK465487	555
15.	*Daldinia eschscholtzii*	*Calophyllum inophyllum*	leaf, Malaysia	ON041211	529
16.	*Macrophomina phaseolina*	*Pometia pinnata*	leaf, Malaysia	OK465197	537
		Parasitic plants			
17.	*Cuscuta australis*	–	seed, China	ON041206	608
18.	*Cassytha filiformis*	*Justicia gendarussa*	stem, Malaysia	OK210073	791

HCl, 20 mM acetic acid, 1 mM EDTA), pH 8.0, containing SYBR Safe DNA gel stain (Invitrogen, USA). The gel was visualised on a BluPAD Dual LED Blue/White light transilluminator (Bio-Helix Co., Ltd.).

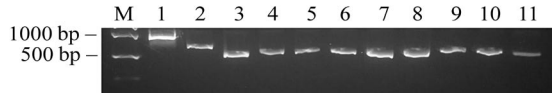
To purify the PCR amplicons from agarose gels, the following steps were involved in the process: a hole was made at the bottom of a 500-µL tube. To provide a cushion, the 500-µL tube, which had been inserted with a round cotton pad (10 mm diameter), was inserted into a 1.5-ml microcentrifuge tube. Before being used, the assembled tubes were autoclaved. A piece of DNA-containing gel was excised from agarose gel and placed on the cotton. The lid of 500-µL tube was closed prior to the centrifugation at 5,000 rpm for 5 min. The collected liquid was then quantified spectrophotometrically before sending for Sanger sequencing conducted at a commercial service provider (Apical Scientific, Malaysia). The PCR products of fungi and parasitic plants were sequenced, and sequence homology search was conducted using the NCBI GenBank database.

Results and discussion

In this study, a simple and rapid fungal and parasitic plant genomic DNA extraction and PCR amplification methods were described. The proposed method does not only reduce the workload substantially but is also time-saving. A combination of mechanical, lysis buffer, and heat disruption on the fungal and plant tissue has the edge over the conventional methods. The study showed that the DNA extraction of fungi and parasitic plants can be performed without a liquid nitrogen or a centrifuge machine, a hazardous chemical such as 2-mercaptoethanol, phenol, and chloroform.

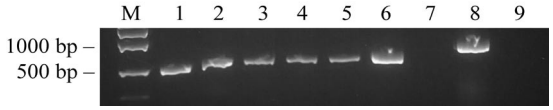
In the initial lysis step, Tris and EDTA in the lysis buffer function to lyse the cell wall and nuclear membrane chemically and as a preventative to inhibit DNA nucleases from DNA degradation (Chauhan, 2018). NaCl removes polysaccharides (Fang et al., 1992) and regulates the pH and osmolarity of the lysate. To break the cell wall of mycelium and plant tissue to enhance the DNA extraction process, after the lysis step, a sterilised stick was used. In the following step, heating was applied to break the tissues, to accelerate the chemical reactions, to denature proteins, and to inactivate nuclease (Fujimoto et al., 1974).

The genomic DNA from the phytopathogenic fungi except *P. thaliae* and *C. australis* were amplified using the primer pair ITS1/ITS4 (Figures 1 and 2). *P. thaliae* was amplified using the primer pair Rust28SF/LR5 (Figure 1); the genomic DNA of *C. filiformis* was amplified using the primer pair MatK-3FKim/MatK-1RKim (Figure 2).



Note. M – DNA marker (VC 1kb); lanes 1–11 are *P. thaliae*, *A. rolfsii*, *T. siamensis*, *N. dimidiatum*, *N. sphaerica*, *N. aurantiaca*, *C. siamense*, *L. theobromae*, *N. cubana*, *E. sorghinum*, and *F. oxysporum*, respectively.

Figure 1. PCR amplification of the genomic DNA of *Puccinia thaliae* using the primer pair Rust28SF/LR5 and other fungi using the primer pair ITS1/ITS4



Note. M – DNA marker (VC 1kb); lanes 1, 2, 3, 4, 5, 6, and 8 are *D. ueckerae*, *H. hypomiltum*, *F. proliferatum*, *D. eschscholtzii*, *M. phaseolina*, *C. australis*, and *C. filiformis*, respectively; lanes 7 and 9 are the negative control for the PCR amplification using the primer pair ITS1/ITS4 and MatK-3FKim/MatK-1RKim.

Figure 2. PCR amplification of the genomic DNA of fungi and *Cuscuta australis* using the primer pair ITS1/ITS4, and *Cassytha filiformis* using the primer pair MatK-3FKim/MatK-1RKim

The sequencing results revealed that the amplicon size of *P. thaliae* was 901 bp, *A. rolfsii* 625 bp, *C. filiformis* 791 bp, *T. siamensis* 464 bp, *N. dimidiatum* 535 bp, *N. sphaerica* 525 bp, *N. aurantiaca* 539 bp, *C. siamense* 568 bp, *L. theobromae* 503 bp, *N. cubana* 545 bp, *E. sorghinum* 507 bp, *F. oxysporum* 507 bp, *D. ueckerae* 566 bp, *H. hypomiltum* 576 bp, *F. proliferatum* 555 bp, *D. eschscholtzii* 529 bp, *M. phaseolina* 537 bp, and that of *C. australis* was 608 bp. The results were consistent with the details summarised in the Table.

In the PCR amplification of genomic fungal and parasitic plant DNA, a KOD One PCR Master Mix was used, and the amplification took around 15 min to complete. According to the Toyobo instruction manual, *UKOD* polymerase in the Master Mix performed the ultra-fast PCR with the inclusion of a unique elongation enhancer while providing the highest efficiency, specificity, fidelity, and yield. At the same time, the *UKOD* polymerase is also resistant to inhibition and is largely unaffected by the unpurified crude sample. The concentration and purity of crude samples were from 100 to 300 ng and 1.6 to 1.8, respectively. The PCR was working although using the unpurified crude samples as templates. The positive amplicons were clearly visualised when using the crude extract of fungi and parasitic plants as templates in the PCR amplification.

Conclusions

1. The proposed DNA extraction and PCR amplification methods are versatile and suitable for diverse fungi and parasitic plants as they are capable to perform diagnosis rapidly.

2. Further research might explore the potential application of these methods on the samples infected with bacteria, phytoplasma, spiroplasma, viroid, and virus.

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Grybų bei parazitinių augalų DNR ekstrakcijos ir pagausinimo PGR būdu greitųjų metodų kūrimas

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Santrauka

Fitopatogeniniai grybai ir parazitiniai augalai yra pagrindiniai didelių žemės ūkio nuostolių ir pasėlių sunaikinimo kaltininkai. Siekiant kovoti su augalų patogenais, būtina paprasta ir greita ligų diagnozė. Eksperimento tikslas – sukurti paprastus ir greitus grybų bei parazitinių augalų genominės DNR ekstrakcijos ir pagausinimo polimerazės grandininės reakcijos (PGR) būdu metodus. Atliekant DNR ekstrakciją, 16-os grybų rūšių grybiena ir dviejų parazitinių rūšių augalų fragmentai buvo laikyti irimo buferyje ir homogenizuoti naudojant sterilizuotą medinę lazdelę. Homogenatai 10 minučių inkubuoti 95° C temperatūroje. Kaip DNR pagausinimo PGR būdu šablonas su *UKOD* polimeraze naudoti neapdoroti ekstraktai. Irimo buferio, mechaninio ir šiluminio irimo taikymo rezultatas – greita DNR ekstrakcija iš grybų ir parazitinių augalų. Naudojant Master Mix su *UKOD* polimerazę, sutrumpinamas DNR pagausinimo laikas.

Tyrimo rezultatai patvirtino, kad šiuos greitus DNR ekstrakcijos ir PGR stiprinimo metodus galima taikyti įvairių rūšių grybams ir parazitiniams augalams.

Reikšminiai žodžiai: irimo buferis, patogeniniai grybai, mechaninis ir šiluminis irimas, parazitiniai augalai, pagausinimas PGR būdu.