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A rapid fungal DNA extraction method suitable for PCR screening fungal mutants, infected plant tissue and spore trap samples

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ABSTRACT

Current DNA extraction methods from filamentous fungi take from an hour to a full day. These methods require buffers and tools for grinding fungal tissues. Although commercial extraction kits can reduce the amount of time spent preparing and extracting genomic DNA from fungal samples, these can be expensive.

Here we describe a quick and inexpensive sonication technique to extract genomic DNA from filamentous fungi without buffer which can be used to perform PCR in under an hour. DNA was extracted from *Zymoseptoria tritici, Fusarium graminearum* and *Botrytis cinerea in vitro* cultures, *Z. tritici* asexual fruiting bodies and directly from spore trap tapes.

1. Introduction

The economic impact of pathogenic fungi on crop yields, and the danger for human and animal health caused by fungal mycotoxins makes the detection of fungal pathogens a top priority [10,11,23]. The filamentous fungal pathogens *Zymoseptoria tritici, Fusarium graminearum* and *Botrytis cinerea* are among the most damaging plant pathogenic fungi recorded to date causing disease on wheat and the latter is able to infect more than 200 different species of commercial crops around the world [34]. DNA based methods can be used to detect and diagnose the presence of fungal pathogens [14]. However, fungal cell walls are strong structures, mostly composed of chitin and glucans that enhance their toughness [17]. These structures usually require a combination of freezing in liquid nitrogen with grinding and strong buffers for the cell walls to be broken and DNA to be successfully extracted [7,27].

The CTAB (cetyltrimethylammonium bromide) method for fungal DNA extraction is well established [2,28]. In addition to commercially available DNA extraction kits, there have been other methods reported including *Basidiomycete* colony-PCR with the use of a microwave and extraction buffers, filamentous fungal colony-PCR using a lysis buffer and the Ampdirect® Plus buffer or alternatively using FTA (Flinders Technology Associates) cards [3,16,30]. However, to date, DNA extraction techniques for filamentous Ascomycete fungi are time-consuming and/or expensive.

We have developed an efficient and quick method to extract DNA from different fungal tissues using sonication which can be successfully used to perform downstream PCR analysis for detection and screening of fungal pathogens. Using a sonication water bath, fungal tissue is exposed to ultrasonic frequencies waves (>20 kHz). The sonic waves pass through the water-fungal suspension in the tube, breaking the cell walls and disrupting cell membranes. This subsequently allows the release of the cell contents including fungal genomic DNA. This method can simplify the diagnosis and identification of fungal pathogens that cause crop disease.

In this work, we analysed fungal DNA from different tissue of fungal pathogens that cause crop disease: 1) fungal mycelium of *Z. tritici, F. graminearum,* and *B. cinerea* from *in vitro* cultures; 2) mature *Z. tritici* asexual fruiting bodies (pycnidia) from infected wheat leaves and 3) spores including sexual ascospores from spore trap tapes installed in wheat fields.

2. Materials and methods

2.1. Rapid DNA extraction from Z. tritici, F. graminearum and B. cinerea mycelium

Cultures of *Z. tritici* (IPO323) provided by Dr A. Bailey, University of

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Abbreviations

A260/280	260 nm/280 nm absorbency ratio
Bc	*Botrytis cinerea*
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribonucleic acid
EFII	Elongation Factor II
Fg	*Fusarium graminearum*
FTA	Flinders Technology Associates
HYG	Hygromycin-trpC gene
ITS	Nuclear Internal Transcribed Spacer
JGI	Joint Genome Institute
KO	Knockout(s)
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
UK	United Kingdom
UV	Ultraviolet
WT	Wild type
YPDA	Yeast Peptone Dextrose Agar
Zt	*Zymoseptoria tritici*
ΔZt	*Zymoseptoria tritici* knockout mutants

Bristol, UK [19], *F. graminearum* (HUGR2) provided by Prof. F. Doohan, University College Dublin, Ireland [5] and *B. cinerea* (CBS 120092) was provided by Prof. Trevor Hodkinson and Dr Brian Murphy at Trinity College Dublin, Ireland [25] were grown on Yeast Peptone Dextrose Agar (YPDA) (yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/L and technical agar 20 g/L) or Potato Dextrose Agar (PDA) (potato dextrose broth 24 g/L, technical agar 20 g/L) medium for five to six days in a growth cabinet with a temperature of 20 °C and 16:8 light/dark cycles.

Approximately 10 mg of fungal mycelium was harvested with a small sterile 0.5–10 μL pipette tip. The fungal tissue was suspended in 200 μl of Sterile Distilled Water (SDW) in a safe seal 1.5 ml tube. The tubes containing the fungal suspension were then placed in a floating rachet and exposed to sonication in a Sonication Bath (Ultrasonic Cleaners Elmasonic™ S 100 H). The sonicator was filled with a 50:50 mix of distilled water and ice, and sweep mode was selected for 25 min. After sonication, the samples were incubated on ice for 10 min to allow the fungal mycelia to settle to the bottom of the tube. The supernatant containing the DNA was transferred to a clear tube. DNA was quantified by UV spectrophotometry using a nanodrop (Thermo Scientific, NanoDrop™ Lite) for quantity (ng/μl) and quality (A260/280) of the samples (Table 1).

2.2. Rapid DNA extraction from *Z. tritici* asexual fruiting bodies from wheat infected leaves

Seedlings of the susceptible wheat variety Longbow were grown in John Innes no.2 compost. The plants were kept in a controlled environment with a temperature of 20 °C, 16:8 light:dark cycles and 80–85% humidity until tillering stage (14–16 days). Vegetative cells from *Z. tritici* IPO323 YPDA cultures were suspended in 0.01% Tween20 at a concentration of 1×10^6 spores/ml. The wheat seedlings were then sprayed with 20 ml of the *Z. tritici* cell suspension using a plastic hand-held spray bottle.

The wheat plants were sealed in a clear bag to maintain humidity for the initial 72 h of infection and incubated at 20 °C and 16:8 light:dark cycles. Following 72 h, bags were removed, and the plants remained in the growth chamber at 20 °C, 16:8 light:dark cycles, relative humidity 80 ± 5%.

At 32 days post-infection, the mature asexual fruiting bodies (pycnidia) were visible on the infected plants. Using a piece of Sellotape (2 cm^2) a single lesion of the leaf was touched, allowing the oozing spores (cirrhi) from the pycnidia to remain attached to the tape. Single tape sections were cut in half (1 cm^2) and then placed in a sterile 1.5 ml Eppendorf tube and 500 μl of SDW was added. The samples were then sonicated following the same protocol as for the mycelial tissue above.

2.3. Rapid DNA extraction from spore trap tapes

Mellinex® adhesive (silicone fluid) tapes from volumetric air-spore

Table 1
DNA concentration ng/μl and ratio of the absorbance at 260 and 280 nm absorbance of samples.
Mellinex(R) tape and Sellotape pycnidia samples were suspended in 500 μl of SDW and mycelia were suspended in 200 μl of SDW. Yield % was calculated by [Total Yield (μg)/Initial yield (10 mg–>10,000 μg)]*100%.

MATERIAL	SAMPLE	SAMPLE NAME	ng/μl	TOTAL YIELD μg	% YIELD	A260/280
SPORES MELINEX® TAPE	Bishop Burton week 1	B_1	9.1	4.5	NA	1.2
	Carnoustie week 1	C_1	5.9	2.9	NA	1.28
	Swindon week 1	S_1	3.8	1.9	NA	1.23
	Lenham week 1	L_1	8.1	4	NA	0.78
	Bishop Burton week 3	B_3	9.4	4.7	NA	1.21
	Carnoustie week 3	C_3	29.7	14.8	NA	1.21
	Swindon week 3	S_3	12.4	6.2	NA	1.33
	Lenham week3	L_3	23.4	11.7	NA	1.21
FUNGAL PYCNIDIA SELLOTAPE	*Z. tritici* pycnidia	Zt_py_1	2.8	1.4	NA	1.41
	Z. tritici pycnidia	Zt_py_2	6.1	3	NA	1.03
	Z. tritici pycnidia	Zt_py_3	4.9	2.4	NA	1.05
	Z. tritici pycnidia	Zt_py_4	3.1	1.5	NA	1.02
FUNGAL MYCELIUM	*Z. tritici*	Zt_1	28.6	5.7	0.057	1.21
	Z. tritici	Zt_2	54.2	10.8	0.108	1.17
	F. graminearum	Fg_1	58.7	11.7	0.117	2.45
	F. graminearum	Fg_2	79	15.8	0.158	1.54
	B. cinerea	Bc_1	8.7	1.7	0.017	1.21
	B. cinerea	Bc_2	3.2	0.64	0.006	1.21
	Z. tritici knockout mutant 1	ΔZt_1	75.6	15.1	0.151	2.21
	Z. tritici knockout mutant 2	ΔZt_2	81.1	16.2	0.162	2.22
	Z. tritici knockout mutant 3	ΔZt_3	80.3	16	0.16	2.07
	Z. tritici knockout mutant 4	ΔZt_4	100.7	20	0.2	1.88
	Z. tritici knockout mutant 5	ΔZt_5	110.5	22.1	0.221	1.9
	Z. tritici knockout mutant 6	ΔZt_6	80	16	0.16	2.63
	Z. tritici knockout mutant 7	ΔZt_7	74.3	14.9	0.149	2.15
	Z. tritici knockout mutant 8	ΔZt_8	77.5	15.5	0.155	2.03

traps Lanzoni and Burkard brands were collected from four wheat field sites in the UK during June 2019: Carnoustie, Angus; Bishop Burton, Yorkshire; Swindon, Wiltshire and Lenham, Kent. Different airborne particles including fungal spores present in the air were captured on the tape's surface. *Z. tritici* ascospores were identified on tapes using light microscopy (Leica DM5500B). Sections approximately 2 cm^2 in size from each of the tapes were cut into smaller pieces, inserted into 1.5 ml tubes, and suspended in 500 μl SDW for sonication and DNA extraction as above, followed by quantification (Table 1).

2.4. PCR amplification and primers

The PCR cycles used for fragments between 100bp-1kb, fragments over 1 kb and multiplex PCR are listed in Table 2. The PCR reaction mix was prepared following the DreamTaq Green PCR protocol: 10X DreamTaq Buffer (5 μl); dNTP Mix 2 mM each (5 μl); Forward Primer (0.1–1.0 μM); Reverse Primer (0.1–1.0 μM); Template DNA (10 pg–1 μg); DreamTaq DNA Polymerase (1.25 U); Nuclease Free Water (to 50 μl) (Thermo Fisher Scientific), all primers used in this study are shown in Table 3.

The mycelia sonicated DNA was analysed with PCR primers for the nuclear internal transcribed spacer (*ITS*) region [33] (Table 3) using the PCR conditions for fragments over 1 kb (Table 2). An agarose gel, 1% was run to visualize the PCR products.

Z. tritici specific primers were designed for the *Z. tritici* housekeeping gene *Elongation Factor II* (*EF2*), and primers for a multiplex-PCR were designed to target *Z. tritici* virulence effector genes such as *Avr3D1* and *AvrStb6* [24,35]; *Mg3LysM* [22]; *ZtSSP2* [18] and *Zt-11* [36]. All primers were designed based on the gene annotations from the Joint Genome Institute (JGI) genome portal for *Z. tritici* [13]. Multiplex-PCR primers were designed using the online tool Oli2go [15]. PCR products from sellotaped sonicated pycnidia were visualized on 2% agarose gels. PCR products from the multiplex and *EFII* were purified using the Invitrogen PureLink™ quick PCR purification kit (Thermo Fisher Scientific) and sequenced by Macrogen Europe B.V. to confirm the correct target was amplified for each primer set.

DNA from spore trap tape was used to perform PCR for the *ITS* region to assess the presences of fungal DNA and *EFII Z. tritici* specific primers to confirm the presence of the pathogen on the spore trap tape (Tables 2 and 3), products were run on a 1% agarose gel.

Table 2
PCR program for fragments between 100bp and 1kb
The three different PCR programs were designed and used depending on the PCR product final size, fragments between 100bp and 1 kb, fragments longer than 1 kb, and a separate program for the Multiplex.

PCR program for fragments between 100bp and 1 kb			
Reaction	**Temperature**	**Time**	**Cycles**
Initialization	94 °C	2 min	**x1**
Denaturation	94 °C	30 s	**x35**
Annealing	56 °C	30 s	
Elongation	72 °C	45 s	
Final Elongation	72 °C	10 min	**x1**
Hold	4 °C	∞ hold	**∞ hold**
PCR program for fragments longer than 1 kb			
Reaction	**Temperature**	**Time**	**Cycles**
Initialization	94 °C	2 min	**x1**
Denaturation	94 °C	30 s	**x35**
Annealing	56 °C	30 s	
Elongation	72 °C	1½ - 2 min.	
Final Elongation	72 °C	10 min	**x1**
Hold	4 °C	∞ hold	**∞ hold**
PCR program for Multiplex			
Reaction	**Temperature**	**Time**	**Cycles**
Initialization	95 °C	2 min	**x1**
Denaturation	95 °C	30 s	**x35**
Annealing	57 °C	30 s	
	58 °C		
	59 °C		
Elongation	72 °C	1½ - 2 min.	
Final Elongation	72 °C	10 min	**x1**
Hold	4 °C	∞ hold	**∞ hold**

2.5. Knockout generation protocol

To further investigate the application of this method, the protocol was tested for *Z. tritici* knockout mutant generation and screening [31, 37].

The knockout (KO) plasmid was constructed by yeast-based homologous recombination using the *Saccharomyces cerevisiae* Y10,000 strain. The *pCAMBIA0380_YA* vector was used as a backbone for the KO plasmids. A 1.5 kb upstream (Left Flank, L.F.) and downstream (Right Flank, R.F.) flanking regions were selected for the effector gene candidates based on the genome database available for *Z. tritici (Zt)* IPO323 [13]. Amplification of these regions was carried out by PCR using sonicated DNA from IPO323 with specific primers (Additional file 1). The *Hygromycin-trpC* (*HYG*) antibiotic resistance gene cassette was PCR amplified from *pCAMBIA_PKS1* plasmid [20] using Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and PCR product purification. Successive recovery of the plasmids from *S. cerevisiae* was performed then transformed into *Escherichia coli DH5α* cells. Colony PCR on *E. coli* were performed to confirm the presence of the correct KO plasmid. In the second phase, *Agrobacterium tumefaciens EHA105* strains cells were transformed with the KO plasmid recovered from *E. coli*. *A. tumefaciens* transformants were used to mediate *Zt* transformation. The *ΔZt* obtained from the transformation were grown in selective antibiotics media with Hygromycin-B and Timentin™ for 14–20 days, then the obtained colonies were sub-cultured on YPDA, a more nutrient-rich media as before under antibiotics conditions for an additional selection. Colonies that survived the antibiotic selection were PCR screened for presence/absence of the Wild Type (WT) gene and the KO amplicon using forward primers designed 2 kb upstream, reverse primers were designed within our target gene for the WT and within the *HYG* gene resistance for the KO (Additional file 1).

3. Results

In this work, we developed a sonication-based PCR method by analysing fungal DNA from different tissues (Fig. 1). These include yeast-like growth of cultured *Z. tritici* and fungal mycelium of *F. graminearum* and *B. cinerea* (Fig. 1.1a), mature *Z. tritici* asexual fruiting bodies (pycnidia) from infected wheat leaves (Fig. 1.1b) and spores including sexual ascospores, from spore trap tapes installed in wheat fields across the UK (Fig. 1.1c).

3.1. DNA extracted from fungal hyphal and yeast-like growth using sonication

Sonication was performed on three cultured plant pathogenic fungi: *Z. tritici, F. graminearum* and *B. cinerea.* Nanodrop analyses (Thermo Fisher Scientific, NanoDrop™ Lite) to check the quality and quantity of the DNA extracted demonstrates that the sonication method can obtain genomic DNA concentrations up to ~100 ng/μl and yields of 0.221% (Table 1). The DNA from sonicated mycelia was successfully analysed with PCR primers for the nuclear internal transcribed spacer (*ITS*) region [33] for *Z. tritici. F. graminearum* and *B. cinerea* (Fig. 2). Therefore, this method can be used to obtain DNA from the fungal hyphal and yeast-like growth *in vitro*.

3.2. DNA extracted from pycnidia using sonication

It was also possible to carry out the sonication method for DNA extraction from asexual spores (pycnidiospores) of *Z. tritici* from infected leaves. Using a piece of Sellotape (2 cm^2) a single lesion of the leaf was touched allowing fungal tissue from the pycnidia and pycnidiospores to

Table 3
Primers used in this study:
PCR products obtained from primers of novel design were sequenced to confirm the correct gene was amplified. *Multiplex primers designed with the online tool Oli2go. † White et al., 1990.

Gene	Name	Primer	Sequence	Tm °C	Product size (bp)
Elongation Factor II	Fwr_EF2_Zt	Forward	CGGTCGTCTCTTCACAGCTT	60.04	286
Elongation Factor II	Rvs_EF2_Zt	Reverse	GGCAGTCGACTTGATGGTCA	60.04	
ITS region	ITS1	Forward†	TCCGTAGGTGAACCTGCGG	68.5	1200–1500
ITS region	ITS4	Reverse†	TCCTCCGCTTATTGATATGC	61.5	
AvrStb6	Fwr_mpx_AvrStb6	Forward*	CTCCATTCTACAAGGCCTCCT	65.2	212
AvrStb6	Rvs_mpx_AvrStb6	Reverse*	CTGTGACACCTTGGATATTG	59.7	
Avr3D1	Fwr_mpx_Avr3D1	Forward*	CTCGATTACTCTTTCCTTCTTTCTT	63.1	545
Avr3D1	Rvs_mpx_Avr3D1	Reverse*	CGAAACGCAATGGACTCCT	64.2	
Mg3LysM	Fwr_mpx_Mg3LysM	Forward*	AGATTCCCTGTTCCGCGTCA	67.8	478
Mg3LysM	Rvs_mpx_Mg3LysM	Reverse*	TGGCGGGAATGCTGAGG	65.4	
Zt11	Fwr_mpx_104	Forward*	CAATTACCCCACACCATCAA	61.8	301
Zt11	Rvs_mpx_104	Reverse*	CCGCCAAGTCGACGAGA	64.9	
ZtSSP2	Fwr_mpx_105	Forward*	AACCACCCATCAATTTCAAGA	61.9	405
ZtSSP2	Rvs_mpx_105	Reverse*	GGTGGCGTCATTGGGGTTG	67.7	

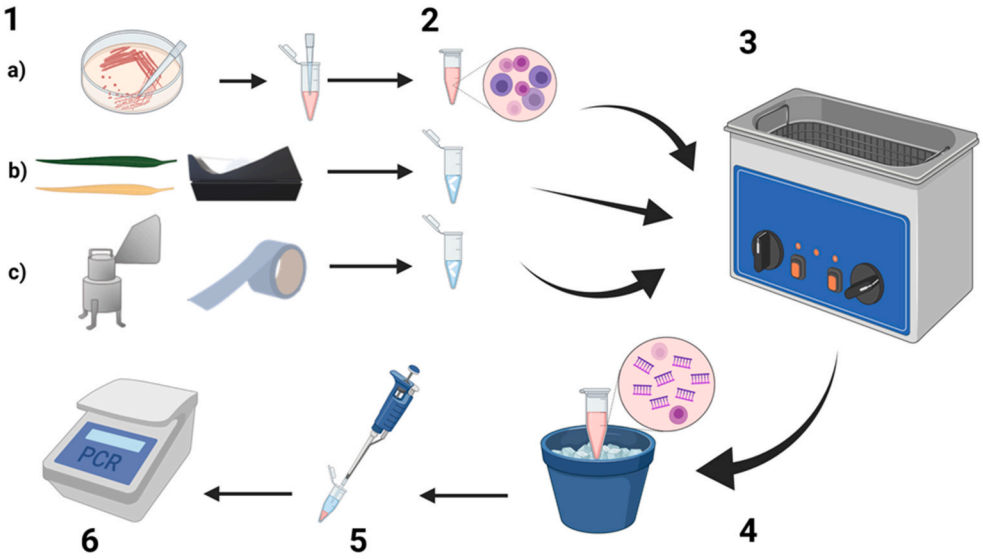

Fig. 1. Overview of the sonication method steps: 1. Samples can be acquired from a) 10 mg of fungal mycelia from 5 to 7 day old cultures on PDA/YPDA plates (petri dish image of a *Z. tritici* culture, pink yeast-like growth *in vitro*); b) pycnidia from leaves with 2 cm^2 of Sellotape; c) spore trap melinex tape sections 2 cm2 2. Suspend the sample in a 1.5 ml tube with sterile distilled water. 3. Place the tubes in the Sonication bath with 50:50, ice:water. 4. Let the samples settle on ice. 5. Use the supernatant as DNA template and 6. Perform PCR analysis. Image created with BioRender (Licence for publication number JM22VOE55J). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

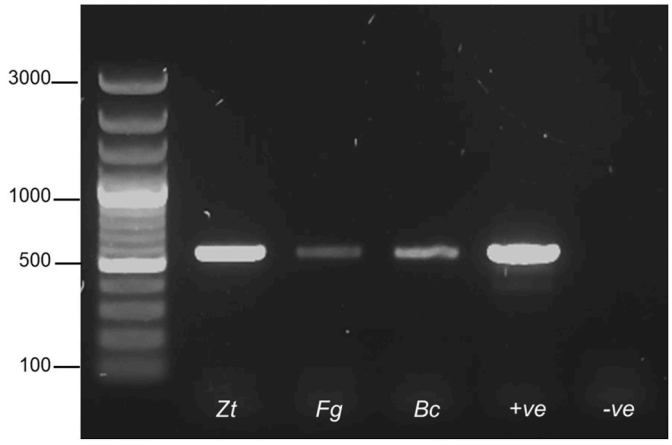

Fig. 2. PCR products of the Nuclear *ITS* region from *Zymoseptoria tritici (Zt)*, *Fusarium graminearum (Fg)* and *Botrytis cinerea (Bc)*. Positive control *Z. tritici* DNA extracted with DNeasy Plant Mini Kit (Qiagen) (+ve), negative control (-ve) sterile distilled water. 1% Agarose gel electrophoresis run at 100V for 25 min. GeneRuler 100bp plus DNA ladder.

be captured. The fungal DNA extracted (Table 1) was used to successfully amplify products from the *Z. tritici* housekeeping gene *elongation factor II* (*EFII*) and virulence/effector gene products from *AvrStb6* [35], *Avr3D1* [24], *Mg3LysM* [22], *ZtSSP2* [18] and *Zt11* [36] (Fig. 3a) using the conditions in Table 2 and primers in Table 3. These effector gene primers (Table 3) were subsequently used in a multiplex-PCR which amplified all expected effector product sizes (Fig. 3b).

3.3. DNA extracted from spore trap tapes using sonication

Spore trap tapes can collect a representative sample of the fungal spores present in the air in the specific location where the machine is installed. We used the sonication method to extract DNA directly from spore trap tapes collected from four wheat field sites in the UK during June 2019 (Carnoustie, Angus; Bishop Burton, Yorkshire; Swindon, Wiltshire and Lenham, Kent). The extracted DNA was used for PCR analysis using primers specific to the fungal *ITS* region (Fig. 4, Table 3) which can be used to distinguish different fungal species [33].

Furthermore, to test for the presence of *Z. tritici* ascospores in the field we used primers specific for the *Z. tritici* housekeeping gene *EFII*. Two of the spore trap tapes tested from Swindon (S) and Lenham (L) field sites were positive for the presence of *Z. tritici* (Fig. 5).

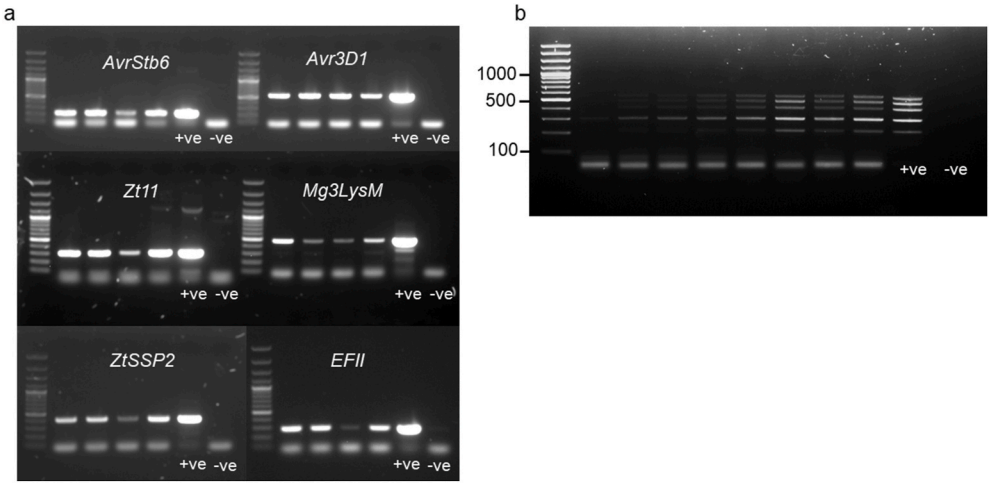

Fig. 3. a) Products of *AvrStb6* [35], *Avr3D1* [24], *Mg3LysM* [22], *ZtSSP2* [18] and *Zt11* [36] and the housekeeping gene *EFII* were amplified using gDNA extracted by the sonication method with Sellotape following contact with *Z. tritici* pycnidia from four leaves (1–4). Positive control *Z. tritici* gDNA extracted with DNeasy Plant Mini Kit (Qiagen) (+ve), negative control (-ve) sterile distilled water. Electrophoresis with 2% agarose gel was run at 100V for 30 min. Thermo Scientific™ GeneRuler 100bp plus DNA ladder. **b)** Multiplex PCR of *AvrStb6, Avr3D1, Mg3LysM, ZtSSP2* and *Zt11* for one of the four pycnidia samples (Py1), Lane 1–8 PCR with different volumes of extracted DNA from 1 μl to 8 μl. Genomic *Zt* DNA (gDNA) as a positive control was extracted with DNeasy Plant Mini Kit (Qiagen) (+ve) and negative control sterile distilled water (-ve). Electrophoresis with 2% agarose gel was run at 100V for 30 min. Thermo Scientific™ GeneRuler 100bp plus DNA ladder.

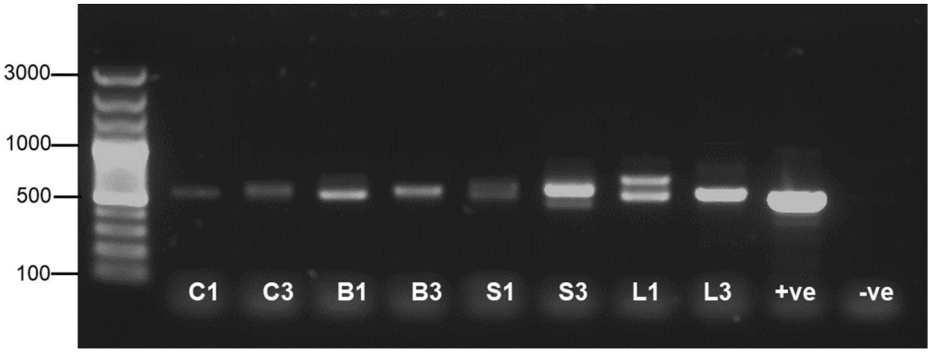

Fig. 4. PCR products with fungal *ITS* region primers on DNA extracted using sonication method from spore trap tapes sites in the UK. These include Carnoustie (C), Bishop Burton (B), Swindon (S) and Lenham (L). Samples from 2019, the 1st (1) and 3rd week of June (3), respectively. 1% Agarose gel electrophoresis run at 100V for 25 min. Genomic DNA (gDNA) extracted with DNeasy Plant Mini Kit (Qiagen) (+ve) and negative control sterile distilled water (-ve). GeneRuler 100bp plus DNA ladder.

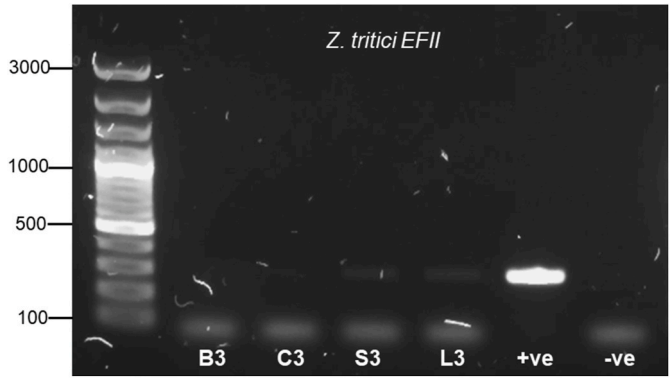

Fig. 5. PCR products with *Z. tritici* specific housekeeping gene *EFII* primers on DNA extracted using the sonication methods from spore trap tapes over 4 UK sites. Carnoustie (C), Bishop Burton (B), Swindon (S) and Lenham (L). Samples from 2019, the 3rd week of June (3). 1% Agarose gel electrophoresis run at 100V for 25 min. Genomic DNA (gDNA) extracted with DNeasy Plant Mini Kit (Qiagen) (+ve) and negative control SDW (-ve). GeneRuler 100bp plus DNA ladder.

3.4. DNA extracted using the sonication method can be used for fungal knockout generation and screening

To further investigate the application of this method, the protocol was tested for *Z. tritici* knockout mutant generation and screening [31, 37]. One of the preliminary stages in *Z. tritici* knockout generation is the synthesis of the knockout vector. This contains a 1.5 kb region upstream of the gene of interest; a 1.5 kb region downstream of the gene of interest; and a *Hygromycin-TrpC* (*HYG*) resistance gene cassette. The 1.5 kb flanking regions upstream and downstream of the gene of interest were obtained using a PCR reaction performed using *Z. tritici* genomic DNA obtained with the sonication protocol from mycelium. Specific primers were used to amplify the flanking regions designed from the *Z. tritici* IPO323 reference genome using the JGI genome portal [13,26] (Additional file 1) (Fig. 6a).

Following the successful transformation of *Z. tritici* by *A. tumefaciens*, the potential knockout mutants were screened to confirm that the gene of interest had been deleted and substituted with the antibiotic resistance gene (*Hygromycin-trpC*). The mutant colonies of *Z. tritici* were analysed using the colony sonication-PCR method (Fig. 6b, Additional file 1). With this technique, the time needed to screen the presence/absence of both genes was reduced. Complete screening of at least forty mutants could be completed in one day.

4. Discussion

Most fungal DNA extraction methods disrupt the fungal cell wall using buffers such as the non-ionic detergent CTAB (cetyltrimethylammonium bromide) [2,28]. The sonication technique described here can be used for fast and inexpensive fungal DNA extraction and PCR screening. There is no cost in terms of extraction buffers per sample other than the original purchase of the sonicator. The cost of which, (€80 upwards) could be repaid in a short time (after ~20

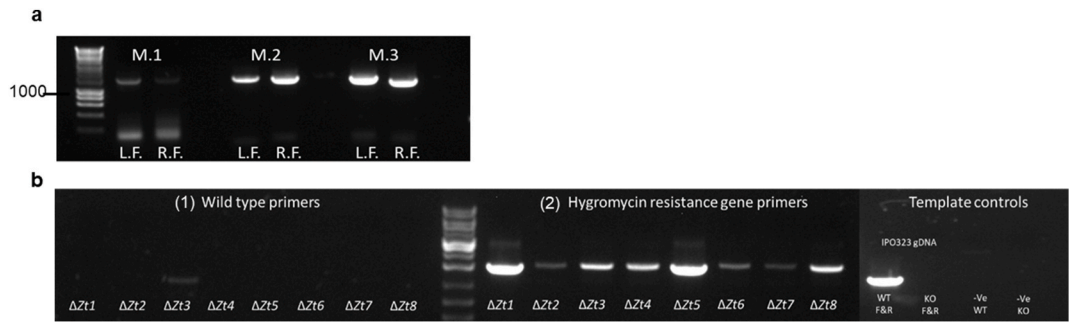

Fig. 6. a) PCR isolation of flanking regions from sonicated IPO323 DNA (Upstream = Left Flank: L.F.; Downstream = Right Flank: R.F.), for the plasmid assembly of 3 mutant candidates (M.1, M.2, M.3) on 1% Agarose gel electrophoresis run at 100V for 25 min. Hyperladder™ 1 Kb. **b)** PCR products from screening of eight candidates *Z. tritici* knockout mutants (*ΔZt1-8*) on 1% Agarose gel electrophoresis run at 100V for 25 min. The presence/absence of bands shows (1) absence of a band (size ~ 1 Kb) indicates the wild-type gene has been deleted or (2) presence of a band (size ~1.5 Kb) indicates the gene has been successfully substituted with the *Hygromycin-trpC* cassette. Positive control IPO323 gDNA extracted with DNeasy Plant Mini Kit (Qiagen) WT-F&R (Band = +ve), KO–F&R (No Band = +ve), negative control (-ve) SDW. Hyperladder™ 1 Kb.

samples) in comparison to around €4 per sample for extraction of fungal genomic DNA using commercial kits that have expiration date of 6–12 months once opened.

Previous reports of using sonication for fungal DNA extraction (from the Ascomycete *Aspergillus fumigatus*) were performed using CTAB buffer with and without glass beads at 55 °C [32]. The percentage yield for this study was around five times lower (0.028%) than the average yield percentage we obtained using the sonication method described here (0.13%) (Table 1). The same study compared different methods of DNA extraction using glass beads and vortexing which could extract DNA in 2–3 h [32]. Commercial kits (eg. Qiagen® Plant Mini Kit), take around an hour for DNA extraction. Previous work on fungal knockout screening [4] used a rapid mini preparation method from Ref. [21]; which involves the use of five different buffers and also takes around 1 h per reaction. These methods are therefore more time consuming compared to the sonication method described here, which can be completed in 35 min without the need for buffers.

Using this sonication method the range of DNA yield from the Mellinex® spore trap tapes and Sellotape was lower (3.1–29.7 ng/μl) than gained from the fungal mycelia (3.2–100.7 ng/μl) (Table 1). This could be due to lower amounts of starting material from the fungal spores or *Z. tritici* pycnidia or it is possible that the silicone/acrylic adhesive on the surface of the Mellinex® tape and Sellotape respectively interfered with the DNA extraction.

This technique has the potential to be applied to other important filamentous Ascomycete fungi and further studies should be carried out on using this technique in other species or phylum such as Basidiomycetes. Previously, *Z. tritici* DNA extraction from spore trap tapes was performed with glass beads in a high-speed benchtop homogenizer (FastPrep machine), to assay for fungicide resistant ascospores [12]. This sonication extraction method could be used with spore trap tapes in combination with species-specific primers to monitor pathogens in the field.

The method described was also successfully used for multiplex PCR where more than one target sequence is amplified using multiple pairs of primers (Fig. 3b). The quality and quantity of the DNA isolation technique impacts the PCR outcome [6]. Multiplex PCR is normally sensitive to contaminants such as DNases and other enzymes which can inhibit the reaction [29]. The successful multiplex PCR conducted here for our samples suggests that the sonication-method is a valid tool to obtain high-quality DNA. Our positive Multiplex PCR results using Sellotape following contact with pycnidia suggests that this technique could be developed for monitoring fungal pathogens in the field. For example, diagnostic assays using multiplex PCR have been used to detect fungal pathogens of wheat *Puccinia graminis f. sp. tritici, Puccinia triticina,* and *Blumeria graminis f. sp. tritici*, which cause wheat stem rust, wheat leaf rust, and wheat powdery mildew respectively [8]. Up to 12 fungi associated with fruit rot in cranberry could be identified using multiplex PCR with the use of *ITS-LSU* and *TEF-1α* gene regions [9]. While for yellow/stripe rust (caused by *Puccinia striiformis f. sp. tritici*) of wheat multiplex PCR has been used for genotyping pathogen populations [1]. This sonication-PCR method has also proved to be an efficient screening tool for the generation of knockout mutants allowing the amplification of fragments between 100 bp and up to 2 Kb in size.

In conclusion, the sonication method is a fast, inexpensive, and convenient tool in everyday laboratory PCR screening. It also has the possibility to be developed for the early detection of fungi from spore traps or for field-based assays.

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Authors' contributions

Paola Pilo: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Anna Tiley: Methodology, Writing – Review & Editing, Colleen Lawless and Sujit Karki: Validation, Investigation, Writing – Review & Editing. James Burke: Funding acquisition. Angela Feechan: Conceptualization, Supervision, Writing – Review & Editing. All the authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pmpp.2021.101758.

References

[1] S. Ali, A. Gautier, M. Leconte, J. Enjalbert, C. De Vallavieille-Pope, A rapid genotyping method for an obligate fungal pathogen, Puccinia striiformis f. sp. tritici, based on DNA extraction from infected leaf and Multiplex PCR genotyping, BMC Res. Notes 4 (2011) 1–5.

[2] G.C. Allen, M.A. Flores-Vergara, S. Krasynanski, S. Kumar, W.F. Thompson, A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide, Nat. Protoc. 1 (2006) 2320–2325, https://doi.org/10.1038/nprot.2006.384.

[3] M.M. Alshahni, K. Makimura, T. Yamada, K. Satoh, Y. Ishihara, K. Takatori, T. Sawada, Direct colony PCR of several medically important fungi using Ampdirect plus, Jpn. J. Infect. Dis. 62 (2009) 164–167.
[4] A.M.M. Tiley, H.J. White, G.D. Foster, A.M. Bailey, The ZtvelB gene is required for vegetative growth and sporulation in the wheat pathogen Zymoseptoria tritici, Front. Microbiol. 10 (2019).
[5] J. Brennan, B. Fagan, A. Van Maanen, B. Cooke, F. Doohan, Studies on in vitro growth and pathogenicity of European Fusarium fungi, Eur. J. Plant Pathol. 109 (2003) 577–587.
[6] K. Cankar, D. Štebih, T. Dreo, J. Žel, K. Gruden, Critical points of DNA quantification by real-time PCR–effects of DNA extraction method and sample matrix on quantification of genetically modified organisms, BMC Biotechnol. 6 (2006) 1–15.
[7] Y. Chen, B.A. Prior, G. Shi, Z. Wang, A rapid PCR-based approach for molecular identification of filamentous fungi, 49, 675-679, https://dx.doi.org/10.1007/s12275-011-0525-3, 2011.
[8] S. Chen, Y.-Y. Cao, T.-Y. Li, X.-X. Wu, Simultaneous detection of three wheat pathogenic fungal species by multiplex PCR, Phytoparasitica 43 (2015) 449–460, https://doi.org/10.1007/s12600-014-0442-1.
[9] M. Conti, B. Cinget, J. Vivancos, P. Oudemans, R.R. Bélanger, A molecular assay allows the simultaneous detection of 12 fungi causing fruit rot in cranberry, Plant Dis. 103 (2019) 2843–2850, https://doi.org/10.1094/pdis-03-19-0531-re.
[10] C. Dweba, S. Figlan, H. Shimelis, T. Motaung, S. Sydenham, L. Mwadzingeni, T. Tsilo, Fusarium head blight of wheat: pathogenesis and control strategies, Crop Protect. 91 (2017) 114–122.
[11] M. Figueroa, K.E. Hammond-Kosack, P.S. Solomon, A review of wheat diseases-a field perspective, Mol. Plant Pathol. 19 (2018) 1523–1536, https://doi.org/10.1111/mpp.12618.
[12] B. Fraaije, H. Cools, J. Fountaine, D. Lovell, J. Motteram, J. West, J. Lucas, Role of ascospores in further spread of QoI-resistant cytochrome b alleles (G143A) in field populations of Mycosphaerella graminicola, Phytopathology 95 (2005) 933–941.
[13] S.B. Goodwin, S. Ben M'barek, B. Dhillon, A.H. Wittenberg, C.F. Crane, J.K. Hane, A.J. Foster, T.A. Van Der Lee, J. Grimwood, A. Aerts, Finished Genome of the Fungal Wheat Pathogen Mycosphaerella Graminicola Reveals Dispensome Structure, Chromosome Plasticity, and Stealth Pathogenesis, 2011.
[14] G. Hariharan, K. Prasannath, Recent advances in molecular diagnostics of fungal plant pathogens: a mini-review, Frontiers in Cellular and Infection Microbiology 10 (2021) 829.
[15] M. Hendling, S. Pabinger, K. Peters, N. Wolff, R. Conzemius, I. Barišić, Oli2go: an automated multiplex oligonucleotide design tool, Nucleic Acids Res. 46 (2018) W252–W256.
[16] K. Izumitsu, K. Hatoh, T. Sumita, Y. Kitade, A. Morita, C. Tanaka, A. Gafur, A. Ohta, M. Kawai, T. Yamanaka, H. Neda, Y. Ota, Rapid and simple preparation of mushroom DNA directly from colonies and fruiting bodies for PCR, Mycoscience 53 (2012) 396–401.
[17] X. Kang, A. Kirui, A. Muszyński, M.C.D. Widanage, A. Chen, P. Azadi, P. Wang, F. Mentink-Vigier, T. Wang, Molecular architecture of fungal cell walls revealed by solid-state NMR, Nat. Commun. 9 (2018), https://doi.org/10.1038/s41467-018-05199-0.
[18] S.J. Karki, A. Reilly, B. Zhou, M. Mascarello, J. Burke, F. Doohan, D. Douchkov, P. Schweizer, A. Feechan, A small secreted protein from Zymoseptoria tritici interacts with a wheat E3 ubiquitin ligase to promote disease, J. Exp. Bot. 72 (2020) 733–746, https://doi.org/10.1093/jxb/eraa489.
[19] G.H.J. Kema, C.H. Van Silfhout, Genetic variation for virulence and resistance in the wheat-mycosphaerella graminicola pathosystem III. Comparative seedling and adult plant experiments, Phytopathology 87 (1997) 266–272, https://doi.org/10.1094/PHYTO.1997.87.3.266.
[20] B.T. Koay, Investigating Polyketide Synthase Genes of the Wheat Pathogenic Fungus Mycosphaerella Graminicola, University of Bristol, 2010.
[21] D. Liu, S. Coloe, R. Baird, J. Pederson, Rapid mini-preparation of fungal DNA for PCR, J. Clin. Microbiol. 38 (1) (2000) 471, https://doi.org/10.1128/JCM.38.1.471-471.2000.
[22] R. Marshall, A. Kombrink, J. Motteram, E. Loza-Reyes, J. Lucas, K.E. Hammond-Kosack, B.P.H.J. Thomma, J.J. Rudd, Analysis of two in planta expressed LysM effector homologs from the fungus mycosphaerella graminicola reveals novel functional properties and varying contributions to virulence on wheat, Plant Physiol. 156 (2011) 756–769, https://doi.org/10.1104/pp.111.176347.
[23] M. Mcmullen, G. Bergstrom, E. De Wolf, R. Dill-Macky, D. Hershman, G. Shaner, D. Van Sanford, A unified effort to fight an enemy of wheat and barley: Fusarium head blight, Plant Dis. 96 (2012) 1712–1728.
[24] L. Meile, D. Croll, P.C. Brunner, C. Plissonneau, F.E. Hartmann, B.A. Mcdonald, A. Sánchez-Vallet, A fungal avirulence factor encoded in a highly plastic genomic region triggers partial resistance to septoria tritici blotch, New Phytol. 219 (2018) 1048–1061, https://doi.org/10.1111/nph.15180.
[25] B.R. Murphy, E. Soldi, M.J. Jadwiszczak, T.R. Hodkinson, Synergy between fungal endophytes improves fruit production in strawberry cultivar, Emergent Life Sciences Research 5 (2019) 29–41.
[26] H. Nordberg, M. Cantor, S. Dusheyko, S. Hua, A. Poliakov, I. Shabalov, T. Smirnova, I.V. Grigoriev, I. Dubchak, The genome portal of the department of energy Joint genome Institute: 2014 updates, Nucleic Acids Res. 42 (2014) D26–D31, https://doi.org/10.1093/nar/gkt1069.
[27] S.-Y. Park, S.-H. Jang, S.-O. Oh, J.A. Kim, J.-S. Hur, An easy, rapid, and cost-effective method for DNA extraction from various lichen taxa and specimens suitable for analysis of fungal and algal strains, MYCOBIOLOGY 42 (2014) 311–316, https://doi.org/10.5941/myco.2014.42.4.311.
[28] S.O. Rogers, A.J. Bendich, Extraction of DNA from plant tissues. Plant Molecular Biology Manual, Springer, 1989.
[29] M. Sidstedt, P. Rådström, J. Hedman, PCR inhibition in qPCR, dPCR and MPS—mechanisms and solutions, Anal. Bioanal. Chem. 412 (2020) 2009–2023, https://doi.org/10.1007/s00216-020-02490-2.
[30] S. Suzuki, H. Taketani, K.-I. Kusumoto, Y. Kashiwagi, High-throughput genotyping of filamentous fungus Aspergillus oryzae based on colony direct polymerase chain reaction, J. Biosci. Bioeng. 102 (2006) 572–574. http://www.sciencedirect.com/science/article/pii/S1389172307700142.
[31] A.M.M. Tiley, G.D. Foster, A.M. Bailey, Exploring the genetic regulation of asexual sporulation in Zymoseptoria tritici, Front. Microbiol. 9 (2018), https://doi.org/10.3389/fmicb.2018.01859.
[32] J.-A. Van Burik, R. Schreckhise, T.C. White, R. Bowden, D. Myerson, Comparison of six extraction techniques for isolation of DNA from filamentous fungi, Med. Mycol. 36 (1998) 299–303.
[33] T.J. White, T. Bruns, S. Lee, J. Taylor, Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR protocols: a guide to methods and applications 18 (1990) 315–322.
[34] B. Williamson, B. Tudzynski, P. Tudzynski, J.a.L. Van Kan, Botrytis cinerea: the cause of grey mould disease, Mol. Plant Pathol. 8 (2007) 561–580. https://bsppjournals.onlinelibrary.wiley.com/doi/abs/10.1111/j.1364-3703.2007.00417.x.
[35] Z. Zhong, T.C. Marcel, F.E. Hartmann, X. Ma, C. Plissonneau, M. Zala, A. Ducasse, J. Confais, J. Compain, N. Lapalu, J. Amselem, B.A. Mcdonald, D. Croll, J. Palma-Guerrero, A small secreted protein in Zymoseptoria tritici is responsible for avirulence on wheat cultivars carrying the Stb6 resistance gene, New Phytol. 214 (2017) 619–631, https://doi.org/10.1111/nph.14434.
[36] B. Zhou, H.R. Benbow, C.J. Brennan, C. Arunachalam, S.J. Karki, E. Mullins, A. Feechan, J.I. Burke, F.M. Doohan, Wheat encodes small, secreted proteins that contribute to resistance to septoria tritici blotch, Front. Genet. 11 (2020). https://www.frontiersin.org/article/10.3389/fgene.2020.00469.
[37] L.H. Zwiers, M.A. De Waard, Efficient Agrobacterium tumefaciens-mediated gene disruption in the phytopathogen Mycosphaerella graminicola, Curr. Genet. 39 (2001) 388–393. https://www.ncbi.nlm.nih.gov/pubmed/11525415.