Manuscript Details

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Title	Development of a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) based on rDNA IGS sequence analysis for the detection of Macrophomina phaseolina in soil
Article type	Research paper

Abstract

The ‘Nucleic Acid Lateral Flow Immunoassay’ (NALFIA) using a generic ‘Lateral Flow Device’ (LFD), combined with PCR employing labelled primers (PCR-NALFIA), enables to circumvent the use of electrophoresis, making the diagnostic procedure more rapid and easier. If the specific amplicon is present in the sample, a coloured band, with an intensity proportional to the amplicon concentration, will develop on the LFD strip in addition to the control band. Species-specific primers for M. phaseolina based on the rDNA intergenic spacer (IGS) were developed and their specificity was checked and confirmed using 20 isolates of M. phaseolina and other 16 non-target fungi. A DNA extraction protocol based on a bead-beating technique using silica beads, skimmed milk and PVP was also developed. The M. phaseolina specific primers MP102F/MP102R, 5’ labelled with biotin and FITC respectively, were used in the PCR-NALFIA assay to identify the pathogen starting from mycelium or microsclerotia. Microsclerotia of M. phaseolina (1, 10, 100 and 200) were manipulated under a stereomicroscope and their DNA was extracted using microsclerotia alone or mixed with different types of soil. The resulting DNA, used for the PCR-NALFIA assay, provided positive results for all the samples tested. A semi-quantitative grey-scale reference card based on the PCR-NALFIA assay using intervals corresponding to microsclerotia soil number was developed. For this purpose, the normalized pixel grey volumes obtained after a densitometric analysis of the test line intensity generated by the LFD dipsticks were used.

Keywords	Botryosphaeriaceae; Molecular diagnosis; Nucleic acid lateral flow immunoassay (NALFIA); PCR; Soil-borne pathogens; rDNA-IGS region.
Taxonomy	Fungi, Microbiological Method, Biosensor, Molecular Biology

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Prof. M.J. Bailey
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Journal of Microbiological Methods

COVER LETTER FOR REVISION OF MANUSCRIPT N. MIMET_2018_202

Dear Prof. Bailey,

enclosed please find our manuscript number MIMET_2018_202 entitled "**Development of a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) based on rDNA IGS sequence analysis for the detection of *Macrophomina phaseolina* in soil**" revised according to reviewers' comments and suggestions which we would like to submit for publication as **Full length Original Research Article** in **Journal of Microbiological Methods**.

The manuscript is being submitted by me, Susanna Pecchia (Corresponding author), on behalf of all the authors.
With the submission of this manuscript, I would like to undertake that:

- All authors of this research paper have directly participated in the planning, execution, or analysis of this study;
- All authors of this paper have read and approved the final version submitted;
- The contents of this manuscript have not been copyrighted or published previously;
- The contents of this manuscript are not now under consideration for publication elsewhere;
- The contents of this manuscript will not be copyrighted, submitted, or published elsewhere, while acceptance by the Journal is under consideration;
- My Institute's (University of Pisa, Department of Agriculture, Food and Environment) representative is fully aware of this submission.
- We submitted sequences to GenBank and we will release them as soon as possible because in the meantime we received the acceptance of our Italian Patent Deposit (we have to modify only one claim) and we deposited the European Patent.

We shall look forward to hearing from you at your earliest convenience.

Yours sincerely

Date: 22.06.2018

Susanna Pecchia

Responses to the reviewer's comments

We sincerely thank the reviewers for constructive criticisms and valuable comments, which were of great help in revising the manuscript. Accordingly, the revised manuscript has been systematically improved with new information and additional interpretations. Our responses to the reviewers comments are given below in blue color.

Reviewer 1

In this manuscript the authors describe a PCR-NALFIA assay for the specific detection of an important fungal plant pathogen. The work was well planned and executed, the results seem to be scientifically sound and warrant the conclusions drawn. The authors did a good job in validating their assay. In my opinion this manuscript presents results worthy of being published in the Journal of Microbiological Methods. I have only some minor comments and suggestions that can be found in the attached revised manuscript file.

All comments and suggestions have been accepted.

Among the non-target organisms we tested 3 *Diplodia seriata* isolates. Anyway, please consider that we analyzed *in silico* 17 IGS sequences of fungi belonging to the family *Botryosphaeriaceae*.

The sentence regarding the patent has been moved to the Acknowledgements.

Reviewer 2

The Authors, with the manuscript entitled "Development of a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) based on rDNA IGS sequence analysis for the detection of *Macrophomina phaseolina* in soil", describe a new and fast method for the detection of this important pathogen.
The length of the manuscript is in some way related with the robust number of experiments reported: I have to remark that are not available in GenBank the sequence information related to Accession No. MH058030-MH058036 and in some way should be released before the publication.

The sequences will be released as soon as possible. The method and primers described in the manuscript are patent pending at Italian and European level. Few days ago we received the acceptance of our Italian Patent Deposit (we have to modify only one claim) and we deposited the European Patent. When we will have the final publication of the Italian patent, we will be able to release the sequences

Reviewer 3

This manuscript describes the detection of the fungal pathogen *Macrophomina phaseolina* using a combination of PCR and Nucleic Acid Lateral Flow Immunoassay (NALFIA) techniques and defines primers specific for this species. It also describes a bead-beating based DNA extraction method for

extracting DNA from mycelium or microsclerotia as well as various types of soils spiked with microsclerotia. In general, it is a good effort towards simplifying the detection of a plant pathogen by using a previously known combination of techniques (PCR & NALFIA) and customizing it specifically for this fungal species. In this manuscript, the use of English language and grammar needs significant improvement. Here are my specific comments:

Lines 93 – 95: This statement, in its own paragraph seems out of context.

Line 96 – 98: Again, there seems a disconnect with the previous statement. Use of 'Moreover' is making it complicated.

We changed the order of the paragraphs and we tried to better connect them.

Line 194: …and the resulting DNA pellet was rinsed…

Done as suggested.

Lines 197 – 202: Too many paragraphs. Try merging those.

Done as suggested.

Line 292: 10 µL of what? Please explain.

Done. 10 µL of PCR product.

Line 303: Is this serial dilution concentration for per µL?

Yes

Line 301 – 303: Strange sentence. Please rephrase. As an example you could say "PCR-NALFIA sensitivity was tested using isolate 10726 mycelium DNA as a PCR template serially diluted from 1.73 ng to 1.73 fg/µL".

Done as suggested

Line 341: Which results? The e is no evidence presented in the manuscript that the protocol did not cause severe shearing

We agree. There is no evidence presented in the manuscript. Anyway, we performed gel electrophoresis of total genomic DNA of isolates to verify their integrity as described in Materials and Methods (lines 212-216). In all the isolates tested a high molecular weight band, above the

10,000 bp band of the marker is well visible. We added the Figure S1 as supplement and we included a short sentence in the text.

Lines 369 – 371: Rephrasing required. You can try ..."DNA extraction using lysis buffer alone generated a brown supernatant resulting into a dark-brown DNA solution following DNA precipitation and pellet dissolving.

Done as suggested.

Lines 369 – 371: Considering that brown coloured DNA prep was obtained most probably due to the presence of humic acid and/or phenolic compounds, did you try removing those compounds by using some desalting slurry, or a PCR inhibitor removal method? For example, something like, OneStep™ PCR Inhibitor Removal Kit (Zymo-Research) may be helpful.

We evaluated different protocols in order to avoid the problem. We try to add BSA at different concentrations, cellulose powder, and a CTAB-urea protocol that includes the use of NaCl 5 M and guanidine hydrochloride 7 M. Anyway, these protocols did not provide good results.

Many thanks for the suggestion, however one of our goals was to set up a protocol with the following characteristics: simple, economic and safe (avoiding the use of toxic reagents and the use of commercial kits).

The solution of chloroform-isoamyl alcohol (24:1 v/v) represents the only toxic component of our extraction protocol.

The extraction method developed in this study requires 180 minutes (10-12 samples) overall and the extraction cost of a single sample was estimated to be around € 0.87. Estimated reagent cost was based on web available prices.

We also compared our method to 7 commercially-available kits for soil DNA extraction. Considering their use on 10-12 samples, the extraction procedure requires from 30 to 90 minutes with an estimated cost of about € 4.60 per reaction. The maximum amount of soil sample that can be processed is between 0.1 and 1 g of soil per reaction, exceptionally 10 g of soils can be used (MoBio PowerMax® Soil DNA Isolation Kit), however the cost for a single reaction was estimated to be considerably higher than other soil extraction kits with an average cost of € 22.63 per reaction.

Although the cost could be a limitation to the use of these kits, another major constraint is that they can only process small volumes of soil samples. Our extraction method (in our opinion) is flexible on the homogenization and extraction steps and can be adapted to process more than 10 g of soil per reaction.

Lines 388 – 390: Confusing sentence; rephrasing is required. ...but did not generate a PCR product for any of the 16 non-target species confirming their specificity for M. phaseolina.

Done as suggested.

Lines 391 – 393: What do you mean by 'have been previously tested'? Do you mean, in this study or a previous study?

In this study.

I would recommend deleting lines 387 – 393 and merge any new information from these lines with lines 396 – 404 in section 3.5. It seems a repetition here.

It seems a repetition, anyway in section 3.4 we used non labelled primer and in section 3.5 we used labelled primer. All the experiments performed with non labelled primer were performed again with labelled primer comparing two detection systems: NALFIA and agarose gel electrophoresis.

Line 405: Please also describe the DNA concentration used here as well for the quantity (1µL) used for each of the microsclerotia DNA prep groups.

Done as suggested.

Line 414: Again, DNA concentration in that 1 µL volume would be helpful here.

Done as suggested.

Lines 514 – 516: Please rephrase using correct grammar. This sentence is very confusing.

Necessary changes have been made and this part is now rewritten

Line 520: It is the first time in the manuscript that the volume of DNA prep (75 µL) is mentioned. This info should be included in the DNA extraction method section.

Done as suggested

Lines 531 – 533: Inappropriate use of English language again… Please rephrase.

Necessary changes have been made and this part is now rewritten

Fig 2: What is sample 10176? This has not been mentioned in the manuscript text. Also, I don't see sample 10726 in this gel. Is this a typo?

Yes, many thanks, it is a typo. We apologize for the mistake.

Fig 3: Including DNA concentrations of the template DNA used (µg or ng/µL) and the volumes of the PCR products used on the gel and for the NALFIA would be helpful here in the figure legend.

Done as suggested

Fig S3: Please include an explanation for multiple bands in the figure legend or in the manuscript text.

We included the explanation in the manuscript.

Fig S4: Please show both, with and without milk results side-by-side for the gel as well.

The gel with the use of milk is shown in Fig S3. Anyway we modified the Figure S4 as you suggested and we eliminated the Figure S3.

Highlights

- A PCR-NALFIA assay for the rapid detection of *M. phaseolina* in soil was developed.
- Species-specific primers for *M. phaseolina* targeting the rDNA IGS region were used.
- The assay showed high sensitivity and specificity for *M. phaseolina*.
- Detection level for *M. phaseolina* in soil samples was 1 microsclerotium g^{-1} of soil.
- A semi-quantitative grey-scale reference card from PCR-NALFIA results was created.

Development of a rapid PCR-Nucleic Acid Lateral Flow Immunoassay (PCR-NALFIA) based on rDNA IGS sequence analysis for the detection of *Macrophomina phaseolina* in soil

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ABSTRACT

The '*Nucleic Acid Lateral Flow Immunoassay*' (NALFIA) using a generic '*Lateral Flow Device*' (LFD), combined with PCR employing labelled primers (PCR-NALFIA), enables to circumvent the use of electrophoresis, making the diagnostic procedure more rapid and easier. If the specific amplicon is present in the sample, a coloured band, with an intensity proportional to the amplicon concentration, will develop on the LFD strip in addition to the control band.

Species-specific primers for *M. phaseolina* based on the rDNA intergenic spacer (IGS) were developed and their specificity was checked and confirmed using 20 isolates of *M. phaseolina* and other 16 non-target fungi.

A DNA extraction protocol based on a bead-beating technique using silica beads, skimmed milk and PVP was also developed.

The *M. phaseolina* specific primers MP102F/MP102R, 5' labelled with biotin and FITC respectively, were used in the PCR-NALFIA assay to identify the pathogen starting from mycelium or microsclerotia. Microsclerotia of *M. phaseolina* (1, 10, 100 and 200) were manipulated under a stereomicroscope and their DNA was extracted using microsclerotia alone or mixed with different types of soil. The resulting DNA, used for the PCR-NALFIA assay, provided positive results for all the samples tested. A semi-quantitative grey-scale reference card based on the PCR-NALFIA assay using intervals corresponding to microsclerotia soil number was developed. For this purpose, the normalized pixel grey volumes obtained after a densitometric analysis of the test line intensity generated by the LFD dipsticks were used.

Keywords: *Botryosphaeriaceae,* Molecular diagnosis, Nucleic acid lateral flow immunoassay (NALFIA), PCR, Soil-borne pathogen, rDNA-IGS region.

1. Introduction

Macrophomina phaseolina (Tassi) Goid. is a soil- and seed-borne generalist fungal pathogen that has a global distribution and can infect more than 500 plant species including monocot and dicot hosts (Farr *et al*., 1995; Kaur *et al*., 2012; Farr and Rossman, 2016). The fungus is likely to become more important under climate change scenarios of increased heat and drought stress (Ali and Dennis 1992, Saleh *et al.*, 2010, Manici *et al.*, 2012, 2014).

The pathogen produces microsclerotia in root and stem tissues of host plants, which enable it to survive in soil for 2–15 years and act as primary source of inoculum (Meyer *et al.*, 1974; Papavizas, 1977; Short *et al.*, 1980; Baird *et al.*, 2003). The disease occurrence and severity have been directly related to the population of viable sclerotia in soil (Khan, 2007).

There are many reports on the isolation and quantification of *M. phaseolina* from soil: flotation techniques (Watanabe *et al*., 1970), selective or semiselective media (McCain and Smith, 1972; Meyer *et al.*, 1973; Papavizas and Klag, 1975; Mihail and Alcorn, 1982; Cloud and Rupe, 1991). However, these soil bioassays are cumbersome, insufficiently sensitive or selective, very labor-intensive, soil dependent, and in many cases require the use of toxic chemicals. In addition, *M. phaseolina* is considered a poor competitor on agar plates (Cook *et al.*, 1973) and the presence of colonies of different fungi reduced its growth or inhibited sclerotia production needed for identification.

Hence, to overcome shortcomings related to culture-based diagnostic methods, nucleic acid (NA)-based techniques are definitely among the most powerful for rapid and sensitive detection of the pathogen.

The most commonly used DNA region targeted to design primers for PCR-based identification and detection of plant pathogenic fungi is the nuclear ribosomal DNA (rDNA), organised in units repeated many times in the genome, allowing a very sensitive detection. This region contains the 18S, 5.8S and 28S rRNA genes separated by two internal transcribed spacers (ITS1 and ITS2) and one intergenic spacer (IGS) (White *et al*., 1990).

79 The ITS regions have been extensively sequenced and shown to be useful to identify numerous
80 fungi at species or sub-species level. The IGS region on the other hand is known to evolve faster than
81 the ITS region, resulting to be the main source of polymorphisms in the rDNA gene complex in other
82 fungi (Jackson *et al*., 1999; Pecchia *et al*., 1998, 2004; Pramateftaki *et al.*, 2000; Pantou *et al*., 2003),
83 however, no IGS sequences are currently published for *M. phaseolina.* Compared to ITS region, IGS
84 poses more difficulties for amplification and sequencing, however, it can be useful when there are
85 not enough differences available across the ITS (Schena *et al.*, 2004a).
86 Since the higher copy number of IGS increases the sensitivity of PCR-based techniques, this
87 region has been used to develop specific diagnostic assays for many plant pathogenic fungi and
88 oomycetes such as *Botrytis cinerea* and *Botrytis* spp. (Suarez *et al*., 2005; Sanzani *et al.*, 2012; Khan
89 *et al.*, 2013), *Fusarium circinatum* (Schweigkofler *et al.*, 2004), *F. oxysporum* f. sp. *vasinfectum*
90 (Zambounis *et al.*, 2007), *Fusarium oxysporum* f. sp. *cubense* (Dita *et al.*, 2010), trichothecene-
91 producing *Fusarium* spp. (Jurado *et al.*, 2005), *Phytophthora medicaginis* (Liew *et al.*, 1998),
92 *Verticillium dahliae* and *V. alboatrum* (Schena *et al.*, 2004b; Bilodeau *et al.*, 2012).
93 The early diagnosis and screening of *M. phaseolina* from different soils needs a simple, rapid
94 and cost-effective test, without the use of sophisticated and expensive equipment and reagents not
95 fully affordable in ordinary laboratories. Recently, a molecular method has been exploited to develop
96 specific primers for a real-time qPCR assay using a sequence characterized amplified region (SCAR)
97 for the detection of *M. phaseolina in planta* and in rhizosphere soil (Babu *et al*., 2011).
98 A simplification of the methodology could be achieved by using simple and cheap read-out
99 systems, such as the nucleic acid lateral flow immuno-assay (NALFIA) (Amerongen and Koets, 2005;
100 Posthuma-Trumpie *et al.,* 2009), which can circumvent the use of electrophoresis or the purchase of
101 expensive real-time PCR machines.
102 The use of NALFIA as biosensor for detecting specific amplified nucleic acid genes is not new
103 (Rule *et al*., 1996) and has been widely used in the point-of-care (POC) devices for medical
104 diagnostics. To date, this technology has also reached many fields of research such as veterinary

105 diagnostic, food and environment monitoring and plant disease diagnosis, with a few cases limited to
106 fitovirus (Posthuma-Trumpie *et al.,* 2009; Ngom *et al.*, 2010; O'Farrel, 2013).

107 In NALFIA, nucleic acids are captured on lateral flow test strips in an antibody-dependent
108 format: the biosensor employs an antibody capture line and a labelled amplicon. In this case, an
109 amplified double-stranded nucleic acid sequence specific to a target organism can be detected by
110 using primers with two different tags (*e.g.* biotin and fluorescein isothiocyanate). The analyte is
111 recognized by binding to a tag-specific antibody (anti-fluorescein antibody) previously sprayed on a
112 nitrocellulose membrane, and gold nanoparticles labelled with avidin are used as reporter, enabling
113 the visualization (Amerongen and Koets, 2005).

114 Results are fast and can be directly observed by naked eye. Moreover, the utilization of a
115 membrane strip as immunosorbent provides an analytical platform that permits one-step, rapid and
116 low-cost analyses (O'Farrel, 2013). Furthermore, plant pathogen nanodiagnostic techniques will
117 enable to understand and control factors involved in plant diseases resulting in eco-friendly diagnostic
118 measurements (Khiyami *et al*., 2014).

119 This study describes the development of a species-specific PCR assay based on IGS sequences
120 combined with NALFIA for the rapid, highly sensitive, reproducible and specific detection of *M.*
121 *phaseolina* directly from pure cultures and soil samples (patent application pending). In this method,
122 a small volume of the final PCR solution is directly added to the one-step assay device and the
123 appearance of a grey/black line reveals the presence of the specific amplicon, detection is visual and
124 requires less than 5 min.

125 This is the first study, to our knowledge, to develop a PCR-NALFIA assay for detection of the
126 fungal pathogen *M. phaseolina* in agricultural soil samples.

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2. Materials and methods

2.1. Fungal cultures and microsclerotia production

Isolates used in this study are summarised in Table 1. Purified fungal cultures were routinely grown on Potato Dextrose Agar (PDA, Difco Laboratories, Detroit, MI, USA) and were maintained on PDA slants covered with mineral oil at 4°C for long-term storage.

Microsclerotia were produced following the protocol described by Papavizas and Klag (1975) with some modifications. Isolates of *M. phaseolina* were grown on PDA at 30°C for 7 days in the dark. The medium surface was covered with a sterilized permeable cellophane disc.

A sterile blade was used to scrape *M. phaseolina* from the cellophane to harvest microsclerotia into Falcon® 50 ml high-clarity polypropylene (PP) conical centrifuge tubes. Fungal materials were comminuted with a homogenizer/disperser (IKA Ultra-Turrax ® Type 18 Basic) in 20 ml tap water for four 10-s pulses, and then filtered through a nested set of Nylon Net Filters (Merck Millipore, Darmstadt, Germany) of 100 μm and 40 μm opening.

Microsclerotia retained on the 40 μm nylon filter were air dried and a sample was assessed under a stereo microscope to determine whether further blending was needed.

When dry, microsclerotia could be easily manipulated by sprinkling them onto small pieces of paper and moving them around with an artist's brush. Microsclerotia were picked up individually on the tip of a sterile needle under a stereo microscope according to the method of Ben-Yephet and Pinkas (1977).

Petri plates containing PDA were placed on a grid of 25 squares, and one microsclerotium was placed on the surface of the medium within each square. Four replicates were used for each experiment. Plates were incubated at 30°C in the dark for 7 days and the number of germinated microsclerotia was counted. In order to determine the viability of microsclerotia, dry sclerotia were plated 5 days and 6 months after their harvest.

157 *2.2 Soil sampling*

158 Five 200 g soil samples were randomly collected from three organic horticultural field crops
159 (A, B, and C respectively from spinach, melon and tomato) located in the Interdepartmental Center
160 for Agro-Environmental Research of University of Pisa where the presence of *M. phaseolina* was not
161 previously reported. Soil samples were dried at room temperature, ground with a mortar and pestle,
162 mixed well and sieved through a 2-mm pore-size screen.

163 Soil A (spinach) was a loamy sand soil (9.3% clay, 8.0% silt, and 82.7% sand), pH 8.3, 1.1%
164 organic matter. Soil B (melon) was a sandy loam soil (9.2% clay, 29.0% silt, and 61.9% sand), pH
165 8.1, 2.4% organic matter, and soil C (tomato) was a loam soil (20.7% clay, 28.8% silt, and 50.5%
166 sand), pH 8.1, 1.6% organic matter.

167 The study was carried out in a sequence first with assays using microsclerotia alone, then with
168 soil samples infested with microsclerotia. By doing so, we aimed to gradually increase the complexity
169 of substrate used in the DNA extraction procedure.

170

171 *2.3. DNA extractions*

172 Fungal mycelium for DNA extraction was grown in 200 ml of Yeast Extract Malt Extract
173 Glucose Broth (YMB – 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose) at 150 rpm
174 for 2–4 days at 30±1°C in the dark. Mycelium was harvested by filtration through sterile Miracloth
175 (Calbiochem, San Diego, CA), washed thoroughly using sterile distilled water and pressed dry
176 between sterile paper towels. The harvested mycelium was either used immediately for DNA
177 extraction or stored at -20°C until use.

178 Total genomic DNA was extracted by the SDS-CTAB method (Kim *et al.,* 1990) with some
179 modifications. Mycelium (200 mg) was placed into a 2 ml extraction tube prefilled with 0.5 mm Silica
180 glass beads (acid washed) (Benchmarck Scientific Inc., NJ), 50 mg of PVP40 (Sigma-Aldrich, Saint
181 Louis, USA) and 400 µl of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl pH
182 7.4, 30 µg ml^{-1} Proteinase K).

183 The mycelium was homogenized by a bead-beating method using the BeadBug™ Microtube
184 homogenizer (Benchmark Scientific Inc., NJ). Tubes were subjected to three beating cycles of 30-s
185 at 4000 rpm followed by a 30-s interval. During the interval and after the cycle, the samples were
186 cooled down in ice. SDS was added to a final concentration of 2% (w/v) and the mixture was
187 incubated at 65°C for 40 min.

188 The lysed suspension was centrifuged for 10 min at 4°C and 2500 *g*, the volume of supernatant
189 was measured and the NaCl concentration was then adjusted to 1.4 M and 1/10 volume of 10% CTAB
190 buffer (10% CTAB, 500 mM Tris-HC1, 100 mM EDTA, pH 8.0) was added. After thorough mixing,
191 the solution was incubated at 65°C for 10 min. After cooling at 15°C for 2 min, extraction with
192 chloroform-isoamyl alcohol (24:1 v/v) was conducted for 10 min at 4°C and 6700 *g*. DNA was
193 precipitated with two volumes of 95% cold ethanol. Samples were stored at -20°C (for a minimum of
194 1 h), centrifuged for 1 min at 4°C and 11,600 *g*, and the resulting DNA pellet was rinsed once with
195 70% cold ethanol, vacuum-dried and redissolved in sterile nuclease-free water. DNA solutions were
196 stored at -20°C until use.

197 DNAs from microsclerotia alone (MS) and microsclerotia mixed to three different agricultural
198 soil samples (MS_{soil}) were also extracted. Microsclerotia (1, 10, 100, and 200) of the *M. phaseolina*
199 isolate 10726 were placed into the lid of a 2 ml extraction tube prefilled with 0.5 mm Silica glass
200 beads (acid washed). The lid contained a drop of PDA to ensure they remained securely in place.
201 DNA from MS was extracted following the procedure described above for fungal mycelium except
202 that the three beating cycles were of 60-s at 4000 rpm followed by a 60-s interval.

203 DNA from MS_{soil} was extracted with a method based on the protocol described by Volossiuk
204 *et al.* (1995). Extraction tubes were filled with 1 g of soil, 50 mg of PVP40 (Sigma-Aldrich, Saint
205 Louis, USA) and 800 µl of 3.2% (w/v) commercial skimmed milk (Plasmon, 73% fat free) suspended
206 in sterile distilled water. MS_{soil} were homogenized by a bead-beating method described above for
207 MS. The mixture was centrifuged at 12,000 *g* for 30 min at 4°C to remove soil. After centrifugation,
208 supernatant was transferred to a fresh tube and Proteinase K was added to a final concentration of 50

209 µg ml^{-1}, then the extraction method continued from the SDS addition step as described above for
210 fungal mycelium. DNA was resuspended in 75 µl of sterile nuclease-free water.
211 The DNA concentrations were estimated by a GeneQuant II spectrophotometer (Pharmacia
212 Biotech, Cambridge, UK) whereas its integrity was examined visually by gel electrophoresis on 0.8%
213 (w/v) agarose gels run in 0.5× TBE buffer followed by GelRed™ staining (Biotium Inc., CA)
214 according to manufacturer's instructions. Following quantification, the genomic DNA was diluted to
215 a final concentration of 25-50 ng μl^{-1}.
216
217 *2.4. Amplification, sequencing and analysis of the ribosomal IGS spacer*
218 The complete IGS region of the ribosomal DNA was amplified using the primers CNL12 (5'-
219 CTGAACGCCTCTAAGTCAG-3') and CNS1 (5'-GAGACAAGCATATGACTACTG-3') with
220 priming sites at the 3' end of the 28S gene and the 5' end of the 18S gene respectively (Anderson and
221 Stasovsky, 1992, White *et al*., 1990).
222 The amplification reactions were carried out in a 50 µl volume containing 25-50 ng of template
223 DNA, 0.5 µM of each oligonucleotide primer and 25 µl of GoTaq® Green Master Mix (Promega,
224 Madison, WI, USA). An initial denaturation step of 94°C for 2 min was followed by 35 amplification
225 cycles of a 45-s 94°C denaturation step, a 45-s 57°C annealing step and a 3-min 72°C extension step.
226 After 35 cycles, samples were incubated for 10 min at 72°C (final extension step). Negative controls
227 (no DNA) were included for each set of reactions.
228 After agarose gel electrophoresis, the resulting PCR products were excised from the gel and
229 purified using the MiniElute Gel Extraction Kit (Qiagen).
230 Eluted PCR products were sent to BMR Genomics (Padova, Italy) for sequencing with the same
231 primers used for PCR. Partial nucleotide sequences initially obtained were used to design the internal
232 forward primer MP885F.
233 Partial IGS region (885 bp) of seven *M. phaseolina* isolates (10169, 10170, 10171, 10172,
234 10726, CM1 and PVS-Mp1) was amplified using the primer pair MP885F/CNS1 and PCR products

were purified using the QIAquick PCR purification Kit (Qiagen, Italy) and sequenced in both directions to confirm the nucleotide sequence. These amplification experiments were performed as described for the complete IGS region.

All amplifications were carried out in a GeneAmp PCR System 2400 (Perkin-Elmer). PCR products were analyzed by electrophoresis in 0.5× TBE buffer with 1% (w/v) agarose gels and detected by UV fluorescence after GelRed™ staining (Biotium Inc., CA) according to manufacturer's instructions. The 1-kb DNA ladder (Promega, Madison, WI, USA) was used as molecular size marker.

Sequences were assembled and edited using the BioEdit program (v.7.2.5) (Hall, 1999) and deposited in GenBank (Accession No. MH058030-MH058036).

Analyses using the BLASTn algorithm on the genome sequences of fungi listed in Table 2 were conducted using the *M. phaseolina* partial IGS sequences as a reference. Contigs containing sequences having high levels of similarity were identified and used in a multiple sequence alignment analysis.

The sequences from *M. phaseolina* isolates and other reference sequences retrieved from GenBank database were aligned using the MAFFT program (v. 7) (Kato and Standley, 2013) and were visually checked for regions having homologies among isolates of *M. phaseolina* but not among other fungi belonging to family *Botryosphaeriaceae*.

2.5. Development of specific oligonucleotide PCR primers

The regions conserved among the isolates and specific for *M. phaseolina* were selected to design species-specific oligonucleotides. Two primers, forward and reverse were designed using Primer3Plus online software with default options (Untergasser *et al*., 2007).

The forward (MP102F) and reverse (MP102R) primers yield a product of 102 bp. The theoretical specificity of the primer set was checked with the sequences from other fungi in GenBank by using BLASTn analysis.

261 *2.6. Development of a specific PCR assay for* M. phaseolina

262 The specific primers MP102F and MP102R, designed during this study, were used for
263 amplification.

264 The optimized PCR amplifications protocol described here was performed in a total volume of
265 25 µl containing 25-50 ng of template DNA, 0.2 µM of each oligonucleotide primer and 12.5 µl of
266 GoTaq® Green Master Mix (Promega, Madison, WI, USA). An initial denaturation step of 94°C for
267 2 min was followed by 35 amplification cycles of a 30-s 94°C denaturation step, a 30-s 57°C
268 annealing step and a 30-sec 72°C extension step. After 35 cycles, samples were incubated for 4 min
269 at 72°C (final extension step).

270 Templates were represented by: i) DNA from fungal mycelium of *M. phaseolina* isolates and
271 non-target species (Table 1); ii) DNA from microsclerotia alone (MS); iii) DNA from microsclerotia
272 mixed with three different agricultural soil samples (MS_{soil}). Negative controls (no DNA) were
273 included for each set of reactions. DNA from *M. phaseolina*-free soils (without microsclerotia) were
274 used as positive control in the microsclerotia assays.

275 PCR products were analyzed by electrophoresis in 0.5× TBE buffer with 2% (w/v) agarose gel
276 and detected by UV fluorescence after GelRed™ staining (Biotium Inc., CA) according to
277 manufacturer's instructions. The 100 bp DNA ladder (Promega, Madison, WI, USA) was used as
278 molecular size marker.

279 To exclude false negative results all template DNAs were initially tested for PCR amplification
280 using universal primers ITS5/ITS4.

281 Further, the specific PCR product obtained in soil experiments was eluted by gel extraction,
282 and sequenced to confirm that the designed primers amplified the expected consensus region of the
283 target organism.

284

285

286

287 *2.7. PCR-NALFIA assay*

288 Specific primers for *M. phaseolina* MP102F and MP102R were labelled with biotin and with
289 fluorescein isothiocyanate (FITC) at the 5′ end respectively. After the PCR reaction, conducted as
290 described before, the resulting double-labelled PCR product was divided into 2 portions for analysis
291 using gel electrophoresis and NALFIA (Fig 1a).

292 10 µl of PCR product were mixed with 70 µl of the assay buffer followed by dipping the
293 universal lateral flow dipstick (Milenia® HybriDetect, MGHD1, Milenia Biotec, Germany) into the
294 mix for 1-2 min to detect the specific PCR product.

295 The Biotin/FITC-labelled amplicon binds first to the gold-labelled FITC-specific antibodies in
296 the sample application area of the dipstick. Driven by capillarity the gold complexes diffuse over the
297 membrane. Only the amplicon that captured the gold nanoparticles will bind to the immobilized
298 biotin-ligand molecules at the test line, generating a red-blue band over the time. Not-captured gold
299 particles flow over the control line and will be fixed there by species-specific antibodies. With
300 increasing incubation time, the formation of an intensely coloured control band appears (Fig. 1b).

301 PCR-NALFIA sensitivity was tested using isolate 10726 mycelium DNA as a PCR template
302 serially diluted from 1.73 ng to 1.73 fg μl^{-1}. To determine the concentration of the DNA solution a
303 Qubit™ fluorometer (Invitrogen) was used according to manufacturer's protocols.

304

305 *2.8. Dipstick sensitivity*

306 In order to determine the sensitivity of the universal lateral flow dipstick, a PCR product using
307 DNA of *M. phaseolina* isolate 10726 as template was purified using the QIAquick PCR purification
308 kit and quantified with a Qubit™ fluorometer. Dilutions of the purified PCR products, from 120 ng
309 to 1.92 pg, were used for the NALFIA assay.

310

311

312

313 *2.9. Analysis of the PCR-NALFIA dipsticks*

314 Quantification of the signal generated by the test line of a PCR-NALFIA dipstick was analysed
315 and compared *in silico* employing the method proposed by Blažková *et al.* (2009a) and Noguera *et*
316 *al.* (2011), with some modifications.

317 The colour intensity of the test line was visually evaluated and quantified using grey scale
318 densitometry after the dipstick was fully air-dried. Dry PCR-NALFIA dipsticks were photographed
319 using a PowerShot G12 (Canon Inc., Tokyo, Japan) digital camera combined with a DI-HD Digimage
320 System (Major Science, Saratoga, CA, USA) hood. The resulting JPG file was converted to a 16-bit
321 black-and-white image and analysed using the ImageJ (v. 1.50i, default options) software (Abramoff
322 *et al*., 2012) to convert the test line colour density in pixel grey volumes. For statistical analysis,
323 measured test line intensities were normalized to the internal controls.

324 Quantification of the test line of the PCR-NALFIA assay was performed on the dipsticks of
325 PCR products of 1, 10, 100 and 200 microsclerotia mixed with the three agricultural soils (spinach,
326 melon and tomato). Each experiment was repeated twice.

327 For each set of microsclerotia (1, 10, 100 and 200), the average value of the pixel grey volumes
328 was used to develop a grey-scale reference card.

329

330 **3. Results**

331

332 *3.1. Microsclerotia germination test*

333 The germination rate of *M. phaseolina* isolate 10726 microsclerotia on PDA plates was 100%
334 for all four replicates in both experiments: microsclerotia plated 5 days or 6 months after their harvest.
335 In most cases, a single microsclerotium was able to produce multiple germination hyphae after 2 days
336 of incubation at 30°C, with a visible white mycelium.

337

338

339 *3.2. DNA extraction from mycelium and microsclerotia*

340 Total genomic DNA was successfully extracted from all the samples tested: 20 *M. phaseolina*
341 isolates, 16 non-target fungi and microsclerotia (1, 10, 100, and 200) of the *M. phaseolina* isolate
342 10726.

343 A high molecular weight band, above the 10,000 bp band of the marker, was well visible in all
344 isolates (Fig. S1). These results suggest that the beat-beating extraction protocol did not cause severe
345 shearing of DNA. The DNA solutions were very clear in colour and had, in average, a high A_{260}/A_{280}
346 ratio equal to 2.0 ± 0.12 for all the extracted samples. DNA concentration varied from 12.3 ± 0.2 ng
347 μl^{-1} to 553.8 ± 0.18 ng μl^{-1}. The highest concentration was obtained from the mycelium of *M.*
348 *phaseolina* isolate 10726.

349 PCR amplification of the ITS region, with the primer pair ITS5/ITS4, was performed using as
350 template the DNA extracted from the mycelium of *M. phaseolina* isolates and from the other non-
351 target fungi (Table 1) in order to verify the potential for amplification of extracted DNA, thereby
352 excluding false negative results. As expected, single amplicons were obtained from all the tested
353 fungi and they varied approximately between 550 and 700 bp (Figs. S2 and S3).

354

355 *3.3. DNA extraction from soil*

356 DNA extraction from soil has three requirements: extraction of high molecular weight DNA,
357 extraction of DNA free from inhibitors for subsequent molecular biological manipulations and
358 representative lysis of microorganisms within the sample.

359 Amplification of the ITS region of fungal DNA present in soil, using the primer pair ITS5/ITS4,
360 confirms the presence of PCR-amplifiable DNA in the sample. Multiple ITS bands of expected length
361 (~600-750 bp) were obtained. A 1:2 dilution of the soil DNA resulted in amplification of all the three
362 soil samples. Therefore, 1:2 dilution of the samples was chosen as standard to amplify the DNA
363 extracted from soils in subsequent experiments (Fig. S4, left panel).

364 The A_{260}/A_{280} ratio of DNA solutions extracted from soils were 1.56 ± 0.03 for spinach soil,
365 1.77 ± 0.16 for melon soil and 1.58 ± 0.02 for tomato soil. Although these values were lower than the
366 ratios of DNA solutions from pure cultures, primarily due to the presence of absorbing contaminants
367 in the preparation, they are sufficient for successful PCR amplification.
368 Significant differences in DNA yield did occur among soils. DNA concentration, alone or
369 mixed with microsclerotia, was 113.5 ± 3.5 ng μl^{-1} for spinach soil, 48.5 ± 2.1 ng μl^{-1} for melon soil
370 and 165.5 ± 2.1 ng μl^{-1} for tomato soil.
371 The DNA solutions from the beat-beating extraction protocol from soils, performed in the
372 experiments using skimmed milk treatment, were clearer in colour compared to those obtained with
373 lysis buffer alone. DNA extraction using lysis buffer alone generated a brown supernatant resulting
374 into a dark-brown DNA solution following DNA precipitation and pellet dissolving (Fig. S4, middle
375 panel).
376 PCR amplification of the ITS region, with the primer pair ITS5/ITS4 and the DNA obtained
377 using the lysis buffer alone, was performed using the undiluted DNA solution and the 10, 20 and 50-
378 fold diluted solutions. The 20-fold diluted sample showed a faint single band of about 600 bp and the
379 intensity of the band increased as the 50-fold dilution sample was used as template (Fig. S4, right
380 panel).
381
382 *3.4. Development of* M. phaseolina *specific oligonucleotide PCR primers*
383 PCR amplification of ribosomal IGS spacer, using the CNL12/CNS1 primer pair on *M.*
384 *phaseolina* isolates, resulted in fragments of approximately 3.0 kbp in length (Fig. S5).
385 DNA sequencing of the IGS 3′ region (891 bp-length), followed by multiple sequence
386 alignment, revealed polymorphic regions for designing species-specific PCR primers. Multiple
387 sequence alignment was produced using IGS sequences available in the GenBank for fungi belonging
388 to the family *Botryosphaeriaceae*: *M. phaseolina, Botryosphaeria dothidea*, *Lasiodiplodia*
389 *theobromae*, *Diplodia corticola, D. sapinea, D. scrobiculata, Neofusicoccum parvum* and

390 *Neoscytalidium dimidiatum*. The primers were designed from 100% sequence homology region of *M.*
391 *phaseolina* isolates and from regions of the greatest sequence dissimilarity among other species.
392 The species-specific primer set MP102F/MP102R successfully amplified the expected 102 bp
393 product of all *M. phaseolina* isolates, but did not generate a PCR product for any of the 16 non-target
394 species, confirming their specificity for *M. phaseolina* (Fig. 2).
395 Quantity and quality of the fungal DNA extracts have been previously tested by a PCR assay
396 targeted to the fungal ribosomal ITS region, in which all isolates had given the expected PCR
397 products, indicating successful amplification.
398
399 *3.5. PCR-NALFIA for* M. phaseolina*: specificity and sensitivity*
400 *Macrophomina phaseolina* specific primers MP102F and MP102R labelled with biotin and
401 fluorescein in 5', respectively, were used for PCR-NALFIA. DNA of *M. phaseolina* isolates and of
402 non-target fungi was amplified and detected using both gel electrophoresis and the nucleic acid lateral
403 flow immuno-assay (NALFIA).
404 NALFIA confirmed the specificity of the primers as MP102F and MP102R successfully
405 amplified the specific 102 bp fragment from all *M. phaseolina* isolates (coloured test line) while
406 NALFIA from other non-target fungi was always negative (blank test line). A negative control (no
407 DNA) was included in the reactions and its response was negative either tested by electrophoresis or
408 by NALFIA (blank test line) (Fig. 2 and Fig. S6).
409 Microsclerotia DNA amplification using primer pair MP102F/MP102R was performed using 1
410 µl of DNA extracted from 1, 10, 100 and 200 microsclerotia of *M. phaseolina* isolate 10726 (MS).
411 The DNA concentration (pg μl^{-1}) of each microsclerotia sample was as follows: 74 (1 MS), 91 (10
412 MS), 136 (100 MS) and 176 (200 MS).
413 On gel electrophoresis the amplicon was visible up to 10 microsclerotia while using NALFIA,
414 a positive response (coloured test line) was also accomplished using as analyte the amplicon obtained
415 from 1 microsclerotium DNA extraction (Fig. 3).

A negative control (no DNA) was included in the reactions and its response was negative by both electrophoresis and NALFIA (blank test line).

Amplification using the specific primer pair MP102F/MP102R was also performed on DNA samples extracted from 1, 10, 100 and 200 microsclerotia of *M. phaseolina* isolate 10726 mixed with 1 g of three different soils on which spinach, melon and tomato were previously grown. 1 µl of a 1:2 dilution was used as template for PCR reactions. DNA concentration (ng μl^{-1}) of each soil sample was on average (1-200 MS) as follows: 56.8 (spinach), 24.3 (melon) and 82.8 (tomato).

The amplicons were detected using either electrophoresis or NALFIA. In the first case, the amplicon was visible up to 10 microsclerotia for spinach and tomato soils, while melon soil was detected on the gel up to 100 microsclerotia. NALFIA test was positive up to 1 microsclerotium for all three soil samples (coloured test line) while control soil (without microsclerotia) gave negative results (blank test line) (Fig. 4).

PCR reactions performed using 10-fold serial dilutions of the template DNA (mycelial DNA of *M. phaseolina* isolate 10726), from 1.73 ng to 17.3 fg, were detected using gel electrophoresis and NALFIA. 25 µl of PCR product of each reaction were used for gel electrophoresis; the amplicon was visible on the gel up to 17.3 pg of template DNA. 10 µl of PCR product of each reaction were also used for NALFIA. The test confirmed the amplification of the target DNA up to 17.3 fg of template DNA (Fig. 5).

Sensitivity of the universal lateral flow dipstick was assessed using serial dilutions – from 120 ng to 1.92 pg – of purified 102 bp amplicon resulting from a single PCR reaction using the labelled primer pair MP102F/MP102R and the mycelial DNA of *M. phaseolina* isolate 10726 as template. As low as 9.6 pg of analyte were visually detectable with the dipstick, on which a faint black line was visible (Fig. S7).

442 *3.6. Grey scale densitometric analysis of PCR-NALFIA dipsticks*

443 The intensities of the dipstick test line of the twelve samples (1, 10, 100 and 200 microsclerotia
444 mixed with the three soils) were analysed using the ImageJ software and the normalized pixel grey
445 volumes of each test line generated by the PCR-NALFIA assay were calculated. The following
446 average values of pixel grey volumes were obtained: 1.0439 ± 0.0125 using 1 microsclerotium;
447 1.3438 ± 0.0152 using 10 microsclerotia; 1.5330 ± 0.0180 using 100 microsclerotia; 1.8286 ± 0.0142
448 using 200 microsclerotia. Each value represents the mean of 6 data ± standard deviation.

449 A semi-quantitative grey-scale reference card was developed based on the dipstick test line
450 analysis (Fig. 6). Each interval represents a given microsclerotia number (1, 10, 100 and 200).

451

452 **4. Discussion**

453 This paper describes the development of a species-specific PCR primer pair based on rDNA
454 IGS sequences and a PCR-NALFIA assay for the specific detection of *M. phaseolina* in soil. Together
455 with the DNA extraction protocol, the diagnostic method allows for a rapid and accurate assessment
456 of soil contamination by the pathogen.

457 Microsclerotia of *M. phaseolina* are resilient structures formed by the fungus in the soil. Our
458 results shown that microsclerotia conserved vital in dry conditions at air temperature, as they were
459 viable and able to germinate after six months. These results are in accordance with many authors who
460 reported the ability of *M. phaseolina* microsclerotia to persist in soil for many months or years (Sheick
461 and Ghaffar, 1979; Ijaz *et al.*, 2013; Kaur *et al.*, 2012). Their ability to germinate after several years
462 and the polyphagous behaviour of this fungus pose a severe threat to crop production as the fungus
463 remains quiescent for a long time in contaminated soils or crop residues without the presence of
464 susceptible hosts.

465 Separation of *M. phaseolina* microsclerotia from soil by sieve methods and counting them using
466 dilutions is reported in literature, however these methods, although being practical, are not as accurate
467 as the single manipulation of microsclerotia technique described in this study.

We isolated and studied the smaller fraction (from 41 to 99 μm) of microsclerotia size as the average diameter of *M. phaseolina* microsclerotia is reported to be 50 – 150 μm (Kaur *et al.*, 2012). The range of microsclerotia number used in this study (1-200) is representative of a real *M. phaseolina* soil infestation and was deduced considering numerous literature studies on soils naturally infested by the pathogen. Even a single sclerotium of *M. phaseolina* per gram of soil is able to cause disease (Sheikh and Ghaffar, 1979; Young and Alcorn, 1984; Khan, 2007).

A simple and inexpensive extraction method has been used in this study, with a small number of efficient lysis and purification steps to allow rapid processing of many samples. The usefulness of the DNA extraction method was confirmed by testing three different types of soil. The extraction procedure was based on the physical disruption of microsclerotia in the soil sample with a bead-beating fungal cell disruption method and the use of both skim milk and PVP combined with a CTAB/Proteinase K extraction method (Kim *et al*., 1990). Bead-beating is considered a very effective method for cell disruption to isolate fungal DNA and RNA (Moré *et al*., 1994; Kuske *et al*., 1998; Miller *et al*., 1999; Griffin *et al.*, 2002; De Boer *et al*., 2010; Leite *et al*., 2012).

One of the crucial step in the diagnosis of soil-borne pathogens is the direct extraction of DNA from soil organisms without prior purification or culturing. Many factors affect DNA extraction efficiency and contaminants in the DNA extracts which inhibit PCR amplification and losses of DNA by degradation or absorption have proven to be the major limitations (Schrader *et al.*, 2012).

Skim milk was reported to reduce the co-extraction of PCR inhibitors, improving the efficacy of DNA extraction from different type of soils (Hoshino and Matsumoto 2004, 2005), and not affecting PCR because of its removal in the purification process. Many soil-borne fungi were successfully detected in different soil types by PCR after direct DNA extraction with skim milk (Volossiouk *et al.*, 1995; García-Pedrajas *et al.*, 1999; Kageyama *et al.*, 2003; Pérez-Artés *et al.*, 2005). PVP forms complex hydrogen bonds with phenolics, co-precipitates with cell debris upon cell lysis and significantly improves the PCR product yield with little loss of target DNA (Volossiouk *et al.*, 1995).

494 The standardized extraction method yielded good quantity of pure, high molecular weight DNA
495 from both pure cultures and soil samples, minimizing handling of hazardous organic solvents. The
496 quality of the extracted DNA from mycelium and soil samples was further confirmed by the
497 amplification of the ITS region, which gives a confirmation of the presence of PCR-amplifiable DNA
498 in the sample, as described by different Authors (Yeates *et al*., 1997; Suarez *et al*., 2005).
499 The whole intergenic spacer (IGS) region (~3.0 kb) of *M. phaseolina* was successfully
500 amplified and partially sequenced using the universal primer pair CNL12/CNS1 and the primer
501 MP885F developed in this study. Sequencing and analysis of the IGS region of *M. phaseolina* have
502 not been previously reported in literature.
503 The IGS region is commonly considered the most variable part of the rDNA unit both in
504 sequence and in length since it evolves faster than ITS region and, as such, more sequence
505 polymorphisms are present. This region occurs in multiple copies increasing the sensitivity of PCR-
506 based diagnostics compared with single-copy target sequences and allowing its amplification using
507 generic PCR primers. Therefore, the use of the IGS region in the development of species-specific
508 primers can be considered as an attractive target for molecular diagnosis for pathogenic fungi such as
509 *M. phaseolina* (Kumar *et al.*, 2016).
510 We focused our attention on a target diagnostic IGS region of about 900 bp, located at the 3'
511 end of the intergenic spacer, for developing the *M. phaseolina* species-specific primer pair
512 MP102F/MP102R. A specific 102 bp PCR product was successfully obtained for all the isolates of
513 the pathogen. This primer pair resulted specific both in *in silico* and *in vivo* tests using DNA from
514 mycelium/microsclerotia and DNA from different soils containing microsclerotia of *M. phaseolina*.
515 Sequencing of the specific fragment purified from PCR reactions from soils confirmed the expected
516 sequence. MP102F/MP102R primer specificity was also tested on non-target fungi with positive
517 results as no PCR product was detected using as template the genomic DNA of the isolates.
518 PCR products obtained with the species-specific primer pair MP102F/MP102R were also
519 detected using the nucleic acid lateral flow immuno-assay (NALFIA). Positive results (double band

520 on the dipstick) were obtained using DNA from mycelium/microsclerotia and DNA from different
521 soils containing microsclerotia of *M. phaseolina* and negative results were obtained using as template
522 for PCR reactions the DNA from non-target fungi.

523 The sensitivity threshold of the PCR reaction was assessed by testing serial dilutions of *M.*
524 *phaseolina* isolate 10726 mycelium DNA as template. The detection limit of the NALFIA assay was
525 17.3 fg using 10 µl of PCR reaction, whereas that of agarose gel electrophoresis was 17.3 pg using
526 25 µl of PCR reaction.

527 Detection level for *M. phaseolina* in soil samples was 1 microsclerotium g^{-1} of soil. The
528 sensitivity of the PCR assay described here is further highlighted when the number of microsclerotia
529 included per PCR reaction is considered. Assuming a 100% recovery, for 1 microsclerotium g^{-1} of
530 soil (DNA resuspended in 75 µl and a 1:2 dilution used as template for PCR reactions) 1 µl of template
531 DNA would contain the equivalent of 0.0067 microsclerotia in a reaction. DNA dilution was taken
532 into consideration also by Cullen *et al.* (2001) in order to exploit the sensitivity of PCR for the specific
533 detection of *Helminthosporium solani* in seeded soils.

534 These results validate the high detection sensitivity of the developed PCR-NALFIA assay.
535 There is no loss of sensitivity when the PCR reaction is performed with DNA from different soils
536 containing microsclerotia of *M. phaseolina*.

537 Depending on the quality of the DNA template and the primer system used, the detection limits
538 of the PCR with DNA isolated from pure fungal cultures on agarose gels stained with ethidium
539 bromide were reported to be 1 to 10 pg for multicopy genes (Bluhm *et al.*, 2002) and 5 to 1000 pg for
540 single copy genes (Möller *et al.*, 1999).

541 Therefore, the sensitivity of the PCR-NALFIA assay developed in this study was over 1000-
542 times higher than agarose gel electrophoresis.

543 Babu *et al.* (2011) developed a real-time qPCR assay targeted on a sequence characterized
544 amplified region (SCAR) of *M. phaseolina* with a minimum detection limit of 30 fg μl^{-1} of DNA.

They estimated that the detection of ≥10 pg μl^{-1} in the sample indicates the presence of at least 1 (≥ 1) CFU/vegetative hyphae/sclerotium.

DNA Detection Test Strips ™ were used for the detection of *Fusarium graminearum* contamination in cereal samples and the method was compared to agarose gel electrophoresis. In this system, a 5'digoxigenin labelled primer pair targeting the galactose oxidase gene was used for PCR and the limit of detection was found to be 260 pg of template DNA. The detection was performed in only 20 min without the need of special technical equipment or hazardous fluorescent dyes (Knoll *et al*., 2002).

In order to further investigate the detection limit of the dipstick, a sensitivity test was performed using serial dilutions of the 102 bp purified amplicon from mycelial DNA of *M. phaseolina*. Detection limit of the LFD was ≤9.6 pg of PCR product, where a faint colouration was still visible on the test line. These results confirm a higher sensitivity of the universal lateral flow dipstick used.

The use of the LFD assay to detect pathogens is rapidly expanding to many diagnostic fields, including plant pathogens, because of the need of a rapid and sensitive detection method using a point of care (POC) diagnostic approach. Manufacturers are trying to develop successful POC products; however, these products are not easy to produce, as they must be cost-effective and meet the market demand at the same time. Nevertheless, in recent years lateral flow immunoassays have achieved broad penetration in a variety of markets (O'Farrell, 2009).

In conclusion, our diagnostic method was developed considering the economic losses caused by the fungus and its potential threat over countries like Italy, which never experienced epidemic levels of this fungus but may face it in consideration of a climate change scenario.

In a future climatic scenario compared to the current conditions (2030 vs. 2000) over Europe, *M. phaseolina* may find favourable conditions in Italy, Spain, France and in central Europe under future summer temperature regimes (Manici *et al.*, 2014). Confirming the climate change trend in Italy, in the last 15 years, as a response to an increase of the average temperature, soil-borne pathogens

570 like *Colletotrichum coccodes*, *Athelia rolfsii* and *M. phaseolina* have become of economic
571 importance, causing serious damages on many different crops (Rete Rurale Nazionale, 2012).
572 The diagnostic protocol described herein proved to be reproducible and could be used to
573 determine the presence of *M. phaseolina* in different types of infected soils and for pathogen detection
574 and identification in plant tissues. The amount of microsclerotia can be estimated using densitometric
575 analysis on the LFD strips and the semi-quantitative grey-scale interpretation card provided in this
576 study.
577 In addition to its higher sensitivity compared to the electrophoresis gel detection, the LFD stick
578 involves several other advantages: (i) the test is rapid, as can be performed in less than 10 minutes;
579 (ii) it is simple to perform; (iii) does not require skilled personnel and expensive instrumentation; (iv)
580 results can be easily interpreted.
581 In future applications, this protocol can be further improved eliminating the PCR technique,
582 which requires specialized personnel and equipment, replacing it, for example, with the recombinase
583 polymerase amplification (RPA) technique in order to make the diagnostic procedure more applicable
584 at point-of-care. Based on these improvements, this protocol might be applied in the development of
585 commercial diagnostic kits to rapidly detect the presence of *M. phaseolina* in naturally infected soils,
586 reducing its potential threat over the crops.
587

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603
604 **Conflict of interest**
605 No conflict of interest declared.
606
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836
837
838 **SUPPORTING INFORMATION LEGENDS**

839 **Fig. S1** Gel electrophoresis of genomic DNA of some *M. phaseolina* isolates. A high molecular
840 weight band, above the 10,000 bp band of the marker, is well visible. The other fragments can be
841 attributed to the presence of extrachromosomal elements. Marker = 1 kb DNA ladder.

842 **Fig. S2** ITS PCR products of 5 *M. phaseolina* isolates using the mycelial DNA as template. Marker
843 = 100 bp DNA ladder. Negative control = no DNA.

844 **Fig. S3** ITS PCR products of 16 non-target fungi used in this study using the mycelial DNA as
845 template. Marker = 100 bp DNA ladder. Negative control = no DNA.

846 **Fig. S4** Left panel = ITS PCR products using the DNA extracted from three different soils (spinach,
847 melon and tomato) with the skimmed milk solution. The starting DNA solutions were diluted 1:2 and
848 1:3. Middle panel = Comparison between supernatants obtained using the skimmed milk solution (A)
849 and the lysis buffer (B). Right panel = ITS amplification using serial dilutions (1:10, 1:20 and 1:50)

of the DNA extracted from spinach soil using the lysis buffer. Marker = 100 bp DNA ladder. Negative control = no DNA.

Fig. S5 Amplification products of the entire IGS region using CNL12/CNS1 primer pair on some *M. phaseolina* isolates. Marker = 1 kb DNA ladder. Negative control = no DNA.

Fig. S6 Specificity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC respectively for the detection of *M. phaseolina*. Marker = 100 bp DNA ladder. Negative control = no DNA. Upper panel = agarose gel electrophoresis. Lower panel = PCR-NALFIA assay.

Fig. S7 Sensitivity of the lateral flow dipstick using serial dilutions (from 120 ng to 1.92 pg) of the 102 bp purified amplicon. The PCR assay was performed with the species-specific primer pair MP102F/MP102R and mycelial DNA of *M. phaseolina* isolate 10726 as template.

FIGURE LEGENDS

Fig. 1 PCR-NALFIA assay for *M. phaseolina*. **a.** A standard PCR reaction using the *M. phaseolina*-specific primer pair labelled with biotin and FITC. If the fungal specific target is present, at the end of the PCR reaction, a biotin-FITC labelled amplicon is formed. **b.** The double-labelled amplicon is mixed with a buffer and poured on the sample pad of a Lateral Flow Dipstick. The amplicon migrates toward the wicking pad by capillarity and the FITC molecule is recognized by gold-labelled FITC-specific antibodies, fixed on the membrane of the dipstick. The biotin on the opposite side of the amplicon is recognised by a biotin-specific ligand, localised on the test line of the dipstick, which blocks the amplicon on this area generating a red-blue band. The excess of gold nanoparticles is blocked over the control line, whose antibody is recognised by specific anti-rabbit antibodies, giving a coloration to the control line. **c**. Evaluation of results using the LFD.

873 **Fig. 2** Specificity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC
874 respectively for the detection of *M. phaseolina*. Lanes 1-7 = *M. phaseolina* (10169, 10170, 10171,
875 10172, 10176, CM1, PVS-Mp1); Lane 8 = *Alternaria brassicicola*; Lane 9 = *Aspergillus* sp.; Lane
876 10 = *Botrytis cinerea*; Lanes 11-13 = *Diplodia seriata* (9990, 9996, 9998); Lane 14 = *Fusarium*
877 *graminearum*; Lane 15 = *F. oxysporum* f. sp. *basilici*; Lane 16 = *F. solani*; Lane 17 = *Penicillium* sp.;
878 Lane 18 = *Rhizoctonia solani*; Lane 19 = *Rhizopus* sp.; Lane 20 = *Sclerotinia sclerotiorum*; Lane 21
879 = *Sclerotium rolfsii*; Lane 22 = *Trichoderma asperellum*; Lane 23 = *Verticillium dahliae*; Lane 24 =
880 negative control (no DNA); M = 100 bp DNA ladder.
881 Upper panel = agarose gel electrophoresis. Lower panel = PCR-NALFIA assay.
882
883 **Fig. 3** Gel electrophoresis (upper panel) and PCR-NALFIA assay (lower panel) of the 102 bp PCR
884 amplicon using the specific primer pair MP102F/MP102R labelled with biotin and FITC respectively
885 and DNA template from 1, 10, 100 and 200 microsclerotia of *M. phaseolina* isolate 10726 (MS).
886 DNA concentration (pg μl^{-1}) of each microsclerotia sample was as follows: 74 (1 MS), 91 (10 MS),
887 136 (100 MS) and 176 (200 MS). 10 µl of PCR products were used for both tests. M = 100 bp DNA
888 ladder. C = Negative control (no DNA).
889
890 **Fig. 4** Gel electrophoresis (upper panel) and PCR-NALFIA assay (lower panel) of the 102 bp PCR
891 amplicon using the specific primer pair MP102F/MP102R labelled with biotin and FITC respectively
892 and DNA template from 0, 1, 10, 100 and 200 *M. phaseolina* microsclerotia mixed with tomato soil
893 (left), melon soil (centre) and spinach soil (right). M = 100 bp DNA ladder. C = Negative control (no
894 DNA).
895
896 **Fig. 5** Sensitivity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC
897 respectively for the detection of *M. phaseolina*. The assay was performed using 10-fold dilutions
898 ranged between 1.73 ng and 1.73 fg of template DNA (mycelial DNA of *M. phaseolina* isolate

899 10726). M = 100 bp DNA ladder. C = Negative control (no DNA). Upper panel = agarose gel
900 electrophoresis. Lower panel = PCR-NALFIA assay.
901
902 **Fig. 6** Semi-quantitative grey-scale reference card based on the lateral flow dipstick test line analysis
903 performed with the ImageJ software. Test lines generated by the PCR-NALFIA assays corresponding
904 to 1, 10, 100 or 200 microsclerotia were reported.

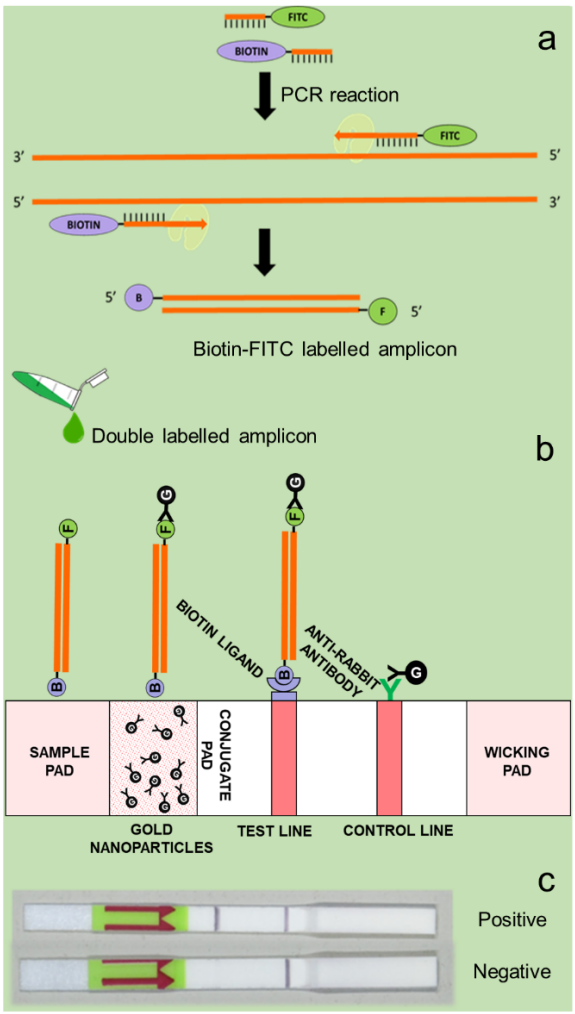

Fig. 1 PCR-NALFIA assay for *M. phaseolina*. a. A standard PCR reaction using the *M. phaseolina*-specific primer pair labelled with biotin and FITC. If the fungal specific target is present, at the end of the PCR reaction, a biotin-FITC labelled amplicon is formed. **b.** The double labelled amplicon is mixed with a buffer and poured on the sample pad of a Lateral Flow Dipstick. The amplicon migrates toward the wicking pad by capillarity. Primarily, the FITC molecule is recognized by gold-labelled FITC-specific antibodies, fixed on the membrane of the dipstick. The biotin on the opposite side of the amplicon is recognised by a biotin-specific ligand, localised on the test line of the dipstick, which blocks the amplicon on this area generating a red-blue band. The excess of gold nanoparticles is blocked over the control line, whose antibody is recognised by specific anti-rabbit antibodies, giving a coloration to the control line. **c**. Evaluation of results using the LFD.

Fig. 2 Specificity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC respectively for the detection of *M. phaseolina*. Lanes 1-7 = *M. phaseolina* (10169, 10170, 10171, 10172, 10726, CM1, PVS-Mp1); Lane 8 = *Alternaria brassicicola*; Lane 9 = *Aspergillus* sp.; Lane 10 = *Botrytis cinerea;* Lanes 11-13 = *Diplodia seriata* (9990, 9996, 9998); Lane 14 = *Fusarium graminearum*; Lane 15 = *F. oxysporum* f. sp. *basilici*; Lane 16 = *F. solani*; Lane 17 = *Penicillium* sp.; Lane 18 = *Rhizoctonia solani*; Lane 19 = *Rhizopus* sp.; Lane 20 = *Sclerotinia sclerotiorum*; Lane 21 = *Sclerotium rolfsii*; Lane 22 = *Trichoderma asperellum*; Lane 23 = *Verticillium dahliae*; Lane 24 = negative control (no DNA); M = 100 bp DNA ladder.

Upper panel = agarose gel electrophoresis. Lower panel = PCR-NALFIA assay.

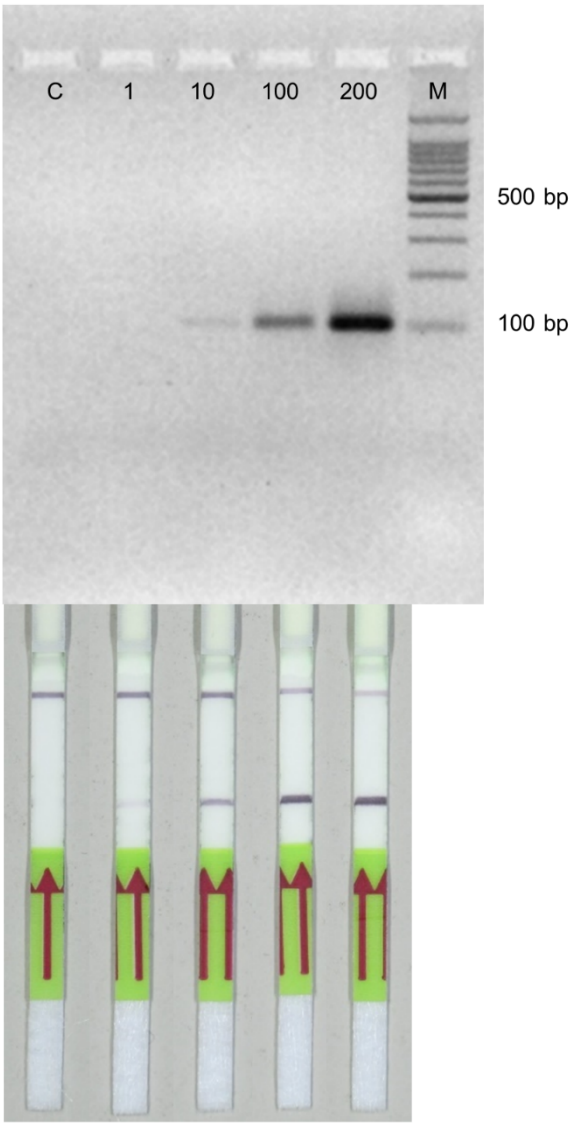

Fig. 3 Gel electrophoresis (upper panel) and PCR-NALFIA assay (lower panel) of the 102 bp PCR amplicon using the specific primer pair MP102F/MP102R labelled with biotin and FITC respectively and DNA template from 1, 10, 100 and 200 microsclerotia of *M. phaseolina* isolate 10726 (MS). DNA concentration (pg μl^{-1}) of each microsclerotia sample was as follows: 74 (1 MS), 91 (10 MS), 136 (100 MS) and 176 (200 MS). 10 µl of PCR products were used for both tests. M = 100 bp DNA ladder. C = Negative control (no DNA).

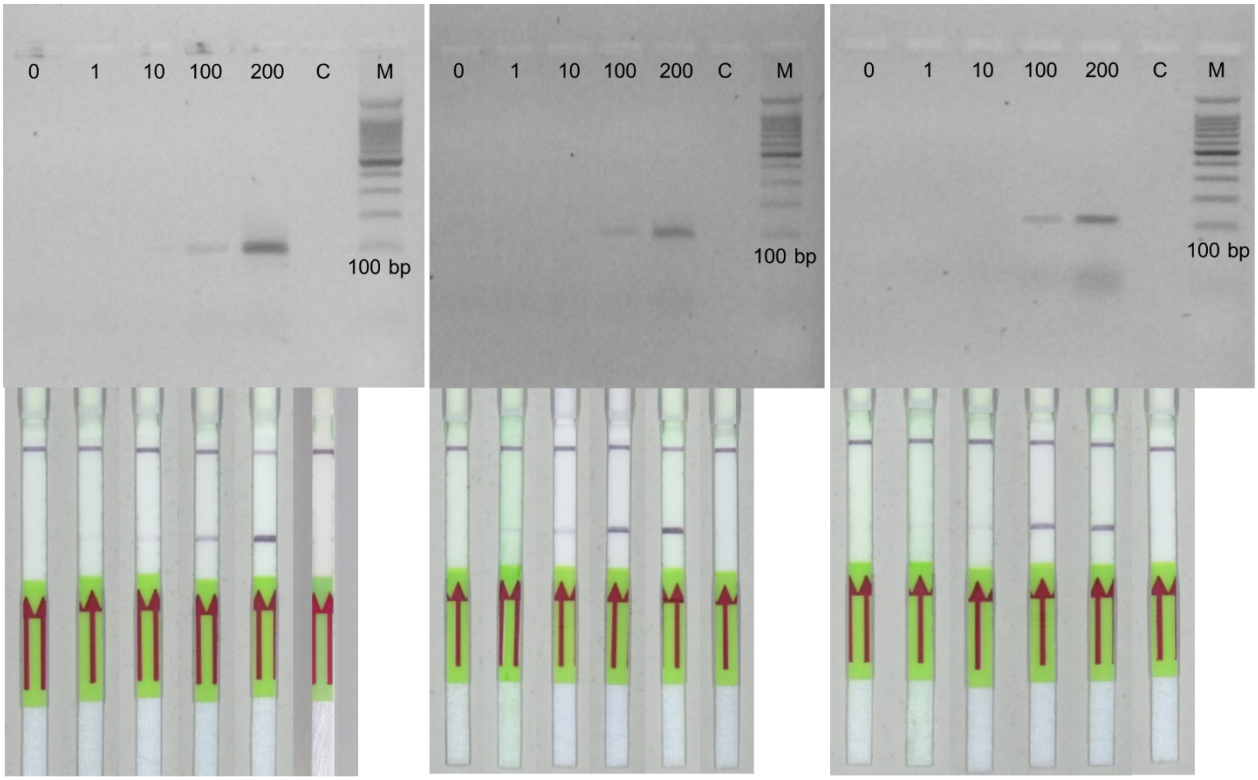

Fig. 4 Gel electrophoresis (upper panel) and PCR-NALFIA assay (lower panel) of the 102 bp PCR amplicon using the specific primer pair MP102F/MP102R labelled with biotin and FITC respectively and DNA template from 0, 1, 10, 100 and 200 *M. phaseolina* microsclerotia mixed with tomato soil (left), melon soil (centre) and spinach soil (right). M = 100 bp DNA ladder. C = Negative control (no DNA).

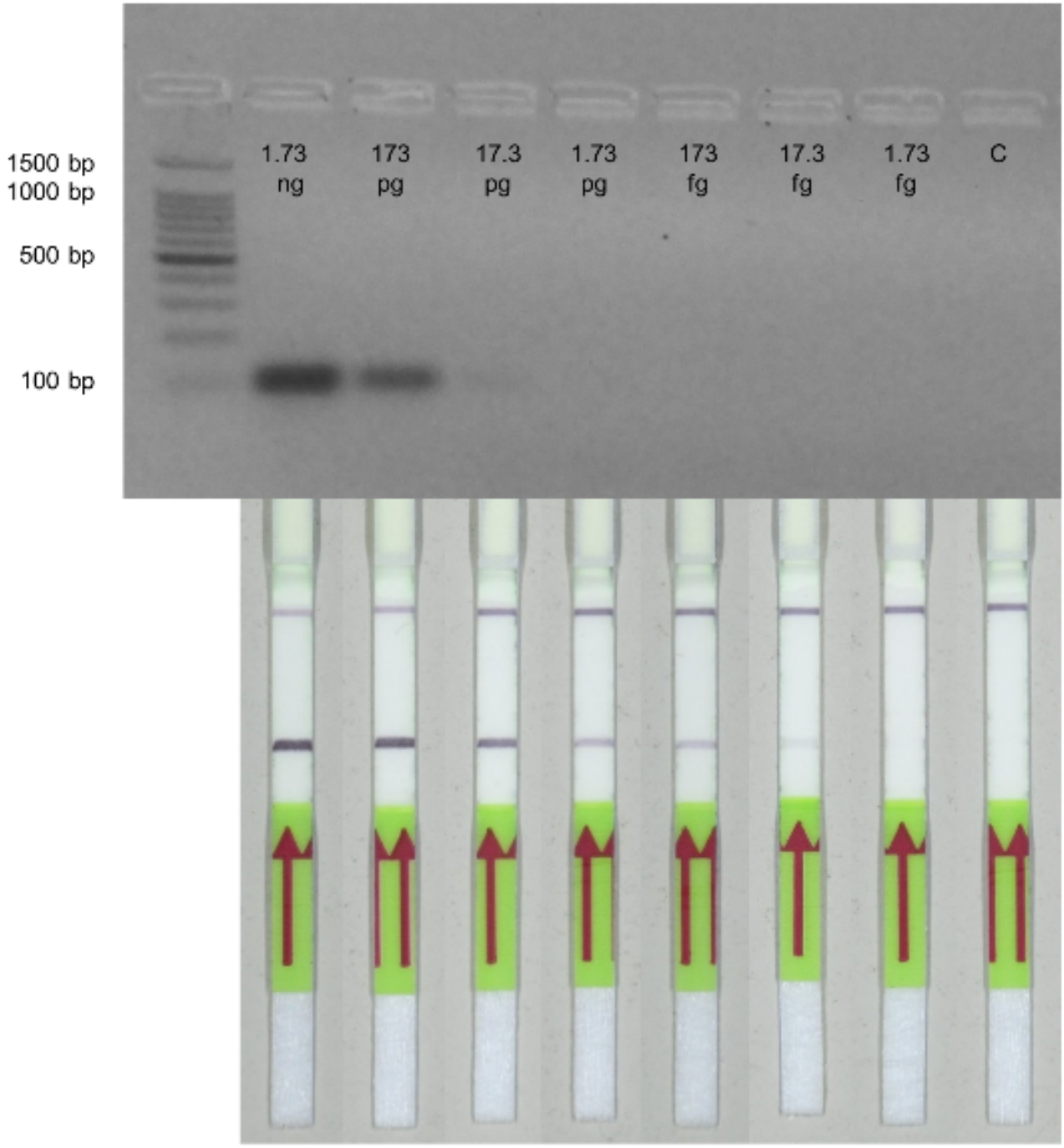

Fig. 5 Sensitivity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC respectively for the detection of *M. phaseolina*. The assay was performed using 10-fold dilutions ranged between 1.73 ng and 1.73 fg of template DNA (mycelial DNA of *M. phaseolina* isolate 10726). M = 100 bp DNA ladder. C = Negative control (no DNA). Upper panel = agarose gel electrophoresis. Lower panel = PCR-NALFIA assay.

Fig. 6 Semi-quantitative grey-scale reference card based on the lateral flow dipstick test line analysis performed with the ImageJ software. Test lines generated by the PCR-NALFIA assays corresponding to 1, 10, 100 or 200 microsclerotia were reported.

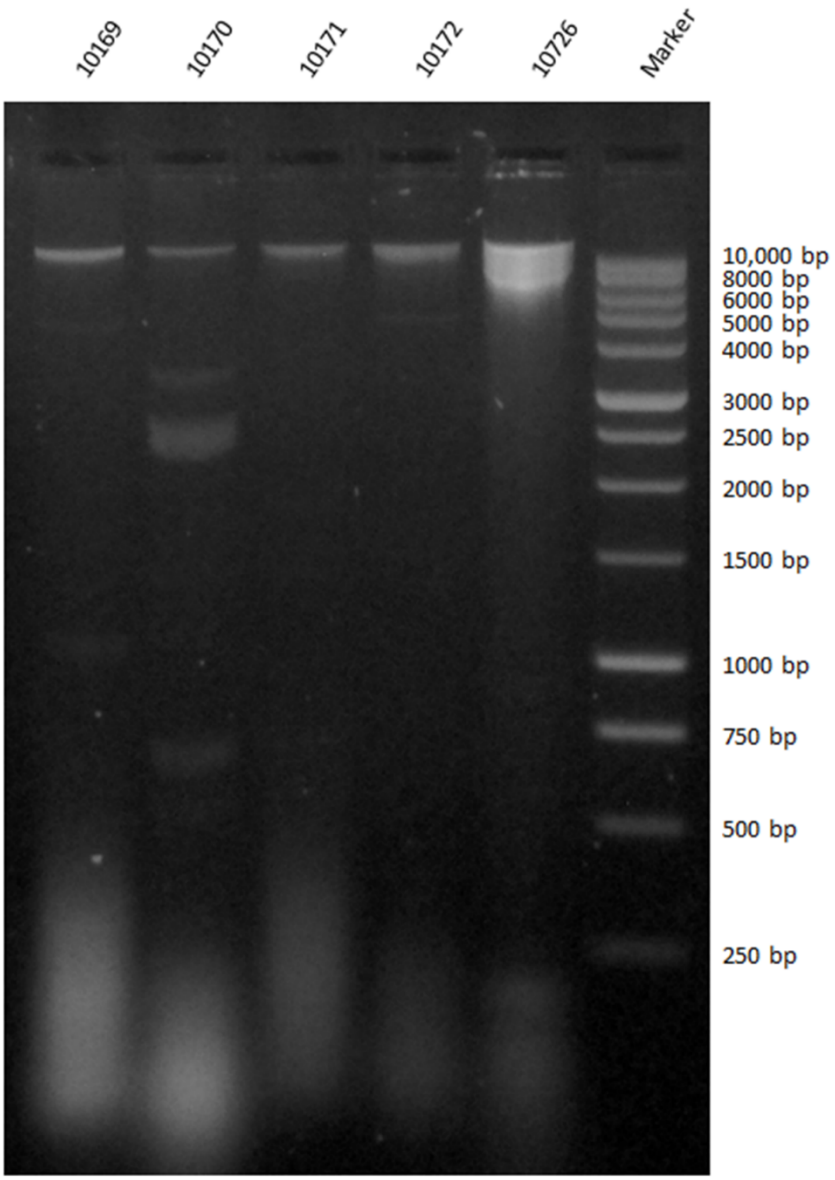

Fig. S1 Gel electrophoresis of genomic DNA of some *M. phaseolina* isolates. A high molecular weight band, above the 10,000 bp band of the marker, is well visible. The other fragments can be attributed to the presence of extrachromosomal elements. Marker = 1 kb DNA ladder.

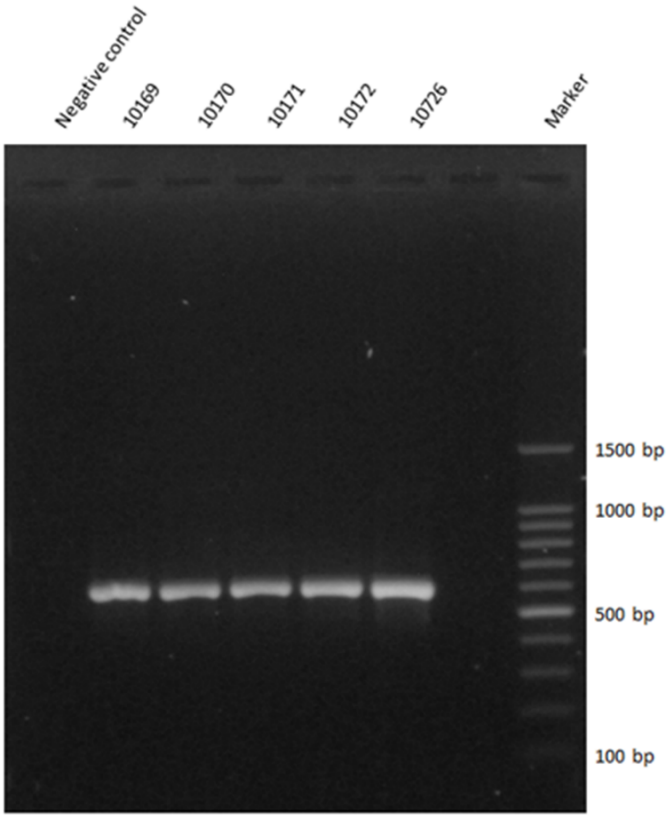

Fig. S2 ITS PCR products of 5 *M. phaseolina* isolates using the mycelial DNA as template. Marker = 100 bp DNA ladder. Negative control = no DNA.

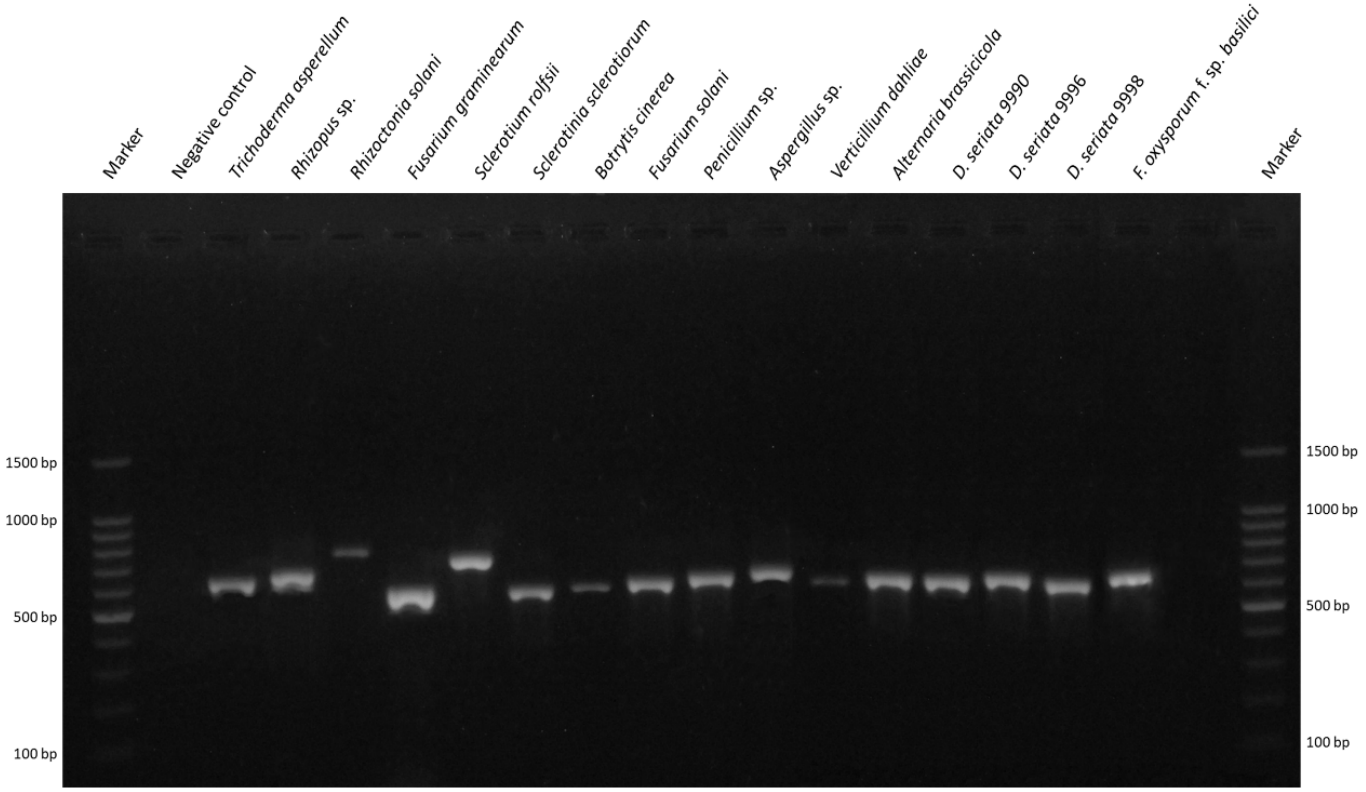

Fig. S3 ITS PCR products of 16 non-target fungi used in this study using the mycelial DNA as template. Marker = 100 bp DNA ladder. Negative control = no DNA.

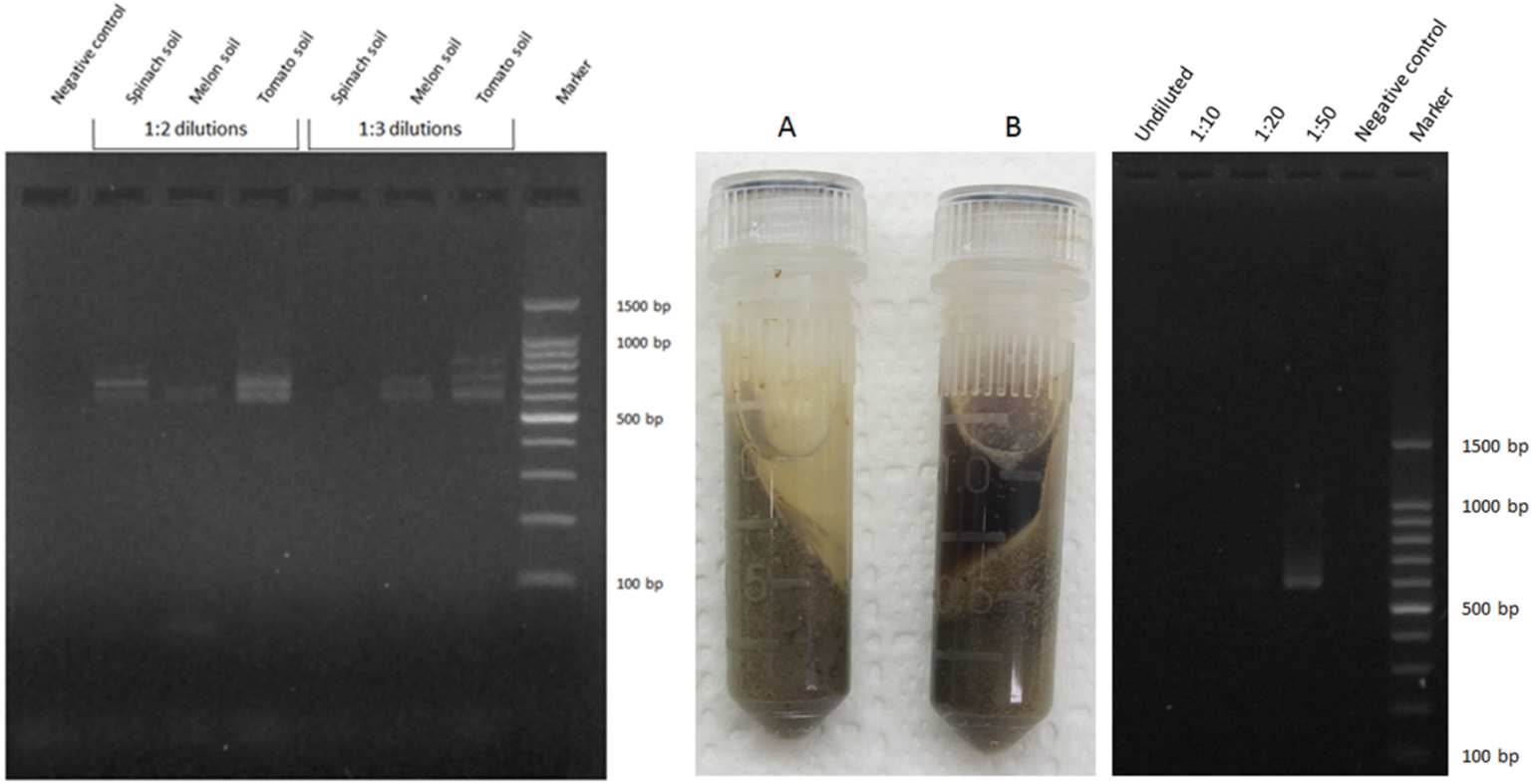

Fig. S4 Left panel = ITS PCR products using the DNA extracted from three different soils (spinach, melon and tomato) with the skimmed milk solution. The starting DNA solutions were diluted 1:2 and 1:3. Middle panel = Comparison between supernatants obtained using the skimmed milk solution (A) and the lysis buffer (B). Right panel = ITS amplification using serial dilutions (1:10, 1:20 and 1:50) of the DNA extracted from spinach soil using the lysis buffer. Marker = 100 bp DNA ladder. Negative control = no DNA.

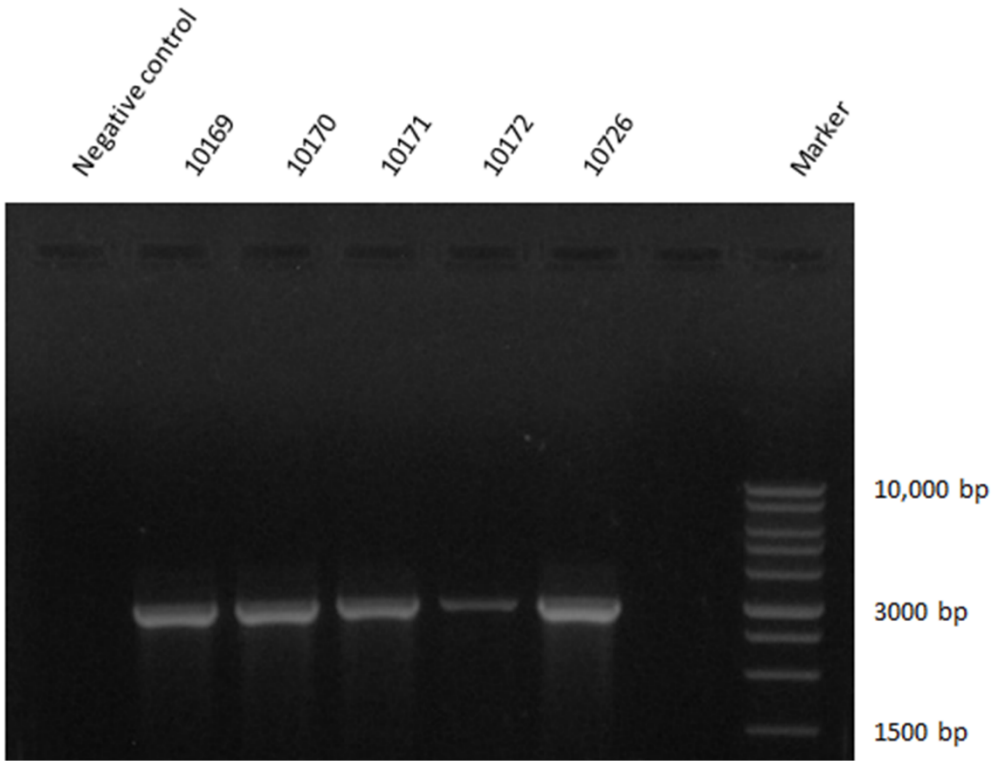

Fig. S5 Amplification products of the entire IGS region using CNL12/CNS1 primer pair on some *M. phaseolina* isolates. Marker = 1 kb DNA ladder. Negative control = no DNA.

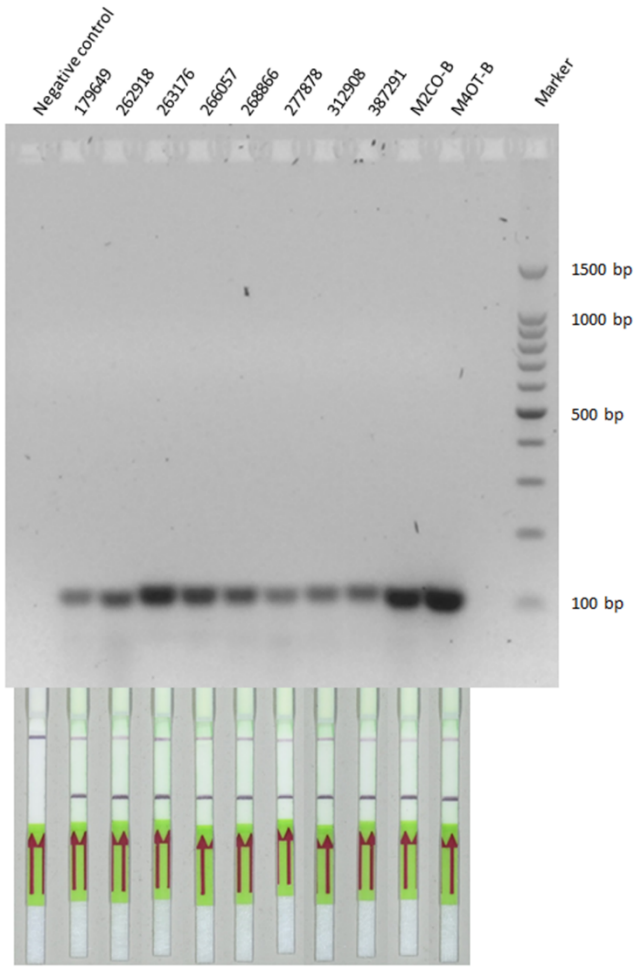

Fig. S6 Specificity of PCR using the primer pair MP102F/MP102R labelled with biotin and FITC respectively for the detection of *M. phaseolina*. Marker = 100 bp DNA ladder. Negative control = no DNA. Upper panel = agarose gel electrophoresis. Lower panel = PCR-NALFIA assay.

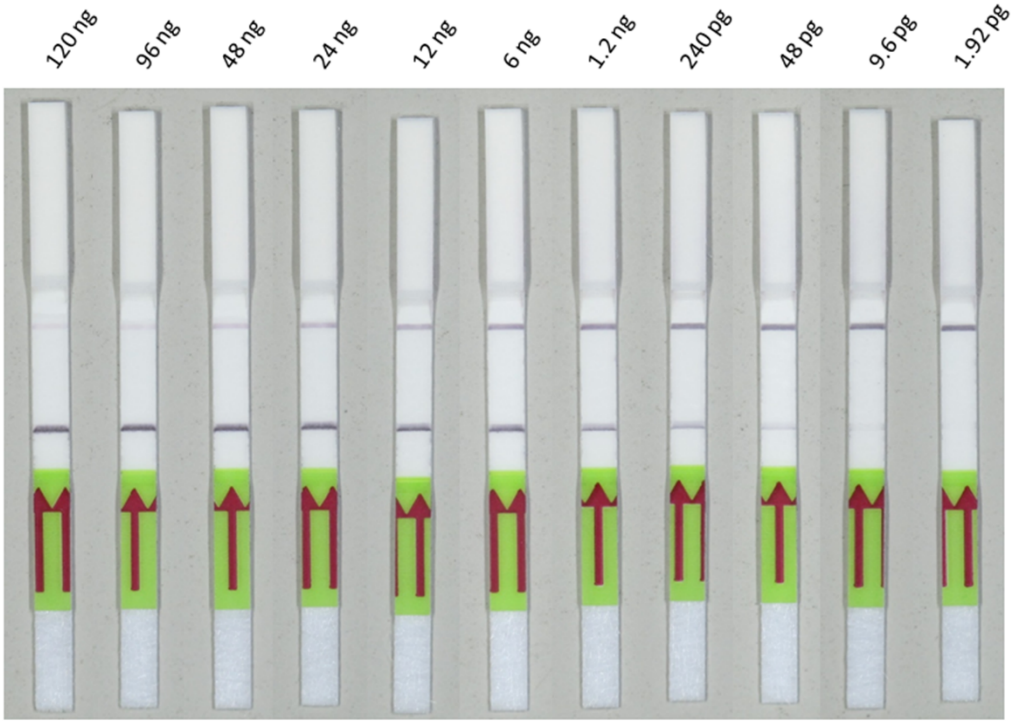

Fig. S7 Sensitivity of the lateral flow dipstick using serial dilutions (from 120 ng to 1.92 pg) of the 102 bp purified amplicon. The PCR assay was performed with the species-specific primer pair MP102F/MP102R and mycelial DNA of *M. phaseolina* isolate 10726 as template.

Table 1 Isolates of *Macrophomina phaseolina* and of different pathogenic and non-pathogenic fungi used in this study.

Species	Isolate	Hosts	Geographic origin	PCR-NALFIA signal
Macrophomina phaseolina	10169[a]	Dandelion	Italy	+
M. phaseolina	10170[a]	Dandelion	Italy	+
M. phaseolina	10171[a]	Dandelion	Italy	+
M. phaseolina	10172[a]	Dandelion	Italy	+
M. phaseolina	10726[a]	Lupin	Italy	+
M. phaseolina	10830[a]	Melon	Italy	+
M. phaseolina	PVS-Mp1[b]	Melon	Italy	+
M. phaseolina	CM1[c]	Melon	Italy	+
M. phaseolina	IMI387291[d]	Safed musli	India	+
M. phaseolina	IMI3312908[d]	Sesame	India	+
M. phaseolina	IMI277878[d]	Sesame	India	+
M. phaseolina	IMI268866[d]	Common bean	Sri Lanka	+
M. phaseolina	IMI268866[d]	Sesame	Mexico	+
M. phaseolina	IMI263176[d]	Moth Bean	India	+
M. phaseolina	IMI262918[d]	Groundnut	India	+
M. phaseolina	IMI179649[d]	Okra	India	+
M. phaseolina	M2CO-B[e]	Cantaloupe melon	Chile	+
M. phaseolina	M4OT-B[e]	Cantaloupe melon	Chile	+
M. phaseolina	M1[f]	Strawberry	Israel	+
M. phaseolina	M2[f]	Strawberry	Israel	+
Alternaria brassicicola	1484[a]	Cabbage	Italy	-
Aspergillus sp.	640[a]	Soil	Switzerland	-
Botrytis cinerea	B05.10[g]	Unknown	Germany	-
Diplodia seriata	9990[a]	Grapevine	Italy	-
D. seriata	9996[a]	Grapevine	Italy	-
D. seriata	9998[a]	Grapevine	Italy	-
Fusarium graminearum	ITEM124[h]	Rice	Italy	-
F. oxysporum f.sp. *basilici*	6100[a]	Basil	Italy	-
Fusarium solani	10728[a]	Gardenia	Italy	-
Penicillium sp.	8037[a]	Peat	Estonia	-
Rhizoctonia solani	RT32[i]	Tobacco	Italy	-
Rhizopus sp.	8485[a]	Soil	Italy	-
Sclerotinia sclerotiorum	724[a]	Chrysanthemum	Italy	-
Sclerotium rolfsii	398[b]	Soil	Italy	-
Trichoderma asperellum	4207[a]	Soil	Israel	-
Verticillium dahliae	10754[a]	Eggplant	Italy	-

[a] Department of Agriculture, Food and Environment (DAFE), University of Pisa, Italy

[b] Department of Agriculture, University of Sassari, Italy

[c] Department Land, Environment, Agriculture and Forestry (TESAF), University of Padova, Italy

[d] Culture collection of CABI Europe UK Centre, Egham, UK

[e] Pontificia Universidad Católica de Chile, Santiago, Chile

[f] Department of Plant Pathology and Weed Research, ARO, The Volcani Center, Bet Dagan, Israel

[g] Institute for Sustainable Plant Protection (CNR), Portici, Napoli, Italy

[h] Institute of Sciences of Food Production (ISPA-CNR), Bari, Italy

[i] Research Unit CRA-CAT, Scafati, Salerno, Italy

Table 2 Genome sequences of *Macrophomina phaseolina* and other fungi belonging to family *Botryosphaeriaceae* used in this study.

Species	Isolate	Hosts	Geographic origin	Genome project GenBank accession No.
Botryosphaeria dothidea	LW030101	*Vitis vinifera*	USA	MDSR00000000.1
Diplodia corticola [a]	CBS112549	*Quercus suber*	Portugal	MNUE00000000.1
Diplodia sapinea [b]	CMW39103	*Pinus patula*	South Africa	JHUM00000000.1
Diplodia sapinea [b]	CMW190	*Pinus radiata*	USA	AXCF00000000.1
Diplodia scrobiculata [c]	CMW30223	*Pinus patula*	South Africa	LAEG00000000.1
Diplodia seriata [d]	F98.1	*Vitis vinifera*	France	MSZU00000000.1
Diplodia seriata [d]	DS831	*Vitis vinifera*	USA	LAQI00000000.1
Lasiodiplodia theobromae	CSS-01s	*Vitis vinifera*	China	MDYX00000000.1
Macrophomina phaseolina [e]	MS6	*Corchorus olitorius*	Bangladesh	AHHD00000000.1
Macrophomina phaseolina	MO00014	*Glycine max*	USA	LHTN00000000.1
Macrophomina phaseolina	MP00003	*Panicum virgatum*	USA	LHTM00000000.1
Macrophomina phaseolina	MP00325	*Lespedeza capitata*	USA	LHTP00000000.1
Macrophomina phaseolina	MP00065	*Zea mays*	USA	LHTO00000000.1
Macrophomina phaseolina	MP00327	*Helianthus* sp.	USA	LHTQ00000000.1
Macrophomina phaseolina	MRf1	*Ricinus communis*	India	LFIX00000000.1
Neofusicoccum parvum [f]	UCRNP2	*Vitis vinifera*	USA	AORE00000000.1
Neoscytalidium dimidiatum	UM880		Malaysia	FLVB00000000.1

[a] Fernandes *et al.,* 2014; [b] van der Nest *et al.*, 2014; [c] Wingfield *et al.*, 2015; [d] Morales-Cruz *et al.*, 2015; [e] Islam *et al*., 2012; [f] Blanco-Ulate *et al.*, 2013.