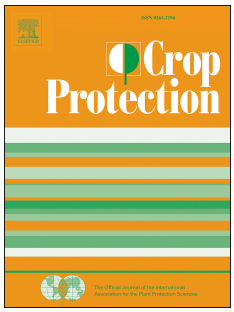


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PII: S0261-2194(20)30396-3

DOI: https://doi.org/10.1016/j.cropro.2020.105463

Reference: JCRP 105463

To appear in: *Crop Protection*

Received Date: 18 February 2020

Revised Date: 9 November 2020

Accepted Date: 11 November 2020

Please cite this article as: Valetti, L., Cazón, L.I., Crociara, C., Pastor, S., Early detection of Ascochyta blight (Ascochyta rabiei) of chickpea by traditional PCR, *Crop Protection*, https://doi.org/10.1016/j.cropro.2020.105463.

# Early detection of Ascochyta blight (*Ascochyta rabiei*) of chickpea by traditional PCR

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**Keywords:** *Ascochyta rabiei, molecular diagnostic, Ascochyta blight, chickpea*

**Abstract**

Ascochyta blight is the major disease affecting chickpea (*Cicer arietinum*) around the world. Since the first report of *Ascochyta rabiei*'s isolation in Argentina in 2012, the pathogen has caused severe economic losses in crop production; so, the detection and rapid identification of the pathogen in early stages is key for the management of the disease. In this work, a traditional PCR procedure for detection of *A. rabiei* directly from plant tissues has been described based on beta-tubulin gene. The TP-6/TP-9 specific primers designed, amplified only a single PCR band of 770 bp from *A. rabiei*. The specificity of the primers was checked using 12 isolates of *A. rabiei* and DNA from 10 other different fungi including common pathogens of chickpea as *Alternaria alternata*, *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Phoma medicaginis* that cause similar symptoms. The detection sensitivity with primers was 2 x $10^4$ ng.µl$^{-1}$ genomic DNA. In inoculated plant

material, PCR amplification gave a band of the expected size and no amplification was observed when DNA was from healthy and uninoculated plants. The results suggested that the assay detected the pathogen more rapidly and accurately than standard isolation methods. The PCR-based method developed here can simplify both plant disease diagnosis, and pathogen monitoring in an early phase, as well as aid in effective management practices that avoid the disease advance and minimize losses.

## 1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important food legumes around the world and is a prominent source of protein principally in central Asia and Africa (Gan et al., 2006; Harveson et al., 2011; Kanouni et al., 2011). Its cultivation area is currently approximately 11.5 million ha, primarily in developing countries (Chen et al., 2016). Argentina leads South American chickpea production, being considered a leader in the international market of chickpea producers (Garzon, 2013; Calzada and Treboux, 2019). In Córdoba province, chickpea production contributes with more than 50 % for exports. The cultivated area and its production is constantly increasing with a production of 139000 tonnes in the 2016/17 season (Carreras et al., 2016; BCC 2017).

One of the most devastating chickpea fungal diseases and economically important throughout the world is the Ascochyta blight (Nene, 1982; Nene et al., 1991; Shahid et al., 2008) caused by *Ascochyta rabiei* (Pass.) Labr. [teleomorph: *Didymella rabiei* (Kovacheski) von Arx (synonym: *Mycosphaerella rabiei* Kovacheski)], class Dothideomycetes, order Pleosporales, family Didymellaceae (Akamatsuet al., 2012). Ascochyta blight affects the leaves, stems and pods of the plants producing lesions, and shoot breakage (Pande *et al*., 2005). In wet and cool weather conditions, blight disease can develop rapidly, with the initial spore germination occurring in single leaves of the chickpea plant and quickly spreads across all chickpea plants and even to the whole crop

(Chen et al., 2016; Manjunatha et al., 2018). Once infection has been established within the field, asexual spores cause secondary spread of the disease (Wiese et al., 1995). Dissemination and development of Ascochyta blight disease can occur through splash and airborne conidia and/or ascospores as well as by commercial distribution of plant material or seeds (Tivoli et al., 2006). The disease significantly reduces chickpea seed yield and quality. The yield losses for susceptible cultivars can reach 100 % when environmental conditions favor the pathogen (Shahid et al., 2008). In Argentina, the first report of *Ascochyta rabiei* causing Ascochyta blight in chickpea was in 2011. This phenomenon caused losses that reached 100 % in some lots (Viotti et al., 2012). The symptoms are easily detectable in an advanced stage of the disease, however, in the initial phase of infection, they may be taken for other pathogens (*Alternaria alternata., Phoma medicaginis, Botrytis cinerea*) and even masked as abiotic damage such as frost or phytotoxicity (Chen et al., 2011). Traditional methods of isolation and identification of *A. rabiei* are time-consuming, consequently limits management options. Therefore, development of effective management practices depends on the rapid detection and precise identification of the pathogen in early stages (Taylor et al., 2007). Polymerase chain reaction (PCR) techniques offer advantages over traditional plant disease diagnosis because organisms do not need to be cultured prior detection by PCR. This technique, apart from being sensitive and fast, provides a powerful tool for disease management. (White et al., 1990; Atkins and Clark 2004; Taylor et al., 2007). The aim of this research was to develop an early diagnostic method by traditional PCR with specific primers for amplification of *A. rabiei* DNA in infected chickpea tissues in order to detect Ascochyta blight in early stages, and be able to take management decisions so as to prevent the spread of the disease.

## 2. Materials and Methods

*2.1. Plant and Fungal Material*

Isolates of *Ascochyta rabiei* used in this study were obtained from harvested seed and naturally infected chickpea plants (cv. Kiara) from Córdoba province in 2017 season. The seeds were washed under tap water, and incubated on agar plate supplemented with 0.15 g $L^{-1}$ of streptomycin sulphate at 21 °C under 12 h alternation of white/black (UV-400 nm) light (Navarro Martinez, 1992; Khan et al 1999). Developed pycnidia on seed tegument were transfered to plates contained chickpea seed meal agar (CSMDA) (chickpea meal 40 g, dextrose 20 g, agar 20 g, distilled water 1L) supplemented with 0.15 g $L^{-1}$ of streptomycin sulphate and incubated at 21 °C with a 12/12 h fluorescent light/dark cycle. Leaves and stems that showed Ascochyta blight symptoms were surface sterilized with 70 % ethanol 1 min, 0.5 % NaClO 1 min and washed three times with sterile water. Samples were cut aseptically in pieces of 5 mm and placed on Petri dishes CSMDA supplemented with 0.15 g $L^{-1}$ of streptomycin sulphate and incubated as described above (Azizpour and Rouhrazi, 2014). Isolates identified as *A. rabiei* by morphologic and microscopic characteristic (Basandrai et al., 2005) were subcultured in CSMDA and single-spored cultures were obtained and stored in glycerol 20 % at -20°C until used. For greenhouse trials, Facultad de Ciencias Agrarias (Universidad Nacional de Córdoba, Argentina) provided seeds of chickpea cv. Chañarito S-156, which has known susceptibility.

*2.2. DNA extraction*

Fungal DNA extractions were performed according to the manufacturer's protocol of Easy pure Genomic DNA kit (Transgene Biotech, Beijing, China). DNA of symptomatic leaves and stems of plants collected from fields and infected plants of greenhouse was extracted following the CTAB protocol with modifications (Doyle and Doyle 1990; Conforto et al.,

2013). Frozen plant tissues were crushed in liquid nitrogen, placed in sterile 1.5 ml microcentrifuge tubes and 500 µl 2 % CTAB supplemented with of 0.2 % of β–mercapto ethanol was added just before use. Tubes were vortexed and incubated at 65 °C for 20 min. One volume of chloroform:isoamyl alcohol (24:1) was added to each tube, which were then centrifuged for 15 min at 13000 g at room temperature. The aqueous phase was transferred to a new tube and the chloroform extraction was repeated. Then 0.7 vol. of cold (-20°C) isopropanol was added and incubated at -20°C for 1 h. Tubes were centrifuged for 30 min at 13000 g at 4°C. DNA was precipitated by the addition of 500 µl 70 % ethanol at room temperature. After centrifugation, the supernatant was discarded; the pellet was washed with 1 ml of 70 % ethanol, and dried at room temperature. DNA was resuspended in 50 µl of distilled water. DNA quality was assessed with electrophoresis in 1 % agarose gel, stained with GelRed™ (Biotium, CA, USA), quantified spectrophotometrically (NanoDrop ND-1000 V3.5; NanoDrop Technologies, USA) and stored at -20 °C.

*2.3. PCR amplification and primers design*

Isolates of *A. rabiei* were identified by using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') (Gardes and Bruns 1993) – ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990). PCR reaction mixture contained 0.5 U GoTaq® DNA polymerase and 5x buffer (Promega, USA), 0.25 µM of each primer, 0.25 µM dNTP, and 2 µl DNA (300 pg) in a final volume of 25 µl. PCR conditions were as follow: initial denaturation at 94 ºC for 5 m, followed by 32 cycles at 94 ºC for 45 s, 58 ºC for 45 s and 72 ºC for 45 s, with a final extension at 72 ºC for 10 min. PCR products were separated by 1.5 % agarose gel electrophoresis and stained with GelRed™ (Biotium, CA, USA). The PCR product obtained was purified via Wizard® columns (Promega, USA) and sent to SICVyA (Unidad Genómica, Instituto de Biotecnología-INTA, Argentina) for

sequencing. Analysis of the obtained sequences were performed by using Pregap4 and Mega6 software and compared with the GenBank database using the BLASTN algorithm (Altschul et al., 1990) in BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST). Sequences of *Ascochyta rabiei* specific primers were designed, based on the existing sequences in GenBank for *Didymella rabiei* isolate AR628 beta-tubulin (KM244529.1) and *Didymella rabiei* strain ATCC 76502 MAT1-1-1 (MAT1-1-1) (DQ341313.1) using Primer 3 software (Untergasseret al., 2012; http://primer3.ut.ee/). The primers sequences were checked by BLAST analysis and PCR amplification for their specificity.

*2.4. Primer specificity and sensitivity test*

A first screening of specificity was determined via PCR using the DNA extracted from *Phoma medicaginis*. The primers that did not amplify *P. medicaginis* DNA, were selected and evaluated with DNA of 10 other different fungi including common pathogens of chickpea (*Fusarium* oxysporum., *Colletotrichum acutatum*., *Phytophtora megasperma*, *Phoma medicaginis*., *Botrytis cinerea*, *Thecaphora frezii*, *Alternaria alternata*., *Phomopsis longicolla*, *Macrophomina phaseolina* and *Valsa ceratosperma*) available in our laboratory. The experiment was carried out twice.

Sensitivity of the primers selected was determined using a dilution series of DNA (2 to 2e-8 ng.$\mu l^{-1}$) of *A. rabiei* as DNA templates for PCR amplification. PCR reaction mixture contained 0.5 U GoTaq® DNA polymerase and 5x buffer (Promega, USA), 0.25 µM of each primer, 0.25 µM dNTP, and 2 µl DNA (300 pg) in a final volume of 25 µl. PCR conditions were as follows: initial denaturation at 95 ºC for 5 min, followed by 37 cycles at 95 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min, with a final extension at 72 ºC for 5 min. PCR products were separated by 1.5 % agarose gel electrophoresis and stained with GelRed™ (Biotium, CA, USA).

155 *2.5. PCR amplification from diseased plant tissue in greenhouse assay and field*

156 To determine if the designed primers were able to detect *A. rabiei* in infected plants, total
157 DNA extraction was carried out from chickpea plants artificially infected in the greenhouse
158 and from plants with symptoms collected from the field. For the artificial infection, two-
159 week-old plants of susceptible chickpea cultivar (cv Chañarito) were inoculated with *A.*
160 *rabiei* OS-8 using a hand atomizer according to Pande et al., (2011). The fungal culture
161 grown on CSMDA medium incubated for 7 days at 20±1°C with a 12-h photoperiod was
162 flooded with sterile distilled water (SDW) and spores were scraped with a sterile Drigalsky
163 spatula. Then the spores were filtered through sterile gauze to remove mycelial fragments
164 and the concentration of the spore suspension was adjusted to 1 x $10^5$ spore $mL^{-1}$ with
165 water. The inoculated plants were covered with transparent polythene sheet and high
166 relative humidity was maintained up to 100 % by humidifiers for 48 hs after inoculation.
167 After 14 days of incubation, the symptomatic plants were harvested and stored to -20 °C
168 for the detection of the pathogen. DNA was extracted from: 1) symptomatic leaves mixed
169 with healthy plant material; 2) necrotic diseased tissue (stem and leaves); 3) a single leaf
170 with a single symptomatic spot; 4) a healthy leaf of a diseased plant and as a negative
171 control, (NC) plant tissue from uninoculated plants.
172 Plants naturally infested with suspicious early symptoms were collected during 2017 and
173 2018 seasons from commercial lots of 14 different locations of the producing area of
174 Córdoba province. Fields were monitored every 15 days throughout the crop cycle to
175 confirm if the disease was present. DNA extraction and PCR were performed as described
176 above using the *A. rabiei* specific primers chosen for detecting *A. rabiei* in vegetal tissue.
177 Asymptomatic plants were used as negative control.
178
179 *2.6. Sequence data analysis*

The obtained fragments with TP6-F/TP9-R specific primers of *A. rabiei* OS-8 colony, a symptomatic plant artificially infected of greenhouse assay and another of field randomly chosen, were purified via Wizard® columns (Promega, USA) and were sent to SICVyA (Unidad Genómica, Instituto de Biotecnología-INTA, Argentina) for sequencing using TP6-F/TP9-R primers. Analysis of the sequences obtained were performed using the BLASTN algorithm (Altschul et al., 1990) and compared with the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST). The consensus sequences were assembled using the Staden program package (Staden et al., 2000), and deposited in the GenBank (NCBI/EMBL) database.

## 3. Results

### *3.1. Fungal isolation and primers design*

Twelve isolates of *A. rabiei* with different morphological characteristics obtained from different field sites were selected. The results of the sequence analysis of the ITS region are shown in Table 1.

A total of seven potential *A. rabiei* specific primers were designed from beta-tubulin gene and five from MAT1-1-1 gene of *Didymella rabiei* and synthesized (Table 2).

### *3.2. Specificity*

Sixteen primer combinations were tested in a first screening for specificity with DNA of *A. rabiei* and *P. medicaginis* because both are closely related (Chen et al., 2015). The results of this specificity test are summarized in Table 3. Healthy and uninoculated plant tissue DNA was used as a negative control, showing that except for TP7-F/TP9-R and MT2-F/MT6-R, the tested primers did not amplify plant tissue. All tested primers amplified *A. rabiei* except for TP1-F/TP4-R which did not show an amplification product. Four primer combinations showed no amplification with either *Phoma* DNA or plant DNA. Two of them

corresponded to the beta-tubulin gene (TP6F/TP1R and TP6F/TP9R) and two to the mating type gene (MT2-F/MT4-R and MT8-F/MT6-R). These primers were tested with 10 other different fungi. Universal primers ITS1 and ITS4 were used as amplification positive control (Table 4). DNA of all genera tested showed no amplification product with the primer combination TP6-F/TP9-R (Table 4); so, it was checked with the DNA of all isolates of *A. rabiei* obtained from field. The pairs of primers TP6-F/TP9-R was able to amplify a unique DNA fragment of approximately 770 bp (Fig. 1) from all *A. rabiei* isolates tested. DNA of *Phoma medicaginis* was used as negative control. All fungal DNA tested gave a positive PCR reaction using ITS universal primers ITS1 and ITS4 (Table 4, Fig 1).

*3.3. Sensitivity*

Sensitivity of the method was evaluated using DNA extracted from a pure culture of *A. rabiei* OS-8 using primers combination TP6-F/TP9-R. The results of this study revealed that TP6-F/TP9-R primers were able to detect $2x10^{-4}$ $ng.\mu l^{-1}$ of *A. rabiei* genomic DNA diluted in sterile water (Fig. 2).

*3.4. PCR amplification from diseased plant tissue in greenhouse assay*

Fourteen days after inoculation, all infected plants showed symptoms on leaf and stem. PCR amplification of DNA extracted from all the artificially infested plants gave an amplification band of the expected size using primers combination TP6-F/TP9-R. No amplification was observed when DNA of healthy and uninoculated plants was used as a template for the amplification (Fig. 3). The presence of *A. rabiei* in the diseased plants was confirmed by isolating the fungi from the tissue to a pure culture.

*3.5. Detection of A. rabiei in symptomatic plants of field*

Fifty sites, 40 in 2017 and 10 in 2018 season, were monitored. A total of 105 samples with suspicious symptoms of *A. rabiei* were analyzed by PCR using TP6-F/TP9-R primers. In the sites where the PCR analysis was positive, it was possible to confirm the presence of the disease from the appearance of the typical symptoms in the pods at the end of the crop cycle. In contrast, in the sites where *A. rabiei* was not detected, the presence of these symptoms was not observed. In the 2017 season, 70 % of the sites analyzed for the early detection of *A. rabiei* using the molecular method tested positive, while in 2018 it was detected in only 10 % of the lots (Fig. 4).

*3.6. Sequence data analysis*

The results of the sequence analysis from PCR product of *A. rabiei* OS-8 DNA, a greenhouse assay symptomatic plants and a randomly picked positive field sample, confirmed that the fragments obtained with TP6-F/TP9-R specific primers correspond to the beta-tubulin gene of *Dydimella rabiei* isolate AR628 (genbank accession: KM244529) (data not shown). Those sequences were deposited in genbank (NCBI) with accession number MN244700, MN244701 and MN244699 respectively.

## 4. Discussion

Ascochyta Blight of chickpea is one of the most important diseases of the crop principally in cold and wet regions. An effective disease management depends among others on the rapid detection and precise identification of the pathogen. Field diagnosis of the disease is currently based on symptoms such as leaf, stem or pod lesions with, or without, pycnidial formation (Reddy 1993; Manjunatha et al., 2018). However, these methods require a lot of time and have not always been adequate due to the superposition of morphological characters; and phenotypic variation, both among related species as well as under different environmental conditions (Taylor et al., 2007). For these reasons a fast detection

by molecular methods is necessary. This helps to quickly manage the disease before pathogen severe dispersal occurs. Phan et al., (2002) developed an efficient PCR–RFLP method for detecting *A. rabiei* infection in chickpea seed that could be used to assess samples of seed prior to distribution and planting. Bayraktar et al. (2016) described a real-time PCR procedure for the detection and quantification of *A. rabiei* directly from plant tissues based on genetic variability of EF gene. In addition, the assay was used to monitor the progression of pathogen infection in infected plant material for efficient selection of resistant breeding material in an early stage of infection as an alternative to the visual disease assessment. However, it is an expensive method to apply massively. Although there are works that describe molecular methods to detect *A. rabiei*, they do not focus on disease diagnosis in the field, with natural infection and in presence of fungi causing similar symptoms. In this work, we propose a simple method, since it needs a single reaction of traditional PCR, and no use of restriction enzymes nor in vitro fungus isolation. It is a quick and inexpensive diagnosis, suitable for field scouting of Ascochyta blight.

In fungal diagnosis based on molecular methods it is well described that ITS region is the main DNA target (Atkins and Clark 2004). Nevertheless, other genes are being more widely studied, in particular the beta-tubulin gene (Fraaijeet al., 1999, Hirischet al., 2000), and mating type genes (Dyer et al., 2001, Foster et al., 2002). In the literature there are several examples where they have developed specific primers based on these genes (McCartney et al., 2003). This work describes the development of a rapid, sensitive, and effective molecular method to detect *A. rabiei* of symptomatic plant tissues in early stages of the disease by traditional PCR based on the specific primers designed from the *Didymella rabiei* beta-tubulin and MAT1-1-1 gene.

A suitable diagnostic assay needs to be both sensitive and specific in order to avoid cross-reactions with other fungi (Atkins and Clark 2004). The present study demonstrates that primers designed of beta-tubulin geneTP6-F and TP9-R resulted in a product of

283 approximately 770 bp with all 12 isolates of *A. rabiei* and not amplify *P. medicaginis* which
284 is the most similar specie, nor another 10 different fungi genera that may be present in the
285 chickpea crop. The sensitivity of PCR assays is an important concern in the molecular
286 detection of plant pathogens in field plants. The results demonstrated that the PCR assay
287 could be used to detect the pathogen in plant tissue with a single symptomatic spot or at a
288 level of $2x10^{-4}$ ng.µl$^{-1}$ of DNA. As regards field monitoring to detect *A. rabiei* in suspicious
289 early symptoms, in the 2018 season less commercial fields than in the 2017 were
290 evaluated. It was due to the fact that in the 2018 season, the environmental conditions
291 were not favorable for the development of the disease, so that suspicious symptoms were
292 observed only in a few sites. This was also reflected in a lower incidence of the disease in
293 the samples analyzed (10 %) as regards the 2017 season (70 %) (Fig. 4). With the method
294 developed in the present work, the pathogen can be detected in early symptoms (not
295 typical symptoms). In that moment these could be taken by those caused by other
296 pathogens present in our production area (Scandolo et al., 2018) or by abiotic stresses.
297 Early symptoms are the most difficult to identify but are also the most important.
298 Intervention with fungicides at the seedling stage is key to limit disease development for
299 the entire season and early Ascochyta blight prevention and management (Doken-
300 Bouchard et al., 2010). The detection of *A rabiei* in the chickpea crop determines the
301 management strategy. If *A. rabiei* is not present, foliar applications of fungicides are not
302 carried out (Sillon and Viotti, 2014).
303 In summary, through this study a highly sensitive and specific PCR diagnostic assay was
304 developed to detect *A. rabiei* in chickpea plants from field compared with the traditional
305 culture isolation method and does not require complicated preparation of samples. A rapid
306 detection of *A. rabiei*, plays an important role in epidemic tracking of Ascochyta blight,
307 especially since it is a fast spreading disease. It could also be used to scout and prevent
308 the development of this pathogen at early stages of disease. This is a critical phase to get

309 an effective integral management, keep plant diseases below economically damaging
310 levels and reduce the important yield losses that it produces.
311
312 **Declaration of competing interest**
313 The authors declare that they have no known competing financial interests or personal
314 relationships that could have appeared to influence the work reported in this paper.
315
316 **Acknowledgements**
317 Thanks are due to Mg. Sc. Laura Torres (Agronomical faculty of Córdoba), Dr. Franco
318 Fernandez, Dr. Nelson Bernardi Lima and Ing. Agr. Mauro Paccioretti (IPAVE-INTA) for
319 writing assistance and general and technical support. Thanks to Ing. Agr Julia Carreras
320 (Faculty of agricultural sciences, national university of Córdoba, Argentina) for kindly
321 providing chickpea seeds. This research was supported by Federal Projects of Productive
322 Innovation (PFIPESPRO 2017). COFECYT. (EX-2017-18920446-APN-DDYME# MCT.
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469

470 **Figure legends**

471 **Figure 1:** Agarose gels with the PCR reaction products of different *A. rabiei* isolates and
472 *Phoma medicaginis*. using specific primers designed for *A. rabiei* TP6-F/TP9-R (A);
473 and universal primers ITS1/ITS4 as a positive control (B). 1) RCA; 2) RCB; 3)
474 RCC,4) RCD; 5) ARK9; 6) TYCM1; 7) MRM1; 8) FCAM1; 9) OS-1;10) OS-8; 11)
475 OS-15; 12) OS-16; 13) *Phoma medicaginis*.; NC) negative control; PC) positive
476 control; M) molecular marker.
477

478 **Figure 2:** Sensitivity PCR test with specific primers TP6-F/TP9-R designed in this study
479 using different concentrations of DNA. The purified DNA of *A. rabiei* OS-8 was
480 used as a template. Lanes 2-10: DNA dilutions from 2 ng to 2 x $10^{-8}$ ng
481 respectively. M: 100 bp DNA ladder
482

**Figure 3:** PCR amplification from artificially infected plants with *A. rabiei* OS-8 using specific primers TP6-F/TP9-R. Line 1: DNA extracted from symptomatic leaves mixed with healthy plant material; Line 2: necrotic diseased tissue (stem and leaves); Line 3: a single leaf with a single symptomatic spot; Line 4: a healthy leaf of a diseased plant; NC: plant tissue from uninoculated plants as negative control; M: 100 bp DNA ladder.

**Figure 4:** Detection of *A. rabiei* by molecular diagnostic method in field plants with suspicious symptoms in 2017 and 2018 seasons.

**Table 1:** *A. rabiei* isolates identification by ITS rDNA region analysis

| Isolate | Location | GenBank number |
|---|---|---|
| RCA | Rayo Cortado | MT835113 |
| RCB | Rayo Cortado | MT835114 |
| RCC | Rayo Cortado | MT835115 |
| RCD | Rayo Cortado | MT835116 |
| ARK9 | Jesus Maria | MT835119 |
| TYCM1 | Cañada de Luque | MT835117 |
| MRM1 | Jesus Maria | MT835108 |
| FCAM1 | Capilla de los Remedios | MT835107 |
| OS1 | Rio Cuarto | MT835109 |
| OS8 | Rio Cuarto | MT835110 |
| OS15 | Rio Cuarto | MT835111 |
| OS16 | Rio Cuarto | MT835112 |

**Table 2.** List of specific primers design to *A. rabiei*

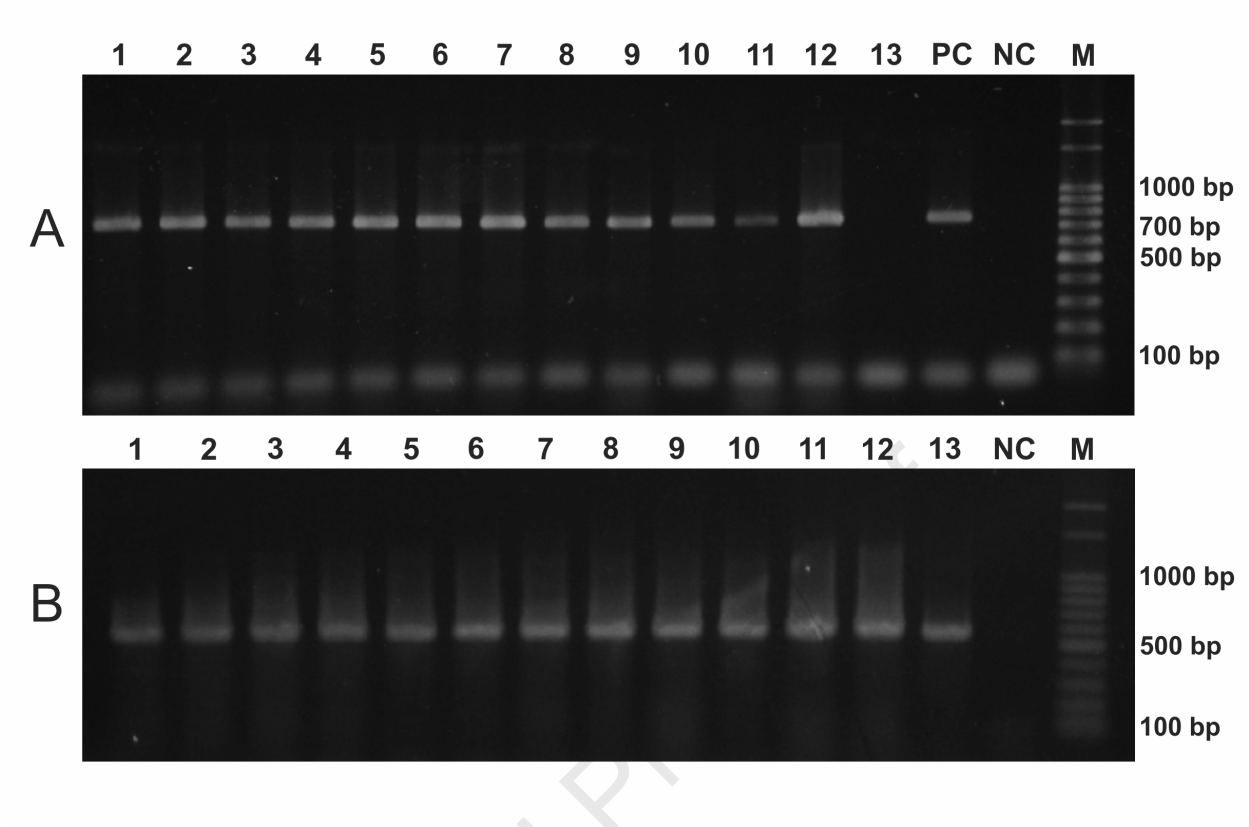
| ***Didymella rabiei* isolate AR628 beta-tubulin gene (KM244529.1)** | | | | | | |
|---|---|---|---|---|---|---|
| **Forward primer** | **Sequence (5'–3')** | **Length** | **Start** | **Stop** | **Tm** | **GC%** |
| TP1-F | GCCTTACAACGCCACTCTCT | 20 | 384 | 403 | 60.04 | 55.00 |
| TP3-F | TGCCGTCCTCGTCGATTTAG | 20 | 27 | 46 | 59.90 | 55.00 |
| TP6-F | GTGCCGTCCTCGTCGATTTA | 20 | 26 | 45 | 60.18 | 55.00 |
| TP7-F | GAGTTCCCTGACCGCATGAT | 20 | 313 | 332 | 59.82 | 55.00 |
| **Reverse primer** | | | | | | |
| TP1-R | GGTCAGAGGAGCGAAACCAA | 20 | 663 | 644 | 59.97 | 55.00 |
| TP2-R | CAAGTGAGGTAGCGACCGTT | 20 | 779 | 760 | 60.04 | 55.00 |
| TP4-R | CTGGTCACCGATACGCTTGA | 20 | 993 | 974 | 59.83 | 55.00 |
| TP9-R | ACGGAAGTAGGCAGAGCAAG | 20 | 795 | 776 | 59.75 | 55.00 |
| ***Didymella rabiei* strain ATCC 76502 MAT1-1-1 (MAT1-1-1) gene (DQ341313.1)** | | | | | | |
| **Forward primer** | **Sequence (5'–3')** | **Length** | **Start** | **Stop** | **Tm** | **GC%** |
| MT2-F | CATCCGCGATCAGATAGGCA | 20 | 147 | 166 | 59.76 | 55.00 |
| MT3-F | CCTTGAGCGTTACGGATGGA | 20 | 240 | 259 | 59.83 | 55.00 |
| MT8-F | CCGTCATCCGCGATCAGATA | 20 | 143 | 162 | 59.48 | 55.00 |
| **Reverse primer** | | | | | | |
| MT4-R | AAGGCGGCCATTGTGAGTAG | 20 | 528 | 509 | 60.39 | 55.00 |
| MT6-R | AGAGCTTGCGAGTGGAGTTT | 20 | 510 | 491 | 59.61 | 50.00 |

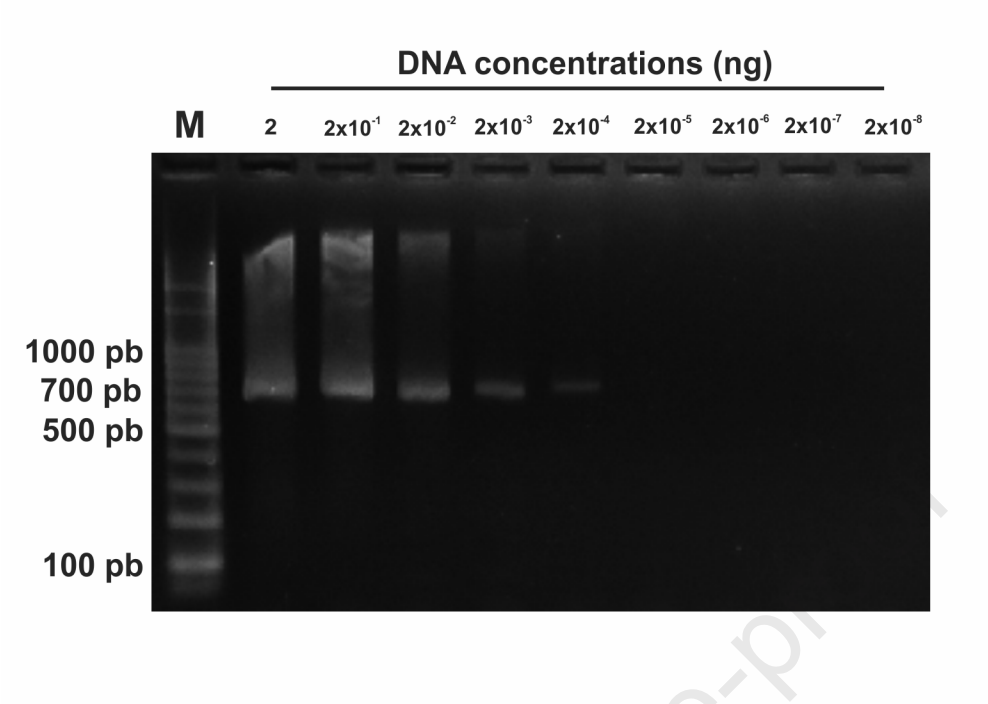
**Table 3.** Result of PCR amplification with different primers design for *Ascochyta rabiei* with DNA of *A. rabiei*, *Phoma* sp. and plant tissue.

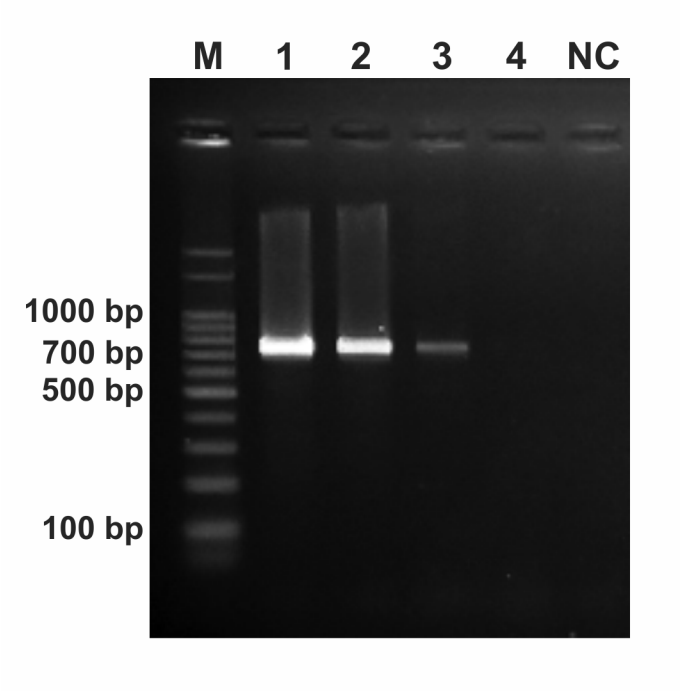
| Primers Combination | Amplicon (pb) | PCR amplification | | |
|---|---|---|---|---|
| | | ***A. rabiei* OS-8** | ***Phoma* sp.** | **Plant tissue** |
| TP1-F/TP4-R | 613 | - | - | - |
| TP3-F/TP1-R | 636 | + | + | - |
| TP6-F/TP1-R | 637 | + | - | - |
| TP7-F/TP1-R | 350 | + | + | - |
| TP3-F/TP2-R | 752 | + | + | - |
| TP3-F/TP9-R | 768 | + | + | - |
| TP6-F/TP2-R | 753 | + | + | - |
| TP6-F/TP9-R | 769 | + | - | - |
| TP7-F/TP9-R | 482 | + | + | + |
| TP7-F/TP2-R | 466 | + | + | - |
| MT2-F/MT4-R | 381 | + | - | - |
| MT2-F/MT6-R | 363 | + | - | + |
| MT3-F/MT4-R | 288 | + | + | - |
| MT3-F/MT6-R | 270 | + | + | - |
| MT8-F/MT4-R | 306 | + | + | - |
| MT8-F/MT6-R | 367 | + | - | - |

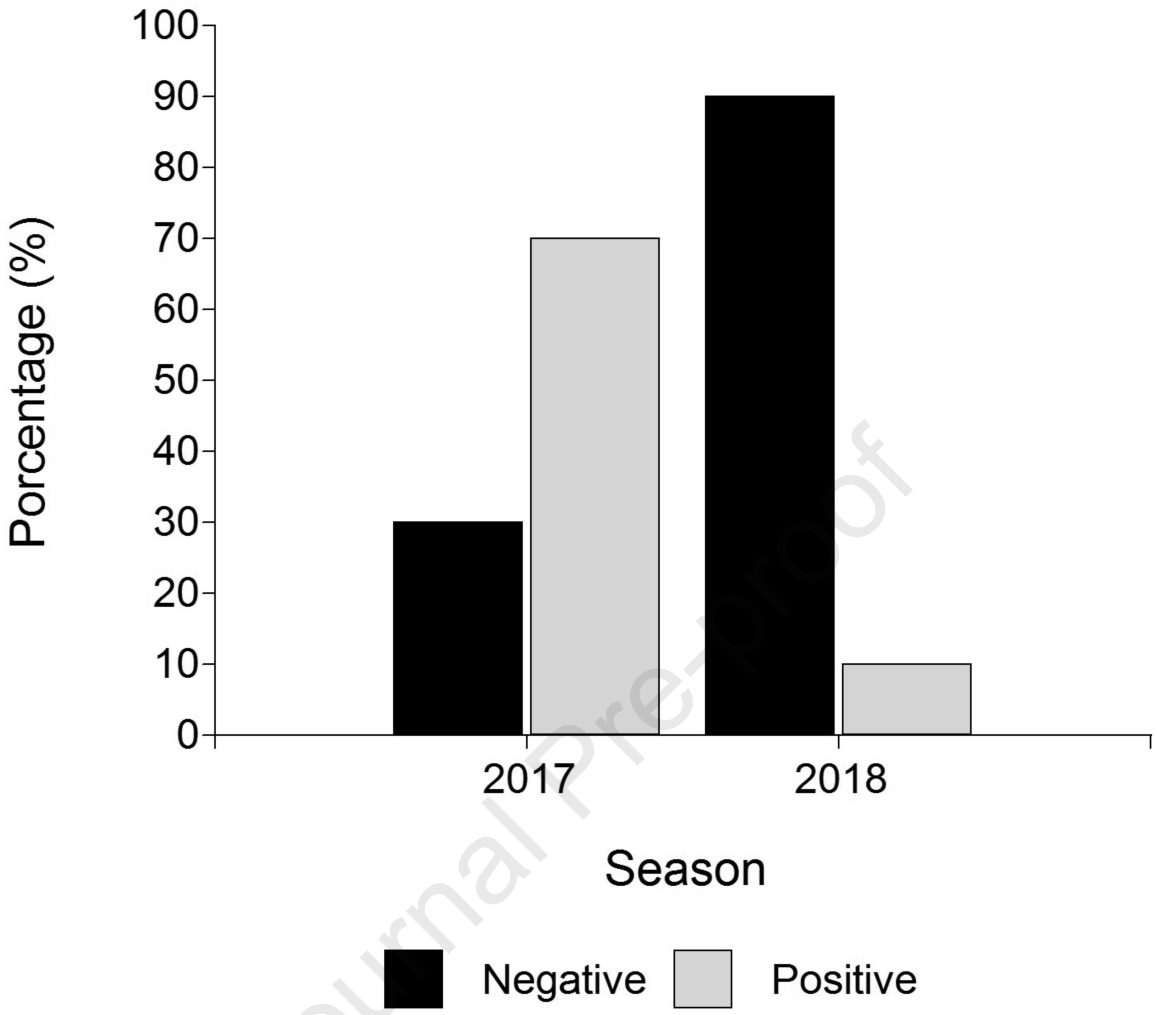
**Table 4.** Result of PCR amplification of specificity test with specific primers selected for *Ascochyta rabiei* using DNA of different pathogens as template.

| | Primers Combination | | | | |
|---|---|---|---|---|---|
| **Tested fungal DNA** | **ITS-1/ ITS4** | **TP6-F/ TP1-R** | **TP6-F/ TP9-R** | **MT2-F/ MT4-R** | **MT8-F/ MT6-R** |
| *Ascochyta rabiei* OS-8 | + | + | + | + | + |
| *Fusarium oxysporum* | + | - | - | - | + |
| *Colletotrichum acutatum* | + | - | - | + | + |
| *Phytophtora megasperma* | + | - | - | + | - |
| *Phoma medicagini* | + | - | - | - | - |
| *Botrytis cinerea* | + | - | - | - | + |
| *Techaphora frezii* | + | - | - | - | + |
| *Alternaria alternata* | + | - | - | - | - |
| *Phomopsis longicolla* | + | - | - | - | - |
| *Macrophomina phaseolina* | + | - | - | - | - |
| *Valsa ceratosperma* | + | + | - | - | - |









Highlights

- A rapid, sensitive, and effective molecular method to detect *A. rabiei* of symptomatic plant tissues in early stages of the disease was developed.
- *A. rabiei* specific primers were designed from beta-tubulin gene.
- Field samples with incipient symptoms of Ascochyta bligth of 50 sites were monitored and analyzed for the early detection of *A. rabiei* using the molecular method developed.

**Declaration of interests**

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: