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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.

1. Introduction

Macrophomina phaseolina (Tassi) Goid. is a soil- and seed-borne generalist fungal pathogen that has a global distribution and can infect > 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 Jr., 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 assays 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

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spacers (ITS1 and ITS2) and one intergenic spacer (IGS) (White et al., 1990).

The ITS regions have been extensively sequenced and shown to be useful to identify numerous fungi at species or sub-species level. The IGS region on the other hand is known to evolve faster than the ITS region, resulting to be the main source of polymorphisms in the rDNA gene complex in other fungi (Jackson et al., 1999; Pecchia et al., 1998, 2004; Pramateftaki et al., 2000; Pantou et al., 2003), however, no IGS sequences are currently published for *M. phaseolina*. Compared to ITS region, IGS poses more difficulties for amplification and sequencing, however, it can be useful when there are not enough differences available across the ITS (Schena et al., 2004a).

Since the higher copy number of IGS increases the sensitivity of PCR-based techniques, this region has been used to develop specific diagnostic assays for many plant pathogenic fungi and oomycetes such as *Botrytis cinerea* and *Botrytis* spp. (Suarez et al., 2005; Sanzani et al., 2012; Khan et al., 2013), *Fusarium circinatum* (Schweigkofler et al., 2004), *F. oxysporum* f. sp. *vasinfectum* (Zambounis et al., 2007), *Fusarium oxysporum* f. sp. *cubense* (Dita et al., 2010), trichothecene-producing *Fusarium* spp. (Jurado et al., 2005), *Phytophthora medicaginis* (Liew et al., 1998), *Verticillium dahliae* and *V. alboatrum* (Schena et al., 2004b; Bilodeau et al., 2012).

The early diagnosis and screening of *M. phaseolina* from different soils needs a simple, rapid and cost-effective test, without the use of sophisticated and expensive equipment and reagents not fully affordable in ordinary laboratories. Recently, a molecular method has been exploited to develop specific primers for a real-time qPCR assay using a sequence characterized amplified region (SCAR) for the detection of *M. phaseolina in planta* and in rhizosphere soil (Babu et al., 2011).

A simplification of the methodology could be achieved by using simple and cheap read-out systems, such as the nucleic acid lateral flow immuno-assay (NALFIA) (van Amerongen and Koets, 2005; Posthuma-Trumpie et al., 2009), which can circumvent the use of electrophoresis or the purchase of expensive real-time PCR machines.

The use of NALFIA as biosensor for detecting specific amplified nucleic acid genes is not new (Rule et al., 1996) and has been widely used in the point-of-care (POC) devices for medical diagnostics. To date, this technology has also reached many fields of research such as veterinary diagnostic, food and environment monitoring and plant disease diagnosis, with a few cases limited to fitovirus (Posthuma-Trumpie et al., 2009; Ngom et al., 2010; O'Farrel, 2013).

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

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

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

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	–

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[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.

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

2. Materials and methods

2.1. Fungal cultures and microsclerotia production

Isolates used in this study are summarised in Table 1. Purified