SHORT COMMUNICATION

DEVELOPMENT OF SCAR MARKERS AND PCR ASSAY FOR *FUSARIUM OXYSPORUM* f. sp. *MELONIS* RACE 2-SPECIFIC DETECTION

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SUMMARY

Fusarium oxysporum f. sp. *melonis* (FOM) is the cause of vascular wilting of melon, the most severe infectious disease of this cucurbit. Four races (0, 1, 2, and 1,2) of FOM have been identified all of which occur in Italy. A PCR assay based on race-specific SCAR markers for FOM race 2 identification was developed which proved specific, sensitive, and reliable, regardless of the origin of isolates. SCAR primers developed from RAPD markers were able to identify unambiguously FOM race 2, that causes symptoms similar to those elicited by the other FOM races. These are morphologically indistinguishable from one another and from the other *formae speciales* of the same fungus. The specificity of these primers, which did not amplify the most common melon pathogens, makes them a reliable tool for diagnosis. This finding may have additional practical applications in the epidemiology and management of Fusarium wilt of melon caused by this race in areas where the disease is widespread.

Key words: melon, soil-borne, wilting, vascular disease, RAPD, diagnosis.

Fusarium oxysporum f. sp. *melonis* Snyd. et Hans. (FOM) is the cause of a severe vascular wilt of melon (*Cucumis melo*), the most important disease of this cucurbit worldwide. It occurs throughout Europe as well as in North and Central America (Zuniga *et al*., 1997), Asia (Abou-Jawdah and Al-Khoury, 1996; Erzurum *et al*., 1999; Namiki *et al*., 1998) and Africa (Schreuder *et al*., 2000). Wilting symptoms and plant death caused by FOM can be devastating, with losses as high as 100% (Wechter *et al*., 1995; Belisario and Corazza, 2003).

With about 27,000 ha given over to melon, Italy is the second largest producer in Europe after Spain. Once introduced into the field, FOM can persist even after rotation with non-host crops, due to the production of chlamydospores (resting and durable spores) and its ability to colonize crop residues and roots of most crops grown in rotation (Gordon *et al*., 1989; Suarez-Estrella *et al*., 2004). Effective control can be achieved only through host resistance (Mas *et al*., 1981; Zink, 1983, 1992; Belisario and Corazza, 2003; Ficcadenti *et al*., 2005). Although many *Fusarium* species can penetrate cortical tissues of the roots, only host-specific strains can enter the vascular elements by mycelial growth and the formation of microconidia, transported in the sap stream (Di Pietro *et al*., 2003).

Four races of the pathogen (0, 1, 2 and 1,2) have been identified, according to the host resistance genes overcome by variants of the pathogen (Risser *et al*., 1976). Race 0 is pathogenic to melon genotypes that lack *Fom* resistance genes. Two dominant, independently-inherited resistance (R) genes (*Fom-1* and *Fom-2*) provide resistance to races 0 and 2, and races 0 and 1, respectively (Risser *et al*., 1976). Race 1,2 overcomes these two resistance genes and has been further subdivided into race 1,2w, which causes wilting, and 1,2y, which causes yellowing.

All four races, including race 2, are present in Italy (Tamietti *et al*., 1994), tough race 1,2 is becoming prevalent (Belisario and Corazza, 2003).

In many *formae speciales* of *F. oxysporum* there is a complex relationship between pathogenic races and vegetative compatibility groups (VCGs) and a given race may be associated with more than one VCG and some VCGs with more than one race (Gordon and Martyn, 1997). For instance, FOM VCG 0134, which is the most represented VCG in Europe and Italy, is associated with all four known races. In turn, the association of one race with many VCGs, such as in the case of FOM race 2, may indicate that genetic changes generating a particular virulence phenotype have occurred on multiple occasions (Gordon and Martyn, 1997). In Israel, FOM race 2 represents a relatively recent discovery (Netzer and Weintal, 1989) compared to race 0 which has a longer history in this country (Netzer and Weintal, 1979). Race 0 is widespread, whereas race 2 has a more localized distribution, occurring together with race 0 in some areas. Race 2 and race 0 are both associated with VCG 0138. FOM race 2 may have been introduced into

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Table 1. Fungal isolates used in this study.

Isolate	Host	Race	Geographic origin
F. o. f. sp. *melonis* ISPaVe1289	Melon	0	Italy
F. o. f. sp. *melonis* ISPaVe1673	Melon	0	Italy
F. o. f. sp. *melonis* ISPaVe1676	Melon	0	Italy
F. o. f. sp. *melonis* ISPaVe1069	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1070	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1074	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1077	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1085	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1296	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1297	Melon	1	Italy
F. o. f. sp. *melonis* ISPaVe1404[a]	Melon	1	USA
F. o. f. sp. *melonis* ISPaVe1018	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1083	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1679	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1680	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1681	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1684	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1689	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1692	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1693	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1694	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1697	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1698	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1700	Melon	1,2	Italy
F. o. f. sp. *melonis* ISPaVe1406[a]	Melon	2	USA
F. o. f. sp. *melonis* ISPaVe1408[a]	Melon	2	USA
F. o. f. sp. *melonis* ISPaVe1409[a]	Melon	2	USA
F. o. f. sp. *melonis* ISPaVe1411[a]	Melon	2	USA
F. o. f. sp. *melonis* ISPaVe1417[a]	Melon	2	USA
F. o. f. sp. *melonis* ISPaVe2068[b]	Melon	2	Israel
F. o. f. sp. *melonis* ISPaVe2069[b]	Melon	2	Israel
F. o. f. sp. *melonis* ISPaVe1703	Melon	2	Italy
F. o. f. sp. *basilici* ISPaVe2097[c]	Basil	-	Switzerland
F. o. f. sp. *ciclaminis* ISPaVe1762	Cyclamen	-	Italy
F. o. f. sp. *cucumerinum* ISPaVe2098[d]	Watermelon	-	Greece
F. o. f. sp. *radicis-cucumerinum* ISPaVe2100[d]	Watermelon	-	Greece
F. o. f. sp. *lentis* ISPaVe ER1395[e]	Lentil	-	Algeria
F. o. f. sp. *lycopersici* ISPaVe2102	Tomato	-	Italy
F. o. f. sp. *radicis-lycopersici* ISPaVe ER1553[f]	Tomato	-	Italy
F. o. f. sp. *vasinfectum* ISPaVe1859[g]	Cotton	-	Italy
F. oxysporum ISPaVe904	Watermelon		Italy
F. oxysporum ISPaVe18	Artichoke		Italy
Plectosporium tabacinum ISPaVe835	Zucchini		Italy
Monosporascus cannonballus ISPaVe ER1327[f]	Melon		Italy
Monosporascus cannonballus ISPaVe ER1328[f]	Melon		Italy
Monosporascus cannonballus ISPaVe ER1329[f]	Melon		Italy
Acremonium cucurbitacearum ISPaVe ER1179[f]	Melon		Italy
Acremonium cucurbitacearum ISPaVe ER1516[f]	Watermelon		Italy
Acremonium cucurbitacearum ISPaVe ER1517[f]	Melon		Italy
Rhizopycnis vagum ISPaVe ER925[f]	Tomato		Italy
Pyrenochaeta lycopersici ISPaVe ER1320[f]	Tomato		Spain
Pyrenochaeta lycopersici ISPaVe ER1278[f]	Tomato		Italy
Pyrenochaeta lycopersici ISPaVe ER876[f]	Tomato		Italy

[a]Strain supplied by Dr. T.R. Gordon, Department of Plant Pathology, University of California, Davis, California.
[b]Strain supplied by Dr. R. Perl-Treves, The Mina & Everard Goodman Faculty of Life Sciences, Bar-llan University, Ramat-Gan, Israel.
[c] Strain supplied by Dr. G. Tamietti, Università di Torino, DIVAPRA, Torino, Italy.
[d]Strain supplied by Dr. D. J. Vakalounakis, National Agricultural Research Foundation, Plant Protection Institute, Heraklion, Greece.
[e]Strain supplied by Dr. L. Riccioni, CRA-Centre of Plant Pathology Research, Rome, Italy.
[f]Strain supplied by Dr. A. Infantino, CRA-Centre of Plant Pathology Research, Rome, Italy.
[g]Strain supplied by Dr. E. Turco, University of Florence, Dipartimento di Biotecnologie Agrarie, Florence, Italy.

Israel from other parts of the world such as North America and Japan where it is well established (Zink, 1983; Namiki *et al*., 1998).

By comparative genomics Ma *et al.* (2010) showed that *F. oxysporum* f. sp. *lycopersici* contains lineage-specific (LS) chromosomes enriched for genes related to host-pathogen interaction and demonstrated the transfer of these chromosomes between strains of *F. oxysporum* converting a non-pathogenic into a pathogenic strain. Horizontal transfer of host specificity factors between distant and genetically isolated lineages of *F. oxysporum* may explain the apparent polyphyletic origins of host specialization which is often observed in this fungus, as in the case of FOM race 2.

Disease diagnosis and pathogen identification of *formae speciales* by traditional methods based on the reaction of inoculated differential hosts are labour-intensive and time-consuming. So the availability of a rapid, specific and sensitive method to detect pathogenic races is important for the development of sustainable pest management strategies. Molecular techniques can be useful for this purpose and they have been widely applied as reported by Lievens *et al.* (2008). Sequences of highly conserved genes like translation elongation factor 1α (TEF-1α), calmodulin, and beta-tubulin are suitable genetic markers to distinguish fungal species, including *Fusarium* spp. (Kim *et al*., 2005; Mbofung *et al*., 2007; O'Donnell *et al*., 1998; Carbone and Kohn, 1999; Glass and Donaldson, 1995), though they may have a low resolving power for discriminating sub-species grouping like *formae speciales* and races. In turn, random amplified polymorphic DNA analysis (RAPD) may be useful in the identification of such fungal sub-specific groups by providing genetic information associated with random punctiform DNA changes. Different diagnostic methods using RAPD have been successfully developed to differentiate races in several *formae speciales* of *F. oxysporum* (Skovgaard *et al.*, 2001; Migheli *et al.*, 1998; Jimenez-Gasco *et al.*, 2001). Nevertheless, RAPD assays have the major disadvantage in their poor interlaboratory reproducibility. For this reason, the diagnostic DNA fragments from RAPD need to be converted into more reliable molecular markers, such as the sequence-characterized amplified region (SCAR) primers. This approach has been used for the identification of different *formae speciales* and races of *F. oxysporum* (Alves-Santos *et al*., 2002; Jimenez-Gasco and Jimenez-Diaz, 2003; Ciocchetti *et al*., 2001).

In this study, a PCR assay based on race-specific SCAR markers was first developed for the identification of FOM race 2, then employed for its detection in diseased melon plants, for providing a specific, reliable, sensitive and rapid diagnostic protocol.

Previously, we had looked for polymorphism among FOM races using internal transcribed spacer (ITS), intergenic spacer (IGS), inter-simple sequence repeat (ISSR), TEF1-α, calmodulin, and β-tubulin and tested 89 Operon RAPD primers for differentiating FOM races. However, only one Operon RAPD primer amplified a band that clearly distinguished FOM race 2 from the other three.

The present investigation involved 32 FOM isolates whose race designation (Risser *et al*., 1976) had previously been determined on differential hosts. Since, SCARS markers had been developed for diagnostic purpose, the specificity of the primer pair was tested on a broad range of fungal pathogens of melon associated with collapse syndromes, i.e. *Plectosporium tabacinum* (van Beyma) M.E. Palm, W. Gams, et Nirenberg, the causal agent of pumpkin blight also implicated in melon "sudden collapse", *Monosporascus cannonballus* Pollack et Uecker, *Acremonium cucurbitacearum* Alfaro-García, W. Gams et García-Jim., *Rhizopycnis vagum* D.F. Farr and *Pyrenochaeta lycopersici* R.W. Schneid. et Gerlach, the cause of corky root disease of melon and tomato (Infantino *et al*., 2000). In addition, eight different *F. oxysporum formae speciales* other than *melonis*, and two isolates of *F. oxysporum*, were tested (Table 1). Single-spore cultures were maintained on filter paper at -20°C in our Institute.

For DNA extraction, mycelium was scraped from 15–day-old pure cultures grown on potato dextrose agar (PDA, Oxoid, UK) at 25°C in the dark and ground to a fine powder in liquid nitrogen with a sterile mortar and pestle. Total DNA was extracted following the protocol of the Purgene DNA purification kit (Gentra, USA). Among 89 arbitrary RAPD PCR 10-mers (Operon Technologies, USA) tested, only one primer OP-F15 (5'-CCAGTACTCC-3') gave rise to a polymorphic repeatable band for FOM race 2 isolates (Fig. 1). RAPD PCR reactions were performed in a Gene Amp System 9700 (Applied Biosystems, USA). Approximately 10 ng of genomic DNA were used in 25 µl of amplification reaction. Amplifications, which included negative controls (sterile double-distilled H_2O) were repeated at least three times. PCR cycling parameters were: a denaturation step for 2 min at 94°C, followed by 45 cycles of 1 min at 94°C, 1 min at 36°C, 2 min at 72°C, and a final extension of 5 min at 72°C. The entire 25 µl PCR reaction were resolved by electrophoresis in a 1.5% agarose gel in 0.5X Tris-borate EDTA (TBE) buffer, stained with ethidium bromide, and visualized under UV light. Gel images were acquired with a Gel Doc 2000 System (Biorad, USA). The polymorphic target RAPD fragment of approximately 420 bp (Fig. 1) was excised from the gel and purified with a gel extraction kit (Invitrogen, USA) following the manufacturer's instructions. The purified fragment was ligated to the pCR2.1 vector (Invitrogen, USA), according to the manufacturer's protocol, and the recombinant plasmid was used to transform *E. coli* One Shot TOP10 Chemically Competent (Invitrogen, USA). Recombinant colonies were identified by

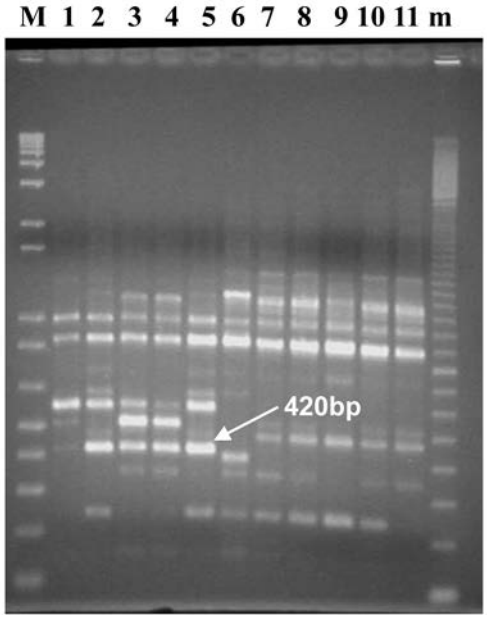

Fig. 1. Agarose gel electrophoresis of RAPD PCR of genomic DNA of *Fusarium oxysporum* f. sp. *melonis* (FOM) by Operon primer OP-F15 (5'-CCAGTACTCC-3'). Lanes 1 to 5 = FOM race 2 isolates; lane 6 and 7 = FOM race 1 isolates; lanes 8 to 11 = FOM race 1,2 isolates; M = 1Kb ladder marker; m = 100 bp ladder marker.

blue-white colour selection after 12-16 h of growth at 37°C on Luria-Bertani (LB) agar medium (sodium chloride 1%, tryptone 1%, yeast extract 0.5% and 1.2% agar-agar) containing kanamycin and X-Gal (5-bromo-4-chloro-3-indolyl ß-D-galactopyranoside). Plasmi DNA extraction and amplifications were done following standard procedures (Sambrook *et al*., 1989), using universal primers M13F/M13R (Invitrogen, USA). PCR products were cleaned using the PCR clean-up Gel Extraction (Macherey-Nagel, Germany).

The nucleotide sequence of the cloned RAPD DNA insert was custom sequenced (Bio-Fab Research, Italy). From the race-specific DNA fragment sequence data, PCR primers were designed using primer 3 software (http://frodo.wi.mit.edu/primer3/) on a 397 bp DNA fragment sequence, resulting in the primer pair Fa15F (5'-TAGGGATGATAGCGGTCTGG-3') and Fa15R (5'-GCTAGTTCGAGGCAATTGGA-3') which amplifies a sequence of 301 bp (NCBI accession No. JN183059). No similarity to this sequence was found with the BLASTN program of NCBI network service, nor in the Cogeme Phytopathogenic Fungi and Oomycete EST Database (http://cogeme.ex.ac.uk/) and the Fusarium Comparative Database (http://www.broadinstitute.org). The specificity of this primer pair was tested against 8 isolates of FOM race 2 from different geographic origin, plus a total of 45 isolates of *F. oxysporum* and other melon pathogens, including the three other FOM races (Table 1). For PCR runs the primer pair was adjusted to a concentration of 0.25 µM using approximately 2 ng of DNA samples in a 25 µl reaction. PCR cycling conditions using 0.25 mM of dNTPs and 2.0 mM $MgCl_2$ comprised an initial denaturation at 94°C for 2 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at 60°C annealing, 1 min at 72°C extension; and 1 min at 72°C for final extension.

The primer pair was specific and amplified a single SCAR fragment of DNA extracted from colonies of FOM race 2 from Italy, USA, and Israel (Fig. 2 and 3). No amplification was observed using DNA either from other FOM races (Fig. 2) or other *F. oxysporum formae speciales* as well as from the other melon pathogen used for comparison (Fig. 3). The sensitivity of the PCR assay, determined with ten-fold serial dilutions of FOM race 2 (ISPaVe2069) genomic DNA in sterile distilled water, yielded a detection threshold of 2 pg DNA (Fig. 4). When FOM race 2 and melon DNA were mixed, increasing host DNA concentration caused a decrease in the intensity of the PCR band likely because of the inhibitory effect of polysaccharidic contaminants present host DNA (Demeke and Adams, 1992; Larsen *et al*., 2002).

The efficiency of the newly developed race-specific primer pair Fa15F/Fa15R was evaluated for FOM detection in diseased plants. Single-spore cultures of the isolate ISPaVe2069 were subcultured on PDA and incu-

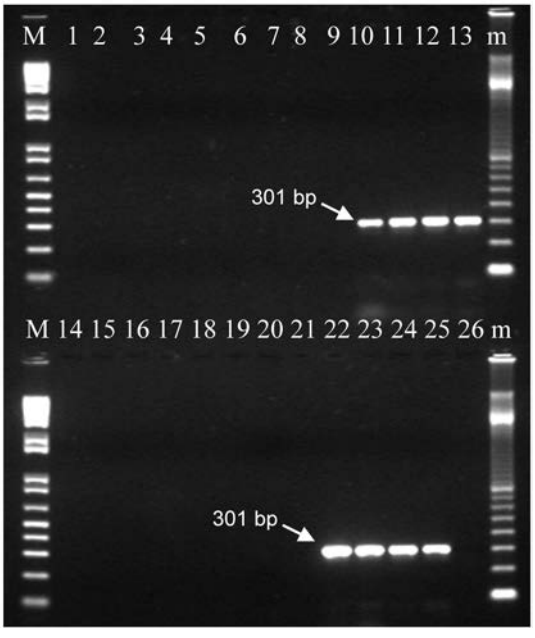

Fig. 2. Agarose gel electrophoresis of PCR-amplified products from mycelial DNA of different races of *F. oxysporum* f. sp. *melonis* by SCAR primers Faf15/Far15 specific to FOM race 2. Lanes 1 to 3 = FOM race 0 isolates; lanes 4 to 9 = FOM race 1 isolates; lanes 10 to 13 = FOM race 2 isolates; lane 14 and 15 = FOM race 1 isolates; lanes 16 to 21 = FOM race 1,2 isolates; lanes 22 to 25 = FOM race 2 isolates; lane 26 = sterile distilled water; M = 1 Kb ladder marker; m = 100 bp ladder marker.

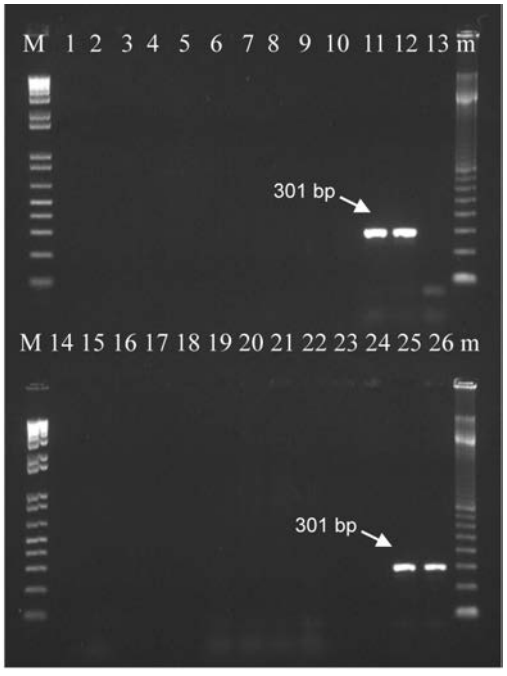

Fig. 3. Agarose gel electrophoresis of PCR-amplified products with specific SCAR primers Faf15/Far15 to *F. oxysporum* f. sp. *melonis* race 2 on mycelial DNA of different *formae speciales* of *F. oxysporum*, and other fungi associated with melon collapse and melon corky root disease. Lane 1 = *F. o.* f. sp. *basilici*; lane 2 = *F. o.* f. sp. *lycopersici*; lane 3 = *F. o.* f. sp. *ciclaminis*; lane 4 = *F. o.* f. sp. *cucumerinum*; lane 5= *F. o.* f. sp. *radicis-cucumerinum*; lane 6 = *F. o.* f. sp. *lentis*; lane 7 = *F. o.* f. sp. *radicis-lycopersici*; lane 8 = *F. o.* f. sp. *vasinfectum*; lane 9 and 10 = *F. oxysporum* isolates; lane 11 and 12 = FOM race 2 isolates; lane 13 = sterile distilled water; lane 14 = *Plectosporium tabacinum* isolate; lanes 15 to 17 = *Monosporascus cannonballus* isolates; lanes 18 to 20 = *Acremonium cucurbitacearum* isolates; lane 21 = *Rhyzopycnis vagum* isolate; lanes 22 to 24 = *Pyrenochaeta lycopersici* isolates; lane 25 and 26 = FOM race 2 isolates; M = 1 Kb ladder marker; m = 100 bp ladder marker.

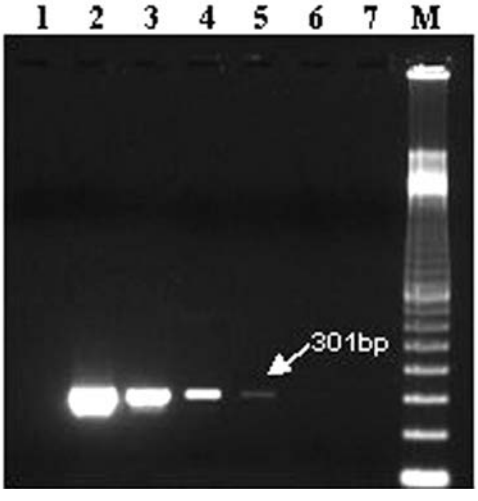

Fig. 4. Sensitivity of PCR-specific primers Faf15/Far15 at different DNA concentration of FOM race 2 ISPaVe2069. Lane 1 = no template control (water); lane 2= 2 ng/µl of DNA; lane 3 = 200 pg/µl DNA; lane 4 = 20 pg/µl of DNA; lane 5 = 2 pg/µl of DNA; lane 6 = 200 fg/µl of DNA; lane 7 = 20 fg/µl of DNA; M = 100 bp ladder marker.

bated at 25°C for 2 weeks to favour sporulation. Two melon genotypes were used, i.e. Charentais *Fom-2* resistant to races 0 and 1 and susceptible to race 2 and 1,2, and Charentais *Fom-0* susceptible to all races. Test plants were inoculated at the 3- to 4-true leaf stage by dipping the roots in a conidial suspension containing 1×10^6 conidia ml^{-1} (Sestili *et al.*, 2011). Control seedlings were dipped in sterile distilled water. Plants were collected 18 days post inoculation, when clear-cut symptoms of wilting were visible. DNA extraction and purification were performed as described above with the Purgene DNA purification kit (Gentra, USA) and PDA was used for re-isolations from stem fragments of infected and control plants.

PCR carried out on total DNA extracts from infected seedlings gave the expected amplicon of 301 bp, which was detected down to a threshold of 20 pg/µl of total DNA. There was no amplification from DNA extracted from healthy tissues (Fig. 5). FOM was readily re-isolated from stem fragments of inoculated seedlings, but not from controls.

The present work has achieved the objective of developing a rapid and sensitive molecular assay for the identification of FOM race 2, using a race-specific PCR-based technique, in agreement with the alleged efficiency of RAPD for generating SCAR markers for the detection of several fungal genera and oomycetes (Wiglesworth *et al.*, 1994; Schilling *et al.*, 1996; Larsen *et al.*, 2002; Causin *et al.*, 2005).

Currently, no other diagnostic methods are available to distinguish FOM races. The specificity of our primers,

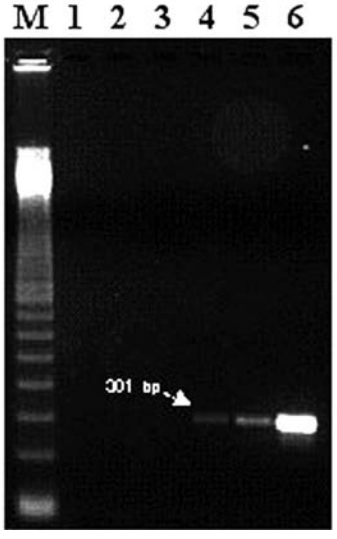

Fig. 5. Specific PCR detection of *F. oxysporum* f. sp. *melonis* race 2 with primers Faf15/Far15 on DNA from artificially infected melon plants Charentais *Fom-0* or Charentais *Fom-2*. M = 100 bp ladder; lane 1 = no template control (water); lane 2 = Charentais *Fom-0* mock inoculated; lane 3= Charentais *Fom-2* mock inoculated; lane 4 = Charentais *Fom-0* inoculated (20 pg/µl of total DNA); lane 5 = Charentais *Fom-2* inoculated (20 pg/µl of total DNA); lane 6 = DNA of FOM race 2 ISPaVe2069 (positive control).

which did not amplify any of the other *formae speciales* tested, nor the most common melon pathogens, make them a reliable diagnostic tool. The *SIX* genes screened by Lievens *et al.* (2009) failed to distinguish *Fom* from other *formae speciales* including *lycopersici*, *lilii*, and *radicis-cucumerinum*, though *SIX1-SIX5* are exclusively present in *F. oxysporum* f. sp. *lycopersici*.

It is worth noting that our SCAR primer pairs amplified a single PCR product from FOM race 2, regardless of the geographic origin of the isolates This finding may have practical applications in the epidemiology and management of Fusarium wilt of melon caused by this race in areas where it is widespread.

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