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Development of a PCR-based assay for the detection of *Fusarium oxysporum* strain FT2, a potential mycoherbicide of *Orobanche ramosa*

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ABSTRACT

Orobanche ramosa is an important parasitic weed of several agriculturally important crops. A fungal strain identified as *Fusarium oxysporum* (named FT2) proved to be specific and highly virulent to *O. ramosa* plants and was proposed as a mycoherbicide for the biological control of this weed to be applied at the soil level. Considering that detecting and tracking the strain is of utmost importance to know the fate of the strain after its release, an amplified fragment length polymorphism (fAFLP) analysis was chosen and utilized with the aim to develop a molecular marker to specifically identify this strain. A wide population of *F. oxysporum* strains isolated from different hosts was screened against the mycoherbicide strain in order to identify specific fragments belonging to this strain. Two specific fragments were found and their DNA sequences were utilized for primer design. A primer pair (named $FT2_{230}F/FT2_{230}R$) proved to be strain–specific and it amplified a 232 bp DNA fragment of FT2. These primers were used to monitoring the presence of the *F. oxysporum* strain in the soil. Amplicons were detected from all the soil samples artificially infected by using known amounts of FT2 inoculum, whereas none of the primer sets amplified DNA from soils not infected by FT2.

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1. Introduction

Orobanche is a genus that includes many parasitic weed species, commonly called broomrapes, which attack vegetables, legumes, and sunflower in Europe, the Middle East, and North Africa (Parker, 2009). One of the main species of this genus is *Orobanche ramosa* L., which is distributed mainly in the Mediterranean basin, North Africa, and Asia, where it infests millions hectares cropped to plants mainly in the Solanaceae, particularly tobacco, potato, tomato, and eggplant (Linke et al., 1989). Difficulties in control of broomrape are due to the large amount of seeds produced that can remain viable in the soil for many years. The seed germinates only if it is stimulated by host root exudates and produces a germ tube that, if it attaches to the host root, develops a haustorium that penetrates the root and forms a tubercle. This is followed by the withdrawal of nutrients, water, and photosynthates from the host by the parasite. Furthermore, broomrapes have a long underground phase, so that when they emerge most of the damage has already been produced (Joel et al., 2007).

Traditional control methods have been proposed but none has proved to be effective (Amsellem et al., 2001a). *Orobanche* spp. is not usually amenable to control by persistent selective herbicides, since herbicides cannot differentiate between crop and the parasite, except on herbicide-tolerant transgenic crops (Joel et al., 1995; Surov et al., 1997). Multiple applications of low rates of crop-degraded herbicides can provide a modicum of control (Hershenhorn et al., 1998). Seed eradication by solarization can be partially effective but it is expensive, whereas soil fumigation with methyl bromide and ethylene dibromide is very effective but it is almost completely banned due to environmental risks (Foy et al., 1989). These weeds cannot be controlled mechanically being attached to the host roots, except by removing their flower stalks to reduce seed accumulation and dispersal. For those reasons, broomrapes are considered a good target for biological control and some promising fungal pathogens have been proposed (Sauerborn et al., 2007; Amsellem et al., 2001b).

Among the several fungal species and strains isolated and proposed as biocontrol agents, *Fusarium oxysporum* Schlecht.:Fr. strain FT2 isolated from diseased broomrape tubercles proved to be highly virulent on *O. ramosa* plants and was considered a good candidate as mycoherbicide (Boari and Vurro, 2004). The fungus, applied in pot in greenhouse experiments, strongly reduced the number and weight of emerging broomrape shoots and the number of tubercles attached to the host roots (Boari and Vurro,

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2004). Applied by drip irrigation in field experiments, the fungus confirmed its efficacy in reducing number and weight of emerging broomrapes (Boari and Vurro, unpublished). Due to the parasitic plant life cycle, multiple applications of FT2 at the soil level would be necessary. Moreover, in order to carefully evaluate the fate of the strain after its release into the environment, a study of persistence, risk of dispersal, effect toward not target organisms, and movement along the soil profile, for FT2 was needed, mainly through its monitoring into the soil. Since tracking a biocontrol agent applied to the soil is highly difficult due to the presence of a number of microorganisms living in that environment, some of which may be very similar to the strain released, the need to develop a specific marker for tracking such agent was of utmost importance. Traditional microbiological methods for the isolation of fungi from plants and soil may be used, but molecular tools that have been developed for specific biocontrol strains greatly aid in the detection process. This is even more important for strains belonging to *F. oxysporum*, a species characterized by a great biodiversity (Leslie and Summerell, 2006). *F. oxysporum* is a plant pathogen causing a wide range of plant diseases mainly related to vascular wilts. However, within the species, many populations isolated mainly from soil have been shown as non pathogenic and some of them are used as biocontrol agents against several diseases also caused by pathogenic *Fusarium* species (Edel et al., 2001). Morphologically, these soil strains cannot be differentiated from pathogenic strains, although a wide genetic diversity of the population originated from soils has been reported (Edel et al., 2001). Amplified fragment length polymorphism (AFLP) is a powerful DNA fingerprinting technique based on total genomic restriction, ligation of primer adapters, and unselective followed by selective PCR amplification of anonymous DNA fragments from the entire genome (Vos et al., 1995). AFLP markers are recognized as more reproducible compared to RAPDs (Random Amplified Polymorphic DNA) and ISSRs (Inter-Simple Sequence Repeats), and are also able to give a higher resolution (Mueller and Wolfenbarger, 1999; Karwasra et al., 2002). This technique is highly versatile for the discrimination of organisms with different genomic complexity by changing the oligo design in selective amplification.

The aims of this study were: (a) to use fluorescent AFLP (f-AFLP) in order to screen the genetic variability of a wide population of *F. oxysporum* strains compared to FT2; (b) to identify a specific DNA marker to be used in the development of molecular assay for the detection of FT2 strain in the soil.

2. Materials and methods

2.1. Fungal strains

The research was focused on one strain of *F. oxysporum* Schlecht.: Fr. named FT2, isolated from diseased *O. ramosa* plants parasitizing tobacco, proposed as mycoherbicide for specifically controlling this weed (Boari and Vurro, 2004). The strain is stored in the mycological collection of the authors' Institute (Institute of Sciences of Food Production, ISPA) labelled as ITEM 5107.

For comparison studies, twelve strains of *F. oxysporum* available in the ISPA fungal collection, isolated and studied as potential agents for biological control of *O. ramosa* were used. Furthermore, fifty isolates of *F. oxysporum* from different hosts were also chosen from the ISPA mycological collection as comparing strains. Origin, collection number and host of the strains are listed in Table 1.

For the production of mycelium to be used for the DNA studies, fungal strains were grown first on PDA plates and then in Wikerham liquid medium according to Mulè et al. (2004).

2.2. DNA extraction and sequencing

Fungal genomic DNA extraction was performed according to Mulè et al. (2004). DNA samples were stored at −20 °C. Concentrations of DNA were determined by gel electrophoresis, by measuring the ultraviolet-induced fluorescence emitted by ethidium bromide molecules intercalated into DNA, and comparing the fluorescent yield of the samples with the standard (Sambrook et al., 1989).

The PCR reaction and sequencing of ribosomal internal transcribed spacers and a portion of the calmodulin gene were amplified and sequenced according to Mulè et al. (2004).

2.3. Fluorescent AFLP (fAFLP) analysis

AFLP assay was carried out according to the manufacturer's protocol (AFLP Microbial Fingerprinting, Applied Biosystems, Foster City, California, USA). Four separate primer combinations were utilized for the selective amplification: *Eco*RI-AC and *Mse*I-CC; *Eco*RI-AT and *Mse*I-CG; *Eco*RI-AC and *Mse*I-CA; *Eco*RI-G and *Mse*I-CT. The *Eco*RI primers were labelled with fluorescent dye (Applied Biosystems).

PCR cycling conditions for this reaction were set according to the AFLP microbial fingerprinting protocol by using a model 9700 GeneAmp PCR system.

AFLP fragments were separated on an ABI Prism 310 automated DNA sequencer to resolve fragments from 35 to 500 bp size with GeneScan-500 (ROX) as a size standard (Applied Biosystems). The capillary electrophoresis mix contained 1 μl of selective PCR product, 25 μl of deionised formamide and 0.5 μl of GeneScan-500 (ROX) size standard (Applied Biosystems). The capillary sample mix was denatured for 2 min at 95 °C and rapidly cooled on ice prior to loading the instrument. Subsequent preparations such as the set-up of the ABI PRISM 310 Genetic Analyzer (Applied Biosystems) were done according to the manufacturer's instructions with reference to using Performance Optimized Polymer 4 (Applied Biosystems). GeneScan collection version 3.1.2 software (PE Applied

Table 1
Strains of *Fusarium oxysporum* used for the present research.

Accession No.	No. of strains	*Forma specialis*	Host
388	1	—	*Zea mays*
2771–2773; 2775; 2776; 2778–2784	12	*dianthi*	*Dianthus caryophyllus*
2793–2797	5	—	*Lycopersicon esculentum*
3278–3284; 3286–3292	14	*melonis*	*Cucumis melo*
3340	1	*opuntiarum*	*Delichothele* sp.
3368	1	*opuntiarum*	*Rhipsalidopsis* sp.
3335; 3346; 3353; 3358; 3361; 3362; 3364; 3365; 3375	9	*asparagi*	*Asparagus officinalis*
3671; 3676; 3796; 3800; 3805; 3867; 3871–3873	9	—	*Oryza sativa*
ACb6, BPo2[a], CPo4, DPo3[b], DT1, DT3, ET10, ET3, ET7, ET8, FT2, HPo1	12	—	*Orobanche ramosa*

[a] According to the genetic analysis, this strain was reclassified as *F. proliferatum*.
[b] According to the genetic analysis, the strain was reclassified as *F. solani*.

Biosystems) was used to automatically size and quantify individual fragments by using the internal lane standards.

2.4. Isolation and sequencing of AFLP fragments

The PCR selective reaction conditions in 50 μl was as follow: 0.2 mM dNTP, 0.5 μM primer of the selective primer combinations; 0.05 U/μl Taq (Sigma–Aldrich), 5 μl Buffer and 10 μl of the eluted DNA from preselective mixture. The PCR cycling conditions for this reaction were set according to the AFLP microbial fingerprinting protocol (Applied Biosystem). PCR products were loaded and separated electrophoretically in a 2% agarose gel to isolate the FT2-specific peaks. These fragments were excited from the gel and eluted using Quantum Prep™ Freeze 'N Squeeze DNA gel Extraction Spin Columns (BIO-RAD, Germany) according to the manufacture's instruction. The purified PCR products were directly sequenced in both directions using Big Dye Terminator cycle sequencing kit (Applied Biosystems).

2.5. PCR assay and validation

Based on data sequence two specific primer pairs were designed (see above) using Primer express software (Applied Biosystems). These primers were used to perform the amplification reaction (final volume 50 μl) containing the same components as the abovementioned assay.

The cycle PCR conditions were as follow: denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension 72 °C for 60 s; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the sample. Amplification products were assessed on 2% agarose gel stained with ethidium bromide. The specificity of the primers was tested against 64 strains of *F. oxysporum* (Table 1).

2.6. Survival of FT2 in soil

2.6.1. Soil type

A 30 Kg sample of sandy-clay soil, representative of the predominant soil type of the area infested by broomrapes, was collected from a field located in Altamura (province of Bari, Italy). Sampling depth was the plough layer (0–25 cm). The soil sample was dried at 35 °C for about 1 week to constant weight and then sifted through a 4 × 4 mm mesh sieve. The soil had the following characteristics: organic matter 2.52%, N: 1.23 μ/g, K: 218.0 μ/g, P: 8.73 μ/g, field capacity: 31.9%.

2.6.2. Soil inoculation

Two inoculum levels were used and experiments were carried out with both non-sterile and sterile soils. For the experiments carried out with sterile soil, about 5 kg of soil were placed in polypropylene bags and sterilized by autoclaving at 120 °C for 1 h, the soil was cooled at room temperature and the sterilization was repeated once again. Then the bags were opened under a sterile hood and aerated for a few hours. The bags were closed again and the sterile soil was stored at 4 °C until used. Experiments were performed in 500 ml jars containing 150 g (dry weight) of soil. An appropriate volume of a conidial suspension of FT2 in sterile distilled water was poured into the jars to obtain a final concentration of 10^3 (low inoculum level) or 10^6 (high inoculum level) colony forming units per gram of soil (CFUs/g) and the field water capacity of soil. The soil was mixed thoroughly, the jars were closed loosely with their screw lids, and incubated at 20 ± 1 °C in a Lab-Line Instruments (Melrose Park, ILL) incubator. The experiments were carried out in triplicate.

Soil samples were collected 1, 15, 30, 60, and 90 days after inoculation (d.a.i.) for determination of population level by enumeration of CFUs/g with the dilution plate method. At each sampling time, two 5 g soil samples were taken from each of three replicated jars. One sample was oven dried at 105 °C for 48 h to determine the soil dry weight. The other sample was introduced in a centrifuge tube containing 45 ml of sterile distilled water, placed into a rotary shaker and shaken for 1 h at 200 rpm to obtain an even suspension. Then, a series of subsequent 1/10 dilutions was prepared with sterile distilled water. Each dilution (0.1 ml) was plated in triplicate on the selective media PCNB: (per litre: peptone 15.0 g, KH_2PO_4 1.0 g, $MgSO_4 \cdot 7H_20$ 0.5 g, penthachloronitrobenzene 0.75 g, agar 20.0 g. Autoclaved at 0.75 atm for 20 min and cooled to 55 °C before adding, in 10 ml sterile water: streptomycin sulfate 1.0 g, neomycin sulfate 0.12 g). The colonies were counted after incubation at 25 ± 1 °C for 3–8 days in a Forma Scientific (Marietta, OH) incubator and the CFUs/g of dry soil were calculated, based on soil dry weight previously determined. The CFU/g values of replicates were averaged and the standard deviation (SD) of the means was calculated.

At every sampling time, an additional 2 g sample of soil was taken from each jar inoculated with *F. oxysporum* FT2 and utilized for the experiments on molecular detection of this biocontrol strain.

2.7. DNA extraction from the soil and PCR conditions

Total genomic DNA was extracted from the soil using FastDNA® Spin Kit for soil (Q BIO gene, Resnova) according to the manufacturer's protocol. The PCR reaction in 50 μl final volume, containing: 50 pmol of strain–specific primers, 1.25 U of Taq Polymerase (Sigma–Aldrich), 10 nmol dNTP (Sigma–Aldrich), 2.5 mM of $MgCl_2$, 2 μg of BSA (Biolabs New England) and 10 μl of total DNA. PCR program was the following: 1 cycle of denaturation at 94 °C for 5 min, 40 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s, the final extension 72 °C for 10 min. The amplicons were visualized on 2% agarose gel stained with ethidium bromide.

3. Results and discussion

3.1. Molecular characterization of Fusarium spp. with rDNA and calmodulin genes

The strategy of amplifying and sequencing conserved genes common to all fungi (O'Donnell et al., 2000; Mulè et al., 2004) was utilized to characterize 12 strains of *Fusarium* (Table 1) previously evaluated as biological control agents for *O. ramosa* (Boari and Vurro, 2004).

DNA was amplified by PCR with ITS1/ITS4 primers and CL1/CL2 primers, resulting in 550–570 pb, 650–770 pb fragments, respectively (data not shown). The amplification products were sequenced in both directions and the similarities of the rDNA to sequences in the GenBank database were determined by using BLASTn (NCBI) for both genes. One strain (BPo2) showed 100% identity with *Fusarium proliferatum* (Matsush.) Nirenberg NRRL31071 Acc. No. AF291061 and with *Gibberella monilifirmis* Wineland IFM 54323 Acc. No. AB 369908. One strain (DPo3) showed 99% identity with *Fusarium solani* (Martius) Saccardo IFM 54324 Acc. No. 369907. FT2 and all the other nine chosen species showed 100% identity with *F. oxysporum* (Acc. No. EU839400) for ITS data sequences.

The Neighbor clustering analysis grouped all the *F. oxysporum* strains in one distinct group with a similarity level of 98% (data not show), whereas the *F. proliferatum* strain BPo2 and the *F. solani* strain DPo3 were clearly separated from the main cluster.

With regard to calmodulin data sequences, strain BPo2 showed 99% identity with *F. proliferatum* ITEM 1475 Acc. No. AJ60771. Strain DPo3 showed 75% identity con *F. oxysporum* ITEM 2367

Acc. No. AJ60774 with a gaps of 79/660 (11%) All the other ten strains, including FT2, had 98–100% identity with *F. oxysporum* ITEM 2367 Acc. No. AJ60774. Also in this case the dendrogram obtained by using the Neighbor analysis grouped the *F. oxysporum* strains in one cluster with a similarity level of 99%, and separated *F. proliferatum* (BPo2) and *F. solani* (DPo3) strains from that cluster.

3.2. Sequencing of AFLP fragments and primer design

Fifty-two *F. oxysporum* strains from different hosts, selected from the ISPA collection, were examined, together with the 12 strains isolated from *O. ramosa*. Clear polymorphisms were obtained by AFLP analysis for each of the four primers pairs used.

Visual inspection of the FT2 fingerprinting was compared to all isolates analyzed and showed two specific peaks in the electrophoretic profiles of FT2 obtained with the primer pair *Eco*RI-AT/*Mse*ICG (Fig. 1) and the size of the fragments identified was 304 and 378 bp, respectively.

The two fragments were named FT2-ETA/MGA_{380} and FT2-ETA/MGA_{300} respectively, according to strain acronym, primer pair and size (Fig. 2). The nucleotide sequences of two DNA fragments were examined for similarity with deposited sequences using the BLASTn or BLASTx programmes (Altschul et al., 1997), available form the National Centre for Biotechnology Information (http://ncbi.nlm.nih.gov/BLAST). The region ranging from 1 to 166 bp of the FT2-ETA/MGA_{380} fragment showed 87% identity with the sequence of mRNA of hypothetical protein of *Gibberella zeae* (Schwein.) Petch, PH-1 strain (Acc. No. XM389811). The region ranging from 100 to 272 bp of the FT2-ETA/MGA_{300} fragment showed a 75% identity with the sequence of mRNA for Esterase STE1 of *Metarhizium anisopliae* var. *anisopliae* (Metsch.) Sorokin (Acc. No. AJ251924).

Based of these results, the strain/specific primers, named $FT2_{270}F$ (5′-TTCATTGACATACAGCCAGCG-3′), $FT2_{270}R$ (5′-CATGG AGAGGACATTGGCG-3′) and $FT2_{230}F$ (5′-TTCCCGTCTTCCTTCGCAC-3′), $FT2_{230}R$ (5′-GCTAACGCATCGACTAAGGAGC-3′), were designed on the DNA sequence of FT2-ETA/MGA_{380} and FT2-ETA/MGA_{300} fragments, respectively. The size of the amplification products was 271 and 232 pb, respectively.

All primers were designed to operate at high annealing temperatures (60 °C), thereby preventing the amplification of non-specific

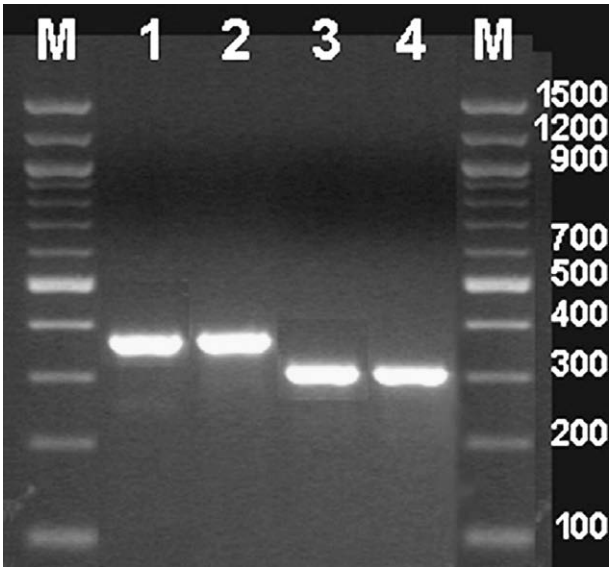

Fig. 2. AFLP fragments of FT2 strain obtained with the primer pair EcoRI + AT/MseI + CG and separated on agarose gel. Lanes 1 and 2: DNA fragment corresponding to the electrophoresis peak of 378 bp size. Lanes 3 and 4: DNA fragment corresponding to the electrophoresis peak of 308 pb size. Lane M: 100 pb DNA ladder (New England, BioLabs).

DNA targets. These pairs of primers were tested on DNA extracted from the *Fusarium* spp. isolated from *Orobanche*. After amplification we observed that only the primer pair $FT2_{230}F/FT2_{230}R$ was strain–specific and it amplified a 232 bp fragment from FT2 strain (Fig. 3) In order to confirm primer specificity, the fragment derived from the studied strain was sequenced. Comparison of its sequence with that of the FT2-ETA/MGA_{300} fragment confirmed the strain specificity of the primers, since the sequence homology was 100%.

The PCR assay specificity was confirmed by testing $FT2_{230}F/FT2_{230}R$ primers towards the *F. oxysporum* strains listed in Table 1 and no bands were obtained from all those strains. Primer sequences were compared also against existing sequences in GenBank, and the results of BLASTn showed 100% homology of primers with *Aspergillus* spp. sequences. Consequently, to confirm

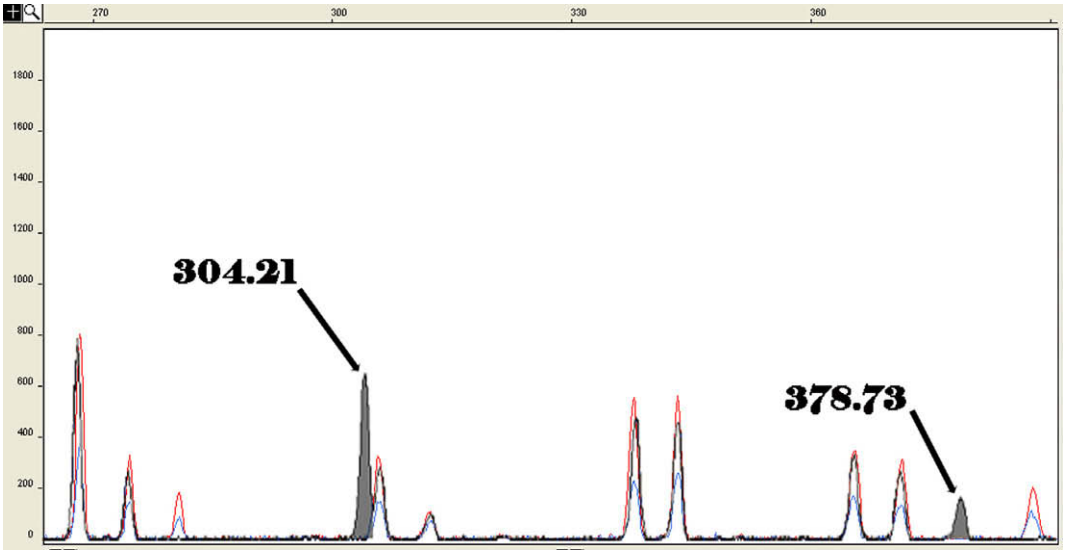

Fig. 1. Fingerprinting profiles, approximately in the range 260–390 bp of electropherograms, corresponding to DNA sized fragments generated by the selective primer pair EcoRI-AT-MseI-CG in the fAFLP reaction. The following profiles are shown: FT2 (in black); a representative *Fusarium oxysporum* strain from *Orobanche ramosa* (in red); a representative *Fusarium oxysporum* strain from a different host (in blue). Peaks of DNA fragments that appear to be specific for FT2 are filled in grey.

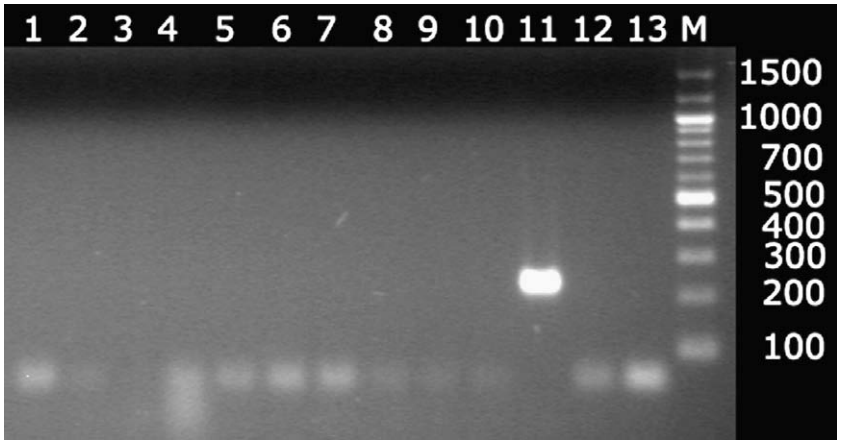

Fig. 3. PCR amplification products obtained with $FT2_{230}F/FT2_{230}R$ primer pairs designed on the DNA sequences of FT2-ETA/MGA300 fragment. Lanes 1–12: ACb6, BPo2, CPo4, DPo3, DT1, DT3, ET3, ET7, ET8, ET10, FT2, HPo1; Lane 13: H_2O, negative control; Lane M: 100 pb DNA ladder (New England, BioLabs).

the PCR assay specificity, the primer pair was also tested against *Aspergillus* spp. available in the ISPA collection. None of these tests showed positive results (Fig. 4).

3.3. Survival and PCR-based detection of FT2 in soil

The changes in microbial population occurring in the soil, inoculated with 10^6 or 10^3 CFU/g dry weight are shown in Fig. 5. In both sterile (Fig. 5A) and non-sterile soil (Fig. 5B), at high inoculum level of FT2, the population of the biocontrol agent showed a slight decline (about one order of magnitude) within the first 30 d.a.i. Subsequently, the population of FT2 remained constantly at a level comprised between 10^4 and 10^5 CFU/g up to 90 d.a.i (Fig. 5B).

Considerable differences were found between sterile and non-sterile soils when low inoculum levels were used. Predictably, in sterile soil (Fig. 5A) the population of FT2 increased with a rather high pace (from about 10^3 to more than 10^4 CFU/g during the first 30 days), and more gradually in the following days, reaching 3.8×10^4 CFU/g 90 d.a.i. Conversely, in non-sterile soil (Fig. 5B) population of FT2 showed a steep decline (from 2.0×10^3 to 2 CFU/g) within the first 30 days, and 60 d.a.i. it was not longer recovered.

At low inoculum level (10^3 CFU/g), no amplification product was obtained except for a faint signal detected immediately after inoculation (1 d.a.i.) (Fig. 6A), while PCR products were detected at high inoculum level (10^6 CFU/g) (Fig. 6B). The PCR signal was very intense up to 15 d.a.i, when the population of FT2 was around 5.2×10^4 CFU/g. Later, a decrease in the intensity of the PCR signal was observed. Nevertheless, the signal was still clearly visible 90 d.a.i., when the concentration of propagules of FT2 was 2.2×10^4 CFU/g. The fragment obtained from the soil DNA amplification was sequenced and aligned with the $FT2\text{-}ETA/MGA_{300}$ nucleotide sequence. The identity of the two sequences, together with the absence of cross-reactivity or interference with non-target DNA of a complex matrix, such as a soil, clearly indicated the high specificity of the method. Based on these results, the limit of detection of FT2 by means of this PCR-based method can be assumed comprised between 10^3 and 10^4 CFU/g of soil dry weight. Similar results were obtained with sterile soil (data not shown).

Beside the effectiveness of biocontrol agents, their release in the environment raises a number of questions regarding the environmental impact, the effects to non-target organisms, the persistence and the possible risks of dispersal. These aspects are much more important and also difficult to evaluate when the biocontrol organism is released into the soil and it is similar to other saprophytic organisms commonly occurring in that environment.

In order to overcome these problems, a reliable tracking method to follow the biocontrol agent is needed. Molecular biology offers now techniques and methods that allow us to identify microorganisms both at species and even at the strain level. Indeed in our work, we have set up an efficient assay that allows to detect the *F. oxysporum* strain FT2 proposed as a mycoherbicide for the bio-

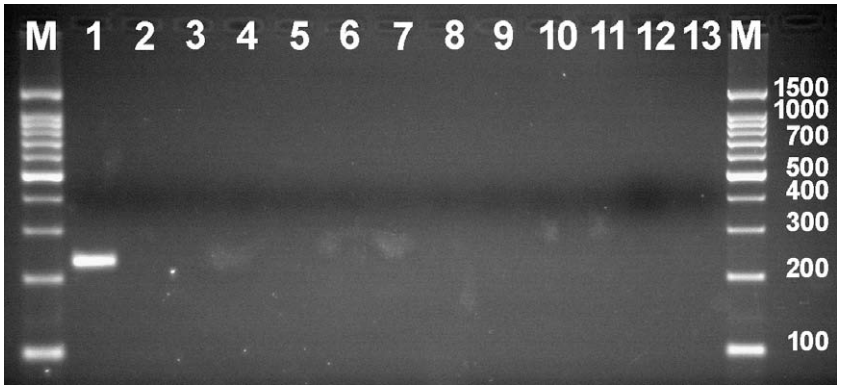

Fig. 4. PCR assay specificity with the $FT2_{230}F/FT2_{230}R$ primer pairs tested against different soil fungal species. Lane 1: FT2, positive control. Lanes 2–12: *Aspergillus japonicus* K. Saito (ITEM 7034). *A. clavatus* Desm. (ITEM 7039), *A. niger* van Tieghem (ITEM 7098), *A. carbonarius* Bainier (ITEM 7475), *A. flavus* Link:Fries (ITEM 7526), *A. parasiticus* Speare (ITEM 7531), *A. tamarii* Kita (ITEM 7833), *A. fumigatus* Fresenius (ITEM 8011), *Erotium rubrum* Jos. Konig et al. (ITEM 8012), *Emericella nidulans* (Eidam) Vuillemin (ITEM 8015), *A. terreus* Thom (ITEM 8048). Lane 13: H_2O, negative control. Lane M: 100 plus Ladder (Quiagen, Germany).

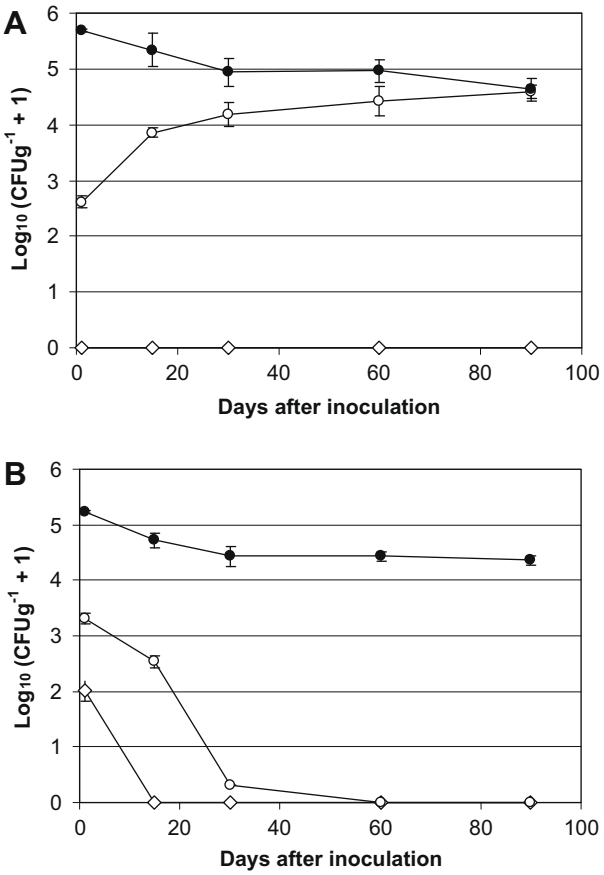

Fig. 5. Population dynamics of FT2 inoculated in sterile (A) or not sterile (B) soils, at two different concentrations: 10^3 (white dots) and 10^6 (black dots) CFU/g soil dry weight. White diamonds = non inoculated controls. Experiments were carried out in jars with a sandy-clay soil, incubated at 20 ± 1 °C. Values are the means of three replicates ± SD.

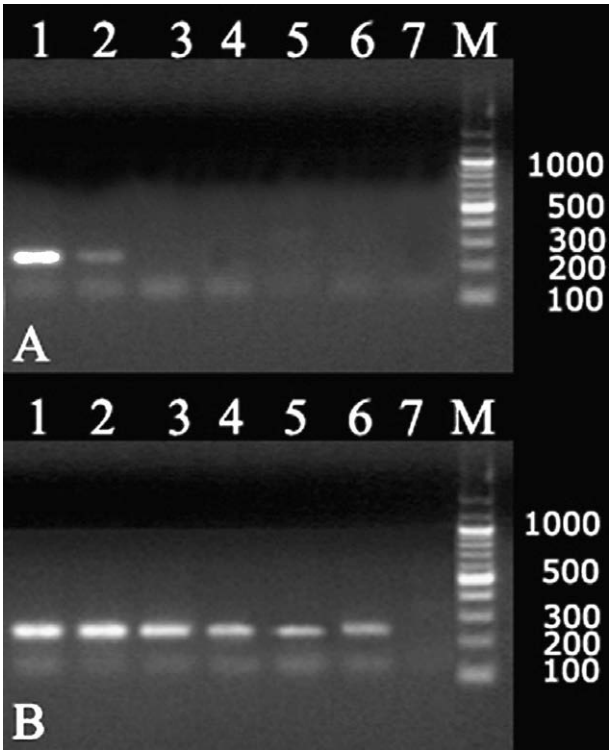

Fig. 6. PCR assay with the $FT2_{230}F/FT2_{230}R$ primer pairs used to monitor the presence of FT2 in non-sterile soil, inoculated with 10^3 (A) or 10^6 CFU/g dry weight (B). Lane 1: FT2 positive control; Lanes 2–6: amplification products obtained 1, 15, 30, 60, and 90 days after inoculation; Lane 7: negative control, non inoculated soil; Lane M: 100 pb DNA ladder (New England, BioLabs).

logical control of the parasitic weed *O. ramosa*. The method proved to be very useful also when applied at the soil level.

The development of methods for the detection of microorganisms at a strain level could also have interesting perspectives from a commercial point of view, even for agents other than mycoherbicides. For example, it could allow recognition of commercial strains and protect them from illegal production or trading, or could be decisive in case of controversies due to supposed non-target effects, e.g. the appearance of diseases on crop plants.

A quantitative PCR assay study based on this method is in progress, in order to quantify the presence of the FT2 strain in the soil. If a direct correlation between FT2 population in the soil and level of protection from *Orobanche* does exist, then the quantification of the biocontrol agent in a given field would give an estimation of the level of protection, addressing the choice of crops sensitive to the parasitic weeds, if the protection is sufficient, or the need of further fungal applications, in order to maintain the adequate level of protection.

A number of papers reported the development of specific markers to detect fungal biocontrol strains to be distributed to the soil by using approaches different from ours. For example, some studies were based on sequence-characterized amplified regions (SCAR), as obtained by Rubio et al. (2005) from a RAPD fragment of a strain of *Trichoderma harzianum* Rifai; other studies developed specific primers designed from specific sequences of genes such as entochitinase genes, used for tracing a *Trichoderma atroviride* P. Karsten strain applied as a open-field biocontrol agent (Savazzini et al., 2008). On the other hands, as far as we are aware, we used for the first time the AFLP analysis to develop a fungal strain–specific marker, showing that this technique can be a powerful tool for tracking microorganisms in the environment.

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