Morphological and molecular identification of seedborne fungi in squash (*Cucurbita maxima*, *Cucurbita moschata*)

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Abstract

Squash is one of the most important crops of tropical and temperate regions, and it can be affected by several fungal pathogens. Most of these infect the seeds, which become an efficient vehicle to disperse seedborne pathogens over long distances, with consequent severe crop losses. The main objective of this study was the identification of the principal seedborne fungi in seeds extracted from 66 samples of asymptomatic and symptomatic squash fruit (*Cucurbita maxima*, *Cucurbita moschata*) collected in two countries, Tunisia and Italy. The symptoms of fruit decay were identified and classified according to lesion size. Following the blotter test, 14 fungal species were detected from the seeds. Seedborne fungi were identified in all fruit samples tested, including asymptomatic fruit. The most frequent fungi from Tunisia seeds were *Alternaria alternata* (25.1%), followed by *Stagonosporopsis cucurbitacearum* (24.6%), *Fusarium solani* (16.6%), *Rhizopus stolonifer* (13.3%), *Fusarium fujikuroi* (7.8%), *Albifimbria verrucaria* (3.3%), and *Stemphylium vesicarium* (2.3%). For the fruits from Italy, the most frequently identified fungal species in seed samples were: *A. alternata* (40.0%), followed by *F. fujikuroi* (20.8%), *S. vesicarium* (3.0%), and *Curvularia spicifera* (2.1%). Morphological identification was confirmed by molecular diagnosis using the available species-specific primers. Furthermore, specific primers were designed to identify *A. verrucaria*, *Paramyrothecium roridum* and *S. vesicarium*. Application of seed-health testing methods, including such conventional and molecular diagnostic tools, will help to improve seed quality and crop yields.

Keywords: β-tubulin, diseases of *Cucurbita* species, EF1α, histone H3, ITS, rDNA

Introduction

Cucurbita L. (pumpkins, squash, gourds) is a widely cultivated genus in agricultural regions worldwide. According to the Food and Agriculture Organization of the United Nations (FAO), a total yield of nearly 20,000,000 tonnes was produced in Asia and Europe, plus 2,932,611 tonnes in the Americas (FAO 2014). Squash (*Cucurbita maxima*, *Cucurbita moschata*) is one of the most important vegetables in tropical and temperate regions. In Italy and Tunisia, the total production of squash is 580,188 tonnes and 90,080 tonnes of fresh fruit, respectively (FAO, 2016).

Cucurbita spp. can be affected by many diseases, including gummy stem blight, Fusarium fruit rot, and Alternaria leaf spot (Gannibal 2011; Keinath 2011; Mehl and Epstein 2007). Gummy stem blight (GSB) (foliar symptoms) and black rot (BR) (fruit symptoms) are caused by three species of *Stagonosporopsis*: *S.* cucurbitacearum (Fr.) Aveskamp, Gruyter & Verkley (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, *S. caricae* (Syd. & P. Syd.) Aveskamp, Gruyter & Verkley (synonym *Mycosphaerella caricae* Syd. & P. Syd.), and *S. citrulli* M.T. Brewer & J.E. Stewart (Stewart et al. 2015). GSB and BR are the most important diseases of cucurbits. Under conducive climatic conditions for the disease, which occur especially in warm and humid environments, severe outbreaks can cause 15% to 50% yield losses, lead to rapid death of the cucurbit plants and reduce yields (Boughalleb et al. 2007; Keinath et al. 1995; Yao et al. 2016; Zitter and Kyle 1992). Pumpkin and winter squash are particularly susceptible to black rot (Brewer et al. 2015), where the seeds can become infested or infected through flower and fruit infection (de Neergard 1989).

Fusarium fruit rot is caused by *Fusarium solani* f. sp. *cucurbitae* W.C. Snyder & H.N. Hansen (Fsc) teleomorph *Nectria haematococca* Berk. & Broome. This pathogen can infect the seeds, and in this way it can be spread over long distances (Boughaleb and Mahjoub 2006; Farrag and Moharam 2012). Mehl and Epstein (2007) reported a significant relationship between infections of *F. solani* f. sp. *cucurbitae* in pumpkin fruit tissue and incidence of infected seeds. Fusarium fruit rot is an economically important problem for pumpkin growers, with 30% of fruit reported as infected in California, USA (Mehl and Epstein 2007). In the field and greenhouse, *Fusarium* spp. can cause important yield losses, that may reach 80% (Blanco and Aveling 2018).

The genus *Alternaria* can affect several crops during their growing stages and after harvest (Kgatle and Aveling 2018; Mamgain et al. 2013). *Alternaria cucumerina* and

Alternaria alternata are pathogens of cucurbits, and they can cause severe crop losses (Gannibal 2011; Vakalounakis 1990). Furthermore, *Alternaria brunsii* has also been detected on seeds of *C. maxima* (Paul et al. 2015). Moreover, within the Cucurbitaceae family, leaf spot diseases caused by seedborne pathogens like *Pleospora herbarum* (anamorph *Stemphylium vesicarium*), *Paramyrothecium roridum*, and *Albifimbria verrucaria* can result in significant production losses (Fish et al. 2012; Petzer 1958; Sultana and Ghaffar 2009). All of these are necrotrophic fungi that can infect cucurbits and can be transmitted by seeds. Furthermore, the obligate biotrophic fungus *Pseudoperonospora cubensis* is a major pathogen of cucurbits; it can be fruit-borne, seed-borne, and seed-transmitted in butternut gourds (*Cucurbita moschata*) (Cohen et al. 2014). Low percentages of seed infection can still result in severe crop losses (Mancini et al. 2016; Vannacci et al. 2014; Walcott et al. 1998).

Seeds represent a particularly efficient vehicle for introducing and spread seedborne pathogens over long distances into new niches (Ahmad et al. 2016; Elmer, 2001; Özer and Coşkuntuna 2016; Pellegrino et al. 2010).

Therefore, early detection of seedborne fungi is a key step to prevent introduction of infected seeds, to use high standard quality of seeds and to define integrated disease management strategies (Majumder et al. 2013; Mancini and Romanazzi 2014; Yao et al. 2016). Conventional methods for seed health testing include the blotter test, which promotes mycelium growth and formation of fruiting bodies on the seed surface, to allow pathogen identification under the microscope (Tsopmbeng and Fomengia 2015). The main problem related to the presence of saprophytic microorganisms on the seed surface can be easily bypassed through surface decontamination of seeds (Du Toit et al. 2005; El-Nagerabi and Elshafie 2000; Rodrigues and Menezes 2005). For detection of *S. cucurbitacearum* and *A. alternata* in seeds, the blotter method proved to be more suitable (Ahmad et al. 2016; Lee et al. 1984). However, when these diagnostic methods are used, some fungal species have a high degree of similarity based on their morphology, and so distinguish among these closely related organisms can be difficult, such as between *D. bryoniae* and *Phoma* sp. (Keinath et al. 1995), *A. alternata* and *S. vesicarium* (Pryor and Gilbertson 2000), and *Bipolaris* spp. and *Curvularia* spp. (Kusai et al. 2015), and also among different *Fusarium* spp. (Chehri et al. 2011). Therefore, diagnostic methods can also be based on polymerase chain reaction (PCR) with specific primers to provide high analytical sensitivity to detect and identify different strains of fungi (Babu et al. 2015; Carneiro et al. 2017).

The main objectives of the present study were: (i) to estimate the incidence of seedborne fungi identified by morphological features, for seeds extracted from symptomatic

and asymptomatic squash fruit; (ii) to carry out molecular identification of the seedborne fungi using specific primers for *S. cucurbitacearum*, *A. alternata*, *C. spicifera*, *F. solani*, and *Fusarium oxysporum*; and (iii) to design species-specific primers for identification of *P. roridum*, *A. verrucaria*, and *S. vesicarium*.

Materials and Methods

Field sites and sample collection

Between 2015 and 2018, 66 samples of asymptomatic and symptomatic squash fruits were sampled in two countries, Tunisia and Italy (Fig. 1). A total of 37 fruit samples were collected from the cultivars 'Batati', 'Bjaoui', and 'Galaoui' in Tunisia. These samples were collected between July and November 2015 and 2016 from multiple farms in Tunisia's five major squash production regions. A total of 29 fruit samples were collected from the cultivars ‘Aspen’, ‘Butternut’ and ‘Naples long’ in Italy. These samples were collected between September and October 2018 from different farms and fields in five locations in Italy (Table 1). These squash cultivars from both Tunisia and Italy are local varieties, and they represent the most commonly produced squash cultivars in the respective countries. The fruit samples were taken from seeds produced on-farmer and from squash seed lots in their respective years.

Fruit sample evaluation

The day after the sampling, the fruits were examined for fungal disease symptoms. Fungi were identified based on the presence of clear signs and symptoms. When the symptoms were unclear, isolation was carried out from small pieces of skin that were cut from the squash fruit with symptoms. These small pieces (~2 mm) were immersed in 1% sodium hypochlorite solution for 5 min, and three washes with sterilized distilled water, the samples were air dried for 30 min on sterile paper toweling in a laminar flow hood. The pieces of squash skin were placed on potato dextrose agar (PDA, 42 g/L; Liofilchem Srl, Roseto degli Abruzzi, Italy) and incubated at 22 ±2 °C for 14 days. The plates were checked daily, and the colonies grown from the pieces of squash skin were transferred to PDA plates to obtain pure cultures. From each fruit sample, the fruit rot, if present, was evaluated according to three levels (Fig. 2): A, asymptomatic fruit; B, infected fruit showing symptoms of rot on the squash skin without reaching the seed cavity; and C, infected fruit showing symptoms of rot that had reached the seed cavity.

Detection and identification of seedborne fungi using classical tools

The seeds were separated, washed with tap water, dried on sterile blotter sheets overnight (10 hours) at room temperature (20-24 °C), and stored in paper bags at 4 °C until use. Each sample comprised seeds collected from a single fruit and two hundred seeds per sample were tested using the standard blotter method of the International Seed Testing Association (Mathur and Kongsdal, 2003). The seeds were surface sterilized using the method of sterilization described above. Two-hundred seeds (10 seeds/plate) were placed onto eight pieces of sterile blotter paper that was moistened with 5 mL sterile distilled water (Whatman no. 4 filter papers; diameter, 110 mm) in Petri dishes (diameter, 110 mm), these were incubated for 14 days at 22 ±2 °C with a 12/12 h dark/ ultraviolet light photoperiod (TL-D 36W BLB 1SL, PHILIPS, Dublin, Ireland)**.**

Fungus identification was carried out first by examination of the fungal fruiting bodies and the mycelia and spores that developed on the seeds under a stereomicroscope (M125; Leica Microsystems CMS, Wetzlar, Germany). Then the spores, conidiophores, pycnidia, and perithecia of the fungi were examined under a microscope (DM 2500; Leica). To support the initial identification under the microscope, single-spore isolates of each fungus were collected and transferred into PDA in Petri dishes, to obtain fungal colonies (Choi et al. 1999). After 8 to 15 days at 22 ±2 °C, morphological identification was carried out according to the colors and shapes of the colonies, with measurements of the fungal structures (i.e., pycnidia, perithecia, conidia) using the LAS V3.8 software (Leica DFC 295), which was applied to 50 units of each structure for each fungus. The fungal species identification was based on the key of Aveskamp et al. (2010), Booth (1971), Champion (1997), Jeon et al. (2015), Lombard et al. (2016), and Mathur and Kongsdal (2003).

Molecular identification of seedborne fungi

DNA extraction from mycelia

On the basis of morphological identification, 93 isolates representative of 14 species isolated were used to set up a protocol of detection based on molecular tools (Table 2). The isolates were grown in PDA Petri dishes until the fungi reach the edge of the plate. A modified DNA extraction by Varanda et al. (2016) was used for these samples. In particular, the mycelia were collected, lyophilized, and ground in 1.5 mL microcentrifuge tubes with the addition of 600 µL extraction buffer (20 mM EDTA, 0.1 M Tris-HCl, pH 8.0, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, 4% polyvinylpyrrolidone, 0.1% sodium metabisulfite added just before use), and 60 mg silicon dioxide (Sigma), to promote mycelium

fragmentation. The quality and quantity of the extracted DNA was directly checked on 1% agarose gels, with evaluation using a BioPhotometer (Eppendorf, Hamburg, Germany). The DNA was finally diluted to 20 ng/µL for further amplification.

PCR amplification using universal primers and sequence analysis

Amplification of the internal transcribed spacer (ITS) and partial sequences of the β-tubulin gene, histone H3, the translation elongation factor (EF1α) region, and *calmodulin* gene in the ribosomal (r)DNA of the isolates (Table 1) was performed. These 20 µL PCR reactions contained 2 µL genomic DNA of the fungal isolate, 10 µL Green Plus Econo Master Mix 2× (Lucigen, WI, USA), and 0.5 µL of each primer (10 µM). The primers used were ITS1 and ITS4 (White et al.1990) for ITS, Bt2a and Bt2b (Glass and Donaldson 1995) for *tub2*, CYLH3F and CYLH3R (Crous et al. 2004) for *his3*, and EF1-728F/EF1-986R (Carbone & Kohn, 1999) for *EF1α*. The PCR reactions were run in a thermal cycler (MyCycler; Bio-Rad Laboratories, Hercules, CA, USA) following the specific parameters published for TUB, HIS, and EF1α. For ITS, some modifications were made: initial denaturation was at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 40 s, and annealing at 57 °C for 30 s for *A. alternata* (Konstantinova et al. 2002), at 55 °C for 1 min for *S. cucurbitacearum*, *P. roridum*, and *A. verrucaria* (Orawan et al. 2014; Babu et al., 2015), at 58 °C for 1 min for *C. spicifera* and *F. solani* (Chehri et al. 2011; Dela Paz et al. 2006), and at 57 °C for 1 min for *S. vesicarium* (Câmara et al. 2002; Dela Paz et al. 2006). Extension was carried out at 72 °C for 50 s, with final extension at 72 °C for 7 min at the end of the amplification The PCR products (9 µL per sample) were separated by electrophoresis in 1.5% agarose gels stained with Red Gel (Biotium, Hayward, CA, USA), and visualized and captured using an imagining system (Gel Doc XR; BioRad).

Bidirectional sequence analysis was conducted on select amplified isolate fragments at Genewiz (UK) (Table 2). The forward and reverse nucleotide sequences were read and edited using the Chromas version 2.33 software, and were assembled using the CAP3 software, to obtain a consensus sequence. The Bioedit software (version 7.0.0) was used (http://www.mbio.ncsu.edu/Bioedit/bioedit.html) to cut-off 20 bp to 30 bp of the terminal end sequence. Finally, nBlast analysis (http://www.ncbi.nlm.nih.gov/BLAST/) was carried out to verify the identities of the amplicons.

After an accurate research in the literature, we selected and used already published specific primer pairs for the molecular identification of *S. cucurbitacearum*, *A. alternata*, *C. spicifera*, *F. oxysporum*, and *F. solani*, as summarized in Table 3.

Design of specific primers for molecular identification of *A. verrucaria*, *P. roridum*, and *S. vesicarium*

The ITS nucleotide sequences for the genera *Paramyrothecium*, *Myrothecium*, *Albifimbria*, *Fusarium*, *Stagonosporopsis*, *Curvularia*, *Stemphylium*, and *Pleospora*, available in NCBI GenBank (Supplementary Table S1), were downloaded in the FASTA format and aligned using ClustalX (version 1.83) (Thompson et al. 1994). The specific nucleotide regions that characterized each fungal genus and the conserved intra-genera nucleotide regions were selected to design a new specific primer pair for *A. verrucaria*, *P. roridum*, and *S. vesicarium* respectively (Table 4). The new set of primers that were designed were submitted and validated to Primer-BLAST software (Ye et al. 2012) developed at the NCBI.

After optimization of the reaction mixture, the PCR amplifications were performed in 25 µL reaction mixture that contained 2 µL genomic DNA (about 20 ng/µL) of the fungal isolates, 200 µM dNTP mixture, 0.5 µM each primer, 1.2 mM $MgCl_2$, and 1.25 U Taq polymerase (Promega). The details of the cycling conditions are reported in Table 5 for each primer pair. PCR was carried out with serially diluted DNA extracted from *A. verrucaria, P. roridum*, and *S. vesicarium* isolates M144, M123, and P164, respectively (4×10 ng to 4×10^{-5} ng), to determine the analytical sensitivity of the tests.

Results

Fruit symptom evaluation

In Tunisia, in the Sidi Hamada area, five fruits showed an infection level of 'A, asymptomatic fruit', one of 'B, infected fruit showing symptoms of rot on the squash skin without reaching the seed cavity' and two of 'C, infected fruit showing symptoms of rot that had reached the seed cavity', with three fruits with symptoms related to *S. cucurbitacearum*, and one to *A. alternata*. In the Kalâat El-Andalous area, two fruits showed an infection level of 'A' and eight of 'B', with one fruit with symptoms related to *F. solani*, one to *C. spicifera*, and seven to *A. alternata*. All of the samples collected in the other three localities of Utique, Sbeïtla, and Sahline areas showed infection levels of 'A' (Table 6).

In Italy, in the Castelfidardo area, two fruits showed an infection level of 'A', three of 'B', and three of 'C', with six with symptoms related to *A. alternata*, and two to *C. spicifera*. In the Osimo area, three fruits showed an infection level of 'A' and nine of 'B', with eight with symptoms related to *S. cucurbitacearum* and nine to *A. alternata*. In the Recanati area,

one fruit showed an infection level of 'A', three of 'B', and two of 'C', with two fruits with symptoms related to *C. spicifera* and five to *A. alternata*. All of the samples collected in the Monopoli and Baranello areas showed infection levels of 'A' (Table 6).

Identification of seed-borne fungi using morphological criteria and molecular tools

After 14 days of incubation using the blotter test at 22 ±2 °C with a 12/12 h dark/ ultraviolet light photoperiod, all of these seeds were examined under a stereomicroscope for the presence of fungal fruiting bodies. The fruiting bodies and fungal structures (i.e., pycnidia, sporodochia, perithecia, conidia) were also analyzed under microscopy, to evaluate the shape and size. For each fungal species identified by morphological criteria, there was a parallel molecular identification (sequence analysis and amplification by specific primers). The main fungi isolated from seeds were *S. cucurbitacearum*, *A. verrucaria*, *P. roridum*, *S. vesicarium*, *A. alternata*, *C. spicifera*, *F. solani*, and *F. oxysporum*, whose descriptions are reported in the following subsections.

***Stagonosporopsis cucurbitacearum (*Fr.) Aveskamp, Gruyter & Verkley (Aveskamp et al. 2010) (Syns. *Didymella bryoniae* (Fuckel) Rehm)**

Pycnidia were observed on squash seeds with diameters ranging from 116 to 131 µm (Fig. 3A-C). These spores were cylindrical with rounded ends 4.0 to 8.0 µm × 1.6 to 3.4 µm (Fig. 3D). The mycelia of the colonies cultured on PDA were white on the top and black on the bottom, and after 10 days they produced pycnidia with pycnidiospores (Fig. 3E). These morphological traits are consistent with *S. cucurbitacearum*.

By sequence analysis, isolates D33, D29, D49, D12, and D83, showed high nucleotide identity with *S. cucurbitacearum* (Table 2) and also the molecular tools proposed by Brewer et al. (2015) corroborated the morphological identification (data not shown). Finally, the amplification with RG-specific primer pair, able to yield a specific fragment of about 450 bp, allowed to molecularly characterized all analyzed isolates as *S. cucurbitacearum* belonging to the RG group II (Fig. 5A) (Somai et al. 2002).

***Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous (Lombard et al. 2016) (Syns. *Myrothecium verrucaria* (Alb. & Schwein.) Ditmar)**

On squash seeds there was an erumpent crowded cluster of conidiophores that formed a viscus stroma in the form of a cushion. These fruiting bodies are known as sporodochia, and their color was green to dark green, they were surrounded by white cotton mycelia on the seeds (Fig. 3I), and they had a large number of elliptical one-celled conidia with acute ends

4.1 to 5.7 µm × 1.5 to 2.2 µm (Fig. 3J). On PDA, this fungus produced white colonies that became greenish black with time, to form black rings of sporodochia. These morphological criteria attributed this fungus to the species *A. verrucaria*, and it was confirmed by the sequence analysis for isolates M144, M135, IAV2, and IAV4 (Table 2).

For the molecular identification of *A. verrucaria*, specific primers were designed. The primer pair Myroverr F1/R1 was used in PCR reactions in a gradient thermal cycler, to determine its maximal annealing temperature; then 60 °C was applied. These conditions yielded a specific fragment of 553 bp amplicons (Fig. 4A). The minimum concentration of the target DNA that could be detected with this primer was 4×10^0 ng (data not shown).

***Paramyrothecium roridum* (Tode) L. Lombard & Crous (Lombard et al. 2016) (Syns. *Myrothecium roridum* Tode)**

In other cases, the sporodochia were black to black green and globular, with a rounded contour and without obvious mycelia (Fig. 3K). Conidia taken from these sporodochia were one-celled and cylindrical, with rounded ends (Fig. 3L). On PDA, this fungus formed white colonies that with time produced successive greenish black and black rings of sporodochia filled with the cylindrical conidia 5.0 to 7.4 µm × 1.2 to 2.6 µm. These morphological features indicated that this fungus corresponded to *P. roridum*, and it was confirmed by the sequence analysis for isolates M123, M138, M141, IPR2, and IPR9 (Table 2).

For the molecular identification of *P. roridum*, specific primers were again designed. The primer pair Myroror F1/R1 was used in PCR reactions in a gradient thermal cycler, to determine its maximal annealing temperature; then 58 °C was applied. These conditions yielded a specific fragment of 562 bp amplicons (Fig. 4B). The minimum concentration of target DNA that could be detected with this primer was 4×10^0 ng (data not shown).

***Stemphylium vesicarium* (Wallr.) E.G. Simmons (Simmons, 1969) (Syns. *Pleospora herbarum* (Pers.) Rabenh.)**

Small black fruiting bodies (perithecia) that were flask-shaped or globose appeared on the squash seed samples (Fig. 3O), with the size of 147 to 282 µm × 131 to 243 µm. The crushed perithecium discharged several bitunicate clavate asci that measured 50 to 116 µm × 8.4 to 18.3 µm (Fig. 3P). These contained eight ellipsoidal monoseriate to biseriate ascospores that were rounded at the ends, 14.7 to 20.0 µm × 6.1 to 9.5 µm, with four to six transverse, and one to three longitudinal, septa (Fig. 3Q). After 4 to 6 days on PDA, this fungus produced one muriform conidium per conidiophore (anamorph). The length of the conidia was 8.4 to 18.7

μm × 6.0 to 11.1 μm, with three transverse and one to three longitudinal septa. After 10 to 15 days on PDA, the fungus started to differentiate perithecia (teleomorph). The isolates that showed these morphological features were closely related to the fungal species *S. vesicarium*, and it was confirmed by the sequence analysis of isolates P164, P66, and IP4 (Table 2).

For the molecular identification of *S. vesicarium*, the primer pairs Pleo F/R (Fig. 4C) and Pleo F1/R1 (Fig. 4D) were designed, and these successfully amplified the target DNA from the five isolates of *S. vesicarium*. The annealing temperature was 60 °C, and under these conditions a specific fragment of 547 bp was yielded. The detection limit for these primers was ~4 $\times 10^{-1}$ ng of fungal DNA input (data not shown).

***Alternaria alternata* (Fr.) Keissl. (1912)**

Black conidia were observed in long chains on all of the seed samples, except for sample Bj52 (Fig. 3F, G). Microscopic examination showed conidia that were highly variable in shape, 5.0 to 17.0 μm × 4.0 to 8.5 μm, with both transverse and longitudinal septa (Fig. 3H). The conidial beaks were also highly variability in shape, with lengths of 1.7 to 14.0 μm. On PDA cultures, the mycelia of this fungus were at first gray, but then became nearly black when sporulation was abundant. The morphological features of this fungus corresponded to the fungal species *A. alternata*, and it was confirmed by the sequence analysis of isolates A38, A15, IA1, and IA3 (Table 2).

For the molecular identification of *A. alternata*, the set of primers AAF2/AAR3 (Konstantinova et al. 2002) yielded a specific fragment of about 350 bp (Fig. 5B).

***Curvularia spicifera* (Bainier) Boedijn (Boedijn, 1933) (Syns. *Bipolaris spicifera* (Bainier) Subram.)**

Other conidia were found in groups on squash seed samples (Fig. 3M). Microscopic examination showed straight conidia with rounded ends, with three distosepta, which measured 12.0 to 23.4 μm × 5.1 to 8.0 μm (Fig. 3N). These morphological features belong to the fungal species *C. spicifera*, and it was confirmed by the sequence analysis of isolates B170, IB41, IB1 (Table 2).

For the molecular identification of *C. spicifera*, the primer pair Bipol-1F/Bipol-1R amplified a specific fragment of 200-bp amplicons for the two isolates tested (Fig. 4C) (Ünal et al. 2011).

***Fusarium solani* (Mart. 1842) (Syns. *Neocosmospora solani* (Mart.) L. Lombard & Crous (Lombard et al. 2015))**

A fungus characterized by the presence of water droplets was found on seed samples (Fig. 3R). Microscopic examination showed long phialides (Fig. 3S), unicellular and bicellular oval microconidia, with size 6.0 to 14.6 µm × 2.0 to 4.1 µm, and cylindrical and slightly curved macroconidia, three to five septate, with mean size of 16.6 to 33.4 µm × 3.0 to 5.2 µm (Fig. 3T). On PDA culture, the fungus produced white to cream colored mycelia. These morphological features indicated that this belonged to the fungal species *F. solani*, and it was confirmed by the sequence analysis of isolate F174 (Table 2).

For the molecular identification, the amplification carried out with the specific primers TEF-Fs4f/TEF-Fs4r (Arif et al. 2012) (Fig. 5D) yielded a specific fragment of about 650 bp amplicons. We did not have any amplification with the primer pairs Fsc1EF1/Fsc1-EF-2 and Fsc2-EF1/Fsc2-EF3.

***Fusarium oxysporum* Schltdl. 1824**

Microscopic examination showed unicellular and bicellular microconidia produced on short monophilides. The macroconidia were generally three to five septate. There were chlamydospores in the mycelial cultures, which were round unicellular and bicellular, and surrounded by a thick cell wall. The fungus developed on PDA culture had white mycelia that became salmon in color, with a tendency to purple. These morphological features indicated that it belonged to the fungal species *F. oxysporum*.

For the molecular identification, the amplification carried out with the specific primers Fc-1/Fc-2 (Zhang et al. 2012) (Fig. 5E) yielded a specific fragment of ~400 bp.

Frequency of seedborne fungi

The seedborne fungi isolated from the seed samples collected in Tunisia were *A. alternata* (25.1%), followed by *S. cucurbitacearum* (24.6%), *F. solani* (16.6%), *R. stolonifer* (13.3%), *F. fujikuroi* (7.8%), *A. verrucaria* (3.3%) and *S. vesicarium* (2.3%). *A. alternata* was detected in all of the localities surveyed and in most of the symptomatic fruits (Table 6, T8, T34, T47, T71, T35, T63, T45, T69) with 6 to 50% infection level. Moreover, *A. alternata* was also detected in 22 asymptomatic fruits collected from these five localities in Tunisia, with incidence ratings from 1% to 38%. *S. cucurbitacearum* was detected in three Tunisia localities (Sidi Hmada, Sbeïtla, Utique), from three symptomatic fruits (T7, T8, T9) collected from the Sidi Hmada area (Table 6). The seed samples extracted from these fruits were highly

infected by *S. cucurbitacearum*, with infection rates from 20% to 60%. Furthermore, *S. cucurbitacearum* was also identified in the seed samples from 12 asymptomatic fruits collected from these three localities, with infection rates from 1% to 44%. *F. solani* was detected in 17 seed samples collected from all five localities in Tunisia (46% of samples). *F. solani* was isolated from one symptomatic fruit (Table 6, T52), with an infection rate of 7%. Moreover, *F. solani* was isolated and identified in 13 asymptomatic fruits, with infection rates from 1% to 60%. *A. verrucaria* and *P. roridum* were isolated from four seed samples obtained from asymptomatic fruits collected from the Sidi Hmada area. Seed sample T18 in Table 7 was highly infected by *A. verrucaria* (15.5%) and *P. roridum* (5.0%). *S. vesicarium* was isolated from 18 seed samples (48.6% of samples), with infection rates from 1% to 13%. *C. spicifera* was isolated from 12 seed samples (32.4% of samples) collected from all five localities, with incidence rates of 1% to 7%.

In the seed samples from Italy, *A. alternata* was also the most frequent, at 40.0%, followed by *F. fujikuroi* (20.8%), *S. vesicarium* (3.0%) and *C. spicifera* (2.1%) (Table 7). *A. alternata* was detected for all of the localities included. This pathogen was isolated from all of the symptomatic fruit samples and also from the seeds extracted from these fruits, with infection rates from 1% to 70%. Moreover *A. alternata* was identified in eight asymptomatic fruits, with infection rates from 1% to 37%. *S. vesicarium* was detected in 12 of the seed samples collected (41.3% of samples), with incidence rates from 1% to 9%. The seeds obtained from three symptomatic fruit samples with infection level of 'C' were infected by *C. spicifera* (Table 6, I2, I5, I31), with infection rates from 11% to 85%. Moreover, another symptomatic fruit sample with an infection level of 'B' was infected with *C. spicifera* (Table 6, I29), with an infection rate of 2%. *C. spicifera* was also identified in the seed samples of two asymptomatic fruits (Table 6, I23, I37), with an infection rate of 1%.

Discussion

In the present study, a survey was carried out to assess the phytosanitary status of squash seeds collected from fruit samples produced in Tunisia and Italy. The survey allowed to isolate and identify several fungi in squash seeds, including *S. cucurbitacearum*, *A. alternata*, *F. solani*, *A. verrucaria*, *P. roridum*, *S. vesicarium*, and *C. spicifera*.

We observed a correlation between the symptoms caused on these fruits collected in Tunisia and Italy, and the fungal species isolated from seeds of these fruits. Our data show that *A. alternata* and *S. cucurbitacearum* were detected and isolated from fruit lesions in both

countries. *A. alternata* is a pathogen of cucurbits, where it can cause severe crop losses (Vakalounakis 1990). *S. cucurbitacearum* has a world-wide distribution and it can infect at least 12 genera and 23 species of Cucurbitaceae (Stewart et al. 2015; Rennberger and Keinath 2018). *S. cucurbitacearum* was reported in Italy in 1885 on *Cucumis melo* (Corlett 1981) and in 2019 on *C. moschata* (Moumni et al. 2019). In Tunisia, *S. cucurbitacearum* was detected only on watermelon (*Citrullus lanatus*) in 2007 (Boughalleb et al. 2007). To our knowledge, our work represents the first report of *S. cucurbitacearum* as a pathogen of squash seed in Tunisia.

The pathogenic fungi *A. alternata*, *S. cucurbitacearum*, and *C. spicifera* were present in high percentages for seeds obtained from symptomatic fruits. Therefore, seeds can be infected indirectly through the fruit, when a lesion extends to the seed cavity, or during the process of seed extraction, when the seeds are mixed with the inoculum present on the external part of the fruit, as demonstrated by Mehel and Epstein (2007).

In addition, seedborne fungi were detected also in the asymptomatic fruits. *S. cucurbitacearum* was detected in 12 asymptomatic fruits collected from the Tunisia areas. Similar data were obtained for *A. alternata*, which was present in all of the 30 asymptomatic fruits collected in Tunisia and Italy. Furthermore, *F. solani* was isolated from 13 seed samples extracted from asymptomatic fruits. As shown for *Stagonosporopsis* sp., *Fusarium* sp., *Alternaria* sp., even if the fruit does not show any symptoms, the seeds inside the fruit can be infected (El-Meleigi 1991; Keinath 2011; Petkar and Ji 2017). This shows that there are other ways of pathogen penetration. Fusarium wilt of watermelon (*Fusarium oxysporum* f. sp. *niveum*) can infect watermelon seeds by direct invasion through vascular bundles or indirect invasion through the pistil, which can lead to infestation of seeds in asymptomatic fruit (Petkar and Ji 2017). Meiri and Rilsky (1983) indicated that conidia of *A. alternata* can germinate on stigmas of pepper flowers, ingress the ovary through the style in the form of hyphae and establish in pepper seeds. Similarly, De Neergaard (1989) showed that *Stagonosporopsis* sp. can infect seeds of Cucurbitaceae via the stigmas. Moreover, the majority of growers in Tunisia and Italy extract the seeds from the fruit, and the presence of seeds contaminated within asymptomatic fruits might contribute to the large-scale spread of these pathogens and to their introduction into new planting area. Therefore, cultural practices, such as visual inspection for absence of lesions on fruit is not sufficient to ensure that seeds are not infected. Thomas-Sharma et al. (2017) showed that the selection of healthy plants in the field is an important step to obtain good quality of seeds. In addition, in Italy, eight symptomatic fruits were infected by *S. cucurbitacearum*, but the seeds obtained from these

fruits were not infected by this pathogen. The likely explanation for this is that the spatial development of a pathogen is influenced by many factors, such as inoculum source (e.g., infested soil) and environmental conditions, such as relative humidity; e.g., *S. cucurbitacearum* is influenced by the environmental conditions during sampling (Rennberger et al. 2018).

Correct identification of fungal pathogens is a key factor for crop protection and for the development of disease management strategies (Kusai et al. 2015). For this reason, ITS-rDNA, β-tubulin, histone H3, and *EF1α* sequence data were used here to confirm the morphological identification by Blast analysis and the homology of sequence in NCBI database (Peay et al. 2008; White et al. 1990). In particular, for *S. cucurbitacearum*, *A. alternata*, *C. spicifera*, *F. oxysporum*, and *F. solani*, the molecular detection was carried out using species-specific primers.

In our study, the application of RG-specific primers, allowed to determine that all *S. cucurbitacearum* isolates from Tunisia and Italy belonged to the genetic group RGII, so none belonged to the genetic group RGI, which is prevalent in Florida and Georgia (USA) (Babu et al. 2015) and in Brazil (Santos et al. 2009). For the molecular identification of the genera *Albifimbria, Paramyrothecium*, and *Stemphylium*, based to the best of our knowledge, there were no specific primers already available. Hence, in this study, species-specific primers were designed to identify *P. roridum*, *A. verrucaria*, and *S. vesicarium*. The ITS region of nuclear rDNA is the main genomic region targeted for PCR primer development (Guillemette et al. 2004). *Myrothecium* spp. have been detected in cucurbits, with identification through their morphological characteristics and through ITS sequence analysis (Fish et al. 2012; Sultana and Ghaffar 2009). In the present study, ITS specific primers for *P. roridum*, *A. verrucaria*, and *S. vesicarium* were used to identify the target microrganisms. The annealing temperatures of 60 °C for *A. verrucaria* and *S. vesicarium* and 58 °C for *P. roridum* allowed these pathogens to be specifically detected while preventing the amplification of other pathogens. To determine the sensitivity of each primer set designed in this study, serial dilutions of fungal genomic DNA of *A. verrucaria*, *P. roridum*, and *S. vesicarium* revealed that 40 ng DNA was necessary to produce a clear results on agarose gels. Using less than 4 ng for *A. verrucaria* and *P. roridum* and 0.4 ng for *S. vesicarium*, the molecular tools set up were not able to clearly identify the specific pathogens.

The present study started from a phytosanitary survey that led to the identification of the main fruit rot and seedborne pathogens of squash through conventional and molecular diagnoses. The principal fungi present in the squash seeds in this study included *S.*

cucurbitacearum, *A. alternata*, *A. verrucaria*, *P. roridum*, and *S. vesicarium*, and these were detected for the first time in Tunisia and Italy for seeds of *C. maxima* and *C. moschata*. These fungi are both seedborne and soilborne pathogens. The use of quality seeds is important for improving yields and conservation of genetic material (Duan et al. 2007), and consequently, the sanitary control of seeds is necessary to limit the spread of these pathogens. This can be achieved through application of seed-health testing methods, including conventional and molecular diagnostic tools. This technique has numerous positive characteristics, including rapidity, specificity, sensitivity, and ease of interpretation, which allow its application to the detection of seedborne pathogens (Mancini et al. 2016; Vannacci et al. 2014; Walcott 2003; Ward et al. 2004). Such data are useful for the identification of seedborne fungi directly in seed samples, and to clarify the risk of infection for the following crop. This study illustrates how fruit and plant selection can reduce the amount of seedborne pathogen inocula and to obtain high-quality seeds, which can be a critical step in the management strategies for sustainable agriculture.

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728 Zitter, T. A., and Kyle, M. M. 1992. Impact of powdery mildew and gummy stem blight on
729 collapse of pumpkins (*Cucurbita pep*o. L.). Cucurbit Genit. Cooper. 15:93-96.
730
731

733 **Table 1.** Field sites and fruit samples collected from Tunisia and Italy.

Country	Province	Locality	Fruit sample	Host	Cultivar [a]	Year of collection
Tunisia	Ariana	Kalâat El-Andalous	T34	*C. maxima*	Bjaoui	2015
		(37°03′45″N, 10°07′06″E)	T35	*C. maxima*	Galaoui	2015
			T47	*C. maxima*	Bjaoui	2015
			T52	*C. maxima*	Bjaoui	2015
			T63	*C. maxima*	Galaoui	2015
			T71	*C. maxima*	Bjaoui	2015
			T45	*C. maxima*	Galaoui	2015
			T69	*C. maxima*	Galaoui	2015
			T1	*C. maxima*	Galaoui	2015
			T3	*C. maxima*	Batati	2015
	Siliana	Sidi Hmada	T8	*C. maxima*	Bjaoui	2015
		(35°57'28"N, 9°32'57"E)	T4	*C. maxima*	Bjaoui	2015
			T13	*C. maxima*	Bjaoui	2015
			T9	*C. maxima*	Bjaoui	2015
			T7	*C. maxima*	Bjaoui	2015
			T18	*C. maxima*	Bjaoui	2015
			T22	*C. maxima*	Bjaoui	2015
			T14	*C. maxima*	Bjaoui	2015
	Bizerte	Utique	T6	*C. maxima*	Bjaoui	2015
		(37°03′25″N, 10°03′43″E)	T16	*C. maxima*	Bjaoui	2015
			T38	*C. maxima*	Bjaoui	2015
			T40	*C. maxima*	Bjaoui	2015
			T58	*C. maxima*	Bjaoui	2015
			T66	*C. maxima*	Bjaoui	2015
			T70	*C. maxima*	Bjaoui	2015
			T81	*C. maxima*	Bjaoui	2016
	Kasserine	Sbeïtla	T76	*C. maxima*	Bjaoui	2016
		(35°14′00″N, 9°08′00″E)	T77	*C. maxima*	Bjaoui	2016
			T78	*C. maxima*	Bjaoui	2016
			T79	*C. maxima*	Bjaoui	2016

			T80	*C. maxima*	Bjaoui	2016
	Monastir	Sahline	T23	*C. maxima*	Batati	2015
		(35°45′02″N, 10°42′44″E)	T24	*C. maxima*	Bjaoui	2015
			T26	*C. maxima*	Bjaoui	2016
			T28	*C. maxima*	Bjaoui	2016
			T29	*C. maxima*	Bjaoui	2016
			T30	*C. maxima*	Bjaoui	2016
Italy	Ancona	Castelfidardo	I2	*C. maxima*	Aspen	2018
		(43°27′51″N, 13°32′46″E)	I3	*C. maxima*	Aspen	2018
			I4	*C. moschata*	Naples long	2018
			I5	*C. maxima*	Aspen	2018
			I8	*C. maxima*	Aspen	2018
			I12	*C. moschata*	Naples long	2018
			I13	*C. moschata*	Naples long	2018
			I16	*C. moschata*	Naples long	2018
	Ancona	Osimo	I17	*C. moschata*	Butternut	2018
		(43°29′00″N, 13°29′00″E)	I18	*C. moschata*	Butternut	2018
			I19	*C. moschata*	Butternut	2018
			I20	*C. moschata*	Butternut	2018
			I21	*C. moschata*	Butternut	2018
			I22	*C. moschata*	Butternut	2018
			I23	*C. moschata*	Butternut	2018
			I24	*C. moschata*	Butternut	2018
			I25	*C. moschata*	Butternut	2018
			I26	*C. moschata*	Butternut	2018
			I27	*C. moschata*	Butternut	2018
			I28	*C. moschata*	Butternut	2018
	Macerata	Recanati	I29	*C. moschata*	Butternut	2018
		(43°24′00″N, 13°33′00″E)	I30	*C. moschata*	Butternut	2018
			I31	*C. moschata*	Naples long	2018
			I32	*C. maxima*	Aspen	2018
			I33	*C. moschata*	Butternut	2018
			I34	*C. maxima*	Aspen	2018

Bari	Monopoli (40°57′00″N, 17°18′00″E)	I35	*C. moschata*	Butternut	2018
		I36	*C. maxima*	Aspen	2018
Campobasso	Baranello (41°32′00″N, 14°33′00″E)	I37	*C. moschata*	Butternut	2018

734 [a] The three squash cultivars from Tunisia use the local Tunisian names ('Galaoui', 'Bjaoui',
735 'Batati') and represent the squash cultivars that are most commonly produced there
736

Table 2. Codes assigned to the isolates of each fungal species from Tunisia (TN) and Italy (IT) that were used during the DNA extraction and PCR analyses of the ITS, EF1α, HIS, and TUB sequences.

Fungal species	Isolate	Location	Source	Host species	Identification method		GenBank accession number			
					Sequencing	Species-specific PCR	ITS	EF1*α*	HIS	TUB
Stagonosporopsis cucurbitacearum	D33*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401569	-*	-	MK497768
	D35	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D43	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D45	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D40	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D36	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D3	Sbeïtla, TN	Seed	*C. maxima*		+				
	D23	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D24	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D29*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MK497779	-	-	MK497766
	D21	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D5	Sbeïtla, TN	Seed	*C. maxima*		+				
	D42	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D49*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401570	-	MK497771	MK497767
	D48	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D45	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D39	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D2	Sbeïtla, TN	Seed	*C. maxima*		+				

	D3	Sbeïtla, TN	Seed	*C. maxima*		+				
	D5	Sbeïtla, TN	Seed	*C. maxima*		+				
	D10	Sbeïtla, TN	Seed	*C. maxima*		+				
	D12*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401568	-	-	-
	D27	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D62	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D69	Sidi Hmada, TN	Seed	*C. maxima*		+				
	D83*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401571	-	-	-
	DBF1	Osimo, IT	Fruit	*C. moschata*		+				
	DBF2	Osimo, IT	Fruit	*C. moschata*		+				
	DBF3	Osimo, IT	Fruit	*C. moschata*		+				
Phoma sp.	Ph39*	Sidi Hmada, TN	Seed	*C. maxima*	+		MF401572	-	-	-
Alternaria	A38*	Sahline, TN	Seed	*C. maxima*	+	+	MK497774	MK497789	MK497770	-
alternata	A15*	Kalâat El-Andalous, TN	Seed	*C. maxima*	+	+	MK497773	MK497788	MK497769	-
	A58	Kalâat El-Andalous, TN	Seed	*C. maxima*		+				
	A17	Kalâat El-Andalous, TN	Seed	*C. maxima*		+				
	A5	Kalâat El-Andalous, TN	Seed	*C. maxima*		+				
	A59	Sidi Hmada, TN	Seed	*C. maxima*		+				
	IA1*	Recanati, IT	Seed	*C. moschata*	+	+	MK497775	-	-	-
	IA2	Recanati, IT	Fruit	*C. moschata*		+				
	IA3*	Osimo, IT	Seed	*C. moschata*	+	+	MK497776	-	-	-
	IA4	Osimo, IT	Seed	*C. moschata*		+				
	IA5	Osimo, IT	Fruit	*C. moschata*		+				

	IA6	Osimo, IT	Seed	*C. moschata*		+				
	IA7	Monopoli, IT	Seed	*C. moschata*		+				
	IA8	Monopoli, IT	Seed	*C. moschata*		+				
	IA9	Castelfidardo, IT	Seed	*C. maxima*		+				
	IA10	Castelfidardo, IT	Seed	*C. maxima*		+				
Albifimbria	M140	Sidi Hmada, TN	Seed	*C. maxima*		+				
verrucaria	M149	Sidi Hmada, TN	Seed	*C. maxima*		+				
	M148	Sidi Hmada, TN	Seed	*C. maxima*		+				
	M144*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MK497782	-	-	MK497761
	M146	Sidi Hmada, TN	Seed	*C. maxima*		+				
	M155	Sidi Hmada, TN	Seed	*C. maxima*		+				
	M135*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MK497783	-	-	MK497762
	IAV1	Osimo, IT	Seed	*C. moschata*		+				
	IAV2*	Osimo, IT	Seed	*C. moschata*	+	+	MK497785	-	-	MK497764
	IAV3	Osimo, IT	Seed	*C. moschata*		+				
	IAV4*	Osimo, IT	Seed	*C. moschata*	+	+	MK497784	-	-	MK497763
	M73	Sidi Hmada TN	Seed	*C. maxima*		+				
	M78	Sidi Hmada, TN	Seed	*C. maxima*		+				
Paramyrothecium	M123*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401575	-	-	MK497759
roridum	M138*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401576	-	-	-
	M167	Sidi Hmada, TN	Seed	*C. maxima*		+				
	M141*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MK497786	-	-	MK497765
	IPR1	Baranello, IT	Seed	*C. moschata*		+				

	IPR2*	Baranello, IT	Seed	*C. moschata*	+	+	-	-	-	MK497760
	IPR3	Baranello, IT	Seed	*C. moschata*		+				
	IPR4	Recanati, IT	Seed	*C. moschata*		+				
	IPR5	Recanati, IT	Seed	*C. moschata*		+				
	IPR6	Recanati, IT	Seed	*C. moschata*		+				
	IPR7	Recanati, IT	Seed	*C. moschata*		+				
	IPR8	Recanati, IT	Seed	*C. moschata*		+				
	IPR9*	Recanati, IT	Seed	*C. moschata*	+	+	MK497780	-	-	-
Fusarium	F59	Sidi Hmada, TN	Seed	*C. maxima*		+				
oxysporum	F16	Sbeïtla, TN	Seed	*C. maxima*		+				
	F19	Kalâat El-Andalous, TN	Seed	*C. maxima*		+				
Fusarium solani	F82	Sbeïtla, TN	Seed	*C. maxima*		+				
	F30	Sidi Hmada, TN	Seed	*C. maxima*		+				
	F174*	Sbeïtla, TN	Seed	*C. maxima*	+	+	MF401578	MK497790	MK497772	-
	F142	Sidi Hmada, TN	Seed	*C. maxima*		+				
Curvularia	B172	Sbeïtla, TN	Seed	*C. maxima*		+				
spicifera	B170*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401577	-	-	-
	IB41*	Castelfidardo, IT	Seed	*C. maxima*	+	+	MK497777			
	IB4	Castelfidardo, IT	Fruit	*C. maxima*		+				
	IB1*	Castelfidardo, IT	Seed	*C. maxima*	+	+	MK497778	-	-	-
	IB2	Recanati, IT	Seed	*C. moschata*		+				
	IB3	Recanati, IT	Seed	*C. moschata*		+				
Stemphylium	P164*	Kalâat El-Andalous, TN	Seed	*C. maxima*	+	+	MF401574	MK497787	-	-

vesicarium	P66*	Kalâat El-Andalous, TN	Seed	*C. maxima*	+	+	MF401573	MK497791	-	-
	IP41	Baranello, IT	Seed	*C. moschata*		+				
	IP4*	Osimo, IT	Seed	*C. moschata*	+	+	MK497781	-	-	-
	IP5	Osimo, IT	Seed	*C. moschata*		+				

* -, sequence not available.

Table 3. Nucleotide sequence primers already published for the detection of *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*. *Curvularia spicifera*, *Fusarium oxysporum*, and *Fusarium solani*.

Fungal species	**Primer pair***	**Sequence (5'-3')**	**Reference**
Stagonosporopsis cucurbitacearum	RGI F	TGTCGTTGAC ATCATTCCAGC	Somai et al. 2002; Babu et al. 2015
	RGI R	ACCACTCTGCTTAGTATCTGC	
	RGII F	GCTAAGCCTT AATCTAGCTGC	
	RGII R	GAGAGTAAGCTAACCTAAAGG	
Stagonosporopsis spp. (*S. cucurbitacearum*, *S. citrulli*, *S. caricae*)	Db01F	CACGCCAGCAAATCTCACTA	Brewer et al. 2015
	Db01R	CGGTCCGGTCAACCTACTAC	
	Db05F	TATGACGTTGGGCAAGTGAG	
	Db05R	TTTGCTGGGATGGTGTTGTA	
	Db06F	GGTGACATCTTGCGTGAATG	
	Db06R	TGGTTGTTTGGTTGTTTGGA	
Alternaria alternata	AAF2	TGCAATCAGCGTCAGTAACAAAT	Konstantinova et al. 2002
	AAR3	ATGGATGCTAGACCTTTGCTGAT	
Curvularia spicifera	Bipol-1F	CAGTTGCAATCAGCGTCAGT	Ünal et al. 2011
	Bipol-1R	AAGACAAAAACGCCCAACAC	
Fusarium oxysporum	FC-1	CATACCACTTGTTGCCTC	Zhang et al. 2012
	FC-2	ATTAACGCGAGTCCCACC	
Fusarium solani	TEF-Fs4 F	ATCGGCCACGTCGACTCT	Arif et al. 2012
	TEF-Fs4 R	GGCGTCTGTTGATTGTTAGC	

F. solani f. sp. *cucurbitae* race 1	Fsc1EF1	GCTAACAATCATCTACAGAC	Mehl and Epstein 2007
	Fsc1-EF-2	GACGGATGAGAGAGCAAC	
F. solani f. sp. *cucurbitae* race 2	Fsc2-EF1	GTTGGTGACATATCTCCC	
	Fsc2-EF3	GAGTGAGAGACATGACGG	

*F, forward; R, reverse

Table 4. Characteristics of the genus-specific and species-specific primers designed for *Albifimbria verrucaria*, *Paramyrothecium roridum*, and *Stemphylium vesicarium*.

Target sequence species	Primer name	Sequence (5' to 3')	Tm (°C)	Bases	GC content (%)
Albifimbria verrucaria	Myroverr F1	5'-TGTGAACCTTACCATATTGTTGC-3'	62.4	23	39.1
	Myroverr R1	5'-CGTTCCAACTGCGAGGTTGT-3'	67.4	20	55.0
Paramyrothecium roridum	Myroror F1	5'-CCCTTTGTGAACCTTACCTAT-3'	58.5	21	42.8
	Myroror R1	5'-AGCTCCAATGCGAGTTGTG-3'	64.1	19	52.6
Stemphylium vesicarium	Pleo F	5'-TACACAATATGAAAGCGGGTTG-3'	63.7	22	40.9
	PleoR	5'-AAGGCTGATTCAAAGTGCAAG-3'	63.2	21	42.8
	Pleo F1	5'-ATTCACCCATGTCTTTTGCG-3'	64.7	20	45.0
	PleoR1	5'-AAATGTGGTCTTGATGGATGC-3'	63.7	21	42.8

Table 5. Cycling conditions for the newly designed primers.

Target sequence species	**Designed primer pair**		**Validated PCR program**		
	Forward	**Reverse**	**Initial denaturation**	**Denaturation: 25 cycles**	**Annealing**
Albifimbria verrucaria	Myroverr F1	Myroverr R1	95 °C, 2 min	95 °C 30 s, 60 °C 30 s, 72 °C 30 s	72 °C, 5 min
Paramyrothecium roridum	Myroror F1	Myroror R1	95 °C, 2 min	95 °C 30 s, 58 °C 30 s, 72 °C 30 s	72 °C, 5 min
Stemphylium vesicarium	Pleo F Pleo F1	PleoR PleoR1	95 °C, 2 min	95 °C 30 s, 60 °C 30 s, 72 °C 30 s	72 °C, 5 min

Table 6. Incidence of different seedborne fungi detected on all of the squash samples using blotter tests.

Country	Sample code	Region[a]	Fungal species on fruit[b]	Level of symptoms[c]	Incidence of fungal species (% ±SE)[d]														
					Total[e]	*Aa*	*As*	*Ab*	*Cs*	*Sc*	*Ph* sp	*Ff*	*Fo*	*Fi*	*Fs*	*Pr*	*Av*	*Sv*	*Rs*
Tunisia	T4	SH	-	A	46.5 ±2.9	19.5 ±3.3	0.0	0.0	0.0	44.0 ±4.5	2.5 ±2.5	8.5 ±2.3	2.0 ±1.4	0.5 ±0.5	9.0 ±2.5	0.0	0.0	0.5 ±0.5	2.5 ±2.5
	T7	SH	*Sc*	B	73.5 ±4.3	2.5 ±1.4	0.0	0.0	0.0	57.0 ±4.1	3.0 ±1.3	10.5 ±2.5	1.5 ±1.1	0.0	13.5 ±4.2	1.0 ±1.0	2.0 ±1.2	0.0	1.5 ±1.5
	T8	SH	*Aa*/*Sc*	C	25.0 ±3.0	6.0 ±2.5	0.0	0.0	0.0	21.5 ±4.5	0.0	8.5 ±2.9	0.0	0.0	6.0 ±3.4	0.0	0.0	0.0	6.5 ±2.1
	T9	SH	*Sc*	C	64.0 ±7.2	0.5 ±0.5	0.0	0.0	0.0	62.0 ±4.2	1.0 ±1.0	2.0 ±2.0	1.0 ±0.7	0.0	0.5 ±05	0.0	0.0	0.0	0.0
	T13	SH	-	A	54.5 ±6.1	17.0 ±4.5	0.0	0.0	0.0	4.0± 4.0	1.0 ±1.0	5.5 ±3.4	0.0	2.0 ±1.2	6.0 ±3.8	1.0 ±1.0	1.5 ±1.5	4.5 ±4.5	8.5 ±6.0
	T14	SH	-	A	75.0 ±5.9	18.0 ±4.0	4.0 ±2.5	2.0 ±1.6	3.5 ±1.3	4.5 ±1.5	0.0	16.0 ±5.9	0.0	0.0	9.5 ±4.9	0.0	0.0	3.5 ±1.1	3.5 ±1.8
	T18	SH	-	A	57.0 ±4.7	16.0 ±4.7	2.0 ±2.0	1.5 ±1.5	2.0 ±2.0	0.0	0.0	4.5 ±2.2	1.0 ±1.0	0.0	10.5 ±3.0	4.5 ±2.1	15.5 ±4.6	3.0 ±1.3	0.5 ±0.5
	T22	SH	-	A	49.0 ±5.7	11.0 ±4.1	0.0	0.0	0.0	3.5 ±1.7	1.0 ±0.7	7.0 ±3.0	1.0 ±0.7	1.5 ±1.1	0.0b	3.0 ±1.8	7.0 ±1.9	1.0 ±0.7	9.5 ±3.8
	T34	KA	*Aa*	B	72.0 ±6.3	28.0 ±5.7	0.0	1.0 ±0.7	0.0	0.0	0.0	1.5 ±0.8	0.0	0.0	0.0	0.0	0.0	12.5 ±0.7	29.5 ±7.6
	T47	KA	*Aa*	B	73.0 ±4.4	35.0 ±3.3	0.0	1.5 ±0.8	1.0 ±0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ±0.7	32.0 ±4.6
	T52	KA	*Fs*	B	14.0 ±2.9	0.0	0.0	0.0	0.0	0.0	0.0	4.0 ±1.5	3.0 ±1.8	0.5 ±0.5	6.5 ±2.0	0.0	0.0	0.0	0.0

T71	KA	*Aa*	B	62.0 ±4.9	28.5 ±4.1	0.0	6.0 ±1.7	0.0	0.0	0.0	2.5 ±1.0	0.0	0.0	0.0	0.0	0.0	1.0 ±0.7	30.5 ±5.4
T35	KA	*Aa*	B	77.0 ±6.7	49.5 ±5.2	0.0	0.0	0.0	0.0	0.0	7.5 ±3.0	0.0	0.0	0.0	0.0	0.0	2.5 ±1.0	17.5 ±4.9
T63	KA	*Aa*	B	21.5 ±7.0	8.0 ±3.5	0.0	0.5 ±0.5	0.5 ±0.5	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	0.0	12.0 ±4.7
T45	KA	*Aa*/*Cs*	B	38.4 ±4.1	34.8 ±3.7	0.0	0.5 ±0.5	3.6 ±1.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T69	KA	*Aa*	B	17.8 ±4.0	14.9 ±3.4	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0 ±1.2	6.0 ±2.0
T1	KA	-	A	11.5 ±4.0	3.0 ±1.7	0.0	0.0	0.0	0.0	0.0	1.0 ±0.6	0.0	0.0	0.0	0.0	0.0	3.5 ±1.3	5.0 ±3.0
T3	KA	-	A	49.0 ±2.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	48.5 ±2.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ±1.0
T76	Sb	-	A	12.5 ±4.2	3.0 ±1.0	0.0	3.0 ±3.0	0.0	0.5 ±0.5	0.0	0.5 ±0.5	0.0	0.0	4±3	0.0	0.0	0.0	1.5 ±0.8
T77	Sb	-	A	62.0 ±5.0	37.5 ±5.0	0.0	0.0	6.5 ±2.0	9.0 ±2.5	0.0	0.0	0.0	0.0	6.5 ±2.0	0.0	0.0	4.5 ±1.8	0.0
T78	Sb	-	A	37.0 ±4.0	10.5 ±2.1	0.0	0.0	0.0	2.5 ±1.2	0.0	9.0 ±3.0	1.0 ±1.0	1.0 ±0.6	13.0 ±4.2	0.0	0.0	0.0	1.5 ±1.0
T79	Sb	-	A	18.5 ±4.7	0.0	0.0	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	18.0 ±4.7	0.0	0.0	0.0	0.0
T80	Sb	-	A	63.0 ±5.2	1.5 ±1.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ±1.0	0.0	59.5 ±6.0	0.0	0.0	0.0	1.5 ±1.0
T6	Ut	-	A	10.5 ±5.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5 ±5.3

T16	Ut	-	A	15.5 ±3.2	1.5 ±0.8	0.0	0.0	0.0	3.0 ±1.2	0.0	4.0 ±1.6	0.0	0.0	0.0	0.0	0.0	0.5 ±0.5	6.5 ±2.2
T38	Ut	-	A	5.0 ±1.3	1.0 ±0.6	0.0	0.0	1.0 ±0.6	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	0.5 ±0.5	2.5 ±1.2
T40	Ut	-	A	4.5 ±2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5 ±2.4
T58	Ut	-	A	27.0 ±4.9	17.0 ±4.0	0.0	5.0 ±2.2	1.5 ±0.8	0.5 ±0.5	0.0	0.0	0.0	0.5 ±0.5	2.5 ±2.5	0.0	0.0	0.0	0.5 ±0.5
T66	Ut	-	A	33.5 ±5.6	11.5 ±3.5	0.0	0.0	0.0	1.0 ±0.6	0.0	4.5 ±1.1	2.0 ±1.5	2.5 ±1.7	12.0 ±3.6	0.0	0.0	0.0	0.5 ±0.5
T70	Ut	-	A	32.5 ±4.5	17.0 ±4.7	0.0	0.0	0.0	0.5 ±0.5	0.0	11.5 ±2.8	0.0	1.5 ±0.8	1.0 ±0.6	0.0	0.0	1.0 ±1.0	1.0 ±0.6
T81	Ut	-	A	35.0 ±5.7	7.0 ±2.4	0.0	0.5 ±0.5	0.0	0.0	0.0	25.0 ±5.1	0.0	0.0	0.0	0.0	0.0	0.5 ±0.5	1.0 ±1.0
T23	Sa	-	A	21.5 ±6.3	0.0	0.0	0.0	0.0	0.0	0.0	20.0 ±6.3	0.0	0.0	0.0	0.0	0.0	0.0	1.5 ±1.4
T24	Sa	-	A	6.0 ±1.6	2.0 ±1.1	0.0	0.0	0.5 ±0.5	0.0	0.0	1.5 ±1.0	0.0	0.0	0.0	0.0	0.0	0.5 ±0.5	1.5 ±1.0
T26	Sa	-	A	9.0 ±1.6	5.5 ±1.5	0.0	0.5 ±0.5	0.5 ±0.5	0.0	0.0	0.0	0.0	0.5 ±0.5	1.0 ±0.6	0.0	0.0	0.0	1.5 ±0.8
T28	Sa	-	A	14.0 ±3.0	4.5 ±1.5	0.0	0.0	0.0	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	0.0	7.0 ±1.9
T29	Sa	-	A	8.5 ±2.0	5.0 ±1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0 ±1.4
T30	Sa	-	A	13.0 ±4.4	1.5 ±0.8	0.0	0.5 ±0.5	0.5 ±0.5	0.0	0.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	2.0 ±0.9	8.0 ±4.0

Italy	I2	Ca	*Aa*/*Cs*	C	85.0 ±2.6	9.5 ±3.3	0.0	0.0	85.0 ±2.6	0.0	0.0	9.5 ±2.5	0.0	0.0	0.0	0.0	0.0	0.0
	I3	Ca	-	A	2.5 ±2.0	0.5 ±0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0 ±2.0
	I4	Ca	*Aa*	B	19.0 ±3.3	1.5 ±1.5	0.0	0.0	0.0	0.0	0.0	12.0 ±2.5	0.0	0.0	0.0	0.0	0.0	4.5 ±2.7
	I5	Ca	*Aa*/*Cs*	C	65.7 ±8.4	4.6 ±1.8	0.0	0.0	11.4 ±2.5	0.0	0.0	53.9 ±7.2	0.0	0.0	0.0	0.0	0.0	0.0
	I8	Ca	*Aa*	C	36.0 ±3.5	2.0 ±0.9	0.0	0.0	0.0	0.0	0.0	17.2 ±3.8	0.0	0.0	0.0	0.0	0.0	0.0
	I12	Ca	*Aa*	B	53.5 ±5.9	10.5 ±3.4	0.0	0.0	0.0	0.0	0.0	13.5 ±5.2	0.0	0.0	0.0	0.0	1.0 ±0.6	29.0 ±5.1
	I13	Ca	*Aa*	B	40.0 ±3.7	6.8 ±2.4	0.0	0.0	0.0	0.0	0.0	21.5 ±4.1	0.0	0.0	0.0	0.0	0.0	11.5 ±3.5
	I16	Ca	-	A	29.0 ±4.1	1.0 ±1.0	0.0	0.0	0.0	0.0	0.0	27.0 ±4.0	0.0	0.0	0.0	0.0	0.0	1.5 ±1.5
	I17	Os	*Sc*/*Aa*	B	6.0 ±3.2	3.1 ±1.7	0.0	0.5 ±0.5	0.0	0.0	0.0	0.6 ±0.6	0.0	0.4 ±0.4	0.0	0.0	0.0	1.1 ±1.1
	I18	Os	*Sc*/*Aa*	B	33.8 ±4.1	19.7 ±3.7	0.0	1.5 ±1.1	0.0	0.0	0.0	9.3 ±3.1	0.0	1.5 ±1.1	0.0	0.0	0.5 ±0.5	0.0
	I19	Os	*Sc*/*Aa*	B	30.7 ±4.1	20.3 ±5.2	0.0	0.0	0.0	0.0	0.0	8.8 ±2.9	0.0	1.5 ±1.0	0.0	0.0	1.0 ±0.6	0.0
	I20	Os	*Sc*/*Aa*	B	38.9 ±5.2	32.2 ±4.3	0.0	0.0	0.0	0.0	0.0	0.9 ±0.6	0.0	0.9 ±0.6	0.0	0.0	2.4 ±0.8	0.0
	I21	Os	*Sc*/*Aa*	B	27.7 ±6.0	9.0 ±3.0	0.0	0.4 ±0.4	0.0	0.0	0.0	10.0 ±3.9	0.0	0.0	0.0	0.0	0.4 ±0.4	7.6 ±4.8

I22	Os	-	A	22.8 ±3.2	11.1 ±2.0	0.0	0.0	0.0	0.0	0.0	10.5 ±2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
I23	Os	-	A	21.7 ±4.2	13.5 ±4.1	0.0	2.2 ±1.7	0.4 ±0.4	0.0	0.0	4.0 ±1.9	0.0	0.0	0.0	0.0	0.0	0.4 ±0.4	0.0
I24	Os	-	A	22.6 ±3.1	11.7 ±2.4	0.0	0.0	0.0	0.0	0.0	9.5 ±3.1	0.0	0.4 ±0.4	0.0	0.0	0.4 ±0.4	0.0	0.4 ±0.4
I25	Os	*Sc*/*Aa*	B	25.1 ±3.7	6.9 ±2.1	0.0	1.6 ±1.0	0.0	0.0	0.0	14.9 ±3.8	0.0	0.0	0.0	0.0	0.0	0.0	1±1
I26	Os	*Sc*/*Aa*	B	20.3 ±3.1	3.6 ±1.5	0.0	0.0	0.0	0.0	0.0	15.3 ±3.6	0.0	1.3 ±0.9	0.0	0.0	0.0	0.0	0.0
I27	Os	*Sc*/*Aa*	B	12.7 ±3.4	3.4 ±1.8	0.0	3.9 ±2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.3 ±2.5
I28	Os	*Aa*	B	14.9 ±3.8	9.5 ±3.0	0.0	2.7 ±2.2	0.0	0.0	0.0	2.2 ±1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.4 ±0.4
I29	Re	*Aa*/*Cs*	B	73.0 ±3.3	62.0 ±4.3	0.0	0.0	2.0 ±1.1	0.0	0.0	22.0 ±3.8	0.0	0.0	0.0	1.0 ±1.0	0.0	8.5 ±2.3	0.0
I30	Re	*Aa*	C	77.5 ±3.9	69.5 ±5.6	0.0	0.0	0.0	0.0	0.0	46.0 ±6.1	0.0	1±1	0.0	0.5 ±0.5	0.0	1.0 ±0.6	0.0
I31	Re	*Aa*/*Cs*	C	65.0 ±4.2	52.0 ±4.4	0.0	0.0	11.0 ±2.2	0.0	0.0	9.5 ±3.5	0.0	0.0	0.0	0.0	0.0	2.5 ±1.2	0.0
I32	Re	-	A	18.0 ±2.8	0.0	0.0	0.0	0.0	0.0	0.0	18.0 ±2.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
I33	Re	*Aa*	B	30.5 ±2.9	24.5 ±3.1	0.0	0.0	0.0	0.0	0.0	3.5 ±1.5	0.0	0.0	0.0	3.0 ±1.4	0.0	0.5 ±0.5	0.0
I34	Re	*Aa*	B	48.5 ±3.5	32.5 ±3.3	0.0	0.0	0.0	0.0	0.0	21.5 ±4.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0

I35	Mo	-	A	21.0 ±3.6	13.5 ±2.2	0.0	3.0 ±1.6	0.0	0.0	0.0	1.5 ±1.5	0.0	0.0	0.0	0.0	0.0	1.0 ±1.0	0.0
I36	Mo	-	A	38.5 ±2.8	36.5 ±3.2	0.0	1.0 ±0.9	0.0	0.0	0.0	14.5 ±3.4	0.0	0.0	0.0	0.0	1.5 ±1.0	0.0	0.0
I37	Ba	-	A	47.5 ±3.2	36.0 ±3.9	0.0	0.0	1.0 ±0.6	0.0	0.0	8.0 ±1.3	0.0	0.0	0.0	1.5 ±1.0	0.0	3.0 ±1.2	0.0

[a], SH, Sidi Hmada; KA, Kalâat El-Andalous; Ut, Utique; Sb, Sbeïtla; Sa, Sahline; Ca, Castelfidardo; Os, Osimo; Re, Recanati; Mo, Monopoli; Ba, Baranello

[b]*Aa*, *Alternaria alternata; As, Alternaria solani; Ab, Aspergillus brasiliensis; Cs, Curvularia spicifera: Sc, Stagonosporopsis cucurbitacearum: Ph sp, Phoma* sp.; Ff, *Fusarium fujikuroi; Fo, Fusarium oxysporum; Fi, Fusarium incarnatum; Fs, Fusarium solani; Pr, Pramyrothecium roridum; Av, Albifimbria verrucaria; Sv, Stemphylium vesicarium; Rs, Rhizopus stolonifer*

[c], A, asymptomatic fruit; B, infected fruit showing lesion on squash skin without colonization of fruit cavity; C, infected fruit showing lesion that has colonized fruit cavity

[d], Data are means ±standard error

[e], The same seed can be infected by more than one fungus

Table 7. Incidence of seedborne fungi detected in the squash seed samples collected in Tunisia and Italy.

Fungal species	**Disease incidence (%)[a]**									
	Tunisia[b]					**Italy[b]**				
	SH	**KA**	**Sb**	**Ut**	**Sa**	**Ca**	**Os**	**Re**	**Mo**	**Ba**
Alternaria alternata	11.3 ±1.3	25.1 ±1.8	10.5 ±1.8	6.8 ±1.0	3.0 ±0.5	4.2 ±0.7	13.0 ±1.2	40.0 ±2.6	25 ±2.7	36.0 ±4
Stagonosporopsis cucurbitacearum	24.6 ±2.3	0.0	2.5 ±0.6	0.6 ±0.2	0.0	0.0	0.0	0.0	0.0	0.0
Rhizopus stolonifer	4.1 ±1.0	13.3 ±1.5	0.9 ±0.3	3.3 ±0.8	3.7 ±0.8	6.6 ±1.2	1.3 ±0.5	0.0	1.25 ±1.25	0.0
Fusarium fujikuroi	7.8 ±1.2	6.5 ±1	2.0 ±0.6	6.3 ±1	3.7 ±1.2	20.8 ±1.8	7.1 ±0.8	20.0 ±2.0	8.0 ±2.1	8.0 ±1.3
Fusarium solani	6.9 ±1.2	0.6 ±0.2	16.6 ±2.7	1.9 ±0.6	0.1 ±0.1	0.0	0.0	0.0	0.0	0.0
Albifimbria verrucaria	3.3 ±0.8	0.0	0.0	0.0	0.0	0.0	0.2 ±0.1	0.0	0.75 ±0.5	0.0
Stemphylium vesicarium	1.6 ±0.6	2.3 ±0.7	1.0 ±0.4	0.3 ±0.1	0.4 ±0.1	0.1 ±0.09	0.4 ±0.1	2.0 ±0.5	0.5 ±0.5	3.0 ±1.2
Pramyrothecium roridum	1.2 ±0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.7 ±0.3	0.0	1.5 ±1.0
Phoma sp.	1.1 ±0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Aspergillus brasiliensis	0.8 ±0.4	1 ±0.2	0.6 ±0.6	0.06 ±0.06	0.1 ±0.1	0.0	1.0 ±0.3	0.0	2.0 ±0.9	0.0
Fusarium oxysporum	0.9 ±0.3	0.3 ±0.1	0.4 ±0.3	0.2 ±0.1	0.0	0.0	0.0	0.0	0.0	0.0
Curvularia spicifera	0.7 ±0.3	0.5 ±0.2	1.3 ±0.5	0.3 ±0.1	0.2 ±0.1	7.6 ±2.0	0.03 ±0.03	2.1 ±0.5	0.0	1.0 ±0.6
Alternaria solani	0.8 ±0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Fusarium incarnatum	0.5 ±0.2	0.2 ±0.1	0.2 ±0.1	0.5 ±0.2	0.08 ±0.08	0.0	0.5 ±0.1	0.1 ±0.1	0.0	0.0
Mean of infected seeds[c]	**55.6 ±2.2**	**45.3 ±2.2**	**36.6 ±2.7**	**20.2 ±1.7**	**11.6 ±1.5**	**37.6 ±2.4**	**23.0 ±1.3**	**52.0 ±2.4**	**29.7 ±2.7**	**47.5 ±3.2**

[a], Data are means ±standard error.

[b], SH, Sidi Hmada; KA, Kalâat El-Andalous; Ut, Utique; Sb, Sbeïtla; Sa, Sahline; Ca, Castelfidardo; Os, Osimo; Re, Recanati; Mo, Monopoli; Ba, Baranello.

[c], The same seed can be infected by more than one fungus.

Figure Legends

Figure 1. **Fruit rot symptoms on squash fruit**. (**A**) Apparently healthy fruit (cv. Galaoui). (**B**, **C**) Fusarium rot with white mycelia, caused by *F. solani* (arrow 1) (B, cv. Galaoui; C, cv. Bjaoui). (**D**, **E**) Black mycelia of *C. spicifera* on squash fruit (arrow 2) (D, cv, Galaoui; E, cv.Aspen). (**F**, **G**) *Alternaria* fruit spot caused by *A. alternata* (arrow 3) (F, cv. Aspen; G, cv. Galaoui). (**H**, **I**) Black rot caused by *S. cucurbitacearum* (arrow 4) (H, cv. Butternut; I, cv. Bajaoui). Scale bars: 5 cm.

Figure 2. Evaluation of symptoms on squash fruit collected from growers. (**A**) Asymptomatic fruit. (**B**) Infected fruit showing symptoms of rot on squash skin without reaching the seed cavity. (**C**) Infected fruit showing symptoms of rot that has reached the seed cavity. Scale bars: 5 cm.

Figure 3. (**A**, **B**) Pycnidia of *S. cucurbitacearum* on a seed from a squash, as seen under the stereomicroscope, with the ooze of pycnidiospores indicated (arrow 1). (**C**) Pycnidia under the microscope. (**D**) Pycnidiospores: cylindrical, mostly non-septate, few uniseptate (arrow 2) and biseptate (inset, arrow 3). (**E**) Ten-day-old colony on PDA at 22 ±2 °C. (**F, G**) Long chains of conidia of *A. alternata* on seeds. (**H**) Conidia of *A. alternata*. (**I**) Sporodochia of *A. verrucaria* on seed. (**J**) Elliptical conidia of *A. verrucaria*. (**K**) Sporodochia of *P. roridum* on a squash seed. (**L**) Cylindrical conidia of *P. roridum*. (**M**) Conidia of *C. spicifera* on a seed, as seen under the stereomicroscope. (**N**) Conidia and vegetative hyphae of *C. spicifera*. (**O**) Perithecia of *S. vesicarium* on a seed. (**P**) An ascus of *S. vesicarium*, with short, broad pedicel bearing eight ascospores. (**Q**) Ascospores of *S. vesicarium*. (R) *F. solani* on a seed. (**S**) Long phialide of *F. solani* (arrow 4). (**T**) Microconidia and macroconidia of *F. solani*. Scale bars: 200 µm (A and B); 100 µm (C); 10 µm (D); 1 cm (E); 100 µm (F); 400 µm (G); 20 µm (H); 200 µm (I); 10 µm (J); 100 µm (K); 10 µm (L); 200 µm (M); 10 µm (N); 400 µm (O); 20 µm (P); 5 µm (Q); 200 µm (R); 25 µm (S); 20 µm (T).

Figure 4. Gel electrophoresis of PCR products generated with the designed specific primers for the detection of the fungi. (**A**) *A. verrucaria*, with the primer pair Myroverr F1/Myroverr R1. Lanes 1 to 10: *A. verrucaria* (isolates IAV1, IAV2, IAV3, IAV4, M149, M155, M144, M140, M135, M146, respectively); lanes 11 to 13, *P. roridum* (isolates M123, M138, M141, respectively); and lane 14, water control. (**B**) *P. roridum*, with the primer pair Myroror

F1/MyrororR1. Lanes 1 to 10: *P. roridum* (isolates IPR1, IPR4, IPR5, IPR6, IPR9, M73, M123, M138, M141, M167, respectively); lanes 11, 12, *A. verrucaria* (isolates M149, M155, respectively); and lane 13, water control. (**C**) *P. herbarum*, with the primer pair Pleo F/ Pleo R. Lanes 1 to 5: *P. herbarum* (isolates IP4, IP5, P164, P66, respectively); lanes 6, 7, *A. alternata* (isolates A38, A15, respectively); lane 8, *C. spicifera* (isolate B170); lane 9, water control. (**D**) *P. herbarum*, with the primers pair Pleo F_1/Pleo R_1. Lanes 1 to 5, *P. herbarum* (isolates IP4, IP5, P164, P66, respectively); lane 6, *A. alternata* (isolate A38); lane 7, water control. M: molecular weight markers (100-bp intervals).

Figure 5. (**A**) Specificity of the primer pair RGII F/RGII R used for detection of *S. cucurbitacearum* in group RGII. Lanes 1 to 10, *S. cucurbitacearum* (isolates D33, D29, D21, D5, D42, D49, D45, D12, DBF1, DBF2, DBF3, respectively); lane 11, *Phoma* sp. (isolate Ph39); lane 12, *A. alternata* (isolate A15); lane 13, *C. spicifera* (isolate C170); lane 14, water controls. (**B**) Specificity of the primer pair AAF2/AAR3 for detection of *A. alternata*. Lanes 1 to 11, *A. alternata* (isolates A38, A15, A17, A5, A59, IA1, IA3, IA7 IA10, IA2, IA5, respectively); lane 12, *S. vesicarium* (isolate P66); lane 13, water control. (**C**) Specificity of the primer pair Bipol-1F/Bipol-1R for detection of *C. spicifera*. Lanes 1 to 6, *C. spicifera* (isolates B172, B170, IB41, IB2, IB3, IB4, respectively); lane 7, *F. solani* (isolate F174); lane 8, water control. (**D**) Specificity of the primer pair TEF-Fs4 F/TEF-Fs4 R for detection of *F. solani*. Lanes 1 to 4, *F. solani* (isolates F174, F82, F30, F142, respectively); lane 5, *F. oxysporum* (isolate F19); lane 6, *S. cucurbitacearum* (isolate D33); lane 7, *S. vesicarium* (isolate P66); lane 8, water control. (**E**) Specificity of the primer pair FC-1/FC-2 for detection of *F. oxysporum*. Lanes 1 to 3, *F. oxysporum* (isolates F59, F16, F19, respectively); lane 4, *F. solani* (isolate F174); lane 5, *S. cucurbitacearum* (isolate D33); lane 6, *S. vesicarium* (isolate P66); lane 7, *Paramyrothecium roridum* (isolate M123); lane 8, water control. Lane M: molecular weight markers (100-bp intervals).

737 **Supplementary Table S1.** GenBank accession numbers of the six fungal genera used to determine the conserved sequence from the ITS region
738 that allows specific identification.
739

Species	NBCI sequence code	Host	Locality	GenBank accession number
Paramyrothecium roridum	LG7	Wood	India	KC414758.1
	KUAB1MRCD	*Cynodon dactylon*	India	KF171528.1
	MA-83	Soybean	Brazil	JF724154.1
	ITS4	*Valerianella olitoria*	Italy	KT354921.1
	LH-5	-	China	HQ433334.1
	A553	*Pogostemon cablin*	China	KJ813720.1
	MTM-522	*Hydrangea*	USA	HM215150.1
	CBS 331.51	*Foeniculum vulgare*	The Netherlands	HQ115647.1
	MRtp-049	*Dendrobium officinale*	China	KU257608.1
	MRtp-013	*Dendrobium officinale*	China	KU257606.1
	MRtp-012	*Dendrobium officinale*	China	KU257605.1
	MRtp-023	*Dendrobium officinale*	China	KT928648.1
	MRtp-055	*Dendrobium officinale*	China	KU257609.1
	MRtp-047	*Dendrobium officinale*	China	KU257607.1
	TVD_Fungal-Culture147	Tomato	-	KF494145.1
	MA-20	Soybean	Brazil	JF724152.1

CLNP RV10 75	*Pinus albicaulis*	USA	GQ152603.1
JL-3	*Tribulus terrestris*	China	JX867215.1
UB:2246	*Begonia* x *hiemalis*	Brazil	KJ494661.1
DGM01	*Hemionitis arifolia*	China	JF343832.1
CM 2246	*Begonia elatior*	Brazil	KJ776790.1
YPE-SH9	*Dendrobium candidum*	China	KM986033.1
myr5	*Anubias barteri*	China	KJ572115.1
DUCC4002	*Anthurium*	Korea	KC581914.1
CD08072303	*Phaseolus vulgaris*	China	GQ381291.1
FQ07090401	Tomato	China	GQ162434.1
XDL07091402	*Abutilon megapotamicum*	China	KF761294.1
KACC 93161P	*Peperomia quadrangularis*	Korea	KJ174523.1
myr2-2	*Dieffenbachia picta*	Taiwan	KC469695.1
HZ07080302	*Anthurium*	China	KF761292.1
AQN07091401	*Petunia hybrida*	China	KJ018792.1
CDA725	*Coffea canephora*	Brazil	KJ815095.1
781	Soybean	Brazil	JF724155.1
CICR	Cotton	India	EU927366.1
KKFC408	*Eichhornia crassipes*	Thailand	AB823655.1
MKSVu119	Cowpea	India	KT819765.1
MKSVu118	Cowpea	India	KT819764.1

KKFC403	*Eichhornia crassipes*	Thailand	AB823654.1
KKFC483	*Eichhornia crassipes*	Thailand	AB857223.1
KKFC470	*Eichhornia crassipes*	Thailand	AB857221.1
KKFC406	*Eichhornia crassipes*	Thailand	AB857216.1
KKFC447	*Eichhornia crassipes*	Thailand	AB857217.1
KKFC448	*Eichhornia crassipes*	Thailand	AB857218.1
KKFC400	*Eichhornia crassipes*	Thailand	AB823652.1
KKFC390	*Eichhornia crassipes*	Thailand	AB823651.1
KKFC402	*Eichhornia crassipes*	Thailand	AB823653.1
KKFC457	*Eichhornia crassipes*	Thailand	AB857219.1
KKFC462	*Eichhornia crassipes*	Thailand	AB857220.1
KKFC509	*Eichhornia crassipes*	Thailand	AB857228.1
KKFC499	*Eichhornia crassipes*	Thailand	AB857227.1
KKFC497	*Eichhornia crassipes*	Thailand	AB857226.1
KKFC496	*Eichhornia crassipes*	Thailand	AB857225.1
KKFC492	*Eichhornia crassipes*	Thailand	AB857224.1
KKFC519	*Eichhornia crassipes*	Thailand	AB857229.1
KKFC480	*Eichhornia crassipes*	Thailand	AB857222.1
MA-73	Soybean	Brazil	JF724153.1
782	Melon	Brazil	JF724156.1
LGHT07091402	*Hiemalis begonia*	China	KJ018794.1

	-	*Salvia* sp.	USA	EF151002.1
	-	Tomato	Italy	KR709186.1
	RDCCT07091402	Swedish ivy	China	KP942366.1
	794	Soybean	Brazil	JF724158.1
	802	Soybean	Brazil	JF724150.1
	801	Soybean	Brazil	JF724151.1
	784	Melon	Brazil	JF724157.1
	MTL07081001	*Zantedeschia aethiopica*	China	KF761293.1
	IMI 394934	*Eichhornia crassipes*	Nigeria	GQ853401.1
	HXC15051716	Water spinach	China	KU312191.1
	HXC15051715	Water spinach	China	KU312190.1
	HXC15051715	*Ipomoea aquatica*	China	KT943519.1
	IFB-E091	*Artemisia annua*	China	GU074399.1
Myrothecium inundatum	IN-5	*Acalypha indica*	India	HQ165763.1
	A2S4-D45	Soil	Malaysia	KJ767119.1
	77F	*Homo sapiens*	Vietnam	AB704784.1
	SCSAAF0024	*Antipathes dichotoma*	China	JQ647903.1
	SCSGAF0095	*Melitodes squamata*	China	JN851023.1
	A1S2-2	Soil	Malaysia	KJ767118.1
	C45	Soybean	Brazil	JQ936268.1
	C13.1	Soybean	Brazil	JQ936267.1

	F216	*Glycine max*	Brazil	KM979993.1
	CBS 582.93	-	Spain	AY254152.1
	GHJ-2	-	China	FJ797514.1
	CBS 582.93	-	Germany	AJ302005.1
Myrothecium leucotricha	ZM0902-9	Soil	China	JX077071.1
	ZM0902-23	Soil	China	JX077070.1
	CBS 131.64	-	Germany	AJ302000.1
	BBA 65577	-	Germany	AJ301992.1
Albifimbria verrucaria	23221	*Taxus chinensis*	China	KF574891.1
	wxm94	Wood	China	HM037988.1
	F0705	Apple	Japan	AB693919.1
	E21	*Ferula*	China	KF887115.1
	G340	Milk thistle leaf	-	KM215639.1
	MYRver2	Soil	Italy	GQ131886.1
	ITS6	Spinach	Italy	KT354922.1
	KASHMIR	*Pinus* sp.	India	KP310497.1
	I-5	Wood	China	KT305924.1
	A-304	Soil	Iran	KC140223.1
	QWKF1	-	China	KJ589551.1
	NRRL 52420	*Zea mays*	USA	GU183129.1
	Hmp-F73	*Hymeniacidon perleve*	China	HQ625520.1

F17	Tomato	Egypt	KU681402.1
F12	Pigeon Pea	China	KJ026704.1
PTCC 799	Soil	Iran	KC140228.1
HGUP 0731	Soil	China	KC806230.1
E16	Soybean	China	JQ356542.1
VKKSP1	Soil	India	HM358041.1
NIi	-	Malaysia	KM246762.1
A-284	Soil	Iran	KC140222.1
CNXY-007	*Houttuynia cordata*	China	KF750592.1
AR346	Soil	India	HQ596904.1
A-115	Soil	Iran	KC140221.1
F16	Pigeon Pea	China	KJ026703.1
A-336	Soil	Iran	KC140225.1
NJR102-16	Sediment	China	JX077018.1
KAUEF26	*Calotropis*	Saudi Arabia	HF548712.1
A-70	Soil	Iran	KC140220.1
MYCver2	Soil	Italy	EF017211.1
C-1	Wood	China	KT305923.1
D2	*Zingiber officinale*	Japan	AB778924.1
XZ04-18-2	Soil	China	JF812340.1
M2	Soil	France	AY303603.1

Myrothecium atroviride	wb256	-	Austria	AF455507.1
Myrothecium gramineum	CY176	-	USA	HQ608010.1
	LCJ 177	Tree trunk	India	KF414681
	A243	Cotton	China	GQ373154.1
Stemphylium vesicarium	1664	Pear	Japan	LC056844.1
	1680	Pear	Japan	LC056845.1
	CT09AMS6S	*Ammi majus*	Japan	AB938190.1
	FF51	*Pyrus commuis*	South Africa	KR912336.1
	FF50	*Pyrus commuis*	South Africa	KR912335.1
	OT3-175.1	Madural cultivar	Portugal	KT804104.1
	EA	Wood	Italy	KF482449.1
	H09-007	-	Spain	KC009768.1
	AFTOL-ID 940	-	USA	DQ491516.1
	ATCC 11681	-	USA	AF229479.1
	-	-	China	AF383967.1
	ICMP 5620-77	Carrot	UK	Y17068.1
	E. G. Simmons 08-069	-	-	AF071345.1
	4248	*Eucalyptus globulus*	Spain	FR667974.1
	EGS48-095	-	New Zealand	AY329232.1
	EGS29-089	-	USA	AY329229.1
	EGS36-088	-	Australia	AY329171.1

	EGS36-138	-	India	AY329169.1
	CT09AMS1S	*Ammi majus*	Japan	AB938189.1
	CBS 191.86	*Medicago sativa*	India	KC584239.1
	MH955	Soil	Czech Republic	LN901148.1
	MAFF 306801	*Asparagus officinalis*	Japan	AB979880.1
	MAFF 305562	*Asparagus officinalis*	Japan	AB979878.1
	MAFF 241964	*Allium tuberosum*	Japan	AB979877.1
	EPS26	Pear	Spain	GU065719.1
	EGS 40-038	*Medicago sativa*	USA	AF442776.1
Stemphylium solani	bgr1	*Avicennia marina*	China	KJ767499.1
	LS2	Lettuce	Malaysia	KC796636.1
	LS1	Lettuce	Malaysia	KC796635.1
	LT5	Lettuce	Malaysia	KC796634.1
	LT4	Lettuce	Malaysia	KC796633.1
	LT3	Lettuce	Malaysia	KC796632.1
	LT2	Lettuce	Malaysia	KC796631.1
	LT1	Lettuce	Malaysia	KC796630.1
	LM	Lettuce	Malaysia	KC796629.1
	LKR2	Lettuce	Malaysia	KC796628.1
Stemphylium botryosum	CBS 714.68	*Medicago sativa*	Canada	KC584238.1
Stemphylium paludiscirpi	EGS31-016	-	USA	AY329231.1

Stemphylium eturmiunum	EGS29-099	-	New Zealand	AY329230.1
	Riv-St	Onion	Puerto Rico	DQ323706.1
	EGS29-099	-	New Zealand	AY329230.1
Stemphylium lycopersici	THYB1	*Aegiceras corniculatum*	China	KU518355.1
	EGS17-137	-	New Caledonia	AY329206.1
Stemphylium majusculum	EGS16-068	-	USA	AY329228.1
Pleospora gigaspora	EGS37-017	-	Switzerland	AY329177.1
Stemphylium triglochinicola	EGS36-118	-	United Kingdom	AY329175.1
Curvularia sesuvii	Bp-zj 03	*Sesuvium portulacastrum*	China	EF175942.1
Curvularia spicifera	L3	Sugarcane	China	JN695636.1
	L2	Sugarcane	China	JN695635.1
	L1	Sugarcane	China	JN695634.1
	FBA-1	*Sorghum bicolor*	Turkey	HQ538774.1
	MH12073	*Panicum virgatum*	USA	HQ015445.1
Curvularia lunata	JGS10	-	China	GU966505.1
	IP 2328.95	-	France	DQ836800.1
	DSM-63137	*Crotalaria juncea*	Burkina Faso	KF897859.1
	NBAIR-NEF10	Maize	India	KU158873.1
	MP03	Sorghum	India	KT598350.1
Fusarium oxysporum	FusO-JSB63	*Cucurbita pepo*	India	JQ665266.1
	FO	Squash	New Zealand	AF055220.1

	-	Squash	Spain	AM940070.1
Fusarium solani f. sp. *cucurbitae*	PCl-511	Zucchini squash	Spain	KF372878.1
	Fsm711	*Cucumis melo*	Spain	KC711040.1
	Fsm731	*Cucumis melo*	Spain	KC711041.1
Fusarium solani	-	Squash	Spain	AM940071.1
	FRC#s1195	Pumpkin	USA	DQ094744.1
Stagonosporopsis cucurbitacearum	Di-4 (426)	Watermelon	Tunisia	EF107642.1
	Di-3 (425)	Watermelon	Tunisia	EF107641.1
	NY1	*Cucumis melo*	USA	AF495850.1
	C76	*Cucumis melo*	USA	AF495849.1
	T153	-	China	FJ462750.1
	ATCC 16241	*Cucumis melo*	USA	AF297228.1
	MA71	Mangrove	Thailand	GU592001.1
	FG58	*Vitis vinifera*	China	EU030365.1
	TMK-4	Muskmelon	China	EF160076.1
	TMK-3	Muskmelon	China	EF160075.1
	TMK-2	Muskmelon	China	EF160074.1
	TMK-1	Muskmelon	China	EF160073.1

740 CBS: Westerdijk Fungal Biodiversity Institute; UB: University of Brasilia Herbarium; CICR: Crop Improvement Division, Central Institute for
741 Cotton Research; ATCC: The Global Bioresource Center; MAFF: Ministry of Agriculture Forestry and Fisheries.

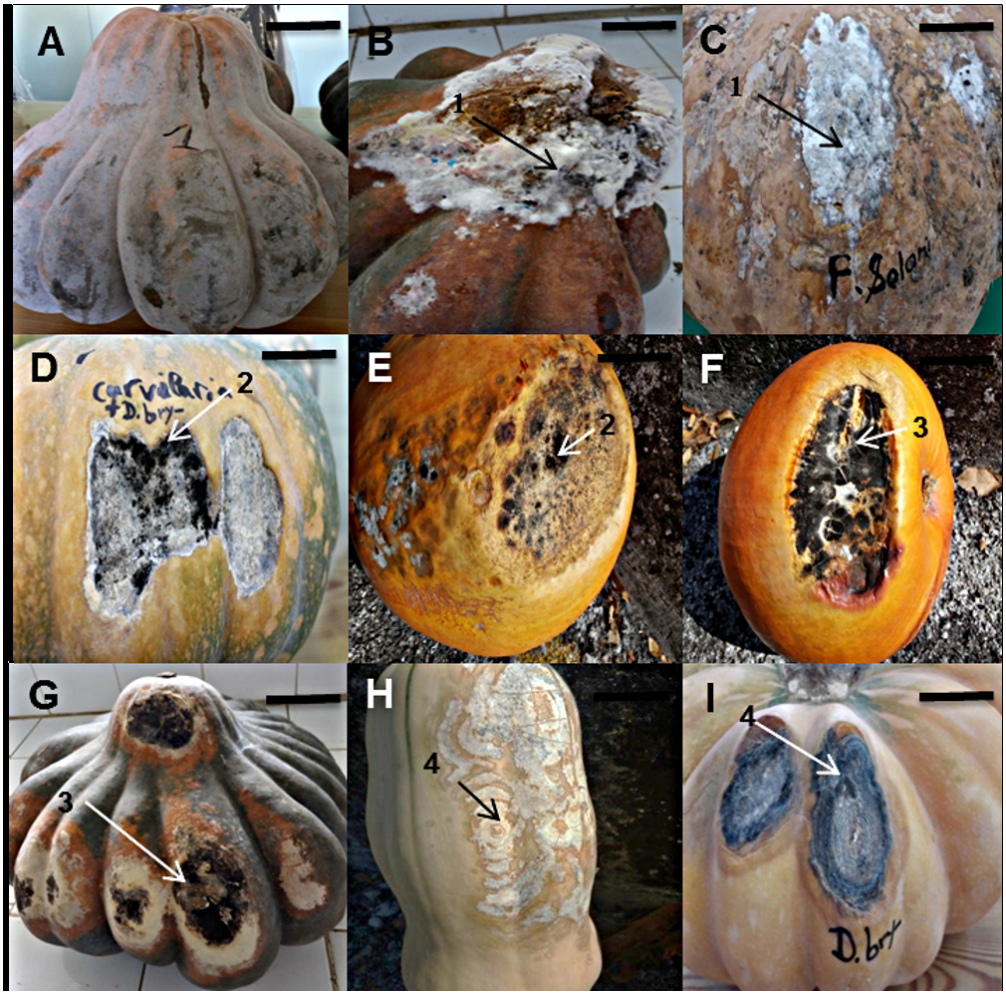

Figure 1. Fruit rot symptoms on squash fruit. (A) Apparently healthy fruit (cv. Galaoui). (B, C) Fusarium rot with white mycelia, caused by F. solani (arrow 1) (B, cv. Galaoui; C, cv. Bjaoui). (D, E) Black mycelia of C. spicifera on squash fruit (arrow 2) (D, cv, Galaoui; E, cv.Aspen). (F, G) Alternaria fruit spot caused by A. alternata (arrow 3) (F, cv. Aspen; G, cv. Galaoui). (H, I) Black rot caused by S. cucurbitacearum (arrow 4) (H, cv. Butternut; I, cv. Bajaoui). Scale bars: 5 cm.

85x83mm (300 x 300 DPI)

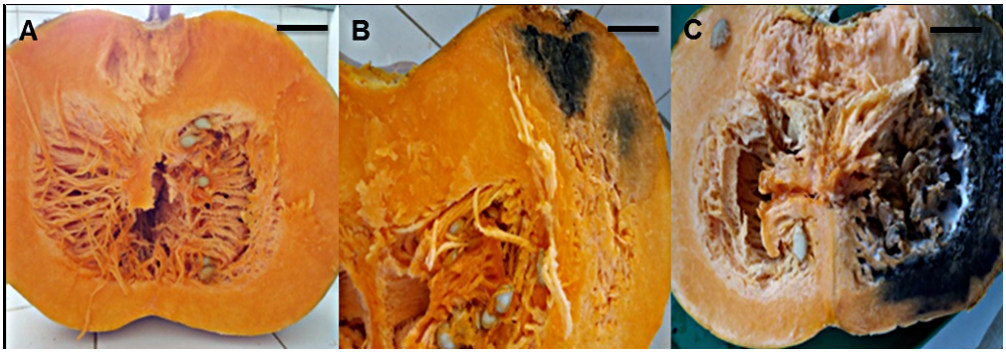

Figure 2. Evaluation of symptoms on squash fruit collected from growers. (A) Asymptomatic fruit. (B) Infected fruit showing symptoms of rot on squash skin without reaching the seed cavity. (C) Infected fruit showing symptoms of rot that has reached the seed cavity. Scale bars: 5 cm.

85x29mm (300 x 300 DPI)

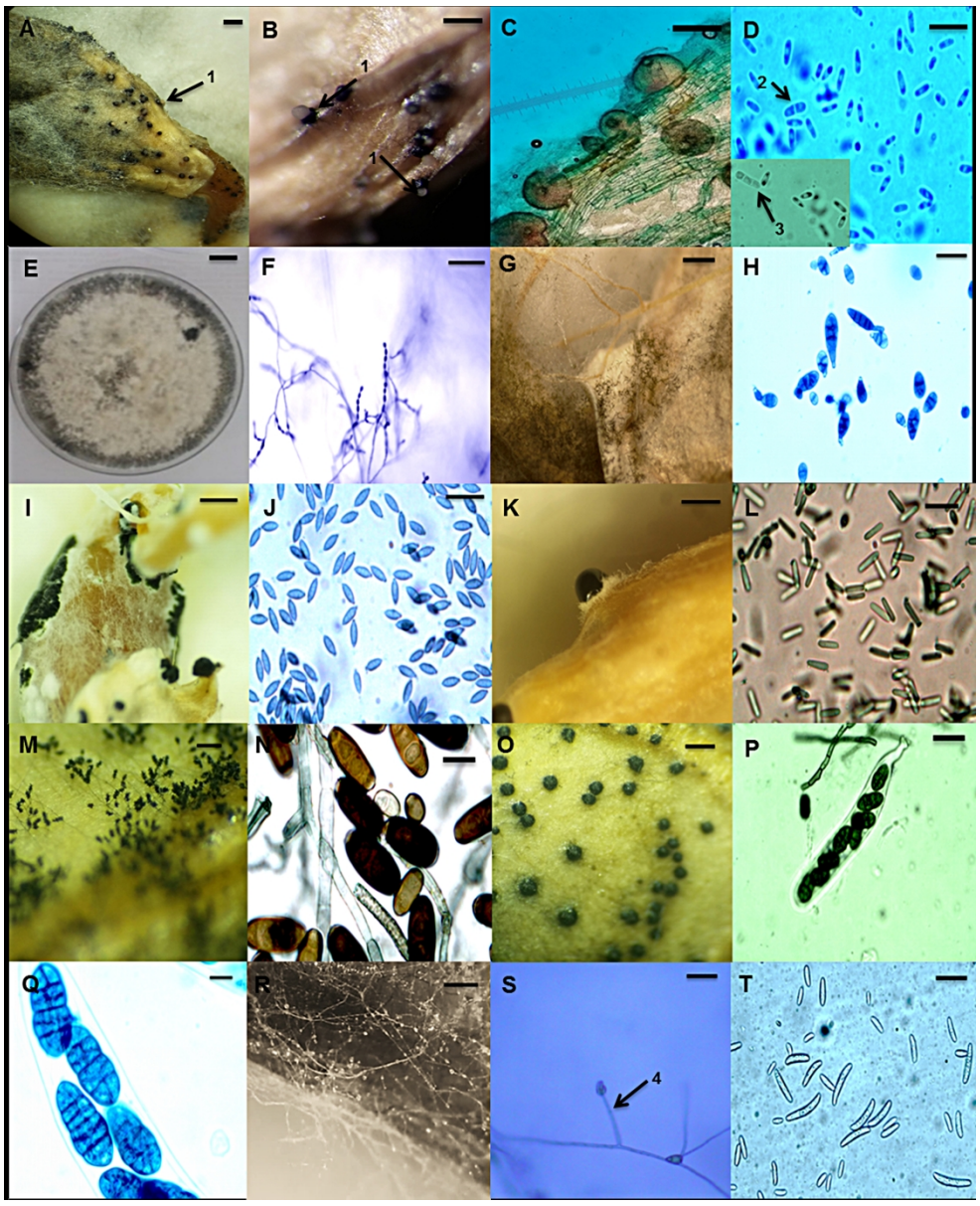

Figure 3. (A, B) Pycnidia of S. cucurbitacearum on a seed from a squash, as seen under the stereomicroscope, with the ooze of pycnidiospores indicated (arrow 1). (C) Pycnidia under the microscope. (D) Pycnidiospores: cylindrical, mostly non-septate, few uniseptate (arrow 2) and biseptate (inset, arrow 3). (E) Ten-day-old colony on PDA at 22 ±2 °C. (F, G) Long chains of conidia of A. alternata on seeds. (H) Conidia of A. alternata. (I) Sporodochia of A. verrucaria on seed. (J) Elliptical conidia of A. verrucaria. (K) Sporodochia of P. roridum on a squash seed. (L) Cylindrical conidia of P. roridum. (M) Conidia of C. spicifera on a seed, as seen under the stereomicroscope. (N) Conidia and vegetative hyphae of C. spicifera. (O) Perithecia of S. vesicarium on a seed. (P) An ascus of S. vesicarium, with short, broad pedicel bearing eight ascospores. (Q) Ascospores of S. vesicarium. (R) F. solani on a seed. (S) Long phialide of F. solani (arrow 4). (T) Microconidia and macroconidia of F. solani. Scale bars: 200 µm (A and B); 100 µm (C); 10 µm (D); 1 cm (E); 100 µm (F); 400 µm (G); 20 µm (H); 200 µm (I); 10 µm (J); 100 µm (K); 10 µm (L); 200 µm (M); 10 µm (N); 400 µm (O); 20 µm (P); 5 µm (Q); 200 µm (R); 25 µm (S); 20 µm (T).

177x218mm (300 x 300 DPI)

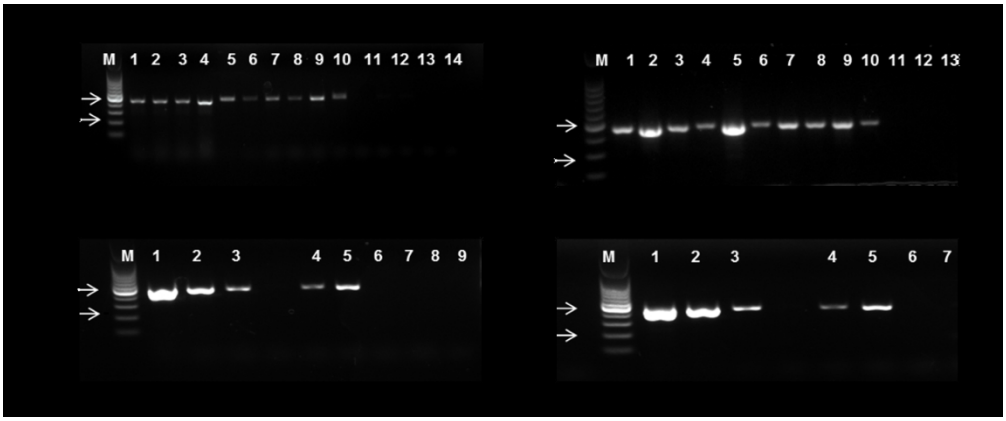

Figure 4. Gel electrophoresis of PCR products generated with the designed specific primers for the detection of the fungi. (A) A. verrucaria, with the primer pair Myroverr F1/Myroverr R1. Lanes 1 to 10: A. verrucaria (isolates IAV1, IAV2, IAV3, IAV4, M149, M155, M144, M140, M135, M146, respectively); lanes 11 to 13, P. roridum (isolates M123, M138, M141, respectively); and lane 14, water control. (B) P. roridum, with the primer pair Myroror F1/MyrororR1. Lanes 1 to 10: P. roridum (isolates IPR1, IPR4, IPR5, IPR6, IPR9, M73, M123, M138, M141, M167, respectively); lanes 11, 12, A. verrucaria (isolates M149, M155, respectively); and lane 13, water control. (C) P. herbarum, with the primer pair Pleo F/ Pleo R. Lanes 1 to 5: P. herbarum (isolates IP4, IP5, P164, P66, respectively); lanes 6, 7, A. alternata (isolates A38, A15, respectively); lane 8, C. spicifera (isolate B170); lane 9, water control. (D) P. herbarum, with the primers pair Pleo F1/Pleo R1. Lanes 1 to 5, P. herbarum (isolates IP4, IP5, P164, P66, respectively); lane 6, A. alternata (isolate A38); lane 7, water control. M: molecular weight markers (100-bp intervals).

178x73mm (300 x 300 DPI)

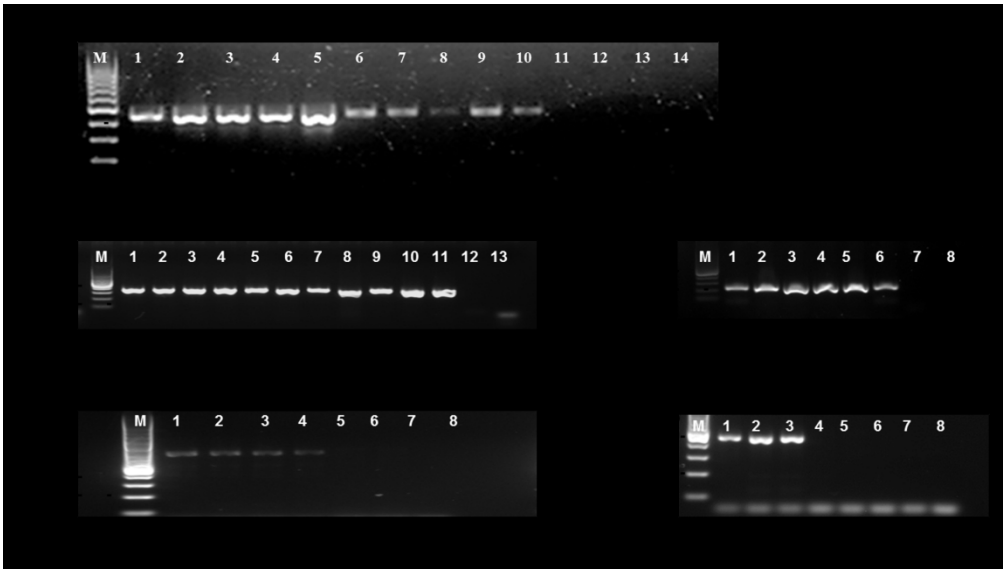

Figure 5. (A) Specificity of the primer pair RGII F/RGII R used for detection of S. cucurbitacearum in group RGII. Lanes 1 to 10, S. cucurbitacearum (isolates D33, D29, D21, D5, D42, D49, D45, D12, DBF1, DBF2, DBF3, respectively); lane 11, Phoma sp. (isolate Ph39); lane 12, A. alternata (isolate A15); lane 13, C. spicifera (isolate C170); lane 14, water controls. (B) Specificity of the primer pair AAF2/AAR3 for detection of A. alternate. Lanes 1 to 11, A. alternata (isolates A38, A15, A17, A5, A59, IA1, IA3, IA7 IA10, IA2, IA5, respectively); lane 12, S. vesicarium (isolate P66); lane 13, water control. (C) Specificity of the primer pair Bipol-1F/Bipol-1R for detection of C. spicifera. Lanes 1 to 6, C. spicifera (isolates B172, B170, IB41, IB2, IB3, IB4, respectively); lane 7, F. solani (isolate F174); lane 8, water control. (D) Specificity of the primer pair TEF-Fs4 F/TEF-Fs4 R for detection of F. solani. Lanes 1 to 4, F. solani (isolates F174, F82, F30, F142, respectively); lane 5, F. oxysporum (isolate F19); lane 6, S. cucurbitacearum (isolate D33); lane 7, S. vesicarium (isolate P66); lane 8, water control. (E) Specificity of the primer pair FC-1/FC-2 for detection of F. oxysporum. Lanes 1 to 3, F. oxysporum (isolates F59, F16, F19, respectively); lane 4, F. solani (isolate F174); lane 5, S. cucurbitacearum (isolate D33); lane 6, S. vesicarium (isolate P66); lane 7, Paramyrothecium roridum (isolate M123); lane 8, water control. Lane M: molecular weight markers (100-bp intervals).

177x100mm (300 x 300 DPI)

Supplementary Table S1. GenBank accession numbers of the six fungal genera used to determine the conserved sequence from the ITS region that allows specific identification.

Species	**NBCI sequence code**	**Host**	**Locality**	**GenBank accession number**
Paramyrothecium roridum	LG7	Wood	India	KC414758.1
	KUAB1MRCD	*Cynodon dactylon*	India	KF171528.1
	MA-83	Soybean	Brazil	JF724154.1
	ITS4	*Valerianella olitoria*	Italy	KT354921.1
	LH-5	-	China	HQ433334.1
	A553	*Pogostemon cablin*	China	KJ813720.1
	MTM-522	*Hydrangea*	USA	HM215150.1
	CBS 331.51	*Foeniculum vulgare*	The Netherlands	HQ115647.1
	MRtp-049	*Dendrobium officinale*	China	KU257608.1
	MRtp-013	*Dendrobium officinale*	China	KU257606.1
	MRtp-012	*Dendrobium officinale*	China	KU257605.1
	MRtp-023	*Dendrobium officinale*	China	KT928648.1
	MRtp-055	*Dendrobium officinale*	China	KU257609.1
	MRtp-047	*Dendrobium officinale*	China	KU257607.1
	TVD_Fungal-Culture147	Tomato	-	KF494145.1
	MA-20	Soybean	Brazil	JF724152.1

CLNP RV10 75	*Pinus albicaulis*	USA	GQ152603.1
JL-3	*Tribulus terrestris*	China	JX867215.1
UB:2246	Begonia x hiemalis	Brazil	KJ494661.1
DGM01	*Hemionitis arifolia*	China	JF343832.1
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	-	*Salvia* sp.	USA	EF151002.1
	-	Tomato	Italy	KR709186.1
	RDCCT07091402	Swedish ivy	China	KP942366.1
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	801	Soybean	Brazil	JF724151.1
	784	Melon	Brazil	JF724157.1
	MTL07081001	*Zantedeschia aethiopica*	China	KF761293.1
	IMI 394934	*Eichhornia crassipes*	Nigeria	GQ853401.1
	HXC15051716	Water spinach	China	KU312191.1
	HXC15051715	Water spinach	China	KU312190.1
	HXC15051715	*Ipomoea aquatica*	China	KT943519.1
	IFB-E091	*Artemisia annua*	China	GU074399.1
Myrothecium inundatum	IN-5	*Acalypha indica*	India	HQ165763.1
	A2S4-D45	Soil	Malaysia	KJ767119.1
	77F	*Homo sapiens*	Vietnam	AB704784.1
	SCSAAF0024	*Antipathes dichotoma*	China	JQ647903.1
	SCSGAF0095	Melitodes squamata	China	JN851023.1
	A1S2-2	Soil	Malaysia	KJ767118.1
	C45	Soybean	Brazil	JQ936268.1
	C13.1	Soybean	Brazil	JQ936267.1

	F216	*Glycine max*	Brazil	KM979993.1
	CBS 582.93	-	Spain	AY254152.1
	GHJ-2	-	China	FJ797514.1
	CBS 582.93	-	Germany	AJ302005.1
Myrothecium leucotricha	ZM0902-9	Soil	China	JX077071.1
	ZM0902-23	Soil	China	JX077070.1
	CBS 131.64	-	Germany	AJ302000.1
	BBA 65577	-	Germany	AJ301992.1
Albifimbria verrucaria	23221	*Taxus chinensis*	China	KF574891.1
	wxm94	Wood	China	HM037988.1
	F0705	Apple	Japan	AB693919.1
	E21	*Ferula*	China	KF887115.1
	G340	Milk thistle le	-	KM215639.1
	MYRver2	Soil	Italy	GQ131886.1
	ITS6	Spinach	Italy	KT354922.1
	KASHMIR	*Pinus* sp.	India	KP310497.1
	I-5	Wood	China	KT305924.1
	A-304	Soil	Iran	KC140223.1
	QWKF1	-	China	KJ589551.1
	NRRL 52420	*Zea mays*	USA	GU183129.1
	Hmp-F73	*Hymeniacidon perleve*	China	HQ625520.1

F17	Tomato	Egypt	KU681402.1
F12	Pigeon Pea	China	KJ026704.1
PTCC 799	Soil	Iran	KC140228.1
HGUP 0731	Soil	China	KC806230.1
E16	soybean	China	JQ356542.1
VKKSP1	Soil	India	HM358041.1
NIi	-	Malaysia	KM246762.1
A-284	Soil	Iran	KC140222.1
CNXY-007	*Houttuynia cordata*	China	KF750592.1
AR346	Soil	India	HQ596904.1
A-115	Soil	Iran	KC140221.1
F16	Pigeon Pea	China	KJ026703.1
A-336	Soil	Iran	KC140225.1
NJR102-16	sediment	China	JX077018.1
KAUEF26	*Calotropis*	Saudi Arabia	HF548712.1
A-70	Soil	Iran	KC140220.1
MYCver2	Soil	Italy	EF017211.1
C-1	Wood	China	KT305923.1
D2	*Zingiber officinale*	Japan	AB778924.1
XZ04-18-2	Soil	China	JF812340.1
M2	Soil	France	AY303603.1

Myrothecium atroviride	wb256	-	Austria	AF455507.1
Myrothecium gramineum	CY176	-	USA	HQ608010.1
	LCJ 177	Tree trunk	India	KF414681
	A243	Cotton	China	GQ373154.1
Stemphylium vesicarium	1664	Pear	Japan	LC056844.1
	1680	Pear	Japan	LC056845.1
	CT09AMS6S	*Ammi majus*	Japan	AB938190.1
	FF51	*Pyrus commuis*	South Africa	KR912336.1
	FF50	*Pyrus commuis*	South Africa	KR912335.1
	OT3-175.1	Madural cultivar	Portugal	KT804104.1
	EA	Wood	Italy	KF482449.1
	H09-007	-	Spain	KC009768.1
	AFTOL-ID 940	-	USA	DQ491516.1
	ATCC 11681	-	USA	AF229479.1
	-	-	China	AF383967.1
	ICMP 5620-77	Carrot	UK	Y17068.1
	E. G. Simmons 08-069	-	-	AF071345.1
	4248	*Eucalyptus globulus*	Spain	FR667974.1
	EGS48-095	-	New Zealand	AY329232.1
	EGS29-089	-	USA	AY329229.1
	EGS36-088	-	Australia	AY329171.1

	EGS36-138	-	India	AY329169.1
	CT09AMS1S	*Ammi majus*	Japan	AB938189.1
	CBS 191.86	*Medicago sativa*	India	KC584239.1
	MH955	Soil	Czech Republic	LN901148.1
	MAFF 306801	*Asparagus officinalis*	Japan	AB979880.1
	MAFF 305562	*Asparagus officinalis*	Japan	AB979878.1
	MAFF 241964	*Allium tuberosum*	Japan	AB979877.1
	EPS26	Pear	Spain	GU065719.1
	EGS 40-038	*Medicago sativa*	USA	AF442776.1
Stemphylium solani	bgr1	*Avicennia marina*	China	KJ767499.1
	LS2	Lettuce	Malaysia	KC796636.1
	LS1	Lettuce	Malaysia	KC796635.1
	LT5	Lettuce	Malaysia	KC796634.1
	LT4	Lettuce	Malaysia	KC796633.1
	LT3	Lettuce	Malaysia	KC796632.1
	LT2	Lettuce	Malaysia	KC796631.1
	LT1	Lettuce	Malaysia	KC796630.1
	LM	Lettuce	Malaysia	KC796629.1
	LKR2	Lettuce	Malaysia	KC796628.1
Stemphylium botryosum	CBS 714.68	*Medicago sativa*	Canada	KC584238.1
Stemphylium paludiscirpi	EGS31-016	-	USA	AY329231.1

Stemphylium eturmiunum	EGS29-099	-	New Zealand	AY329230.1
	Riv-St	Onion	Puerto Rico	DQ323706.1
	EGS29-099	-	New Zealand	AY329230.1
Stemphylium lycopersici	THYB1	*Aegiceras corniculatum*	China	KU518355.1
	EGS17-137	-	New Caledonia	AY329206.1
Stemphylium majusculum	EGS16-068	-	USA	AY329228.1
Pleospora gigaspora	EGS37-017	-	Switzerland	AY329177.1
Stemphylium triglochinicola	EGS36-118	-	United Kingdom	AY329175.1
Curvularia sesuvii	Bp-zj 03	*Sesuvium portulacastrum*	China	EF175942.1
Curvularia spicifera	L3	Sugarcane	China	JN695636.1
	L2	Sugarcane	China	JN695635.1
	L1	Sugarcane	China	JN695634.1
	FBA-1	*Sorghum bicolor*	Turkey	HQ538774.1
	MH12073	*Panicum virgatum*	USA	HQ015445.1
Curvularia lunata	JGS10	-	China	GU966505.1
	IP 2328.95	-	France	DQ836800.1
	DSM-63137	*Crotalaria juncea*	Burkina Faso	KF897859.1
	NBAIR-NEF10	Maize	India	KU158873.1
	MP03	Sorghum	India	KT598350.1
Fusarium oxysporum	FusO-JSB63	*Cucurbita pepo*	India	JQ665266.1
	FO	Squash	New Zealand	AF055220.1

	-	Squash	Spain	AM940070.1
Fusarium solani f. sp. *cucurbitae*	PCl-511	Zucchini squash	Spain	KF372878.1
	Fsm711	*Cucumis melo*	Spain	KC711040.1
	Fsm731	*Cucumis melo*	Spain	KC711041.1
Fusarium solani	-	Squash	Spain	AM940071.1
	FRC#s1195	Pumpkin	USA	DQ094744.1
Stagonosporopsis cucurbitacearum	Di-4 (426)	Watermelon	Tunisia	EF107642.1
	Di-3 (425)	Watermelon	Tunisia	EF107641.1
	NY1	*Cucumis melo*	USA	AF495850.1
	C76	*Cucumis melo*	USA	AF495849.1
	T153	-	China	FJ462750.1
	ATCC 16241	*Cucumis melo*	USA	AF297228.1
	MA71	Mangrove	Thailand	GU592001.1
	FG58	*Vitis vinifera*	China	EU030365.1
	TMK-4	Muskmelon	China	EF160076.1
	TMK-3	Muskmelon	China	EF160075.1
	TMK-2	Muskmelon	China	EF160074.1
	TMK-1	Muskmelon	China	EF160073.1

4 CBS: Westerdijk Fungal Biodiversity Institute; UB: University of Brasilia Herbarium; CICR: Crop Improvement Division, Central Institute for
5 Cotton Research; ATCC: The Global Bioresource Center; MAFF: Ministry of Agriculture Forestry and Fisheries.