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 pathogens 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 Tunisian seeds were *Alternaria alternata* (25.1%), followed by *Stagonosporopsis cucurbitacearum* (24.6%), *Fusarium solani* (16.6%), *Rhizopus stolonifer* (13.3%), *F. 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 *Alternaria alternata* (40.0%), followed by *F. fujikuroi* (20.8%), *Stemphylium 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 *Albifimbria verrucaria*, *Paramyrothecium roridum*, and *Stemphylium vesicarium*. Application of seed-health testing methods, including such conventional and molecular diagnostic tools, will help to improve seed quality and crop yields.

Keywords: *Albifimbria*, asymptomatic, blotter test, β -tubulin, detection, diseases of *Cucurbita* species, EF1 α , histone H3, ITS, *Paramyrothecium*, rDNA, seed-health test, species-specific primers, *Stemphylium*

Cucurbita L. (pumpkins, squash, and gourds) is a widely cultivated genus in agricultural regions worldwide. According to the Food and Agriculture Organization of the United Nations (2016), a total yield of nearly 20,000,000 tonnes was produced in Asia and Europe, plus 2,932,611 tonnes in the Americas, in 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 was 580,188 and 90,080 tonnes of fresh fruit, respectively, in 2016 (Food and Agriculture Organization of the United Nations 2016).

Cucurbita spp. can be affected by many diseases, including gummy stem blight (GSB), Fusarium fruit rot, and Alternaria leaf spot (Gannibal 2011; Keinath 2011; Mehl and Epstein 2007). GSB (foliar symptoms) and black rot (BR) (fruit symptoms) are caused by three *Stagonosporopsis* species: *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley [anamorph *Phoma cucurbitacearum* (Fr.) Sacc., syn. *Didymella bryoniae* (Fuckel) Rehm]; *Stagonosporopsis caricae* (Syd. & P. Syd.) Aveskamp, Gruyter &

Verkley (syn. *Mycosphaerella caricae* Syd. & P. Syd.); and *Stagonosporopsis 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 BR (Brewer et al. 2015), where the seeds can become infested or infected through flower and fruit infection (de Neergaard 1989).

Fusarium fruit rot is caused by *Fusarium solani* f. sp. *cucurbitae* W.C. Snyder & H.N. Hansen (Fsc) teleomorph *Nectria hematococca* Berk. & Broome. This pathogen can infect the seeds and can thus be spread over long distances (Boughalleb and El 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 in the United States (Mehl and Epstein 2007). In the field and greenhouse, *Fusarium* spp. can cause important yield losses, which may reach 80% (Blanco and Aveling 2018).

The genus *Alternaria* can affect several crops during their growing stages and after harvest (Kgatle et al. 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 *Cucurbita maxima* (Paul et al. 2015). Moreover, within the Cucurbitaceae family, leaf spot diseases caused by seedborne pathogens such as *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 fruitborne, seedborne, and seed

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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 spreading 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 2001; Rodrigues and Menezes 2005). For detection of *Stagonosporopsis cucurbitacearum* and *Alternaria 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, so distinguishing among these closely related organisms can be difficult, such as between *D. bryoniae* and *Phoma* sp. (Keinath et al. 1995), *Alternaria alternata* and *Stemphylium 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 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 this study were as follows: (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 *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*, *Curvularia spicifera*, *F. solani*, and *F. oxysporum*; and (iii) to design species-specific primers for identification of *Paramyothecium roridum*, *Albifimbria verrucaria*, and *Stemphylium 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 farms and from squash seed lots in their respective years.

Fruit sample evaluation. The day after 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 (approximately 2 mm) were immersed in 1% sodium hypochlorite solution for 5 min and were washed three times 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/liter; Liofilchem Srl, Roseto degli Abruzzi, Italy) and incubated at $22 \pm 2^\circ\text{C}$ 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 h) at room temperature (20 to 24°C), and stored in paper bags at 4°C until use. Each sample comprised seeds collected from a single fruit and 200 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 on eight pieces of sterile blotter paper that was moistened with 5 ml of sterile distilled water (Whatman no. 4 filter papers; 110 mm in diameter) in Petri dishes (110 mm in diameter), and they were incubated for 14 days at $22 \pm 2^\circ\text{C}$ 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 $22 \pm 2^\circ\text{C}$, morphological identification was carried out according to the colors and shapes of the colonies, with measurements of the fungal structures (i.e., pycnidia, perithecia, and conidia) using LAS software (version 3.8; 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 of extraction buffer (20 mM of EDTA, 0.1 M of Tris-HCl, pH 8.0, 1.4 M of NaCl, 2% cetyltrimethylammonium bromide, 4% polyvinylpyrrolidone, and 0.1% sodium metabisulfite added just before use) and 60 mg of 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 (TUB) gene, histone H3 (HIS), the translation elongation factor (EF1 α) region, and *calmodulin* gene in the ribosomal (r)DNA of the isolates (Table 1) was performed. These 20- μl PCR reactions contained 2 μl of genomic DNA of the fungal isolate, 10 μl of Green Plus Econo Master Mix 2 $\times$ (Lucigen, Middleton, WI), 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 and Kohn 1999) for EF1 α . The PCR reactions were run in a thermal cycler (MyCycler; Bio-Rad Laboratories, Hercules, CA) following the specific parameters published for TUB, HIS, and EF1 α . For ITS, the following 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 *Alternaria alternata* (Konstantinova et al. 2002), at 55°C for 1 min for *Stagonosporopsis cucurbitacearum*, *Paramyothecium roridum*, and *Albifimbria verrucaria* (Babu et al. 2015; Orawan et al. 2014), at 58°C for 1 min for *Curvularia*

spicifera and *F. solani* (Chehri et al. 2011; Dela Paz et al. 2006), and at 57°C for 1 min for *Stemphylium vesicarium* (Cámara et al. 2002; Dela Paz et al. 2006). Extension was carried out at 72°C for 50 s, with a 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) and were visualized and captured using an imaging system (Gel Doc XR; Bio-Rad).

Bidirectional sequence analysis was conducted on select amplified isolate fragments at Genewiz (Takeley, United Kingdom) (Table 2). The forward and reverse nucleotide sequences were read and edited using Chromas software (version 2.33) and they were assembled using CAP3 software to obtain a consensus sequence. Bioedit software (version 7.0.0) was used (<http://www.mbio.ncsu.edu/Bioedit/>

bioedit.html) to cut off 20 to 30 bp of the terminal end sequence. Finally, nBlast analysis (<https://www.ncbi.nlm.nih.gov/BLAST/>) was carried out to verify the identities of the amplicons.

We selected and used already published specific primer pairs for the molecular identification of *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*, *Curvularia spicifera*, *F. oxysporum*, and *F. solani*, as summarized in Table 3.

Design of specific primers for molecular identification of Albifimbria verrucaria, *Paramyrothecium roridum*, and *Stemphylium 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 FASTA format and aligned using ClustalX (version 1.83) (Thompson et al.

Fig. 1. Fruit rot symptoms on squash fruit. **A**, Apparently healthy fruit (cultivar Galaoui). **B** and **C**, *Fusarium* rot with white mycelia, caused by *Fusarium solani* (arrow 1), in cultivars Galaoui and Bjaoui, respectively. **D** and **E**, Black mycelia of *Curvularia spicifera* on squash fruit (arrow 2) in cultivars Galaoui and Aspen, respectively. **F** and **G**, *Alternaria* fruit spot caused by *Alternaria alternata* (arrow 3) in cultivars Aspen and Galaoui, respectively. **H** and **I**, Black rot caused by *Stagonosporopsis cucurbitacearum* (arrow 4) in cultivars Butternut and Bjaoui, respectively. Scale bars = 5 cm.

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 (37°03'45"N, 10°07'06"E)	T34	<i>Cucurbita maxima</i>	Bjaoui	2015
			T35	<i>C. maxima</i>	Galaoui	2015
			T47	<i>C. maxima</i>	Bjaoui	2015
			T52	<i>C. maxima</i>	Bjaoui	2015
			T63	<i>C. maxima</i>	Galaoui	2015
			T71	<i>C. maxima</i>	Bjaoui	2015
			T45	<i>C. maxima</i>	Galaoui	2015
			T69	<i>C. maxima</i>	Galaoui	2015
			T1	<i>C. maxima</i>	Galaoui	2015
			T3	<i>C. maxima</i>	Batati	2015
	Siliana	Sidi Hmada (35°57'28"N, 9°32'57"E)	T8	<i>C. maxima</i>	Bjaoui	2015
			T4	<i>C. maxima</i>	Bjaoui	2015
			T13	<i>C. maxima</i>	Bjaoui	2015
			T9	<i>C. maxima</i>	Bjaoui	2015
			T7	<i>C. maxima</i>	Bjaoui	2015
			T18	<i>C. maxima</i>	Bjaoui	2015
			T22	<i>C. maxima</i>	Bjaoui	2015
			T14	<i>C. maxima</i>	Bjaoui	2015
			T6	<i>C. maxima</i>	Bjaoui	2015
			T16	<i>C. maxima</i>	Bjaoui	2015
	Bizerte	Utique (37°03'25"N, 10°03'43"E)	T38	<i>C. maxima</i>	Bjaoui	2015
			T40	<i>C. maxima</i>	Bjaoui	2015
			T58	<i>C. maxima</i>	Bjaoui	2015
			T66	<i>C. maxima</i>	Bjaoui	2015
			T70	<i>C. maxima</i>	Bjaoui	2015
			T81	<i>C. maxima</i>	Bjaoui	2016
			T76	<i>C. maxima</i>	Bjaoui	2016
			T77	<i>C. maxima</i>	Bjaoui	2016
			T78	<i>C. maxima</i>	Bjaoui	2016
			T79	<i>C. maxima</i>	Bjaoui	2016
	Kasserine	Sbeïtla (35°14'00"N, 9°08'00"E)	T80	<i>C. maxima</i>	Bjaoui	2016
			T23	<i>C. maxima</i>	Batati	2015
			T24	<i>C. maxima</i>	Bjaoui	2015
			T26	<i>C. maxima</i>	Bjaoui	2016
			T28	<i>C. maxima</i>	Bjaoui	2016
			T29	<i>C. maxima</i>	Bjaoui	2016
			T30	<i>C. maxima</i>	Bjaoui	2016
			T23	<i>C. maxima</i>	Batati	2015
			T24	<i>C. maxima</i>	Bjaoui	2015
			T26	<i>C. maxima</i>	Bjaoui	2016
			T28	<i>C. maxima</i>	Bjaoui	2016
			T29	<i>C. maxima</i>	Bjaoui	2016
			T30	<i>C. maxima</i>	Bjaoui	2016
Italy	Ancona	Castelfidardo (43°27'51"N, 13°32'46"E)	I2	<i>C. maxima</i>	Aspen	2018
			I3	<i>C. maxima</i>	Aspen	2018
			I4	<i>Cucurbita moschata</i>	Naples Long	2018
			I5	<i>C. maxima</i>	Aspen	2018
			I8	<i>C. maxima</i>	Aspen	2018
			I12	<i>C. moschata</i>	Naples Long	2018
			I13	<i>C. moschata</i>	Naples Long	2018
			I16	<i>C. moschata</i>	Naples Long	2018
	Ancona	Osimo (43°29'00"N, 13°29'00"E)	I17	<i>C. moschata</i>	Butternut	2018
			I18	<i>C. moschata</i>	Butternut	2018
			I19	<i>C. moschata</i>	Butternut	2018
			I20	<i>C. moschata</i>	Butternut	2018
			I21	<i>C. moschata</i>	Butternut	2018
			I22	<i>C. moschata</i>	Butternut	2018
			I23	<i>C. moschata</i>	Butternut	2018
			I24	<i>C. moschata</i>	Butternut	2018
			I25	<i>C. moschata</i>	Butternut	2018
			I26	<i>C. moschata</i>	Butternut	2018
	Macerata	Recanati (43°24'00"N, 13°33'00"E)	I27	<i>C. moschata</i>	Butternut	2018
			I28	<i>C. moschata</i>	Butternut	2018
			I29	<i>C. moschata</i>	Butternut	2018
			I30	<i>C. moschata</i>	Butternut	2018
			I31	<i>C. moschata</i>	Naples Long	2018
			I32	<i>C. maxima</i>	Aspen	2018
			I33	<i>C. moschata</i>	Butternut	2018
			I34	<i>C. maxima</i>	Aspen	2018
	Bari	Monopoli (40°57'00"N, 17°18'00"E)	I35	<i>C. moschata</i>	Butternut	2018
			I36	<i>C. maxima</i>	Aspen	2018
	Campobasso	Baranello (41°32'00"N, 14°33'00"E)	I37	<i>C. moschata</i>	Butternut	2018

^a The three squash cultivars from Tunisia use the local Tunisian names (Galaoui, Bjaoui, and Batati) and represent the squash cultivars that are most commonly produced there.

1994). The specific nucleotide regions that characterized each fungal genus and the conserved intragenera nucleotide regions were selected to design a new specific primer pair for *Albifimbria verrucaria*, *Paramyothecium roridum*, and *Stemphylium 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 of reaction mixture that contained 2 µl of genomic DNA (about 20 ng/µl) of the fungal isolates, 200 µM of dNTP mixture, 0.5 µM of each primer, 1.2 mM of MgCl₂, and 1.25 U of Taq polymerase (Promega, Madison, WI). 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 *Albifimbria verrucaria*, *Paramyothecium roridum*, and *Stemphylium vesicarium* isolates M144, M123, and P164 (4 × 10 to 4 × 10⁻⁵ ng), respectively, to determine the analytical sensitivity of the tests.

Results

Fruit symptom evaluation. In the Sidi Hamada area of Tunisia, 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 *Stagonosporopsis cucurbitacearum* and one to *Alternaria 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 *Curvularia spicifera*, and seven to *Alternaria alternata*. All of the samples collected in the other three localities of Utique, Sbeitla, and Sahline areas showed infection levels of A (Table 6).

In the Castelfidardo area of Italy, two fruits showed an infection level of A, three of B, and three of C, with six with symptoms related to *Alternaria alternata* and two to *Curvularia spicifera*. In the Osimo area, three fruits showed an infection level of A and nine of B, with eight with symptoms related to *Stagonosporopsis cucurbitacearum* and nine to *Alternaria 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 *Curvularia spicifera* and five to *Alternaria alternata*. All of the samples collected in the Monopoli and Baranello areas showed infection levels of A (Table 6).

Identification of seedborne 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 *Stagonosporopsis cucurbitacearum*, *Albifimbria verrucaria*, *Paramyothecium roridum*, *Stemphylium vesicarium*, *Alternaria alternata*, *Curvularia 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, B, and C). These spores were cylindrical with rounded ends (4.0 to 8.0 × 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 they produced pycnidia with pycnidiospores after 10 days (Fig. 3E). These morphological traits are consistent with *Stagonosporopsis cucurbitacearum*.

By sequence analysis, isolates D33, D29, D49, D12, and D83 showed high nucleotide identity with *Stagonosporopsis cucurbitacearum* (Table 2) and the molecular tools proposed by Brewer et al. (2015) also corroborated the morphological identification (data not shown). Finally, the amplification with the RG-specific primer pair, able to yield a specific fragment of about 450 bp, allowed molecular characterization of all analyzed isolates as *Stagonosporopsis cucurbitacearum* belonging to the RG group II (Fig. 4A) (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 × 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 *Albifimbria verrucaria*, which was confirmed by the sequence analysis for isolates M144, M135, IAV2, and IAV4 (Table 2).

For the molecular identification of *Albifimbria 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. 5A). The minimum concentration of the target DNA that could be detected with this primer was 4 × 10⁰ ng (data not shown).

Paramyothecium 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

Fig. 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.

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 × 1.2 to 2.6 µm). These morphological features indicated that this fungus corresponded to

Paramyrothecium roridum, which was confirmed by the sequence analysis for isolates M123, M138, M141, IPR2, and IPR9 (Table 2).

For the molecular identification of *Paramyrothecium 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

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 internal transcribed spacer (ITS), translation elongation factor (EF1α), histone H3 (HIS), and β-tubulin (TUB) sequences

Fungal species	Isolate	Location	Source	Host species	Identification method ^a		GenBank accession number ^b			
					Sequencing	Species-specific PCR	ITS	EF1α	HIS	TUB
<i>Stagonosporopsis cucurbitacearum</i>	D33	Sidi Hmada, TN	Seed	<i>Cucurbita maxima</i>	+	+	MF401569	–	–	MK497768
	D35	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D43	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D45	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D40	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D36	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D3	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D23	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D24	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D29	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MK497779	–	–	MK497766
	D21	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D5	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D42	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D49	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401570	–	MK497771	MK497767
	D48	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D45	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D39	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D2	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D3	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D5	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D10	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	D12	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401568	–	–	–
	D27	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D62	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D69	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	D83	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401571	–	–	–
	DBF1	Osimo, IT	Fruit	<i>Curcubita moschata</i>		+				
	DBF2	Osimo, IT	Fruit	<i>C. moschata</i>		+				
	DBF3	Osimo, IT	Fruit	<i>C. moschata</i>		+				
<i>Phoma</i> sp. <i>Alternaria alternata</i>	Ph39	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401572	–	–	–
	A38	Sahlne, TN	Seed	<i>C. maxima</i>	+	+	MK497774	MK497789	MK497770	–
	A15	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>	+	+	MK497773	MK497788	MK497769	–
	A58	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>		+				
	A17	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>		+				
	A5	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>		+				
	A59	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	IA1	Recanati, IT	Seed	<i>C. moschata</i>	+	+	MK497775	–	–	–
	IA2	Recanati, IT	Fruit	<i>C. moschata</i>		+				
	IA3	Osimo, IT	Seed	<i>C. moschata</i>	+	+	MK497776	–	–	–
	IA4	Osimo, IT	Seed	<i>C. moschata</i>		+				
	IA5	Osimo, IT	Fruit	<i>C. moschata</i>		+				
	IA6	Osimo, IT	Seed	<i>C. moschata</i>		+				
	IA7	Monopoli, IT	Seed	<i>C. moschata</i>		+				
	IA8	Monopoli, IT	Seed	<i>C. moschata</i>		+				
	IA9	Castelfidardo, IT	Seed	<i>C. maxima</i>		+				
	IA10	Castelfidardo, IT	Seed	<i>C. maxima</i>		+				

(Continued on next page)

^a A plus sign indicates a positive result with the identification method.

^b A dash indicates that the sequence is not available.

conditions yielded a specific fragment of 562 bp amplicons (Fig. 5B). 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 a size of 147 to 282×131 to 243 μm . The crushed perithecium discharged several bitunicate clavate asci that measured 50 to 116×8.4 to 18.3 μm (Fig. 3P).

These contained eight ellipsoidal monoseriate to biseriate ascospores that were rounded at the ends (14.7 to 20.0×6.1 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 18.7×6.0 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

Table 2. (Continued from previous page)

Fungal species	Isolate	Location	Source	Host species	Identification method ^a		GenBank accession number ^b			
					Sequencing	Species-specific PCR	ITS	EF1 α	HIS	TUB
<i>Albifimbria verrucaria</i>	M140	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M149	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M148	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M144	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MK497782	–	–	MK497761
	M146	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M155	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M135	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MK497783	–	–	MK497762
	IAV1	Osimo, IT	Seed	<i>C. moschata</i>		+				
	IAV2	Osimo, IT	Seed	<i>C. moschata</i>	+	+	MK497785	–	–	MK497764
	IAV3	Osimo, IT	Seed	<i>C. moschata</i>		+				
<i>Paramyrothecium roridum</i>	IAV4	Osimo, IT	Seed	<i>C. moschata</i>	+	+	MK497784	–	–	MK497763
	M73	Sidi Hmada TN	Seed	<i>C. maxima</i>		+				
	M78	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M123	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401575	–	–	MK497759
	M138	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401576	–	–	–
	M167	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	M141	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MK497786	–	–	MK497765
	IPR1	Baranello, IT	Seed	<i>C. moschata</i>		+				
	IPR2	Baranello, IT	Seed	<i>C. moschata</i>	+	+	–	–	–	MK497760
	IPR3	Baranello, IT	Seed	<i>C. moschata</i>		+				
<i>Fusarium oxysporum</i>	IPR4	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IPR5	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IPR6	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IPR7	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IPR8	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IPR9	Recanati, IT	Seed	<i>C. moschata</i>	+	+	MK497780	–	–	–
	F59	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	F16	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	F19	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>		+				
	F82	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
<i>F. solani</i>	F30	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	F174	Sbeitla, TN	Seed	<i>C. maxima</i>	+	+	MF401578	MK497790	MK497772	–
	F142	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
	F142	Sidi Hmada, TN	Seed	<i>C. maxima</i>		+				
<i>Curvularia spicifera</i>	B172	Sbeitla, TN	Seed	<i>C. maxima</i>		+				
	B170	Sidi Hmada, TN	Seed	<i>C. maxima</i>	+	+	MF401577	–	–	–
	IB41	Castelfidardo, IT	Seed	<i>C. maxima</i>	+	+	MK497777			
	IB4	Castelfidardo, IT	Fruit	<i>C. maxima</i>		+				
<i>Stemphylium vesicarium</i>	IB1	Castelfidardo, IT	Seed	<i>C. maxima</i>	+	+	MK497778	–	–	–
	IB2	Recanati, IT	Seed	<i>C. moschata</i>		+				
	IB3	Recanati, IT	Seed	<i>C. moschata</i>		+				
	P164	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>	+	+	MF401574	MK497787	–	–
	P66	Kalâat El-Andalous, TN	Seed	<i>C. maxima</i>	+	+	MF401573	MK497791	–	–
	IP41	Baranello, IT	Seed	<i>C. moschata</i>		+				
	IP4	Osimo, IT	Seed	<i>C. moschata</i>	+	+	MK497781	–	–	–
	IP5	Osimo, IT	Seed	<i>C. moschata</i>		+				

Stemphylium vesicarium, which was confirmed by the sequence analysis of isolates P164, P66, and IP4 (Table 2).

For the molecular identification of *Stemphylium vesicarium*, the primer pairs Pleo F/R (Fig. 5C) and Pleo F1/R1 (Fig. 5D) were designed, and these successfully amplified the target DNA from the five isolates of *Stemphylium 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 approximately 4×10^{-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 and G). Microscopic examination showed conidia that were highly variable in shape (5.0 to 17.0×4.0 to $8.5 \mu\text{m}$), with both

transverse and longitudinal septa (Fig. 3H). The conidial beaks were also highly variability in shape, with lengths of 1.7 to $14.0 \mu\text{m}$. 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 *Alternaria alternata*, which was confirmed by the sequence analysis of isolates A38, A15, IA1, and IA3 (Table 2).

For the molecular identification of *Alternaria alternata*, the set of primers AAF2/AAR3 (Konstantinova et al. 2002) yielded a specific fragment of about 350 bp (Fig. 4B).

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

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

Fungal species	Primer pair ^a	Sequence (5' to 3')	Reference
<i>Stagonosporopsis cucurbitacearum</i>	RGI F	TGTCGTTGAC ATCATTCCAGC	Babu et al. (2015) and Somai et al. (2002)
	RGI R	ACCACTCTGCTTAGTATCTGC	
	RGII F	GCTAAGCCTT AATCTAGCTGC	
	RGII R	GAGAGTAAGCTAACCTAAAGG	
<i>Stagonosporopsis</i> spp. (<i>S. cucurbitacearum</i> , <i>S. citrulli</i> , <i>S. caricae</i>)	Db01F	CACGCCAGCAAACTCTCACTA	Brewer et al. (2015)
	Db01R	CGGTCCGGTCAACCTACTAC	
	Db05F	TATGACGTTGGGCAAGTGAG	
	Db05R	TTTGCTGGGATGGTGTGTGA	
	Db06F	GGTGACATCTGCGTGAATG	
	Db06R	TGGTTGTTTGGTTGTTTGA	
<i>Alternaria alternata</i>	AAF2	TGCAATCAGCGTCAGTAACAAAT	Konstantinova et al. (2002)
	AAR3	ATGGATGCTAGACCTTTGCTGAT	
<i>Curvularia spicifera</i>	Bipol-1F	CAGTTGCAATCAGCGTCAGT	Ünal et al. (2011)
	Bipol-1R	AAGACAAAAACGCCAACAC	
<i>Fusarium oxysporum</i>	FC-1	CATACCCTTGTTGCCTC	Zhang et al. (2012)
	FC-2	ATTAACGCGAGTCCACC	
<i>F. solani</i>	TEF-Fs4 F	ATCGGCCACGTCGACTCT	Arif et al. (2012)
	TEF-Fs4 R	GGCGTCTGTTGATTGTTAGC	
<i>F. solani</i> f. sp. <i>cucurbitae</i> race 1	Fsc1EF1	GCTAACAATCATCTACAGAC	Mehl and Epstein (2007)
	Fsc1-EF-2	GACGGATGAGAGAGCAAC	
<i>F. solani</i> f. sp. <i>cucurbitae</i> race 2	Fsc2-EF1	GTTGGTGACATATCTCCC	
	Fsc2-EF3	GAGTGAGAGACATGACGG	

^a F = forward and 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 ^a	Sequence (5' to 3')	Tm (°C) ^b	Bases (n)	GC content (%)
<i>Albifimbria verrucaria</i>	Myroverr F1	5'-TGTGAACCTTACCATATGTGTGC-3'	62.4	23	39.1
	Myroverr R1	5'-CGTTCCAACCTGCGAGGTTGT-3'	67.4	20	55.0
<i>Paramyrothecium roridum</i>	Myroror F1	5'-CCCTTTGTGAACCTTACCTAT-3'	58.5	21	42.8
	Myroror R1	5'-AGCTCCAATGCGAGTTGTG-3'	64.1	19	52.6
<i>Stemphylium vesicarium</i>	Pleo F	5'-TACACAATATGAAAGCGGGTTG-3'	63.7	22	40.9
	Pleo R	5'-AAGGCTGATTCAAAGTGCAAG-3'	63.2	21	42.8
	Pleo F1	5'-ATTACCCATGTCTTTTGGCG-3'	64.7	20	45.0
	Pleo R1	5'-AAATGTGGTCTTGATGGATGC-3'	63.7	21	42.8

^a F = forward and R = reverse.

^b Tm = melting temperature.

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
<i>Albifimbria verrucaria</i>	Myroverr F1	Myroverr R1	95°C, 2 min	95°C for 30 s, 60°C for 30 s, 72°C for 30 s	72°C, 5 min
<i>Paramyrothecium roridum</i>	Myroror F1	Myroror R1	95°C, 2 min	95°C for 30 s, 58°C for 30 s, 72°C for 30 s	72°C, 5 min
<i>Stemphylium vesicarium</i>	Pleo F	Pleo R	95°C, 2 min	95°C for 30 s, 60°C for 30 s, 72°C for 30 s	72°C, 5 min
	Pleo F1	Pleo R1			

showed straight conidia with rounded ends, with three distosepta, which measured 12.0 to 23.4×5.1 to $8.0 \mu\text{m}$ (Fig. 3N). These morphological features belong to the fungal species *Curvularia spicifera*, which was confirmed by the sequence analysis of isolates B170, IB41, and IB1 (Table 2).

For the molecular identification of *Curvularia 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).

F. 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 (size of 6.0 to 14.6×2.0 to $4.1 \mu\text{m}$), and cylindrical and slightly curved macroconidia, three to five septate (mean size of 16.6 to 33.4×3.0 to $5.2 \mu\text{m}$) (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*, which 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. 4D) 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.

F. oxysporum Schltdl. 1824. Microscopic examination showed unicellular and bicellular microconidia produced on short monophialides. 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. 4E) yielded a specific fragment of approximately 400 bp.

Frequency of seedborne fungi. The seedborne fungi isolated from the seed samples collected in Tunisia were *Alternaria alternata* (25.1%), followed by *Stagonosporopsis cucurbitacearum* (24.6%), *F. solani* (16.6%), *R. stolonifer* (13.3%), *F. fujikuroi* (7.8%), *Albifimbria verrucaria* (3.3%), and *Stemphylium vesicarium* (2.3%). *Alternaria alternata* was detected in all of the localities surveyed and in most of the symptomatic fruits (T8, T34, T47, T71, T35, T63, T45, and T69 in Table 6), with an infection level of 6 to 50%. Moreover, *Alternaria alternata* was also detected in 22 asymptomatic fruits collected from these five localities in Tunisia, with incidence ratings from 1 to 38%. *Stagonosporopsis cucurbitacearum* was detected in three Tunisia localities (Sidi Hmada, Sbeitla, and Utique) from three symptomatic fruits (T7, T8, and T9) collected from the Sidi Hmada area (Table 6). The seed samples extracted from these fruits were highly infected by *Stagonosporopsis cucurbitacearum*, with infection rates from 20 to 60%. Furthermore, *Stagonosporopsis 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 (T52 in Table 6), with an infection rate of 7%. Moreover, *F. solani* was isolated and identified in 13 asymptomatic fruits, with infection rates from 1 to 60%. *Albifimbria verrucaria* and *Paramyrothecium 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 *Albifimbria verrucaria* (15.5%) and *Paramyrothecium roridum* (5.0%). *Stemphylium vesicarium* was isolated from 18 seed samples (48.6% of samples), with infection rates from 1 to 13%. *Curvularia 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, *Alternaria alternata* was also the most frequent (40.0%), followed by *F. fujikuroi* (20.8%),

Stemphylium vesicarium (3.0%), and *Curvularia spicifera* (2.1%) (Table 7). *Alternaria 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, *Alternaria alternata* was identified in eight asymptomatic fruits, with infection rates from 1 to 37%. *Stemphylium 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 an infection level of C were infected by *Curvularia spicifera* (I2, I5, and I31 in Table 6), with infection rates from 11 to 85%. Moreover, another symptomatic fruit sample with an infection level of B was infected with *Curvularia spicifera* (I29 in Table 6), with an infection rate of 2%. *Curvularia spicifera* was also identified in the seed samples of two asymptomatic fruits (I23 and I37 in Table 6), with an infection rate of 1%.

Discussion

In this 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 us to isolate and identify several fungi in squash seeds, including *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*, *F. solani*, *Albifimbria verrucaria*, *Paramyrothecium roridum*, *Stemphylium vesicarium*, and *Curvularia 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 *Alternaria alternata* and *Stagonosporopsis cucurbitacearum* were detected and isolated from fruit lesions in both countries. *Alternaria alternata* is a pathogen of cucurbits, where it can cause severe crop losses (Vakalounakis 1990). *Stagonosporopsis cucurbitacearum* has a worldwide distribution and it can infect at least 12 genera and 23 species of Cucurbitaceae (Rennberger and Keinath 2018; Stewart et al. 2015). *Stagonosporopsis cucurbitacearum* was reported in Italy in 1885 on *Cucumis melo* (Corlett 1981) and in 2019 on *Cucurbita moschata* (Moumni et al. 2019). In Tunisia, *Stagonosporopsis cucurbitacearum* was detected only on watermelon (*Citrullus lanatus*) in 2007 (Boughalleb et al. 2007). To our knowledge, our work represents the first report of *Stagonosporopsis cucurbitacearum* as a pathogen of squash seed in Tunisia.

The pathogenic fungi *Alternaria alternata*, *Stagonosporopsis cucurbitacearum*, and *Curvularia 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 Mehl and Epstein (2007).

Seedborne fungi were also detected in the asymptomatic fruits. *Stagonosporopsis cucurbitacearum* was detected in 12 asymptomatic fruits collected from the Tunisia areas. Similar data were obtained for *Alternaria 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., and *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 finding shows that there are other mechanisms of pathogen penetration. *Fusarium* wilt of watermelon (*F. 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). Halfon-Meiri and Rilsky (1983) indicated that conidia of *Alternaria 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

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 (%) ^d				
					Total ^e	Aa	As	Ab	Cs
Tunisia	T4	SH	–	A	46.5 ± 2.9	19.5 ± 3.3	0.0	0.0	0.0
	T7	SH	Sc	B	73.5 ± 4.3	2.5 ± 1.4	0.0	0.0	0.0
	T8	SH	Aa/Sc	C	25.0 ± 3.0	6.0 ± 2.5	0.0	0.0	0.0
	T9	SH	Sc	C	64.0 ± 7.2	0.5 ± 0.5	0.0	0.0	0.0
	T13	SH	–	A	54.5 ± 6.1	17.0 ± 4.5	0.0	0.0	0.0
	T14	SH	–	A	75.0 ± 5.9	18.0 ± 4.0	4.0 ± 2.5	2.0 ± 1.6	3.5 ± 1.3
	T18	SH	–	A	57.0 ± 4.7	16.0 ± 4.7	2.0 ± 2.0	1.5 ± 1.5	2.0 ± 2.0
	T22	SH	–	A	49.0 ± 5.7	11.0 ± 4.1	0.0	0.0	0.0
	T34	KA	Aa	B	72.0 ± 6.3	28.0 ± 5.7	0.0	1.0 ± 0.7	0.0
	T47	KA	Aa	B	73.0 ± 4.4	35.0 ± 3.3	0.0	1.5 ± 0.8	1.0 ± 0.7
	T52	KA	Fs	B	14.0 ± 2.9	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
	T35	KA	Aa	B	77.0 ± 6.7	49.5 ± 5.2	0.0	0.0	0.0
	T63	KA	Aa	B	21.5 ± 7.0	8.0 ± 3.5	0.0	0.5 ± 0.5	0.5 ± 0.5
	T45	KA	Aa/Cs	B	38.4 ± 4.1	34.8 ± 3.7	0.0	0.5 ± 0.5	3.6 ± 1.7
	T69	KA	Aa	B	17.8 ± 4.0	14.9 ± 3.4	0.0	0.0	0.5 ± 0.5
	T1	KA	–	A	11.5 ± 4.0	3.0 ± 1.7	0.0	0.0	0.0
	T3	KA	–	A	49.0 ± 2.0	0.5 ± 0.5	0.0	0.0	0.0
	T76	Sb	–	A	12.5 ± 4.2	3.0 ± 1.0	0.0	3.0 ± 3.0	0.0
	T77	Sb	–	A	62.0 ± 5.0	37.5 ± 5.0	0.0	0.0	6.5 ± 2.0
	T78	Sb	–	A	37.0 ± 4.0	10.5 ± 2.1	0.0	0.0	0.0
	T79	Sb	–	A	18.5 ± 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
	T6	Ut	–	A	10.5 ± 5.3	0.0	0.0	0.0	0.0
	T16	Ut	–	A	15.5 ± 3.2	1.5 ± 0.8	0.0	0.0	0.0
	T38	Ut	–	A	5.0 ± 1.3	1.0 ± 0.6	0.0	0.0	1.0 ± 0.6
	T40	Ut	–	A	4.5 ± 2.4	0.0	0.0	0.0	0.0
	T58	Ut	–	A	27.0 ± 4.9	17.0 ± 4.0	0.0	5.0 ± 2.2	1.5 ± 0.8
	T66	Ut	–	A	33.5 ± 5.6	11.5 ± 3.5	0.0	0.0	0.0
	T70	Ut	–	A	32.5 ± 4.5	17.0 ± 4.7	0.0	0.0	0.0
	T81	Ut	–	A	35.0 ± 5.7	7.0 ± 2.4	0.0	0.5 ± 0.5	0.0
	T23	Sa	–	A	21.5 ± 6.3	0.0	0.0	0.0	0.0
	T24	Sa	–	A	6.0 ± 1.6	2.0 ± 1.1	0.0	0.0	0.5 ± 0.5
	T26	Sa	–	A	9.0 ± 1.6	5.5 ± 1.5	0.0	0.5 ± 0.5	0.5 ± 0.5
	T28	Sa	–	A	14.0 ± 3.0	4.5 ± 1.5	0.0	0.0	0.0
	T29	Sa	–	A	8.5 ± 2.0	5.0 ± 1.3	0.0	0.0	0.0
	T30	Sa	–	A	13.0 ± 4.4	1.5 ± 0.8	0.0	0.5 ± 0.5	0.5 ± 0.5
Italy	I2	Ca	Aa/Cs	C	85.0 ± 2.6	9.5 ± 3.3	0.0	0.0	85.0 ± 2.6
	I3	Ca	–	A	2.5 ± 2.0	0.5 ± 0.5	0.0	0.0	0.0
	I4	Ca	Aa	B	19.0 ± 3.3	1.5 ± 1.5	0.0	0.0	0.0
	I5	Ca	Aa/Cs	C	65.7 ± 8.4	4.6 ± 1.8	0.0	0.0	11.4 ± 2.5
	I8	Ca	Aa	C	36.0 ± 3.5	2.0 ± 0.9	0.0	0.0	0.0
	I12	Ca	Aa	B	53.5 ± 5.9	10.5 ± 3.4	0.0	0.0	0.0
	I13	Ca	Aa	B	40.0 ± 3.7	6.8 ± 2.4	0.0	0.0	0.0
	I16	Ca	–	A	29.0 ± 4.1	1.0 ± 1.0	0.0	0.0	0.0
	I17	Os	Sc/Aa	B	6.0 ± 3.2	3.1 ± 1.7	0.0	0.5 ± 0.5	0.0
	I18	Os	Sc/Aa	B	33.8 ± 4.1	19.7 ± 3.7	0.0	1.5 ± 1.1	0.0
	I19	Os	Sc/Aa	B	30.7 ± 4.1	20.3 ± 5.2	0.0	0.0	0.0
	I20	Os	Sc/Aa	B	38.9 ± 5.2	32.2 ± 4.3	0.0	0.0	0.0
	I21	Os	Sc/Aa	B	27.7 ± 6.0	9.0 ± 3.0	0.0	0.4 ± 0.4	0.0
	I22	Os	–	A	22.8 ± 3.2	11.1 ± 2.0	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
	I24	Os	–	A	22.6 ± 3.1	11.7 ± 2.4	0.0	0.0	0.0
	I25	Os	Sc/Aa	B	25.1 ± 3.7	6.9 ± 2.1	0.0	1.6 ± 1.0	0.0
	I26	Os	Sc/Aa	B	20.3 ± 3.1	3.6 ± 1.5	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
	I28	Os	Aa	B	14.9 ± 3.8	9.5 ± 3.0	0.0	2.7 ± 2.2	0.0
	I29	Re	Aa/Cs	B	73.0 ± 3.3	62.0 ± 4.3	0.0	0.0	2.0 ± 1.1
	I30	Re	Aa	C	77.5 ± 3.9	69.5 ± 5.6	0.0	0.0	0.0
	I31	Re	Aa/Cs	C	65.0 ± 4.2	52.0 ± 4.4	0.0	0.0	11.0 ± 2.2
	I32	Re	–	A	18.0 ± 2.8	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
	I34	Re	Aa	B	48.5 ± 3.5	32.5 ± 3.3	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
	I36	Mo	–	A	38.5 ± 2.8	36.5 ± 3.2	0.0	1.0 ± 0.9	0.0
	I37	Ba	–	A	47.5 ± 3.2	36.0 ± 3.9	0.0	0.0	1.0 ± 0.6

(Continued on next page)

^a SH = Sidi Hmada, KA = Kalâat El-Andalous, Sb = Sbeitla, Ut = Utique, Sa = Sahline, Ca = Castelfidardo, Os = Osimo, Re = Recanati, Mo = Monopoli, and Ba = Baranello.

^b Sc = *Stagonosporopsis cucurbitacearum*, Aa = *Alternaria alternata*, Fs = *F. solani*, and Cs = *Curvularia spicifera*.

^c A = asymptomatic fruit, B = infected fruit showing lesion on squash skin without colonization of fruit cavity, and C = infected fruit showing lesion that has colonized fruit cavity.

^d Data are means ± SE. As = *Alternaria solani*, Ab = *Aspergillus brasiliensis*, Ph sp. = *Phoma* sp., Ff = *Fusarium fujikuroi*, Fo = *F. oxysporum*, Fi = *F. incarnatum*, Pr = *Paramyrtetium roridum*, Av = *Albifimbria verrucaria*, Sv = *Stemphylium vesicarium*, and Rs = *Rhizopus stolonifera*.

^e The same seed can be infected by more than one fungus.

Table 6. (Continued from previous page)

Incidence of fungal species (%) ^d									
Sc	Ph sp.	Ff	Fo	Fi	Fs	Pr	Av	Sv	Rs
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
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
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
62.0 ± 4.2	1.0 ± 1.0	2.0 ± 2.0	1.0 ± 0.7	0.0	0.5 ± 0.5	0.0	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
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
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
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
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
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ± 0.7	32.0 ± 4.6
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
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
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
0.0	0.0	0.5 ± 0.5	0.0	0.0	0.0	0.0	0.0	0.0	12.0 ± 4.7
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	0.0	0.0	0.0	0.0	0.0	3.0 ± 1.2	6.0 ± 2.0
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
0.0	0.0	48.5 ± 2.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0 ± 1.0
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
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
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
0.5 ± 0.5	0.0	0.0	0.0	0.0	18.0 ± 4.7	0.0	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
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5 ± 5.3
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
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
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	4.5 ± 2.4
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
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
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
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
0.0	0.0	20.0 ± 6.3	0.0	0.0	0.0	0.0	0.0	0.0	1.5 ± 1.4
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
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
0.0	0.0	0.5 ± 0.5	0.0	0.0	0.0	0.0	0.0	0.0	7.0 ± 1.9
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.0 ± 1.4
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
0.0	0.0	9.5 ± 2.5	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	0.0	0.0	0.0	2.0 ± 2.0
0.0	0.0	12.0 ± 2.5	0.0	0.0	0.0	0.0	0.0	0.0	4.5 ± 2.7
0.0	0.0	53.9 ± 7.2	0.0	0.0	0.0	0.0	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	0.0
0.0	0.0	13.5 ± 5.2	0.0	0.0	0.0	0.0	0.0	1.0 ± 0.6	29.0 ± 5.1
0.0	0.0	21.5 ± 4.1	0.0	0.0	0.0	0.0	0.0	0.0	11.5 ± 3.5
0.0	0.0	27.0 ± 4.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5 ± 1.5
0.0	0.0	0.6 ± 0.6	0.0	0.4 ± 0.4	0.0	0.0	0.0	0.0	1.1 ± 1.1
0.0	0.0	9.3 ± 3.1	0.0	1.5 ± 1.1	0.0	0.0	0.0	0.5 ± 0.5	0.0
0.0	0.0	8.8 ± 2.9	0.0	1.5 ± 1.0	0.0	0.0	0.0	1.0 ± 0.6	0.0
0.0	0.0	0.9 ± 0.6	0.0	0.9 ± 0.6	0.0	0.0	1.9 ± 1.4	2.4 ± 0.8	0.0
0.0	0.0	10.0 ± 3.9	0.0	0.0	0.0	0.0	0.0	0.4 ± 0.4	7.6 ± 4.8
0.0	0.0	10.5 ± 2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.0	4.0 ± 1.9	0.0	0.0	0.0	0.0	0.0	0.4 ± 0.4	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
0.0	0.0	14.9 ± 3.8	0.0	0.0	0.0	0.0	0.0	0.0	1 ± 1
0.0	0.0	15.3 ± 3.6	0.0	1.3 ± 0.9	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	0.0	5.3 ± 2.5
0.0	0.0	2.2 ± 1.8	0.0	0.0	0.0	0.0	0.0	0.0	0.4 ± 0.4
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
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
0.0	0.0	9.5 ± 3.5	0.0	0.0	0.0	0.0	0.0	2.5 ± 1.2	0.0
0.0	0.0	18.0 ± 2.8	0.0	0.0	0.0	0.0	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
0.0	0.0	21.5 ± 4.1	0.0	0.0	0.0	0.0	0.0	0.0	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
0.0	0.0	14.5 ± 3.4	0.0	0.0	0.0	0.0	1.5 ± 1.0	0.0	0.0
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

large-scale spread of these pathogens and to their introduction into new planting areas. Therefore, cultural practices such as visual inspection for absence of lesions on fruit are 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 seeds of good quality. In addition, eight symptomatic fruits in Italy were infected by *Stagonosporopsis cucurbitacearum*, but the seeds obtained from these fruits were not infected by this pathogen.

Fig. 3. **A and B**, Pycnidia of *Stagonosporopsis 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 nonseptate, few uniseptate (arrow 2) and bisepate (inset, arrow 3). **E**, Ten-day-old colony on potato dextrose agar at $22 \pm 2^\circ\text{C}$. **F and G**, Long chains of conidia of *Alternaria alternata* on seeds. **H**, Conidia of *Alternaria alternata*. **I**, Sporodochia of *Albifimbria verrucaria* on seed. **J**, Elliptical conidia of *Albifimbria verrucaria*. **K**, Sporodochia of *Paramyothecium roridum* on a squash seed. **L**, Cylindrical conidia of *Paramyothecium roridum*. **M**, Conidia of *Curvularia spicifera* on a seed, as seen under the stereomicroscope. **N**, Conidia and vegetative hyphae of *Curvularia spicifera*. **O**, Perithecia of *Stemphylium vesicarium* on a seed. **P**, An ascus of *Stemphylium vesicarium*, with a short, broad pedicel bearing eight ascospores. **Q**, Ascospores of *Stemphylium vesicarium*. **R**, *Fusarium solani* on a seed. **S**, Long phialide of *F. solani* (arrow 4). **T**, Microconidia and macroconidia of *F. solani*. Scale bars = 200 μm in A, B, I, M, and R; 100 μm in C, F, and K; 10 μm in D, J, L, and N; 1 cm in E; 400 μm in G and O; 20 μm in H, P, and T; 5 μm in Q; and 25 μm in S.

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 (e.g., relative humidity); for example, *Stagonosporopsis 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

Fig. 4. A, Specificity of the primer pair RGII F/RGII R used for detection of *Stagonosporopsis cucurbitacearum* in group RGII. Lanes 1 to 10, *Stagonosporopsis cucurbitacearum* (isolates D33, D29, D21, D5, D42, D49, D45, D12, DBF1, and DBF2, respectively); lane 11, *Phoma* sp. (isolate Ph39); lane 12, *Alternaria alternata* (isolate A15); lane 13, *Curvularia spicifera* (isolate C170); and lane 14, water controls. B, Specificity of the primer pair AAF2/AAR3 for detection of *Alternaria alternata*. Lanes 1 to 11, *Alternaria alternata* (isolates A38, A15, A17, A5, A59, IA1, IA3, IA7, IA10, IA2, and IA5, respectively); lane 12, *Stemphylium vesicarium* (isolate P66); and lane 13, water control. C, Specificity of the primer pair Bipol-1F/Bipol-1R for detection of *Curvularia spicifera*. Lanes 1 to 6, *Curvularia spicifera* (isolates B172, B170, IB41, IB2, IB3, and IB4, respectively); lane 7, *Fusarium solani* (isolate F174); and 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, and F142, respectively); lane 5, *F. oxysporum* (isolate F19); lane 6, *Stagonosporopsis cucurbitacearum* (isolate D33); lane 7, *Stemphylium vesicarium* (isolate P66); and 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, and F19, respectively); lane 4, *F. solani* (isolate F174); lane 5, *Stagonosporopsis cucurbitacearum* (isolate D33); lane 6, *Stagonosporopsis cucurbitacearum* (isolate P66); lane 7, *Paramyrtrothecium roridum* (isolate M123); and lane 8, water control. M = molecular weight markers (100-bp intervals).

Fig. 5. Gel electrophoresis of PCR products generated with the designed specific primers for the detection of the fungi. A, *Albifimbria verrucaria*, with the primer pair Myrovrr F1/Myrovrr R1. Lanes 1 to 10, *Albifimbria verrucaria* (isolates IAV1, IAV2, IAV3, IAV4, M149, M155, M144, M140, M135, and M146, respectively); lanes 11 to 13, *Paramyrtrothecium roridum* (isolates M123, M138, and M141, respectively); and lane 14, water control. B, *Paramyrtrothecium roridum*, with the primer pair Myroror F1/Myroror R1. Lanes 1 to 10, *Paramyrtrothecium roridum* (isolates IPR1, IPR4, IPR5, IPR6, IPR9, M73, M123, M138, M141, and M167, respectively); lanes 11 and 12, *Albifimbria verrucaria* (isolates M149 and M155, respectively); and lane 13, water control. C, *Pleospora herbarum*, with the primer pair Pleo F/Pleo R. Lanes 1 to 5, *Pleospora herbarum* (isolates IP4, IP5, P164, and P66, respectively); lanes 6 and 7, *Alternaria alternata* (isolates A38 and A15, respectively); lane 8, *Curvularia spicifera* (isolate B170); and lane 9, water control. D, *Pleospora herbarum*, with the primer pair Pleo F1/Pleo R1. Lanes 1 to 5, *Pleospora herbarum* (isolates IP41, IP4, IP5, P164, and P66, respectively); lane 6, *Alternaria alternata* (isolate A38); and lane 7, water control. M = molecular weight markers (100-bp intervals).

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

Fungal species	Disease incidence (%) ^a									
	Tunisia					Italy				
	SH	KA	Sb	Ut	Sa	Ca	Os	Re	Mo	Ba
<i>Alternaria alternata</i>	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
<i>Stagonosporopsis cucurbitacearum</i>	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
<i>Rhizopus stolonifer</i>	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
<i>Fusarium fujikuroi</i>	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
<i>F. solani</i>	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
<i>Albifimbria verrucaria</i>	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
<i>Stemphylium vesicarium</i>	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
<i>Paramyothecium roridum</i>	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
<i>Phoma</i> sp.	1.1 ± 0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>Aspergillus brasiliensis</i>	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
<i>F. oxysporum</i>	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
<i>Curvularia spicifera</i>	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
<i>Alternaria solani</i>	0.8 ± 0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>F. incarnatum</i>	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 ^b	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 ± SE. SH = Sidi Hmada, KA = Kalâat El-Andalous, Sb = Sbeïtla, Ut = Utique, Sa = Sahline, Ca = Castelfidardo, Os = Osimo, Re = Recanati, Mo = Monopoli, and Ba = Baranello.

^b The same seed can be infected by more than one fungus.

(Kusai et al. 2015). For this reason, ITS-rDNA, TUB, HIS, and *EF1α* sequence data were used here to confirm the morphological identification by Blast analysis and the homology of sequences in the NCBI database (Peay et al. 2008; White et al. 1990). For *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*, *Curvularia spicifera*, *F. oxysporum*, and *F. solani* in particular, molecular detection was carried out using species-specific primers.

In our study, the application of RG-specific primers allowed us to determine that all *Stagonosporopsis cucurbitacearum* isolates from Tunisia and Italy belonged to the genetic group RGII; thus, none belonged to the genetic group RGI, which is prevalent in Florida and Georgia (Babu et al. 2015) and in Brazil (Santos et al. 2009). To our knowledge, there were no specific primers already available for the molecular identification of the genera *Albifimbria*, *Paramyothecium*, and *Stemphylium*. In this study, species-specific primers were thus designed to identify *Paramyothecium roridum*, *Albifimbria verrucaria*, and *Stemphylium 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 this study, ITS-specific primers for *Paramyothecium roridum*, *Albifimbria verrucaria*, and *Stemphylium vesicarium* were used to identify the target microorganisms. The annealing temperatures of 60°C for *Albifimbria verrucaria* and *Stemphylium vesicarium* and 58°C for *Paramyothecium 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 *Albifimbria verrucaria*, *Paramyothecium roridum*, and *Stemphylium vesicarium* revealed that 40 ng of DNA was necessary to produce clear results on agarose gels. When we used <4 ng for *Albifimbria verrucaria* and *Paramyothecium roridum* and 0.4 ng for *Stemphylium vesicarium*, the molecular tools were not able to clearly identify the specific pathogens.

This 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 *Stagonosporopsis*

cucurbitacearum, *Alternaria alternata*, *Albifimbria verrucaria*, *Paramyothecium roridum*, and *Stemphylium vesicarium*, and these were detected for the first time in Tunisia and Italy for seeds of *Cucurbita maxima* and *Cucurbita moschata*. These fungi are both seedborne and soilborne pathogens. The use of quality seeds is important for improving yields and conserving genetic material (Duan et al. 2007); 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 for clarification of 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 can be used to obtain high-quality seeds, which can be a critical step in management strategies for sustainable agriculture.

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