REPUBLIQUE TUNISIENNE

MINISTERE DE L'AGRICULTURE, DES RESSOURCES HYDRAULIQUES ET DE LA PECHE

MINISTERE DE L'ENSEIGNEMENT SUPERIEUR ET DE LA RECHERCHE SCIENTIFIQUE

Institut National de la recherche Agronomique de Tunisie (INRAT)

Università Politecnica Delle Marche (UNIVPM)

INSTITUT NATIONAL AGRONOMIQUE DE TUNISIE

Ecole Doctorale Sciences et Techniques de L'Agronomie et de l'Environnement

THESIS IN AGRICULTURAL SCIENCES

Specialty: Phytiatrie

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

Publicly presented by

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25 February 2020 at INAT

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Dedication

I would be honor to dedicate this work to my sweet and loving father "**Yahia Moumni**" and mother "**Amel Bennour**". My parents gave for me the tools and values necessary to be where I am standing today. I will never finish to thank my father and my mother for all the opportunities that they have offer to me, for all the teachings that they have told me and for every advise that come out of their mouth. I am so graceful with them for trusting me that I would do a good job and letting me come to achieve a higher education.

I would like to thank my sister **Imen Moumni** for the endless support and my two brothers **Noomen Moumni** and **Mohamed Amine Moumni** for their constant encouragement.

Thanks to my husband, **Kaies Mezrioui**, you have been an inspiration. Thank you for support and encouragement every day. Thanks to my loving son **Adam** for keeping spirit up with all the innocence, for your nice words that encourage me a lot, you are my child, my life, and my dreams for tomorrow.

Finally, I dedicate this work to my best friends **Hajer Ben Amara** and **Valeria Manccini** for the help and making life enjoyable

Acknowledgments

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

Immeasurable appreciation and deepest gratitude for the help and support are extended to the following persons who in one way or another have contributed in making this study possible.

I would like to first say a very big thank you to my supervisor Prof. **Mohamed Bechir Allagui** for all the support and encouragement he gave me. Without his precious guidance and constant feedback, this PhD would not have been achievable.

Many thanks also to my co-supervisor **Prof. Gianfranco Romanazzi** for his patience, optimism, motivation, and enthusiasm. His guidance helped me in all the time of research. It would never have been possible for me to take this work to completion without their incredible support and encouragement.

I would also like to thank each member of my thesis committee for generously accepting to evaluate the study: **Prof**. **Matilde Tessitori** for being the president member of my thesis; **Prof. Naima Boughalleb** for being the examiner member; **Prof. Mohamed Ali Triki** for being the reviewer of the thesis, his precious corrections, advices and scientific guidance and **Prof. Giuseppe Lima** for the effort as reviewer of the thesis, his constructive revisions and his precious corrections.

My thanks are extended to **Prof. Neji Tarchoun**, for his guidance, advices, and his encouragement helped me to work on seedborne fungi of squash.

I thank my colleagues and friends in UNIVPM (Department of Agricultural, Food and Environmental Sciences): **Dr. Sergio Murolo**, **Dr. Valeria Mancini**, for providing a good working environment, and for the help during the work and for assistance in lab work; **Prof. Lucia Landi**, **Dr. Renzo Foglia**, and **Prof. Dharini Sivakumar**, for their respect and support.

My acknowledgment also goes for my professors, in IRESA: **Prof. Elyes Hamza** for that he always kept his office door open to me and his encouragement and support during my studies and trainings. **Prof. Ali Rhouma** is also thanked because of his kindness and scientific support during my study.

I am very grateful to my colleagues and friend in the Ministry of Agriculture and Water Resources: **Manel Skander essid**, **Fawzia Trabelsi**, **Afef Saffar**.

Many thanks to my professors, colleagues and friends in INRAT: **Dr. Samia Gargouri**; **Prof. Amed Jemmali**, **Dr. Ikbel Chaieb**, **Sonia Derouiche**; **Hager Ben Amara** for their support.

Thanks are expressed to **Prof. Sebastiano Delfine** for providing samples from Campobasso, and squash growers from the Italian and Tunisian regions for their kind cooperation.

ABSTRACT

In the Mediterranean basin, Squash is one of the most important crops, and it can be affected by several fungal pathogens. Between 2015 and 2018, 66 samples of asymptomatic and symptomatic squash fruits (*Cucurbita maxima*, *Cucurbita moschata*) were 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 different primers amplifying ITS region, β-tubulin, histone H3, EF 1α, and CALM genes. Moreover, in this study specie specific primers were designed to identify *S. cucurbitacearum*, *A. verrucaria*, *Paramyrothecium roridum* and *S. vesicarium*. *S. cucurbitacearum* was the main fungal species found in the present study, and it was described for the first time in Tunisia and Italy on seeds and fruits of *C. maxima* and *C. moschata*. The transmission of this pathogen showed a significant regression and a high correlation level (R^2= 0.78; P≤0.001) was observed among seed contamination and seedling mortality. Furtehrmore, there was a significant positive correlation (R^2= 0.74; P≤0.001) between the seed infected with *S. cucurbitacearum* and the incidence of infected plantlets. Using the conventional PCR method, 17 seed samples were tested for *S. cucurbitacearum* localization. The results showed that this pathogen was detected in all of seed parts (tegument, cotyledon, and embryo). The antifungal activity of seven essential oils (EOs) (two Marjoram, Bay laurel, Lavender, Lemongrass, Lavandin, and Tea tree) have been studied by tests performed *in vitro* and *in vivo* conditions. Both assays showed that Lemongrass EO was the most effective to reduce seedborne inoculum of the tested fungal pathogen.

Keywords: asymptomatic, blotter test, detection, essential oils, localization, PCR, species specific primers, *Stagonosporopsis cucurbitacearum*, transmission, treatment.

RESUME

La courge (*Cucurbita maxima* et *Cucurbita moschata*) est une culture maraichère importante dans le bassin méditerranéen. Les fruits de cette plante présentent souvent des symptômes variés sous forme de nécroses et de pourritures qui sont généralement associées à des infections produites par de nombreuses espèces fongiques. Pendant 2015 et 2018, nous avons collecté 66 fruits de courge (symptomatique et asymptomatique) provenant de différentes régions en Tunisie et en Italie. Un classement de ces fruits a été établi en fonction de la taille de la lésion avant de procéder à l'identification des espèces fongiques associées aux semences extraites des différents fruits. Après incubation séparées des semences de chaque fruit sur papier buvard et analyse morphologique sous microscopie, 14 espèces fongiques ont été détectés dans les semences de fruits symptomatique et asymptomatique. Dans les semences de courge de Tunisie, les espèces fongiques les plus fréquentes étaient *Alternaria alternata* (25,1%) suivi par *Stagonosporopsis cucurbitacearum* (24,6%), *Fusarium solani* (16,6%), *Rhizopus stolonifer* (13,3%), *Fusarium fujikuroi* (7,8%), *Albifimbria verrucaria* (3,3%) et *Stemphylium vesicarium* (2,3%). En ce qui concerne les analyses sur semences de fruits d'Italie, les espèces fongiques les plus fréquemment identifiées étaient *A. alternata* (40,0%) suivi par *F. fujikuroi* (20,8%), *S. vesicarium* (3,0%), et *Curvularia spicifera* (2,1%). Cette identification morphologique a été confirmée par des analyses moléculaires utilisant différents gènes (β-tubuline, histone H_3, EF1α et CALM) et la région ITS de l'ADN ribosomique. Par ailleurs, des amorces ont été conçues pour identifier spécifiquement *S. cucurbitacearum*, *A. verrucaria*, *Paramyrothecium roridum* et *S. vesicarium*. Il est important de signaler que notre étude décrit pour la première fois en Tunisie et en Italie l'espèce *S. cucurbitacearum* dans les semences et les fruits de *C. maxima* et *C. moschata.* La transmission de *S. cucurbitacearum* de la semence à la plantule a montré une forte corrélation significative (R^2= 0.78; P≤0.001) entre son incidence dans les semences et le taux de mortalité des plantules. En outre, il y avait une corrélation positive significative (R^2= 0.74; P≤0.001) entre le taux des semences infectées par *S. cucurbitacearum* et l'incidence de plantules infectées. La localisation de *S. cucurbitacearum* au niveau des semences de 17 échantillons a été déterminée en utilisant la technique PCR. Les résultats montrent que ce champignon a une aptitude de se localiser dans le tégument, le cotylédon et l'embryon des semences. L'activité antifongique de sept huiles essentielles (deux marjolaines, laurier, lavande, citronnelle, lavandin et arbre à thé) a été également étudiée en réalisant des tests *in vitro* et *in vivo*. Les résultats montrent que l'huile essentielle de citronnelle est la plus efficace quant à une réduction significative des champignons transmis par les semences de courge.

Mot clé: asymptomatique, amorces spécifique, détecter, huiles essentielles, papier buvard, localisation, PCR, *Stagonosporopsis cucurbitacearum*, transmission, traitement.

RIASSUNTO

Identificazione con tecniche classiche e molecolari e strategie di controllo biologico di patogeni fungini trasmessi per seme in zucca (*Cucurbita maxima* Duchesne, *Cucurbita moschata* Duchesne)

Nel bacino del Mediterraneo, la zucca è una delle colture più importanti e può essere soggetta ad infezione da parte di diversi agenti patogeni fungini trasmessi per seme. Tra il 2015 ed il 2018, 66 campioni di semi estratti da frutti di zucca (*Cucurbita maxima*, *Cucurbita moschata*) asintomatici e sintomatici sono stati raccolti in Italia e in Tunisia. I sintomi di malattia sono stati classificati in base alla dimensione della lesione. I patogeni fungini sono stati identificati mdiante il blotter test, l'isolamento in piastra e la successiva osservazione al microscopio. I funghi più frequenti rinvenuti sui campioni tunisini sono stati *Alternaria alternata* (25,1%), *Stagonosporopsis cucurbitacearum* (24,6%), *Fusarium solani* (16,6%), *Rhizopus stolonifer* (13,3%), *Fusarium fujikuroi* (7,8%), *Albifimbria verrucaria* (3,3%) e *Stemphylium vesicarium* (2,3%). Nei campioni italiani sono stati trovati *A. alternata* (40,0%), *F. fujikuroi* (20,8%), *S. vesicarium* (3,0%) e *Curvularia spicifera* (2,1%). Per i frutti provenienti dall'Italia, i funghi più frequentemente sono A. alternata (40,0%), seguite da F. fujikuroi (20,8%), S. vesicarium (3,0%) e Curvularia spicifera (2,1%).L'identificazione morfologica è stata confermata da una identificazione molecolare utilizzando primer che amplificano la regione ITS, nonché i geni β-tubulina, histone H3, EF 1α e CALM. Inoltre, in questo studio sono stati disegnati primer specifici per *S. cucurbitacearum*, *A. verrucaria*, *Paramyrothecium roridum* e *S. vesicarium*. *S. cucurbitacearum* è stata una delle specie fungine più frequenti, ed è stata rinvenuta per la prima volta in Tunisia e in Italia su semi e frutti di *C. maxima* e *C. moschata*. La trasmissione di questo patogeno ha mostrato una regressione significativa, ed un'elevata correlazione (R^2 = 0,78; P≤0,001) è stata osservata tra contaminazione dei semi e mortalità delle piantine. Inoltre, una significativa correlazione (R^2 = 0,74; P≤0,001) è emersa tra la percentuale di seme infetto da *S. cucurbitacearum* e l'incidenza di plantule infette. Utilizzando la PCR convenzionale, sono stati testati 17 campioni di semi per la localizzazione di *S. cucurbitacearum*. Questo patogeno è stato rilevato in tutte le parti del seme (tegumento, cotiledone ed embrione). L'attività antifungina di sette oli essenziali (EO) (due di maggiorana e uno di alloro, lavanda, citronella, lavandino e albero del tè) è stata studiata *in vitro* ed *in vivo*. L'olio essenziale di citronella è risultato il più efficace per ridurre l'inoculo dei patogeni fungini trasmessi per seme. Nel complesso, le attività svolte contribuiranno ad una migliore conoscenza dei patogeni trasmessi per seme in zucca e alla messa a punto di strategie di controllo.

Parole chiave: asintomatico, blotter test, localizzazione, oli essenziali, PCR, primer specifici, rilevamento, *Stagonosporopsis cucurbitacearum*, trasmissione, trattamento.

الملخص

يعتبر القرع من أهم المحاصيل الزراعية في حوض البحر الأبيض المتوسط و هو عرضة للعديد من الأمراض الفطرية.

جمعت في تونس و إيطاليا بين سنتي 2015 و 2018 ستة وستون عينة من ثمار القرع (*Cucurbita maxima*, *Cucurbita moschata*)سواء المصابة أو الخالية ظاهريا من اعراض التعفن. تم تصنيف الثمار وفقاً لحجم التعفن عليها (خالية من التعفن،تعفن محصوربين القشرة و اللب، تعفن بلغ جوف الثمار). بإستخدام إختبار ورق الترشيح في حقة بيتري و المجهرتم تمييز 14 صنفا فطريا في البذور المستخرجة من الثمارالمتعفنةو الخالية من التعفن.

أثبتت الدراسة أن الفطريات الأكثر تواجدا في البذور التونسية كانت *Alternaria alternata* (25.1%) و *Stagonosporopsis cucurbitacearum*(24.1%) و *Fusarium solani* (16.6%) و*Rhizopus stolonifer* (13.3%) و *Fusarium fujikuroi* (7.8%) و *Albifimbria verrucaria* (3.3%) و *Stemphylium vesicarium* (2.3%). أما عينات الثمار من إيطاليا، فإن اصناف الفطريات الاكثر تواجداً في بذورها كانت *A. alternaria* (40.0%) و يليها *F. fujikuroi* (20.8%) و *S. versicarium* (3.0%) و *Curvularia specifera* (2.1%). تم تأييد التحديد الشكلي للأجناس الفطرية الآنفة من خلال التشخيص الجزيئي المبني على المشرعات لمضاعفة منطقة ITS والجينات β-tubulin، histone H3، EF 1α و CALM.

بالإضافة إلى ذلك مكنت هذه الدراسة من تصميم مشرعات مختصة لتحديد أجناس *S.cucurbitacearum* و *A. verrucaria* و *S. vesicarium* و *Paramyrothecium rirodum*. يعتبر *S.cucurbitacearum*من أهم الفطريات في بذور القرع، حيث يكتشف للمرة الأولى في تونس وإيطاليا .اظهر إختبار انتقال هذا الفطر أن نسبة ذبول الشتلات شديد الإرتباط بنسبة تواجد الفطريات في البذور (R^2 = 0.78; P≤0.001) .كما كان هناك ارتباط إيجابي (R^2=0.74; P≤0.001) بين نسبة البذور المصابة بـ*S.cucurbitacearum* و نسبة النبتات المصابة به.

بإستخدام طريقة تفاعل البلمرة المتسلسل (PCR) مكنت التحاليل من تحديد موقع مسبب المرض في البذور، وذلك بإكتشاف تواجد *S. cucurbitacearum* في الغشاء و الفلقة و بصفة أقل في الجنين. بالإضافة إلى ذلك تمت دراسة قدرة تثبيط نمو الفطريات المنقولة بالبذور وذلك بإختبارفعالية سبعة من الزيوت الروحية (اثنان من المردقوش و الغار و الخزامى و الإذخر الليموني و اللافاندين و وشجرة الشاي) أجريت في وسط نمو إصطناعي وبالبذور في التربة. أظهر كلا الاختبارين الفاعلية المتميزة للزيت الروحي للإذخر الليموني في الحد من تكاثر الفطريات المنقولة بواسطة البذور.

الكلمات المفاتيح: *Stagonosporopsis cucurbitacearum*، PCR ، إختبار الورق النشاف، تفاعل البلمرة المتسلسل، مشرعات محددة للاصناف، الكشف، التموقع، الانتقال، زيوت روحية.

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General Introduction

Cucurbits are important groups of *Cucurbitaceae* family, among the main cultivated vegetable with a production value equal to $ 95.94 billion worldwide in 2016 (FAO 2019). In the Mediterranean basin, vegetables are components essential of the Mediterranean diet and constitute an important source of income for countries of this area. *Cucurbita* L. (pumpkins, squashes, gourds) is a widely cultivated genus in agricultural regions worldwide. According to the Food and Agriculture Organization of the United Nations (FAO), in Italy and Tunisia, the total production of squash is 590.450 tonnes and 94.780 tonnes of fresh fruit, respectively, in 2017 (FAO 2019).

In the last decade, emerging plant diseases have been reported worldwide. In particular, viruses, bacteria, and fungi diseases have a considerable impact on productions. Diseases are one of the biggest challenges in plant production because they are difficult to control and the only way is the prevention and the early treatments. *Cucurbita* spp. can be affected by many fungal diseases, including gummy stem blight (caused by *Stagonosporopsis cucurbitacearum*), Fusarium fruit rot (*Fusarium solani* f. sp. *cucurbitae*), and Alternaria leaf spot (*Alternaria alternata* and *Alternaria cucumerina*) (Boughaleb and Mahjoub 2006; Gannibal 2011; Keinath 2011; Mehl and Epstein 2007); Bacterial spot of pumpkin (*Xanthomonas cucurbitae*) (Zhang and Babadoost 2018) and viral disease including Squash mosaic virus (SqMV) (Li et al. 2015).

All of these pathogens responsible of the main diseases on cucurbits can be present on seed. Seedborne pathogens limit production in many crops can result in severe economic losses to growers. In Tunisia, few studies have been done one the seedborne pathogens of *Cucurbita maxima*, despite the fact that the quantities of seeds self-produced in the farm are very high and the phytosanitary problems seem serious.

In addition, the association between seed and pathogen is an important means for the pathogens to spread on a large scale and a way to guarantee their survival in nature (Ahmad et al. 2016; Özer and Coşkuntuna 2016; Pellegrino et al. 2010). Almost 90% of the world's food crops are grown from seeds (Abdulsalam and Shenge 2011). Therefore, sowing healthy seeds with high quality is essential to improve crop yields and increase food production (Mathur and Kongsdal 2003). The use of seed certified to be disease-free or certified to have disease levels below a threshold is often recommended as the primary management strategy (Thomas-Sharma 2017). Therefore, seed health testing to detect seedborne pathogens is a fundamental step in the management of crop diseases (Ora et al. 2011). Conventional methods for seed

health testing are easy to perform and relatively inexpensive, except the cost of microscope and incubation facilities. Their main problem is that some fungal species have a high degree of similarity based on their morphology, so distinguishing among these closely related organisms can be difficult. Therefore, molecular tools have become increasingly effective, allowing to set up assays that satisfy specific technical aspects (e.g., specificity, sensitivity, robustness) (Walcott 2003; Lievens and Thomma 2005). In addition, the study of the localization and transmission of the pathogen can lead to improve management of related disease (Sudisha et al. 2006; Zhang et al. 2018).

Another critical and important step for the production of high quality seeds is the seed treatment. Integrated pest management strategies can provide more environmentally sound and economically feasible alternatives for seedborne disease management. These strategies are needed to minimize the inoculum of potential pathogens on seeds, drawing on management components that are currently available to farmers, or can be made available in the near future (Thomas-Sharma et al. 2017).

Objectives of the thesis

The overall objective of this research was to identify seedborne fungi of *Cucurbita maxima* and *Cucurbita moschata* in Tunisia and Italy, to determine their localization and transmission on seeds, and to evaluate different natural compounds for seed treatment. The specific objectives were:

- Collection of fruit samples from different field sites in Tunisia and Italy and evaluation of fruit samples according to the symptoms;
- Detection of seedborne fungi in squash seeds using conventional and molecular tools;
- Design of species-specific primers for detection of *Stagonosporopsis cucurbitacearum* on squash seeds, and for its localization on seed;
- Assessment of the transmission of pathogens from seed to fruit;
- Evaluation of the effectiveness of different seed treatments natural compounds (essential oils).

CHAPTER 1

Literature review

Part I

1. Importance of *Cucurbita* species

Squash, pumpkin, and gourds are grown all over the world for culinary and medicinal uses. The fruit of this genus is rich in nutrients, *C. maxima* flesh contains vitamin C (9-20 mg·100g-1), thiamine (0.05 mg), riboflavin (0.11 mg), niacin (0.6 mg), vitamin B6 (0.06 mg), folates (0.16-0.20 mg), vitamin E (0.06 mg), vitamin K (1.1 μg), B-carotene (2-10 mg·100g-1), and nutrients such as potassium, phosphorus, magnesium, iron and selenium (Poštić et al. 2018). Due to growing concerns about the health of humans over the last year, Indrian et al. (2019) evaluated the antioxidant activity, morphological, physicochemical and chemical properties of three components of *C. maxima* Duchesne*, C. moschata* Duchesne (the pulp, seed, and rinds). They demonstrated that the higher antioxidant activity measured by DPPH (1,1-diphenyl-2- picrylhydrazyl) assays was found in the rinds of *C. maxima* as of 33.8%, followed by the pulp of *C. maxima* and seed of *C. moschata* as of 29.6% and 25.4% respectively.

2. Squash crop production

Pumpkin, squash, and gourds are grown throughout the world and approximately there is an increase of 1 million tonnes of production in the world every year (Figure 1) (FAO, 2019). The biggest *Cucurbita* producing countries are china and India with 7563412 and 5028655 tonnes of totally production respectively (Figure 2). Squash (*C. maxima*, *C. moschata*) is one of the most important vegetables in tropical and temperate regions. Italy is ranked 9th in the world with 590.5 tonnes in terms of total production (Figure 2) (FAO 2017). In Tunisia, the total production of squash is 97.7 tonnes of fresh fruit (DGPA 2018). The top squash-producing states in Tunisia are Nabeul and Ariana with 19125.0 and 7858.5 tonnes respectively in 2018 (DGPA 2018) (Table 1).

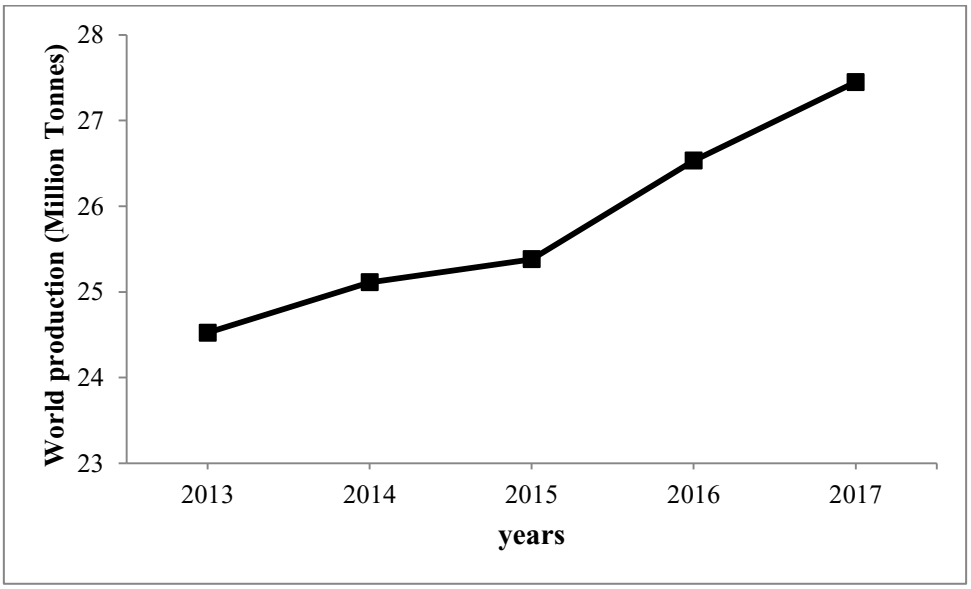

Figure 1. Production of Pumpkins, squash and gourds in world between 2013 and 2017 (FAO 2019).

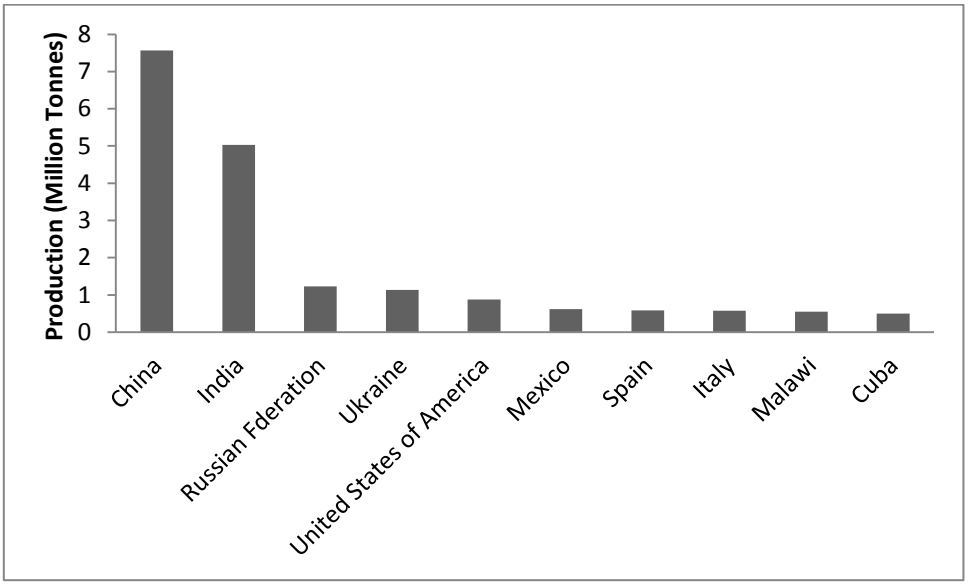

Figure 2. Production of pumpkin, squash and gourds in top ten world producers between 2013 and 2017 (FAO 2019).

Table 1. Squash production in the important producer provinces of Tunisia (DGPA 2019)

Regions	Production (tonnes/ year)		
	2016	**2017**	**2018**
1. Ariana	13475	12180	7858,5
2. Nabeul	26500	18500	19125
3. Kasserine	6200	10625	8125
4. Gabes	6500	6175	7260
5. Bizerte	6000	6000	6250
6. Gafsa	10000	4000	3000
7. Monastir	1240	2200	2060
8. Sousse	2080	3000	2040
9. Zaghouan	1700	1600	2470
10. Siliana	1500	1800	1710

3. The main seedborne diseases of cucurbits

3.1.Bacteria

3.1.1. Angular leaf spot

Angular leaf spot of cucurbits caused by *Pseudomonas syringae* pv. *lachrymans* is worldwilde distributed and emerged under humid and wet condition associated with rainfall. Newbery et al. (2016) reported that the host range of *P. syringae pv. lachrymans* included the larger Cucurbitaceae including watermelon, cantaloupe, and squash in Florida, Georgia, and California. The pathogen can cause infection on cotyledons, leaves, petioles, stems, fruits, and seeds. The pathogen primarily appeared on contaminated seed, where bacterial cells survive under the seed coat. During germination, primary infections appear. The pathogen spreads by penetrating through stomata, hydathodes, and small wounds (Mortensen and Fatmi 2019). Previous epidemics of angular leaf spot and similar cucurbit diseases have resulted in 100% yield loss due to bacterial blight (Harighi 2007; Langston et al. 2003; Morriset al. 2000). The

symptoms may vary according to the host and environmental conditions and are initially observed as circular necrotic lesions on leaves (Figure 3 A). Over time, lesions may become irregular in shape and limited by leaf venation, thus producing an angular appearance, hence the name of disease. The damage on fruit is manifested by small lesions with necrotic spot located in the center of the lesion, giving a bull's-eye appearance (Figure 3 B). Large numbers of warts were observed on the fruit (Figure 3 C) (Sharrock et al. 1997; Tymon and Inglis 2017).

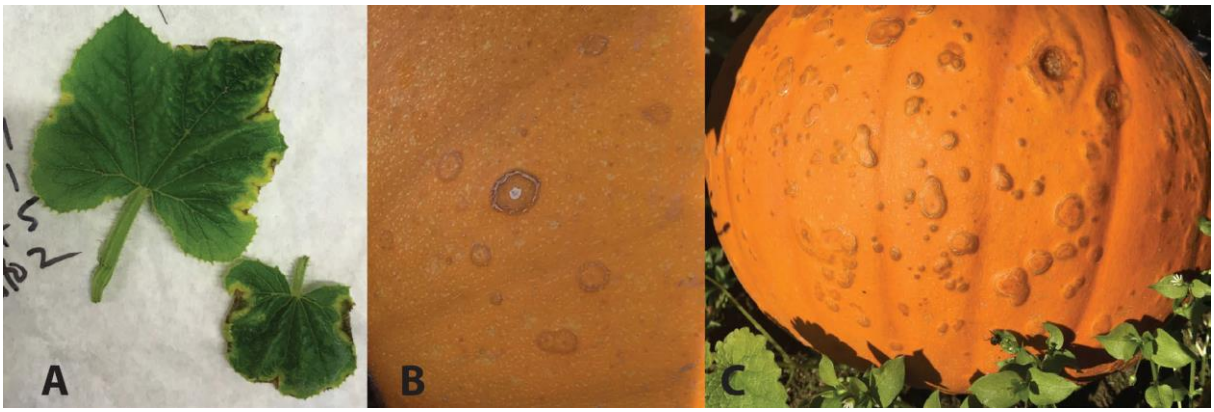

Figure 3. Symptoms of angular leaf spot on cucurbits (A) A necrotic leaf lesion on *Cucurbita pepo* leaves, (B,C) Bull's-eye lesions and raised warts on *C. maxima* fruit (Tymon and Inglis 2017).

3.1.2. Bacterial spot of pumpkin

Bacterial spot of pumpkin is caused by *Xanthomonas cucurbitae* (syn.: *Xanthomonas campestris* pv. *cucurbitae*) (Babadoost and Zitter 2009; Pruvost et al. 2008; Saddler and Bradbury 2005; William and Zitter 1996). This bacterium can be a harmful disease of several cucurbit species in tropical environments (watermelon, melon, cucumber, butternut squash). *X. cucurbitae* has become an important threat to pumpkin (*Curcurbita maxima* and *C. moschata*) production in Illinois and in some pumpkin productions throughout the world (Babadoost et al. 2012; Babadoost and Zitter 2009; Egel et al. 2017; Lamichhane et al. 2010; Pruvost et al. 2008; Ravanlou and Babadoost 2015). Major symptoms of the disease were on leaves and fruits of pumpkins (Ravanlou and Babadoost 2015; Williams and Zitter 1996). Leaves displayed small (2 to 4 mm), angular, water soaked, yellow lesions (Ravanlou and Babadoost 2015), while fruit symptoms include lesions that are smaller (1 to 3 mm in diameter), circular, and slightly sunken with a beige center and brown halo (Figure 4) (Liu et

al. 2016; Ravanlou and Babadoost 2015). Sometimes young stems and petioles may be attacked, which develop water-soaked lesions (Bryan 1930; William and Zitter 1996).

The disease causes up to 100% yield losses in pumpkin fields (Babadoost and Ravanlou 2012; Liu et al. 2016; Ravanlou and Babadoost 2015). Seed play an important role in dispersal of the pathogen, especially over long distances (Zhangand and Babadoost 2018).

Figure 4. Water-soaked lesions caused by *Xanthomonas cucurbitae* on the fruit of pumpkin (*Cucurbita maxima*) (Jarial et al. 2015).

3.1.3. Bacterial fruit blotch

Bacterial fruit blotch (BFB) of cucurbits is caused by *Acidovorax avenae* subsp. *citrulli*. The disease was discovered in the late 1980s, in watermelon fields in several states in the US (Wall and Santos 1988). Then, BFB has spread worldwide, and has been reported in other cucurbits such as melon, pumpkin, squash, and cucumber. BFB can be detected on seed, fruits, leaves and vines (Feng et al. 2013; Schaad et al. 1978; Walcott 2005). *A*. *avenae* subsp. *citrulli* infected seed and considered as an important primary source of inoculum (Dutta et al. 2014; Rane and Latin 1992; Walcott and Gitaitis 2000). Dutta (2011) has shown that *A*. *citrulli* survived significantly better in seeds when the bacterium was localized in the embryo compared to when it was localized on the testa and the perisperm–endosperm (PE) layer of the seed. On leaves, this bacterium causes small, dark brown, angular and water-soaked lesions (Hopkins and Thompson 2002). On the fruit, the spots are dark and olive-green (or blotch) on the upper side of the fruit. These lesions usually become apparent shortly after fruit ripening.

3.2. Viruses

Viruses are among the seed borne pathogens that causes serious damages to yield and quality on cucurbit, and in particular squash and pumpkin crops. The most prevalent and economically important seed borne viruses of cucurbits worldwide are Cucumber mosaic virus (CMV), Zucchini yellow mosaic virus (ZYMV), Squash mosaic virus (SqMV), Tobacco ring spot virus (TRSV), and Cucumber green mottle mosaic virus (CGMMV) (Provvidenti 1996).

3.2.1. Squash mosaic virus (SqMV)

SqMV, is a Comovirus that has been first reported on pumpkin in California, in 1934 (Sherf and MacNab 1986), then spread and has been reported as seed-borne virus infecting cucurbit worldwide (Li et al. 2015). The symptoms of this virus can be a severe systemic mosaic, with leaf and fruit deformation. Blancard and Lecoq (1996) cited that 10% of SqMV infected seed can cause the transmission and the early spreading of the virus in the field and that result serious yield losses.

3.2.2. Cucumber mosaic virus (CMV)

CMV is one of the most important cucurbits viruses in the world (Chua et al. 2003). Seed transmission of this virus would have very serious implications for seed growers, and others, since this devastating disease has caused widespread losses (Sevik and Balkaya 2015; Smith 1972). The symptoms of this virus can be leaf mosaic or mottling, yellowing, ringspots, stunting, deformations of flowers, fruits and leaves (Carrère et al. 1999). Many variants of the virus occur, and it is difficult to identify CMV from symptoms alone. CMV produces a systemic infection in most host plants. Older tissues and organs that developed prior to infection usually are not affected by the virus, but newer cells and tissues that develop after infection may be affected with varying severity. The concentration of the virus increases for several days following inoculation, then decreases until the plant dies (Agrios 1997).

3.2.3. Zucchini yellow mosaic virus (ZYMV)

ZYMV was first detected in North Italy (Lisa et al. 1981). Then the virus was reported worldwide as infecting and damaging squash and pumpkin crops (Desbiez and Lecoq 1997; Svoboda and Polák 2002). ZYMV is transmitted in a non-persistent way by aphid transmission (Adlerz 1987; Dodds et al. 1984; Purcifull et al. 1984). It can also be transmitted vertically through seed. The leaves eventually yellows and the fruits are stunted, twisted and deformed.

3.3.Fungi

3.3.1. Gummy stem blight

Gummy stem blight (GSB) (arial part of plant; foliar symptoms) and black rot (BR) (fruit symptoms) are caused by three species of *Stagonosporopsis*: *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, *Stagonosporopsis caricae* (Syd. & P. Syd.) Aveskamp, Gruyter & Verkley (synonym *Mycosphaerella caricae* Syd. & P. Syd.), and *Stagonosporopsis citrulli* M.T. Brewer & J.E. Stewart (Stewart et al. 2015). *S. cucurbitacearum* and *S. citrulli* can infect several species of Cucurbitaceae, including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Rennberger and Keinath 2018; Babu et al. 2015; Huang and Lai 2019), muskmelon (*Cucumis melo* L.) (Nuangmek et al. 2018), squash (*Cucurbita maxima* L., *Cucurbita moschata* Duch) (Keinath 2014), and pumpkin (*Cucurbita pepo* L.) (Grube et al. 2011). Under conducive climatic conditions for the disease, which occur especially in warm and humid environments, severe outbreaks can cause 15% to 50% yield losses, leading to rapid plant death and reducing yields (Boughalleb et al. 2007; Keinath et al. 1995; Yao et al. 2016; Zitter and Kyle 1992). On cantaloupe, field losses due to *S. cucurbitacearum* can reach 100% under conditions conducive to infection (Nuangmek et al. 2018). Pumpkin and winter squash are particularly susceptible to black rot (Brewer et al. 2015), whose seeds can become infested or infected through flower and fruit infection (de Neergard 1989). In Tunisia, *S. cucurbitacearum* was detected only on grafted watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) in 2007 (Boughalleb et al. 2007) and this pathogen has only been reported once in Italy, in 1885 on *C. melo* L., and it was described as *Didymella melonis* Pass. by Giovanni Passerini (Corlett 1981). GSB is an important foliar disease of cucurbits. Necrotic spots are the common symptoms that can affect all aboveground plant parts (leaves, petioles, vines, and stem) (Figure 5). Cankers on stem and crowns of cucurbits begin as a large water-soaked lesion that become dry, tan colors and then the surface cracks covered with pycnidia and pseudothecia (Figure 5 A). BR can reduce preharvest and postharvest yields (de Neergaard 1989), and cause up to 15% fruit loss (Keinath 2000). Infected fruit manifest large irregular-shaped spots and black rot (Choi et al. 2010). Fruiting bodies are found in the oldest parts of lesions, because *S. cucurbitacearum* is a necrotrophic fungus (Keinath 2014). Keinath (2011) reported that *S. cucurbitacearum* produces black mycelia inside melon and giant pumpkin (*C. maxima*) fruit.

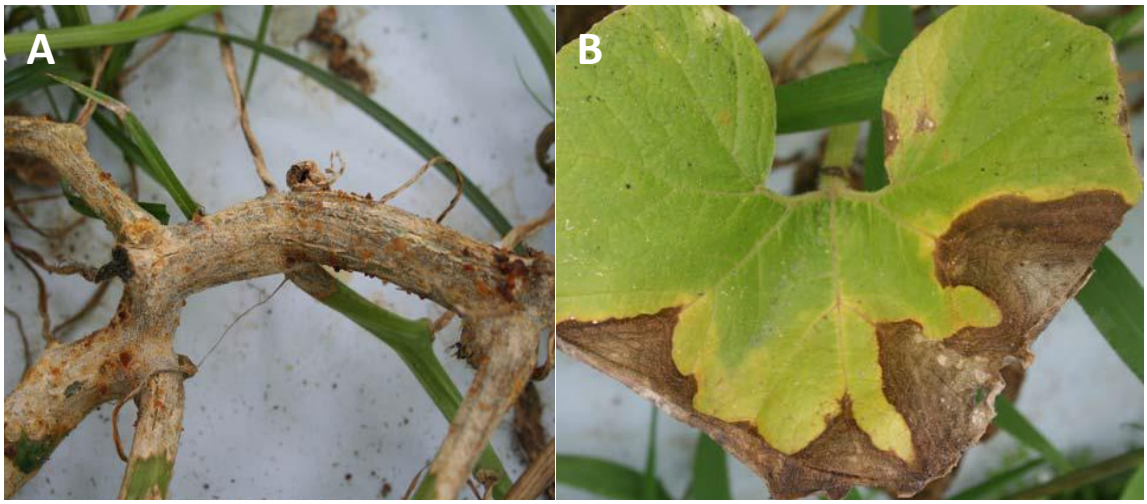

Figure 5. Symptoms of gummy stem blight on cucurbits (A) Tan canker on the main stem and vines (B) Necrotic spots symptoms of gummy stem blight on leaves of *Cucurbita argyrosperma* (Keinath 2014).

3.3.2. Fusarium fruit rot

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 is one of the main diseases affecting several cucurbits (Boughalleb et al. 2006a; Pérez-Hernández et al. 2017). Davis et al. (2006) reported that generally only pumpkin (*Cucurbita pepo* L.) and winter squash (C. *pepo* L., *C. moschata* Duchesne, and *C. maxima* Duchesne) are affected by *F. solani* f. sp. *cucurbitae* in the field. This is an economically important problem for pumpkin growers, with 30% of fruit reported as infected in California, USA. In addition, it has been demonstrated that the pathogen can infect the seeds, and by this way it can be spread over long distances (Boughalleb and El Mahjoub 2006b; Farrag and Moharam 2012). Mehl and Epstein (2007) were the first to demonstrate a significant relationship between infections of *F. solani* f. sp. *cucurbitae* in pumpkin fruit tissues and incidence of infected seeds. This pathogen has two races: race 1 attacks hypocotyls, crown, roots, and fruits, while race 2 infects only fruits. Boughalleb et al. (2005) were the first to identify the two races of *Fusarium solani* f. sp. *cucurbitae* in the watermelon production areas in Tunisia and they demonstrated that race 1 is widely distributed whereas race 2 has a lower incidence but it is present in northern, central, and southern Tunisia. In Italy, *F. solani* f. sp. *cucurbitae* race 1 has been reported on zucchini (*Cucurbita pepo*) in a greenhouse in Tuscany Region (Vitale et al. 2007). The pathogen can infect seedlings, plants, and fruit at any age. In the most severe cases, the plants eventually wilt until they get completely dry (Figure 6 and 7). The infected

fruit presents soft circular lesions and a wet and soft rot develops on the part in contact with the soil (Gómez et al. 2008).

Figure 6. Wilt symptoms in melon plants inoculated with *Fusarium solan*i f. sp. *cucurbitae* (Gómez et al. 2008).

Figure 7. Zucchini plants at the fruit-bearing stage, growing in a commercial plastic house in Almería, exhibiting symptoms of crown rot (Gómez et al. 2008).

3.3.3. *Alternaria* spp.

The genus *Alternaria* can affect several crops during their growing stages and in postharvest (Kgatle and Aveling 2018; Mamgain et al. 2013). The diseases caused by *Alternaria* are the most responsible factors for low production of cucurbitaceous. *Alternaria cucumerina* (Ellis & Everh.) J.A. Elliott and *Alternaria alternata* (Fr.) Keissl. are pathogens of cucurbits, and they can cause severe damage that can reach up 50% (Egel et al. 2001; Gannibal 2011; Vakalounakis 1990). *A. cucumerina* of muskmelon is a serious disease of muskmelon crop in Indiana. The leaves of muskmelon plants can be severely blighted, leading to lower yield

(Latin 1992), reduced sugars (Latin et al. 1994), or both. In addition, no commercial muskmelon cultivar possesses an acceptable level of resistance or tolerance to *A. cucumerina* (Egel 1999). *A. alternata* infection has become the most important postharvest disease. Disease symptoms first appear on older leaves as circular brown to dark brown color spots (Figure 8) (Valkonen and Koponen 1990). In presence of serious infection, rapidly expanding lesions coalesce and cause defoliation and sun-scalding of the fruit, which becomes a source of conidia that are spread via wind and water. This fungus causes a fruit rot on squash; the fruit becomes brown and later black and mummified. Furthermore, *Alternaria brunsii* Uppal, Patel & Kamat has also been detected on *C. maxima* seeds (Paul et al. 2015) and *A. alternata* has been isolated from *Cucumis sativus* L. and *Cucurbita pepo* L. (Avinash and RavishankarRai 2013).

Figure 8. *Alternaria* leaf blight on watermelon leaves (Kehinde 2013).

3.3.4. Leaf spot diseases

Species of the genera *Paramyrothecium* and *Albifimbria* (formerly in the genus *Myrothecium*; Lombard et al. 2016) are saprophyte soil inhabitant. Only *Paramyrothecium roridum* (Tode) L. Lombard & Crous and *Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous are at present considered important plant pathogens and caused leaf spot diseases (Belisario et al. 1999; Bharath et al. 2006a; Garibaldi et al. 2016b,c ; Saira et al. 2017; Tulloch 1972). *P. roridum* affects diverse economically important crops, including tomato, cotton, cucurbit, soybean, lamb's lettuce, pepper, and others (Ben et al. 2015a, b, 2017; Farr and Rossman

2018; Fish et al. 2012; Garibaldi et al. 2016a; Quezado Duval et al. 2010). *A. verrucaria* infects many plant species such as beet, potato, cucurbit, spinach, sunflower, peanut, cotton, and ornamental plants (Abbas et al. 2001; Farr and Rossman 2018; Garibaldi et al. 2016b, c). *P. roridum* and *A. verrucaria* generally cause circular gray to brown spots on vegetable crops tissues. Spots gradually expand and coalesce to form blighted areas on the leaves (around 30 mm), which assume a water-soaked consistency (Figure 9) (Matić et al. 2019). These two species have been frequently isolated from cucurbits seeds (Bharath et al. 2006a; Bharath et al. 2006b).

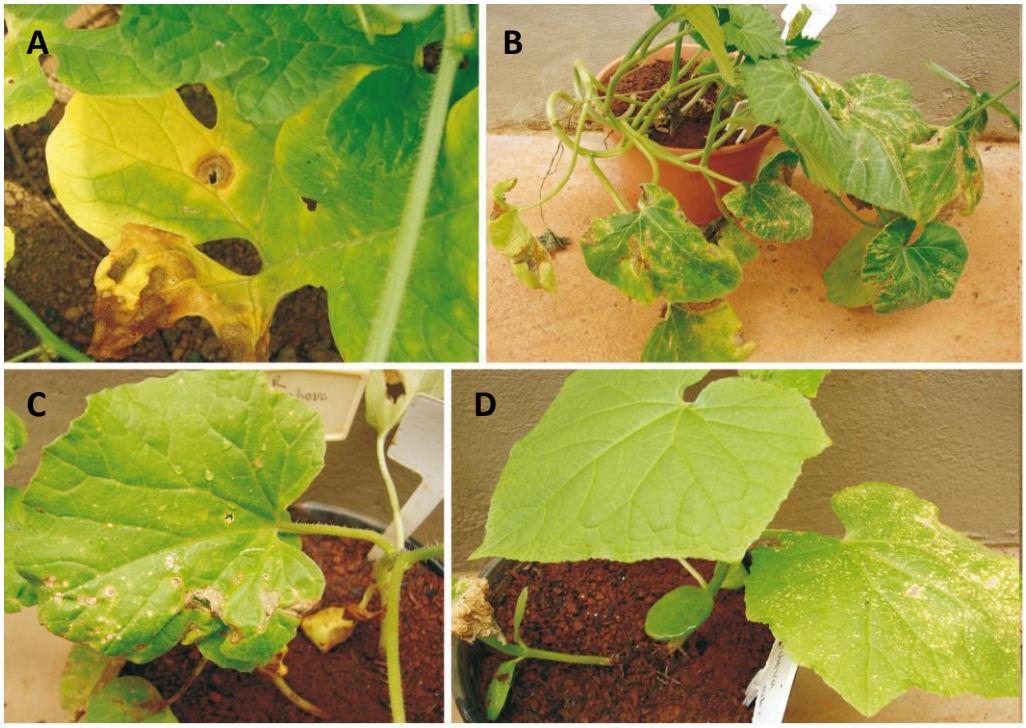

Figure 9. Leaf spots caused by *Paramyrothecium roridum* on cucurbits: **A**. gherkin (*Cucumis anguria*) in the field; **B**. squash (*Cucurbita moschata*); **C**. melon (*Cucumis melo*) and **D**. cucumber (*C. sativus*) plants inoculated without wounds in greenhouse (Cabral et al. 2009).

4. Seed transmission of fungal diseases

4.1.Direct invasion

The seeds can be infected by direct invasion through vascular bundles. Therefore, the xylem of mother plant can be infected through the embryo of seeds. Petkar and Ji (2017) reported that *Fusarium oxysporum* f. sp. *niveum* infects seeds by direct invasion.

4.2.Indirect invasion

Indirect invasion occurs following the pathogen penetration through the stigma, which follows the same path of the pollen grain. The fungal spores reach the stigma, germinate and produce a hypha to reach the ovary through the style, where they can remain as dormant mycelium until seed germination. Halfon-Meiriand Rylsky (1983) demonstrated 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* spp. invades *Cucumis sativus* through the stigma and style of the flower.The pathogen can also penetrate through the wall of the ovary, flower and fruit. Most fungi penetrate through the peduncle of fruits until they reach the seeds (Agarwal et al. 1996). Therefore, seeds can be infected indirectly through the fruit, when a lesion extends to the seed cavity, as demonstrated by Mehel and Epstein (2007).

4.3.Seed contamination

There are several sources responsible for seed infestation after fruit maturation. Besides, the pathogen can stick to the seed coat during harvesting, extraction, drying, and packing process. In addition, seeds can be infested when it is mixed with contaminated soil and debris. Many researches demonstrated that the majority of seedborne pathogens are localized mostly on seed coat, such as *A. alternata*, *Cercospora guizoticola* (Nagaraja and Krishnappa 2016), *Alternaria radicina* (Shakir et al. 2000), *S. cucurbitacearum* (Sudisha et al. 2006), *Verticillium dahliae* (Rampersad 2010), *Fusarium oxysporum* (Farrag and Moharam 2012), *Cladosporium variabile* and *Stemphylium botryosum* (Du Toit and Hernandez-Perez 2005).

Part II

5. Detection of seedborne pathogens

International organisations such as the European and Mediterranean Plant Protection Organization (EPPO), the International Seed Health Initiative (ISHI), the International Seed Testing Association (ISTA) and the International Society for Plant Pathology (ISPP) are responsible to develop and publish the standard seed health test methods to be used in international trade.

5.1.Conventional methods for seed health testing

5.1.1. Visual examination using stereomicroscope

The inspection of dry seeds is a method which consists in examining the presence of any signs of pathogens (sclerotia, spore masses, pycnidia, perithecia) and disease symptoms (discoloration, pigmentation, etc.) on seeds with the naked eye or under stereomicroscope. For example, chickpea seeds infected with *Fusarium oxysporum* f. sp. *ciceri* (Padwick) Snyd. & Hans. are generally smaller, wrinkled and discoloured (Mathure book) and the pycnidia of *Septoria apii* embedded in the seedcoat of celery seeds (Horst 2008). This examination usually displays low detection sensitivity. Moreover, few pathogens can be found in this way because seed can be infected by fungi do not show macroscopic symptoms (Walcott 2003).

5.1.2. Seed washing techniques

The washing test consists of shaking the seeds in distilled water (Reeves 1998). This technique allows the removal of fungal structures present on seed surface e.g. spores, oospores, sclerotia. The obtained suspension can be directly examined under stereomicroscope or centrifuged and the obtained pellet examined with the help of a microscope (Agarwal et al. 2006; Duan et al. 2007; Rao et al. 2006). The seed washing techniques should be applied only for those fungi with surface-borne spore, otherwise the sensitivity is poor if the fungus is also within seed tissues (Vanacci et al. 2014)

5.1.3. Incubation methods

Incubation methods are widely used for detecting a wide range of seed-transmitted pathogens. Seeds can be incubated either on water-soaked blotter/filter paper (blotter test) or on agar media (agar plate test). A standard sample for testing uses 200 seeds (Marcinkowska 2002; Rao et al. 2006), although this will depend on the seed size.

5.1.3.1.Blotter test

The blotter paper method is the most popular and effective in the seed control (Duan et al., 2007). Seeds of different kinds of crops are arranged in the petri dishes containing blotter paper moistened with sterile water. The number of seeds, layers of blotter paper and the size of petri dishes are fixed by ISTA according to the crops (Table 1.2). The blotter test promotes mycelium growth and formation of fruiting bodies on seed surface and allows the pathogen identification under the stereomicroscope (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). Different surface disinfectants can be used, such as 0.5% to 1.0% sodium hypochlorite, which is most commonly applied (Peres et al. 2002; Roy et al. 2000), 0.1% mercuric chloride (El-Nagerabi and Elshafie 2000; Ora et al. 2011), or 10% to 90% ethyl alcohol, for few seconds to several minutes (Vallad et al. 2005). For the 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). This diagnostic method is difficult to apply when the fungal species have a high degree of similarity based on their morphology. Distinguishing among these closely related organisms can be difficult, such as between *S. cucurbitacearum* 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). The blotter test method is certainly economically advantageous but it has the disadvantage of not allowing the identification of all the fungi. There is a drawback in this method: after incubation, seed germination can interfere with the growth of seedborne fungi and can disturb the identification of pathogens. So, the deep-freeze blotter method can be used. This method consists in adding another step to the blotter method: after 24h of seed incubation at 22°C, the seeds are maintained at -20°C for 24h to kill the embryo and stop the emergence of root and shoot (Du Toit et al. 2005; Elwakil et al. 2009).

Table 1.2. Different parameters of blotter test by ISTA according to the crops (Mathur and Kongsdal 2003).

Scientific name of crops	Size of blotter paper (mm)	Number of layers of blotter paper	Period of incubation (days)
Cucumis sativus	110	8	14
Cucurbita maxima	110	12	14
Cucurbita pepo	110	12	14
Lactuca sativa	90	8	8
Cucumis melo	110	8	14
Allium cepa	90	8	8
Citrillus lanatus	110	12	14

5.1.3.2.Agar plate tests

This is the most commonly used method for identifying seedborne fungi. Different agar media may be used, selective or non-selective potato dextrose agar and malt extract agar for specific fungi (Marcinkowska 2002; Nasir 2003; Ora et al. 2011; Roy et al. 2000). The fungus identification with this technique is based on visual inspection such as colony characteristics, sporulation, presence of pigments, etc.. The method is time consuming, because the fungi have to be examined carefully under a microscope. Farrag and Maharam (2012) applied this method to detect *Fusarium* spp. *Rhizoctonia* sp., *Penicillium* spp., *Alternaria* sp. and *Helminthosporium* sp. on cucumber seeds. One of the drawbacks of this technique is that it can be used for many bacteria and fungi, but it cannot be used for non-culturable parasites, such as obligate fungi.

5.2.Innovative methods

5.2.1. Polymerase chain reaction

PCR is a useful molecular tool for identification and detection of seedborne pathogens on different crops. The conventional PCR is able to amplify the specific fragments from tiny quantities of DNA genome, which are visualized after electrophoresis in ethidium-bromide-stained agarose gels (Walcott 2003). Since its introduction in the mid-1980s, it has improved the genomic techniques. This technique has many beneficial characteristics, including high analytical sensitivity and specificity to detect and identify different strains or race of the pathogen, and easy to be interpreted by personnel with no specialized taxonomical expertise

(Amaral Carneiro et al. 2017; Babu et al. 2015; Walcott 2003; Ward et al. 2004). Therefore, PCR technique is a great help in monitoring seed health in international seed trade (quarantine), in seed testing laboratories and in research laboratories and a large number of samples can be processed within a short period of time (Mbofung and Pryor 2010). The transcribed internal spacer region (ITS1, 5,8S and ITS2 sequence) of rDNA is widely used to design species-specific primers (small oligonucleotide probes) to detect the presence of seed-borne fungi. There are many published papers reporting the PCR protocols for the detection of *Alternaria radicina* from carrot seeds, *Alternaria brassicae* from cruciferous seeds, *Ascochyta lentis* from lentil seeds, *Leptosphaeria maculans* from canola seeds, and *Phoma valerianella* from lamb's lettuce seeds (Chen et al. 2010; Guillemette et al. 2004; Hussain et al. 2000; Landa et al. 2007; Pellegrino et al. 2010; Pryor and Gilbertson 2001). Despite the above reports, PCR methods have some disadvantages such as the detection even of dead fungal spores, which have no pathological importance. Furthermore, the major limitation in the use of PCR is that it is not possible to quantify the pathogen and to determine the number of infected seeds in a lot. One of the major obstacles to the adoption of nucleic acid–based seed health tests is the presence of inhibitors in the seeds, which determines false negatives also from highly infected samples. These inhibitors are compounds commonly found within seeds that can inhibit the PCR reaction, such as polyphenolics, proteins, polysaccharides, fats, and salts (Arbeli and Fuentes 2007; Iacomi-Vasilescu et al. 2002; Murillo et al. 1998; Reeves 1998; Rogers et al. 1996; Terry et al. 2002).

5.2.2. Nested polymerase chain reaction

Nested PCR increases sensitivity of conventional PCR to detect low levels of the target fungus on seeds. After an initial PCR, a second round of amplification is applied using primers designed to anneal the amplification produced by the first round of amplification. Nested PCR has been used to detect DNA levels of 10 fg for *Colletotrichum lindemuthianum* in bean seeds (Chen et al. 2007), and of 1 fg for *F. oxysporum* f. sp. *lactucae* in lettuce seeds (Mbofung and Prior 2010). This technique increases the sensitivity of the assay but also increases the false positives due to cross-contamination (Tomlinson et al. 2005).

5.2.3. Multiplex PCR

Multiplex PCR allows amplifying several different DNA and RNA targets in a single reaction, helping in reducing the number of required tests (Majumder et al. 2013). Multiplex real-time PCR found successful application particularly for routine seed inspections in the

spinach seed industry, which needs to certify the absence of at least *Peronospora effusa*, *Stemphylium botryosum*, *V. dahliae*, and *Cladosporium variabile* (Feng et al. 2014).

5.2.4. Real-time polymerase chain reaction

As previously mentioned, the risks of cross contamination with conventional PCR, have made the technique unattractive. In the early 1990s, with the development of the TaqMan® chemistry by Applied Biosystems (Foster City, CA, USA) (Holland et al. 1991), real-time PCR technology (qPCR) has been developed instead of conventional PCR assays and promise to eliminate many of these barriers and make PCR more accessible for seed detection. Besides, the costs for real-time PCR machines and reagents limited its use to research activities rather than for diagnostic purposes (Cullen et al. 2001; Gachon et al. 2004; McCartney et al. 2003), although these costs have been reduced over time. Real-time PCR assays have been implemented for the detection of fungal plant pathogens in several recent studies (Guillemette et al 2004; Martin et al. 2007). Real-time PCR combines the sensitivity and specificity of conventional PCR with the quantification of a microorganism based on nucleic acid sequences and concentrations. Guillemette et al. (2004) reported that this technique could soon be useful for seed health testing. This technique is faster than conventional seed detection and conventional PCR assays (Guillemette et al. 2004; Chilvers et al. 2007), and sensitive such that Chen et al. (2013) detected 5 fg of genomic DNA of a target fungal pathogen in the DNA extracted from seeds. Real-time PCR-based methods have been applied for the identification, detection, and quantification of seedborne pathogens by using SYBR Green I (Molecular Probes Inc., Eugene Ore.) that binds double-stranded DNA indiscriminately or with the use of specific reporter probes like TaqMan (Applied Biosystems, Foster City, Calif.) (Taylor et al. 2002). Many recent researches report the use of real-time PCR to detect and quantify *Botrytis* spp. in onion seed (Chilvers et al. 2007), *Alternaria brassicae* in cruciferous seed (Guillemette et al. 2004); *Rhynchosporium secalis* in barley seed (Lee et al. 2002); *V. dahliae* in spinach seeds (Duressa et al. 2012), *C. lindemuthianum* in dry bean seeds (Chen et al. 2013).

5.2.5. Loop-mediated isothermal amplification

The LAMP assay is one of the most powerful molecular techniques for pathogen detection and disease diagnosis. The PCR techniques require a long operation time, laboratory instrumentation and reagents and expert technicians, besides being expensive (Francois et al. 2011; Moradi et al. 2014; Shen et al. 2016). These drawbacks limit its routine use, especially

in quarantine stations, plant protection organizations, poorly resourced laboratories and in rural areas of developing countries. Thus, a more convenient, rapid, sensitive, simple and economical method of diagnosing pathogen is needed (Chen et al. 2013; Li et al. 2013; Zhang et al. 2013). Recently, a novel DNA amplification technique named Loop-mediated isothermal amplification (LAMP) has been first reported by Notomi et al. (2000) (Nagamine et al. 2001). This new technique allows having results in less than 60 min (Nagamine et al. 2002; Notomi et al. 2000). The entire procedure is not difficult to perform and requires only a simple instrument. The results can be easily visualized by the naked eye by adding colorimetric indicators, such as calcein, which produces a green fluorescent signal if the LAMP reaction is positive (Zhang et al. 2013). Therefore, with these numerous advantages, LAMP technique has been used to detect pathogens, including viruses (Zhao et al. 2014), bacteria, fungi (Li et al. 2013) and nematodes (He et al. 2013). Over the last years, several LAMP assays have been developed for the molecular detection of seedborne pathogens: *Fusarium fujikuroi* in rice seeds (Zhang et al. 2019; Ortega et al. 2018); *Acidovorax citrulli* in cucurbits seeds (Yan et al. 2019); *Fusarium graminearum* in wheat seeds (Abd-Elsalam et al. 2011); *Colletotrichum gloeosporioides* in soybean seeds (Wang et al. 2017); *Ascochyta rabiei* L. in chickpeas seeds (Chen et al. 2017); *S. cucurbitacearum* in cucurbits seeds (Tian et al. 2017). This method may serve as a valuable tool for the rapid, simple, and sensitive detection of other seedborne pathogens in quarantine inspections, commercial and laboratory fields, especially for resource-limited laboratories in small seed companies.

Part III

6. Seed treatments

6.1. Fungicide treatments

Seed treatment with fungicides is a very common practice worldwide. Chemical seed treatment consists of the application of pesticides (fungicides, insecticides, nematicides and rodenticides) to seed to control diseases and pests affecting seeds and seedlings (White and Hoppin 2004). There are three classes of seed treatment fungicides. The first group includes fungicides that act by contact; these are only effective to control fungal spores localized on the surface seeds. The second groups of fungicides are locally systemic and target both seed surface-borne and internally seed-borne pathogens. Finally, the third group of fungicides includes those that are xylem mobile and thus are systemically translocated. Captan (N-trichloromethylthio-4-cyclohexene-1,2-dicarboximide) and thiram (tetramethylthiuramdisulfide) are contact fungicides widely used in seed treatment (Nasraoui, 2006). Systemic fungicides are effective against fungal diseases located inside the seed. These fungicides can present less risk to crops, animals and the environment because they may be easily degraded by soil microorganisms, which prevent their accumulation in the soil. Correct application of the fungicide seed treatment is required to obtain the greatest net benefit. These benefits include improved seed germination, seedling emergence and prevented seed transmission of seed-borne pathogens (da Silva et al. 2017; Durrant and Mash 1991, Khanzada et al. 2002). Nowadays fungicide seed treatment is very common and worldwide practiced. Sudisha et al. (2006) evaluated the efficacy of three contact and systemic fungicides on melon seed against *S. cucurbitacearum*. Among these fungicides there are Dithane M-45 75% WP (Manganese ethylene bis-dithiocarbamate plus zinc) at a concentration of 0.2%, Wanis at a concentration of 0.3% and Captaf (Captan 50% WP; *N*-(Trichlorométhylthio)cyclohex-4-ène-1,2-dicarboximide) at the concentration 0.3%. Recently, Kandel et al. (2019) reported that the treatment of soybean seed with fluopyram provided the highest level of control of root rot and foliar symptoms of Sudden death syndrome, caused by *Fusarium virguliforme*. Although seed treatments have important benefits, they also pose certain risks for environment, phytotoxicity and selection of resistant pathogen populations (Pimentel and Levitan 1986; Nostro et al. 2000). Aimin et al. (1994) have shown that the treatment of watermelon seeds by the two fungicides Thirame and Captan has a negative effect on the fixation of minerals on the roots of plants, resulting poor growth of the plant.

6.2.Heat treatments

Heat treatments are physical seed treatments with hot water, hot air, and electron treatments. Hot water treatments are classical thermo physical methods of plant protection and are reported to reduce the incidence of seedborne fungi, without affecting the viability of seeds, when it carried out using precise treatment parameters (Aveling et al. 1993; Hermansen et al. 2000; Pryor et al. 1994;). At the end of the 19th century, hot water treatment was frequently applied to control seedborne fungi of cereal and this method now receiving new importance for organic farming (Koch et al. 2010; Schmitt et al. 2009). Du toit and Hernandez-Perez (2005) demonstrated that spinach seed treatments in 1.2% NaOCl for 10 to 60 min, or hot water (40, 45, 50, 55, and 60ºC) for 10 to 40 min, were evaluated for eradication of *Cladosporium variabile*, *Stemphylium botryosum*, and *V. dahliae*. Amein et al. (2011) reported that hot water and electron seed treatment are effective in the control of *A. brassicicola* on naturally infested cabbage seeds. Bennett and Colyer (2010) investigated the potential of low- and high-temperature dry heat, and hot water treatments, for controlling *Fusarium oxysporum* f. sp. *vasinfectum* on cotton seed.

6.3.Treatments with natural compounds

6.3.1. Chitosan seed treatment

Chitosan is a naturally-occurring compound, which derives from crab-shell chitin. It is a biopolymer with antiviral, antibacterial, and antifungal properties (El Hadrami et al. 2010). This natural compound has been explored for many agricultural uses and it helps to reinforce host plant defenses (Reddy et al. 1999). In addition, chitosan was reported to improve seed germination and plant vigor (Agrawal et al. 2002; Bhaskarareddy et al. 1999; Devlieghere et al. 2004; Guan et al. 2009; Muzzarelli and Muzzarelli 2005). Recently, fungicidal activity of chitosan has been demonstrated against several species of seed borne fungi. El Gahouth et al. (1993) reported that chitosan is an ideal antifungal agent against greenhouse diseases. In the same way, Nandeeshkumar et al. (2008) demonstrated that treatment of sunflower seeds with 5% chitosan decreased downy mildew severity caused by *Plasmopara halstedii* and offered 46 and 52% protection under greenhouse and field conditions respectively.

6.3.2. Plant extracts and essential oil seed treatment

Recently, several studies have reported the use of essentials oils and plant extracts for the control of seedborne pathogens. They have shown antifungal activities with no side effects on humans and animals (Table 1.3). Generally, essential oils are obtained from different plant

parts such as flowers, buds, seeds, leaves bark, woods, roots, and fruits. They can be used as a natural seed treatment to control fungal pathogens causing superficial infections. In addition, for pathogens localized within the seeds, products able to penetrate the seeds are needed. Therefore, Gullino et al. (2014) reported that physical treatments (e.g. hot treatment) combined with some essential oils seem to have this ability.

Table 1.3. Main antifungal compounds and their effect against the pathogens.

Scientific name of plant	Antimicrobial compound	Against	References
Chrysanthemum coronarium	Camphor; α-pinene; β-pinene; lyratylacetate	*Alternaria* sp., *Aspergillus favus*, *Pythium ultimum*, *Botrytis cinerea* and *Sclerotinia sclerotiorum*.	Alvarez-Castellanos et al. 2001
Artemisia absinthium *A. santonicum* *A. spicigera*	Camphor; 1,8-cineole; chamazulene; nuciferol propionate; nuciferolbutanoate; caryophyllene oxide; borneol; α -terpineol; spathulenol; cubenol; β-eudesmol; terpinen	*Alternaria alternata*, *Fusarium oxysporum*, *F. sambucinum*, *F. solani*, *Penicillium jensenii*, *Rhizoctania solani*, *Sclerotium minor*, *S. sclerotiorum*, *Verticillium albo-atrum*, and *V. tenerum*	Kordali et al. 2005
Tagetes patula	Piperitone; piperitenone; terpinolene; dihydro tagetone; cis-tagetone; limonene; allo-ocimene	*Botrytis cinerea* and *Penicillium digitatum*	Romagnoli et al. 2005
Chrysactinia mexicana	Eucalyptol; piperitone; linalyl acetate	*Aspergillus flavus*	Cárdenas-Ortega et al. 2005
Helichrysum italicum	α-terpinolene, trans-cariophyllene and nerylacetate	*Pythium ultimum*	Tundis et al. 2005

Thymus vulgaris	*γ*-terpinene; *p*-cymene; carvacrol; thymol	*Penicillium digitatum*; *Alternaria padwickii*; *Bipolaris oryzae*; *Fusarium moniliforme*; *Ascochyta lentis*; *Colletotrichum goleosporioides*	Daferera et al. 2000; Nguefack et al. 2008; Marinelli et al. 2012
Origanum heracleoticum and *O. majorana*	Carvacrol; p- cymene	*Aspergillus niger*; *Botrytis cinerea*; *Monilinia fructicola*: *Penicillium expansum*	Della Pepaet al. 2019
Melaleuca alternifolia	terpinen-4-ol, α -terpinen and 1,8-cineole (eucalyptol)	*Fusarium graminearum, Fusarium culmorum and Pyrenophora graminea, Ascochyta rabiei, Colletotrichum lindemuthianum, Drechslera avenae, Alternaria radicina and A. dauci*	Riccioni and Orzali 2011; Terzi et al. 2007
Laurus nobilis	1,8-Cineole (Eucaliptol), sabinene, α-Terpinenyl acetate	*Fusarium culmorum and Fusarium verticillioides*	Roselló et al. 2015; Chahal et al. 2017

Cymbopogon citratus	Geranial; neral; myrcene	*Alternaria padwickii, Bipolaris oryzae and Fusarium moniliforme; Aspergillus niger; A. flavus; F. oxysporum, F. solani.*	Nguefack et al. 2008; Gbenou et al. 2013; Aoudou and Childeric 2017

6.4. Genetic resistance

Host plant resistance is an essential section of integrated disease management. The development and use of resistant cultivars is the most effective, environmentally sustainable, and economical method. Several researchers over the world have reported good tolerant varieties and introduced various resistance genes in crops. Oumouloud et al. (2002) recorded three varieties of muskmelon i.e. Charentais-Fom1, Charentais-Fom-2, and Dinero F1 resistant against *F. oxysporum* f. sp. *melonis*. In tomato, IIHR-305, IIHR-308, IIHR-2266, IIHR-2285 and IIHR-2288 are cultivars resistant to Alternaria early blight (Matharu et al. 2006). GSB is the major pathogen among all the cucurbits growing areas, but unfortunately Keinath (2017) reported that to date no commercially varieties of cucurbit with resistance to GSB are available.

CHAPTER 2

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

Plant Disease • 2020 • 0:1-16 • https://doi.org/10.1094/PDIS-04-19-0741-RE

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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, EF1α, histone H3, ITS, *Paramyrothecium*, rDNA, seed-health test, species-specific primers, *Stemphylium*

1. 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 seed, which then become an efficient vehicle to disperse seedborne pathogens over long distances resulting in severe crop losses. The main objective of this study was to identify seedborne fungi in seeds extracted from 66 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: Seed health test, blotter test, asymptomatic, species specific primers, detection, *Albifimbria, Paramyrothecium*, *Stemphylium*

2. Introduction

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, 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* (Ellis & Everh.) J.A. Elliott and *Alternaria alternata* (Fr.) Keissl. are pathogens of cucurbits, and they can cause severe crop losses (Gannibal 2011; Vakalounakis 1990). Furthermore, *Alternaria brunsii* Uppal, Patel & Kamat 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* (Pers.) Rabenh. (anamorph *Stemphylium vesicarium* (Wallr.) E.G. Simmons), *Paramyrothecium roridum* (Tode) L. Lombard & Crous, and *Albifimbria verrucaria* (Alb. & Schwein.) L. Lombard & Crous 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* (Berk. & M.A. Curtis) Rostovzev 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 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 identify 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. Distinguishing 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, from 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*.

3. Materials and Methods

3.1. 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 (Figure 10). 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 4). 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 before planting in their respective years.

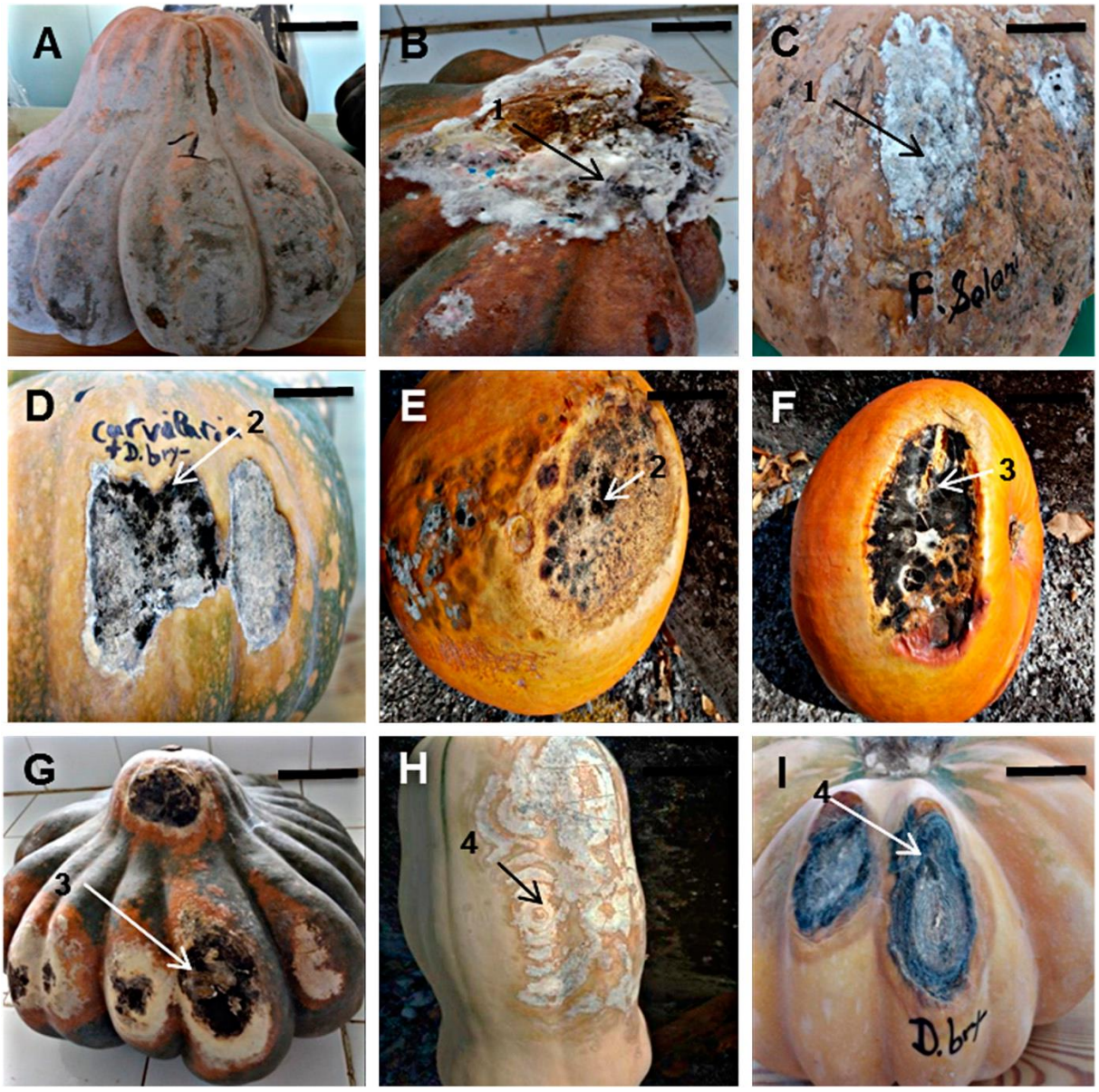

Figure 10. **Fruit rot symptoms on squash fruit**. (**A**) Apparently healthy fruit (cv. Galaoui). (**B**, **C**) Fusarium rot with white mycelia, caused by *F. solani* (arrow 1) (B, cv. Galaoui; C, cv. Bjaoui). (**D**, **E**) Black mycelia of *Curvularia spicifera* on squash fruit (arrow 2) (D, cv, 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 *Stagonosporopsis cucurbitacearum* (arrow 4) (H, cv. Butternut; I, cv. Bajaoui). Scale bars: 5 cm.

Table 4. Field sites and squash 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

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

3.2. 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 symptoms. The 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, rinsed in three washes of sterilized distilled water, and 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 (Figure 11): 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.

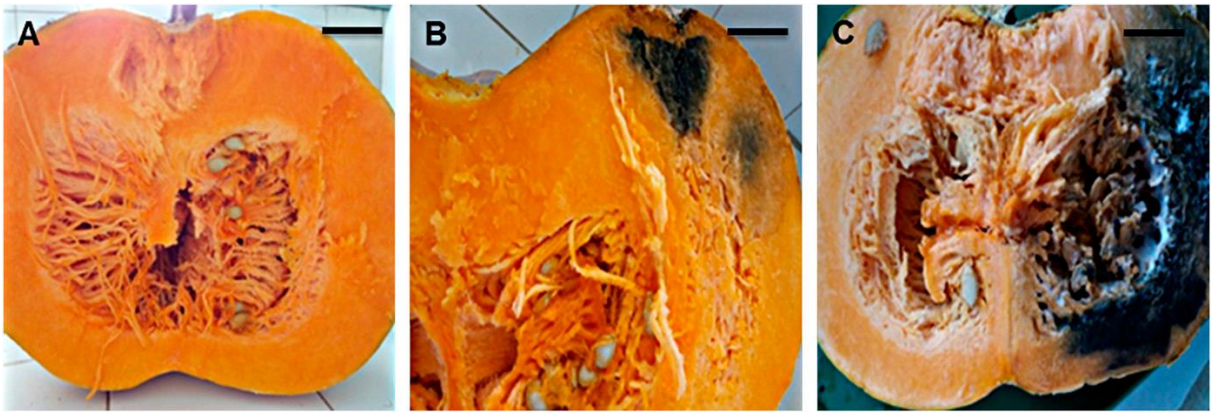

Figure 11. Evaluation of symptoms on squash fruit collected from Tunisia and Italy. (**A**) Asymptomatic fruit. (**B**) Infected fruit showing symptoms of rot on squash skin without reaching the seed cavity. (**C**) Infected fruit showing symptoms of rot that has reached the seed cavity. Scale bars: 5 cm.

3.3. 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 (Whatman no. 4 filter papers; diameter, 110 mm) that was moistened with 5 mL sterile distilled water in Petri dishes (diameter, 110 mm). These were then 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). The spores, conidiophores, pycnidia, and perithecia of the fungi were then examined under a microscope (DM 2500; Leica). To support this initial identification, single-spore isolates of each fungus were collected and transferred into PDA in Petri dishes, to obtain pure cultures (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 keys of Aveskamp et al. (2010), Booth (1971), Champion (1997), Jeon et al. (2015), Lombard et al. (2016), and Mathur and Kongsdal (2003).

3.4. Molecular identification of seedborne fungi

3.4.1. 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 5). The isolates were grown in PDA Petri dishes until the fungi reached the edge of the plate. A modified DNA extraction method 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 verified on 1% agarose gels, with evaluation using a BioPhotometer (Eppendorf, Hamburg, Germany).The DNA was finally diluted to 20 ng/µL for further amplification.

Table 5. Codes assigned to the isolates of each fungal species isolated from fruits and seeds collected from Tunisia (TN) and Italy (IT) that were used during the DNA extraction and PCR analyses of the ITS, EF1α, HIS, TUB, and CALM sequences.

Fungal species	**Isolate**	**Location**	**Source**	**Host species**	**Identification method**		**GenBank accession number**				
					Sequencing	**Species-specific PCR**	**ITS**	**EF1*α***	**HIS**	**TUB**	**CALM**
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	MN599712
	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	MN599713
	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	-	-	MN630223	MN599711
	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	-	-	MN630222	-
	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	MN599708
roridum	M138*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MF401576	-	-	-	MN599709
	M167	Sidi Hmada, TN	Seed	*C. maxima*		+					

	M141*	Sidi Hmada, TN	Seed	*C. maxima*	+	+	MK497786	-	-	MK497765	MN599710
	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	-	-	MN630219	MN599704
	IB41*	Castelfidardo, IT	Seed	*C. maxima*	+	+	MK497777	-	-	MN630221	MN599706
	IB4	Castelfidardo, IT	Fruit	*C. maxima*		+					
	IB1*	Castelfidardo, IT	Seed	*C. maxima*	+	+	MK497778	-	-	MN630220	MN599705
	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	-	-	MN599707	
	IP41	Baranello, IT	Seed	*C. moschata*		+						
	IP4*	Osimo, IT	Seed	*C. moschata*	+	+	MK497781	-	-	-	-	
	IP5	Osimo, IT	Seed	*C. moschata*		+						

* -, sequence not available.

[a] Positive result with identification method.

3.4.2. 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, and the translation elongation factor (EF)1α region in the ribosomal (r)DNA of the isolates (Table 5) 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*, EF1-728F/EF1-986R (Carbone & Kohn, 1999) for *EF1α*, and CAL228F/737R (Carbone & Kohn, 1999) for *calmodulin* gene. The PCR reactions were run in a thermal cycler (MyCycler; Bio-Rad Laboratories, Hercules, CA, USA) following the specific parameters published for TUB, HIS, EF1*α*, and CALM. 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, Hercules, California).

Bidirectional sequence analysis was conducted on select amplified isolate fragments at Genewiz (UK) (Table 5). The forward and reverse nucleotide sequences were read and edited using the Chromas version 2.33 software (Technelysium Pty Ltd, Australia), 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.

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 6.

Table 6. 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, revers

3.4.3. 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, See annexes), 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 7). The new set of primers that were designed were submitted to and validated by Primer-BLAST software (Ye et al. 2012) developed at the NCBI GenBank.

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 8 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 (4 ×10 ng to 4 $\times 10^{-5}$ ng), respectively, to determine the analytical sensitivity of the tests.

Table 7. 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	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

[a] **F =** forward and **R** = reverse**.** [b] **Tm =** melting temperature.

Table 8. Cycling conditions for the newly designed primers for the detection of *Albifimbria verrucaria*, *Paramyrothecium roridum*, and *Stemphylium vesicarium*.

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

4. Results

4.1. 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 associated with *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 9).

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 fruits to *A. alternata*. All of the samples collected in the Monopoli and Baranello areas showed infection levels of 'A' (Table 9).

Table 9. Incidence of different seedborne fungi detected on all of the squash samples using the blotter test.

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	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	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	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	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	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	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	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	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	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	0.0	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	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	1.9 ±1.4	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	0.0	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	13.5	0.0	3.0	0.0	0.0	0.0	1.5	0.0	0.0	0.0	0.0	0.0	1.0	0.0
				±3.6	±2.2		±1.6				±1.5						±1.0	
I36	Mo	-	A	38.5	36.5	0.0	1.0	0.0	0.0	0.0	14.5	0.0	0.0	0.0	0.0	1.5	0.0	0.0
				±2.8	±3.2		±0.9				±3.4					±1.0		
I37	Ba	-	A	47.5	36.0	0.0	0.0	1.0	0.0	0.0	8.0	0.0	0.0	0.0	1.5	0.0	3.0	0.0
				±3.2	±3.9			±0.6			±1.3				±1.0		±1.2	

[a] SH = Sidi Hmada, KA = Kalˆaat El-Andalous, Sb = Sbe¨ıtla, Ut = Utique, Sa = Sahline, Ca = Castelfidardo, Os = Osimo, Re = Recanati, Mo =Monopoli, and Ba =Baranello.
[b] Fungal species isolated from fruit sample. 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. = *Phom*a sp., Ff = *Fusarium fujikuroi*, Fo = *F. oxysporum*, Fi = *F. incarnatum*, Pr = *Pramyrothecium roridum*, Av = *Albifimbria verrucaria*, Sv = *Stemphylium vesicarium*, and Rs = *Rhizopus stolonifera*.
[e] The same seed can be infected by more than one fungus.

4.2. 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 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 using microscopy, to determine shapes and sizes. 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 (Figure 12 A-C). These pycnidiospores were cylindrical with rounded ends 4.0 to 8.0 µm × 1.6 to 3.4 µm (Figure 12D). 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 (Figure 12E). These morphological traits were consistent with *S. cucurbitacearum*.

By sequence analysis, isolates D33, D29, D49, D12, and D83, showed high nucleotide identity with *S. cucurbitacearum* (Table 5) and the molecular tools proposed by Brewer et al. (2015) corroborated the morphological identification (data not shown). The amplification with the RG-specific primer pair, able to yield a specific fragment of about 450 bp, allowed molecularly characterization of all analyzed isolates of *S. cucurbitacearum* belonging to the RG group II (Figure 14A) (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 known as sporodochia. They were green to dark green, surrounded by white cotton mycelia on the seeds (Figure 12I), and had a large number of elliptical one-celled conidia with acute ends 4.1 to 5.7 µm × 1.5 to 2.2 µm (Figure 12J). 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 this was confirmed by the sequence analysis for isolates M144, M135, IAV2, and IAV4 (Table 5).

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 (Figure 13A). 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 (Figure 12K). Conidia taken from these sporodochia were one-celled and cylindrical, with rounded ends (Figure 12L). 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 this was confirmed by the sequence analysis for isolates M123, M138, M141, IPR2, and IPR9 (Table 5).

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 (Figure 13B). 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 with the size of 147 to 282 µm × 131 to 243 µm appeared on the squash seed samples (Figure 12O). The crushed perithecium discharged several bitunicate clavate asci that measured 50 to 116 µm × 8.4 to 18.3 µm (Figure 12P). 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 (Figure 12Q). 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 were confirmed by the sequence analysis of isolates P164, P66, and IP4 (Table 5).

For the molecular identification of *S. vesicarium*, the primer pairs Pleo F/R (Figure 13C) and Pleo F1/R1 (Figure 13D) 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 (Figure 12 F, 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 (Figure 12H). 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 was confirmed by the sequence analysis of isolates A38, A15, IA1, and IA3 (Table 5).

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 (Figure 14B).

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

Other conidia were found in groups on squash seed samples (Figure 12M). 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 (Figure 12N). These morphological features belong to the fungal species *C. spicifera*, with confirmation by the sequence analysis of isolates B170, IB41, IB1 (Table 5).

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 (Figure 14C) (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 (Figure 2.3R). Microscopic examination showed long phialides (Figure 12S), 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 (Figure 12T). 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 5).

For the molecular identification, the amplification carried out with the specific primers TEF-Fs4f/TEF-Fs4r (Arif et al. 2012) (Figure 14D) 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) (Figure 14E) yielded a specific fragment of ~400 bp.

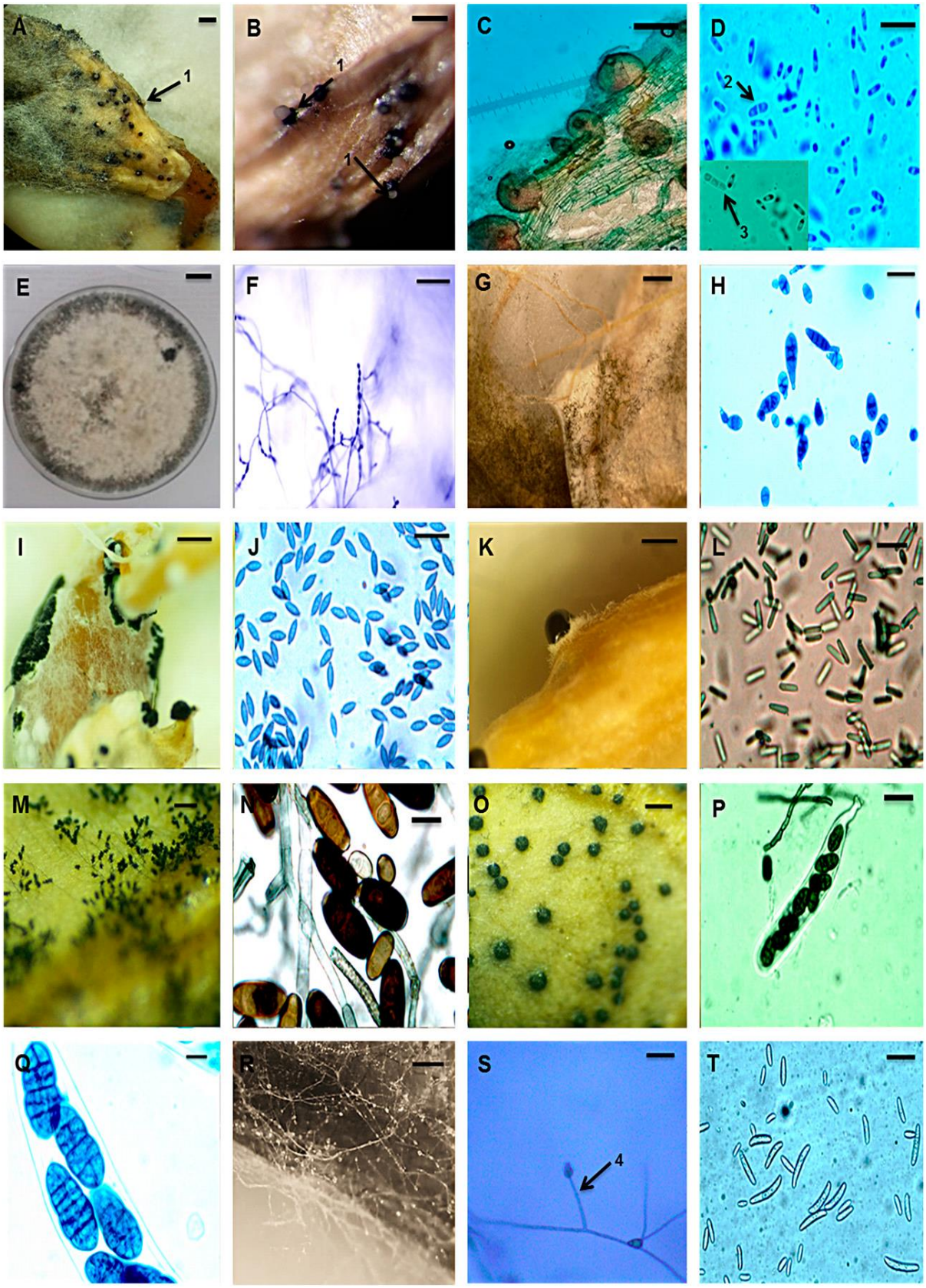

Figure 12. (**A**, **B**) Pycnidia of *Stagonosporopsis cucurbitacearum* on a seed from a squash, as seen under the stereomicroscope, with the ooze of pycnidiospores indicated (arrow 1). (**C**) Pycnidia under the microscope. (**D**) Pycnidiospores: cylindrical, mostly non-septate, few

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 *Alternaria alternata* on seeds. (**H**) Conidia of *A. alternata*. (**I**) Sporodochia of *Albifimbria verrucaria* on seed. (**J**) Elliptical conidia of *A. verrucaria*. (**K**) Sporodochia of *Paramyrothecium roridum* on a squash seed. (**L**) Cylindrical conidia of *P. roridum*. (**M**) Conidia of *Curvularia spicifera* on a seed, as seen under the stereomicroscope. (**N**) Conidia and vegetative hyphae of *C. spicifera*. (**O**) Perithecia of *Stemphylium vesicarium* on a seed. (**P**) An ascus of *S. vesicarium*, with short, broad pedicel bearing eight ascospores. (**Q**) Ascospores of *S. 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 mm in A, B, I, M, and R; 100 mm in C, F, and K; 10 mm in D, J, L, and N; 1 cm in E; 400 mm in G and O; 20 mm in H, P, and T; 5 mm in Q; and 25 mm in S.

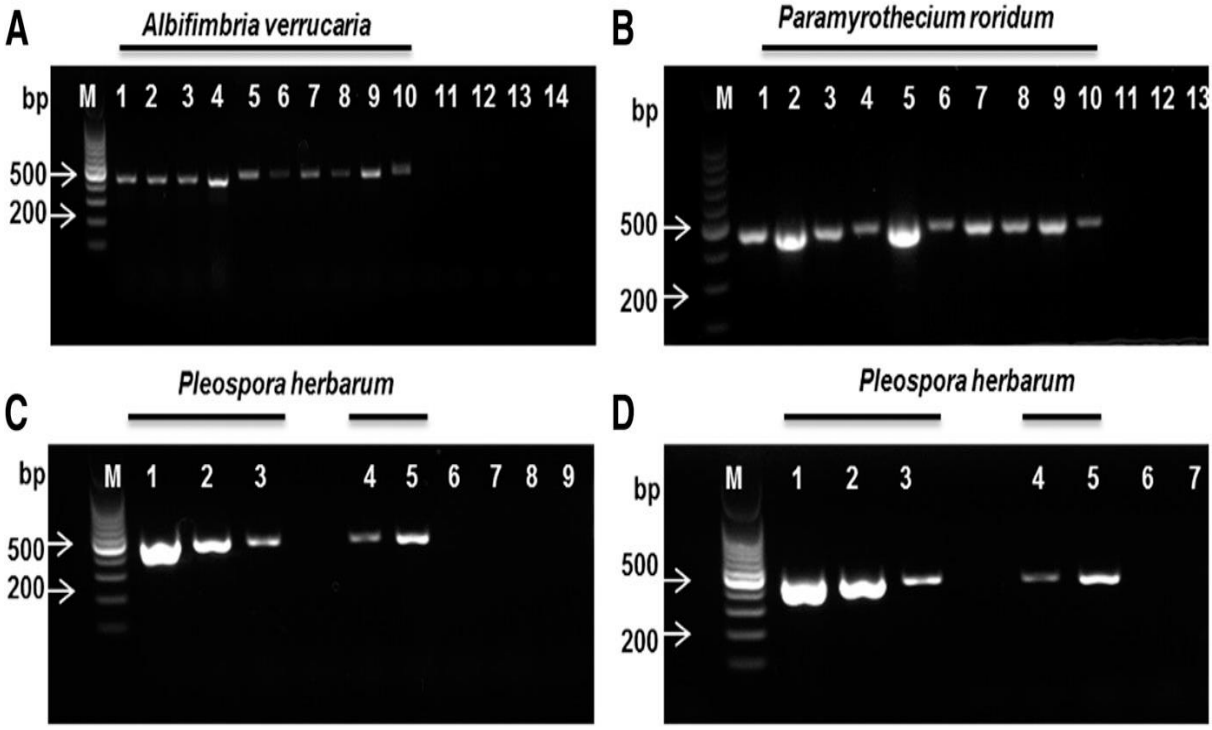

Figure 13. Gel electrophoresis of PCR products generated with the designed specific primers for the detection of the fungi isolated from seed collected from Tunisia and Italy. (**A**) *Albifimbria verrucaria*, with the primer pair Myroverr F1/Myroverr R1. Lanes 1 to 10: *A. verrucaria* (isolates IAV1, IAV2, IAV3, IAV4, M149, M155, M144, M140, M135, M146, respectively); lanes 11 to 13, *Paramyrothecium 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**) *Pleospora herbarum*, with the primer pair Pleo F/ Pleo R. Lanes 1 to 5: *P. herbarum* (isolates IP41, IP4, IP5, P164, P66, respectively); lanes 6, 7, *Alternaria alternata* (isolates A38, A15, respectively); lane 8, *Curvularia 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).

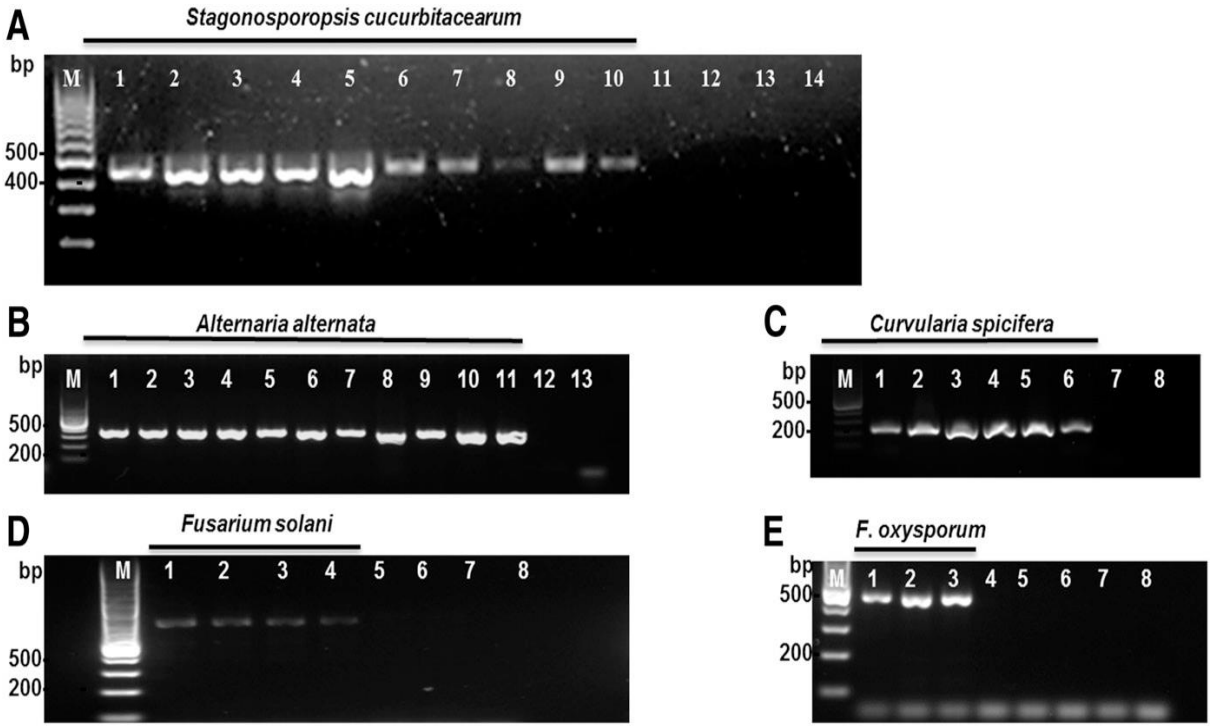

Figure 14. (**A**) Specificity of the primer pair RGII F/RGII R used for detection of *Stagonosporopsis cucurbitacearum* in group RGII. Lanes 1 to 10, *S. cucurbitacearum* (isolates D33, D29, D21, D5, D42, D49, D45, D12, DBF1, and DBF2, espectively); lane 11, *Phoma* sp. (isolate Ph39); lane 12, *Alternaria alternata* (isolate A15); lane 13, *Curvularia 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, *Stemphylium 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, *Fusarium 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, *Fusarium 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).

4.3. 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%). *Alternaria alternata* was detected in all of the localities surveyed and in most of the symptomatic fruits (Table 9, 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%. *Stagonosporopsis 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 9). 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 9, 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%. *Albifimbria 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 9 was highly infected by *A. verrucaria* (15.5%) and *P. 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, *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 10). *A. alternata* was detected in all of the included localities. 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%. *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 infection level of 'C' were infected by *C. spicifera* (Table 9, I2, I5, I31), with infection rates from 11% to 85%. Another symptomatic fruit sample with an infection level of 'B' was infected with *C. spicifera* (Table 9, I29), with

an infection rate of 2%. *Curvularia spicifera* was also identified in the seed samples of two asymptomatic fruits (Table 9, I23, I37), with an infection rate of 1%.

Table 10. Incidence of seedborne fungi detected in 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.

5. 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. This work identified 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. *Alternaria alternata* is a pathogen of cucurbits, where it can cause severe crop losses (Vakalounakis 1990). *Stagonosporopsis 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). *Stagonosporopsis cucurbitacearum* was reported in Italy in 1885 on *Cucumis melo* L. (Corlett 1981) and in 2019 on *C. moschata* (Moumni et al. 2019). In Tunisia, *S. cucurbitacearum* was detected only on watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) 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 finding shows that there are other ways of pathogen infestation. 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). Halfon-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 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 good quality 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 sequences in the 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, determined 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. When we used <4 ng for *A. verrucaria* and *P. roridum* and 0.4 ng for *S. vesicarium*, the molecular tools 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 on 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. The molecular diagnostic has numerous positive characteristics, including rapidity, specificity, sensitivity, and ease of interpretation, which allows for its application in the detection of seedborne pathogens (Mancini et al. 2016; Vannacci et al. 2014; Walcott 2003; Ward et al. 2004).

6. Conclusion

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 that just the fruit selection is not sufficient to reduce the amount of seedborne pathogen inoculum. Therefore, it is very important for the farmers to select healthy plants, and fruits, which can be very important for producing high-quality seeds and a critical step in the management strategies for sustainable agriculture.

CHAPTER 3

Black rot of squash (*Cucurbita moschata*) caused by *Stagonosporopsis cucurbitacearum* reported in Italy

OPEN ACCESS

Citation: Moumni M., Mancini V., Allagui M.B., Murolo S., Romanazzi G. (2019) Black rot of squash (*Cucurbita moschata*) caused by *Stagonosporopsis cucurbitacearum* reported in Italy. *Phytopathologia Mediterranea* 58(2): 379-383. doi: 10.14601/Phytopathol_Mediter-10624

Accepted: April 3, 2019

Published: September 14, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Competing Interests: The Author(s) declare(s) no conflict of interest.

Editor: Josep Armengol Forti, Polytechnical University of Valencia, Spain.

Black rot of squash (*Cucurbita moschata*) caused by *Stagonosporopsis cucurbitacearum* reported in Italy

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Summary. *Stagonosporopsis cucurbitacearum* (syn. *Didymella bryoniae*) can affect cucurbits through induction of black rot. This pathogen produces irregular white spots covered with pycnidia on infected cucurbit fruit. Twenty squash fruit (cv. Butternut) with black rot symptoms were collected in Italy from two locations: Osimo (AN) and Montacuto (AN), in the Marche region. Several fungal colonies were isolated from these fruit, the morphological features of which corresponded to *S. cucurbitacearum*. This identification was confirmed using multiplexing of three microsatellite markers and by sequence analysis using internal transcribed spacers. The sequence identity for the internal transcribed spacer regions was greater than 98% compared with sequences of *S. cucurbitacearum* in the NCBI database. This is the first report of *S. cucurbitacearum* on *Cucurbita moschata* fruit with black rot symptoms in Italy.

Keywords. Butternut squash, black rot, ITS sequencing, microsatellite markers.

1. Abstract

Stagonosporopsis cucurbitacearum (syn. *Didymella bryoniae*) can affect cucurbits through induction of black rot. This pathogen produces irregular white spots covered with pycnidia on infected cucurbit fruit. Twenty squash fruit (cv. 'Butternut') with black rot symptoms were collected in Italy from two locations: Osimo (AN) and Montacuto (AN), in the Marche region. Several fungus colonies were isolated from these fruit, the morphological features of which corresponded to *S. cucurbitacearum*. This identification was confirmed using multiplexing of three microsatellite markers and by sequence analysis using internal transcribed spacers. The sequence identity for the internal transcribed spacer regions was greater than 98% compared with sequences of *S. cucurbitacearum* in the NCBI database. This is the first report of *S. cucurbitacearum* on *Cucurbita moschata* fruit with black rot symptoms in Italy.

Key words: Butternut squash; black rot; ITS sequencing; microsatellite markers

2. Introduction

Black rot (BR) is caused by the fungal pathogen *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (Aveskamp et al. 2010) (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, which is one of the most important pathogens on cucurbits worldwide (Li et al. 2015; Yao et al. 2016). *Stagonosporopsis cucurbitacearum* and *S. citrulli* can infect several species of Cucurbitaceae, including watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) (Rennberger and Keinath,2018; Babu et al. 2015; Huang and Lai 2019), muskmelon (*Cucumis melo* L.) (Nuangmek et al. 2018), squash (*Cucurbita maxima* L., *Cucurbita moschata* Duch) (Keinath 2014), and pumpkin (*Cucurbita pepo* L.) (Grube et al. 2011). These fungi can cause infection of the stems, leaves, roots, seeds, and fruit of these host plants (Keinath 2011).

Infected fruit manifest large irregular-shaped spots and black rot (Choi et al. 2010). Fruiting bodies are found in the oldest parts of lesions, because *S. cucurbitacearum* is a necrotrophic fungus (Keinath 2014). Keinath (2011) reported that *S. cucurbitacearum* produces black mycelia inside melon and giant pumpkin (*C. maxima*) fruit. The ideal conditions for disease development include humidity greater than 90%, leaf wetness and temperatures from 16 to 24°C (Park et al. 2006; Seebold 2011). BR can reduce preharvest and postharvest yields (de Neergaard 1989), and cause up to 15% fruit loss (Keinath 2000). *Stagonosporopsis cucurbitacearum* has only been reported once in Italy, in 1885 on *C. melo* L., and it was described as *Didymella melonis* Pass. by Giovanni Passerini (Corlett 1981). Our investigations aimed to identify the causal agent of black rot symptoms on squash fruit.

3. Materials and methods

3.1. Fungal isolation

Twenty fruit of squash (*C. moschata*; cv. 'Butternut') with symptoms of black rot were collected from Osimo (AN) and Montacuto (AN), in the Marche region of Italy in September and October 2018. Small infected pieces of squash peel (≈2 mm) were cut from the fruit, sterilized with 1% sodium hypochlorite solution for 2 min, washed three times with sterilized distilled water, placed into 90 mm diam. Petri dishes containing water agar (Bacteriological agar; Liofilchem), and incubated at 24°C. After 7 d, pycnidia were excised from developing fungus colonies, placed into Petri dishes containing potato dextrose agar (Liofilchem), and incubated at 24°C. Identification of the fungus was carried out according to the colour and shape of the colonies, and to the size of the conidia produced from pycnidia (50 conidia measured).

3.2.DNA amplification and phylogenetic studies

The fungal genomic DNA was extracted from 100 mg of mycelia of isolates grown on potato dextrose agar as pure cultures, following the protocol proposed by Varanda et al. (2016). The DNA was amplified in a rapid PCR based assay for distinguishing the three morphologically similar species (*S. cucurbitacearum*, *S. citrulli*, and *S. caricae*) by multiplexing of the three microsatellite markers *Db01*, *Db05* and *Db06* (Brewer *et al*., 2015). The primer pairs ITS_1 and ITS_4 (White *et al.*, 1990) were then used to amplify the internal transcribed spacers (ITS). The PCR products were separated on 1.5% agarose electrophoresis gels, stained with Red Gel (Biotium), and visualized, with images captured using an imagining system (Gel Doc XR; BioRad). Selected PCR amplicons were purified and sequenced by Genewiz, and the sequences have been deposited with Genebank (accession numbers: isolates ID1, MK330934; ID3, MK330935; ID9, MK330936; for ITS regions). The nucleotide sequences were subjected to Blast analysis to determine the relative similarities with other sequences available in Genebank.

4. Results and discussion

In the two surveyed locations in Italy, black rot symptoms occurred on butternut squash fruit. The initial symptoms were brown circular spots with exudates on the fruit surfaces (Figure 15A). Over time, the spots became white and were covered with black fruiting bodies (Figure 15B, C). After 8 d incubation on water agar, some pycnidia were seen (using a stereomicroscope) to be developing in rows on the fruit peel (Figure 16A). On crushing the pycnidia, the conidia inside were cylindrical, mostly non-septate and a few one-septate, and measuring 4 to 11 µm × 2 to 5 µm (Figure 16B, C). The isolates on potato dextrose agar showed white mycelia on the colony upper surfaces top and black mycelia on the undersides. These morphological characteristics coincided with those known for *S. cucurbitacearum* (Choi et al. 2010; Keinath 2013; Keinath et al. 1995; Koike, 1997).

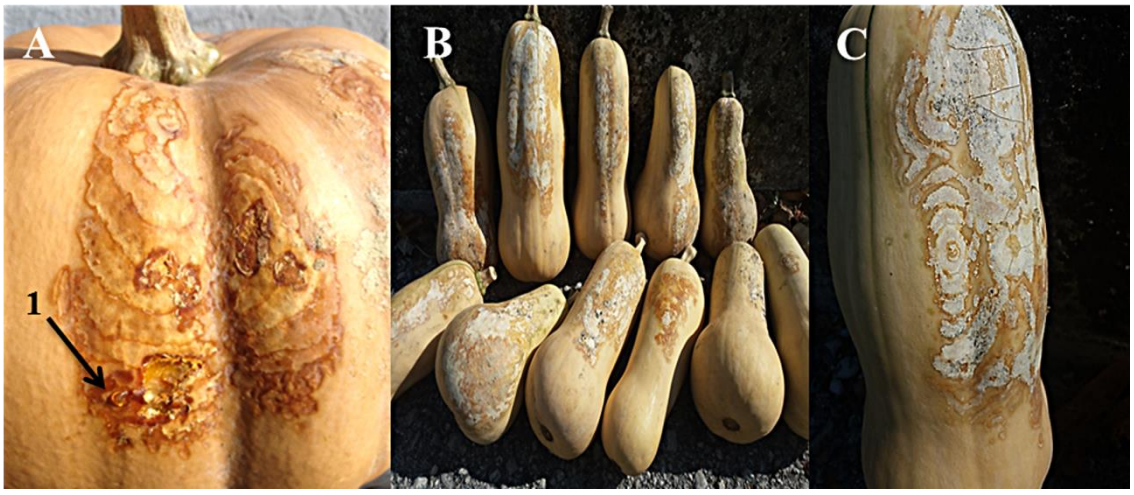

Figure 15. Typical symptoms of black rot caused by *Stagonosporopsis cucurbitacearum* on butternut squash fruit. A, Infected fruit showing exudate (arrow 1). B and C, Irregularly circular and white spots covered by pycnidia.

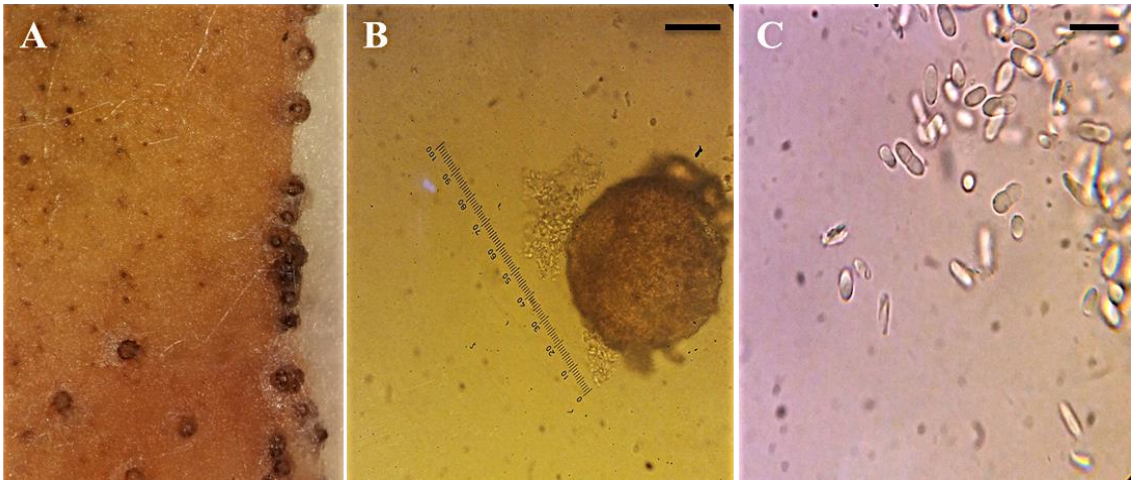

Figure 16. Morphological characteristics of *Stagonosporopsis cucurbitacearum*. A, Row of pycnidia on peel from a squash fruit under the stereomicroscope. B, Pycnidia under the microscope. Scale bar = 50 µm. C, Aseptate and one-septate pycnidiospores. Scale bar = 10 µm.

Morphological identification was supported by the multiplex amplification using the primers *Db01*f/r, *Db05* f/r and *Db06* f/r, which yielded two amplicons (220 bp and 280 bp), characteristic for *S. cucurbitacearum*. The presence of an amplicon of 280 bp and the lack of an amplicon of about 360 bp indicated that three isolates (ID1, ID3, and ID9) were *S. caricae* or *S. citrulli*, as reported by Brewer *et al*. (2015) (Figure 17). Blast analysis showed 98% to 99% similarity for the ITS regions compared to other sequences of *S. cucurbitacearum* already in the NCBI database, as shown in Table 11. Therefore, the isolates ID1, ID3, and ID9 from butternut squash are confirmed as *Stagonosporopsis cucurbitacearum*.

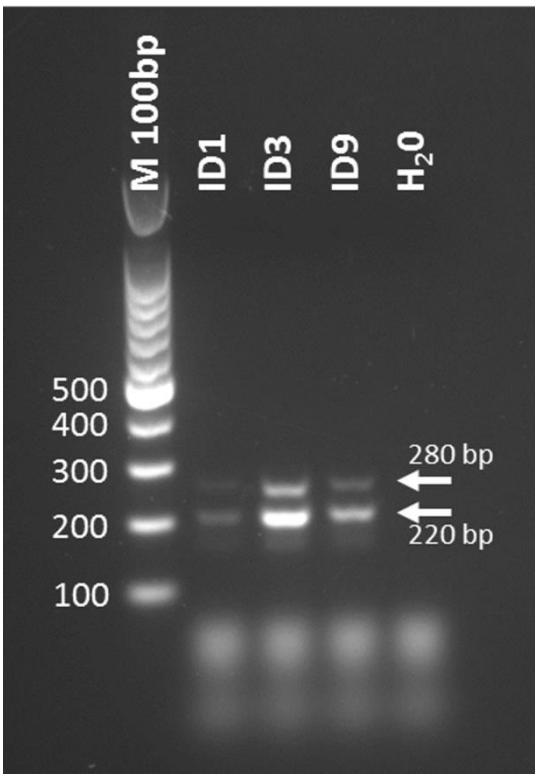

Figure 17. Polymerase chain reaction (PCR)-based marker for distinguishing *Stagonosporopsis cucurbitacearum*. Lane M is a 100-bp ladder with sizes of visible fragments indicated. Three fungal isolates (ID1, ID3 and ID9) from butternut squash fruit were analyzed with PCR-based markers using three sets of primers (Db01, Db05 and Db06) in a single reaction. Two amplicons of 220 and 280 bp were produced and no fragment of 360 bp was visible, despite the presence of the microsatellite locus Db01.

Table 11. Comparison of sequence similarities of *Stagonosporopsis cucurbitacearum* isolates with sequences already in the NCBI database.

Fungal species	Isolate number	NCBI accession no.	Nucleotide similarity (%)	Query cover
S. cucurbitacearum	ID1	EU167573	99%	100%
S. cucurbitacearum	ID3	MG009202	98%	100%
S. cucurbitacearum	ID9	KF990402	99%	99%

Stagonosporopsis spp. is a major pathogen of cucurbits worldwide, and it occurs everywhere these crops are grown (Mancini et al. 2016; Nuangmek et al. 2018; Stewart et al. 2015).

Gummy stem blight and BR can affect all part of cucurbit plants, including stems, leaves, roots, seeds, and fruit. This pathogen is seed- and soil-borne, and it can remain for long periods in the seeds and in the soil. Infected seed has continued to spread the pathogen around the world (Keinath 2011). Seed-borne pathogens can reduce of the quantity and quality harvested fruits and/or seeds, and their management is crucial for profitable production (Mancini et al. 2014). On cantaloupe, field losses due to *S. cucurbitacearum* can reach 100% under conditions conducive to infection (Nuangmek et al. 2018), and on watermelon, Gummy stem blight and BR can cause significant production losses, both in the field and post-harvest (Maynard and Hopkins 1999). No commercial cultivar of any cucurbit species has resistance to Gummy stem blight (Keinath 2017).

5. Conclusion

Somai et al. (2002) have already reported *S. cucurbitacearum* for butternut squash in the United States of America. In Italy, this pathogen has been reported on *C. melo* (Corlett 1981). To our knowledge, this is the first report of *Stagonosporopsis cucurbitacearum* on squash in Italy.

CHAPTER 4

PCR detection and transmission of *Stagonosporopsis cucurbitacearum* in squash (*Cucurbita maxima*) seed

1. Abstract

Stagonosporopsis cucurbitacearum is an important and serious seedborne disease of squash (*Cucurbita maxima*). Sanitary control of seed is necessary to limit the spread of this pathogen. Thirty asymptomatic and symptomatic squash fruit showing or not specific symptoms were collected from 9 fields in north-west of Tunisia. Blotter method, based on morphological identification of the fungus was carried out to determine the incidence of *S. cucurbitacearum* on seeds extracted from symptomatic and asymptomatic fruits. A polymerase chain reaction (PCR) based diagnostic assay was developed and species-specific primers were designed for the detection of *S. cucurbitacearum* in squash seed. The assay detected infested seed sample down to an incidence of 1.5%. Using the conventional PCR method, 17 seed samples were tested for *S. cucurbitacearum* localization. The results showed that the pathogen was detected in all the seed parts, such as tegument, cotyledon, and embryo. Naturally infected seeds were subjected to transmission studies. Significant regression and high correlation (R^2= 0.78; P<0.001) between the pathogen incidence within seeds and seedling mortality. Also, there was a significant positive correlation (R^2= 0.74; P<0.001) between the seed infected with *S. cucurbitacearum* and the incidence of infected plantlets. No significant correlation was observed between infected seeds and pre-emergence mortality (R^2=0.06; P= 0.336). Infected seed in asymptomatic fruit can provide an important source of inoculum for introducing the pathogen and initiating epidemics in areas where the pathogen did not occur previously. Further research is required to better understand the mechanism through which the fungus invades inner parts of squash seeds.

Keywords: blotter method, PCR detection, *Stagonosporopsis cucurbitacearum*, transmission.

2. Introduction

In 2017, the annual worldwide production of cucurbit crops (cucumber, gherkins, melon, pumpkins, squash and gourds) was 261.6 millions tons of which 830.000 tons produced in Tunisia (FAO 2019). The seeds used for assuring these annual important productions could be from seed companies or self-produced in farm carrying in many cases different pathogens that act as a primary inoculum of disease of these crops.

Gummy stem blight (GSB) and black rot (BR) are re-emergent diseases of cucurbits in several countries caused by three species which are *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, *Stagonosporopsis caricae* (Syd. & P. Syd.) Aveskamp, Gruyter & Verkley (synonym *Mycosphaerella caricae* Syd. & P. Syd.), and *Stagonosporopsis citrulli* M.T. Brewer & J.E. Stewart (Stewart et al. 2015). *S. cucurbitacearum* and *S. citrulli* can affect *Cucurbita*, *Cucumis*, and *Citrullus* genera, whereas *S. caricae* infects also papaya. These three species of *Stagonosporopsis* are found on at least 24 species of cucurbits (Keinath 2011; Rennberger and Keinath 2018).

Cucurbita spp. are especially susceptible to BR, whose symptoms appear on the fruits as white circular lesions covered by black fruiting bodies (Keinath et al., 1995; Moumni et al, 2019). BR can decrease the fruit yield both in preharvest and postharvest (Keinath et al. 1995) and the economic losses can reach 100% in watermelon production (Jensen et al. 2011). Nuangmek et al. (2018) reported that, under conditions conducive to S. *cucurbitacearum*, losses in cantaloupe can reach 100%. GSB is a serious foliar disease, whose symptoms on cucurbit plants are necrotic lesions on cotyledons, leaves, petioles, hypocotyls, and stems. In addition, GSB could appear as cankers on crown and stem. Such symptoms are common on pumpkin, squash, bottle gourd, cucumber, watermelon, and muskmelon. Humidity over 90% and temperatures from 16 to 24 °C increase the risk of epidemic (Park et al. 2006; Seebold 2011). *S. cucurbitacearum* is homothallic in nature (Punithalingam and Holliday 1972) propagated to plant hosts as airborne, soilborne or seedborne pathogen (Keinath 2011). Hence, a better knowledge of seed infestation/infection process by this seedborne fungus is required to prevent introduction and release of inoculum. Among the various pathways of seed infection, De Neergaard (1989) provides evidence of the mode of transmission through seed and fruit by showing S. *cucurbitacearum* invades *Cucumis sativus* through the stigma and style of the flower.

Accordingly, studies on cucurbits and *S. cucurbitacearum* were interested in the localization on the seed and showed that the pathogen was found mainly in the seed coats, then in cotyledon and in a lesser extend in embryo (Lee et al. 1984; Sudisha et al. 2006). More studies are needed to understand how this pathogen is localized on/in the seed and what is their incidence on the future plant growth. In addition, early diagnosis, sensitive specific and rapid detection of *S. cucurbitacearum* is very important to limit the spread of GSB. Currently, polymerase chain reaction (PCR), and PCR-enzyme-linked immunosorbent assay (PCR-ELISA) were the common methods used for the rapid detection of *S. cucurbitacearum* (Keinath et al. 2001; Somai et al. 2002). Specific primers set (RGI and RGII) have been used by Somai et al. (2002) to detect two genotypes of *S. cucurbitacearum*, the primer set was only specific for detection of isolates of *S. cucurbitacearum*, but was not tested in a direct detection from seedling or seed (Yao et al. 2016). In this study, specific primer to dected this pathogen on squash seed will be designed.

The objective of this study were (i) to estimate the incidence of *S. cucurbitacearum* on seeds extracted from symptomatic and asymptomatic fruits; (ii) to design species-specific primer for detection of *S. cucurbitacearum* on seeds; and (iii) to investigate the relationship between seed infection and subsequent plantlets disease.

3. Materials and methods

A total of 30 squash fruits (*C. maxima*, cv. Bjaoui), with or without symptoms of *S. cucurbitacearum*, were collected from 9 fields in the north-west of Tunisia in October of three consecutive years (2015, 2016 and 2017) (Table 12).

Table 12. Incidence of *Stagonosporopsis cucurbitacearum* detected on 30 seed samples extracted from asymptomatic and symptomatic fruits in 2015, 2016, and 2017.

Years	Farmer	Code samples	Fruit symptoms*	Incidence (%) of *S. cucurbitacearum* on seeds		Results of morphologic detection
				Mean	Range	
2015	Field 1	T4	A	44.0	10.0-80.0	Moumni et al. 2020
		T82	A	50.0	10.0-70.0	This study
		T12	A	4.4	0.0-50.0	This study
	Field 2	T7	S	57.0	10.0-90.0	Moumni et al. 2020
	Field 3	T8	S	21.5	0.0-70.0	Moumni et al. 2020
	Field 4	T9	S	62.0	30.0-90.0	Moumni et al. 2020
		T5	A	4.2	0.0-50.0	This study
	Field 5	T13	A	4.0	0.0-20.0	Moumni et al. 2020
	Field 6	T14	A	4.5	0.0-20.0	Moumni et al. 2020
	Field 7	T18	A	0.0	0.0	Moumni et al. 2020
		T19	A	9.0	0.0-30.0	This study
		T11	A	29.2	0.0-83.3	This study
	Field 8	T22	A	3.5	0.0-30.0	Moumni et al. 2020
	Field 1	T83	S	49.0	10.0-100	This study
2016		T84	S	26.5	0.0-60.0	This study
		T85	A	65.4	30.0-100	This study
		T94	A	9.0	0.0-40.0	This study
	Field 5	T86	A	25.0	10.0-40.0	This study
		T92	S	63.0	20.0-100	This study
	Field 6	T87	A	11.0	0.0-40.0	This study
		T91	S	10.0	0.0-70.0	This study
		T95	S	15.0	0.0-40.0	This study
	Field 7	T89	A	2.0	0.0-10.0	This study
		T93	A	6.1	0.0-50.0	This study
	Field 9	T90	A	1.5	0.0-20.0	This study
2017	Field 5	T96	S	11.0	0.0-30.0	This study
	Field 6	T98	A	1.5	0.0-10.0	This study
	Field 7	T97	S	1.5	0.0-10.0	This study
		T100	S	26.0	0.0-60.0	This study
	Field 9	T99	A	8.0	0.0-30.0	This study

* A= Asymptomatic fruits; S= Fruits with symptoms of *S. cucurbitacearum*

3.1. Morphological identification

3.1.1. Fungal detection by blotter method

Each seed sample was analyzed to verify the presence of *S. cucurbitacearum* using the standard blotter method of the International Seed Testing Association (ISTA) (Mathur and Kongsdal 2003). Two hundred seeds per sample were soaked during 3 min in 1% sodium hypochlorite solution then triple-rinsed with sterile distilled water. The seeds were dried under laminar flow hood on sterile paper towels for 2 minutes. Ten seeds were placed in a glass Petri plate (110 mm) on eight overlapping sterile layers (Whatman no. 4 filter papers), moistened with 5 ml of sterile distilled water, and incubated at 25 ºC under 12h/12h day/night cycle (MASTER TL-D Super 80 58W/830). For each sample, 20 Petri plates containing each 10 seeds were used. From the 7^{th} to the 15^{th} day after plating, all the plates were examined daily under a stereomicroscope to verify the presence of *S. cucurbitacearum* fungal structures. The pycnidia on the seeds were excised and examined under a compound microscope for morphological identification. For each sample, the percentage of seed infected by *S. cucurbitacearum* was calculated. The fungal structures on seeds, morphologically identified as *S. cucurbitacearum*, were transferred on potato dextrose agar (PDA, Liofilchem Srl, Roseto degli Abruzzi, Italy) in Petri dishes. After 10 days at 23 ± 2 °C in PDA, morphological identification was carried out according to the color and shape of the colonies, and the characteristics of the pycnidia and spores.

3.2. Molecular identification

3.2.1. DNA extraction from purified fungi

The mycelia of each isolate (Table 13) of *S. cucurbitacearum* (10-day-old) were harvested and lyophilized. DNA was extracted from 100 mg of lyophilized mycelia, folowing the protocol proposed by Varanda et al. (2016).

Table 13. Sources of fungal species and code of isolates.

Species	**Code of Isolates**	**Host**	**Source**	**Year of isolation**
Stagonosporopsis cucurbitacearum	D33	squash	Siliana,Tunisia	2015
S. cucurbitacearum	D29	squash	Siliana,Tunisia	2015
S. cucurbitacearum	D49	squash	Siliana,Tunisia	2015
S. cucurbitacearum	12016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	22016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	32016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	42016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	52016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	62016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	72016	squash	Siliana,Tunisia	2016
S. cucurbitacearum	12017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	22017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	32017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	42017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	52017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	62017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	72017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	82017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	92017	squash	Siliana,Tunisia	2017
S. cucurbitacearum	102017	squash	Siliana,Tunisia	2017
Alternaria alternata	A38	squash	Monastir,Tunisia	2015
Albifimbria verrucaria	M144	squash	Siliana,Tunisia	2016
Paramyrothecium roridum	M123	squash	Siliana,Tunisia	2016
Fusarium solani	F174	squash	Kasserine,Tunisia	2015
Curvularia spicifera	B170	squash	Siliana,Tunisia	2016
Stemphylium vesicarium	P164	squash	Ariana,Tunisia	2015
Phoma sp.	Ph39	squash	Siliana, Tunisia	2015
Fusarium oxysporum	F59	squash	Siliana, Tunisia	2015

3.2.2. DNA extraction from seeds

Total DNA was extracted from 17 samples of squash seeds proven infested by *S. cucurbitacearum* and 2 healthy seed samples (Tu1 and Tu2) from local market analysed by conventional blotter assay. One hundred seeds/sample were ground with liquid nitrogen, and then 100 mg were taken and put in 2 mL microcentrifuge tubes with the addition of 900 µL of CTAB 2% and 20 mg of sodium metabisulphite. The solution was incubated at 65 °C for 20 min, then the samples were centrifuged at 8,000 × *g* for 7 min; the supernatant was then transferred to a fresh tube with 0.5 of volume of phenol and 0.5 of volume of chloroform (2:1:1). The tubes were centrifuged at 10,000 × *g* for 5 min; the aqueous phase was then transferred to a fresh tube with the same volume of chloroform (1:1). The tubes were centrifuged at 12,000 × *g* for 8 min; the supernatant was then transferred to a fresh tube following the addition of 2.5 volumes of 100 % cold EtOH (-20 °C) with 0.1 volume of Sodium Acetate 3M. The samples were gently mixed by inversion and stored overnight at -20°C; then they were centrifuged at 12,000 × *g* for 25min; the liquid solution was removed and the DNA pellet washed with 300 µL of 70 % cold EtOH. The matrix pellet was dried and resuspended in 60 µL of ultrapure water and stored at -20 °C. The quality and quantity of the extracted DNA was directly checked using BioPhotometer (Eppendorf, Hamburg, Germany) and finally diluted to 20 ng/µL for further amplification.

3.2.3. DNA extraction from seedling parts

Healthy and infected squash seedlings, germinated from seeds placed on blotter paper and incubated under lights for 15 days, were collected keeping aside the seed coats. These seedlings were cut into three parts: root, hypocotyl and cotyledon. The DNA extraction was done from the seed coat and each part of seedling, following the same protocols described above for DNA extraction from seed.

3.2.4. *S. cucurbitacearum* localization in squash seed

The method used to localize *S. cucurbitacearum* in seed was as given by Sudisha et al. (2006) with some modification. Thirty seeds for each of the 17 samples were soaked in distilled water for 24 h. The soaked seeds were then dissected into three parts, cotyledon, coat and embryo, using sterilized forceps and scalpels under laminar flow hood. Then, the DNA extraction was done from each part, following the same protocol described above for DNA extraction from seed.

3.2.5. Design of species-specific primers for *S. cucurbitacearum*

In a previous study, based on random amplification of polymorphic DNA (RAPD) markers, Ling et al. (2010) developed sequence characterized amplified regions (SCAR) primers (DB17 primer set) with broad-spectrum specificity that amplified a conserved sequence region common to both genotypes (RGI and RGII) of *S. cucurbitacearum*. This SCAR, common to both genotypes of *D. bryoniae*, was identified in PCR products generated using the DB17 primer set (Ling et al., 2010). According to this conserved SCAR (GenBank accession n. GQ872461 and GQ872462), a set of inner primer pair (DBF1/DBR1) (Table 14) was designed by Primer3Plus software. The primers were synthetized and purified by Sigma–Aldrich.

Table 14. Characteristics of the species-specific primers designed for *Stagonosporopsis cucurbitacearum*

Primer name	Sequence (5' to 3')	Tm (°C)	Bases	GC content (%)
DB F1	5'-TCGAATGGCTCAGAGAAGGT-3'	63.9	20	50
DB R1	5'-AAGTCCACGTCAGACCCATC-3'	64.1	20	55

3.2.6. PCR assay

To avoid the existence of false negatives and estimate the specificity, the primer pair DBF1/R1 was firstly evaluated by a bioinformatics approach that included a BLASTn query at NCBI database. Then, the specificity was tested in PCR using as template the DNA extracted from *S. cucurbitacearum* (isolates D49, D33, D29, 12016, 42017), and *Phoma* sp. (isolate Ph39), *Alternaria alternata* (isolate A38), Fusarium solani (isolate F174), F. oxysporum (isolate F59), *Curvularia spicifera* (isolate B170), *Paramyrothecium roridum* (isolate M123), *Albifimbria verrucaria* (isolate M144), *Stemphylium vesicarium* (isolate P164).

The PCR amplifications were performed in 25 µL reaction mixture that contained 2 µL genomic DNA (about 20 ng/µL) of the fungal isolate, 200 µM dNTP mixture, 0.5 µM each primer, 1.2 mM $MgCl_2$, 1.25 U Taq polymerase (Promega). PCR was carried out in the MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) programmed for the following parameters: initial denaturation at 95°C for 2 min, followed by 30 cycle for mycelia

and 40 cycles for seeds of denaturation at 95°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 30 sec. A final extension at 72° for 7 min was performed at the end of the amplification. The PCR products (9 µL per sample) were fractionated by electrophoresis in 1.5% agarose gel stained with Red Gel (Biotium, Hayward, CA, USA), and visualized and captured using the Gel Doc XR imagining system (BioRad).

3.2.7. Sensitivity of *S. cucurbitacearum* primer pair

To determine the limit of detection of the primer set, PCR was carried out with serially diluted (40 ng to 1×10^{-7} ng) DNA extracted from seed sample highly infested (T85, 68% infestation). Thirteen seed samples, with different levels of *S. cucurbitacearum* infestation (ranged from 0% to 56%) were used to assess the limit of detection of the PCR-based seed assay.

3.3. Seed transmission of *S. cucurbitacearum*

Fifteen seed samples, with a range of *S. cucurbitacearum* incidence from 2.0 to 65.4 %, were used in transmission studies. Sixty seeds from each tested sample were sown on autoclaved soil in sterile speedling trays. All trays were incubated at room temperature (22 ± 4 ºC). After 20 days the first visual inspection was done, checking: (i) asymptomatic seedlings; (ii) symptomatic seedlings and (iii) dead seedlings. Then, all affected seedlings were collected and examined under stereomicroscope (Leica M125, Leica Microsystems CMS, Wetzlar, Germany) to verify the presence of *S. cucurbitacearum* pycnidia. Each affected seedling was sliced in three parts, root, hypocotyl and cotyledon, using flame-sterilized scalpels. Each part was surface-disinfected in 1% sodium hypochlorite for 2 min, triple-rinsed in sterile distilled water, and dried on sterile blotter papers. Each part was cut in pieces of about 2 mm, plated on PDA and after 7 days of incubation at 25 °C morphological identification was carried out. A second inspection was done ten weeks after planting, collecting five types of samples: (i) non-germinated seed, (ii) affected seedling, (iii) healthy-looking plantlets, (iv) plantlets with symptoms, and (v) seed coats. Number of leaf and length of plantlets were measured. Symptomatic and asymptomatic plantlets, seeds and seed coats were examined under stereomicroscope, and then surface sterilized and put to incubate on PDA. The plantlets were cut in four parts, roots, stems, cotyledon and true leaf, as described above for seedlings, before putting them on PDA.

4. Statistical analysis

Analysis of variance was calculated using SPSS (version 20). Data were analyzed by analysis of variance (ANOVA), Means were compared using Fisher's Test protected least significant

difference at $P \leq 0.05$. Pearson correlations were computed to investigate interactions between percentage of seed contamination and infected seedling, incidence of infected plantlets, number of true leaves, and length of plantlets.

5. Results

5.1. Morphological identification

5.1.1. Symptom of *S. cucurbitacearum* and incidence in squash seed

Symptoms of black rot were obeserved in 19 squash fruits to a total of 30 fruits examined. The symptoms were irregular circular white areas on skin of the fruit. Older lesions were dried, cracked and covered by black pycnidia. Some pycnidia were also seen after removing the skin below the symptoms (Figure 18). The level of seed contamination varied in relation to the fruit from which the seeds were taken, if symptomatic or not. Seed samples harvested in 2016, T85 from symptomatic fruit and T92 from asymptomatic fruit, showed the highest incidence, 65.4% and 63% respectively. The infection rates, in the seeds obtained from asymptomatic fruit, varied from 0% to 65.4%, while those from symptomatic fruit from 1.5% to 63% (Table 12).

Figure 18. Symptoms of black rot caused by *Stagonosporopsis cucurbitacearum* on squash fruits. A, bleached large spots (arrow 1). B, C, D, E, and F, white irregular circle (arrow 2). G, black pycnidia below the symptoms (arrow 3). H, irregular circular spots cover by pycnidia (arrow 4). I, pycnidia cover the symptomatic fruit surface under stereomicroscope (arrow 5).

5.2. Molecular identification

5.2.1. Specificity of primers

The species-specific primer pair DB F1/R1 was designed for the molecular identification of *S. cucurbitacearum*. DB F1/R1 amplified a specific fragment of 208 bp of five isolates of *S. cucurbitacearum* (Figure 19). No amplification was recorded in water control and in the other DNA samples belonging to the main genus of fungi potentially infecting squash fruits and seeds, reported in Table 13. Furthermore, it was able to identify the pathogen in 20 ng/µl

DNA extracted from 17 naturally infected seed samples, while no amplification was observed for healthy seeds (data not shown). These results confirm the specificity of the designed primers for *S. cucurbitacearum*.

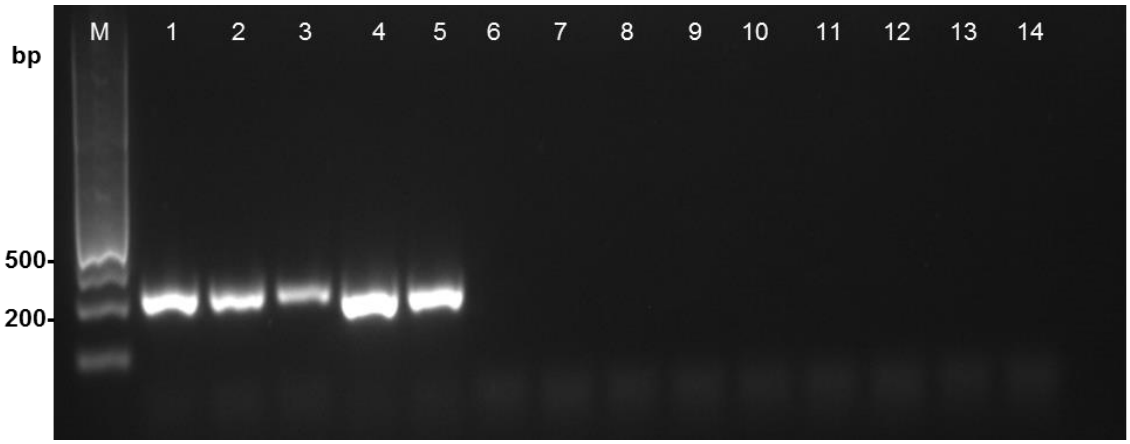

Figure 19. Gel electrophoresis of PCR products using the primers (DBF1/DBR1) for the detection of the *Stagonosporopsis cucurbitacearum*. Lanes 1 to 5: *S. cucurbitacearum* (isolates D49, D33, D29, 12016, 42017, respectively). Lane 6: *Alternaria. alternata* (isolate A38); lane 7: *Fusarium oxysporum* (isolate F59); lane 8: *Stemphylium vesicarium* (isolate P164); lane 9: *Curvularia spicifera* (isolate B170); lane 10: *Fusarium solani* (isolate F174); lane 11: *Paramyrothecium roridum* (isolate M123); lane 12: *Albifimbria verrucaria* (isolate M144); lane 13: *Phoma* sp. (isolate Ph39); lane 14: water control. M: molecular weight markers (100-bp intervals).

5.2.2. Sensitivity of primers and PCR detection

On other hand, the sensitivity of DB F1/R1 primers was evaluated after a series of DNA dilution from the seed sample T85 highly infected by *S. cucurbitacearum* (65.4 %) according to the blotter method. PCR amplification fragments were strongly visualized when DNA concentration of *S. cucurbitacearum* ranged from 40 to 10 ng and slightly spotted with 1 ng. Very high concentration (without dilution of DNA, 1524 ng) and very low concentration (from 1×10^{-1} ng to 1×10^{-7} ng) of DNA didn't give amplification by the primers (Figure 20). These primers were able to amplify DNA from seed naturally contaminated by *S. cucurbitacearum*, till a threshold of 4.5% incidence of the fungus in 100 seeds. The incidence of 1.5% gave rise to a very thin amplicon compared to the amplicon resulted from purified *S. cucurbitacearum* (Figure 21). No amplification was observed in the case of purified *C.*

spicifera and *Phoma* sp. (20 ng). A positive relation is apparent between the incidence of seed contamination and the intensity of the amplicons on gel. The PCR primers consistently showed strong bands for seed samples with incidence ranging from 44 to 65.44%, moderate bands between 21.5 and 29.11%, weak bands between 4.17 and 4.5%, and a very low band with 1.5% infection (Figure 21).

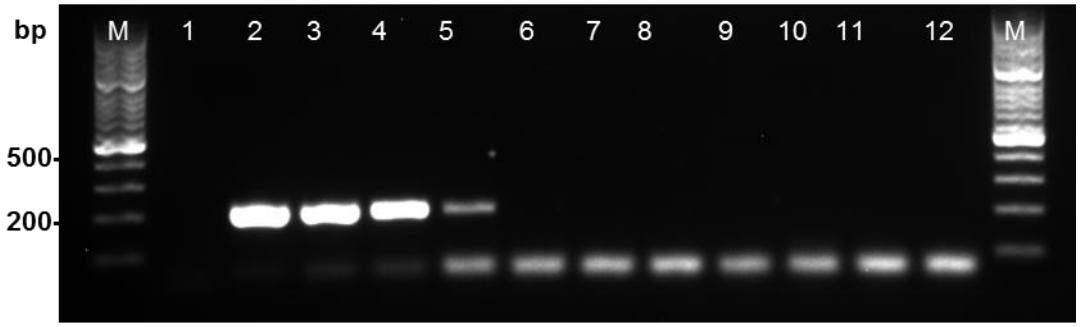

Figure 20. Detection limit of *Stagonosporopsis cucurbitacearum* primer pair (DBF1/DBR1). The primer pair was used in the polymerase chain reaction with serial dilutions of DNA extracted from the seed sample T85 (65.4%, incidence of infestation). Lanes 1 to 12: 1524 ng (DNA without dilution), 40 ng, 20 ng, 10 ng, 1 ng, 1×10^{-1} ng, 1×10^{-2} ng, 1×10^{-3} ng, 1×10^{-4} ng, 1×10^{-5} ng, 1×10^{-6} ng, 1×10^{-7} ng. Lane 12, water control. M: molecular weight markers (100-bp intervals).

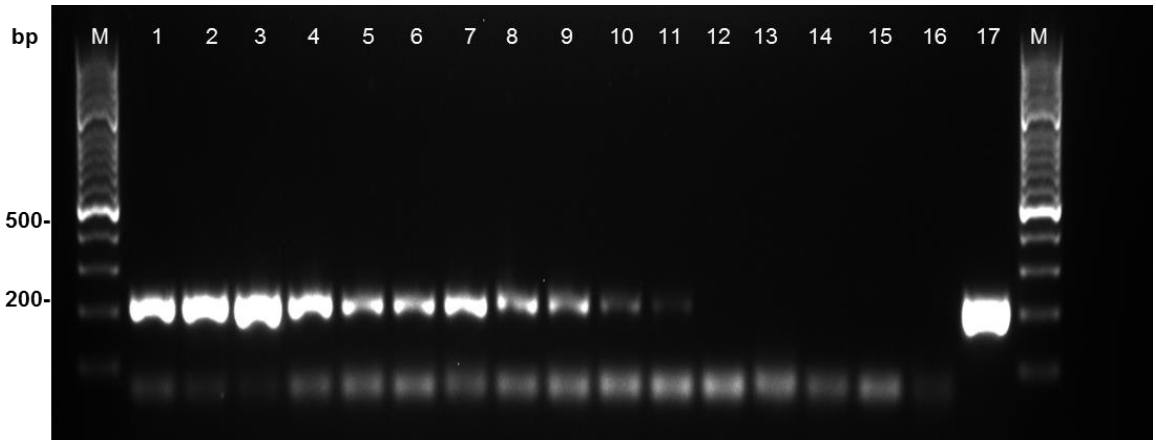

Figure 21. Detection of *Stagonosporopsis cucurbitacearum* from squash seed samples with different levels of infestation using polymerase chain reaction (PCR) with primer pair DBF1/DBR1. Lanes 1 to 11 show DNA of seed samples with incidences of: lane 1, (65.4 %); lane 2, (62 %); lane 3, (44 %); lane 4, (57 %); lane 5, (25 %); lane 6, (21.5 %); lane 7, (29.1 %); lane 8, (4.2 %); lane 9, (4.4 %); lane 10, (4.5 %); lane 11, (1.5 %). Lanes 12 and 13, healthy squash seed (Tu1 and Tu2); lane 14, water control; lane 15, *Curvularia spicifera*

(isolate B170); lane 16, *Phoma* sp. (isolate Ph39); lane 17, *S. cucurbitacearum* (isolate D33). M: molecular weight markers (100-bp intervals).

5.2.3. Pathogen localization in squash seed using PCR method

The contaminated seed samples were tested for the localization of *S. cucurbitacearum* in squash seeds, using the conventional PCR method. *S. cucurbitacearum* was detected in the tegument of all the 17 seed samples, in the cotyledon of 7 seed samples and in the embryo of 3 seed samples (Table 15) (Figure 22).

Table 15. *Stagonosporopsis cucurbitacearum* localization in seed (tegument, cotyledon and embryo) through PCR detection and incidence of pre-emergence mortality.

N° sample	PCR detection for *S. cucurbitacearum* localization in seed[1]			Pre-emergence damping-off (%)
	Tegument	cotyledon	embryo	
T85	+++	-	-	18.3 b
T4	+++	-	-	15.0 b
T83	+++	-	-	3.3 a
T5	++	-	-	NT
T7	++	-	-	6.7 a
T93	++	-	-	0.0 a
T8	++	-	-	1.7 a
T9	++	++	-	NT
T13	++	++	-	5.0 a
T11	++	+	-	18.3 b
T84	++	+	+	20.3 b
T87	++	+	+	21.7 b
T12	++	-	++	15.0 b
T14	+	-	-	1.7 a
T89	+	+	-	NT
T86	+	+	-	NT
T90	+	-	-	3.3 a
T92	NT	NT	NT	3.3 a
T91	NT*	NT	NT	0.0 a
Healthy seeds (Tu1)	-	-	-	0.0 a

[1] -, no amplification; +, weak amplification, ++, moderate amplification; +++, strong amplification. *NT, no tested.

Numbers followed by the same letter within a column are not significantly different based on Fisher's protected least significant difference ($P \leq 0.05$).

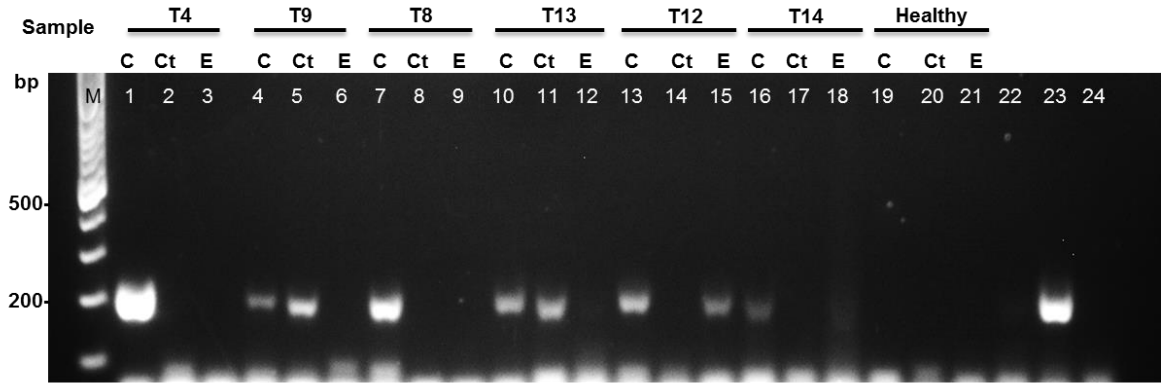

Figure 22. Detection of *Stagonosporopsis cucurbitacearum* in different parts of squash seed using polymerase chain reaction (PCR) with primer pair DBF1/DBR1. DNA of the different seed parts (C, Coat; Ct, Cotyledon; E, Embryon) from contaminated (T4, T9, T8, T13, T12, T14) and healthy (Tu1) seed samples was amplified. Lane 22, *Alternaria alternata* (isolate A38); lane 23, *S. cucurbitacearum* (isolate D49); lane 24, water control. M: molecular weight markers (100-bp intervals).

5.3. Process of *S. cucurbitacearum* transmission from seed to plantlets

The first damage caused by *S. cucurbitacearum* is seed mortality before and after germination (Figure 23 A). Later, symptoms were observed on the cotyledon as brown or gray spots with pycnidia (Figure 23 B-D). Generally, seed coats remain attached to the cotyledon several days after germination. We note that this property favoured progression of the fungus from the cotyledon to petiole and hypocotyle causing by this way seedling mortality (Figure 23 F). As shown in Figure 23 G, the pathogen on the hypocotyl infected the aerial part of the seedling which become dried and covered by black pycnidia. In other cases, the fungus on seed coat yet on cotyledon induced on the hypocotyl bleached a longitudinal burst covered by pycnidia (Figure 23 H-J). This burst widens with time until covering entirely the stem (Figure 23 K), conducing to observe splitting on the stem (Figure 23 L) and on the primary root (Figure 23 M). Gray angular lesions with pycnidia appeared on the leaves (Figure 23 N-O).

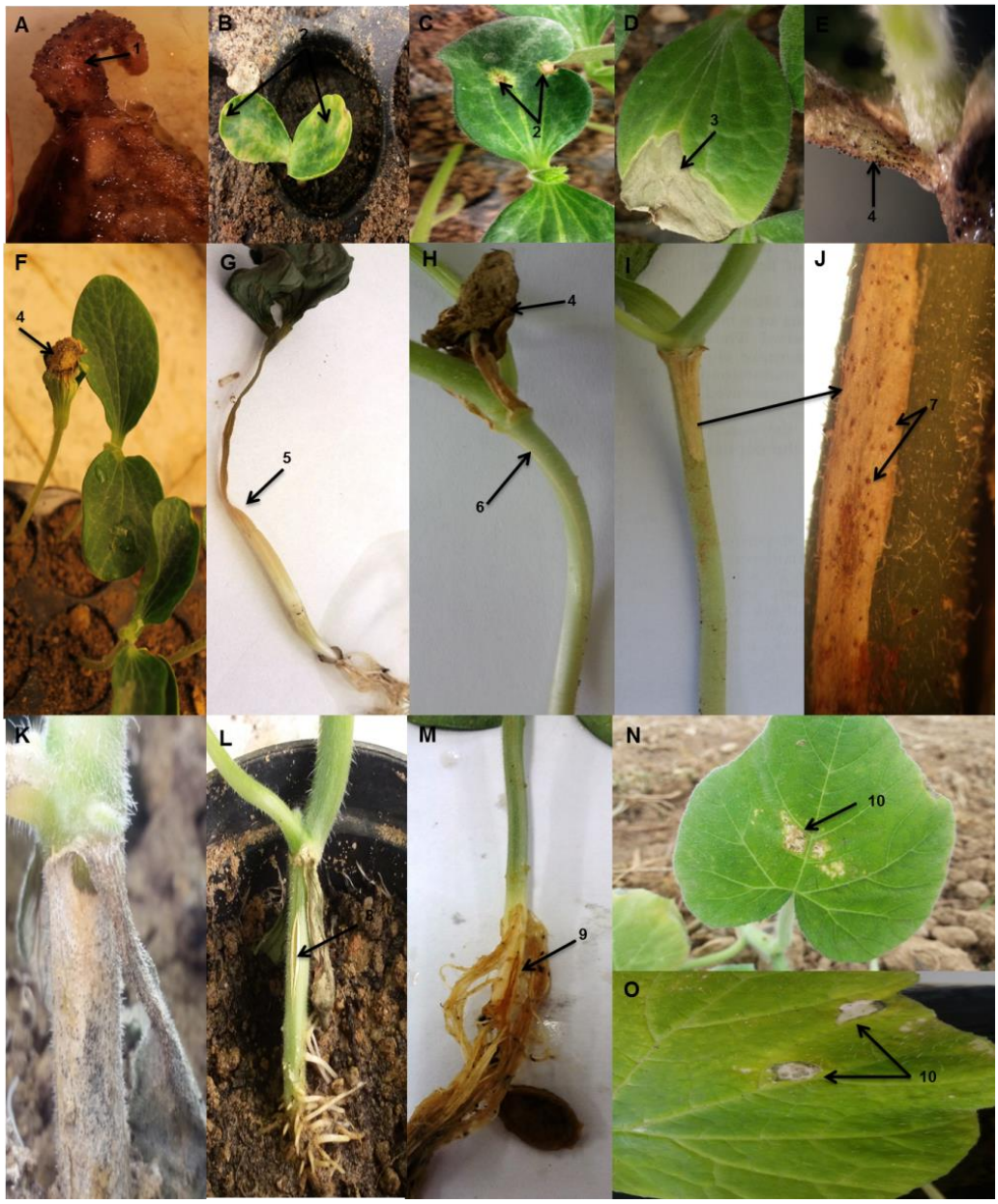

Figure 23. Symptoms of *Stagonosporopsis cucurbitacearum* on seedlings and plantlets. (A) Germinated seed covered with pycnidia. (B, C, D) Brown and gray spots on the seedling cotyledon. (E) Pycnidia on the cotyledon of an infected seedling. (F) Infected seed coat attached to the cotyledon. (G) Fungal stem constriction inducing dryness of the above plantlet part. (H) Infected seed coat attached to the cotyledon and bleached burst on the seedling hypocotyl. (I, J) Widening of the brust covered by pycnidia, as seen under the stereomicroscope (arrow). (K) Tan canker on the stem covered by fruiting bodies. (L, M) Fissured, bleached canker on the stem (arrow) and on the primary root (arrow). (N, O) Angular lesion on the leaves (arrow 10).

Correlation was analyzed between level of infected seeds by *S. cucurbitacearum* and seedling growth criteria. A significant regression and high coefficient of correlation (R^2= 0.78; P≤0.001) identified between the pathogen incidence within seeds and seedling mortality (Figure 24 B). Moreover, there was a significant positive correlation (R^2= 0.74; P≤0.001) between the seed infected with *S. cucurbitacearum* and the incidence of infected plantlets (Figure 24A). Low coefficients of determination occurred between seed infection and other plant growth parameters, length of plantlets and number of true leaves, with R^2 value of 0.34 and 0.35 respectively (Figure 24 C-D). No significant correlation was observed between infected seeds and pre-emergence mortality (R^2=0.06; P=0.336). There was not significantly difference relating to the pre-emergence mortality between the seed sample high infected by *S. cucurbitcearum* (T85) and the low infected one (T12) (Table 15). Likewise, the pre-emergence damping off is important in the seed samples T87, T84, T11, and T12 (21.66, 20.33, 18.33, 14.99%, respectively) where the pathogen was detected in the cotyledon and embryo. Otherwise, the incidence of *S. cucurbitcearum* ranged from 4.41% to 29.17% in these samples (Table 12).

70 days after sowing, 62.2 % of symptomatic plantlets showed typical symptoms caused by *S. cucurbitacearum*. While 36.8% of healthy-looking plantlets were put to incubate on PDA and the results shown that 44% of these plantlets were infected by *S. cucurbitacearum* and seed coats, found on the soil surface or attached to cotyledon and roots of these plantlets, were infested. The pathogen was detected in all parts of plantlets with high incidence in the roots and stems (36.46 and 31.63%, respectively) (Table 16). On other hand, when the seed coats were healthy, the plantlets were healthy. Results were confirmed using PCR assay with the DBF1/DBR1 designed primer pairs, used to detect the presence of *S. cucurbitcearum* in the infected seed coat and all of seedlings parts (roots, stem, and cotyledon) (data not shown).

Table 16. Incidence of *Stagonosporopsis cucurbitacearum* (%) within different parts of plantlet after 70 days of sowing.

Incidence of *S. cucurbitacearum* (%)	**Code samples**															
	T13	**T91**	**T83**	**T11**	**T7**	**T93**	**T92**	**T90**	**T84**	**T14**	**T4**	**T12**	**T8**	**T85**	**T87**	**Total**
Roots	22.0a	38.4a	52.7a	78.5a	56.4a	32.1a	47.3a	7.0a	15.9a	4.51a	75.5a	21.2a	35.7a	58.3a	29.0a	36.5a
Stem	20.3a	24.0ab	48.8a	56.8b	44.3a	17.1b	37.1a	5.4a	19.5a	2.1a	75.3a	15.23ab	29.9a	37.0a	22.3a	31.6a
Cotyledon	15.5a	13.3b	23.2b	43.5bc	43.3a	5.0b	14.3b	1.6a	18.2a	1.7a	28.0b	8.4ab	14.0b	34.8b	20.0a	19.45b
True leaves	14.2a	21.7ab	5.8b	28.3c	18.7c	5.0b	18.3b	5.3a	11.5a	0a	3.7c	3.3b	8.1b	18.6b	15.7a	11.9c

Numbers followed by the same letter within a column are not significantly different based on Fisher's protected least significant difference ($P \leq 0.05$).

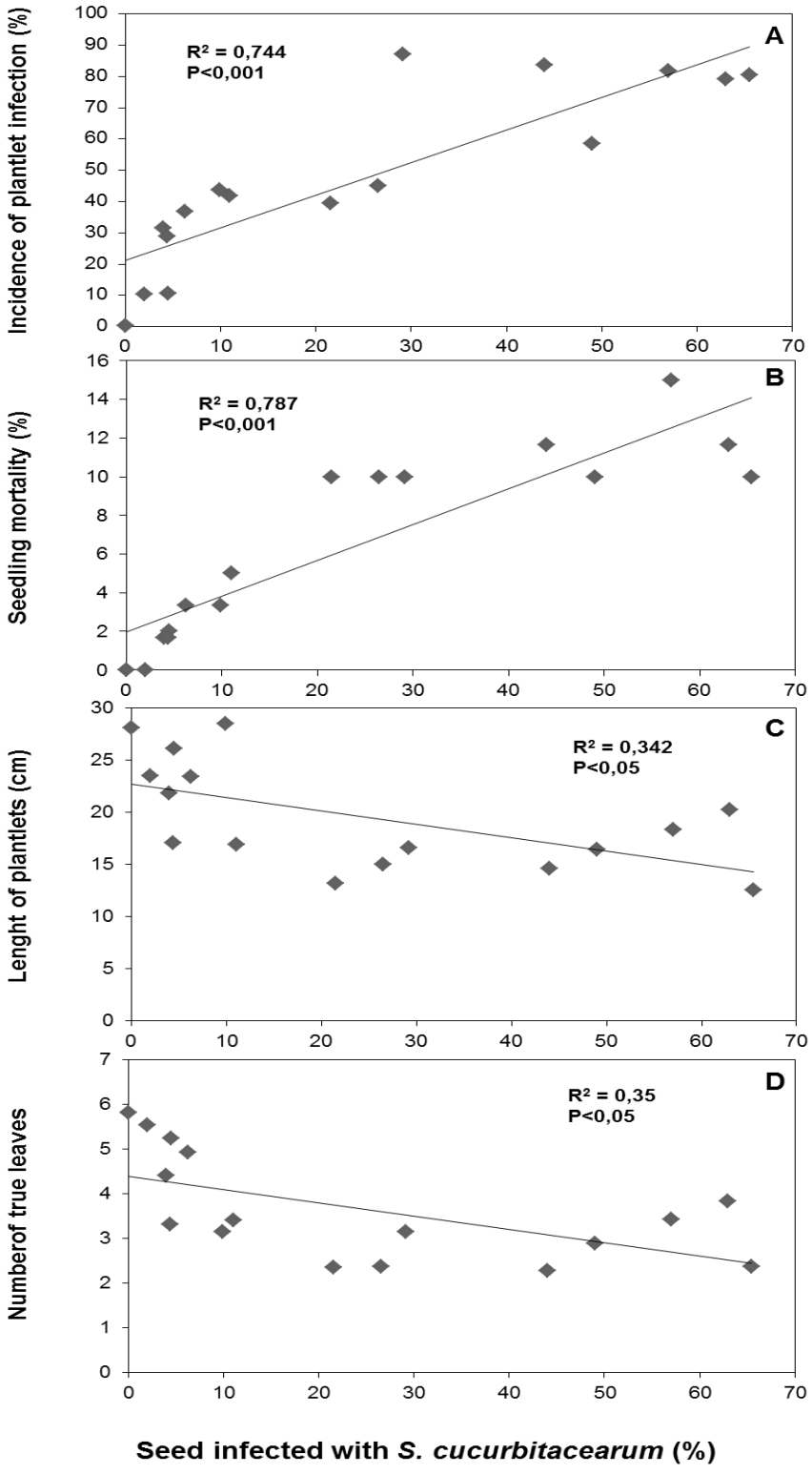

Figure 24. Correlation between level of seed infestation with *Stagonosporopsis cucurbitacearum* and (A) incidence of infected plantlets, (B) seedling mortality, (C) length of plantlets, and (D) number of true leaves when significant ($P \leq 0.05$) regression models were detected.

6. Discussion

Healthy seeds is an essential practice to safeguard the productivity of crops (Zhang et al. 2018). Gummy stem blight caused by *S. cucurbitcearum* is a highly widespread disease and leads to significant losses in yield and quality on cucurbit crops worldwide (Keinath, 2011; Li et al. 2015; Yao et al. 2016). In addition, the use of grafted cucurbits further increases the risk of GSB development from seedborne inoculum. GSB was observed on grafted watermelon in Tunisia, causing severe yield losses (Boughalleb et al. 2007). In 2019, *S. cucurbitcearum* was repoted on squash in Tunisia and Italy (Moumni et al. 2019, Moumni et al. 2020). Therefore, it was necessitated a re-evaluation of the disease epidemiology of this disease including the role of seed as a means for pathogen survival and spread (Brewer et al. 2015). The pathogen reproductive biology is critical for effective disease management.

Our surveys detected a high incidence of *S. cucurbitacearum* in seeds extracted from symptomatic and also asymptomatic fruits. So, we assume that fruit symptom is not usually the criterion by which it is stated the squash seeds are fungal free. This was confirmed by Keinath (2011) and Moumni et al. (2020) working on the pathogen *S. cucurbitacearum* indicating that seeds from apparent healthy fruit gave rise to infected seeds. Blotter method was appropriate in this study to detect *S. cucurbitacearum* in seed and fruit. However, the drawback of this conventional method is that morphological identification requires mycological skills and time-consuming. Morphological characterization of *S. cucurbitacearum* and *Phoma* spp. are similar and the distinction can be difficult (Keinath et al. 1995). In addition, the main problem related to the contamination of seeds with other saprophytic fungi which prevents the development of the pathogen. Hence, several molecular methods have now been published to detect fungal pathogens in various (Lee et al. 2001; Pryor and Gilbertson 2001; Samac et al. 1998). In order to constitute efficient substitutes for more traditional techniques, such methods need to be specific, sensitive, rapid and adaptable to routine analysis. In this study, species-specific primers DBF1/DBR1 were designed to detect in squash seed *S. cucurbitacearum*. This primer set successfully amplified the predicated size of the DNA fragment in infected material. To our knowledge this is the first work that design primers to detect and localize pathogen on squash seeds. The results revealed that *S. cucurbitcearum* was dominant in the seed tegument followed by cotyledons and embryo. Similar report have been demonstrated by Sudisha et al. (2006) in muskmelon. The advantages of PCR detection method that was developed requires 1 day for completion compared to 10 days required for the blotter method. Thus, it can be used to examine both

greater number and larger sample sizes with high reliability. Several studies have been already reported primer pairs for identification of *S. cucurbitcearum* in plant fragments after pure isolations (Brewer et al. 2015; Keinath et al. 2001; Somai et al. 2002). Therefore, this method of detection has potential application to target the location of the pathogen before seed treatment. Localization of pathogen in seed is a very important step for the success of seed treatments, to ensure seed quality and increased yields (Mancini and Romanzzi 2014). The results showed that six seed samples obtained from asymptomatic fruit were infected by *S. cucurbitacearum* and in these samples the pathogen was detected on three parts of seeds (tegument, cotyledon, and embryo); this can be explain by the indirect infection of *S. cucurbitacearum* via stigma through the style and funiculus to the ovules which provided evidence of how cucurbit seed becomes infected (De Neergaard, 1989; Keinath, 2011). Our study showed that transmission of *S. cucurbitacearum* is important via seeds, infected seed tegument remain attached to the cotyledon causing seedling mortality. This explains the significant regression and the high coefficient of correlation identified between pathogen incidence within seeds and seedling mortality. According to Brown et al. (1970) and Lee et al. (1984) who noted that seedling infections with *S. cucurbitacearum* in greenhouses are owed to contaminated seed. One surprising result was the high rate of pre-emergence mortality in the samples with low incidence of *S. cucurbitacearum* and when the pathogen is localized in the cotyledon and embryo of these seed samples. Therefore, no significant correlation was observed between infected seeds and pre-emergence mortality. Accordingly, Keinath (2011) reported that the pathogen can be detected with very low incidence in seed, and infect transplant seedling in the greenhouse. In present study, squash plantlets showed, 70 days post-seeding leaf-spot, and stem cankers, which are similar to results found by Lee et al. (1984) after inoculation of pumpkin and cucumber seeds. The pathogen was detected in all parts of plantlets with high incidence on roots and stems. Keinath (2011) reported that *S. cucurbitacearum* was found on all parts of cucurbits, such as leaves, petioles, vines, stem, tendrils, pedicels, flowers, peduncles, fruits, seeds, and roots. While in the present study, 36.8% of healthy-looking plantlets were shown that 44% of these plantlets were infected by *S. cucurbitacearum* and seed coats, found on the soil surface or attached to cotyledon and roots of these plantlets, were infested after incubation on PDA. Therefore, visual inspection for absence of lesions on plantlets and fruit are not sufficient to ensure that seeds are not infected.

7. Conclusion

This study demonstrates that under experimental condition infested seeds are the source of primary inoculum and *S. cucurbitacearum* can be detected in or on seeds extracted from asymptomatic and symptomatic fruits. In addition, this infected seed cannot cause any apparent symptoms in plantlets. Further research is required to better understand the mechanism through with the fungus invades squash seeds. This finding will point out the importance of treating seed for controlling squash gummy stem blight. High quality of seed is necessary management strategy for sustainable agriculture production.

CHAPTER 5

Investigation of *in vitro* and *in vivo* antifungal activities of different essential oils against seedborne fungi of squash (*Cucurbia maxima*)

1. Abstract

Chemical composition of seven essential oils (Eos) was analyzed by GC–MS. A total of 41 components were identified from these EOs. Eucalyptol was the main component of *Lavandula dentata* and *Laurus nobilis* EOs, citral was the major component of *Cymbopogon citratus* EO, *Melaleuca alternifolia* and *Origanum majorana* EOs were rich in terpinen-4-ol, and linalool was the main component of *Lavandula hybrida* EO. The antifungal activities of these essential oils (EOs) have been studied by tests performed under *in vitro* and *in vivo* conditions. The *in vitro* test indicated that lemongrass EO showed great antifungal activity and completely inhibited mycelial growth of *Alternaria alternata* and *Stagonosporopsis cucurbitacearum* at 600 ppm and 900 ppm concentration, respectively. EOs seed treatments at 500 ppm concentration were performed for the disinfestation of squash seed that had been naturally infested. The *C. citratus* EO was the most effective, significantly reducing the incidence of infected seed by 68%. Moreover, EOs of *M. alternifolia, O. majorana* 1*, L. nobilis, O. majorana* 2*, L. hybrida,* and *L. dentata,* reduced significantly the level of seed infection by 60%, 59%, 58%, 56%, 55% and 52%, respectively. Seed treatment EOs had no significant effect on either seed germination. The *in vivo* assays showed that *C. citratus* (500 ppm) seed treatment significantly increased seedling emergence ($P < 0.002$) and reduced by 40% *S. cucurbitacearum* incidence in the plantlets ($P < 0.0001$) compared with the control. Forty day after seeding, the plantlet length was 30.5, 30.2, and 23.3 cm for *C. citratus*, Celest Extra 50 FS, and untreated control. More, future studies are needed to test the effect of separate chemical components of effective EO in managing seedborne fungi on cucurbits.

Keywords: *Alternaria alternata*, Essential oil, *Cymbopogon citratus*, seedborne, squash, *Stagonosporopsis cucurbitacearum*.

2. Introduction

Cucurbits are an important source of income for countries in the Mediterranean basin. Squash (*Cucurbita maxima* Duchesne, *Cucurbita moschata* Duchesne) is among the major cucurbits grown in tropical and temperate regions. *Cucurbita* spp. can be affected by one or more fungal pathogens, most of which seedborne fungi, causing economic losses. Gummy stem blight (GSB) (foliar symptoms), or black rot (BR) (fruit symptoms), is caused by *Stagonosporopsis cucurbitacearum* (Fr.) Aveskamp, Gruyter & Verkley (anamorph *Phoma cucurbitacearum* (Fr.) Sacc.), synonym *Didymella bryoniae* (Fuckel) Rehm, and it is a serious disease and a major constraint to cucurbits production worldwide. Infected plants show different types of leaf-spot, stem canker, vine wilt and yield reduction (Lee et al. 1984; Boughalleb et al. 2007; Keinath et al. 1995). Nuangmek et al. (2018) reported that, under conditions conducive to S. *cucurbitacearum*, losses in cantaloupe can reach 100%. *Alternaria alternata* (Fr.) Keissl is among the major factor responsible for low production of cucurbits and causes leaf spot disease. The genus *Alternaria* affects seedlings, leaves, stalks, stems, flowers and fruits. Yield losses of 50% and more can occur in weather conditions conducive to the disease, 25 to 32°C temperatures associated with 40% relative humidity during the day and 95% at night (Töfoli and Domingues, 2004). Furthermore, many pathogens have been detected on cucurbits seeds, *Fusarium solani* (Boughalleb and El Mahjoub 2006b; Farrag and Moharam 2012), *Alternaria cucumerina* (Gannibal 2011), *Paramyrothecium roridum*, and *Albifimbria verrucaria* (Fish et al. 2012; Sultana and Ghaffar 2009). The most important unit of the squash crop is the seed, which should be of high quality and pathogen free. Seedborne fungi are generally controlled by chemical treatment (Farrag and Moharam 2012; Boughalleb et al. 2006c; Yates et al. 2003). Many attempts on chemical control of *S. cucurbitacearum* were determined by several studies (Keinath 2000; Keinath et al. 1998, Johnson et al. 1995). Sudisha et al. (2006) reported that the seeds treatment with Dithane M-45 (Mancozeb 75% WP) fungicide can reduce the incidence of GSB in muskmelon crop. Chemical fungicides are the most adopted for disinfestation, disinfection and protection of seeds. However, these chemicals can also cause environmental pollution by the high persistence in soil and water because of their slow degradability (Barnard et al. 1997; Misra and Pavlostathis 1997). However, in recent years, other alternatives to synthetic fungicides are investigated, considering the extensive use of fungicides for plant and seed treatment, the problem of pathogens resistance to fungicides, and the increased demand for organic and free-residues vegetables (Antunes and Cavaco 2010; Djioua et al. 2010; Shahi et al. 2003). Unfortunately,

there are no commercial cultivars of cucurbit resistant to GSB (Keinath 2017). Organic natural compounds such as plant extracts, essential oils (EOs), and chitosan are among the environmental-friendly alternatives which are being developed or tested for their antifungal activities against seedborne pathogens (Mancini and Romanazzi 2014). EOs are a rich source of broad-spectrum antifungal plant-derived metabolites that inhibit both fungal growth and production of toxic metabolites (Kishore and Pande 2004). The *in vitro* use of EOs and their principal active components have been shown to be effective at low concentration in the control of seedborne fungal pathogens. Tea tree EO, terpinen-4-ol, 1,8-cineole and c-terpinen at 2% have shown a high reduction of *Fusarium graminearum, Fusarium culmorum* and *Pyrenophora graminea* mycelial growth (Terzi et al. 2007). Riccioni and Orzali (2012) have reported that Tea tree EO could be a potential source of sustainable eco-friendly botanical fungicides, on the basis of its efficacy to control seedborne fungi. The evaluation of *in vitro* efficacy of lemongrass (*Cymbopogon citratus*) EO to control the main seedborne pathogens of cucurbits have shown a total inhibition of mycelia growth of *A. alternata and S. cucurbitacearum* at low concentration (Fiori et al. 2000; Guimarães et al. 2011; Seixas et al. *20*12). Moreover, the melon seeds treatment with lemongrass EO at low concentration (below 0.3%) reduced the plant disease severity caused by *S. cucurbitacearum* (Dalcin et al. 2017). Van der Wolf et al. (2008) found that thyme, oregano, cinnamon, and clove EOs reduced fungi on cabbage seeds in blotter tests. Schmitt et al. (2009) have demonstrated that treatment with 1% of thyme EO reduced *Phoma valerianellae* lettuce seeds infection. Chitosan is a biopolymer derived from crab-shell chitin that considered as seed coating agent with antifungal properties. In addition, chitosan has shown the capacity to induce resistance to several plant pathogenic fungi (Mancini and Romanazzi 2014). Benhamou et al. (1994) cited that tomato seed treatment with chitosan reduced *Fusarium oxysporum* plant infection.

The objectives of this study were: (i) to evaluate the *in vitro* inhibitory effects of 7 EOs, differing in chemical composition, (ii) to determine their broad-spectrum antifungal activity against seedborne fungi; (iii) to test *in vivo* the effect of essential oils against *S. cucurbitacearum*.

3. Materials and methods

3.1. Essential Oils

Sweet marjoram, lavender, lemongrass and bay laurel EOs were provided by different labs (Table 17) where the dried aerial part of plants was hydro-distilled using Clevenger apparatus as recommended by the European Pharmacopeia. On the contrary, *Lavandula hybrida* and *Melaleuca alternifolia* (*Myrtaceae*) EOs were purchased from Flora srl (Lorenzana, Pisa, Italy). EOs were selected on the basis of the literature data, and on the *in vitro* efficacy previously observed versus the pathogen growth (Alves et al. 2019; Black-Solis et al. 2019; Soylu and Kose 2015).

Table 17. List of the tested essential oils.

Family	Species	Code	Common name	Provenience
Lamiaceae	*Origanum majorana* L. 1	*Orig.1*	Marjoram	Medicinal Plants Laboratory, National Institute of Agronomy of Tunisia, University of Carthage, 43, avenue Charles-Nicolle, 1082 Cité Mahrajène, Tunisia.
Lauraceae	*Laurus nobilis* L.	*L.nob*	Bay laurel	
Lamiaceae	*Origanum majorana* L. 2	*Orig.2*	Marjoram	Biopesticides Laboratory, Regional Center for Research in Horticulture and Organic Agriculture (CRRHAB), Chott Meriem 4042 Sousse Tunisia.
	Lavandula dentata L.	*L.dent*	Lavender	
Poaceae	*Cymbopogon citratus* (DC.) Stapf	*C.cit*	Lemongrass	
Lamiaceae	*Lavandula hybrida* E.Rev. ex Briq	*L.hyb*	Lavandin	Batch N°:161808
Myrtaceae	*Melaleuca alternifolia* (Maiden & Betche) Cheel	*M.alt*	Tea tree	Batch N°:161960

3.2. GC-MS Analysis

Volatile constituents of each EO were analysed by GC-MS as previously reported (Najar et al. 2019). The equipment included an Agilent 7890B gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent HP-5MS (Agilent Technologies Inc., Santa Clara, CA, USA) capillary column (30m×0.25 mm; coating thickness 0.25µm) and an Agilent 5977B single quadrupole mass detector (Agilent Technologies Inc., Santa Clara, CA, USA). Analytical conditions were as follows: injector and transfer line temperatures 220 and 240 °C, respectively; oven temperature programmed from 60 to 240 °C at 3 °C/min; carrier gas helium at 1 mL/min; injection of 1 µl (0.5% HPLC grade n-hexane solution); split ratio 1:25. The acquisition parameters were as follows: full scan; scan range: 30–300 m/z; scan time: 1.0 sec. Identification of the constituents was based on a comparison of the retention times with those of the authentic samples, comparing their linear retention indices relative to the series of n-hydrocarbons. Computer matching was also used against commercial (NIST 14 and ADAMS) and laboratory-developed mass spectra library built up from pure substances and components of known oils and MS literature data (Adams 2007, Davies 1990, Jennings and Shibamoto 1982, Masada 1976, Stenhagen et al. 1974, Swigar and Silverstein 1981).

3.3. Collection of squash seed samples

Twenty-nine seed samples were collected from Tunisia (Siliana; 35°57'28"N, 9°32'57"E). These seed samples were extracted from asymptomatic and symptomatic squash fruits (*Cucurbita maxima* cv. Bjaoui). All of these seed samples were mixed together, stored in paper bags at 4 °C until use in the experiments.

3.4. *In vitro* assay

3.4.1. Fungal strains

Strains of *A. alternata* (Isolate number: A38; GenBank accession: MK497774) and *S. cucurbitacearum* (Isolate number: D33; GenBank accession: MF401569) used in this study were isolated from infected squash seed, identified by morphological methods based on macroscopic and microscopic characteristics, then identification was confirmed by molecular tools. According to Moumni et al. (2020), the main pathogens of these naturally infected seeds are *S. cucurbitacearum* and *A. alternata*. Single spore cultures were maintained in PDA tubes and kept at 4°C until use. For the *in vitro* essay pure cultures were transferred into (90 mm, diameter) Petri dishes with PDA and incubated at 22 ±2 °C with a 12/12 h dark/ ultraviolet light photoperiod (TL-D 36W BLB 1SL, PHILIPS, Dublin, Ireland).

3.4.2. Antifungal activity of essential oils on mycelial growth *in vitro*

The antifungal activity of the *C. citratus, O. majorana* (1)*, O. majorana* (2)*, L. hybrida, L. dentata, M. alternifolia,* and *L. nobilis* EOs was assessed for investigating its contact phase effects on the mycelial growth of *A. alternata* and *S. cucurbitacearum.* For this tests, the EOs were dissolved in a solution composed by sterilized distilled water and Tween 20 (0.1% v/v) (Sigma Aldrich, Steinheim, Germany), to obtain homogenous emulsion. Autoclaved PDA medium, cooled to 40°C, was amended by the corresponding EOs emulsions to obtain the final concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 ppm. The negative control was just PDA containing Tween 20 solution (0.1 % v/v). Positive control was three concentrations (100, 500, and 1000 ppm) of fungicide Celest Extra 50 FS (25 g/L Difenoconazole + 25 g/L Fludiovonil). The PDA was mixed and poured immediately into (90 mm, diameter) Petri dishes (20 mL/plate) and, after medium solidification, inoculated under aseptic conditions with 6 mm plugs of *A. alternata* or *S. cucurbitacearum* from the edge of actively growing cultures. There were three replicates per concentration and treatment. The inoculated plates were then sealed with parafilm and incubated for seven days at 22 ±2 °C with a 12/12 h dark/ ultraviolet light photoperiod (TL-D 36W BLB 1SL, PHILIPS, Dublin, Ireland)**.** The orthogonal diameter of colony was measured daily until the control plates were completely covered by the mycelium. Mycelial growth inhibition was calculated based on the following formula: MGI (%) = [(dc - dt) / dc] × 100, where dc and dt represent the average of mycelial growth diameter of control and of treated fungal strain, respectively.

Moreover, the median inhibitory concentration (IC_{50}) for the reduction of 50% of mycelial growth of the fungi was determined from the linear regression between and the EO concentrations and the mycelial growth inhibition percentages.

In addition, transfer experiment was performed to differentiate between the fungicidal and fungistatic activity of an elevated EO concentration against fungi. the completely inhibited fungal plugs, if any were transferred to fresh PDA plates to note their viability after seven days of incubation in the same above conditions.

3.4.3. Seed treatment with essential oils

Naturally infected seeds were surface sterilized in 1% sodium hypochlorite solution for 5 min, rinsed with three washes of sterilized distilled water, and air dried for 2 hours on sterile paper toweling in a laminar flow hood before treatment. For the seed treatment with seven EOs, three concentrations were chosen (250; 500; and 1000 ppm) to determine the most effective concentration. The naturally infected seeds were immersed in 40 mL of each concentration of EO solution, in fungicide (Celest Extra 50 FS; 17%), and two negative control of Tween 20 (0.1%) and distilled water for 6 h, mixing them every 30 min. Then, seeds were dried on sterile blotter sheets overnight (12 h) at room temperature (25- 28°C). Treated seeds were placed in glass Petri dishes containing eight pieces of sterile blotter paper (Whatman no. 4 filter papers; diameter, 110 mm) that were moistened with 5 mL sterile distilled water, and 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)**.** For each concentration of the different EOs one hundred seeds were treated and the experimental trial was repeated twice. To compare the efficacy of the different EOs on fungi naturally infecting seeds, the intermediary 500 ppm concentration was chosen to perform this additional trial. So, 200 seeds were treated separately with each of the seven EOs at 500 ppm as described above. The naturally infected seed immersed in the solution of Tween 20 (0.1%) were used as control. This experimental was repeated twice. After 14 days of incubation, fungi identification was carried out first by examination of the fungal fruiting bodies and of the congregated mycelia and spores on the seeds under a stereomicroscope (M125; Leica Microsystems CMS, Wetzlar, Germany). Then, the individual spores, conidiophores, and pycnidia were examined under a microscope (DM 2500; Leica). Assessments ware based on relative frequencies of seedborne fungi, germination rate of seeds and length of the groing radicle. The percentage of infected seeds was calculated by the following formula:

$$IS\ (\%) = \frac{\text{Number of infected seeds} \times 100}{\text{Total seeds tested}}$$

3.4.4. Seed treatment with the mixture of essential oils and chitosan

Commercial chitosan-based formulation, known as Chito Plant (ChiPro GmbH, Bremen, Germany) was prepared by dissolving the powder (1%, w/v) directly in distilled water. For experimental use, the 1% solution (w/v) of chitosan was dissolved in 2 mL acetic acid and 0.1 mL of Tween 20. This solution was shaked and heated at 30 °C during 6 h of shaking. The pH of the chitosan dissolved solution was adjusted to 5.5 using NaOH (1 ×N).

Six mixtures were used for seed treatment: chitosan (1%); chitosan (1%) + *C. citratus* EO (500 ppm); chitosan (1%) + *O. majorana* 2 EO (500 ppm); two EOs *C. citratus* and *O. majorana* 2 at 500 ppm; and fungicide (Celest Extra 50 FS; 17%). One hundred seeds per treatment were used applying the method described in the previous paragraph. The naturally infected seed immersed in the solution of Tween 20 (0.1%) were used as the control. This experimenta was repeated twice.

3.5. *In vivo* assay

3.5.1. Effects of *Cymbopogon citratus* essential oil on emergence of seedling and disease incidence of *S. cucurbitacearum* in squash plantlets.

Naturally infected seeds were treated separately with *C. citratus* EO at 500 ppm and a fungicide (Celest Extra 50 FS; 17%). A solution of Tween 20 (0.1%) was used as control. For each treatment, 50 seeds were used applying the method described previously and the experiment was repeated twice. Treated seeds were sown on pasteurized soil in desinfected seedling trays. Seeds in trays were incubated at room temperature (22 ± 4 ºC). Seedling emergence was recorded based on the number of emerged seedlings every 2 days until 14 days after sowing. On the 14th day after sowing, stand establishment dead and germinated seedlings were assessed. On the 40th day after sowing, length of plantlets was measured. At the same time, 30 symptomatic and asymptomatic plantlets from each treatment were collected and sliced in three parts, root, stem and leaf, using flame-sterilized scalpels, in order to determine disease incidence of *S. cucurbitacearum*. Each vegetative section was surface-disinfected in 1% sodium hypochlorite for 2 min, triple-rinsed in sterile distilled water, and then dried on sterile blotter papers. Each part was cut in pieces of about 2 mm, plated on PDA and observed for fungal colony development after 7 days of incubation at 25 °C. Morphological identification of the colony was carried out to look for *S. cucurbitacearum* growth. The percentage of *S. cucurbitacearum* transmission was calculated using the following formula:

$$\text{Sc transmission (\%)} = \frac{\text{Number of infected section of plantlet} \times 100}{\text{Total section of plantet}}$$

Statistical analysis

Analysis of variance was calculated using Spss (version 20). Data (IC50, Incidence of infected seeds, Germination, Radicle length, Plantlet infection, Plantlet lengths) were

analyzed by analysis of variance (ANOVA) was calculated using SPSS (version 20). Means were compared using Fisher's Test with significant difference at $P \leq 0.05$.

4. Results

4.1. GC-MS Analyses

The composition of the EOs is reported in Table 18. Overall, 41 components were identified representing a range between 97.7% in *O. majorana* 1 to a 100% in *L. hybrid*. Oxygenated monoterpenes dominated in all the studied species even though they belonged to different families. They represented more than 67% of the identified fraction in three out of 4 species from *Lamiaceae*: *O. majorana* 2 (66.8%), *L. hybrida* (90.8%) and *L. dentata* (81.1%). This latter chemical class prevailed also in *L. nobilis* (70.3%) and *C. citratus* (88.5%). *M. alternifolia* and *O. majorana* 1 EOs showed a composition divided mainly between oxygenated monoterpenes, which were always the main class (48.1% and 49.7%, respectively), and monoterpene hydrocarbons present with almost the same amount (40.4% and 44,3%, respectively).

More in detail of composition, both terpenen-4-ol and *p*-cymene accounted more than 45% of the total composition of *Origanum* species (44.8% in *O. majorana 1* and 68.2% in *O. majorana* 2) followed by γ-terpinene (12.6%) in case of *O. majorana 1* and α-terpineol (5.4%) in *O. majorana* 2. Always in the *Lamiaceae*, sixty-four percent of the identified fraction of *Lavandula dentata*, was due to eucalyptol (63.5%) followed by β-Selinene (4.1%). More than fifty percent of the identified fractions of *L. nobilis* EO was characterized by both eucalyptol (47.9%) and α-terpinyl acetate (10.2%). *C. citratus* EO showed α-citral (geranial) and β-citral (neral) (51.6% and 26.0%, respectively) as major compounds which represented together more than 78% of the total composition. Concerning the commercial EOs and starting by *L. hybrida* we noted that linalool (33.7%) and linalyl acetate (27.7%) were the main compounds of lavandin EO followed by camphor (9.3%). Regarding others plant belong *M. alternifolia*, eighty-six percent of the oxygenated monoterpene was represented by terpinene-4-ol (41.1%) while the total of γ-terpinene (16.0%), *p*-cymene (9.3%) and α-terpinene (6.1%) showed the seventy eight percent of monoterpene hydrocarbons.

Table 18. Volatile constituents of different EOs extracted from seven plant species (*Melaleuca alternifolia*, *Lanvandula hybrida*, *Origanum majorana* 1, *Origanum majorana* 2, *Laurus nobilis*, *Lavandula dentata*, and *Cymbopogon citratus*) and determined by GC-MS analyses.

			Myrta -ceae	**Lamiaceae**				**Lauraceae**	**Poaceae**
	Compounds[a] (chemical class)	**LRI[b]**	***M.alt***	***L.hyb***	***L.dent***	***Orig.1***	***Orig.2***	***L.nob***	***C.cit***
1	α-Pinene (mh)	937	2.7	0.5	0.6	0.6	0.3	5.6	0.1
2	Sabinene (mh)	974	-	0.1	-	4.5	2.2	6.7	0.1
3	β-Pinene (mh)	979	0.7	0.5	3.1	0.4	0.2	5,0	-
4	β-Myrcene (mh)	991	0.6	0.4	-	1.1	0.6	1.2	5.3
5	α-Terpinene (mh)	1017	6.1	-	-	5.7	0.8	0.8	-
6	*p*-Cymene (mh)	1025	9.3	0.2	0.9	11.3	17.8	0.6	0.3
7	Limonene (mh)	1030	1.0	0.8	1.1	3.5	2.4	1.7	0.4
8	Eucalyptol (om)	1032	2.8	6.5	63.5	0.7	0.2	47.9	0.6
9	γ-Terpinene (mh)	1060	16.0	-	-	12.6	3.8	1.4	-
10	Terpinolene (mh)	1088	3,0	0.2	-	3.4	1.2	0.3	-
11	Linalool (om)	1099	-	33.7	1.8	1.1	3.6	7.4	0.8
12	*cis-p*-Menth-2-en-1-ol (om)	1122	0.1	-	-	1.3	0.7	-	-
13	*trans*-Pinocarveol (om)	1139	-	-	2.9	-	-	-	-
14	*trans-p*-Menth-2-en-1-ol (om)	1141	0.1	-	-	1.2	0.7	-	-
15	Camphor (om)	1145	-	9.3	0.5	-	0.1	-	-
16	*γ*-Terpineol (om)	1166	-	-	1.3	-	-	0.3	-
17	*endo*-Borneol (om)	1167	-	4.4	0.4	0.1	0.2	-	-
18	*p*-Mentha-1,5-dien-8-ol (om)	1170	-	-	-	-	-	-	2.5
19	Terpinen-4-ol (om)	1177	41.1	4.5	1.3	32.4	50.1	1.5	0.1
20	*p*-Cymen-8-ol (om)	1183	-	-	0.3	0.3	0.4	-	1.1
21	Cryptone (nt)	1186	-	-	1.3	-	-	-	-
22	α-Terpineol (om)	1189	3.7	1.1	1.8	6.0	5.4	1.6	-
23	Myrtenal (om)	1198	-	-	2.7	-	-	-	-
24	*trans*-Piperitol (om)	1208	-	-	-	1.0	0.6	-	-
25	*β*-Citral (om)	1240	-	-	-	-	-	-	26.0
26	Carvone (om)	1243	-	-	1.6	0.2	0.6	-	0.9
27	Geraniol (om)	1253	-	-	-	-	-	-	2.7
28	Linalyl acetate (om)	1257	-	27.7	-	2.7	2.2	0.2	-
29	α-Citral (om)	1270	-	-	-	-	-	-	51.6
30	2-Undecanone (nt)	1294	-	-	-	-	-	-	1.2
31	4-Terpinyl acetate (om)	1300	-	-	-	1.5	1,0	-	-

32	Lavandulyl acetate (om)	1304	-	3.0	-	-	-	-	-
33	α-Terpinyl acetate (om)	1350	-	-	-	0.1	-	10.2	-
34	Methyleugenol (pp)	1404	-	-	-	-	-	3.1	-
35	β-Caryophyllene (sh)	1419	0.5	2.0	-	2.4	1.6	0.5	-
36	Aromandendrene (sh)	1440	2.2	-	-	-	-	-	-
37	β-Selinene (sh)	1486	0.1	-	4.1	-	-	-	-
38	Eremophyllene (sh)	1498	1.5	-	-	-	-	-	-
39	δ-Cadinene (sh)	1524	1.7	-	-	-	-	0.1	-
40	Caryophyllene oxide (os)	1583	-	0.1	1.9	0.2	0.3	-	0.2
41	β-Eudesmol (os)	1651	-	-	2.1	-	-	-	-

	M. alt	***L. hyb***	***L.dent***	***Orig.1***	***Orig.2***	***L. nob***	***C. cit***
Monoterpene Hydrocarbons (mh)	40.4	3.6	5.9	44.3	29.7	24.0	6.5
Oxygenated Monoterpenes (om)	48.1	90.8	81.1	49.7	66.8	70.3	88.5
Sesquiterpene Hydrocarbons (sh)	9.3	4,0	5.5	3.3	2,0	1.2	0,0
Oxygenated Sesquiterpens (os)	1.8	0.2	4.6	0.6	1.1	-	0.3
Penylpropanoids (pp)	-	-	-	-	-	3.6	-
Non-terpene Derivatives (nt)	-	1.4	2.2	-	-	0.4	3.1
Total Identified	**99.6**	**100**	**99.3**	**97.7**	**99.6**	**99.5**	**98.4**

[a] Compounds present for a percentage ≥ 1% at least in one of the analysed EO. Orig 1: *Origanum majorana*; Orig 2: *Origanum majorana*; M. alter: *Melaleuca alternifolia*; L. hyb: *Lavandula hybrida*; L. dent: *Lavandula dentata*; C. cit: *Cympobogon citratus*; L. nob: *Laurus nobilis*

[b] LRI Linear retention index.

4.2. Inhibitory effect of essential oils on fungal growth *in vitro*

The effects of concentrations of seven EOs on the mycelial growth of *A. alternata* and *S. cucurbitacearum* are shown in Figure 25. All EOs inhibited the growth of *A. alternata* and *S. cucurbitacearum* in a dose-dependent manner. The results indicate that the most relevant EOs was of *C. citratus* showing 100% inhibition of mycelial growth of *A. alternata* and *S. cucurbitacearum* at 600 and 900 ppm respectively (Figure 25). In addition *C. citratus* EO showed a fungicidal effect against *S. cucurbitacearum* from 900 ppm (Table 19). This oil showed against *A. alternata,* a fungistatic effect at 600 and 700 ppm but a fungicidal effect from 800 ppm.

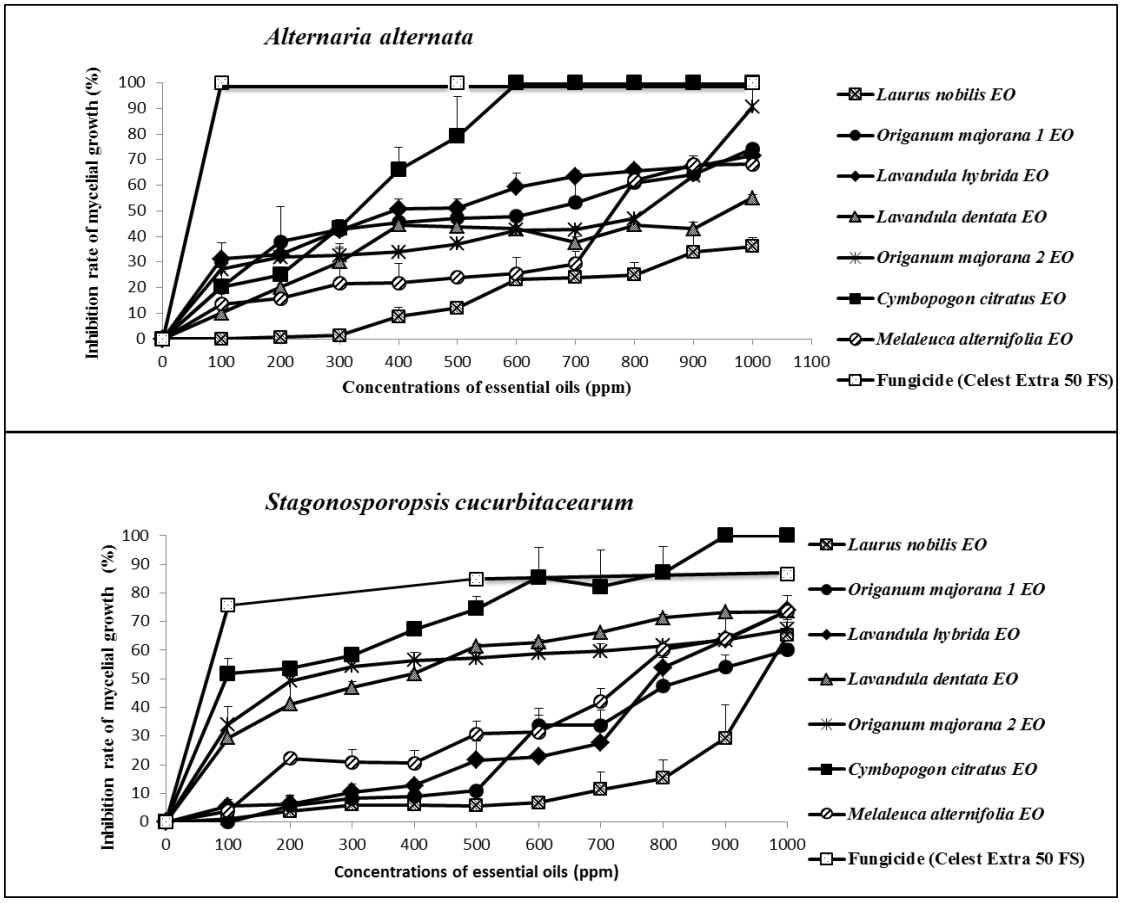

Figure 25. Inhibition rates (%) of *Alternaria alternata* and *Stagonosporopsis cucurbitacearum* with different concentration of seven essential oils: *Melaleuca alternifolia*, *Lanvandula hybrida*, *Origanum majorana* 1, *Origanum majorana* 2, *Laurus nobilis*, *Lavandula dentata*, and *Cymbopogon citratus* after seven days of incubation on PDA at 22 ±2 °C (Data are means±SD).

Table 19. Fungistatic or fungicidal activities of *Cymbopogon citratus* essential oil on the mycelia growth of *Stagonosporopsis cucurbitacearum* and *Alternaria alternata* after 7 days of incubation

Essential oil concentration (ppm)	*A. alternata*	*S. cucurbitacearum*
600	fs	-
700	fs	-
800	fc	-
900	fc	fc
1000	fc	fc

(-) not studied; fs: fungistatic activity; fc: fungicidal activity

The EOs from *O. majorana* 2*, O. majorana* 1*, L. hybrida, M. alternifolia, L. dentata,* and *L. nobilis* inhibited at 1000 ppm the mycelia growth of *A. alternata* by 90, 74, 71, 68, 54 and 36% respectively (Figure 26). For *S. cucurbitacearum* the EOs at 1000 ppm of *L. hybrida, L. dentata, M. alternifolia, O. majorana* 2*, L. nobilis* and *O. majorana* 1*,* reduced the radial growth of by 74, 73, 73, 67, 65 and 60%, respectively (Figure 27). The percentage of mycelial growth inhibition gradually increased when the concentration of EO increased from 100 to 1000 ppm. TheCelest Extra inhibited the mycelial growth of *A. alternata* at all concentrations. For *S. cucurbitacearum* the fungicide at 100, 500, and 1000 ppm inhibited the mycelial growth by 75.7%, 84.9%, and 86.7% respectively. No inhibition of mycelial growth of the two pathogens was observed in the negative control.

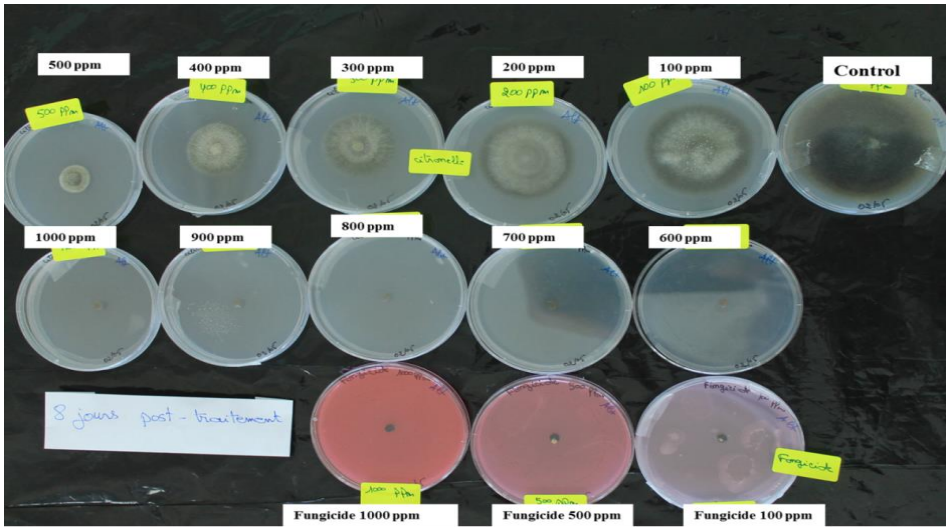

Figure 26. Inhibition of *Alternaria alternata* mycelial growth by *Cymbopogon citratus* essential oil at different concentrations ranging from 100 to 1000 ppm and fungicide (Celest Extra 50 FS) at 100, 500 and 1000 ppm after 8 days of incubation.

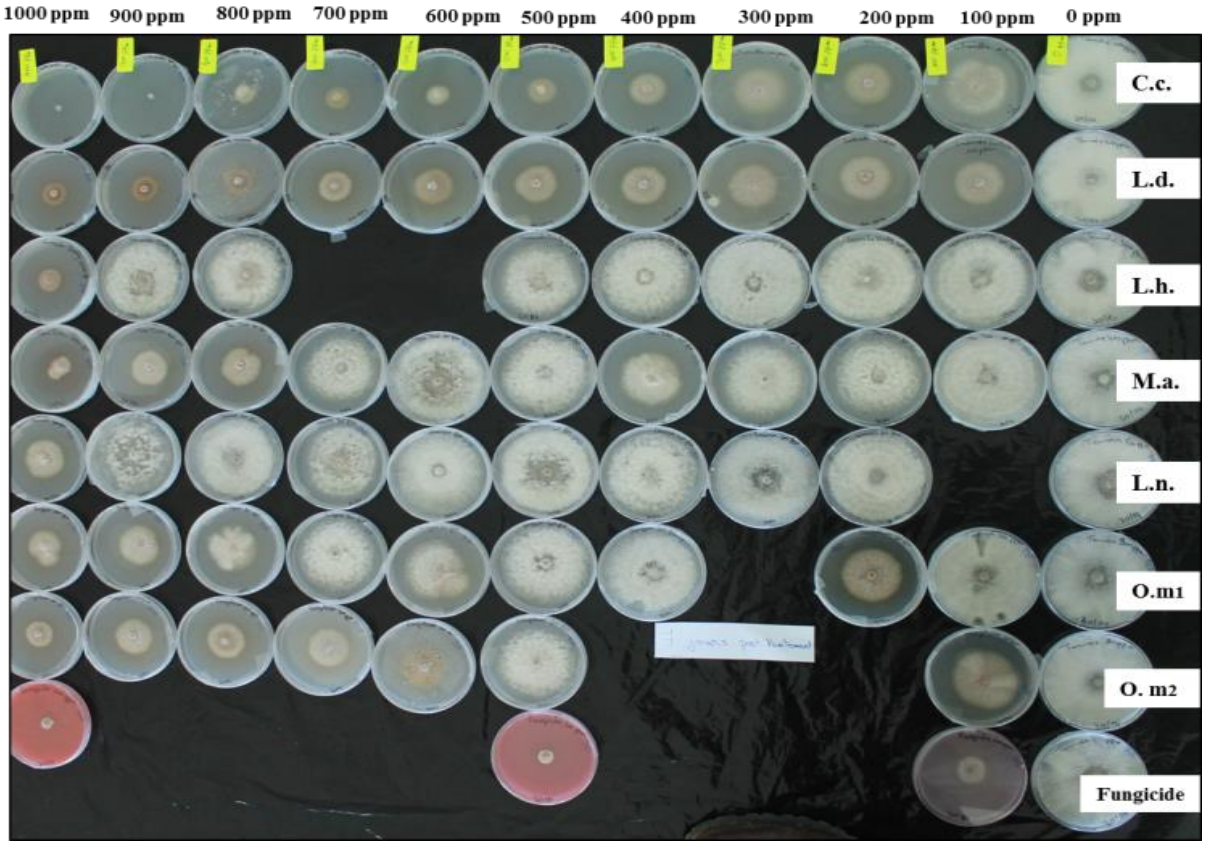

Figure 27. Inhibition of *Stagonosporopsis cucurbitacearum* mycelial growth by seven essential oils: (C.c) *Cymbopogon citratus*; (L.d.) *Lavandula dentata*; (L.h.) *Lavandula hybrida*; (M.a.) *Melaleuca alternifolia*; (L.n.) *Laurus nobilis*; (O.m1) *Origanum majorana 1*; (O.m2) *Origanum majorana* 2 at different concentrations ranging from 100 to 1000 ppm and by fungicide (Celest Extra 50 FS) at 100, 500 and 1000 ppm after 7 days of incubation.

The results showed that the *C. citratus* EO had a strong antifungal activity against *S. cucurbitacearum* and *A. alternata* with IC_{50} of 101 and 315 ppm, respectively (Figure 28). The EOs of *O. majorana* 2*, L. dendata, M. alternifolia, L. hybrida,* and *O. majorana* 1*,* showed a moderate antifungal activity against *S. cucurbitacearum* with IC_{50} varying between 322, and 884 ppm. Almost the same was found against *A. alternata* with IC_{50} varying beetween 474, and 893 ppm. However, *L. nobilis* EO showed a weak antifungal activity against *S. cucurbitacearum* and *A. alternata* with a relatively high IC_{50} of 1249 and 1310 ppm respectively (Figure 28).

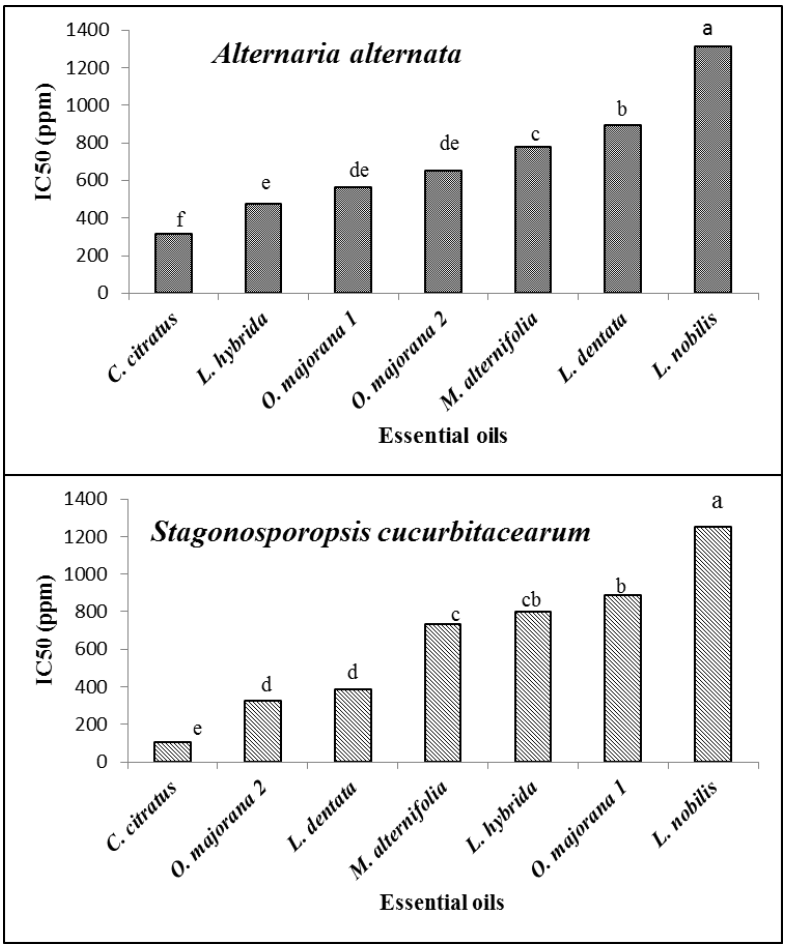

Figure 28. Inhibitory concentration reducing 50% of fungal mycelial growth (IC_{50}) of seven essential oils: *Melaleuca alternifolia*, *Lanvandula hybrida*, *Origanum majorana* 1, *Origanum majorana* 2, *Laurus nobilis*, *Lavandula dentata*, and *Cymbopogon citratus* against *Alternaria alternata* and *Stagonosporopsis cucurbitacearum*. Data with different letters are significantly different between treatments according to Fisher's least significant difference (LSD) ($P \leq 0.05$).

4.3. Efficacy of essential oils on seed health

The tested EOs were used for seed treatment at three concentrations (250, 500 and 1000 ppm) using standard blotter method. The result showed that the effect of the EOs was significant on reducing seed infection compared to the negative treatment (Figure 29). Accordingly, *C. citratus* and *L. dentata* EOs at 1000 ppm were highly effective in reducing the incidence of infected seeds by 88% and 83% respectively in the same way as do Clest Extra. Moreover, the incidence of infected seeds was significantly reduced by treatments with *O. majorana* 2*, L. nobilis, L. hybrida, M. alternifolia* and *O. majorana* 1 EOs by 74, 66, 66, 61 and 55%, respectively. Furthermore, the effect of the concentrations 500 and 1000 ppm on the incidence of seed infection was statistically the same ($p > 0.05$) whatever the EOs.

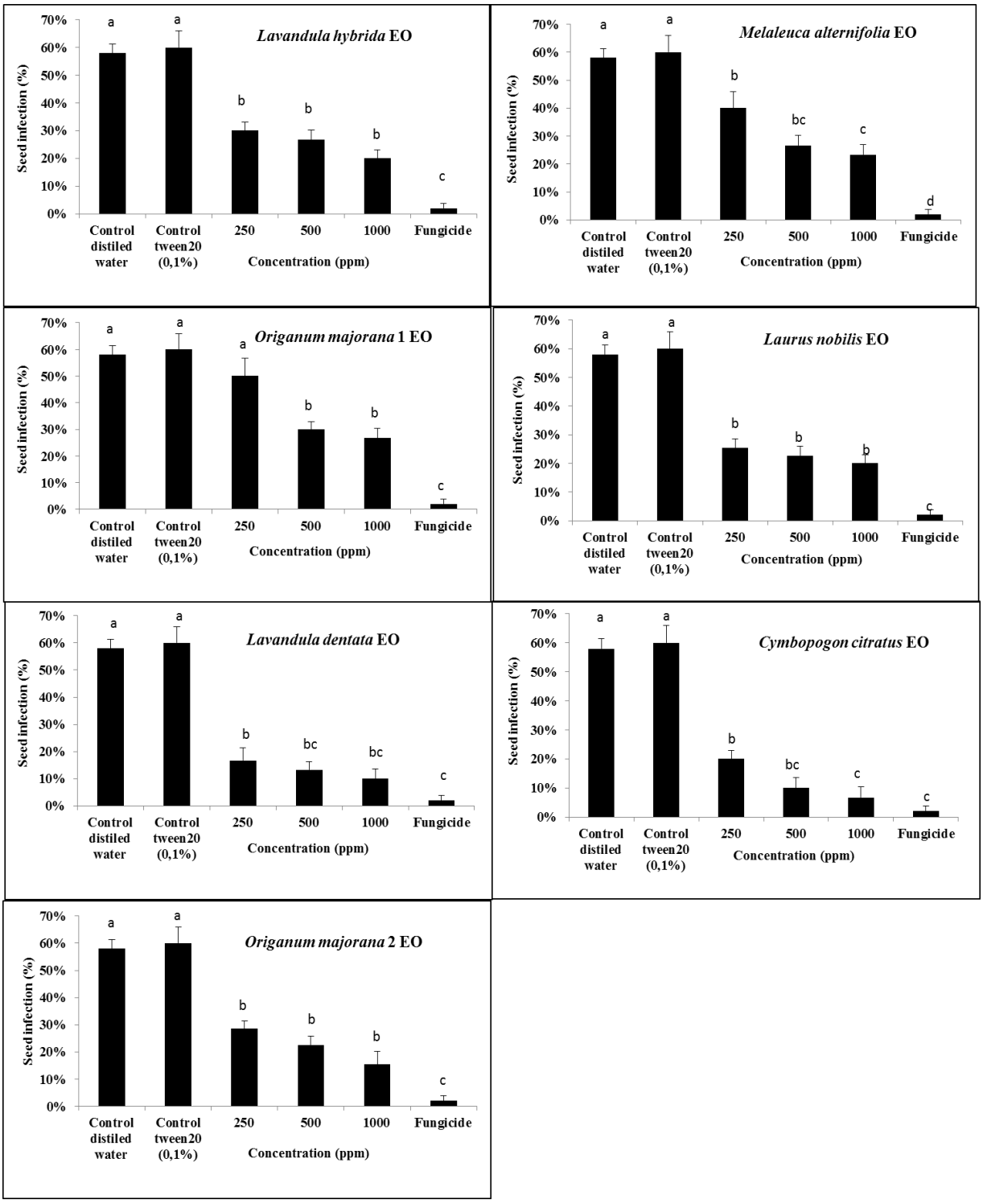

Figure 29. Incidence of infected seeds after treatment with three concentration (250, 500, 1000 ppm) of different essential oils: *Lavandula hybrida*; *Melaleuca alternifolia*; *Origanum majorana* 1; *Laurus nobilis*; *Lavandula dentate*; *Cymbopogon citratus*; *Origanum majorana* 2 compared to two negative controls (seed treated with distilled water and seed treated with Tween 20 (0.1%)) and positive control fungicide (Celest Extra) using the blotter test method (Data are means±SD). Data with different letters are significantly different between treatments according to Fisher's least significant difference (LSD) ($P \leq 0.05$).

In agreement with the results mentioned above, re-evaluation of the efficacy of the different EOs at 500 ppm on seedborne infections is represented in Table 20. The *C. citratus* EO was the most effective since it reduced significantly the incidence of seed infection by 68%. Moreover, EOs of *M. alternifolia, O. majorana* 1*, L. nobilis, O. majorana* 2*, L. dentata,* and *L. hybrida* reduced significantly the level of seed infection by 60, 59, 58, 56, 55 and 52%, respectively. Otherwise, the results showed that *C. citratus* EO reduced significantly the seed infection by *A. alternata* and *F. solani* by 87% followed by *A. verrucaria*, *F. fujikuroi*, *C. spicifera*, *R. stolonifer*, *S. cucurbitacearum* and *P. roridum* that were reduced by 83, 76, 74, 74, 72 and 70% respectively. In case of seeds treated with *M. alternifolia* EO, *F. solani* was completely eliminated and *C. spicifera*, *P. roridum, A. alternata, S. cucurbitacearum, F. fujikuroi* and *R. stolonifer* were significantly reduced by 92, 87, 81, 75, 64 and 53% respectively, while no significant effect was registred on *A. verrucaria*. The treatment with *Origanum majorana* 1 eliminated completely *P. roridum* and reduced significantly *F. solani, A. alternata, S. cucurbitacearum, C. spicifera*, *F. fujikuroi*, and *R. stolonifer* by 88, 70, 68%, 62, 61 and 51% respectively, while *A. verrucaria* was no significantly reduced by this treatment. In case of seeds treated with *L. nobilis,* significant reduction of *A. alternata, F. solani, P. roridum, C. spicifera*, *A. verrucaria, S. cucurbitacearum, R. stolonifer* and *F. fujikuroi,* was observed by 89, 88, 78, 76, 69, 67, 61 and 58% respectively. The seedborne fungi *C. spicifera*, *A. alternata, S. cucurbitacearum, A. verrucaria, P. roridum, F. fujikuroi,* and *R. stolonifer* were significantly reduced when treated with *O. majorana* 2 EO by 90, 86, 84, 70, 69, 55, 49% respectively, while *F. solani* was completely eliminated. In case of the treatment with *L. dentata, C. spicifera* was completely eliminated; *A. alternata, F. solani* and *F. fujikuroi* were significantly reduced by 89% compared to the control. *P. roridum, R. stolonifer* and *S. cucurbitacearum* were reduced by 82, 77 and 67% respectively, while there was no significant reduction of *A. verrucaria.* Moreover significant reduction of *A. verrucaria, A. alternata, S. cucurbitacearum, C. spicifera, F. fujikuroi, R. stolonifer* and *P. roridum* was observed when the seeds were treated with *L. hybrida* EO by 92, 75, 73, 68, 64, 62 and 60% respectively, while there was no significant reduction of *F. solani*.

The effect of the EOs at 500 ppm on seed germination and radicle length is shown in Table 20. No significant difference in germination rates was noted between seed treated with the different EOs and untreated seeds. On the other hand, the germination of seeds treated with the fungicide was significantly reduced by 11.7% compared to the control. Moreover, the results showed that *C. citratus* and *O. majorana* 1 EOs did not affect significantly the radicle

length compared with untreated seeds. But radicle length of seeds treated with *M. alternifolia, L. dentata, O. majorana* 2, *L. nobilis,* and *L. hybrida* EOs were significantly decreased by 34, 28, 27, 25 and 23% respectively. On the contrary, radicle length of seeds treated with the fungicide was significantly increased by 24% compared to untreated seeds.

Table 20 Effect of different essential oils at 500 ppm on seed germination, length of radicle and on seedborne fungi reduction compared to control.

Treatment	Germination (%)	Radicle Length (cm)	Infected seed (%)	Fungi (%)							
				S.c	*A.a*	*F. f*	*F.s*	*P.r*	*A.v*	*C.s*	*R.s*
C. cit	86.5 a ±1.9	21.0 b ±0.9	20.6 c ± 2.2	4.5 cd ±0.9	1.1 bc ±0.5	5.3 cdef ±1.2	0.5 b ±0.3	1.7 bc ±0.6	0.5 b ±0.3	0.85 b ±0.4	4.2 bc ±1.4
L. hyb	85.6 a ±2.2	16.0 c ±0.6	30.5 b ±2.2	4.3 cd ±0.9	2.3 bc ±0.8	8.0 cd ±1.4	3.0 a ±1.6	2.3 bc ±0.8	0.2 b ±0.2	1.0 b ±0.6	6.1 bc ±1.9
Orig 1	87.1 a ±1.5	20.5 b ±0.9	26.2 bc ±2.2	5.0 bcd ±1.3	2.7 bc ±0.7	8.7 bc ±1.6	0.5 b ±0.3	0.0 c ±0.0	1.7 ab ±0.6	1.2 b ±0.5	8.1 b ±1.9
Orig 2	85.8 a ±1.4	15.1 c ±0.7	28.5 b ±2.8	2.5 de ±0.7	1.2 bc ±0.5	10.1 b ±1.6	0.0 b ±0.0	1.7 bc ±0.6	1.0 b ±0.4	0.3 b ±0.3	8.3 b ±2.4
M. alter	85.8a ±2.4	13.7 c ±0.7	25.5 bc ±2.5	3.9 cde ±1.2	1.7 bc ±0.6	7.9 cd ±1.3	0.0 b ±0.0	0.7 c ±0.4	1.7 ab ±0.8	0.2 b ±0.2	7,60 bc ±2.0
L. dent	86.1 a ±1.7	15.0 c ±0.7	28.6 b ±2.4	5.2 bcd ±1.2	1.0 bc ±0.4	2.4 fe ±0.8	0.5 b ±0.5	1.0 c ±0.4	1.7 ab ±0.6	0.00 b ±0.0	3.7 bc ±1.1
L. nob	86.6 a ±1.9	15.5 c ±0.6	26.7 bc ±2.4	5.3 bcd ±1.1	1.0 bc ±0.4	9.3 bc ±1.4	0.5 b ±0.3	1.2 bc ±0.5	1.0 b ±0.4	0.7 b ±0.4	6.3 bc ±1.8
Fungicide	75.6 b ±2.2	26.0 a ±1.1	1.4 d ±0.5	0.2 e ±0.2	0.0 c ±0.0	1.21 f ±0.5	0.0b ±0.0	0.0 c ±0.0	0.0 b ±0.0	0.0 b ±0.0	0.0 c ±0.0
Control	85.4 a ±1.4	20.9 b ±0.7	64.7 a ±2.8	16.1 a ±2.3	9.3 a ±1.8	22.6 a ±2.4	4.6 a ±1.0	5.8 a ±1.6	3.3 a ±1.6	3.3 a ±1.7	16.4 a ±3.9

Sc,*Stagonosporopsis cucurbitacearum*; Aa, *Alternaria alternata*; Ff, *Fusarium fujikuroi*; Fs*, Fusarium solani;* Pr, *Pramyrothecium roridum;* Av, *Albifimbria verrucaria;* Cs, *Curvularia spicifera;* Rs, *Rhizopus stolonifer*.

Orig 1: *Origanum majorana* 1; Orig 2: *Origanum majorana 2*; M. alter: *Melaleuca alternifolia*; L. hyb: *Lavandula hybrida*; L. dent: *Lavandula dentata*; C. cit: *Cympobogon citratus*; L. nob: *Laurus nobilis*

Data are means±SD. Data with different letters are significantly different between treatments according to Fisher's least significant difference (LSD) ($P \leq 0.05$).

4.4. Effect of the mixture of essential oils with chitosan on seed health

The effect of two essential oils mixed to chitosan on seed health is shown in Figure 30. Considering all seedborne pathogens isolated from squash seeds, the results showed that the fungicide was able to reduce completely their growth (100%). However, EOs were relatively less powerful. The EOs of *O. majorana* 2 EO*, C. citratus* EO and chitosan combined to *O. majorana* 2 EO decreased significantly the incidence of seed infection compared to negative control by 55, 54 and 33%, respectively, while chitosan alone or mixed with *C. citratus* EO were statiscally the same as the negative control. Considering *S. cucurbitacearum* and *A. alternata,* Eos of *O. majorana* 2 EO, *C. citratus* EO*,* chitosan mixed with *O. majorana* 2 EO or with *C. citratus* EO reduced significantly the incidence of *S. cucurbitacearum* by 76, 87, 58 and 58% respectively, and the incidence of *A. alternata* by 74, 88, 77 and 70%, respectively. The chitosan alone at 0.5% was next to negative control. In this assay there was no significant difference (p>0.05) between EOs and chitosan mixed with either EOs.

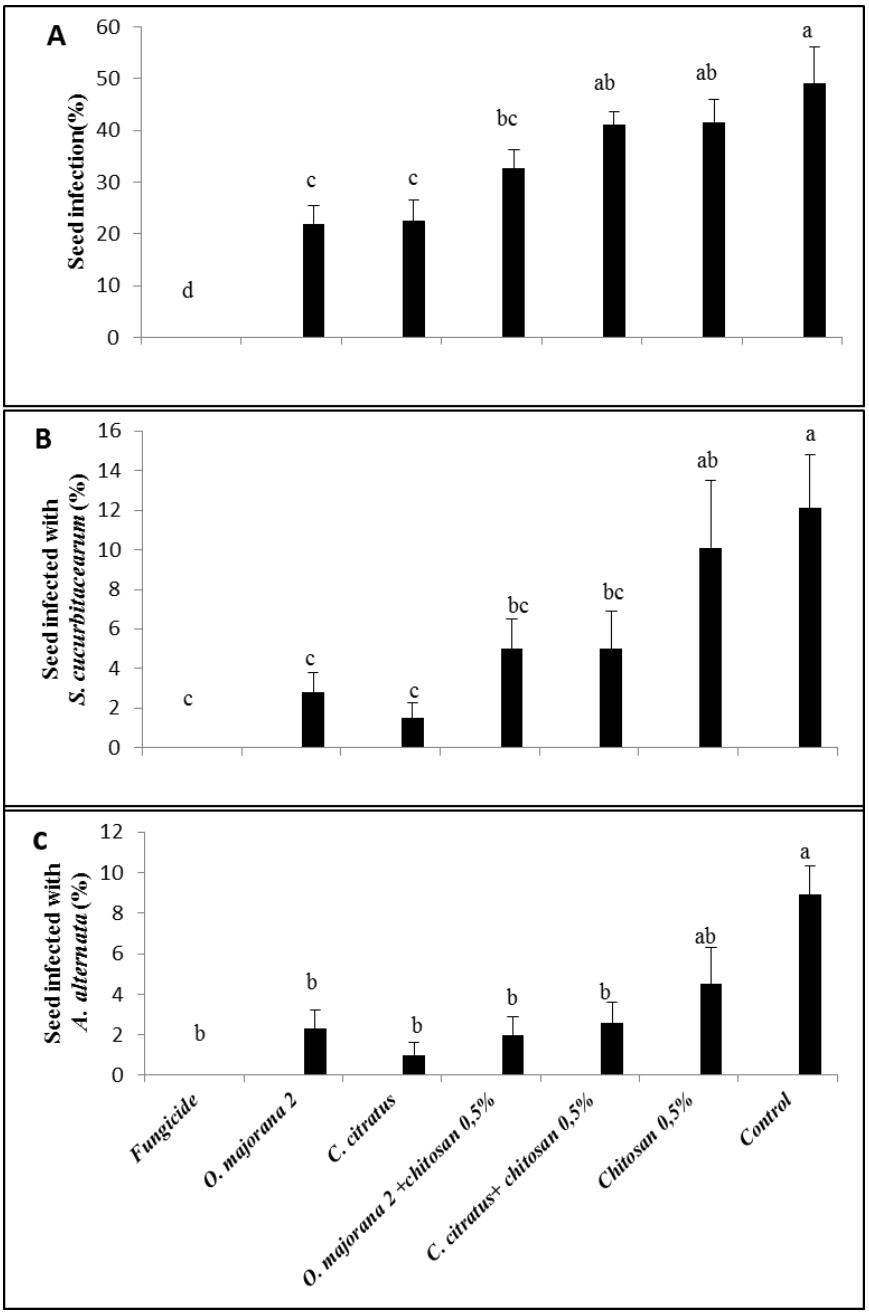

Figure 30. The effect of *Cymbopogon citratus* (500 ppm) and *Origanum majorana* 2 (500 ppm) essential oils, applied alone or with chitosan (0.5%) and with chitosan (0.5%) in the reducing (A) Total seed infection, (B) infection by *Stagonosporopsis cucurbitacearum*, (C) *Alternaria alternata*. Data with different letters are significantly different between treatments according to Fisher's least significant difference (LSD) (P≤0.05). Data are means±SD.

4.5. Effect of essential oil on plantlets health

Seed treatment with *C. citratus* EO (500 ppm) significantly increased seedling emergence ($P < 0.002$) in comparison with negative control and with fungicide. Four days after sowing, the seeds treated with *C. citratus* EO showed 30% of emergence, while those treated with water or fungicide 20 and 10% respectively. The results demonstrated that the seeds treated with fungicide took more days to emerge (Figure 31).

In this trial seed treatments with fungicide or EO were very effective in improving plantlets health. Accordingly, forty days after seeding, seed treated with the EO of *C. citratus* showed significant reduction (40%) of *S. cucurbitacearum* on the plantlets ($P < 0.0001$) compared with the negative control (Table 21 and Figure 32). In this assessments, the infection of leaves, stems and roots with *S. cucurbitacearum* was not significantly different compared to *C. citratus* EO and fungicide, which were significantly different from the control. Values of plantlets lengths were significantly increased ($P < 0.0001$) by whatever seed treatments in comparison with the negative control (Table 21).

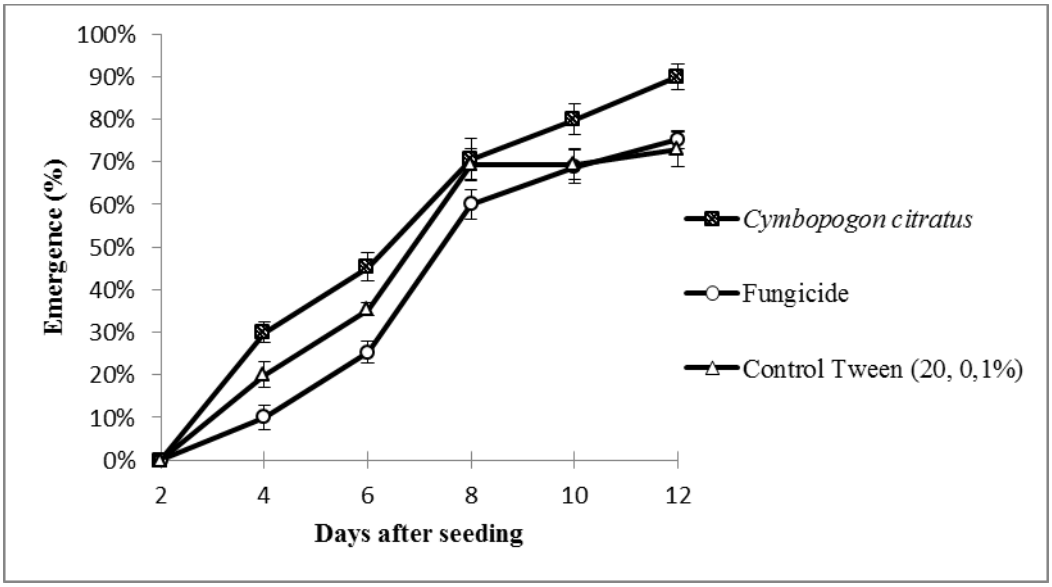

Figure 31. The effect of *Cymbopogon citratus* (500 ppm) EO and fungicide (Celest Extra 50 FS) on seedling emergence. Data are means±SD.

Table 21. The effect of *Cymbopogon citratus* EO (500 ppm), fungicide and negative control on the incidence of *Stagonosporopsis cucurbitacearum* in the plantlets after 30 days of seeding.

Treatment	**Plantlets infection (%)**	**Disease incidence (%)**			**Plantlets lengths (cm)**
		Leaves	stems	roots	
C. citratus	30.0±4.4 b	18.7±3.3 a	28.3±4.5 a	18.1±4.3 a	30.5±1.1 b
Fungicide	16.8±1.9 a	9.7±1.6 a	17.3±1.9 a	17±2.1 a	30.2± 1.2 b
Control	49.9±2.9 c	30.3±5.2 b	43±4.4 b	43.1±3.2 b	23.3±1.2 a

Data are means±SD. Values within a column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) ($P \leq 0.05$).

Figure 32. *Stagonosporopsis cucurbitacearum* symptoms on the plantlets of squash emerged from untreated (left) and treated seeds with *Cymbopogon citratus* EO at 500 ppm (right) after 30 days of seeding.

5. Discussion

In vitro assays performed on antifungal activity of EOs measured with mycelial growth and seed infestation showed that Lemongrass EO was the most effective. The mycelial growth of *A. alternata* was totally inhibited by 600 ppm of lemongrass EO whereas that of *S. cucurbitacearum* was completely inhibited at 900 ppm. This EO had fungicidal activity on both fungi. In addition, evaluation of different EOs at 500 ppm showed that lemongrass oil was the best in controlling seedborne fungi. Guimarães et al. (2011) confirmed the inhibition of *A. alternata in vitro* by the lemongrass EO. Seixas et al. (2012) found that this EO inhibited entirely the mycelial growth of *S. cucurbitacearum* at 250 ppm. Fiori et al. (2000) found the same results, 100% inhibition of mycelial growth and spore germination of *S. cucurbitacearum*. The Lemongrass EO had the highest inhibition effect on mycelial growth of *S. cucurbitacearum* and *A. alternata* (Guimarães et al. 2011).

Our chemical analyses illustrated a high content of citral and myrcene in Lemongrass EO which confirms previous studies in Indonesia and Spain (Bermúdez-Vásquez et al. 2019; Supardan et al. 2019). Brügger et al. (2019) reported that in addition to its richness in citral, the analysed of commercial *C. citratus* EO displayed a significant amount of nonan-4-ol (6.5%) and camphene (5.2%) which were completely absent in our analyses. Brazilian commercial EO of *C. citratus* seems rich in non-terpene compounds especially 4,8-dimethyl-3,7-nonadien-2-one (25%), 1-heptadec-1-ynyl-cyclopentanol (9.6%) and 7,7-dimethyl-bicycloheptan-2-ol (8%), while citral represented an amount around 37% (Macedo et al. 2019).

According to several reports, citral inhibited the mycelia of *F. oxysporum*, *Colletotrichum gloeosporioides*, *Bipolaris* spp. and *A. alternata* (Dalcin et al. 2017; Kishore et al. 2007) owing to antioxidant and antimicrobial properties (Farias et al. 2019). Kurita et al. (1981) stated that citral is fungicidal compound due to electrons transfer from the fungal cell to citral via a donor electron present in fungal cell inducing the death of the fungus.

Concerning the other EOs, the *in vitro* inhibition was partial. However in seeds treatment, *M. alternifolia* and *O. majorana* 2 EOs exhibited excellent activities against *S. cucurbitacearum* and *F. solani*, the latter was completely eliminated. It was considered that high antifungal potential of these EOs is the result of the high content of terpinen-4-ol, monoterpene alcohol, showing in previous studies good antifungal activity against *Fusarium* spp. (Sahab et al. 2014; Szczerbanik et al. 2007). For Tea tree EO, Ebani et al. (2018) registred a similar composition as in our work even though with a difference in percentages including, terpinene-

4-ol (30.2%) and γ-terpinene (16.9%) as the main components followed by α-terpineol (4.4%) and eucalyptol (4.0%). These latter two compounds were present in this work with lesser amount. Italian and Brazilian commercial EOs of Tea tree confirmed the domination of terpinene-4-ol (41.5% and 43.1%, respectively) and γ-terpinene (20.6% and 22.8%, respectively) which were followed by α-terpinene (9.6% and 9.3%, respectively) and α-terpineol (4.4% and 5.2%, respectively) (Elmi et al. 2019; Silva et al. 2019). Terpinene-4-ol was also the main compound of Indian and Tunisian *O. majorana* EOs (31.2% and 23.2%, respectively) (Hajlaoui et al. 2016; Raina and Negi 2012).

In agreement with what we found herein, Tahmasebi et al. (2016) during their investigation on the ontogenetic effect of *O. majorana* EO, deduced that terpinene-4-ol, γ- and α-terpinene, as well as α-terpineol were the main compounds. Results of Busatta et al. (2017) on the EO extracted by hydro-distillation from Egyptian dried leaves *O. majorana* were in accordance with the above-cited analyses (terpinen-4-ol (31.8%) and γ-terpinene (13.0%)) as the most abundant compounds. Both *O. majorana* 1 and *O. majorana* 2 EOs, collected from different provenience, presented the same components but with a different rate. The terpinene-4-ol compound was high in *O. majorana* 2 compared to *O. majorana* 1. This can explain the efficacy of *O. majorana* 2 against some seedborne fungi. Other recent researches on EOs of the species here studied confirmed oxygenated monoterpene class as the most dominant one (Fikry et al. 2019; Jan et al. 2018; Nardoni et al. 2018; Semiz et al. 2018) although the rate of their major compounds varied.

Seed treatment with *L. nobilis* and *L. dentata* showed significant reduction of *A. alternata*. These two EOs were characterized by high eucalyptol as the main compound. Eucalyptol (known also as 1,8-cineole), was the major compound in Brazilian and Mexican lavender (63.0% and 68.6%, respectively) (Martins et al. 2019; Silva-Flores et al. 2019). A similar behaviour was also noted in Moroccan lavender where camphor (64.4%), linalool (5.0%) and β-pinene (4.0%) predominated (Boubaker et al. 2019). On the contrary, Rahmouni et al. (2019) showed a completely different composition in Lavender EO where linalool (30.2%), linalyl anthranilate (23.5%) and eucalyptol (9.2%) were the predominant ones. All these compounds were present in different percentages herein except for linalyl anthranilate which was missing. The composition of *L. nobilis* was in total agreement with the results obtained by Fidan et al. (2019) who investigated the Bulgarian species (33.3% eucalyptol and 10.3% α-terpinyl acetate) as well as what reported by Dammak et al. (2019). Damasceno et al. (2019) cited in the review on chemical composition of the Lauraceae eucalyptol (11 times) followed by α-terpinyl acetate (8 times), sabinene (5), methyleugenol (4) and linalool (3) as the most

representative compounds in the plant family EOs. The same behaviour was also evidenced in this study where linalool (7.4%), sabinene (6.7%) and methyleugenol (3.1%) were present in appreciable amounts toghether with the prevalence of the first two compounds. Xu et al. (2016) showed excellent antifungal activity of *L. nobilis* EO against *A. alternata*.

Lavandin EO (*L. hybrida*) was characterized by the relatively high content of linalool followed by linalyl acetate and camphor. A similar composition was reported in the commercial EO of *L. hybrida* (Ebani et al. (2018); Nardoni et al. 2018)). Moreover, several EOs of lavandin cultivars highlighted a similar behaviour such in Super A and Grosso EOs analysed by Pistelli et al (2017) (33.5>21.1>7.8% and 33.8>17.0>8.5%, respectively). More recently, Tardugno et al. (2018) and Blažeković et al. (2018) confirmed the prevalence of linalool and linalyl acetate in both Italian and Croatian lavandin (43.1% *vs* 57.1%; 12.3% *vs* 9.83%, respectively), while Kıvrak (2018) pointed out a reversed order of these constituents. In this study, *L. hybrida* EO exhibited a moderate action on seedborne infestation. There was significant reduction of *A. verrucaria* and *A. alternata*, while no significant reduction was assessed for *F. solani* compared to control. Daferera et al. (2002) reported that Lavandin EO presented less inhibitory activity against *Botrytis cinerea* and *F. solani* compared to oregano and thyme EOs.

The main compounds of *Salvia sclarea* L. EO were linalyl acetate and linalool (like Lavandin) and this EO presented a good antifungal activity against *A. alternata* (Džamić et al. 2008). Indeed, Kishore et al. (2007) showed that the antifungal activity of linalool was fungus specific, because it completely inhibited the growth of *A. alternata*, *C. lunata,* and *F. moniliforme*. In our study performed in a controlled environment, the EOs were all effective against seedborne fungi when applied on seeds naturally infested. Although, these EOs significantly outperformed the non-treated seeds but none of them was as effective as Celest Extra fungicide.

Chitosan is considered as an alternative product especially used in organic farming. Chitosan hydrochloride was approved for use in agriculture as a plant protection product by European Commission Regulation (EU) No 563/2014 of 23 May, 2014, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and Council. Many researches have demonstrated the fungicidal activities of chitosan against various fungi. Among its several application areas, chitosan has been shown to control preharvest and postharvest plant diseases (Romanazzi et al. 2016). In our study, seed treatment with chitosan (0.5%) alone was not significantly effective against *A. alternaria* and *S. cucurbitacearum* in comparison with control. However, mixture of, chitosan with *O. majorana* 2 or with *C. citratus* EOs reduced

significantly the incidence of *S. cucurbitacearum* and *A. alternata* on seeds. It seems that thid observed efficacy is due more to the Eos than to chitosan itself.

As previously mentioned, citral was the main component of lemongrass EO, which was the most powerful in antifungal. This compound was missing in the other tested Eos. In addition, *C. citratus* EO was significantly harmless to otherwise promoting, the radicule length unlike other Eos and untreated seeds. For these reasons, this EO was selected to be tested *in vivo*. Seed treatment with *C. citratus* (500 ppm) EO significantly increased seedling emergence and reduced the incidence of *S. cucurbitacearum* in the plantlets. Dalcin et al. (2017) reported that lemongrass EO reduced the severity of *S. cucurbitacearum* in plants of melon.

6. Conclusion

The management of plant diseases with natural compounds is highly needed nowadays. Therefore, these EOs are a novel alternative to control seedborne fungi and can be applied with physical methods to control fungal pathogens localized within the seeds (Gullino et al. 2014). The results of this study evidenced that the chemical composition of EOs are of interest since some chemical configurations had greater potency, while others less. Future studies are needed to adjust the appropriate EOs concentrations and to test the effect of some components in controlling separately seedborne fungi.

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General Conclusion

The pathogens associated with seeds are considered one of the major means to disseminate diseases from infected to non-infected areas around the world by international trade. Seeds are an important nutrient to survive pathogens in nature.

Our research highlights the importance seedborne fungi of local varieties of *C. maxima* and *C. moschata* in Tunisia and Italy. In particular, the results showed a positive correlation between the symptoms on squash fruit and the fungal species isolated from seeds. Some fungi, such as *S. cucurbitacearum* and *A. alternata*, were present in seeds although their fruits were asymptomatic. This research proves that the cultural practices, the routine visual inspection to look for rot on the fruits are not enough to guarantee that the seeds inside these fruits are healthy. Therefore, it is very important to test seeds for disease organisms before they are sown in the field.

Conventional method (Blotter method) was used in this work to detect pathogens on seeds and fruits. However, morphological identification is time consuming and requires mycological skills. This study showed that the molecular tool (PCR) was highly sensitive to detect *S. cucurbitacearum* on seeds and in the three parts of seeds (tegument, cotyledon, and embryo). Study the transmission and the localization of pathogen help to address the challenge of understanding the role of inoculum in disease risk within a field. In addition, knowledge improvement of pathogen epidemiology initiated from seeds could lead to improve the management of such disease.

The management of plant diseases with natural compounds is highly needed nowadays. This research was performed to study the antifungal activity of seven essential oils under *in vitro* and *in vivo* conditions. The results showed that *Cymbopogon citratus* EO was the most effective since it significantly reduced multiple pathogens on seeds. This EO was able to reduce the incidence of *S. cucurbitacearum* in both seeds and plants. Ultimately, reducing the amount of seedborne inoculum will help disease management in short- and long-term.

In many low-income Countries, 80 to 95% of seeds is routinely obtained from informal seed sources with poor or unknown seed health status (Thomas-Sharma et al. 2016). This study represents the first base-line characterization of seedborne fungi of squash in different Mediterranean Countries as Tunisia and Italy, where the majority of growers extract themselves the seeds. Such information could be used as a model to deepen researches on seedborne fungi. Uses of healthy seeds are the best weapon to prevent many diseases, and sowing seeds of local varieties is the best weapon against climate change and hunger. On-

farm seed selection can perform as well as certified seed, if the rate of success in selecting healthy soil, plants, and fruit for seed production is high.

Early diagnosis of fungal infection is critical to effective treatment. The goal of the present work was to move from good results of research to sustainable production. If we hope to meet that goal, we must embrace more innovative tools of seed detection with high specificity and sensitivity (e.g. digital PCR). Moreover, the success of seed treatments depends on the pathogen localization and a better understanding of the fungal invasion mechanism. Furthermore, the success for the application of non-chemical alternatives requires an integrated approach involving combinations of multiple control strategies according to the pathogen on the seed. We need to further develop principles and methods of biological and physical control as relevant pest management tools for sustainable agricultural production.

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Annexes

Supplementary Table S1. GenBank accession numbers of *Paramyrothecium*, *Myrothecium*, *Albifimbria*, *Stemphylium*, *Pleospora*, *Curvularia*, *Fusarium*, *Stagonosporopsis* 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
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 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

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

Poster 1:

ICPP BOSTON Plant Health in a Global Economy JULY 29 - AUGUST 3 2018

ABSTRACT

275-P: Morphological and molecular identification of seedborne fungi in squash (*Cucurbita maxima*)

Monday, July 30, 2018 16:00 - 16:00

Squash (*cucurbita maxima*) is one of the important vegetable in tropical and temperate regions. This crop can be affected by several diseases transmitted by seeds, which become a responsible for the dispersion of many pathogens. Low percentages of seed infection can still result in severe economic losses. Early detection of seedborne fungi is the first step to control diseases. Squash fruits were collected from two regions in Tunisia and were evaluated according to three levels of lesion extension: asymptomatic fruit, infected fruit showing lesions on the squash skin without colonization of the fruit cavity, and infected fruit showing lesions that had colonized the fruit cavity. Using blotter method, morphological identification of seedborne fungi was showed that *Didymella bryoniae*, *Alternaria alternata*, *Fusarium moniliforme*, *Fusarium solani*, *Myrothecium verrucaria*, *Myrothecium roridum* and *Pleospora herbarum* were the most frequent fungi. Morphological identification was confirmed by molecular diagnosis using the available genus-specific and species-specific primers. Furthermore, specific primers were designed from DNA sequence in the internal transcribed spacer (ITS) region of nuclear rDNA, to identify *Myrothecium* spp., *M. verrucaria*, *M. roridum* and *Pleospora* spp. Those results can be useful to prevent squash seedborne pathogen dispersal and related losses of production later occurring in the field.

Presenting Author

Gianfranco Romanazzi

Marche Polytechnic University

Co-authors

Marwa Moumni

National Agricultural Institute of Tunisia

Mohamed Bechir Allagui

National Institute for Agronomic Research of Tunisia

Valeria Mancini

Marche Polytechnic Univ

Membership Sipav and winner young researcher.

sipav201 <sipav201@mta01.univpm.it>
À : moumni_marwa@yahoo.fr

31 mai 2018 à 16:24

Dear Dr Marwa Moumni,

the Committee evaluated your abstract and curriculum, and we are pleased to tell you that you resulted winner of Young Researcher Award to join next SIPaV meeting, including the free admission to the congress and social dinner, and SIPaV membership for the following 3 years. Your work will be presented as a poster. Moreover, you will have the chance to show briefly (3 min for presentation and 2 for eventual questions) your work to the audience in the afternoon of the first day of the congress.

Congratulations for the achievement

Kind regards
Secretariat of SIPaV 2018 Meeting

Poster 2 and Présentation orale 1

XXIV SIPaV Congress, Ancona 5-7 September 2018 - *Factory of Ideas – Young Researcher Session*

Morphological and molecular identification of seed-borne fungi in squash (*Cucurbita maxima*)

M. Moumni1,2,3, M.B. Allagui3, V. Mancini1, S. Murolo1, N. Tarchoun4, G. Romanazzi1

1Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, 60131 Ancona, Italy; 2National Agricultural Institute of Tunisia, 43 Avenue Charles Nicolle, 1082 Tunis, Tunisia; 3Laboratory of Plant Protection, National Institute for Agronomic Research of Tunisia, University of Carthage, Rue Hédi Karray, 2080 Ariana, Tunisia; 4Laboratory of Vegetable Crops, High Agronomic Institute of Chott Mariem, Sousse, Tunisia. E-mail: moumni_marwa@yahoo.fr

Squash (*Cucurbita maxima*) is one of the most important vegetables grown in tropical and temperate regions. This crop can be affected by several diseases transmitted by the seeds, as an efficient vehicle to disperse pathogens over long distances. Even low percentages of seed infection can result in severe economic losses. Early detection of seed-borne fungi is the first step in the control of such diseases. Squash fruit were collected from two regions of Tunisia and were evaluated according to three levels of lesion extension: asymptomatic fruit; infected fruit showing lesions on the squash skin without colonization of the fruit cavity; and infected fruit showing lesions that colonized the fruit cavity. Following the blotter test, 16 fungal species were detected in the seeds. Seed-borne fungi were identified for all of the tested fruit samples, including the asymptomatic fruit. The most frequent seedborne fungi were *Didymella bryoniae* (24.6%), *Alternaria alternata* (24.8%), *Fusarium moniliforme* (7.8%), *Fusarium solani* (6.9%), *Rhizopus* spp. (20.3%), *Myrothecium verrucaria* (3.3%), *Pleospora herbarum* (2.8%), and *Myrothecium roridum* (1.2%). Morphological identification was confirmed by molecular diagnosis using the available genus-specific and species-specific primers. Furthermore, specific primers were designed from DNA sequences in the internal transcribed spacer (ITS) regions of the nuclear rDNA to identify *Myrothecium* spp., *M. verrucaria*, *M. roridum*, and *Pleospora* spp. Correct identification of seed-borne fungi is a key factor for crop protection. Application of seed health testing methods, including conventional and molecular diagnostic tools, will help to improve seed quality and crop yields.

Poster 3:

Preliminary detection of necrotrophic fungal species associated with local caraway and cumin seeds in the Cap Bon region of Tunisia

S. Hassouna, M.B. Allagui, M. Moumni

Laboratory of Plant Protection, National Institute for Agronomic Research of Tunisia,

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syrine.hasouna@gmail.com

This study was performed in order to determine the importance of seed-borne fungi associated with local seeds of caraway (meridian fennel) and cumin. Six samples of caraway and five of cumin were collected from farmers in the Cap Bon region during 2017 to be analyzed by the blotter method to isolate the associated seed-borne fungi. Based on morphological criteria, seven species of nectrotrophic fungi were isolated and identified in caraway and cumin seeds, namely *Rhizoctonia* sp., *Alternaria alternata*, *Stemphylium botryosum*, *Cladosporium* sp., *Chaetomium globosum*, *Ulocladium* sp. and *Aspergillus* sp. The main species associated with caraway seeds were *Rhizoctonia* sp., *Alternaria alternata* and *Stemphylium botryosum* with an incidence of 28.5%, 21.8% and 18.6%, respectively. The most frequent fungi in cumin seeds were *Rhizoctonia* sp. followed by *Chaetomium globosum,* with an incidence of 19.7% and 6.25%, respectively. These preliminary results could lead to the carrying out of studies about the implication of such fungal species in seedling mortality in the field, and on the presence of harmful mycotoxins into grains harmful for consumers.

Membership in APS

APS Global Membership Program

Ginnie O'Neill <goneill@scisoc.org>
À : moumni_marwa@yahoo.fr

27 déc. 2018 à 21:47

December 2018

Dear Marwa Moumni,

On behalf of the APS Office of International Programs we are pleased to inform you that you have been selected for the APS Global Membership Fund. You will receive two years of free membership, starting January 1, 2019. This program is managed collaboratively by APS Foundation and the APS Office of International Programs. APS hopes that this free membership will be of value to you and that you will share the benefits of membership with colleagues. With this membership, your benefits include:

* Voting privileges
* Eligibility to apply for awards and programs
* Bi-Weekly APS News Capsule
* Online access to Phytopathology News for APS news and updates
* Significant discounts on the annual meeting, APS PRESS items, and journal subscriptions
* Access to the online member directory and member-only areas of the APS website
* Networking opportunities with other APS members
* Access to webinars free to members
* Discounted rate on publishing in all journals
* Job Center services
* Opportunities for committee involvement and professional development

Only APS membership in Tunisia:

Megan Petersen <phytonewseditor@scisoc.org>
À : moumni_marwa@yahoo.fr

View Mobile or Web Version

Phytopathology News

Dear Marwa Moumni,

APS has recently launched a member spotlight series called "APS Membership Matters" in *Phytopathology News*. I am writing to invite you to contribute a written interview as part of this series. Our goal is to highlight the benefits of APS membership through the eyes of our members. In particular, we are interested in your perspective as one of the only APS members in TUNISIA.

Please let me know if you would like to participate. The interview questions are below. The total word count—including a short biography—should be around 900 words at most. Please also include a headshot and a photo or two of your work in action! We would need the interview questions back by March 15. If you would need more time, please let me know.

Please let me know if you have any questions. Check out past issues of *Phytopathology News* if you would like some examples. We would love to feature you in an upcoming issue!

Sincerely,

Megan Petersen
Membership Communications Coordinator
Editor, *Phytopathology News*
American Phytopathological Society
651.994.3802
www.apsnet.org

APS Interview:

APS Membership Matters

MARWA MOUMNI

Resources for Collaboration and Education

Marwa Moumni is a PhD student at the National Institute of Agronomy of Tunisia (INAT). In 2011, she received an engineering degree at the Higher School of Agriculture of Kef (ESAK) in Tunisia for a project involving virus of artichoke. In 2014, she received a master's degree in integrated pest management, focusing on the topic of seedborne fungi on watermelon and muskmelon. From 2013 to 2019, her research continued in the Tunisia Department of Plant Protection and the National Institute of Agronomical Research in Tunisia (INRAT) under the guidance of **Bechir Allagui** and in Italy in the Department of Agricultural, Food, and Environmental Sciences of Marche Polytechnic University (UNIVPM) under the guidance of **Gianfranco Romanazzi**. Her work focused on detection, morphological, and molecular identification of seedborne fungi on cucurbits (watermelon, muskmelon, and squash). She also studied seed transmission of the main pathogens of cucurbits (e.g., *Stagonosporopsis cucurbitacearum*, *Alternaria alternata*, *Paramyrothecium roridum*, and *Albifimbria verrucaria*) and their control using essential oils and chitosan. Recently, she investigated *Aspergillus* contamination and mycotoxin production in caraway and cumin seeds. Marwa has been awarded memberships in the Italian Phytopathological Society (SIPaV) and The American Phytopathological Society (APS), and she was proposed to serve as a member of the ISPP Seed Pathology Committee.

> *"... It is very important to be member of APS. This opportunity can open for me ways to meet experts in plant pathology within my field of study."*

As a plant pathologist based in Tunisia, what prompted you to become a member of The American Phytopathological Society?

Many things prompted me to become a member of The American Phytopathological Society (APS). In my research, for the design of experiments, I used APS scientific papers, which provided the necessary details to help me to understand the work and to repeat it. For me, APS is an excellent reference that is recognized worldwide. For this reason, I feel that it is very important to be member of APS. This opportunity can open for me ways to meet experts in plant pathology within my field of study: seedborne fungi. In 2018, thanks to APS, I presented a poster on detection of squash seedborne pathogens at ICPP2018, and I was considered to be part of the ISPP Seed Pathology Committee. I am confident that these chances that APS offers me will improve my career, skills, and knowledge.

What is the most valuable benefit of APS membership?

There are many benefits of APS membership. The most valuable is to have the opportunity to participate in the meeting and have a great connection with all the participants and opportunities for collaboration. Meeting other staff and members of professional bodies allows everyone to share experiences and learn from each other. APS encourages student members to be more motivated and creative.

How has your APS membership supported and enriched your work in the plant pathology field?

My APS membership can support and enrich my career in several ways. I can benefit from following conferences, communities, and publications, and I can participate in courses that will help me learn more about phytopathology. I can have discounts to publish in APS journals, and I can receive travel awards and participate in meetings to present my research and show the results of my investigations. I can benefit by sharing my results and collaborating with other APS members, who believe in the importance of studying plant diseases and can teach me about the tools for understanding their etiology, epidemiology, detection, and management strategies. APS publications (journals, books, etc.) are precious resources for research. By attending APS conferences, I can know all the trends in plant pathology, such as new pathogens, new tools, and new scientific techniques. Improving my skills in plant pathology will make me more motivated.

What are the benefits of an APS membership for plant pathologists in Tunisia and beyond?

Having an APS membership allows any plant pathologist the opportunity to interact with other plant pathologists worldwide in the niches of seedborne fungi, as well as broader fields. Networking opportunities include participating in APS-sponsored events. Plant pathologists in Tunisia can be up to date with the new research carried out, and the whole Tunisian phytopathological and agricultural community will benefit from such cooperation. ■

Menbership in ISPP Subject Matter Committee: Seed Pathology

ISPP Subject Matter Committee: Seed Pathology

Chair: Prof Theresa (Terry) Aveling (Pr. SC.Nat.)
Department of Plant and Soil Sciences
Forestry and Agricultural Biotechnology Institute
University of Pretoria
Pretoria 0002
South Africa
Email: terry.aveling@fabi.up.ac.za

Seed Pathology

The ISPP Subject Matter Committee on Seed Pathology is to be revived. The Committee will address seed pathology in its broadest possible meaning. The aim is to have committee members from chemical and seed companies, universities, research institutions, government departments and international bodies such as ISTA, ISF, EPPO, NSHS etc. The production of pathogen-free seed is essential for global food security and safety. Due to economics and the importance of seed-borne diseases and pathogen-free seed in international seed trade, renewed interest in the ability to identify and detect seed-transmitted pathogens using the latest technological advances has occurred. Seed treatments have also become more feasible and environmentally friendly. With the update of the "Annotated list of seed-borne diseases" currently taking place, the input of a revived ISPP Seed Pathology Committee will be invaluable. This is a call for all interested colleagues to participate in the ISPP Seed Pathology Committee.

Please contact Theresa (Terry) Aveling at terry.aveling@fabi.up.ac.za.

2018 ISPP Seed Pathology Committee Meeting.pdf

Global Impact of International Seed Movement IPPC session.pdf

International Society for Plant Pathology Seed Pathology Committee (2014)

Name	Country	Organization	Email	Key words
Theresa (Terry) Aveling	South Africa	University of Pretoria	terry.aveling@fabi.up.ac.za	Seed-borne diseases, seed pathology, seed vigour, seed treatments
Guro Brodal	Norway	Bioforsk	guro.brodal@bioforsk.no	Seed borne diseases cereals, oil seed crops, legumes, grasses, seed treatment, mycotoxins, organic seed
Quenton Kritzinger	South Africa	University of Pretoria	quenton.kritzinger@up.ac.za	mycotoxins, seed health, seed treatments, biopesticides
Jose da Cruz Machado	Brazil	Federal University of Lavras	machado@dfp.ufla.br	Seed Pathology
Lindsey du Toit	USA	Washington State University	dutoit@wsu.edu	Vegetables, small-seeded, dry-seeded
Marie-Agnes Jacques	France	INRA	marie-agnes.jacques@angers.inra.fr	*Xanthomonas, Clavibacter*, Bacteria', Microbial community plant-microbe interaction
Gary Munkvold	USA	Iowa State University	munkvold@iastate.edu	Maize, soybean, vegetables
Gianfranco Romanazzi	Italy	Marche Polytechnic University	g.romanazzi@univpm.it	Vegetables, seed-borne diseases, seed pathology, diagnosis, control measures
Dorota Szopińska	Poland	Polish Phytopathological Society	dorota.szopinska@up.poznan.pl	Seed pathology, fungi associated with seeds, methods of seed health evaluation
Casiana M. Vera Cruz	Philippines	International Rice Research Institute	c.veracruz@irri.org	Rice, *Xanthomonas oryzae*
Shuxian Li	USA	United States Department of Agric.	shuxian.li@ars.usda.gov	Soybean seed diseases, soybean Phomopsis seed decay, resistance
Gerbert Hiddink	The Netherlands	Enza Zaden Seed Operations		Seed health testing.
Carlos Bolanos-Carriel	USA	University of Nebraska- Lincoln	cbolanos@huskers.unl.edu	Propagation of biocontrol agents
Marwa Moumni	Tunisia	National Institute of Agronomy		Mycotoxins in Caraway and Cumin seeds

Marwa Moumni an Agriculture engineer. In 2014, she got Master degree in Integrated Pest Management at National Institute of Agronomy of Tunisia (INAT). In 2015, her research continued in Tunisia in the 'Department of Plant Protection, National Institute of Agronomical Research in Tunisia (INRAT)' and in Italy in the 'Department of Agricultural, Food and Environmental Sciences of Marche Polytechnic University' and focused on detection, morphological and molecular identification of seed-borne fungi on cucurbits (watermelon, muskmelon and squash). She also studied seed-transmission of the main diseases of cucurbits (e.g. *Stagonosporopsis cucurbitacearum*) and their control using essential oils and chitosan. She is a member of the Italian Phytopathological Society (SIPaV). In recent years her main focus has been on *Aspergillus* and mycotoxins in Caraway and Cumin seeds.