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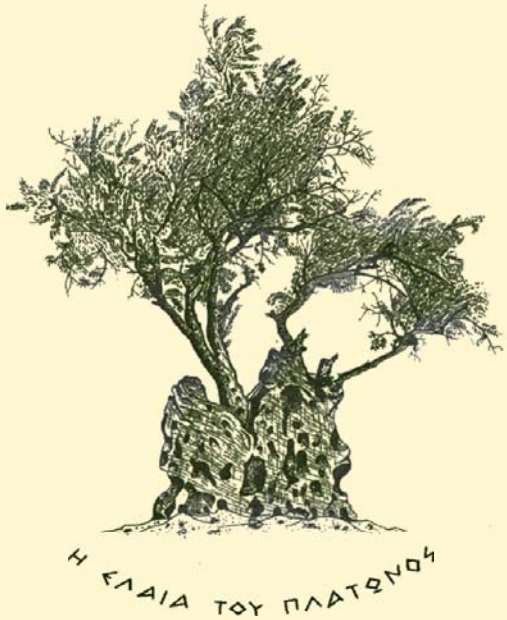

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REVIEW ARTICLE

Late blight of potato: From the great Irish potato famine to the genomic era – An overview

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Summary Late blight of potato and tomato, one of the most widely reported diseases of plants, is a significant curb in global agriculture which poses severe problems in terms of yield and economic losses, and environmental pollution due to pesticides use. The disease is caused by *Phytophthora infestans* -an oomycete - which first drew the considerable attention of plant pathologists during the mid-1840s when the pathogen incited historic starvation in Ireland – the great Irish potato famine - as a consequence of substantial potato losses due to late blight disease. Since that period, late blight has triggered several epidemics of potato and tomato of profound intensity in different regions. Over the course, synthetic fungicides have been proved effective management practice for late blight control; nonetheless, the evolution of new genotypes with increased virulence to hosts and resistance to fungicides has been greatly regarded as an agricultural problem. Breakthroughs in genome sequencing of *P. infestans* and identification of resistance genes in some plants have opened ways for the development of resistant genotypes. However, there still exist numerous challenges to deal with this noxious pathogen. This review aims to highlight the historical significance of late blight disease, its chemical control strategies and associated challenges, and resistance breeding programs by employing genetic approaches.

Additional Keywords: Breeding, pesticides sensitivity, qualitative and quantitative resistance, resistance genes, virulence

Introduction

Late blight of potato, induced by an oomycete pathogen, *Phytophthora infestans,* is a significant curb in global potato production which has severe effects on the crop, yield, and quality of tubers. The disease has been an important limiting factor for potato production worldwide and a major contributing element in financial losses in terms of fungicide application (Hijmans *et al*., 2000). Outcomes of late blight are propounded in temperate and moist regions which facilitate *P. infestans* and proliferation of the disease with hastened intensity (Nowicki *et al*., 2012). The disease first appeared in the US during 1842-43 (Peterson *et al*., 1992). However, devastating effects were observed in Ireland during 1845 when the pathogen wiped out potato crop throughout the country triggering famine – the Great Irish famine- which is believed to have caused the death of more than two million Irish population (O'Grada, 1999; Scholthof, 2007).

Due to the popularity and importance of potato – the third most cultivated crop following rice and wheat – understanding and controlling the limiting factors which have profound impacts on potato yield are crucial for avoiding future food issues. Advances in synthetic chemistry have greatly contributed to minimal losses of potato by the development of fungicide compounds which have shown good efficiency in controlling the disease epidemic throughout the world for almost a century. However, the

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use of such fungicides is of grave concern because they may cause environmental issues and increased resistance of *P. infestans* strains to oomyceticides (Qin *et al*., 2016; Majeed *et al*., 2017a; Zhang *et al*., 2017).

To address environmental challenges posed by fungicide application and the emerging strains of the pathogen which are less responsive to some of the fungicides, research activities during the last few years have been diverted to find out alternative substances of natural origin, biocontrol agents (BCAs), and introgression of resistance genes of interest in the hosts from some wild sources, which could efficiently reduce high reliance on fungicides in future (Daayf *et al*., 2003; Dorn *et al*., 2007; Śliwka *et al*., 2014). Several efforts have been made in this regard to introduce natural formulations and BCAs as alternative sources of fungicides and breeding for resistance development in host crops (Lenman *et al*., 2016; Ippólito *et al*., 2017; Roman *et al*., 2017; O'Brien, 2017; Tala *et al*., 2018). However, identification, isolation, preservation, and consistency in the effectiveness of natural compounds and BCAs in laboratory and field remain great challenges for researchers (Velivelli *et al*., 2014). Moreover, qualitative resistance breeding approaches in cultivated potatoes have been proven less effective due to the potential of *P. infestans* to overcome such single gene-controlled resistance (Rietman *et al*., 2012). On the other hand, the search for quantitative resistance and introgression of multiple resistance genes in potatoes against the pathogen seems a promising quest to endure late blight resistance (Rietman *et al*., 2012; Sarkar, 2015). Although we have not been able to develop a durable resistant potato cultivar, nor have formulated an efficient biocontrol agent alternative to fungicides, still there exists a great hope for sustainable management of late blight of potato through further expansion in the genomic understanding of the host and pathogen interaction. This review aims to highlight historical perspectives of late blight, its chemical control and challenges, and resistance breeding in the genomic era.

Late blight in history

There is sufficient documentary evidence that late blight (LB) had occurred in some parts of the United States during 1842-43 (Peterson *et al*., 1992; Andrivon, 1996; Tateosian *et al*., 2017), reaching to Canada, Belgium, Holland, Italy, Germany, Scotland, and the UK during 1844 and 1845. However, first profound effects of the disease appeared by the mid of 1845 in Ireland where it caused a devastative catastrophe which triggered food starvation and consequent Great Irish Famine (Ristaino, 2002; Scholthof, 2007). It is believed that the famine resulted in a population drop in Ireland from 8.2 to 5.5 million due to human deaths and migrations (Cantwell, 2017). At the time of famine and years later, the exact causal agent of the disease was cynical and different views were expressed about the calamity of the famine arbitrating towards environmental conditions and a fungus called *Botrytis infestans*. It was de Bary in 1870 who first described the causal agent as *P. infestans* and opened ways for further research and subsequent formulations of antimicrobial compounds (Turner, 2005). In succeeding years after the famine, late blight disease remained major havoc in most parts of the world until the introduction of the Bordeaux mixture in the late 1880s which significantly contributed to the management of blight incited crop damages (Haverkort *et al*., 2008). For instance, due to rare chemical control approaches in Finland, the disease had major epidemics in the country until the 1970s (Hannukkala *et al*., 2007). During 1916-17, substantial potato losses occurred in Germany due to poor management of late blight disease as a result of a shortage of Bordeaux mixture which led to famine - generally attributed to causing several deaths of the German soldiers (Mizubuti and Fry, 2006). Outbreaks of late blight occurred with severe intensity in different parts of the USA particularly Oregon and Washington during 1947, 1974, and 1990s (Johnson *et al*., 1998) and the USA and Canada during 1980-1990 which resulted in considerable losses of both po-

tato and tomato (Fry and Goodwin, 1997). In 2009, the USA experienced another episode of late blight causing significant crop damage to tomatoes (Fry *et al*., 2013). In the Netherlands, a comprehensive analysis of data covering the period between 1950 and 1996 revealed the different intensity of late blight disease in the region (Zwankhuizen and Zado, 2002). In northern and southern parts of India, Oman, Tunisia, Nigeria, China, and Chile considerable losses for potato and tomato growers were incurred by *P. infestans* during a different period of times from 1953 to 2012 (Chowdappa *et al*., 2015; Fry *et al*., 2015). The occurrence of late blight and its devastations are not limited to the aforementioned regions; rather it has recurrently appeared in different parts throughout the world with a differential intensity of damages depending on the climatic conditions, the potato varieties, and growers' approaches to manage the disease in those regions. The disease remains a challenging problem for agriculturalists throughout the world in climates suitable for the spread and establishment of the pathogen (Majeed *et al*., 2017a).

Phytophthora infestans is generally believed to have its origin in Mexico from where the pathogen migrated to several parts of the world (Andrivon, 1996). The migrations are thought to have occurred either from Mexico to the USA and subsequently to Europe or from Andean regions to the USA and Europe or probably from Mexico to Peru, USA, and then to Europe with the further general perception that the disease could have spread to several other parts of the world through those migrations after the 1850s when potato production had been established in the world (Andrivon, 1996; Mizubuti and Fry, 2006).

An important factor linked with emerging threats of the late blight pathogen is its changing population structure globally. Frequent studies suggest that before the 1980s, the global population of late blight pathogen consisted of a single clonal lineage (A1) which reproduced asexually, while in Mexico both lineages (A1 and A2) co-occurred with the tendency of asexual and sexual reproduction (Andrivon, 1996; Mizubuti and Fry, 2006; Runno-Paurson *et al*., 2014). Probable migration of A2 mating strains of *P. infestans* from Mexico to Europe during 1976 could have resulted in sexual reproduction, the formation of oospores, diversity in population structure, the virulence of the newly introduced strains, and difficulties in disease control methods (Hannukkala *et al*., 2007; Runno-Paurson *et al*., 2014). The dominant method of reproduction of *P. infestans* is asexual where sporangia and zoospores are produced. Sexual reproduction occurs following the mating of the compatible strains and results in the formation of oospores which are resistant to unfavorable environmental conditions (Nowicki *et al*., 2012; Lehsten *et al*., 2017; Rekad *et al*., 2017).

Evolution of fungicides and emerging challenges

Until the 1880s, potato late blight appeared to be a challenging threat to potato growers throughout the world because there was no curative treatment to control the disease. The first-ever fungicide, the Bordeaux mixture (a mixture of lime and copper sulfate) when formulated in the mid-1880s, opened ways for the efficient control of late blight and several other plant pathogens (Ayres, 2004; Lemire *et al*., 2013). Since the development of copper fungicides, late blight was successfully managed by the exhaustive application of these compounds. After the 1950s, several other fungicides containing different chemical ingredients and diverse modes of actions were progressively developed which further strengthened the disease control methods and contributed to greater yields of potato crop (Haverkort *et al*., 2008). However, successive use of newly developed fungicides, particularly metalaxyl compounds, appeared gradually less effective in controlling the disease during the 1980s as a result of selection pressure and resistance induction in population of *P. infestans* in several parts of the world (Cooke

et al., 2011; Haesaert *et al*., 2015). Reports about the insensitivity of *P. infestans* strains to commonly used fungicides from the USA, Europe, Asia, Middle East and the rest of the world were documented in the 1980s (Goodwin *et al*., 1996; Cooke *et al*., 2011; Fry *et al*., 2015). The resistance development to fungicides in isolates of *P. infestans* is yet to be fully explored, however, studies suggest that mutation, sexual recombination, the role of semi-dormant locus, the influence of quantitative trait (controlled by major and minor genes), and changes in ribosomal RNA could greatly contribute to the insensitivity of the new strains of the pathogen to fungicides (Lee *et al*., 1999; Randall *et al*., 2014; Fry *et al*., 2015; Qin *et al*., 2016).

Besides stimulation of fungicidal resistance, the use of fungicides for late blight control has drastic implications on the environment, ecosystem, and human economy. Under environmental conditions favorable for the disease spread, cultivation of susceptible cultivars may substantially increase the input of synthetic chemicals into the agricultural fields. For instance, in Netherlands, Belgium, and other countries where wet and cool climate prevails during potato growth, the spray frequencies of oomycetices may exceed 20 applications per growing season (Haesaert *et al*., 2015). Estimated economic costs mainly incurred by fungicide application in the USA are reported to be 0.287 billion dollars, in Netherlands and Belgium 68-124 billion while such estimates exceed 6.7 billion dollars globally on annual basis (Haverkort *et al*., 2008; Haesaert *et al*., 2015; Liu *et al*., 2016). These estimates are generally variable because climatic conditions in different countries vary significantly and there is no proper monitoring system to collect exact data regarding actual costs and damages to potato crops due to late blight disease and the volumes of used fungicides. The first use of copper-related fungicides raises issues of risks for environmental pollution and ecosystem stability and these risks have even arisen over time with the development of more sophisticated compounds (Aktar *et al*., 2009).

Resistance induction: successes and challenges

In the context of sustainable use of pesticides for the control of late blight, forcasting systems and integrated disease control measures can work in reducing the use of fungicide applications and their input (Cooke *et al*., 2011; Majeed *et al*., 2017b). Seemingly, one of the most efficient quests for late blight control is to develop resistance in potato crop to the pathogen. For durable resistance induction, identification of genes and loci with potential resistance-conferring abilities is the key step in breeding for resistance efforts. However, such a goal may not be easy to accomplish because of the evolution of pathogens to overcome such resistance with time and thus, more systematic approaches are consistently required which may employ wild sources for discovering resistance genes (Nelson *et al*., 2018).

Efforts have been made through decades by employing conventional and molecular breeding approaches for induction of the required resistance in potatoes against *P. infestans*. Genome sequencing of *P. infestans* (Haas *et al*., 2009; Martin *et al*., 2013) and potato (Xu *et al*., 2011) has fostered a wide scientific interest in looking for several durable disease resistance strategies, i.e., manipulating crosses between susceptible and resistant *Solanum* species, introgression of genes from resistant donors, understanding the pathogen and host proteins involved in host-pathogen interaction, using biotechnological approaches to enhance the immunity of potato against *P. infestans*.

To date, many blight-resistant genes (BRG) from wild species and hybrids of *Solanum* such as *S. demissum* (*R1-R11*), *S. bulbocastanum* (Rpi-blb1-Rpi-blb3), *S. stoloniferum* (*Rpi-pta* and *Rpi-sto1; Rpi-sto2*), *S. michoacanum* (*Rpi-mch1*), *S. americanum* (*Rpi-amr3*), *S. ruiz-ceballosii* (*Rpi-rzc1*) and several others have been identified and successfully mapped into different chromosomes of cultivated potato (Sliwka *et al*., 2006; Bradshaw *et al*., 2006; Hein *et al*., 2009; Sliwka *et al*., 2012a,b; Du and Vleeshouwers, 2017; Agu-

ilera-Galvez *et al*., 2018). The introduced *R genes* particularly R1-R11 in domestic potato proved effective against *P. infestans* for long period until the evolution of new strains of the pathogen which have overcome those *R-genes*, thus making qualitative resistance a non-durable approach (Sliwka *et al*., 2006; Vleeshouwers *et al*., 2011; Du *et al*., 2017). Qualitative resistance which relies on major *R-genes* offers wide-ranging resistance against the pathogen by the production of proteins in host tissues which recognize the pathogen; defeating of major *R-genes* by the newly evolved strains of *P. infestans* however, suggests quantitative disease resistance conferred by many genes (often termed as minor genes) framed into quantitative trait loci as a better strategy, although difficult to be accomplished (Du *et al*., 2015; Nelson *et al*., 2018).

Since resistance based on single *R-genes* corresponds to hypersensitive response in a host but such durability is not persistent, there is a strong need for isolation and employment of more *R-genes* in pyramids (Aguilera-Galvez *et al*., 2018). A large number of wild species of *Solanum* with efficient resistance to *P. infestans* have been described which may serve as valuable donors of *R-genes* however major challenges emerge when it comes to cross them with a cultivated potato because of variation in ploidy level (Smyda *et al*., 2013). Quantitative resistance conferred by minor genes may either be deployed in potato after successful identification and isolation or the potato may itself harbor such type of resistance. However, those minor genes may interfere with major *R-genes*, influencing the quantitative resistance ability of the host, thus posing another challenge to durable resistance quests (Ewing *et al*., 2000). Further, dependence only on *R-genes*, focus on other plant genes which encode defense-responses and pattern recognition immune receptors in the host can work better in achieving durable late blight resistance (Du and Vleeshouwers, 2017). Moreover, staking numerous *R-genes* and quantitative trait loci in a single genome can enhance the resistance potential of the host to the pathogen (Nelson *et al*., 2018).

In addition to *R-genes* searches, effectoromics, transcriptomics, metabolomics and cis-genic approaches may greatly foster our knowledge about resistance induction in potatoes against the pathogen. Recently Gromadka *et al*. (2018) described that calcium-dependent protein kinases had a strong relation with host-defense against *P. infestans*, whose elevation in potato seems an ideal approach to suppress pathogenic stress. Domazakis *et al.* (2017) argued that effectoromics could potentially boost our efforts for blight-resistance development because the employment of such techniques assists in the identification of pathogen-recognition receptors. Aguilera-Galvez *et al*. (2018) believed that effectoromics screening of the interaction between avirulence (*Avr*) and *R-genes* in *Phytophthora-Solanum* pathosystem could lead to broad-spectrum late blight resistance in potato. Haverkort *et al.* (2016) proved that cis-genic introgression of *R-genes* into potato clones exhibited a high degree of late blight resistance in field trials.

In conclusion, late blight, one of the costliest plant diseases, is generally managed by the widespread application of fungicides. Environmental, ecological and health risks associated with fungicide application have resulted in an increased search for sustainable disease management. Induction of resistance against *P. infestans* in cultivated potato from wild sources has been long practiced; however, introgression of *R-genes* which confer only race-specific resistance has been overcome by new races of the pathogen. Therefore, quantitative resistance by employing many genes could offer durable resistance. Moreover, search for genes associated with host immunity other than *R-genes* can boost disease resistance approaches. Although there are several challenges in the accomplishment of late blight resistance programs, a comprehensive understanding of the pathogen and its host genome has the spacious potential for further improvement in resistance breed-

ing. Identification, isolation, and pyramiding of new sources of genes with durable resistance to *P. infestans* into potato would be possible in near future with the help of effectoromics, transcriptomics, metabolomics, and cis-genic approaches.

List of abbreviations:
(Avr): avirulence
BCAs: Biocntrol agents
BRG: Blight resistance genes
LB: late blight

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Περονόσπορος της πατάτας: Από τον Ιρλανδικό λιμό της πατάτας στη γονιδιωματική εποχή - Μια ανασκόπηση

A. Majeed, S. Siyar και S. Sami

Περίληψη Ο περονόσπορος της πατάτας και της τομάτας, μια από τις πλέον ευρέως διαδομένες ασθένειες των φυτών, αποτελεί σημαντικό περιορισμό στη γεωργία παγκοσμίως προκαλώντας σοβαρά προβλήματα στην παραγωγή με οικονομικές απώλειες και επιβάρυνση του περιβάλλοντος με τη χρήση φυτοφαρμάκων. Η ασθένεια οφείλεται στον ωομύκητα *Phytophthora infestans*, ο οποίος τράβηξε για πρώτη φορά την προσοχή των φυτοπαθολόγων στα μέσα της δεκαετίας του 1840, όταν προκάλεσε τον ιστορικό λιμό στην Ιρλανδία -τον μεγάλο Ιρλανδικό λιμό της πατάτας- ως αποτέλεσμα των σημαντικών απωλειών στην παραγωγή πατάτας από την ασθένεια. Έκτοτε, ο περονόσπορος έχει πυροδοτήσει αρκετές επιδημίες μεγάλης έντασης στις καλλιέργειες πατάτας και τομάτας σε διάφορες περιοχές. Με την πάροδο του χρόνου, τα συνθετικά μυκητοκτόνα έχουν αποδειχτεί αποτελεσματική πρακτική για την αντιμετώπιση του περονοσπόρου. Εντούτοις, η εμφάνιση νέων γονοτύπων με αυξημένη παθογόνο δύναμη και αντοχή στα μυκητοκτόνα θεωρείται σε μεγάλο βαθμό ως πρόβλημα στη γεωργία. Σημαντικές ανακαλύψεις στην αλληλουχία του γονιδιώματος του *P. infestans* και ο προσδιορισμός γονιδίων ανθεκτικότητας σε ορισμένα φυτά έχουν ανοίξει δρόμους για τη δημιουργία ανθεκτικών γονοτύπων. Εντούτοις, πολλές προκλήσεις εξακολουθούν να υφίστανται αναφορικά με το συγκεκριμένο επιβλαβές παθογόνο. Η παρούσα ανασκόπηση έχει στόχο να υπογραμμίσει την ιστορική σημασία του περονοσπόρου, τις στρατηγικές χημικής αντιμετώπισής του και τις σχετικές προκλήσεις, και τα προγράμματα δημιουργίας ανθεκτικών γονοτύπων χρησιμοποιώντας γενετικές προσεγγίσεις.

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Temporal development of stem rot caused by *Athelia rolfsii* in peanut fields in Iran

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Summary Stem rot caused by *Athelia rolfsii* (syn. *Sclerotium rolfsii*), is an important disease of peanut in Iran. Soil samples were collected from 15 peanut fields before the 2014 growing season in Guilan province, Iran and the viable sclerotia were counted using the soil-tray technique. The 15 selected fields were also evaluated for disease incidence at three intervals during the growing seasons of 2013 and 2014. The disease incidence at the end of the growing season ranged from 0 to 29.3% and from 0 to 45% in 2013 and 2014, respectively, depending on the field. The disease progress model, which was introduced for each field based on the disease incidence, showed good fitness with the monomolecular model in both years, but especially in 2014. Disease severity was evaluated in three out of the 15 peanut fields at three intervals during the growing season of 2014. The monomolecular model could describe more than 88% of the data. There was a significant linear relationship between the disease incidence or severity at the end of the growing season and the inoculum density in the soil. A positive linear relationship was also observed between the disease progress rate and the inoculum density.

Additional keywords: *Arachis hypogaea*, *Sclerotium rolfsii*, southern blight, temporal analysis, viable sclerotia, white rot

Introduction

Groundnut or peanut (*Arachis hypogaea* L.) as an annual legume crop is cultivated in more than 80 countries in the tropics, subtropics and warm temperate locations (Hammons, 1994). It is a main source of edible oil, vitamins and amino acids and is extensively used for feed and food (Savage and Keenan, 1994). Groundnut is also a main crop in Guilan province of Iran with about 3500 hectares cultivation area.

Southern blight, stem rot or white rot, caused by *Athelia rolfsii* (Curzi) Tu and Kimbrough (syn: *Sclerotium rolfsii* Sacc.), is an important disease of peanut. The disease is found in all peanut-growing areas worldwide, where it causes great yield losses (up to 80%), especially when the disease incidence is high and the edaphic conditions, such as soil temperature and humidity, are favorable for fungal development (Kolte, 1984; Kokalis-Burelle *et al.,* 1997; Le, 2004; Nguyen *et al.,* 2004).

Athelia rolfsii is one of the most common soil-borne plant pathogenic fungi in warm temperate and subtropical regions of the world. More than 500 plant species, mostly dicotyledonous, are hosts of the pathogen (Punja *et al*., 1985). Several symptoms are produced by *A. rolfsii* on its hosts, such as crown and root rot, stem canker and damping-off with the resulting diseases being called southern wilt, blight or stem rot (Punja, 1985). Yield losses were reported between 10 and 25% and in some cases even more than 81% (Mehan *et al*., 1995).

Athelia rolfsii overwinters in soil and infected plant tissues as mycelium or sclerotia. Germinating sclerotia or hyphae infect the plant under favorable conditions and then colonize and invade the root and stem tissues through its typical silky white mycelium (Mullen, 2001). Infected plants become yellow and then wilt; the collar root turns brown and rots. In groundnut, *A. rolfsii* also infects the pegs and pods leading to high yield losses. Control of *A. rolfsii* is difficult by physical and cultural practices because of its

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wide host range (Aycock, 1966; Punja, 1985) and persistent sclerotia (Punja, 1985; Lakpale, 2007). Knowledge of the disease epidemiology, especially temporal analysis of the disease and its relationship with the inoculum density, is essential to successfully implement management practices (e.g., chemical and biological).

Stem rot incidence has been mostly considered in studies on the pathogenicity of fungal isolates or as a tool for assaying resistant lines or cultivars. However, only a few precise studies exist on the progression of the disease under field conditions. Backman *et al.* (1981) developed regression equations to relate populations of viable sclerotia to the percentage of sugar beet roots infected by *A. rolfsii*. Punja (1986) studied the stem rot incidence and its progression on processing carrots in naturally infested fields in Georgia and North Carolina and reported that the disease increased almost linearly until harvest time with the final incidence ranging 39-48%. In addition, there was a significant correlation between the percentage of dead plants and inoculum density up to 15 sclerotia / 300 cm^3 dried soil. Tomasino and Conway (1987) observed a positive linear relationship between sclerotial densities and disease incidence in apple nurseries in Oklahoma. Okabe and Matsumoto (2000) reported that 10-40% of the peanut plants in four fields in Tsukuba, Japan, were infected by *A. rolfsii.* Le *et al.* (2012) investigated the stem rot incidence in eight groundnut-growing areas in central Vietnam and showed that 5-25% of the groundnut plants were infected by *A. rolfsii.*

The stem rot severity has been assessed on various hosts mainly for screening cultivars for resistance in breeding programs and not for studying the disease progress under filed conditions. Shokes and Gorbet (1998) evaluated the resistance of 11 peanut genotypes to stem rot disease at the North Florida Research and Education Center (NFREC) in 1991-1993 using an agar disk inoculation method and a 1-6 scale for assessing disease severity. Fery and Dukes (2005; 2011) determined the resistance of some pepper and cowpea cultivars and lines to stem rot based on the disease severity. Eslami *et al.* (2015) also evaluated some peanut genotypes for resistance to *A. rolfsii* based on the disease severity.

The aim of the current study was to determine the temporal progress of stem rot and its correlation with the inoculum density in the peanut fields of Astaneh-Ashrafiyeh county in Guilan province, which is the main peanut cultivation area in Iran. The results will contribute to better management of the disease and the reduction of yield losses.

Materials and Methods

Determination of soil inoculum density

Fifteen peanut fields in different locations of Astaneh-Ashrafiyeh county were selected based on the history of stem rot disease. In one of the fields (field 7), two main hosts of *A. rolfsii,* bean and peanut, were cultivated simultaneously. Selected fields were divided into 250-500 m^2 plots. In 2014 and before the growing season, 10 soil core samples 15 cm deep and 7 cm wide were randomly collected from each plot (Punja *et al.,* 1985) and transferred to the laboratory. The number of viable sclerotia in each soil sample was determined using the soil-tray technique (Rodriguez-Kabana *et al.,* 1980).

Evaluation of the disease parameters

During the 2013 and 2014 growing seasons, the plots of the 15 selected fields were monitored for disease symptoms. Thirty randomly selected plants in each plot were evaluated and disease incidence was estimated as the percentage of infected plants per plot. The plant which either showed stem lesions covered with white mycelium and sclerotia or was associated with the presence of sclerotia on the soil surface around the stem base was recorded as infected plant. In total 222 plots were surveyed. This evaluation was done at three intervals during the growing season. Final recording was done at harvest. The mean disease incidence in each evaluation time point was calculated for each field.

In 2014, three peanut fields (fields 2, 6 and 7) of 0.6-0.9 ha each in Noghredeh village in Astaneh-Ashrafiyeh county were selected for evaluating the disease severity. In total, 43 plots and 30 plants in each plot were evaluated. Disease severity was recorded for all the stems of each evaluated plant based on the presence or absence of symptoms on stems, stem area affected (percentage of stem circumference which was surrounded by lesions) and mycelium outgrowth on the lesions, as introduced by Le *et al.* (2012) with some modifications (Table 1). Subsequently, the mean disease severity was calculated for each plant and plot. This evaluation was done at three intervals during the growing season of 2014, except for field 7 where only two assessments were done, because the crop was harvested earlier than in the other two fields.

Statistical analysis

Linearized disease progress curves were drawn for each of the evaluated fields based on the disease incidence or severity using Excel software. For the temporal analysis of the disease, the monomolecular growth model $\ln[1/(1-y)] = r_m t+C$, in which, y is the disease incidence or severity at time t, r_m is the disease growth rate and C is y-intercept and equal to $\ln[1/(1-y_0)]$ when t=0, fitted with the incidence and severity data using Statgraphics v. 2.1. The relationship between the disease incidence or severity and the inoculum density as well as the relationship between the growth rate and the inoculum density were determined using Statgraphics v. 2.1.

Results

Soil inoculum density

In 2014, a total of 203 soil samples from the 15 selected peanut fields were analyzed for viable sclerotia using the soil-tray technique. Mycelia resulted from germination of viable sclerotia (Fig. 1) were observed in 20 soil samples from 8 peanut fields, with the lowest and the highest numbers of viable sclerotia being recorded in fields 6 and 1, respectively (Table 2).

Symptom development

All studied fields showed disease symptoms except for field 10 which had zero dis-

Table 1. Characteristics used to evaluate the stem rot severity caused by *Athelia rolfsii* in peanut plants.

Scale	Stem area affected (%)	Mycelium outgrowth on the lesions	Stem appearance	Severity
0	0%	No	Healthy	0
1	>0-15%	No	Healthy	10%
2	16-30%	No or Yes	Healthy	25%
3	31-60%	Yes	Healthy	45%
4	61-75%	Yes	Partially wilted	60%
5	76-99%	Yes	Partially wilted	75%
6	100%	Yes	Dead	100%

Figure 1. Germination of a viable sclerotium of *Athelia rolfsii* and mycelia formation on the soil surface using the soil-tray technique.

ease incidence in both years. The mean disease incidence at harvest time in the 15 studied fields ranged between 0 and 29.3% and between 0 and 45% in 2013 and 2014, respectively (Fig. 2, Fig. 3). The mean disease incidence at the beginning of the growing season in 2014 was 0.65% higher than that in 2013. Also, the mean disease incidence at the second evaluation time point (middle of the growing season when the plants were at anthesis stage) in 2014 was 1.64% higher than that in 2013. The final mean disease incidence at harvest time was approximately the same in 2013 (12.88%) and 2014 (12.90%),

Table 2. Number of viable *Athelia rolfsii* sclerotia in soil samples collected from peanut fields in Astaneh-Ashrafiyeh county, Iran in 2014 and the respective stem rot incidence in those fields at harvest.

Field No	Area (ha)	Size of soil sample (g)	Number of viable sclerotia / 500 g soil	Disease incidence (%)
1	1	1000	4	45
2	0.6	500	2	25.66
3	0.7	1050	1.5	17.10
4	1.5	1450	1.3	14.48
5	0.8	800	0.625	11.16
6	0.9	900	0.45	11.48
7	1.2	1100	0.55	12.87
8	0.7	500	2	10

Figure 2. Symptoms (lesions, cankers) and sclerotia of *Athelia rolfsii* on peanut stems (a) and gynophores (b).

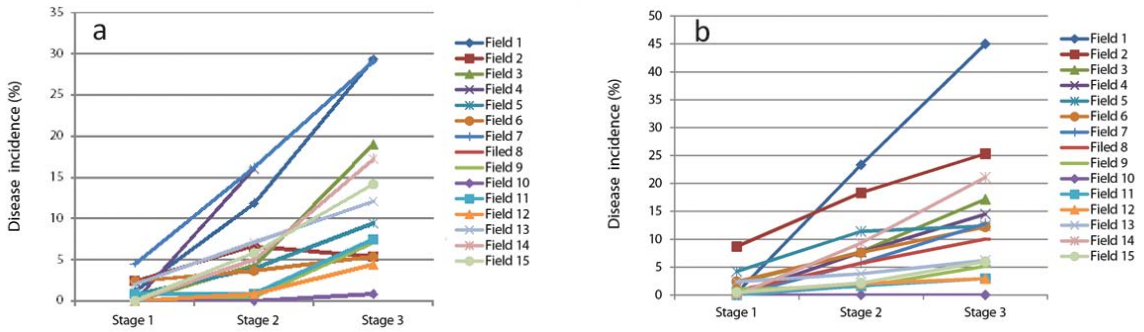

Figure 3. Stem rot disease progress in each evaluated peanut field based on the incidence data in 2013 (a) and 2014 (b). Stage 1: Beginning of the growing season; Stage 2: Middle of the growing season (anthesis stage); Stage 3: Harvest time.

although the final evaluation in 2014 was done 8 days earlier than in 2013.

Modeling the disease incidence progress in 2014 for the plots of 8 out of the 15 experimental fields (Fig. 4) showed that the disease progress had good fitness (≥90%) with the monomolecular model in more than 65% of the plots, while ignoring the plots with zero incidence (Table 3 and Fig. 4). The disease progress model was also introduced for each field based on the mean of incidence in the plots in each evaluation time point in 2013 and 2014 (Table 4) and again the data showed good fitness with the monomolecular model in both years, but especially in 2014. The disease growth rate in seven fields (fields 1, 2, 3, 5, 6, 8 and 14) was higher in 2014 than in 2013, whereas in the other six fields, the growth rate in 2014 was lower than that in 2013 (Table 4). In field 4, the third evaluation was not done in 2013 because the crop was harvested earli-

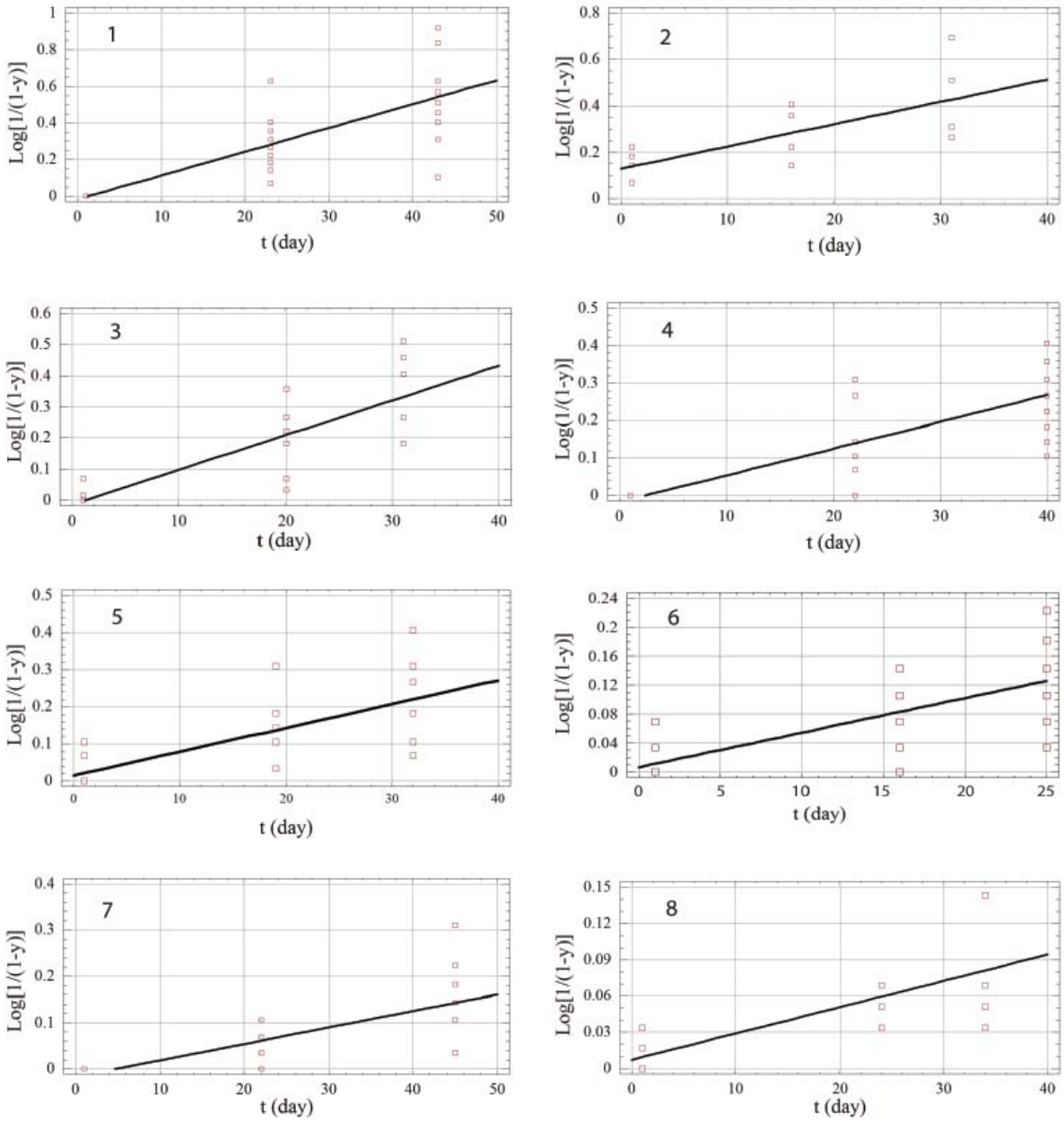

Figure 4. The linearized models for stem rot disease incidence progress in eight peanut fields (1-8) based on 2014 data. y: stem rot incidence; t: time (day) after first evaluation.

Table 3. Stem rot disease progress model for different plots of field 1 based on 2014 data.

Plot	Progress model	R^2 (%)	SE
1	ln[1/(1-y)] = -0.0289+0.0108*t	97.96	0.0464
2	ln[1/(1-y)] = 0.0167+0.0079*t	80.89	0.1141
3	ln[1/(1-y)] = -0.0485+0.0148*t	96.30	0.0865
4	ln[1/(1-y)] = -0.0302+0.0198*t	99.80	0.0265
5	ln[1/(1-y)] = -0.1449+0.0235*t	83.45	0.3121
6	ln[1/(1-y)] = -0.0615+0.0148*t	93.12	0.1200
7	ln[1/(1-y)] = 0.213+0.0097*t	92.92	0.0800
8	ln[1/(1-y)] = -0.0127+0.0122*t	99.99	0.0015
9	ln[1/(1-y)] = -0.0135+0.0074*t	99.48	0.0157
10	ln[1/(1-y)] = -0.163+0.026*t	82.54	0.3528
11	ln[1/(1-y)] = -0.057+0.0084*t	79.90	0.1248
12	ln[1/(1-y)] = -0.0608+0.0217*t	97.62	0.1006
13	ln[1/(1-y)] = -0.0095+0.0135*t	99.93	0.0102
14	ln[1/(1-y)] = 0.0404+0.0200*t	93.62	0.1555
15	ln[1/(1-y)] = 0.0190+0.0095*t	93.71	0.0738
16	ln[1/(1-y)] = -0.01953+0.0545*t	95.00	0.3624
17	ln[1/(1-y)] = 0.0606+0.0151*t	84.20	0.1948
18	ln[1/(1-y)] = -0.0192+0.0096*t	99.26	0.0245
19	ln[1/(1-y)] = -0.013+0.0121*t	99.99	0.0015
20	ln[1/(1-y)] = -0.0018+0.0025*t	97.78	0.0112

y: stem rot incidence; R^2: coefficient of determination; SE: standard error; t: time

er compared to the other fields and thus no model was applied to the data.

Mean of stem rot severity in the first evaluation of the fields 2, 6 and 7 in 2014 was 2.84%, 0.60% and 1.45%, respectively (Fig. 5). The disease severity of these selected fields at harvest (final disease severity) was 16.20%, 5.90% and 10.56%, respectively, although in field 7, the final disease severity corresponded to that assessed in the middle of the growing season as the crop was harvested 3 days later (Fig. 5).

Modeling the disease severity in fields 2 and 6 showed that the monomolecular model had the ability to describe more than 88% of the data: ln[1/(1-y)]=0.0048*t+0.0312 (R^2=96.69) for field 2 and ln[1/(1-y)]=0.0018*t-0.00047 (R^2=88.48) for field 6 (data not shown). In field 7, the crop was harvested 3 days after the second evaluation of the disease and thus no model was fitted to the data, as only two sets of data were collected.

Effect of soil inoculum density on disease symptoms

Viable sclerotia were detected in the soil of 8 out of the 15 experimental fields in 2014, with fields 1 and 8 showing the highest and the lowest final disease incidence, respectively (Table 2). There was a significant ($P \leq 0.01$) linear relationship between the final disease incidence (DI) and soil inoculum density (Fig. 6), which fitted to the model: DI=0.0419+0.0908*Q (R^2 = 78.72), where Q is the number of viable sclerotia in 500 g dried soil.

Table 4. Stem rot disease progress model for each peanut field based on the mean disease incidence in the plots at each evaluation time in 2013 and 2014.

Field	Village	Year	Progress model	R^2 (%)	SE
1	Noghredeh	2013	ln[1/(1-y)] = -0.0172 + 0.0054*t	88.90	0.0802
		2014	ln[1/(1-y)] = -0.0248 + 0.0145*t	99.33	0.0344
2	Noghredeh	2013	ln[1/(1-y)] = -0.0007 + 0.0026*t	78.46	0.0586
		2014	ln[1/(1-y)] = 0.0876 + 0.0067*t	99.59	0.0091
3	Noghredeh	2013	ln[1/(1-y)] = -0.0244 + 0.0040*t	74.27	0.0800
		2014	ln[1/(1-y)] = 0.0045 + 0.0055*t	91.12	0.0357
4	Noghredeh	2013	(-)	(-)	(-)
		2014	ln[1/(1-y)] = -0.005 + 0.0038*t	99.91	0.0032
5	Noghredeh	2013	ln[1/(1-y)] = 0.00015 + 0.0017*t	89.99	0.0206
		2014	ln[1/(1-y)] = 0.0444 + 0.0038*t	93.15	0.0180
6	Noghredeh	2013	ln[1/(1-y)] = 0.0223 + 0.00047*t	86.64	0.0080
		2014	ln[1/(1-y)] = 0.0181 + 0.0031*t	98.12	0.0102
7	Noghredeh	2013	ln[1/(1-y)] = 0.0264 + 0.0055*t	88.14	0.0730
		2014	ln[1/(1-y)] = -0.0054 + 0.0031*t	99.70	0.0053
8	Salestan	2013	ln[1/(1-y)] = -0.0116 + 0.0013*t	77.50	0.0287
		2014	ln[1/(1-y)] = -0.0014 + 0.0028*t	96.37	0.0132
9	Salestan	2013	ln[1/(1-y)] = -0.0121 + 0.0012*t	73.33	0.0304
		2014	ln[1/(1-y)] = 0.0052 + 0.00108*t	61.77	0.0203
10	Khoshkarvandan	2013	Zero incidence		
		2014	Zero incidence		
11	Khoshkarvandan	2013	ln[1/(1-y)] = 0.0016 + 0.00105*t	51.63	0.0395
		2014	ln[1/(1-y)] = 0.0019 + 0.0007*t	97.40	0.0032
12	Khoshkarvandan	2013	ln[1/(1-y)] = -0.0056 + 0.0007*t	67.97	0.0193
		2014	ln[1/(1-y)] = 0.0069 + 0.00076*t	93.84	0.0042
13	Khoshkarvandan	2013	ln[1/(1-y)] = 0.0141+0.0017*t	93.60	0.0194
		2014	ln[1/(1-y)] = 0.0214 +0.0009*t	97.73	0.0043
14	Noghredeh	2013	ln[1/(1-y)] = -0.0194 + 0.0036*t	79.97	0.0618
		2014	ln[1/(1-y)] = -0.0153 + 0.0056*t	97.56	0.0260
15	Noghredeh	2013	ln[1/(1-y)] = -0.0119 +0.0030*t	88.58	0.0367
		2014	ln[1/(1-y)] = -0.0011 +0.0016*t	87.16	0.0148

y:stem rot incidence; R^2: coefficient of determination; SE: standard error; t: time; (-): no model was fitted to the data because only two disease assessments were done.

There was also a significant linear relationship between the soil inoculum density and disease growth rate, which fitted to the model: r_m=0.0043+0.0024*Q (R^2 = 76.72), where r_m is the disease growth rate and Q is the number of viable sclerotia / 500 g dried soil (Fig. 7). Based on this model, the effective factor (R) for the monomolecular model is equal to 0.0024 that shows the impact of the environment, host, etc. on the disease development. A positive linear relationship was also observed between the final disease severity (DS) and the number of viable sclerotia / 500 g dried soil for fields 2, 6 and 7, where the disease severity was evaluated in 2014 (Fig. 8). The number of viable sclerotia /

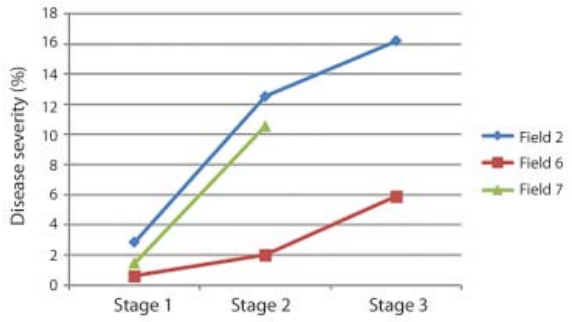

Figure 5. Stem rot disease progress for three peanut fields based on the severity data in 2014. Stage 1: Beginning of the growing season; Stage 2: Middle of the growing season (anthesis stage); Stage 3: Harvest time.

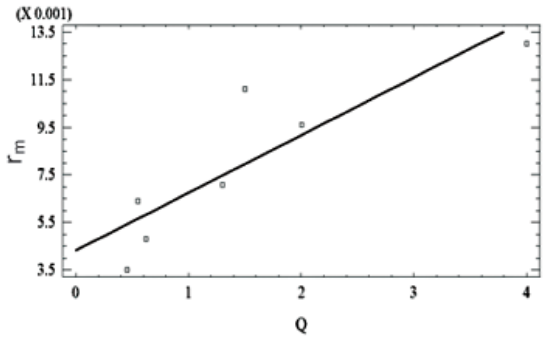

Figure 7. Linear relationship between the number of viable sclerotia and the disease growth rate in eight evaluated peanut fields in 2014. r_m: Disease growth rate (multiply at 0.001); Q: Number of viable sclerotia / 500 g dried soil.

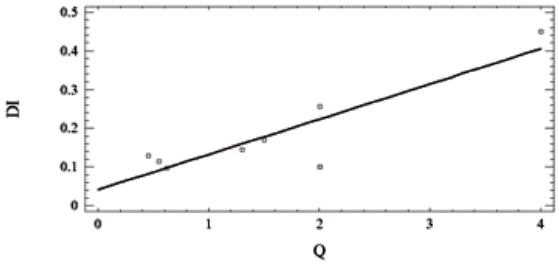

Figure 6. Linear relationship between the number of viable sclerotia and the stem rot incidence in eight evaluated peanut fields in 2014. DI: Disease incidence at harvest time (based on 0-1); Q: Number of viable sclerotia / 500 g dried soil.

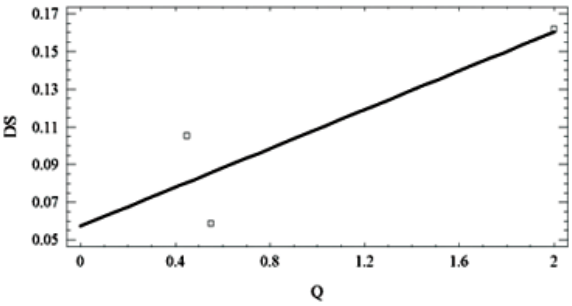

Figure 8. Linear relationship between the number of viable sclerotia and the disease severity in eight evaluated peanut fields in 2014. DS: Disease severity at harvest time (based on 0-1); Q: Number of viable sclerotia / 500 g dried soil.

500 g dried soil was 2, 0.55 and 0.45 and the final DS was 16.20%, 5.90% and 10.56% for the fields 2, 6 and 7, respectively. The data fitted to the model: DS=0.0575+0.0514*Q (R^2 = 74.76).

Discussion

As noted above, the lowest and the highest number of viable sclerotia were counted in the fields 6 and 1, respectively. The final disease incidence in the 15 peanut fields studied at the present study varied between 0-29.3% and 0-45% in 2013 and 2014, respectively. Okabe and Matsumuto (2000) reported that the stem rot disease incidence in peanut fields in Japan ranged between 10 and 40% during the period 1994-1997. According to Le *et al.* (2012), the disease incidence in peanut fields of eight regions in Vietnam was 5-25%.

The disease progress model introduced in the current study for each field based on the mean disease incidence in the plots in each evaluation time point in 2013 and 2014 showed good fitness with the monomolecular model in both years, but especially in 2014. The disease growth rate in 7 fields was higher in 2014 than in 2013. There was a significant (P≤0.01) linear relationship between the final disease incidence and the inoculum density in the soil and between the inoculum density and the disease growth rate.

Backman *et al.* (1981) estimated the number of viable sclerotia of *A. rolfsii* in soil samples from sugar beet (*Beta vulgaris* L.) fields to predict the stem rot incidence in the following season and reported 0-36 viable sclerotia in 500 g dried soil samples based on soil-tray technique. They reported a significant linear relationship between inoculum den-

sity and infected sugar beet plants (P≤0.01) and stated that the disease can be forecasted based on this model. They declared that one viable sclerotium in 500 g dried soil is nearly equivalent with 1% disease incidence at harvest time. Punja (1986) reported a relationship between the disease incidence and inoculum density for carrot fields. Based on his study, there was a linear increase in disease incidence related to inoculum population increment from 0 to15 viable sclerotia / 300 cm^3 dried soil; in addition, the number of the dead plants was significantly (P≤0.05) related to inoculum density.

Tomasino and Conway (1987) identified a positive linear relationship between inoculum density and disease incidence on apple roots in Oklahoma and stated that 5 viable sclerotia / 1000 g dried soil resulted in 19, 5 and 35% disease incidence in pot, micro-plot and field studies, respectively.

Because of the low precipitation in the 2014 peanut growing season in Astaneh-Ashrafiyeh, the farmers had to harvest the crop 7-10 days earlier for preventing yield loss. As a result, the final evaluation of the disease incidence was done 8 days earlier than in 2013. Although the final evaluation of the disease incidence was done earlier in 2014 compared with 2013, the mean of the final disease incidences in both years were approximately the same (i.e., 12.88% for 2013 and 12.90% for 2014).

Based on the results of the study conducted by Bowen (2003) in Alabama, the higher than the normal amount of precipitation during the growing season and the higher than the field capacity soil moisture lead to decreased stem rot disease incidence. Punja (1986) also considered the increased soil moisture as an inhibiting factor for mycelial growth of *A. rolfsii*. In the current study and based on the weather data, the consecutive rainfall occurred in June and July 2013 compared with the rarely rainy weather during the same period in 2014 resulted in increased field capacity and to some extent decreased disease incidence.

As shown in the present study, the inoculum densities in fields 2 and 8 were equal. However, the final disease incidences in these two fields were not the same. This difference could be related to different soil textures in these fields. Punja and Jenkins (1984) reported that the stem rot disease incidence is higher in sandy soils compared to loamy or clay soils. The second factor could be the plant density, as field 2 was more densely populated and resulted in a faster plant to plant spread of the disease compared to field 8. In densely populated fields, even few foci can lead to high disease incidence (Punja, 1986). Sconyers *et al*. (2007) investigated the effect of cropping pattern on the stem rot distribution in peanut fields and reported that the physical distance among the plants is a critical factor in disease development.

In the current study, during the first evaluation of field 2, infection was observed in 70% of the studied plots whereas, only 44% of the plots in field 6 were infected. Punja (1986) pointed to the importance of disease foci in the stem rot incidence during the next growing season. In the present study, the high number of disease foci in field 2 at the beginning of the growing season may have resulted in higher disease severity during the first evaluation. However, the final disease severity in field 2 was 5.7-fold of that at the beginning of the growing season, whereas in filed 6, it was 9.8-fold. It seems that the drought stress related to the soil texture in field 6 was responsible for the increase in disease severity during the growing season. Although higher numbers of viable sclerotia were counted in field 6 compared to field 7, field 6 showed lower disease severity at harvest time. Soil texture and plant density as well as simultaneous cultivation of two hosts of *A. rolfsii* (bean and peanut in field 7) are probably responsible for this difference. This is the first time that the stem rot severity progress has been studied under field conditions.

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Ανάπτυξη σήψης στελέχους *Athelia rolfsii* σε καλλιέργεια αραχίδας στο Ιράν

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Περίληψη Η σήψη στελέχους, η οποία προκαλείται από τον φυτοπαθογόνο μύκητα *Athelia rolfsii* (συν. *Sclerotium rolfsii*), είναι μια σημαντική ασθένεια της αραχίδας στο Ιράν. Σε 15 αγρούς με καλλιέργεια αραχίδας στην επαρχία Guilan του Ιράν έγιναν δειγματοληψίες εδάφους πριν από την καλλιεργητική περίοδο του έτους 2014 και μετρήθηκε ο αριθμός των βιώσιμων σκληρωτίων. Επίσης αξιολογήθηκε η συχνότητα εμφάνισης της ασθένειας σε τρία μεσοδιαστήματα κατά τη διάρκεια των καλλιεργητικών περιόδων 2013 και 2014. Στο τέλος της καλλιεργητικής περιόδου η συχνότητα εμφάνισης της ασθένειας κυμαινόταν από 0 έως 29,3% και από 0 έως 45%, το 2013 και το 2014, αντίστοιχα, ανάλογα με τον αγρό. Το μοντέλο προόδου της ασθένειας, το οποίο βασίστηκε στη συχνότητα εμφάνισης της ασθένειας σε κάθε αγρό, έδειξε καλή προσαρμοστικότητα με το μονομοριακό (monomolecular) μοντέλο και στα δύο χρόνια, αλλά ιδιαίτερα το 2014. Η σοβαρότητα της ασθένειας αξιολογήθηκε σε τρεις από τους 15 αγρούς σε τρία μεσοδιαστήματα κατά την καλλιεργητική περίοδο του 2014. Το μονομοριακό μοντέλο περιέγραψε περισσότερο από το 88% των δεδομένων. Διαπιστώθηκε σημαντική γραμμική συσχέτιση μεταξύ της συχνότητας εμφάνισης ή της σοβαρότητας της ασθένειας στο τέλος της καλλιεργητικής περιόδου και της πυκνότητας του μολύσματος (σκληρωτίων) στο έδαφος. Επιπλέον, παρατηρήθηκε θετική γραμμική συσχέτιση μεταξύ του ρυθμού προόδου της ασθένειας και της πυκνότητας του μολύσματος.

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Entomopathogenic fungus, *Metarhizium anisopliae,* anchored onto MCM-41 silica nanomaterial: A novel and effective insecticide against potato tuber moth in stored potatoes

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Summary *Phthorimaea operculella* (PTM) is an economically significant invasive potato insect pest in tropical and sub-tropical regions. Due to many problems concerning chemical insecticides, biocontrol agents such as entomopathogenic fungi have attracted researchers' interest regarding their application as a part of integrated potato tuber moth management strategies in several countries. Hence, in the present study, we examined the lethal effects of entomopathogenic fungus, *Metarhizium anisopliae* (used as pure culture (PEF) or formulated with MCM-41 silica nanomaterial (MCM-41@fungus)) on eggs and neonate larvae of PTM. The MCM-41was completely characterized by Fourier transform infrared (FT-IR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDS), X-ray diffraction (XRD), and Thermogravimetric analysis (TGA) techniques. The morphology of MCM-41@fungus was evaluated by scanning electron microscope (SEM). The morphology of the mesoporous structure was exhibited to be homogeneous, regular, and spherical. LC_{50} values of PEF and MCM-41@fungus were estimated to be 1.7×10^7 and 2×10^5 conidia/ml for eggs and 1.8×10^6 and 1.5×10^4 conidia/ml for neonate larvae, respectively. Hence, bioassays demonstrated that MCM-41@fungus was more toxic than the pure culture at egg and neonate larval stages of the pest. The results exhibited that pure *M. anisopliae* and its nano-formulation could play key roles as biopesticides in management programmes of *P. operculella*.

Additional keywords: bio-pesticide**,** MCM-41, *Metarhizium anisopliae*, nano-formulation, *Phthorimaea operculella*, toxicity

Introduction

Potato (*Solanum tuberosum* L. (Solanaceae)) is one of the most important agricultural products that plays a major role as a high value crop for human health and food industry. Among foods, it has the fourth rank in the world after wheat, rice, and corn (Germchi *et al*., 2011). Potato tuber moth (PTM), *Phthorimaea operculella* Zeller (Lepidoptera: Gelechiidae) is considered as the most damaging potato insect pest in the world (Radcliffe, 1982). PTM occurs in tropical and sub-tropical regions where potato is cultivated and it causes notable crop yield losses (Kroschel and Sporleder, 2006). Since potato plants resist some PTM damages, protection of stored potatoes against larvae of the pest is more important than in the field (Lacey, 2012). When tubers infested by PTM enter storage without chemical treatment, the larvae continue to develop, make tunnels and pollute the tubers with their frasses and finally the decaying organisms can enter the plant (Radcliffe, 1982).

Extensive use of chemical insecticides has resulted in adverse effects on environment and human health. Particularly, excessive application of non-selective chemical insecticides can destroy natural enemies of the pest and beneficial organisms and induce problems such as development of pest resistance (Sharma and Gupta, 2009). These adverse effects urged the development of alternative pest management tactics in which microbial controls may play principal

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roles (Collantes *et al.,* 1986; Llanderal-Cázares *et al*., 1996).

Entomopathogenic fungi are efficient microbial control agents of several pests (Goettel *et al*., 2005), including some key insect pests of potato (Lacey *et al*., 1999; Wraight and Ramos, 2005). The entomopathogenic filamentous fungus *Metarhizium anisopliae* is a common pathogen of arthropods applied in biological control strategies against numerous serious insect pests (Santi *et al*., 2010). In spite of the potential of entomopathogenic fungi in pest control, these biocontrol agents have some disadvantages including sensitivity to environmental factors such as moisture, light, and temperature that have limited their applications under storage and field conditions. However, these problems can be overcome through recent technological advances such as nano-formulation approaches which can improve their efficacy and pathogenicity and will permit future use of entomopathogenic fungi in crop production systems. In this respect, the hexagonal array of uniform mesoporous MCM-41 with particular properties such as exceptionally high surface areas and high pore volumes (Chen *et al*., 1993; Rath and Parida, 2011) attracted our interest in applying it in plant protection and insect pest management. Since it has 1-D uniform mesopores, the entomopathogenic fungi can be grafted into MCM-41. Consequently, we selected MCM-41 as a support which belongs to M41S family and is mainly made up of silica, SiO_2, as a carrying agent (Shylesh and Singh, 2005; Rath and Parida, 2011). Silica has unique benefits such as extraordinary thermal and chemical stability, ease of handling, and abundance of exposed silanol (Si-OH) groups (Abdollahi-Alibeik and Pouriayevali, 2011).

Materials and Methods

Phthorimaea operculella rearing, *M. anisopliae* culture, nano-formulation

Insect rearing

A colony of potato tuber moth was obtained from the University of Mohaghegh Ardabili, Ardabil, Iran. The colony was continuously reared on Agria potato cultivar. Experiments were carried out under laboratory conditions at 26 ± 1ºC, 60 ± 5% RH and photoperiod of 8:16 (L:D). To achieve cohort eggs of *P. operculella*, 30 male-female pairs of newly emerged moths were kept inside cylindrical containers. The adults were fed using a piece of cotton imbued with a 10% honey-water solution. Cylindrical containers were covered with a fine mesh netting on the heads. Filter papers were placed on the head of containers which provided an oviposition site for moths (Golizadeh and Zalucki, 2012).

Microorganism and culture media

The entomopathogenic fungus, *M. anisopliae* (DS12) was provided from Mycology Collections of Urmia University, Urmia, Iran. This isolate was originally obtained from wheat samples collected from Urmia, Iran. Stock cultures of the isolate were grown on potato dextrose agar slants (PDA; Merck, Germany) and stored at 4°C for future uses. Subcultures were prepared by transferring pieces of stock cultures onto new PDA plates and incubated at 25 ± 1ºC for 14 days before use in experiments. Conidia were harvested by flooding the cultures with sterile distilled water containing 0.02% Tween-80 and scraping with a sterile L-shaped glass rod. Concentrations of the resulting stock suspension were determined using a Neubauer hemocytometer (Fuchs-Rosenthal 0.0025 mm 2, depth 0.100 mm, VWR, Sweden). Germination was surveyed under a light microscope (400X). A conidium was considered germinated when the germ tube was expanded beyond its width (Inglis *et al*., 2012). The mean germination rate for *M. anisopliae* was 96%.

Preparation of siliceous MCM-41

Mesoporous Si-MCM-41 was synthesized through Sol-gel method according to Cai *et al*. (2001).

Preparation of siliceous MCM-41-(CH2)3Cl

The resulted MCM-41 powder (4.8g) with 5.0g 3-chloropropyltrimethoxysilane (CPT-

MS) was added to n-hexane (96 ml) and the mixture was stirred under refluxing and nitrogen atmosphere for 24 h. The obtained sediments were collected by filtration, were washed with n-hexane for several times, and eventually dried under vacuum to obtain MCM-41-(CH2)3-Cl.

Preparation of MCM-41-entomopathogenic fungus

In a 100ml round bottom flask, a mixture of MCM-41-Cl (1g), entomopathogenic fungus (10ml of determined concentrations) and Et_3N (1.5ml) in H_2O (50ml) were stirred under room temperature for 24h. Then, the final product was dried at room temperature.

Particle morphology studies

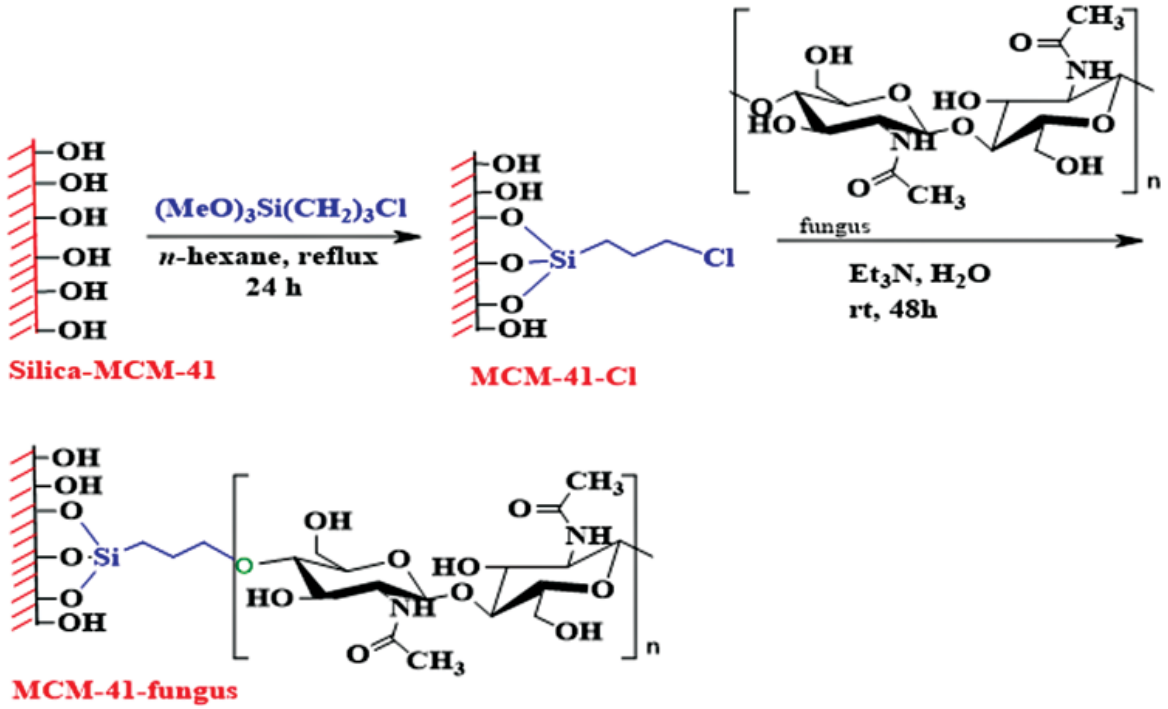
Particle morphology was surveyed by a scanning electron microscope (SEM) (Day Petronic Company, Tehran, Iran), using FESEM-TESCAN MIRA3. Thermogravimetric analysis (TGA) curves were recorded on a Shimadzu DTG-60 instrument (University of Kurdistan, Sanandaj, Iran). Fourier transform infrared (FT-IR) spectra were recorded with KBr pellets on a Nexus 670 FT-IR spectrometer (Medical Sciences of Urmia University, Urmia, Iran). X-ray power diffraction (XRD) patterns were collected on Scintag PAD V X-ray diffractometer using a Co radiation source with wavelength λ = 1.78897 Å, 40 kV (Shahid Beheshti University, Tehran, Iran). The samples were scanned in the range of 2θ =1–10°.

M. anisopliae was grafted into MCM-41 which led to the synthesis of MCM-41@fungus. The details of MCM-41@fungus preparation procedure are presented in Scheme 1.

Bioassays

Effect on larvae

To examine larval mortality, each potato was dipped in suspensions of pure entomopathogenic fungus and MCM-41@fungus, separately, which were determined by preliminary dose setting experiments. Concentrations of PEF and MCM-41@fungus were



Scheme 1. Synthesis of MCM-41@fungus. CPTMS was anchored onto MCM-41 and *Metarhizium anisopliae* was grafted to CPTMS via its hydroxyl groups.

in the ranges of 10^4-10^8 and 10^2-10^6 conidia/ml, respectively. In MCM-41@fungus treatments, 0.1g of each nano-composite was dispersed in 100ml distilled water containing 0.02% Tween-80 until water absorbance was stabilized. After shaking and product dispersion, potato tubers were dipped in the corresponding solutions for 15s. When water was evaporated and tubers were dried, potato tubers were transferred into plastic containers with ventilated lids that were kept at 26 ± 1°C, 60 ± 5% RH and a photoperiod of 8 L:16 D. Then, 20 newly emerged larvae (<5 h old) were placed on each tuber using a soft hair brush. Criterion of larval penetration was the number of adult emergence in all experiments. Each treatment and dose level (5 concentrations) was replicated three times.

Effect on egg hatching

PEF and MCM-41@fungus were tested at 10^5-10^9 and 10^3-10^7 conidia/ml, respectively. For treatments, filter papers containing 20 one-day-old eggs/each concentration were separately dipped in solutions of PEF and MCM-41@fungus. Preparation of MCM-41@fungus treatments was carried out in a similar way to the previous one. When filter papers dried, they were transferred into plastic containers with ventilated lids containing intact potato tubers to stimulate egg hatching. The containers were kept under laboratory conditions (26 ± 1°C, 60 ± 5% RH and a photoperiod of 8L: 16D). Egg hatch was investigated using a light microscope after eight days. Each treatment was replicated three times.

Statistical analysis

In order to determine LC_{50} values, the data were analyzed utilizing probit procedures with software SPSS for Windows® version16.

Results and Discussion

Characterization of MCM-41 substrate and its use as a carrying agent in nano-formulation of *M. anisopliae*

Fungus was supported on mesoporous MCM-41 which was completely characterized by Fourier transform infrared (FT-IR), Scanning Electron Microscopy (SEM), Energy Dispersive X-ray (EDS), X-ray diffraction (XRD), and Thermogravimetric analysis (TGA) techniques.

Fourier transform infrared (FT-IR) spectra, recorded separately at different steps of MCM-41@fungus synthesis, can be observed in curves a-b of Figure 1. As presented in curve a, for MCM-41 sample, the absorption bands at 1230, 1058.07, 812.10, and 463.30 cm^{-1} were assigned to asymmetric and symmetric stretching vibrations of mesoporous framework (Si-O-Si). The band at 3431.91 cm^{-1} was attributed to the stretching vibration of O-H groups. Curve b demonstrated stretching vibrations at 1520.45 (C-N), and 1690.89 cm^{-1} (C=O) and a broad band at around 3440.07 cm^{-1} (O-H and N-H). The band at 1375.95 cm^{-1} corresponded to the bending vibration of CH_3 group, and the presence of the anchored CPTMS group was confirmed by C-H stretching vibrations at 2950 and 2865 cm^{-1} for MCM-41@fungus.

The Energy Dispersive X-ray (EDS) of mesoporous MCM-41@fungus displays the grafting of fungus onto MCM-41 surface (Fig. 1). The results showed that there were only C, Si, O, and N elements in the nano-pores with relative mass percentages of 15.50, 48.81, 32.15, and 3.54%, respectively (Fig. 2). Scanning electron microscope (SEM) micrograph of mesoporous MCM-41@fungus is demonstrated in Figure 3, showing that the particles were in nano range (<100 nm). The morphology of the mesoporous MCM-41@fungus was homogeneous, regular, and spherical. Figure 4 demonstrates slow angle X-ray powder diffraction pattern of the samples (MCM-41 and MCM-41@fungus). XRD pattern of MCM-41 displayed three reflection peaks corresponding to d100 plane (very strong reflection) at 2θ=2.37° and two peaks at d110 and d200 planes (weaker reflections) at 2θ=4.3° and 4.48°, respectively, which can be attributed to the hexagonal structure of MCM-41. The values of spacing

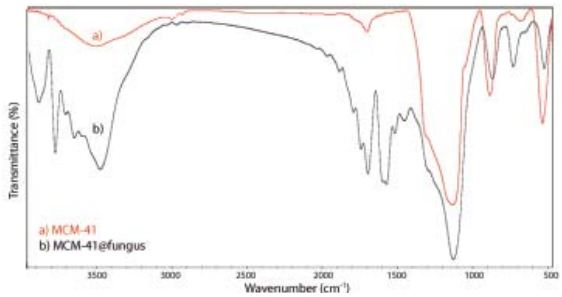


Figure 1. (a) Fourier transform infrared (FT-IR) spectra of MCM-41 and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*.

In curve a, for MCM-41 sample, the absorption bands at 1230, 1058.07, 812.10, and 463.30 cm^{-1} were assigned to asymmetric and symmetric stretching vibrations of mesoporous framework (Si-O-Si). The band at 3431.91 cm^{-1} was attributed to the stretching vibration of O-H groups.

Curve b demonstrated stretching vibrations at 1520.45 (C-N), and 1690.89 cm^{-1} (C=O) and a broad band at around 3440.07 cm^{-1} (O-H and N-H). The band at 1375.95 cm^{-1} corresponded to the bending vibration of CH_3 group, and the presence of the anchored CPTMS group was confirmed by C-H stretching vibrations at 2950 and 2865 cm^{-1} for MCM-41@fungus.

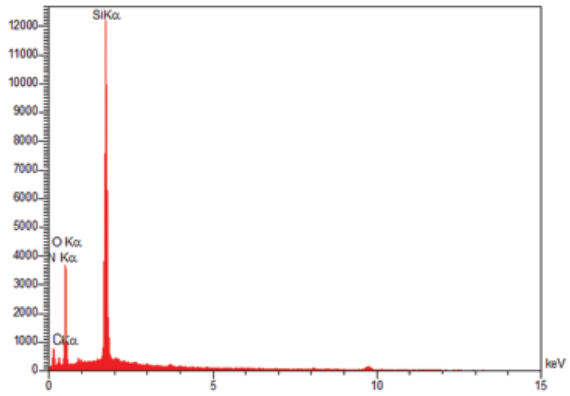


Figure 2. Energy Dispersive X-ray (EDS) spectrum of MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: There were only C, Si, O, and N elements in the nano pores with relative mass percentages of 15.50, 48.81, 32.15, and 3.54%, respectively.

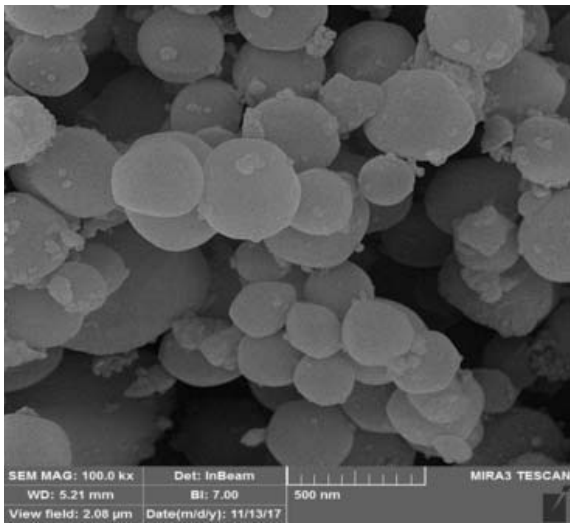


Figure 3. SEM image of MCM-41@fungus mesoporous of the entomopathogenic fungus *Metarhizium anisopliae*: The particles are observed to be in the nano range (<100 nm). The mesoporous presents homogeneous, regular, and spherical morphology.

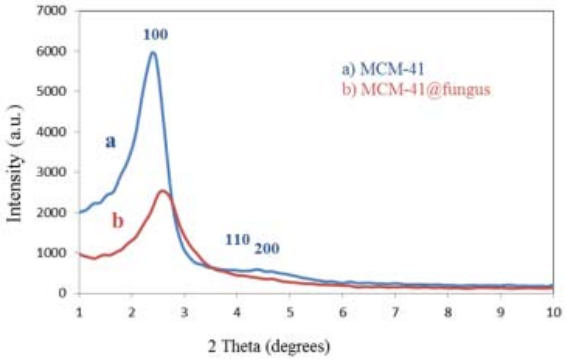


Figure 4. (a) X-ray diffraction (XRD) patterns of MCM-41 and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: The values of spacing (100) for XRD patterns of MCM-41 and MCM-41@fungus were 37.14 and 32.98Å, respectively. As demonstrated, after functionalization steps, a considerable decrease in XRD peak intensities was also observed.

(100) for XRD patterns of MCM-41 and MCM-41@fungus were 37.14 and 32.98Å, respectively. As demonstrated, after functionalization steps, a considerable decrease in XRD peak intensities was observed which caused variations in wall thickness and might be due to the reduction of scattering contrast between the channel wall of silicate framework and fungus. Therefore, it can be concluded that the grafting of fungus occurred in the inner pore channels of Si-MCM-41.

Thermogravimetric analysis (TGA) was employed to select the weight changes of functionalized mesoporous MCM-41. TGA curves for MCM-41(a) and MCM-41@fungus (b) are depicted in Figure 5. According to the TGA curve b, an initial weight loss was seen at temperatures below 200°C because of the removal of physically and chemically adsorbed water molecules inside the pores

channels and surface hydroxyl groups. Additionally, at temperatures above 200°C, large weight losses occurred which were mainly due to the decomposition of covalently bonded organics (200-500°C) and silanol groups (>500°). This shows that the grafting of fungus had occurred on the inner pore channels of Si-MCM-41. MCM-41 has attracted great attention because of its large pore size and extremely high surface area especially above 1000 m^2/g (Nikoorazm *et al*., 2015). The exceptionally high surface area and porous structure of MCM-41 make it as a potential efficient host material for a variety of supported catalysts (Nikoorazm *et al*., 2015). According to our results, the entomopathogenic fungus *M. anisopliae* was grafted to MCM-41 through the hydroxyl group of the fungus leading to the synthesis of MCM-41@fungus.

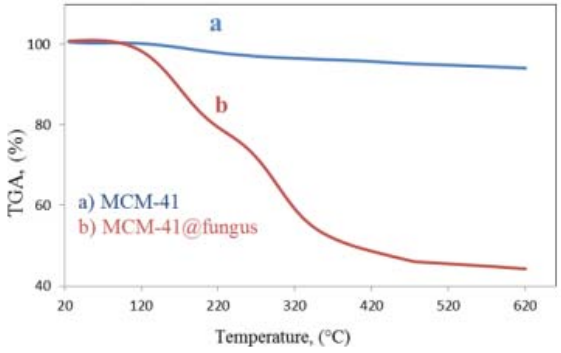


Figure 5. (a) TGA thermograms of MCM-41(a) and (b) MCM-41@fungus of the entomopathogenic fungus *Metarhizium anisopliae*: At temperatures above 200°C, large weight losses occurred which were mainly due to the decomposition of covalently bonded organics (200-500°C) and silanol groups (>500°), indicating grafting of fungus on the inner pore channels of Si-MCM-41.

MCM-41@fungus against eggs and neonate larvae of *P. operculella*

The results of the bioassays for PEF and MCM-41@fungus against eggs and neonate larval penetration of PTM are presented in Table 1. LC_{50} values of PEF and MCM-41@fungus were 1.7×10^7 and 2.0×10^5 conidia/ml on eggs and 1.8×10^6 and 1.5×10^4 conidia/ml on neonate larvae, respectively. Hence, at both life stages, LC_{50} values of nano-formulated entomopathogenic fungus against *P. operculella* were significantly lower thus more effective than pure *M. anisopliae*, as indicated by the absence of overlapping of confidence limits. Sabbour (2014) also found similar results on the efficacy of nano-formulated destruxin of *M. anisopliae* against adult females of *Hetiracris littoralis* Rambur (Orthoptera: Acrididae) under laboratory conditions, i.e., in nano-formulated samples, LC_{50} values were significantly decreased.

Susceptibility of potato tuber moth larvae to *Baeuveria bassiana* and *M. anisopliae* and the role of chemical additives in enhancing the action of entomopathogenic fungi were assessed by Sabbour (2002) who noted that oxalic and citric acids demonstrated the highest improvement of *M. anisopliae* efficacy against first instar lar-

Table 1. Toxicity of *Metarhizium anisopliae* and MCM-41@fungus to eggs and first instar larvae of *Phthorimaea operculella* under laboratory conditions.

Growth stages	Treatments	Slope ± S. E.	χ^2 (df)	LC_{50} (conidia/ml)	LC_{90} (conidia/ml)
Egg	PEF	3.74 ± 0.05	1.47 (3)	1.7×10^7 (6.3×10^6-5.2×10^7)	9.2×10^{10} (8.9×10^9-6.9×10^{12})
	MCM-41@fungus	4.26 ± 0.08	1.26 (3)	2.0×10^5 (7.1×10^4-6.6×10^5)	1.5×10^9 (1.2×10^8- 1.9×10^{11})
Neonate larvae	PEF	3.94 ± 0.06	1.00 (3)	1.8×10^6 (6.6×10^5- 6.1×10^6)	1.4×10^{10} (1.2×10^9- 1.7×10^{12})
	MCM-41@fungus	4.57 ± 0.09	1.63 (3)	1.5×10^4 (5.3×10^3-5.0×10^4)	1.4×10^8 (1.1×10^8-1.8×10^{10})

PEF: Pure Entomopathogenic Fungus (=non-formulated fungus)
95% fiducial limit (FL) is shown in parenthesis

vae of *P. operculella* (LC_{50} values=1.20×10^7 and 1.30×10^7conidia/ml, respectively, compared with LC_{50}=8.61×10^7 conidia/ml for *M. anisopliae* alone). All LC_{50} values were higher than the LC_{50} value of PEF obtained from our results on larvae, possibly due to variations in the isolates and experimental procedures, however the chemical additives increased the efficiency of *M. anisopliae* against the pest. On the other hand, Khorrami *et al*. (2018) found a lower LC_{50} value (= 1.9×10^5 conidia/ml) relatively to our results for pure *M. anisopliae*, which could be also attributed to variation in the entomopathogenic fungus isolate. Other studies have also revealed the efficacy of *M. anisopliae* in pest control (Lacey *et al*., 1994; Ansari *et al*., 2004, 2007; Marannino *et al*., 2006; Meyling and Eilenberg, 2007; Baker *et al*., 2018) while Khorrami *et al*. (2018) evaluated the pathogenicity of *M. anisopliae* against the potato tuber moth in comparison with *Nomuraea rileyi* and *Paecilomyces tenuipes*. In the latter study, *N. rileyi* presented the highest toxicity against neonate larvae of PTM in laboratory conditions (LC_{50} value equivalent to 1.0×10^3 conidia/ml) whereas *M. anisopliae* and *P. tenuipes* had lower activity (LC_{50} values equivalent to 1.9×10^5 and 2.4×10^6 conidia/ml, respectively).

Based on our findings on the effect of pure *M. anisopliae* and MCM-41@fungus, neonate larvae are more susceptible to infection by *M. anisopliae* than its eggs, which is consistent with the conclusions of Khorrami *et al*. (2018). Similarly, Sewify *et al*. (2000) reported that *M. anisopliae* has a potential for control of potato tuber moth larvae. They also reported that the combination of *M. anisopliae* and phoGV had a synergistic effect for larval control. Pandey *et al*. (2015) examined efficacy of *M. anisopliae* against PTM under laboratory conditions and exhibited that the fungus showed high biological activity against *P. operculella* larvae with LC_{50} values, ranging from 2.94×10^5 to 7.16×10^7 conidia/ml. In addition, Marannino *et al*. (2006) found that *M. anisopliae* isolates were more virulent to neonate larvae of *Capnodis tenebrionis* Eschscholtz than the eggs of the pest. The results are promising since the first larval stage is the only free-living stage exhibiting a potential practical target in storage. To our knowledge, MCM-41 has not been used as carrying agent in bio-pesticides and this is the first report of using of MCM-41 as carrying agent that can significantly improve the effectiveness of the *M. anisopliae* on eggs and neonate larvae of *P. operculell*a. The experimental protocol followed in the present study allowed us to provide evidence for MCM-41@entomopathogenic fungus infection.

Conclusion

Laboratory bioassays have clearly demonstrated the pathogenicity of *M. anisopliae* and MCM-41@*M. anisopliae* for neonate larvae and eggs of *P. operculella*. These biocontrol agents should be considered for the development of a new and environmentally compatible approach for potato tuber moth management in terms of preventing PTM infestations. Further research is required to determine the biological activities of MCM-41-@*M. anisopliae* and its persistence after store applications and other factors that may improve its performance against *P. operculella* throughout its egg and larval stages.

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Σκεύασμα του εντομοπαθογόνου μύκητα *Metarhizium anisopliae* με νανοϋλικό πυριτίου MCM-41: Ένα νέο και αποτελεσματικό εντομοκτόνο κατά της φθοριμαίας της πατάτας σε αποθηκευμένους κονδύλους

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Περίληψη Η φθοριμαία της πατάτας, *Phthorimaea operculella*, αποτελεί οικονομικής σημασίας εντομολογικό εχθρό της πατάτας σε τροπικές και υποτροπικές περιοχές. Λόγω πολλών προβλημάτων σε σχέση με τη χημική καταπολέμηση του εντόμου, οι παράγοντες βιολογικής αντιμετώπισης, όπως οι εντομοπαθογόνοι μύκητες, έχουν προσελκύσει το ενδιαφέρον των ερευνητών σχετικά με την εφαρμογή τους ως μέρος ολοκληρωμένων στρατηγικών διαχείρισης του εντόμου σε πολλές χώρες. Ως εκ τούτου, στην παρούσα εργασία εξετάστηκε η εντομοκτόνος δράση του εντομοπαθογόνου μύκητα *Metarhizium anisopliae* (ως καθαρή καλλιέργεια ή σε σκεύασμα με νανοϋλικό πυριτίου MCM-41 (MCM-41@fungus)) σε ωά και νεοεκκολαφθείσες προνύμφες του εντόμου. Το νανοϋλικό MCM-41 χαρακτηρίστηκε πλήρως με τεχνικές Φασματοσκοπίας Υπερύθρου Μετασχηματισμού Fourier (FT-IR), Ηλεκτρονική Μικροσκοπία Σάρωσης (SEM), Ακτίνες Χ Διασποράς Ενέργειας (EDS), Περίθλαση ακτίνων Χ (XRD) και Θερμοβαρυμετρική ανάλυση (TGA). Η μορφολογία του νανο-σκευάσματος MCM-41@fungus αξιολογήθηκε με ηλεκτρονικό μικροσκόπιο σάρωσης (SEM). Η μορφολογία της μεσοπορώδους δομής του βρέθηκε ότι είναι ομοιογενής, κανονική και σφαιρική. Οι τιμές LC_{50} της καθαρής καλλιέργειας του μύκητα και του νανο-σκευάσματος MCM-41@fungus υπολογίστηκαν ότι είναι $1,7\times10^7$ και 2×10^5 κονίδια/ml για τα ωά και $1,8\times10^6$ και $1,5\times10^4$ κονίδια/ml για τις νεοεκκολαφείσες προνύμφες, αντίστοιχα. Επομένως, οι βιοδοκιμές έδειξαν ότι το σκεύασμα MCM-41@fungus ήταν πιο δραστικό από την καθαρή καλλιέργεια έναντι και των δύο σταδίων του εντόμου. Η καθαρή καλλιέργεια του *M. anisopliae* και το νανο-σκεύασμα αυτού θα μπορούσαν να αξιοποιηθούν ως βιο-εντομοκτόνα σε προγράμματα διαχείρισης της φθοριμαίας της πατάτας.

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Antioxidant and antifungal activities of essential oils from Algerian spontaneous plants against five strains of *Fusarium* spp.

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Summary The present study evaluates the antioxidant and antifungal effects of essential oils (EOs) from *Thymus vulgaris*, *Thymus algeriensis*, *Mentha piperita*, *Mentha pulegium*, *Artemisia herba-alba* and *Artemisia campestris*, on five strains of *Fusarium*. The chemical composition of EOs of *T. vulgaris*, *T. algeriensis*, *A. herba-alba*, *M. piperita* and *M. pulegium* is characterized by a dominance of the family of oxygenated monoterpenes with 73.85%, 59.41%, 70.01%, 60.01% and 87.2%, respectively. On the other hand, the EO of *A. campestris* showed a diverse composition by similar percentages between all families. The two strains BD17 and INRA 349 were found to be resistant to low concentrations of EOs from *T. algeriensis*, *A. campestris* and *A. herba-alba*, sensitive to EOs of *M. pulegium* and *M. piperita* and very sensitive to *T. vulgaris* EO (0.25 µl/ml). A high antioxidant effect was recorded by *T. vulgaris* EO in BCB assay with an effective concentration (0.5 mg/ml) 3 to 60 times higher compared to the other EOs tested. This antioxidant capacity of *T. vulgaris* EO was also recorded with DPPH assay at an EC50=1.41 mg/ml.

Additional keywords: *Thymus*, *Mentha*, *Artemisia*, antifungal activity, antioxidant activity, *Fusarium*

Introduction

The dangers of intensive use of chemical pesticides in agriculture have forced the search for new natural alternatives based on secondary metabolites of plant origin which have shown significant protective power of crops with no side effects or toxic residues in treated products and the environment by their slow degradation (Kordali *et al*., 2009; Sumalan *et al*., 2013; Gordon, 2017). This has led to the emergence of new eco-based products consisting mainly of terpenoids such as DMC Base Natural, Eugenol-Tween®, Protecta, Herbalox® and Pycnogenol® (Prakash and Kiran, 2016). Furthermore, the use of EOs from spontaneous or cultivated plants for their antifungal and antioxidant properties in tests against phytopathogenic agents has been the objective of several studies (Luchesi *et al*., 2020; Chibane *et al*., 2020). These natural extracts have shown significant capacity to fight against spoilage and pathogenic microorganisms which gives them an interest with regard to their possible use as alternatives to the food preservatives currently used (Velluti *et al*., 2004).

Fusarium wilt is a disease that affects crops before harvest, reducing their quality and yield, while it can also develop after harvest on seeds, causing quality degradation and contamination by mycotoxins dangerous for humans and animals (Gordon, 2017). Several studies have focused on the capacity of some food-grade antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), trihydroxybutyrophenone (THBP) and propylparaben (PP) to inhibit fungal growth and production of mycotoxins in an alternative and corrective strategy, the results of which have showed potential for controlling germination and growth of mycotoxigenic strains of *Fusarium* and *Penicillium* (Etcheverry *et al*., 2002; Torres *et al*., 2003). The EOs of six plants (*Melissa officinalis*, *Salvia officinalis*, *Coriandrum sativum*, *Thymus vulgaris*, *Mentha piperita* and *Cinnamomum zeylanicum*) at a range of 500 to 2000 ppm, have shown a protective power of wheat seeds against *Fusarium* and *Asper-*

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gillus fungi after five days of treatment. On the other hand, the antioxidant powers have negatively correlated with seed contamination index (SCI) suggesting that essential oils with high antioxidant characteristics induce low fungal contamination and cannot have a crucial role in the expression of the antimycotoxin effect (Sumalan *et al*., 2013).

In this study, essential oils of six plants which belong to two families; *Lamiaceae* and *Asteraceae* were compared for their fungal growth inhibiting effects on five *Fusarium* strains using concentrations between 0.25 and 4 µl/ml as well as their antioxidant powers. In this respect, this first comparison of local botanical sources of terpenoids in Algeria allows their biochemical valorization for economic and health interests in agriculture and food industry.

Materials and Methods

Plant material and extraction of essential oils

Four species of *Lamiaceae*: *Mentha piperita* L., *Mentha pulegium* L., *Thymus vulgaris* L. and *Thymus algeriensis* Boiss. & Reut. and two species of *Asteraceae*: *Artemisia herba-alba* Asso and *Artemisia campestris* L. were collected in June 2017 from the region of ElHadjeub (Laghouat) and ElGhicha in the south of Djebel Amour in the Algerian hautes plaines. Collected samples were dried at room temperature, away from light and humidity. Essential oils were obtained by hydro-distillation during 2.5 to 3 h, using a Clevenger type apparatus (Clevenger, 1928). The obtained oil was treated by anhydrous sodium sulfate, filtrated then stored at +4°C, until analysis.

Gas chromatography analysis

Chromatographic analyses of volatile compounds were carried out at the Research Laboratory of Fundamental Sciences at the University of Laghouat in Algeria, using a gas chromatograph GC-5400 equipped with a flame ionization detector (FID) and a fused silica capillary column of type DB-5 (30 m × 0.32 mm, film thickness = 0.10 µm). The vector gas used is hydrogen with a flow rate of 1 ml/min. Temperature of the column is programmed at a rate of 5°C/min from 50°C to 250°C. The temperature of injector and detector was set at 250°C and 280°C, respectively. Essential oil solutions are prepared by dissolving 10 µL of each in 1 ml of pentane organic solvent. Linear retention indices of the constituents are based on a series of alkane (C8–C20) analyzed under the same operating conditions as those of the samples.

Determination *in vitro* of the antifungal activity of the obtained essential oils

The antifungal power of EOs was tested on five strains; INRA 349 of *F. graminearum* from CBS collection 185.32 (Centraalbureau voor Schimmelcultures, Netherlands), BD17 of *F. culmorum* from the collection of Touati-Hattab Sihem (Touati-Hattab*et al*., 2016), *Fusarium oxysporum* f.sp. *lycopersici* (FOL) from National Institute of Agronomy (El Harrach), *Fusarium oxysporum* f.sp. *albedinis* (FOA) from Regional Plant Protection Station (SRPV Ghardaïa) and *Fusarium oxysporum* f.sp. *pisi* (FOP) obtained from Mycology laboratory of agronomy department (University of Blida).

The methodology of the bioassays for the evaluation of the effect of EOs on mycelial growth and the determination of inhibition rates in solid culture medium are described in a previous study (Elhouiti *et al*., 2017). In brief, 1/5th–1/200th dilutions of the EOs were prepared in an agar solution (2%). In tubes, each containing 13.5 ml of sterile PDA medium (45°C), 1.5 ml of each EO dilution was added to obtain final concentrations ranging from 0.5 to 20 µl/ml. The effect of the EOs on mycelial development was evaluated by calculating the inhibition percentage of the mycelium after 7 days of incubation at 25 ± 2°C.

Evaluation of Antioxidant Activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay

DPPH radical scavenging assay was carried out according to the method of Brand-

Williams *et al*. (1995). In order to test their antioxidant power, dilutions of essential oils were prepared in absolute ethanol. A volume of 1 ml of the ethanolic solution of DPPH (200 µM) is added to 1 ml of the solutions of extracts or of the standard antioxidant (BHT, Vit E, Vit C) at different concentrations, the mixture is vigorously stirred. After 30 minutes of incubation in the dark and at room temperature, the absorbances were read at 517 nm by a UV-Vis spectrophotometer (Shimadzu 1601) against a blank. DPPH inhibition in terms of percentage was calculated using the following equation: Percent scavenged (DPPH)=[(A0-AS)/A0]x100, where A0 is the absorbance of DPPH solution without EO and AS is the absorbance in presence of EO. Antioxidant activity of EOs was expressed as IC50 defined by the concentration (in mg/ml) of tested EOs inhibiting the formation of DPPH radicals by 50%.

β-Carotene Bleaching (BCB) Assay

A modified method of β-carotene bleaching (BCB) assay described by Marco (1968) and Miller (1971). 1 mg of β-carotene is dissolved in 1 ml of chloroform. 1 ml of this solution (1 mg/ml) is mixed with 200 mg of Tween 20 and 20 mg of linoleic acid. Then chloroform is evaporated under vacuum at 45°C for 3 minutes, and then 75ml of distilled water was added to the emulsion to form the final reagent.

For each experiment a fresh solution of the reagent was prepared. A volume of 0.2 ml of each extract and of the synthetic antioxidant BHT (at different concentrations) is added to a volume of 2 ml of the emulsion of β-carotene/linoleic acid. The absorbance of the extracts is measured at 470 nm after incubation at 50°C for 120 min. The percentage of antioxidant activity of EOs was calculated with the following equation: I(%)=[(AE-AC)/(A0-AC)]x100. Where AE is the absorbance in presence of EOs at t = 120 min, AC is the absorbance of control at t = 120 min and A0 is the absorbance of control at t_0.

Statistical analysis

All experiences were performed in triplicate. Data processing was carried out with Origin b9.6.5.169. The determination of IC50 and MIC was performed with dose-response curve with variable Hill slope at error probability≤0.05.

Results and discussion

Chemical composition analysis

The essential oil yields of *Thymus vulgaris*, *Thymus algeriensis*, *Mentha piperita*, *Mentha pulegium*, *Artemisia herba-alba* and *Artemisia campestris* were 2.38%, 0.72%, 2%, 1.3%, 1.02% and 0.2% (w/w) respectively. The chemical composition of essential oils of *T. vulgaris*, *T. algeriensis*, *A. herba-alba*, *M. piperita* and *M. pulegium* is characterized by a dominance of the family of oxygenated monoterpenes with 73.85%, 59.41%, 70.01%, 60.01% and 87.2% respectively (Fig. 1). EO of *A. campestris* showed a diverse composition by similar percentages between all families. The elemental composition of EOs of the genus *Thymus* revealed high percentages of Carvacrol (63.42%), γ-Terpinene (9.10%) and *p*-Cimene (6.73%) in *T. vulgaris* EO and Limonene (11.49%), α-Pinene (9.26%) and Carvacrol acetate (14.16%) in *T. algeriensis* EO. On the other hand, high percentages of Carvone (70.8%), Piperitenone (4.94%), and Menthofurane (6.8%) have characterized *M. pulegium* EO, contrary to *M. piperita* EO which is dominated by Piperitone (57.51%) and Limonene (28.29%). *A. herba-alba* EO contained high percentages of Camphor (36.48%), Chrysanthenone (13.99%) and Limonene (10.44%), while the composition of *A. campestris* EO did not show high percentages of distinct compounds but Sabinene (5.71%), γ-Muurolene (5.08%), Bicyclogermacrene (16.8%) and (Z) -3-Hexenyl benzoate (25.72%) generally characterized its composition.

The composition of *T. vulgaris* EO suggests that it belongs to Carvacrol chemotype (Iten *et al*., 2009) which has shown significant antiinflammatory and insecticidal potential (Fachini-Queiroz *et al*., 2012; Szczepanik *et al*., 2012). The variability of EOs chemical composition in the populations of

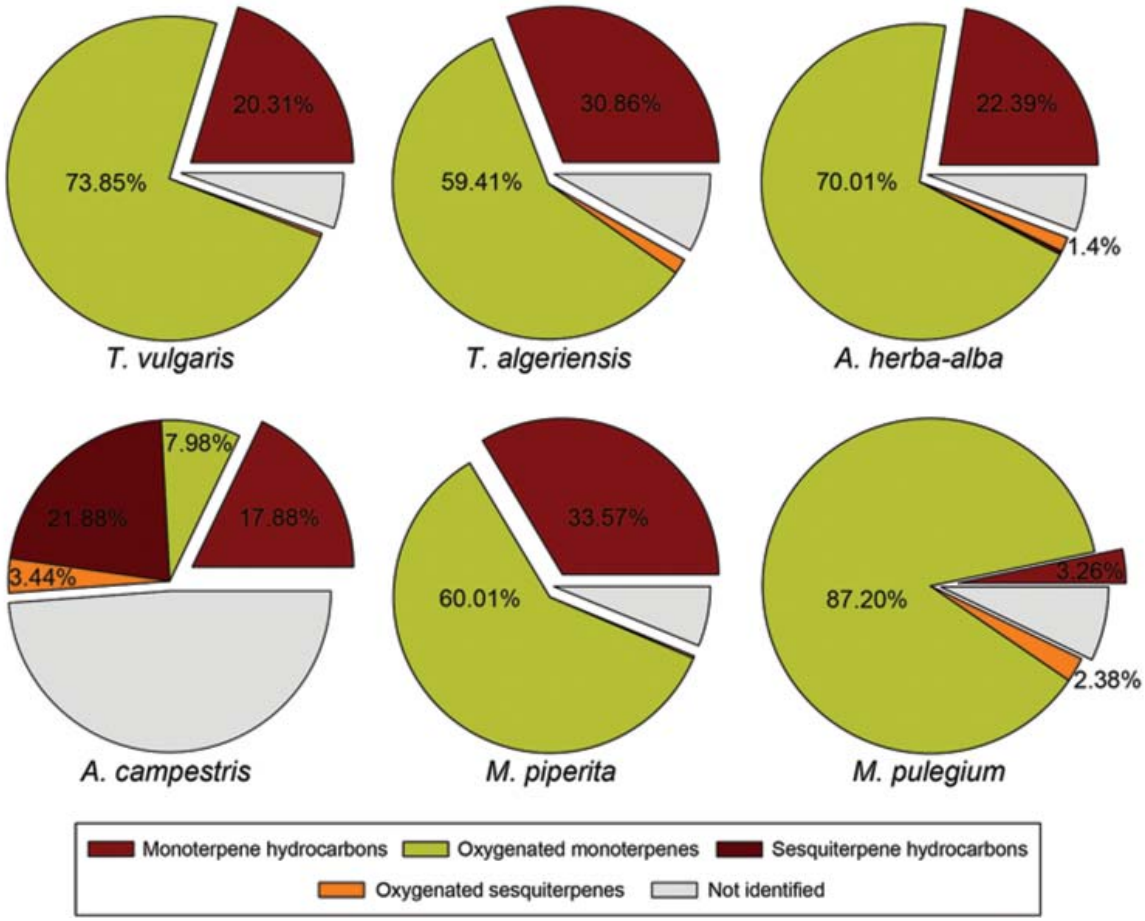

Figure 1. Chemical compositions by families of terpenoids in essential oils of *Thymus vulgaris*, *Thymus algeriensis, Mentha piperita, Mentha pulegium, Artemisia herba-alba* and *Artemisia campestris*.

Tunisian *T. algeriensis* has shown the presence of five different chemical groups (Ben El Hadj Ali *et al*., 2012) and this high variation was also shown in the composition of the samples analyzed in other studies (Dob *et al*., 2006, Amarti *et al*., 2010). In the composition of *M. pulegium* EO, the high percentage of Carvone also characterized Tunisian samples from semi-arid bioclimatic stage and may represent Carvone-pulegone chemotype (Mkaddem *et al*., 2007). The high percentage of Piperitone in the composition of *M. piperita* EO has not been reported previously, but the form D of this ketone was isolated from essential oils of the genus *Mentha* and played a role in the biosynthesis cycle of (-)-menthol (Bergman *et al*., 2019). A high percentage of oxygenated monoterpenes and the percentage of Camphor also characterized the composition of *A. herba-alba* EO in other studies (Dob and Benabdelkader, 2006; Mohsen and Ali, 2009), unlike *A. campestris* EO which showed high levels of hydrocarbon monoterpenes (Akrout *et al*., 2001, 2003).

Antifungal activity

Kinetics of fungal growth of *Fusarium* strains in the presence of different concentrations of the tested plant EOs showed a remarkable sensitivity of the strains of *F. oxysporum*. The growth inhibition curves are shown in Figure 2. The two strains BD17 and INRA 349 were found to be resistant to low concentrations of EOs from *T. algeriensis*, *A. campestris* and *A. herba-alba*, sensitive to EOs of *M. pulegium* and *M. piperita*, very sensitive to the EO of *T. vulgaris*. At the minimum concentration tested 0.25 µl/ml of *T. vulgaris* EO, the growth of strains FOA, FOL and FOP

of *F. oxysporum* was completely inhibited.

In Figure 3, the IC50 and MIC values show very high bioactivity of *T. vulgaris* EO against all strains. In general, IC50 values of the other EOs are between 0.01 and 0.6 µl/ml against the strains of *F. oxysorum* which are more sensitive to *T. vulgaris* and *A. campestris* EOs, on the other hand, the strains of *F. culmorum* and *F. graminearum* are only sensitive in the presence of *T. vulgaris* EO. A

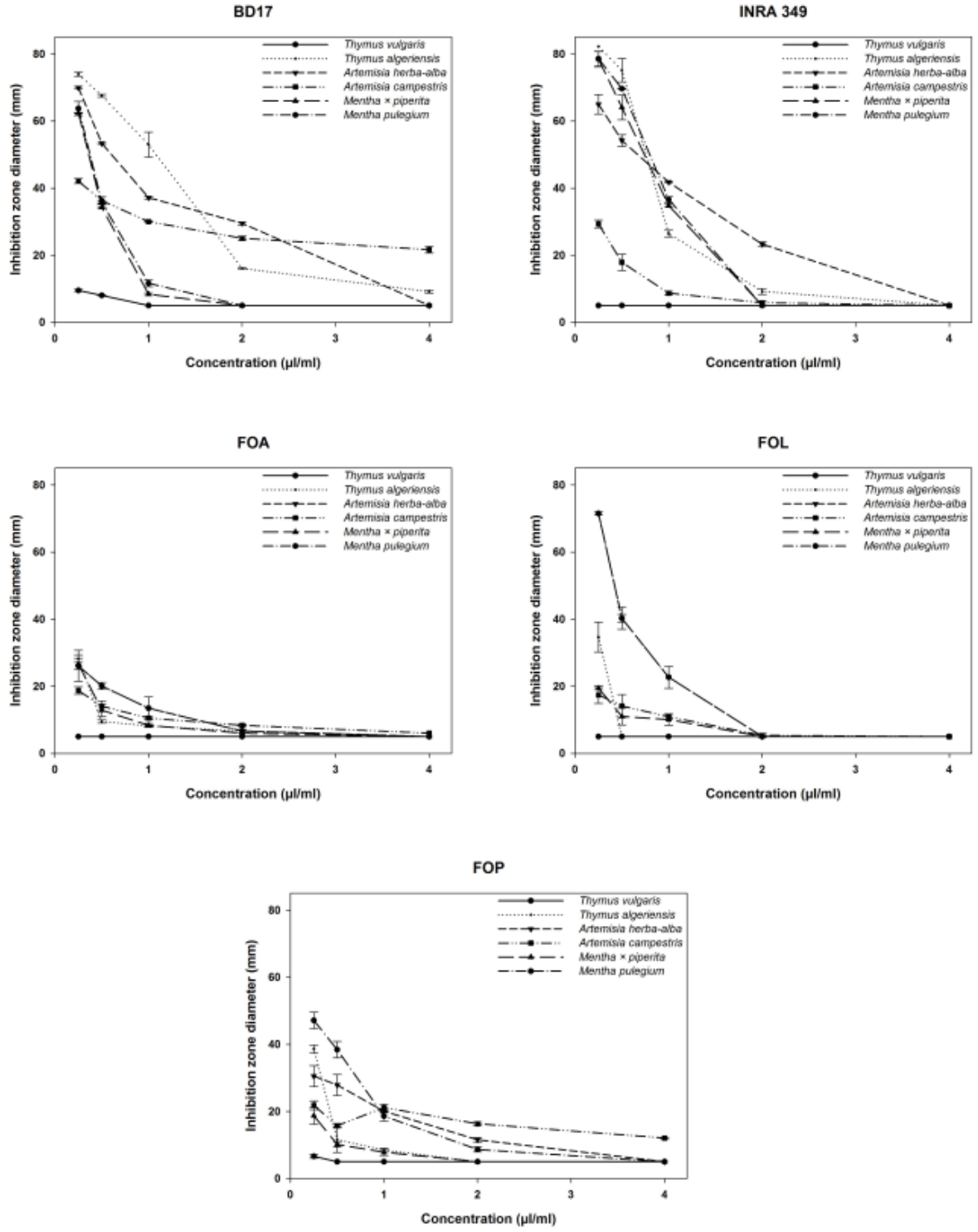

Figure 2. Growth inhibition of the strains BD17, INRA 349, FOA, FOL and FOP by different concentrations of tested essential oils.

weak inhibitory effect of EOs of *A. campestris* and *A. herba-alba* compared to other oils resulted in high MIC values against the strains BD17, INRA 349, FOA and FOP. Potency and efficiency of EOs of *M. pulegium* and *M. piperita* were important especially against the strains of *F. oxysporum*.

Against strains BD17 and INRA 349, IC50 and MIC values recorded by these tested EOs are more effective than the EO of *Rhanterium adpressum* (0.7 and 1.54 for IC50, 12.81 and 12.73 µl/ml for MIC for the two strains respectively), an endemic Algerian plant that was the subject of a previous study (Elhouiti *et al*., 2017), and even more effective against FOA than the EO of this same plant (2 µl/ml for IC50 and 10 µl/ml for MIC) (Elhouiti *et al*., 2016). MIC value of *A. campestris* EO against the growth of INRA 349 is similar to that recorded by Houicher *et al*. (2016) of 1.25 µl/ml against the strain MUCL 53452. Moreover, at 1 µl/ml, growth inhibition of the strains Fc107 and Fc289 of *F. culmorum* was complete (100%) with *T. vulgaris* EO mainly constituted by p-Cymene (21.94%), γ-terpinene (7.8%) and Thymol (44.6%) (Matusinsky *et al*., 2015). On the other hand, the EO of *M. piperita* from two different regions also showed a strong inhibition of *F. graminearum* in the presence of MIC>0.32 µl/ml (Rekanović *et al*., 2012).

Antioxidant activity

The antioxidant potential of the six EOs was investigated with their capacity to donate hydrogen by DPPH radical scavenging assay and with quenching linoleate free radicals capacity by *β*-carotene bleaching assay (BCB) was shown by the IC50 values in Table 1. A high antioxidant effect has been recorded by *T. vulgaris* EO with an effective concentration only 5 times more than the concentration of BHT in BCB test, the other oils

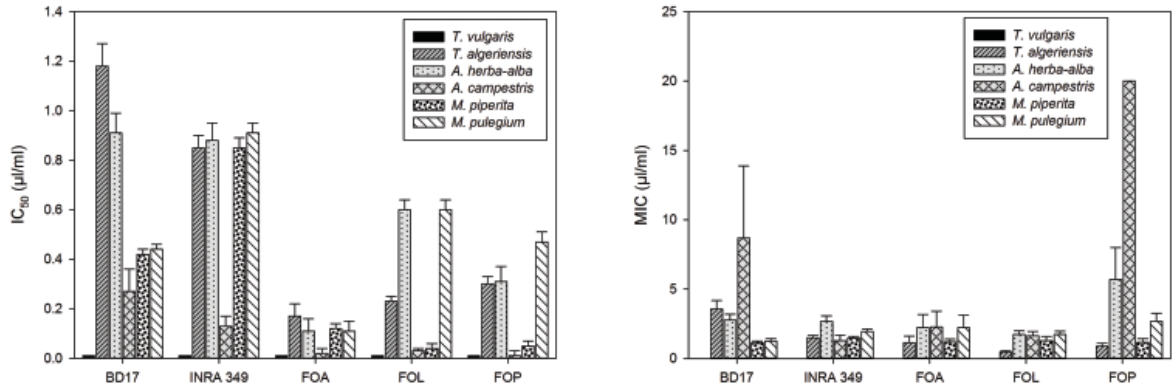

Figure 3. IC50 and MIC for essential oils of six plant species (*Thymus vulgaris, Thymus algeriensis, Mentha piperita, Mentha pulegium, Artemisia herba-alba* and *Artemisia campestris*) against five strains of *Fusarium*.

Table 1. Antioxidant activity by the BCB and DPPH assays of tested essential oils.

Extracts and references	BCB assay (mg/ml)	DPPH assay (mg/ml)
T. vulgaris	0.5±0.130	1.41±0.012
T. algeriensis	7.68±0.245	8.86±1.13
A. herba-alba	7.36±1.968	50.6±7.9
A. campestris	1.74±1.128	18.45±1.33
M. × piperita	5.52±2.107	40.65±5.44
M. pulegium	30.01±1.903	33.76±1.94
BHT	0.096±0.02	0.086±0.003
VIT C		0.006±0.0004
VIT E		0.009±0.001

showed weak inhibition compared to *T. vulgaris* EO with IC50 3 to 60 times less. DPPH test showed the weak antioxidant capacity of all oils compared to the standards. However, the capacity of *T. vulgaris* EO is 6 to 35 times higher than the other oils.

Compared to other studies, by BCB test, *T. vulgaris* is more active at a concentration of 0.5 mg/ml than three other species (*T. kotschyanus*, *T. eriocalyx* and *T. daenensis* ssp. *lancifolius*) studied by Amiri (2012), unlike *M. piperita* and *M. pulegium* compared to six species of *Mentha* from Brazil, concentrations of 5.52 and 30.01 mg/ml were required to reach IC50 (de Sousa Barros *et al*., 2015). As for *A. herba-alba*, the activity revealed a significant effect in comparison with the activity of four Tunisian chemotypes (Mighri *et al*., 2010). By DPPH test, essential oils of *T. algeriensis* collected from other regions of Algeria showed stronger activities (0.8-1 mg/ml) than in our study (Hazzit *et al*., 2009). Furthermore, with IC50 of 0.005 mg/ml, *M. piperita* EO from Turkey was more active than that of our study (Kizil *et al*., 2010). However, the activity of *T. vulgaris* EO is significant compared to other EOs from Morocco (4.57 mg/ml) (Ismaili *et al*., 2017).

This first report on the comparison of fungal power of some local aromatic plants reveals the significant potential of their essential oils against phytopathogenic strains of *Fusarium*. Our results show that these essential oils exert a significant fungicidal effect starting with low concentrations of 0.25 µl/ml and reaching inhibition rates of 80 to 100%. In addition to this activity, a strong antioxidant capacity has been shown by *T. vulgaris* EO unlike other EOs which have medium or low activity. In this study, *T. vulgaris* EO was identified as a Carvacrol chemotype which may explain its activity by the dominance of this compound and also by its synergistic effect with other components of the EO.

In the study of Saghrouchni *et al*. (2020), the MIC values for Carvacrol against *F. oxysporum*, *F. nivale* and *F. solani* were 1/1000, 1/2000 and 1/2000 respectively. Moreover, the effect of Carvacrol on topsoil disinfection during 4 weeks of treatment was able to reduce the fungal load to 0 for 3 weeks with a concentration of 0.8 g/l. However, the use of a single antifungal and antioxidant product in high doses and long term causes health and environmental problems. Thus, natural extracts with multiple antifungal and antioxidant components by their synergistic effect can be used as preservatives and dietary supplements to promote health safety (Weng and Yen, 2015).

Conclusions

The studied EOs have shown significant antifungal activity against *F. oxysporum* strains. The fungal growth of all *Fusarium* strains was strongly inhibited by *T. vulgaris* EO based on its bioactivity in DPPH radical scavenging assay and β-carotene bleaching assay. The EOs of *T. algeriensis*, *A. herba-alba*, *A. campestris*, *M. piperita* and *M. pulegium* exerted a moderate inhibitory activity on the strains of *F. culmorum* and *F. graminearum* with a weak antioxidant power revealed by the two assays. The antifungal and antioxidant activities may be due to monoterpenes which are the major components of the chemical composition of all oils. Overall, the EOs of the investigated Algerian spontaneous plants represent a source of natural antioxidants and antifungals in food industry as good alternative preservatives for foodstuffs.

List of abbreviations:

BCB: β-Carotene Bleaching
BHA: Butylated HydroxyAnisole
BHT: Butylated HydroxyToluene
DB-5: Dimethylsilicone stationary phase with 5% phenyl groups
DPPH: 2,2-DiPhenyl-1-PicrylHydrazyl
EO(s): Essential Oil(s)
FID: Flame Ionization Detector
Foa: *Fusarium oxysporum* f .sp. *albedinis*
Fol: *Fusarium oxysporum* f .sp. *lycopersici*
Fop: *Fusarium oxysporum* f .sp. *pisi*
GC: Gas Chromatograph
PDA: Potato Dextrose Agar

PP: PropylParaben
SCI: Seed Contamination Index
THBP: TriHydroxyButyroPhenone
Vit C: Vitamin C
Vit E: Vitamin E

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Αντιοξειδωτική και αντιμυκητιακή δράση αιθερίων ελαίων από αυτοφυή φυτά της Αλγερίας έναντι πέντε στελεχών *Fusarium* spp.

F. Elhouiti, K.H. Benabed, D. Tahri, M. Ouinten και M. Yousfi

Περίληψη Η παρούσα εργασία αξιολογεί την αντιοξειδωτική και αντιμυκητιακή δράση αιθερίων ελαίων από αυτοφυή φυτικά είδη, *Thymus vulgaris, Thymus algeriensis, Mentha piperita, Mentha pulegium,*

Artemisia herba-alba και *Artemisia campestris,* σε πέντε στελέχη *Fusarium*. Στα αιθέρια έλαια των ειδών *T. vulgaris, T. algeriensis, A. herba-alba, M. piperita* και *M. pulegium* κύρια χημική ομάδα είναι τα οξυγονωμένα μονοτερπένια με ποσοστά 73,85%, 59,41%, 70,01%, 60,01% και 87,2 %, αντίστοιχα. Το αιθέριο έλαιο του *A. campestris* παρουσίασε ποικιλομορφία στη χημική του σύσταση, με παρόμοια ποσοστά μεταξύ των χημικών ομάδων. Τα στελέχη BD17 και INRA 349 βρέθηκαν ανθεκτικά σε χαμηλές συγκεντρώσεις αιθερίων ελαίων των φυτικών ειδών *T. algeriensis, A. campestris* και *A. herba-alba*, ευαίσθητα στα αιθέρια έλαια των *M. pulegium* και *M. piperita*, και πολύ ευαίσθητα στο αιθέριο έλαιο του *T. vulgaris* (0,25 μl/ml). Σε δοκιμές BCB διαπιστώθηκε υψηλή αντιοξειδωτική δράση του αιθερίου ελαίου του *T. vulgaris*, με αποτελεσματική συγκέντρωση (0,5 mg/ml) 3 έως 60 φορές υψηλότερη σε σύγκριση με τα άλλα αιθέρια έλαια που δοκιμάστηκαν. Ανάλογη αντιοξειδωτική ικανότητα του αιθερίου ελαίου του *T. vulgaris* καταγράφηκε και σε δοκιμές DPPH, με EC50= 1,41 mg/ml.

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Dissipation of spiroxamine residues in open field cucumber and dietary risk assessment

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Summary Spiroxamine is one of the most used fungicides in the Mediterranean region, in significant uses such as open field cucumber. Residue trials in the Northern part of Egypt were conducted to investigate the dissipation of spiroxamine (SPX) in cucumbers according to the authorized Good Agricultural Practice (GAP) (1 x 75 g a.i. ha^{-1} at BBCH 85 to 89) and more critical use patterns. SPX was extracted from cucumbers using a modified QuEChERS protocol and residues were analyzed by liquid chromatography with tandem mass spectrometry (LC–MS/MS). The method was successfully validated with an LOQ of 0.001 mg kg^{-1}. A steep decline of SPX residues in cucumbers fit a first-order decay process with a calculated $t_{1/2}$ of approximately 2 days and almost complete degradation (99%) after 14 days. Chronic and acute exposure calculations were performed for cucumbers treated with SPX according to all tested GAPs employing two different approaches. In all cases a health risk after consumption of cucumbers was not identified.

Additional keywords: consumer exposure, field trial, kinetic model, LC-MS-MS, pre-harvest interval, spiroxamine

Introduction

Cucumber (*Cucumis sativus* L., Cucurbitaceae) is a popular vegetable, and a significant part of the Mediterranean diet, being a source of vitamins and minerals, and is consumed mostly raw. The cultivation of the vegetable in warm climates is susceptible to various disease infestation, especially under open field conditions, with fungi diseases such as *Fusarium oxysporum* f.sp. *cucumerinum, Alternaria alternata, Verticillium* sp., *Botrytis cinerea, Pseudoperonospora cubensis, Erysiphe cichoracearum, Sphaerotheca fuliginea* and *Leveillula taurica* being some of the most serious problems, effecting both yield and quality of the final product.

To protect cucumber cultivation in Egypt, several fungicides (active substances) are authorized by the Egyptian Agricultural Pesticide Committee (APC) with spiroxamine (SPX) being one of them. SPX is authorized in many countries for various uses such as *Uncinula necator* in grapes (Hellenic Ministry of Rural Development and Food), bananas (European Food Safety Authority, 2015) and cereals in combination with triazole fungicides, like prothioconazole (European Food Safety Authority, 2010). Although, these authorized uses are supported by residue data provided by the industry, in open literature, research studies investigating the residue behavior of SPX in cucurbits are not available. However, studies are available on the investigation of the residue behavior in grapes (Tsiropoulos, 2005a; Tsiropoulos, 2005b), strawberries (Malhat, 2020) or on the metabolism in various crops (Buerge *et al*., 2016) and soil (Rosales-Conrado, 2009; Sukul *et al*., 2010; Baćmaga *et al*., 2019). In addition, several studies are available assessing parameters related to the efficacy of the active substance, in general (Miller and Gubler, 2004) or in specific crops like citrus (D'Aquino *et al*., 2011).

Residues of SPX are detected in various food commodities of plant origin like bananas, peppers, grapes, wheat and melons

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(European Food Safety Authority, 2020), animal origin like eggs (Pereira, 2014) and environment samples like pollen of field bean, strawberry and raspberry (Tosi, 2018; David, 2020), and water (Tokatli, 2020). Exposure to this compound might have detrimental health effects on humans, thus dietary toxicological reference values are set. An acceptable daily intake (ADI) of 0.025 mg/kg bw/d by applying an assessment factor of 100 to the no-observed-adverse-effect level (NOAEL) from the 1-year dog study and an acute reference dose (ARfD), is 0.1 mg/kg bw derived from the acute neurotoxicity study in rat, with a safety factor of 100 are adopted at European (EU) level (European Food Safety Authority, 2010; European Commission, 2020). Therefore, it is important to minimize these residues to a reasonable level by establishing maximum residue limits (MRLs) in cucumbers. After a search by the authors in several databases (BCGlobal's Pesticide Database; Codex Pesticides Residues in Food Online Database; European Commission 2020), established MRLs on SPX in cucumber were not identified. In the EU the default value of 0.01 mg kg^{-1} is used for enforcement (European Commission, 2016).

In this study, the dissipation kinetics of SPX in Egypt following several use patterns, was investigated. A risk assessment of the residues resulting from these practices was conducted and the safety of the maximum residues was estimated at several Pre-Harvest intervals (PHIs).

Materials and Methods

SPX was applied using a commercial EC formulation, in outdoor field trials following several GAPs at Matshtul district (Sharkia governorate, Egypt) in one growing season (2018-2019). According to regulatory guidelines (OECD, 2009) in the case of outdoor crops the results on pesticide residues can be extrapolated to other regions with the same climate, this being a common practice in the EU (European Commision, 2017; European Commission, 2013). Although the Matshtul district is on the Mediterranean part of Egypt, residue studies of SPX in other Mediterranean counties, especially EU countries, were not available from the literature and hence a comparison of the results was not possible.

A randomized block design (15 m^2 plot size) was employed for the plot/crop layout with triplicate treatments including a buffer area with no pesticide application. An additional plot was included in the design as blank control plot. The pesticide was applied once, twice or three times at a single (75 g a.i. ha^{-1}) or double (150 g a.i. ha^{-1}) dose using a high–pressure knapsack sprayer. The first spray was applied at BBCH 85 – 89 (half of the size of fruit maturation) and with a 14 days interval for the GAPs with more applications. The authorized GAP (1x75 g a.i. ha^{-1}) and additional more critical use patterns (double dose rate or 2-3 applications or both) were investigated as to estimate the final residue and risk to the consumer from a potential misuse or emergency applications due to unexpected disease or pattern that may be authorized in the future.

For each field, fruit samples (2 kg) were randomly collected at 3 and 7 days, except from the fields in which application was conducted according to the authorized (1 x 75 g a.i. ha^{-1}) and the double dose pattern (1 x 150 g a.i. ha^{-1}). In the latter fields, samples were collected at 0, 1, 3, 7, 10 and 14 days after the final application, packed in well-aerated bags and brought to the laboratory in the same day. The storage period between harvest and analysis was less than 1 week, thus investigation of the stability of residues during storage is not required (European Commision 1995; OECD 2007). For the analysis of the samples the (Quick, Easy, Cheap, Effective, Rugged and Safe) QuEChERS extraction procedure (EN-15662 2009) as previously modified by the authors (Malhat et al. 2020) was used. Related to the materials used in the analysis, SPX certified reference standard (99%) was bought from Dr Ehrenstorfer (Augsburg, Germany), all solvents (acetonitrile and methanol) were HPLC grade and purchased from Sigma Al-

drich Chemical Co., analytical grade sodium chloride (NaCl), magnesium sulfate anhydrous ($MgSO_4$) (dried at 450°C overnight before use), trisoduim citrate dehydrates and disodium hydrogen citrate sesquihydrate were supplied from Merck (Darmstadt, Germany). The sample preparation is described below for completeness:

Ten grams of the homogenized subsample was weighted into 50 mL centrifuge tube along with 10 mL of acetonitrile and vortexed for 2 min. Hereafter, 4.0 g of $MgSO_4$ anhydrous, 1.0 g of NaCl, 1.0 g tri-sodium citrate (dihydrate) and 0.5 g sodium hydrogencitrate sesquihydrate were added and the tube was shaken intensively for 2 min. by hand. The sample was then centrifuged at 4800 rpm for 5 min for phase separation and an aliquot of 200 µL of supernatant was diluted with 800 µL acetonitrile, filtered with a PTFE (0.2 µm) syringe filter and the samples were ready for injection in the LC–MS/MS. Based on this procedure, the C (mg kg^{-1}) in the sample correspond to 5 times the concentration in the extract (µg mL^{-1}) analyzed. An LC-MS/MS system with an Exion LC system coupled to QTRAP mass spectrometer (QTRAP 6500^{+}, AB SCIEX) was used for the analysis of SPX residues. A Synergi C_{18} column, 2.5 µm Fusion-RP 100Å, 3.0 x 50 mm (Phenomenex) was used for chromatographic separation. The column temperature was held at 40°C. Mobile phases consist of water (A) and methanol (B) both containing 5 mM ammonium formate.

Elution was performed at gradient mode as follows: a ratio of 20% for eluent B, running for (0-1.0 minutes), 20-100% B (1-8 min.), and then 20% B (8.05–10 min.). at a flow rate of 0.4 mL/min. The injection volume was 2 µL. SPX was eluted at 5.5 min. The LC-MS/MS was operated using electrospray ionization (ESI) in the positive ion mode with multiple reaction monitoring (MRM) in the scan mode. Sources and gas parameter were optimized as follows: ion spray voltage 5500 V for ESI (+); ion source temperature 400°C; curtain gas 20 psi; collision gas medium; nebulizer gas and auxiliary gas 35 psi. The Analyst software (Version 1.7.1, AB Sciex) was used for the instrument setting, data acquisition and processing. The specific parameters are given in Table 1.

The method was validated, determining the limit of quantitation (LOQ), accuracy and precision (recoveries from fortified samples), matrix effect (due to co-extractives) and linearity (in solvent and matrix) as per SANTE guideline (Commission 2017). The matrix effect (ME) was evaluated according to the following equation:

$$\%ME = 100\left(\frac{M_{matrix} - M_{solvent}}{M_{matrix}}\right)$$

where *%ME* is the % estimation of the matrix effect, M_{matrix} and $M_{solvent}$ are the slopes of calibration curves in cucumber extract and acetonitrile, respectively. If the ME value is between the threshold of ±20 %, matric effect can be considered not significant (Kmellár *et al*., 2008; Pizzutti *et al*., 2009). In addition to the above threshold, according to Ferrer *et al.* (2011) matrix effect can be considered as soft when ME is calculated between 20- 50% and strong above 50 % or below -50%.

The dissipation rate of SPX in cucumber was evaluated by subjecting the data to a first-order kinetics equation:

$$C_t = C_0 e^{-kt}$$

Table 1. LC-MS/MS optimization parameters for SPX.

Precursor ion (Q1)	Product ions (Q3)	Retention time	Declustering potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)	Purpose
298.3	144.2	5.5	80	10	27	4	quantitation
298.3	100.2	5.5	80	10	50	4	qualifier

where C_t represents the concentration (mg kg^{-1}) of SPX at time (t), C_0 represents the initial concentration (mg/kg) of SPX after application, and k is the dissipation coefficient in day 0.

The persistence of SPX is generally expressed in terms of half-life, $t_{1/2}$ or DT_{50}, i.e., time for the disappearance of pesticide to 50 % of its initial concentration and was calculated from the k value as following:

$$t1/2 = ln\ 2\ /k$$

To ensure that a health risk is not related to the current use, a dietary consumer risk assessment was performed, including as input values for the exposure the residue levels from all PHIs related to the authorized GAP; for the hazard the ADI (0.025 mg/kg bw/d) and ARfD (0.1 mg/kg bw/d) was taken into consideration (European Commission 2020; European Food Safety Authority, 2010).

The long-term dietary exposure to SPX from cucumbers consumption was estimated using the following equations (Wang *et al*., 2015; Zhu Xiaodan *et al*., 2016) (FAO, 2009; FAO/WHO, 1988):

$$\mathrm{NEDI} = \mathrm{CRL} \times \frac{\mathrm{F}}{60}$$

where NEDI is national estimated daily intake (mg/kg, bw), CRL is residue levels (mg/kg); F is the cucumber average consumption data (g/d) according to WHO cluster diet G06 (IEDI 2014; WHO 2013a; WHO 2013b) and 60 is the body weight (kg).

and

$$\mathrm{RQ} = \frac{\mathrm{NEDI}}{\mathrm{ADI}}$$

where RQ is the risk quotient and ADI is the acceptable daily intake (mg/kg, bw). Whereas RQ value higher than 1 (≥100% of ADI) an unacceptable chronic risk to the consumer is identified.

Additionally to the above approach, since EU is a significant market for Egyptian cucumbers, the deterministic model EFSA PRIMo revision 3.1 (European Food Safety Authority, 2019) which is the calculation model used on EUMRLs setting, was also employed.

Results and Discussion

The applied method is considered suitable for the determination of SPX in cucumbers at an LOQ of 0.001 mg kg^{-1} according to EU standards (SANTE/11813/2017, 2017). The LOQ is 10 times below the default EUMRL of 0.01 mg kg^{-1} and is suitable for investigation of the degradation of the compound at very low levels. The mean recovery values were between 99 and 105%. For the precision, both repeatability and reproducibility were estimated and were found acceptable, with RSD_r of below 17% for repeatability (at all fortification levels) and RSD_R of 11% (calculated at 0.1 mg kg^{-1}) for "*day to day*" reproducibility. Good linearity using 5 levels (0.001, 0.01, 0.02, 0.05, and 0.1 mg L^{-1}) was obtained in both solvent and matrix with the coefficients of determination (R^2) higher than 0.99. Matrix effect was found to be negligible (ME below 20%). Recovery and precision data are presented in Tables 2 and 3. A visual comparison of the chromatograms in blank cucumber and spiked sample at 0.001 mg kg^{-1} (validated LOQ) is presented in Fig. 1.

The dissipation rate of SPX in cucumbers was calculated based on the experiment performed with the authorized GAP from residues measured at the day of the last application (0 day), and 1, 3, 7, 10 and 14 days later. Residues were not detected in the control samples. In the samples col-

Table 2. Recovery percentage and relative standard deviation of SPX in cucumber (*n*=6).

Spiking level (mg/kg^{-1})	Recovery (%)	RSD (%)
0.001	98	17
0.01	96	15
0.1	97	10

Table 3. Recovery RSD_r and RSD_R values obtained from analysis of cucumber samples spiked with SPX at 0.1 mg kg $^{-1}$ (*n*=6**).**

Analysis day	Recovery %	RSD_r%	RSD_R %
1	95	11	
2	99	6	11
3	94	3	

lected at the day of the last application (day 0) residues were 0.67 mg kg^{-1}. In the 1st day a degradation of 13% was observed with a rapidly and gradually decrease of the residues until day 7, with residue levels declining down to 0.041 mg kg^{-1} (decline of 94% compared to the initial concentration). After this point and with a stable but less steep degradation, the residue levels dropped to

A. Blank cucumber matrix

B. Spiked cucumber sample 0.001 mg L^{-1}

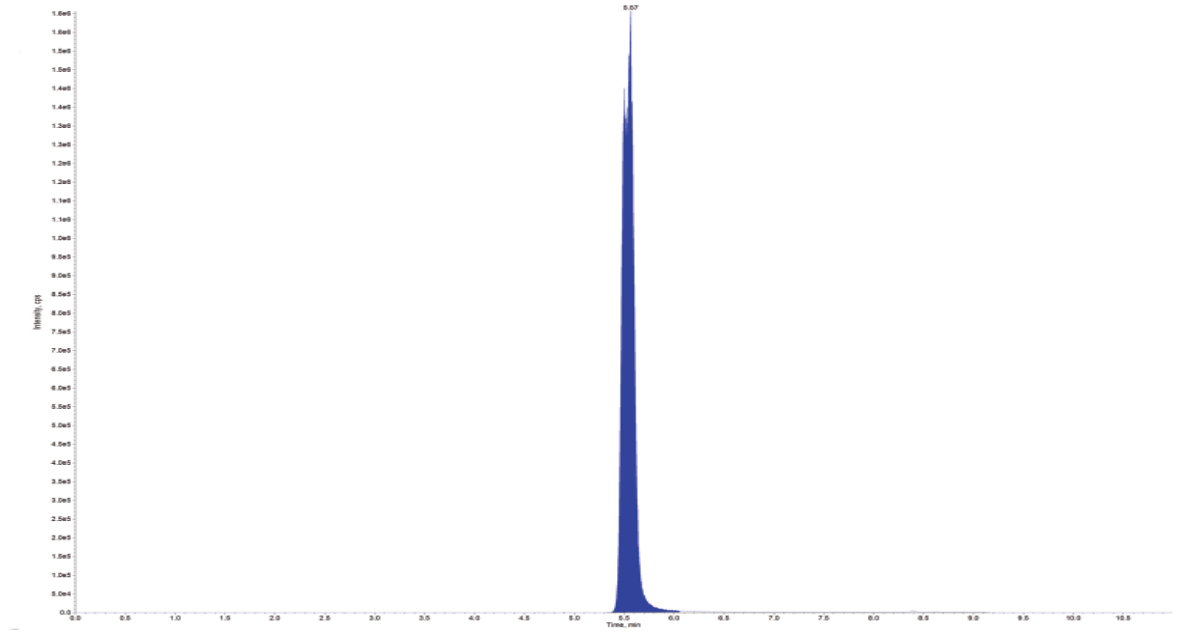

Figure 1. LC-MS/MS chromatograms of SPX (A) Cucumber blank (B) Spiked cucumber sample (0.01 mg L^{-1}).

0.003 mg kg^{-1}, representing almost complete degradation (>99%) at 14 days. Based on this behavior the half-life ($t_{1/2}$) was calculated at approximately 2 days (1.95) with a good exponential correlation between concentration and time ($r^2 > 0.9953$) to be observed. A graphical visualization of the dissipation pattern is presented in Fig. 2. The published work on the application of SPX in crops is scarce. In the few studies available under Mediterranean conditions, its half-life is 7 days in grapes (Tsiropoulos, 2005a) and 4.71 days in strawberries (Malhat, 2020).

The effect of the different rates and applications was also examined by comparison of the residue levels at PHI of 3 and 7 days from different use patterns. The results are presented in tabular form in Table 4. In some cases, despite the increase of dose or number of applications, the residue levels were significantly lower compared to the less critical cases, whereas in principle an increase should have been observed. Since from the experimental conditions no error was observed or an extreme environmental parameter was not recorded, this is considered as a variability of the data set as a whole, therefore not rejected. At a PHI of 3 days, residue levels were 0.23±0.005 mg kg^{-1} when applied at a use pattern of 2x75 g a.i. ha^{-1} and 0.43±0.026 mg kg^{-1} at twice the dose rate, indicating 47% increase in residues due to dose increase. Similar in the case with 3 applications, in which the increase was higher (68-75%). A correlation between the number of applications and the residue levels cannot be identified. In both levels in the use patterns with 3 applications, the residue levels at 3 days were lower or similar compared to the ones with 2 applications. Based on the above, the number of applications seem not to have a significant effect on the final residue. This can be confirmed by the results from both sampling days (3 and 7 days PHI).

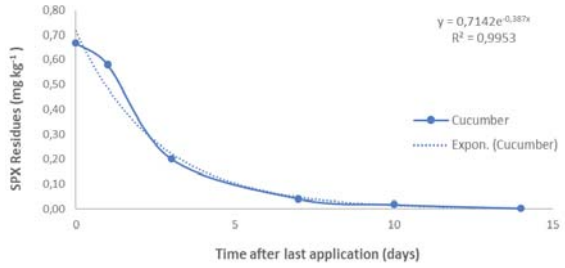

Figure 2. Dissipation pattern of SPX in/on cucumbers at the authorized GAP.

Due to the absence of established MRL for SPX in cucumbers, a comparison is only

Table 4. Residues and % comparison of SPX in cucumbers at 2 different dosage and number of applications, more critical that the authorized GAP (1 x 75 g a.i ha^{-1}).

Dosage (g a.i/ha)	n[a]	PHI	Residues (mg/kg ±SD)	Residue decline from previous sample point[b]	Residue increase due to dose rate[c]	Residue increase due to number of applications[d]
75	2	3	0.23±0.005			
		7	0.012±0.001	95%		
	3	3	0.11±0.009			-116%[e]
		7	0.014±0.011	87%		14%
150	2	3	0.43±0.026		47%	
		7	0.010±0.002	98%	-20%[f]	
	3	3	0.42±0.032		75%	-4%[e]
		7	0.044±0.001	89%	68%	77%

[a] Number of applications.
[b] The decline was calculated as the difference of the residues in comparison to the previous PHI.
[c] Increase was calculated based on the comparison with the previous pattern with application of 150 g a.i/ha.
[d] Increase was calculated based on the comparison with the previous pattern with 2 applications.
[e] In these cases, residues at the GAP with 3 applications were lower than the ones in the pattern with 2 applications despite the addition residue burden due to an additional application.
[f] In these case, residues at the pattern with double the dose rate were lower than the ones in the pattern with the recommended dose despite the addition residue burden due to high dose.

Table 5. Exposure calculations based on the FAO/WHO and EFSA approach, following the authorized GAP (1 x 75 g a.i/ha).

PHI (days)	C (mg kg-1)	FAO/WHO		EFSA PRIMo rev 3.1	
		NEDI[a] (mg/kg bw/d)	RQ	Highest % of ADI	Highest % of ARfD
0	0.67	3.89×10^{-4}	0.02	4.0	44
1	0.58	3.38×10^{-4}	0.01	4.0	38
3	0.20	1.18×10^{-4}	<0.01	1.0	13
7	0.041	2.39×10^{-5}	<0.01	0.3	3
10	0.018	1.05×10^{-5}	<0.01	0.1	1
14	0.003	1.75×10^{-5}	<0.01	<0.1	0.2

[a] consumption of cucumbers (34,92 gr/bw/day or 0.0349 kgr/bw/day was used.

feasible with the default value of 0.01 mg kg^{-1}. Applying the authorized GAP and based on the sampling points, residue levels in cucumbers can be considered compliant with this value after 14 days and from the dissipation pattern, after 11 days residues can be expected to be at 0.01 mg kg^{-1} or lower.

Results of dietary consumer risk assessment are presented in Table 5. In all cases, NEDI values were far below the ADI with RQ at or below 0.02. By applying the EFSA PRIMo, the estimated chronic exposure was calculated up to 4% of the ADI. Using the same model, short term exposure was estimated at 44% of the ARfD. Based on the above, a chronic and acute risk is not identified. However, it should be noted that the above conclusion is derived from values based on a single independent field trial and although no shortcomings were observed further independent trials would be desirable to have a more robust estimation (Malhat *et al*,. 2020).

In conclusion, a decline of SPX residues in cucumbers fit a first-order decay process with a calculated $t_{1/2}$ of approximately 2 days with almost a complete degradation (<99%) after 14 days and residues up to 0.003 mg kg^{-1} when SPX is applied according to the authorized GAP. An increase of the dose level will result in a significant increase of the residue levels, however the contribution of the increase of the number of applications in the residues is uncertain. From the available data, cucumbers treated according to the authorized or higher dose present up to 4% of the ADI and 44% of the ARfD using FAO/WHO and EFSA models.

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Μελέτη διασποράς υπολειμμάτων σπιροξαμίνης σε υπαίθρια καλλιέργεια αγγουριού και εκτίμηση διατροφικής επικινδυνότητας

F. Malhat, Ch. Anagnostopoulos, S. El-Sayed και S. Abdelsalam Shokr

Περίληψη Η σπιροξαμίνη είναι ένα από τα πλέον ευρέως χρησιμοποιούμενα μυκητοκτόνα στη Μεσόγειο με χρήση σε σημαντικές καλλιέργειες όπως του αγγουριού. Σκοπός της παρούσας μελέτης είναι η διερεύνηση της υπολειμματικής συμπεριφοράς της σπιροξαμίνης στην καλλιέργεια του υπαίθριου αγγουριού σε ξηρά κλίματα. Η ουσία εφαρμόστηκε σύμφωνα με την εγκεκριμένη ορθή γεωργική πρακτική και με πιο κρίσιμες πρακτικές σε υπαίθριο αγγούρι στο βόρειο μέρος της Αιγύπτου. Ο προσδιορισμός των υπολειμμάτων έγινε με την μέθοδο QuEChERS σε συνδυασμό με υγρή χρωματογραφία συζευγμένη με σύστημα διαδοχικής φασματομετρίας μάζας LC–MS/MS. Το όριο ποσοτικοποίησης LOQ της μεθόδου υπολογίστηκε στα 0.001 mg kg^{-1}. Ο χρόνος ημιζωής των υπολειμμάτων υπολογίστηκε στις 2 ημέρες με αποδόμηση περίπου 99% μετά από 14 ημέρες. Πραγματοποιήθηκαν υπολογισμοί χρόνιας και οξείας διατροφικής έκθεσης και σε όλες τις περιπτώσεις δεν παρατηρήθηκε κίνδυνος για τον καταναλωτή.

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