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Photo of the cover page: Thrips damage on orange fruit (Courtesy *Imen Belaam-Kort*)

Guest Editorial

Nanotechnology: A Future for Pesticides Manufacturing

Nanotechnology is science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers. Nanoscience and nanotechnology are the study and application of extremely small things and can be used across all the other science fields, such as chemistry, biology, physics, materials science, and engineering (Physicist Richard Feynman, the father of nanotechnology). The ideas and concepts behind nanoscience and nanotechnology started with a talk entitled "There's Plenty of Room at the Bottom" by physicist Richard Feynman at an American Physical Society meeting at the California Institute of Technology (CalTech) on December 29, 1959, long before the term nanotechnology was used. In his talk, Feynman described a process in which scientists would be able to manipulate and control individual atoms and molecules. Over a decade later, in his explorations of ultraprecision machining, Professor Norio Taniguchi coined the term nanotechnology. It was not until 1981, with the development of the scanning tunneling microscope that could "see" individual atoms, that modern nanotechnology. It is hard to imagine just how small nanotechnology is. There are 25,400,000 nanometers in an inch, and a sheet of newspaper is about 100,000 nanometers thick.

Pesticides are widely used in agricultural production throughout the world to protect plants against pests. Therefore, residues of pesticides are extensively contaminating our food and dispersed in drinking waters, groundwaters, and soils. Pesticides are indispensable to modern agricultural production. They can effectively reduce plant pests and diseases to improve crop yields. However, traditional pesticide formulations have several disadvantages, such as

high organic solvent contents, dust drift, and poor dispersibility, wherein most of the pesticide is lost to the environment and less than 1% remains on the target. This low effectiveness contributes to serious environmental pollution. Hence, efforts should be taken to reduce waste, production costs, and environmental pollution associated with pesticides while also extending the duration of pesticide activity on crops.

The development of nanomaterials and related technologies has provided new ways of creating intelligent nano-pesticides targeting surface properties, a small size, and quantum size effects. Nano-microcapsules or nanospheres have been prepared using light-sensitive, thermo-sensitive, humidity-sensitive, and enzyme- and soil pH-sensitive high polymer materials to deliver pesticides. These nanostructured materials are prepared via processes such as adsorption, coupling, encapsulation, and embedding. Such formulations can protect pesticides' active ingredients and enhance stability, control the release of core materials, reduce or obscure odors, and decrease volatility. In recent years, the application of nanomaterials and technologies in the field of pesticides has made considerable strides. Controlled release of nanomaterials can improve pesticide utilization and reduce residue and pollution. Pesticide nanocapsule formulations have slow release and protection performance and, due to their small size, improvable pesticide droplet ductility, wettability, and target adsorption when spraying fields; these methods provide efficient and environmentally friendly advantages. If a pesticide microcapsule can be properly applied, the pesticide will be released only at the target tissue. This process allows the pesticide to be used more effectively while ensuring that affected plant tissues receive the optimum therapeutic effect, thus reducing environmental pollution. Therefore, the application of microcapsule materials in pesticides and the development of new environmentally intelligent and responsive pesticide formulations represent new directions in pesticide formulation.

Although nanotechnology developments could increase pesticide-loading, improve formulation of active pesticide ingredients into emulsions with increased efficiency, dispersibility and stability of active ingredients, while allowing for lower dosing which therefore are better for the environment, and promote target ability. It is also cover seed

treatments, improved water utilization through increased irrigation efficiency, improved utilization of fertilizers with slow release and with improved nitrates control, and improve energy efficiency, e.g. in glass panels used in greenhouses.

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Review Article

Comprehensive Mini-Review for the New Virulence Type of *Pyrenophora tritici-repentis* Identified in Algeria

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ABSTRACT

Benslimane, H. 2019. Comprehensive mini-review for the new virulence type of *Pyrenophora tritici-repentis* identified in Algeria. Tunisian Journal of Plant Protection 14 (1): 1-9.

Pyrenophora tritici-repentis (Ptr) is the causal agent of tan spot disease on wheat, which causes important losses worldwide. Currently, eight races of this fungus are known according to their reactions on a bread wheat differential set. When durum wheat genotypes were added to the differential set, a new virulence pattern was identified. The isolates among this virulence pattern were unable to attack bread wheat in the differential set (Glenlea, 6B365 and 6B662), while they produced necrosis on durum wheat. A molecular characterization of these isolates showed the presence of the two virulence genes; *ToxA* and *ToxB* which control toxins synthesis, responsible for Glenlea and 6B-662 sensitivity, respectively. *ToxA* and *ToxB* coding regions sequences in the new virulence type isolates were similar to those of functional genes. This paper discusses and explains how the isolates among this new virulence pattern are unable to attack bread wheat differential set, even they harbored *ToxA* and *ToxB* genes. Several hypotheses are made about the possibility of PtrToxA and PtrToxB biosynthesis process alteration, which are encoded by *ToxA* and *ToxB* genes. These include any step in the pathway leading from DNA to protein; transcription, post-transcription, translation, or proteins maturation steps. Each hypothesis is supported by similar molecular event, reported previously in filamentous fungi.Then, it highlights the consequences of this finding on Ptr/wheat interaction. In fact, the results lead to suppose a new basic race, able to produce a unique new toxin, unknown yet. They also allow discussing the horizontal gene transfer hypothesis of *ToxA* to Ptr, which is supported mainly by *ToxA* conservation.

Keywords: New virulence type, *Pyrenophora tritici-repentis*, *ToxA*, *ToxB*

Introduction.

Pyrenophora tritici-repentis (Ptr), is the causal agent of tan spot, an ascomycete which can attacks both bread and durum wheats. The foliar disease, caused by this pathogen is spread worldwide, and causes losses up to 50% (Shabeer and Bockus 1988). Eight races of this fungus are known (Lamari and Strelkov, 2010). Three host-selective toxins PtrToxA, PtrToxB, and PtrToxC have been isolated to date; two of them have been purified and characterized. PtrToxA is responsible for the development of necrosis symptom in susceptible wheat genotypes (Ballance et al. 1989; Lamariand Bernier 1989; Tomas et al. 1990; Tuorï et al. 1995). The toxin encoding gene, *ToxA*, was cloned and sequenced (Ballance et al. 1996; Ciuffetti et al. 1997); it is found only in races 1, 2, 7, and 8, that possess PtrToxA toxin

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activity (Ciuffetti et al. 1997; Lamari et al. 2003). The second host-specific toxin, PtrToxB, was shown to be associated with development of the chlorotic symptoms (Orolaza et al. 1995), and it was purified by Strelkov et al. (1999). This toxin is encoded by *ToxB*, a multiple-copy gene (Martinez et al., 2004; Strelov 2002). This gene has been identified in races 5, 6, 7 and 8 of Ptr isolates, which are known to produce PtrToxB (Lamariet al. 2003; Martinez et al.2001). A form of *ToxB* (termed *toxb*) was also reported in an avirulent race 4 isolate that did not appear to produce the toxin (Martinez et al. 2004). Effertz et al. (2002) partially purified the third host-selective toxin designated as PtrToxC. It also induces chlorosis but not on the same host genotypes as PtrToxB (Gamba et al. 1998). PtrToxC is produced by race 1, 3, 6 and 8 (Lamari et al. 2003).

In wheat, three independent loci which confer sensitivity/insensitivity to each of the toxins (and susceptibility/resistance to the isolates which produce them) have been discovered (Gamba et al. 1998). To be fully resistant, a wheat cultivar must carry insensitivity genes for both chlorosis and necrosis (Lamari and Bernier 1991). Hence, the wheat/Ptr pathosystem does not fit the gene-for-gene incompatibility model (Loegering 1978), in which the interaction of a pathogen avirulence gene with the corresponding host resistance gene confers resistance (Lamari and Bernier 1991). In fact, the wheat/Ptr pathosystem conforms to the toxin model, in which compatibility results from an interaction between a pathogen-produced toxin and its putative receptor in the host (Lamari et al. 2002).This relationship is a mirror image (Ellingboe 1976) of the one described by the classical gene-for-gene model (Flor 1955). The main difference resides in the fact that compatibility is the basis of specificity in tan spot of wheat as opposed to incompatibility in the classical gene-for-gene model (Strelkov and Lamari 2003).

According to their ability to produce necrosis and/or chlorosis on a set of four differentials bread wheat, Ptr isolates were currently grouped into eight races (Lamari and Strelkov 2010). However, when three durum wheat genotypes were added to the differential set, a novel virulence pattern was identified in Algeria (Benslimane et al. 2011). Isolates showing this new virulent pattern are able to produce necrosis only in durum wheat genotypes, whereas the bread wheat genotypes in a differential set were resistant (Benslimane et al. 2011). Molecular characterization of the isolates with a new virulence pattern, showed the presence of *ToxA* and *ToxB* in their genomes (Benslimane 2018). Coding regions sequences of both of them, were similar to those of functional genes, suggesting point mutation(s) in unanalyzed genes sequences (Benslimane 2018).

The current paper discusses the hypothesis of an alteration of toxins synthesis process despite the highlighting of *ToxA* and *ToxB* genes and the consequences of this finding in Ptr/wheat interaction.

Toxin synthesis may be affected.

The biosynthesis way of PtrToxA and PtrToxB toxins, suggests several factors which could cause *ToxA* and *ToxB* genes inhibition suspected on the new virulence type isolates. In fact, both toxins encoded by the two virulence genes, undergo a proteolytic processing to reach the mature products which are allowed to induce the known symptoms (Ballance et al. 1996; Ciuffetti et al.1997; Martinez et al. 2001; Strelkov 2002;Tuori et al. 1995). All cell mechanisms, used for

proteins synthesis by eukaryotes, should be considered.

Inhibition of any step in the pathway leading from DNA to protein, may affect gene products, because all steps are regulated. Changing in enzymes concentration (such as RNA polymerases, aminoacyl-tRNA synthetases) or in nucleic acid (DNA or RNA) sequences, may increase or decrease genes expression.

The first level to consider is the chromatin structure of *ToxA* and *ToxB* genes, because DNA transcription processes are dictated by its structure. Acquisition of the "active" structure is the first step in gene expression. Genes that are "active" in eukaryotic organisms have a particular chromatin conformation, which makes them more accessible to RNA polymerase or transcription protein regulators (Keplan and Delpech 1992; Moore and Novak Fraze 2002). Regulation of eukaryotic gene expression may be under the control of a remodeling of the DNA primary structure; this could be a simple chemical modification such as methylation or reconfiguration (Keplan and Delpech 1992).

DNA accessibility can be altered as a result of many modifications which may up- or down-regulate the expression of genes; a failure at this level could repress the expression of these genes. Results of several studies showed that when a DNA region is methylated (a methyl group is attached to 5-position of cytidine), the associated gene is transcriptionally silent; when the same region is under-methylated, the gene is transcriptionally active. Methylation seems to be a signal of eukaryotic genes repress. This phenomenon has already been reported in filamentous fungi; the elongation of nascent mRNA transcript is reduced when there is methylation of chromosomal DNA encoding them in *Ascobolus immersus* (Barry et al. 1993) and in *Neurospora crassa* (Rountree and Selker 1997). In *Schizophyllum commune*, reduction of gene expression associated with methylation of DNA also affects transcription (Schuurs et al. 1997).

The transcriptional control of *ToxA* and *ToxB* is also taken into account. In most studied filamentous fungi, transcription is regulated on at least at two different levels: a pathway-specific one and a more global one (e.g., carbon or nitrogen regulation). The interaction between these different levels is the primary factor in determining the final yield of a protein (MacKenzie et al. 1993). For this reason, several causes could be suggested to explain *ToxA* and *ToxB* transcription process inhibition.This is most likely due to several pathway-specific activator proteins that have now been identified from filamentous fungi. Furthermore, a large proportion of DNA-binding proteins involved in the transcription of eukaryotic genes have been identified (Moore and Novak Frazer 2002). The failure of one of the transcription factors could easily lead to the inhibition of genes that we studied.

Post-transcriptional controls are crucial for the regulation of many genes. There are many mechanisms that modulate gene expression following transcription in filamentous fungi, which can act to switch off *ToxA* and *ToxB* genes or to produce proteins functionally different than PtrToxA and PtrToxB.

The more dramatic mechanism is alternative splicing; in fact, a little is known about this mechanism in filamentous fungi (Sachs 1998), although they possess a mechanism for mRNA splicing similar to that encountered in other eukaryotes (Tollervey and Mattaj1987; Tollervey et al. 1990; Séraphin 1995). An alternative splicingof an intron can occur at different splice

junctions allowing the elaboration of different forms of a protein. In *Tolypocladium inflatum*, the alternative splicing of the ARN of *restless*, a transposable element produced two transcripts with different coding capacity (Kempken and Kück 1996). Otherwise, either the presence of an unspliceable intron or the absence of a spliceable intron would have affected *ToxA* and *ToxB* gene expression. This was already shown in filamentous fungi; for example, a mutation that eliminates *Ustilago REC1* intron splicing results in a gene product that cannot complement the radiation-sensitive phenotype of *rec1* mutant (Onel et al. 1995). Similarly, a 3'-splice site mutation in a *Neuropspora crassa am* intron, eliminates *am* gene function (Kinnaird et al. 1992).

Also, at this level of regulation, it should be remembered that changes in the expression of specific genes are manifested by changes in the steady-state of individual mRNA. Consequently, the regulation of mRNA stability seems to bean important step in the control of gene expression (Resnekov and Von-Gabain 1996). For filamentous fungi, some results showed that sequences in the three prime Untranslated Transcribed Region (3 'UTR) are involved in controlling mRNA stability, as it was described in *Aspergillus nidulan*s for which *areA* gene expression was analyzed. A deletion of sequences in the 3'UTR modifies this gene expression (Platt et al. 1996). Additionally, another post transcriptional control was also reported in *Ustilago maydis*, for which two of RNA-binding proteins, Khd4 and Rrm4, were reported as important for filamentous growth (Becht et al., 2005). The loss of Khd4 causes pleiotropic effects such us altered cell and filament morphology, as well as defects in pheromone response and plant infection (Becht et al., 2005).

Hence, because in eukaryotes, both mRNA stability and translatability are linked to the level of mRNA polyadenylation (Wickens et al. 1997), inhibition of both studied genes could occur, at this stage.

Moreover, "gene silencing" is not excluded from our investigation. In this way, interruption or suppression of the expression of *ToxA* and *ToxB* genes is an epigamic process that cannot be explained by changes in gene sequences, but could be an RNA degradation mechanism. A number of gene-silencing phenomena that occur at the post-transcriptional level were discovered in fungi (Catalanotto et al. 2000; Fagard et al. 2000). In *Neurospora crassa*, there is strong evidence that gene silencing by the presence of extra copies of genes or gene segments can occur post-transcriptionally and likely affects RNA stability (Cogoni et al. 1996).

To perform its function, and translate the sequences encoded in *ToxA* and *ToxB* genes to the two proteinaceous toxins (PtrToxA and PtrToxB), mRNA needs several protein factors. Variation of translation factors can alter the translation efficiency of mRNAs. Indeed, it has been established that in eukaryote cells, translation may decrease considerably in response to several conditions which can inhibit the initiation factors of translation (Malacinski and Freifelder 1998). In heat-shocked *Neurospora* cultures, the translation of most cellular mRNAs is reduced, and major translation products are from induced mRNAs specifying heat shock proteins (Curle and Kapoor 1988; Plesofsky-Vig and Brambl 1985, 1987). The translation efficiency of *ToxA* and *ToxB* in products could also be determined by *cis* acting sequences in the mRNA. Indeed, investigation of translation control through uORFs (upstream ORFs) in fungi has been

established in several studies. For example, in *Saccharomyces cerevisiae*, some of these sequences are critical for translation control, while others have important roles in modulating the ability of ribosomes to reinitiate translation (Hinnebush 1996, 1997). Implication of uORFs in the control of translation has been demonstrated in *Neurospora crassa* and *Aspergillus nidulans* (Delbecq et al. 1994; Luo et al. 1995; Werner et al. 1987). According to the effects described before, we can suggest that putative mutations occurred in *cis* mRNA sequences and could cancel the translation process allowing the production of PtrToxA and PtrToxB in the analyzed isolates of Ptr.

Once the polypeptide toxins have been synthesized, various post-translational modifications subsequently could have affected their function, as it can happen in eukaryote organisms. These include binding of substrates, coenzymes and metabolic products, as well as covalent modification reactions (e.g. oxidation, acetylation, or phosphorylation).

Conclusion and consequences.

Results of Benslimane (2018) and Benslimane et al. (2011) have highlighted a new virulence pattern which strongly suggests the existence of an unknown race of Ptr in Algerian wheat fields. The presence of this putative new race involves a significant impact on some bases of wheat/Ptr pathosystem. It is recognized that races 2, 3 and 5 are considered basic races because they are able to produce a single toxin, while races 1, 6, 7 and 8 are combined from basic races (Strelkov and Lamari 2003). The discovery of this new virulence type, which seems to be able to produce a single toxin completely different from those known, implies the existence of a new basic race (Benslimane et al. 2011). Consequently, the model proposed by Strelkov and Lamari (2003) establishing the relationship between different races is permissible only when the host/pathogen interaction is considered for bread wheat. The introduction of durum wheat in the host differential set makes the hypothesis invalid. Otherwise, these results give us a good support to open a serious reflection about the horizontal gene transfer of *ToxA* gene. Indeed, according to Friesen et al. (2006), this gene, which encodes a host-selective toxin (PtrToxA), was transferred from *Stagnospora nodorom* to *Ptr*. The main evidence given by the authors was that *ToxA* sequences analyzed in 57 isolates of Ptr collected in a large geographical area showed that they were almost identical. Our results showed that *ToxA* could be less conserved; four isolates originally from the east and center of Algeria harbored *ToxA* in their genome, whereas they were unable to produce necrosis in Glenlea, when they were inoculated to the differential set.

Further investigations are necessary to shed light on the consequences of these findings. This is important because gene expression is the essential mechanism of fungal virulence, and therefore it should be evaluated carefully with regard to future projects to combat fungal disease (Kaclkanci et al. 2011). These primary data indicated that Ptr/wheat pathosystem should be further investigated and revised. In fact, changes such as loss-of-function mutations can potentially lead to virulence of a pathogen. Host-selective toxins and their corresponding sensitivity genes have likely coevolved in a similar way as avirulence and resistance genes. Although, most studies have focused on the function of host-selective toxins, little is known about the evolution of their interaction (Walton 1996).

RESUME

Benslimane H. 2019. Mini-revue compréhensive sur le nouveau profil de virulence chez *Pyrenophora tritici-repentis* identifié en Algérie. Tunisian Journal of Plant Protection 14 (1): 1-9.

Pyrenophora tritici-repentis (Ptr) est l'agent causal de la maladie de la tache bronzée affectant le blé; il est à l'origine d'importantes pertes de rendements dans le monde. Actuellement 8 races de ce champignon sont connues; elles ont été définies en fonction de la réaction produite sur une gamme d'hôtes différentielle, composée de blé tendre. Lorsque le blé dur a été ajouté à la gamme d'hôtes différentielle, un nouveau profil de virulence a été identifié. Les isolats apparentant à ce profil de virulence ont été incapables d'attaquer le blé tendre de la gamme différentielle (Glenlea, 6B365 et 6B662), alors qu'ils ont produit des nécroses sur le blé dur. La caractérisation moléculaire de ces isolats a montré la présence des gènes de virulence *ToxA* et *ToxB*, codant la synthèse des toxines responsables de la sensibilité de Glenlea et 6B-662. Les séquences des régions codantes au niveau des gènes *ToxA* et *ToxB* chez les isolats du nouveau profil de virulence étaient identiques à ceux des gènes fonctionnels. Le présent article discute et explique comment les isolats du nouveau profil de virulence sont incapables d'attaquer le blé tendre de la gamme différentielle, malgré la présence des gènes *ToxA* et *ToxB*. Plusieurs hypothèses sont émises; elles sont relatives à l'altération du processus de biosynthèse des toxines PtrToxA et PtrToxB, codées par les gènes *ToxA* and *ToxB*. Celles-ci incluent toute étape dans la voie menant de l'ADN à la protéine: transcription, post-transcription, traduction ou étapes de maturation de la protéine. Chaque hypothèse est justifiée par des événements moléculaires similaires, rapportés chez les champignons filamenteux. Par la suite le présent article révèle les conséquences possibles de cette découverte dans l'interaction Ptr/blé. En effet, les résultats supposent la présence d'une nouvelle race de base, capable de produire une toxine unique non identifiée encore. Ils permettent également de discuter l'hypothèse du transfert horizontal de *ToxA* vers Ptr, justifié principalement par la conservation de *ToxA*.

Mots clés :Nouveau profil de virulence, *Pyrenophora tritici-repentis*, *ToxA*, *ToxB*

ملخص

بن سليمان، حميدة. 2019. مراجعة استيعابية مختصرة لنمط شراسة جديد لدى الفطر *Pyrenophora tritici-repentis* مُشخص في الجزائر. Tunisian Journal of Plant Protection 14 (1): 1-9.

يعد الفطر *Pyrenophora tritici-repentis* العامل المسبب لمرض التبقع البرنزي، أحد الأمراض التي تصيب القمح والتي تؤدي إلى خسائر معتبرة في المردود .يعرف حاليا ثماني سلالات لهذا الفطر، تم تحديدها اعتمادا على الأعراض المسببة على مجموعة اختلافية من القمح اللين/الطري. أدت إضافة طرز عرقية من القمح الصلب/القاسي إلى تشخيص نمط شراسة جديد لدى هذا الفطر. تختص العزلات التي تظهر هذا النمط من الشراسة بعدم القدرة على إصابة القمح اللين في هذه المجموعة الاختلافية (Glenlea, 6B662, 6B365)، بينما تنتج نكرزة على القمح الصلب. أظهر التوصيف الجزيئي لهذه العزلات وجود المورثتين/الجينتين *ToxA* و *ToxB* اللتين تنظمان انتاج التوكسين، هما المسؤولتان عادة على حساسية Glenlea و 6B-662 على التوالي. وبيّن تسلسل الحمض النووي في المناطق المشفرة للمورثتين *ToxA* و *ToxB* لدى هذه العزلات تطابق مع تلك الموجودة لدى المورثات الوظيفية. يناقش هذا المقال ويشرح لماذا العزلات صاحبة هذا النمط الجديد من الشراسة لا تقدر على إصابة القمح اللين رغم تضمنها للمورثتين *ToxA* و *ToxB*.قُدمت عدة فرضيات لتفسير التغيرات التي تحصل في طريقة التركيب الحيوي للتوكسنتيْن PtrToxA و PtrToxB اللتين تشفرهما المورثتين *ToxA* و *ToxB*. وهذه التغييرات تضم أي مرحلة من مسار الحمض النووي إلى البروتين:مراحل النسخ أو ما بعد النسخ أو الترجمة أو مرحلة نضج البروتين. تم الاستشهاد في كل فرضية بظواهر جزيئية مماثلة سجلت سابقا عند الفطريات الخيطية. كما ركز هذا المقال على الآثار الناتجة عن هذا الاكتشاف حول التفاعل بين الفطر-*P.tritici repentis* وعائله القمح، حيث أن النتائج المتوصّل إليها تقود إلى افتراض ظهور سلالة قاعدية جديدة منتجة لتوكسين

وحيد غير معروف إلى الآن. كما أنها تفتح المجال لمناقشة فرضية النقل الأفقي للمورثة *ToxA* إلى الفطر *P. tritici-repentis*، وهذه الفرضية يدعمها أساسا حفظ المورثة *ToxA*.

كلمات مفتاحية: نمط شراسة جديد، *Pyrenophora tritici-repentis*، *ToxB*، *ToxA*

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In vitro Antifungal Activities of Essential Oils of Three Lamiaceae Plant Species against *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*)

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ABSTRACT

Kalai-Grami, L., Bahri, B.A., Chemekh, M., Hammami, M., Limam, F., and Hajlaoui, M.R., 2019. Evaluation of antifungal activities of essential oils of three Lamiaceae plants against *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*). Tunisian Journal of Plant Protection14 (1): 11-32.

The mal secco (meaning dry dieback) caused by *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*) is a very serious and incurable disease on citrus. In this study, we investigated a biological control as strategy against this disease. The antifungal activity of the essential oils (EO) of thyme (*Thymbra capitata*), rosemary (*Rosemarinus officinalis*) and sage (*Salvia officinalis*) extracted by hydrodistillation, was evaluated on mycelial growth, sporulation rate and mycelial biomass of two *P. tracheiphilus* isolates. The results showed that thyme EO is the most effective product that completely stopped mycelial growth of the fungus in solid medium. For rosemary and sage EO, mycelial growth inhibition reached up to 69 and 58%, respectively. In addition, the thyme EO had the highest percentage of mycelial biomass inhibition of *P. tracheiphilus* in liquid medium which reached 91% in average, followed by rosemary and sage EO with 75and 58%, respectively. The inhibition of sporulation rate in liquid medium was also the highest under thyme EO application with 88% and the lowest with sage EO with 52% in average. These results highlighted the good in vitro antifungal activity of thyme EO on the development of *P. tracheiphilus.* Qualitative and quantitative analysis of the EO volatile profile showed that the main constituent of thyme EO is carvacrol (76. 71%). The phytotoxicity test revealed that thyme EO is toxic at a dose exceeding 60 ppm on *Citrus aurantium* detached leaves. On the other hand, the determination of the minimum inhibitory concentration (MIC) of thyme EO (400ppm) was found to be higher than the phytotoxic dose. Further analyses are needed to optimize in vivo application conditions against *P. tracheiphilus* at doses above the MIC without causing phytotoxicity.

Keywords: Antifungal activity, essential oils, *Plenodomus tracheiphilus* (syn. *Phomatracheiphila*), *Rosmarinus officinalis, Salvia officinalis, Thymbra capitata*

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Citrus are an important part of fruit production worldwide. In Tunisia, citrus fruit production still plays an important role in the country; however, this sector continues facing problems at both production and marketing levels. Indeed, the intensification of lemon orchards resulted in the upsurge of mal secco disease *Plenodomus tracheiphilus* (Former *Phoma tracheiphila*) (De Gruyter et al. 2012) which has become the main threat to the extension of citriculture in Tunisia.

In the absence of effective chemical control and the increased sensitivity of the Eureka variety, which dominates over 90% of citrus orchards, this fungal disease can lead to considerable yield losses creating an unbalance between the low production and the high lemon summer demand.

This acropetal tracheomycosis, caused by *P. tracheiphilus* induces the drying of leaves and branches leading to the apoplexy of the entire tree. The mal secco disease infects the genera *Citrus*, *Poncirus*, *Severinia,* and *Fortunella*. However, it is more virulent on the *Citrus limon* trees and on the *Citrus medica* trees (Whiteside et al. 1988). Generally, all species belonging to the genus cited above are susceptible to artificial infections with *P. tracheiphilus* but in orchards, their degree of infection is variable (Anonymous 2003).

Measures to prevent the spread of this pathogen are mainly based on prophylaxis; however, these control measures are not fully effective, and the pathogen is continuously spreading and infecting new orchards. The use of resistant lemon varieties is probably the most effective and long-lasting way to control large-scale mal secco disease, but this strategy is not currently feasible due to the lack of resistant varieties satisfying both quantitative and qualitative levels of the production (Migheli et al. 2009). Facing the difficulties in managing the disease by traditional measures, the use of essential oils (EO) to control *P. tracheiphilus* appears to be a promising alternative, especially at the nursery level.

Several researches have been conducted to determine the chemical composition and biological applications of EO, representing a source of natural products economically important for food, drug, and cosmetic industry. Among the aromatic plants characterized by an antimicrobial activity on plant pathogens, *Thymbra capitata* (thyme), *Rosmarinus officinalis* (rosemary) and *Salvia officinalis* (sage) are the most important members of the Lamiaceae family (Pierozan et al. 2009). In fact, the anti-fungal potential of *S. officinalis* EO was demonstrated against *Botrytis cinerea*, *Fusarium* sp. and dermatophyte strains (Rus et al. 2015). *T. capitata* and *R. officinalis* also inhibited the growth of *Candida albicans* (Matsuzaki et al. 2013), *Aspergillus* sp. (Moghtader et al. 2011) and *Fusarium* sp. (Da silva Bomfim et al. 2015).

In this context, the EO of thyme, rosemary and sage were studied for their antifungal properties against *P. tracheiphilus* by (i) evaluating their activities on mycelial growth, biomass production and sporulation rate, (ii) investigating the minimal inhibitory concentration and the phytotoxic dose of the best EO, and iii) determining the chemical composition of the two most effective EO through GC-MS analysis.

MATERIALS AND METHODS

Plant material and extraction.

Three aromatic plants from three species of the Lamiaceae family, thyme, rosemary and sage were collected respectively from different regions of North of Tunisia (Sidi Thabet, Ariana,

and Zaghouan). Aerial parts of dry (thyme) or fresh (rosemary and sage) individual leaves were hydro distilled for two hours using a Clevenger-type system; this distillation was carried out only one time. Collected EO were kept in amber vials at 4°C. The efficiency of extraction was calculated according to Boudia et al. (2012) as follow: E = (EW/PW) × 100, with E: efficiency of extraction, W: EO weight (g), and PW: Plant weight (g).

Isolation and identification of fungal isolates.

Two *P. tracheiphilus* isolates were collected from twigs of *Citrus limon* trees located in symptomatic orchards in Bouargoub (Ptba) and Dar Chaabane El Fehri (Pt36) Nabeul, Tunisia. Isolation and identification were performed as described by Kalai et al. (2010) based on morphological (Punithalingam and Holliday, 1973) and molecular criteria using specific primer pairs *Pt*-FOR2 and *Pt*-REV2 (Balmas et al. 2005).

In vitro antifungal activities of essential oils on mycelial growth.

Well diffusion method. The three EO of thyme, rosemary and sage were screened for their antifungal activities on mycelia growth toward two Tunisian *P. tracheiphilus* isolates (Ptba and Pt36), by well diffusion method. Briefly, a fungal mycelial plug was placed in the center of a PDA plate and 50 µl of EO were loaded onto wells placed 3 cm apart from the mycelial plug. Dishes were incubated at 25°C during 20 days and inhibition diameters were measured when *P. tracheiphilus* mycelium reached the edge of the plate in the negative control. Wells filled with sterile distilled water were used as control. The percentage of inhibition was evaluated, on four replicates for each treatment, according to Whipps (1987) as follow: G = [(R1-R2)/R1] × 100, with G: percentage of growth inhibition where R1 is the furthest radial distance grown by the pathogen in the direction of the antagonist (a control value) and R2 is the distance grown on a line between inoculation positions of the pathogen and antagonist (an inhibition value).

Mixing method. The antifungal effect of the EO was also determined by mixing directly 400 ppm of the product (diluted in DMSO) to the PDA medium before solidification. A disc plugs of Ptba and Pt36 were then deposited in the center of the plates and incubated at 25°C during 20 days. Controls were grown on PDA supplemented with DMSO. The evaluation of growth inhibition was performed according to Whipps (1987), on four replicates for each treatment.

In vitro antifungal activities of essential oils on sporulation rate and mycelial biomass.

The effect on mycelial biomass and on sporulation rate of the two isolates (Ptba and Pt36) of *P. tracheiphilus* was performed by adding 400 ppm of the EO to liquid cultures of the fungus. To assess the mycelial biomass, *P. tracheiphilus* was grown on 50 ml of PDB medium and to assess sporulation, it was grown on 50 ml of carrot liquid medium. Each treatment was replicated thrice. After 10 days of incubation at 22°C and 135 rpm centrifugation, the number of spores/ml was evaluated by Malassez cell counting and the mycelium was recovered by filtration. It was dried during four days at 60°C and the percentage of mycelial development was evaluated as follow: Mycelial development (%) = (Dry weight of mycelium in presence of oil/Dry weight of control) × 100.

Determination of minimal inhibitory concentration of thyme EO.

The minimal inhibitory concentration (MIC) of thyme EO was determined by mixing method as previously described with dilutions performed with DMSO as follow: 30, 50, 70, 100, 200, 300, and 400 ppm. Only Ptba isolate was tested and controls were grown on PDA medium supplemented with DMSO. The evaluation of growth inhibition was performed according to Whipps (1987) after 15 days incubation until control reached the edge of the plate, on five replicates for each tested concentration.

Evaluation of in vitro inhibitory activity of Thyme EO with reference fungicide.

The in vitro antifungal activity of thyme EO was compared to the effect of two fungicides commonly used on fruit and citrus trees (Score and Methylthiophanate). *P. tracheiphilus* radial growth was evaluated on PDA medium supplemented with the MIC for thyme EO (400 ppm), and with the concentration recommended by manufacturer on fruit trees for Score (Difenoconazole 250 g/l), and for Methylthiophanate (Thiophanate methyl 1 g/l). A volume of 400 ppm of DMSO alone was added to the medium for negative control. Five replicates were performed for each treatment.

Determination of minimal non-phytotoxic concentration.

The minimal non-phytotoxic concentration was determined for the EO showing the strongest antifungal activity. This assay was performed on *Citrus aurantium* detached leaves sterilized by ethanol 70% and placed on humid and sterile filter paper on Petri dishes at 25°C. The EO is then diluted with DMSO and applied with decreasing concentrations (pure EO, 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 ppm) directly on leaves.

Qualitative and quantitative analysis of the essential oils.

The two EO showing the best anti *P. tracheiphilus* activities were subjected to GC-MS analysis. It was performed on a gas chromatograph HP 7890 (A) interfaced with an HP 5972 mass spectrometer (Agilent Technologies, Palo Alto, CA, USA) with electron impact ionization (70eV). A HP-5MS capillary column (30 m × 0.25 mm, 0.25 mm film thickness) was used. The column temperature was programmed to rise from 60 to 260°C at a rate of 5°C/min. The carrier gas was He with a flow rate of 0.9 ml/min. Scan time and mass range were 1 s and 50-550 m/z, respectively. The injected volume was 1µl and the total run time was approximately 42 min. Identification was made by matching their recorded spectra with those stored in the Wiley NIST 2011 mass spectral library of the GC-MS data system and other published mass spectra.

Statistical Analysis.

Main effects of isolate and treatment (three EO and control) and the interaction isolate × treatment were tested on *P. tracheiphilus* mycelial growth in vitro on solid medium at 25°C (using well diffusion and mixing methods) and, on *P. tracheiphilus* mycelial biomass and sporulation rate in vitro on liquid medium at 22°C. All data were subjected to analysis of variance (ANOVA) using the statistical package STATISTICA version 6.0 (StatSoft Inc. 2001). Pairwise mean comparisons were performed using Newman and Keuls test at $P < 0.05$. An ANOVA comparing mycelial growth using the well diffusion and the mixing methods and taking into account the

effects of isolate, treatment, method and all interactions between the factors, was also performed. In addition, a regression analysis was used to test the correlation between the averages of mycelial growth of each treatment and each isolate using the well diffusion and the mixing methods.

RESULTS

Yield of essential oils.

The yield of EO is an important criterion to select plant to be hydrodistilled. The determination of this parameter revealed that thyme is the richest in EO as it has the highest yield with1.8% followed by rosemary (0.65%) while sage registered the lowest yield (0.24%).

Identification of *P. tracheiphilus* isolates.

The two fungal isolates Ptba and Pt36 were successfully identified as *P. tracheiphilus* by the production of typical pycnio- and phialoconidia as described by Punithalingam and Holliday (1973) on PDA solid medium (Data not shown). Besides, specific primers Pt-FOR2 and Pt-REV2 generated an amplicon of 378 bp characteristic of *P. tracheiphilus* species for the two isolates while the two negative controls did not show any amplification product (Fig. 1).

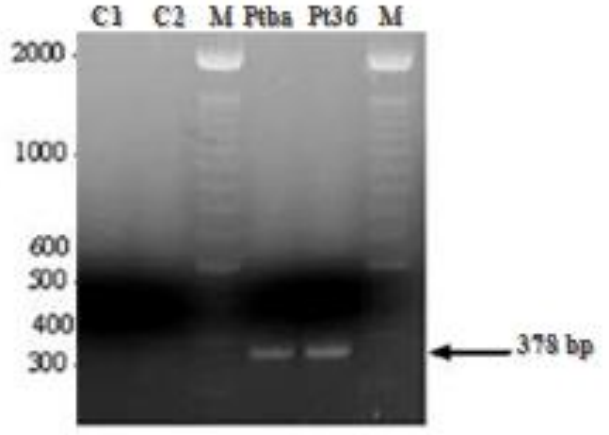

Fig. 1. Molecular identification of the two *Plenodomus tracheiphilus* isolates by specific PCR. C1: Negative control *Colletotrichum gloeosporioides;* C2: Negative control (*Fusarium* sp.); M: DNA marker (Tracklt TM 100 bp DNA ladder, Invitrogen); Ptba: Ptba isolate; Pt36: Pt 36 isolate.

Effect of essential oils on *P. tracheiphilus* mycelial growth using well diffusion method.

All tested products showed antifungal activity against *P. tracheiphilus* with different inhibition percentages (Figs. 2, 3; Table 1-A). Statistical analysis allowed the classification of thyme EO as the most significantly effective treatment showing a total inhibition of mycelial growth of *P. tracheiphilus* followed by rosemary EO with 60 to 70% than by the sage EO with 54 to 59% of inhibition after 20 days of incubation. There was also a significant difference between mycelial growth of the two isolates as Pt36 grew faster than Ptba ($P < 0.001$). Also, Pt36 isolate was

less inhibited by rosemary EO than Ptba while they were inhibited with the same intensity using thyme and sage EO, revealing a significant interaction isolate × treatment effect ($P < 0.001$) on *P. tracheiphilus* mycelial growth.

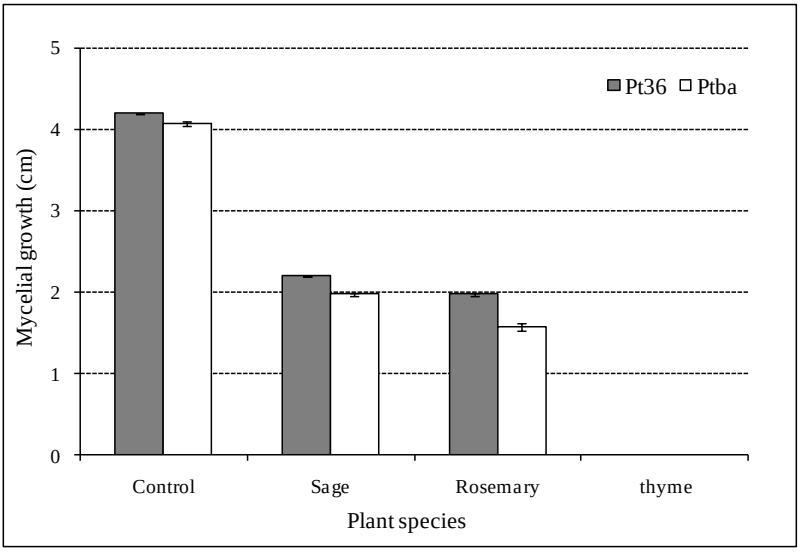

Fig. 2. Effect of sage, rosemary and thyme essential oils (50 µl/well) on mycelial growth of two *Plenodomus tracheiphilus* isolates on PDA media after 20 days of incubation at 25°C, using well diffusion method. The control was treated with sterile water. Each value represents the mean of four observations. Segments are standard errors.

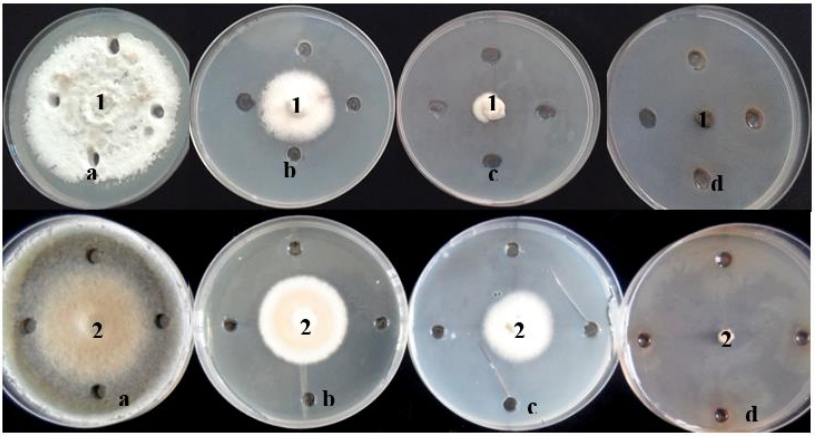

Fig. 3. In vitro antifungal activities of essential oils on mycelial growth of two *Plenodomus tracheiphilus* isolates on PDA media after 20 days of incubation at 25°C,using well diffusion method. 1: Ptba; 2: Pt36; a: sterile distilled water; b: Sage EO; c: Rosmary EO; d: Thyme EO (50 µl/well).

Table 1. Inhibition effects (%) of sage, rosemary and thyme essential oils, compared to the control treated with DMSO, on mycelial growth using well diffusion method (A), on mycelial growth using mixing method (B), on mycelial biomass (C) and on sporulation rate (D) of two isolates of *Plenodomus tracheiphilus*

Treatment	Isolate	Sage	Rosemary	Thyme
A	**Pt36**	54	60	100
	Ptba	59	70	100
B	**Pt36**	10	13	100
	Ptba	7	11	100
C	**Pt36**	30	56	85
	Ptba	87	94	97
D	**Pt36**	19	48	85
	Ptba	86	88	91

Each value represents the mean of four observations.

Effect of essential oils on *P.tracheiphilus* mycelial growth using mixing method.

Using mixing method, the mycelial growth of the two isolates was significantly lower (3.5-100%) for all the EO treatments compared to the control ($P < 0.001$). Thyme EO totally inhibited *P. tracheiphilus* mycelial growth similarly to the well diffusion method. However, the inhibition of mycelial growth didnot exceed 13% with rosemary EO and reached only 7-10% with sage EO. A significant interaction isolate × treatment effect ($P < 0.001$) on *P. tracheiphilus* mycelial growth was also observed using mixing method. Indeed, mycelial growth of the two isolates was significantly different (13.25% for Pt36 and 11.26% for Ptba) under rosemary EO treatment (Figs. 4, 5; Table 1-B).

Comparison of mycelia growth between the well diffusion method and the mixing method for two *P. tracheiphilus* isolates.

An ANOVA analysis showed a highly significant difference ($P < 0.001$) between the two methods adopted to evaluate the effect of EO for the two tested isolates. The mixing method showed overall 1.4 to 6.2 times lower mycelial growth inhibition than the well diffusion method probably due to difference of EO used volume which is less important for the latter method compared to the other. Under rosemary and sage EO, mixing method had 4-fold lower mycelial growth inhibition. The analysis also showed a high positive correlation of 97% for isolate Pt36 and 93% for isolate Ptba between the two methods (Fig. 6).

Effect of essential oils on *P. tracheiphilus* mycelial biomass.

Significant effects of isolates ($P < 0.001$), treatments ($P < 0.001$) and isolates × treatments ($P = 0.001$) on *P. tracheiphilus* mycelial biomass were observed. The effect of tested EOs on mycelial biomass showed that under thyme EO treatment, *P. tracheiphilus* had

the significantly higher mycelial biomass inhibition which reached 97% (Ptba) and 85% (Pt36). It is followed by rosemary and sage EOs, which registered higher mycelial biomass inhibition of Ptba (94 and 87% respectively) compared to Pt36 (56 and 30% respectively) (Fig. 7; Table 1-C).

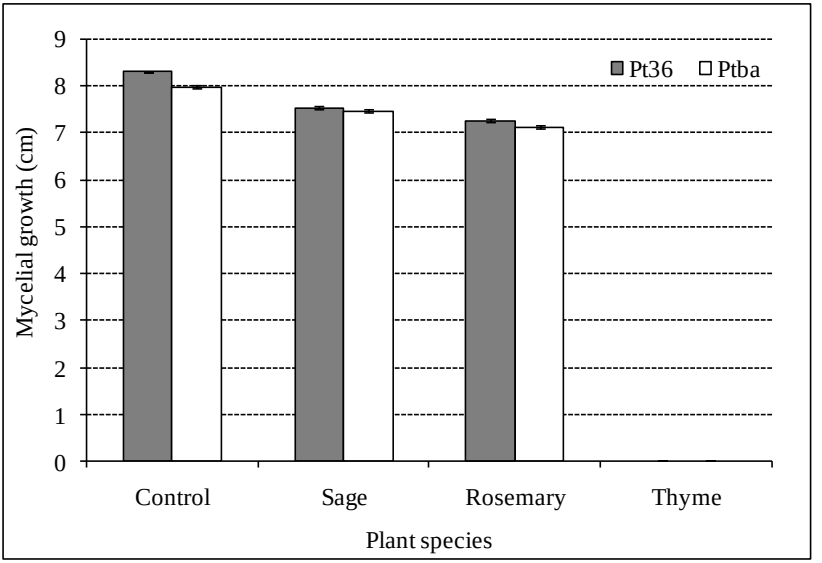

Fig.4. Effect of sage, rosemary and thyme essential oils (400 ppm) on mycelial growth of two *Plenodomus tracheiphilus* isolates after 20 days of incubation at 25°C using mixing method. The control was treated with DMSO. Each value represents the mean of four observations. Segments are standard errors.

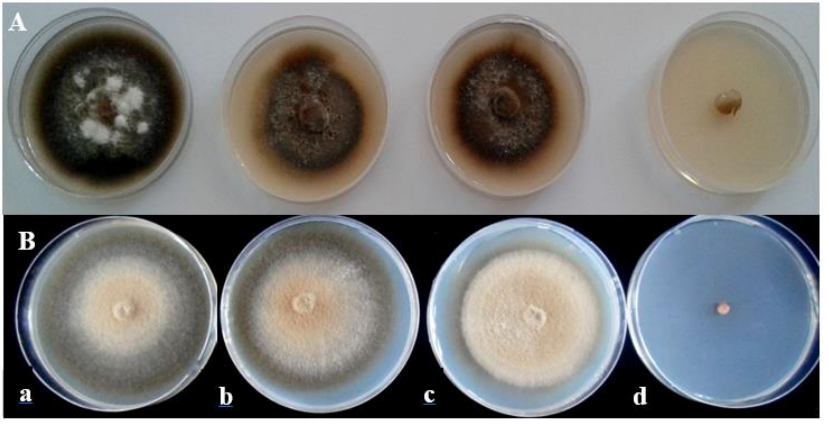

Fig. 5. In vitro antifungal activities of essential oils (400 ppm) on two *Plenodomus tracheiphilus* isolates after 20 days of incubation at 25°Cusing mixing method. A: Ptba; B: Pt36; a: negative control; b: sage; c: rosemary, d: thyme essential oils.

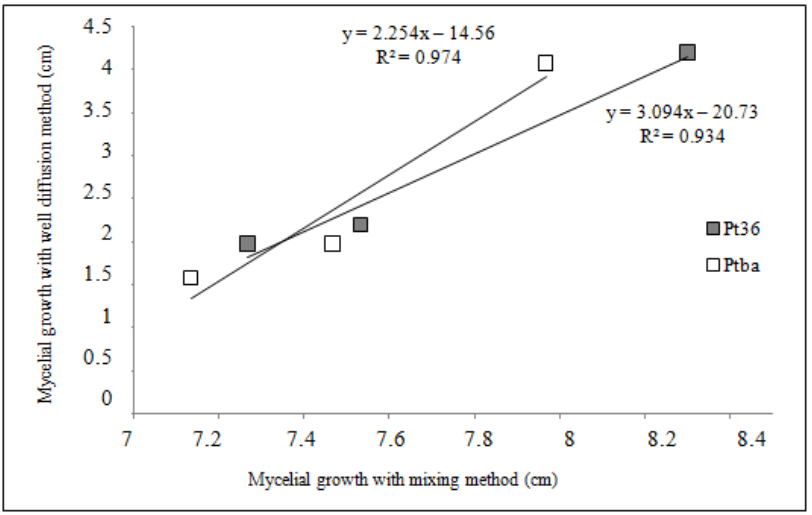

Fig. 6. Correlation of mycelial growth between the well diffusion method and the mixing method for two *Plenodomus tracheiphilus* isolates. Each value represents the mean of four observations.

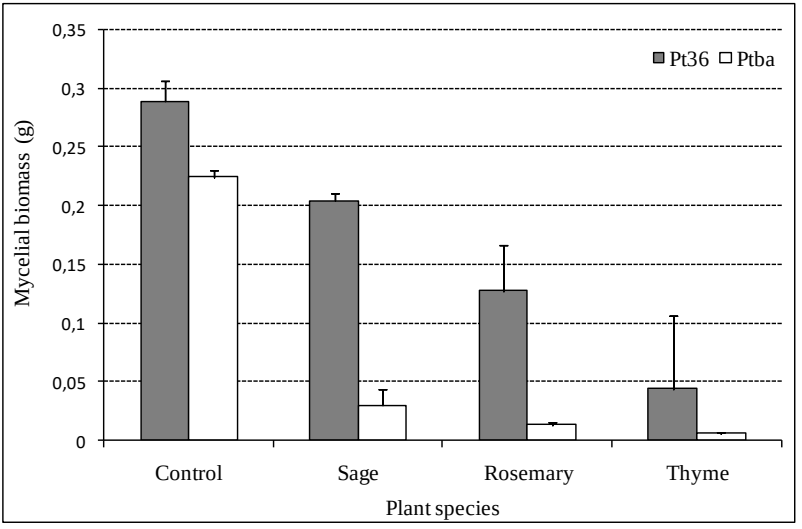

Fig. 7. Effect of sage, rosemary and thyme essential oils (20 µl/l) on mycelial biomass of two *Plenodomus tracheiphilus* isolates on PDB media after 10 days of incubation at 22°C. The control was treated with DMSO. Each value represents the mean of three observations. Segments are standard errors.

Effect of essential oils on *P.tracheiphilus sporulation* rate.

The addition of thyme EO to liquid cultures of *P. tracheiphilus*, induced a highly significant effect on sporulation rate ($P < 0.001$), with inhibition values approximately of 85% (Pt36) and over 90% (Ptba). The use of

sage and rosemary EO, also led to a significant reduction of sporulation rate with values of 86 and 88% (Pt36 and Ptba, respectively). For Pt36, the decrease of sporulation rate was less important reaching 48% with rosemary EO and, only 19% with sage EO (Fig. 8, Table 1-D). Again, the interaction isolates × treatments effect was significant on *P. tracheiphilus* sporulation rate ($P = 0.001$).

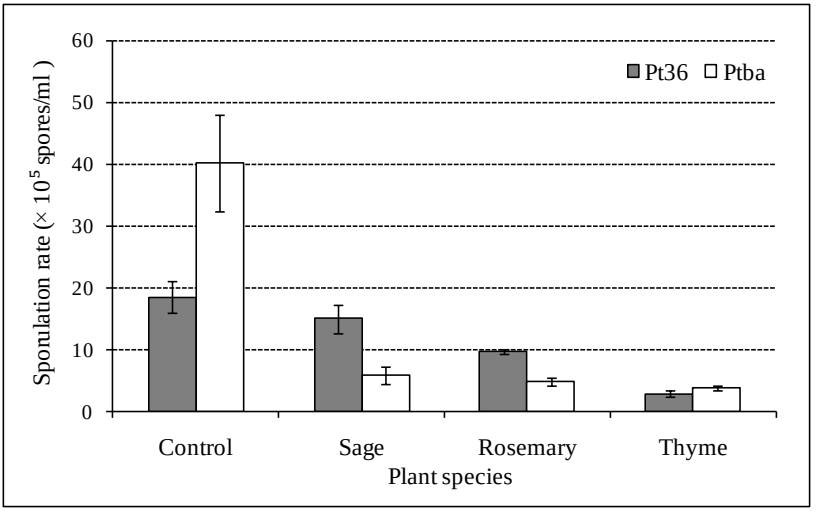

Fig. 8. Effect of sage, rosemary and thyme essential oils (20 µl/l) on sporulation rate of two *P. tracheiphilus* isolates on carrot liquid media after 10 days of incubation at 22°C. The control was treated with sterile distilled water. Each value represents the mean of three observations. Segments are standard errors.

Determination of minimal Thyme EO non-phytotoxic concentration.

The application of thyme EO on *Citrus aurantium* leaves revealed that this product is phytotoxic when applied pure or at a concentration higher than 60 ppm. Indeed, at 70 ppm, leaves showed a localized necroses; these necroses extended as the dose increased (Fig. 9).

Fig. 9. Determination of minimal non phytotoxotic thyme essential oil concentration by direct application of different dilutions on *Citrus aurantium* leaves. a : Pure essential oil of thyme ; b :100 ppm; c : 90 ppm; d :80 ppm; e :70 ppm ; f :60 ppm; g :50 ppm; h :40 ppm; i :30 ppm; j :20 ppm; k :10 ppm.

Determination of minimal inhibitory concentration.

The minimal inhibitory concentration of thyme EO not allowing the growth of *P. tracheiphilus* was determined at 400 ppm in comparison with negative control showing 100% of growth. The fungus expansion inhibition reached 22.57% at 100 ppm, 64.28% at 200 ppm and 89.71% at 300 ppm (Fig. 10, Table 2).

Table 2. *Plenodomus tracheiphilus* mycelial growth inhibition on PDA medium after 15 days of incubation at 25°C, according to increasing thyme essential oil dilutions

Treatment	Growth (cm)	Inhibition (%)
Control	3.5 +/-0.86	0
30 ppm	3.5 +/- 0.39	0
50 ppm	3.63 +/- 0.14	0
70 ppm	3.55 +/- 0.34	0
100 ppm	2.71 +/- 0.45	22.57
200 ppm	1.25+/- 0.20	64 .28
300 pm	0.36+/- 0.09	89.71
400 ppm	0+/- 0	100

Each value represents the mean of five observations.

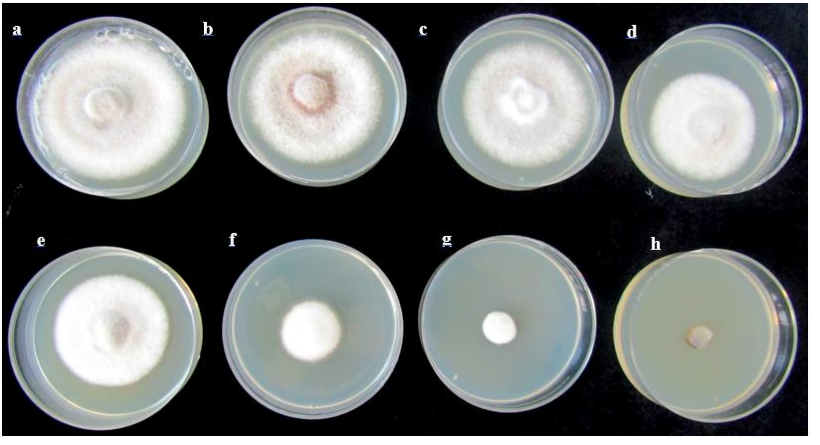

Fig. 10. Effect of different dilutions of thyme essential oil on *Plenodomus tracheiphilus* mycelial growth on PDA medium after 15 days of incubation at 25°C. a: control DMSO; b: 30 ppm thyme EO; c: 50 ppm thyme EO; d: 70 ppm thyme EO; e: 100 ppm thyme EO; f: 200 ppm thyme EO; g: 300 ppm thyme EO; h: 400 ppm thyme EO.

Evaluation of in vitro inhibitory activity of thyme EO with reference fungicide.

The thyme EO showed 100% in vitro inhibition of *P. tracheiphilus* growth in comparison with negative control and was as active as the Methylthiophante when tested at 1 g/l. However, thyme EO presented 24% higher inhibitory effect than Score (used at 250 g/l) which inhibit 76% of *P. tracheiphilus* development (Fig. 11, Table 3).

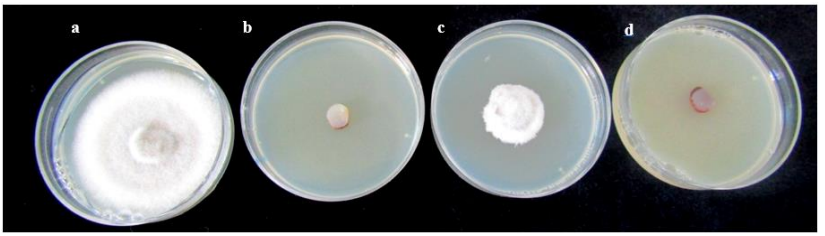

Fig. 11. Fungicidal effect of thyme essential oil on *Plenodomus tracheiphilus*mycelial growth in comparison with two fungicides after 15 days of incubation in PDA medium at 25°C. a: control DMSO; b: Thyme Essential Oil (400 ppm); c: Score (250 g/l); d: Methylthiophanate (1 g/l).

Table 3. Comparison of antifungal activity of thyme essential oilagainst *Plenodomus tracheiphilus* with reference fungicide

Treatment	Growth (cm)	Inhibition (%)
Control	3.5 +/- 0.86	0
400 ppm thyme EO	0+/- 0	100
Methylthiophanate	0+/- 0	100
Score	0.9 +/- 0.26	74.28

Each value represents the mean of five observations.

Qualitative and quantitative analysis of the essential oils.

Qualitative and quantitative analysis of the EO volatile profile are listed in Tables 4 and 5. These results showed that the main constituents of the thyme EO were carvacrol (76. 71%), γ-terpinene (6.94%), *p*-cymene (5.74%) and β-caryophyllene (2.79%), whereas the major constituents of rosemary EO were 1.8-Cineole (42.17%), α-pinene (8.85%), Bornyl acetate (7.18%), Camphor (7.08%), β-pinene (6.71%), endo-Borneol (5.92%) and β-Caryophyllene (5.38%). Other components were present in amounts less than 2%.

There are 17 similar compounds between the two analyzed plant EO, however, the components displayed different amounts.

Table 4. Thyme essential oil composition measured with GC-MS analysis

Peak #	Kovats index	Area (%)	Identification	Class
1	935	0.55	α-thujene	Monoterpenehydrocarbon
2	939	0.50	α-pinene	Monoterpenehydrocarbon
3	940	0.10	Camphen	Monoterpenehydrocarbon
4	980	0.13	β-pinene	Monoterpenehydrocarbon
5	991	0.87	β-Myrcene	Monoterpenehydrocarbon
6	993	0.07	3-Octanol	Aliphaticalcohol
7	1005	0.20	α-Phellandrene	Monoterpenehydrocarbon
8	1011	0.07	Δ-3-carene	Monoterpenehydrocarbon
9	1018	1.51	α-Terpinene	Monoterpenehydrocarbon
10	1026	5.74	p-Cymene	Monoterpenehydrocarbon
11	1031	0.45	β-Phellandrene	Monoterpenehydrocarbon
12	1033	0.14	1,8-Cineole	Oxygenatedmonoterpene
13	1062	6.94	Γ-terpinene	Monoterpenehydrocarbon
14	1072	0.12	cis-Ocimene	Monoterpenehydrocarbon
15	1088	0.11	α-terpinolene	Monoterpenehydrocarbon
16	1098	0.63	Linalool	Oxygenatedmonoterpene
17	1145	0.41	4-Terpineol	Oxygenatedmonoterpene
18	1163	0.08	β-fenchol	Oxygenatedmonoterpene
19	1290	0.22	Thymol	Oxygenatedmonoterpene
20	1298	76.71	Carvacrol	Oxygenatedmonoterpene
21	1391	1.08	Carvacrylacetate	Oxygenatedmonoterpene
22	1421	2.79	β-Caryophyllene	Sesquiterpenehydrocarbon
23	1454	0.09	α-Humulene	Sesquiterpenehydrocarbon
24	1581	0.49	Caryophylleneoxide	OxygenatedSesquiterpene

DISCUSSION

In this study, the antifungal activity of the essential oils (EO) of thyme, rosemary and Sage extracted by hydrodistillation, was evaluated on mycelial growth, sporulation rate and mycelial biomass of two *P. tracheiphilus* isolates. The results showed that thyme EO is the most effective product that completely stopped mycelial growth of the fungus in solid medium.

Table 5. Rosemary essential oil composition evaluated with GC-MS analysis

Peak #	Kovats index	Area (%)	Identification	Class
1	912	0.13	Tricyclene	Monoterpenehydrocarbon
2	935	0.26	α-thujene	Monoterpenehydrocarbon
3	939	8.85	α-pinene	Monoterpenehydrocarbon
4	940	4.10	Camphene	Monoterpenehydrocarbon
5	966	0.12	Sabinene	Monoterpenehydrocarbon
6	980	6.71	β-pinene	Monoterpenehydrocarbon
7	991	0.89	β-Myrcene	Monoterpenehydrocarbon
8	1005	0.14	α-Phellandrene	Monoterpenehydrocarbon
9	1011	0.09	δ-3-carene	Monoterpenehydrocarbon
10	1018	0.42	α-Terpinene	Monoterpenehydrocarbon
11	1026	0.95	p-Cymene	Monoterpenehydrocarbon
12	1033	42.17	1,8-Cineole	Oxygenatedmonoterpene
13	1072	0.27	cis-Ocimene	Monoterpenehydrocarbon
14	1088	0.74	α-terpinolene	Monoterpenehydrocarbon
15	1089	0.25	transSabinene hydrate	Oxygenatedmonoterpene
16	1098	0.51	Linalool	Oxygenatedmonoterpene
17	1110	7.08	Camphor	Oxygenatedmonoterpene
18	1149	5.92	endo-Borneol	Oxygenatedmonoterpene
19	1145	0.74	4-Terpineol	Oxygenatedmonoterpene
20	1179	2.19	δ-Terpineol	Oxygenatedmonoterpene
21	1285	7.18	Bornylacetate	Oxygenatedmonoterpene
22	1298	1.44	Carvacrol	Oxygenatedmonoterpene
23	1376	0.10	α-copaene	Sesquiterpenehydrocarbon
24	1401	0.96	Methyleugenol	Oxygenatedmonoterpene
25	1421	5.38	β-Caryophyllene	Sesquiterpenehydrocarbon
26	1430	0.06	β-copaene	Sesquiterpenehydrocarbon
27	1443	0.03	aromadendrene	Sesquiterpenehydrocarbon
28	1454	0.52	α-humulene	Sesquiterpenehydrocarbon
29	1477	0.14	γ-muurolene	Sesquiterpenehydrocarbon
30	1493	0.17	Ledene	Sesquiterpenehydrocarbon
31	1494	0.12	Valencene	Sesquiterpenehydrocarbon
32	1499	0.12	δ-amorphene	Sesquiterpenehydrocarbon
33	1507	0.30	δ-cadinene	Sesquiterpenehydrocarbon
34	1578	0.08	Spathulenol	OxygenatedSesquiterpene
35	1581	0.85	Caryophylleneoxide	OxygenatedSesquiterpene
36	1632	0.02	Longifolene	Sesquiterpenehydrocarbon

Several researchers have focused on the potential of plant extracts including essential oils that have a broad spectrum of activities in the control of phytopathogens (Flamini 2012; Isman, 2000). Lamiaceae is a relatively common botanical family, which members are spread in the temperate regions worldwide and many of them are potentially used as medicinal or aromatic herbs due to their volatile oil composition. Among them rosemary, sage and thyme which are commonly and naturally growing in Tunisia, are well-known for their EO content and their antimicrobial properties (Hanana et al. 2017).

The hydrodistillationof these plants showed that the output of thyme EO was 1.8% which concord with the literature where the yield of thyme EO was cited to be between 0.6%-1.8 (GuedriMkaddem, 2010) and 2.8-3.25% (Aouadhi, 2013) with a maximum of 6% (Bounatirou et al. 2007) obtained during the post-flowering phase. In our study, rosemary EO yield was 0.65% which also corroborate literature data where the maximum output of rosemary essential oils was of 3.2% (Moghtader et al. 2011) and the minimum of 0.44% (Boutekedjiret et al. 2003). The lowest income of EO was obtained with sage (0.24%) which is below the minimum yield (0.33%) (Zheljazkov et al. 2014), knowing that the mean value of yield is around 1.2-2.8% (Fellah et al. 2006; AbuDarwich et al. 2013). These divergences about richness in essential oils between plants of the same species could be attributed to differences of developmental stage of plant material and their geographical origin (Hedili et al.2002; Bounatirou et al. 2007).

The study of the antifungal activity showed that all EO displayed significant inhibition of *P. tracheiphilus* mycelial growth and sporulation rate. The results also indicated that the antifungal activity depended on the fungal strain (Ptba or Pt36), on the tested EO and on the method of application.

Indeed, the statistical comparison between the two methods used for the evaluation of the EO effect on the mycelial growth suggested that both are effective although the mixing method (or poisoned medium method) presented lower percentages of mycelial growth inhibition than the well diffusion method either due to less volume used or to the unequal distribution of EO into the medium depending on its relative hydrophilicity, as claimed by El Ouadi (2015). The well diffusion method and the mixing method are the most adapted techniques for evaluating antifungal and antimicrobial effect of EO (Pierozan et al. 2009; Russo et al. 2013; Miladinović and Miladinović 2000; Rus et al. 2015; Moghtader et al. 2011). The choice of the technique depends on the oils and the micro-organism tested as suggested by Donaldson et al. (2005).

Regardless the method of application of the EO, the two isolates had their mycelial growth and their sporulation reduced by at least 85% by thyme EO displaying the strongest inhibitory effect followed by rosemary EO. These results corroborate with previous studies indicating that Lamiacae family exhibit antifungal activities and in particular thyme and rosemary EO which had already shown strong activities against *Sclerotium cepivorum* (Russo et al. 2013), *Candida albicans* (Matsuzaki 2013), *Apergillus* sp. (Moghtader et al. 2011), *Fusarium*sp. (Da Silva Bomfimetal. 2015). Bounatirou et al. (2007) have also reported antibacterial and antioxidant activities of Tunisian thyme EO collected from different provenances. However, it is the first time

that *P. tracheiphilus* is reported to be inhibited by thyme EO. The minimal inhibitory concentration of Tunisian thyme EO not allowing any *P. tracheiphilus* mycelial growth was determined at 400 ppm. It is approximately the MIC of thyme EO (300 ppm) towards *Sccharomyces* (Konuk and Ergüden 2017) and slightly higher than MIC of thyme EO (200 ppm) reported to inhibit otherphytopathogenic fungi (*Pythium. aphanidermatum, Rhizoctonia. solani, Fusarium graminearum, Sclerotinia sclerotiorum* (Amini et al. 2012) and *Pythium italicum* (130 ppm) (Vitoratos et al. 2013)),whereas it is lower than MIC necessary to stop the growth of *Staphylococcus aureus* (1000 ppm) (Sienkiewicz et al. 2012) and *Candida albicans* (1460 ppm) (Omran et al. 2010). These variations in the minimal inhibitory concentrations enabling antimicrobial activities are probably associated to the composition of each essential oil and its interaction with the pathogen.

Indeed, the antifungal activity could be attributed to the high proportions of oxygenated monoterpenes such as carvacrol, thymol, pulegone and 1.8-cineole. In fact, there are many qualitative similarities between the thyme and rosemary EO although the amounts of some corresponding compounds are different. In fact, thyme EO is mainly constituted of carvacrol (76. 71%), whereas in rosemary EO the major compound is 1.8-Cineole (42.17%). However, other major or trace components in the EO can also be associated to the antifungal activity. Possible synergistic and antagonistic interactions among and between these components were also suggested (Sokovic and Griensven 2006). The mode of action of EO was investigated by many authors who implied that the antimicrobial activity resulted from the phenols, which at low concentrations, interact with the enzymatic systems associated with energy production and the synthesis of structural compounds of the microbial cells (Omidbeygi et al. 2007; Russo 2013), whereas at greater concentrations, they cause protein denaturation (Russo 2013). Both thymol and carvacrol isomers and their precursor p-cymene were also shown to depolarize microbial cell membranes, increasing thus membrane permeability causing the loss of its integrity and leakage of cellular material, disturbing ion homeostasis and leading to metabolic activity disruption (Da Silva Bomfim et al. 2015; Lima et al. 2013; Rao et al. 2010).

The antifungal activity of rosemary EO is reported to be highest in monoterpens combination and in particular α-pinene (Moghtader et al. 2011), but not in camphor or 1.8-cineole (Matsuzaki et al. 2013). This assumption could explain the stronger antifungal activity of thyme (carvacrolchemotype) in comparison with rosemary analyzed as a 1.8 cineole chemotype. These results are consistent with previous findings stipulating that 1.8 cineole was the major component of rosemary EO (Jalali-Heravi et al. 2011; Jiang et al. 2011; Da Silva Bomfim et al. 2015), whereas it is in contradiction with other works where α-pinene, p-cymene or camphor were the major compounds of rosemary EO (Jamshidi et al. 2009; Santoyo et al. 2005; Ozcan and Chalchat 2008; Belkhodja et al. 2016).

Indeed, the chemical composition of the tested essential oils is reported to be dependent on its origin. It is also the case for thyme EO whose composition differs from one locality to another even in the same country suggesting that the environmental factors strongly influence its chemical composition (Aouadhi et al. 2013). In

fact, thyme growing in Jendouba, Haouaria and Ain Tounine were characterized by a carvacrolchemotype EO (Bounatirou et al. 2007) which is in concordance with our results and with the chemotype of Morocco (Karousou et al. 2005) and Greece (Ouariachi et al. 2011). Although these conclusions were in contradiction with Guedri Mkaddem et al. (2010) results reported that thyme EO of Matmata was a thymol chemotype. The soil and the climate factors (temperature, pluviometry, and altitude) has also been associated to these important differences between the two chemotypes. Bounatirou et al. (2007) agreed with this assumption and asserted that the composition of thyme EO can also diverge according to the harvesting season, the geographical locality, the genotype, the climate, the soil composition, the drying procedure and the distilled part of the plant. Besides, Hedhilli et al. (2002) showed that the composition of thyme EO is also linked to phases of the vegetative cycle. At flowering time, the EO is rich in carvacrol; p-cymene showed maximum values when carvacrol is minimum (Aouadhi et al. 2013).

In this study, concentration over 70ppm of thyme EO induced phytotoxicity symptoms on *C. aurantium* leaves at 25°C. This concentration represents the 1/6 of the MIC of thyme EO that showed *P. tracheiphilus* inhibition. Main components of EO identified as thymol and carvacrol seems also to be responsible of the phytotoxicity observed on *C. aurantium* leaves reported in our work, asthey have been well documented as potential herbicides against various plants (Batishet al. 2012; Rolliet al. 2014). Phytotoxicity testing is very important when studying the potential of an EO as an alternative disease control method. However, these disadvantages may be overcome by optimizing treatment conditions, such as formulation (Plotto et al. 2003), combination with other compounds and temperature of application (Tsao and Zhou, 2000).

Inthis context, previous field studies have shown that at certain concentrations EO tends to "burn" the plants. The level of phytotoxicity depends on the adjuvant used, the EO evaluated, the species and the variety on which the substance is tested and the phenological stage of application (Vidal et al. 2018).

Indeed, many studies demonstrated that the emulsion of the EO could reduce its phytotoxicity (Plottoet al. 2003). The way and temperature of application also determinate the success of the treatment. In fact, Tsao and Zhou, (2000) ascertained that phytotoxicity of monoterpenoids (thymol and carvacrol) was negligible at 2°C when used as fumigant against postharvest pathogens.

The combination of the EO with other biological compounds could also reduce the phytotoxicity. The use of thymol or carvacrol with methyl jasmonate as co-fumigant in sweet cherryshowed reduced side effect of stem browning in fruits by 73% while maintaining the disease suppression, knowing that methyl jasmonate do not reduce disease symptoms by itself (Tsao and Zhou, 2000).The association of *Tagetes filifolia L.*, *Laurus nobilis* (Camilettiet al. 2016), *Origanum vulgare*, *Citrus sinensis*, *Citrus limon*, *Citrus maxima*, *Syzygium aromaticum*, *Melaleuca alternifolia*, and *Eucalyptus globulus* (Vidal et al. 2018) EO with fungicide in order to avoid phytotoxicitywhilereducing the chemical approach in agriculture, was also attempted in order to reduce *Sclerotium cepivorum* in garlic crop (Camiletti et al. 2016) or *Plasmopara viticola* in

grapevine (Vidal et al. 2018), respectively.

In this study, analysis of the EO volatile profile revealed the carvacrol (76. 71%) as the main constituent of thethyme EO which appeared to be very efficient in inhibiting mycelial growth, mycelial biomass and sporulation rate of *P. tracheiphilus*. Results suggest that this high anti-fungal activity is attributed to the carvacrol. Further analyses are needed to identify the bioactive compoundand to adjust final concentrations and formulation to be applied in field crops.

RESUME

Kalai-Grami L., Bahri B.A., Chemekh M., Hammami M., Limam F. et Hajlaoui, M.R., 2019. Evaluation des activités anti-fongiques d'huiles essentielles de trois espèces de plantes *Lamiaceae* contre *Plenodomus tracheiphilus* (syn. *Phomatracheiphila*). Tunisian Journal of Plant Protection14 (1): 11-32.

Le mal secco (signifiant dépérissement sec) causé par *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*) est une maladie très grave et incurable touchant les agrumes. Dans cette étude, nous nous sommes proposés de chercher une stratégie de lutte biologique contre cette maladie. Pour cela, le pouvoir antifongique des huiles essentielles (HE) du thym (*Thymbra capitata*), du romarin (*Romarinus officinalis*) et de la sauge (*Salvia officinalis*) extraites par hydrodistillation, a été évalué sur la croissance mycélienne, le taux de sporulation et la biomasse mycélienne de deux isolats de *P. tracheiphilus*. Les résultats montrent que HE du thym est le produit le plus efficace permettant de stopper entièrement la croissance mycélienne du champignon sur milieu artificiel solide. HE du romarin et de la sauge présentent des taux d'inhibition de la croissance mycélienne pouvant atteindre respectivement 69 et 58%. De plus, HE de thym ont présenté le pourcentage d'inhibition de la biomasse mycélienne de *P. tracheiphilus* le plus élevé en milieu liquide, atteignant 91% en moyenne, suivi par HE du romarin et de la sauge avec respectivement 75 et 58%. L'inhibition du taux de sporulation était également la plus importante sous l'effet des HE du thym avec 88% et la plus faible avec HE de la sauge avec 52% en moyenne. Ces résultats mettent en évidence la bonne activité antifongique des HE du thym sur le développement de *P. tracheiphilus in vitro*. L'analyse qualitative et quantitative du profil volatil des HE a montré que le carvacrol est le constituant principal des HE du thym (76,71%). Le test de phytotoxicité a révélé que HE du thym est toxique à des doses dépassant 60ppm sur feuilles détachées de *Citrus aurantium*. D'un autre côté, la concentration minimale inhibitrice (CMI) chez HE du thym (400 ppm) s'est avérée supérieure à la dose phytotoxique. D'autres analyses complémentaires sont nécessaires pour optimiser les conditions d'application *in vivo* contre *P. tracheiphilus* à des doses supérieures à la CMI sans provoquer de phytotoxicité.

Mots clés: Activité antifongique, huiles essentielles, *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*), *Rosmarinus officinalis, Salvia officinalis, Thymbra capitata*

ملخص

القلعي- قرامي، ليلى وبشرى أمينة بحري ومهجة الشامخ ومجدي الهمامي وفريد الإمام ومحمد رابح الحجلاوي. 2019. تقييم الأنشطة المضادة للفطريات لدى الزيوت الروحية لثلاثة أنواع نباتات من عائلة الشفويات ضد الفطر *Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*).

Tunisian Journal of Plant Protection14 (1): 11-32.

يعتبر مرض مالسيكو (بمعنى التدهور الجاف) المتسبب عن الفطر *Pleunodomus tracheiphilus* (syn. *Phoma tracheiphila*) مرض خطير جدا لا يعالج، يصيب أشجار القوارص/الحمضيات.في هذه الدراسة تم اقتراح البحث عن

استراتيجية للمكافحة البيولوجية لهذا المرض. لذلك وقع اختبار تأثير الزيوت الروحية المستخرجة بواسطة التقطيرالمائي من الأنواع النباتية الزعتر(*Thymbra capitata*) والإكليل (*Romarinus officinalis*) والمريمية (*Salvia officinalis*) على النمو الغزلي ونسبة التبوغ والكتلة الحيوية الغزلية لعزلتين للفطر *P.tracheiphilus*. أظهرت النتائج أن الزيوت الروحية للزعترهي الأكثر فاعلية للحد من النمو الغزلي للفطر في المستنبت الصلب. أما زيوت الإكليل والمريمية فتمكنت من الحد من النمو الغزلي بنسبتي 69 و 58%، على التوالي. كما تبين أن الزيوت الروحية للزعتر هي التي أظهرت أقوى نسبة تثبيط لكتلة الفطرالحيوية الغزلية في المستنبت السائل حيث بلغت 91%، تليها زيوت الإكليل بنسبة 75% ثم زيوت المريمية بنسبة 58%. وكانت أقوى نسبة تثبيط لتبوغ الفطر تحت تأثير الزيوت الروحية للزعتر أي أقل بـ 88% بينما كانت الأدنى مع زيوت المريمية بـ 52%.هذه النتائج تبين مدى قدرة الزيوت الروحية للزعتر على الحد من نمو الفطر في جسم العائل. بينت التحاليل الكيميائية النوعية والكمية أن Carvacrol هو المكون الأساسي للزيوت الروحية للزعتر (76,71%). وأظهر اختبار السمية النباتية أن الزيوت الروحية للزعتر سامة لأوراق النوع *Citrus aurantium* إذا تجاوزت الجرعة 60 جزء من مليون. من ناحية أخرى، اتضح أن الحد الأدنى للتركيز المثبط عند الزيوت الروحية للزعتر (400 جزء من مليون) يتجاوز الجرعة السمية النباتية. هناك حاجة إلى مزيد من التحاليل التكميلية لتحسين ظروف تطبيق الزيوت الروحية للزعترعلى النبتة ضد الفطر بجرعات أعلى من الحد الأدنى للتركيزالمثبط دون التسبب في السمية النباتية.

كلمات مفتاحية: النشاط المضاد للفطريات، زيوت روحية، (*Plenodomus tracheiphilus* (syn. *Phoma tracheiphila*، *Rosmarinus officinalis*، *Salvia officinalis*، *Thymbra capitata*

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Variations in Essential Oils Composition and Potential as Fumigants against Stored Date Moths *Ectomyelois ceratoniae* and *Ephestia kuehniella*

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ABSTRACT

Yousfi, S., Haouel-Hamdi, S., Bessi, H., Assoudi, C., Elimem, M., Messaoud, C., Flamini, G., and Mediouni-Ben Jemâa, J. 2019. Variations in essential oils composition and potential as fumigants against stored date moths *Ectomyelois ceratoniae* and *Ephestia kuehniella*. Tunisian Journal of Plant Protection 14 (1): 33-53.

Rosmarinus officinalis and *Eucalyptus viminalis* essential oils (EO) were analysed using Solid Phase Micro-Extraction (SPME) and Gas Chromatography-Mass Spectrometry (GC-MS) techniques and assessed for their fumigant toxicity against eggs and last instar larvae of *Ectomyelois ceratoniae* and *Ephestia kuehniella*. Principal Components Analysis (PCA) and Hierarchical Cluster Analysis (HCA) revealed quantitative and qualitative differences in oil composition in relation to plant species and the technique that was used in the analysis. The major common compounds were identified as 1,8-cineole, α-pinene, β-pineneand and p-cymene. Ovicidal and larvicidal activities were highly dependent upon insect species and oils. *E. viminalis* EO was more effective compared to that of *R. officinalis*. In larval bioassays, LC_{50} values were respectively 25.80 µl/l air and 33.05 µl/l air for *E. ceratoniae* versus 12.92 µl/l air and 12.47 µl/l air for *E. kuehniella*. Moreover, ovicidal activity was lower against eggs of *E. kuehniella* than that of *E. ceratoniae*. This work clearly defends the interest in the efficacy of EOs both as ovicidal and larvicidal insecticides against stored date moth pests.

Keywords: *Ectomyelois ceratoniae*, Essential oils, *Eucalyptus viminalis*, GC-MS, *Rosmarinus officinalis*, SPME

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In Tunisia, date palm (*Phoenix dactylifera*) is the most important fruit tree in the southern part of the country. Dates are primarily intended for export; 43.75% of the date crops were exported in 2014 (GIF 2015). Dates are subjected to many pests and diseases both in field and

in storage after harvest (Mediouni et al. 2004). The carob moth *Ectomyelois ceratoniae* (Lepidoptera: Pyralidae) and the Mediterranean flour moth *Ephestia kuehniella* (Lepidoptera: Pyralidae) are among the major destructive insect pests causing serious damage during storage (Mediouni Ben Jemâa et al. 2012). Losses caused by insects consist of the direct consumption of food stuff in addition to the accumulation of leftovers such as silk, exuviae, body fragments and dead insects (Shankar and Abrol 2012).

Generally, the post-harvest control of these pests is based on the use of synthetic insecticides (organophosphates and pyrethroids) and fumigants (such as methyl bromide and phosphine) that induced multiple harmful effects on the environment and human health (Azelmat et al. 2006; Kim et al. 2003; Mbata and Shapiro-Ilana, 2010). In the last few years, more than 504 resistant mite and pest species have been registered and there is still a steady increase in resistance to other chemicals (Georghiou 1990; Lee 2002) in addition to the accumulation of chemical residues in food, as well as human exposure to pesticides (Arthur and Phillips 2003; Attia 1977; Phillips et al. 2010). Moreover, methyl bromide has been declared an ozone-depleting fumigant and consequently is being phased out completely (Collins et al. 2002; Taylor 1989;). Thus, the search for effective and environmental-friendly alternatives becomes a necessity. In this respect, since old times, plants and their based-products were used for stored product pest control. However, their use against pests diminished since the development of chemical products. Concerns regarding public health and environmental safety of synthetic insecticidal products prompted research natural products that may be used against insect pests. In addition, the use of essential oils (EO) has proved its efficacy against various insect pest species for example Coleoptera (Kim et al. 2003) and Lepidoptera (Tripathi et al. 2009). Many studies were done on the effect of EO on coleopteran pests such as *Tribolium castaneum*, *Sitophilus zeamais* and *Callosobruchus maculatus* (Bouda et al. 2001; Haider et al. 2017; Kéïta et al. 2000 ;). Only a few studies have focused on Lepidopteran species such as *Helicoverpa armige*r*a*, *E. ceratoniae* and *E. kuehniella* (Al-Izzi and Al-Maliky 1996; Karabörklü et al. 2011; Koul et al. 2003;).

Bachrouch et al. (2010) showed that the EO of *Pistacia lentiscus* had a significant ovicidal effect on *E. kuehniella* and *E. ceratoniae*. Mediouni-Ben Jemâa et al. (2013) reported the EO of *Eucalyptus* to be effective against the last instars larvae of *E. ceratoniae* under various space occupation with dates.

Analysis of EO usually involves the separation, identification and quantitative determination of their components.The preferred technique for analysis of EO components is capillary gas chromatography (GC) due to their volatility and polarity. The reason being that EO in general are complex mixtures of components with similar physicochemical characteristics (Rubiolo et al. 2010). However, Solid-Phase Micro-Extraction (SPME) is a simple, fast and effective sampling technique that was developed in 1990's. As an analytical technique, SPME is widely accepted in combination with other techniques such as GC and GC-MS (Aulakh et al. 2005). These have great advantages over the classical analytical techniques, which are time consuming and require larger samples and solvents (Aulakh et al. 2005). The SPME coupled with gas chromatography-mass spectrometry (GC-MS) has been developed to analyse the

components in many plants (Li et al. 2012; Ruíz-Ramón et al. 2014). Huang et al. (1994) have demonstrated that the technique of analyses used affected the composition of the EO of two varieties of *Perilla frutescens*. In the same context, Shakeel et al. (2013) showed that the quantity of components of *Melissa officinalis* essential oils varied according to the technique used (HS-SPME or GC-MS). Therefore, the present study was carried out to determine the chemical composition of *Rosmarinus officinalis* and *Eucalyptus viminalis* EO using GC-MS and SPME analyses and to determine their insecticidal activity against eggs and fifth instar larvae of *E. ceratoniae* and *E. kuehniella.*

MATERIALS AND METHODS

Plant materials.

R. officinalis leaves were collected from natural populations located at Siliana (North-west, Tunisia), while, *E. viminalis* leaves were collected from the arboretum of Souiniet (Ain-drahem Jendouba, North-west, Tunisia) during the same season. The collected material was air-dried at a room temperature (20 to 25°C) for one week and then kept for the extraction. The plant species was identified according to the morphological description presented in Tunisian flora (Pottier-Alapetite 1979). Voucher specimens (P.I.03 and P.I.04) were deposited in the Laboratory of Biotechnology Applied to Agriculture (National Agricultural Research Institute of Tunisia, INRAT).

EO extraction.

EO extractions were obtained using a modified Clevenger-type apparatus. The weight of 100 g of dry leaves were hydro-distilled in 500 ml of distilled water for 90 min. The oils were dried over anhydrous sodium sulphate and then stored at 4°C in sealed glass vials. Yields were averaged over three experiments and calculated according to dry weight of the plant materials.

SPME analyses

Supelco SPME (Solid Phase Micro-extraction) devices coated with polydimethylsiloxane (PDMS, 100 µm) were used to sampling the headspace of living flowers inserted into a 50 ml glass container and allowed to equilibrate for 30 min.

SPME sampling was performed using the same new fibre, preconditioned according to the manufacturer instructions, for all the analyses. Sampling was accomplished in an air-conditioned room (22 ± 1°C) to guarantee a stable temperature.

After the equilibration time, the fibre was exposed to the headspace for 10 s. Once sampling was finished, the fibre was withdrawn into the needle and transferred to the injection port of the GC and GC-MS system.

All the SPME sampling and desorption conditions were identical for all the samples. Furthermore, blanks were performed before each first SPME extraction and randomly repeated during each series. Quantitative comparisons of relative peaks areas were performed between the same chemicals in the different samples.

GC-EIMS analyses were performed with a Varian CP-3800 gas-chromatograph equipped with a DB-5 capillary column (30 m x 0.25 mm; coating thickness 0.25 µm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions: injector and transfer line temperatures 220 and 240°C respectively; oven temperature programmed from 60 to 240°C at 3°C/min; carrier gas helium at 1 ml/min; for essential oils: injection of 0.2 ml (10%

hexane solution); split ratio 1:30; for SPME: injector temperature 250°C, splitless injection. Identification of the constituents was based on comparison of the retention times with those of authentic samples, comparing their linear retention indices relative to the series of *n*-hydrocarbons, and on computer matching against commercial (NIST 2000 and Adams 2007) and home-made library mass spectra built up from pure substances and components of known oils and MS literature data (Adams, 2007; Davies, 1990; Joulain and König, 1998;).

Volatile compounds were grouped into monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpeneoxygenated, sesquiterpenehydrocarbons and other compounds. Three replica injections were done in order to verify the SPME and GC-MS obtained data.

Insect rearing.

For bioassays, fresh eggs (0-24 h) and fifth instar larvae were collected from the rearing colonies initiated under laboratory controlled conditions. Carob moth *E. ceratoniae* was reared on an artificial diet based on wheat bran, yeast, sucrose salt mixture, vitamin C, aureomycine, methylparaben, lysine, glycerine and distilled water (Mediouni and Dhouibi 2007). The rearing conditions were: 28 ± 1°C, a photoperiod of 16:8 h (L:D) and relative humidity (65 ± 5%).The Mediterranean flour moth *E. kuehniella* was reared on bread wheat flour (*Triticum aestivum*). Rearing was conducted in plastic boxes in darkness at 25 ± 1°C and 65 ± 5% relative humidity.

Fumigant toxicity assays.

To assess the fumigant toxicity of *E. viminalis* and *R. officinalis* EO, 2 cm diameter filter papers (Whatman No. 1) were impregnated with the different oil doses 10, 20 and 50 µl. Doses were converted to give equivalent fumigant concentrations of respectively 21.27, 42.55 and 106.38 µl/l air. Experiments were conducted in screw-cap sealed of 50 ml Plexiglas bottles. Five replicates of thirty eggs and twenty larvae were used. Untreated filter papers served as a control.

Treated eggs were checked daily for hatching. After fourteen days, the numbers of hatched and non-hatched eggs were counted. The percentage of hatched eggs was calculated as the number of hatched eggs/the total number of exposed eggs × 100. Then, emerged adults from survived eggs were counted. The percentage of adult emergence was determined as number of emerged adults/the number of hatched eggs × 100.

The percentage larval mortality was calculated as the number of dead larvae/the total number of exposed larvae × 100. The number of adults that survived from the surviving larvae was also counted. The percentage adult emergence was calculated as number of adults that emerged/the number of surviving larvae × 100.

In cases where control mortality occurred, the data were corrected using Abbott's formula (Abbott 1925) as follows: Mortality (%) = [(test - control)/control] × 100.

In order to estimate the lethal concentrations LC_{50} and LC_{95} values, Probit analysis (Finney 1971) was performed. Delayed development of eggs and larvae due to EO exposures was calculated as the number of adults that emerged from surviving eggs and larvae, respectively.

Statistical analysis.

All data are expressed as mean values. Data were subjected to one way

analysis of variance (ANOVA) followed by Duncan tests at the 0.05% level.

To evaluate whether the identified components may be useful in reflecting differences between both analysis techniques, all compounds detected in the oil samples either with GC-MS or HS-SPME-GC-MS methods were selected and used for this purpose. All identified compounds were subjected to a principal component analysis (PCA) and to hierarchical cluster analysis (HCA).The influence of fumigation parameters (insect species: *E. ceratoniae* and *E. kuehniella*, essential oils: *R. officinalis* and *E. viminalis*, concentration: 0, 21.27, 42.55, 106.38 and 170.2 µl/l air and exposure time: 7, 14, 21 and 28 days as independent variables) on mortality of eggs and larvae of both species were analysed by means of simple correlation analyses. In order to investigate the simultaneous influence of fumigation parameters on insect mortality, multiple linear regression analysis were used. Statistical significance was defined as $P < 0.05$. All these statistical analyses were performed using the SPSS Inc., software (Version 20.0).

RESULTS

EO yield and chemical composition.

The hydrodistillation of leaf parts of *R. officinalis* and *E. viminalis* yielded yellow oils with a distinct sharp odour and yield of 1.01% and 2.52% (w/w) on a dry weight basis respectively. Moreover, the hydrodistillation of *E. viminalis* offered significantly higher essential oil yields than *R. officinalis* (Table 1).

Table 1. Yields (%) of *Rosmarinus officinalis* and *Eucalyptus viminalis* essential oils

Essential oils	Yield (%)
Rosmarinus officinalis	1.01 ± 0.10 b
Eucalyptus viminalis	2.52 ± 0.02 a

Values presented are means of ten replications. Means followed by different letters are significantly different by ANOVA test ($P < 5\%$).

The list of the different constituents of oils identified from the plant species are provided in Table 2. The essential oil composition varied according to the analysis techniques used. Both techniques produced high percentages of oxygenated monoterpenes, though GC-MS gave more constituents than SPME (Table 2).

Table 2. Emitted volatiles (HS-SPME-GC-MS) and essential oil composition (GC-MS) of *Rosmarinus officinalis* and *Eucalyptus viminalis*

N°	Compounds	LRI	SPME		Essential oils (%)	
			Rosmarinus officinalis	***Eucalyptus viminalis***	***Rosmarinus officinalis***	***Eucalyptus viminalis***
Monoterpenehydrocarbons			**15.80**	**5.80**	**24.39**	**6.06**
1	Tricyclene	921	-	-	0.14±0.04	-
2	α-pinene	941	6.95±0.05	3.95±0.09	13.55±0.32	4.39±0.18
3	Camphene	955	2.85±0.07	-	4.11±0.09	-
4	β-pinene	982	2.40±0.20	0.40±0.14	2.09±0.21	0.32±0.03
5	Myrcene	993	1,10±0.03	.70±0.25	-	-
6	3-octanone	988	0,10±0.04	-	-	-
7	β-Myrcene	1 003	-	-	0.91±0.07	0.38±0.02
8	α-phellandrene	1 006	0.20±0.03	-	-	0.10±0.04
9	δ-3-carene	1 013	-	-	0.24±0.09	-
10	α-terpinene	1 020	0.50±0.04	-	0.68±0.07	-
11	l-Phellandrene	1 021	-	-	0.25±0.09	-
12	*p*-cymene	1 028	1.30±0.07	0.35±0.03	1.26±0.04	0.32±0.04
13	*(Z)*-β-ocimene	1 042	0,10±0.04	-	-	-
14	γ-terpinene	1 063	-	0.40±0.14	0.78±0.13	0.53±0.04
15	α-Terpinolene	1 085	-	-	0.37±0.03	-
16	Terpinolene	1 090	0.40±0.04	-	-	-
Non-terpenederivatives			**0.10**	**0.30**	**-**	**-**
17	Isopentylisovalerate	1 104	-	0.30±0.11	-	-
Oxygenatedmonoterpenes			**78.45**	**62.95**	**66.89**	**78.53**
18	1,8-cineole	1 034	42.45±2.03	60.80±1.21	50.36±0.11	77.47±0.20
19	Linalool	1 101	1.20±0.04	-	0.39±0.03	-
20	*exo*-fenchol	1 118	0.10±0.02	-	-	-
21	Camphor	1 145	15.15±0.66	-	8.20±0.26	-
22	Borneol	1 167	12.60±0.48	-	4.88±0.10	-
23	δ-terpineol	1 172	-	0.20±0.07	-	-
24	terpinen-4-ol	1 178	-	-	-	0.39±0.09
25	4-terpineol	1 179	1.50±0.14	0.75±0.05	0.55±0.05	-
26	α-terpineol	1 191	5.45±0.40	1.20±0.15	-	-
27	β-Fenchylalcohol	1 197	-	-	1.94±0.11	0.56±0.04

28	Isoamylisovalerate	1 285	-	-	-	0.10±0.04
29	Bornylacetate	1 287	-	-	0.51±0.03	-
30	1-Octen-3-ol	1 670	-	-	0.07±0.02	-
Sesquiterpenehydrocarbons			**3.35**	**8.90**	**8.54**	**4.75**
31	α-ylangene	1 369	-	-	0.10±0.04	-
32	α-copaene	1 377	-	-	0.42±0.02	-
33	α-gurjunene	1 410	-	0.65±0.04	-	0.37±0.05
34	β-caryophyllene	1 419	2.50±0.17	-	-	-
35	Caryophyllene	1 424	-	-	5.79±0.26	-
36	β-copaene	1 430	-	0,20±0.72	-	-
37	Aromadendrene	1 440	-	3.70±0.20	0.08±0.03	2.40±0.35
38	α-humulene	1 456	0.55±0.12	-	0,73±0.05	-
39	Alloaromadendrene	1 462	-	1.05±0.06	-	0.61±0.00
40	β-Patchoulene	1 475	-	-	0.12±0.05	-
41	γ-muurolene	1 479	-	-	0.23±0.03	-
42	β-selinene	1 487	-	0.20±0.07	-	0.45±0.06
43	Viridiflorene	1 495	-	0.75±0.04	-	-
44	α-muurolene	1 500	-	-	0.13±0.04	-
45	β-bisabolene	1 508	-	-	0.06±0.02	-
46	*trans*-γ-cadinene	1 514	0.10±0.04	-	-	-
47	*α*-Selinene	1 521	-	-	-	0.52±0.02
48	δ-cadinene	1 524	0.20±0.02	-	0.58±0.04	-
49	germacrene B	1 557	-	2.35±0.08	-	-
50	β-Cubenene	1 662	-	-	-	0.11±0.03
51	Valencene	1 884	-	-	-	0.21±0.02
52	α-Amorphene	1 969	-	-	0.29±0.03	-
53	Dehydroaromadendrene	2 287	-	-	-	0,08±0.03
Oxygenatedsesquiterpenes			**1.15**	**18.45**	**0.09**	**10.66**
54	Spathulenol	1 577	-	0.40±0.14	-	0.19±0.03
55	Caryophylleneoxide	1 582	0.70±0.25	-	0.09±0.02	-
56	Globulol	1 584	-	12.25±0.26	-	7.38±0.13
57	*epi*-globulol	1 588	-	-	-	1.22±0.07
58	Viridiflorol	1 591	-	2.40±0.36	-	1.70±0.10

59	Guaiol	1 596	-	0.70±0.25	-	-
60	5-*epi*-7-*epi*-α-Eudesmol	1 603	-	1.45±0.18	-	-
61	humuleneepoxide II	1 607	0.15±0.05	-	-	-
62	1-*epi*-cubenol	1 628	-	0.15±0.05	-	-
63	caryophylla-4(14),8(15)-dien-5-ol	1 636	0.20±0.07	-	-	-
64	T-cadinol	1 641	0.10±0.04	0.55±0.21	-	-
65	T-muurolol	1 642	-	0.55±0.03	-	0.16±0.01
Total (%)			**98.85**	**96.40**	**99.91**	**100**

- : compound not detected; LRI are Linear Retention Indexes calculated on a HP-5MS capillary column (30 m x 0.25 mm x 0.25 µm).

SPME analysis of *R. officinalis* and *E. viminalis* EO led to the identification of 25 compounds for both oils (Table 2). Essential oils were rich in monoterpenes (94.25% for *R. officinalis* and 68.74% for *E. viminalis*). The main common constituents were 1,8-cineole (42.45% and 60.80%) and α-pinene (6.95% and 3.95%). Other notable common compounds were α-terpineol, β-pinene, myrcene and *p*-cymene. Comparing these results with those of GC-MS, 30 components were identified for *R. officinalis* and 23 for *E. viminalis*. Essential oils were rich in monoterpenes (91.28% for *R. officinalis* and 84.59% for *E. viminalis*). The main common constituents were 1,8-cineole (50.36% and 77.47%) and α-pinene (13.55% and 4.39%). This technique confirmed the results obtained by means of the SPME procedure.

Qualitatively, as expected, the results obtained from the two analytical techniques were different; the main common compounds were α-pinene, β-pinene, *p*-cymene and 1,8-cineole. In this respect, for *R. officinalis* essential oils, with major constituents such as myrcene (1.1%), α-terpineol (5.45%) and β-caryophyllene (2.5%) were identified using SPME, while β-myrcene (0.91%), γ-terpinene (0.78%), β-fenchyl alcohol (1.94%), bornylacetate (0.51%), α-capaene (0.42%) and caryophyllene (5.79%) were identified by essential oil injection. SPME revealed the identification of the following major compounds from *E. viminalis* essential oil: myrcene (0.70%), 4-terpineol (0.75%), α-terpineol (1.2%), viridiflorene (0.75%), germacrene B (2.35%), guaiol (0.70%) and 5-*epi*-7-*epi* (1.45%). Through GC-MS injection of the essentil oils the following major constituents were identified: β-fenchyl alcohol (0.56%), α-selinene (0.52%) and *epi*-globulol (1.22%).

The results of the headspace analysis performed by SPME may be useful to know which volatiles can better reach the samples submitted to fumigation.

Principal components analysis (PCA) and hierarchical cluster analysis (HCA).

The content of the 65 selected components did not differ significantly ($P < 0.05$) between *R. officinalis* and *E. viminalis* essential oils and analysis techniques. These 65 components were used for the PCA and the HCA analysis. The PCA horizontal axis explained 52.7%

of the total variance while the vertical axis a further 32.0% (Fig. 1). The HCA based on the Euclidean distance between groups indicated two groups (A and B) (with a dissimilarity of 25.0) (Fig. 2), identified by plant species (eucalyptus or rosemary). Group A (SPME-eucalyptus and GC-MS-eucalyptus) clearly stood out forming a separate group in the PCA (Fig. 1). It was correlated negatively with the axes 1 and 2. Group A was further divided into two subgroups A1 and A2 with dissimilarity < 5.

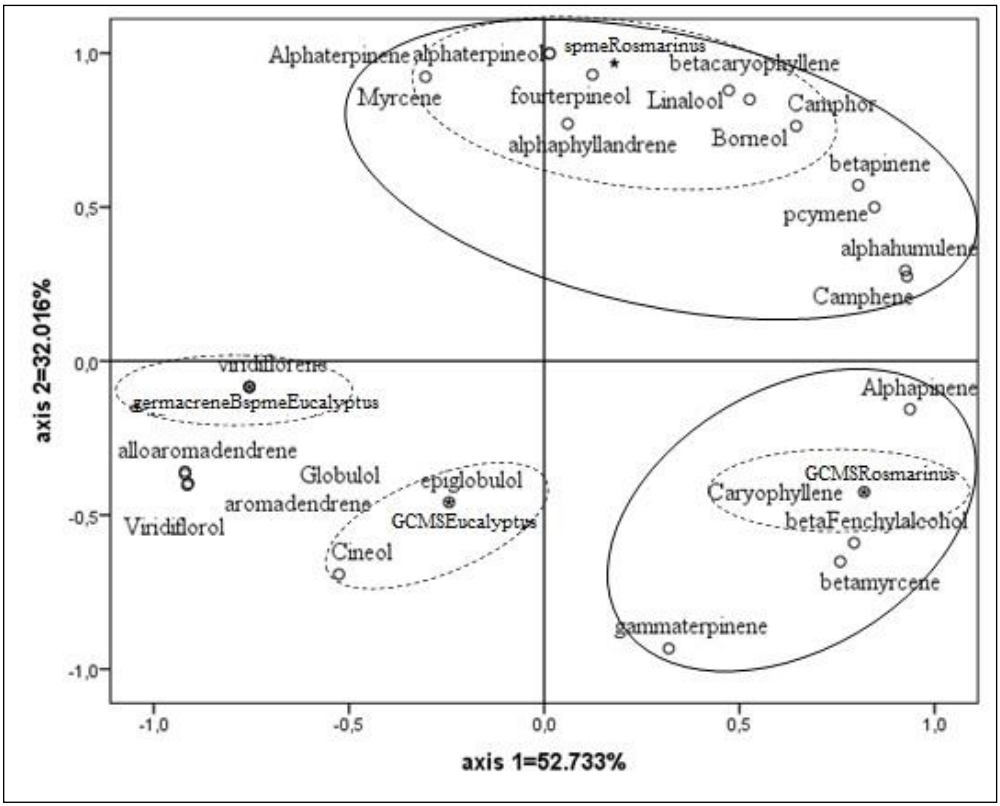

Fig. 1. Representation of *Eucalyptus viminalis* and *Rosmarinus Officinalis* essential oil compounds and analytical technique used (GCMS and SPME) according to the ACP results.

Subgroup A1 was constitued by *E. viminalis* essential oil analysed using SPME. This subgroup was characterized by the presence of compounds such as viridiflorene, germacrene and 5-*epi*-7-*epi*-α-eudesmol. On the other hand, subgroup A2 was presented by *E. viminalis* essential oils analysed by means of GC-MS. Subgroup A2 showed the highest percentage of 1,8-cineole (77.47%) and *epi*-globulol (7.38%) (Table 1 and Fig.2).

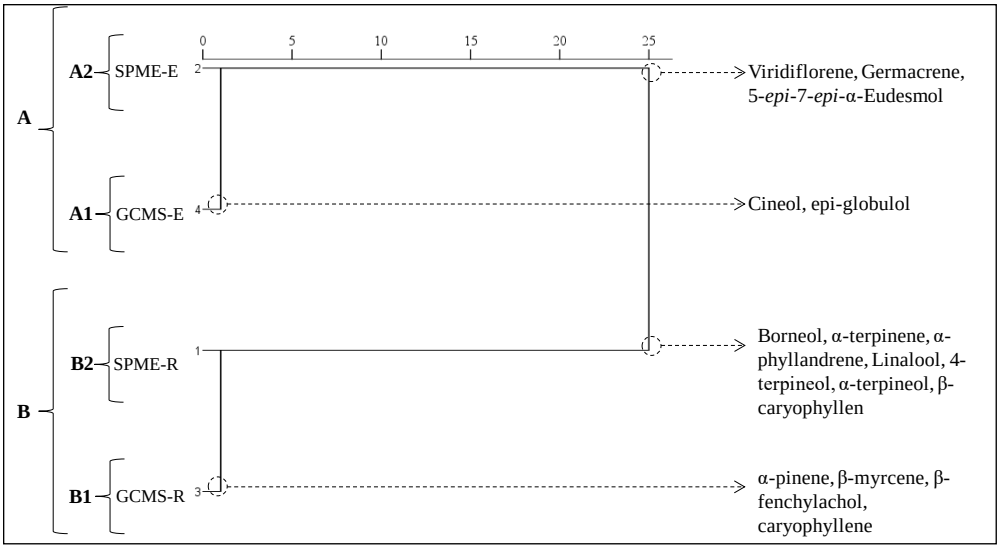

Fig. 2. Dendrogram obtained by hierarchical duster analysis based on Euclidean distance between *Eucalyptus viminalis* and *Rosmarinus officinalis* essential oil compounds and analytical technique.

Group B was positively correlated with axis 1 and was further divided into two subgroups (B1 and B2) with dissimilarity < 5. Their separation was mainly due to axis 2. Subgroup B1 was constituted by essential oils of *R. officinalis* analysed by means of GC-MS. This essential oils contained the highest percentages of α-pinene (0.954%), β-myrcene (0.909 %), β-fenchylachol (0.958 %) and caryophyllene (1 %). In addition, subgroup B2, made up of *R. officinalis* essential oils analysed by SPME was characterized by borneol (0.922 %), α-terpineol (0.976%), α-phyllandrene (0.870%), linalool (0.945%), 4-terpineol (0.859%) and β-caryophyllene (1%).

PCA results showed that the differences observed between results obtained with the two different analytical techniques could be largely ascribed to the ability of a technique to identify more components. Results showed that in both techniques, the oils were identified 47 different constituents, while by SPME 43 were identified in the headspace (Table 2).

Fumigant toxicity bioassays.

A dose-response relationship was determined for *R. officinalis* and *E. viminalis* EO applied to fifth instar larvae (Fig. 3, Table 3) and fresh eggs (Fig. 4, Table 3) of *E. ceratoniae* and *E. kuehniella* respectively.

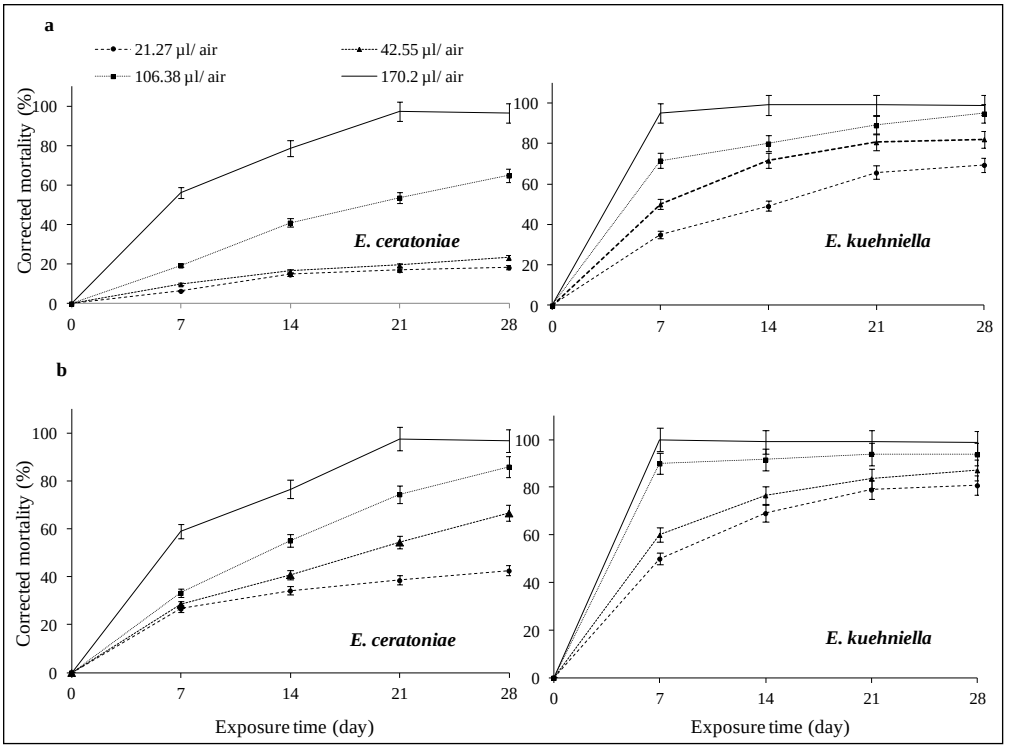

Fig. 3. Corrected mortality (%) of *Ectomyelois ceratoniae* and *Ephestia kuehniella* larvae exposed for various periods of time to EOs from *Rosmarinus officinalis* (a) and *Eucalyptus viminalis* (b). Data represent the means and standard error of the means.

Larvicidal potential.

Both *R. officinalis* and *E. viminalis* oils were toxic to *E. ceratoniae* and *E. kuehniella* fifth instar larvae (Fig. 3, Table 3). Preliminary tests showed that the insecticidal activity varied with plant species, oil concentration and exposure time. With *R. officinalis* essential oils, 100% mortality of larvae was achieved after exposure periods of 14 and 21 days for *E. ceratoniae* and *E. kuehniella* respectively, compared to 7 and 21 days when exposed to *E. viminalis* oils at a concentration of 170.2 µl/l air. Oils were more toxic to *E. kuehniella* than to *E. ceratoniae* (Fig. 3). The essential oils of *E. viminalis* was proved to be more effective than *R. officinalis* essential oils with larval mortalities of 45.9 and 82.0% compared to 21.7 and 70.7% achieved with *R. officinalis* oils at the lowest concentration (21.27 µl/l air), against *E. ceratoniae* and *E. kuehniella* (Fig. 3). These findings are confirmed with Probit analysis which also showed *E. viminalis* oils to be more toxic than *R. officinalis* oils. LC_{50} values of *E. viminalis* oils ranged between 12.92 and 25.8 µl/l air compared to 12.47 and 33.05 µl/l air with *R. officinalis* oils for *E. Kuehniella* and *E. ceratoniae* respectively (Table 3).

Ovicidal potential.

The mortalities of *E. ceratoniae* and *E. kuehniella* eggs (nonhatched eggs) exposed to various concentrations from *R. officinalis* and *E. viminalis* essential oils are indicated in Fig. 4.

Table 3. LC_{50} values of *Rosmarinus officinalis* and *Eucalyptus viminalis* essential oils against *Ectomyelois ceratoniae* and *Ephestia kuehniella* larvae and eggs

Pest	Plant	Property	Larvae (L5)	Eggs (0-24h)
Ectomyelois ceratoniae	***Rosmarinus officinalis***	LC_{50} (µl/l air)	33.05	17.65 (12.41-21.28)
		Slope±SEM	3.18±1.09	2.00±0.30
		Degrees of freedom	6	6
		X^2	13.55	2.18
	Eucalyptus viminalis	LC_{50} (µl/l air)	25.80 (20.45-30.83)	15.469 (11.21-19.46)
		Slope±SEM	2.33±0.25	1.42±0.30
		Degrees of freedom	6	6
		X^2	4.99	3.19
Ephestia kuehniella	***Rosmarinus officinalis***	LC_{50} (µl/l air)	12.47	60.65 (52.30-69.14)
		Slope±SEM	1.34±0.42	3.13±0.29
		Degrees of freedom	6	6
		X^2	6.75	4.04
	Eucalyptus viminalis	LC_{50} (µl/l air)	12.92 (7.78-17.24)	21.45
		Slope±SEM	2.32±0.37	1.80±0.43
		Degrees of freedom	6	6
		X^2	0.65	6.81

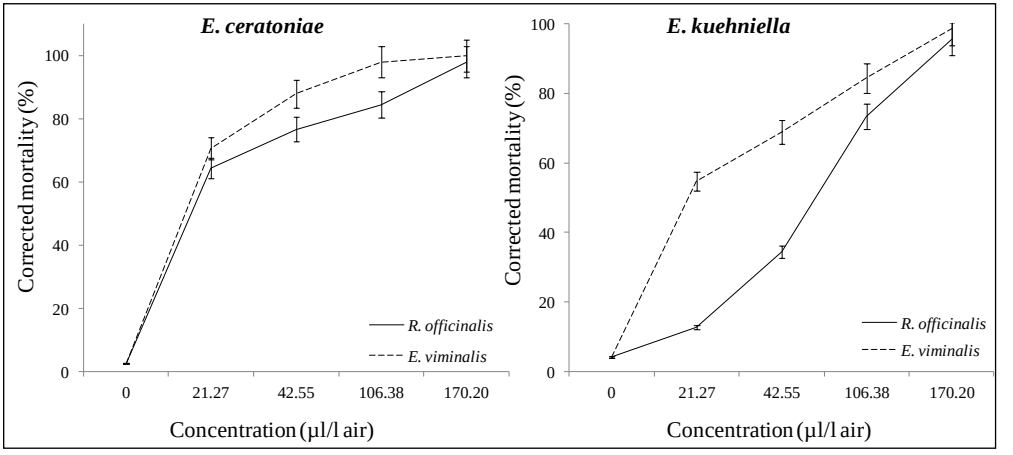

Fig. 4. Effect of fumigation with *Rosmarinus officinalis* and *Eucalyptus viminalis* essential oils at different concentrations on egg mortalities of *Ectomyelois ceratoniae* (a) and *Ephestia kuehniella* (b). Data represent the means and standard error of the means.

Both plant oils exhibited an ovicidal activity against eggs of both moth species, but *E. viminalis* proved to have the highest ovicidal potential compared to *R. officinalis*. At the highest concentration (170.2 µl/l air), *E. viminalis* oils accomplished respectively 100% and 98.7% of mortality for *E. ceratoniae* and *E. kuehniella* versus 98 and 95.7% with *R. officinalis* oils. Contrary to results observed in the study on larvicidal toxicity, the LC_{50} bioassay results showed that *E. ceratoniae* eggs were more sensitive than *E. kuehniella.* The corresponding LC_{50} values ranged between 15.47 and 17.65 µl/l air for *E. ceratoniae* and 21.45 and 60.65 µl/l for *E. kuehniella* treated with *E. viminalis* and *R. officinalis* respectively (Table 3). Nevertheless, for both insecticidal activities (larvicide and ovicide), *E. viminalis* was more toxic. Probit analysis showed that *E. viminalis* oils were more toxic than *R. officinalis* oils against both *E. ceratoniae* and *E. kuehniella* larvae. Moreover, the LC_{50} bioassay results also showed a higher efficacy for *E. viminalis* oils compared to *R. officinalis*.

Regression model.

The relationships between larval as well as egg mortalities and fumigation variables are shown in Fig. 5. The fitted regressions were highly significant for larvae (*E. ceratoniae: F* = 294.715, *P* = 0.001 explaining 88.4% of the total variance and *E. kuehniella: F* = 155.544, *P* = 0.001 explaining 80.1% of the total variance) and eggs mortalities (*E. ceratoniae: F* = 48.124, *P* = 0.001 elucidating 78.1% of the total variance and *E. kuehniella: F* = 281.662, *P* = 0.001 explaining 95.4% of the total variance). Eggs mortality also varied according to insect species. *E. ceratoniae* eggs were more sensitive to both oils (R^2 = 0.781) than *E. kuehniella* (R^2 = 0.954). Contrarily, E. *kuehniella* larvae were more susceptible (R^2 = 0.801) than *E. ceratoniae* larvae (R^2 = 0.884) (Fig. 5). These results were confirmed by the LC_{50} values (Table 2) and are in accordance with those reported by Delkhoon et al. (2013) and Eliopoulos et al. (2007).

Effects on adults' emergence.

The effect that EO fumigation of egg and fifth instar larvae has on adult emergence of both pyralid species is reported in Fig. 6. Both plant essential oils significantly influenced adult emergence when eggs or larvae were treated.

Results indicated that when eggs of *E. ceratoniae* were treated at the concentration of 106.38 µl/l air of *R. officinalis* oils, only 3% of larvae survived to adults. No larvae completed their development after being treated with *E. viminalis* oils. However, at this same concentration, 7.3 and 1% of adult emergence were obtained for *E. kuehniella* with *R. officinalis* and *E. viminalis* oils, respectively.

Higher concentrations of essential oils applied to larvae reduced adult emergence. There was a significant negative correlation between larval survival and dose. Adult emergence after larval treatments with *R. officinalis* and *E. viminalis* essential oils reduced by 78.15 and 88.72% for *E. ceratoniae* and 96 and 94.77% for *E. kuehniella* compared to the control treatments (Fig. 6).

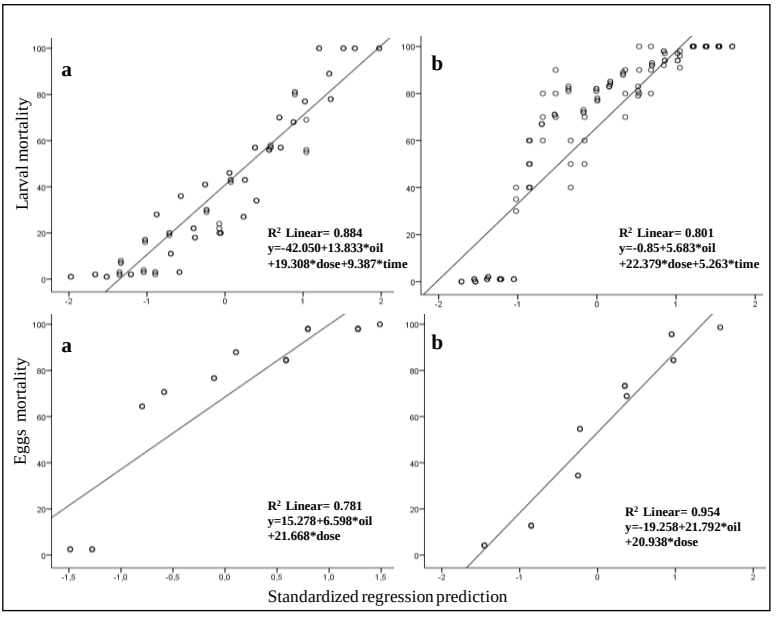

Fig. 5. The relationship between larval and egg mortalities of *Ectomyelois ceratoniae* (a) and *Ephestia kuehniella* (b) and fumigation parameters (essential oils, concentration and exposure time).

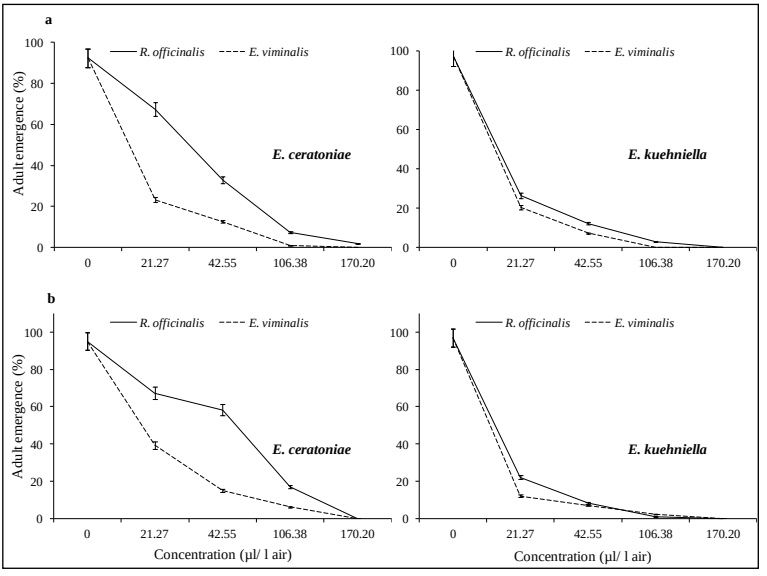

Fig. 6. Percentage adult emergence 60 days after fumigation of eggs (a) and larvae (b) of *Ectomyelois ceratoniae* and *Ephestia kuehniella* exposed to various concentrations of *Rosmarinus officinalis* and *Eucalyptus viminalis* essential oils. Data represent the means and standard error of the means.

Effect of essential oils fumigation on the duration of the developmental cycle.

The duration of the development was significantly increased for all tested concentrations (Table 4). A dose-response relationship was determined for both insect species and EO applied.

E. kuehniella was more susceptible than the *E. ceratoniae* to both plant oils. In all cases, *E. viminalis* caused a longer developmental period than *R. officinalis* for both insects. With the application of a concentration of 42.55 µl/l air, the developmental duration of *E. ceratoniae* were 42.85 and 40.7 days for *E. viminalis* and *R. officinalis* respectively, compared to 36.33 days for the control. However, for *E. kuehniella*, durations were 33 and 30 days after exposure to *E. viminalis* and *R. officinalis* oils respectively compared to 27 days for the control (Table 4). Our study showed a delay in the developmental period of both moth species after application of essential oils compared to the control. For *E. kuehniella*, the delay ranged between 6 to 14 days with *E. viminalis* oil whereas *R. officinalis* treatments resulted in a delay of 3 to 10 days in development. *E. ceratoniae* larvae took 4 to 6 days longer to reach adulthood when they were fumigated with *E. viminalis* oil (adults emerged only from sub-lethal concentrations 21.27 and 42.55 µl/l air) while *R. officinalis* oils caused delays of 2 to 8 days.

Table 4. Effect of egg fumigation with *Eucalyptus viminalis* and *Rosmarinus officinalis* essential oils on the developmental cycle durations of *Ephestia kuehniella* and *Ectomyelois ceratoniae* (mean ± SE; n = 5 replicates each of 30 eggs)

Pest	Cycle duration (day)					
	Concentration (µl/ air)	**0 (Control)**	**21.27**	**42.55**	**106.38**	**170.20**
Ephestia kuehniella	***Eucalyptus viminalis***	27±0.66 A,a	33±1 B,b	38±0.66 B,c	41±0.33 B,d	-
	Rosmarinus officinalis	27±0.66 A,a	30±0.33 A,b	33±1.33 A,c	35±1.66 A,d	37±1.23 e
Ectomyelois ceratoniae	***Eucalyptus viminalis***	36.33±1.50 A,a	40.32±0.89 B,b	42.85±1.5 B,c	-	-
	Rosmarinus officinalis	36.33±1.50 A,a	38.25±1.2 A,b	40.66±1.22 A,c	44.22±0.45 d	-

For each insect species, for each oil, within columns, comparisons were made between concentrations (letter in lowercase). Means followed by the same letters were not statistically different according to Duncan test ($P < 0.05$). For each insect species, for each concentration, within rows, comparisons were made between essential oils (letters in uppercase). Means followed by the same letters were not statistically different according to Student test ($P < 0.05$).

DISCUSSION

Our study showed that 1,8-cineol is the most common major compound of *R. officinalis* and *E. viminalis* EO. In previous investigations, 1,8-cineol was also reported as the main constituent of *E. viminalis* oils (Alzogaray et al. 2011; Maghsoodlou et al. 2015). Likewise, 1,8-cineol and α-pinene, the major constituents of *R. officinalis* in our

analysis, have also been reported as the most prominent compounds in *R. officinalis* in other studies (Akbari et al. 2015; Roomiani et al. 2017).

Indeed, great quantitative and qualitative differences have been found in the chemical composition of both analysed samples depending on the technique of analysis. In this context, results from this study are supported by those reported by Pirbalouti et al. (2013) who ascribed the qualitative and quantitative variation in essential oil components to differences in the analytic methods used.

Concerning our PCR results, they are similar to those reported by Yang et al. (2010) who showed that the quantity of the chemical composition of *Pinus armandii* essential oils depended on the techniques used for analysis. It is also in agreement with the findings of Sellami et al. (2011) that qualitative and quantitative differences found in the essential oil content depend on the methods of extraction and analysis.

The findings of larvicidal potential are in accord with results reported by Mediouni-Ben-Jemâa et al. (2012) who indicated that the essential oils of *E. camaldulensus* and *E. leucoxylon* have 20.6% and 17.6% 1,8-cineole and cause 86.6% and 80.3% larval mortality of *E. ceratoniae*, respectively. High rich cineol oils from *E. dumosa* (79.44 %) and *E. transcontinentalis* (82.82 %) are toxic to final instar larvae of *E. ceratoniae* (Khemira 2012). Mortalities of 100% and 65% at a concentration of 142.86 µl/l air for these respective cineol oils were reported. Furthermore, Yazdani et al. (2013) showed that *R. officinalis* essential oils containing a high percentage of 1,8-cineole (20.02%) resulted in high mortality of *Glyphodes pyloalis* with LC_{30} and LC_{50} values of 1.18% and 1.59% (v/v), respectively. *Lavandula stoechas* and *R. officinalis* EO have also strong larvicidal activity against *Orgyia trigotephras* (Badreddine et al., 2015).

For the ovicidal potential, at all concentrations, *E. kuehniella* eggs were more tolerant to EO fumigation than larvae, a trend also observed by Eliopoulos et al. (2015) with EO from *Ocimum basilicum* and *Mentha spicata* against *E. kuehniella* and *Plodia interpunctella*. In this context, Shadia et al. (2007) showed that the EO of *Ocimum americanum* reduced the hatching rate of *Agrotis ipsilon*. Tunç et al. (2000) reported that the EO of *E. globulus* has an ovicidal effect against the Mediterranean flour moth. In addition, Erler (2005) showed that 1,8-cineol and thymol had fumigant activity against eggs and adults of *Tribolium confusum*, and eggs and larvae of *E. kuehniella*.

This study revealed that larvae of both species are more tolerant to essential oil vapors than eggs. Amri et al. (2014) demonstrated that *R. officinalis* essential oils reduced adult emergence of *E. ceratoniae* issued from treated eggs to 49.9% after 10 days of exposure and it caused 55% of fifth instar larval mortality at the dose 8µl/ml after 24 h of exposure. Essential oils of *E. Transcontinalis* only resulted in 89% mortality of *E. ceratoniae* eggs after exposure to a concentration of 57.14 µl/l air for 10 days, while 37% of larval mortality was obtained for the same concentration after 12 days of exposure (Khemira 2012).

As with chemical insecticides, the larval tolerance to essential oils could be explained by three primary mechanisms. These are dehydrochlorination, microsomal detoxication and reduced penetration (Georghiou 1972); enzymes (Esterase, Cytochrome P450 and Glutathion S-

transférase) (Ishaaya 1993) as well as relationships that may exist between the insect's physiology and the mode of action of the active ingredient. These volatile compounds act directly on the nervous system of insects which is primitive for larvae and explains their resistance to essential oils (Majdoub et al. 2014). Generally the egg stage has been reported to be tolerant to essential oil vapour due to the fact that it is considered as a quiescent stage (Ducom 1996).

Adult emergence reduction after issus from larval treatments with *R. officinalis* and *E. viminalis* essential oils is in accordance with the trend demonstrated by Karaborklu et al. (2011) for *E. kuehniella*.

Our results pointed out a delay in the developmental period of both moth species after application of essential oils compared to the control. These outcomes agree with those reported by Vasantha-Srinivasan et al. (2016) proved that larval and pupal duration of *Spodoptera litura* increased after fumigation with essential oils of *Piper betle*. The EO of *Vitex trifolia* and *V. agnus-castus* caused extended larval and pupal periods, higher larval mortality and adult deformity and decreased adult emergence as well as reduced fecundity and fertility of *Spilosoma obliqua* (Tandon and Mittal 2008). Deka and Singh (2005) ascribed this delay in development to interference of EO with theapolytic and molting processes in insects.

To summarize, this study indicates that essential oils of *R. officinalis, E. viminalis* and their components (1,8-cineol and α-pinene) have potent toxicity against larvae of *E. ceratoniae* and *E. kuehniella* that could be developed as control agents against these pests. These findings proved that Tunisian *E. viminalis* and *R. officinalis* EO can be used as alternatives to chemical fumigants for treating stored-date commodities. Specially, that residues of essential oil pesticides are actually beneficial to human health (Huang et al. 2011). The effect of high doses of these EO on date quality should, however, be further investigated as well as the bioactivity of individual compounds in these Eos that could be used in Integrated Pest Management Programs. Formulations to ameliorate their efficacy and stability and to reduce their cost should also be determined.

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RESUME

Yousfi S., Haouel-Hamdi S., Bessi H., Assoudi C., Elimem M., Messaoud C., Flamini G. et Mediouni-Ben Jemâa J. 2019. Variations de la composition des huiles essentielles et leurs potentiels en tant que fumigants contre les ravageurs des dattes stockées *Ectomyelois ceratoniae* et *Ephestia kuehniella*. Tunisian Journal of Plant Protection 14 (1): 33-53.

Les huiles essentielles de *Rosmarinus officinalis* et d'*Eucalyptus viminalis* ont été analysées à l'aide de la technique de micro-extraction en phase solide (MEPS) et celle de chromatographie en phase gazeuse couplée à la spectrométrie de masse (CPG-SM) pour évaluer leur toxicité contre les œufs et les larves du dernier stade d'*Ectomyelois ceratoniae* et d'*Ephestia. Kuehniella*. L'analyse en composantes principales (ACP) et l'analyse en clusters hiérarchiques (ACH) ont révélé des différences quantitatives et qualitatives dans la composition de l'huile essentielle en fonction des espèces végétales et de la

technique utilisée dans l'analyse. Le 1,8-cinéole, l'α-pinène, le β-pinène et le p-cymène ont été identifiés comme étant les principaux composés communs. Les activités ovicides et larvicides dépendent fortement des espèces d'insectes et des huiles. L'huile essentielle d'*E.viminalis* était plus efficace que celle de *R. officinalis*. Lors d'essais biologiques sur des larves, les valeurs de CL_{50} étaient respectivement de 25,80 et de 33,05 µl/l d'air pour *E. ceratoniae* contre 12,92 et 12,47 µl/l d'air pour *E. kuehniella*. De plus, l'activité ovicide était plus faible chez les œufs d'*E. Kuehniella* que chez ceux d'*E. ceratoniae*. Ce travail défend clairement l'intérêt de l'efficacité des huiles essentielles à la fois comme insecticides à effets ovicides et larvicides contre les ravageurs des dattes stockées.

Mots-clés : CPG-SM, *Ectomyelois ceratoniae, Eucalyptus viminalis,* Huiles essentielles, MEPS, *Rosmarinus officinalis*

ملخص

يوسفي، سندس وسمية حوال-حمدي وهيثم بسي وشريفة أسودي ومحمد ليمام وشكري مسعود وجيدو فلاميني وجودة مديوني-بن جماعة. 2019. إختلاف في تركيبة الزيوت العطرية وفاعليتها كمبيدات تبخير ضد فراشتيْ التمور المخزنة *Ephestia kuehniella* و *Ectomyelois ceratoniae*.

Tunisian Journal of Plant Protection 14 (1): 33-53.

تم تحليل الزيوت العطرية لنبتتيْ *Rosmarinus officinalis* و*Eucalyptus viminalis* باستخدام تقنية الاستخلاص الجزئي (SPME) وتقنية قياس الغازالكروماتوغرافي (GC-MS) وتقييم فاعليتها على بيض ويرقات الطور الأخيرمن حشرتيْ *Ectomyelois ceratoniae* و*Ephestia kuehniella*. كشف تحليل المكونات الرئيسية (PCA) وتحليل الكتلة الهرمية (HCA) أن هناك إختلافات كمية ونوعية في تكوين الزيوت حسب نوع النبتة والتقنية المستخدمة في التحليل. تم تحديد 1,8 cineole و β-pinene و α-pinene و p-cymene كمركبات رئيسية مشتركة. تتأثر النشاطات المبيدة للبيض واليرقات كثيرا بأنواع الحشرات والزيوت المستخدمة. كانت الزيوت العطرية لنبتة *E. viminalis* أكثرفاعلية مقارنة بزيوت النبتة *R. officinalis*. خلال الاختبارات البيولوجية لليرقات، كان تقييم LC_{50} يعادل على التوالي 25,8 و 33,05 مكل/ل هواء لدى نبتة *E. Ceratoniae* مقابل 12,92 مكل/ل هواء لدى نبتة *E. kuehniella*. كان نشاط الزيوت أقل تأثيرا على بيض *E. kuehniella* منه على *E. ceratoniae*. يدافع هذا العمل بوضوح على فاعلية الزيوت العطرية كمبيدات حشرية ضد بيض ويرقات آفات التمرالمخزن.

كلمات مفتاحية: تقنية قياس الغاز الكروماتوغرافي، تقنية الاستخلاص الجزئي، زيوت عطرية، *Ectomyelois ceratoniae*، *Eucalyptus viminalis*، *Rosmarinus officinalis*

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Assessment of Thrips Damage in Citrus Orchards in Tunisia

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ABSTRACT

Belaam-Kort, I., and Boulahia-Kheder, S. 2019. Assessment of thrips damage in citrus orchards in Tunisia. Tunisian Journal of Plant Protection 14 (1): 55-68.

Before the 2000s, damage produced by thrips have been considered rare or absent in Tunisian citrus orchards. However, during these ten last years and since the first report of the species *Pezothrips kellyanus*, fruit scars attributed to thrips are increasingly being reported. This study aimed to assess thrips damage on citrus and susceptibility of different citrus species and orange varieties to these pests. The relationship between thrips damage and frequency of pesticide use was also studied. The assessment of thrips damage was achieved by visual observation of 200 to 1000 mature fruits from each of the 101 visited orchards located in different regions in Cap-Bon, Bizerte and Mornag during December, January and February from 2015 to 2017. The examined citrus species and orange varieties were Lemon, Bergamot, Grapefruit, Clementine, Mandarin, Navel, Maltaise, Valentia Late and Double Fine oranges. Frequency of insecticide treatments and type of active ingredients in visited citrus orchards were noted in relation with damage rate. Fruit scars caused by thrips were 20% on average for all citrus species and orange varieties. Bergamot and Lemon seem to be the most sensitive citrus species to *P. kellyanus*, while Maltaise and Navel oranges were the most orange varieties affected by marbling caused by other thrips species. Data provided by 94 citrus orchards showed that damage increases with the rise of the number of pesticide applications per year. In fact, thrips are currently common in citrus orchards in Tunisia. However, their harmfulness may become more severe as the management of citrus pests is based mainly on broad-spectrum insecticides that eliminate the beneficial insects and could enhance thrips populations. The introduction of new invasive species could also contribute to increase economic importance of thrips.

Keywords: Chemical treatments, citrus, *Frankliniella occidentalis*, marbling, *Pezothrips kellyanus*, ring, sensitivity, Thrips, *Thrips major*

Thrips are an increasing threat to citrus production in the world and in the Mediterranean region (Marullo and De Grazia 2012). Many species are known for their feeding damage on flowers, leaves and fruits of citrus on which they cause silvery scars on the tissues around the calyx or other lesions located in the lateral parts or in the basis. The most important thrips pests on citrus are *Scirtothrips citri* in California (Tanigoshi et al.1985), *S. aurantia* in South Africa (Grove et al. 2000), *S. dorsalis* in East Asia (Masui 2007), *Pezothrips kellyanus* in New Zealand (Blank & Gill 1996), Australia (Smith et al. 1997), and in some Mediterranean countries such as Italy (Conti et al. 2001), Spain (Navarro-Campos et al. 2011) and Cyprus (Vassiliou 2007). The species *Chaetanaphothrips orchidii* is also known

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as an important citrus pest in Florida and Argentina (Goane et al. 2013). It was recently reported in Spain causing rind blemishes damaging 70% of citrus fruits in some orchards (Campos-Rivela et al. 2017).

In Tunisia, an inventory of thrips in citrus orchards showed 21 species but only one is known as a pest of citrus: *P. kellyanus*, that was first detected in citrus groves in 2008 (Trabelsi and Boulahia-Kheder 2009). In 2012, it represented 3.3% of the thrips fauna causing less than 2% of damage in 22 citrus orchards visited in the regions of Mornag, Bizerte and Cap-Bon (Belaam-Kort and Boulahia-Kheder 2017a). The polyphagous species *Thrips major* and *Frankliniella occidentalis* were the most abundant in citrus orchards of Tunisia with 90 % and 2.6 % of thrips fauna respectively and are considered as potential pests of citrus (Belaam and Boulahia-Kheder 2012; Belaam-Kort and Boulahia-Kheder 2017a). This study dealing with damage assessment follows determination of thrips species and their abundance in citrus orchards (Belaam and Boulahia-Kheder 2012; Belaam-Kort and Boulahia-Kheder 2017a).

However, there is a difficulty with assessment of thrips damage because they can be confused with many abiotic and biotic agents that cause scarring very similar to those produced by thrips. The wind, for example, is an important abiotic agent that makes young fruits rub against branches, leaves and twigs. This leaves scars on fruits that can be superficial in the case of low wind or more severe in case of strong dust or sand wind causing fruit abrasions. Other abiotic agents are phytotoxicity, sunburns, and hail that may cause spotting and yellow to brown leathery scars on fruits (Dreistadt 2012; Garcia-Marí and Palacios 1999; Grafton-Cardwell et al. 2003). Moreover, some other insects produce silvery or brownish scars that may be confused with those caused by thrips. For example, the larva of Tortricidae (Lepidoptera) feed under the calyx causing circular brown scars around the calyx similar to that caused by citrus thrips, but deeper. The larvae of the citrus leaf miner *Phyllocnistis citrella*, although not very common on fruits, feed in pale tunnels beneath the surface of rinds and green stems. Besides caterpillars, other chewing insects like grasshoppers or crickets (Orthoptera) can cause on young fruits a single corky and deep scar around the midsection of fruit. Leafhoppers can also cause roundish discoloring on fruits by puncturing and feeding on rind cells that can be confused with scars of citrus thrips (Dreistadt 2012; Garcia-Marí and Palacios 1999; Grafton-Cardwell et al. 2003).

Hence the interest of this study which aims to evaluate accurately thrips damage after being able to distinguish thrips symptoms from others that are very similar; to determine the sensitivity of citrus species and orange varieties to thrips, and to correlate the frequency of chemical treatments with thrips damage.

MATERIALS AND METHODS

Experimental sites.

This study was carried out from 2015 to 2017, in 101 citrus orchards; 44 located in different regions in the governorate of Bizerte (Ghar El Melh, El Alia and Ras-Jebel), 21 in the region of Mornag and 36 in the peninsula of Cap-Bon situated in Beni-Khaled, Menzel-Bouzelfa, Takelsa and Bou-Argoub (Table 1).

Table 1. Geographic coordinates of the visited localities

Regions	Localities	Geographic coordinates
Bizerte	GharMelh	37° 10′ 26″ N, 10° 11′ 31″ E
	El Alia	37° 10′ 08″ N, 10° 02′ 00″ E
	Ras-Jebel	37° 12′ 54″N, 10° 07′ 26″ E
Cap-Bon	Beni-Khaled	36° 38′ 57″ N, 10° 35′ 29″ E
	Menzel Bouzelfa	36° 41′ N, 10° 35′ E
	Takelsa	36° 47′ N, 10° 38′ E
	BouArgoub	36° 32′ N, 10° 33′ E
Mornag	Mornag	36° 40′ 51″ N, 10° 17′ 25″ E

Thrips damage assessment.

Assessment of thrips damage was achieved on mature fruits during harvest. In each orchard, thrips damage was assessed by randomly selecting samples of 200 to 1000 mature fruits for each species and variety. The examined crop fruits were those of the citrus species Lemon, Bergamot, Grapefruit, Clementine, Mandarin, and of the orange varieties Navel, Maltaise, Valentia Late and Double Fine (Table 2).

Table 2. Number of fruits and orchards of the citrus species and orange varieties checked for thrips damage

Species/Varieties	Number of orchards	Number of sampled fruits
Lemon	37	6700
Bergamot	15	3200
Grapefruit	25	4500
Navel Orange	91	77000
Maltaise Orange	69	48300
Mandarin	42	8400
Clementine	22	6600
Valentia Late	3	600
Double fine	7	1400

The rate of damage for each species and variety was calculated according the following formula: Thrips damage (%) = Number of damaged fruits × 100 / Total of fruits.

Thrips damage and frequency of chemical treatments.

Frequency of chemical treatments and pesticides employed in 94 citrus orchards visited were noted and were correlated with the level of observed thrips damage. Statistical analyses were performed using the statistical software XLSTAT Pearson Edition (Addinsoft 1995-2014).

RESULTS

Scars confused with those caused by thrips.

As pointed out in the introduction, thrips damage evaluation was difficult because there were many abiotic and biotic agents that may cause scars very similar to those produced by thrips. The Fig. 1 shows some of the most common types of physical (Fig. 1a,b,c,e), chemical (Fig. 1d) or biotic symptoms (Fig. 1f,g,h,i) observed on citrus fruits in visited orchards.

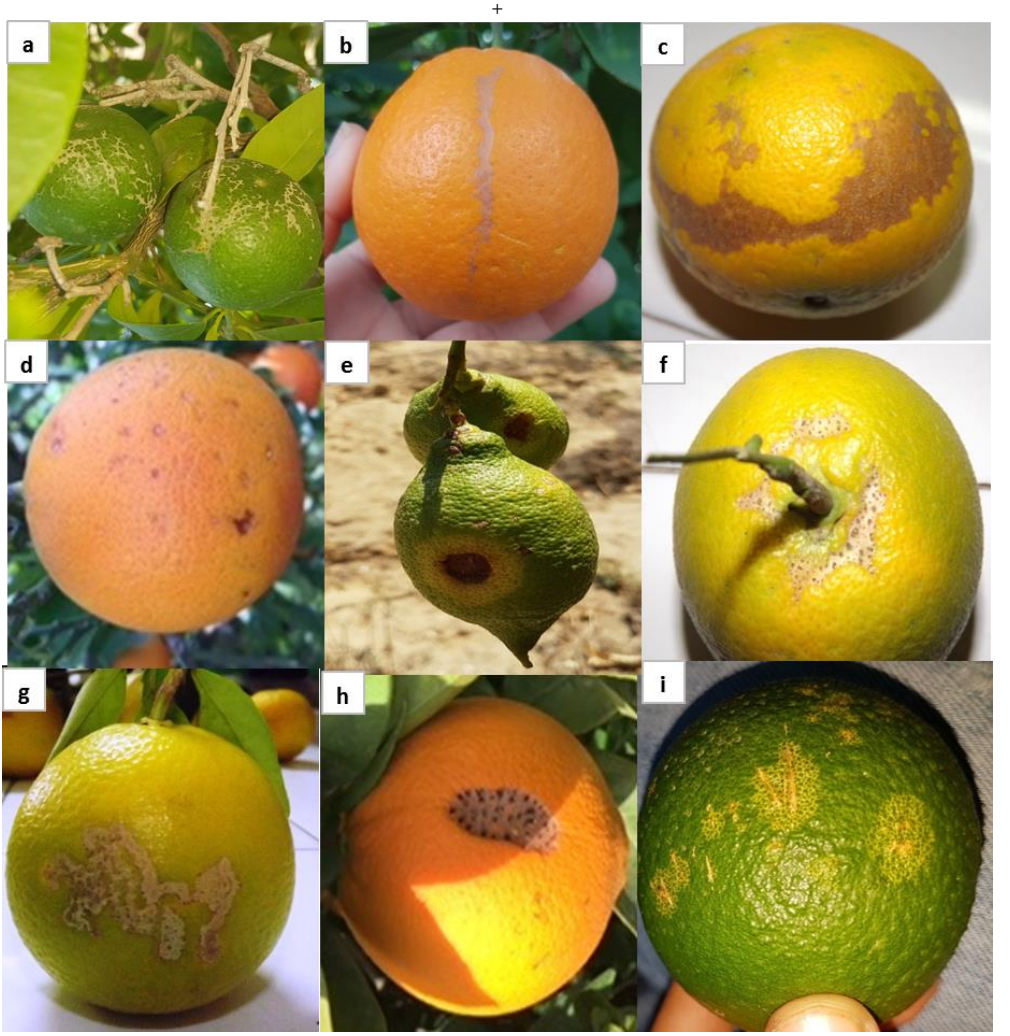

Fig. 1. Citrus symptoms that can be confused with thrips scars, a and b: Scars resulted from rubbing between fruit and other plant organ caused by wind; c: Strong dust wind effect; d: phytotoxicity; e: sunburn effect; f: damage probably of Amorbia larva; g: tunnels caused by the citrus leaf-miner; h: scars caused by chewing insects probably Orthoptera; i: scars of leafhoppers.

Thrips damage.

Two types of thrips damage were observed in the 101 visited citrus orchards: 1/ a silvery partial or complete ring at the base of the fruit or fruit peduncle (Fig. 1a,b) and 2/ marbling more or less developed (Fig. 2c).

The ring depreciation is caused specifically by *P. kellyanus*. It affects 12.17% of fruits for all citrus species and orange varieties (Table 3). Regarding the marbling scars, they are probably produced by *Frankliniella occidentalis* and *Thrips major*, which are major species, found in citrus orchards (on flowers and fruits) in Tunisia (Belaam-Kort and Boulahia-Kheder 2017). They were estimated on 5.13% of fruits for all citrus species and orange varieties (Table 3).

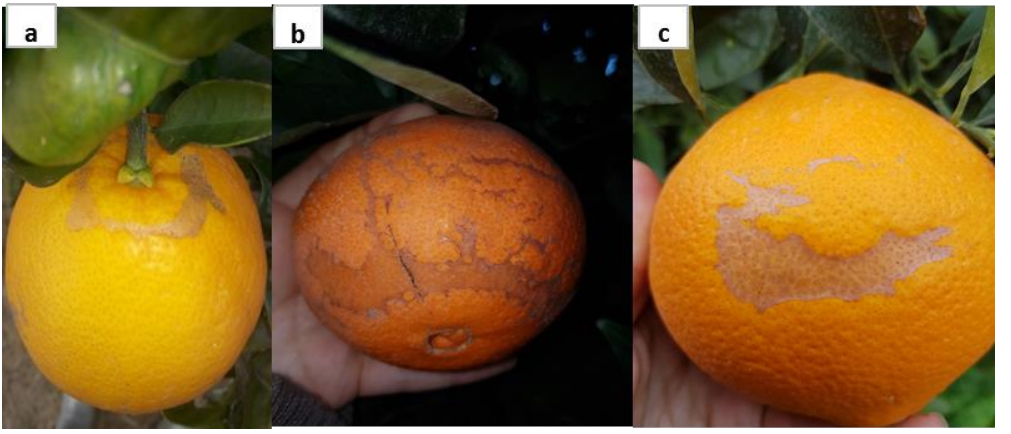

Fig. 2. Thrips damage, a: ring around the peduncle of a fruit; b: ring at the base of a fruit; c: marbling on Navel Orange.

Table3. Thrips damage on citrus fruits for all checked citrus species and orange varieties

Parameter	Type of damage		Total of checked fruits
	Marbling	Ring	
Total of fruits	8039	19090	156700
Damaged fruits (%)	5.13	12.17	100
[Min-Max] (%)	0.41-78.9	0.1-87.6	-

Sensitivity of citrus to thrips damage.

Fig. 3 shows that Bergamot and Lemon are the most sensitive species to *P. kellyanus* with 58.96% and 48% of damaged fruits respectively in all visited citrus orchards (Fig. 4a,b). Grapefruit, Navel orange, Clementine and Mandarin are less sensitive, with less than 1% of affected fruits (Fig. 4c). The Maltaise, Double Fine and Valencia Late oranges did not show any symptoms due to *P. kellyanus* (Fig. 3).

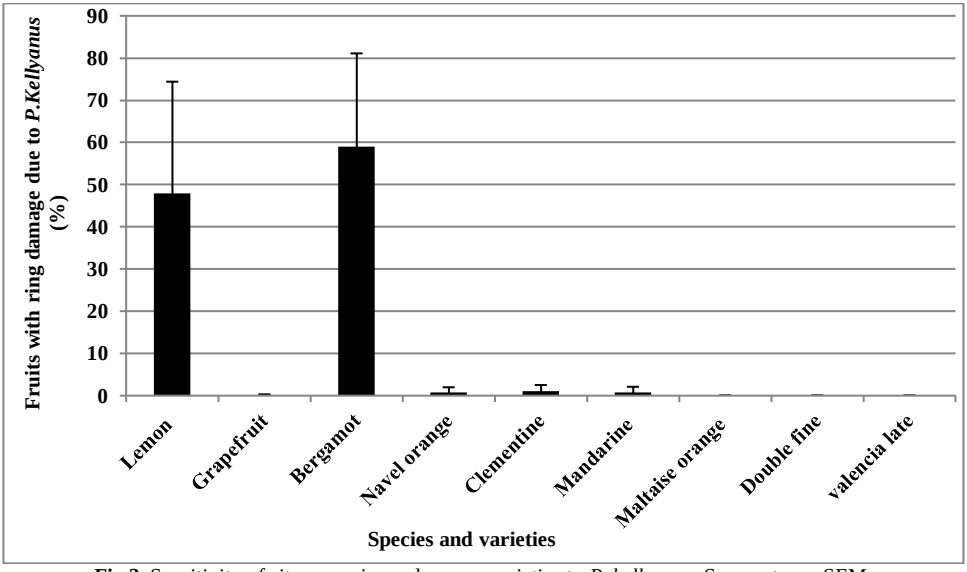

Fig 3. Sensitivity of citrus species and orange varieties to *P. kellyanus.* Segments are SEM.

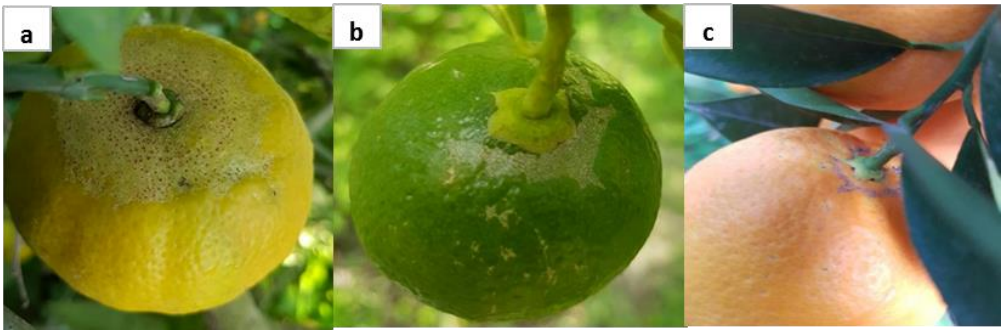

Fig. 4. Silvery ring caused by *P.kellyanus* on Bergamot (a), Lemon (b) and Navel orange (c).

Fig. 5 shows that Navel and Maltaise oranges are the most sensitive to marbling damages with 24.33 and 17% of damaged fruits respectively in all citrus visited orchards (Fig. 6a,b). Grapefruit, Lemon, Double fine orange, Clementine and Mandarin are less sensitive with less than 2% of affected fruits. Bergamot and Valencia Late oranges did not show any marbling (Fig. 5).

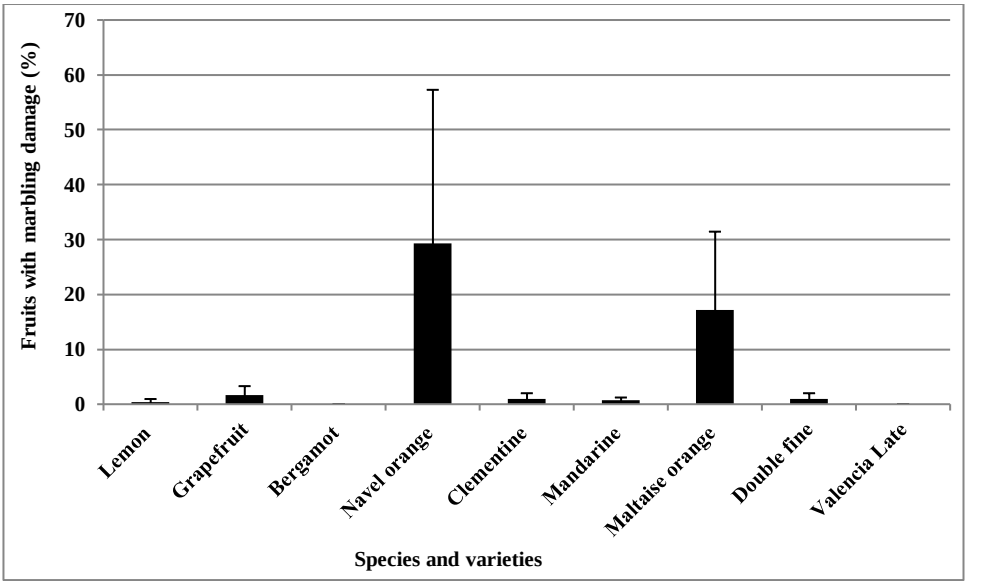

Fig. 5. Sensitivity of citrus species and orange varieties to marbling damage caused by thrips species different from *P. kellyanus*. Segments are SEM.

Fig. 6. Marbling on Navel (a) and Maltaise oranges (b)

Thrips damage and frequency of chemical treatments.

The frequency of chemical treatments in 94 citrus orchards visited in 3 regions of Tunisia (Bizerte, Mornag and Cap-Bon) was between 1 and 25 treatments per year. Among the surveyed orchards, only two organic orchards did

not receive any chemical treatments. Many insecticides, mostly Organophosphates (OP), were used in visited citrus orchards. About 83.78% of orchards were sprayed with malathion, methidathion, deltametrin, thiacloprid, acetamiprid and only 16.21% by organic products: spinosad, abamectin and azadiracthin (Table 4).

Table 4. Frequency of chemical treatments in the citrus orchards visited (n = 94)

Insecticide treatments (number/ year)	Scarred fruits (%)	Organic insecticides		Synthetic insecticides	
		Active ingredient	Sprayed orchards	Active ingredient	Sprayed orchards
<4	14%			Malathion	81
		Spinosad	17	Methidathion	69
4-10	25%	Abamectin	9	Deltametrin	53
		Azadiractin	22	+ Thiacloprid	
>10	48%			Acetamiprid	45

The correlation between damage level of thrips and frequency of chemical interventions shows that the infestation increases with high number of treatments used in citrus orchards (Table 4 and Fig. 6). Data shows a correlation (Pearson) positive (R = 0.836) and significant ($P < 0.01$) between the level of thrips damage and the number of phytosanitary treatments.

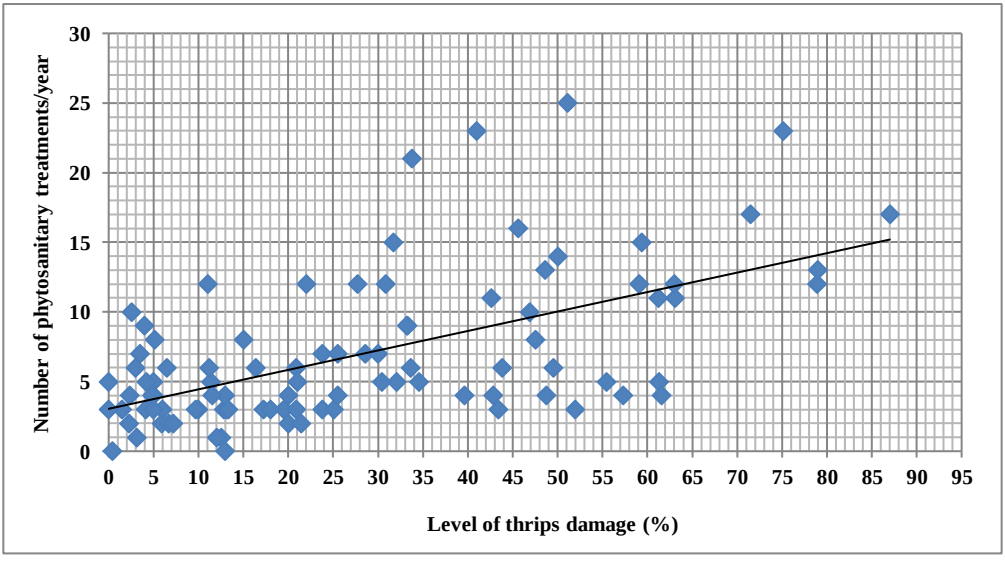

Fig. 6. Regression between thrips damage and frequency of chemical treatments in citrus orchards (n = 94)

DISCUSSION

In Tunisia, thrips were previously not considered as citrus pests. However, recently many farmers have complained about some scarring on citrus fruits that seems to be caused by thrips. This is why we considered it important to assess accurately thrips damage on citrus as well as to identify species living in citrus orchards. Several prospections had shown that 21 species were present in citrus orchards, either on citrus and/or herbaceous plants. The most abundant thrips were *F. occidentalis*, *T. major* and *P. kellyanus* (Belaam-Kort and Boulahia-Kheder 2017a). Regarding damage, field observations showed that scars caused by thrips can be easily confused with injuries caused by other biotic or abiotic agents. This difficulty already cited by other authors (Garcia-Marí and Palacios 1999; Grafton-Cardwell et al. 2003), has been overcome by repeated rigorous observations. The obtained results showed that in Tunisia, thrips symptoms are becoming more noticeable, as they affect about 18% of fruits for all surveyed citrus species and orange varieties. The percentage of injured fruits varies between 0.1 and 87.6%. Two types of fruit damage caused by thrips were observed in the visited citrus orchards: silver ring scars caused by *P. kellyanus* on 12.17% of all citrus species and orange varieties and marbling scars most probably caused by *T. major* or *F. occidentalis* on 5.13% of all citrus species and orange varieties. Bergamot and Lemon were the most sensitive to ring scars with 60 and 50% of damaged fruits, respectively; however, Navel and Maltaise oranges were the most affected by marbling scars with 25 and 17% of damaged fruits, respectively.

This work indicates that the level of thrips damage recorded in Tunisia for 2015-2017, close to 20% for all citrus species and orange varieties, should be considered with attention. Especially for the species *P. kellyanus*, an important citrus pest in some areas of the Mediterranean region, whose damage has increased compared to previous surveys. Indeed, in this study, *P. kellyanus* was detected in 41 citrus orchards over 101 visited, causing injuries on 12.17% of all citrus species and orange varieties, while in 2012, this species damaged no more than 2% of fruits (Belaam-Kort and Boulahia-Kheder 2017). The same results were observed in Turkey, where fruits damaged by thrips ranged between 3.8% and 9.1% depending on varieties (Elekcioğlu 2013). Regarding the specific symptoms of *P. kellyanus*, in 2009 then in 2013, Elekcioğlu (2013) and Teksam and Tunç (2009), reported that they did not exceed 10%. In contrast, in other countries such as Spain, Italy, Cyprus, Australia and New Zealand, the damage caused by *P. kellyanus* was significantly more severe, ranging between 20 and 80% (Baker 2016; Blank and Gill 1997; Conti et al. 2001; Navarro et al. 2010; Vassiliou 2007). Among the factors that increase *P. kellyanus* populations and injuries, is the presence of several alternative host plants in citrus agro-ecosystem that can sustain *P. kellyanus* breeding, as was observed in Spain (Navarro et al. 2013), Cyprus (Vassiliou 2010) and New Zealand (Froud et al. 2001) where *Jasminum* spp., *Lonicera* spp., *Gardenia jasminoides* and *Araujia sericifera* play a determinant role for thrips presence year-round. This is unlike in Tunisia where only 2 alternative host plants for *P. kellyanus* were found: *Jasminum officinale* and *Bunium pachypodium* (Belaam-Kort and Boulahia-Kheder 2017b). Regarding the sensitivity to *P. kellyanus*, Bergamot and

Lemon appear to be the most susceptible with respectively 58.96 and 48% of severe or fine scars on fruits. Grapefruit, Clementine, Mandarin citrus, and Navel, Maltaise, Double Fine, Valencia Late oranges, are much less sensitive to *P. kellyanus* with less than 1% of damaged fruits. Similar results were observed in other Mediterranean countries (Italy, Spain and Turkey) where Lemon was the most commonly attacked species by *P. kellyanus*, followed by orange, then by clementine (Conti et al. 2003; Elekcioğlu 2013; Navarro et Garcia-Mari 2017). Interestingly, based on these results, we see that Lemon is very sensitive to *P. kellyanus*, while it is resistant to the polyphagous fruit fly *Ceratitis capitata* (Bodenheimer 1951; Papachristos et al. 2009). Papachristos et al. (2009) explained the practical immunity of Lemon to *C. capitata* by the high percentage of acid in the Lemon pulp that acts as a protective agent. This component does not seem to be a deterrent factor for thrips. However, regarding the sensitivity/resistance of citrus to thrips, no much research has been conducted. Brodbeck et al. (2001) demonstrated that seasonal trends of *F. occidentalis* on tomato were correlated to the number of flowers per host plant as well as the concentrations of total nitrogen in flowers. Some authors suggested the difference in ovipositional preferences and the suitability of fruit skin for nymphal survival and development (Sreedevi and Rajulu 2008). Other researchers thought that the size of flowers and the presence of aromatic amino acids may play a role in the nutritional ecology of thrips (Brodbeck*et* al. 2001; Marullo 1998). Therefore, investigations on the morphological and biochemical basis of resistance to thrips for genotypes would be one of the promising future topics of research.

Regarding marbling, this depreciation is probably produced by *Frankliniella occidentalis* or *Thrips major*, species that are abundant and common in citrus orchards of Tunisia and living on citrus fruits and flowers (Belaam-Kort and Boulahia-Kheder 2017). Marbling affects about 5% of all citrus species and orange varieties, with a higher sensitivity in Navel and Maltaise oranges for which respectively 24 and 17% of injured fruits have been recorded in all visited citrus orchards. The other citrus species and orange varieties are less sensitive with less than 2% of affected fruits. Currently, this damage poses problems particularly to Navel and Maltaise varieties, since they occupy the first position in citrus production in Tunisia. Concerning the orange Maltaise that is the most exported variety, a percentage of 1% of thrips damage can reduce its marketability, especially that Tunisian citrus exportations are decreasing and lower than the quota set by the UE estimated to 34 000 t for Tunisia (Hassen Daly, personal communication 2018), and this is due to diseases and pests including thrips that affect the quality of citrus fruits.

In addition, this study demonstrates that thrips damage increases with increasing number of treatments used in citrus orchards and this may be explained by the fact that thrips are associated with the phenomenon of secondary pest outbreak. This phenomenon has been known to occur in response to a reduction or destruction of natural enemy populations, releasing the pest population from regulation (Dutcher 2007). This phenomenon could explain the increasing of other pests in Tunisia such as *Icerya purchasi* and *Planococcus citri*. For example, *I. purchasi* spreads the last years at a harmful level in citrus orchards most probably as a result of the

elimination of its natural enemies, especially *Rodolia cardinalis*, by the abusive use of chemicals to control the Mediterranean fruit fly. In fact, malathion which is the main insecticide used is known to be toxic to many beneficials such as *Cryptolaemus montrouzieri* and *Leptomastix dactilopii*, natural enemies of the mealybug *P. citri* (Abbes et al. 2018; Rahmouni et al. 2015).

This study shows that currently, the thrips damage in Tunisian citrus orchards is increasing. Hence, these pests require regular monitoring in order to prevent population outbreak and more importantly to detect early new species that could be very aggressive and cause severe damage. Regarding the species *P. kellyanus*, it needs to be monitored in Tunisia at least on Lemon and Bergamot because of its extensive damage and the increase of its area of invasion in recent years. Further investigation is required to determine the status of the species *T. major* and *F. occidentalis* in citrus orchards and to confirm their harmfulness and role in causing marbling scars. Furthermore, chemical treatments in citrus groves must be managed in order to preserve the natural enemies of thrips: those hosted by the foliage of citrus or by herbaceous plants as well as those living into the ground (Belaam-Kort et al. 2018).

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RESUME

Belaam-Kort I. et Boulahia-Kheder S. 2019. Evaluation des dégâts des thrips en vergers d'agrumes en Tunisie. Tunisian Journal of Plant Protection 14 (1): 55-68.

Avant les années 2000, les dégâts causés par les thrips sur agrumes en Tunisie, étaient considérés comme rares ou absents. Cependant, ces dix dernières années et depuis le premier signalement de l'espèce *Pezothrips kellyanus* en 2008, diverses cicatrices sur fruits attribuées aux thrips, sont de plus en plus enregistrées. Suite à l'inventaire des espèces de thrips en vergers d'agrumes, ce travail vise à évaluer les dégâts causés par ces ravageurs sur différentes espèces d'agrumes et variétés d'orange, afin d'étudier la sensibilité des ces espèces et variétés à ces insectes. L'utilisation des insecticides a également été recensée en relation avec les dégâts des thrips. Ces derniers ont été estimés par l'observation de 200 à 1000 fruits mûrs dans les 101 vergers visités des régions du Cap-Bon, Bizerte et Mornag, de décembre à février, pendant les années 2015 à 2017. Les espèces d'agrumes et variétés d'orange examinées étaient les suivantes : citron, bergamote, pamplemousse, clémentine, mandarine et oranges Navel, Maltaise, Valentia Late et Double Fine. Les insecticides ainsi que leur fréquence d'utilisation dans chaque verger ont été notés. Les dégâts des thrips sur fruits tous types confondus étaient en moyenne de 20% pour toutes les espèces d'agrumes et variétés d'orange. La bergamote et le citron étaient les plus sensibles à l'espèce *P. kellyanus* causant des anneaux argentés, tandis que les oranges Maltaise et Navel étaient plus affectées par les marbrures causées par d'autres espèces. Les données fournies par 94 agrumiculteurs ont montré que les dégâts augmentent avec le nombre de traitements chimiques/an. Cette étude montre que les dégâts des thrips sont de plus en plus fréquents dans les vergers d'agrumes en Tunisie. Ils pourraient dans l'avenir prendre plus d'ampleur car la gestion des nuisibles des agrumes repose principalement sur des insecticides à large spectre qui en éliminant les insectes utiles, risquent de renforcer les populations des thrips, d'où la nécessité de rationaliser les interventions chimiques et de généraliser le recours aux méthodes alternatives.

Mots clés: Agrumes, anneaux, *Frankliniella occidentalis*, marbrures, *Pezothrips kellyanus*, sensibilité, Thrips, *Thrips major*, traitements chimiques

ملخّص

بالعم-قرط، إيمان وسندة بولحية-خذر. 2019. تقييم أضرار حشرة التربس في بساتين القوارص/الحمضيات بتونس.
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قبل سنوات 2000، كان الضرر الناجم عن التربس يعتبر نادرًا أو غير موجود في بساتين القوارص/الحمضيات التونسية. مع ذلك، خلال السّنوات العشر الأخيرة ومنذ تسجيل لأول مرّة لنوع *Pezothrips kellyanus* سنة 2008، تمّ تسجيل العديد من أعراض ندوب الفاكهة المنسوبة إلى آفة التربس بنسق تصاعدي. بعد حصر أنواع التربس في بساتين القوارص، يهدف هذا العمل إلي تقييم الأضرار النّاجمة عن هذه الآفة على أنواع مختلفة من القوارص وأصناف مختلفة من البرتقال. تمّت مراقبة 200 إلي 1000 فاكهة ناضجة من 101 بستان في الوطن القبلي وبنزرت ومرناق وذلك منذ ديسمبر إلي فيفري من سنوات 2015 إلي 2017. تمّ فحص أنواع القوارص اللّيمون والليمون الهندي/الزّنباع والبرغموت والكليمنتين والماندرين وأصناف البرتقال نافل وفالنسيا لايت ودبل فين ومالطي. بلغ معدّل نسبة الأضرار النّاجمة عن التربس 20% على جميع أنواع القوارص وأصناف البرتقال. كان نوعيْ اللّيمون والبرغموت لهما حساسية لحشرة *P. kellyanus* الّتي تسبّب حلقات فضّية علي الفاكهة في حين كان صنفيْ البرتقال مالطي ونافل الأكثر حساسية لأنواع أخري من التربس. لوحظ أيضا من خلال بيانات 94 فلاح قوارص، أنّ أضرار التربس تزداد مع تكرار المعاملات بالمبيدات الحشرية وتبيّن من خلال هذا العمل، أنّ أضرار التربس في تصاعد في تونس وقد تصبح الأكثر أهمّية لأنّ إدارة آفات القوارص تعتمد بشكل رئيسي على مبيدات حشرية واسعة الطيف التي تقضي على الحشرات النافعة ومن هنا تتبين الحاجة إلي ترشيد التّدخّلات الكيميائيّة وتعميم استخدام الطّرق البديلة.

كلمات مفتاحية: أعراض حلقات، أعراض مرمرية، تربس، حساسية، قوارص/حمضيات، معاملات كيميائية، *Frankliniella occidentalis*، *Pezothrips kellyanus*، *Thrips major*

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Screening Chickpea Lines and Varieties for a Possible Resistance or Tolerance to the Pod Borer *Helicoverpa armigera*

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ABSTRACT

Bouslama, T., Laarif, A., Soltani, A., Chaieb, I., Amri, L., and Rhouma, A. 2019. Screening chickpea lines and varieties for a possible resistance or tolerance to the pod borer *Helicoverpa armigera*. Tunisian Journal of Plant Protection 14 (1): 69-81.

Helicoverpa armigera is a polyphagous moth that causes substantial economic losses on various crops in the world. The use of resistant or tolerant varieties is one of the most important components of integrated pest management and can play major role in its control. The flight activity of male moths was monitored using Delta traps baited with sexual pheromone installed in a chickpea field planted with 27 chickpea lines and varieties in the northern of Tunisia. During the experimental period, weather conditions did not affect the flight activity of *H. armigera* males. However, it was affected by the different crop development stages. Screening the different chickpea lines and varieties for resistance or tolerance to *H. armigera* allowed us detecting a resistant variety which is cv. Kasseb. Kasseb has shown the minimum level of damage in pods and seeds per plant which did not exceed 2.5%.

Keywords: Chickpea, damage, *Helicoverpa armigera*, flights, tolerance/resistance

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The cotton bollworm or the chickpea pod borer, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is a regular economic pest of many important crops in the world, and is widely distributed in Europe, Africa, Asia, and Oceania (EPPO 2006; Guo 1997; Zaluki

et al. 1986). *H. armigera* is a polyphagous agricultural pest and was reported in more than 180 cultivated crop species, which include pigeon pea (*Cajanus cajan*), cotton (*Gossypium hirsutum*), soybean (*Glycine max*), tomato (*Solanum lycopersicum*), chickpea (*Cicer arietinum*), green pepper (*Capsicum annuum*), okra (*Abelmoschus esculentus*) (Goyal and Rathore 1988; Kakimoto et al. 2003).

Females lay eggs on the flowering and fruiting structures of these crops, where larval feeding leads to substantial economic loss (Reed and Pawar 1982).

Management methods used against this insect are essentially chemical; however, resistance to several chemical insecticides has already been reported for this pest (Ahmad et al. 2006; Xinjun et al. 2005).

The resistance problems have increased interest in other control methods such as plant extracts (Bisen and Bansal 2014; Gupta 2007; Hussain et al. 2016; Pachundkar et al. 2013), biological agents such as viruses (Gupta et al. 2007; Thakur 1998) and bacteria (Ahmad et al. 2012),cultural practices such as intercropping (Ahmad 2003; Pimbert 1990) and trap crops (Reddy and Manjunatha 2000).

The use of resistant or tolerant varieties is one of the most important component of integrated pest management and can play major role in the management of *H. armigera* (David and Easwaramoorthy 1988; Hossain et al. 2008; Jeffree 1986; Peter et al. 1995; Ujagir and Khere 1987).

Chickpea is one of the most popular vegetables in many regions of the world. In addition, to its importance in human and animal feeding, chickpea plays a substantial role in improving soil fertility by fixing the atmospheric nitrogen and can also tolerate high temperatures during and after flowering (Bakr et al. 2004; Cumming and Jenkins 2011).

In Tunisia, chickpea is grown over an area of 6500 hectares (0.05% of global grown area) for a production of 5000 tons (0.04% of global production) and a productivity of 769 kg/ha in 2016 (FAOSTAT 2016). *H. armigera* is responsible for causing important damage in chickpea due to its regular occurrence from the vegetative growth to the pod formation stage (Manjunath et al. 1989; Reed and Pawar 1982; Sharma 2001).

Because of limited studies on *H. armigera* in Tunisia (Boukhris-Bouhachem et al. 2007; Cherif and Grissa-Lebdi 2015), the present research was conducted to detect *H. armigera* flights in the region of Beja in the north of Tunisia and to determine resistance or tolerance of chickpea lines and varieties against this insect under field conditions.

MATERIALS AND METHODS

Field trial description.

The trial was conducted during growing season of 2016/17, from 23 March to 28 June 2017, in the experimental station (Oued Beja) of the Regional Field Crop Research Center of Beja, Tunisia (CRRGC) (36°44'N; 9°11'E). In total, 27 winter and spring chickpea lines and varieties, initially set for a varietal trial, were tested in this study (Table 1). Among the 27 studied genotypes, 16 advanced lines and released varieties were planted the third week of February 2016 according to a Randomized Complete Block Design (RCBD). Each genotype was planted in 4 rows plot of 4 m length and 0.5 m inter-row spacing at a density of 30 seeds/m². Nor herbicides neither insecticides were applied over the trial after plant emergence and only hand weeding was

carried out.

Table 1. List of the chickpea lines and varieties used in the study

Entry/Variety/Pedigree	Characteristics
FLIP09-75C	Advanced line
FLIP09-68C	Advanced line
FLIP09-145C	Advanced line
FLIP09-151C	Advanced line
FLIP09-191C	Advanced line
FLIP09-356C	Advanced line
FLIP09-372C	Advanced line
FLIP08-38C	Advanced line
FLIP08-42C	Advanced line
FLIP08-53C	Advanced line
FLIP09-189C	Advanced line
FLIP85-1C	Advanced line
FLIP97-503C	Advanced line
FLIP07-3C	Advanced line
FLIP07-254C	Advanced line
FLIP09-342C	Advanced line
ILC 464	Advanced line
X96TH62-A4-A1-W1-A1-A1-A1-A1	Advanced line
Joud	Variety released on 2017, high yielding, large seed size (HSW=46-47g)
Nayer (FLIP84-92C)	Variety released on 2017, high yielding, small seed size (HSW=34-36g). Recommended for winter and spring planting
Bouchra (FLIP84-79C)	Variety released on 2017, high yielding, small seed size (HSW=34-36g). Recommended for winter planting
Chetoui (ILC3279)	Variety released on 2017, high yielding, small seed size (HSW=32-34g). Recommended for winter planting
Kasseb (FLIP83-46C)	Variety released on 2017, high yielding, small seed size (HSW=31-34g). Recommended for winter planting
Rebha	Variety released on 2017, high yielding, large seed size (HSW=46-47g). Recommended for winter and spring planting
Beja 1	Variety released on 2003, high yielding, medium seed size (HSW=36-38g). Recommended for winter planting
Nour	Variety released on 2011, high yielding, large seed size (HSW=44-45g). Recommended for winter and spring planting
Amdoun1	Variety released on 1987, large seed size (HSW=46-47g). Recommended for spring planting

Adult monitoring by pheromone traps and damage evaluation.

Two delta traps (Koppert) baited with lures of Pherodis *H. armigera* (Koppert) were installed in the site and kept about 10 cm above the top of the plant height. *H. armigera* males were counted weekly and removed from the sticky inserts which were replaced when needed. The pheromone dispensers were changed every 4 weeks.

Pod damage was estimated from six randomly collected plants per plot after counting the numbers of total pods and damaged pods.

Data analysis.

Based on the crop development stage, flights data were grouped in 4 sub-groups (emergence to flowering stage, flowering stage, pod setting stage and crop physiological maturity stage); sub-groups were compared with each other

using analysis of variance (ANOVA) at *P*=0.05 and subjected to Student, Newman and Keuls multiple-range test. Flights data were also correlated with climatic data using Pearson correlation. Based on the chickpea winter/spring planting, and the different lines and varieties, data on the percentage of damage caused by *H. armigera* on different lines and varieties of chickpea were subjected to analysis of variance (ANOVA) at *P*=0.05 and subjected to Student, Newman and Keuls multiple-range test. All the statistical tests were performed using SPSS V 21 software.

RESULTS

H. armigera field monitoring in relation with weather conditions.

The study area was classified as Csa climate type of the Köppen classification system, characterized by hot summer; coldest month averaging above 0 °C, at least one month average temperature above 22 °C, and at least four months averaging above 10 °C. At least three times as much precipitation in the wettest month of winter as in the driest month of summer, and driest month of summer receives less than 30 mm (Köppen 1984; Peel et al. 2007). The average minimum temperature during the trial period was 11.73±4.6°C, the average maximum temperature was 28.68±6.22°C, relative humidity was 55.34±12.64% and precipitations were 1.66±0.76 mm; the weekly climate data of the governorate of Beja are shown in Table 2.

Table 2. Climatic data per week of the governorate of Beja (INM 2017)

Study period (week)	Temperature (°C)		Relative humidity (%)	Precipitation (mm)
	Min	Max		
March 4th	6.40	21.63	70.25	8.33
April 1st	7.61	19.31	78.14	39.37
April 2nd	7.30	22.26	71.13	1.02
April 3rd	5.46	22.24	58.63	0.00
April 4th	9.93	26.89	58.43	2.29
May 1st	10.94	28.34	60.00	0.00
May 2nd	11.64	29.19	48.63	0.00
May 3rd	11.44	28.66	53.00	0.00
May 4th	11.93	31.81	45.57	0.00
June 1st	15.41	31.27	54.29	19.81
June 2nd	16.14	35.74	44.57	0.00
June 3rd	18.00	35.43	43.43	0.00
June 4th	20.32	40.04	33.40	0.00

The relationship between the number of collected *H. armigera* males per week and weather conditions (mean weekly minimum temperature, mean weekly maximum temperature, mean weekly humidity and mean weekly rainfall) from 23 March to 28 June 2017 was evaluated. Results showed that there is no significant correlation between flights and climatic data (Table 3).

Table 3. Correlation matrix between climate data of the tested period and the average number of *H.armigera* caught males

Matrix parameters		NMC	Tmin	Tmax	H	PP
NMC	Correlation of Pearson	1	-	-	-	-
	Sig. (bilateral)		-	-	-	-
	N	13	-	-	-	-
Tmin	Correlation of Pearson	-.382	1	-	-	-
	Sig. (bilateral)	.198		-	-	-
	N	13	13	-	-	-
Tmax	Correlation of Pearson	-.296	**.959****	1	-	-
	Sig. (bilateral)	.327	.000		-	-
	N	13	13	13	-	-
H	Correlation of Pearson	.131	**-.842****	**-.943****	1	-
	Sig. (bilateral)	.669	.000	.000		-
	N	13	13	13	13	-
PP	Correlation of Pearson	.178	-.221	-.444	**.578***	1
	Sig. (bilateral)	.560	.468	.129	.038	
	N	13	13	13	13	13

*. The correlation is significant at $P< 0.05$ (bilateral).
**. The correlation is significant at $P< 0.01$ (bilateral).
NMC: Average number of caught *H. armigera* males per trap; Tmax: Average maximum temperature per counting period; Tmin: Average minimum temperature per counting period; H: Average humidity per counting period; PP: Average precipitations per counting period.

H. armigera field monitoring in relation with chickpea development stages.

First incidence of pod borer was observed on the 4th week of March. In total, 92 *H. armigera* males were caught during the study period, from the fourth week of March to the fourth week of June.

H. armigera flights were detected during the different chickpea development stages. A maximum of 10 males/trap/week was recorded at the flowering stage against a minimum of only one male/trap recorded at the crop physiological maturity stage. At the flowering stage, number of males/trap/week (an average of 6 males/trap/week) is greater than that during the other stages of crop development.

According to these results, flights are less intense during the crop physiological maturity stage; this is probably due to the lack of food for both adults and young larvae (Figs. 1,2).

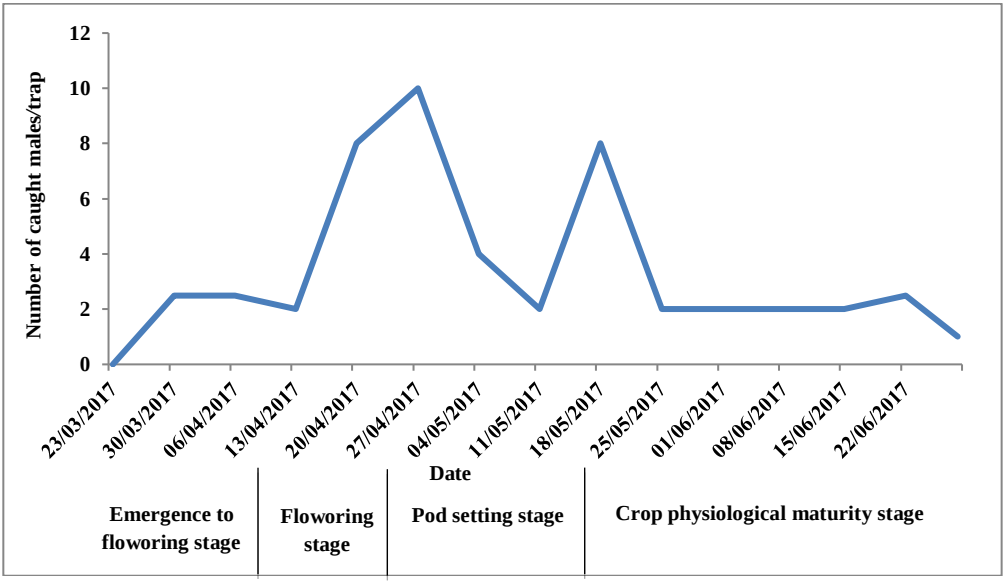

Fig.1. Evolution of the number of caught males of *H. armigera* at different stages of chickpea development.

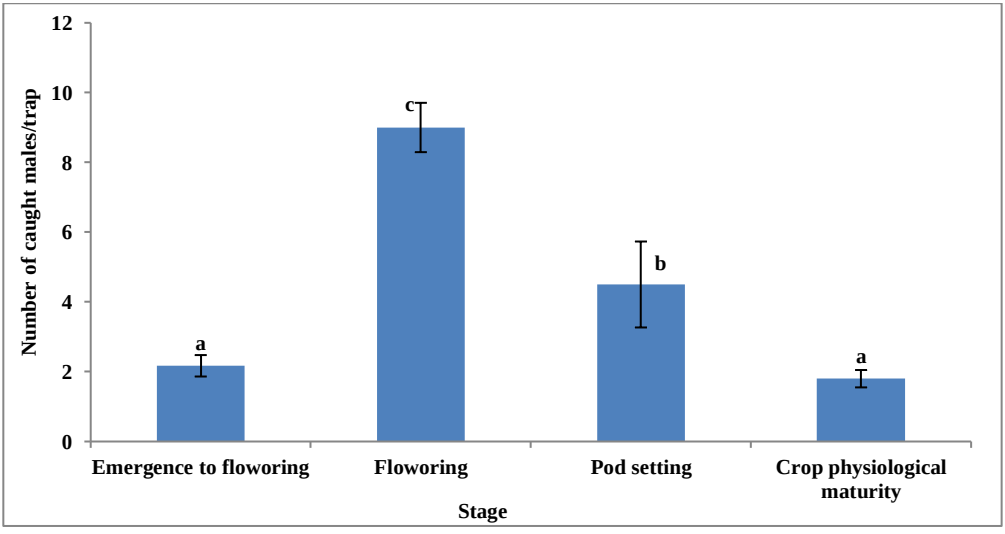

Fig.2. Comparison between numbers of males of *H. armigera* at different stages of chickpea development. Mean comparison with Student, Newman and Keuls test: Averages with different letters (a, b and c) are significantly different at $P<0.05$. Segments are standard errors.

Screening chickpea lines and varieties for resistance or tolerance to *H. armigera.*

During the test, the damage of *H. armigera* on chickpea is shown by bored pods (Fig. 3).

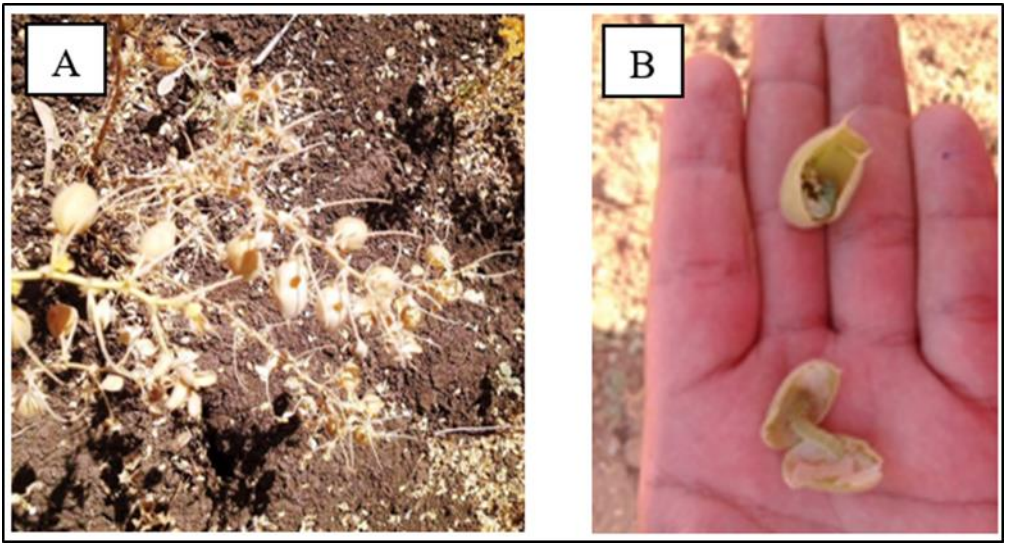

Fig.3. Damage caused by *Helicoverpa armigera* on chickpea pods (A: Pods bored by larvae; B: larvae feeding on chickpea pods).

Studied performance of chickpea lines and varieties against the pod borer, showed that there is no significant difference at $P<0.05$ between winter and spring planting of chickpea genotypes. Comparison of resistance among the chickpea lines and varieties against the insect showed that none was completely resistant against this pest. But results showed also that there is a significant difference at $P=0.05$ threshold between the different studied genotypes. Table 4 indicates that the level of damage to the pods and seeds per plant varied from a minimum of 2.5% for cv.Kasseb to a maximum of 14.33% observed for the advanced line FLIP09-151C. Cv.Kasseb is, therefore, the most resistant variety to *H. armigera* attacks and can be an interesting genotype to use in a breeding program to control the pod borer (Table 4).

DISCUSSION

The life cycle of *H. armigera* takes about 30-34 days from egg to adult (Zaluki et al. 1986). Many studies reported that the 1st, 2nd, and 3rd instar larvae of *H. armigera* in chickpea initially feed on leaves and flowers (Reed and Pawar 1982). Larvae shift from foliar feeders to developing seeds as larval instars development progresses (Reed and Pawar 1982). The larvae (1st, 2nd, and 3rd instars) occasionally enter the pod and feed upon the developing chickpea grains, but more often they feed on outside the pod with the anterior part of its body in the pod (Saxena 1978; Singh and Singh 1987).

Table 4. Comparison between the percentage of attacked chickpea pods of the different lines andvarieties

Chickpea line/variety	Percentage of damage
Kasseb	2.5±0.92a
Chetoui	3.45±0.76ab
FLIP85-1C	5.33±0.4abc
Amdoun 1	5.67±0.76abc
FLIP07-3C	6.00±1.24abc
FLIP09-191C	6.33±1.36abc
FLIP09-75C	6.67±1.08abc
ILC 464	7.33±0.67abcd
Nayer	7.83±0.17abcde
Béja 1	8.17±0.17abcdef
Joud	8.67±1.15bcdef
FLIP08-53C	8.67±0.21bcdef
FLIP09-189C	8.83±0.31bcdef
FLIP09-372C	9.17±0.54bcdef
Nour	9.5±0.31bcdef
FLIP08-42C	9.5±2.23bcdef
FLIP08-38C	9.83±0.92cdef
FLIP09-145C	10.33±0.61cdef
Bouchra	10.67±1.7cdef
X96TH62-A4-A1-W1-A1-A1-A1-A1	10.67±0.65cdef
Rebha	11.17±1.9cdef
FLIP97-503C	11.67±2.43cdef
FLIP09-68C	11.67±1.05def
FLIP07-254C	13.17±1.9def
FLIP09-356C	13.67±2.6def
FLIP09-342C	13.83±1.85ef
FLIP09-151C	14.33±1.32f

Mean comparison with Student, Newman and Keuls test: Averages with different letters (a, b, c, d, e and f) are significantly different at $P< 0.05$.

From vegetative to pod formation stages, there is at least two successive generation of *H. armigera.*The beginning of the pod maturity stage

coincides with a new generation, since adults are nectar-feeding (Jervis et al. 2005) and young larvae rarely feed on chickpea pods (Saxena 1978; Singh and Singh 1987); this may explain the fact that flights are less intense during the crop physiological maturity stage (Chatar et al. 2010).

H. armigera is widely distributed in Asia, Africa, the Mediterranean region, and Oceania (EPPO 2006). This insect is causing on average 30-40% damage to chickpea pods (Hashmi 1994; Luckmann and Metcalf 1975; Rahman 1990; Saleem and Younis 1982), which may increase to 80-90% in conducive environments (Sachan and Katti 1994; Sehgal and Ujagir 1990). For example, 10-85% yield losses in chickpea have been recordedin India (Ahmed 1984; Das 1987; Lal et al. 1985; Qadeer and Singh 1989; Reed 1983). In Bangladesh and Nepal, pod borer damage in unprotected chickpea fields has been in the range of 5-15% (Musa 2000; Pandeand Narayana Rao 2000). In northern Pakistan, up to 90% pod damage due to *H. armigera* has been recorded in unprotected chickpea fields (Ahmed et al. 1986).

Many morphological characteristics which contribute to antixenosis have been used to breed pod borer-resistant varieties. Morphological traits such as pod trichome length and density, pod wall thickness, pod length, breadth and area, and the number of pods per plant showed influence on pod borer resistance in chickpea (Hossain et al. 2008; Ujagir andKhere 1987). Trichomes and trichome exudates on plant surfaces play an important role in the host selection process of herbivorous insects. The types of trichomes and their orientation, density, and length have been correlated with reduced insect damage in several crops (David and Easwaramoorthy 1988; Jeffree 1986; Peter et al. 1995).

Kasseb was registered in 1987; it comes from a cross made at ICARDA to obtain performing cultivars and tolerant to abiotic stress. This variety is moderately resistant to Ascochyta blight and susceptible to Fusarium wilt. It is characterized by a large number of secondary branches, a short stem and a high number of pods, which can reach 80 pods per plant but with a quite weak weight of 100 seeds (Khamassi et al. 2014).

According to Khamassi et al. (2014), Kasseb also has the particularity to reduce the duration of flowering and lengthen that of maturation. This property could be responsible for the tolerance/resistance of cv. Kasseb to *H. armigera*. Adults are nectar-feeding, which promotes egg maturation (Jervis et al. 2005). Therefore, the absence of flowers would result in low egg production and maturation. Furthermore, *H. armigera* larvae are considered to be flower, fruit and seed feeders in preference to leaf feeders (Fitt 1989; Rajapakse and Walter 2007; Wilson and Waite 1982; Zalucki et al. 1986), and although larvae will feed on leaves, their movements on plants will primarily take them to flower structures (Johnson and Zalucki 2005; Perkins et al. 2008; Yang et al. 2008). Considering that young *H. armigera* larvae rarely bore into pods (Saxena 1978; Singh and Singh 1987) and in the absence of flower structures, the larval population is expected to decrease due to the lack of food in quality and quantity.

More advanced trials are needed to confirm the resistance of the Kasseb variety to *H. armigera* and to identify the factors that govern this resistance.

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RESUME

Bouslama T., Laarif A., Soltani A., Chaieb I., Amri L. et Rhouma A. 2019. Criblage de lignées et de variétés de pois chiche en vue d'une éventuelle résistance ou tolérance à la noctuelle de la tomate, *Helicoverpa armigera*. Tunisian Journal of Plant Protection 14 (1): 69-81.

Helicoverpa armigera est une noctuelle polyphage qui provoque des pertes économiques importantes sur diverses cultures dans le monde. L'utilisation de variétés résistantes ou tolérantes est l'une des composantes les plus importantes de la lutte intégrée contre ce ravageur. Les vols de la noctuelle ont été suivis par piégeage des mâles avec une phéromone sexuelle dans un champ de pois chiche. Au cours de la période expérimentale, les conditions météorologiques n'ont pas affecté l'activité des mâles de *H. armigera*. Cependant, cette activité a été affectée par les différents stades de développement de la culture. Le criblage des différentes variétés et lignées de pois chiche pour la résistance ou la tolérance à *H. armigera* a permis de détecter une variété résistante qui est cv. Kasseb. Le pourcentage de dégâts sur lesgousses et les graines par plante n'a pas dépassé 2,5% pour cette variété.

Mots clés: Dégâts, *Helicoverpa armigera*, pois chiche, tolérance/résistance, vols

ملخص

بوسلامة، ثامر وأسماء العريف وعبير السلطاني وإقبال الشايب ومعز العمري وعلي رحومة. 2019. فحص أصناف وسلالات من الحمص بحثا عن مقاومة أو تحمّل لدودة القطن *Helicoverpa armigera*.
Tunisian Journal of Plant Protection 14 (1): 69-81.

دودة القطن (*Helicoverpa armigera*) هي حشرة متعددة العوائل تتسبب في خسائر اقتصادية كبيرة لمحاصيل مختلفة حول العالم. يعد استخدام أصناف مقاومة أومتحملة أحد أهم مكونات الإدارة المتكاملة لهذه الآفة. تمت متابعة طيران الحشرة من خلال مصائد الذكور باستعمال فيرومون جنسي في حقل حمص. خلال فترة التجربة، لم تؤثر الظروف المناخية في نشاط ذكور الحشرة. لكن هذا النشاط تأثر بمختلف مراحل نمو النبتة. مكنت عملية فحص مختلف الأصناف والسلالات المزروعة بحثا عن مقاومة أو تحمّل للآفة من تشخيص صنف مقاوم وهو صنف "كساب" الذي سجل نسبة قرون وبذور متلفة لا تتعدى 2.5%.

كلمات مفتاحية: أضرار، حمص، طيران، مقاومة/تحمّل، *Helicoverpa armigera*

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Short Communication

Variability and Host Specificity of *Striga hermonthica* in Response to in situ Root Exudates of *Pennisetum glaucum*

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ABSTRACT

Dafaallah, A.B., Babiker, A.T., and Hamad Elneel, A.H. 2019. Variability and host specificity of *Striga hermonthica* in response to in situ root exudates of *Pennisetum glaucum*. Tunisian Journal of Plant Protection 14 (1): 83-92.

Field surveys and a laboratory experiment were conducted during the seasons 2012/13 and 2013/14 in *Striga hermonthica* endemic areas in Sudan to investigate variability and host specificity in the early developmental stages of *S. hermonthica* parasitism in response to in situ root exudates of millet; cv. Ugandi, cv. Ashana and cv. Sudan II. Field surveys were conducted to collect *S. hermonthica* seeds from sorghum and millet fields. Fifteen *S. hermonthica* populations were collected. An in vivo experiment was conducted to study the effects of in situ root exudates of the three millet varieties on percentage of seed germination, haustorium initiation, attachment and penetration. The results revealed that in situ root exudates of all millet cultivars induced seed germination and haustorium initiation in *S. hermonthica* tested populations. Seed germination, haustorium initiation, attachment and penetration of *S. hermonthica* collected from parasitized millet in response to millet in situ root exudates were significantly higher compared to *S. hermonthica* collected from parasitized sorghum. It is noteworthy that for some individuals of the *S. hermonthica*, sorghum populations displayed limited attachment and penetration into millet roots. This study suggests two levels of physiological specialization in *S. hermonthica* in Sudan; intercrop specialization and intracrop specialization. Moreover, two strains of *S. hermonthica* are suggested, one specific to sorghum and another to millet. The existence of variability and host specificity within *S. hermonthica* populations seem to be based almost entirely on differential response of *S. hermonthica* isolates to in situ root exudates from host.

Keywords: Host, millet, sorghum, specificity, *Striga hermonthica*, variability

Striga spp., Orobanchaceae, are obligate root parasites on important cereal and leguminous crops in Sub-Saharan Africa (Olmstead et al. 2001). Existence of physiological strains, ecological variants and races of the parasite together with variability in size of the seed bank represents serious obstacles for development of simple and effective control measures. Co-evolution of the parasite with its hosts has resulted in both specificity and non-specificity within the genus (Babiker 2007; Haussmann et al.

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2000). Existence of biological strains was recorded since 1930s. However, this area of host specificity and host specialization in *Striga* is not fully explored (Musselman 1987). Two levels of physiological specialization have been suggested in *Striga*; inter- and intracrop specialization (Ramaiah and Parker 1982). Ramaiah and Parker (1982) suggested the existence of physiological strains of *S. hermonthica* in Africa, where it was observed that sorghum cultivars resistant in one location were susceptible in others. King and Zummo (1977) reported the existence of physiological specialization in *S. hermonthica* from West Africa following their analysis of parasite virulence on different host crops.

Several experiments were undertaken to study diversity and host specificity within *S. hermonthica* populations and their interactions with selected hosts. However, little work has been done in Sudan. Therefore, this study was conducted to investigate diversity and host specificity in early developmental stages of *S. hermonthica* parasitism in response to in situ root exudates of 3 millet cultivars (Ugandi, Ashana and Sudan II).

MATERIALS AND METHODS

Collection of *S. hermonthica* seeds.

Field surveys were conducted during the rainy season 2012/13 (2013/14) in *S. hermonthica* endemic areas in Gadarif, Gezira and Kordofan, i.e. Eastern, Central and Western Sudan, respectively. Field surveys were conducted to collect seeds from *S. hermonthica* plants growing under their respective hosts; sorghum and millet. Twelve populations of *S. hermonthica* were collected from sorghum fields at Galabat, Sumsum, Gadarif, Butana, El Fau (Gadarif area); Hasaheisa, Abu-Haraz, Hag-Abdalla, Barakat and Wad-Rabia (Gezira area); Um-Rawaba and El-Rahad (Kordofan area) and three populations were collected from millet field at Kadugli, Khour-Tagat and El Obied (Kordofan area). *S. hermonthica* seeds were surface sterilized by sodium hypochlorite (NaOCl), 1% solution, for 3 min with continuous agitation. Subsequently, they were thoroughly washed with sterilized distilled water for several times. Floating seeds were discarded and the remaining was stored at room temperature until used.

Seed conditioning.

The disc technique described by Dafaallah (2008) was used. About 80-100, Glass Fiber Filter Paper (GFFP) (Whatman GF/C) discs (0.5 mm diameter) were placed one layer GFFP in a glass Petri-dish (GPD), 9 cm internal diameter (i.d.). *S. hermonthica* seeds (25-50) were sprinkled on each disc and the GPDs, moistened with distilled sterilized water (4.5 ml), sealed with parafilm, covered with black polyethylene bag, were incubated at 30°C in the dark for 12 days.

In vivo experiment.

Disposable Petri-dishes (DPD) 9 cm i.d. were filled with Gezira soil that is typical haplusten, line smectitic, isophyperthemic and characterized by heavy clay soil (clay 60%), with pH 8-8.5, low organic matter and nitrogen, adequate potassium and low available phosphorous (Elbasher 2016). Soil was sterilized in an oven at 105°C for 24 h. Fiber filter paper (Whatman F/C) was placed on top of soil. Soil was moistened with 30 ml distilled sterilized water. Three millet cultivars (Ugandi, Ashana and Sudan II) obtained from the Millet Program, Agricultural Research Corporation (ARC), were raised in paper rolls for five days. Crop seedlings were

transferred and placed on GFFP in DPD with a lateral opening to allow shoots emergence. Conditioned *S. hermonthica* seeds that placed on glass fiber filter discs, were placed underneath the roots. Sterilized-distilled water was added to each Petri dish as needed. The Petri dishes, covered with a black glass fiber filter paper, sealed with parafilm and placed in black polyethylene bags, were incubated at room temperature in continuous light. Treatments (factorial combination of 12 *S. hermonthica* populations and three millet cultivars) were arranged in a completely randomized design with three replicates. *S. hermonthica* seeds and/or germlings were then examined under a binocular (40 × magnification) for germination, haustorium initiation, attachment and penetration 24, 72, 144 and 192 h after initial incubation. Experiments were repeated twice.

Statistical analysis.

Data were collected and subjected to analysis of variance (ANOVA) procedure. Means were separated for significance using DMRT at $P \leq 0.05$.

RESULTS

Effects of in situ root exudates of millet cultivars on seed germination.

Seeds of *S. hermonthica* exhibited relatively high germination (65.3, 68.1 and 70.0%) when placed in the vicinity of roots of millet cv. Ugandi, cv. Ashana and cv. Sudan II, respectively (Fig. 1). There were significant differences in seed germination in response to millet cultivars.

On placement of seeds in the vicinity of roots of millet cv. Ashana, germination of *S. hermonthica*, millet populations, was high and ranged between 73.0% seeds collected from El Obied to 76.2% for seeds collected from Kadugli (Table 1). When seeds of *S. hermonthica*, millet populations, were placed in close proximity to roots of millet cv. Ugandi, germination was high and ranged between 76.0% for seeds collected from El Obied to 78.7% for seeds collected from Kadugli. With placement of seeds in close proximity to roots of millet cv. Sudan II, germination of *S. hermonthica*, millet populations, was high and ranged between 79.3% for population collected from Khour-Tagat (Table 1). Moreover, there were significant differences among *S. hermonthica* populations. When seeds of *S. hermonthica*, sorghum populations, were placed near roots of millet cv. Ashana, germination was relatively high and ranged between 57.3% for seeds collected from El Fau to 66.7% for seeds collected from El-Rahad. When seeds of *S. hermonthica*, sorghum populations, placed near roots of millet cv. Ugandi, germination ranged between 58.0% for population collected from El Fau to 71.3% for population collected from Abu-Haraz. When seeds of *S. hermonthica*, sorghum populations, placed near roots of millet cv. Sudan II, germination ranged between 60.0% for seeds collected from El Fau to 79.3% for seeds collected from El-Rahad. There were significant differences among *S. hermonthica* populations.

Effects of in situ root exudates of millet cultivars on haustorium initiation.

S. hermonthica achieved low haustorium initiation (33.7, 34.2 and 34.9%) when seed were placed in the vicinity of roots of millet cv. Sudan II, cv. Ashana and cv. Ugandi, respectively (Fig. 1). However, there were significant differences among *S. hermonthica* populations.

Table 1. Effects of in situ root exudates of millet cultivars on seed germination of *Striga hermonthica* populations

S. hermonthica population		Germination (%)		
		Millet cultivar		
Area	Location	cv. Ashana	cv. Ugandi	cv. Sudan II
Gadarif	Galabat*	64.9 klm	67.0 ijk	69.0 ghi
	Sumsum*	64.9 klm	66.7 jk	69.0ghi
	Gadarif*	64.9 klm	67.0 ijk	69.0 ghi
	Butana*	61.7 opq	65.0 klm	66.7 jk
	El Fau*	57.3 s	58.0 rs	60.0 qr
Gezira	Hasaheisa*	61.6 opq	64.0 lmn	68.0 hij
	Abu-Haraz*	63.5 lmno	71.3 ef	73.0 de
	Hag-Abdalla*	63.0 mnop	65.0 klm	67.7 hij
	Barakat*	60.3 qr	63.0 mnop	66.3 jk
	Wad-Rabia*	61.0 pq	62.3 nop	65.3 kl
Kordofan	Kadugli**	76.2 c	78.7 b	80.0 ab
	Khour-Tagat**	74.1 cd	78.3 b	81.0 a
	Um-Rawaba*	66.7 jk	68.0hij	69.7 fgh
	El Obied**	73.0 de	76.0 o	79.3 ab
	El-Rahad*	67.0 ijk	71.0 efg	79.3 ab
SE (±)		0.69		
CV (%)		3.7		

*, ** = *Striga* populations collected under sorghum and millet, respectively. Means in columns and rows followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

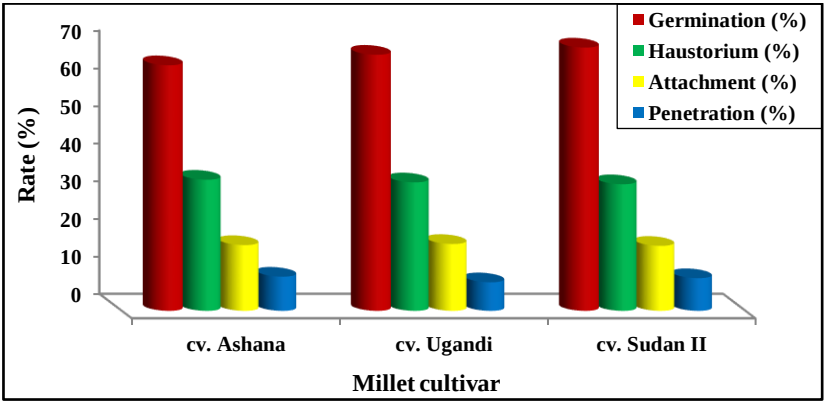

Fig. 1. Effects of in situ root exudates of millet cultivars on early developmental stages of *Striga hermonthica* parasitism.

With placement of seeds in close proximity to roots of millet cv. Ashana, haustorium initiation of *S. hermonthica*, millet populations, was significantly high and ranged between 77.0% for population collected from El Obied to 80.0% for population collected from Kadugli (Table 2).

Table 2. Effects of in situ root exudates of millet cultivars on haustorium initiation of *Striga hermonthica* populations

S. hermonthica population		Haustorium initiation (%)		
		Millet cultivar		
Area	**Location**	**cv. Ashana**	**cv. Ugandi**	**cv. Sudan II**
Gadarif	Galabat*	24.4 kl	23.0 lm	24.0 kl
	Sumsum*	24.3 kl	21.0 no	21.7 mn
	Gadarif*	33.0 ef	30.3 h	31.0 gh
	Butana*	25.0 jk	26.0 j	23.3 l
	El Fau*	17.3 s	18.0 qrs	17.3 s
Gezira	Hasaheisa*	21.0 no	19.3 opqr	20.3 nop
	Abu-Haraz*	19.3 opqr	18.0 qrs	18.0 qrs
	Hag-Abdalla*	21.0 no	20.0 op	18.3 qrs
	Barakat*	19.7 opq	17.7 rs	18.7 pqrs
	Wad-Rabia*	17.7 rs	20.0 op	20.0 op
Kordofan	Kadugli**	80.0 ab	79.7 ab	79.0 ab
	Khour-Tagat**	78.3 bc	80.3 a	79.7 ab
	Um-Rawaba*	33.0 ef	30.0 hi	28.7 i
	El Obied**	77.0 cd	79.3 ab	76.7 d
	El-Rahad*	34.7 e	29.7 ab	29.7 ab
SE (±)		0.54		
CV (%)		4.3		

*, ** = *Striga* populations collected from under sorghum and millet, respectively. Means in columns and rows followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

When *S. hermonthica*, millet populations, was placed in close proximity to roots of millet cv. Ugandi, haustorium initiation was high and ranged between 79.3% for population collected from El Obied to 80.3% for population collected from Khour-Tagat (Table 2). With placement of seeds in the vicinity of roots of millet cv. Sudan II, haustorium initiation of *S. hermonthica*, millet populations, was high and ranged between 76.7% for population collected from Khour-Tagat (Table 2). When *S. hermonthica*, sorghum populations, was placed near roots of millet cv. Ashana, haustorium initiation was significantly low and ranged between 17.3% for seeds collected from El Fau to 34.7% seeds collected from El-Rahad (Table 2). With seeds of *S. hermonthica*, sorghum populations, placed near roots of millet cv. Ugandi, haustorium initiation was low and ranged between 17.7% for seeds collected from Barakat to 30.3% for seeds collected from Gadarif. When seeds of *S. hermonthica*, sorghum populations, were placed near roots of millet cv. Sudan II, haustorium initiation was low and ranged between 17.3% population collected from El Fau to 31.0% for population collected from Gadarif.

Effects of in situ root exudates of millet cultivars on attachment.

S. hermonthica showed low attachment (17.3, 17.5 and 17.8%) to roots of millet cv. Sudan II, cv. Ugandi and cv. Ashana, respectively (Fig. 1). *S. hermonthica*, millet populations, exhibited relatively high attachment to roots of millet cv. Ashana and ranged between 58.9% for population collected from El Obied to 62.8% for populations

collected from Kadugli (Table 3). *S. hermonthica*, millet populations, achieved relatively high attachment to roots of millet cv. Ugandi and ranged between 59.0% for population collected from El Obied to 62.0% for population collected from Khour-Tagat. *S. hermonthica*, millet populations, showed high attachment to roots of millet cv. Sudan II and ranged between 60.0% population collected from Khour-Tagat to 61.7% for population collected from El Obied. Sorghum population displayed significantly very low attachment to roots of millet cv. Ashana and ranged between 5.5% for population collected from Galabat and Wad-Rabia to 7.9% population collected from Sumsum (Table 3). Sorghum populations displayed very low attachment to roots of millet cv. Ugandi and ranged between 6.0% for populations collected from Sumsum and Barakat to 8.0% population collected from Gadarif. Sorghum populations exhibited very low attachment to roots of millet cv. Sudan II and ranged between 6.0% for populations collected from Sumsum, El Fau, Hasaheisa, Wad-Rabia and El-Rahad to 7.7% population collected from Gadarif.

Table 3. Effect of in situ root exudates of millet cultivars on attachment of *Striga hermonthica*

***S. hermonthica* population**		**Attachment (%)**		
		Millet cultivar		
Area	**Location**	**cv. Ashana**	**cv. Ugandi**	**cv. Sudan II**
Gadarif	Galabat*	5.8 f	7.0 def	6.3 def
	Sumsum*	7.9 d	6.0 ef	6.0 ef
	Gadarif*	6.3 def	8.0 d	7.7 de
	Butana*	6.0 ef	7.0 def	6.7 def
	El Fau*	6.3 def	7.3 def	6.0 ef
Gezira	Hasaheisa*	7.2 def	7.0 def	6.0 ef
	Abu-Haraz*	7.2 def	6.7 def	6.3 def
	Hag-Abdalla*	7.0 def	7.3 def	6.7 def
	Barakat*	7.0 def	6.0 ef	7.0 def
	Wad-Rabia*	5.8 f	7.0 def	6.0 ef
Kordofan	Kadugli**	62.8 a	61.0 ab	61.0 ab
	Khour-Tagat**	62.0 a	62.0 a	60.0 bc
	Um-Rawaba*	6.0 ef	7.7 de	6.3 def
	El Obied**	58.9 bc	59.0 c	61.7 a
	El-Rahad*	6.0 ef	7.3 def	6.0 ef
SE (±)		0.50		
CV (%)		7.4		

*, ** = *Striga* populations collected under sorghum and millet, respectively. Means in columns and rows followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

Effects of in situ root exudates of millet cultivars on penetration.

S. hermonthica displayed negligible penetration (7.6, 8.7, and 9.1%) into roots of millet cv. Ashana, Sudan II and cv. Ugandi, respectively (Fig. 1).

S. hermonthica, millet populations, showed low to relatively moderate penetration into roots of millet cv. Ashana and ranged between 39.0%

for population collected from El Obied to 40.2% for population collected from Kadugli (Table 4). *S. hermonthica*, millet populations, exhibited low penetration into roots of millet cv. Ugandi and ranged between 35.0% for population collected from El Obied to 37.3% for population collected from Kadugli. *S. hermonthica*, millet populations, showed moderate penetration into roots of millet cv. Sudan II and ranged between 44.0% for population collected from Khour-Tagat to 40.0% for population collected from El Obied (Table 4). Sorghum populations displayed no penetration into roots of millet cv. Ashana with exceptional cases of Galabat, Sumsum and Gadarif that supported 5.7% penetration (Table 4). Sorghum populations displayed no penetration into roots of millet cv. Ugandi with exceptional case of Um-Rawaba population that sustained 5.7% penetration. Sorghum populations displayed no penetration into roots of millet cv. Sudan II.

Table 4. Effect of in situ root exudates of millet cultivars on penetration of *Striga hermonthica* populations

***S. hermonthica* population**		**Penetration (%)**		
		Millet cultivar		
Area	**Location**	**cv. Ashana**	**cv. Ugandi**	**cv. Sudan II**
Gadarif	Galabat*	5.7 f	0.0 g	0.0 g
	Sumsum*	5.7 f	0.0 g	0.0 g
	Gadarif*	5.7 f	0.0 g	0.0 g
	Butana*	0.0 g	0.0 g	0.0 g
	El Fau*	0.0 g	0.0 g	0.0 g
Gezira	Hasaheisa*	0.0 g	0.0 g	0.0 g
	Abu-Haraz*	0.0 g	0.0 g	0.0 g
	Hag-Abdalla*	0.0 g	0.0 g	0.0 g
	Barakat*	0.0 g	0.0 g	0.0 g
	Wad-Rabia*	0.0 g	0.0 g	0.0 g
Kordofan	Kadugli**	40.2 c	37.3 de	43.3 b
	Khour-Tagat**	40.1 c	35.7 e	41.0 c
	Um-Rawaba*	0.0 g	5.7 f	0.0 g
	El Obied**	39.0 cd	35.0 e	46.0 a
	El-Rahad*	0.0 g	0.0 g	0.0 g
SE (±)			0.75	
CV (%)			34.4	

*, ** = *Striga* populations collected under sorghum and millet, respectively. Means in columns and rows followed by the same letter(s) are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

DISCUSSION

Results revealed that in situ root exudates of all millet cultivars induced seed germination and haustorium initiation in *S. hermonthica* tested populations. Also, results revealed that, seed germination, haustorium initiation, attachment and penetration of *S. hermonthica* collected under millet in response to millet in situ root exudates was significantly high compared to *S. hermonthica* collected under sorghum. It is noteworthy that some of the *S. hermonthica*, sorghum populations, displayed limited attachment and penetration into sorghum roots.

These findings agree with those of Ali (2008) who reported that, root extracts and exudates from sorghum, millet and maize were able to induce germination and haustorium initiation, attachment and penetration of sorghum, millet and maize. However, the magnitude of germination, haustorium initiation, attachment and penetration varied with the parasite population and the host in question. These findings are, also, in agreement with the results of Rao (1984) who suggested that the earlier stages of parasite establishment may have greater importance in determining host specificity.

These findings are consistent with observation made by Wilson-Jones (1955) who suggested that two strains of *S. hermonthica* exist in Sudan, one prevailing in Eastern and Central Sudan and attacks only sorghum while in Western Sudan, both millet and sorghum were attacked. Furthermore, the strain on millet did not attack sorghum and vice versa. Sorghum was usually heavily attacked by *S. hermonthica* in the clay soils of Central Sudan whereas millet was particularly immune, but the reverse was true on sandy soils.

The observed differential response of the two *S. hermonthica* strains to haustorium inducing factor(s) from sorghum and millet may indicate specificity of the haustorium factors. Such specificity may be related to differences in quality, identity and/or quantity of the haustorium factor. The observed differential response is consistent with a previous report by Astatt and Hansen (1978) who reported that the potential number of haustoria is a product of the concentration and/or quality of haustoria inducing factor and the parasite individual ability to respond. It is concluded that existence of two levels of physiological specialization in *S. hermonthica* in Sudan: intercrop specialization and intracrop specialization. Furthermore, the results confirm the existence of two host-specific strains.

RESUME

Dafaallah A.B., Babiker A.T. et Hamad Elneel A.H. 2019. Variabilité et spécificité à l'hôte de *Striga hermonthica* en réponse aux exsudats racinaires *in situ* de *Pennisetum glaucum*. Tunisian Journal of Plant Protection 14 (1): 83-92.

Des enquêtes de terrain et une expérience au laboratoire ont été conduites pendant les saisons 2012/13 et 2013/14 dans des régions du Soudan endémiques pour *Striga hermonthica*, pour étudier la variabilité et la spécificité a l'hôte des stades précoces de développement du parasitisme de *S. hermonthica* en réponse aux exsudats racinaires *in situ* du millet; cv. Ugandi, cv. Ashana and cv. Sudan II. Les enquêtes de terrain ont été conduites pour collecter des semences de *S. hermonthica* des champs de millet et de sorgho. Quinze populations de *S. hermonthica* ont été collectées. Une expérience a été conduite *in vivo* pour étudier les effets des exsudats racinaires des trois cultivars de millet sur le pourcentage de germination des semences, l'initiation des hostauries, l'attachement et la pénétration. Les résultats ont révélé que les exsudats racinaires *in situ* de tous les cultivars induisent la germination des semences et l'initiation des hostauries chez les populations testées de *S. hermonthica*. La germination des semences, l'initiation des hostauries, l'attachement et la pénétration de *S. hermonthica* collectée du millet parasité en réponse aux exsudats racinaires *in situ*, étaient significativement plus élevées comparées à *S. hermonthica* collectée du sorgho parasité. Il est utile de noter que pour certains individus de *S. hermonthica*, les populations du sorgho montraient un attachement et une pénétration limitée dans les racines du sorgho. Cette étude suggère

deux niveaux de spécialisation physiologique chez *S. hermonthica* au Soudan; spécialisation intercultures et spécialisation intraculture. En plus, deux souches de *S. hermonthica* sont suggérées, une spécifique au sorgho et une autre au millet. L'existence de la variabilité et de la spécificité à l'hôte dans les populations de *S. hermonthica* semblent être basées presque totalement sur la réponse différentielle des isolats de *S. hermonthica* aux exsudats racinaires *in situ* de l'hôte.

Mots cles: hôte, millet, sorgho, spécificité, *Striga hermonthica*, Variabilité

ملخص

دفع الله، عوض الله بلال وعبد الجبار الطيب بابكر وأحمد حامد حمد النيل. 2019. التباين وتخصصية العائل لدى النبتة الطفيلية *Striga hermonthica* كاستجابة للإفرازات الموضعية لجذور الدخن (*Pennisetum glaucum*).
Tunisian Journal of Plant Protection 14 (1): 83-92.

أُجريت دراسات حقلية وتجربة معملية خلال فترة موسميي 2013/2012 و 2014/2013 في مناطق موبوءة داخليا بالنبتة الطفيلية *Striga hermonthica* في السودان لبحث التباين وتخصصية العائل في المراحل المبكرة لتطور الطفيل كاستجابة للإفرازات الموضعية لجذور الدخن (*Pennisetum glaucum*)؛ أصناف cv. Ugandi و cv. Ashana و cv. Sudan II. أُجريت الدراسات الحقلية بجمع بذور الطفيل *S. hermonthica* النامي على عوائله الخاصة؛ الدخن والذُرة البيضاء. تم جمع خمس عشر عشيرة من الطفيل *S. hermonthica*. أُجريت تجربة في الجسم الحي لدراسة تأثير الإفرازات المباشرة لجذور أصناف الدخن الثلاثة على إنبات البذور وإنشاء المماص والالتصاق والاختراق. بينت النتائج بأن الإفرازات الموضعية لجميع الأصناف تحث إنبات البذور وإنشاء المماص لدى العشائر المجربة للطفيل *S. hermonthica*. كان إنبات البذور وإنشاء المماص والالتصاق والاختراق للطفيل *S. hermonthica* المجمّع من الدخن المتطفَّل عليه، كاستجابة للإفرازات الموضعية، أعلى معنوياً بالمقارنةً مع الطفيل *S. hermonthica* المجمّع من الذرة البيضاء المتطفَّل عليها. الجدير بالملاحظة أن من بين نباتات الطفيل *S. hermonthica*، أظهرت بعض أفراد عشائر الذُرة البيضاء التصاقاً واختراقاً محدوداً على جذور الذرة البيضاء. يقترح هذا البحث مستويين من التخصصية الفسيولوجية للطفيل *S. hermonthica* في السودان: تخصصية بين أنواع المحاصيل وتخصصية داخل نوع المحصول. كذلك، يُقترح اعتبار سلالتين للطفيل *S. hermonthica*، واحدة خاصة بالذُرة البيضاء والأخرى بالدخن. يبدو أن وجود التباين وتخصصية العائل لدى عشائر الطفيل *S. hermonthica* ، ترتكز تقريباً كلياً على الاستجابة التفضيلية لعزلات الطفيل *S. hermonthica* للإفرازات الوضعية لجذور العائل.

كلمات مفتاحية: تباين، تخصصية، عائل، دخن، ذُرة بيضاء، *Striga hermonthica*

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Plant Protection Events

Report

on

Information and awareness-raising day on the bacterium *Xylella fastidiosa*

Tunis, Tunisia, 18th February 2019

Introduction

An information and awareness-raising day on *Xylella fastidiosa*, was held at the National Institute of Agronomic Research of Tunisia (INRAT), Tunis, Tunisia, 18th February 2019. It was realized in the framework of the project "Capacity Building and Raising Awareness in Europe and in Third Countries to Cope with *Xylella fastidiosa* (CURE-XF)", funded by the EU H2020 program in collaboration with SYNAGRI and CSNSP. CURE-XF involves 18 partners from the following countries:

Italy, France, Greece, Spain, Belgium, UK, Egypt, Morocco, Tunisia, Lebanon, Palestine and Iran.

Objectives

The objectives of the information day are:

- Raising the awareness of the concerned stakeholders (researchers in the agricultural field, nurserymen, farmers and ministry decision-makers) on this dangerous plant bacterium;
- Strengthen the knowledge and the know-how on *X. fastidiosa*;
- Present the measures to be taken to prevent the introduction of this bacterium in Tunisia.

Expectations

It is expected to:

- Acquire information on this bacterium;
- Understand the harmful effect of the introduction of this bacterium;
- Establish an action plan between the different actors.

Participants

During the day, 5 presentations were given on the bacterium, its vectors, the legislation, the control activities and the FAO regional strategy. More than 140 participants from different sectors took part in the event: researchers (54%), ministry 0fficers (15%), and especially professionals, i.e. nurserymen and farmers (31%).

Recommendations

- Share knowledge and establish a dialogue between all the stakeholders working in the agricultural sector (researchers, nurserymen, farmers, extension agents, and airport and border officers);
- Start up activities of surveillance of the territory and control of plant movements at the gateways of the country;
- Establish a national communication plan;
- Start monitoring activities and develop an emergency action plan. It is necessary to promote a general mobilization against the introduction of this bacterium since its known vectors are present in Tunisia;
- It is the responsibility of everyone to fight against this bacterium which represents a threat to our tree growing activities: grapevine, citrus, olive and almond trees, together with ornamental plants and forests.

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Recent Doctorate Theses in Plant Protection (2018/19)

Ilhem, Selmi. 2018. Identification, molecular characterization and study of transmission of viruses associated with rugose wood disease of grapevine in Tunisia. Doctorate Thesis in Agronomic Sciences (Plant Pathology), INAT, University of Carthage, Tunis, Tunisia, 126 pp. *(Public Defense: 03 August 2018)*

Grapevine virus A (GVA), *Grapevine virus B* (GVB), *Grapevine virus D* (GVD), *Grapevine virus E* (GVE), *Grapevine virus F* (GVF) and *Grapevine rupestris stem pitiing-associated virus* (GRSPaV) are associated with Rugose wood disease (RW); one of the most important grapevine diseases worldwide. To study the prevalence of RW in Tunisia, the genetic diversity of GRSPaV, GVA and GVD and possible transmission of GVA and GVD by mealybug *Planococcus ficus*, surveys were conducted in the main Tunisian grapevine growing areas. A total of 487 vines were collected from autochthonous, spontaneous, table and wine grapes, and rootstocks. All samples were analyzed by RT-PCR to test the presence of RW-associated viruses using specific primers. Molecular analysis showed that 77.8% of the tested samples were infected with at least one virus. GRSPaV was the most widespread virus (51.3%), followed by GVA (42.1%), GVD (26.7%), GVF (19.3%), GVB (14.8%) and finally GVE (5.9%). According to grapevines typology, wine grapes were the most infected (93.9%) vines, followed by table grapes (87.8%) and rootstocks (75.0%). Autochthonous grapevine varieties and spontaneous grapes were relatively less infected showing infection rates of about 65.9% and 63.1%, respectively. The study of genetic diversity of GVA, GVD and GRSPaV using sequencing and phylogenetic analysis showed large sequence variability among Tunisian isolates. GRSPaV isolates were separated in seven phylogenetic groups, of which two are new putative. GVA and GVD isolates were grouped in three (I, III and IV) and four (I, II, III and V) phylogenetic groups, respectively. Results obtained in this work showed that *P. ficus* is able to acquire and transmit Tunisian GVA and GVD under experimental conditions.

The etiological and epidemiological study of viruses associated with rugose wood disease in Tunisian vineyards provided results which are reported for the first time.

Otherwise, the present thesis provides for the first time the prevalence of RW-associated viruses in autochthonous and spontaneous grapevines and rootstocks, the first report on the presence of GVE and GVF in Tunisian vineyards, the first information about genetic diversity of some viruses associated with RW and the first report of a natural vector of GVD.

Mannai, Sabrine. 2018. The decline of apple and peach seedlings in nurseries: Diagnosis, morphological and molecular characterization of causal agents and management methods. Doctorate Thesis in Agronomic Sciences (Plant

Protection and Environment), ISA Chott-Mariem, University of Sousse, Chott-Mariem, Tunisia, 226 pp. *(Public Defense: 31 December 2018)*

The diagnosis made in Tunisian apple and peach tree nurseries showed the presence of decline symptoms. Morphological and molecular characterization allowed to identify five Fusarium species (*F. oxysporum*, *F. solani*, *F. equiseti*, *F. proliferatum* and *F. chlamydosporum*) and four species of Pythiaceae (*Pythium ultimum*, *Phytopythium mercuriale*, *Phytopythium helicoides* and *Phytophthora citrophthora*). The results showed that the percentages of isolation of different species are not related to the vigor index. The sequences have been deposited in the GenBank. The pathogenicity tests on apple seedlings showed that the most virulent species were those of the genera *Pythium* and *Phytopythium* and that most isolates of *F. oxysporum* and *F. solani* were non-pathogenic. On peach seedlings, pathogenesis tests revealed that *P. citrophthora* and *P. ultimum* are pathogenic on both Carnival and Royal glory varieties and that the Carnival variety is the most sensitive. Similarly, *F. oxysporum* and *F. solani* were virulent on the Garnem rootstock and the two peach varieties used. The evaluation of the MM106 rootstock response has shown that it is susceptible to the identified pathogens. In contrast, the Garnem peach rootstock was resistant to the majority of *P. citrophthora*, *P. mercuriale* and *P. ultimum* isolates and susceptible to isolates of *F. solani* and *F. oxysporum*. The study of the seasonal variation of the population of Fusarium spp. showed a positive significant correlation of the Fusarium population with the temperature and a negative significant correlation with the relative humidity in four prospected nurseries in different regions. For the physicochemical characteristics of the soil, the total population of Pythiaceae is positively correlated with silt content and soil pH and negatively with nitrogen content. The population of *P. ultimum* is negatively and significantly correlated with silt and clay content as well as electrical conductivity and positively significantly with sand, organic matter and organic carbon content. The *P. mercuriale* population is significantly negatively correlated with pH, silt and clay content of the soil. The in vitro test of different doses of the active ingredient of different fungicides showed a variation in efficacy depending to the dose, the chemical product and the pathogen. Indeed, fosetyl-Al, hymexazol and chinosol were the most effective in vitro on all species. In vivo, the fosetyl-Al and Metalaxyl-M reduced significantly the root browning in peach seedlings inoculated with *P. ultimum* and *F. oxysporum*. The in-vitro and in vivo study of some antagonistic fungi, isolated from the rhizosphere of apple and peach plants from surveyed nurseries, against Pythiaceae and *Fusarium*, revealed that among the means of control biological, *Trichoderma* spp., *Aspergillus* spp. as well as *Bacillus* spp., appear effective against some causal agents. Similarly, the use of composts and their extracts has shown their effectiveness in vitro as well as in vivo. Indeed, their effectiveness varies according to the pathogenic species studied. They stimulated plant growth and reduced the severity of the symptoms induced by the majority of pathogens. Regarding the test of two plants *Raphanus raphanistrum* and *Asphodelus microcephalus* extracts, in-vitro, it has been found that the aqueous extract is more effective than the methanolic extract. Indeed, the aqueous extracts of the two plants totally inhibited the mycelial growth of different pathogens. This study also proved that there is an interaction between the extract used and the treatment for the majority of the tests on the parameters of the severity of the symptoms as well as the growth of the plants. The application of *R. raphanistrum* 8 weeks before inoculation is more effective than treatment before one week. On the other hand, the efficacy of different parts of *A. microcephalus* varies between the different pathogens tested. The

study of the chemical composition of *R. raphanistrum* and its correlation with the efficiency showed that unsaturated hydrocarbons could be responsible to the antifungal activities in synergy with carboxylic acids against *F. oxysporum*, in synergy with esters, amines, ketones and dimethylsulfoxides and in antagonism with nitriles against *F. solani*, in synergy with alcohols, ethers and amides against *P. ultimum*. The esters could be responsible for the inhibition activity of *P. mercuriale* in synergy with saturated hydrocarbons, dimethylsulfoxides and amines and antagonism with carboxylic acids and ethers.

Haifa, Ben Gharsa. 2019. Ecology of complex of *Agrobacterium tumefaciens* in relationship to its plant host. Doctorate Thesis in Biological Sciences, Faculty of Sciences of Tunis, University of Tunis El-Manar, Tunisia, 181 pp. *(Public Defense: 02 March 2019)*

The dynamics of agrobacteria inoculated in natural soils, the colonization of the rhizosphere, the occupation of the gall and the exchange of genetic traits with other soil bacteria are areas not yet well understood in detail and require the use of strains easy to distinguish in the complex soil environment. For this purpose, chromosomal tagging with gentamicin and kanamycin is performed for two strains of *Agrobacterium fabrum*, C58 and C58C1. After verification of the stability and efficiency of this labeling, these strains are co-inoculated with different genomovars (G1, G4, G7 and G8), in the soil and rhizosphere of tomato and maize under controlled conditions. The plasmid, pTi, is unnecessary for C58 strain multiplication. Statistically, the total agrobacterial population is always higher in the rhizosphere than in the bulk soil, which proves the rhizosphere nature of agrobacteria, the proportion of C58Gmr differs according to the compartment and the treatment. The difference in soil was significant only with the genomovars G1 and G4. In both rhizospheres, the proportion of C58Gmr is more variable in the presence of genomic species G1, G4 and G7. From the data obtained, it is clear that the plant has a significant effect on the competition between bacteria from different genomovars.

To understand the conjugal transfer of plasmid Ti, a study of plasmid exchanges was carried out between strain C58C1Knr with different genomic species of A. tumefaciens, *Agrobacterium vitis* and two species identified as *Raoultella* spp. in the rhizosphere and in the gall of tomato. The highest conjugal transfer rate was observed between the C58C1Knr strain and the C58 wild-type strain both in rhizosphere and in gall. Transconjugants resulted from the mating of the pathogenic and non-pathogenic strains of Agrobacterium from the same genomovar and also with strains from different genomovars except G4 where there was no conjugal transfer either at the rhizosphere or on the gall. Transfer pTi is also possible with other species (*A. vitis*) and with strains belonging to other family than Rhizobiaceae (Enterobacteriaceae). The frequency of pTi exchange from one bacterium to another differs according to the type of plasmid, the genomovar, the species, the family and the biosystem (rhizosphere or tumor).

To find an alternative to chemical control of disease caused by agrobacteria, biological control assays were conducted using the strain *Bacillus velezensis* (M2). This study was focused on the potential of this strain for the control of crown gall disease. Thus, the antagonistic effect of this strain was evaluated on the gall and rhizosphere of tomato and maize plants. The evolution of the C58 strain was followed in the presence of strain M2 under sterile and non-sterile soil conditions. The results showed a significant decrease in the

pathogen population under both conditions, compared to negative and positive control treatments. However, the antagonistic activity of the M2 strain, against the pathogenic strain, was better under sterile soil conditions. The ability of the strain M2 to control C58-induced gall development has also been demonstrated on the stems of tomato and almond plants. Molecular analyzes allowed for the determination of a wide range of bacteriocins, lipopeptides and polyketides which are probably involved in this antagonistic activity.

Ammar, Nawaim. 2019. Effects of *Sargassum vulgare* (C. Agardh, 1820) aqueous and organic extracts against *Fusarium* spp. and *Pythium aphanidermatum* the agents of potato tubers' rots. Doctorate Thesis in Biological Sciences, Faculty of Sciences of Bizerte, University of Carthage, Tunisia, 181 pp. *(Public Defense: 10 April 2019)*

Extracts from the brown alga of Sargassum vulgare have been exploited as a potential source of bioactive compounds against agents involved in potato tuber rots. Aqueous and organic extracts of S. vulgare (from four sites) were tested in vitro and in vivo for their antifungal potential against *Fusarium sambucinum, F. oxysporum, F. solani* (dry rot causal agents) and *Pythium aphanidermatum* (watery rot causal agent). The antifungal activity noted varied significantly according to the targeted agents, the alga sampling sites (Tunis, Monastir, Mahdia1 and Mahdia2), the nature of the extracts used (aqueous or organic) and the concentrations tested (ranging from 1 to 100 mg/ml). Aqueous and organic extracts of S. vulgare collected from Tunis and Monastir were found to be the most active in vitro against all targeted rot agents.

All extracts were also tested as tuber treatments 2 hours prior to their inoculation with rots causal agents. The obtained results showed that rots' severity, estimated through the external diameter and the mean penetration, was reduced in a variable manner according to pathogen involved in the disease, the alga sampling sites, the types of extracts used and the concentrations tested. Aqueous and organic extracts were also shown to be effective in reducing the severity of rots. Following a double infection with *F. sambucinum* and *F. solani*, tubers treated with methanolic extracts of S. vulgare showed a significant reduction by 61% in dry rot severity compared to 3.1-27.1% noted with carbendazim. Thus, compared to this fungicide, the efficiency gain obtained using methanolic extracts of S. vulgare at 50 mg/ml, based on lesion diameter and mean penetration, was estimated at more than 47 and 62%, respectively. It should be noted that these extracts were found to be also active against *F. sambucinum*, which was shown to be resistant to carbendazim.

All organic extracts were subjected to a dosing of total polyphenols according to the Folin-Ciocalteau method but only the methanolic extracts (from Tunis, Monastir and Mahdia1) were analyzed by HPLC-DAD where more than 64 components were detected including phenolic acids and flavonoids. Thus, S. vulgare can be exploited for the isolation of compounds active against these rot pathogens and probably other potato-associated fungi.

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Photo of the cover page: Thrips damage on orange fruit (Courtesy *Imen Belaam-Kort*)

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Plantae Senae in Terra Sena