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Contents

Photo of the cover page: *Potosia opaca* (Courtesy *Mohamed Lahbib Ben Jamâa*)

Guest Editorial

New European Union plant health regime: A more stringent regulation that could impact trade from developing countries in the near future

Outbreaks of plant diseases may have devastating effects on the quality of our lives and our economy. Plant diseases may affect the livelihoods of farmers, nursery owners or traders, the quality and prices of our food as well as the condition of our forests and parks. The example of the recent outbreak of Xylella fastidiosa *in Italy is very symptomatic. Notification on the presence of this pest was received for the first time in 2013 when it was already widely spread in the region of Apulia, the heart of Italy's olive production area. The disease has seriously damaged the agricultural economy, as well as the traditional landscape of the region. The conclusion of the European Commission was an urgent need to allocate more resources at an early stage in order to prevent future heavy losses due to the destruction of the European agricultural production or the environment by the entry or spread of plant pests within the European Union (EU) territory. Plant pests currently fall under different legal acts (seven EU directives) depending on their quarantine status or whether they affect the quality of plant reproductive material. This can lead to confusion among the users of those acts, within and also outside of the EU. It was thus important to ensure clarity and transparency for all affected parties, and notably for the competent authorities and the professional operators concerned.*

A new regulation for plant health (Regulation (EU) 2016/2031) was adopted on October 2016. These new rules on protective measures against pests of plants will replace the current directive (2000/29/EC) and will be applicable on 14 December 2019 after a transition period. This delay will give competent authorities in Europe, but also in developing countries, and professional operators time to adjust to the new rules, as well as for delegated and implementing acts to be adopted. In the meantime, Directive 2000/29/EC on harmful organisms and annexes remain applicable. The new legal framework should be seen as a major overhaul of the EU's Plant Health legislation.

The new "Plant Health Law" provides more comprehensive and clearer rules for the prevention of entry into, and spread within, the EU territory of pests injurious to plant health but this regulation also includes regulating pests on the basis of pre-established criteria for risk assessment and prioritising of pests with the most serious consequences. It means that more focus is being placed on "high-risk trade" coming from developing countries, namely African, Caribbean and Pacific countries (ACP Group of States) where the inspection services are weak and public or private resources rather limited. The new "Plant Health Law" introduces further requirements, or codifies existing practices, which concern imports into the EU. The European Commission considers that certain plants, plant products and other objects pose an unacceptable risk due to their likelihood

of hosting an EU quarantine pest. Depending on the availability of acceptable risk-mitigation measures, their introduction into, or movement within, the EU territory should be either prohibited or subject to special requirements. Those plants, plant products and other objects will be listed. The new "Plant Health Law" has thus introduced the concept of "high risk plants, plant products and other objects", namely commodities whose import will be prohibited into the EU until a full risk assessment (Pest Risk Assessment (PRA)) confirms their phytosanitary status. The European Commission is further required to adopt within two years (December 2018) a list of so-called high risk plants or plant products. This list will take into account the specific criteria in Annex III to the new Regulation. The import of most plants and plant products from non-EU countries will in principle be allowed, subject to certain conditions. Some will be prohibited or subject to very strict requirements if a risk assessment indicates that this is necessary due to the pests they might host. The new Regulation sets out more precise rules about the risk assessment and risk management supporting such measures. As an example, wood packaging material should only be imported into, or exported out of the EU if it bears the ISPM 15 mark.

Moreover, this new Regulation introduces also the concept of "priority pests". These are the EU quarantine pests with the most severe potential impacts on the economy, environment and/or society of the EU. They will be subject to enhanced measures concerning surveys, action plans for their eradication, contingency plans and simulation exercises. But for specific cases, where there is little experience with trade of certain plants or plant products and where related pest risks are still unknown, the new Regulation sets out the possibility to introduce temporarily phytosanitary import restrictions… or even a prohibition until more scientific information becomes available! The import of these commodities will be prohibited as long as no detailed risk assessment has been carried out to determine if such imports should be acceptable and, if yes, under which conditions.

Finally, all living plant material (namely entire plants, fruits, vegetables, cut flowers, seeds, etc.) will only be imported into the EU if accompanied by a phytosanitary certificate confirming their compliance with the EU legislation. Under the new Regulation, all plant passports will be issued using a common format, thus facilitating their visibility and making them more easily recognisable throughout the EU. The European Commission will adopt within two years a list of plant materials to be exempted from that certification if they are deemed safe for the EU territory.

Therefore, in many developing countries such as African countries exporting flowers, fruit and vegetables onto the European market, there is an urgent need for capacity building programs PRA. People, both from the private and public sectors, should be informed and trained on the FAO ISPM N°11 (PRA for quarantine pests, including analysis of environmental risks and living modified organisms), on the EPPO Standard 5/2 (Pest risk analysis) and should be able to use the EPPO Probabilistic Risk Assessment software platform (CAPRA 4.2) which has been designed to assist pest risk analysts in running the decision-support scheme for pest risk analysis. PRAs take time and resources, so it is essential for ACP stakeholders (public and private) to be

fully prepared and in a position to take any necessary action to prevent an impact on trade. Key dates that will clarify what needs to be done will be the listing of high risk commodities (December 2018), the exemptions to plant passports (December 2018), and the listing of priority pests (2019).

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Allelopthic Effects of *Ziziphus jujuba* and *Z. lotus* Leaf Extracts on *Triticum durum* and *Lens culinaris*

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ABSTRACT

Elaloui, M., Ghazghazi, H., Ennajah, A., Ben Youssef, I., Ben Othman, N., and Laamouri, A. 2017. Allelopthic effects of *Ziziphus jujuba* and *Z. lotus* leaf extracts on *Triticum durum* and *Lens culinaris.* Tunisian Journal of Plant Protection 12: 1-10.

Ziziphus species were known for their widespread uses in folk medicine. This work aimed to determine the secondary metabolites (total phenols, flavonoïds, and tannins) of aqueous leaf extracts of two *Ziziphus* species (*Z. jujuba* and *Z. lotus)* from different origins (Mahres, Mahdia, Kairouan, or Rouhia) and their allelopathic effects on *Triticum durum* and *Lens culinaris*. The germination percentage, plumule and radicle lengths were recorded after seven days. Total phenols and flavonoïds varied from 10 to 14.03 mg EAG/g DW (total phenols) and from 4.63 to 7 mg QE/g DW (flavonoïds) for *Z. jujuba* and *Z. lotus,* respectively. Tannin contents varied from 4.4 (*Z. jujuba)* to 6 mg CE/g DW (*Z. lotus*). The radicle length was strongly inhibited by 69.38% in *T. durum* and by 43.29% in *L. culinaris* especially when treated with *Ziziphus* spp. leaf extracts at 100 mg/ml concentration. Root length of *T. durum* was more inhibited (86.75%) by *Z. lotus* leaf extract than that of *L. culinaris*. High levels of phenolic compounds detected especially in *Z. lotus* leaf extract could justify its inhibitory effect on germination rate and seedling length. *Z. lotus* leaf extract could be used as herbicide to delete undesirable weeds.

Keywords: Allelopathy, leaf extract, phenolic compounds, *Ziziphus* species

The investigation of the natural products from medicinal plants became a great need in the pharmaceutical industry. Those products used as a source of therapeutic agents attempted to discern many plant potentialities such as antioxidant and antimicrobial activities (Amri et al. 2013). The allelopathy was one of these activities that gained much attention as shown by numerous reports on the subject (Elaloui et al. 2016; Grichi et al. 2016; Prasad et al. 2016). Therefore, an integration of natural allochemical systems has become a necessity. It was defined as harmful or beneficial interaction caused by a plant to neighboring species (plant, algae, and microorganisms) by secreting allelochemical compounds. These plants could reduce mitotic activity in roots and hypocotyls and inhibit seed germination and growth of other plants.

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In agriculture, pesticides and particularly herbicides were widely used in order to improve production and to delete undesirable weeds without considering their negative effects on human health and environment (Benmeddour 2010).

In Tunisia, many people included different *Ziziphus* parts in their nutritional preparations and traditional medicines (Elaloui et al. 2016). *Ziziphus* species especially *Z. jujuba* offered rich fruits that could be eaten fresh, dried or incorporated in cakes (Elaloui et al. 2016a). *Ziziphus* leaves could be also used in infusion teas or powders (Elaloui et al. 2016a). Allelopathic activity of *Z. spina-christi* grown in Tunisia was widely studied (Elaoui et al. 2016). However, no previous studies were reported on *Z. jujuba* and *Z. lotus* activities (Neffati et al. 2017). Most studies dealt with their chemical, antibacterial and nutritional composition (Benmeddour 2010; Elaloui et al. 2016; Ghazghazi et al. 2014). The present study was conducted to determine the chemical composition of these two *Ziziphus* species collected from different origins and to assess the allelopathic effects of their aqueous leaf extracts against *Triticum durum* (durum wheat) and *Lens culinaris* (lentil).

MATERIALS AND METHODS

Plant material.

Z. jujuba and *Z. lotus* leaves (Fig. 1) were sampled in summer 2014 (the maximum foliation period) from 25 years old plants. Durum wheat cv. Karim and lentil cv. Medik were used as test plants.

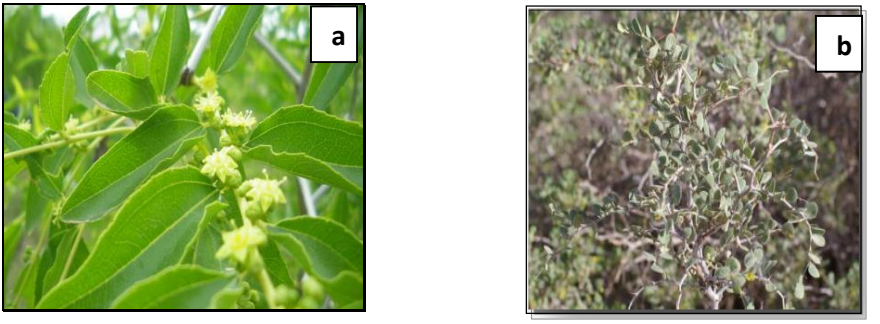

Fig. 1. *Z. jujuba* (a) and *Z. Lotus* (b) trees cultivated in Tunisian areas.

Four locations were selected and identified by Prof. Mohamed Boussaid (Laboratory of Plant Biotechnology (01/UR 0-9-10), INSAT, University of Carthage, Tunis, Tunisia) and a voucher specimen of leaves (N° Pn 959) was deposited at the Herbarium of INRGREF (Tunisia): ZL1: *Z. lotus* (Kairouan): ZL2: *Z. lotus* (Rouhia), ZJ1: *Z. jujuba* (Mahdia), and ZJ2: *Z. jujuba* (Mahres).

Collected leaves were air-dried and subsequently grinded. Leaf powders (5, 20, 40, 60 and 100 g) were macerated each with 1000 ml of distilled water for 24 h. The extracts were filtered through Whatman N°1 filter paper, pooled and concentrated under vacuum. Allelopathic bioassays were conducted using the obtained supernatant.

Determination of secondary metabolites.

Total phenols contents. Total phenol contents (TPC) in leaf extracts were determined using Folin-Ciocalteu method as described by Singleton et al. (1999) based on colorimetric method. The absorbance was measured at 760 nm using the standard curve. Calibration solutions were prepared using gallic acid. The results were given as mg gallic acid equivalent per 1 g of dry weight (mg GAE/g DW).

Flavonoïd contents. The total flavonoïd contents (TFC) were estimated using the aluminum chloride colorimetric method as described by Popova et al. (2004). The absorbance was measured at 430 nm and the concentration of total flavonoïds in the test sample was determined from the calibration curve using quercetin as standard. Results were expressed as mg quercetin equivalent per 1 g of dry weight (mg QE/g DW).

Tannin contents. Condensed tannins, determined according to the method of Sun et al. (1998), were expressed as mg catechin equivalents per 1 g of dry weight (mg CE/g DW).

Allelopathy bioassays.

After sterilization for 2 min with sodium hypochlorite (5%), *T. durum* and *L. culinaris* seeds were rinsed with distillated water. Then, 25 seeds were arranged in Petri plates (9 cm in diameter) lined with two discs of Whatman N°1 filter paper. Each Petri plate was moistened with 2 ml of the aqueous extract tested. Control seeds were similarly treated using 2 ml of deionized water. Bioassays were conducted under laboratory conditions during 7 days.

Parameters recorded were:

*The germination rate (%)

$$Gp=\frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100$$

*The shoot length in cm (SL)

*The root length in cm (RL)

*The rate of inhibition or stimulation (%)

$$\text{Inhibition(-)/Stimulation(+)}=\frac{\text{(Treated seeds)-Control}}{\text{Control}} \times 100$$

Control: Untreated seeds

Statistical analysis.

In this study, trial was carried out according to a completely randomized design with 3 studied fixed variables (factors) i.e. *Ziziphus* species (*Z. lotus* and *Z. jujuba*), geographical origin (JL1, JL2, ZJ1, and ZJ2) and concentration tested (5, 20, 40, 60 and 100 g/l). Results were statistically analyzed using STATISTICA (Statsoft, 1998). Each data presented was mean of three replicates (± standard deviation). Multiple mean comparisons were performed using the Student-Newman Keuls test with a significance level of $P < 0.05$.

RESULTS

Secondary metabolites contents.

The concentration of total phenolic contents (TPC) in *Ziziphus* leaves varied depending species and their geographical origins. The highest level of the total phenols, flavonoïds and condensed tannin was recorded in *Z. lotus* grown in Kairouan (Table 1) (TPC = 14.03 mg GAE/g DW; TFC = 7.6 mg QE/g DW; CTC = 6 mg CE/g DW) while the lowest contents in phenolics and tannins were observed in *Z. jujuba* collected from Mahdia (TPC = 10.1mg GAE/g DW, CTC = 4.4 ± 0.4 mg CE/g DW) and lowest flavonoïd contents were noted in *Z. lotus* from Rouhia (TFC = 4.63 ± 0.09 mg QE/g DW).

Table 1. Levels of phenolic compounds detected in *Z. lotus* and *Z. jujuba* leaf extracts depending on their geographical sampling sites

Plant	Phenols (mg GAE/g DW)	Flavonoïds (mg QE/g DW)	Tannins (mg CE/g DW)
ZL1	14.03 ± 0.13 c	7.60 ± 0.08 c	6.00 ± 0.46a
ZL2	10.87 ± 0.04 a	4.63 ± 0.09 b	5.64 ± 0.50a
ZJ1	10.10 ± 0.15 a	5.30 ± 0.27 a	4.40 ± 0.40b
ZJ2	12.10 ± 0.23 b	5.59 ± 0.12 a	5.45 ± 0.50a

Data are mean values of three replicates ± SD (standard deviation). Confidence intervals were calculated at the threshold of 5%. ZL1: *Z. lotus* (Kairouan); ZL2: *Z. lotus* (Rouhia); ZJ2: *Z. jujuba* (Mahres); ZJ1: *Z. jujuba* (Mahdia); DW: Dry weight; GAE: Gallic acid equivalent; QE: Quercetin equivalent; CE: Catechin equivalents. Values within each column followed by the same letter are not significantly different ($P < 0.05$) using Student-Newman Keuls test.

Allelopathic effect of *Ziziphus* spp. extracts on seed germination.

Seed germination of both target species (*T. durum* and *L. culinaris*) was influenced by many factors like extract concentration, *Ziziphus* species and their sampling sites (Fig. 2). About 20% of *L. culinaris* seeds failed to germinate when treated using ZL2 leaf extracts at the concentration 100 g/l. In fact, the germination rate decreased from 100% (at 5 g/l) to 80% (at 100 g/l) following treatments using this extract. Also, germination of *L. culinaris* seeds treated with ZJ2 extracts decreased from 100% (at 5 g/l) to 90% (at 100 g/l). These seeds were indifferent to ZJ1 leaf extracts treatment.

For *T. durum* seed germination, the effect of *Z. lotus* and *Z. jujuba* leaf extracts became less obvious with a level of inhibition reaching -15%. The highest concentrations of leaf extracts (60 and 100 g/l) gave the greatest inhibitory effects.

Allelopathic effect of *Ziziphus* spp. extracts on shoot length.

L. culinaris shoot elongation was significantly inhibited especially when treated by *Z. lotus* extracts (Table 2). The greatest inhibition occurred using *Z. lotus* extract where *T. durum* shoot length was lowered from 42 to 5.5 mm.

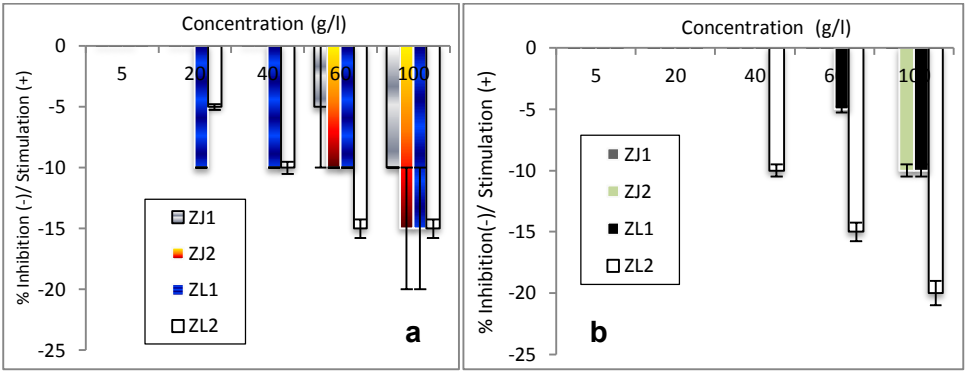

Fig. 2. Inhibitory effects of *Ziziphus jujuba* and *Z. lotus* leaf extracts on *Triticum durum* (a) and *Lens culinaris* (b) seed germination noted after 7 days of incubation. Data are mean values of three replicates ± SD (standard deviation); Confidence intervals were calculated at the threshold of 5%; ZL1: *Z. lotus* (Kairouan); ZL2: *Z. lotus* (Rouhia); ZJ2: *Z. jujuba* (Mahres); ZJ1: *Z. jujuba* (Mahdia).

Table 2. Inhibitory and stimulatory effects of *Z. lotus* leaf extracts on *Lens culinaris* and *Triticum durum* shoot length (in mm) noted 7 days after seed germination

Concentration (g/l)	Species	ZL1	ZL2
0	*L. culinaris*	25.0 ± 2 d	15.0 ± 0 a
	T. durum	46.0 ± 1 e	42.0 ± 3 d
5	*L. culinaris*	50.5 ± 4.5 j	33.0 ± 3 f
	T. durum	50.0 ± 2 j	43.5 ± 2.5 de
20	*L. culinaris*	27.5 ± 2.5 d	15.5 ± 0.5 a
	T. durum	29.0 ± 1 c	30.5 ± 1.5 c
40	*L. culinaris*	19.5 ± 0.5 e	13.75 ± 0.8 ac
	T. durum	10.5 ± 1.5 b	13.5 ± 1.5 f
60	*L. culinaris*	14.0 ± 1 ac	10.0 ± 0 bc
	T. durum	8.5 ± 1.5 ab	9.5 ± 0.5 b
100	*L. culinaris*	9.0 ± 3 b	8.0 ± 2 b
	T. durum	6.0 ± 0 a	5.5 ± 0.5 a

Data are mean values of three replicates ± SD (standard deviation); Confidence intervals were calculated at the threshold of 5%; ZL1: *Z. lotus* (Kairouan); ZL2: *Z. lotus* (Rouhia). Values within each column followed by the same letter are not significantly different ($P < 0.05$) using Student-Newman Keuls test.

The effect was more important in *T. durum*. In fact, shoot length decreased from 53 to 7.5 mm in response to treatments based on *Z. jujuba* extracts (Table 3).

Table 3. Inhibitory and stimulatory effects of *Z. jujuba* leaf extracts on *Lens culinaris* and *Triticum durum* shoot length (in mm) noted 7 days after seed germination

Concentration tested (g/l)	Species	ZJ1	ZJ2
0	*L. culinaris*	20.5 ± 4.5 ac	20.0 ± 5 ac
	T. durum	53.0 ± 1 a	50.0 ± 0 a
5	*L. culinaris*	40.5 ± 3.5 e	38.0 ± 1 e
	T. durum	62.5 ± 3.5 e	52.0 ± 1 a
20	*L. culinaris*	28.5 ± 1.5 d	24.5 ± 2.5 cd
	T. durum	49.0 ± 1 a	35.0 ± 4 d
40	*L. culinaris*	17.5 ± 2.5 a	17.0 ± 3 a
	T. durum	14.5 ± 2.5 b	19.0 ± 3 c
60	*L. culinaris*	15.0 ± 0 ab	16.5 ± 2.5 a
	T. durum	11.0 ± 1 ac	9.5 ± 1.5 a
100	*L. culinaris*	10.0 ± 2 b	10.5 ± 0.5 b
	T. durum	7.5 ± 1.5 a	8 ± 1 a

Data are mean values of three replicates ± SD (standard deviation); Confidence intervals were calculated at the threshold of 5%; ZJ2: *Z. jujuba* (Mahres); ZJ1: *Z. jujuba* (Mahdia). Values within each column followed by the same letter are not significantly different ($P< 0.05$) using Student-Newman Keuls test.

All concentrations of tested leaf extracts (except 5 g/l,) had inhibited *L. culinaris* shoot length. The most important values were obtained with ZL1. This inhibition ranged between -21.65% (40 g/l) and -64.73% (100 g/l).

Shoot lengths of *T. durum* were inhibited especially when treated with 100 g/l of *Z. lotus* leaf extract. This inhibition reached levels of -84 and -86% for ZJ2 and ZJ1, respectively. The highest inhibitions were obtained at the highest concentrations.

Allelopathic effect of *Ziziphus* spp. extracts on root length.

T. durum root length was highly affected by leaf extracts from *Ziziphus* species applied at 100 g/l concentration where this parameter decreased from 72

to 22 mm (Table 3). Less inhibitory effects were recorded using *Z. jujuba* leaf extracts. Roots of *T. durum* and *L. culinaris* reached 28 mm in length. Results showed also that all tested extracts had stimulatory effects at lowest concentration 5 g/l.

The inhibitory potential of *Ziziphus* extracts confirmed the superiority of *Z. lotus* leaf extracts in suppressing root growth than *Z. jujuba* (Fig. 3).

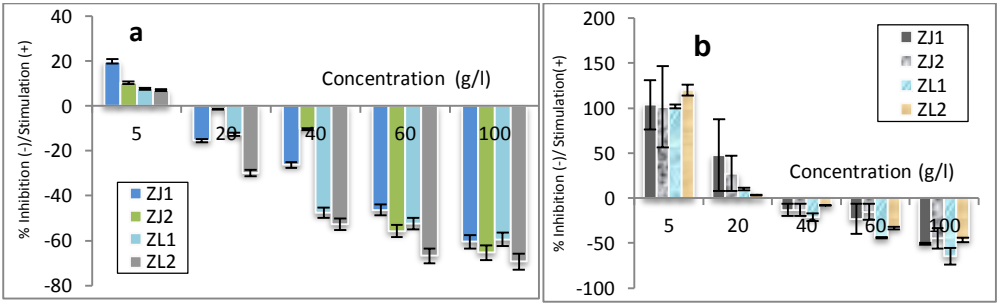

Fig. 3. Inhibitory and stimulatory effects of *Ziziphus jujuba* and *Z. lotus* leaf extracts on *Triticum durum* (a) and *Lens culinaris* (b) root length noted 10 days after seed germination. Data are mean values of three replicates ± SD (standard deviation); Confidence intervals were calculated at the threshold of 5%; ZL1: *Z. lotus* (Kairouan); ZL2: *Z. lotus* (Rouhia); ZJ2: *Z. jujuba* (Mahres); ZJ1: *Z. jujuba* (Mahdia).

DISCUSSION

Secondary metabolite contents obtained for the *Ziziphus* leaf extracts showed an interspecific and intraspecific variability between the two species (*Z. lotus* and *Z. jujuba*) and the four geographical origins (ZJ1, ZJ2; ZL1 and ZL1). For *Z. jujuba*, the phenol contents obtained for ZJ2 were higher than those of ZJ1. This variability may be related to different climate or soil composition of sampling sites (Mahdia and Mahres). Similar idea was also reported by Muller-Riebau et al. (1997).

The comparison between the two *Z. lotus* sampling sites showed that the highest phenolic compounds were noted for ZL1 (14.03 mg GAE/g) compared to ZL2 (10.87 mg GAE/g DW). This result could be explained by the fact that ZL1 leaves were collected from younger trees compared to those collected from ZL2. Similar findings were obtained by Shrivastava et al. (2016). Results showed also that the majority of analyzed compounds were stocked in *Z. lotus* leaves compared to those of *Z. jujuba*. In fact, the environment conditions of *Z. lotus* sampling sites (Kairouan and Rouhia) could not be adequate. In this case, the plant promoted the synthesis of secondary metabolites in order to adapt and survive (Tim and lamb 2005).

Total phenolic content found in *Z. jujuba* leaf extracts in this study were more abundant (14.03 mg GAE/g) than those found (6.04 mg GAE/g) in a previous work (Elaloui et al. 2016) using the acetone-water (4:1, v/v) as solvent extract. This variability depending on the experimental condition justified that the acetone could not be used as the suitable

solvent of phenolic compounds from *Ziziphus* leaves (Elaloui et al. 2016a).

Inhibitory effect on germination and seedling development on *L. culinaris* and *T. durum* were obtained especially at higher concentrations (60 and 100 g/l). Similar findings were also recorded by many researchers (Arora et al. 2015; Prasad et al. 2016; Turker et al. 2008). The degree of inhibition was largely dependent on the concentration of the aqueous extracts (Turk et al. 2002). The root lengths were more inhibited than shoot lengths. These results are in agreement with those of Patil and Kore (2016). This inhibition could be caused by the presence of certain allelochemicals such as terpenoids that contributed to inhibition of gibberellins and indole acetic acid function in meristimatic cells (Asharafi et al. 2008). In fact, the allelochemicals present in the leaf extract could reduce the amylase activity and prevent seed germination (Hegab et al. 2016).

T. durum seeds were more sensitive than those of *L. culinaris* to all tested treatments. A similar effect was recorded on *Trigonella foenum-graecum* and *L. culinaris* seeds treated with *Z. spina-christi* leaves (Elaloui et al. 2016a).

The results obtained in this study showed that different extracts of *Ziziphus* leaves inhibited germination and growth of *T. durum* and *L. culinaris* seedlings. Therefore, it was not possible to determine the principal or direct action of the allelochemicals present in *Ziziphus* extracts. However, these effects were probably related to the biosynthetic metabolism, concentration and/or sensitivity of the various plant hormones. It would also be interesting to explore the composition of *Zizphus* extracts using HPLC method.

These results were obtained under in vitro conditions and constituted a first step in the search of a natural product. Thus, in vivo study of leaf aqueous extracts of the two *Ziziphus* trees would be desirable, in order to improve the valorization of these products on the improvement of agricultural production.

RESUME

Elaloui M., Ghazghazi H., Ennajah A., Ben Youssef I., Ben Othman N. et Laamouri, A. 2017. Effets allélopathiques des extraits des feuilles de *Ziziphus jujuba* et *Z. lotus* sur la germination et la croissance de *Triticum durum* et de *Lens culinaris*. Tunisian Journal of Plant Protection 12: 1-10.

Les espèces de *Ziziphus* sont connues pour leurs utilisations répandues en médecine traditionnelle. Ce travail a pour objectif de déterminer les métabolites secondaires (phénols totaux, flavonoïdes et tannins) des extraits aqueux des feuilles de deux espèces de *Ziziphus* (*Z. jujuba* et *Z. lotus*) collectées à partir de différentes origines (Mahdia, Mahres, Kairouan ou Rouhia) et évaluer leurs effets allélopathiques contre *Triticum durum* et *Lens culinaris*. Le taux de germination, la longueur des plumules et des radicules ont été notés après sept jours. Les phénols totaux et les flavonoïdes ont varié respectivement de 10 à 14,03 mg EAG / g DW (phénols totaux) et de 4,63 à 7 mg QE / g DW (flavonoïdes) pour *Z. jujuba* et *Z. lotus*, respectivement. Les teneurs en tannins ont varié de 4,4 (*Z. jujuba*) à 6 mg CE / g DW (*Z. lotus*). Les longueurs des radicules ont été fortement inhibées à des taux de 69,38% chez *T. durum* et de 43,29% chez *L. culinaris* surtout lorsqu'elles ont été traitées par des extraits des feuilles de *Ziziphus* à une concentration de 100 mg/ml. Les longueurs des tiges de *T. durum* ont été les plus inhibées (86,75%) que celles de *L. culinaris*. Des taux élevés en composés phénoliques ont été détectés dans les extraits de feuilles de *Z. lotus*; ce qui pourrait justifier leurs effets inhibiteurs

sur la vitesse de germination et la longueur des plantules. L'extrait aqueux des feuilles de *Z. lotus* pourrait être utilisé comme un herbicide pour supprimer les mauvaises herbes.

Mots clés: Allélopathie, composés phénoliques, espèces de *Ziziphus,* extraits des feuilles

ملخص

العلوي، مريم وحنان الغزغازي وأمال النّاجح وإيمان بن يوسف وندى بن عثمان وعبد الواحد لعموري.2017التأثير المجاهضي لمستخلصات أوراق السّدر والعنّاب على القمح الصّلب و العدس.
Tunisian Journal of Plant Protection 12: 1-10.

اشتهر السّدر باستخدامه على نطاق واسع في الطّب التقليدي. يهدف هذا العمل إلى تقدير بعض المستقلبات الثّانويّة (الفينولات الجملية والفلافونويد والعفص) من مستخلصات أوراق نوعين من السّدريات: السّدر (*Ziziphus lotus*) والعنّاب (*Ziziphus jujuba*) والتي جمعت من أماكن مختلفة (المهدية والمحرس والقيروان والروحية) وتأثيرها المجاهضي على القمح الصّلب والعدس.سجلت نسبة الإنبات وطول الجذير وطول الجذع بعد سبعة أيام. تراوحت نسب الفينولات الجملية والفلافونويد بين 10 و 14.03 ملغ من معادل الحامض الغاليكي/غ من الوزن الجاف بالنسبة للفينولات الجملية ومن 4.63 إلى 7 ملغ من معادل كيرسيتين/غ من الوزن الجاف في مستحضرات العنّاب والسّدر، على التوالي. وتراوحت نسبة العفص بين 4.4 (العنّاب) و 6 (السّدر).ملغ من معادل كاتشين/غ من الوزن الجاف .لوحظ أعلى تأثير سمّي خاصّة على الجذور بنسب 69.38% (القمح الصلب) و 43.29% (العدس) عند تركيز 100 غ/ل. كانت سيقان القمح الصّلب الأكثر تأثّرا (86.75%) عند معالجتها بمستحضرات السّدر مقارنة بسيقان العدس. إنّ النّسب العالية المسجلة من مركّبات الفينولات وخاصة تلك المتأتية من مستخلصات أوراق السّدر يمكن أن تبرر تأثيرها على نموّ الجذور وطول البادرات التي تم اختبارها.يمكن أن تستخدم مستخلصات أوراق السّدر كمبيد لمقاومة الأعشاب الضارة.

كلمات مفتاحية: سدريات، مجاهضة، مركبات فينولية، مستخلصات ورقية

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Short Communication

Effect of Ploidy Level of *Trigonella foenum-graecum* on its Allelopathic Potential

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ABSTRACT

Omezzine, F., and Haouala, R. 2017. Effect of ploidy level of *Trigonella foenum-graecum* on its allelopathic potential. Tunisian Journal of Plant Protection 12: 11-18.

The present study was conducted to assess allelopathic activity of diploid and mixoploid fenugreek (*Trigonella foenum-graecum*) aqueous extracts of dried shoots (10, 20, 30 and 40 g/l) against lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*), and peganum (*Peganum harmala*). Results showed that fenugreek allelopathic potential was significantly enhanced by polyploidization. Mixoploid plant extracts have more significant effects ($P < 0.05$) on all target species than that of diploid plants at all tested concentration levels. At the high concentration of 40 g/l, mixoploid plant extract induced 9% more inhibition on lettuce root growth, 21% more on peganum, and 36% more on alfalfa than that from diploid plant. Based on the whole-range inhibition index for growth, the allelopathic potential of mixoploid plants was enhanced by 24.4% on lettuce, 79.7% on peganum, and 37% on alfalfa. There was a significant difference in sensitivity towards fenugreek allelopathy among the target species with lettuce being the most sensitive and alfalfa the least affected. Such differences were consistent for both mixoploid and diploid plants. Fenugreek may be favorably used in sustainable weed management. Moreover, it seems that polyploidy induction is an effective tool to increase fenugreek allelopathic activity.

Keywords: Allelopathic potential, fenugreek, inhibition index, mixoploidy

Currently, there is a growing interest in searching for novel natural plant products to develop bio-herbicides and bio-pesticides (Haig et al. 2009; Seal et al. 2009; 2010). Numerous plants have been reported to possess allelopathic potential and efforts have been made to apply them for the control of weeds or phytopathogenic fungi. Although most common allelopathic plants have potential for weed suppression, their effects are usually short-lived and weeds re-emerge after application (Xuan et al. 2005). Furthermore, to successfully suppress the initial growth of weeds, a large amount of plant materials needs to be applied to the soil, which also requires a large labor force. Isolation and identification of allelochemicals in higher

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plants have been attempted but often the identified allelochemicals with strong biological activities were in low concentrations. Therefore, enhancement of plant allelochemical contents remains a great challenge. Allelopathic substances have potential either as herbicides or templates for new synthetic herbicide classes (Duke et al. 2000). These allelochemicals are, particularly, prone to qualitative and quantitative variations, depending on genetic drift and ploidy level (Te Beest et al. 2012), physiological conditions, season, harvesting time and analytical method used for sample preparation (Çirak et al.2008).

Fenugreek (*Trigonella foenum-graecum*) is a diploid annual legume that has been used as an effective medicinal plant as well as fodder plant with economic repercussions. The species is reported to have insecticidal and antifungal activity and have strong allelopathic potential (Evidente et al. 2007; Omezzine and Haouala 2013; Omezzine et al. 2014 a). The allelopathic potential of various parts of fenugreek were evaluated on seed crops and weeds and extracts from the shoots were found to be the most active and showed selective activity (Haouala et al. 2008).

The objectives of this study were (1) to assess the allelopathic potential of resulted polyploid plants, and (2) to explore alternatives for enhancing natural allelopathic potential of plants via improving their genetic make-up for better and effective weed control in field.

The mixoploid plants of fenugreek were obtained following seed treatment with 0.05% colchicine solution, according to the method of Omezzine et al. (2012). Fenugreek treated seeds were sown in field under natural conditions. The mixoploidy confirmation was done by flow cytometry and stomata and pollen grain size (Omezzine et al. 2012). Fresh plants were washed with tap water, then oven-dried at 60°C for 72 h, powdered and used for extraction.

Forty grams of dried shoots of fenugreek diploid and mixoploid plants were separately extracted by soaking in 1 liter of distilled water at 24°C for 24 h in shaker to give a concentration of 40 g dry tissue per liter for final stock extract. The extracts were filtered through two layers of cheesecloth to remove fiber debris, and centrifuged at 4530 g for 2 h. The supernatants were vacuum filtered again through Whatman N°42 filter paper and stored at 4°C until the commencement of bioassay.

Each extract was diluted with sterile distilled-water to give final concentrations between 10 and 40 g/l. They were tested on lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*) and peganum (*Peganum harmala*). Seeds of test species were surface sterilized by immersing in 0.525 g/liter sodium hypochlorite for 15 min, rinsed four times with deionized water, then imbibed in deionized water at 22°C for 12 h, and carefully blotted using folder paper towel. Twenty swollen seeds were evenly placed on filter paper wetted with extract in each Petri dish and kept in a growth chamber [24°C, 14 h light 400 µmol photons/m^2s photo synthetically active radiation (PAR), 10 h dark and 22°C]. A volume of 5 ml of respective extract was applied for each treatment. Distilled water was used as control. Three replicates were incubated in a randomized complete block design. Germination was recorded at 24 h intervals until 144 h. A seed was considered germinated when radical protruded ≥ 2 mm (Hou and Romo 1998). Data were transformed to percentage of control for further analysis. At the 7th day, the shoot and root length and dry weight of test species seedlings were measured

and weighed. Data were transformed to percentage of control for analysis.

All data were subject to ANOVA and Duncan–test with SPSS 13.0 for Windows. Whole-range assessment method by An et al. (2005) and Liu et al. (2007) was employed to assess the overall allelopathic potential of fenugreek.

Seed germination of test species was significantly delayed and reduced, particularly at high concentrations of the extracts. These effects were enhanced in presence of extracts from mixoploid plants (Fig. 1). Lettuce was the most sensitive species and its germination was reduced up to 63.4 and 86.7% by diploid and mixoploid plant extracts of 40 g/l, respectively. At the same concentration, the germination of other test species was reduced by 28.4-35.0% by diploid plant extract and by 26.7-37.0% by that from mixoploid plants.

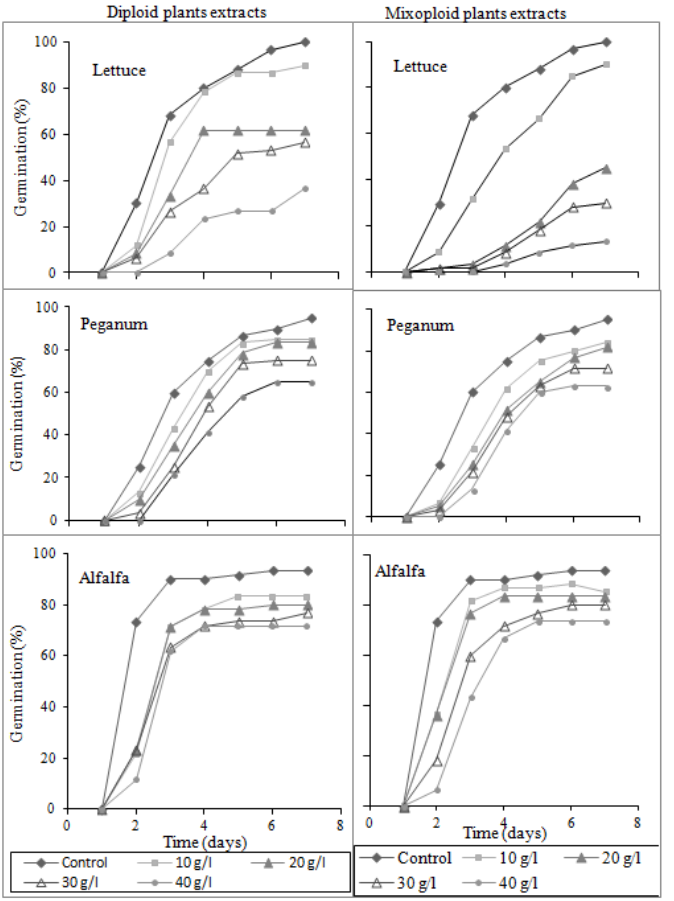

Fig. 1. Effect of aqueous extracts of diploid and mixoploid *Trigonella foenum-graecum* at different concentrations on germination rate of lettuce, peganum and alfalfa.

Seedling growth of all test species was significantly reduced by both diploid and mixoploid plant extracts (Fig. 2). Alfalfa showed great tolerance to fenugreek aqueous extracts, whereas lettuce was the most sensitive since its root and shoot growth were inhibited by 82 and 99% with diploid and mixoploid plants extracts, respectively.

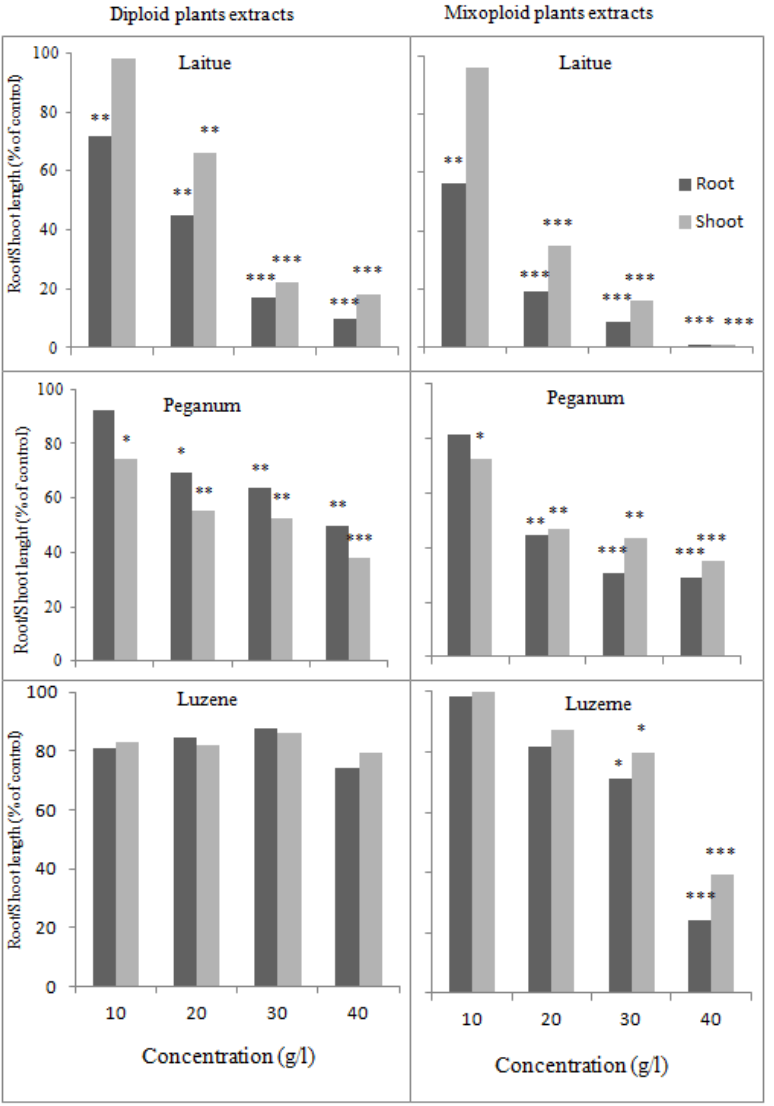

Fig. 2. Effect of aqueous extracts of diploid and mixoploid *Trigonella foenum-graecum* on root and shoot growth of lettuce, peganum and alfalfa, noted 7 days after germination. Statistical differences (Duncan test) from control are marked with one ($P \leq 0.05$), two ($P \leq 0.01$) or three asterisks ($P \leq 0.001$).

The comparison of the effects of fenugreek aqueous extracts (diploid and mixoploid) on the growth of target plants shows that, overall, the mixoploidy biomass is more toxic. At the concentration of 40 g/l, root growth inhibition of all test species was of an average of 56.5% by diploid plants extract compared to 81.9% by mixoploid plants. Measured by root inhibition index of the whole-range assessment, the allelopathic potential of fenugreek was enhanced by mixoploidy 24.4% on lettuce root growth in comparison with diploid plants, 79.7% on peganum, and 37.0% on alfalfa. However, as estimated based on shoot inhibition index, enhancement was 26.3% on lettuce, 11.8% on peganum, and 23.0% on alfalfa (Table 1). Average enhancement across all test species was greater than 20%.

Table 1. Phytotoxicity of diploid and mixoploid *Trigonella foenum-graecum* aqueous extracts on lettuce, peganum and alfalfa growth, assessed by inhibition index (I) estimated from WESIA (Whole-range Evaluation of the Strength of Inhibition in Allelopathic-bioassay).

Test species	Root inhibition			Shoot inhibition		
	Diploid plant (%)	Mixoploid plant (%)	Allelopathy enhancement (%)	Diploid plant (%)	Mixoploid plant (%)	Allelopathy enhancement (%)
Lettuce	53.3	66.3	24.4	43.8	55.3	26.3
Peganum	24.8	44.6	79.7	37.8	42.3	11.8
Alfalfa	17.3	23.6	37.0	15.3	18.8	23.0

This study showed that germination of lettuce, peganum, and alfalfa was inhibited by fenugreek plant extracts, and such inhibition was largely dependent on extract concentration. Our findings are consistent with those reported elsewhere for other species in a variety of plant families (Gatti et al.2008; Liu et al. 2008; Omezzine et al. 2011). Shoot extracts of fenugreek also significantly delayed seed germination with the increase of extract concentration. The delay was more apparent with mixoploid plant extract. This result is in agreement with those of Omezzine and Haouala (2013) and Omezzine et al. (2014 b) who showed that the aqueous extracts of fenugreek aerial parts (diploid and mixoploid) harvested at the vegetative stage strongly inhibited the germination of lettuce. Similarly, Haouala et al. (2008) showed that crude extracts of fenugreek plant parts exhibited a strong allelopathic potential on seed germination of test crops and that extracts from the aerial parts were the most inhibitory. In addition, Dong et al. (2013) reported that the germination of *Orobanche minor* depended on ploidy level, growth stage and different concentrations of *Triticum aestivum* aqueous extracts. The toxicity variation of fenugreek aqueous extracts could be explained by the variation in their chemical composition.

For growth, roots were more sensitive than shoots and fenugreek extract phytotoxicity was much stronger when concentration increased. These results are in agreement with previous findings reporting that water extracts of allelopathic plants generally have more pronounced effects on radicles, rather than hypocotyl growth (Turk et al. 2002) because radicles are directly exposed to allelochemicals. Furthermore, the increase in the allelopathic potential of fenugreek mixoploid plants may have contributed to an increase in the content of allelochemical compounds or to synthesis of new toxic molecules, which would be ascribable to the mixoploidy. It was reported that the genetic load of

species is a very significant factor in the differences in the behavior and the morphological, physiological and biochemical characters of plants. Colchicine could affect the primary and the secondary metabolism of plants. This finding corroborates with that of Omezzine et al. (2014 b) who showed that the toxicity of fenugreek biomass varied with the development stage, for diploid and mixoploid plants, and that the highest toxicity was recorded in presence of biomass extracts harvested at the vegetative and flowering stages for mixolpoid plants.

In conclusion, our results showed that the aqueous extracts from mixoploid plants were more toxic than those from diploid ones. Similarly, it seems that polyploidy induction is an effective method to increase the production of secondary metabolites since extract toxicity of diploid and mixoploid plants was different.

RESUME

Omezzine F. et Haouala R. 2017. Effet du niveau de ploïdie de *Trigonella foenum-graecum* sur son potentiel allélopathique. Tunisian Journal of Plant Protection 12: 11-18.

La présente étude a été menée afin d'évaluer l'activité allélopathique des extraits aqueux de plantes diploïdes et mixoploïdes du fenugrec *Trigonella foenum-graecum* (10, 20, 30 et 40 g/l) sur la laitue (*Lactuca sativa*), la luzerne (*Medicago sativa*) et le peganum (*Peganum harmala*). Les résultats ont montré que le potentiel allélopathique du fenugrec a été significativement amélioré par la polyploïdisation. Les extraits de plantes mixoploïdes ont eu des effets plus significatifs ($P < 0,05$) sur toutes les espèces cibles comparées aux plantes diploïdes à tous les niveaux de concentration testés. A la concentration la plus élevée (40 g/l), l'extrait de plantes mixoploïdes a induit une inhibition 9% de plus sur la croissance racinaire de la laitue, 21% de plus sur le peganum et 36% de plus sur la luzerne que celui des plantes diploïdes. En se basant sur l'indice d'inhibition de la croissance, le potentiel allélopathique des plantes mixoploïdes a été amélioré de 24,4% pour la laitue, de 79,7% pour le peganum et de 37% pour la luzerne. Il y avait une différence significative dans la sensibilité des espèces cibles vis-à-vis de la toxicité du fenugrec où la laitue a été la plus sensible et la luzerne a été la moins touchée. De telles différences ont été importantes pour les plantes mixoploïdes et diploïdes. *T. foenum-graecum* peut être favorablement utilisé pour une gestion durable des mauvaises herbes. En outre, il semble que l'induction de la polyploïdie est un outil efficace pour augmenter l'activité allélopathique du fenugrec.

Mots clés: Fenugrec, indice d'inhibition, mixoploidie, potentiel allélopathique

ملخص

أم الزين، فاتن وربيعة حوالة. 2017. تأثير مستوى الصيغة الصبغية للحلبة *Trigonella foenum-graecum* على إمكانياتها في المجاهضة. Tunisian Journal of Plant Protection 12: 11-18.

أجريت هذه الدراسة لتقييم نشاط المجاهضة للمستخلصات المائية للنباتات مضاعفة ومختلطة الصيغ الصبغية من الحلبة *Trigonella foenum-graecum* (10 و 20 و 30 و 40 غ/ل) على الخس *Lactuca sativa* والفصة *Medicago sativa* والحرمل *Peganum harmala*. أظهرت النتائج أن إمكانية المجاهضة للحلبة تحسنت بشكل معنوي بواسطة تغيير الصيغ الصبغية. للمستخلصات المائية لنباتات مختلطة الصيغ الصبغية تأثيرات أكثر معنوية (احتمال < 0.05) على جميع الأصناف المستهدفة من النباتات مضاعفة الصيغ الصبغية مع جميع التركيزات التي تم اختبارها. مع تركيز عال (40 غ/ل)، أدّت مستخلصات نباتات مختلطة الصيغ الصبغية إلى تثبيط أكثر بـ 9% على نمو جذور الخس و 21% على الحرمل و 36% على الفصة مقارنة بنباتات الحلبة المضاعفة. بالاعتماد عل مؤشر التثبيط،

تحسنت إمكانيات المجاهضة أكثر عند نباتات مختلطة الصيغ الصبغية (24.4% للخس و 79.7% للحرمل و 37% للفصة). كان هناك اختلاف معنوي في حساسية الأنواع المستهدفة فيما يتعلق بسُميّة الحلبة حيث كان الخس أكثر حساسية والفصة الأقل حساسية. وكان هذا الاختلاف مهم للنباتات مختلطة ومضاعفة الصيغ الصبغية. يمكن استخدام الحلبة بشكل إيجابي في الإدارة المستدامة للأعشاب الضارة.وعلاوة على ذلك، يبدو أن تعدد الصيغ الصبغية هو أداة فعالة لزيادة نشاط المجاهضة لدى الحلبة.

كلمات مفتاحية: إمكانية المجاهضة، اختلاط الصيغ الصبغية، حلبة، مؤشر التثبيط

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In vitro Antifungal Activity of Different Plant Extracts against *Phytophthora infestans* the Causal Agent of Potato Late Blight

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ABSTRACT

Messgo-Moumene, S., Boukhalfa, R., Belaïdi, D., Beninal, L., Haddadj-Hamdi, S., and Bellatreche, M. 2017. In vitro antifungal activity of different plant extracts against *Phytophthora infestans* the causal agent of potato late blight. Tunisian Journal of Plant Protection 12: 19-33.

Our study aimed to evaluate the in vitro antifungal activity of aqueous extracts prepared from seven medicinal plants (*Carya illinoinensis*, *Equisetum arvense*, *Rosmarinus officinalis*, *Pistacia lentiscus*, *Mentha suaveolens*, *Punica granatum*, and *Posidonia oceanica*) against A_1 and A_2 isolates of *Phytophthora infestans* the causal agent of potato late blight. The crude (100%) plant extracts were prepared by decoction and tested at various concentrations (70, 50, 30 and 10% v/v) for determining their relative effectiveness against target pathogen. Their antifungal potential was assessed based on their ability to inhibit pathogen mycelial growth, sporulation, germination and their capacity to affect pathogen in vitro and in vivo survival after treatment. Tested aqueous extracts showed a variable efficiency. For all noted parameters, the greatest inhibition rates were recorded using aqueous extracts from *P. granatum* bark and from *P. lentiscus* leaves and berries (88%) used at the concentration of 10% v/v. Their antifungal potential was expressed by lysis of mycelia and sporangia as well as inhibition of *P. infestans* mycelial growth. Chemical analysis of phenolic compounds of tested aqueous extracts revealed a close relationship between their contents in total polyphenols and the observed antifungal activity. This study clearly demonstrated that pomegranate bark and leaves or berries mastic can be explored as potential sources of bioactive molecules for potato late blight control.

Keywords: Biocide effect, *Phytophthora infestans*, plant aqueous extracts, polyphenols, *Solanum tuberosum*

Late blight caused by the pseudo-fungus *Phytophthora infestans* is the most important disease of potato crops worldwide. It can become pandemic under low temperatures below 20ºC and high relative humidity (over 90%). Under favorable conditions, total destruction of potato crops may occur within 5 days (Namanda et al. 2004).

Commercially grown potato cultivars are moderately or extremely

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susceptible to disease. Thus, chemical control is still being the mostly used tool for late blight management (Fry and Goodwin 1997). However, the abusive use of metalaxyl, active ingredient in various systemic fungicides, has led to the development of highly resistant *P. infestans* strains, thus, rendering disease control more difficult and increasing crop production costs (Bashan et al. 1989; Daayf and Platt 2000; Daayf et al. 2000).

In response to public concerns about fungicide toxicity and health risks together with environmental pollution, the tendency to substitute these chemicals with naturally derived products was timely in plant protection. Thus, natural plant-derived products seem to be the most interesting alternatives.

In this concept, seven herbal aqueous extracts were evaluated in vitro against *P. infestans* to determine their antifungal potential against mycelial growth, sporulation and germination as well as pathogen survival after treatment, followed by chemical identification of polyphenols in the different tested plant extracts.

MATERIALS AND METHODS

Plant material.

Seven medicinal plants were selected for this study. Plant parts collected for extraction were randomly removed during autumn 2015, air-dried and stored in dark glass jars until use. For the study of the in vivo pathogen survival after treatments, healthy detached leaves of potato cv. Spunta were used.

Table 1. Plants selected for this study and their sampling sites

Tested plant	Code	Common name	Organ used	Sampling site
Carya illinoinensis	CI	Pecan	Leaves	Regional station of the National Plant Protection of Boufarik city
Equisetum arvense	EA	Horsetail	leaves and stems	The experimental university station of Blida 1
Rosmarinus officinalis	RO	Rosemary	Leaves	The experimental university station of Blida 1
Pistacia lentiscus	PLL PLB	Mastic	Leaves and berries	The forest of Fouka marine city
Mentha suaveolens	MS	Fragrant Mint	Leaves	The fields in Kolea city
Punica granatum	PG	Pomegranate	Bark	The fields in Kolea city
Posidonia oceanica	PO	Posidonia	Leaves	The sea in Cherchell

Pathogen inoculum.

Two purified isolates of *P. infestans* were used. They were originally isolated from potato-producing areas at El Abbadia (Department of Ain Defla) and Bourkika (Department of Tipasa) sites. They were previously identified as belonging to A_1 and A_2 *P. infestans* mating types (Messgo-Moumene et al. 2015).

Preparation of plant aqueous extracts.

Plant aqueous extracts were prepared according to Kalbende and Dalal (2013) protocol with some modifications. A 100 g-sample of plant material powder was mixed with 1000 ml of sterile distilled water (SDW) and held on a rotary shaker for 12 h. The mixture was subsequently filtered through a double-layered tissue and then centrifuged at 4000 rpm for 30 min.

The supernatant was filtered through Whatman filter paper No. 1 and sterilized by autoclaving at 100°C for 10 min. The resulting crude plant aqueous extracts were separately stored aseptically at 4°C for later use.

Assessment of the in vitro antifungal activity of plant extracts against *P. infestans.*

Inhibition of pathogen mycelial growth. The in vitro antifungal activity of tested plant extracts was assessed based on Farooq and Nasreen (2015) technique. It consists of placing 2 ml of each crude (100%) extract in Petri plates (90 mm in diameter) to which 20 ml of molten (cooled at 48°C) Agar Pea medium (PPA) were added just before solidification. A series of dilutions (70, 50, 30 and 10% (v/v)) were prepared for each plant extract. Control plates were poured with PPA medium only.

After medium solidification, 5 mm of mycelial plugs, removed from 21-day-old cultures of either A1 or A2 *P. infestans* isolates, were deposited on medium surface. After 7 days of incubation at 20°C, the diameter of pathogen colony was measured. The antifungal activity of aqueous plant extracts was determined by calculating mycelial growth inhibition rates according to Mishra and Dubey (1992) formula:

$$\mathrm{I}\ (\%) = \frac{(\mathrm{DT} - \mathrm{Dt})}{\mathrm{DT}} \times 100$$

where I: Inhibition rate of mycelial growth (%), DT: Diameter of pathogen colony in control plates (mm), and Dt: Diameter of pathogen colony grown on medium amended with tested aqueous extract (mm).

Inhibition of pathogen sporulation and germination. After 21 days of incubation at 20°C, 10 ml of SDW were poured onto each culture of pathogen isolates growing on PPA medium amended or not with different concentrations (the same mentioned above) of the tested crude plant aqueous extracts. Colonies were scraped with a sterile Pasteur pipette and the obtained sporangial suspension was suspended in sterilized test tubes and stirred using a Vortex. The sporangial suspensions of A_1 and A_2 *P. infestans* isolates were used for the determination of the concentration of sporangia and germinating sporangia using a Malassez haemocytometer. The inhibition of sporulation was calculated based on Hmouni et al. (1996):

$$\mathrm{IS}\ (\%) = \frac{(\mathrm{ST} - \mathrm{St})}{\mathrm{ST}} \times 100$$

where IS: Inhibition rate of pathogen sporulation (%), ST: Concentration of sporangia (Number of sporangia/ml) in control tubes, and St: Concentration of sporangia in treated tubes.

In addition, the inhibition rate of sporangia germination (IG) was calculated for each isolate using Berber et al. (2009) formula:

$$\mathrm{IG}(\%) = \frac{(\mathrm{GT} - \mathrm{Gt})}{\mathrm{GT}} \times 100$$

where IG: Inhibition rate of sporangia germination (%), GT: Concentration of germinated sporangia in control tubes (number of germinated sporangia/ml), and Gt: Concentration of germinated sporangia in treated tubes.

Survival of P. infestans isolates after treatments. The survival of A_1 and A_2 *P. infestans* isolates grown on PPA medium amended or not with tested plant aqueous extracts was assessed in vitro on unamended PPA medium and in vivo on potato leaf discs.

The in vitro trial was carried out according to Mahanta et al. (2007) technique with slight modification. This method relies on the recovery or not of mycelial growth previously inhibited by the extract after re-inoculation of explants on

fresh PPA medium and incubation under the same conditions. The mycelial growth was followed daily during one week.

Trial performed on potato leaf discs was carried out according to Klarfeld et al. (2009) method. Potato leaves were cut into discs of 3 cm in diameter, washed with water, disinfected for 10 min in 2% sodium hypochlorite solution and then rinsed with SDW.

Sterile filter papers impregnated with SDW were placed in plastic boxes containing potato leaf discs. Explants of control isolates and those previously treated with crude aqueous plant extracts, at the tested concentrations, were added.

Leaf discs were regularly examined for disease development. Results were expressed as the rate of infected leaf areas. Inhibition rate of late blight infection was determined according to Berber et al. (2009) formula:

$$\text{Inf}\,(\%) = \frac{(\text{InfT} - \text{Inf(t)})}{\text{InfT}} \times 100$$

where Inf: Leaf infection rate (%), Inf T: Infection rate in control leaf discs (%), Inf (t): Infection rate in treated leaf discs (%).

Determination of total polyphenols in plant aqueous extracts.

The total polyphenol contents in plant aqueous extracts were determined using the Folin-Ciocalteu method (Singleton and Rossi 1965) where 200 µl of the 1/50 diluted extract were mixed with 1 ml of Folin-Ciocalteu reagent at 10% and 0.8 ml of Na_2CO_3 at 7.5%. The mix was incubated at room temperature for 30 min. Absorbance was read at 765 nm using a spectrophotometer. The results were expressed in mg equivalent of gallic acid/µg of dry plant material.

Statistical analysis.

In order to validate the eventual efficiency of plant aqueous extracts against *P. infestans* isolates and to compare their antifungal activity based on the various pathogen biological parameters, statistical analysis were carried out using the SYSTAT software Version. The differences were considered significant at $P \leq 0.05$ (Philipeau 1989).

Analysis of the variance was carried out by the ANOVA test. It concerned the inhibition rates of mycelial growth, sporulation and germination of *P. infestans* at five replicates for each parameter.

The total polyphenol contents of each of the tested crude aqueous plant extracts (100%) were also analyzed by the ANOVA test at three replicates.

RESULTS

Effects of plant aqueous extracts on mycelial growth of *P. infestans* isolates.

The inhibition of *P. infestans* mycelial growth varied significantly (at $P \leq 0.05$) depending on the crude (100%) tested plant aqueous extracts, targeted pathogen isolates (A_1 and A_2) and their interactions. Meanwhile, mycelial growth inhibition rates were higher for A_2 *P. infestans* isolate (90-72%) than for A_1 mating type (90-25%) for all plant extracts excepting those from posidonia (5-15%). The inhibition was more important using mastic leaf and berry extracts, pomegranate bark (90%) for the two isolates and pecan leaf extracts (90%) just for A2 isolate, good for horsetail (80%), fragrant mint (75%) and rosemary plant extracts, concerning A2 isolate, but moderate for the common fragrant mint (53%) concerning the A1 isolate and remained low for all the other plant extracts (Fig. 1).

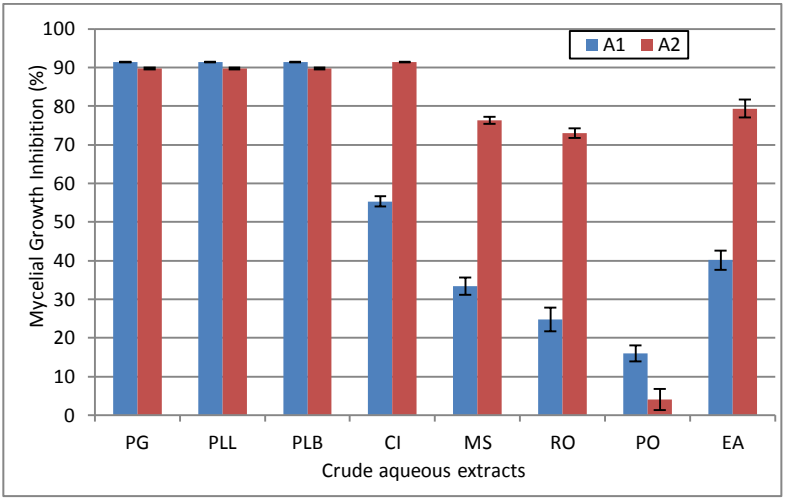

Fig. 1. Effect of tested plant aqueous extracts on the mycelial growth of *Phytophthora infestans* isolates cultured on Agar Pea Medium noted after 7 days of incubation at 20°C. Bar segments mean standard deviation. CI: *Carya illinoinensis*; EA: *Equisetum arvense*; RO: *Rosmarinus officinalis*; PL: *Pistacia lentiscus* (PLL: leaves & PLB: berries); MS: *Mentha suaveolens*; PG: *Punica granatum*; PO: *Posidonia oceanica*.

The three effective aqueous extracts of pomegranate bark, mastic leaves and berries showed variability in their potential to inhibit the mycelial growth of each A1 and A2 *P. infestans* isolates depending on used concentrations. All plant extracts tested at different concentrations induced a strong inhibition of the mycelial growth of the A2 isolate. It seems to be maximal for both *P. infestans* isolates at the following concentrations: 100, 70, 50 and 30% share for A1 isolate for pomegranate bark (75%). The analysis of variance revealed a significant difference ($P \leq 0.05$) between the two isolates of *P. infestans* with respect to the aqueous extracts of the pomegranate bark and the mastic leaves (PLL), but not significant for the mastic berries (PLB, P = 0.082).

A strong pathogen inhibition (90-82%) was induced by extracts applied at 100, 70, 50 and 30% for A2 isolate but just for the third first concentrations for A1 isolate. It should be highlighted that the inhibition rates recorded at the lowest concentration (10%) of all tested plant extracts exceeded 60% for isolate A2 and 70% for A1 isolate. Thus, these plant aqueous extracts can be recommended for the important reduction (90%) of pathogen growth at the inhibitory concentration of 30% (Fig. 2).

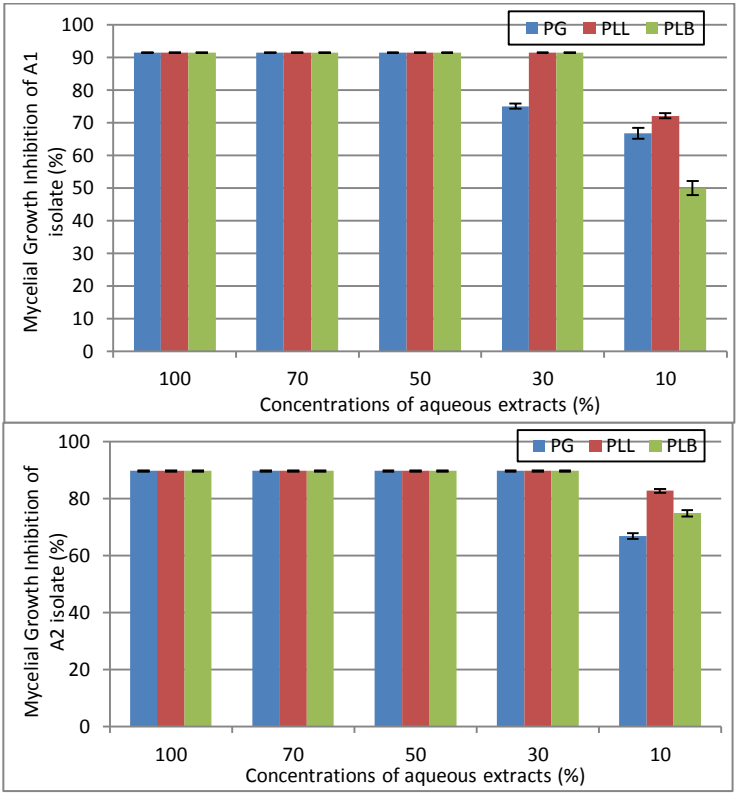

Fig. 2. Effect of three tested plant aqueous extracts at five concentrations on the mycelial growth of A1 (above) and A2 (below) *Phytophthora infestans* isolates cultured on Agar Pea Medium noted after 7 days of incubation at 20°C. Bar segments mean standard deviation. PG: *Punica granatum*; PLL: *Pistacia lentiscus* leaves; PLB : *Pistacia lentiscus* berries.

Effects of plant aqueous extracts on sporulation and germination of *P. infestans* isolates.

Analysis of the variance of sporulation and germination inhibition rates showed a significant difference ($P \leq 0.05$) between the extracts of tested plants but not significant between A1 and A2 of *P. infestans* isolates ($P = 0.976$ and $P = 0.392$ for the sporulation and germination trials, respectively).

Inhibition of *P. infestans* sporulation induced by tested extracts ranged between 15 and 100%. In fact, leaf (100%) and berry mastic (98%), pomegranate bark (100%) and pecan (80-95%) extracts triggered high levels of inhibition of pathogen sporulation whereas this effect appeared to be moderate using horsetail (58%) aqueous extract and remained low for the other tested plant extracts (Fig. 3). Moreover, all the tested extracts had inhibited the germination of *P. infestans* sporangia. The highest inhibition rates were recorded using extracts from mastic (berries and leaves) (100-94%), pomegranate bark (100%), pecan leaves (78-95%) but they were moderate for common horsetail leaves (55%) and low for the other plant extracts (Fig. 3).

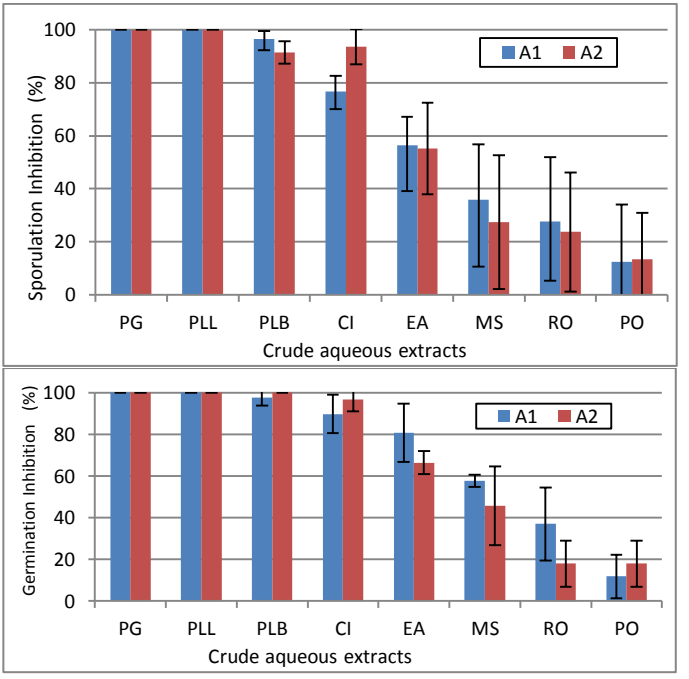

Fig. 3. Inhibition of sporulation (above) and germination (below) of A1 and A2 isolates of *Phytophthora infestans*, noted after 21 days of incubation 20°C on Agar Pea Medium, depending on plant aqueous extracts. Bar segments mean standard deviation. CI: *Carya illinoinensis*; EA: *Equisetum arvense*; RO: *Rosmarinus officinalis*; PL: *Pistacia lentiscus* (PLL: leaves & PLB: berries); MS: *Mentha suaveolens*; PG: *Punica granatum*; PO: *Posidonia oceanica*.

Survival of *P. infestans* isolates following treatments using plant aqueous extracts.

Analysis of the variance of inhibition rates of mycelial growth and infection of potato leaf discs of *P. infestans* isolates previously inhibited in vitro by the tested aqueous extracts showed a significant difference ($P \leq 0.05$) between the extracts of plants but not in vitro and in vivo significant difference ($P = 0.687$ and $P = 0.987$) between A1 and A2 *P. infestans* isolates, respectively.

Inhibition of mycelial growth of *P. infestans* isolates previously inhibited by crude (100%) plant aqueous extracts, noted after one week of incubation at 20°C, varied depending on tested plant extracts (60-91%). In fact, very high levels of inhibition were observed using pomegranate bark extracts, mastic leaves and berries as well as for pecan (91%) (Fig. 4). This confirms their high fungicidal potential recorded in vitro.

Furthermore, all tested aqueous plant extracts had inhibited disease

development on potato leaf discs. A total suppression of disease expression (100%) was recorded after one week of the incubation using extracts from mastic (berries and leaves), pomegranate bark and pecan leaves. Also, the rate of infected leaf areas was more than 40% using treatments based on common horsetail (70%), fragrant mint (50-67%) and rosemary (45-50%) extracts compared to 20% noted on leaf discs treated with *Posidonia* aqueous extracts (Fig. 4).

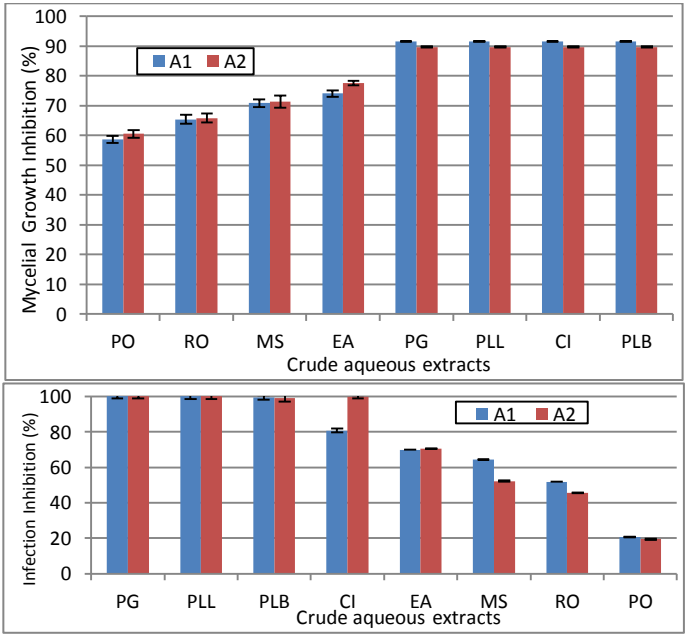

Fig. 4. In vitro (above) and in vivo (below) inhibition of survival of A1 and A2 isolates of *Phytophthora infestans* previously inhibited by plant aqueous extracts noted after 7 days of incubation at 20°C. Bar segments mean standard deviation. CI: *Carya illinoinensis*; EA: *Equisetum arvense*; RO: *Rosmarinus officinalis*; PL: *Pistacia lentiscus* (PLL: leaves & PLB: berries); MS: *Mentha suaveolens*; PG: *Punica granatum*; PO: *Posidonia oceanica*.

Content of total polyphenols from aqueous plant extracts.

The total polyphenol contents of plant extracts varied significantly ($P \leq 0.05$) depending on tested plants. The highest levels were found in extracts from pomegranate bark (344 µg/ml) and mastic leaves (126 µg/ml) and mastic berries (98 µg/ml). However, they were detected in lower levels in the other plants (Fig. 5).

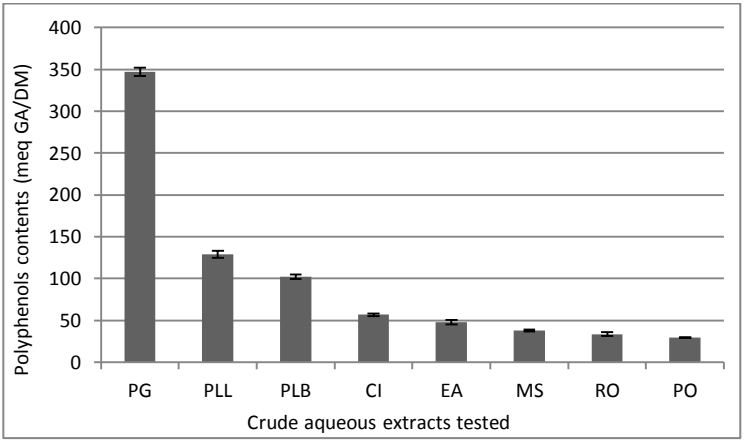

Fig. 5. Total polyphenol contents detected in tested plant aqueous extracts against *Phytophthora infestans*. Bar segments mean standard deviation. GA: Gallic acid; DM: dry material; CI: *Carya illinoinensis*; EA: *Equisetum arvense*; RO: *Rosmarinus officinalis*; PL: *Pistacia lentiscus* (PLL: leaves & PLB: berries); MS: *Mentha suaveolens*; PG: *Punica granatum*; PO: *Posidonia oceanica*.

DISCUSSION

One of the major originalities of plants is their ability to produce highly diversified natural substances. They frequently accumulate these secondary metabolites which represent an important source of bioactive molecules (Haddouchi and Benmansour 2008) intensively examined and used in plant protection (Daferera et al. 2000). Some plant extracts (Davidson and Naidu 2000) and essential oils (Kurita et al. 1981) showed activity against a wide range of plant pathogenic fungi.

In the present study, aqueous extracts from pomegranate bark, pecan leaves and mastic leaves and berries have shown an excellent inhibitory effect but low antifungal potential was noted for the fragrant mint, rosemary and common horsetail leaves and very low for Posidonia ones. Our results are in agreement with those of many authors reporting the efficiency of some plant extracts in the control of potato late blight (Bekele et al. 2006; Maharjan et al. 2010; Yanar et al. 2011).

The inhibitory effect of extracts from *P. granatum* bark against the mycelial growth of other plant pathogenic fungi has been already reported by Shuhua et al. (2010). Their results have shown the efficiency of methanolic and aqueous extracts against *Penicillium digitatum*. These extracts may contain active antifungal compounds such as punicalagine, castagalagine, granatine, catechin, gallocatechin, kaempferol, and quercetin (Jayaprakasha et al. 2006). The synergistic interactions of these compounds could increase their antifungal activity. Indeed, the punicalagine isolated from the pomegranate bark displayed an antimicrobial activity against *Candida albicans* (De et al. 1999). Pomegranate bark extract showed antifungal activity against *Penicillium citrinum*, *P. patulum*, *P. roquefortii* and *Aspergillus ochraceus*.

Moreover, the high frequency of use of *P. lentiscus* leaves may be explained by the fact that they are rich in secondary

metabolites responsible for its interesting biological properties (Bigendako-Polygenis and Lejoly 1990). Several studies have reported significant antibacterial and antifungal activities in this plant (Gardeli et al. 2008; Kordali et al. 2003). However, antifungal activity seems to be much more interesting (Iauk et al. 1996). Its efficiency lies much more in the synergistic effect of some of its active compounds (Derwich et al. 2008). Studies on the effect of *P. lentiscus* extracts on plant pathogens are limited. Kordali et al. (2003) showed the inhibitory effect of crude extracts from *P. vera*, *P. terebinthus*, and *P. lentiscus* against the mycelial growth of *Pythium ultimum* and *Rhizoctonia solani* but these extracts did not inhibit *Fusarium sambucinum*.

Pecan leaf extract was extensively tested against several phytopathogenic agents. In fact, Osorio et al. (2009) confirmed the high antifungal potential of its acetonic extracts against *R. solani*. Goussous et al. (2010) also demonstrated the important in vitro inhibitory effect of crude extracts from *Rosmarinus officinalis* against *Alternaria solani* when applied at various concentrations. Also, Yanar et al. (2011) and Dellavalle et al. (2011) observed complete inhibition of mycelial growth of *P. infestans* and *Alternaria* spp. Its antibacterial and antifungal capacities can be explained by the chemical composition of its extracts as demonstrated by Pinto et al. (2006). Indeed, the same effect was noted with extracts from *P. oceanica* against *Fusarium* spp. (Kouki et al. 2012) and those from *Carya illinoinensis* toward *R. solani* (Hernández-Castillo et al. 2010).

The different aqueous herbal extracts exhibited low antifungal activity for the respective dilutions compared to the crude extracts. These results are in agreement with those of Banso et al. (1999) claiming that high concentrations of antimicrobial substances induce greater inhibition. In addition, our results showed that all tested plant extracts, even at the lowest tested concentration (10%), had efficiently inhibited *P. infestans* sporulation and germination. These inhibition rates recorded have generally exceeded 84% and even reached 100% using aqueous extracts of mastic, pecan and pomegranate bark. These are consistent with the work of Itako et al. (2008) and Goussous et al. (2010) who noted a complete inhibition of sporulation and germination of *A. solani* conidia following treatments with *R. officinalis* extracts. Blaeser and Steiner (1998) have also shown that extracts from different plants had also prevented germination and affected the outflow and mobility of *P. infestans* zoospores. Indeed, the inhibitory effect of the aqueous extracts of the tested plants is in agreement with the work of Omidbeygi et al. (2007) suggesting that the components of essential oils and plant extracts are able to cross the cell membrane of fungal pathogens and interact with their enzymes and proteins. It is the production of a proton flux at the external cell part which induces cellular changes and finally the death of targeted microorganisms.

Sharma and Tripathi (2006) demonstrated the antifungal activity of compounds of essential oils and plant extracts on hyphae, leading to the release of cytoplasmic inclusions, loss of rigidity and integrity of the cell wall, culminating at the end to the destruction and the death of the fungus. Concerning the survival of *P. infestans* isolates after in vitro or in vivo treatments, all the tested aqueous plant extracts showed a fungistatic effect at low concentrations and a fungicidal effect with the increase of extract concentrations. However, plant aqueous extracts prepared from the pomegranate bark, aerial parts of mastic and pecan have prevented the resumption of growth of *P. infestans*

isolates and completely inhibited the development of late blight symptoms on potato leaf discs. In the same way, Blaeser and Steiner (1998) demonstrated the reduction of the severity of disease using plant extracts.

The inhibitory capacity of tested aqueous plant extracts on both isolates of *P. infestans* can be supported by findings of Burt (2004), Lahlou (2004) and Davicino et al. (2007) who affirmed that the biological activity of a plant extract is related to its chemical composition, the functional groups of its major compounds, their synergistic effect and their proportions. In this concept, the total polyphenol contents detected in each plant extract could be related to its recorded antifungal activity. Pomegranate bark aqueous extracts, mastic leaves and berries and pecan leaves contain high levels of polyphenols, in contrast to rosemary, common horsetail, posidonia and fragrant mint aqueous extracts. Also, Prasad and Kapoor (2004) found that the quantity and quality of these active ingredients depend on plant, plant tissues and several exogenous factors (Demo and Oliva 2008; Webster et al. 2008). Several authors have also asserted that antifungal broad-spectrum chemical compounds belong mainly to these three major families: phenylpropanoids and phenolic substances (Cakir et al. 2004; Chuang et al. 2007), terpenoids and steroids (Grande et al. 1992), alkaloids and nitrogen compounds. In this sense, several studies have shown that plant extracts rich in phenol groups displayed high inhibitory activity toward fungal sporulation (Inouye et al. 1998).

In conclusion, this study demonstrated the antifungal activities of plant aqueous extracts of seven medicinal plants as part of research for identification of new biological control methods for potato late blight control. All tested aqueous extracts showed variability in their mycelial growth inhibitory activity of the two isolates A_1 and A_2 of *P. infestans*. The inhibition was very important (88%) even at the concentration of 10% using extracts from pomegranate bark and from *P. lentiscus* leaves and berries, and from *C. illinoinensis* (74%). A moderate activity was displayed by *E. arvense* (58%), *M. suaveolens* (51%) and *R. officinalis* (45%) compared to the weak inhibitory effect (9%) expressed by *P. oceanica* extracts.

Sporulation and germination of the two *P. infestans* isolates were also affected by all treatments with plant aqueous extracts. Significant levels of inhibition were recorded using extracts from mastic leaves and berries (99%), pomegranate bark (99%) and pecan leaves (85%).

The fungicidal effect of plant extracts was also confirmed in vitro and in vivo, respectively, by the lack of resumption of mycelial growth and the lack of development of symptoms of late blight on potato leaf discs. Furthermore, the determination of the phenolic compounds of the crude plant aqueous extracts was strongly linked to their fungicidal potential. In this context, pomegranate bark, mastic leaves and berries as well as pecan leaves aqueous extracts should be tested under field conditions with different sprays for their effects against *P. infestans* inoculum and consequently late blight incidence and severity.

RESUME

Messgo-Moumene S., Boukhalfa R., Belaïdi D., Beninal l, Haddadj-Hamdi S. et Bellatreche M. 2017. Activité antifongique *in vitro* de différents extraits de plantes contre *Phytophthora infestans*, l'agent causal du mildiou de la pomme de terre. Tunisian Journal of Plant Protection 12: 19-33.

Notre étude a visé l'évaluation de l'activité antifongique *in vitro* des extraits aqueux préparés à partir de sept plantes médicinales (*Carya illinoinensis*, *Equisetum arvense*, *Rosmarinus officinalis*, *Pistacia lentiscus*, *Mentha suaveolens*, *Punica granatum* et *Posidonia oceanica*) contre les isolats A1 et A2 de *Phytophthora infestans*, agent causal du mildiou de la pomme de terre. Les extraits aqueux bruts (100%) de plantes ont été préparés par décoction et aux concentrations de 70, 50, 30 et 10%, pour les extraits les plus efficaces. Leur potentiel antifongique a été évalué *in vitro* et *in vivo* après traitement, en se basant sur l'inhibition de la croissance mycélienne, de la sporulation, de la germination et de la survie des isolats pathogènes de *P. infestans.* Les extraits aqueux des plantes testés ont montré une efficacité variable. Les taux d'inhibition les plus élevés ont été enregistrés pour l'ensemble des paramètres en utilisant des extraits aqueux préparés d'écorce de *P. granatum* et de feuilles et de baies de *P. lentiscus* (88%), à la concentration de 10%. Leur potentiel antifongique a été exprimé par la lyse mycélienne et la digestion du contenu des sporanges ainsi que par l'inhibition de la croissance mycélienne de *P. infestans*. L'analyse des composés phénoliques des extraits aqueux des plantes testés a révélé une relation étroite entre le contenu des polyphénols totaux et l'activité antifongique observée. Cette étude a clairement démontré que l'écorce de grenade, les feuilles et/ou les baies du pistachier lentisque peuvent être exploités comme sources potentielles de molécules bioactives pour la lutte contre le mildiou de la pomme de terre.

Mots clés: Effet biocide, extraits aqueux végétaux, *Phytophthora infestans*, polyphénols, *Solanum tuberosum*

مـلـخص

مسقو-مومن، سعيدة وريم بوخالفة وجازية بلعيدي و الياس بنينال وسهام حمدي-حداج ومحمد بلطرش.2017. النشاط المضاد للفطريات في المخبر لعدة مستخلصات نباتية ضد شبه الفطر *Phytophthora infestans* العامل المسبب لمرض اللفحة المتأخرة على البطاطا/البطاطس. Tunisian Journal of Plant Protection 12: 19-33.

هدفت دراستنا إلى تقييم النشاط المضاد في المختبر للمستخلصات المائية التي أعدت من سبعة أعشاب طبية هي *Carya illinoinensis* و *Equisetum arvense* و *Rosmarinus officinalis* و *Posidonia oceanica* و *Mentha suaveolens* و *Punica granatum* و *Posidonia oceanica* ضد العزلات (A1 / A2) لشبه الفطر *Phytophthora infestans* العامل المسبب لمرض اللفحة المتأخرة على البطاطا. تم إعداد المستخلصات النباتية الخامة (100%) بواسطة الاستخلاص بالإغلاء وبالتركيزات 70 و 50 و 30 و 10% بالنسبة إلى المستخلصات الأكثر فعالية. تم تقييم النشاط المضاد للفطريات في التجارب البلورية والإحيائية بعد المعاملة، على أساس تثبيط نمو شبه الفطر وتبوغه وإنباته وبقاء السلالات الممرضة لـ *P. infestans*. أظهرت المستخلصات المائية للنباتات المجربة فعالية متفاوتة. سجلت أعلى نسبة تثبيط لمجموعة من المعلمات باستخدام المستخلصات المائية المحضرة من لحاء *P. granatum* ومن أوراق وثمرة *P. lentiscus* (88٪) بالنسبة إلى تركيز 10%. بينت القدرة المضادة للفطريات عن طريق تدمير مشائج شبه الفطر والأبواغ وعن طريق تثبيط نمو *P. infestans*. أظهر تحليل المركبات الفينولية في المستخلصات المائية للنباتات المختبرة علاقة وثيقة بين المحتوى الإجمالي للبوليفينولات والنشاط المضاد للفطريات. وأظهرت هذه الدراسة بوضوح أن لحاء P. *granatum* وأوراق و/أو ثمار *P. lentiscus* يمكن استغلالها كمصادر محتملة للجزيئات النشطة بيولوجيا لمكافحة مرض اللفحة المتأخرة للبطاطا.

كلمات مفتاحية: بوليفينولات، مستخلصات نباتية مائية، نشاط ضد حيوي، *Phytophthora infestans*، *Solanum tuberosum*

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Investigations into Physiological, Biochemical, and Histological Modifications in a Vine Decline Associated with Biotic and Abiotic Factors

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ABSTRACT

Bahri, B.A., Mabrouk, H., Chebil, S., and Kallel, S. 2017. Investigations into physiological, biochemical, and histological modifications in a vine decline associated with biotic and abiotic factors. Tunisian Journal of Plant Protection 12: 35-52.

Tunisian table grape production has significantly increased since two decades due to vineyards regional expansion and yield improvement. But, since several years, decline symptoms on *Vitis vinifera* have been recorded in some areas. A study case of a vineyard in Naassen area (near to Tunis) was chosen to investigate the disease origin and the physiological, biochemical, and histological modifications associated with vine decline. The investigation revealed characteristic symptoms on leaves, old and young shoots similar to decline symptoms of Grapevine Trunk Diseases. Based on cultural characteristics, laboratory investigations revealed the presence of *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., *Diplodia seriata* and *Botryosphaeria dothidea*, from root and shoot samples, respectively. These fungi are known as the main pathogens responsible for the Esca, Black dead arm and Excoriose. Molecular analysis confirmed the identification of *Diplodia seriata*. Beside morphological alterations on leaves and shoots, symptomatic vines presented significant reductions of 30 and 20% in trunk diameter and bud break rate, respectively, and delayed spring growth compared to healthy ones. Furthermore, roots and stems from declined vines contained 3 times more starch than those from asymptomatic ones. Decline survey revealed a heterogeneous dispersion of symptoms in the vineyard in accordance with water supply. The vines along the edge of vineyards are usually less watered and show more decline symptoms. Decline dynamics in time and space scales have to be considered in order to develop effective management strategies.

Keywords: *Botryosphaeria dothidea*, *Diplodia seriata*, Esca, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, *Vitis vinifera*.

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The grapevine is a relatively warm growing species which can prosper over a wide range of soil textures. Regions around the Mediterranean sea distinguished with an international reputation for viticulture and winemaking. Mediterranean vineyards are

planted with a number of varieties of the *Vitis vinifera*, common grapevine, species native from Western Europe to the Persian shores of the Caspian Sea (Bonnier and De Layens 1894). *V. vinifera* has been threatened by dieback diseases with various origins. Two major decline events on *V. vinifera* have been recorded; the first was associated with attack of phylloxera (Granett et al. 2001) and the second with fungal pathogens causing Grapevine Trunk Diseases (GTD) (Graniti et al. 2000; Morton 2000). These diseases were at first observed in old vineyards and are associated with different fungal species inducing Esca, Eutypa and Botryosphaeria diebacks (Edwards and Pascoe 2004; Graniti et al. 2000; Morton 2000; Mundy and Manning 2010; Pitt et al. 2010; Surico 2009; Surico et al. 2006). Symptoms are variable and inconsistent but generally involve wood and leaf necrosis, reduced growth, and in severe cases, vine death. Esca, associated with especially some *Phaeoacremonium* species and several other species, is a widely reported disease, and more has recently become a problem in young grapevines. Symptoms include tiger-striped pattern on leaves, small chlorotic leaves, reduced shoot growth and shoot tip dieback, superficial brown spotting or measles on berries, berry shrivel, and acrid-tasting fruit. Other symptoms are streaking of the woody cylinder and stunted growth (Calzarano and di Marco 2007; Surico 2009; Surico et al. 2006). Foliar symptoms are commonly related to infection by the vascular fungi *Phaeomoniella chlamydosporum* and *Phaeoacremonium aleophilum*, leading to the Grapevine Leaf Stripe Disease. *Fomitiporia mediterranea*, known as true Esca, is associated with a particular wood necrosis, the white rot, and is frequently observed in Esca-diseased plants (di Marco et al. 2011; Surico 2009). Interaction between these pathogens themselves and with their environment and host plants appears to be complex and sometimes species specific , geographical region and environmental stress, making the disease more difficult to control (van Niekerk et al. 2011a). Pruning wounds are the principle point of entry, and control measures are limited to preventing spread and the severe surgery of infected or entire vines (Rolshausen et al. 2010; van Niekerk et al. 2011b).

Tunisia is an important vine-growing country of North Africa and Tunisian viticulture areas are considered as one of the most significant of the Mediterranean vineyards (Mabrouk 2002, Mabrouk et al. 2007). Tunisian vineyards began to show symptoms of decline over the last decade. Etiology of the disease was determined (Chebil et al. 2014; 1997) but knowledge on the epidemiology of the causal agents, the pathogenicity of Tunisian isolates and the physiological modifications in vine are still needed. A case study on a 10 year-old vineyard in Naassen area (near to Tunis) was chosen for further investigation into physiological, biochemical, and histological modifications associated with the vine decline. Fungal species involved in the vine dieback were identified.

MATERIALS AND METHODS

Vineyard description: case study.

Shoots and root systems were investigated in a 10 year-old vineyard of Muscat d'Italie cultivar grafted on Paulsen 1103 rootstock. The vineyard is located in Naassen (36.64N, 10.21E) area suitable for growing grapevines and characterized by a sub-humid climate (Fig. 1a). The vineyard (1.9 ha) has a planting density of 1600 vines/ha and is drip irrigated. Vines have manifested unknown decline symptoms since 4 years and the vineyard was the first attacked in

the region. Decline symptoms were investigated according to the incidence and severity of attacks and their distribution in the vineyard in 2011 and 2013. Dieback symptoms were classified into three categories (Fig. 2): Stage 0 (no visible symptom); stage 1 (slight to moderate damage: leaf chlorosis, red brown spots on leaves, leaving only a narrow strip of green tissue along the main veins; reduction in trunk circumference and shoot growth; dieback of up to 50% of shoots; stem with internal white heart rot surrounded by black dots); stage 2 (severe damage: leaf-drop, shoot and stem death (100 %)). The overall severity S of the decline symptom in the vineyard was evaluated for each year according to the following formula suggested by Dimenteva (1970):

$$S = \sum_{n=0}^{2} (N_n \times n) \times 100 \div (N \times 2)$$

where N_n is the number of vines at stage n and N the total number of vines.

To measure the degree of randomness of decline symptoms in the vineyard, the dispersion index "D", defined as the ratio of the variance σ^2 to the mean μ, was calculated. When the dispersion index "D" is less than 1, the dataset is said to be "under-dispersed"; this condition is related to patterns of occurrence that are more regular than the randomness associated with a Poisson process (D =1). If the dispersion index "D" is larger than 1, the dataset is said to be over-dispersed; this corresponds to the existence of clusters of occurrence of the decline symptoms in the vineyard (Fig. 1b).

Physiological modification assessment: trunk circumference, bud break rate and shoots growth.

Differences in growth patterns were assessed on 20 symptomatic (moderate damage: stage 2) and 20 asymptomatic (healthy vine: stage 0) vines during the spring. Vines were chosen randomly in order to cover the whole vineyard and according to their visual disease diagnostic. Bud break rate was recorded for each vine. Evolution of the shoot growth was also assessed every week during 6 weeks starting April, the 20th. Shoot growth is recorded on the 7th bud of each pruning cane. In addition, the trunk circumference was measured on the evaluated vines as an approximation of vine vigor.

Biochemical modification assessment: Starch content in primary roots and stems.

Starch content in primary roots and stems from declined and symptomless trees was assessed based on Nielson's iodine method (Nielson 1943). Tissue samples were dried during 3 days at 70°C, then crushed. Three hundred mg of the dried crushed material were suspended into 20 ml of ethylic alcohol 70% during 48 h. The volume of the preparation was then adjusted to 250 ml by distilled water. The supernatant was discarded and the pellet dried at 60°C. The dry extract was weighed then hydrolyzed by 70% perchloric acid solution at the rate of 5.7 ml for 300 mg of dry extract. The volume of this preparation was fitted to 250 ml by distilled water. After 48 h, 7 ml of every solution were mixed in a test tube with 0.5 ml of a solution of iodine prepared at the rate of 0.1% of KI and 0.02% of KIO_3. At the end of 5 h reaction time, the reading of the absorbance was made with the spectrophotometer Bauch and Lamber (CAMSPEC M330) at 675 nm.

The amount of starch was afterward determined for root and stem samples according to the standard curve (Fig. 3) established by several quantities (between 1 and 25 mg) of pure soluble

starch with 20 ml of 70% perchloric acid. The volume of the preparation was adjusted to 250 ml by distilled water. After 48 h of hydrolysis, 7 ml of every solution undergo the same approach made for the studied samples and the reading of the absorbance in the spectrophotometer (λ = 675 nm) was made after 5 h of reaction.

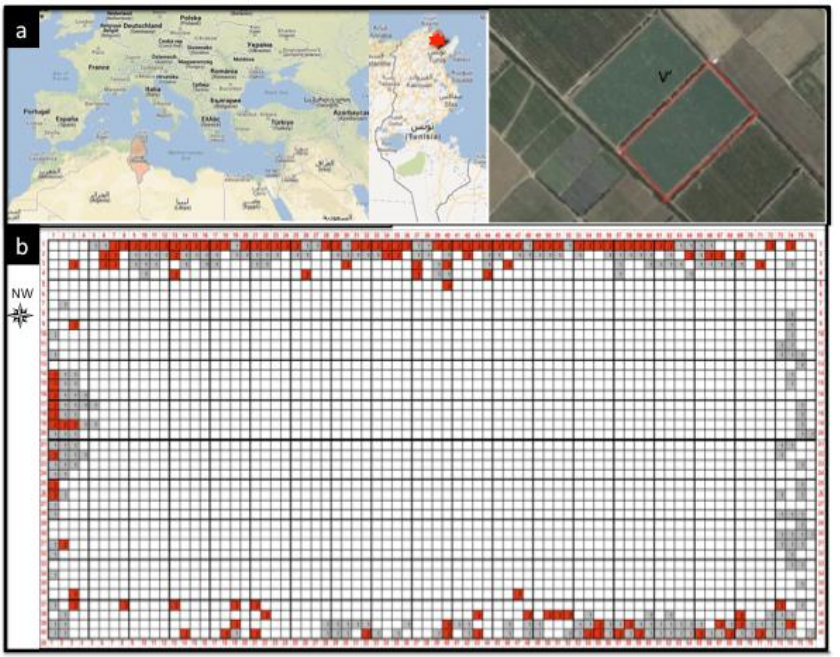

Fig. 1. A-10 year-old vineyard of Muscat d'Italie cultivar grafted on the rootstock Paulsen 1103, a: The vineyard is located in Naassen (36.64N, 10.21E: region of Mornag, governorate of Ben Arous, Tunisia) area with a sub-humid climate and a northwest prevailing winds; b: The vineyard (1.9 ha) has a planting density of 1600 vines/ha. Symptoms dispersion over the vineyard are represented by white (Healthy vine: stage 0), gray (declined vine: stage 1) and red (dead vine: stage 2) squares.

Fig. 2. Vine decline symptoms classified into three categories, a: stage 0 = no visible symptom; b, c and d: stage 1 = slight to moderate damage: leaf chlorosis and red brown spots on leaves, leaving only a narrow strip of green tissue along the main veins; reduction in shoot circumference and shoot length; dieback of up to 50% of shoots; stem with internal white heart rot surrounded by black dots; e: stage 2 = severe damage: leaf-drop symptom, shoot and stem death (100%).

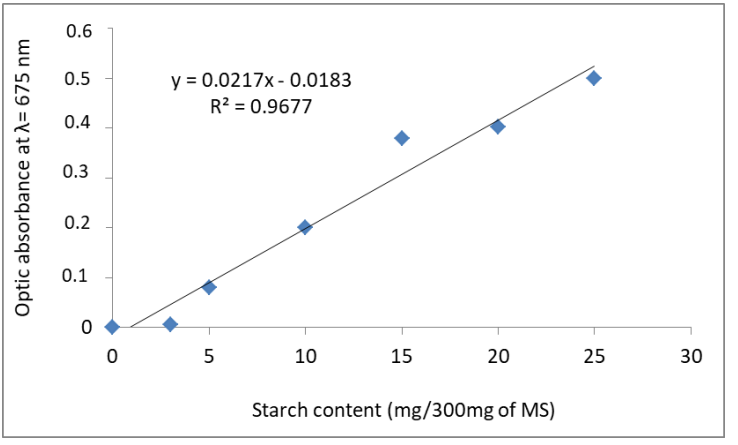

Fig. 3. Standard curve of the amount of starch in root and stem samples established by several quantities (between 1 and 25 mg) of pure soluble starch with 20 ml of 70 % perchloric acid and adjusted to 250 ml by distilled water. Absorbance reading at 675 nm by spectrophotometer was made after 5 h. DM: dry matter.

Histological modification assessment: root and shoot transverse sections.

In order to analyze the cellular organization of roots and shoots from infected vines, longitudinal and transverse sections were prepared. The conventional histological methods involving the processes of fixation, embedding and microtome sectioning were used. Shoot and root fragments from healthy and infected vine trees were cut into small sizes and washed twice with distilled water prior to fixation. Fixation was done by the fixed tissue method with paraffin. The fragments were first fixed in Navachine liquid for 18 h at the dark, followed by successive water washes for 24 h. The fragments were then dehydrated successively with 70° and 95° absolute alcohol baths for 1 h each. The alcohol was removed by placing the fragments in a xylene: paraffin (1:1 v/v) bath for 1 h at 58°C followed by a pure hot liquid paraffin bath. The fragments were finally treated by a continuous release pump under pressure for 4 h. The hardened paraffin block with the fixed tissue was then cut with the microtome usually around 12 to 14 µm thick. Thin slices of tissue were then applied to microscopic slides after colorations with Lugol's liquid and Schiff's reagent contrasted with Picro-indigo carmine.

Isolation and identification of fungi from root and shoot samples.

Pure cultures of fungi were obtained from 3 × 0.5 cm wood pieces taken from roots and shoots of asymptomatic and symptomatic vines. The pieces were cut out, washed in sterile water, sterilized in open fire and plated on Petri dishes containing potato dextrose agar (PDA) medium. All samples were incubated at 24°C for two weeks. Fungal pure cultures were grouped depending on culture and conidia morphologies. Isolates were identified based on morphological and cultural characteristics of mycelium, spores and colony three to four weeks after incubation at 20°C. One isolate was chosen for molecular confirmation based on the ITS region using the standard ITS1 and ITS4 primers; the sequence obtained was blasted against the NCBI nucleotide database for identification.

Statistical analysis.

The one-way analysis of variance (ANOVA), using the software SPSS 16.0 (SPSS Inc. Released 2007), was used to determine whether there were significant differences between the means of samples from declined and healthy vines for trunk circumference, starch content, frequency of fungi isolation and bud break rate. Means were separated using the Duncan's Multiple Range (DMR) test at 5% level of probability.

For the distribution pattern of the decline symptoms, the chi-square (χ^2) test statistic was used to test the validity of a distribution assumed for a random phenomenon at a 5% significance level.

RESULTS

Isolation and identification of fungi from root and shoot samples.

Isolation from root and shoot samples in culture media revealed the presence of multiple fungi. Based on cultural characteristics, the fungi were identified as *Phaeomoniella chlamydospora*, *Phaeoacremonium* spp., and *Diplodia seriata*: these fungal pathogens are known to induce decline symptoms and to cause Esca, Black dead arm, and *Phomopsis cane* diseases respectively. In addition, *Botryosphaeria dothidea* (causal agent of Excoriose), *Fusarium* sp. and *Alternaria* sp. were also isolated (Figs. 4 and 5). *Fomitiporia mediterranea,* pathogen also involved in

Esca disease, was not found. Based on ITS region, one of the isolate was molecularly identified as *Diplodia seriata* (Supplementary material 1). Since trunk necrosis have not been investigated and Koch's postulates criteria have not been checked, we cannot clearly establish from this study a causative relationship between the isolated fungi and the GTD and prove that these pathogens cause wood necrosis.

In addition, laboratory diagnosis revealed a significantly higher frequency of fungal isolation from declined vines compared to healthy ones (Fig. 6). On average, 27% of root and shoot samples from asymptomatic vines revealed the presence of latent fungal infection . Early diagnosis of the decline and the check of asymptomatic trees constitute probably the best strategy for the constraints of the decline.

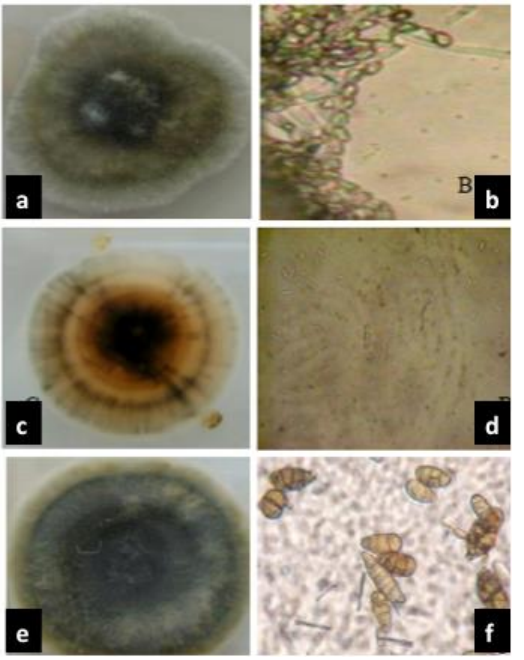

Fig. 4. Fungi isolated from shoot samples and associated to the vine decline symptoms observed in a Tunisian vineyard; a: Colony of *Diplodia setaria* on PDA medium; b: Conidia of *D. setaria* observed under the light microscope (× 400); c: Colony of *Phaeoacremonium* spp. on PDA medium; d: Conidia of *Phaeoacremonium* spp. observed under the light microscope (× 200); e: Colony of *Alternaria alternata* on PDA medium; f: Conidia of *A. alternata* observed under the light microscope (× 500).

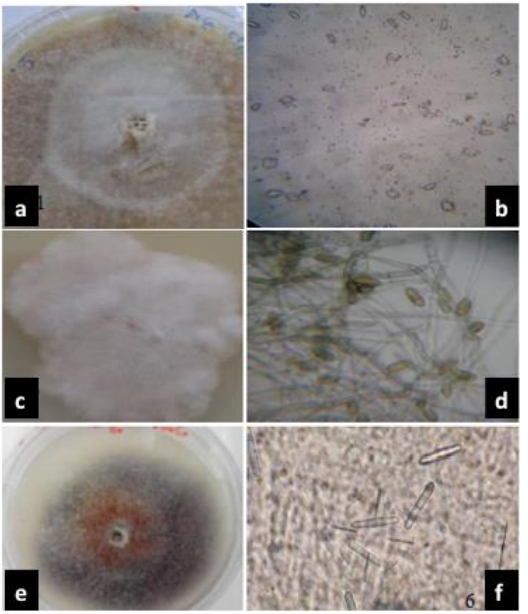

Fig. 5. Fungi isolated from root samples and associated to the vine decline symptoms observed in the investigated vineyard, a: Colony of *Phaeomoniella chlamydospora* on PDA medium; b: Conidia of *Phaeomoniella chlamydospora* observed under the light microscope (× 400); c: Colony of *Fusarium* sp. on PDA; d: Conidia of *Fusarium* sp. under the light microscope (× 400); e: Colony of *Botryosphaeria dothidea* on PDA; f: Conidia of *B. dothidea* under the light microscope (× 500).

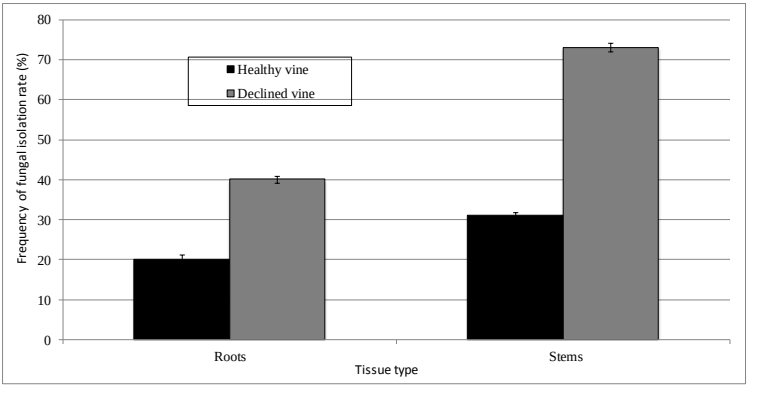

Fig. 6. Frequency of isolation of pathogens in healthy (stage 0) and declined vines (stage 2) from roots and shoots. Each histogram represents the means and SE calculated over 20 root or shoot samples.

Decline severity and distribution in the vineyard.

The decline symptoms affected 11% of the vines in 2011 and showed 2 different severity levels named stage 1 (slight symptoms) and stage 2 (severe symptoms). Stages 1 and 2 affected 7 and 4% of vines, respectively. An overall decline symptom severity of 15% was assessed in the vineyard in 2011. This severity increased by 36% in 2 years. This was mainly due to more intense decline symptoms (stage 1 to stage 2) than to the observation of new infected plants.

The disease survey revealed significantly heterogeneous dispersions of the declines symptoms at the vineyard scale. The dispersion index "D", evaluated at 6.34 in the vineyard for 2011, indicates over-dispersion of the symptoms. This support the existence of clusters of occurrences of the decline symptoms in the vineyard which means that the hypothesis of a completely random spatial structure is rejected with the χ^2 test statistic at a risk $\alpha = 0.05$; the disease presents an aggregate structure (Fig. 1b).

Physiological, biochemical and histological modifications assessments.

Effects of vine decline on plant physiology, biochemical composition and plant histology were assessed.

Symptomatic vines were characterized by a reduction of 22% on average in spring growth as measured by primary shoot elongation during April and May (Fig. 7), which corresponds to the period of maximum shoot growth during the season in Tunisia. In addition, bud break rate and trunk circumference of symptomatic vines were respectively 20 and 30% less than in asymptomatic vines.

From another part, starch content was assessed in primary roots and shoots during both dormancy and growth period of the vine. Our results showed that roots and shoots from declined vines contained 3 times more starch than those from asymptomatic vines (Fig. 8). These differences were significant at 5%. Finally, histological sections realized on symptomatic and asymptomatic roots and shoots explained the dried vine states (Fig. 9). The internal anatomy of infested wood showed discoloration and necrosis of the vessels. The gum deposits in the heartwood vessels observed in symptomatic vines supports the vascular wilt disease and the decline symptoms. In addition, root sections showed exudations near epidermis cells indicating the presence of pathogens. Moreover, the microscopic observations revealed the presence of chitinous structures on root and shoot sections confirming fungus attacks on declined vine.

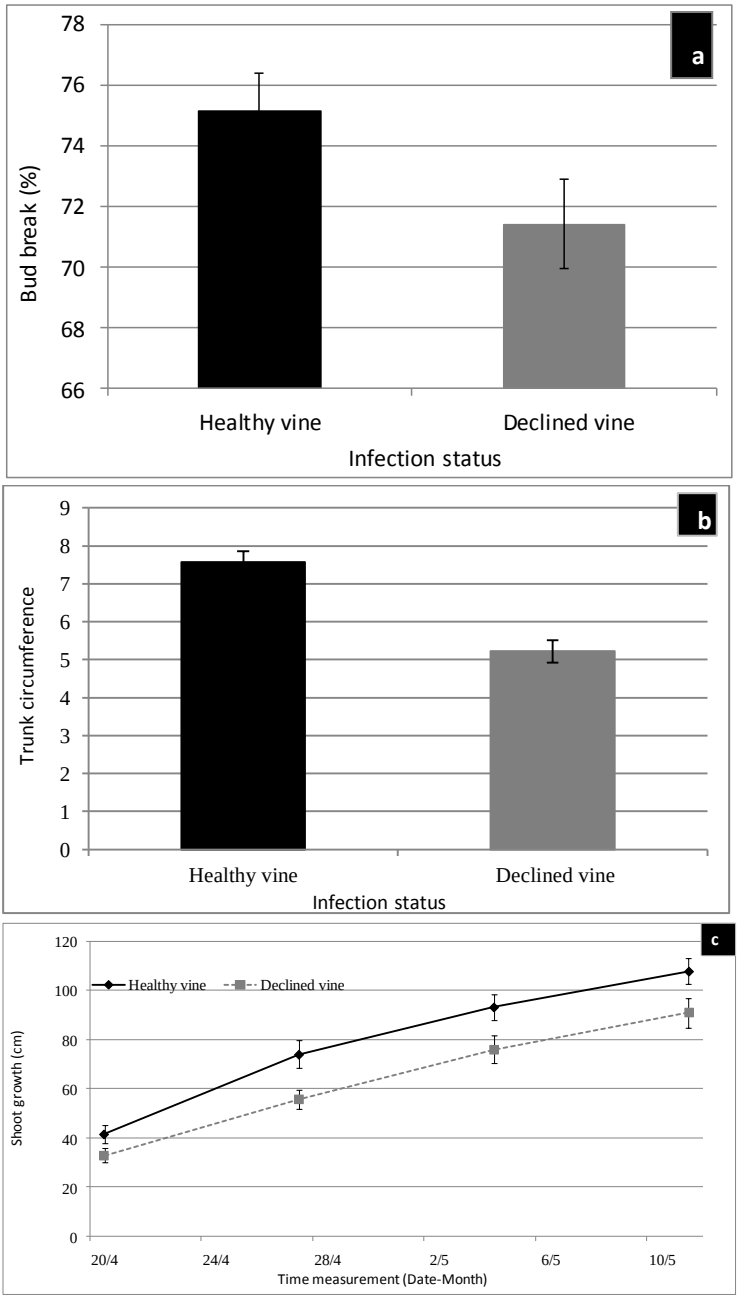

Fig. 7. Physiological modification assessment in healthy (stage 0) and declined vines (stage 2). a: Bud break rate (%); b: Trunk circumference (cm); c: Evolution of the shoot growth assessed every week during 6 weeks starting April 20th (cm). Each histogram or point represents the means and SE calculated over 20 samples.

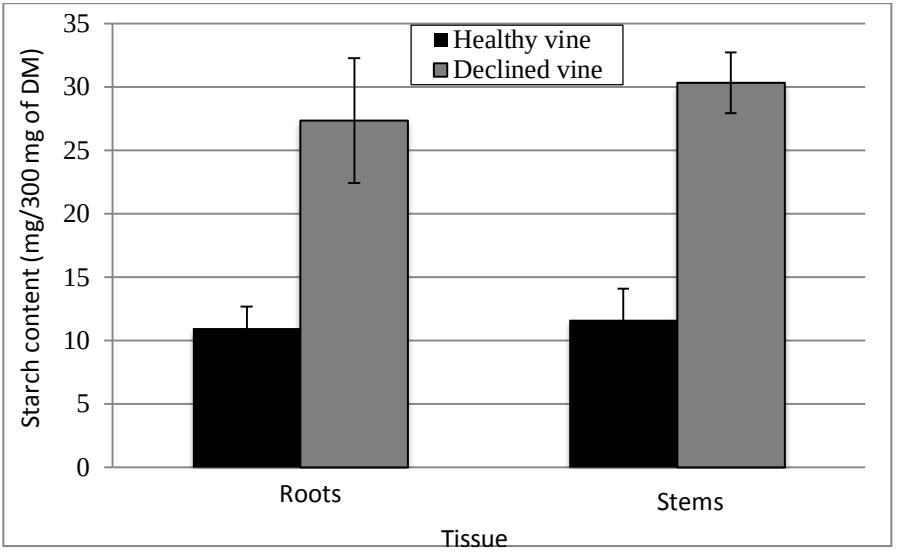

Fig. 8. Biochemical modification assessment in healthy (stage 0) and declined vines (Stage 2): Starch content (mg/300 mg of DM) in stems and in primary roots. The amount of starch was determined for root and stem samples according to the standard curve (Fig. 3). Each histogram represents the means and SE calculated over 20 samples. DM: dry matter

DISCUSSION

Our study revealed the presence of a severe decline in a productive Tunisian young vineyard located in Naassen where vine trees developed tiger-striped pattern on leaves and reduced shoot growth and shoot tip dieback. Biotic factors involving fungal attacks could be associated to this vine decline. Based on cultural characteristics, the fungal pathogens isolated from roots and shoots have been identified as *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp., *Diplodia seriata*, and *Botryosphaeria dothidea*, known to be responsible for Esca, Black dead arm and Phomopsis cane diseases. This study also showed that *Eutypa lata*, responsible for Eutypa dieback was not found in this declined vineyard. A molecular identification on the ITS region performed on one isolate confirmed the presence of *D. seriata*. Even if our current study cannot clearly establish it, we can suggest the causative relationship between the isolated fungi and the Grapevine Trunk Diseases. In fact, this vineyard has been further studied in 2014 and was part of an extensive sampling program on GTD (grapevine trunk diseases) in Tunisia held by the Center of Biotechnology of Borj Cédria (CBBC) where visual diagnosis (presence of necrosis in the trunk), laboratory (isolation of fungi), and molecular (phylogenetic analysis of ITS region) investigations confirmed the presence in this vineyard of a fungal pathogen complex known to cause Grapevine Trunk Diseases (Samir Chebil, data not shown). Further studies have to be done to demonstrate the influence of the pathogenic fungi on the development of GTD-like symptoms. Grapevine Trunk Diseases have been widely described all over the word in declined vineyard. Major

pathogens include *Botryosphaeria* spp., *Cylindrocarpon* spp., *Eutypa lata*, *Phaeomoniella chlamydospora*, *Phaeoacremonium aleophilum*, *Phomopsis viticola*, *D. seriata* and others (Edwards and Pascoe 2004; Graniti et al. 2000; Morton 2000; Surico et al. 2016). The Grapevine Trunk Diseases are emerging diseases of vine in Tunisia and Chebil et al. (2014) was the first to report *D. seriata* in young Tunisian grapevine.

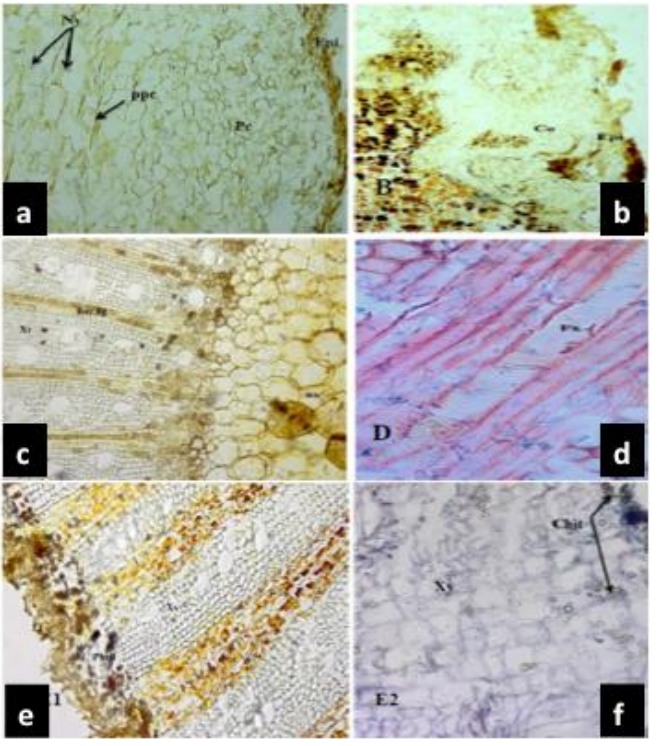

Fig. 9. Histological modification assessment in healthy (stage 0) and declined vines (stage 2), a: Longitudinal section of primary root in declined vine after colorations with Lugol's liquid (× 200); Ep: Epidermis; Pc: Cortical parenchyma; ppc: Pecto cellulosic wall; ny: Core; b: Transverse section of primary root in declined vine after colorations with Lugol's liquid (× 100); Ep: Epidermis; Co: Collenchym; c: Transverse section of shoot in declined vine after colorations with Lugol's liquid (× 100); Xs: Secondary xylem; mo: Pith; Ray.lig: wood beam; d: Longitudinal section of shoot in declined vine after coloration contrasted with Picro-indigo carmine (× 400); Fx: xylem vessels; e, f: Transverse section of shoot in declined vine after colorations with Lugol's liquid (× 200); Li: cork; Phell: phellogen; Xs: secondary xylem; and f: after coloration contrasted with Picro-indigo carmine (× 400); Xy: xylem; chit: Chitin structure.

The identified fungi may act alone, in combination or in succession to cause vine decline and death of the trees. Several examples of associations between fungi have been reported to cause heavy damage to various annual and perennial crops (Al-Naimi et al. 2005; de Jesus et al. 2001; Lopes and Berger 2001; Nolan et al. 1999; Oliveira et al. 2013; Weber et al. 1994). For example, Fusarium ear blight, a disease caused by several species acting in synergistic interactions can severely damage wheat (Xu et al. 2005). Although widely varied statements have been recorded, few studies investigated deeply the fungi associations especially in vine decline. In the current context, it is important to consider that knowledge in pathogen interactions might be the key factors for a successful integrated decline management strategy in vine. In our study, *Fusarium* sp. and *Alternaria* sp. were also isolated. As it was recorded in South Africa and Poland (Ferreiral et al. 1989; Krol 2006), *Alternaria alternata*, *Fusarium oxysporum* as well as *Aspergillus* sp. and *Penicillium* sp. can be isolated from vines presenting Esca and/or Phomopsis cane decline. These fungi may predispose vine cultivars to severe Esca infections.

The decline survey revealed significantly heterogeneous dispersions of the symptoms at the vineyard scale. In fact, decline symptoms were exclusively confined to the vineyard edges (Fig. 1). This edge pattern of the decline is probably due to an irregular distribution of irrigation water in the vineyard. In fact, vines on the edges are irrigated with only one dripper (8 l/h) while the inner vines are irrigated with 2 drippers meaning that they received twice more water during the season. This is because edge vines are pruned to 2 canes instead of 4 canes as for the rest of the vineyard. So their crop load is theoretically half of the other vines and so they need less water. Our hypothesis here is that edge vines were under water stress conditions in summer that predispose them to decline symptoms and fungus infection. Fischer and Kassemeyer (2012) concluded, similarly, that water supply plays a statistically significant role; i.e. plants under "stress" regime were showed to be more affected by *P. chlamydospora* on grapevine. Although, moisture is important for spore germination and pathogen infection and dispersion (Caffarra et al. 2012; Eastburn et al. 2011; Garrett et al. 2006; Gessler et al. 2011); many studies reported an increase of disease severity under drought conditions (Desprez-Loustau et al. 2006; Schoeneweiss 1975). Forest trees exhibit higher symptoms of canker/dieback diseases, caused by pathogens such as *Botryosphaeria*, *Sphaeropsis*, *Cytospora* and *Biscognauxia*, when they are subject to drought stress (Desprez-Loustau et al. 2006). Drought conditions predispose rice plants to *Bipolaris oryzae*, causing brown spot disease (Mainul et al. 2011). In addition, in our study, the edge exposed to the predominant wind (North-West) exhibited the most severe symptoms (stages 2 and 3 more frequent). Vine suffering from wind damage are usually more exposed to disease infections and are more vulnerable to wind dispersed conidia released from an inoculum source (Pautasso et al. 2012). This edge pattern of the decline was also observed in a neighbor vineyard supplied with a similar irrigation water system (data not shown).

The decline symptoms also involved modifications of the chemical composition to the vine. Our results revealed lower amounts of starch in roots and stems of healthy vine compared to decline vine, in accordance with Garcion et al. (2007) and Lebon (2005) studies. In fact, sugar and other nutrients are stored as starch and other material (e.g. amino

acids and proteins) in woody organs during the growing season and are remobilized in spring to support bud break and the initial shoot and root growth before the new leaves start to export assimilates. Declined vine has its biochemical pathways changed. The reduction of the starch into reducing sugars in the roots is stopped, inducing its accumulation in a high amount in the infected roots. The starch is also accumulated in shoots due to the reduction of foliar photosynthesis process in declined vine. In addition, in a recent study Oliveira et al. (2013) showed clearly that both excess of salt and fungi inoculations with *P. chlamydospora*, negatively affect photosynthesis and sucrose metabolism in vines under controlled conditions. These vines showed also a decrease in amylase activity, which may play an important role in the increased levels of starch found in inoculated plants. Secondly, our results on vine physiology are consistent with previous works reported for grapevine decline in California (Rooney-Latham et al. 2005). Reduced spring growth of the symptomatic vines for several years probably resulted in a 30% reduction in vine trunk circumference, used here as a vigor index. Similarly, Andreini et al. (2013) investigated the effect of Esca disease on bud break of grapevine in relation to different rootstock combinations. Their results showed that Esca disease significantly delayed bud break of symptomatic vines for several years. Finally, similar observations on histological modifications have been assessed in declined vines (Calzarano and di Marco2007; Larignon 2011; Surico et al. 2006).

Vine decline is spreading and threatening wine and grape productions in Tunisia. Up to now, no efficient control method has been available and further studies have to be done to demonstrate the influence of pathogenic fungi on the development of GTD-like symptoms in Tunisia. The vine decline may be associated to GTD and caused by the coexistence of multi infectious fungi including *P. chlamydospora* and *Phaeoacremonium* spp., *D. seriata* and *B. dothidea* causal agents of Esca, Black dead arm and Phomopsis cane (Excoriosis) diseases, respectively. These pathogenic agents cause the dysfunction of conductive vessels and induce plant biochemical, physiological and histological modifications. Prevalence and spread of diseases may be affected by a variety of factors. In this study, the observed vine decline present a distribution structured at a micro-geographical scale. The dynamics of the decline appears to be tightly related to biotic and abiotic stresses. Drought pressure, wind damage, fungi complex and association may have potential effects in the spatial distribution of the decline inside the vineyard. In our study, the decline severity increased over time providing evidence for its high dynamics. Both time and geographic scale at regional level have to be considered in order to define vine decline dynamics and develop effective control strategies.

RESUME

Bahri B.A., Mabrouk H., Chebil S. et Kallel S. 2017. Investigation sur les modifications physiologiques, biochimiques et histologiques associées à des facteurs biotiques et abiotiques accompagnant le dépérissement de la vigne. Tunisian Journal of Plant Protection 12: 35-52.

La production tunisienne de raisin de table a considérablement augmenté depuis deux décennies en raison de l'expansion régionale de vignobles et l'amélioration du rendement. Néanmoins, depuis plusieurs années, des symptômes de dépérissement de *Vitis vinifera* ont été observées dans certains domaines. Une étude de cas d'un vignoble dans la région de Naassen (près de Tunis) a été choisie pour identifier les origines de cette maladie et les modifications physiologiques, biochimiques et histologiques associées au dépérissement de la vigne. L'étude a révélé des symptômes caractéristiques des maladies du bois de la vigne sur les feuilles et les vieilles et les jeunes pousses. La présence de champignons associés à l'Esca (*Phaeomoniella chlamydospora*, *Phaeoacremonium* spp.), au Black-Dead Arm (*Diplodia seriata*) et au Phomopsis (*Botryosphaeria dothidea*), sur les racines et les pousses jeunes ou âgées de vignes est en faveur d'une infection par ces champignons sur les ceps dépérissants. L'identification de *Diplodia seriata* a été confirmée par l'analyse moléculaire. En conséquence du dépérissement, les vignes symptomatiques présentent une réduction significative de 30% du diamètre du tronc et de 20% du taux du débourrement et un retard de la croissance printanière par rapport aux vignes asymptomatiques a été observé. De plus, les racines et les tiges de vignes dépérissantes contenaient trois fois plus d'amidon que les racines et les tiges des vignes asymptomatiques. La répartition du dépérissement dans le vignoble parait hétérogène et les symptômes sur les ceps de vigne sont liés à la disponibilité de l'eau d'irrigation. Les ceps en bordure de la parcelle, mis dans des conditions de sécheresse, étaient plus sensibles au dépérissement. Ceci indique que les dynamiques du dépérissement dans le temps et dans l'espace doivent être prises en compte afin d'élaborer des stratégies de gestion efficaces.

Mots clés: *Botryosphaeria dothidea*, *Diplodia seriata*, esca, *Phaeoacremonium* spp., *Phaeomoniella chlamydospora*, *Vitis vinifera*

ملخص

بحري، بشرى أمينة وحاتم مبروك وسمير شبيل وصدر الدين قلال. 2017. دراسة حول التغيرات الفزيولوجية والبيوكيميائية والنسيجية المرتبطة بالعوامل الإحيائية واللاإحيائية المصاحبة لتدهور الكروم.
Tunisian Journal of Plant Protection 12: 35-52.

ارتفع الإنتاج التونسي من عنب المائدة بشكل كبير خلال العقدين الماضيين بسبب توسع المساحات من كروم العنب وارتفاع الإنتاجية. ومع ذلك، لوحظت منذ عدة سنوات أعراض تدهور شائعة على كروم في بعض المناطق. وقد تم اختيار دراسة حالة من الكروم في منطقة نعسان (قرب تونس العاصمة) لتحديد أسباب المرض والتغيرات الفسيولوجية والبيوكيميائية والنسيجية المرتبطة بهذا التدهور. أظهرت الدراسة وجود الأعراض المميزة لأمراض خشب الكروم على الأوراق والبراعم الكبيرة والصغيرة. إن وجود فطريات مرتبطة بمرض "إيسكا" مثل *Phaeomoniella chlamydospora* و *Phaeoacremonium* spp. والعامل الفطري المتسبب في الذراع الأسود *Diplodia* (*seriata*) وكذلك العامل الممرض *Botryosphaeria dothidea* على الجذور والبراعم الصغيرة والكبيرة هي المسببة في تدهور عنب الكروم. وتم تأكيد تشخيص *Diplodia seriata* عن طريق التحليل الجزيئي . وعند تدهور الكروم نلاحظ انخفاض كبير بـ 30 % في قطر الجذع وبنسبة 20% في البراعم وبالتالي فإن النمو خلال فصل الربيع يتأخر عند الكروم المتدهورة بالمقارنة مع الكروم بدون أعراض. بالإضافة إلى ذلك، فإن جذور وجذع شجرة الكروم المتدهورة يحتويان على ثلاث مرات أكثر من النشاء من الكروم بدون أعراض. يبدو انتشار المرض غير منتظم و تعود الأعراض إلى عدم توفر مياه الري. فنباتات الكروم المزروعة على حواشي البستان في ظروف أكثر جفاف، كانت أكثر عرضة للتدهور. لذلك يجب أن تؤخذ بعين الاعتبار ديناميكيات التدهور في الزمان والمكان حتى يتم وضع استراتيجيات ناجعة لمقاومة الأمراض.

كلمات مفتاحية: إسكا، كروم، *Phaeoacremonium* spp.، *Botryosphaeria dothidea*، *Diplodia seriata*، *Phaeomoniella chlamydospora*، *Vitis vinifera*

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Insecticidal and Synergistic Activities of Two Essential Oils from *Pistacia lentiscus* and *Mentha pulegium* Against the Green Peach Aphid *Myzus persicae*

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ABSTRACT

Behi, F., Bachrouch, O., Ben Fekih, I., and Boukhris-Bouhachem, S. 2017. Insecticidal and synergistic activities of two essential oils from *Pistacia lentiscus* and *Mentha pulegium* against the green peach aphid *Myzus persicae*. Tunisian Journal of Plant Protection 12: 53-65.

Chemical composition of two essential oils (EOs) extracted from *Mentha pulegium* and *Pistacia lentiscus* was investigated. Volatile compounds were characterized. Major ones were pulegone (45.89%), cis-menthone (23.25 %) and trans-menthone (14.73 %) for *M. pulegium* and α-pinene (28.57%), β-myrcene (21.03%) and L-limonene (6.97%) for *P. lentiscus*. Then, the insecticidal and synergistic activities of the EOs were studied against *Myzus persicae*. The results showed that both EOs were toxic against the target pest. Aphid mortality caused by *M. pulegium* and *P. lentiscus* Eos was 86 ± 11.4 and 76 ± 11.4%, respectively. LC_{50} of the latest EO was lower than that of *P. lentiscus* with 596 and 876 ppm, respectively. In addition, no synergism was observed when both oils were mixed and used against the same aphid. Interestingly, there are no differences between toxicity of both EOs and that of the chemical insecticide leading to 70 ± 10% mortality. This study suggested that the EOs have a great potential to be used in agriculture against *M. persicae*.

Keywords: Contact toxicity, essential oils, *Mentha pulegium, Myzus persicae, Pistacia lentiscus*

The green peach aphid *Myzus persicae* (Hemiptera: Aphididae) is one of the most polyphagous and harmful insect pests of various crops (Blackman and Eastop 2000). It has a cosmopolitan distribution and can be a vector of more than 100 plant viruses (Blackman et al. 2007; Kennedy et al. 1962). The control of this species is becoming more and more problematic because of the insecticide resistance which now extends to most classes of insecticides, including organophosphates, carbamates and pyrethroids (Bass et al. 2014). This is due to the extensive and repeated use, since several decades, of chemical insecticides leading to a frequent risk of emergence of new resistances. In Tunisia, *M. persicae* is well distributed particularly in the north and the center of the country and cause

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serious damage to various crops such as peach, potato, pepper, citrus, etc. (Boukhris et al. 2007). Moreover, as in the rest of the world, it is proved that the insecticide resistance is well established in Tunisia (Charaabi et al. 2016). This situation has stimulated interest in developing eco-friendly alternative methods to control this aphid and to promote Integrated Pest Management (IPM). In this context, some studies had reported the insecticidal activities of essential oils (EOs) and plant extracts to control aphids. Digilio et al. (2008) found that several Mediterranean Eos are able to cause mortality of *Acyrthosiphon pisum* and *M. persicae*. Other studies also underlined the toxic effect of *Illicium verum* fruit extracts against *M. persicae* and its ability to inhibit the acetylcholinesterase and glutathione S-transferase activities (Li et al. 2016). The present work was performed to investigate the EO composition and the insecticidal and synergistic activities of two Tunisian medicinal and aromatic plants: mastic *Pistacia lentiscus* (Sapindales: Anacardiaceae) and pennyroyal *Mentha Pulegium* (Lamiales: Lamiaceae) against *M. persicae*. These plant species were chosen because of their abundance in the Tunisian mountain and the Mediterranean area (Bonnier and Douin 1990; Pottier-Alapetite 1981). They also are cited in the literature for their insecticidal potential (Bachrouch et al. 2010a, 2010b; Petrakis et al. 2014).

MATERIALS AND METHODS

Plant material.

M. pulegium and *P. lentiscus* leaves were collected at flowering stage in September 2014 from natural forestry populations. *M. pulegium* were collected in Marja Lekbouch site (36°11′10″N8°42′00″E) in the locality of Kef (North-West, Tunisia) and *P. lentiscus* in Korbous (36°48′59″N10°34′07″E) in the site of Nabeul (North-East, Tunisia). Samples were air-dried at ambient temperature. EOs were extracted from 70 g of leaves by hydrodistillation for 90 min using modified Clevenger-type apparatus (Craveiro et al. 1976). Anhydrous sodium sulfate was used to remove water after extraction. The obtained EO were quantified to calculate the EO yields using the folowing formula: Y= (Wo/Wp) × 100, where Y: EO yield, Wo: weight of EO and Wp: dry weight of plant (Akrout 2004). Three replicates were adopted to calculate the average of EO yield. The oils were then conserved at 4°C.

Essential oil composition.

EO analysis by GC-MS was performed on an Agilent 7890A GC system, coupled to an Agilent 5972C mass spectroscopy detector with electron impact ionization (70 eV). A HP-5 MS capillary column was used (30 m × 0.25 mm, coated with 5% phenyl methyl silicone, 95% dimethylpolysiloxane, 0.25 mm film thickness; Hewlett-Packard, CA, USA). The column temperature was programmed to rise from 40 to 240°C with a 5°C/min rate, the carrier gas was helium N60 with a 0.9 ml/min flow rate; split ratio was 100:1. Scan time and mass range were 1 s and 50-550 m/z, respectively. The identification of compounds was based on mass spectra (compared with Wiley Registry 9th Edition/NIST 2011 edition mass spectral library) and by comparison of their Kovats retention indices (Ri) with either those in the literature (Papachristos and Stamopoulos 2002; Pavela 2006) or with those of authentic compounds available in our laboratories. Kovats retention indices were determined in relation to a homologous series of n-alkanes (C8-C40) under the same conditions.

Insect rearing.

M. persicae individuals were collected at different stages (larvae, winged and wingless adults) from citrus orchards in Cap Bon region (North-East, Tunisia). Rearing was performed on young radish plants in cages under controlled conditions of a growth chamber (22°C ± 1; 60% ± 10 RH; 16:8 h photoperiod).

Bioassay.

Contact insecticidal activity. The insecticidal activities of *P. lentiscus* and *M. pulegium* EOs were tested on apterous adult aphids of 2-3 day-old age. This stage is known to be the most harmful for plants. A preliminary test was conducted in order to select the doses to be used. The adopted doses for both extracted oils were 400, 500, 600, 800, 900 and 1100 ppm (corresponding to 0.4, 0.5, 0.6, 0.8, 0.9 and 1.1 µl/ml of water with 2% tween 20 and in practical application to 40, 50, 60, 80, 90, 110 ml/hl). Distilled water with 2% tween 20 was used for dilution. Whatman filter paper discs (9 cm in diameter) were placed in Petri dishes before being impregnated afterward by 1 ml of each tested dose of EO. Ten adults of *M. persicae* were placed carefully using a fine brushin the Petri dishes containing the treated filter papers to perform the contact toxicity assays. During the experiment, fresh leaves of *R. sativus* were given as a food resource for aphids. Treatment with water solution mixed with 2% tween 20 was considered as control. A chemical insecticide namely imidacloprid as active molecule, was used as control and tested at the manufactory recommended dose (60 ml/hl). Incubation of the different Petri dishes was carried out in a growth chamber under the same conditions cited above. Five replications were used for each individual treatment. Mortality of aphids was observed at 2, 4, 6, 8 and 24 h after aphid exposure to EOs. The corrected mortality (Mc) was calculated using the modified Abbott formula (Abott 1925) considering the mortality in the treated Petri dishes (Mo) and the natural mortality in control ones (Mt): $Mc = [(Mo – Mt) / (100 – Mt)] \times 100$. An aphid was considered dead when no leg or antennal movements were observed.

Spray assays and synergistic effect. The synergism between both oils was checked as a complementary assay. Five test groups were conducted: *M. pulegium* EO, *P. lentiscus* EO and three binary mixtures of these oils applied at three ratios (1:1, 1:2 and 2:1 (w/w) ratios). The doses used were based on the previously obtained LC_{50}. Aphid sprays were performed in Petri dishes. Incubation conditions were same as cited above. Five replications were used for each individual treatment. Aphid's mortality was observed at 2, 8 and 24 h after aphid exposure to EOs. Abbott formula (Abott 1925) was used to calculate the mortality.

Statistical analysis.

Data were analyzed with SPSS software (version 20). The lethal concentrations LC_{50} and LC_{95} were calculated using the mortality rates obtained after 24 h in the bioassay with PROBIT analysis (Finney 1971). The mortalities of aphids induced by both EO were compared by ANOVA test at $\alpha = 0.05$.

RESULTS

Yields of tested essential oils and their chemical composition.

The average EO yield was estimated at 1.76% for *M. pulegium* and at 0.19% for *P. lentiscus*. GC-MS analysis revealed a total of 27 compounds

from *M. pulegium* (Table1). The major compounds were pulegone (45.89%), cis-menthone (23.25 %) and trans-menthone (14.73%). Concerning *P. lentiscus* EO, the chemical analysis revealed the presence of 32 compounds (Table1). The most abundant ones were α-pinene (28.57%), β-myrcene (21.03%) and L-limonene (6.97%). Five compounds were detected in both oils namely camphene, sabinene, trans-caryophyllene, α-pinene, and β-myrcene.

Table 1. Chemical composition of *Mentha pulegium* and *Pistacia lentiscus* essential oils

Volatile compounds	*Mentha pulegium*		*Pistacia lentiscus*	
	Rate (%)	Quantity (mg/100 g dry weight)	Rate (%)	Quantity (mg/100 g dry weight)
(+)-Neoisomenthol	0.35	5.73	-	-
1.8-(P-Menthadienone)	0.07	1.10	-	-
1.8-Cineole	0.91	14.93	-	-
1-Menthene	0.56	9.28	-	-
1r-Menthyl Acetate	2.01	33.00	-	-
2- B-Pinene	-	-	1.88	3.65
3-Cyclohexen-1-Carboxaldehyde.3.4-Dimethyl	0.03	0.49	-	-
3-Octanol	0.37	6.01	-	-
3-Octanone	0.10	1.72	-	-
3-Phenylbutyraldehyde	0.16	2.59	-	-
4-Terpineol	-	-	4.60	8.92
Alloaromadendrene	-	-	0.16	0.30
A-Terpinene	-	-	2.12	4.12
Bornyl Acetate	-	-	1.26	2.44
Cadina-1.4-Diene	-	-	0.18	0.35
Camphene	0.06	0.95	3.41	6.62
Camphre	0.26	4.29	-	-
Caryophyllene Oxide			0.16	0.31
Chrysanthenone	0.09	1.54	-	-
Cis-Isopulegone	0.88	14.38	-	-
Cis-Menthone	**23.25**	**381.92**	-	-
Cymene	-	-	0.79	1.52
D-Limonene	0.89	14.55	-	-
Eucarvone	4.62	75.87	-	-

Germacrene-D	-	-	2.39	4.64
Hexahydrofarnesyl Aceton	0.07	1.14	-	-
L-Limonene	-	-	**6.97**	**13.52**
L-Phellandrene	-	-	1.10	2.14
N-Butyl Isovalerate	-	-	0.12	0.24
Piperitone	3.25	53.39	-	-
Pulegone	**45.89**	**753.77**	-	-
Sabinene	0.08	1.36	1.75	3.40
Tau-Cadinol		--	0.50	0.96
Trans-Caryophyllene	0.09	1.43	3.19	6.19
Trans-Menthone	**14.73**	**241.97**	-	-
Tricyclene	-	-	0.83	1.61
A -Amorphene	-	-	0.69	1.34
α -Humulene	0.13	2.14	-	-
α -Terpineol	0.07	1.12	-	-
A-Amorphene	-	-	0.54	1.05
A-Copaene	-	-	0.27	0.52
A-Eudesmol	-	-	0.82	1.60
A-Muurolene	-	-	0.56	1.08
α-Pinene	**0.61**	**10.10**	**28.57**	**55.43**
A-Terpineol	-	-	2.16	4.20
A-Terpinolene	-	-	0.92	1.79
A-Thujene	-	-	0.54	1.05
B-Cis-Ocimene	-	-	4.76	9.24
β-Myrcene	**0.16**	**2.59**	**21.03**	**40.81**
β-Pinene	0.31	5.14	-	-
β-Selinene	-	-	0.61	1.19
B-Trans-Ocimene	-	-	1.79	3.47
γ-Terpinene	-	-	2.70	5.24
δ-Cadinene	-	-	2.61	5.06
Total	**100**	**1642.5**	**100**	**194**

Insecticidal and synergistic activities.

Eos extracted from *P. lentiscus* and *M. pulegium* were found to be toxic against *M. persicae* but no synergistic interaction has been noticed between them. Aphid mortality varied depending on exposure time and concentrations of tested EOs.

Insecticidal activity. Only two hours of exposure to *M. pulegium* EO were sufficient to observe an important mortality in *M. persicae* individuals ranging between 40 ± 25.49% at 600 ppm and 60 ± 14.14% at 1100 ppm. This clearly shows the rapid action of *M. pulegium* EO on aphids. A mortality of 86 ± 11.4% was recorded after 24 h of exposure using the highest dose i.e. 1100 ppm (Fig. 1).

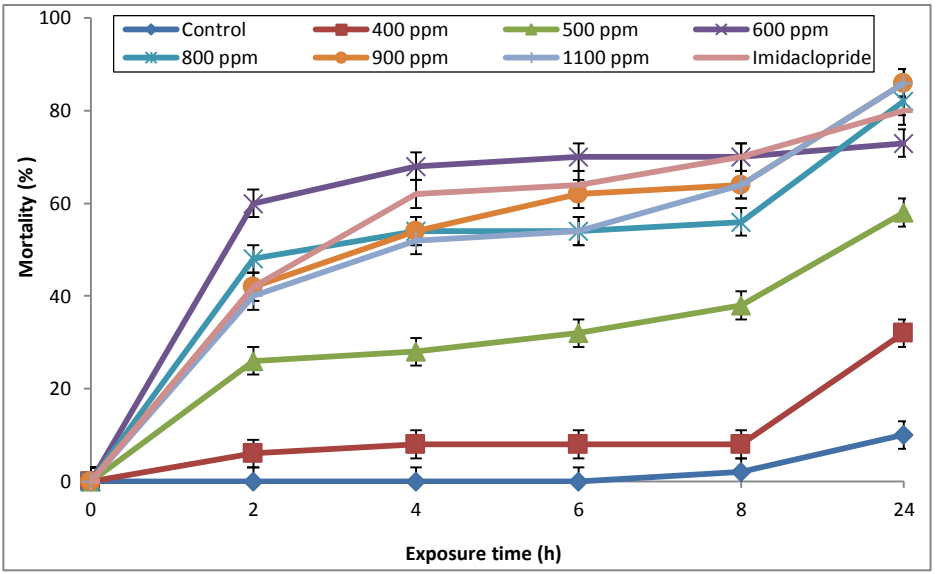

Fig. 1. Effect of *Mentha pulegium* essential oil on *Myzus persicae* mortality noted at different concentrations and exposure times. Error bars represent standard deviations.

P. lentiscus EO-based treatment applied to *M. persicae* has also led to an important aphid mortality which was positively correlated to the increase of tested concentrations. After exposure for 2 h, mortality was 2 ± 0.44% for 400 ppm and 32 ± 10.95% for 1100 ppm. After 24 h, the mortality reached 76±11.4% using *P. lentiscus* EO at the highest concentration (1100 ppm) (Fig. 2).

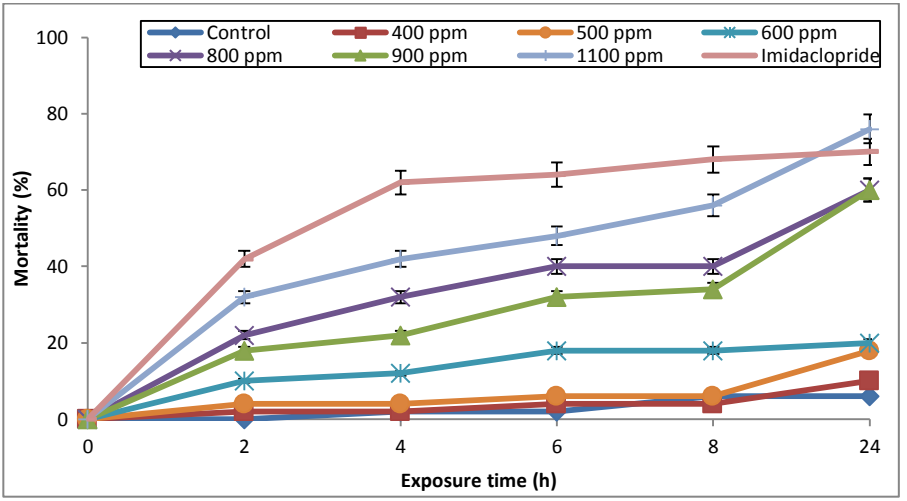

Fig. 2. Effect of *Pistacia lentiscus* essential oil on *Myzus persicae* mortality noted at different concentrations and exposure times. Error bars represent standard deviations.

After 24 h exposure to *M. pulegium* EO, aphid mortality was significantly different from the control. At concentrations varying from 600 to 1100 ppm, no significant differences were observed in aphid mortality (73 ± 8.36 to 86 ± 11.4%, respectively) neither between concentrations nor compared to the reference insecticide imidacloprid. However, after 24 h of exposure to *P. lentiscus* EO, the observed aphid mortality was low and statistically equal to the control at concentrations ranging between 600 and 1100 ppm. The recorded mortality varied between 60 ± 18.7 and 76 ± 11.4% (Fig. 3). However, in the same conditions the mortality caused by *M. pulegium* was always higher than *P. lentiscus*.

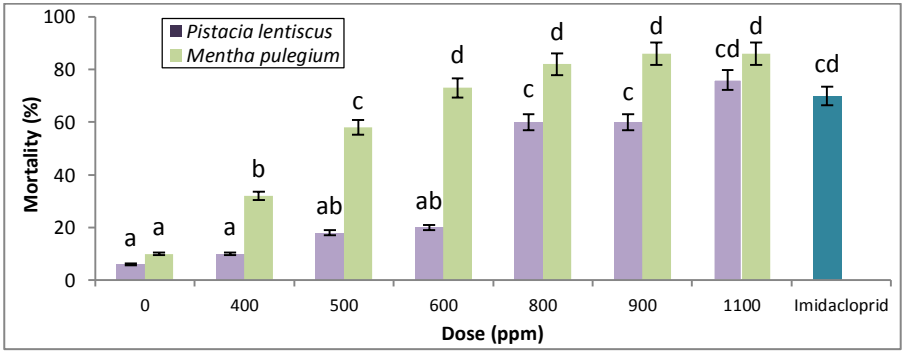

Fig. 3. Effect of *Mentha pulegium* and *Pistacia lentiscus* essential oils applied at different concentrations on *Myzus persicae* mortality noted after 24 h of exposure. Error bars represent standard deviations. For each essential oil tested, bars sharing the same letters are not significantly different based on Duncan's Multiple Range test (at $P < 0.05$).

This higher efficiency of *M. pulegium* EO is confirmed by its LC_{50} and LC_{95} which are much lower than those of *P. lentiscus*. The lethal concentrations of *M. pulegium* EO for 50 and 95% of treated aphids are 596 and 1264 ppm, respectively, whereas those of *P. lentiscus* EO's are 876 and 1405 ppm, respectively (Table 2).

Table 2. Lethal concentrations of *Mentha pulegium* and *Pistacia lentiscus* essential oils against *Myzys persicae* noted after 24 h of exposure

Source of EO	LC_{50} (ppm)	LC_{95} (ppm)	χ^2	ddl
Mentha pulegium	596 (427 - 736)	1264 (1036 - 1852)	5.087	5
Pistacia lentiscus	876 (760 - 1062)	1405 (1174 - 2032)	1.393	5

The estimated lethal concentrations values (ppm) were given using probit analysis.

Synergistic effect. Based on the common detection of five compounds in the both tested EOs, the combination of *M. pulegium* and *P. lentiscus* EOs is supposed to improve the mortality of aphids. However, the results of the synergism test showed that the mortality of *M. persicae* varied from 60 ± 2.58% to 66 ± 3.27% using *P. lentiscus* and *M. pulegium* EOs, respectively. The used concentrations corresponded to LC_{50} of both EOs. Those combined induced around 50% of aphid mortality with the spraying test which showed similar results as the contact test with a treated surface. This indicates that both application methods induce the same mortality for the same dose. No synergism was noticed between both oils in all the mixtures. According to the statistical analysis, there is no significant difference between the recorded mortalities caused by the individual oils and their mixtures (Fig. 4).

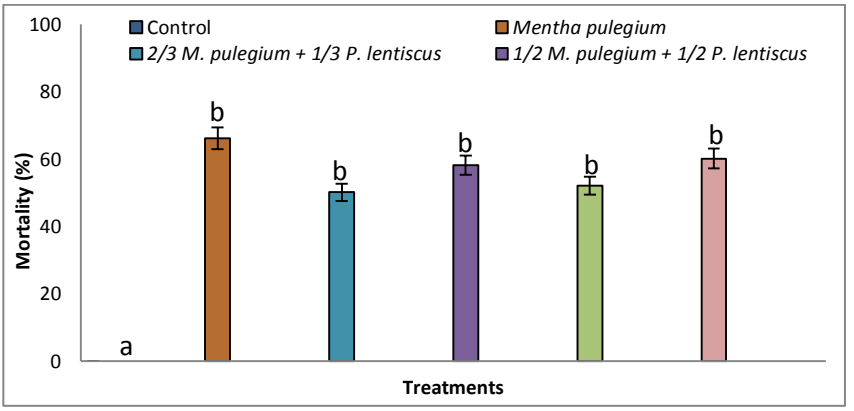

Fig. 4. Effect of different combinations of *Mentha pulegium* and *Pistacia lentiscus* essential oils on *Myzus persicae* mortality noted after 24 h of exposure. Error bars represent standard deviations. Bars sharing the same letters are not significantly different based on Duncan's Multiple Range test (at $P < 0.05$).

DISCUSSION

Essential oils extracted from *M. pulegium* and *P. lentiscus* were investigated for their yield, chemical composition and insecticidal activities against *M. persicae* with contact and spray methods.

The oil extraction yield of *M. pulegium* (1.76%) was higher than that of *P. lentiscus* (0.19%). For the chemical composition, the major compounds identified in both oils are monoterpenoïds. This fact is in accordance with previous studies (Duru et al. 2003; Gradeli et al. 2008; Işcan et al. 2002; Yadegarinia et al. 2006). Tunisian *M. pulegium* EOs are characterized by their abundance of pulegone (45.89%) compared to those from Turkey, India, Washington State, Oregon which are rich in menthol (28-42%) and menthone (18-28%) (Işcan et al. 2002). However, *M. pulegium* EOs from Iran are rich with α-terpinene (19.7%), isomenthone (10.3%) and trans-carveol (14.5%) (Yadegarinia et al. 2006).

P. lentiscus EO was characterized by the abundance of limonene (6.97%), β-myrcene (21.03%) and α-pinene (28.57%) while Turkish *P. lentiscus* EO was more rich in terpinene-4-ol (29.9%) and limonene (10.6%) (Duru et al., 2003). Morocco EO from this plant contained germanicol (12.8%), thunbergol (8.8%), himachalene (7.4%), trans-squalene (6.7%), terpinyl propionate (6.7%), 3,3-dimenthol (6.2%), and cadina-1.4-diene (5.1%) (Mharti et al. 2011). This composition variability with remarkable quantitative and qualitative differences in relation with sampling sites was previously reported by Barra et al. (2007) and Mkaddem et al. (2007) for *P. lentiscus* and *M. pulegium*, respectively. The major compounds detected in both Eos are known for their insecticidal activities (Koul et al. 2008). Several studies have proved that they interfere with the insect physiological functions by a rapid cuticule penetration (Lee et al. 2002; Papachristo and Stamopoulos 2002). Furthermore, it has been shown that some of them have an effect on stomach tissues (Sauvion 1995) and can disturb growth, fecundity and molting process of several insects. In the same context, Chiasson and Beloin (2007) have proved the relation between octopamine (a neurotransmitter responsible of insect metabolism and flight) and some monoterpenoic essential oil compounds.

Based on bioassay results, both tested Eos were found to be toxic to *M. persicae* with some variability in the induced mortality depending on concentrations and exposure times. Moreover, the obtained LC_{50} and LC_{95} confirm the efficiency of both oils against the target aphid with about four times less than the LC_{50} obtained with camphor *Eucalyptus globules* (4070 ppm), cinnamon *Cinnamomum zeylanicum* (3307 ppm), clove *Syzygium aromaticum* (5469 ppm) and mustard *Brassica rapa* (4261 ppm) against*Bruchidius incarnatus* adults (Fouad 2013).The contact toxicity has probably affected the aphid nervous system via a penetration of EO's toxic compounds through the ventral surface as cited by Lee et al. (2002) and Papachristos and Stamoupolos (2002).

After exposure to 500 ppm of *M. pulegium* EO, the aphicidal efficiency was as high as the chemical insecticide used as control (Imidaclopride) where mortality reached 86 ± 11.4%. The toxic oil effect recorded is probably attributed to the major compounds pulegone and (Cis and Trans) menthone which are known for their insecticidal properties (Koul et al. 2008). Gabris et al. (2005) and Dancewikz et al. (2008) also found that pulegone deterred aphid probing and feeding and influenced their growth and

reproduction. In addition, it has been shown that the essential oil of *M. pulegium* reduced the longevity and fecundity of *M. persicae* adults (Petrakis et al. 2014). Furthermore, this EO displayed a strong antimicrobial activity (Oraby and El Borollosy 2013).

P. lentiscus EO showed toxicity against *M. persicae* leading to high morality level (76 ± 11.4%) and exhibited similar efficiency as the chemical insecticide used as control but an activity less than that of *M. pulegium* EOs. The experimental toxicity is in accordance with previous reports on other insect pests such as *Tribolium castaneum*, *Lasioderma serricorne*, *Ectomyelois ceratoniae* and *Ephestia kuehniella* (Bachrouch et al. 2010a, 2010b).

The synergism assay with the tested mixtures of EOs revealed the absence of interaction (neither negative nor positive) between their main compounds against *M. persicae*. However, Liu et al. (2006) found that repellent activity of the mixture of EOs from *Artemisia princeps* and *Cinnamomum camphora* against *Sitophillus oryzae* and *Bruchus rugimanus* adults was significantly higher than that elicited by individual oils. Nevertheless, the mechanisms involved in how the interactions between the components of each EO improve the repellent activities, need further investigations (Nerio at al. 2010).

In conclusion, this study showed a better aphicidal activity of *M. pulegium* EO compared to that of *P. lentiscus* in controlling adult form of *M. persicae*. However, both of them showed the same efficiency as the chemical insecticide used as control. Moreover, it is established that both EOs have a potential as a biopesticide in integrated pest management against this polyphagous aphid species. However, the effect of EO on non-target insects and natural enemies needs to be further elucidated. This work should be completed with the stable formulation of those EOs for practical use in agriculture once their safety toward non-target insects confirmed.

RESUME

Behi F., Bachrouch O., Ben FekihI. et Boukhris-Bouhachem, S. 2017. Effet insecticide et synergique de deux huiles essentielles *Pistacia lentiscus* et *Mentha pulegium* sur le puceron vert du pêcher *Myzus persicae*. Tunisian Journal of Plant Protection 12: 53-65.

La composition chimique de deux huiles essentielles extraites à partir de la menthe pouliot *Mentha pulegium* et du pistachier lentisque *Pistacia lentiscus* a été étudiée. Les composés volatils ont été caractérisés. Les composés majeurs détectés ont été pulégone (45,89%), cis-menthone (23,25 %) et trans-menthone (14,73 %) pour *M. pulegium* et α-pinène (28,57%), β-myrcène (21,03%) et L-limonène (6,97%) pour *P. lentiscus.* Ensuite, les activités insecticides et la synergie des huiles essentielles ont été étudiées contre *Myzus persicae*. Les résultats ont montré que les deux huiles essentielles sont toxiques au puceron cité. La mortalité causée par *M. pulegium* et *P. lentiscus* a été de 86 ± 11,4% et 76 ± 11,4%, respectivement. La LC_{50} de *M. pulegium* a été plus faible que celle de *P. lentiscus* avec des valeurs respectives de 596 et 876 ppm. Aucune synergie n'a été détectée entre les deux huiles lorsqu'elles ont été mixées et testées contre *M. persicae.* Néanmoins, elles ont montré une efficacité équivalente à celle du produit chimique : l'imidaclopride, qui a causé une mortalité de 70 ± 10%. Cette étude a prouvé que les deux huiles essentielles ont un potentiel intéressant avec une efficacité supérieure de *M. pulegium* et peuvent être utilisées en agriculture pour la lutte contre *M. persicae.*

Mots clés: Huiles essentielles, *Mentha pulegium, Myzus persicae*, *Pistacia lentiscus*, toxicité de contact

ملخص

الباهي، فاطمة وألفة بشروش وابتسام بن فقيه وسنية بوخريص-بوهاشم. 2017. فعالية وأنشطة التآزر للزيوت الأساسية لنبتتيْ *Mentha pulegium* و *Pistacia lentiscus* ضد حشرة منّ الخوخ الأخضر *Myzus persicae*. Tunisian Journal of Plant Protection 12: 53-65.

تمت دراسة التركيبة الكيميائية لإثنين من الزيوت الأساسية للنعناع (*Mentha pulegium*) والمستكة. (*Pistacia lentiscus*). تم الكشف عن مركبات متبخرة. كانت المركبات الأكثر تواجد من بينهم هي pulegone (45.89%) و cis-menthone (23.25%) و trans-menthone (14.73%) بالنسبة لزيت النعناع و α-pinene (28.57%) و β-myrcene (21.03%) و L-limonene (6.97%) بالنسبة لزيت المستكة. تم اختبار هذه الزيوت ضد حشرة المن الأخضر (*Myzus persicae*). أظهرت النتائج أن كلا الزيتين سام لحشرات المن وتبين أن زيت النعناع أكثر فعالية من زيت المستكة. تسبب زيت النعناع في مقتل 86 ± 11.4% من الحشرات بينما تسبب زيت المستكة في مقتل 76 ± 11.4% من الحشرات. وصلت الجرعة القاتلة لـ 50% من الحشرات إلى 596 ppm بالنسبة لزيت النعناع و 876 ppm لزيت المستكة. لم يظهر اي تآزر بين الزيتين عندما تم خلطهما. تبين أيضا أنه لهما نفس سمية المبيدالكيميائ الحشري Imidaclopride الذي تسبب في قتل 70± 10%. تظهر نتائج هذه الدراسة أن كلا الزيتين لهما نجاعة جيدة، ويمكن استخدامهما في مكافحة المن الأخضر في مختلف الزراعات.

كلمات مفتاحية: زيوت أساسية، سمية التلامس، *Pistacia lentiscus*، *Myzus persicae*، *Mentha pulegium*

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Population Demographic and Reproductive Parameters of the Cowpea Seed Beetle *Callosobruchus maculatus* Infesting Stored Lentil and Chickpea Commodities

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ABSTRACT

Haouel-Hamdi, S., Titouhi, F., Boushih, E., Dhraief, M.Z., Amri, M., and Mediouni-Ben Jemâa, J. 2017. Population demographic traits and reproductive parameters of the seed beetle *Callosobruchus maculatus* infesting stored lentil and chickpea commodities . Tunisian Journal of Plant Protection 12: 67-81.

This paper carried out first exhausted investigations on pest status of the cowpea weevil *Callosobruchus maculatus* on two food legumes namely chickpea (Amdoun 1 variety) and lentil (Ncir variety) during six months of storage. Data on populations' dynamic, demographic traits, reproductive parameters, juvenile and adult fitness, economic injury level (EIL) and damages (impact on germination and weight losses) were studied through this work. Results revealed that *C. maculatus* is a major pest on stored chickpea in Tunisia. Moreover, results indicated that reproductive parameters, the juvenile and adult fitness of *C. maculatus* exhibited great variations among hosts. In this respect, linear regression analysis demonstrated that hosts have significant effects on adult fitness. Results showed that host contributed respectively by 77% for body weight and 80% for body size. Chickpea was more suitable host compared to lentil, since the mortality rate of eggs and larvae and the generation duration means were higher in lentil. In addition, significant differences were observed in the Susceptibility Index of the two food legume hosts showing chickpea seeds as moderately susceptible to *C. maculatus* attacks while, lentil seeds were resistant. *C. maculatus* caused large reductions in seed germination (78% chickpea and 33% lentil for highest infestation level 80%) and seeds weight (45% for chickpea against 8% for lentil after 6 months of storage) of both hosts; the infestation levels and the weight losses were significantly different in the storage periods. Overall, this study provides reasons for farmers and traders to make a decision to take a control action against *C. maculatus* during storage. Moreover, this work pointed out the variability of economic injury levels with host legumes.

Keywords: Adults' and juvenile fitness, *Callosobruchus maculatus*, chickpea, demographic traits, economic injury level, lentil, reproductive parameters

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The cowpea seed beetle *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) is a cosmopolitan polyphagous pest of pulse crops under

storage conditions in the most tropics and subtropics such as Mediterranean's areas and Africa (Ajayi and Lale 2001; Bagheri 1996; Booth et al. 1990). This beetle is reported to be the most damaging pest of legume seeds and its larvae, being internal feeders, infested grains such as bean, chickpea, green gram, lentil, broad bean and green pea (Kazemi et al. 2009). It is a field-to-store pest that causes heavy yield losses in terms of both quantity and quality of stored grain legumes. The neonate larvae penetrate the grains causing serious damage such as grain weight loss, reduction in germination, seed viability and nutritional low quality (Melo et al. 2015; Oke and Akintunde 2013). Furthermore, this pest also causes secondary infestation by preparing appropriate conditions for activity of saprotrophic fungi during seed storage (Loganathan et al. 2011).

In Tunisia, few studies have examined the importance of *C. maculatus* damages on stored food legumes in relation to its population growth and reproductive parameters. Even fewer have examined whether damages depend on the host plants and storage conditions. In this respect, Jarraya (2003), Mabrouk and Belhadj (2012) reported that *C. maculatus* is among the major insect pests attacking chickpea in Tunisia. Additionally, Haouel et al. (2012; 2015) indicated that *C. maculatus* is among the most important stored grain pests commonly found in chickpea in Tunisia. Thus, this work aims to investigate population dynamics, reproductive parameters, demographic traits, juvenile and adult fitness, the Susceptibility Index (SI), damages (weight losses, germination) and the Economic Injury Level (EIL) of *C. maculatus* reared on two host legumes: chickpea and lentil.

MATERIALS AND METHODS

Insect rearing and seed material.

C. maculatus individuals were obtained from infested chickpea seeds provided by the Field Crops' Research Laboratory. The stock colonies were maintained in glass bottles of 1 liter of volume in a growth chamber at 30 ± 5ºC temperature, 65 ± 5% RH and 12:12 Light:Darkness photoperiod.

Two food legumes hosts: a chickpea (*Cicer arietinum*) Amdoun 1 variety (Pedigree Be-sel-81-48) and a lentil (*Lens culinaris*) Ncir variety (Pedigree ILL 4400) served as food substrates for the insect rearing. Flightless-form females were used for this study.

Reproductive parameters and life table study.

The reproductive parameters of *C. maculatus* laid on either chickpea or lentil seeds studied using three replicates of 100 eggs. To determine durations of eggs incubation, larval developmental time and life cycle, the eggs were transferred individually in Petri dishes and were checked daily until the emergence of adults. Moreover, adult longevity was recorded daily until death of last individual. After emergence of adults, each couple was placed in Petri dish containing 20 g of either chickpea or lentil seeds. The duration of ovipositon and post-oviposition periods as well as longevity, daily fecundity (eggs per female day), total fecundity (eggs during reproduction period), sex ratio, emergence rate and juvenile and adult fitness were determined.

Demographic traits study.

The age-stage specific fecundity (f_{xj}), the age-specific survival rate (l_x), the age-specific fecundity (m_x) and the population growth parameters namely:

the net reproduction rate (R_0), the mean generation time (*T*), the intrinsic rate of increase (*r*), the finite rate of increase (λ), and the doubling time (*DT*) were calculated according to Khanamani et al. (2013) and Win et al. (2011).

The age-specific survival rate (l_x) comprising both female and male, was calculated according to Chi and Liu (1985) as:

$lx = \sum_{j=1}^{k} Sxj$ with k: number of stages

The age-specific fecundity (m_x) was calculated as:

$$mx = \frac{\sum_{j=1}^{k} Sxjfxj}{\sum_{j=1}^{k} Sxj}$$

The net reproduction rate (R_0) was calculated according to Carey (1993) as:

$$Ro = \sum_{j=1}^{k} lx\ mx$$

The mean generation time (*T)* can be defined as the average length of time between when an individual is born and the birth of its offspring approximated by the following formula (Birch 1948)

$$T = \frac{\sum xlxmx}{\sum lxmx}$$

The intrinsic rate of increase (*r*) also called the intrinsic rate of natural increase or the innate capacity for increase was after that estimated using Carey (1993) formula as: $r = \frac{Ln\ Ro}{T}$

The doubling time (*DT*) defined as the number of days required by a population to double was as well calculated according to Carey (1993):

$$DT = \frac{Ln\ 2}{rm}$$

The finite rate of increase (λ) characterized by the number of female offspring per female per day was calculated according to Carey (1993) as: $\lambda = e^{rm}$.

The susceptibility index (SI).

The Susceptibility Index was used as the criterion to test the susceptibility of both host substrates toward insect feeding. It was calculated using the method of Dobie and Kilminster (1977) given by the formula:

$$SI = \frac{\log e\ F}{D} \times 100$$

with D: the median development period, F: the total of number of F1 progeny emerged.

The SI was used to classify the two host legumes into susceptibility groups following the scales as follows: scale index of ≤ 4 was classified as resistant; scale index of 4.1-6.0 as moderately resistant; scale index of 6.1-8.0 as moderately susceptible; scale index of 8.1-10 as susceptible and scale index >10 as highly susceptible.

Seed germination.

Germination tests were carried out following the methodology of the International Seed Testing Association employing a representative sample of 50 seeds in three replications of chickpea and lentil with three infestation rates of *C. maculatus* (0, 5, and 80%).

Infestation levels and weight losses.

This trial was conducted over a storage period of six months where weight loss controls were performed each month. The experiment consists on placing five pairs of *C. maculatus* (less than 24 h old) in glass bottles containing 100 g of healthy seeds of chickpea or lentil. The test was replicated three times. The females laying their eggs were left until they died. After laying eggs, the dead adults were removed from bottles. Hatched eggs were allowed to develop until adult emergence.

Each month, infested and healthy seeds were separated, cleaned, counted,

and finally weighed after completion of adult emergence. Seed infestation and weight loss were computed by using the following formulae (Anonymous 1988):

$$A\% = \frac{Nd}{Nd + Nu} \times 100 \; B\% = \frac{WuNd - WdNu}{Wd(Nd + Nu)} \times 100$$

with A% = percent of damage, B% = percentage of weight loss, Nd = number of damaged seeds, Nu= number of undamaged seeds, Wu = weight of undamaged seeds, and Wd = weight of damaged seeds.

Economic injury levels.

C. maculatus Economic Injury Levels (EILs) were determined each month for chickpea and lentil seeds for a period of six moths' storage duration. EILs were calculated according to Pedigo et al. (1986) formula:

$$EIL = \frac{C \times N}{V \times I}$$

where C=management cost per production unit expressed with Tunisian Dinars/kg, I = percent weight loss, N = number of pests causing injury and V = market value per production unit expressed with Tunisian Dinars/kg.

Statistical analysis.

To analyze the possible effects of the rearing substrates (chickpea and lentil) on all biological parameters of *C. maculatus*, statistical analyses were performed using SPSS statistical software version 20.0. All values given were the mean of three replications and were expressed as the mean ± standard deviation ($\bar{x}$ ± SD). Significant differences between the mean values ($P \leq 0.05$) were determined by using Student test.

Adults' fitness component (weight) and number of eggs was log transformed to attain normality. Correlation analyses (Pearson's correlation coefficient) and general linear model were established between the juvenile and adults' fitness and the number of eggs.

RESULTS

Reproductive parameters.

The reproductive parameters of *C. maculatus* reared on chickpea and lentil were illustrated in Table 1. Results showed that *C. maculatus* reproductive parameters varied upon host legumes. Best performances were recorded when the insect was reared on chickpea. In this respect, the mean eggs per female, the fertility rate and the immature development period values were respectively 87.4 egg/female, 38.1% and 38.3 days for chickpea against 25.7 egg/female, 10.1% and 50 days for lentil. However, no statistical differences were detected between the two hosts regarding adult's longevity parameter (F = 4.00, P = 0.12, Table1).

Table 1. Reproductive parameters of *Callosobruchus maculatus* reared on two food legumes (mean ± SD)

Seed	Adult longevity (Days)	Oviposition period (Days)	Immature development period (Days)	Mean eggs per female	Mean progeny per female	Fertility rate (%)
Chickpea	5.3 ± 0.6 a	7.3 ± 0.6 b	38.3 ± 1.5 a	87.4 ± 10.5 b	13.9 ± 5.1 b	38.1 ± 9.9 b
Lentil	4.0 ± 0.0 a	5.3 ± 0.6 a	50.0 ± 5.0 b	25.7 ± 5.0 a	2.7 ± 0.6 a	10.1 ± 3.2 a

For each biological parameter, comparisons were made between the two legume hosts. Means followed by the same letters are not significantly different at the 5% threshold (Student Test).

Juvenile and adults' fitness assessment.

Correlation. Results revealed that the juvenile and adults' fitness of *C. maculatus* exhibited great variations among hosts (Table 2). A highly significant and negative correlation was observed between the host and the body weight and size of the beetle (r_{weight} = -0.88, $P < 0.001$ and r_{size} = -0.89, $P < 0.001$). On the other hand, results showed high significant and positive correlations between juvenile fitness, adults' fitness and number of eggs (fertility) (Table 2).

Table 2. Correlation analyses between juvenile fitness, adults' fitness and number of eggs of *Callosobruchus maculatus*

Pearson's correlation coefficient	Seeds legumes	Juvenile fitness	Adults fitness (log weight)
Juvenile fitness	-0.681**	-	-
Adults fitness (log weight)	-0.877**	0.482**	-
Adults fitness (body size)	-0.895**	0.554**	0.816**
Log number of eggs	-0.915**	0.634**	0.778**

** Highly significant at 1% level

Linear Regression Model. Linear regression analysis revealed that hosts have significant effects on adults fitness (body weight, body size) (F = 75.64, $P < 0.001$, R^2 = 0.66, Fig. 1) confirming results of correlation analysis (Table 2). The importance of the host for explaining significant portions of the independent variable for fitness of *C. maculatus* is also emphasized. The linear regression analysis allowed as investigating the host contribution into the variation of adults' fitness. Results showed that host contributed respectively by 77% for body weight and 80.1% for body size (Fig. 1). Overall, *C. maculatus* laid a larger number of eggs on chickpea than lentils. The interaction between host and body weight explain a significant amount of the variance in fecundity (F = 58.11, $P < 0.001$, R^2 = 0.60). These results pointed out the direct effect of host on fecundity and adults' fitness.

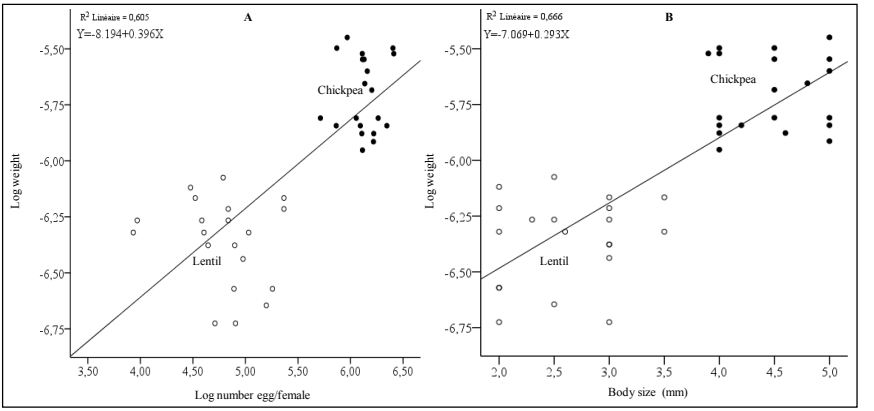

Fig. 1. Relationship between weight-number of laid eggs (A) and weight-body size (B) of *Callosobruchus maculatus*. Linear regression line plotted for significant ($P < 0.005$).

Demographic parameters traits.

Results revealed that the host legumes affect significantly all demographic parameters excepted for the sex-ratio where no differences were detected (F = 0.72, *P* = 4.44) (Table 3).

Demographic traits data revealed that *C. maculatus* performances were dependent on host legumes. Chickpea appeared as the most suitable host compared to lentil.

Table 3. Demographic traits of *Callosobruchus maculatus* reared on chickpea or lentil

Seeds	MGR*	MRE*	MRL*	ER*	SR*	Ro*	GT*	R*	DT*	λ*
Chick-pea	0.8 ± 0.1 b	60.6 ± 10.6 a	4.6 ± 0.6 a	89.8 ± 2.6 b	0.7 ± 0.2 a	11.3 ± 6.2 b	44.3 ± 1.5 a	0.051 ± 0.015 b	14.5 ± 5.2 b	1.050 ± 0.020 b
Lentil	-1.4 ± 1.1 a	89.6 ± 0.7 b	62.8 ± 14.5 b	37.1 ± 14.5 a	0.8 ± 0.3 a	0.2 ± 0.2 a	54.0 ± 5.0 b	-0.035 ± 0.02 a	-	0.960 ± 0.020 a

For each demographic parameter, comparisons were made between hosts. Means followed by the same letters are not significantly different at the 5% threshold (Student Test).

* MGR = mean growth rate, MRE = mortality rate of eggs, MRL = mortality rate of larvae, ER = emergence rate, SR = Sex-Ratio, R = net reproductive rate, T = generation time, r = intrinsic rate of increase, DT = doubling time, λ = finite rate of increase.

C. maculatus could successfully survive and reproduce both on chickpea and lentil, with a food-preference tendency to chickpea (Fig. 2).

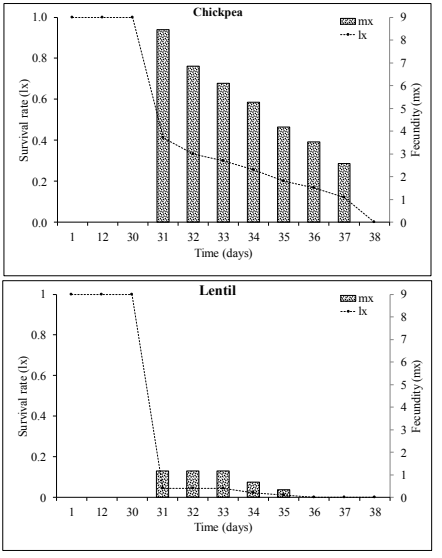

Fig. 2. Effect of time on age-specific survival rate (l_x) and fecundity (m_x) of *Callosobruchus maculatus* on two host legumes chickpea (left) and lentil (right).

The susceptibility index (SI).

Significant differences (F = 47.48, *P* = 0.02) were observed in the susceptibility index of the two food legume hosts (Table 4). The SI values were 3.54 for lentil and 6.06 for chickpea. Chickpea seeds were moderately susceptible to *C. maculatus* attacks, while lentil seeds were not.

Table 4. Susceptibility Index of chickpea and lentil seeds to *Callosobruchus maculatus* after six months of storage

Hosts	SI*	Scale index classification
Chickpea	6.06 ± 0.47 b	Moderately susceptible
Lentil	3.54 ± 0.41 a	Resistant

Comparisons were made between hosts susceptibility index. Means followed by the same letters are not significantly different at the 5% threshold (Student Test). *SI = The Susceptibility Index.

Germination.

C. maculatus caused significant reduction in seeds germination of both hosts, with the highest infestation level (80%) causing reductions of 78% and 33% for chickpea and lentil, respectively (Fig. 3).

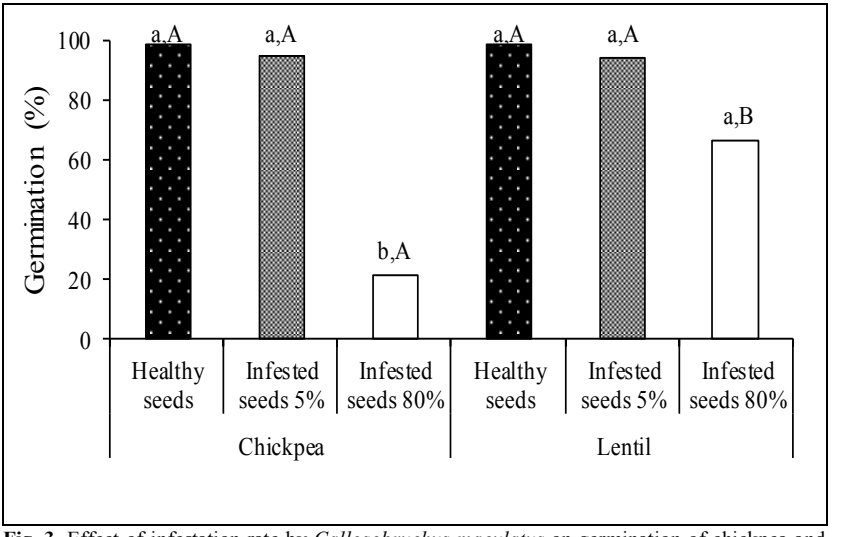

Fig. 3. Effect of infestation rate by *Callosobruchus maculatus* on germination of chickpea and lentil seeds. Bars having different letters are significantly different at 5% level of probability among infested levels for each legume seeds (lowercase letters) and among legume seeds for each infested levels (uppercase letters).

Infestation levels and weight losses.

Infestation levels and weight losses are significantly different among the two hosts and storage periods (Fig. 4). For chickpea, infestation levels ranged from 15 and 49 to 100% after 1, 3 and 6 months of storage, respectively. Similar data for lentil which had infestation levels ranging from 0.3 and 19 to 80% after 1, 3 and 6 months of storage, respectively. For chickpea, weight losses varied between 1, 19 and 45% after 1, 3 and 6 months of storage, respectively. For lentil, weight losses oscillated between 0.3, 4 and 8% after 1, 3 and 6 months of storage, respectively (Fig. 4).

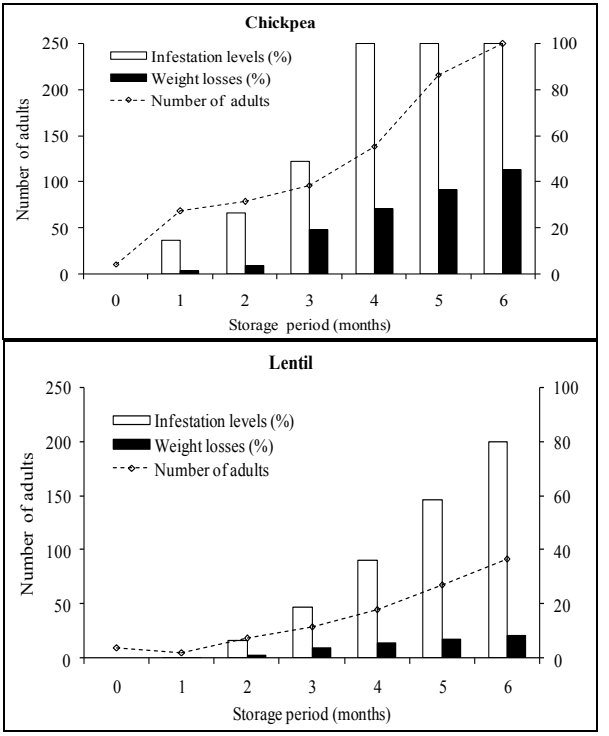

Fig. 4. Relationship between storage period and number of adults, infestation levels (%) and weight losses (%) of *Callosobruchus maculatus* reared on chickpea (left) and lentil (right).

Economic Injury Level (EIL).

EIL depended on food hosts (Fig. 5). The respective values were 63 and 83 insect/kg for chickpea and lentil. These findings further revealed that *C. maculatus* has a host-trophic preference toward chickpea.

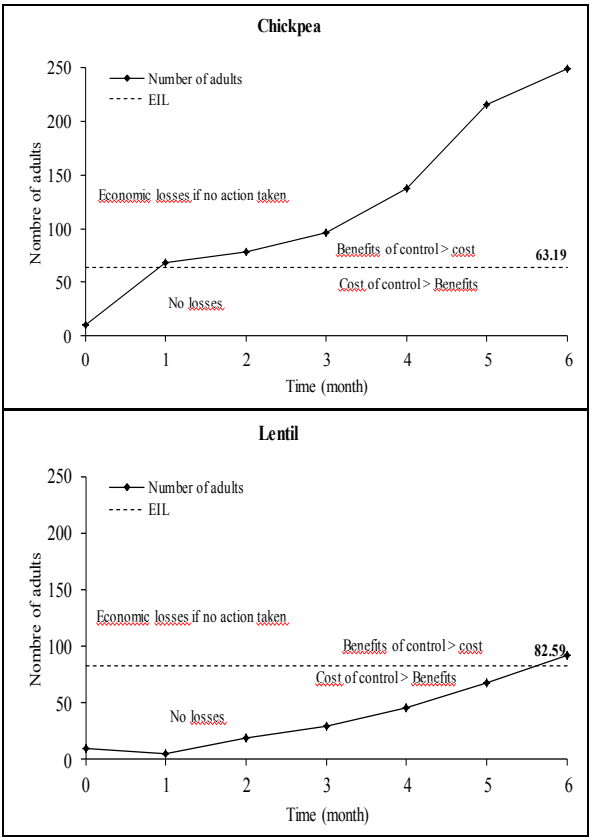

Fig. 5. Representation of the Economic-Injury Level (EIL) of *Callosobruchus maculatus* and its relationship to economic loss, benefits, and costs in chickpea (left) and lentil (right).

DISCUSSION

The cowpea seed beetle *C. maculatus* is considered as the most important pest of stored food legume worldwide. However, in Tunisia data on the pest population dynamic, the growth parameters and demographic traits, the susceptibility index, the economic injury level and its impact on germination and weight losses was poorly documented.

Our results revealed that *C. maculatus* is a major beetle pest on stored chickpea in Tunisia. These findings are consistent with those reported by Iturralde-García et al. (2016), Kedia et al. (2015), Staneva (1982), Van Huis and De Roy (1998) indicating that *C. maculatus* is the main pest of stored chickpea. Previous works reported that *C. maculatus* reproductive parameters varied according to host plants. In this respect, Gokhale (1973), Obopile et al. (2011), and Swella and Mushobozy (2009) specified that the highest mean egg counts and high percent of adult emergence were obtained on chickpea compared to other legume seeds.

Moreover, Kazemi et al. (2009) demonstrated that it was a significant difference in the immature development

period of *C. maculatus* on four legume species (cowpea, lentil, chickpea, green gram). It was the shortest on chickpea (37.43 ± 0.43 days) and the longest on lentil (42.66 ± 0.22 days). Similarly, several studies were achieved on reproduction and fecundity of *C. maculatus*. It was clearly demonstrated that egg production was significantly affected by host (Van Huis and De Roy 1998), seed size and weight (Cope and Fox 2003; Govindarajan and Balasubramanian 1981; Meyer et al. 1986), female weight at the time of mating (Credland et al. 1986; Sibly et al. 1991), male size and density (Giga and Canhao 1997; Savalli and Fox 1999), and egg and larvae density in seeds (Ofuya and Agele 1990; Wijeratne 1998).

The comparative study of the demographic traits between the two hosts legumes performed in our study revealed that a highly significant and negative correlation was observed between hosts and the adults' fitness (body weight and size) of *C. maculatus* whereas high significant and positive correlations between juvenile fitness, adults' fitness and number of eggs were obtained. Thus, we could conclude that the host legumes affect fecundity and adult emergence size. In these regards, our results are in accordance with those described by (Credland et al. 1986; Messina 1991; Smith and Lessells 1985) who point out the relationship between fecundity and the size of adults of *Callosobruchus* beetles. Additionally, Kawecki (1997) demonstrated that females emerging from *Vigna radiate* and *Vigna angularis* had different fecundities but apparently similar weights. According to William and Kirk (1991), there was a correlation between insect size and host size. Body size has many ecological implications (Peters 1983), including those relating to an insect's distribution, abundance, and population variability (Gaston and Lawton 1988).

Our results pointed out the fact that, if individual fitness adaptive traits increased, the population size and reproductive and demographic performances increased. These outcomes agree with those reported by Metz et al. (2008). Additionally, since the seed beetle *C. maculatus* is a cosmopolitan pest of legume seeds, its populations vary substantially in their host associations, and vary in a suite of life history and behavioral traits associated with these host plant differences as reported by Kawecki and Mery (2003), Fox et al. (2004), Messina (1991), Messina and Slade (1997), and Savalli et al. (2000). Similarly, Janzen (1977) specified that the different hosts provide different qualities of resource to the beetles leading to different biological parameter traits.

On the other hand, differences of hosts' susceptibility toward *C. maculatus* could be due to the differences in the ability of particular hosts to resist to *C. maculatus* attack including protein, carbohydrate, ash, tannin and saponin (Desroches et al. 1995; Janzen 1977; Mogbo et al. 2014; Onuh and Onyenekwe 2008). Lentil has the lowest weight losses, infestation levels and the highest germination rate. In fact, our results are in agreement with those described by Jackai and Asante (2003), Keba and Sori (2013), and Redden and McGuire (1983) who indicated that variables such as adult emergence, growth index, developmental period and weight loss are the most reliable indicators for resistance of cowpea to damage by *C. maculatus*.

In addition, Tadesse (1995) and Tefera et al. (2011) indicated that the extent of damage during storage depends on the number of emerging adults during each generation, the duration of each life cycle and host varieties. These authors

specified that varieties allowing more rapid and higher levels of adult emergence are more seriously damaged.

EIL is defined as the lowest population density of a pest that will cause economic damage or the amount of pest injury which will justify the cost of control. However, no economic studies were undertaken to assess the injury of *C. maculatus* on food legumes stored commodities in Tunisia. Earlier works by Mi et al. (1998), Pedigo et al. (1986), and Stern et al. (1959) reported that the EILis a basic component of decision making in pest management. Thus, this study will provide reasons for farmers and traders to make a decision to take a control action against *C. maculatus* during storage. Moreover, this work pointed out the variability of EIL with host legumes. Consequently, a complementary study on the economic threshold is required for a best postharvest management of *C. maculatus* populations.

To summarize, this study demonstrated that *C. maculatus*is a serious problem for food stored legumes in Tunisia, mainly chickpea. Therefore, an appropriate technical panel on the beetle pest status on various host legumes together with adequate control approaches is required either for farmers and traders. In addition, further studies are required to determine the extent of infestation and damage in fields.

RESUME

Haouel-Hamdi S., Titouhi F., Boushih E., Dhraief M.Z., Amri M. et Mediouni-Ben Jemâa J. 2017. Les traits démographiques et les paramètres reproductifs des populations des bruches du niébé *Callosobruchus maculatus* infestant la lentille et le pois chiche stockés .Tunisian Journal of Plant Protection 12: 67-81.

Cet article est une première étude exhaustive concernant le statut de nuisibilité du coléoptère du niébé *Callosobruchus maculatus* sur deux légumineuses alimentaires le pois chiche (Variété Amdoun 1) et la lentille (Variété Ncir) durant six mois de stockage. Les données relatives à la dynamique des populations, les traits démographiques, les paramètres reproductifs, les performances juvéniles et adultes, le niveau de nuisibilité économique et les dégâts (impact sur la germination et les pertes de poids) ont été étudiés à travers ce travail. Les résultats ont révélé que *C. maculatus* est un ravageur majeur du pois chiche stocké en Tunisie. En outre, les résultats ont montré une grande variation des paramètres biologiques ainsi que les performances juvéniles et adultes selon les hôtes. Dans ce contexte, l'analyse de la régression linéaire a démontré que les hôtes affectent significativement les performances des adultes de *C. maculatus*. Les résultats ont également prouvé que l'hôte contribue par 77% du poids corporel et 80% de la taille du corps. Les données des traits démographiques ont montré que le pois chiche apparait comme un hôte plus favorable que la lentille puisque les taux de mortalités des œufs et des larves ainsi que la durée d'une génération ont été supérieurs chez la lentille. De plus, des différences significatives ont été observées pour l'index de sensibilité aux attaques de *C. maculatus* des deux hôtes montrant ainsi que les graines du pois chiche sont modérément sensibles aux attaques tandis que les graines de lentille ne le sont pas. En outre, les résultats ont indiqué que *C. maculatus* induit des réductions significatives de la germination (pois chiche 78%, lentille 33% pour le taux d'infestation le plus élevé 80%) et le poids des graines (pois chiche 45%, lentille 8% après 6 mois de stockage) des deux hôtes et que les niveaux d'infestation et des pertes de poids diffèrent significativement selon la durée du stockage. En somme, cette étude fournit des arguments pour les agriculteurs et les commerçants pour entreprendre des décisions de lutte contre *C. maculatus* durant le stockage. En outre, ce travail révèle la variabilité des niveaux de nuisibilité économique selon les hôtes.

Mots clés: *Callosobruchus maculatus*, lentille, niveau de nuisibilité économique, paramètres reproductifs, performances juvéniles et adultes, pois chiche, traits démographiques

ملخص

حوال-حمدي، سمية وفاتن تيتوحي وآمنة بوصحيح ومحمد زياد ظريف ومعز عمري وجودة مديوني-بن جماعة. 2017. الصفات الديموغرافية والخصائص الإنجابية لمجتمعات حشرة خنفساء اللوبيا *Callosobruchus maculatus* التي تصيب العدس والحمص المخزنيْن. Tunisian Journal of Plant Protection 12: 67-81.

يقدم هذا المقال أول دراسة شاملة على وضعية أضرار خنفساء اللوبيا *Callosobruchus maculatus* على بقوليتين غذائيتين هما الحمص (الصنف عمدون 1) والعدس (الصنف نصير) خلال ستة أشهر من التخزين. تمت دراسة المعطيات المتعلقة بديناميكية مجتمعات الحشرة والصفات الديموغرافية والخصائص الإنجابية وكفاءات الطور اليرقي والطور البالغ ومستوى الضرر الاقتصادي والخسائر (تأثيرها علي الإنبات و فقدان الوزن). أثبتت النتائج أن خنفساء اللوبيا تمثل آفة حشرية مهمة على الحمص المخزن في تونس. بالإضافة إلي ذلك، أشارت النتائج إلى أن الخصائص الإنجابية وكفاءات الطور اليرقي والطور البالغ تتغير بصفة كبيرة حسب العائل. في هذا السياق، أظهرت تحاليل الانحدار الخطي أن العوائل تؤثر بطريقة معنوية علي خصائص الطور البالغ للخنفساء. بينت النتائج أن العائل يساهم بنسبة 77% في وزن الجسم وبنسبة 80% في حجم الجسم. أثبتت بيانات الصفات الديمغرافية أن الحمص يمثل العائل الأكثر ملائمة بالمقارنة مع العدس بما أن نسب وفيات طور البيض وطور اليرقة وطول مدة الجيل كانت الأعلى بالنسبة للعدس. لوحظت اختلافات معنوية بخصوص مؤشر القابلية لهجمات للخنفساء لدى العائليْن بما أن بذور الحمص أظهرت قابلية معتدلة للهجمات بينما بذور العدس لم تكن كذلك. من جهة أخرى، أشارت النتائج أن الخنفساء خفضت بشكل ملحوظ نسبة الإنبات للعائليْن (78% للحمص و33% للعدس بالنسبة إلى أعلى معدل إصابة 80%) ووزن البذور (45% للحمص و8% للعدس بعد ستة أشهر من التخزين) وأن نسبة الإصابة وفقدان الوزن يختلفان بشكل معنوي حسب مدة التخزين. في الخلاصة، وفرت هذه الدراسة الحجج للمزارعين والتجار لاتخاذ القرارات لمكافحة خنفساء اللوبيا خلال التخزين وإضافة إلى ذلك كشف هذا العمل عن الاختلاف في مستوى الضرر الاقتصادي بحسب العائل.

كلمات مفتاحية: حمص،خصائص إنجابية، صفات ديموغرافية،عدس،كفاءات الطور اليرقي والطور البالغ، مستوى الضرر الاقتصادي، *Callosobruchus maculatus*

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Short Communication

Potosia opaca, an Insect Newly Found on Canary Palm (*Phoenix canariensis*) in Tunisia

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ABSTRACT

Ben Jamâa, M.L., Boudhina, S., Dhahri, S., and Hdidi, S. 2017. *Potosia opaca*, an insect newly found on Canary palm (*Phoenix canariensis*) in Tunisia. Tunisian Journal of Plant Protection 12: 83-90.

Ornamental palm tree *Phoenix canariensis* has been introduced in Tunisia at late 1800's and becomes a symbol of the city landscape in different localities. *P. canariensis* was seriously attacked since 2011 by the red palm weevil (RPW), *Rhynchophorus ferrugineus*. Recently a new pest *Potosia opaca* was detected in North of Tunisia on living *P. canariensis* trees solely or associated with RPW. *P.opaca* develops one generation per a year; adult mating takes place in June-July and females lay in humus inside decayed trunks of living palms tree. Larva lives in most accumulations of organic matter inside trunks between fibers and sheaths into close proximity with live wood. The pupa stage lasts on average 50 days. *P.opaca* lives on living trees and seems not to be associated with tree mortality. However it is essential to consider a number of indirect damage as this insect is likely becoming a vector of many pathogens.

Keywords: Biology, *Phoenix canariensis*, *Potosia opaca*, repartition area, Tunisia

Palms (Arecaceae), widely distributed plants in the world, are commonly used for landscaping, decorations and households items (Zhang et al. 2015). There are about 2600 species of palm trees in the world. *Phoenix canariensis* (Canary palm or ornamental palm) is endemic to the Canary Islands and has been widely propagated for centuries and became one of the most commonly grown and appreciated ornamental palms of the world. *P. canariensis* is a massive palm with a single trunk growing typically up to 20 meters, and a thick trunk up to nearly a meter across with a wide, bulbous flaring base.

In the Mediterranean region, *P. canariensis* is extensively cultivated as a street tree or garden plant and is also widespread in all Tunisia (Sosa et al. 1998). In North of Tunisia, it has been introduced at the late 1800's and becomes a symbol of the city landscape such as Bizerte, Menzel Bourguiba, Jendouba, and Tunis.

In Tunisia, *P. canariensis* is well-grown and remains fairly free of damaging insect pests until the end of 2011, when red palm weevil (RPW), *Rhynchophorus ferrugineus* was detect (Chebbi 2011) in City Hall of Carthage

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Township, Tunis, where 30 to 40 of Canary palm trees were dried and the apexes of these declined trees were seriously affected. In 2014, about 254 palms were died (Chihaoui 2014).

The aim of this study was (i) to report a new pest of *P. canariensis* detected for the first time in Tunisia on Canary palm, (ii) to establish its area distribution and (iii) to describe its main biology traits.

This research was conducted on old and young Canary palms, from November 2015 to July 2016 in different localities belonging to the North part of Tunisia (Table 1).

Table 1. Review of investigated localities planted by *Phoenix canariensis* living tree

Region	Locality	Station	Date	Age
Bizerte	Bizerte City	Municipality	30 November 2015	Old palms: more than 100 years
	Menzel Bourguiba	Hall Sport	24 March 2016 2 June 2016 19 July 2016	Young palms
		Hospital	24 March 2016 2 June 2016 19 July 2016	Old palms: more than 100 years
	Utique	Private farmers	24 March 2016	
Tunis	Agronomy complex	INRGREF Park	20 April 2016	Old palms: more than 100 years
		City of Sciences	17 June 2016	
	Tunis	Carthage	April 2016	Young palms

In each locality, we observed the sanitary state of palms, mainly the green palm leaves. Careful observations were done inside stem into the base of the attacked leaves to detect larvae and around the trunk to check fallen larvae and cocoons (Fig. 1).

Fig.1. Investigated Canary palm for insect infestation

Larvae collected in the field (Fig. 2a) were reared to the adult stage in plastic breeding cages (11 cm high, 10.5 cm wide and 10.5 cm length) (Fig. 2b) under laboratory conditions (Temperature 20°C; HR 50%) and were observed daily to note if they are still alive and to check the next stages (pre-nymph, nymph and adult).

Fig. 2. Laboratory rearing of *Potosia opaca*

Identification of the insect species was performed through numerous morphological and anatomical characters analyses using the keys of Mico and Galante (2003) and Tauzin (2007) and the consulting of the reference collection of Dr. Normand of beetles at the National Institute of Agronomy of Tunisia (INAT, University of Carthage).

Diagnosis of larvae and adults from the field and laboratory rearing, and the collection of Dr. Normand showed that this species is the rose chafer *Potosia opaca* (Coleoptera: Cetoniidae).

The larval description is based on third instars larvae collected at Menzel Bourguiba (Bizerte), 24 March 2016. The third instar is light yellow and the head capsule width varied from 3.78 to 4.86 mm, the mean is about 4.4 ± 0.45 mm. However, the adult description was based on eight adults (4 collected in the field, 4 reared from larvae collected in the field). Adult has dorsal surface black. The body size varied from 2.2 to 2.5 cm.

No attack was noted on old trunk palm. However, on young palm trees, inside trunk and at the base of the palm leaves, we found numerous larvae. In INRGREF Park, we found larvae of *P. opaca* associated with larvae, cocoons and adults of *R. ferrugineus* (Fig. 4).

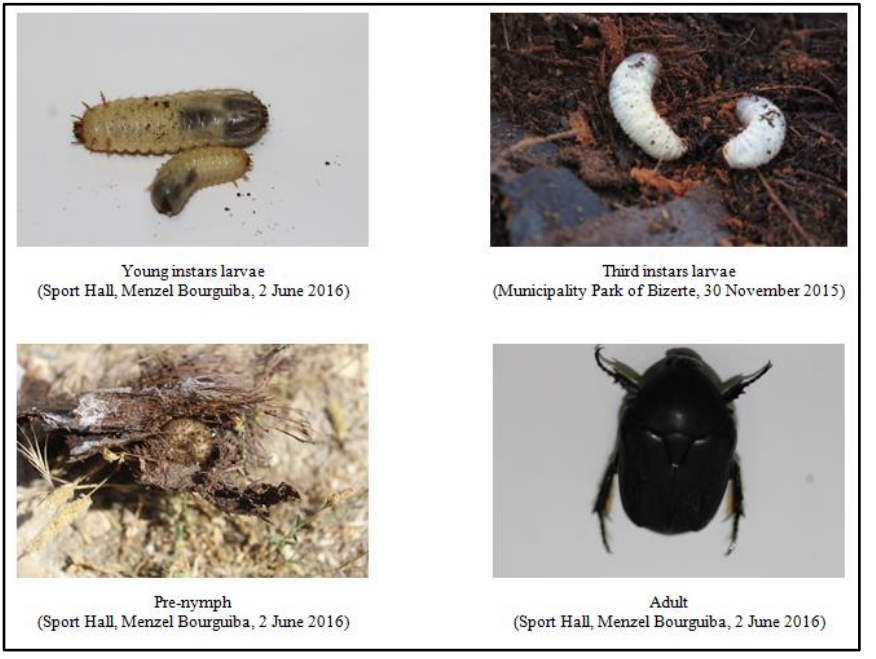

Fig. 3. Developmental stages of *Potosia opaca*.

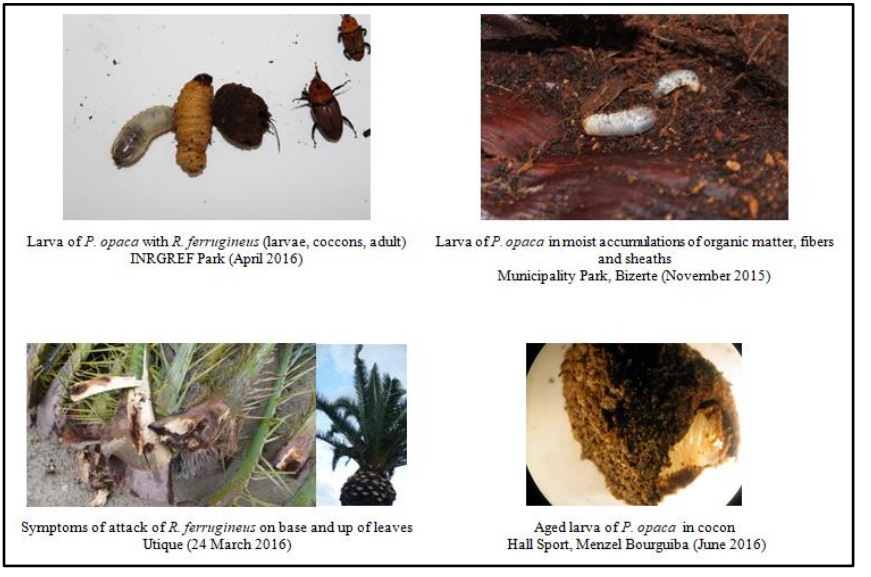

Fig. 4. Damage caused by *Potosia opaca* and *Rhynchophorus ferrugineus* on Canary palm.

In Bizerte region, the rose chafer *P.opaca* was noted in all surveyed localities (Bizerte City, Menzel Bourguiba), but it seems to be absent in Utique. In Tunis region, however, this pest was observed only in Ariana (INRGREF Park, City of Sciences), and absent in Carthage. The Cetoniidae is present solely in Bizerte City and Menzel Bourguiba, but in Utique there is only *R. ferruginus*, and in Tunis *P. opaca* co-exists with *R. ferrugineus.*

We did not observe neither eggs nor first larva in our investigation. But we collected mainly larvae at third instar and adults of *P. opaca* (Table 2).

Table 2. Field collects of *Potosia opaca*

Locality	Date	Larvae			Pre-nymph	Nymph	Adult
		1st instar	2nd instar	3rd instar			
Bizerte City	30 November 2015	0	0	10	0	0	0
Menzel Bourguiba	24 March 2016	0	0	11	0	0	0
	02 June 2016	0	4	13	1	1	1
	19 July 2016	0	0	0	0	0	3
INRGREF Park	20 April 2016	0	2	4	0	0	0
	17 June 2016	0	0	0	1	1	1

Two larvae of the third instar collected in 24 March 2016, turn into pupae in 10 May and 13 May respectively, and the adults emerge in 26 and 29 June 2016, respectively. Each one lasts 48 and 51 days, the mean nymph period is about 50 days under laboratory conditions.

The insects observed on living *P. canariensis* trees were identified as *P. opaca.* The genus *Potosia* is among the most diverse Cetoniidae genera of the Palaearctic region, comprising 29 species (GBIF, Database). The first *Potosia* taxon was described by Fabricius in 1787. The Gyllenhal Cardui taxon was described earlier in 1871. The Cretica taxon was described by Kraatz in 1880. The Paulianiana was described by Bedel in 1889. According to Tauzin (2007), the four species are the same.

This is the first record of a Cetoniidae species on *P. canariensis* trees in Tunisia and North Africa. The *P. opaca* attack *P. canariensis* solely or in association with *R. ferruginus*. In Algeria, three Cetoniidae's species pests were reported on *Phoenix dactilifera*: *Oxytherea funesta*, *O. pantherena* and *Cetonea faveata* (Anonymous 2015).

P. opaca occurs in the occidental Mediterranean basin. It was observed in Algeria, Egypt, France, Greece, Italy, Libya, Morocco (Atlas Mountain, Maamora), Portugal, Spain, Turkey, and Tunisia (Tauzin 2007). In Tunisia, *P. opaca* exists from the North to the South and in all bioclimatic area on different

host species (Tauzin 2007). On living Canary palm, our field investigations showed that the *P. opaca* was recorded in Bizerte City, Menzel Bourguiba, INRGREF Park and City of Sciences, but it seems to be absent in Utique and Carthage. However, it was noted solely in Bizerte City, Menzel Bourguiba, but with *R. ferrugineus*, in INRGREF Park and City of Sciences. This result is similar to that of Mico and Galate (2003) finding, who reported that in Spain *P. opaca* occurs in living *P. dactilifera* trees. *P. opaca* occurs also in decaying wood of *Ficus carica*, *Ceratonia siliqua* and *P. dactilifera*, where it consumes the wood and promote more rapid decay (Mico and Galante 2003).

In March 2016, the Bizerte Agricultural Authority indicates that they detected 55 Canary palms attacked by the CPR where 22 palms were cut; meanwhile, 1400 palms were treated preventively. However, our observations showed that *R. ferrugineus* is absent in Bizerte City and Menzel Bourguiba where *P. opaca* exists solely.

Third instars larvae were collected around the trunk of the old Canary palm. However, on young palm trees they were found inside the trunks between fibers and sheaths into close proximity with live wood. This medium is often full of soil and organic matter; these moist accumulations of organic matter are optimal for the development of *P. opaca* larvae (Mico and Galante 2003). Palms differ greatly from broadleaf (Dicotyledonous) and coniferous (Gymnosperm) trees in their overall form and external structure (morphology) and in their internal structure (anatomy) (Broschat 2013). The palm tree has no trunk but a stem full of marrow or fibers. In general, palm fibber interweaves into network thus forming palm barks and covering palm trunks, layer upon layer (Zhang et al. 2015). In *Phoenix*, the dorsal portion of a sheath is thickened whereas the remainder is fibrous, and the woody dorsal portion may persist as a stub on the trunk after a leaf in shed (Tomlinson, 1990).

The development cycle of *P. opaca* (from egg laying to adult flying) lasts from 13 to 25 months (Dutt 2006). According to Dutto (2006), adult mating takes place in Jun-July and females lay in humus under old trees or inside decayed trunks. Embryonic development lasts 15-20 days (Dutto 2006). Larvae enter into close proximity with live wood and develop in moist organic substances such as piles of decomposing vegetable matter (Mico and Galante 2003). In the field, *P. opaca* overwinter as a mature larva of the third instar (Dutto 2006; Mico and Galante 2003). First and second instars larvae last on average 35-40 days each one, while the third age is the longest (from a few to more than 12 months). Pupal stage lasts about 30-45 days (Dutto 2006). However, our results show that under laboratory conditions, the pupal stage lasts about 50 days.

A few Cetoniidae are of minor economic importance: adults damage flowers, fruits and other plant parts above the ground, larvae damage roots, tubers, etc. (Krikken 1984). *P. opaca* lives on living trees and seems not to be directly associated with tree mortality. However, according to Dutto (2006), the analysis of the damage caused by this Cetoniidae must never be limited to damages that derive directly from the trophic, but it is essential to consider the indirect damage as well because this insect is likely becoming a vector of many pathogens.

RESUME

Ben Jamâa M.L., Boudhina S., Dhahri S. et Hdidi, S. 2017. *Potosia opaca*, un insecte nouvellement trouvé sur le palmier des Canaries (*Phoenix canariensis*) en Tunisie. Tunisian Journal of Plant Protection 12: 83-90.

Phoenix canariensis (palmier des Canaries ou d'ornement) a été introduit en Tunisie à la fin des années 1800 et devient un symbole du paysage urbain de différentes régions. *P. canariensis* a été sérieusement attaqué depuis 2011 par le Charançon Rouge des Palmiers (CRP), *Rhynchophorus ferrugineus*. Récemment, un nouveau ravageur *Potosia opaca* a été détecté au Nord de la Tunisie sur *P. canariensis* vivant seul ou associé au CRP. *P. opaca* développe une seule génération par an; l'accouplement des adultes a lieu en juin-juillet et les femelles pondent leurs œufs dans l'humus des palmiers sous les palmes. Les larves vivent dans la matière organique décomposée des stipes des palmiers vivants. Le stade nymphal dure environ 50 jours. *P. opaca* vit dans les palmiers vivants et semble ne pas être associé à la mort de ces palmiers, mais il est essentiel de considérer les effets indirects induits car cet insecte peut agir comme vecteurs de plusieurs agents pathogènes.

Mots clés: Biologie, *Phoenix canariensis*, *Potosia opaca*, répartition géographique, Tunisie

ملخص

بن جامع، محمد الحبيب وسارة بوذينة وسمير ظاهري وسفيان حديدي. 2017. السيتونيا *Potosia opaca* حشرة رصدت حديثا على نخيل الزينة الكناري (*Phoenix canariensis*) في تونس.

Tunisian Journal of Plant Protection 12: 83-90.

تم إدخال نخيل الزينة الكناري (*Phoenix canariensis*) إلى تونس في أواخر سنوات 1800 حيث أصبح جزءا من المناظر الطبيعة الحضرية، ولكنه تعرض إلى أضرار كبيرة منذ عام 2011 من قبل سوسة النخيل الحمراء (*Rhynchophorus ferrugineus*). خلال زياراتنا الميدانية الأخيرة، تم العثور عن آفة جديدة هي السيتونيا *Potosia opaca* في شمال تونس على أشجار نخيل الكناري الحية، بمفردها أو مع سوسة النخيل الحمراء. تتميز هذه الحشرة بنمو جيل واحد سنويا، يتم التزاوج خلال شهري جوان/يونيو وجويلية/يوليو يتبعه إخصاب الإناث اللواتي يضعن بيضهن في النخيل على الدبال تحت الجريد. تعيش اليرقات في المواد العضوية المتحللة حيث يستمر طور العذراء حوالي 50 يوما. يعيش *P. opaca* في أشجار النخيل الحية ولا يبدو أنه المتسبب في موتها ولكن لا بد من أخذ بعين الاعتبار أضراره الغير المباشرة لأنه ناقل لعديد العنا صر الممرضة.

كلمات مفتاحية: بيولوجيا، توزيع جغرافي، تونس، *Potosia opaca*، *Phoenix canariensis*

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Three Methods to Assess Levels of Farmers' Exposure to Pesticides in the Urban and Peri-urban Areas of Northern Benin

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ABSTRACT

Lawson, A.J., Akohou, H., Lorge, S., and Schiffers, B. 2017. Three methods to assess levels of farmers' exposure to pesticides in the urban and peri-urban areas of Northern Benin. 2017. Tunisian Journal of Plant protection 12: 91-108.

Small farmers in urban and peri-urban areas of Northern Benin use pesticides without respect of hygiene rules and any personal protective equipment (PPE). Based on observation of the local practices in Djougou, Gogounou and Parakou,field trials have been carried out under similar conditions to evaluate contamination and exposure levels of farmers, using three usual sampling methods (Visual Method, Patch Method and Whole Body Method). Both Visual and Patch Methods used dye and ghost ink as tracers. In the Whole Body trials, deltamethrin (PLAN D 25 EC) was used as insecticide treatment. Deposits were observed on the protective equipment and on the collectors. Tartrazine was determined by colorimetry and deltamethrin by gas chromatography with ECD detector (GC-ECD). The examination of protective equipment (Visual Method) showed that the whole body could be potentially exposed to pesticides. Hands were contaminated during the preparation and the loading of mixture up to sprayer rinsing. The Patch Method was not perfectly able to predict the contamination pattern on the farmers' body. The Whole Body Method results appeared to be more variable and influenced by the skill of each operator compared to the Patch Method. The contamination levels observed were rather higher than the value estimated with a theoretical model (from 368 to 2867 mg of deltamethrin at the total/body). With PPE, the average exposure reached 3.25 mg/kg bw/day. Without PPE, the potential exposure was equal to 32.52 mg/kg bw/day. Both values far exceed the AOEL of deltamethrin (0.0075 mg/kg bw/day) indicating a high risk level for the operator. The theoretical used model (UK-POEM) was unable to predict the potential exposure outcomes measured in these trials.

Keywords: Backpack sprayers, exposure assessment, pesticides, small scale growers

In Benin, horticulture is today one of the main components of urban and suburban agriculture. It represents an important source of incomes for thousands of small producers, mainly aged between 21 and 40 years old (Adorgloh 2006; Allagbé et al. 2014). On the tomato strategic speculation for urban producers, there are no fewer than 37 pests (Chougourou et al. 2012). Face to this threat, farmers use intensively broad spectrum insecticides. Pyrethroids (54% of applications) are often associated with organophosphorus insecticides (25% of applications) (Ahouangninou et al.

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2011; Azandémè-Hounmalon et al. 2014). These chemicals can significantly improve yields, as demonstrated by Cissé et al. (2003) in Senegal, but lack of information on routes of exposure, pesticide toxicity, small resources and intensive use of pesticides lead to a significant contamination of the environment (soil and water) with the destruction of beneficial insects (Deguine and Ferron 2006) in addition to the high exposure of small producer.

During treatment, dermal and inhalation exposure are the main routes of exposure (EFSA 2010; Fenske and Elkner 1990; Kim et al. 2013). Farmers' exposure to pesticides while using backpack sprayers mainly occurs through the dermal route (Machera 2003). The risk of detrimental health effects should be significant for small producers in Northern Benin due to frequent treatments with very toxic and highly concentrated pesticides registered to control resistant cotton pests (Assogba-Komlan et al. 2007).

Moreover, it is known that pesticides are often handled and applied by many operators without hygiene rules or proper personal protective equipment (PPE) (Godeaux et al. 2008; Stimamiglio et al. 1998). Nevertheless, few studies have been dedicated to estimate the potential exposition of the small scale growers when handling insecticides and spraying in situ, with their usual practices and their own backpack sprayers. To assess the risk of exposure in field conditions, a study was undertaken in the outskirts of major cities of Northern Benin (Djougou, Gogounou, and Parakou) in order to identify the most exposed body parts and to characterize the potential levels of exposure.

Exposure to pesticides during field applications can be estimated by measuring the contamination of the skin (Syamimi et al. 2011). To evaluate the distribution of mixture droplets on the body, three methods are currently used (Salyani and Whitney 1988; Tannahill et al. 1996), known as Visual sampling Method, Patch sampling Method and Whole Body sampling Method.

The "Visual sampling Method" consists to mix a dye, an ink or a fluorescent product with water in the spray tank to form a mixture to have after spraying a global view of the distribution pattern of deposits on the whole body (results are only qualitative but indicative). Tartrazine was used by many authors (Koch et al. 2006; Pergher and Lacovig 2005) and the ghost ink by Ncamurwanko (2012).

The "Patch sampling Method" was described in the "*Guidance Document for the Conduct of Studies of Occupational Exposure to Pesticides During Agricultural Application"* (OECD 1997). Collectors (patches) are placed on various body parts to collect during spraying the droplets of mixture with a dye dispersed to the water tank. At the end of work, deposits on the collectors are measured, reported and extrapolated to the surface of the exposed body part. This may be done using standard surface area of body parts such as those proposed by WHO (in 1982), by EPA (in 1987) or, more recently, by OECD (in 1997).

The "Whole Body sampling Method" (Chester 1993 1995; Gonzalez et al. 1999; WHO 1982) consists to dress an operator with a coverall that covers completely the body to serve as a global collector (imitating the "skin") and cutting it in several pieces after spraying to extract and analyze the pesticide deposits (Garrido Frenich et al. 2002; Syamimi et al. 2011).

Those methods, where tracers are used as substitution elements to pesticides, are simple, cheap and easy to

implement without risk for operators during the trials. Therefore, many authors used tracers to evaluate the drift (Stainier 2006) or the potential contamination (Gil et al. 2005; Gil 2007; Kadri et al. 2012; Koch et al. 2003; Yates et al. 1976). However, the results obtained with this method can always be discussed, since the mixture of a tracer does not have the same physicochemical properties (density, viscosity and surface tension) than a mixture with a pesticide formulation and usually the tests with collectors are not performed in real field conditions and by farmers.

Therefore, it was interesting to compare results obtained with the Visual and Patch Methods (with mixtures containing tracers and tests performed by the research team) and results of the Whole Body Method (with a mixture of water and a plant protection product, and tests performed by farmers themselves) in order to check if Visual and Patch Methods could really be reliable to assess the distribution of pesticides on the body under field conditions. The conclusions of this study could help researchers who want to assess the performance of the Visual and Patch Methods compared to the Whole Body Method and, on the other hand, should enable risk managers to take certain measures and to issue appropriate recommendations of personal protection tailored to local economic context.

Furthermore, when measuring insecticide deposits on the body, it should be possible to estimate if the risk level could be considered as acceptable for the small producers according to their usual practices. The risk will be considered as acceptable if the potential exposure (measured on the patches or obtained using an exposure model such as UK-POEM) is lower than the AOEL value (*Acceptable Operator Exposure Level*, expressed in mg as/kg bw/day) (EFSA 2014).

MATERIALS AND METHODS

Study sites.

A careful observation of local growers practices and field trials have been carried out on the outskirts of three large communities of Northern Benin (Djougou, Gogounou, and Parakou). The sites on which the field trials were conducted have similar characteristics: a high population growth (from 3.45% for Djougouup to 4.81% for Parakou) (INSAE 2013), a sustained demand for vegetables and, consequently, a permanent increase of urban and suburban production areas with an intensive use of pesticides. These sites are cultivated since the 80's by growers originating from Cotonou (Allagbé et al. 2014) on very small surfaces with vegetables (tomato is the main crop) grown in the dry and rainy seasons (Adékambi and Adégbola 2008). In Djougou area, the presence of several rivers and valleys are favorable to horticulture which is a very ancient activity with different cropping systems (Simeni et al. 2009). This city is an important crossroads and its frontier markets with Togo constituting an easy outlet for the main local productions. In Parakou and Gogounou, vegetable production areas are concentrated around water points (wells and streams, finished or unfinished). Enforcement practices selected for field trials have been based on observations made in these three sites which were representative of how farmers usually work in Benin when they apply plant protection products on their crops with backpack sprayers.

Methods used to estimate the contamination of the operator's body.

Three different sampling techniques have been implemented successively in this study to assess the exposure of operators' body: Visual, Patch and Whole Body Methods. According to literature, each method alone cannot be sufficient for a reliable evaluation of operators' exposure but their combination should provide a rather good representation of the contamination during application in Benin.

All sprayings were performed with backpack sprayers (flat fan nozzle, about 700 l/ha) by local right-handed voluntary operators or farmers with a walking direction perpendicular to the dominant wind. Farmers were asked to work according to their usual practices, as previously observed. During the field trials, all of them have worn white coveralls (TYVEK type or cotton type for the whole body trials) with a hood, boots, gloves resistant to chemicals and a filter mask. All tests were conducted on 800 m² plots.

An average temperature of 33.5°C and relative humidity of 61.4% were recorded during the field trials using a thermo-hygrometer (TFA, Kat. N°30. 5007). The average wind speed measured during the tests with an anemometer (HMI CFM/CMM SI 6190) was 2.3 m/sec (1.7-3.1 m/sec).

- *The Visual Method*

A dye or a ghost ink was added separately to water tank to obtain spraying mixtures. Tartrazine (E102 code, an azo compound yellow) is a non-toxic food grade coloring (Acros Organics, 89% purity). Added to water (10 g/l), it allows a good visualization of the cover on a white combination (Murray et al. 2000). The ghost ink (110 NORIS UV) is a non-toxic liquid detectable and visible under UV light frequently used for demonstrations during training (Shiffers and Mar 2011). After mixture applications, operators' body were put under visible light (tartrazine) or black light (UV lamp for ghost ink) to take pictures of their coverall and hands, and to view the contaminated parts and equipment (e.g. gloves and boots). Various tests were performed both without vegetables and in chili fields at different working heights (Loquet et al. 2008).

- *The Patch Method*

The number and the distribution of patches were adapted from the WHO Standard Protocol (1982) and OECD guidelines (1997) as described by Kadri et al. (2012). In this study, the tartrazine dye (Acros Organics, 89% purity) was preferred due to its non-toxicity, friendly to use, both easy to extract from collectors and to measure by colorimetry with good sensitivity and linearity of absorbance values. Tartrazine was mixed to the water tank (10 g/l). Two trials were carried out by operators belonging to the research team using a backpack sprayer, at two different heights (0.5 and 1 m). The patches (or collectors) were square pieces of 100 cm² in unbleached cotton spread all over the farmers' body and firmly attached to the coverall (TYVEK type protection suit) to collect the droplets of mixture. After spraying, all collectors were removed from the coverall, transferred to a FALCON® tube to which is added 30 ml of distilled water for extraction and the absorbance was immediately measured with a Macherey-Nagel colorimeter (Nanocolor 500D) at λ = 436 nm. Concentration in the extract was then determined according to a calibration curve ($y = 0.0544\ x$; $r^2 = 0.9994$) previously established with 8 concentrations of dye (from 0.17 to 21.80 µg/ml, to reach a maximum absorbance of about 1 unit). The

absorbance of blancos (white cotton pieces in 30 ml of distilled water) was previously measured and considered negligible. Results were reported in mg/cm² and extrapolated to the body part on which the patch was fixed using the table giving the average area of each part of the body as proposed by the OECD guidelines (results are therefore semi-quantitative).

- *The Whole Body Method*

For this trial, applications of a mixture (insecticide PLAN 25 EC dispersed in water) were performed by three voluntary farmers wearing new unbleached cotton coverall. The duration of work was fixed at fifty minutes after what the coveralls were collected and left to dry in the shade as recommended by Machera et al. (2003). To limit the number of analyses, the coveralls were cut into 5 big pieces according to Fig. 1 and adapted from Garrido Frenich et al. (2002) for analysis: sleeves (shoulder-arm) left and right, thorax (chest and back), legs (thigh and tibia) left and right. The deposits and distribution of PLAN 25 EC (deltamethrin) on the entire body was extracted and determined by gas chromatography. None interference was detected in extracts of blancos (unbleached cotton pieces).

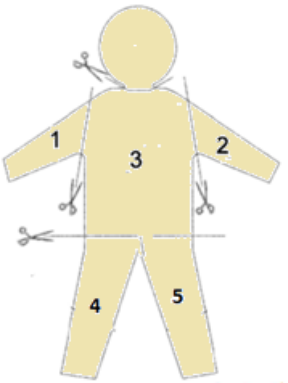

Fig. 1. Cutting of the combination into 5 parts, adapted from Garrido Frenich et al. (2002) (1: right sleeve; 2: left sleeve; 3: thorax (chest and back); 4: right leg; 5: left leg).

Insecticide choice.

PLAN 25 EC is an emulsifiable concentrate packaged in bottles of 250 ml containing 25 g deltamethrin/l, and the best seller insecticide in Benin. Deltamethrin is a contact insecticide. It is a pyrethroidtoxic chemical (oral LD_{50}: 87 mg/kg bw; dermal LD_{50} > 2000 mg/kg bw; ADI 0.01 mg/kg bw/day). The value of the systemic AOEL has been set to 0.0075 mg/kg bw/day (EU Pesticides Database, 2016). Moreover, deltamethrin was also considered particularly suitable for testing because it is very stable (air, light and temperature), does not adsorb textiles irreversibly (it is used for mosquito netting), and is soluble in many organic solvents. Deltamethrin, both

easily adsorbed on patches and extracted, is therefore very suitable for this test. Finally, it can be dosed even at low concentrations by gas chromatography (GC). The analytical method was previously validated internally to be used in routine tests (LOQ $\leq$ 0.01 mg/kg).

Extraction and dosage of deltamethrin extracted from the coverall.

Having determined the mass per cm² (either 13.14 mg/cm²) and the weight of each part of the coverall, their surface were determined accurately. After weighing, they were torn into small pieces using scissors and about 15 g of cut tissue (or 1141.55 cm²) are taken at random and transferred into an Erlenmeyer flask to which 200 ml of dichloromethane (stabilized with approximately 50 mg 2-methyl2-butene/l) were added for extraction for 24 hours. After stirring, the solution is transferred into a ground ball by filtering it on pleated paper. The Erlenmeyer flask is rinsed again with another 100 ml of dichloromethane. Solutions were stored in a refrigerator at 4°C before analysis. The filtrates were dried-out using a rotary evaporator and finally residues were dissolved in 2 ml of trimethyl (or higher volume when dilution is needed). The analysis of deltamethrin was performed by gas chromatography (GC) with an Electron Capture Detector (ECD, constant current), in splitless (at 280°C), by injecting 1 µl on an Optima column (5-30 m × 0.25 mm - 0.25 µm) with a program of temperature (1 min at 90°C; 10°C/min to 320°C for 5 min) and the ECD detector (Ar/CH_4, 1.7 kg/cm², pulse amplitude = 50 V, pulse delay = 1 µs, pulse width = 0.1 µs and reference current = 0.5 nA) at 290°C. Deltamethrin concentration was determined according to a calibration curve established from 0.1 to 2 µg/ml ($y = 2.4203 + 1.3968\ x$; $r^2 = 0.9831$).

Risk assessment using an exposure model.

Potential exposure values can be predicted using various theoretical models (EFSA 2014). The English model UK-POEM (*Predictive Operator Exposure Model*) was selected because this model helps to simply calculate potential exposure for operators using backpack sprayers (hand-held sprayers). This is the only POEM where hand-held sprayers are considered. As the working scenarios can differ from Europe, results will be analyzed with caution and will be only indicative. The model is an Excel® spreadsheet, in which some parameters are introduced and others are set by default (e.g. surface treated, work duration, skin absorption), providing the potential exposure value of an operator (mg as/kg bw/day). Based on this theoretical model, potential exposures were calculated for various working conditions (e.g. with or without protective equipment) and compared not only to the AOEL value of deltamethrin but also to the results of analysis of the coverall.

RESULTS

Distribution of the deposits observed with the Visual Method.

Examination of the gloves and the coverall under visible or UV light allowed visualization of the location of tartrazine deposits or ghost ink spots at the end of the tests. Observations showed that different body parts have been exposed to pesticides, but to varying degrees. As expected, the hands of operators (inside and outside the gloves) have been contaminated (Photo 1) during opening the package and rinsing of the sprayer. The back which supports the sprayer (and the thorax in general) was not spared from contamination, and deposits' spots were visible on 50% of the back surface. Nevertheless, observations indicated that

thelower legs have been heavily exposed during spraying (Photos 2 and 3). The contamination appeared clearly more distributed on the bottom, below the knees. In tests carried out in chili fields where plant height was greater than 1 m, tartrazine or ghost ink were distributed up to the thighs (Photo 3) or almost to the middle of the body.

Photo 1. Operator contaminated hands (ghost ink).

Photo 2. Contaminated lower limbs (ghost ink).

Photo 3. Coloration of the coverall (legs covered by tartrazine).

Distribution of the deposits observed with the Patch Method.

Tables 1 and 2 show the results for the average quantities of tartrazine measured on 11 collectors in two trials with a backpack sprayer, for two heights (0.5 m and 1 m). Theoretical distribution was obtained by extrapolating the deposits/cm² to the body surface using the OECD table (1997). Total quantities collected and distributions on the body were remarkably close between the two repetitions, indicating a good reproducibility of this method. All parts of the body were contaminated (even head and face), but the greater part of the contamination was located on the legs (thighs and tibias). Chest and back (thorax) did not appear heavily contaminated in results obtained with the Patch Method.

Table 1. Quantities of tartrazine measured on the collectors and distribution of deposits on various body parts when applying the mixture with a backpack sprayer at 0.5 m height (conventional surfaces of the body parts are given in the OECD table)

Body part (collector)	Test 1 (µg/cm²)	Test 2 (µg/cm²)	Average (µg/cm²)	Surface (cm²)	Average deposit (µg)	Total distribution (%)
Head and face	0.188	0.193	0.190	1300	247.33 ± 5.07	1.32 ± 0.03
Neck	0.590	0.595	0.593	260	154.05 ± 0.92	0.82 ± 0.00
Shoulder-right arm	0.369	0.398	0.384	2910	1115.99 ± 59.67	5.94 ± 0.32
Right forearm	0.188	0.176	0.182	1210	220.20 ± 9.44	1.17 ± 0.05
Shoulder-left arm	0.535	0.535	0.535	2910	1556.85 ± 0.00	8.29 ± 0.00
Left forearm	0.210	0.210	0.210	1210	253.57 ± 0.00	1.35 ± 0.00
Thorax	0.320	0.320	0.320	3550	1135.48 ± 0.00	6.04 ± 0.00
Right thigh	1.307	1.307	1.307	3820	4992.68 ± 0.00	26.58 ± 0.00
Right tibia	1.903	1.903	1.903	2380	4528.13 ± 0.00	24.10 ± 0.00
Left thigh	0.585	0.585	0.585	3820	2233.01 ± 0.00	11.89 ± 0.00
Left tibia	0.982	0.993	0.987	2380	2349.38 ± 18.56	12.51 ± 0.10
Total	-	-	7.194	-	18786.66	100.00

Table 2. Quantities of tartrazine measured on the collectors and distribution of deposits on various body parts when applying the mixture with a backpack sprayer at 1 m height (conventional surfaces of the body parts are given in the OECD table)

Body part (collector)	Test 1 (µg/cm²)	Test 2 (µg/cm²)	Average (µg/cm²)	Surface (cm²)	Average deposit (µg)	Total distribution (%)
Head and face	0.392	0.408	0.400	1300	520.00 ± 14.71	3.16 ± 0.09
Neck	0.866	0.850	0.858	260	223.08 ± 2.94	1.36 ± 0.02
Shoulder-right arm	0.755	0.755	0.755	2910	2197.05 ± 0.00	13.36 ± 0.00
Right forearm	0.358	0.689	0.524	1210	633.44 ± 283.20	3.85 ± 1.72
Shoulder-left arm	0.397	0.667	0.532	2910	1548.12 ± 555.57	9.41 ± 3.38
Left forearm	0.369	0.369	0.369	1210	446.49 ± 0.00	2.71 ± 0.00
Thorax	0.425	0.424	0.425	3550	1506.98 ± 2.51	9.16 ± 0.02
Right thigh	1.048	1.408	1.228	3820	4690.96 ± 972.41	28.52 ± 5.91
Right tibia	0.540	0.540	0.540	2380	1285.20 ± 0.00	7.81 ± 0.00
Left thigh	0.474	0.474	0.474	3820	1810.68 ± 0.00	11.01 ± 0.00
Left tibia	0.667	0.667	0.667	2380	1587.46 ± 0.00	9.65 ± 0.00
Total	-	-	6.771	-	16449.45	100.00

Fig. 2 compares the distribution of the mixture on the body when the treatment is performed at low height (0.5 m, as at the beginning of the crop) or at medium height (1 m when the plants have grown). It was observed that a larger portion of the mixture had contaminated the upper body when the working height increases, especially on the right side of the hose (all tests performed by right-handed operators).

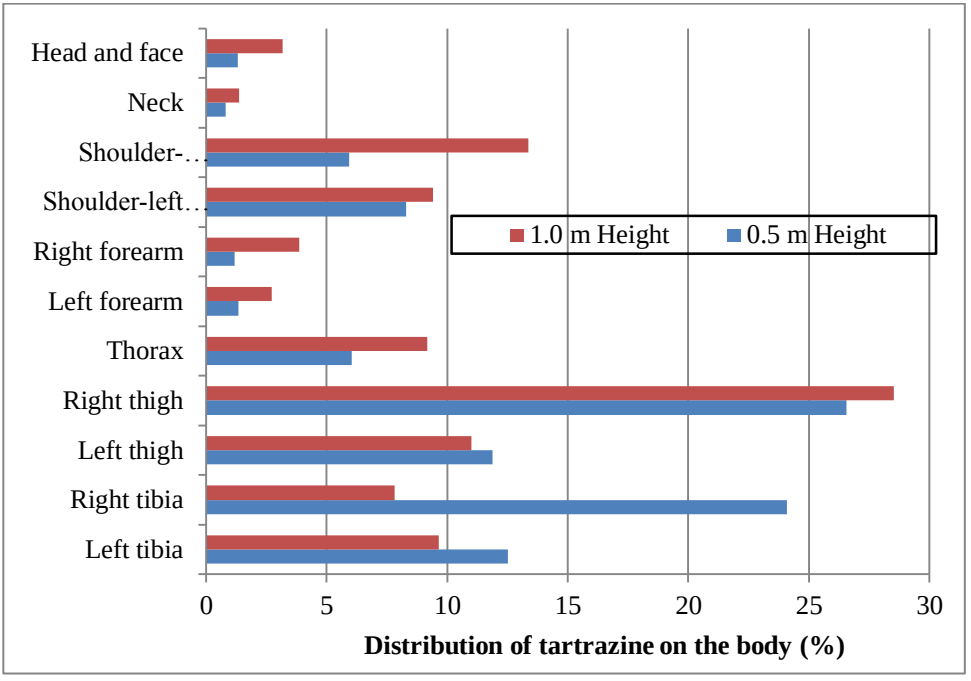

Fig. 2. Distribution of tartrazine on various parts of the operator's body for two heights (0.5 and 1 m) with a backpack sprayer in controlled conditions.

Distribution of the deposits observed with the Whole Body Method.

Table 3 outlines the results relating to the quantities of deltamethrin measured

Fig. 3 compares the distribution of deltamethrin on 5 parts of the body according to three different farmers working in their field, with their own practices and equipment.

on each side of the coverall cut in 5 pieces. Tests were performed by three farmers in their fields and with their own practices and equipment.

From Table 3 and Fig. 3, it appeared clearly that thorax (chest and back) and legs were the most contaminated parts of the body during spraying in field conditions.

Table 3. Deltamethrin distribution on each part of the coverall of four operators (values of deposits/cm² for a sample substantially equal to 15 g, surface of each cut piece and total amount of deltamethrin on each part)

Operator and combination	Cut portion of the coverall	Surface of each piece (cm²)	Deltamethrin (µg/cm²)	Deltamethrin on each part (mg)	Distribution on each body parts (%)
Farmer No. 1	Shoulder-right arm	2058	2.690	5.54	0.2
	Shoulder-left arm	1904	1.200	2.28	0.1
	Right leg	8105	125.540	1017.50	35.4
	Left leg	7846	107.650	844.65	29.5
	Thorax (chest & back)	9810	101.630	996.96	34.8
	Total quantity			2866.94	100%
Farmer No. 2	Shoulder-right arm	1729	0.590	1.02	0.3
	Shoulder-left arm	1684	1.650	2.78	0.7
	Right leg	7511	8.070	60.62	16.5
	Left leg	7367	6.5600	48.33	13.1
	Thorax (chest & back)	12580	20.320	255.62	69.4
	Total quantity			368.37	100%
Farmer No. 3	Shoulder-right arm	1689	28.350	47.90	1.8
	Shoulder-left arm	1825	5.450	9.95	0.4
	Right leg	7755	70.310	545.25	20.8
	Left leg	7953	49.160	390.96	14.9
	Thorax (chest & back)	13280	122.380	1625.21	62.0
	Total quantity			2619.27	100%

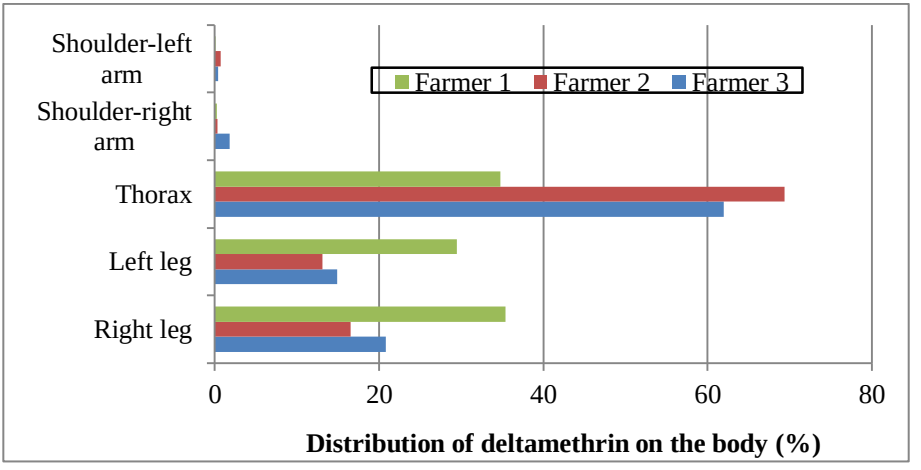

Fig. 3. Deltamethrin distribution on the various body parts of three farmers after spraying with a backpack sprayer in field conditions.

Estimate of the exposure level of operators according to the UK POEM model.

The UK-POEM model was selected for the insecticide PLAN 25 EC. The parameters entered in the calculation model are listed in Table 4.

The potential exposure value provided by the UK POEM model in the absence of personal protective equipment is 0.0240 mg/kg bw/day (> AOEL deltamethrin: 0.0075 mg/kg bw/day) which is not an acceptable level of risk. Wearing all protective equipment the exposure value is reduced by about 90% (Lloyd 1986; Methner and Fenske 1994; Soutar et al. 2000) and then equal to 0.0024 mg/kg bw/day (<AOEL), indicating the theoretical absence of risk when an operator is working in these conditions. Nevertheless, this model cannot be considered reliable enough to assess the potential exposure of the applicator to pesticide. This model may be more appropriate for Europe.

Table 4. Parameters used in the theoretical model UK POEM to estimate the potential exposure of operators (treated surface, working time and exposure reduction of 90% with PPE are default set parameters)

Application equipment	Backpack, hand-held sprayer
Formulation	EC (liquid, emulsifiable concentrate)
Concentration of active substance (deltamethrin)	25 g/l
Type and volume of packaging	Packaging(1 liter unspecified opening)
Dosage (l/ha)	1 liter, EC formulation
Applied volume (l/ha), rounded	260 (volume average: 20.7 l on 800 m²)
Treated surface (ha)	1 ha (default value)
Duration of work (mixture, loading, spraying, rinsing)	6 hours (default value)
Operator's weight (kg)	60 kg (conventional WHO body weight)

DISCUSSION

The evaluation of the occupational exposure of farmers to pesticides is an integral part of the risk assessment for product safety and regulatory purposes. Several methods have been developed to assess exposure to pesticides and comprehensive reviews are available (Chester 1993; Davis 1980; Durham and Wolfe 1962; Van Hemmen and Brouwer 1995). In developing countries, sampling methods for assessment of exposure must be inexpensive and easy to use (Blanco et al. 2008). Qualitative and semi-quantitative methods such as Visual observations, Patch and Whole Body Methods are examples of such simple methods allowing an overall distribution on different body parts.

All of the distribution results obtained in these tests, whatever the method, are consistent with previous works and the results are roughly in agreement with those obtained by other authors using the same methods. Garrido Frenich et al. (2002) reported that the lower limbs were particularly exposed. Fenske (1990) showed that with a backpack sprayer, legs (right and left) were more contaminated with pesticides

than other parts of the body. Syamimi et al. (2011) showed that during phytosanitary treatments in rice fields, the most exposed body areas are the lower parts of both legs. For Kim et al. (2013), the most contaminated body areas are the legs but also the chest when operators work in apple orchards (processing height).

The Visual Method using a dye or a ghost ink can provide a first rough indication but this sampling method tends to show contamination of body parts in reality poorly exposed. Results obtained were only qualitative and it was not possible to make a link between intensity of the coloration or spots and the quantity on the coverall (leading to overestimation of exposure). Observations of protective equipment (including under UV light) allowed an overall view of the distribution of the mixture on the operators' body. In the trials, visualization of deposits indicated that the hands have been heavily contaminated during the preparation and loading of the mixture until the rinsing of the sprayer. The legs, but also the back which supports the sprayer, appeared to be heavily contaminated compared to other parts. These observations are in accordance with those made by Ncamurwanko (2012), but this method should be limited to training demonstrations and cannot be considered as reliable to assess the distribution of pesticide on the body. The Visual Method should therefore be kept for educational demonstrations to risk-based awareness to operators. It can help them to understand that wearing safety equipment is crucial for their health.

Tests done with the Patch Method allowed quantitative observations of the distribution as tartrazine deposits were measured by colorimetry. This method appeared to be friendly and simple to use, inexpensive and had given very reliable measurements between repetitions (good reproducibility), despite extrapolations after deposits measurements. This method has been recommended by OECD (1997) to assess the distribution of pesticide on the body. However, it should be remembered that the Patch Method only estimates the amount of pesticide on the outer suit. This approach assumes uniform distribution of exposure over each body region in order to directly compare inner and outer patches. However, direct deposition through openings in the clothing will result in non-uniform exposure, as will splashes. An overestimation of the amount on the outer suit would lead to lower penetration factors and an underestimation would result in higher penetration factors (Soutar et al. 2000). The results of the trials have indicated a greater distribution of the mixture in the lower limbs (thighs and tibias) compared with other regions of the body. However, if the upper extremities (shoulder, arm and forearm) were far less contaminated than the legs, it appeared in the study that chest, neck and head could also be contaminated, even if they only received a small amount of the mixture. Contamination of various body parts, observable through the patch method, could be explained by the turbulence generated during application by the jet pressure and the forward movement of the operator in line. It was also observed that the legs are even more contaminated than the processing height is low (66.5% of deposits on the legs to 0.5 m against 41.4% at 1 m). Furthermore, the results show that the right leg is more contaminated than the left (the operators are right-handed). These results are in agreement with those obtained by Kadri et al. (2012). Moreover, when using a backpack device, the working height influences in part the

general distribution of the mixture but also the level of contamination. These results corroborate and complement those obtained by previous authors (Hughes et al. 2006; Kadri et al. 2012; Kim et al. 2013; Ndao 2008).

In a study which compared the Patch Method with the Whole Body Method, Tannahill et al. (1996) concluded that the Patch Method was an acceptable method for estimating potential dermal exposure, but because the number of patches is rather limited and their spread cannot be able to represent the whole surface of the body, results were not reliable and not able to predict perfectly the distribution pattern of the pesticide during application. Therefore where a more accurate measurement is required, then a change of approach may be necessary. The Patch Method could be better used to compare various working situations (e.g. wind direction or speed, applied volume, height of plants, etc.) or the influence of the equipment used on contamination (sprayer or nozzle types).

Tests done with the Whole Body Method have produced a different pattern of pesticide distribution compared to the Patch Method. The thorax (chest and back) of the farmers' bodies appeared to be heavily contaminated, in accordance with Hughes et al. (2008) and Kim et al. (2013) findings, indicating that the pesticide could be dispersed directly on the entire body and not only on its bottom even if the legs were also heavily exposed during the operations. Therefore, although the Patch Method is simpler and less costly, the use of the Whole Body Method has been previously recommended (Machera et al. 1998). Results obtained from the Whole Body Method were more variable and influenced by the technique of each farmer, both to the observed contamination levels (from 368.37 to 2867 mg at the total/body) as well as on the distribution on the body (from 35% up to about 70% of the total amount on the thorax). Surprisingly, the arms and shoulders have received very little amount of the insecticide (about 1% of deposits). This observation is interesting because it demonstrates that it is not necessary to spend a lot of money - often limited - to equip operators with cartridges masks rather than to provide them with boots, coverall and waterproof pants. The thorax being very exposed (but without being able to distinguish the back or the torso), it is also necessary to cover it completely. Wearing waterproof apron in this case is a good solution because this equipment is less painful to bear than waterproof suits in hot climates (Nigg et al. 1992).

With quantitative data provided by the Whole Body Method, it has also been possible to understand the risk to an operator by comparing the observed deposits (total deposits on the body) to the value of the AOEL deltamethrin (0.0075 mg/kg bw/day). The average quantity on the body was determined equal to 1951.53 mg (n = 3). Considering an average body weight of 60 kg (WHO reference weight for an adult), the exposure value obtained (32.52 mg/kg bw/day) exceeds the AOEL. As it is admitted today (Fenske 1988; Nigg et al. 1992; Soutar et al. 2000) that wearing full protective equipment reduces exposure by 90%, the average exposure values for farmers wearing PPE is 3.25 mg/kg bw/day which still exceed the acceptable limit. It is interesting to note that the potential exposure value given by the theoretical model UK-POEM without body protection (0.024 mg/kg bw/day) is far away from the observed reality. The model predicts an absence of risk for the protected operators but it is clearly not true. The default parameters set in the

UK-POEM model prevent refining calculations: six consecutive hours are unrealistic, even if experimental results (Soutar 2000) showed that contamination occurs even after very short exposure, lasting as little as six minutes, suggesting that duration of spraying is not an important variable. Moreover, the pesticide distribution on the body included in the model is not consistent with the test results and the model, as well as the recommended patch sampling method, failed to predict the distribution of pesticide on the body: the model provides 25% of hands, 25% on the trunk and 50% on the legs, compared to 55% and 33% of average deposits on the thorax and on the legs respectively in the trial with the Whole Body Method.

The Whole Body dosimetry technique does not require any extrapolation and is far more realistic as mentioned by Soutar et al. (2000), but compared to the Patch Method, it seems more influenced by the way the operator worked. This can explain why the European legislation (Regulation (EC) 1107/2009) had recommended a minimum of 15 tests for GLP testing of operator exposure (Glass et al. 2002). Therefore a 'whole-body' sampling method should be recommended for the measurement of the real dermal exposure. In agreement with Chester (1993), for concurrent exposure and biological monitoring a refined Whole Body Method is recommended which involves the use of clothing representing that which workers normally wear under the prevailing conditions. Biological monitoring is recommended as the most precise means of estimating the absorbed dose of a pesticide, particularly if supported by human metabolism and pharmacokinetic data.

Finally, it should be noted that it is essential for the operator to be well trained to respect hygiene rules and Good Phytosanitary Practices because there are many factors that influence the exposure, such as the operator's skill (Hughes et al. 2008), the personal protective equipment (Ndao 2008), the type and crop height (Hughes 2008), the weather conditions (Hughes 2006; Kim et al. 2013), the type of device used and the orientation of the spray lance (Kadri 2012). This variability inherent in the technique and type of used device, where the human factor is much more decisive than for a large spray nozzles ramp, explains why the theoretical model was unable to predict reliably the level of exposure for a backpack sprayer. The model could be improved if these factors are introduced for a better predictive contamination level. But only practical testing conditions, based on prior observation of farmers' practices and tests performed with their help can give a realistic estimation of the potential exposure.

RESUME

Lawson A.J., Akohou H., Lorge S. et Schiffers B. 2017. Trois méthodes pour l'évaluation de l'exposition des agriculteurs aux pesticides dans des zones urbaines et péri-urbaines au Nord du Bénin. Tunisian Journal of Plant Protection 12: 91-108.

Les petits agriculteurs des zones urbaines et périurbaines du nord du Bénin utilisent des pesticides sans respecter les règles d'hygiène et sans équipement de protection individuelle (EPI). Sur la base de l'observation des pratiques locales à Djougou, Gogounou et Parakou, des essais sur le terrain ont été menés dans des conditions similaires pour évaluer la contamination et les niveaux d'exposition des agriculteurs, en utilisant trois méthodes d'échantillonnage habituelles (la Méthode Visuelle, la Méthode

des Patchs et la Méthode du Corps Entier). Pour la Méthode Visuelle et la Méthode des Patchs, un colorant et de l'encre fantôme ont été utilisés comme traceurs. Dans les essais avec la Méthode du Corps Entier, la deltaméthrine (PLAN D 25 EC) a été utilisée comme traitement insecticide. Des dépôts ont été observés sur les équipements de protection et sur les collecteurs. La tartrazine a été mesurée par colorimétrie et la deltaméthrine par chromatographie en phase gazeuse avec un détecteur DCE (CG-DCE). L'examen des équipements de protection (Méthode Visuelle) a montré que l'ensemble du corps était potentiellement exposé aux pesticides. Les mains ont été contaminées pendant la préparation et le chargement du mélange jusqu'au rinçage du pulvérisateur. La Méthode des Patchs n'a pas été parfaitement capable de prédire la distribution de la contamination sur le corps des agriculteurs. Les résultats de la Méthode du Corps Entier sont apparus être plus variables et influencés par la compétence de chaque opérateur par rapport à la Méthode des Patchs. Les niveaux de contamination observés étaient en général supérieurs aux valeurs estimées avec un modèle théorique (avec un total de 368 à 2867 mg de deltaméthrine pour l'ensemble du corps). Avec le port d'EPI, l'exposition moyenne a atteint 3,25 mg/kg pc/jour. Sans EPI, l'exposition potentielle était égale à 32,52 mg/kg pc/jour. Ces deux valeurs dépassent très largement l'AOEL de la deltaméthrine (0,0075 mg/kg pc/jour) indiquant un niveau de risque élevé pour l'opérateur. Le modèle théorique utilisé (UK-POEM) n'a pas été capable de prédire les résultats d'exposition potentielle mesurés dans ces essais.

Mots clés: Evaluation de l'exposition, pesticides, petits producteurs, pulvérisateurs à dos

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ملخص

لاوسن، أرميل جويل وهرمين أكوجو وستيفاني لورج وبرونو تشيفارس. 2017. ثلاث طرق لتقييم تعرض المزارعين للمبيدات في المناطق العمرانية وشبه العمرانية في شمال البنين.

Tunisian Journal of Plant Protection 12: 91-108.

لحماية محاصيلهم من الآفات والأمراض، يستخدم المزارعون الصغار في المناطق العمرانية وشبه العمرانية في شمال البنين مواد كيميائية لوقاية النباتات. في غياب المعلومات والموارد الكافية، تتم المعاملات من دون التقيد بالحد الأدنى من قواعد السلامة وبدون معدات الحماية الشخصية. بناء على ملاحظات الممارسات المحلية في دجوغو وغغونو وباراكو، أجريت تجارب ميدانية في ظروف مشابهة لتقييم التلوث ومستويات تعرض المزارعين، وذلك باستخدام ثلاثة طرق لأخذ العينات (الطريقة البصرية، طريقة البقع، وطريقة الجسم الكامل) وبمقارنة النتائج التي تم الحصول عليها باستخدام كل من هذه الطرق. تستخدم الطريقة البصرية وطريقة البقع كعنصرين مقتفيين للأثر، الصبغة تارترازين أو الحبر الشبح. وفي اختبار طريقة الجسم الكامل، تستخدم الدلتاميثرين (PLAN D 25 EC). وقد لوحظت الرواسب على معدات الوقاية وعلى الجوامع. تم قيس رواسب التارترازين بواسطة قيس الألوان والدلتاميثرين بواسطة الكروماتوغرافيا الغازية (GC-ECD). وأظهرت ملاحظة معدات الحماية (بالطريقة البصرية) أن الجسم بأكمله يمكن أن يتعرض للمبيدات، ولكن بمستويات مختلفة. وقد تلوثت الأيدي منذ الإعداد وتحميل الخليط إلى الرش وأثناء التنظيف. لم تقدم طريقة البقع وطريقة الجسم الكامل نفس النتائج. فطريقة البقع لم تكن قادرة على التنبؤ بدقة، بتوزيع التلوث على أجسام المزارعين. أما في التجارب التي أجريت بطريقة الجسم الكامل، تبدو النتائج أكثر تغيرا وتأثرا بمهارة كل عامل مقارنة بطريقة البقع. ثبت أن صدر المزارع (البطن والظهر معا) ملوث بشدة بالدلتاميثرين، مع الإشارة إلى أن مبيدات الآفات قد تكون متناثرة على الجسم بأكمله وليس فقط على الجزء السفلي، حتى لو كانت الساقان معرضة خلال العمل. تظهر مستويات التلوث الملاحظة أعلى بكثير من القيمة المقدرة التي أصدرها النموذج النظري (مجموع 368 إلى 2867 مغ للجسم بأكمله) وبالنظر إلى متوسط وزن الجسم 60 كغ (الوزن المرجعي للبالغ الذي حددته منظمة الصحة العالمية)، بلغ متوسط قيمة التعرض 3.25 مغ/كغ من وزن الجسم/يوم (مع معدات الحماية الشخصية) أو 32.52 مغ/كغ من وزن الجسم/يوم (بدون معدات الحماية الشخصية)، الذي يتجاوز كل المستويات المحددة من طرف AOEL لدلتاميثرين (0.0075 ملغ/كغ من وزن الجسم/يوم) والنموذج النظري المستخدم من طرف UK-POEM غير قادر على التنبؤ بالنتائج المتحصل عليها في هذه التجارب الميدانية.

كلمات مفتاحية: آلة الرش الظهرية، تقييم التعرض، مبيدات ، مزارعون صغار

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Plant Protection Events

Report on

The International Conference on the Red Palm Weevil in Tunisia
Tunis, Tunisia, May 3-5, 2017

In 2011, Red Palm Weevil (RPW) (Rhynchophorus ferrugineus) was recorded in Tunisia killing Canary Palms (Phoenix canariensis) in the northern city of Tunis. Management efforts have so far contained this pest in the north of Tunisia but infested areas are increasing.

The International Conference on the Red Palm Weevil in Tunisia (3-5 May 2017) was sponsored by the US embassy in Tunisia. The aim of this US-sponsored event was to assist Tunisian officials and RPW management teams with the development of containment and management programs to restrain populations in the north of Tunisia thereby preventing or greatly slowing the spread of this pest into date production areas. The ultimate goal of RPW management programs is its eradication from northern Tunisia.

The RPW International Conference ran multiple sessions over three days. The Conference had palm weevil experts from Tunisia, USA, Costa Rica, FAO, France, India, and Saudi Arabia who delivered talks on various aspects of date production, RPW biology, behavior, invasion ecology, and management. The third day of the conference was a RPW training day in La Marsa Saâda Park in Tunis an area with very high numbers of dead and dying Canary Palms.

The opening remarks, made by Mr. Elies Hamza, President of the IRESA, Mr.

Khalil Lamiri, Secretary of State to the Tunisian Minister of Higher Education and Scientific Research, Mr. Benjamin Moeling, Deputy Chief of Mission for the US Embassy in Tunis, Tunisia, Mr. Omar El Behi, Secretary of State to the Tunisian Minister of Agriculture, Water Resources and Fisheries, made it clear that they are very concerned about the eradication of this pest. The reason for this concern is potential economic, social, and food insecurity.

In Session 1 titled Overview of RPW in Tunisia, Containment, Control, and Eradication Efforts: Three presentations were given related to: (A) Date production presented by Ms. Dorsaf Ben Ahmad, Principal Agricultural Engineer, DGPA, indicating that date palm growers produce 150,000 tons of dates each year which accounts for 6% of agricultural production in Tunisia. Dates make up 12% of agricultural exports, and 50,000 tons, on average are exported. Although Tunisia accounts for just 4% of global date production, it commands an impressive 24% of global date revenue because of the high quality of this product. (B) Major arthropod pests of date palms presented by Dr. Med Habib Dhouibi, INAT; indicated that the pest complex on date palms in Tunisia includes carob moth, leaf feeding mites, Oryctes beetles and scales. RPW is not yet in Tunisian date production areas. (C) Tunisian RPW control/containment/management strategy presented by Ms. Fethia Hellali, Directorate of Plant Protection and Quality Control of Agricultural Product, indicated that the Tunisian National RPW Campaign relies on mass trapping using bucket traps baited with fermenting fruit and with an aggregation pheromone lure, pesticides, geo-referenced database of Canary palms, and phytosanitary removal of infested palms. Major constraints that were identified during the conference include capacity training, logistical and financial support for maintaining the existing program in Tunis/Carthage/Bizerte and Nabeul.

In Session 2 titled Overview of Biology, Ecology, Invasion ecology, Molecular identification: Two presentations were given related to: (A) The basic RPW biology, ecology, behavior, distribution, and invasion history, presented by Dr. Mark Hoddle, University of California, Riverside, informed the audience about additional invasive palm weevil species in California, R. vulneratus and R. palmarum, and the red ring nematode, a palm killing nematode spread by R. palmarum. Smart traps and drones are novel technologies that are starting to be available for RPW monitoring and deployment of treatment. (B) Identity, phylogeny, and invasion history of RPW presented by Dr. Paul Rugman-Jones, University of California Riverside, indicated that the DNA analyses demonstrated that the Tunisian RPW population is likely originated from existing Mediterranean populations.

In Session 3 titled RPW Pheromones, Synergists, and Trapping: Two presentations were given related to: (A) The discovery of palm weevil aggregation pheromone and their use in pest management presented by Dr. A.C. Oehlschager, the one of the discoverers of the RPW pheromone and vice president of Chem Tica International, Costa Rica, indicated that trapping efficacy is measured in terms of weevil numbers caught in traps and decreasing palm mortality rates. Programs need to be economic. Additionally, the presentation covered new emerging technologies as the no service trapping that could be synergistic if be combined with smart trap technologies and "Attract and Kill". (B)

Synergists for optimal trapping of RPW using aggregation pheromone presented by Dr. Didier Rochat, INRA Versailles, France, indicated that the use of fermenting fruit baits with high sugar content, greatly enhance trap captures and is necessary that the fruit be placed into the water.

In Session 4 titled Area-Wide Management Program for RPW: Two presentations were given by Dr. Jose-Romeno Faleiro, FAO consultant for RPW covered the current RPW-IPM strategy which revolves around four core components: (i) inspecting palms to detect infestations, (ii) capturing adult weevils using food baited pheromone traps, (iii) preventive and curative chemical treatments and (iv) removal/eradication of severely infested palms. Additionally, the presentations covered phytosanitary (quarantine) measures necessary for successful management of RPW. With regard to recent advances in semiochemical mediated technologies for the management of RPW, findings from Saudi Arabia on the use of 'Attract and Kill' (Hook RPW TM) and a dry trap (Electrap TM), were presented. Both these technologies do not need regular servicing (change of food bait and water). Attract and Kill and dry traps generated interest among meeting participants.

In Session 5 titled Future Directions and Emerging New Technologies for RPW Control: one presentation given by Dr. Agenor Mafra-Neto, entomologist and the CEO of ISCA Technologies, California, introduced several innovative technologies to the management of RPW as potential improvements upon the traditional mass-trapping. HOOK RPWTM, SMART Ferrolure + and semiochemical formulations that attract and kill RPW.

The third day of this event, the international experts gave a training for a selected group of Tunisian officers in La Marsa Saâda Park. This field day devoted to detailed discussion and demonstration of pheromone-based trapping programs, management of trap data, and emerging technologies such as "Attract and Kill" that support traditional pesticide applications to palms for RPW population suppression.

The workshop on RPW in Tunis in May 2017 highlighted proper practice in the components of RPW management and introduced new techniques that could make RPW management more effective.

Dr. Saida Slimane-Kharrat
Faculty of Science of Bizerte
University of Carthage
Bizerte, Tunisia

Announcing of

The First Maghreb Symposium on Integrated Pest Management

Sousse, Tunisia, October 30 - November 01, 2017

The Regional Research Center on Horticulture and Organic Agriculture, the Association Tunisienne pour une Agriculture Durable *and the Technical Center of Organic Agriculture jointly organize the First Maghreb*

Introduction

The potential negative environmental impact of the acute use of pesticides in Agriculture is well documented. Dependency on chemical pest control which is an easy and reliable solution has resulted in crop and environmental contamination, detrimental effects on human health and development of resistance to pesticides. In the Maghreb countries, there is reluctance in the adoption and implementation of Integrated Pest Management strategies to control pests despite continuous researches achievements as evidenced by the growing number of published research papers. This symposium follows the first National Symposium on Integrated Pest Management which was held in Sousse (Tunisia) from 20 April to 21 April 2015 and was reached by eminent scientists. The First Maghreb Symposium on Integrated Pest Management will take place in Sousse (Tunisia) from 30 October to 01 November 2017.

Objective of the Symposium

The objective of the symposium will be to give the opportunities to researchers, students, technicians and professionals to share their recent findings and new developments on Integrated Pest Management in cultivated plants and forest. The science motivation for holding a Maghreb Symposium around the Integrated Pest Management will be the establishment of the state-of-the-art of recent development of research in Plant Protection strategies aiming the reduction of the use of chemical pesticides. The symposium topics will include plenary lectures, oral communications, and posters.

Symposium theme

The symposium theme will be the Integrated Pest Management, with the following topics:

1. *Taxonomy of Pests and natural enemies*
2. *Pest emergence and invasive species*
3. *Plant-pest interactions*

4. *Climatic change and plant health*
5. *Pesticides and environment*
6. *Biotechnologies and plant protection*
7. *Pesticide formulation and spraying techniques*
8. *Rational use of chemical pesticides*
9. *Biological protection of plants*
10. *Bio-pesticides and alternative methods for pest control*
11. *Post-harvest technology management of pests*
12. *Legislative framework for plant protection.*

Symposium language

French is the official language of the symposium.

Abstract submission

- Participants should submit their abstracts in one page by email to <sympip2017@gmail.com>.

- After the Symposium, participants who wish to publish their work in a special issue of the "Tunisian Journal of Plant Protection" devoted to SYMPIP2017, can submit the whole paper mandatory in English language. Author instructions can be downloaded at <http://www.tjpp.tn/SiteWeb/Instructions Authors.pdf>.

Important date

Deadline for abstract submission: 31 July, 2017

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