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Guest Editorial

Copper-Resistance in Plant Pathogenic Bacteria

The multiple applications of copper-based bactericides (mainly Bordeaux mixture) on vegetable and fruit crops as a unique chemical mean for control of bacterial pathogens (Silver and Walden 1997; Rogers et al. 1994) has led to the development and prevalence of copper-resistant (Cur) strains of several species of bacteria affecting plants (Adaskaveg and Hine 1985; Bender and Cooksey 1986; Cooksey et al. 1990; Zhang et al. 2017).

Once copper resistance is acquired, the pressure posed by the continual applications of copper sprays gradually increases the frequency of the resistant population of the pathogen and compromises the efficacy of copper (Sundin et al. 1989). Cur strains have been identified in many plant-pathogenic bacterial species, including species of Pseudomonas (Bender and Cooksey 1986; Andersen et al. 1991; Cazorla et al. 2002), Pantoea (Nischwitz et al. 2007), Erwinia (Al-Daoude et al. 2009) and several species of

Xanthomonas, such as Xanthomonas euvesicatoria (formerly X. campestris pv. vesicatoria, X. axonopodis pv. vesicatoria) (Basim et al. 2005; Cooksey 1990; Marco and Stall 1983; Stall et al. 1986), X. arboricola pv. juglandis (Giovanardi et al. 2015; Lee et al. 1994), X. alfalfae subsp. citrumelonis, and X. citri subsp. citri (Behlau et al. 2011).

Horizontal transfer of copper resistance determinants through conjugation is the main mechanism for acquisition of copper resistance by bacteria (Behlau et al. 2012; Cooksey 1990; Stall et al. 1986). It is unlikely that bacteria become resistant to copper through spontaneous mutations because copper resistance is regulated by several genes in these organisms (Cooksey 1990). Although most copper resistance genes characterized from plant-pathogenic bacteria have been shown to be plasmid borne (Mellano and Cooksey 1988; Stall et al. 1986), chromosomal copper resistance genes have also been identified (Lee et al. 1994; Lim

and Cooksey 1993). The most detailed studies of bacterial Cu homeostasis have focused on plasmid-borne resistance genes in *E. coli* and in fluorescent *Pseudomonas* species (Tom-Petersen et al. 2001).

The resistance mechanisms so far discovered are: i) sequestration of copper ions out of cells, ii) relative impermeability of the outer and inner bacterial membranes to copper ions, iii) metallothionein-like copper-scavenging proteins in the cytoplasm and periplasm and iv) active removal of copper from the cell (Grass et al. 2011). Cellular copper sequestration has been suggested as the copper resistance mechanism in resistant strains of *Pseudomonas syringae* (Cooksey 1990). Cooksey (1993) explained that most bacterial species in the environment have acquired at least one of the management systems of copper, and that the development of copper resistance may have come about by the modification of copper uptake genes found on chromosomes. The mechanism of resistance in *E. coli* and *Pseudomonas* is the same, but there are differences in the nomenclature of the genes. The copper resistance system in *E. coli* consists of *pcoABCDE* and *pcoRS* genes. This resistance system is based on an efflux mechanism. The *pco* operon in *E. coli* is closely related to the *cop* operon in *Pseudomonas*, (*copABCD* and *copRS*)

(Bender and Cooksey 1986; Melano and Cooksey 1988). *CopA* is responsible for encoding a copper uptake ATPase and *copB* responsible for detoxification and an efflux ATPase (Silver 1996; Silver and Ji 1994). The *copR* and *copS* genes are regulatory in nature and are required for copper-induced expression of other *Cop* proteins and provide full copper resistance (Cooksey 1994). A second copper resistance operon, located on the chromosome and designated *copJ* (Rogers et al. 1994), has been found in a limited number of *Cur P. syringae* pv. *syringae* strains (Scheck et al. 1996). Although *copJ* shares some structural similarities with *copABCD*, it seems that it mediates resistance by a different mechanism (Rogers et al., 1994; Scheck et al., 1996).

In *P. syringae*, the copper resistance operon is present on plasmid *pPT23D* (Cha and Cooksey 1991; Mellano and Cooksey 1988). Studies have shown that *P. syringae* containing the *cop* operon accumulates more copper than strains lacking the operon (Cha and Cooksey 1991; Cooksey and Azad 1992) and that this operon confers copper resistance to *P. syringae* at least in part by sequestering and accumulating copper in the periplasm with copper binding proteins, which may prevent toxic levels of copper from entering the cytoplasm (Cha and Cooksey 1991).

According to Rouch et al. (1985), genes that confer copper resistance are regulated and induced only by high levels of copper.

*Nakajima and Scortichini groups found that all tested copper-sensitive *P. syringae* pv. *actinidia* strains possess homologous genes for *copA* and *copB* (Ferrante and Scortichini 2010; Marcelletti et al. 2011 Nakajima et al. 2002). Marcelletti et al. (2011) found that the two genes *copR* and *copS* required for (maximum) copper resistance were absent in many strains. These strains appeared to be sensitive to copper compounds (Ferrante and Scortichini 2010). Repeated spraying with copper bactericides resulted in detection of*

*strains of *Psa* with *copR* and *copS* genes in Japan in a few years' time (Nakajima et al. 2002).*

Related to the selection of copper-resistant strains, the efficacy of copper compounds is often limited (Cazorla et al. 2002). For this reason, several attempts have been made to reduce the application of copper compounds to crops and to develop compounds that reduce bacterial resistance to copper bactericides and thereby enhancing the bactericidal effect (Ninot et al. 2002). In this term, the European Union countries have introduced legislation limiting the use of copper compounds by the regulation N°473/2002 (Anon 2002).

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Optimization of Simple DNA Extraction Method Suitable for Diverse Microorganisms

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ABSTRACT

Zammouri, S., Kalai-Grami, L., and Mnari-Hattab, M. 2018. Optimization of simple DNA extraction method suitable for diverse microorganisms. Tunisian Journal of Plant Protection 13 (2): 145-155.

To date no simple DNA extraction method was reported to be efficient and adapted to various organisms and microorganisms. Moreover, this approach is hard, time consuming and rely on the use of liquid nitrogen. In order to obtain highly purified nucleic acids free of contaminants that could interfere with the amplification reaction during PCR, adequate and easy extraction methods should be developed. During this work, an efficient, fast and economical method for the isolation of high-quality DNA from fungi, bacteria and viruses is described. Those DNA extractions were performed without the use of liquid nitrogen. Besides, the protocol used allowed obtaining very good DNA concentrations that can be utilized at 1/50, regardless the origin of the analyzed samples, whether freshly collected, conserved in calcium chloride, or frozen at -80°C for long time (more than 10 years). The quantity and the quality of the extracted DNA by this method are enough high to perform cloning, PCR simplex or multiplex and also other DNA manipulation techniques.

Keywords: DNA, extraction, liquid nitrogen, microorganisms, PCR

Development of PCR-based molecular techniques has become the principal method to detect and characterize pathogens, and to understand the principle factors of molecular evolution (Mishra et al. 2008). The first step in molecular analysis is the preparation of purified, high molecular weight DNA and looking for an adequate method for DNA isolation leading to the development of various protocols.

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Reported DNA isolation methods such as sodium dodecyl sulfate (SDS) and cetyltrimethyl ammonium bromide (CTAB) are limited to certain organisms (Ahmed et al. 2009; Margam et al. 2010), and protocols must to be adjusted to each type of tissue because of the presence of secondary metabolites (polysaccharides, polyphenols, alkaloids and flavanoids) accumulated by plants or insect tissues, bacterial or fungal material which causes damage to DNA and/or inhibit Taq polymerases and restriction endonucleases (Calderón-Cortés et al. 2010; Sahu et al. 2012). Those approaches also rely on long-lasting period (through the need for overnight incubation) and hardworking, and some methods are expensive and/or ineffective (Milligan 1998).

Furthermore, DNA must be purified from cellular material mainly to avoid degradation, the main trouble encountered during DNA extraction (Weishing et al. 1995; Sahu et al. 2012). For this reason, in most cases an initial grinding stage with liquid nitrogen is performed (Rogers and Bendich 1994) to break down cell wall material and allow access to DNA (Tan and Yiap 2009), although liquid nitrogen has many disadvantages related to its availability, cost, transportation, storage and quick evaporation especially in Tunisia where temperatures are relatively high. Hence, in order to simplify classical DNA isolation methods, commercial genomic DNA extraction kits have been developed (Jobes et al. 1995; Cheng et al. 2003). However, these methods are usually either expensive or not readily available, require large amount of cellular material (in grams) to be grounded and are not suitable for a large number of samples (Wang et al. 2011).

A method requiring neither the use of commercial expensive kit nor liquid nitrogen and mainly adapted to divers organisms and microorganisms (plants, insects, fungi and bacteria) need to be developed for DNA isolation.

The aim of this study is the optimization of a protocol based on buffer method described by Carling 2004 without need of liquid nitrogen, and available for different plant tissues and microorganisms. We describe in this paper one consistent protocol to extract DNA from diversified cell origins. The optimized protocol is characterized by the use of high salt concentrations (2M NaCl) to remove polysaccharides (Fang et al. 1992), polyvinyl pyrrolidone (2% PVP) to remove polyphenols, and phenol-chloroform-isoamyl alcohol for extraction and finally DNA precipitation by cold isopropanol and extended RNase treatment.

This method is relatively simple, quick, low cost and suitable for an open laboratory environment, a method that avoids the use of liquid nitrogen and needs only small amount of sample, fresh buffer and mortar.

The results have proved that this protocol is valid for DNA extraction from different plant and insect tissues, bacteria, and fungi and the extracted DNA could be directly used in experiments, such as PCR, enzyme digestion, etc.

MATERIALS AND METHODS

DNA extraction

DNA was extracted from cultured microorganisms (fungi, bacteria) and from virus infected plant organs. A quantity of 50 mg of fresh or conserved samples (ie mycelium, bacterial cell, animal or vegetal tissues) was mixed with 500 µl of freshly prepared and preheated at 65°C grinding buffer composed of 5 ml of extraction buffer (0.35 M Sorbitol, 0.1 M Tris pH 7.5, 5 mM EDTA), 5 ml of lysis buffer (0.2 Tris pH 7.5, 0.05 M EDTA, 2 M NaCl, 2% CTAB), 2 ml of sodium lauryl sarkosyl (NLS at 5% w/v), 0.05 M de sodium bisulfate and 2% w/v of polyvinylpyrrolidone (PVP) 25000-30000). Then, the samples were grinded in mortar by adding sterilized quartz sand. Samples were immediately vortexed and incubated at 65°C for 40 min with agitation every 5 min. After adding an equal volume of phenol chloroform-isoamyl alcohol (24/24/1), tubes were homogenized by gentle tube inverting 30 times and centrifuged at 13200 rpm during 5 min; the supernatant was then recovered in Eppendorf tubes containing 80 µl of sodium acetate 3 M. Precipitation was performed by adding 0.8 volume of isopropanol and mixing gently by inverting the tube 20 times. After incubation during at least 1 h at -80°C, tubes were centrifuged for 5 min at 13200

rpm at 4°C. Precipitated nucleic acids collected were washed twice with 500 µl of ice-cold 70% ethanol and centrifuged for 5 min at 13200 rpm. The pellets were air dried and resuspended in 50 µl bi distilled (DEPC) treated water (0.1% diethylpyrocarbonate) containing 0.5 µg of RNase, then incubated at 37°C for 30 min. Purified DNA samples may be stored for short time at -20°C prior to analysis or for long time at -80°C until use.

Qualitative and quantitative analysis of extracted DNA.

For quality and yield assessments, electrophoresis was done for all kind of samples of DNA extracts, in 0.8% agarose gel, stained with ethidium bromide. Visualization was performed by gel documentation system and DNA quantification was assessed by using 1-kbp DNA ladder (Fermentas). DNA purity was evaluated by calculating the absorbance ratio DO260/280. Good-quality DNA will have an A260/A280 ratio of 1.7-2.0.

DNA concentration was calculated using the relationship that an A260 of 1.0 = 50 µg/ml pure DNA. Thus, concentration in µg/µl = A260 reading × dilution factor × 50µg/1000.

DNA yield (µg) = DNA concentration × total sample volume (µl)

PCR analysis.

Viral DNA amplification. Total DNA extracted using the described method from different plant species (tomato, watermelon, faba bean, pepper and some weeds) showing *Tomato yellow leaf curl virus* (TYLCV) symptoms freshly collected or frozen at -80°C for long time (more than 10 years) was subjected to amplification with two specific primer pairs designed by Davino et al. 2008 to target the IR region. (TY2222 [5'-GTCGTTGGCTGTCTGTTGTC-3'], TY 255 [5'-GGTTCGTAGGTTTCTTCAAC

TAG-3'], TY2463 [5'-GGTTCGTAGGT TTCTTCAACTAG-3'], TY247 [5'-TGG TTCCCCATTCTCGTGG-3']).

Amplification reaction was done in final volume of 20 µl containing 1x PCR reaction buffer (Pol Jena Bioscience), 0.2 mM of each primer, 0.2 mM of each dNTPs, 2 mM MgCl₂, and 1 U of Taq DNA polymerase (Pol Jena, Bioscience). PCR was performed in an Applied Biosystem Thermocycler programmed as follows: initial denaturation at 94°C for 4 min followed by 34 cycles (denaturing at 94°C for 30 s, annealing at 60°C for 90s and extension at 72°C for 90s) and a final extension cycle at 72°C for 10 min.

The second 2 primer pairs (TY209, TY575, TY613, TY1363) amplifying a portion of the CP and the V2 gene of TYLCV was described by Pellegrin et al. 2008. Amplification reactions were done in 20 µl contained 2x Master Mix (Quiagen, France), 0.2 µM of each primer and 1 µl of total DNA (diluted 1/20 to 1/50 depending on the initial concentration of the sample) per reaction mixture. PCR was performed in an Applied Biosystem Thermocycler with an initial denaturation at 95°C for 15 min followed by 34 cycles (denaturing at 94°C for 30 s, annealing at 63°C for 90 s and extension at 72°C for 90 s), and a final extension at 72°C for 7 min).

Fungal DNA amplification. The ITS regions and 5.8rDNA were amplified for *Athelia rolfsii*, *Macrophomina phaseolina* and *Fusarium* spp. isolates using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT GC-3') developed by White et al. 1990. Amplification reactions (50 µl) contained 1x PCR buffer (Promega, USA), 200 µM of each dNTP (Promega, USA), 1.5 mM MgCl₂, 1.0 µM of each primer, 1.0 U of Green GoTaq DNA Polymerase (Promega,

USA) and 10 ng of fungus DNA per reaction mixture. PCR was performed in a Biometra Thermocycler programmed with an initial denaturation at 94°C for 5 min followed by 35 cycles (denaturation at 94°C for 60 s, annealing at 50°C for 90 s and extension at 72°C for 90 s), and terminated with a final extension at 72°C for 7 min.

Bacterial DNA amplification.

16S rDNA region of bacterial DNA was amplified using the fd1 (5-GGAGAG TTAGATCTTGGCTC-3') and rd1 (5-AA G-GAGGTGATCCAGCCGCA-3') primer pair (Weisburg et al. 1991). PCR reactions were carried out in 50 µl reaction mixture containing 1 x PCR buffer (Quiagen) (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl), 200 µM of each dATP (Quiagen), 1.0 µM of each primer fd1 and rd1, 2 µl of DNA, and 0.5 µl of crude recombinant Taq DNA polymerase (Quiagen). PCR reactions were conducted in a Biometra thermocycler programmed with an initial denaturation at 94°C for 5 min; 31 cycles of denaturation at 94°C for 30 s, annealing at 65 °C for 60 s and extension at 72 °C for 90 s followed by a final extension at 72 °C for 5 min.

Visualization of PCR products.

PCR products were separated on 0.8 and 1.5% agarose gels, respectively for DNA extract or amplified product of more than 1200 bp and for PCR amplified fragments of about 800 bp, stained using ethidium bromide and visualized under UV light.

Sequencing.

The PCR products obtained after amplification of viral, fungal, and bacterial

DNA were further purified with a MinElute TMgel extraction kit (Quiagen) or PCR preps DNA purification systems (Promega, USA) according to manufacturer's instructions. Sequencing was performed by Genewiz (England).

Sequences analysis.

Sequences were checked and assembled by the CAP program (<http://pbil.univ-lyon1.fr/cap3.php>). The BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to search for sequences similarities into the DNA databases and sequences were aligned using Geneious software then were deposited in GenBank.

RESULTS

Quantification and qualification of DNA extracted.

After electrophoresis of DNA extracted from different tissues and pathogens, the visualization of agarose gel under UV showed clean pattern of DNA extracted without liquid nitrogen in terms of quantity and quality (Figs. 1, 2). All obtained DNA extracts were very concentrated, and must be diluted 1 to 50 or more before use excepted the DNA purified from *Athelia rolfsii*. The concentration of the extract was relatively low due to the exopolysaccharides that bind to the DNA making it mucilaginous. The problem was resolved by adding one step of purification using ethyl ether before precipitation (Punja and Sun 2001).

The determination of absorbance of nucleic acids confirmed the high quality and quantity of DNA extracted from all tested materials even for sample stored for long time (Fig. 1, Table 1). The ratio 260/280 is ranged from 1.71 to 2.05.

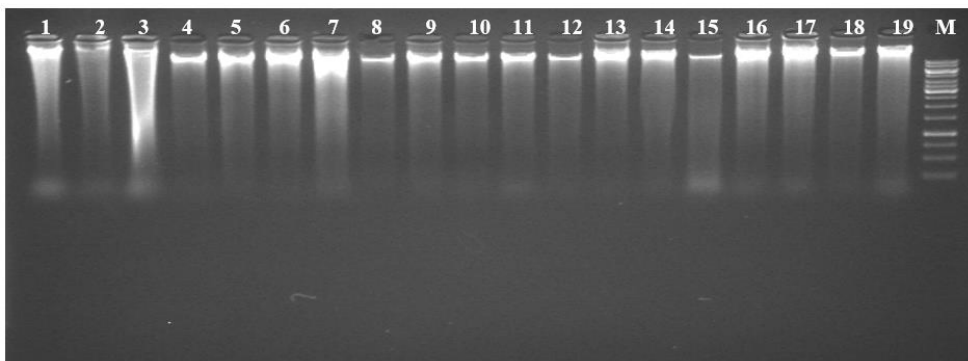


Fig. 1. Agarose gel electrophoresis (0.8%) of 2 μ l of extracted genomic DNA purified from various plant tissues freshly collected or frozen: M: 1 Kb DNA Ladder (GeneRuler) Line 2 and 3: extracted DNA from pepper; Line 1, 4, 6, 7, 18 and 19: extracted DNA from tomato freshly collected; Line 5 and 16: extracted DNA from tomato conserved at -80°C since 2009; Line 8 and 9: extracted DNA from watermelon conserved at -80°C since 2005; Line 12 and 13: extracted DNA from faba bean ; Line 10, 11, 14, 15 and 17: extracted DNA from weeds.

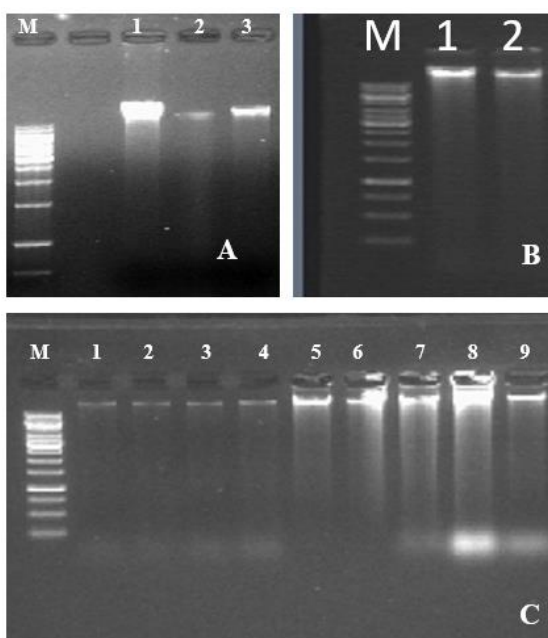


Fig. 2. Agarose gel electrophoresis (0.8%) of 2 μ l extracted genomic DNA purified from various tissues and cells; **A:** DNA purified from *Mayetiola destructor*; **B:** DNA purified from Aphids; **C:** DNA purified from *Athelia rolfsii* (Lane 1-4) and *Macrophomina phaseolina* (Lane 5-9).

Table 1. Quantification and qualification of DNA extracted by assessment of optical density

Samples	Do 260	DO 260/280	Concentration (µg/µl)
Fungus sample DNA extracts	0.091-0.539	1.77 to 1.9	0.182*-1.078
Bacteria sample DNA extracts	0.255-0.312	1.71 to 1.9	1.02-1.248
Plant tissue sample DNA extract	0.337-0.423	1.79 to 2.05	1.348-1.692
Insect tissue sample DNA extract	0.143-0.182	1.91 to 1.99	0.572-0.728

* Concentration of DNA of mucilaginous extract of *A. rolfsii* was low due to a second step of purification using ethyl ether before precipitation.

To confirm the DNA purity and to test amplification ability extracted DNAs were tested for PCR amplification.

Viral DNA amplification.

Multiplex PCR amplifications of extracted DNA from freshly and or stored vegetable sample for more than 10 years to detect TYLCV species was successfully achieved using primers pair dressed by Davino et al. 2008. Respectively a product

of 570 bp for TYLCV and 800 bp for *Tomato yellow leaf curl Sardinia virus* (TYLCSV) were obtained (Fig. 3A). A reproducible PCR product pattern was obtained using different primers (Fig. 3B); there was no sign of degraded DNA after amplification. Moreover, the same extracted DNA was successfully used for a period over 5 years which indicates the reproducibility of the results and the integrity of the DNA.

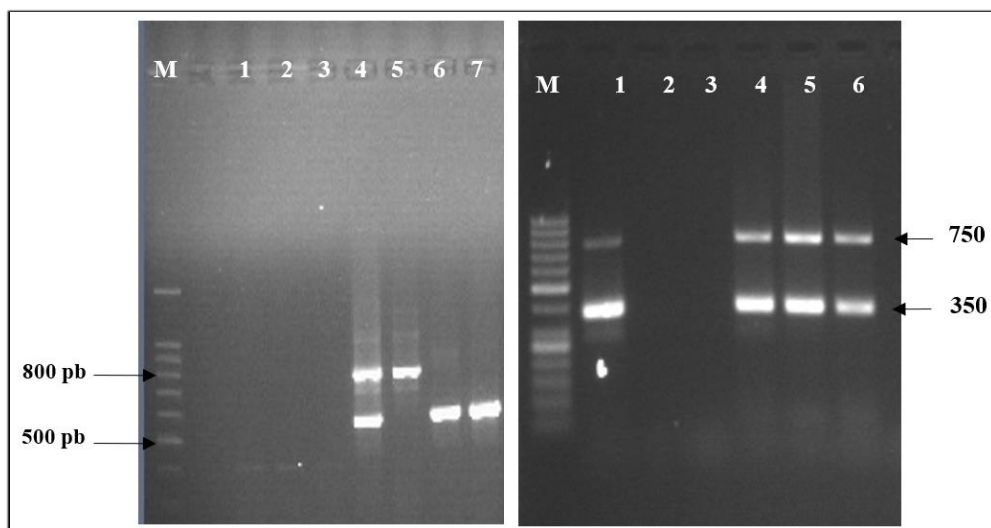


Fig. 3. A: Agarose gel electrophoresis (1.5%) of the PCR products obtained after amplification with the primer pairs TY255, TY2222, TY247 and TY2463. M: GeneRuler™50 bp DNA ladder from Fermentas, Lane 1: Negative control, Lane 2, 3, 4, 5, and 6: tomato samples; Lane7: positive control. B: Agarose gel electrophoresis (1.5%) of the PCR products obtained after amplification with the primer pairs TY209, TY575, TY613, TY1363: M: GeneRuler 100 pb+ DNA ladder. Lane 1: positive control; Lane2: Negative control, Lane 3, 4, 5, and 6: tomato samples.

Fungal DNA amplification (ITS).

The amplification of ITS region of fungal species gave a major single band

of 580 to 600 bp corresponding to four different *Fusarium* species characterized by variable ITS region length (Fig. 4).

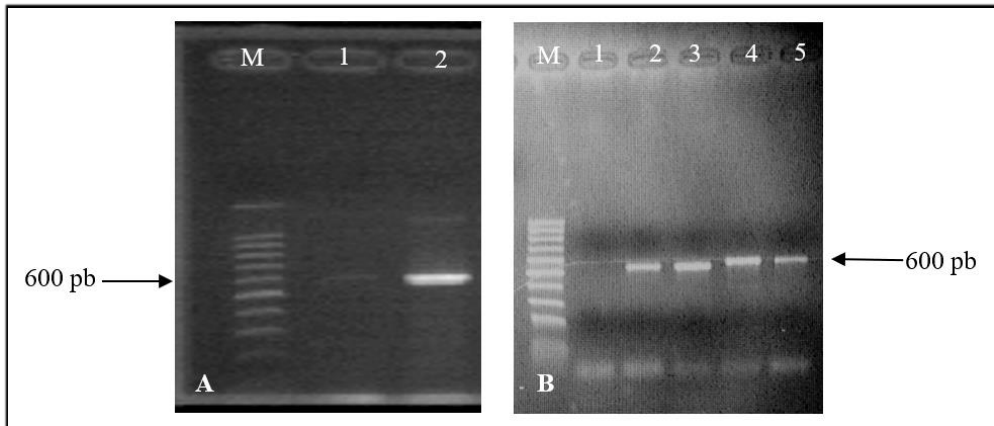


Fig. 4: Amplification of the ITS1-5.8S-ITS2 region for molecular identification. **A:** *Athelia rolfsii*: M: 100 pb molecular marker (invitrogen); Lane 1: negative control; Lane 2: *Athelia rolfsii*; **B:** M: 100 pb molecular marker (invitrogen); Lane 2-5: *Fusarium* spp. The amplification of 16S region of bacterial isolates gave a major single band of 1600 bp (Fig. 5).

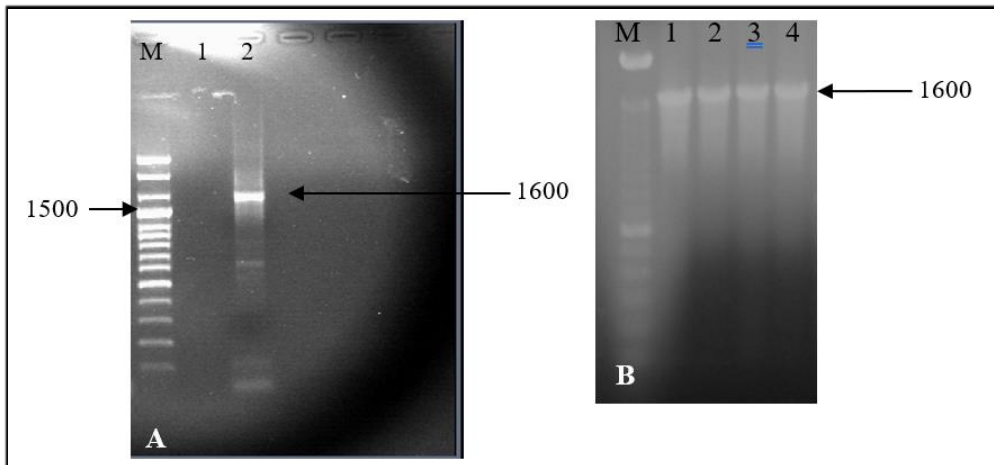


Fig. 5: Agarose gel electrophoresis (1%) of the PCR products obtained after amplification of the 16S rDNA region. **A:** M: Gene Ruler 100pb + DNA ladder; Lane 1: negative control; Lane 2: *Athelia rolfsii*; **B:** M: 100 pb molecular marker (invitrogen); Lane 1-4: *Bacillus* spp.

Sequencing.

The obtained sequences from virus infected vegetable were affiliated to *Tomato yellow leaf curl virus*, and *Tomato leaf curl Sardinia virus* (Zammouri et al. 2014).

Sequencing of bacterial 16S rDNA region and fungal ITS1-5.8S-ITS2 region allowed assigning them respectively to *Bacillus* genus for bacteria and to *Athelia rolfsii* (Kalai-Grami et al. 2013) and *Macrophomina phaseolina* (Hajlaoui et al. 2015) for fungi.

DISCUSSION

Most of all genomic DNA extraction methods from plant species, from bacteria and mostly from fungi or insects are difficult due to high mucilage (Cassago et al. 2002), high concentrations of phenolics and protein content (Mishra et al. 2008; Calderón-Cortés et al. 2010). Consequently, those protocols require the use of liquid nitrogen for tissue grinding (Aljanabi and Martinez 1997) which is difficult to obtain and to maintain in region like Tunisia characterized with very hot summer and moderate winter. Moreover, liquid nitrogen is not always available. The method presented here makes possible to extract and purified high molecular weight DNA from different plant species freshly collected or stored at -80°C for more than 10 years, either from bacteria, insects (Mayetiola and Aphids), or fungi (*Athelia rolfsii* and *Macrophomina phaseolina* and *Fusarium* spp.). All these DNA extractions were done without the use of liquid nitrogen or time-consuming procedures. The protocol used is technically rapid and relevant on a variety of species regardless the complexity of their genomes. The use of sterilized little fine sand is sufficient for grinding a huge variety of tissues. Besides, the efficiency of this method was confirmed with small amount (50 mg) of

fresh or up to 10 years stored samples (-80°C).

The obtained total DNA could be amplified by PCR using different primer pairs and satisfactory amplifications were generated. These results were in concordance to Sharma et al. (2008, 2010) that showed that a good quality of DNA can be isolated without the use of liquid nitrogen.

Moreover, we used the same DNA extracted for PCR amplifications over a period of 5 years and we obtained the same banding pattern which indicates the reproducibility of the results and the integrity of the DNA.

In addition, phenolics, polysaccharides and other secondary compounds accumulated by plants and insect tissues and fungal mycelia induce damage to DNA and/or inhibit restriction endonucleases and Taq polymerases (Calderón-Cortés et al. 2010). In the present study, higher concentrations of cetyltrimethylammonium bromide (2% CTAB) and the addition of antioxidants such as PVP (2%) with lower molecular weight to the extraction buffer further improved the quality of the extracted DNA by removing all phenolics from DNA preparations (Calderón-Cortés et al. 2010). The high quality of obtained DNA could also be attributed to the use of a higher concentration of NaCl. A number of researchers have recommended the use of PVP to face the problem of phenolics (Zidani et al. 2005). The addition of sodium acetate coupled with isopropanol help to purify and precipitate nucleic acid by removing cellular and histone proteins bounded to the DNA (Zidani et al. 2005).

Here we have described a simple, reliable, and cost-efficient DNA extraction method that provides high-quality DNA from fungi, bacteria, insects and plants containing high concentrations of

polysaccharides and polyphenolic compounds. This method allowed also reducing DNA degradation, and the contaminations of extracted DNA and minimize the risk to obtain low yield of DNA due to binding with starches and polysaccharides.

The quantity and the quality of the DNA extracted by this method are high

enough to perform amplification using DNA dilution 1/50 and to make a hundred of PCR-based reactions and also to be used in other DNA manipulation techniques such as restriction digestion.

However, this protocol should be improved to limit as much as possible the use of toxic organic substances.

RESUME

Zammouri S., Kalai-Grami L. et Mnari-Hattab M. 2018. Optimisation d'une méthode d'extraction d'ADN simple et valable pour divers microorganismes. Tunisian Journal of Plant Protection 13 (2): 145-155.

Actuellement aucune méthode simple d'extraction efficace et adaptée n'a été rapportée pour isoler l'ADN à partir de divers organismes et microorganismes. En plus, la plupart des protocoles décrits sont assez difficiles, longs et ayant recours à l'utilisation de l'azote liquide. Afin, d'obtenir des acides nucléiques hautement purifiés exempts de contaminants qui pourraient gêner la réaction d'amplification au cours de la PCR, des méthodes d'extraction adéquates et faciles devraient être mises au point. Au cours de ce travail, nous décrivons une méthode efficace, rapide et économique pour purifier les acides nucléiques totaux à partir de champignons, bactéries et virus et ce, sans avoir recours à l'azote liquide. Cette méthode nous a également permis d'obtenir de très bonnes concentrations d'ADN que nous avons utilisé au 1/50 et ce quel que soit l'origine des échantillons analysés, qu'ils soient frais ou séchés au chlorure de calcium ou même conservés à -80°C depuis plus d'une dizaine d'années. La qualité et la pureté des acides nucléiques obtenues permettent leur utilisation pour le clonage, l'amplification par PCR simplex, multiplexe, et diverses autres techniques.

Mots clés: ADN, azote liquide, extraction, microorganismes, PCR

ملخص

الزمووري، سامية وليلي قلعي-قرامي ومنية المناري-حطاب. 2018. ضبط طريقة مثلى لاستخراج الحامض النووي DNA بسهولة من كائنات دقيقة مختلفة.

Tunisian Journal of Plant Protection 13 (2): 145-155.

لم يتم إلى حد الآن تسجيل طريقة بسيطة، ناجعة ومأقلمة مع استخراج الحامض النووي DNA من أنسجة مختلفة. علاوة على ذلك، إن غالبية البروتوكولات المتداولة، إما أن تكون صعبة أو أن تطبيقها يستغرق وقتاً طويلاً. كما أنها تعتمد على النيتروجين السائل. لذلك، استوجب ضبط طريقة مناسبة وفعالة للحصول على أحماض نووية نقية من كل الشوائب التي يمكن أن تعطل عملية تفاعل البوليميراز التسلسلي PCR. لقد تم من خلال هذا العمل ضبط طريقة سهلة وسريعة وغير مكلفة لتطهير الأحماض النووية الكاملة لعدة عينات من فيروسات وبكتيريا وفطريات، وهذا دون اللجوء إلى استعمال النيتروجين السائل. مكنتنا هذه طريقة من الحصول على أحماض نووية نقية وجيدة التركيز بغض النظر عن نوعية العينة أو طريقة تخزينها (مجففة على استعمال CaCl_2 أو محفوظة منذ أكثر من 10 سنوات في درجة حرارة -80°C). تم استعمال هذه الأحماض بعد تخفيفها إلى حد 50/1 وكانت النتائج إيجابية وجيدة. مكنتنا النوعية الممتازة من الأحماض النووية التي تحصلنا عليها من استعمالها في الاستساخ وتضخيم PCR البسيط والمتعدد وكذلك تقنيات مختلفة أخرى.

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Endophytic Bacteria from *Solanum nigrum* with Plant Growth-Promoting and Fusarium Wilt-Suppressive Abilities in Tomato

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ABSTRACT

Aydi-Ben Abdallah, R., Jabnoun-Khiareddine, H., Nefzi, A., Ayed, F., and Daami-Remadi, M. 2018. Endophytic bacteria from *Solanum nigrum* with plant growth-promoting and Fusarium wilt-suppressive abilities in tomato. *Tunisian Journal of Plant Protection* 13 (2): 157-182.

Fifteen endophytic bacterial isolates from *Solanum nigrum* and *S. nigrum* var. *villosum* stems were screened for their plant growth-promoting potential and antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Isolates SV65, SV68 and SV109 were the most efficient in controlling the development of the disease (77-92%) and in improving tomato growth (32-62%) compared to the controls. They were characterized and identified by using 16S rDNA sequencing genes as being *Bacillus amyloliquefaciens* subsp. *plantarum* for the strain SV65 (KR818073) and *B. methylotrophicus* for the two strains SV68 (KR818074) and SV109 (KR818076). Gas Chromatography-Mass Spectrometry analysis of the n-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 matched phthalic acid, mono(2-ethylhexyl)ester as major compound. The bacterium *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 were shown to be chitinase-, protease-, pectinase-, phosphatase-, and indole 3-acetic acid (IAA)-producing agents. Furthermore, *B. methylotrophicus* SV68 produced chitinase, pectinase, and IAA (28.49 µg/ml), and *B. amyloliquefaciens* subsp. *plantarum* SV65 excreted siderophores and oxalic and malic acids. This study demonstrates that *S. nigrum* and *S. nigrum* var. *villosum* can be potential plant species for isolation of endophytic bacteria serving as biocontrol and biofertilizing agents for the improvement of production of tomato grown in FOL infested and non-infested soils.

Keywords: *Bacillus* spp., biocontrol, *Fusarium oxysporum* f. sp. *lycopersici*, metabolites, *Solanum nigrum*, tomato growth

After potato, tomato (*Solanum lycopersicum*) is one of the most widely grown and consumed vegetable species all over the world (Olaniyi et al. 2010). However, this crop is highly susceptible to various pathogens such as viruses, bacteria

and fungi. Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), is one of the most devastating soil-borne disease occurring in major tomato-producing areas worldwide (Moretti et al. 2008). This phytopathogen is responsible for heavy yield losses both in open field (season crop) and all year under greenhouse crops (McGovern 2015).

Disease control is a difficult task due to the limited range of effective fungicides, the colonization ability of the

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causal agent within the vascular tissues, and to the survival of its resting structures (chlamydospores) in the soil for many years (Vethavalli and Sudha 2012). In addition, using tomato cultivars resistant to FOL race 1 and race 2 failed to control disease in the presence of race 3 emerging in several countries (Reis et al. 2005).

Biological control of Fusarium wilt, using non-pathogenic microbial agents, remains the most promising and environmentally safe method (Moretti et al. 2008). These microbial agents are associated with various plant species and are commonly present in many environments. Associations between microorganisms and botanical species play an important role in plant surviving in diverse environmental conditions either under abiotic or biotic stresses (Andreolli et al. 2017; Compant et al. 2005). Potential uses of plant-associated bacteria as plant growth-stimulating agents and for managing soil and plant health was largely investigated (Botta et al. 2013; Chen et al. 1995; Eleftherios et al. 2004; Hallman et al. 1997; Marcos et al. 2016; Sturz et al. 2000; Welbaum et al. 2004).

The widely recognized mechanisms of biocontrol mediated by plant-associated bacteria are competition for an ecological niche or a substrate, production of inhibitory allelochemicals, and induction of systemic resistance (ISR) in host plants toward a broad spectrum of pathogens (Compant et al. 2005). Microbial-derived allelochemicals included iron-chelating siderophores (Bar-Ness et al. 1992), cell wall-degrading enzymes (Berg et al. 2005), lipopeptide antibiotics (Han et al. 2015) and/or other compounds belonging to the family of phthalic acids (Ramayabharathi and Raguchander 2014). Moreover, endophytic bacteria stimulate plant growth via indole-3-acetic acid (IAA) production, phosphate solubilization, nitrogen fixation

(Gaiero et al. 2013) and/or indirectly through inducing systemic resistance (Compant et al. 2005).

Solanum genus has been explored as a rich source of antimicrobial metabolites (Lin et al. 2011; Nefzi et al. 2016; Tuba et al. 2016). Furthermore, *S. trilobatum*, *S. melongena* and *S. torvum* are known to harbor various endophytic bacteria which were not previously assessed for their antifungal activity or their plant growth-promoting ability (Achari and Ramesh 2014; Bhuvaneswari et al. 2013).

S. nigrum, commonly known as Makoi or black nightshade, is a medicinal plant from the Solanaceae family. This wild plant was widely used in traditional medicine due to its antitumorigenic, antioxidant, anti-inflammatory, hepatoprotective, diuretic and antipyretic properties (Jain et al. 2011). This plant was also explored as potential source of isolation of endophytic fungi with potent plant growth promoting properties as is the case of *Fusarium tricinctum* and *Alternaria alternata* (Khan et al. 2015a). *S. nigrum* has been also used for isolation of endophytic bacteria such as *Serratia* sp. displaying the ability to enhance plant growth and phytoremediation potential under cadmium stress (Khan et al. 2015b). In addition, endophytic *Streptomyces* strains isolated from *S. nigrum* have been used for the biocontrol of *Rhizoctonia solani* damping-off and the promotion of tomato growth (Goudjal et al. 2014).

In this study, healthy *S. nigrum* and *S. nigrum* var. *villosum* plants were explored for the isolation of endophytic bacteria with the ability to suppress Fusarium wilt severity and to stimulate growth of tomato plants under greenhouse conditions. The most effective isolates were screened for their in vitro antifungal potential toward FOL. Antimicrobials

involved in disease suppression and plant growth promotion were also elucidated.

MATERIALS AND METHODS

Preparation of tomato seedlings.

Tomato cv. Rio Grande seeds® (BADDAR semences · seeds), used in this study, were gratefully provided by the Regional Center of Research on Horticulture and Organic Agriculture at Chott-Mariem, Sousse, Tunisia. This cultivar is known by its susceptibility to *Fusarium* wilt disease incited by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) races 2 and 3 (Barker et al. 2005). Peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) was sterilized into individual batches (80 × 120 cm) at 120°C, 1 bar pressure for 30 min before use. Seedlings were grown in alveolus plates (7 × 7 cm) filled with sterilized peat under greenhouse with 16 h photoperiod, 60-70% relative humidity and air temperatures ranging between 20 and 30°C. They were watered regularly until reaching the two-true-leaf growth stage. Seedlings with approximately similar heights were used for all the in vivo bioassays.

Phytopathogen culture.

F. oxysporum f. sp. *lycopersici* (FOL) strain used in this study was originally isolated from tomato plants showing typical *Fusarium* wilt symptoms. Isolation was performed from stem tissues exhibiting vascular discoloration. The phytopathogen was re-isolated from artificially infected tomato plants, fulfilling Koch's postulates, and previously identified based on wilting pattern and phytopathogen morphological and cultural traits on Potato Dextrose Agar (PDA) medium (Aydi Ben Abdallah et al. 2016a).

FOL strain used for in vitro and in vivo bioassays was previously grown

for 7 days on PDA medium supplemented with streptomycin sulfate (300 mg/ml) (w/v) and incubated at 25°C.

Solanum nigrum sampling and isolation of endophytic bacteria.

Healthy wild *S. nigrum* and *S. nigrum* var. *villosum* plants were used for isolation of endophytic bacteria. Plants were sampled, at the fruiting stage, from Chott-Mariem, Tunisia (N35°56'20,451"; E10°33'32,028").

Five stem samples per plant were individually disinfected by soaking in 70% ethanol for 1 min, immersion in 1% sodium hypochlorite solution for 10 min, and then in 70% ethanol for 30 s. They were rinsed thrice in sterile distilled water (SDW) and air-dried on sterile filter papers. Three stem pieces (1 cm in length) were pierced with a sterile-nipper and the liquid exuding from the internal tissues was streaked on Nutrient Agar (NA) medium. The efficiency of surface sterilization of stem sections was determined according to Hallmann et al. (1997) procedure. Plates were incubated at 25°C for 48 h. Bacterial colonies with morphological diversity were selected and conserved in Nutrient Broth (NB) supplemented with 40% glycerol at -20°C. Before being used in the different bioassays, stock cultures were grown onto NA and incubated at 25°C for 48 h.

Evaluation of endophytic colonization ability.

The endophytic colonization ability of bacterial isolates recovered from *S. nigrum* plants was checked according to Chen et al. (1995). Twenty isolates were grown on NA supplemented with streptomycin sulfate and rifampicin (applied at 100 µg/ml each). Isolates exhibiting resistance to these antibiotics were selected and the wild types were used for the inoculation of tomato cv. Rio

Grande seedlings (at the two-true-leaf stage). Seedlings were soaked for 30 min in a suspension of bacterial cells ($\sim 10^8$ cells/ml) prepared by suspending colonies growing in NA after 48 h of incubation in SDW. Control seedlings were dipped in SDW only. Seedlings were transplanted into individual pots (12.5×14.5 cm) filled with sterilized peat. Five seedlings were used for each individual treatment. Seedlings were grown for 60 days under greenhouse conditions as previously described. Re-isolation of bacterial isolates from the internal stem tissues was performed on NA amended with both antibiotics (100 $\mu\text{g/ml}$) as described above. After incubation at 25°C for 48 h, growing bacterial colonies similar to the wild type ones were considered as endophytes.

Evaluation of the hypersensitivity reaction.

Fifteen isolates that shown to be able to colonize the internal stem tissues of tomato cv. Rio Grande seedlings were used for the hypersensitivity test.

Tobacco (*Nicotiana tabacum*) seeds were provided by the laboratory of phytopathology of the Regional Research Center on Horticulture and Organic Agriculture at Chott-Mariem, Sousse, Tunisia. Seeds were disinfected with 0.2% sodium hypochlorite for 3 min, washed several times with SDW and then sown into individual pots (12.5×14.5 cm) filled with sterilized peat. Tobacco seedlings were grown in a growth chamber at $24-26^\circ\text{C}$ with 12 h photoperiod and 70% humidity for 15 days and watered regularly until reaching the two-true-leaf growth stage. A volume of 10 μl of bacterial cell suspension ($\sim 10^8$ cells/ml) was injected to tobacco leaves using a microsyringe (1 ml U-100 BD Micro-Fine™ Plus - 0.33 mm (29 G) \times 12.7 mm). Negative control leaves were treated

similarly using an equal volume of SDW. Tobacco plants (inoculated and non-inoculated) were incubated under ambient room conditions ($\sim 27^\circ\text{C}$) for 24 h. Isolates inducing the formation of chlorotic and/or necrotic zones on inoculated leaf areas were considered as phytopathogens and they were excluded from further biocontrol trials (Nawangsih et al. 2011).

Evaluation of the hemolytic activity.

Fifteen endophytic isolates were tested for their ability to degrade hemoglobin according to Murray et al. (2003). In fact, 100 μl of each bacterial cell suspensions ($\sim 10^8$ cells/ml) were transferred on the Blood Agar® (HiMedia, India) medium and incubated at 25°C for 48 h. Bacterial isolates with positive hemolytic activity, expressed by the formation of clear zones around their colonies, were considered as pathogenic to humans and excluded from the following trials.

Evaluation of the plant growth-promoting ability.

Fifteen endophytic bacterial isolates were screened in vivo for their ability to enhance tomato growth based on Botta et al. (2013) method. Roots of tomato cv. Rio Grande seedlings (at the two-true-leaf stage) were soaked for 30 min in bacterial suspensions adjusted at 10^8 cells/ml. Control seedlings were treated similarly with SDW.

Treated and non-treated (control) seedlings were transferred to individual pots (12.5×14.5 cm) filled with sterilized peat. Five seedlings were used for each individual treatment. Growth conditions were similar to those described above. At 60 days post-planting, plant height, fresh weight of the aerial parts and roots, and the maximum root length were noted.

Evaluation of *Fusarium* wilt-suppressive ability.

Seedling bacterization test was performed for fifteen endophytic isolates according to Nejad and Johnson (2000). Tomato cv. Rio Grande seedlings were separately drenched with 25 ml of bacterial suspensions (10^8 cells/ml). Six days post-bacterial treatment, they were drenched with 25 ml of FOL conidial suspension adjusted at 10^6 conidia/ml using a hemocytometer. Negative control seedlings were non-inoculated with FOL and watered with equal volume of SDW. Positive control seedlings were inoculated with FOL and treated similarly with SDW. Each individual treatment was replicated five times (five seedlings per treatment).

At 30 days post-inoculation (DPI) with FOL, disease severity was rated on 0-4 scale (Amini 2009), where 0 = no symptoms and 4 = 76-100% of leaves showing chlorosis and/or necrosis, and the vascular browning extent (from collar). Plant height, fresh weight of the whole plant and the percentage of FOL re-isolation frequency from stems sections onto PDA were also noted. Isolation frequency was calculated using formula: $IF (\%) = f/F \times 100$, where f = number of stem fragments showing growing FOL colonies and F = total number of stem fragments plated onto PDA (Moretti et al. 2008).

The most efficient bacterial isolates in suppressing *Fusarium* wilt severity and in improving growth of tomato plants challenged or not with FOL were selected for further characterizations, and identification of their metabolites involved in both effects.

Characterization and molecular identification of the most efficient antagonist isolates.

Morphological, biochemical and molecular characterizations were

performed for the most effective bacterial isolates in reducing tomato *Fusarium* wilt severity and in improving tomato growth. Colonies were characterized macro-morphologically based on their form, margin, elevation, surface, opacity and pigmentation on NA medium (Patel et al. 2012). Gram staining was performed using Gram procedure and colonies were observed using light microscopy® (LABORLUX S, Leitz). Biochemical characterization was performed using conventional tests according to Schaad et al. (2001) protocols.

Molecular identification was carried out by 16S rDNA sequencing genes and similarity analysis. Identification was performed after extraction of genomic DNA using the method described by van Soolingen et al. (1994) for Gram positive bacteria. PCR conditions and the two universal eubacterial primers 27f(5'-AGAGTTT GATCMTGGCTCAG-3') and 1492r(5'-TACCTTGTTACGACTT-3') used for amplification of 16S rDNA gene were detailed in Aydi Ben Abdallah et al. (2016b). The similarity of the 16S rDNA sequence of a given isolate was performed using BLAST-N program from GenBank database (<http://www.ncbi.nlm.gov/BLAST/>) and Ez-Taxon-e-server (<http://eztaxon-e.ezbiocloud.net/>). Alignment of the sequences was performed using ClustalX (1.81). Phylogenetic analysis for the aligned sequences was performed using the Kimura two-parameter model (Kimura 1980). The phylogenetic tree was constructed based on the neighbor joining (NJ) method with 1000 bootstrap sampling.

Evaluation of the antifungal activity.

Antifungal activity of the selected endophytic isolates was performed using their whole cell cultures,

cell-free culture filtrates and organic extracts.

Evaluation of the antifungal activity of whole cells.

The antifungal activity of the whole cell suspensions of selected isolates was carried out using the streak method (Sadfi et al. 2001) and the disc diffusion method (Vethavalli and Sudha 2012). Bacterial suspensions ($\sim 10^8$ cells/ml) were streaked along two perpendicular lines crossing the center of a Petri plate (9 cm in diameter) containing PDA medium. Four agar plugs (6 mm in diameter), cut from the actively growing edge of a 7 day-old culture of FOL, were placed at each side of the tested bacterial isolate. Control plates were similarly streaked using SDW. Each individual treatment was replicated four times. After 4 days of incubation at 25°C, diameter of FOL colony was noted. The inhibition rate (IR) of pathogen mycelial growth was calculated according to Tiru et al. (2013) formula: $IR\% = [(C2 - C1) / C2] \times 100$, where C2: diameter of pathogen colony in control plates and C1: diameter of pathogen colony in treated plates.

For the disc diffusion method, FOL suspension (10^6 conidia/ml) was added (at 1% v/v) to molten PDA pre-cooled to around 45°C. Bacterial cell suspensions ($\sim 10^8$ cells/ml) were applied as 20 μ l droplets injected onto sterile Whatman No. 1. filter paper discs (6 mm in diameter). Four paper discs were used per plate. In control plates, paper discs were treated similarly with SDW. Each individual treatment was replicated four times. After incubation at 25°C for 4 days, the inhibition zones formed around bacterial colonies were measured.

Evaluation of the antifungal activity of cell-free culture filtrate.

The antifungal activity of extracellular metabolites in cell-free culture filtrates of the selected bacterial isolates was assessed according to Karkachi et al. (2010) protocol. Liquid cultures, obtained from bacterial suspension ($\sim 10^8$ cells/ml) previously grown for 3 days in NB medium at $28 \pm 2^\circ\text{C}$ under continuous shaking at 150 rpm, were centrifuged at 10.000 rpm for 10 min. The centrifugation was repeated three times. The cell-free filtrates were sterilized by filtration through a 0.22 μ m pore size filter. NB filtrate was used as control treatment. Filtrates were aseptically added (at 20% v/v) to Petri plates containing molten PDA pre-cooled to around 45°C and supplemented with streptomycin sulfate (300 mg/l). This concentration of filtrates was chosen based on previous screening tests in our preliminary experiments (data not shown). After solidification of the mixture, three agar plugs of the phytopathogen (6 mm in diameter) were plated equidistantly in each plate. Each individual treatment was replicated three times. After incubation at 25°C for 4 days, the diameter of FOL colony was measured and the inhibition rate (IR) was calculated as described above.

Evaluation of the antifungal activity of organic extracts.

Organic extraction was performed for only one isolate being the most effective using chloroform (Bhoonobtong et al. 2012) and *n*-butanol (Romero et al. 2007). Sixty milliliters of cell-free culture filtrate of *B. amyloliquefaciens* subsp. *plantarum* SV65 were poured in a separating funnel and 60 ml of solvent (chloroform or *n*-butanol) were added carefully. The funnel was reversed several times by degassing from time to time. The mixture was allowed to settle for few minutes with the

cap open. The organic phase (the lower phase for extraction with chloroform and the upper one with *n*-butanol) was recovered. The aqueous phase was replaced in the funnel and the extraction was repeated two other times. The solvent was evaporated in a rotary evaporator at 35°C for chloroform and 75°C for *n*-butanol with a slight rotation at 150 rpm. The weight of crude extracts obtained using chloroform and *n*-butanol solvents were measured and the yield of each extraction was determined (in mg/ml).

To assess their antifungal activity against FOL, chloroform and *n*-butanol extracts were suspended in ethanol (1:1) (mg/ml) and added separately at two concentrations (2.5 and 5% v/v) to molten PDA medium pre-cooled to about 45°C and amended with streptomycin sulfate (300 mg/l) before being poured in Petri plates. Negative control cultures were treated with similar concentrations of ethanol. Two positive controls carbendazim (Amini and Sidovich, 2010) and *Bacillus thuringiensis* (Choi et al. 2007) were also included in the bioassay used at 2.5 and 5% (v/v) each. After solidification of the mixture, an agar plug (6 mm in diameter) colonized by FOL, removed from 7-day-old cultures, was placed at the center of each plate. Each individual treatment was replicated three times. After incubation for 7 days at 25°C, the diameter FOL colony was measured and the inhibition rate (IR) was calculated as described above.

GC-MS analysis of the most effective organic extract.

The major compounds present in the most bioactive extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 (*n*-butanol extract) were identified using Gas Chromatography coupled to Mass Spectrometry (GC-MS) analysis. This analysis was performed with an Agilent

7890A gas chromatograph equipped with a HP-5MS column (30 m × 0.25 mm; 0.25 µm film thickness), interfaced with an Agilent mass selective detector 5975C inter MSD. Oven temperature program was from 60 to 240°C at 4°C/min; injector temperature was 250°C; helium at 0.8 ml/min was used as carrier gas and interface temperature was 280°C; MS source temperature was 230°C; MS quadrupole temperature was 150°C; mass scan range from 50 to 550 amu at 70 eV; scan velocity was 2.91 scans/s. One microliter of sample was injected. The identification of compounds was performed by comparing the mass spectra with the data from the National Institute of Standards and Technology (NIST) library.

Enzymatic activity of selected endophytic isolates.

The ability of three selected endophytic isolates to produce chitinase, protease and pectinase was checked onto agar plates according to Tiru et al. (2013). Briefly, chitinase activity was assessed on minimum-medium supplemented with chitin® (MP Biomedicals, LLC, ILLKrich, France) by streaking the water bacterial suspensions (~10⁸ cells/ml) onto sterilized chitin-agar medium 0.5% (w/v). Control plates contained chitin-agar medium only. Three plates were used for each individual treatment. After 72 h of incubation at 28 ± 2°C, the presence of clearing zones around bacterial colonies was noted.

For the test of protease activity, isolates were streaked on sterilized skim milk agar (SMA, 3% v/v) medium. Control plates contained SMA only. Treatments were performed in triplicate. The diameter of the clear zone formed around the bacterial spots was measured after 48 h of incubation at 28 ± 2°C.

Pectinase production ability was tested by streaking bacterial suspensions (~10⁸ cells/ml) onto sterilized NA-pectin®

(ICN Biomedicals, Inc, Germany) medium 0.5% (w/v). Control plates contained the NA-pectin medium only. Treatments were performed in triplicate and after 48 h of incubation at $28 \pm 2^\circ\text{C}$, the presence or the absence of clear zones around bacterial colonies was noted.

Characterization of plant growth-promoting traits.

Indole-3-acetic acid (IAA) production. The ability of the three selected isolates to produce IAA was checked using the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. Liquid cultures, obtained from bacterial suspensions previously grown in Luria-Bertani (LB) medium supplemented with L-tryptophan 50 $\mu\text{g/ml}$ and incubated for 48 h under continuous shaking, were centrifuged at 10.000 rpm for 10 min. Two milliliters of Salkowski's reagent and 2-3 drops of orthophosphoric acid were added to 1 ml of the culture supernatant. Non-inoculated growth medium was used as negative control. Absorbance was read at 530 nm. Treatments were performed in triplicate. The concentration of IAA was determined and compared to a standard curve prepared from IAA dilution series at 100 $\mu\text{g/ml}$ in LB medium.

Phosphate solubilization ability.

The phosphate solubilization ability of the selected bacterial isolates was assessed according to Katzenelson and Bose (1959) with some modifications. An agar plug (6 mm in diameter) removed from bacterial cultures previously grown for 48 h on NA medium, was deposited onto Pikovskaya agar medium. Non-inoculated plates were used as control. Treatments were performed in triplicate. After 7 days of incubation at $28 \pm 2^\circ\text{C}$, the clearing zone formed around colonies was measured.

Siderophore production. The siderophore production ability of the three selected isolates was checked qualitatively according to Lacava et al. (2008). An agar plug (6 mm in diameter) removed from bacterial cultures previously grown for 48 h on NA medium, was plated onto chrome azurol S (CAS) agar medium. Control cultures contained the CAS agar medium only. Three plates were used for each individual treatment. After 5 days of incubation at $28 \pm 2^\circ\text{C}$, the clearing zone (yellow halo) formed around colonies was measured.

Organic acids production.

Preparation of root exudates of tomato. *B. amyloliquefaciens* subsp. *plantarum* SV65, which had completely suppressed Fusarium wilt and stimulated the growth of tomato plants challenged or not with FOL using their cells suspensions and filtrates cultures (data not shown), was used for this trial.

Tomato seeds cv. Rio Grande were disinfected with sodium hypochlorite (5% v/v) for 3 min and rinsed six times with SDW. Then, they were placed on sterile filter paper moistened with 2 ml of SDW and incubated in the dark at 26°C for 4 days. After germination, homogeneous seedlings were selected and cultured in 27 ml of Hoagland's nutrient solution (50% v/v) supplemented with the bacterial suspension (adjusted at $\sim 10^4$ cells/ml), issued from cultures previously grown in LB medium for 48 h. Control seedlings were cultivated in the Hoagland nutrient solution 50% (v/v). Bacterized and control seedlings were grown for 14 days in a growth chamber adjusted at 16 h photoperiod, 65% of relative humidity and air temperatures ranging between 20 and 25°C temperatures (Lugtenberg et al. 1999).

Two elementary treatments were tested: (I) seedlings untreated with *B.*

amyloliquefaciens subsp. *plantarum* SV65, and (ii) seedlings treated with *B. amyloliquefaciens* subsp. *plantarum* SV65. Each treatment was replicated 20 times.

Analysis of organic acids by HPLC. The Hoagland's nutrient solution, in which the tomato seedling was grown, was centrifuged at 5000 rpm for 20 min. Root exudates collected from the supernatant were analyzed by high performance liquid chromatography (HPLC). The organic acids used as standards for HPLC analysis are oxalic acid, malic acid, citric acid, and succinic acid. The mobile phase consisted of 90% H₂O and phosphoric acid (H₃PO₄) (pH 2.6) and 10% methanol (MeOH). After the initial equilibration of the column for 1 h, the samples (20 µl each) were eluted for 10 min each at a flow rate of 0.8 ml/min and detected at 216 nm (Kamilova et al. 2006). The standard curve was prepared by a mixture of four organic acids with concentrations ranging from 0.5 to 250 mg/l. The organic acids from each sample were identified by comparing their retention times and peak areas with the acids used as standards.

Data analysis.

Data were subjected to a one-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) software for Windows version 16.0. Each in vitro and/or in vivo experiment was conducted twice yielding similar results. The in vitro assay of organic extracts was analyzed according to a completely randomized factorial model with two factors (treatments and concentrations). For the other in vitro bioassays, data were analyzed according to a completely randomized design. All in vivo trials were performed following a completely randomized model. Means

were separated using LSD or Student Newman Keuls tests to identify significant pair-wise differences at $P \leq 0.05$. For the test of organic acid analysis in root exudates of tomato, means were separated using test-t of Student at $P \leq 0.05$. Correlations between Fusarium wilt severity and plant growth parameters were analyzed using bivariate Pearson's test at $P \leq 0.01$.

RESULTS

Endophytic colonization ability of bacterial isolates recovered from *Solanum nigrum* stems.

Twenty bacterial isolates, exhibiting diversity in their macro-morphological traits on NA medium, were recovered from the internal stem tissues of *S. nigrum* and *S. nigrum* var. *villosum* plants. Among them, 16 isolates were found to be resistant to streptomycin and rifampicin (when grown on NA amended with these antibiotics). Challenged to tomato cv. Rio Grande seedlings, 15 isolates were successfully re-isolated from the internal stem tissues when plated on NA medium amended with these antibiotics. They were classified as endophytes and retained for further screening of their plant growth-promoting and Fusarium wilt-suppressive abilities on tomato plants cv. Rio Grande.

Plant growth-promoting ability displayed by *S. nigrum*-associated endophytic bacteria.

When tested onto pathogen-free tomato seedlings, bacterial treatments had significantly (at $P \leq 0.05$) affected growth parameters (plant height, aerial part fresh weight, maximum root length and root fresh weight) noted 60 days post-treatment. As shown in Table 1, SV60-, SV64-, SV109-, SV68- and SV65- based treatments led to significant increment in plant height over the untreated control

ranging from 29.1 to 37.8 %. The aerial part fresh weight was significantly enhanced from 33.7 to 46.1% by the isolates SV61, SV64, SV60, SV109, SV65 and SV68 in comparison to control.

Assessed for their ability to promote root growth, SV64, SV65, SV68 and SV109 based-treatments had induced

significant increase in the maximum root length which ranged from 27.2 to 43.6% compared to control. Three isolates namely SV65, SV68 and SV109 out of the 15 tested had significantly enhanced the root fresh weight by 50.9-54.1% versus control (Table 1).

Table 1. Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from *Solanum nigrum* stems on tomato cv. Rio Grande growth parameters noted 60 days post-treatment

Plant source	Bacterial isolate	Plant height (cm)	Aerial part fresh weight (g)	Maximum root length (cm)	Root fresh weight (g)
	Control	20 ± 0.7 e	11.08 ± 0.4 e	15 ± 0.3 c	4.2 ± 0.2 b
<i>S. nigrum</i>	SV59	22.4 ± 1.6 de	13.99 ± 0.7 de	18.8 ± 0.3 bc	5.16 ± 0.5 b
	SV60	28.2 ± 1.1 abcd	17.85 ± 0.3 abcd	20 ± 0.3 bc	5.44 ± 0.5 b
	SV61	25 ± 1.2 bcde	16.72 ± 0.7 abcd	19 ± 1.2 bc	4.64 ± 0.2 b
	SV62	22.2 ± 0.8 de	15.11 ± 0.3 cde	17.6 ± 0.6 bc	4.45 ± 0.2 b
	SV63	23.2 ± 0.8 cde	15.72 ± 0.6 bcde	17 ± 1.4 bc	4.05 ± 0.3 b
	SV64	28.2 ± 1.1 abcd	17.06 ± 0.6 abcd	20.6 ± 1.1 b	5.73 ± 0.3 b
	SV65	32.2 ± 0.8 a	19.90 ± 1 ab	25 ± 0.8 a	8.54 ± 0.3 a
	SV66	22.4 ± 0.6 de	14.97 ± 0.4 cde	16.6 ± 0.7 bc	4.99 ± 0.1 b
	SV68	30.6 ± 1.2 ab	20.57 ± 1.2 a	26.6 ± 0.3 a	8.85 ± 0.3 a
	SV69	24.4 ± 1.3 bcde	15.56 ± 0.4 bcde	18.6 ± 0.3 bc	4.47 ± 0.2 b
	SV70	20.4 ± 0.5 e	13.54 ± 1.1 de	16.4 ± 0.6 bc	4.35 ± 0.5 b
<i>S. nigrum</i> var. <i>villosum</i>	SV105	24.4 ± 0.3 bcde	15.46 ± 0.3 bcde	17.6 ± 0.2 bc	5 ± 0.5 b
	SV106	24.4 ± 0.2 bcde	14.75 ± 0.2 cde	17 ± 0.5 bc	4.96 ± 0.2 b
	SV109	29.6 ± 0.4 abc	19.01 ± 0.3 abc	24.8 ± 0.2 a	9.15 ± 0.4 a
	SV111	23.6 ± 1 cde	14.28 ± 0.4 cde	15.8 ± 1.1 bc	4.37 ± 0.3 b

Control: Non-inoculated with pathogen and untreated. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. SD: Standard deviation.

Fusarium wilt-suppressive ability displayed by *S. nigrum*-associated endophytic bacteria.

Fusarium wilt severity, noted on tomato plants 30 DPI with FOL, varied significantly (at $P \leq 0.05$) depending on bacterial treatments tested. Significant decreases by 77.3 to 88.9 % in the leaf damage index (chlorosis and/or necrosis) and by 88 to 92% and in the vascular browning extent were noted on tomato plants already challenged with FOL and treated with the three isolates SV109, SV68 and SV65 as compared to FOL-inoculated and untreated control (Table 2).

Also, FOL re-isolation frequency onto PDA medium was lowered by 20 to 100% relative to positive control when performed for stems tissues issues from tomato plants challenged with FOL and treated with bacterial isolates. The highest decrease (100%) in this parameter was achieved using the isolates SV65, SV68 and SV109 (Table 2).

Furthermore, as given in Table 2, SV109-, SV68- and SV65-based treatments had significantly improved plant height and plant fresh weight by 36.6-38.1% and 61.1-62.4%, respectively, if compared to the FOL-inoculated and

untreated control. It should be also highlighted that tomato plants infected with FOL and treated with these isolates showed significantly similar growth as disease-free and untreated control ones.

Correlation analysis between *Fusarium wilt* severity and plant growth parameters.

Pearson's correlation analysis revealed that the decrease in *Fusarium wilt* severity, as estimated by the leaf damage index (and/or necrosis) and the vascular browning extent, led to an increment in all plant growth parameters. In fact, plant height was significantly and negatively

correlated to the leaf damage index ($r = -0.832$, $P = 3.444 \text{ E-}5$) and to the vascular browning extent ($r = -0.904$, $P = 6.182 \text{ E-}7$). Furthermore, the plant fresh weight was significantly and negatively linked to leaf chlorosis score ($r = -0.928$, $P = 7.464 \text{ E-}8$) and to the vascular browning extent ($r = -0.811$, $P = 7.55 \text{ E-}5$). Similar trend was observed between FOL re-isolation frequency and plant growth parameters where significant and negative correlations were recorded between FOL re-isolation frequency, plant height ($r = -0.739$, $P = 0.0006$), and whole plant fresh weight ($r = -0.877$, $P = 3.806 \text{ E-}6$).

Table 2. Effects of endophytic bacterial isolates recovered from *Solanum nigrum* stems on *Fusarium wilt* severity, plant growth parameters and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) re-isolation frequency from tomato cv. Rio Grande plants, recorded 30 days post-inoculation with FOL as compared to controls.

Plant source	Bacterial isolate	Disease severity (0-4)	Vascular browning extent (cm)	Plant height (cm)	Plant fresh weight (g)	FOL re-isolation* (%)
	NIC	0 ± 0 c	0 ± 0 b	8 ± 0.3 abc	3.43 ± 0.1 a	0
	IC	3.6 ± 0.2 a	5 ± 0.4 a	5.2 ± 0.4 e	1.30 ± 0 d	100
<i>Solanum nigrum</i>	SV59	3 ± 0.3 a	4.8 ± 0.2 a	4.8 ± 0.1 e	1.65 ± 0.1 cd	60
	SV60	2.8 ± 0.4 a	5.4 ± 0.1 a	5.6 ± 0.5 de	2.82 ± 0.1 abc	70
	SV61	2.2 ± 0.4 ab	5 ± 0.1 a	5.2 ± 0.6 e	2.51 ± 0.2 abcd	80
	SV62	2.6 ± 0.3 a	3.9 ± 0.3 a	6.1 ± 0.3 bcde	2.28 ± 0.2 abcd	70
	SV63	2.6 ± 0.5 a	5 ± 0.4 a	6.2 ± 0.2 bcde	2.37 ± 0.1 abcd	80
	SV64	4 ± 0 a	4.6 ± 0.5 a	5.9 ± 0.5 cde	1.29 ± 0.2 d	70
	SV65	0.4 ± 0.2 c	0.4 ± 0.2 b	8.4 ± 0.2 a	3.35 ± 0 ab	0
	SV66	2.2 ± 0.2 ab	4.7 ± 0.2 a	6 ± 0.2 bcde	1.99 ± 0.1 bcd	70
	SV68	0.6 ± 0.2 c	0.6 ± 0.3 b	8.2 ± 0.2 abc	3.47 ± 0.1 a	0
	SV69	2.6 ± 0.3 a	5.4 ± 0.2 a	6.6 ± 0.4 abcde	2.12 ± 0.3 abcd	60
	SV70	2.6 ± 0.5 a	3.8 ± 0.2 a	6 ± 0.1 bcde	2.45 ± 0.1 abcd	50
<i>S. nigrum</i> var. <i>villosum</i>	SV105	3.4 ± 0.4 a	5.2 ± 0.2 a	5.8 ± 0.4 cde	1.96 ± 0.2 bcd	50
	SV106	4 ± 0 a	5.2 ± 0.4 a	5.8 ± 0.3 cde	1.26 ± 0.2 d	40
	SV109	0.8 ± 0.2 bc	0.6 ± 0.3 b	8.3 ± 0.2 ab	3.36 ± 0.1 ab	0
	SV111	4 ± 0 a	5.5 ± 0.1 a	6 ± 0.1 bcde	1.65 ± 0.3 cd	80

NIC: Non-inoculated with pathogen and untreated control. IC: Inoculated with FOL and untreated control. For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. SD: Standard deviation.

*The re-isolation of FOL was carried out from the stems of tomato plants cv. Rio Grande at 0-15 cm height from the collar. Ten stem fragments were plated on Potato Dextrose Agar (PDA) medium and incubated at 25°C for 4 days.

Characterization and identification of the bioactive bacterial isolates.

The three bioactive isolates selected above (SV65, SV68, and SV109) based on their effectiveness in suppressing Fusarium wilt severity and in improving tomato growth even in plants challenged

with FOL were morphologically and biochemically characterized (Table 3). These isolates were also checked for their hypersensitivity reaction and their hemolytic activity and were found negative for these both tests (Table 3).

Table 3. Characterization of the most effective endophytic bacterial isolates from *Solanum nigrum* stems on Fusarium wilt suppressive and plant growth-promoting.

Bacterial characterization	Bacterial isolates		
	SV65	SV68	SV109
Morphological characterization			
Form	Irregular	Irregular	Circular
Margin	Lobed	Irregular	Undulate
Elevation	Humped	Convex	Humped
Surface	Rough	Rough	Smooth
Opacity	Opaque	Opaque	Opaque
Color	White	White	White
Gram's staining	Positive	Positive	Positive
Biochemical characterization			
King A	-	-	-
Catalase	+	+	+
Urease	-	-	+
Lecithinase	-	-	+
Nitrate reductase	+	+	+
Tryptophane deaminase	-	+	-
Lysine decarboxylase	-	-	-
Mannitol	+	+	-
Simmons citrate	-	+	+
Indole	+	+	+
Red of Methyl	-	-	-
Voges-Proskauer	+	+	+
Hydrogen sulfide	-	-	-
Gaz production	+	+	+
Lactose	-	+	-
Glucose	-	-	-
Hypersensitivity reaction			
	-	-	-
Hemolytic activity			
	-	-	-

-: Negative test; +: Positive test; Lactose (+/-): (fermentation/utilization); Glucose (+/-): (fermentation/utilization).

Blast-N analysis of sequenced 16S rDNA gene similarity revealed that the three endophytic isolates belonged to the genus of *Bacillus* with 99.6% of similarity to *B. amyloliquefaciens* subsp.

plantarum Hs-18, 99.8% to *B. methylotrophicus* Ha13 and 100 % of similarity to *B. methylotrophicus* Nk5-1 with isolates SV65, SV68 and SV109, respectively (Table 4). The phylogenetic

analysis of the aligned 16S rDNA sequences of isolates SV65, SV68 and SV109 and the most related species revealed a short distance between the tested isolates and *Bacillus* species indicated above (Fig. 1). Sequences of SV65, SV68 and SV109 were submitted to GenBank and assigned the following

accession numbers: KR818073, KR818074 and KR818076, respectively (Table 4). Based on EzTaxon, the strain SV65 showed a similarity of 99.45% with *Bacillus siamensis* KCTC13613(T) and the strains SV68 and SV109 belonged to *Bacillus velezensis* CR502(T) with 99.62 and 99.27% of similarity, respectively.

Table 4. Identification of the most effective endophytic bacterial isolates from *Solanum nigrum* stems by 16S rDNA sequencing genes

Isolate	Accession number	Most related species	Sequence similarity (%)
SV65	KR818073	<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> Hs-18	99.6
SV68	KR818074	<i>Bacillus methylotrophicus</i> Ha13	99.8
SV109	KR818076	<i>Bacillus methylotrophicus</i> Nk5-1	100

SV65 and SV68: Bacterial isolates recovered from internal stem tissues of *Solanum nigrum*.
SV109: Bacterial isolate issued from internal stem tissues of *S. nigrum* var. *villosum*.

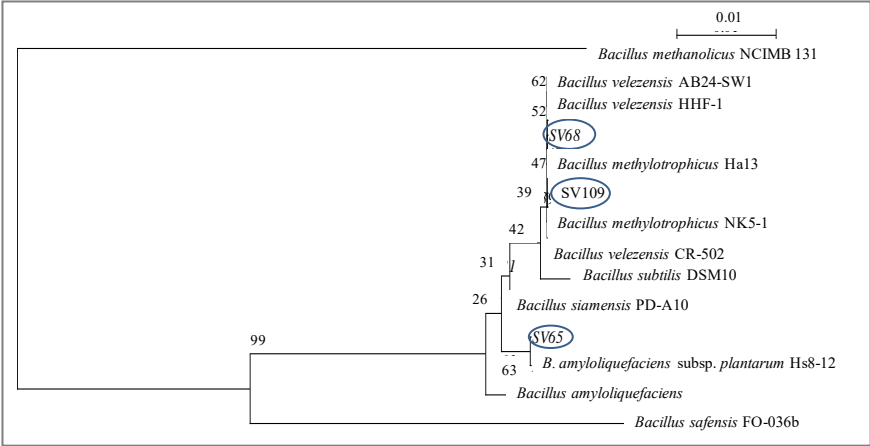


Fig. 1. Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of the endophytic bacterial isolates SV65, SV68 and SV109 recovered from *Solanum nigrum* and *S. nigrum* var. *villosum* and their closest phylogenetic relatives.

The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers: JF899271 (*B. amyloliquefaciens* subsp. *plantarum* Hs8-12), JF899290 (*B. methylotrophicus* Ha13), HQ831395 (*B. methylotrophicus* Nk5-1), AY603658 (*B. velezensis* CR-502), AB112727 (*B. methanolicus* NCIMB), AB006920 (*B. amyloliquefaciens*), GQ281299 (*B. siamensis* PD-A10), AJ276351 (*B. subtilis* DSM10), AF234854 (*B. safensis* FO-036b), MG757677 (*B. velezensis* HHF-1), MG890226 (*B. velezensis* AB24-SW), and for the bacterial isolates tested: KR818073 (SV65), KR818074 (SV68) and KR818076 (SV109). The tree topology was constructed using ClustalX (1.81).

Mycelial growth inhibitory ability displayed by *Bacillus* spp. associated to *S. nigrum*.

Antifungal activity of *Bacillus* spp. whole cell suspensions. Tested using the streak method, metabolites present in the whole cell suspension of tested *Bacillus* spp. isolates induced a significant ($P \leq 0.05$) decrease in FOL mycelial growth, noted after 4 days of incubation at 25°C, as compared to the untreated control (Table 5). Phytopathogen growth was lowered by 40.404 to 55.555%, relative to control, when dual cultured with the three isolates of *B. methylotrophicus* SV109, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV68, respectively. Tested using the disc

diffusion method, the three isolates of *Bacillus* formed, after 4 days of incubation at 25°C, an inhibition zone against FOL growth of about 12-12.62 mm (Table 5).

Antifungal activity of *Bacillus* spp. cell-free culture filtrates. ANOVA revealed a significant ($P \leq 0.05$) variation in the colony diameter of FOL depending on cell-free cultures filtrates of *Bacillus* spp. tested and used at 20% (v/v). The FOL growth inhibition (58.7-62%) was obtained using the extracellular metabolites from *B. methylotrophicus* SV68, *B. methylotrophicus* SV109 and *B. amyloliquefaciens* subsp. *plantarum* SV65 (Table 5).

Table 5. Antifungal activity of *Bacillus* spp. isolates and their extracellular metabolites against *Fusarium oxysporum* f. sp. *lycopersici* noted after 4 days of incubation at 25°C

Bacterial treatment	Whole cell suspension (~10 ⁸ cells/ml)		Extracellular metabolites (20 % v/v)
	Colony diameter (cm) and growth inhibition of FOL (%)	Inhibition zone (mm)	Colony diameter (cm) and growth inhibition of FOL (%)
Untreated control	3.71 ± 0.08 a	0 b	3.63 ± 0.02 a
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> SV65 (KR818073)	1.98 ± 0.1 bc (46.5)	12.62 ± 1.4 a	1.38 ± 0.08 b (62)
<i>B. methylotrophicus</i> SV68 (KR818074)	1.65 ± 0.08 c (55.5)	12.5 ± 0.7 a	1.5 ± 0.1 b (58.7)
<i>B. methylotrophicus</i> SV109 (KR818076)	2.21 ± 0.09 b (40.4)	12 ± 0.3 a	1.45 ± 0.2 b (60)

For each column, values followed by the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$. Values in parenthesis indicate the percentage (in %) of the mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* as compared to the untreated control.

Antifungal activity of organic extracts from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65.

The *n*-butanol extraction performed for the isolate *B. amyloliquefaciens* subsp. *plantarum* SV65 yielded 373 mg of dry residues (estimated at 6.22 mg/ml) compared only to 26 mg

(0.43 mg/ml) using the chloroform extraction.

Analysis of variance revealed a significant ($P \leq 0.05$) variation in the average diameter of FOL colonies depending on organic extracts tested (chloroform and *n*-butanol extracts) and concentrations used, and their interactions.

Results shown in Fig. 2 indicated that chloroform and *n*-butanol extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65, used at 1 mg/ml, had inhibited FOL growth by 43.4-73% and 61.1-90% as compared to ethanol-treated control whatever the concentration used, respectively. Both organic extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65 were more active when used at 5% than at 2.5% (v/v) which reduced FOL growth was reduced by 43.3-61.1% and 73-90%, respectively (Fig. 2). The highest inhibition, of about 90%, was achieved using the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 at 5% (v/v).

The decrease in FOL growth was significantly higher with the chloroform and *n*-butanol extracts from *B. amyloliquefaciens* subsp. *plantarum* SV65 used at 5% (v/v) (73 and 90%, respectively) compared to carbendazim- (39.5%) and *B. thuringiensis*-based treatments (43.2%). Tested at 2.5% (v/v), the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 was more effective (61.1%) than the two commercial used products (31.3-40.9%). The chloroform extract from *B. amyloliquefaciens* subsp. *plantarum* SV65, used at 2.5% (v/v), had significantly reduced FOL radial growth by 43.4% and was more effective than the chemical fungicide (31.3%) (Fig. 2).

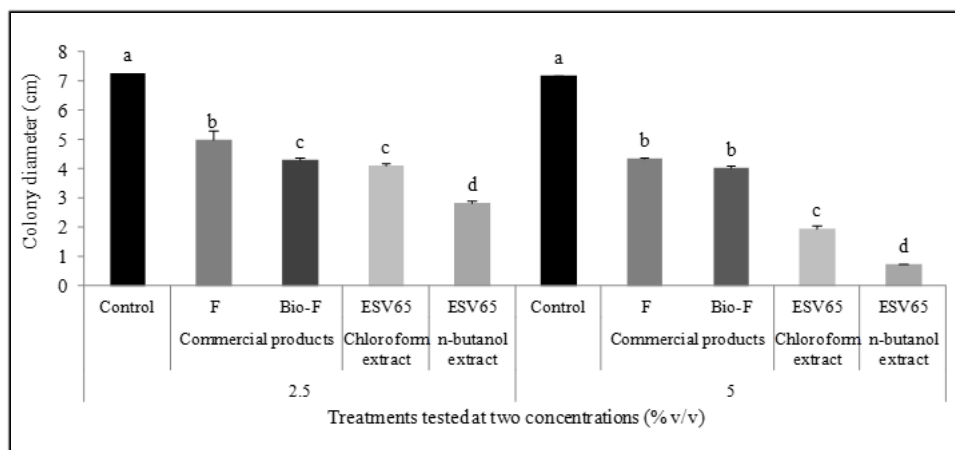


Fig. 2. Effect of chloroform and *n*-butanol extracts from endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 tested at two concentrations on *Fusarium oxysporum* f. sp. *lycopersici* mycelial growth noted after 7 days of incubation at 25°C as compared to positive and negative controls.

ESV65: Organic extract from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 (KR818073) isolated from internal tissues stems of *Solanum nigrum*. Control: Ethanol control. F: Carbendazim used at 2.5 and 5% (v/v); Bio-F: *Bacillus thuringiensis* used at 2.5 and 5% (v/v). LSD (Treatments tested × Concentrations used): 0.74 cm at $P \leq 0.05$. For each concentration, bars sharing the same letter are not significantly different according to Student Newman Keuls test at $P \leq 0.05$.

GC-MS analysis of the *n*-butanol extract from *Bacillus amyloliquefaciens* subsp. *plantarum* SV65.

GC-MS analysis of the most bioactive extract (*n*-butanol) from *B. amyloliquefaciens* subsp. *plantarum* SV65 detected 9 compounds. The peak noted at

the retention time 47.398 min with high percentage area of about 33.2% corresponded to the phthalic acid, mono(2-ethylhexyl)ester (Table 6). The other compounds identified, including 2-methyl butanoic acid, 3-(P-T-butyl)-2-methyl propane-1-thiol, tetradecamethyl-heptasiloxane, tetracosamethyl-

cyclododecasiloxane, *n*-benzyl-*n*-ethyl-4-isopropylbenzamide, pentanoic acid, silicate anion tetramer and 3-isobutylhexahydropyrrolo [1,2-*a*] pyrazine-1,4-dione were detected at 15.47, 10.18, 10.02, 8.76, 6.67, 5.47, 3.55 and 3.69%, respectively (Table 6).

Table 6. Major compounds identified in the *n*-butanol extract from the endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 (KR818073) isolated from *Solanum nigrum* stems by gas chromatography-mass spectrometry analysis

Peak	Retention time (min)	Area (%)	Name
1	3.527	5.47	Pentanoic acid
2	3.711	15.47	2-Methyl butanoic acid
3	34.077	3.55	3-Isobutylhexahydropyrrolo [1,2- <i>a</i>] pyrazine-1,4-dione
4	38.924	3.69	Silicate anion tetramer
5	42.116	6.67	<i>n</i> -Benzyl- <i>n</i> -ethyl-4-isopropylbenzamide
6	45.052	8.76	Tetracosamethyl-cyclododecasiloxane
7	47.398	33.20	Phthalic acid, mono (2-ethylhexyl) ester
8	47.821	10.18	3-(P-T-butyl)-2-methyl propane-1-Thiol
9	51.020	10.02	1,1,3,3,5,5,7,7,9,9,11,11,13,13-Tetradecamethyl - heptasiloxane

Enzymatic activity of endophytic *Bacillus* spp. associated to *Solanum nigrum*.

B. amyloliquefaciens subsp. *plantarum* SV65, *B. methylotrophicus* SV68 and *B. methylotrophicus* SV109 formed clear zones around their colonies when grown on chitin- and pectin-agar medium. This indicates that the three isolates of *Bacillus* spp. tested are able to produce chitinase and pectinase, respectively (Table 7).

Transferred on skim milk-agar medium, clear zones (of 9 and 36.33 mm in diameter) were formed around *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 colonies, respectively. Thus, these two *Bacillus* strains were found to be protease-producing agents. However, *B. methylotrophicus* SV68 cannot produce

protease as no clear zones were observed (Table 7).

Plant growth-promoting traits expressed in *Bacillus* spp. associated to *Solanum nigrum*.

Indole-3-acetic acid production.

When assessed for their ability to produce phytohormones, the three selected *Bacillus* spp. isolates were shown able to produce the indole-3-acetic acid (IAA), involved in plant growth promotion, after 24 and 48 h of incubation (Table 7). This production of IAA rose from 0.11-2.28 µg/ml after 24 h of incubation to 15.48-28.49 µg/ml after 48 h. The highest production of IAA, estimated at 28.49 µg/ml, was recorded after 48 h of incubation using *B. methylotrophicus* SV68.

Phosphate solubilization ability.

Data shown in Table 7 indicated the ability of *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV109 to solubilize phosphate as expressed by the formation of a clear zone of about 9.5 and 10.5 mm in diameter around their colonies, respectively. However, *B. methylotrophicus* SV68 did not produce phosphatase on Pikovskaya agar medium.

Siderophores production.

Regarding their capacity to produce siderophores, *B. amyloliquefaciens* subsp. *plantarum* SV65 grown in CAS agar medium for 5 days showed ability to synthesize these compounds (Table 7) as indicated by the presence of yellow color of about 23.33 mm in diameter around its colonies.

Table 7. Enzymatic activity and plant-growth promoting traits of endophytic *Bacillus* spp. Isolates recovered from *Solanum nigrum* stems

Isolate	Enzymatic activity			PGPB ability		
	Chitinase ^a	Protease ^b	Pectinase ^c	Siderophore production ^d	IAA production ^e	Phosphate solubilization ^f
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> SV65 (KR818073)	+	+	+	+	+ ^v	+ ^y
<i>B. methylotrophicus</i> SV68 (KR818074)	+	-	+	nt	+ ^w	-
<i>B. methylotrophicus</i> SV109 (KR818076)	+	++	+	nt	+ ^x	+ ^z

-: No metabolite production; +: Metabolite production; nt: Not tested.

^aTested on chitin-agar (0.5 % w/v) medium and incubated at 28 ± 2 °C for 72 h.

^bTested on skim milk agar (3 % v/v) medium and incubated at 28 ± 2 °C for 48 h; +: Presence of clear zone (9 mm in diameter), ++: Presence of clear zone (36.33 mm in diameter).

^cTested on pectin-agar (0.5 % w/v) medium and incubated at 28 ± 2 °C for 48 h.

^dTested on Chrome Azurol Sulfonate (CAS) agar medium and incubated at 28 ± 2 °C for 5 days; +: Presence of yellow color: 23.3 mm.

^eIAA (Indole-3-acetic acid) production after 24 and 48 h of incubation at 28 ± 2 °C in Luria-Broth medium; ^{v,w,x}: Production of IAA (2.28 and 18.11, 0.62 and 28.49, 0.11 and 15.48 µg/ml, respectively).

^fTested on Pikovskaya agar medium and incubated at 28 ± 2 °C for 7 days; ^{y,z}: Presence of clear zone (9.5, 10.5 mm in diameter, respectively).

Organic acid production. HPLC analysis performed for root exudates from tomato plants treated with *B. amyloliquefaciens* subsp. *plantarum* SV65 showed the presence of oxalic acid and malic acid (Table 8).

A significant production (about 4448.75 mg/l) of oxalic acid was recorded in the root exudates of plants treated with *B. amyloliquefaciens* subsp. *plantarum*

SV65 as compared to the untreated ones (3587.58 mg/l). The production of malic acid in root exudates of treated tomato plants was of about 18.03 mg/l while no production of this acid was detected in root exudates from untreated control plants. Succinic acid and citric acid were not released by roots of all tomato plants treated or not with *B. amyloliquefaciens* subsp. *plantarum* SV65 (Table 8).

Table 8. Amounts of organic acids detected in root exudates^a of tomato cv. Rio Grande treated with endophytic *Bacillus amyloliquefaciens* subsp. *plantarum* SV65 recovered from *Solanum nigrum* stems compared to the untreated control

Treatment tested	Oxalic acid (mg/l)	Malic acid (mg/l)	Citric acid (mg/l)	Succinic acid (mg/l)
Control	3587.58 ± 126.66	0 ± 0	0	0
<i>B. amyloliquefaciens</i> subsp. <i>plantarum</i> SV65	4448.73* ± 56.24	18.03* ± 4.46	0	0

^a Root exudates were collected after 14 days from tomato plants cultivated in Hoagland solution (50 %). Results are the mean of two high-performance liquid chromatography analyses. SD = Standard deviation. Values with asterisk are significantly different to the control according to test-t of Student at $P \leq 0.05$.

DISCUSSION

Endophytic bacteria have been widely explored as potent biocontrol agents effective against vascular wilt diseases on various crops (Chen et al. 1995; Eleftherios et al. 2004; Ramyabharathi and Raguchander 2014). Furthermore, they were found to be excellent plant growth promoters on several crops such as sugarcane (Marcos et al. 2016) and tomato (Botta et al. 2013).

In the current study, out of 20 isolates recovered from *S. nigrum* and *S. nigrum* var. *villosum* stems, 15 isolates formed endophytic populations on tomato plants cv. Rio Grande. The present study clearly demonstrated the strong Fusarium wilt suppressive ability displayed by three isolates namely SV65, SV68 and SV109. Blast similarity analyses of 16S DNAr sequencing gene revealed that SV65 belonged to *B. amyloliquefaciens* subsp. *plantarum* with 99.6% of similarity compared to 99.45% of similarity to *B. siamensis* noted using Ez-cloud taxon server. Also, based on NCBI database, SV68 and SV109 isolates showed 99.8 and 100% of similarity with *B. methylotrophicus* compared to 99.62 and 99.27% of similarity with *B. velezensis* recorded using Ez-cloud taxon server.

The three selected isolates were also shown to be able to enhance plant growth on tomato plants inoculated or not with FOL. In other studies, various endophytic *Bacillus* species were

recovered from *N. attenuata*, *S. trilobatum* (Bhuvaneswari et al. 2013; Santhanam et al. 2014), *S. melongena* and *S. torvum* (Achari and Ramesh 2014) but they were not tested against FOL nor for their plant growth promoting ability onto tomato plants. In Nawangsih et al. (2011) study, an endophytic *B. amyloliquefaciens* JK-SD002 strain, recovered from tomato stems, was shown to be able to improve height of inoculated tomato plants. Similarly, Algam et al. (2005) found that endophytic *Brevibacillus brevis* B2 and *B. subtilis* (B5, B7 and B8), originally isolated from tomato rhizosphere, had enhanced growth of tomato and had successfully controlled the bacterial wilt disease caused by *Ralstonia solanacearum*. As confirmed through Pearson's correlation analysis, the decrease in Fusarium wilt severity was related to the lowered FOL colonization of vascular tissues leading to the enhancement of plant growth. In the same regards, Ramyabharathi and Raguchander (2014) found that *Bacillus subtilis* EPC016-treated tomato plants grown in presence of FOL showed lowered infection and enhanced growth. In our previous studies, Fusarium wilt-suppressive effect was associated to tomato growth promotion using *Bacillus*, *Stenotrophomonas*, *Pseudomonas* and *Alcaligenes* species recovered from the explored wild Solanaceae plants (Aydi

Ben Abdallah et al. 2016a, 2016b, 2016c, 2016d, 2016e).

The three endophytic *Bacillus* spp. isolates selected in the present study (*B. amyloliquefaciens* subsp. *plantarum* SV65, *B. methylotrophicus* SV68, and *B. methylotrophicus* SV109) were assessed in vitro for their antifungal activity towards FOL. In fact, when tested as whole cell cultures, the three *Bacillus* spp. isolates had inhibited FOL mycelial growth and induced the formation of an inhibition zone. Similar findings were reported for various endophytic *B. mojavensis* isolates inhibiting *F. moniliforme* radial growth through the formation or not of an inhibition zone depending on isolates (Bacon and Hinton 2002). Extracellular metabolites present in the cell-free culture filtrates from *B. amyloliquefaciens* subsp. *plantarum* SV65, *B. methylotrophicus* SV68, and *B. methylotrophicus* SV109 and other *Bacillus* species (*Bacillus* sp. SV101, *B. cereus* S42 and *B. tequilensis* SV104) (Aydi Ben Abdallah et al. 2016b, 2016f) were found to be effective in suppressing FOL in vitro growth. Other studies also reported the inhibitory effects of secondary metabolites from endophytic *Bacillus* spp. being active against *Fusarium* spp. and other plant pathogens (Li et al. 2012).

The inhibitory effect on FOL radial growth may be due in part to the production of hydrolytic enzymes such as chitinases and/or proteases as shown on chitin- and skim milk- agar media. Ability to synthesize hydrolytic enzymes seemed to vary depending on isolates as shown for *B. methylotrophicus* SV68 which failed to produce proteases. Sgroey et al. (2009) also demonstrated variation in protease production between endophytic *Bacillus* species. They found that *B. licheniformis* Ps14 was negative for protease production whereas *B. subtilis* Ps8 and *B. pumilus*

Ps19 were positive. Chitinases may be released by various endophytic *Bacillus* spp. isolates (Berg et al. 2005). However, endophytic *B. velezensis*, *B. mojavensis*, *B. amyloliquefaciens*, and *B. methylotrophicus* isolates recovered from *Citrus* plants were negative for the chitinase activity (Kalai-Grami et al. 2014).

In the present study, we investigated the chemical composition of the most bioactive organic extracts against FOL in order to search for new bioactive metabolites from endophytic *Bacillus* isolates. Chloroform and *n*-butanol extractions were performed for extracellular metabolites present in the cell-free culture of *B. amyloliquefaciens* subsp. *plantarum* SV65. The *n*-butanol extract was found to be more effective than the chloroformic one where FOL growth was inhibited by 61-90% whatever the concentration used. In the same sense, the *n*-butanol extract from endophytic *B. subtilis* ZZ120 (applied at 1 mg/ml) led to 61.4% decrease in *F. graminearum* growth (Li et al. 2012). Identification of the chemical compounds in the *n*-butanol extract from *B. amyloliquefaciens* subsp. *plantarum* SV65 led to the detection of phthalic acid, mono (2-ethylhexyl) ester as major compound. A similar compound was also excreted by an endophytic fungus *Aspergillus flavipes* which displayed antifungal activity against *Sclerotinia sclerotiorum* (Verma et al. 2014). The phthalic acid, bis (2-ethylhexyl) was also produced by *Tsukamurella incheonensis* and *Corynebacterium nitrilophilus* exhibiting antifungal potential toward *Alternaria solani*, *F. oxysporum* and *Penicillium digitatum* (El-Mehalawy et al. 2008). Furthermore, Aydi Ben Abdallah et al. (2016f) reported the presence of phthalic acid dibutyl ester in the chloroform extract from endophytic *B. cereus* S42 isolated from *N. glauca* stems.

Similarly, phthalic acid was also detected in filtrates of an endophytic bacterium (*B. cereus*) isolated from *Azadirachta indica* (Kumar et al. 2015). Indeed, the phthalic acid dibutyl ester and fatty acids are produced by endophytic *B. subtilis* recovered from cotton and exhibiting antifungal activity against FOL (Ramayabharathi and Raguchander 2014). In the current study, the other compounds identified in the *n*-butanol extract from *B. velezensis* SV65 included 2-methyl butanoic acid, 3-(*P*-T-butyl)-2-methyl propane-1-thiol, tetradecamethyl-hepta siloxane, tetracosamethyl-cyclododeca siloxane, *n*-benzyl-*n*-ethyl-4-isopropyl benzamide, pentanoic acid, silicate anion tetramer and 3-isobutylhexahydropyrrolo [1,2-*a*] pyrazine-1,4-dione. These secondary metabolites may be also involved in the antifungal activity recorded towards FOL. In other previous works, *Bacillus* spp. extracts showed the presence of chemical substances belonging to the families of aldehydes, ketones, benzenes (Yuan et al. 2012) and dimethyl disulfide (Huang 2012).

The ability to produce siderophores has been widely reported to be involved in antagonism through competition for iron with phytopathogenic agents during their progression within host tissues (Bacon and Hinton 2011) and/or stimulation of plant growth through iron supply (Bar-Ness et al. 1992). *B. amyloliquefaciens* subsp. *plantarum* SV65 was able to produce siderophores. Several *Bacillus* species including *B. amyloliquefaciens* isolated from three *Citrus* species are also capable to produce siderophores (Kalai-Grami et al. 2014). In addition, *B. tequilensis* has been demonstrated by Verma et al. (2014) as a siderophore-producing agent, by forming a clear zone of 22.5 mm in size compared to 23.3 mm induced by our isolate *B.*

amyloliquefaciens subsp. *plantarum* SV65.

Dastager et al. (2011) found that black pepper seeds treated with *B. tequilensis* NII-0943, a siderophore and IAA producing agent, showed higher root initiation in inoculated plants compared to untreated ones. *B. methylotrophicus* SV109, *B. amyloliquefaciens* subsp. *plantarum* SV65 and *B. methylotrophicus* SV68 selected in the present study as the most active plant growth promoting agents were found able to produce IAA at 15.48, 18.11 and 28.49 µg/ml, respectively. These amounts are higher than those produced by *B. thuringiensis*, *B. subtilis*, *B. arbutinivorans* and *B. fusiformis* (ranging from 4 to 14.46 mg/l) but less than that released by *B. megaterium* i.e. > 50 mg/l (Wang et al. 2013).

Solubilization of bound soil phosphorus and plant supply with phosphate are also involved in plant growth-promoting traits. In the current study, phosphate solubilization ability was confirmed for *B. methylotrophicus* SV109 and *B. amyloliquefaciens* subsp. *plantarum* SV65. Some endophytic bacteria recovered from *Citrus* species namely *B. methylotrophicus*, *B. velezensis* and *B. mojavensis* were also able to solubilize phosphate (Kalai-Grami et al. 2014).

The three selected endophytic *Bacillus* spp. isolates were shown to be able to produce pectinase and this potential may be also involved in the recorded enhancement of tomato growth as reported by Baldan et al. (2003) and in the endophytic colonization of host plant (Hallmann et al. 1997). It should be also mentioned that pectinolytic enzymes act normally as virulence factors for plant pathogenic bacteria but in case of endophytic microorganisms, they might play a role in invasion of host plants by endophytes as demonstrated for *B. cereus*,

B. subtilis and *B. stearothermophilus* (Torimiro and Okonji 2013).

Other substances are detected in root exudates such as organic acids, sugars and amino acid. These allelochemicals may be involved in the promotion of plant growth (Kamilova et al. 2006). Composition of root exudates induced chemotactic responses for endophytic bacteria for early colonization of plant tissues (Bacilio-Jiménez et al. 2003). In the current study, chemical analysis of root exudates from tomato plants treated by *B. amyloliquefaciens* subsp. *plantarum* SV65 revealed the presence of oxalic acid and malic acid which may play a key role in plant colonization. In fact, chemotaxis through root exudates such as malic acid and citric acid is crucial for the colonization of tomato roots by *Pseudomonas* (De Weert et al. 2007). Yuan et al. (2015) assessed the role of banana root exudates (especially oxalic acid, malic acid and fumaric acid) in the colonization of *B. amyloliquefaciens* NJN-6.

In conclusion, *S. nigrum* and *S. nigrum* var. *villosum* were potentially important sources for isolation of endophytic bacteria serving as biocontrol and biofertilizing agents in the production of tomato on both FOL infested and non-infested soils. The most effective agents were identified as *B. amyloliquefaciens* subsp. *plantarum* SV65 (KR818073), *B. methylotrophicus* SV68 (KR818074) and *B. methylotrophicus* SV109 (KR818076).

These isolates displayed interesting enzymatic activity. Their plant growth-promoting traits and colonization ability were achieved through IAA and siderophores production, phosphate solubilization, and release of organic acids in root exudates. *B. amyloliquefaciens* subsp. *plantarum* SV65, recovered from *S. nigrum* stems, was considered as a novel biologically source of phthalic acid, mono (2-ethylhexyl) ester. The extracellular metabolites from these *Bacillus* spp. isolates will be further tested for their in vivo antifungal activity against FOL and probably their ability to induce systemic resistance and to promote growth.

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RESUME

Aydi-Ben Abdallah R., Jabnoun-Khiareddine H., Nefzi A., Ayed F. et Daami-Remadi, M. 2018. Des bactéries endophytes issues de *Solanum nigrum* ayant des capacités promotrices de la croissance des plantes et suppressives de la fusariose vasculaire de la tomate. Tunisian Journal of Plant Protection 13 (2): 157-182.

Quinze isolats bactériens endophytes issus des tiges de *Solanum nigrum* et *S. nigrum* var. *villosum* ont été criblés pour leur potentiel promoteur de la croissance et leur activité antifongique contre *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Les isolats SV65, SV68 et SV109 se sont montrés les plus efficaces dans le contrôle de la maladie (77-92%) et dans l'amélioration de la croissance de la tomate (32-62%)

comparés aux témoins. Ils ont été caractérisés et identifiés après séquençage de leurs gènes d'ADNr 16S comme étant *Bacillus amyloliquefaciens* subsp. *plantarum* pour l'isolat SV65 (KR818073), *B. methylotrophicus* pour les deux isolats SV68 (KR818074) et SV109 (KR818076). L'analyse par chromatographie en phase gazeuse et spectrométrie de masse de l'extrait butanolique de *B. amyloliquefaciens* subsp. *plantarum* SV65 a montré que l'ester mono (2-éthylhexyl) de l'acide phtalique est un composé majeur. Les bactéries *B. amyloliquefaciens* subsp. *plantarum* SV65 et *B. methylotrophicus* SV109 se sont révélés être des agents productifs des chitinases, des protéases, des pectinases, des phosphatases et de l'acide indole 3-acétique (AIA). *B. methylotrophicus* SV68 a produit des chitinases, des pectinases et de l'AIA (28,49 µg/ml), et *B. amyloliquefaciens* subsp. *plantarum* SV65 a excrété des sidérophores, de l'acide oxalique et de l'aide malique. Cette étude a démontré que *S. nigrum* et *S. nigrum* var. *villosum* peut être de bonne espèce de plante pour l'isolement de bactéries endophytes servant d'agents de lutte biologique et de biofertilisant pour l'amélioration de la croissance et la production de tomates cultivées dans des sols infestés et non infestés par FOL.

Mots-clés: *Bacillus* spp., biocontrôle, *Fusarium oxysporum* f. sp. *lycopersici*, *Solanum nigrum*, métabolites, croissance de la tomate

ملخص

العابدي-بن عبد الله، رانية وهيفاء جبنون-خيار الدين وأحلام النفزي وفاخر عياد وماجدة الدعيمي-الرمادي. 2018. بكتيريا داخلية نباتية مجمعة من نبتة *Solanum nigrum* ذات قدرة على تحثيث نمو النباتات وعلى الحد من الذبول الفوزاري على الطماطم. *Tunisian Journal of Plant Protection* 13 (2): 157-182.

تم تقييم قدرة خمسة عشر عزلة بكتيرية داخلية نباتية، مجمعة من جذوع نبتتي *Solanum nigrum* و *S. nigrum* var. *villosum*، على تحثيث نمو النباتات وعلى مكافحة الحيوية ضد *Fusarium oxysporum* f. sp. *lycopersici* (FOL). كانت العزلات SV65، SV68 و SV109 الأكثر فعالية في السيطرة على المرض (92-77%) وتحثيث نمو الطماطم (62-32%) مقارنة بالشواهد. تم تشخيص هذه العزلات وتم تحديد انتمائها باستخدام تسلسل جينات الحمض النووي 16S إلى *Bacillus amyloliquefaciens* subsp. *plantarum* للعزلة SV65 (KR818073) و *B. methylotrophicus* للعزلات SV68 (KR818074) و SV109 (KR818076). وأظهر التحليل الكروماتوغرافي الغازي ومطياف الكتلة للمستخلص البيتانولي من *B. amyloliquefaciens* subsp. *plantarum* SV65 أن ester mono (2-éthylhexyl) من حمض الفثاليك هو من أكثر المركبات. تمكنت *B. amyloliquefaciens* subsp. *plantarum* SV65 و *B. methylotrophicus* SV109 من إنتاج الكايتينات والبروتينات والبكتيناز والفوسفاتاز وحمض الأنندول-3-أساتيك. أظهرت العزلة *B. methylotrophicus* SV68 قدرتها على إنتاج الكايتينات والبكتيناز وحمض الأنندول-3-أساتيك (28،49 مكغ/مل) والعزلة *B. velezensis* SV65 على إنتاج السيداروفور وحمض الأكساليك وحمض الماليك. بينت هذه الدراسة أن نبتتي *S. nigrum* و *S. nigrum* var. *villosum* يمكن أن تكونا نوعا من النباتات الجيدة لعزل البكتيريات الداخلية النباتية بمثابة عناصر للمكافحة البيولوجية والتسميد الحيوي الفعالة في تحثيث نمو وإنتاج الطماطم المزروعة في التربة السليمة والملوثة بالفطر FOL.

كلمات مفتاحية: إفرازات، طماطم، مكافحة حيوية، نمو، *Bacillus* spp.، *Fusarium oxysporum* f. sp. *lycopersici*، *Solanum nigrum*

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Effect of soil management on biodiversity of nematode communities as a biological indicator of soil quality in oasis agro-ecosystem of Kebili

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ABSTRACT

Larayedh-Bettaieb, A., Hajji-Hedfi, L., Sanchèz-Moreno, S., Chihani-Hammas, N., Regaieg, H., Aoun, F., Horrigue-Raouani, N., and Belkadhi, M.S. 2018. Effect of soil management on biodiversity of nematode communities as a biological indicator of soil quality in oasis agro-ecosystem of Kebili. Tunisian Journal of Plant Protection 13 (2): 183-200.

Nematode communities were monitored in 26 oases in Kebili under various agricultural systems. Differences between studied oases consisted in tillage frequency, soil amendment type (manure or manure+ mineral fertilizers), cover crops and field age. In addition, this study evaluated the importance of the C-P (Colonizer-Persistent) triangle and the faunal profile (representation of enrichment index vs. structure index) as a biological indicator and monitoring tools in support of soil quality assessment. The results showed that nematode communities were composed by 10 bacterial feeders (Ba), 3 fungal feeders (Fu), 12 plant parasites (PP), 4 omnivores (O), 1 predator (P) with the dominance of (Ba) and (PP) in all surveyed oases. The nematode communities differed slightly depending on oases age. Bacterial feeders and *Discolaimium* genus were found in both young and old ones. *Plectus* genus (Ba) was found only in young oases while *Xiphinema*, *Criconema* and *Trichodorus* genera (PP) were absent in these oases. Few nematode taxa were affected by soil amendment type and cover crops including some bacterial and fungal feeders. The highest taxa richness was recorded in bare soils and in field with tillage frequency of 2 or 3 years. The lower MI (Maturity index) value was recorded in old oases. Most of studied oases were characterized either by a high soil disturbance level with a high abundance of cp-1 group (Bacterivore

nematodes with c-p value =1) as an indicator of a disturbed food web or by a stressed soil with high abundance of cp-2 group (Bacterivore and fungivore nematodes with c-p value = 2) as an indicator of degraded food web. Only few sites showed a maturing and structured food webs with respectively low to moderate soil disturbance level and undisturbed soil. This study highlighted also that some nematode genera may potentially serve as differential bio-indicators of soil disturbance.

Key words: Agricultural system, biodiversity, date palm, ecological indices, nematode community, oases, soil

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Nematodes are multicellular animals found in all soil and aquatic systems in any environment that provides a source of organic carbon, under all climatic conditions, and in all habitats (Bongers and Ferris 1999). They are playing major roles in component process of ecosystem services. Their populations respond rapidly to disturbance and enrichment and individuals do not rapidly migrate from stressful conditions. In soil and aquatic food webs, nematodes are involved in the transformation of organic matter into mineral and organic nutrients which are available to plants and enhance plant growth and crop productivity (Ferris et al. 2004).

Nematodes are abundant, ubiquitous and diverse, participate in several soil food web links and are sensitive to agricultural disturbance (Yeates 2003). Both in natural areas and under experimental conditions, nematode assemblages are used to assess the effects of pollution (Korthals et al 1996), and as indicators of soil enrichment and disturbance (Berkelmans et al. 2003; Bongers 1990; Ferris et al. 2001). The composition and structure of terrestrial nematode communities vary among ecosystems because of contrasting plant composition and phenology, soil

properties and microclimates (Neher 2010).

In agricultural fields, nematode abundance and diversity are used to infer soil process rates (Ettema et al 1998), soil functions and effects of disturbance on soil fauna (Wardle et al 1995). According to Neher (2001), composition and abundance of the nematode fauna have been used as soil health indicators in many different environments, and it was also reported by Ettema et al. (1998), that the tight relationships between soil characteristics and abundance of nematode in different functional guilds have been used to develop soil assessment criteria. Among soil organisms, nematodes possess several attributes that make them good indicators.

In comparison to many natural ecosystems, the agro-ecosystems receive numerous human actions that may cause serious fauna and flora disruption. Practices such as soil preparation and over use of fertilizers and pesticides can result in a decrease in soil microbial diversity (Timper 2014). In oases, cropping system is conducted under 3 stages of cultures where various fruit trees, vegetables and forage species are cultivated usually in association with date palm. The management of such agro-ecosystem is characterized by frequent soil tillage, a high organic matter (manure) input and the

use of some fertilizers for the growth of date palm associated forage cultures. Thus, the special microclimate, the different agricultural techniques and diversification of host plant species incite the multiplication of many groups of soil nematode populations. Since nematodes are considered as organisms with an appropriate bio-indication potential and relatively good traceable reactions to different changes in the environment Neher (2001) and several researchers described bio-indication of different impacts by nematodes (Pen-Mouratov et al. 2010; Sanchez-Moreno and Navas 2007), we were interested to study the relationship between nematode community structure and different agricultural practices through several ecological indices relative to soil

nematode assemblages in some oases of southern Tunisia.

MATERIALS AND METHODS

Soil sampling.

During October 2015, among 13 localities in Kebili (Fig.1), 2 oases of 1 ha of surface (Old and New in the context of age) were randomly chosen from each locality, and 6 individual date palms were sampled from each oasis to have 156 soil samples in total. The sampling from date palm rhizosphere was carried out with a hand auger (5 cm inside diameter) at 60 cm from the palm trunk and at 50 cm of depth from the top layer of bulk soil after removing surface residues. Each sample was then placed in a plastic bag, labeled and stored in a cold room at 4 °C until processed.



Fig. 1. Geographical localization of the study sites in Kebili, Southern Tunisia

Nematode community analysis.

Soil nematodes were extracted from fresh soil by sieving and Baermann funnel method (Barker et al. 1985). Nematode abundance (N) was expressed as the total individual number per 100 g of dry soil. Collected nematodes from each sample were counted at stereomicroscope (50× magnification). Nematodes were mounted on temporary slides and identified at higher magnification (microscope) to genus or family level using key of Bongers (1988). Nematode taxa were classified into five main trophic groups: bacterial-feeders (Ba), fungal-feeders (Fu), plant parasites/herbivores (PP), omnivores (O), and predators (P) (Yeates et al. 1993).

In addition, they were classified along the colonizer-persister (cp) scale according to Bongers (1990). The cp scale comprises five groups of nematode families, namely microbial feeders with short life cycles and high reproduction rates (cp 1 and cp 2) and predators and omnivores with long life cycles, low reproduction rates (cp 3) and those which are very sensitive to environmental perturbations (cp 4 and cp 5). Taxa richness (S) was expressed as the average number of taxa in each sample.

Ecological indices.

Several ecological indices were calculated including the Maturity Index (MI) and the Plant-Parasitic Index (PPI) (Bongers 1990) as a weighted means of the relative contribution of each cp group to the assemblage of free-living nematodes (MI) and of plant parasitic nematodes (PPI), respectively.

Additionally, four soil food web indices were calculated: (i) The Structure Index (SI), a weighted measure of the proportion of sensitive predator and omnivore nematodes, which is a sensitive indicator of soil food web complexity; (ii)

The Channel Index (CI), based on the ratio of fungivore to bacterivore nematodes, which is an indicator of the prevalence of organic matter decomposition mediated by fungi; (iii) The Basal Index (BI), based on the abundance of general opportunistic nematodes, which is an indicator of basal, perturbed soil food web condition; and (iv) the Enrichment Index (EI), based on the abundance of enrichment opportunistic nematodes, which is an indicator of rapid, bacterial mediated organic matter decomposition. A cp triangle and a graphical representation of the SI vs. the EI allows for the diagnosis of the soil food web as disturbed, maturing, structured, or degraded (Ferris et al. 2001).

Index calculation, the cp triangle and the faunal profile were realized using the NINJA internet tool (<https://sieriebriennikov.shinyapps.io/ninja/>) (Sieriebriennikov et al. 2014).

Data analyses.

Principal component analysis PCA was performed by STATISTICA software package (StatSoft 1996).

RESULTS

Nematode community composition.

Thirty nematode taxa (10 bacterivores, 3 fungivores, 12 Plant parasites, 4 omnivores, 1 predator) were identified in the study fields. Per trophic group, the most abundant nematode taxa were Rhabditidae, *Acrobeloides* and *Panagrolaimus* in bacterial feeder group (Ba), *Aphelenchus* and *Aphelenchoides* in fungal feeder group (Fu), Tylenchidae, *Pratylenchus*, *Meloidogyne*, and *Tylenchorhynchus* in plant parasitic nematode group (PP), Quedsinematidae and Aporcelaimidae in omnivore nematode group (O) and *Discolaimium* in predator nematode group (P) (Table 1). Nematode populations identified from studied soils were dominated by bacterial

feeder group and plant parasitic group. Nematode abundance (N) was about 497 (± 444) individuals/100g of dry soil with a mean of 13 (± 2) taxa/sample. All bacterial feeder (Ba) taxa were found in both old and young oases except *Plectus* which is only identified in some young oases (Table 1).

For fungal feeders, all taxa were identified in both old and young oases. Some plant parasitic nematodes like *Xiphinema*, *Criconea* and *Trichodorus* were absent in young oases, while *Helicotylenchus* was absent from old oases. From omnivore group, only Aporcelaimidae and Quedsinematidae families were identified in old oases while other omnivore taxa like *Mesodorylaimus* and *Eudorylaimus* were found also in young oases (Table 1). *Discolaimium*, the only representing genus for predator group in this study was identified in both old and young oases (Table 1).

Effects of agricultural practices on nematode diversity.

Table 2 shows total taxa richness in fields with different managements. Sampled bare soils showed the higher S value with 28 taxa in comparison to only 23 taxa present in cover-cropped soils. In conventional system (manure + mineral fertilizers) taxa richness was higher (27 taxa) than in organic system (manure) (25 taxa).

Plectus, *Xiphinema*, *Mesodorylaimus* and *Discolaimium* were absent from soils where only manure is used. *Pratylenchoides*, *Criconea* and *Eudorylaimus* were not identified in oases where both manure and mineral fertilisers are incorporated to the soil. In bare studied soils, only *Trichodorus* and *Eudorylaimus* were not found, while other taxa like *Telotylenchus*, *Heterodera*, *Xiphinema*, *Criconea*, *Mesodorylaimus* and

Discolaimium were absent in cover-cropped (alfalfa or barley) soils.

The less taxa richness S values 17 and 20 were relative to soils tilled each year and in recently tilled soils. *Diploscapter*, *Plectus*, *Wilsonema*, *Prismatolaimus*, *Rhabdolaimus*, *Criconea*, *Trichodorus*, *Mesodorylaimus*, *Eudorylaimus* and *Discolaimium* were all absent from fields tilled each year. The highest taxa richness values 27 and 28 were relative to oases where tillage frequency is about 2 or 3 years and in soils tilled 1 year ago before sampling date where only *Trichodorus* and *Xiphinema* were absent (Table 3).

Food web indices in relation with soil management.

It is also obvious that the presence of cover crops associated with date palm trees have decreased nematode abundance in studied fields. Data from Table 4 shows that N was higher (522 ± 500 individuals/100g of dry soil) in bare soils than in cultivated soils (414 ± 110 individuals/100g of dry soil).

Soil tillage has also negatively affected nematode abundance (N) in recently tilled soils and in soils usually tilled each year where this index has the lower values with respectively only 315 and 337 individuals/100g of dry soil. However, in oases where soil is tilled usually each 2 years and only manure is used, nematode abundance showed the highest levels, respectively 567 and 602 individuals/100 g of dry soil. Additionally, in old oases and in oases where soil is tilled just one year before sampling date, nematode abundance showed also higher values, 553 and 534 respectively (Table 4).

The lowest MI value (1.79) was recorded in old oases; this is an indication of the high soil disturbance level. Higher MI values of 2 and 2.09 were registered respectively in young oases and in fields

with cover crops in association with date palm. In soils tilled each year or 2 years and in soils tilled just 1 year before

sampling date, this index showed also high values respectively 1.97, 1.93 and 1.94.

Table 1. Distribution and abundance of different nematode taxa identified from prospected oases

Taxa or Family	Feeding type	cp value	N	SD	Old	Young
Rhabditidae	Ba	1	88.13	±219.37	+	+
<i>Panagrolaimus</i>	Ba	1	32.82	±35.01	+	+
<i>Diploscapter</i>	Ba	2	5.21	±19.31	+	+
<i>Acrobels</i>	Ba	2	22.78	±25.98	+	+
<i>Acrobeloides</i>	Ba	2	68.15	±46.35	+	+
<i>Cervidellus</i>	Ba	2	13.79	±33.38	+	+
<i>Plectus</i>	Ba	2	0.2	±0.74	-	+
<i>Wilsonema</i>	Ba	2	1.09	±3.055	+	+
<i>Prismatolaimus</i>	Ba	3	4.06	±11.42	+	+
<i>Rhabdolaimus</i>	Ba	3	2.85	±5.52	+	+
<i>Aphelenchus</i>	Fu	2	55.61	±91.97	+	+
<i>Aphelenchoides</i>	Fu	2	21.58	±30.77	+	+
<i>Tylencholaimus</i>	Fu	4	3.25	±12.30	+	+
<i>Pratylenchus</i>	PP	3	74.64	±71.47	+	+
<i>Pratylenchoides</i>	PP	3	0.55	±1.58	+	+
Tylenchidae	PP	2	53.95	±68.19	+	+
<i>Tylenchorhynchus</i>	PP	3	11.75	±17.26	+	+
<i>Telotylenchus</i>	PP	2	1.52	±3.49	+	+
<i>Rotylenchus</i>	PP	3	6.19	±12.40	+	+
<i>Helicotylenchus</i>	PP	3	0.28	±1.21	-	+
<i>Heterodera</i>	PP	3	0.34	±1.098	+	+
<i>Meloidogyne</i>	PP	3	16.44	±30.28	+	+
<i>Xiphinema</i>	PP	5	0.074	±0.36	+	-
<i>Criconema</i>	PP	3	0.2	±0.58	+	-
<i>Trichodorus</i>	PP	4	0.053	±0.26	+	-
Quedsinematidae	O	4	4.27	±6.30	+	+
Aporcelaimidae	O	5	4.72	±5.49	+	+
<i>Mesodorylaimus</i>	O	4	0.41	±1.85	-	+
<i>Eudorylaimus</i>	O	4	0.06	±0.28	-	+
<i>Discolaimium</i>	P	5	0.45	±1.46	+	+
					S = 26	S = 27

Presence or absence of each taxa are indicated by + or -; Ba: bacterial feeder group; Fu: fungal feeder group; PP: plant parasitic nematode group; O: omnivore nematode group; P: predator nematode group; S: taxa richness; N: nematodes/100g dry soil; Old: more than 50 years; Young: less than 15 years

Table 2. Effect of soil amendment and cover crops on structure and taxa richness within oasis nematode assemblages

Taxa or Family	N ± SD	Soil amendment		Cover crops	
				0	1
Rhabditidae	88.13	±219.37	+	+	+
<i>Panagrolaimus</i>	32.82	±35.01	+	+	+
<i>diploscapter</i>	5.21	±19.31	+	+	+
<i>Acrobels</i>	22.78	±25.98	+	+	+
<i>Acrobelloides</i>	68.15	±46.35	+	+	+
<i>Cervidellus</i>	13.79	±33.38	+	+	+
<i>Plectus</i>	0.2	±0.74	-	+	-
<i>Wilsonema</i>	1.09	±3.055	+	+	+
<i>Prismatolaimus</i>	4.06	±11.42	+	+	+
<i>Rhabdolaimus</i>	2.85	±5.52	+	+	+
<i>Aphelenchus</i>	55.61	±91.97	+	+	+
<i>Aphelenchoides</i>	21.58	±30.77	+	+	+
<i>Tylencholaimus</i>	3.25	±12.30	+	+	+
<i>Pratylenchus</i>	74.64	±71.47	+	+	+
<i>Pratylenchoides</i>	0.55	±1.58	+	-	+
Tylenchidae	53.95	±68.19	+	+	+
<i>Tylenchorhynchus</i>	11.75	±17.26	+	+	+
<i>Tyleotylenchus</i>	1.52	±3.49	+	+	-
<i>Rotylenchus</i>	6.19	±12.40	+	+	+
<i>Helicotylenchus</i>	0.28	±1.21	-	+	+
<i>Heterodera</i>	0.34	±1.098	+	+	-
<i>Meloidogyne</i>	16.44	±30.28	+	+	+
<i>Xiphinema</i>	0.074	±0.36	-	+	-
<i>Criconema</i>	0.2	±0.58	+	-	-
<i>Trichodorus</i>	0.053	±0.26	+	+	-
Quedsinematidae	4.27	±6.30	+	+	+
Aporcelaimidae	4.72	±5.49	+	+	+
<i>Mesodorylaimus</i>	0.41	±1.85	-	+	-
<i>Eudorylaimus</i>	0.06	±0.28	+	-	+
<i>Discolaimium</i>	0.45	±1.46	-	+	-
		S = 25	S = 27	S = 28	S = 23

0: bare soil, 1: cultivated soil (barley or alfalfa); Presence or absence of each taxa are indicated by + or -; N: nematodes/100g dry soil; S: taxa richness.

Table 3. Effect of soil tillage frequency and the last tillage before sampling on structure and taxa richness within oasis nematode assemblages

Taxa or Family	N \pm SD	Tillage frequency			Last tillage		
		1 Y	2 Y	3 Y	Recent	1 Y	2 Y
Rhabditidae	88.13 \pm 219.37	+	+	+	+	+	+
<i>Panagrolaimus</i>	32.82 \pm 35.01	+	+	+	+	+	+
<i>Diploscapter</i>	5.21 \pm 19.31	-	+	+	-	+	+
<i>Acrobels</i>	22.78 \pm 25.98	+	+	+	+	+	+
<i>Acrobelloides</i>	68.15 \pm 46.35	+	+	+	+	+	+
<i>Cervidellus</i>	13.79 \pm 33.38	+	+	+	+	+	+
<i>Plectus</i>	0.2 \pm 0.74	-	+	+	-	+	+
<i>Wilsonema</i>	1.09 \pm 3.055	-	+	+	+	+	+
<i>Prismatolaimus</i>	4.06 \pm 11.42	-	+	+	+	+	+
<i>Rhabdolaimus</i>	2.85 \pm 5.52	-	+	+	+	+	+
<i>Aphelenchus</i>	55.61 \pm 91.97	+	+	+	+	+	+
<i>Aphelenchoides</i>	21.58 \pm 30.77	+	+	+	+	+	+
<i>Tylencholaimus</i>	3.25 \pm 12.30	+	+	+	-	+	+
<i>Pratylenchus</i>	74.64 \pm 71.47	+	+	+	+	+	+
<i>Pratylenchoides</i>	0.55 \pm 1.58	-	+	-	+	+	-
Tylenchidae	53.95 \pm 68.19	+	+	+	+	+	+
<i>Tylenchorhynchus</i>	11.75 \pm 17.26	+	+	+	+	+	+
<i>Tyleotylenchus</i>	1.52 \pm 3.49	+	+	+	-	+	+
<i>Rotylenchus</i>	6.19 \pm 12.40	+	+	+	+	+	+
<i>Helicotylenchus</i>	0.28 \pm 1.21	-	-	+	-	+	-
<i>Heterodera</i>	0.34 \pm 1.098	+	+	+	-	+	-
<i>Meloidogyne</i>	16.44 \pm 30.28	+	+	+	+	+	+
<i>Xiphinema</i>	0.074 \pm 0.36	-	-	+	-	-	+
<i>Criconea</i>	0.2 \pm 0.58	-	+	+	+	+	+
<i>Trichodorus</i>	0.053 \pm 0.26	-	+	-	+	-	-
Quedsinematidae	4.27 \pm 6.30	+	+	+	+	+	+
Aporcelaimidae	4.72 \pm 5.49	+	+	+	+	+	+
<i>Mesodorylaimus</i>	0.41 \pm 1.85	-	-	+	-	+	-
<i>Eudorylaimus</i>	0.06 \pm 0.28	-	+	-	-	+	-
<i>Discolaimium</i>	0.45 \pm 1.46	-	+	+	-	+	+
		S = 17	S = 27	S = 27	S = 20	S = 28	S = 24

S: Taxa richness; Y: year; Tillage frequency = 1Y: each 1 year, 2Y: each 2 years, 3Y: each 3 years; Last tillage = Recent: same year of sampling, 1 Y: since 1 year, 2 Y: since 2 years; N: nematodes/100g dry soil)

The MI 2-5 is an indicator of more stable environment as nematodes with higher cp values (more sensitive to soil disturbance) are more abundant in the soil sample. This index has the higher

values, 2.34 and 2.35 in respectively young oases and in oases where cover crops are associated with date palm. The less index value 2.13 was found in old oases. In oases where soil is recently tilled,

where soil is usually tilled each 1 year and where only manure is incorporated to soil, the MI (2-5) has relatively low values, 2.17, 2.16 and 2.18 respectively.

Among studied fields (Table 4), oases where only manure is used and where soil tillage frequency is about 2 years, the higher CI values, 29.47 and 28.7 respectively, were recorded as an indicator of fungal organic matter decomposition in these fields. However, the lower CI value was relative to young oases (15.86), to soil where both manure and chemical fertilizers are used (17.36) and to recently tilled soils (17.6). In these fields, it seems that organic matter decomposition was dominated by bacteria. The basal index (BI) is based on the abundance of general opportunistic nematodes predominantly bacterial feeder (Ba) with cp = 2 in the Cephalobidae family and fungal feeders (Fu) with cp = 2 in the Aphelenchidae, Aphelenchoididae families. It is an indicator of basal, perturbed soil food web condition. High BI values indicate a nematode assemblage composed of perturbation-resistant nematodes mainly of lower trophic levels. This index, an indicator of disturbed soil food webs, was higher in oases where soil is tilled each year and where soil was recently tilled. The community structure was, in this case, associated with fungal-dominated organic matter decomposition pathways (Table 4). The EI is based on the weighted abundance of bacterial feeding nematodes enrichment-opportunistic, indicating highly active bacterial-mediated decomposition channels. Higher values were relative to soils tilled each 3 years and to soils tilled 2 years before sampling (68.59 and 66.48, respectively) (Table 4). It showed also high values, 65.9 and 65.03 respectively, in old oases and in oases with no cover crops associated with date palm. The SI is an indicator of soil food web length and connectance, the indicator

guilds of a structured soil food web include the (P) Discolaimidae and (O) Quedsinematidae (cp 5) (often considered larger Dorylaimida). Nematodes in these guilds are large-bodied, and have the lowest fecundity and longest life courses of soil nematodes. They are susceptible to soil disturbance and are often absent from disturbed, polluted, or intensely-managed environments (Bongers 1990; Bongers 1999).

In studied fields, the higher SI values, 43.12 and 42.89, revealing a structured food web where resources are more abundant or where recovery from stress is occurring, were recorded respectively in young oases and in oases with cover crops in association with date palm. However, in old oases and in soils where only manure was incorporated to soil, the SI showed the lowest values, 21.07 and 23.07 respectively (Table 4).

In Fig. 2, results from PCA show that nematode abundance (N) and EI are positively correlated with bare soils and with soil tilled each 3 years. Thus, the increase in nematode abundance in corresponding oases is explained by an increase in enrichment opportunist group (especially cp-1 group).

BI and CI are correlated with old oases, organic fertilizers (manure) and with soils tilled 2 years before sampling date, indicating then fungal organic matter decomposition. However, the MI (2-5) and the SI are correlated with young oases and mineral fertilizers and with soils tilled 1 year or 2 years before sampling date; in these fields food web is more structured and matured. Taxa richness (S), PPI and MI are correlated with cover cropped soils, with soils recently tilled and with soils usually tilled each 1 or 2 years (Fig. 2). The positive correlation between PPI and S reveals that the high taxa richness within studied nematode communities is reported to the high diversity in PPN population.

Table. 4: Overview on means \pm SD for N (nematodes/100g dry soil), MI, MI (2-5), PPI and soil food web index values discriminated based on oasis age and soil management

Oasis Characteristic		N	MI	MI (2-5)	PPI	BI	CI	EI	SI
Field age	Old	553.08 \pm	1.79 \pm	2.13 \pm	2.63 \pm	30.95 \pm	26.11 \pm	65.9 \pm	21.07 \pm
		632.62	0.25	0.10	0.24	14.81	19.98	16.12	12.05
	Young	444.46 \pm	2 \pm	2.34 \pm	2.76 \pm	26.96 \pm	15.86 \pm	62.37 \pm	43.12 \pm
		142.29	0.35	0.22	0.21	11.95	15.93	17.77	20.01
Cover crops	0	522.03 \pm	1.85 \pm	2.2 \pm	2.67 \pm	29.67 \pm	21.85 \pm	65.03 \pm	28.25 \pm
		500.67	0.30	0.16	0.23	14.88	20.11	17.28	18.73
	1	414.92 \pm	2.09 \pm	2.35 \pm	2.7 \pm	30.99 \pm	22.54 \pm	56.67 \pm	42.89 \pm
		110.55	0.32	0.26	0.27	12.51	16.55	16.36	20.47
Fertilizers	Organic	602.87 \pm	1.88 \pm	2.18 \pm	2.62 \pm	31.92 \pm	29.47 \pm	61.16 \pm	23.07 \pm
		677.70	0.39	0.24	0.19	15.49	21.93	18.72	19.02
	Mineral+ organic	431.33 \pm	1.92 \pm	2.27 \pm	2.72 \pm	28.75 \pm	17.36 \pm	64.31 \pm	36.98 \pm
		151.20	0.26	0.16	0.26	13.50	15.86	16.46	18.90
Tillage frequency	1 Y	337.71 \pm	1.97 \pm	2.16 \pm	2.74 \pm	40.31 \pm	25.82 \pm	52.95 \pm	25.7 \pm
		201.19	0.050	0.021	0.17	4.81	11.99	6.54	2.63
	2 Y	567.55 \pm	1.93 \pm	2.23 \pm	2.75 \pm	29.87 \pm	21.45 \pm	61.22 \pm	29.82 \pm
		584.74	0.40	0.24	0.20	13.52	16.70	17.70	21.24
	3 Y	453.88 \pm	1.84 \pm	2.25 \pm	2.58 \pm	27.01 \pm	21.6 \pm	68.59 \pm	35.76 \pm
		203.44	0.22	0.16	0.27	15.88	23.72	17.45	20.78
Last Tillage	Recent	315.86 \pm	1.89 \pm	2.17 \pm	2.86 \pm	36.31 \pm	17.6 \pm	58.18 \pm	27.26 \pm
		135.60	0.12	0.06	0.058	11.59	8.51	13.66	7.53
	1 Y	534.47 \pm	1.94 \pm	2.26 \pm	2.73 \pm	28.82 \pm	19.91 \pm	62.55 \pm	33.45 \pm
		542.26	0.37	0.22	0.23	13.52	19.78	17.58	21.70
	2 Y	490.15 \pm	1.82 \pm	2.2 \pm	2.49 \pm	29.88 \pm	28.7 \pm	66.48 \pm	29.33 \pm
		192.16	0.24	0.16	0.20	16.50	20.05	17.88	19.53

BI: Basal index, CI: Channel index, EI: Enrichment index, SI: Structure index, Y: year; N: nematodes/100g dry soil, MI: Maturity Index, PPI: Plant Parasitic Index.

cp triangle and faunal profile for studied nematode communities.

The classification of nematode assemblages identified in studied oases into the cp scale allows the construction of the cp triangle (Fig.3). To just depict the proportional representation of un-weighted cp-1, cp-2 and cp3-5 taxa, oases were only designated by locality name and no characteristics or agricultural management were used to discriminate them in this case. This figure reveals that most of studied oases regardless any effect of soil management type had relatively stressed soils with a high abundance of cp-

1 group as an indicator of disturbed food web and few oases were characterized by a stable food web where taxa belonging to cp3-5 (susceptible to soil disturbance) were more abundant.

The faunal profile (Fig. 4) is constructed to indicate whether the soil community is basal (and inferred stressed), enriched, or structured and stable. The weighting system allows separation of the condition of food webs at different sites or at different times, based on shifts in presence and abundance of nematode taxa, with greater resolution than through the use of un-weighted cp triangles. The

faunal profile in Fig. 4 representing the soil food web condition through the projection of SI vs EI, shows that among 26 studied oases, most oases had either disturbed soil (Quadrat A) relative to cp-1 group, N-enriched, with a low C/N and a degraded or depleted soil (Quadrat D) relative to cp-2 group with high C/N. Other oases were characterized by a maturing food web with N-enriched soil where the C/N was low and organic matter

decomposition was bacterial; in these oases a shift from stressed to stable soil condition was occurring (Quadrat B). Among all oases only one had a matured food web where C/N was moderated, and decomposition pathway was dominated by both bacterial and fungal groups (Quadrat C). In this case the occurrence of high trophic level groups (O) and (P) is more important as they are sensitive to any soil disturbance.

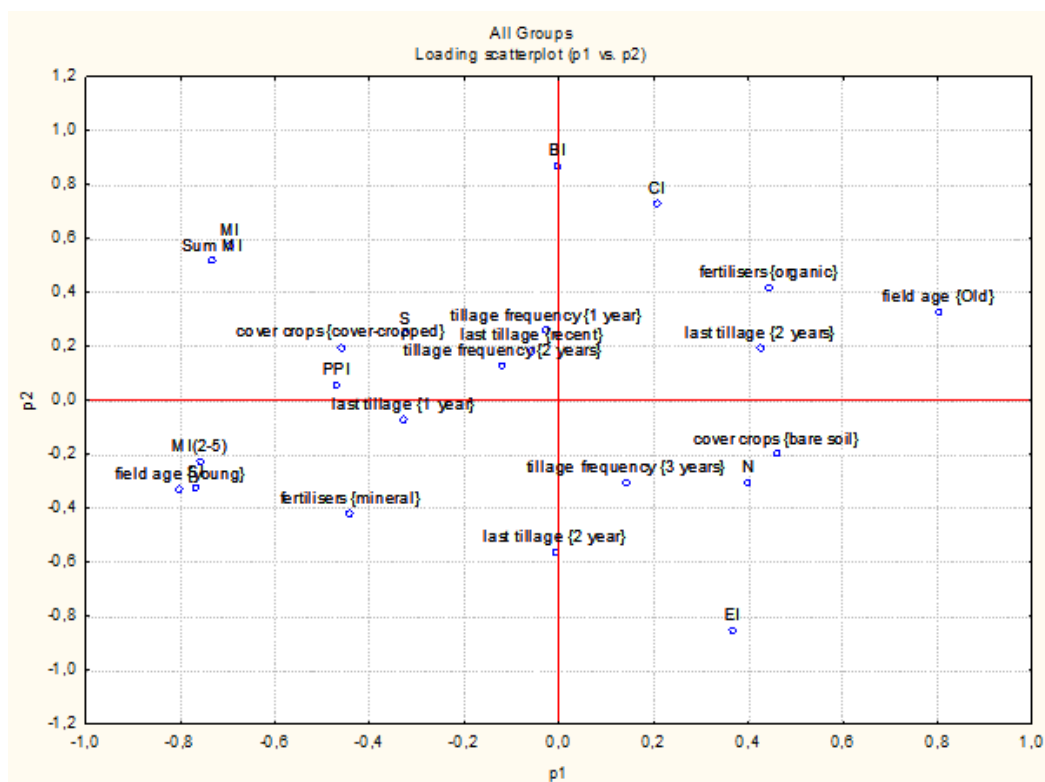


Fig. 2. Ordination of ecological indices on the biplot resulting from the Principal Component Analyses (PCA) based on the different soil managements in studied oases.

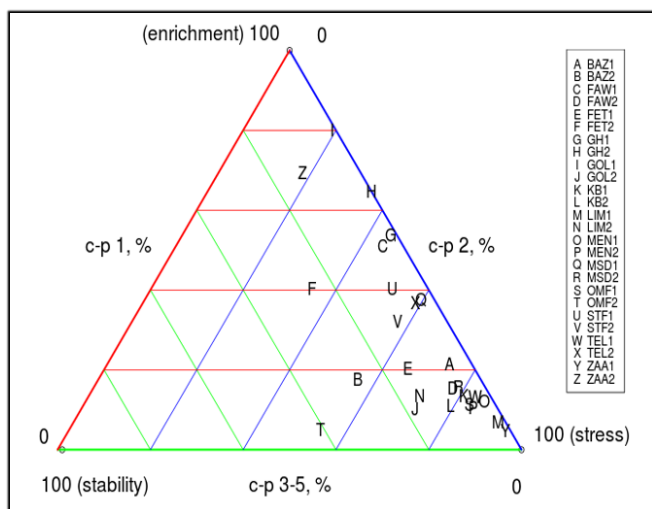


Fig. 3. cp triangle according De Goede et al. (1993) with unweighted proportional representation of cp-1, cp-2 and cp-3-5 groups of the nematode fauna in studied oases. The 13 prospected localities are indicated on the triangle by capital letters. A, B: Bazma; C, D: Fawar; E, F: Fethnassa; G, H: Ghidma; I, J: Golaa; K, L: Kebili; M, N: Limaguès, O, P: Menchiya; Q, R: M'Saaid; S, T: Om'Fareth; U, V: Esteftimi; W, X: Telmin; Y, Z: Zaafran. 1: Old oasis; 2: Young oasis.

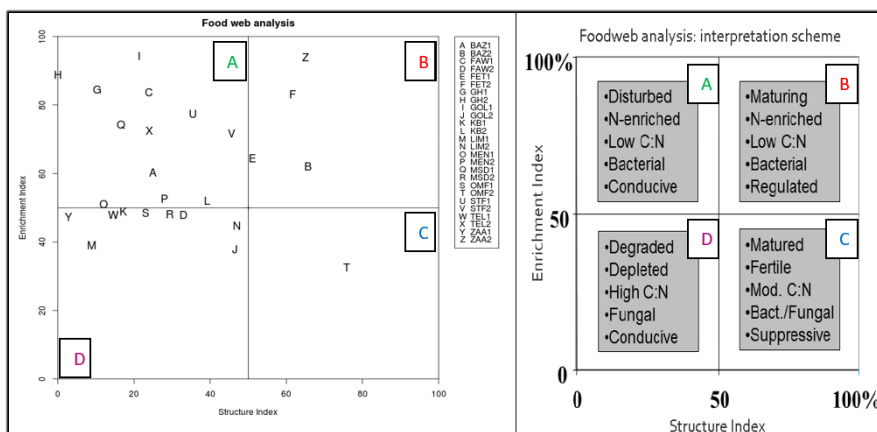


Fig. 4. Faunal profile representing the structure and enrichment conditions of the soil food web for studied oases. The 13 prospected localities are indicated on the graph by capital letters. A, B: Bazma; C, D: Fawar; E, F: Fethnassa; G, H: Ghidma; I, J: Golaa; K, L: Kebili; M, N: Limaguès, O, P: Menchiya; Q, R: M'Saaid; S, T: Om'Fareth; U, V: Esteftimi; W, X: Telmin; Y, Z: Zaafran.

DISCUSSION

Nematode populations identified from studied soils were dominated by (Ba) and (PP), as confirmed by Yeates and Bongers (1999); bacterial feeding nematodes represented the predominant feeding group of nematodes in every ecosystem and plant parasitic nematodes were the second most important group in several studied ecosystems.

In country to results of Dupont et al. (2009) indicating that fallow bare soils had less nematode abundance than cover-cropped soils, our results demonstrate that sampled bare soils showed the higher abundance and taxa richness in comparison to cover-cropped soils.

In bare studied soils, only *Trichodorus* and *Eudorylaimus* were not found, while other taxa like *Tyleotylenchus*, *Heterodera*, *Xiphinema*, *Criconema*, *Mesodorylaimus* and *Discolaimium* were absent in cover-cropped (alfalfa or barley) soils. These observations indicate that the response of the total nematode abundance to managements depends on the assemblage composition, so no general patterns can be discerned without analysing separately the taxa and functional group responses as it was reported by (Sanchèz-Moreno et al. 2006).

According to this survey, it has been confirmed that in oasis agro-ecosystem, soil amendments are usually whether the organic matter from animal source (manure) incorporated alone to soil (date palm rhizosphere) or added to other chemical fertilizers to enhance the growth and the productivity of date palm and forage associated cultures (generally barley or alfalfa)

In conventional system (manure + chemical fertilizers), taxa richness was higher than in organic system (manure). However, total nematode abundance was

negatively correlated with S index in these two systems. Through this result we can conclude that the high abundance in oases under organic management is correlated with the predominance of some specific genera in the whole community. Most oases under organic system are old and characterized by a frequent and high input of manure stimulating (Ba) and (Fu) nematodes feeding on microbes involved in organic matter decomposition and mineralisation. Our observations are consistent with previous studies of (Hole et al. 2005; Liphadzi et al. 2005; Sanchèz-Moreno et al. 2006) but in contradiction with findings of Quist et al. (2016) who reported that total nematode densities in conventional and organic systems were not different. Yeates and Bongers (1999) affirmed also in this context that the nature and quantity of organic material strongly affect nematodes and usually influence the overall composition of the nematode fauna.

Studies of Neher (1999), Quist et al. (2016) and Yeates et al. (1997) confirmed that the genus *Prismatolaimus* was absent from conventional farming systems but abundant in organic systems. This result is not in correlation with that found in the current study as *Prismatolaimus* was recorded in both organic and conventional systems.

The less taxa richness was relative to soils tilled each year and in recently tilled soils. *Diploscapter*, *Plectus*, *Wilsonema*, *Prismatolaimus*, *Rhabdolaimus*, *Criconema*, *Trichodorus*, *Mesodorylaimus*, *Eudorylaimus* and *Discolaimium* were all absent from fields tilled each year. The highest taxa richness was reported in oases where tillage frequency is about 2 or 3 years and in soils tilled 1 year before sampling date. According to Fiscus and Neher (2002), *Plectus* is an indicator of disturbance due to the

concordance between its cp value (cp-2) and its tolerance to chemical and mechanical perturbations, but we found it here more sensitive to tillage than expected from its cp value as it was absent from recently tilled soils and soils tilled each year. Sensitivity of omnivores and the predator nematode *Discolaimium* to soil tillage frequency was observed. This finding is consistent with several studies (Bongers 1999; Kladvico 2001; Mulder et al. 2003 and Sanchez-Moreno et al. 2006) where sensitivity of high trophic level nematodes (omnivorous and predators) to soil disturbance and managements was confirmed. Wardle et al. (1995) revealed that bacterial feeders are usually abundant in cultivated soils while predators and omnivores often disappear with cultivation.

From omnivores only *Mesodorylaimus* and *Eudorylaimus* were affected by soil tillage while other families seem indifferent to this soil disturbance; these different responses in the same trophic group have been reported by Porazinska et al. (1999). In addition, Postma-Blaaw et al. (2005) confirmed that nematode genera within the same trophic group can exhibit asymmetric competition, negatively influencing the abundance of other genera. Responses to perturbation may be influenced by the presence of other nematode taxa (Sanchez-Moreno et al. 2006).

A maturity index for free-living taxa (MI) may be viewed as a measure of disturbance, with lower values being indicative of a more disturbed environment and higher values characteristic of a less disturbed environment. The MI decreases with increasing microbial activity and pollution induced stress (Korenko and Schmidt 2006). Nematode groups with low cp value are considered as enrichment opportunists and therefore indicate resource availability.

Nematode groups with high cp value indicate system stability, food web complexity and connectance (Bongers 1990). The lower MI value (1,79) was recorded in old oases which is an indication of the high soil disturbance level especially where the high manure input is a common and frequent agricultural practice in these oases at Kebili. Bongers et al. (1997) reported that MI have lower value in fertilized soils than in unfertilized soils.

The PPI index responds as the inverse of the MI (Bongers et al. 1997) or has direct relationship with MI (Neher and Campbell 1994). It is based on plant parasitic nematodes. Our results showed that cover-cropped sampled soils have higher PPI than bare soils which is in correlation with findings of Ferris and Bongers (2009) who suggested that high PPI indicates vigorous host plants which reflect system enrichment while low PPI indicates poor host growth supporting nematodes assigned to lower cp values.

As tillage is related to soil amendments incorporation (fertilization) in oasis management system, tillage frequency and last tillage seems have the most important effect on MI and PPI variations in this study. Bongers et al. (1997) found that agricultural practices as tillage and fertilization cause a disturbance of the soil ecosystem resulting in a stimulation of bacterial and fungal activities, subsequently the soil fauna reacts by an increase of opportunist species and for nematodes by a decrease of MI and an increase of PPI, secondary successions result in a decreasing of nutrient conditions and an increasing of MI vs. decreasing of PPI (Bongers et al. 1997).

The Channel Index (CI), based on the ratio of fungivore to bacterivore nematodes, is an indicator of the prevalence of organic matter

decomposition mediated by fungi. High values of the CI indicate slower, fungal-mediated, decomposition pathways while low values indicate that bacterial and rapid organic matter decomposition is occurring. In our study we can conclude that slow and fungal organic material decomposition was related with organic system with 2 years as soil tillage frequency as confirmed by Minoshima et al. (2007) who

reported that no tilled soils has higher CI. Rapid bacterial decomposition was related to conventional system and to recently tilled soils. Most of oases under organic system are old oases and farmers are used to accumulate date palm residues on soil surface and use manure as amendments; this can explain the slow organic matter decomposition mediated by fungi found in these oases.

RESUME

Larayedh-Bettaieb, A., Hajji-Hedfi, L., Sanchèz-Moreno, S., Chihani-Hammas, N., Regaieg, H., Aoun, F., Horrigue-Raouani, N. et Belkadhi, M, S. 2018. Effets de la gestion du sol sur la biodiversité des communautés de nématodes en tant qu'indicateur biologique de la qualité des sols dans l'agro-écosystème oasien de Kebili. Tunisian Journal of Plant Protection 13 (2): 183-200.

Les communautés de nématodes du sol ont été étudiées dans 26 oasis du sud tunisien (Kébili) caractérisées par différentes pratiques culturales. Les oasis étudiées ont été discriminées selon la fréquence du travail du sol, le type d'amendement du sol (fumier ou fumier + engrais), les cultures en association avec le palmier dattier et l'âge de l'oasis. En outre, cette étude a évalué l'importance du triangle C-P (Colonisateur-Persistant) et du profil faunistique (représentation de l'indice d'enrichissement vs. L'indice de structure) en tant qu'indicateur biologique et un outil de surveillance à l'appui de l'évaluation de la qualité du sol. Les résultats ont montré que les communautés de nématodes étaient composées de 10 genres bactériophages (Ba), 3 mycophages (Fu), 12 phytoparasites (PP), 4 omnivores (O) et 1 prédateur (P) avec une dominance de (Ba) et (PP) dans toutes les oasis. Les communautés de nématodes différaient légèrement en fonction de l'âge de l'oasis. Les bactériophages et le genre *Discolaimium* ont été trouvés dans les anciennes et les nouvelles oasis. Le genre *Plectus* (Ba) n'a été trouvé que dans les nouvelles oasis alors que les genres *Xiphinema*, *Criconea* et *Trichodorus* (PP) étaient absents dans ces oasis. Seuls quelques genres ont été affectés par le type d'amendements du sol et les cultures associées, y compris certains bactériophages et mycophages. La richesse générique la plus élevée a été enregistrée dans les oasis à sols nus et dans les oasis où la fréquence de travail du sol était de 2 ou 3 ans. La plus faible valeur de l'indice de maturité (IM) a été enregistrée dans les anciennes oasis. Le profil faunistique représentant les conditions de structure et d'enrichissement de la chaîne alimentaire dans les sols oasiens prospectés montre bien que la plupart des oasis étudiées étaient caractérisées soit par un niveau élevé de perturbation du sol avec une abondance élevée du groupe à valeur c-p = 1 en tant qu'indicateur d'un réseau trophique perturbé, soit par un sol stressé avec une densité élevée du groupe à valeur c-p = 2 comme indicateur d'un réseau trophique dégradé. Seuls quelques sites ont montré des réseaux trophiques structurés ou en cours de maturation avec des niveaux de perturbation du sol respectivement faibles à modérés et des sols non perturbés. Cette étude a mis en évidence que certains genres de nématodes pourraient potentiellement servir comme des bio-indicateurs différentiels de la perturbation du sol.

Mots Clés: Biodiversité, communautés des nématodes, indices écologiques, oasis, palmier dattier, pratiques culturales, sol

ملخص

لعريض-الطبيب، أسماء ولبنى حاجي-هادفي وساره ساتشز-مورينو ونورة شيهاني-حماس وهاجر رقيق وفوزي عون ونجاة حريق-رواني ومحمد الصادق بالقاضي. 2018. تأثير إدارة التربة على التنوع الحيوي لمجتمعات النيماتودا كمؤشر بيولوجي لجودة التربة في النظام البيئي-الزراعي بواحات قبلي.

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تمت دراسة تجمعات نيماتودا التربة في 26 واحة بالجنوب التونسي تحت أنظمة زراعية مختلفة. تكمن الاختلافات بين الواحات المدروسة في كثافة حراثة التربة، نوع الأسمدة (السماد العضوي أو السماد العضوي مع الأسمدة المعدنية)، الزراعات المرتبطة بالنخيل وعمر الواحة. بالإضافة إلى ذلك، قيمت هذه الدراسة أهمية مثلث C-P (المستمر - المستمر) و التتميط المجتمعي (تمثيل مؤشر الإثراء IE مقابل المؤشر الهيكلي IS) لنيماتودا التربة كمؤشر بيولوجي وأداة لتقييم جودة التربة. أظهرت النتائج أن تجمعات نيماتودا التربة كانت تتكون من 5 أصناف مقسمة حسب نوعية النظام الغذائي: 10 أنواع تتغذى على البكتيريا، 3 أنواع تتغذى على الفطريات، 12 نوع يتغذى على جذور النباتات، 4 أنواع تتغذى على الكائنات الدقيقة المختلفة في التربة ونوع واحد مفترس يتغذى على النيماتودا مع ملاحظة هيمنة الصنفين الأولين في جميع الواحات التي تمت دراستها. اختلفت مجتمعات هذه النيماتودا بدرجة قليلة بالاعتماد على عمر الواحة. تم العثور على النيماتودا المتغذية على البكتيريا و النوع *Discolaimium* في كلا الواحات القديمة والحديثة. تم عزل النوع *Plectus* فقط في الواحات الحديثة في حين كانت الأنواع *Xiphinema* و *Criconema* و *Trichodorus* غالبة في هذه الواحات. تأثرت القليل من أنواع النيماتودا بنوع الأسمدة وبالزراعات المرتبطة بالنخيل بما في ذلك بعض أنواع النيماتودا المتغذية على البكتيريا وعلى الفطريات. تم تسجيل أعلى نسبة تنوع صنف في الواحات التي لا توجد بها زراعات مرتبطة بالنخيل وفي الواحات التي تتميز بكثافة حراثة تربة منخفضة (كل عامين أو 3 أعوام). تم تسجيل أدنى معدل للمؤشر MI في الواحات القديمة. اتسمت معظم الواحات المدروسة إما بتربة مضطربة تتميز بوفرة كبيرة للمجموعة cp-1 كمؤشر على شبكة غذائية مضطربة أو بتربة مرهقة تتميز بوفرة كبيرة للمجموعة cp-2 كمؤشر على شبكة غذائية متدهورة. أظهر عدد قليل فقط من الواحات المدروسة شبكات غذائية ناضجة ومنظمة ذات مستوى تآكل تربة منخفض إلى معتدل و تربة غير مضطربة على التوالي. أوضحت هذه الدراسة أن بعض أجناس النيماتودا يمكن أن تستعمل بمثابة مؤشرات بيولوجية تقاضلية لتقييم درجة اضطراب التربة.

كلمات مفتاحية: تجمعات نيماتودا، تربة، تنوع بيولوجي، مؤشرات بيئية، نخيل تمر، نظام زراعي، واحات

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Toxicity of the active fraction of *Pergularia tomentosa* and the aggregation pheromone phenylacetone nitrile on *Schistocerca gregaria* fourth-instar nymph: effects on behavior and acetylcholinesterase activity

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ABSTRACT

Miladi, M., Abdellaoui, K., Ben Hamouda, A., Boughattas, I., Tlili, H., Mhafdhi, M., Acheuk, F., and Ben Halima-Kamel, M. 2018. Toxicity of the active fraction of *Pergularia tomentosa* and the aggregation pheromone phenylacetone nitrile on *Schistocerca gregaria* fourth-instar nymph: effects on behavior and acetylcholinesterase activity. *Tunisian Journal of Plant Protection* 13 (2): 201-216.

Chemical insecticides remain the most used approach in locust control although they present a serious menace to human health and the environment. The search for alternative control methods, efficient and environmentally friendly, has become indispensable. The aim of this work is to study the effect of the aggregation pheromone, phenylacetone nitrile, alone or in combination with the active fraction of *Pergularia tomentosa* on *Schistocerca gregaria* fourth-instar nymph. Toxicity bioassays showed that the combination of phenylacetone nitrile with the active fraction of *P. tomentosa* significantly increased nymph mortality. Results also showed that the aggregation pheromone caused significant mortality especially after 6 hours of exposure. The pheromone also caused neurotoxic effects on *S. gregaria* nymph due to the disturbance of the acetylcholinesterase activity. We also noted the presence of cannibalism phenomenon. Phenylacetone nitrile seems to have an effect on phase polyphenism of *S. gregaria* imago that exhibit specific traits to the solitary phase.

Keywords: Behavior, botanical insecticide, desert locust, enzyme, pheromone

The desert locust, *Schistocerca gregaria* (Orthoptera: Acrididae) is potentially the most destructive pest insects in many semi-arid territories of Africa and the Near East (Seidelmann et al. 2005). This species is able to transform between two phases, solitaria and gregaria, which differ in various behavioral, morphological and physiological characteristics (Pener and Simpson 2009; Tanaka and Nishide 2013). During solitary phase, the locust populations are likely to present no obvious economic threat to the cultivated vegetations (Sword et al. 2010). However, nymphs in the gregarious phase show an aggregating behavior and move in bands to seek food, whereas adults swarm and migrate over long distances causing significant crop and pastures damage (Magor et al. 2008). The consequences of the invasions can be disastrous for food security of many states in affected areas (Abbassi et al. 2003; Chen 2010). To limit damage to crops and pastures, treatment is required with large amounts of broad-spectrum chemical pesticides. Due to fast action, low cost, easy application and efficiency, synthetic insecticides have become an important part of locust and grasshoppers management in modern agricultural systems. However, after decades of use, their negative side effects, such as toxicity to humans and animals, environmental contamination, and toxicity to non-target insects have become apparent and interest in less hazardous alternatives of locust control is therefore being renewed. Plant species with known insecticidal actions are being promoted and research is being conducted to find new sources of botanical insecticides. Plant species have the ability to synthesize a variety of secondary metabolites that are not essential for their growth and development, but are important in the protection against phytophagous insects and microbial pathogens. Many secondary

metabolites have insecticidal, repellent as well as antifeedant activity. Furthermore, they cause reproduction retardation and act as insect growth disruptors (Barney et al. 2005; Al-Mazra'awi and Ateyyat 2009).

Asclepiadaceae (milkweeds) is a large family of about 230 genera and almost 2000 species distributed through the tropics and temperate areas of the world (Arribère et al. 1998). Among these species, *Pergularia tomentosa* is a perennial shrub that occur in the Tunisia flora (Gill 1992). The plant was reported to have effective antifungal activity (Hassan et al. 2007), insecticidal activity against *Locusta migratoria* (Acheuk and Doumandgi Mitiche 2013) and antifeedant effect on *Spodoptera littoralis* (Green et al. 2011).

In the same way of research and in an effort to identify novel pest-control products that are both environmentally acceptable and effective for the management of the desert locust, the study of the pheromonal bouquet of *S. gregaria* is currently one of the most actively investigated areas in chemical ecology, partly owing to its interesting perspectives for the development of new bio-pesticides. A series of studies led to the identification of a number of volatile aromatic compounds released by gregarious desert locusts with phenylacetone nitrile (PAN, also named benzyl cyanide) as the dominant component (Torto et al. 1994). Hassanali et al. (2005) discovered that phenylacetone nitrile significantly affected the behavior of *S. gregaria* nymphs and increased their sensitivity to insecticides. Bal and Sidati (2013) have demonstrated that the addition of small amounts of PAN reduces the quantities of insecticides used for the control of nymphs, while maintaining the same efficiency.

In this paper, we studied (i) the toxicity of the active fraction of *Pergularia tomentosa* (AFP) alone or in

binary combination with PAN against *S. gregaria* nymphs, and (ii) the effect of the PAN and on some behavioral and physiological parameters of treated nymph such as cannibalism, phase polyphenism and neurotoxicity. The attempt results give us additional information on the mode of action of PAN.

MATERIALS AND METHODS

Locust rearing.

Mass rearing of *S. gregaria* was carried out in the Laboratory of Entomology at the Higher Agronomic Institute of Chott-Mariem as previously described (Abdellaoui et al. 2013). Nymphs and adults were held in 50 × 50 × 50 cm cages at 30-32 °C, 50-70% RH, and under a 12 h:12 h, L:D photoperiod. Locusts were fed daily on fresh *Brassica oleracea* leaves supplemented with wheat bran.

Plant material and the active fraction extraction.

The Asclepiadaceae *P. tomentosa* was collected during spring seasons (March-April 2017) from the region of Hergla (N: 36.1°, E: 10.3°), Tunisia. The active fraction preparation was carried out in the Laboratory of Insect Physiology and Molecular Ethology (KU Leuven, Belgium). The methanolic crude extract (18 g) was subjected to column chromatography on a silica gel column (63-200 µm, 70-230 meshes). The column was initially eluted with hexane, followed by EtOAc and hexane mixtures (EtOAc: hexane, 25:75, 50:50, 75:25, 100:0). Then, the eluting solvent mixture was changed to MeOH: EtOAc (25:75, 50:50, 75:25, 100:0, v:v) and finally to 20% aqueous acetic acid: MeOH (5:95, 10:90). 5 ml aliquots from each fraction were concentrated under reduced pressure in a Savant SpeedVac Concentrator (Thermo Scientific), the dried residues were re-

dissolved in 5 ml EtOH and tested for their insecticidal activity against 3rd instar nymph locust. Only the MeOH, the EtOAc (50:50) fraction, which gives mortality rate >70% has been recovered and the solvent was evaporated. The dried active fraction of *P. tomentosa* (AFP) was kept at 4°C.

The aggregation pheromone phenylacetoneitrile.

Phenylacetoneitrile (PAN) or benzyl cyanide (98%) was purchased from Sigma-Aldrich. It is an aromatic organic compound of formula C₆H₅CH₂CN.

Treatments.

The bioassay for insecticidal activity of the active fraction of *P. tomentosa* (AFP) against *S. gregaria* was conducted on newly ecdysed (< 6 h post molting) 4th instar nymph using three concentrations 0.06, 0.24 and 0.96 % chosen in accordance with amounts used in preliminary tests in the laboratory. Locusts were inoculated by applying 5 µl of the corresponding concentration under the pronotal shield. In the control experiment, the insects received the same quantity of ethanol used as solvent for preparing the different concentrations. Control and experimental nymphs (n = 60 for each concentration) were placed in separate cages (30 cm³) under the same conditions described above for the mass rearing. Insect mortality was calculated according the Abbott correction formula (Abbott 1925). Probit analysis (Finney 1971) was conducted to estimate the LC₅₀ values with their 95% confidence limits after 8 days of treatment. Other experiments were conducted to assess the effect of the AFP and PAN in binary combination. Firstly, both products were applied simultaneously by treating nymphs topically with a homogeneous mixture of PAN (at the concentration of

0.5, 1 and 2%) and the AFP (at the LC₅₀) (v:v) formulated in ethanol. In other tests, insects were first exposed to the PAN (0.5, 1 and 2%) for 2, 4 and 6 hours and then treated topically by the AFP (LC₅₀) as described earlier. The mortality was then assessed daily via direct observation for a period of 8 days.

Effect of PAN on cannibalistic behavior.

Four replicates of 20 fourth-instar nymphs that had molted 12 hours earlier were exposed to PAN at the concentrations of 0.5, 1 and 2% for 6 hours and transferred into 30 cm × 30 cm × 30 cm cages as described above. For control, a similar number of replicates treated with paraffin oil were deployed. Fresh food consisting of *B. oleracea* leaves (25 g/cage) was provided early in the morning (8 am). The mortality was assessed daily and dead nymphs were immediately removed and inspected. Number of nymphs that were cannibalized (evidence of having been mauled and partially fed by conspecifics e.g. nymph without abdominal parts) was recorded. The percentage of cannibalized nymphs was computed.

Comparison of the effects of PAN and antennectomy.

The purpose of these experiences is to verify if the gregarious male pheromone has a disruptor behavioral effect on the conspecific fourth-instar nymphs and to check if the insects are able to detect the pheromone in their environment. Replicate groups (three each) of 10 fourth-instar nymphs were either treated with PAN, antennectomised, or left untreated (control) and the number of nymphs engaged in different behaviors (feeding, roosting, moving) at 8 am and 8 pm compared for 3 successive days after treatment was recorded (Bashir and Hassanali 2010).

Morphometric measurements.

Digital calipers were used to measure the following classical morphometric phase characteristics for adults to determine E/F and F/C ratios (E = Length of fore wing, F = Length of hind femur, C = Maximum head width) for female and male imago originating from control (exposed to the paraffin oil) and PAN-exposed fourth-instar nymphs at the concentration of 2% for 6 hours. Locusts were held in a number of 20 individuals/cage to maintain the same phase characteristics. F/C and E/F ratios were reported on morphometric chart according to Duranton and Lecoq (1990). They distinguish the gregarious from the solitary and *transiens* (*dissosians* and *congregans*) forms. For each group, 20 males and 20 females were measured.

Enzymatic assays.

Acetylcholinesterase (AChE) assays was conducted on the fourth-instar nymphs (< 6h post-molting, n = 12) sampled from control and treated groups after 2, 4 and 6 hours of exposure. Heads were separately homogenized in a glass homogenizer in 100 mM K-phosphate buffer (250 mmol/l sucrose, 50 mmol/l Tris-HCl, 1 mmol/l EDTA, and 1 mmol/l DTT at a 1/4 w/v ratio, pH 7.5). The homogenates were centrifuged at 15000 g for 20 min to generate the S9 fraction. After centrifugation, the supernatants were collected and stored at -80 °C until analysis. All procedures were carried out at 4 °C. Proteins in the S9 fraction were quantified according to the Bradford method (1976) using Coomassie Blue reagent. The AChE activity was carried out following the method of Ellman et al. (1961) as previously described (Abdellaoui et al. 2018).

Data analysis.

Results are expressed as means \pm standard deviation (SD). For multiple comparisons, a parametric one-way analysis of variance (ANOVA) was performed on data along with Tukey-test using Graph Pad Prism (Version 6.0.).

RESULTS

Effect of the active fraction of *P. tomentosa* on fourth-instar nymph mortality.

The results reported in Fig. 1 showed that the AFP exhibited insecticidal activity against *S. gregaria*. Treatment of newly emerged fourth-instar nymph resulted in a significant mortality with the highest concentration 0.96% which reached $54.19 \pm 11.05\%$ 8 days after treatment. The influence of AFP is widely correlated to the concentration used and the analysis of variance considering AFP concentrations as classification criterion revealed a significant difference among treatments ($F = 15.19$, $df = 2$, $P = 0.0013$).

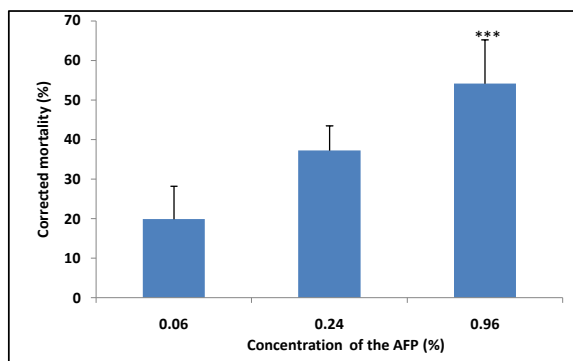


Fig.1. Effect of the active fraction of *P. tomentosa* (AFP) on the corrected mortality of *S. gregaria* fourth-instar nymph. Mean value (\pm SD, %) represents three replicates, each containing 20 insects. Significant differences ($P < 0.001$) are indicated by asterisks *** according to Tukey-test.

The evaluation of the AFP toxicity was also estimated by LC_{50} value, and its 95% confidence limits expressed as percentage for an 8-day period (Table 1).

The corresponding median lethal concentration was 0.8 % (95%, $cl = 0.59-1.27\%$). This concentration was chosen for the subsequent interaction experiments.

Table 1. Toxicity of *P. tomentosa* active fraction to newly emerged 4th instar nymph of *S. gregaria*

Treatment	LC ₅₀ ^{a, b}	Chi square (χ^2)	df	P value
Topical application	0.81 (0.59 - 1.27)	46.36	10	P< 0.0001

^a Units LC₅₀ = %

^b 95% lower and upper fiducial limits are shown in parenthesis.

Effect of PAN/AFP combination on nymphal mortality.

Experiments were conducted on fourth-instar nymphs of *S. gregaria* in order to determine the percent mortality at each concentration of the PAN alone and combined with the AFP at the LC₅₀. As summarized in Table 2, when the PAN is applied alone by topical application, the mortality rates ranged from 23.81 ± 8.9 to $44.26 \pm 15.9\%$ at 8 days after treatment for the average concentration 0.5 and 2% respectively. The analysis of variance considering concentrations as classification criterion showed a significant difference among treatments ($F = 6.96$, $df = 2$, $P = 0.015$). The activity of PAN against nymphs becomes more evident when the locusts are exposed to the pheromone and the mortality rates become higher even with the lowest tested concentration. Indeed, the percentage of corrected mortality reached $79.64 \pm 28.29\%$ after 6 hours of exposure at the concentration of 1%. ANOVA showed a significant difference among treatments for 2 and 6 hours of exposure ($F = 10.18$, $df = 2$, $p = 0.005$; $F = 5.9$, $df = 2$, $P = 0.02$, respectively). The analysis of variance also showed that the mortality rates did not differ significantly between the PAN

concentrations after 4 hours of exposure ($F = 1.81$, $df = 2$, $P = 0.22$) as indicated by the homogeneous groups generated using Tukey-test (Table 2).

Toxicity bioassays also showed that the application of the aggregation pheromone PAN in a binary combination with the AFP at the LC₅₀ significantly increased the nymphal mortality. Indeed, the mortality rates observed in the nymphs treated with the AFP alone at the highest used concentration (0.96%) was $54.19 \pm 11.05\%$ for an 8-day observation period. However, when the LC₅₀ of the AFP was applied simultaneously with the PAN at the concentration of 2%, the percentage of corrected mortality reached $79.64 \pm 16.35\%$. Data also revealed that when the nymphs were first exposed to the aggregation pheromone before receiving the AFP, the mortality rates become higher and reached $92.72 \pm 10.48\%$ after 6 hours of exposure. The analysis of variance, with the PAN concentrations as classification criterion, showed a significant difference among treatments for all the tested exposure times ($F = 4.67$, $df = 2$, $P = 0.04$; $F = 10.88$, $df = 2$, $P = 0.004$; $F = 13.88$, $df = 2$, $P = 0.002$), respectively for 2, 4 and 6 hours of exposure (Table 2).

Table 2. Percentage of corrected mortality (mean \pm SD) of *S. gregaria* fourth-instar nymph treated by the AFP in combination with the aggregation pheromone, phenylacetone nitrile (PAN/AFP) for an 8-day observation period.

Treatment	Topical application		PAN-Exposure		
			2 h	4 h	6 h
PAN alone	0.5%	23.81 \pm 8.90	10.17 \pm 8.17	28.39 \pm 8.49	36.42 \pm 12.48
	1%	13.44 \pm 9.43	37.94 \pm 19.49	33.92 \pm 12.48	79.64 \pm 28.29 *
	2%	44.26 \pm 15.9 *	56.16 \pm 13.6 **	49.37 \pm 9.21	64.19 \pm 4.24
	$F_{(2,9)}$	6.96	10.18	1.81	5.90
	<i>P</i> value	0.015	0.005	0.22	0.02
PAN/AFP (LC ₅₀)	0.5%	64.82 \pm 17.80	36.7 \pm 14.69	36.70 \pm 14.69	53.52 \pm 15.12
	1%	56.79 \pm 17.00	64.71 \pm 9.61 *	57.43 \pm 18.28	89.08 \pm 9.08 **
	2%	79.64 \pm 16.35	57.99 \pm 15.51	85.44 \pm 10.49 **	92.72 \pm 10.48 **
	$F_{(2,9)}$	1.848	4.67	10.88	13.38
	<i>P</i> value	0.2126	0.0405	0.004	0.002

Mean value represents three replicates, each containing 20 insects. Significant and highly significant differences ($P < 0.05$ and $P < 0.01$) are indicated by asterisks * and ** respectively (Tukey-test). For the topical application, the PAN and AFP were applied simultaneously. For the exposure tests, nymphs were first exposed to the PAN for 2, 4 and 6 hours and then treated topically by the AFP (LC₅₀).

Effect of PAN on cannibalistic behavior.

We investigated in this study the relationship between the PAN treatment and cannibalism behavior in *S. gregaria* fourth-instar nymphs. It appears from the results grouped in Fig. 2 that cannibalistic behavior was significantly higher ($F = 8.16$, $df = 2$, $P = 0.0031$) in PAN-treated groups compared with control. Indeed, the percentages of nymphs that were cannibalized were 17.5 ± 5 and $22.5 \pm 5\%$

respectively to the lowest and highest tested concentration of PAN. For the medium concentration (1%), the number of cannibalized nymphs did not exceed $10 \pm 8.16\%$. However, the cannibalistic behavior did not exceed $3.33 \pm 2.71\%$ in the control group exposed to the paraffin oil (Fig. 2). As illustrated in Fig. 3, the cannibalism results in a partially fed by conspecifics e.g., nymphs without abdominal orthoracic parts (Fig. 3).

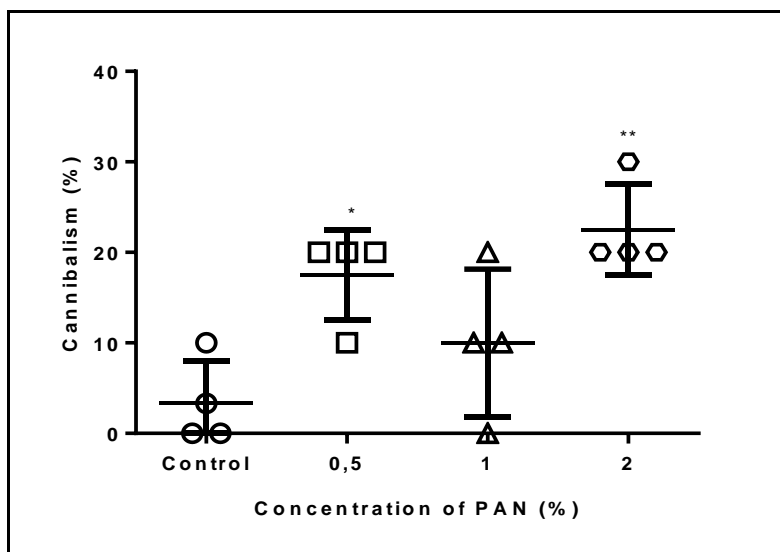


Fig. 2. Effect of the PAN on cannibalistic behavior of *S. gregaria* fourth-instar nymphs. The data are represented as means \pm SD of four replicates each containing 20 insects. Significant and highly significant differences ($P < 0.05$ and $P < 0.01$) are indicated by asterisks * and ** respectively (Tukey-test).



Fig. 3. Cannibalistic behavior of *S. gregaria* fourth-instar nymphs exposed to the aggregation pheromone for 6 hours. — = 0.1cm. Nymphs cannibalized in the abdomen (a), thorax (b) and head (c) parts.

Comparison of the effects of PAN and antennectomy.

The results regrouped in Fig. 4 showed the effects of PAN and antennectomy on the fourth-instar nymphs behavior as reflected in the percentages engaged in feeding, roosting or moving, in the mornings and evenings. Obtained results revealed that the PAN-exposed nymphs demonstrated similar behavioral as those antennectomised. Indeed, we noted that both groups are in moving at the two different periods of the day (8 am and 8 pm). However, the control nymphs showed a normal circadian cycle characterized by an engagement in feeding at 8 am and roosting at 8 pm (Fig. 4a).

Fig. 4b summarised the percentages of nymphs engaged in feeding, roosting or moving in the mornings for three consecutive days. The obtained results showed that $72.22 \pm 10.18\%$ of the control nymphs are engaged in feeding and only $2.22 \pm 1.92\%$ are in roosting which showed normal circadian pattern of behavior. For both PAN-exposed and antennectomised nymphs, the majority of the nymphs are observed moving in the experimental cages with $54.44 \pm 9.62\%$ and $38.88 \pm 5.09\%$ respectively. These results were also evidenced by the ANOVA showing a significant difference among treatments in the fourth-instar nymph behavior ($F = 8.46$, $df = 2$, $P = 0.017$; $F = 8.13$, $df = 2$, $P = 0.019$; $F = 40.61$, $df = 2$, $P = 0.0003$) respectively for moving, roosting and feeding).

Effect of PAN on morphometric measurements of adults.

The morphometric measurements, F/C and E/F ratios used in the determination of the phase polyphenism of the desert locust were calculated on male

and female imagoes originating from control and PAN-exposed fourth-instar nymphs at the concentration of 2% for 6 hours. Results were reported on morphometric chart as illustrated in Fig. 5. These results showed that PAN shifts morphometric variables of adult locusts towards solitary values compared with the control ones especially in the case of males. In Fig. 5b, the F/C ratio was higher, whereas the E/F ratio was lower indicating a shift toward a solitaryness. Furthermore, the majority of the treated locust females were in *transiens dissocians* phase progressing towards solitary phase as shown in Fig. 5b. However, control individuals present a morphometry *transiens*-type with a gregarious tendency (Fig. 5a).

Acetylcholinesterase activity.

The effect of the exposure to PAN at different concentrations on the AChE enzymatic activities of *S. gregaria* fourth-instar nymph is shown in Fig. 6. Results revealed that the PAN significantly increased the AChE activity. The analysis of variance considering concentrations as classification criteria showed a significant difference among ($F = 7.37$, $df = 3$, $P = 0.0046$; $F = 72.61$, $df = 3$, $P < 0.0001$; $F = 19.59$, $df = 3$, $P < 0.0001$) respectively for 2, 4, and 6 hours of exposure. The ANOVA also showed that the effect of the PAN on the AChE activity varied as a function of the exposure time ($F = 62.4$, $df = 2$, $P < 0.0001$; $F = 32.89$, $df = 2$, $P < 0.0001$; $F = 8.61$, $df = 2$, $P = 0.0081$) respectively for the 0.5, 1, and 2%. The AChE values of the control group ranged from $121.6 \pm 35.83\%$ to $964.9 \pm 209.5\%$ and $257.8 \pm 67.76\%$ nmol/min/mg proteins after 2, 4 and 6 hours of exposure to paraffin oil respectively.

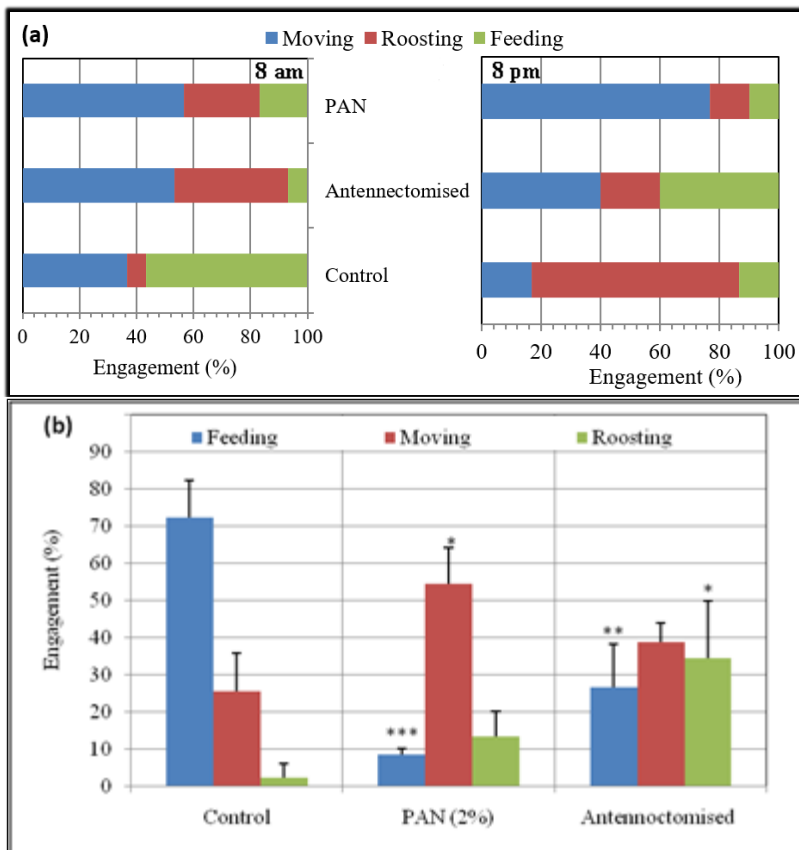


Fig. 4. Percentages of fourth-instar nymphs engaged in feeding, moving or roosting after PAN exposure and antennectomised (a). Nymphs engagement observed in the mornings (8am) and evenings (8pm); (b) cumulative percentages of nymphs engagement observed at 8 am for three consecutive days. Significant differences ($P < 0.05$, $P < 0.01$ and $P < 0.001$) are indicated by asterisks *, ** and *** respectively (Tukey-test).

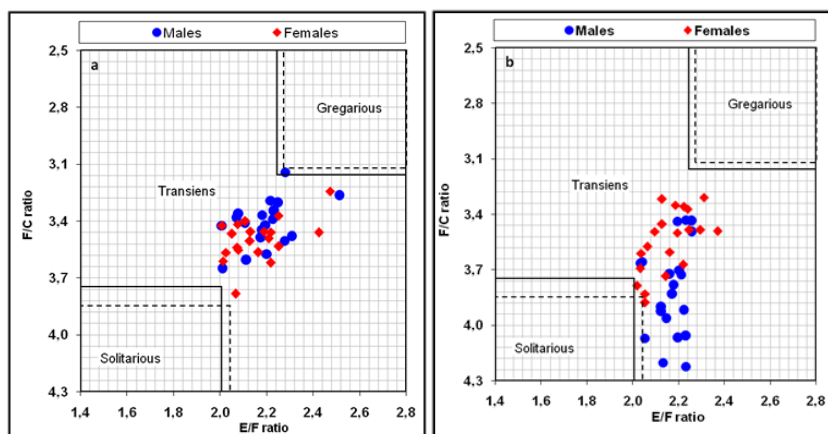


Fig. 5. Dispersion of morphometric parameters in control and treated locusts. a: F/C and E/F ratios of control locusts. b: F/C and E/F ratios of locusts originating from PAN-exposed nymphs. E = length of fore wing, F = hind femur length, C = maximum head width.

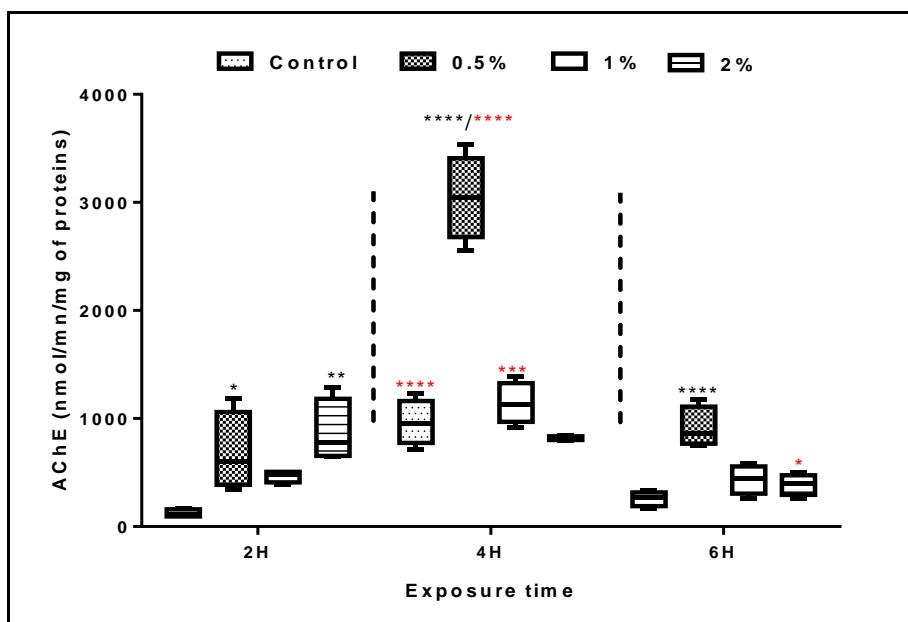


Fig. 6. Effects of PAN on the AChE activity (means \pm SD, $n = 12$) of *S. gregaria* fourth-instar nymph. Comparison was made between concentrations (black asterisks) and between the exposure times for each concentration (red asterisks). *, **, and *** indicated significant differences at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively, (Tukey-test).

DISCUSSION

Considering negative effects of synthetic pesticides, research in recent years has been turning more towards new environment-friendly alternatives for pest control. Botanical insecticides, known as plant secondary metabolites, have proved to be suitable candidates in locust management programs (Acheuk et al. 2012). Experiments were conducted to assess the effect of the active fraction of *P. tomentosa* (AFP) alone and in combination with the aggregation pheromone (PAN) on *S. gregaria* fourth-instar nymph. Results revealed that treatment of newly emerged nymphs by the AFP resulted in a significant mortality with a dose response relationship. The percentage of corrected mortality becomes higher and reached $92.72 \pm 10.48\%$ when the nymphs were first exposed to the pheromone before receiving the AFP. A previous study conducted by Acheuk and Doumandji-Mitiche (2013) showed that alkaloids extracted from the aerial part of *P. tomentosa* exhibited a potent insecticidal effect against the Fifth-instar nymph of *L. migratoria* with a dose-dependent relationship. Similarly, methanol crude extracts of *P. tomentosa* deterred feeding of *Spodoptera littoralis* in a binary-choice bioassay. The anti-feeding activity in *P. tomentosa* was attributed to cardenolides compounds (Green et al. 2011) which are responsible of several physiological effects such as spasms, regurgitation and slowed growth in non-adapted species of insect (Dussourd and Hoyle 2000).

Although semiochemicals have been successfully used in the field for the management of some pests, they are not generally considered to be sufficiently reliable in their action when used alone. They are more effective when used as part of integrated control strategies (Smart et al. 1994). The use of the PAN in locust management programs has been discussed

by several authors. Indeed, Bal and Sidati (2013) showed that the addition of small quantities of phenylacetone nitrile could make possible to decrease by half the quantities of insecticides used in the control of desert locust nymphs, while preserving the same efficiency. Moreover, Bashir and Hassanali (2010) reported that major adult pheromone constituent PAN, in addition to its pheromone character and its effects on communication between individuals, could also have some toxicity on nymphs and can make them more sensitive to insecticides and biological agents treatment. In the same way, Miladi et al. (2018) studied the effects of the latex of *P. tomentosa* and the PAN against *L. migratoria* nymphs. The authors demonstrated that the exposure to PAN reduced the concentration of fresh latex from 100 to 1 % while preserving the same efficiency.

The results of the present investigation also showed that PAN significantly affect the fourth-instar nymphs behavior by disturbing their circadian cycle as reflected in the percentages of nymphs engaged in feeding, roosting or moving in the mornings and evenings. The PAN exposure appears to interfere with the functioning and the physiology of the antennae and the insect will be unable to locate the diet. Indeed, the antennae function primarily as sense organs and they are richly endowed with olfactory sensilla. They are the primary olfactory organs of all insects. Olfaction is a sense that can be used at a distance as well as locally for detecting a variety of odorants in the environment that facilitate the research of feeding sites (Patel et al. 2013). Therefore, any perturbation at this level can be lethal to the target organism. Similarly, Hassanali et al. (2005) showed that when nymphs of *S. gregaria* are exposed to PAN vapor for 6 hours, their

antennal electrophysiological response to odors substantially depressed (by >90%) and recovery of antennal sensitivity occurs only gradually.

Our results also showed that the PAN may be involved in the control of phase-related morphometric ratios of F/C and E/F. Nymphs treated with PAN show a shift toward solitary morphometric ratios at adult stage suggesting that PAN has a solitarising effect on the progeny of treated locusts. Heifetz et al. (1996) reported that loss of olfactory communication between the gregarious individuals is also consistent with behavioral phase change of crowd-reared nymphs resulting from disruption of antennal chemoreception. These findings are consistent with those of Mahgoub et al. (2011) who indicated that the PAN induced solitary behavior. Treated nymphs become confused and disoriented, some lost their appetite completely, while others turned cannibal. Similarly, Bashir and Hassanali (2010) showed that long term contact of field gregarious hopper bands with PAN makes them relatively hyperactive. Few days later, their movements became random and they stopped marching as coherent groups. They started to roost for longer periods on vegetations and they fragmented into smaller and smaller groupings and individuals. There were clear signs of increased predation and cannibalism at the roosting sites. These authors suggest that cannibalistic behavior may be secondary consequences of the reduced food intake. In addition, PAN

could interfere with the metabolic function in nymphs resulting in impaired development that could in turn disrupt the formation and function of olfactory organs such as antennae and inhibits perception by nymphs of their own aggregation pheromone (Ochieng 1997). However, pheromonal communication may be the principal mechanism modulating the aggregation behavior of the desert locust (Obeng-Ofori et al. 1993).

Finally, the obtained results also demonstrated that PAN was found to induce a disturbance in AChE activity. The AChE is a key enzyme that terminates nerve impulses by catalyzing hydrolysis of the neurotransmitter acetylcholine in the nervous system (Aygun et al. 2002).

From this experiment, it may be concluded that the addition of the aggregative pheromone PAN enhanced the toxicity of the LC₅₀ of the active fraction of *P. tomentosa*. The compound seemed to present anti-insect properties by disturbing behavior and reducing the neurotoxicity as evidenced by an increase in AChE activity. This can reflect positively on reducing cost and environment hazards especially that the quantity of the pheromone used in desert locust manipulation is very low. However, the mode of action of PAN on desert locust nymphs and its interference with the nervous system is still imperfectly known and requires further detailed study like possible interaction with some central nervous system neurotransmitters such as serotonin and GABA that accompany phase change in the locust.

RESUME

Miladi M., Abdellaoui K., Ben Hamouda A., Boughattas I., Tlili H., Mhafdhi M., Acheuk F. et Ben Halima-Kamel M. 2018. Toxicité de la fraction active de *Pergularia tomentosa* et de la phéromone d'agrégation phénylacétonitrile contre les larves du quatrième stade de *Schistocerca gregaria*: Effets sur le comportement et l'activité de l'acétylcholinestérase. *Tunisian Journal of Plant Protection* 13 (2): 201-216.

Les insecticides chimiques restent l'approche la plus utilisée dans la lutte antiacridienne bien qu'ils représentent une menace pour la santé humaine et l'environnement. La recherche de méthodes alternatives de lutte, efficaces et respectueuses de l'environnement, est devenue indispensable. Le but de ce travail est d'étudier l'effet de la phéromone d'agrégation, phénylacétonitrile, seule ou en combinaison avec la fraction active de *Pergularia tomentosa* sur les larves de *Schistocerca gregaria*. Les essais biologiques de toxicité ont montré que la combinaison du phénylacétonitrile avec la fraction active de *P. tomentosa* a augmenté significativement la mortalité larvaire. Les résultats ont également montré que la phéromone d'agrégation a entraîné une mortalité significative, en particulier après 6 heures d'exposition. La phéromone a également provoqué des troubles du comportement (locomotion et alimentation) et a augmenté l'activité de l'acétylcholinestérase chez les larves de *S. gregaria*. Nous avons également noté la présence du phénomène de cannibalisme. Le phénylacétonitrile semble avoir un effet sur le polymorphisme phasaire chez les imagos de *S. gregaria* qui ont présenté des caractères morphométriques spécifiques à la phase solitaire.

Mots clés: Comportement, criquet pèlerin, enzyme, insecticide botanique, phéromone

ملخص

الميلادي، مريم وخميس عبد اللاوي وأمال بن حمودة وعتاب بوغطاس وهيثم تليبي ومنى المحافظي وفاطمة عشاق ومنية كامل-بن حليمة. 2018. سمية المستخلص الفعال لنبتة *Pergularia tomentosa* وفيرمون التجميع phenylacetoneitrile على يرقات الطور الرابع ليرقة الجراد الصحراوي *Schistocerca gregaria*: التأثيرات السلوكية ونشاط الأنزيم acetylcholinesterase.

Tunisian Journal of Plant Protection 13 (2): 201-216.

تعتبر المبيدات الحشرية الكيميائية الطريقة الأكثر استخداماً في مكافحة الجراد على الرغم من أنها تشكل تهديداً على صحة الإنسان والبيئة. لذلك أصبح البحث عن طرق مكافحة بديلة فعالة ومنسجمة مع المحيط أمر لا غنى عنه. يهدف هذا البحث إلى دراسة مدى تأثير فيرمون التجميع phenylacetoneitrile بمفرده أو بالاشتراك مع المستخلص الفعال لنبتة *P. tomentosa* على الطور الرابع ليرقات الجراد الصحراوي *S. gregaria*. أظهرت الاختبارات الحيوية أن الجمع بين المستخلص الفعال لنبتة *P. tomentosa* وفيرمون التجميع phenylacetoneitrile يؤدي إلى زيادة كبيرة في معدل الوفيات لدى الطور البرقي المذكور. كما أثبتت النتائج أيضاً أن التعرض إلى phenylacetoneitrile بصفة منفردة لمدة 6 ساعات يؤدي بدوره إلى ارتفاع في نسبة الوفيات كما يتسبب أيضاً في اضطرابات سلوكية وزيادة نشاط أنزيم acetylcholinesterase. لاحظنا أيضاً أن لهذا الفيرمون تأثير على ظاهرة التحول المرحلي لليرقات.

كلمات مفتاحية: أنزيم، جراد صحراوي، سلوك، فيرمون، مبيدات الحشرات النباتية

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Effects of Temperatures and Rainfall Variability on the Abundance and Diversity of Caelifera (Insecta, Orthoptera) in Three Natural Environments in the Mزاب Valley, Septentrional Sahara (Algeria)

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ABSTRACT

Zergoun, Y., Guezoul, O., Sekour, M., Bouras, N., and Holtz, M.D. 2018. Effects of temperatures and rainfall variability on the abundance and diversity of Caelifera (Insecta, Orthoptera) in three natural environments in the Mزاب Valley, Septentrional Sahara (Algeria). *Tunisian Journal of Plant Protection* 13 (2): 217-228.

The climatic condition is assumed as the main factor responsible for development and survival of insects; this investigation was conducted to study the responses of Caelifera to temperatures and precipitation variations during 2017 in three natural environments of Mزاب Valley, Ghardaïa, Algeria. A total of 22 grasshopper species were collected, representing four families and eight subfamilies. The subfamily Oedipodinae was the dominant, followed by Pyrgomorphinae and Thrinchinae. Two species: *Sphingonotus rubescens* and *Sphingonotus savignyi* occurred frequently in the three sites. However, only one accidental species, *Eunapiodes* sp. was found. According to our observations, it is clear that the grasshopper diversity was higher in July and August coinciding with the increase in temperature. In such conditions, the precipitation has less influence on species diversity.

Keywords: Algeria, Caelifera, diversity, precipitation, Mزاب Valley, temperature

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More than 28157 species of Orthoptera are distributed worldwide (Cigliano et al. 2018). This order is among the most recognizable and familiar insects, that includes the grasshoppers, locusts and crickets. Although grasshoppers

(Acrididae) are often thought to be associated with grasslands, many species are currently found in tropical forests, shrub-lands, deserts, wetlands, and alpine regions (Song et al. 2018). Grasshoppers are considered as the main important insect that cause damage to crops, comprise an overwhelming proportion of animal biomass and biodiversity, form a major component of food webs, and play important roles in nutrient cycling and plant production in grassland ecosystems (Hawlena and Schmitz 2010).

Several studies confirmed that the recent climatic changes strongly affect the abundance and geographic distribution of insects (Eo et al. 2017) and the richness of pests, particularly Orthoptera (Weiss et al. 2012). In addition, many researches consider Orthoptera as a bioindicators of the climate change (Báldi and Kisbenedek 1997) due to their sensitivity to microclimatic conditions (Zografou et al. 2009). The high temperature can affect directly and indirectly all arthropods by increasing or decreasing their metabolic rates, changing their activity patterns as well as their developmental rates (Zografou et al. 2017).

The abundance of Orthoptera is generally influenced by high temperatures and dry conditions; however, this is not applicable to all species in this group (Capinera and Horton 1989). Weather in the regional scale, particularly the precipitation, is also a critical factor that shapes the population density of locusts (Wysiecki et al. 2011). Lack of rainfall is usually cited as the main factor limiting population increase in acridid inhabiting tropical semiarid and arid areas, while those species occurring in more temperate or marshy areas are favored by years of subnormal rain (Hunter et al. 2001). Climate change also indirectly affects insects by affecting their host plants. The stage of the vegetation can also impact the

biological traits of locust, in case of dry vegetation, a phenomenon of gregarization appear and swarm formation (Cisse et al. 2013). In addition, species with low mobility are susceptible to climate change as they may not be able to shift their ranges fast enough to keep up with environmental changes (Eo et al. 2017). A clear understanding of the mechanisms and ability of locusts and grasshoppers to overcome limiting factors in their environment is essential for predicting when and where outbreaks are likely to occur (Hunter et al. 2001). A strong economic loss was signaled and a large amounts of vegetation was destroyed resulting to the invasion of this pests. In general, the grasshopper fauna of the Algerian Sahara, and Mزاب in particular, has attracted less attention of entomologists and no serious studies were performed on the pest species diversity of the Mزاب Valley. The intention of the present study is to improve knowledge in the diversity of Orthoptera in the Mزاب Valley. The objectives of this work was (1) to inventory the species of Orthoptera present in the Mزاب Valley, and (2) to compare the attributes of the Caelifera community (abundance, richness and diversity of species).

MATERIALS AND METHODS

Study site.

the Mزاب Valley sites locate in the northern Sahara of Algeria (Fig. 1) at an elevation of 530 m above mean sea level, between 32° to 33° north latitude and 3° to 4° east longitude. These sites are characterized by little-evolved type of alluvial wind soils. The climate is Saharan with a mild winter. Sampling and the study of the Caelifera fauna were conducted in three different localities: Wadi N'tissa (Béni Isguen), Wadi Touzouz (Ghardaïa) and Wadi Mزاب (El Djaoua, El Atteuf).

Wadi N'tissa (S1): Latitude 32° 45' North, and longitude 3° 66' East. The surface of the site is about 4 ha (rocky terrain). The vegetal cover is mainly composed of *Haloxylon scoparium*, *Peganum harmala*, *Pergularia tomentosa*, *Colocynthis vulgaris*, *Pituranthos chloranthus*, *Atractylis serratuloides*, *Echinops spinosus* and *Androcymbium punctatum*. The sandy and dry areas are characterized by the presence of two Gramineae, *Aristida obtusa* and *Stipagrostis pungens*.

Wadi Touzouz (S2): Latitude 32° 51' North and longitude 3° 60' East. The surface of the collection site is 4 ha (the terrain changes into a rocky bottom with scattered patches of sand). Among the plants, *Haloxylon scoparium*, *Ferula communis*, *Zilla spinosa*, *Cleome Arabica*, *Oudneya Africana*, *Fagonia glutinosa* and *Thymelaea microphylla* were found.

El Djaoua (S3): Latitude 32° 46' North, and longitude 3° 73' East. The surface of the collection site is 4 ha (Rocky and sandy terrain). The vegetation is dominated by *Thymelaea microphylla*, *Oudneya africana*, *Echium pycnanthum*, *Cleome arabica*, and *Stipagrostis pungens*.

Climatic data.

The climatic data was obtained from the Regional Weather Service Station of Ghardaïa (32° 40' N, 3° 80' E; elevation 461 m). Average annual maximum temperature was 28.47 °C; with highest temperature of 40.5 °C in summer and lowest temperature of 6.2 °C in winter. The amount and seasonal precipitation varied greatly among years. From the Regional Weather Service Station database, 2017 annual precipitation was 34 mm.

Grasshopper sampling.

Populations of Caelifera were sampled monthly from January to December 2017. Sampling was performed with quadrats, the most frequently used method for biodiversity studies. The quadrant method defines in each collection site an area of 10000 m². Essentially, it consists of laying a 25 m long transect using a string and then placing square 5 m quadrats (25 m²) along the transect (Gillon 1974). Ten quadrats were sampled at each of the three sites on each sampling date. Grasshoppers collected using the sweep-net were taken to the laboratory for species identification based on identification keys for Orthopteroid from North Africa of Chopard (1943), Dirsh (1965) and keys of the Acridomorpha from North West Africa (Louveaux et al. 2018). Grasshoppers were classified according to modern systematic as used in the Orthoptera species file (Cigliano et al. 2018).

Data analysis.

The data collected from the different sites within the study area were analyzed using the ecological indices. Relative abundance of grasshopper species was estimated by the comparison with the total abundance of all species collected from each site. Species richness was quantified as the total number of species present in each habitat. Two of the most common indices have been used to describe and compare the grasshopper diversity in the prospected sites in 2017: the Shannon-Weaver diversity index (H) and the species evenness. The formula of the Shannon-Weaver diversity index is:

$$H = - \sum (ni/N) \times \log_2 (ni/N),$$

where ni is the abundance of the species i in one site and N the total number of species living in the same site. Pielou's evenness (J) is a measure of uniformity. It

describes how evenly the individuals are distributed among the different species:

$$J = H / \log_2 S,$$

where, J = species evenness, H = species diversity and S = number of species (Magurran 2004). The constancy indices were obtained following the formula proposed by Bodenheim:

$$C = p \times 100 / N,$$

Where p is the number of collections containing the particular species studied and N is the total number of collections carried out. Based on the obtained results, the species were classified as: constant (present in more than 50% of the collections), accessory (present in between 25-50% of the collections) or accidental (present in under 25% of the collections) (Gallo et al. 2002).

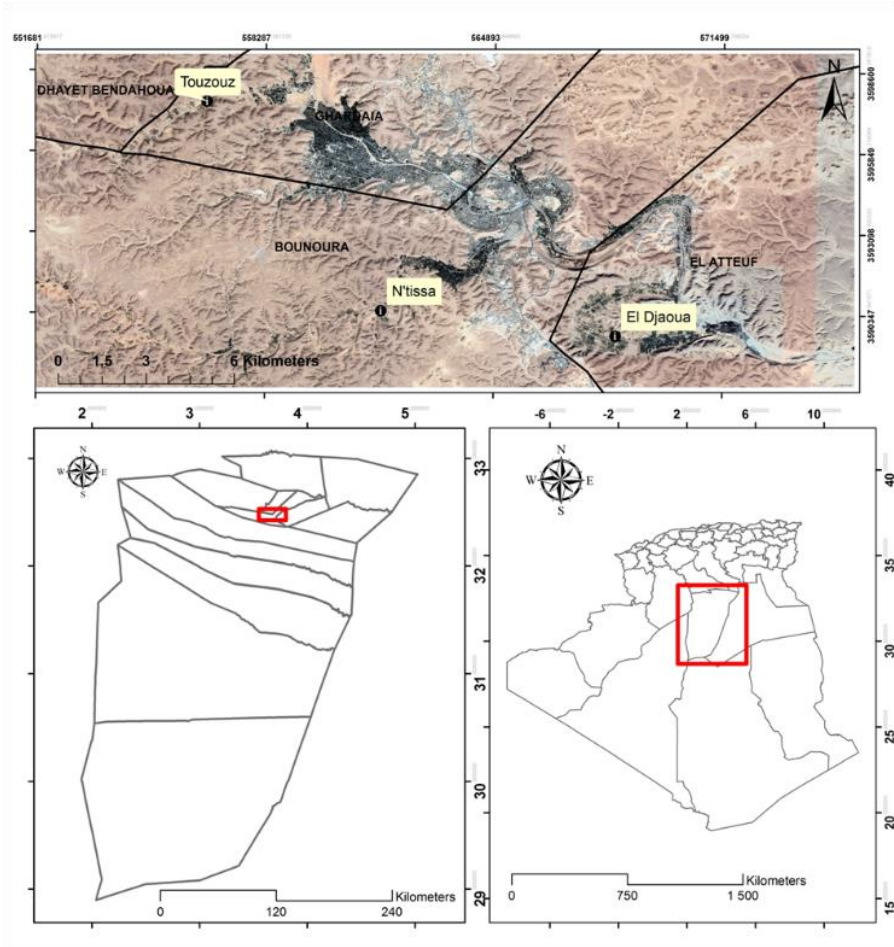


Fig 1. Geographic location of the study sites in the Mzab Valley, Septentrional Sahara, Algeria.

RESULTS

Diversity and abundance of the Caelifera community.

In total, 1385 specimens of grasshoppers were collected during the 12 months. They belonged to 22 species, four families, and eight subfamilies (Table 1). Only 14 species were found in Wadi Mzab, whereas 20 species were found in Wadi N'tissa and 17 species in Wadi Touzouz. From a taxonomic perspective, the Oedipodinae was the most abundant and diverse subfamily (with 12 species), followed by Pyrgomorphinae (with 3 species) and Thrinchinae (with 2 species). There was only one species within Acridinae, Cyrtacanthacridinae, Eremogryllinae, Dericorythinae and Pamphaginae. The abundance of grasshopper species fluctuated between sites (Table 1). *Sphingonotus rubescens* was broadly distributed in the three natural environments [29.74% (S1), 28.82% (S2) and 31.60% (S3)], and *Sphingonotus rubescens* exhibited very high population abundance, representing 29.96% of the total grasshopper relative abundance. *Sphingonotus paradoxus* occurred in Wadi Touzouz but was not found in Wadi N'tissa and Wadi Mzab. Some species were only distributed on rocky terrain. For example, *Tuarega insignis*, *Dericorys millierei* and *Eunapiodes* sp. occurred only in Wadi N'tissa. Nevertheless, *Eunapiodes* sp. (0.36%), *Sphingonotus pachecoi* (0.91%), and *Schistocerca gregaria* (0.91%) had the lowest numbers.

The recorded species were divided into constancy classes (Table 1): in Wadi N'tissa 7 species (35%) are constant species (C), 12 species (60%) are accessory species (Ac) and one species (5%) is an accidental species (Acc). In Wadi Touzouz 8 species (47.06%) are constant species (C) and 9 species (52.94%) are accessory species (Ac). The most numerous are the constant species

(C) in Wadi Mzab: 9 species (64.28 %), and 5 species (35.71%) are accessory species (Ac).

Wadi N'tissa was most diversified (2.49%) with the highest abundance of Caelifera (548), Wadi Touzouz was least diversified (2.34%) with low abundance (451) and Wadi Mzab was least diversified (2.23%) with low abundance (386) of Caelifera. Grasshopper abundance was positively related with species diversity (Table 2). High values of evenness ($0.82 < J < 0.84$) indicated that the Orthoptera community was evenly distributed at all sites.

Species diversity.

During the study period, the highest Shannon-Weaver diversity index (2.45 bits) was observed in August (maximum temperature of 40.3 °C, minimum temperature of 27 °C, average temperature of 33.7 °C) and lowest (0.67 bits) in January (maximum temperature of 14.9 °C, minimum temperature of 4.8 °C, average temperature of 9.8 °C) in Wadi N'tissa. From Wadi Touzouz the highest Shannon-Weaver diversity index (2.27 bits) was observed in August (maximum temperature of 40.3 °C, minimum temperature of 27 °C, average temperature of 33.7 °C) and lowest (0.95 bits) in December (maximum temperature of 16.7 °C, minimum temperature of 6.2 °C, average temperature of 11.5 °C). In Wadi Mzab the highest Shannon-Weaver diversity index (2.13 bits) was observed in April (maximum temperature of 27.7 °C, minimum temperature of 14.9 °C, average temperature of 21.3 °C) and lowest (1.21 bits) in February (maximum temperature of 20.6 °C, minimum temperature of 8.8 °C, average temperature of 14.7 °C). Temperature effect on the Shannon-Weaver diversity index (H) of Caelifera in the three study areas is given in Fig. 2.

Table 1. Listing, abundance (%) and constancy of Caelifera species in natural environments at three localities from the Mزاب Valley, northern Sahara (Algeria) during 2017

Family	Subfamily	Species	S1	S2	S3
Acrididae	Acridinae	<i>Truxalis nasuta</i> (Linnaeus, 1758)	3.28 (C)	2.21 (Ac)	3.37 (C)
	Cyrtacanthacridinae	<i>Schistocerca gregaria</i> (Forskål, 1775)	0.91 (Ac)	1.10 (Ac)	2.07 (Ac)
	Eremogryllinae	<i>Notopleura saharica</i> (Krauss, 1902)	1.46 (Ac)	3.10 (Ac)	4.14 (Ac)
	Oedipodinae	<i>Acrotylus longipes</i> (Charpentier, 1845)	6.20 (Ac)	7.32 (Ac)	10.88 (Ac)
		<i>Acrotylus patruelis</i> (Herrich-Schäffer, 1838)	3.83 (Ac)	-	-
		<i>Hyalorhipis calcarata</i> (Vosseler, 1902)	1.46 (Ac)	-	-
		<i>Sphingoderus carinatus</i> (Saussure, 1888)	3.28 (Ac)	2.44 (Ac)	5.18 (C)
		<i>Sphingonotus azureus</i> (Rambur, 1838)	4.01 (Ac)	2.66 (Ac)	-
		<i>Sphingonotus obscuratus lameerei</i> (Finot, 1902)	3.83 (Ac)	2.88 (Ac)	2.33 (Ac)
		<i>Sphingonotus octofasciatus</i> (Serville, 1839)	5.29 (C)	3.32 (C)	1.81 (Ac)
		<i>Sphingonotus pachecoi</i> (Bolivar, 1908)	0.91 (Ac)	1.33 (Ac)	2.84 (C)
		<i>Sphingonotus paradoxus</i> (Bei-Bienko, 1948)	-	4.65 (C)	-
		<i>Sphingonotus rubescens</i> (Walker, 1870)	29.74 (C)	28.82 (C)	31.60 (C)
		<i>Sphingonotus savignyi</i> (Saussure, 1884)	13.32 (C)	19.29 (C)	16.32 (C)
		<i>Vosseleriana fonti</i> (Bolivar, 1902)	2.74 (Ac)	13.30 (Ac)	-
Dericorythidae	Dericorythinae	<i>Dericorys millierei</i> (Bonnet & Finot, 1884)	1.09 (Ac)	-	-
Pamphagidae	Pamphaginae	<i>Eunapiodes</i> sp. (Bolivar, 1907)	0.36 (Acc)	-	-
	Thrinchinae	<i>Tmethis cisti</i> (Fabricius, 1787) <i>Tuarega insignis</i> (Lucas, 1851)	- 2.55 (Ac)	5.76 (C) -	4.14 (C) -
Pyrgomorphidae	Pyrgomorphinae	<i>Pyrgomorpha cognata</i> (Krauss, 1877)	5.66 (C)	2.88 (C)	5.44 (C)
		<i>Pyrgomorpha conica</i> (Olivier, 1791)	3.83 (C)	2.66 (C)	4.14 (C)
		<i>Tenuitarsus angustus</i> (Blanchard, 1836)	6.20 (C)	8.20 (C)	5.70 (C)

(-): absent species. S1: Wadi N'tissa (Béni Isguen), S2: Wadi Touzouz (Ghardaïa), S3: Wadi Mزاب (El Djaoua, El Atteuf). (C): Constant, (Ac): Accessory, Acc: Accidental.

Table 2. Diversity parameters for three localities from the Mزاب Valley, northern Sahara (Algeria) during 2017

Index	Wadi N'tissa	Wadi Touzouz	Wadi Mزاب
Species number	20	17	14
Species diversity (H)	2.49	2.34	2.23
Species evenness (J)	0.83	0.82	0.84

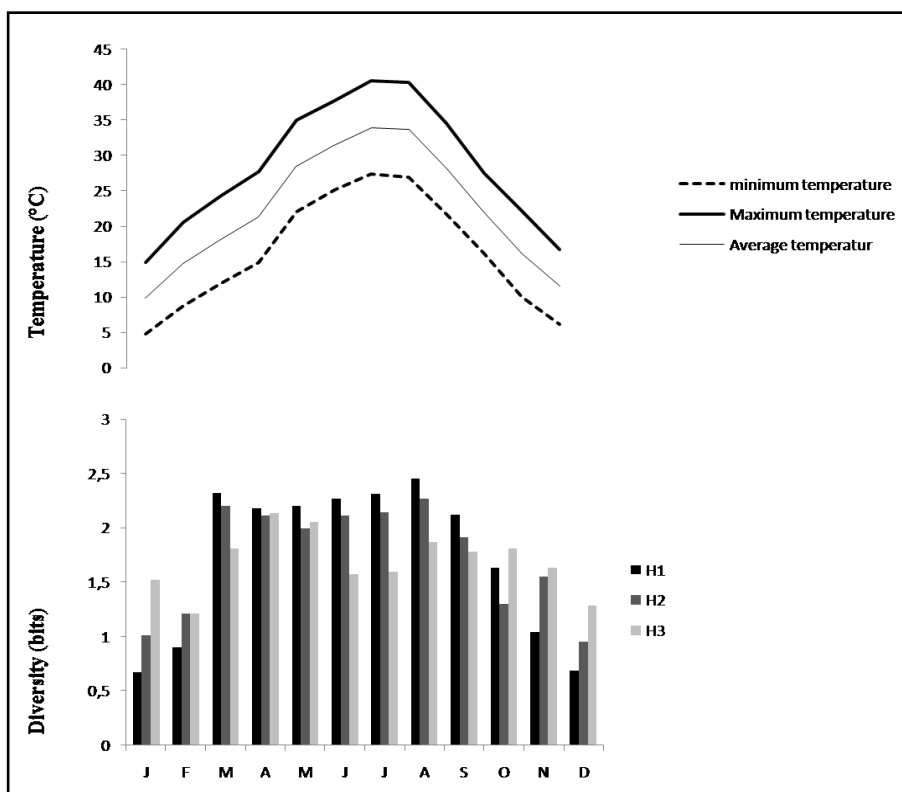


Fig. 2. Effect of temperature on Caelifera species diversity (H) in three localities from the Mzab Valley, northern Sahara, Algeria (during 2017). H1-H3 : Shannon-Weaver diversity index (H) of Wadi N'tissa (Béni Isguen), Wadi Touzouz (Ghardaïa) and Wadi Mzab (El Djaoua, El Atteuf), respectively.

Effect of precipitation on species diversity.

The study of precipitation effect (P) on species diversity (H) of Caelifera shows that the highest Shannon-Weaver diversity index (2.45 bits) was observed in August (P = 0 mm) and lowest (0.67 bits) in January (P = 1 mm) in Wadi N'tissa. From Wadi Touzouz the highest Shannon-Weaver diversity index (2.27 bits) was

observed in August (P = 0 mm) and lowest (0.95 bits) in December (P = 0 mm). In Wadi Mzab the highest Shannon-Weaver diversity index (2.13 bits) was observed in April (P = 0 mm) and lowest (1.21 bits) in February (P = 0 mm). Effect of precipitation on species diversity (H) of Caelifera in the three study areas is given in Fig. 3.

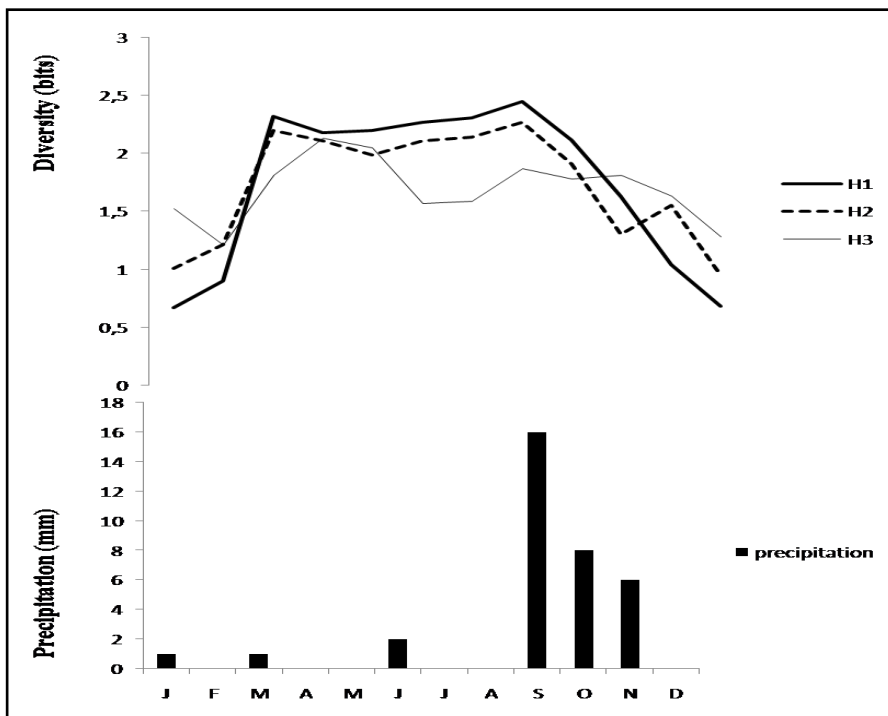


Fig. 3. Effect of precipitation on Caelifera species diversity (H) in three localities from the Mzab Valley, northern Sahara, Algeria (during 2017). H1-H3 : Shannon-Weaver diversity index (H) of Wadi N'tissa (Béni Isguen), Wadi Touzouz (Ghardaïa) and Wadi Mzab (El Djaoua, El Atteuf), respectively.

DISCUSSION

Species richness of grasshoppers noted in this study was about 22 species; however, Zergoun (1991) and Babaz (1992) noted 17 and 16 species, respectively, in the same area, Mzab Valley. Previous studies conducted by Zergoun (1991, 1994), (Babaz 1992) and Yagoub (1995) reported that Oedipodinae was the most abundant in this area. Zergoun (1991, 1994) found *Sphingonotus rubescens* particularly dominant in natural environments of the Mzab Valley. According to Otte (1984), band-winged

grasshoppers (subfamily Oedipodinae) was abundant only in areas with relatively sparse ground cover. In this study, they were most common on rocky terrain. Rock cover seems to have an even greater positive influence on Orthoptera (Zografov et al. 2017). Rocks are important to Orthopterans aiding in thermoregulation as well as providing shelter (Chappell 1983). Members of Oedipodinae and Thrinchinae subfamilies, preferring warm, dry habitats with sparse grass cover, were only found in desert and mountain rangelands (Sun et al. 2015). In

the prospected areas, the diversity was stable as also noted by Aprile (2013) indicating that these zones are the most adapted areas that offer the best conditions for their survival. Only one accidental species, were found, *Eunapiodes* sp. Diversity of Caelifera fluctuates with the seasons. They are abundant for only a few months and absent or rare during the rest of the year. When we compare different months for Caelifera species diversity (H) it is clear that diversity was at a maximum in July and August and lowest in January, February and December. The results were similar to the finding of Zergoun (1994). According Capinera and Horton (1989), warm dry weather is positively associated with grasshopper and locust densities in several areas of the world. In the present study, the lower diversity registered in winter could be related to the weather patterns and decreased vegetal diversity at these sites. According to De Wysiecki (2000), composition and structure of vegetation likely influence habitat selection among grasshoppers. From Fig. 2, it is clear that the diversity of Caelifera is affected by variation in the pattern of rainfall in different months of 2017. From these results, it appears that the grasshopper diversity was higher in July and August concurrent with the decrease in rainfall. In such conditions, precipitation has no influence. Smith and Holmes (1977) suggested that densities of *Melanoplus sanguinipes* and *Camnula pellucida* in Alberta were positively related to summer temperatures and negatively related to amount of precipitation during the previous August and September. In the United States, the populations case of *Dissoteira longipennis* showed rapid increases in numbers following periods of drought, subsiding in numbers during periods of above-average rainfall (Wakeland 1958). Contrary to our results, Usmani et al. (2010) reported that

rainfall also affects diversity in India. According to Hunter et al. (2001), rainfall seems to be a major regulator of Acridid populations in Australia. Precipitation and drought driven effects on food quality and quantity are important for grasshopper population dynamics (Joern et al. 2012). According to Jonas et al. (2015), weather acts on grasshopper populations indirectly by altering host plant species composition, availability and quality.

The present study was conducted to estimate abundance, diversity, constancy and the effect of temperature and precipitation on grasshopper species in Mzab Valley, Ghardaïa, Algeria, during 2017. The grasshoppers were collected from study sites with the majority of specimens belonging to sub-family Oedipodinae (54.54 %) dominated by genus *Sphingonotus*. The family Acrididae contained most collected species (15) followed by Pyrgomorphidae with 3 species. Data on species abundance showed that *Sphingonotus rubescens* was dominant in the three sites. Fluctuation in temperature is vital roles in determining the abundance of Caelifera fauna from Mzab Valley. It is obvious to see that the diversity of Caelifera is affected by temperature variations in different months. It appears that the grasshopper diversity was higher in July and August when temperatures increase. In these months, precipitation is very low or absent. However, the results of this study cannot be generalized to all areas or grasshopper assemblages. Further research is required on the physiological and behavioral responses of dominant species according to climate parameters.

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RESUME

Zergoun Y., Guezoul O., Sekour M., Bouras N. et Holtz M.D. 2018. Effets des températures et de la variabilité des précipitations sur l'abondance et la diversité des *Caelifera* (insecte, Orthoptère) dans trois environnements naturels de la Vallée de Mزاب, Sahara Septentrional (Algérie). *Tunisian Journal of Plant Protection* 13 (2): 217-228.

La condition climatique est supposée être le principal facteur responsable du développement et de la survie des insectes; cette étude a été menée pour étudier les réponses de *Caelifera* aux variations de températures et de précipitations en 2017 dans trois environnements naturels de la Vallée de Mزاب, Ghardaïa, Algérie. Au total, 22 espèces de sauterelles ont été recueillies, représentant 4 familles et 8 sous-familles. La sous-famille *Oedipodinae* est la plus dominante, suivie des *Pyrgomorphinae* et des *Thrinchinae*. Deux espèces: *Sphingonotus rubescens* et *Sphingonotus savignyi* sont les plus fréquentes sur les trois sites. Cependant, une seule espèce accidentelle, *Eunapiodes* sp., a été trouvée. Selon nos observations, il est clair que la diversité des sauterelles était plus élevée en juillet et août, ce qui coïncidait avec l'augmentation de la température. Dans de telles conditions, les précipitations ont moins d'influence sur la diversité des espèces.

Mots clés: Algérie, *Caelifera*, diversité, précipitations, température, Vallée du Mزاب

ملخص

زرقون، يوسف وعمر قزول ومخلوف سكور ونورالدين بوراس ومايكل هولتز. 2018. تأثيرات درجات الحرارة وتقلبات تساقط الأمطار على وفرة وتنوع *Caelifera* (حشرات، مستقيمات الأجنحة) في ثلاث بيئات طبيعية في وادي مزاب، الصحراء الشمالية (الجزائر).

Tunisian Journal of Plant Protection 13 (2): 217-228.

أجريت هذه الدراسة لتقدير وفرة وتنوع وانتظام وتأثير درجة الحرارة والتساقط على مستقيمات الأجنحة تحت رتبة *Caelifera* في عام 2017 في ثلاث بيئات طبيعية في وادي مزاب، غرداية (الجزائر). تم جمع 22 نوعا من الجراد، تمثل أربع فصائل وثمانية تحت فصائل. كانت فصيلة *Oedipodinae* المهيمنة، تلتها *Pyrgomorphinae* و *Thrinchinae*. تمت ملاحظة أن *Sphingonotus rubescens* و *Sphingonotus savignyi* هما نوعان شائعان في جميع المواقع الثلاثة. على مستوى المواقع الثلاثة، تمت ملاحظة أنواع ثابتة وأخرى ملحقة. كما وجد نوع واحد عارض وهو *Eunapiodes* sp. أظهرت النتائج بوضوح أن تنوع الجراد كان أعلى في شهري يوليو وأغسطس، بالتزامن مع الزيادة في درجة الحرارة. في مثل هذه الظروف، كان للتساقط أقل تأثيرا على تنوع الأنواع.

كلمات مفتاحية: الجزائر، انتظام، تساقط، تنوع، درجة حرارة، وادي مزاب، *Caelifera*

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Insecticidal effects of siliceous sands as preservative for maize and cowpea storage

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ABSTRACT

Cissokho, P.S., Welle, F., Gueye, M.T, Diarra, K., Sow, E.H., and Lognay, G. 2018. Insecticidal effects of siliceous sands as preservative for maize and cowpea storage. Tunisian Journal of Plant Protection 13 (2): 229-241.

Siliceous sands were tested in maize and cowpea storage against pests. The purpose of this study is to evaluate the insecticidal activity of two sands applied at increased doses of 1, 2, 3 and 4g/250g of maize and cowpea on *Sitophilus zeamais*, *Callosobruchus maculatus*, *Prostephanus truncatus* and *Tribolium castaneum* adults. Sands (Diobe1 and 2) were sieved and the two particles sizes retained for the study were 1×1 mm and 0.3×0.3 mm. Untreated plots and Actellic® served as control and the experiment was conducted during one month. Each dose was repeated 4 times. Results revealed a high efficiency of siliceous sand against these four pests with greater efficiency of Diobe1. Mortality of 85% was observed with Diobe 1 against 100% for actellic® and 0% for untreated plots. Emergences progressed inversely to the mortality. Damage and losses reached respectively 25% and 6% with untreated plots. *P. truncatus* caused nearly 16% of damages and 3% of losses at lower doses. However, with 4g/250g of stored substances (1.6%, w/w), the losses were below 1%. Insects did not show the same sensitivity to treatment and fineness of particles sands inhibits their action as long as the dose increases.

Keywords: Cowpea, insecticidal activity, maize, pests, siliceous sands, storage

Legumes and cereals are the main staple food sources in many parts of the world especially in Sahel where malnutrition continues to persist with a prevalence rate estimated at 24.8% between 2011 and 2013 (FAO, FIDA, PAM 2009). The cowpea (*Vigna unguiculata*), is a leading global food

legume, particularly in the arid savannahs of West Africa. Its seeds are a valuable source of vegetable protein, vitamins, and incomes for humans and fodder for animals. Furthermore, the cowpea plays an important role as a nitrogen source on cereal crops such as millet, sorghum and maize (Dugye et al. 2009). Louga and Saint-Louis are the main Senegalese growing regions with about 65% of Senegalese production (Cissé and Hall 2001). Maize (*Zea mays*) has become one

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of the main cereals next to rice and wheat. World production of maize in 2016 was estimated at 839 million tons compared to the previous year with 860 million tons (Planetoscope 2015). In the least developed countries, particularly in Africa, maize is a food crop especially intended for human and animal consumption, but also used by agribusiness. In Senegal, maize is the most important cereal after millet and rice. Maize grows in the southern and east regions; it is irrigated in the groundnut basin and the Senegal River Valley (Guèye et al. 2010).

In field, maize is threatened by numerous abiotic and biotic factors (Guèye et al. 2010; Hayma 2004). Insects' damage on stored products is a major problem which can induce significant losses. Also, the cowpea weevil, *Callosobruchus maculatus* and the larger grain borer *Prostephanus truncatus* can cause complete loss within three to six months if the storage is not adequate (Amoivine et al. 2007; Guèye et al. 2012).

To address such problems, pesticides are often used for the protection of stored products. While it is true that pesticides contributed to the increase in food production over the last 50 years, the fact remains that their use is limited by many constraints. Pest resistance (Charaabi et al. 2016), discovery of carcinogenic and environment pollution caused pesticide issues (Maumbe and Swinton 2003; World Health Organization 2008).

All these complaints brought against pesticides require the exploration of alternative methods of storage which are more efficient and less polluting. For this purpose, many studies have been undertaken in recent years in Senegal to offer local ecofriendly alternative solutions based on the biodiversity exploitation (Cissokho et al. 2015, Guèye

et al. 2013; Seck et al. 1993). The objective of this study is to test the effectiveness of two inert dusts (silica sands) from Senegal against adults of *Sitophilus zeamais*, *C. maculatus*, *Tribolium castaneum*, and *P. truncatus*, in the maize and cowpea storage.

MATERIALS AND METHODS

Plant material.

Grains of cowpea and maize were purchased from a local market in Dakar (Senegal). To avoid an insect infestation, the maize and cowpea grains were placed in polyethylene bags and stored in a freezer at a temperature of -4 °C for two weeks. Finally, they were re-exposed to ambient laboratory conditions before use. Grains showing any kind of damage were discarded. Maize grains were used for tests with *S. zeamais*, *T. castaneum* and *P. truncatus* and cowpea grains with *C. maculatus*.

Inert dusts.

Inert dusts in a form of siliceous sands were used from Matam, a region in the north of Senegal. They were collected at Diobe hill (Foumé Hara Diobé) located in the north west of this area (N 15 ° 27.022 / W 13 ° 11.116). Both silica sands are from the same out crop, but from different locations (Fig. 1). Diobe 1 was taken at 2 meters from the up stream of the exposure while Diobe 2 was collected at 2.5 meters from the latter. Both substances were ground, put through a sieve 0,3 mm and 1 mm and kept in the laboratory at room temperature.

Both powdered fractions were kept in individual polyethylene bags and placed at ambient temperature and relative humidity. In addition, chemical analysis by fluorescence was performed.

Insect rearing.

S. zeamais, *C. maculatus*, *P. truncatus*, *T. castaneum* adults came from a breeding ground and maintained in the laboratory at $27 \pm 1^\circ\text{C}$ and $70 \pm 5\%$ RH

for at least 4 generations in one-liter glass jars. Insects used in the tests were young, emerging up to 48 hours before experiment's start.

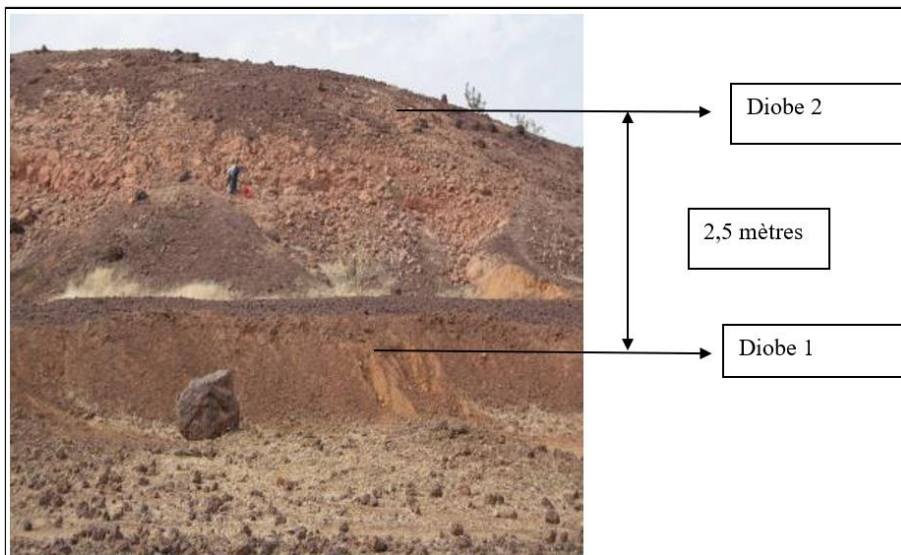


Fig. 1. Dusts collection sites for Diobe 1 and Diobe 2 samples.

Chemical material.

Actellic® Super Dust is a broad-spectrum insecticide composed of 16g/kg pirimiphos-methyl and 3g/kg permethrin served as positive control. It is used as protectant for stored products but also for disinfection of storage facilities. It controls most pests, including beetles, moths, and mites. Actellic® is active on larval and adult forms of pests by contact, ingestion, and inhalation.

Experimental procedures.

Adults of the 4 insect species namely *S. zeamais*, *C. maculatus*, *P. truncatus* and *T. castaneum* were tested. The tests were carried out in jars with a capacity of 1 liter, each containing 250 g of maize grains or cowpea with moisture content below 11% and siliceous sands. To

achieve uniform distribution of the powder on the grains, the jars were agitated manually for 2 to 3 minutes, and then stabilized 8 to 10 minutes, until all the particles settled, then 20 young unsexed adults were added to each jar.

For each type of siliceous sands, Diobe1 and Diobe2 respectively, two sizes refusals sieve (1 and 0.3 mm) were considered and tested doses were 1, 2, 3 and 4 g/250 g of stored substrate. The experimental design consisted of a completely randomized block design with 12 treatments (glass jars) and 4 replicates for the purpose of statistical analysis. The treatments included 2 controls, one treated with Actellic® and the second untreated control. Actellic® insecticide retained to the recommended dose of 50g/100kg whether 0,125 g/250 g (Table 1).

Table 1. Treatment design of siliceous sands

Treatments	Particles size (mm)	Dose (g/250 g maize)
T ₁ (untreated)	—	—
T ₂ (treated with Actellic®)	—	0,125g
T ₃	1	1
T ₄	0,3	1
T ₅	1	2
T ₆	0,3	2
T ₇	1	3
T ₈	0,3	3
T ₉	1	4
T ₁₀	0,3	4

Mortality and first generation evaluation (F₁).

The mortality monitoring was conducted over a period of 14 days. The dead individuals were counted and removed daily.

Evaluation was achieved on the first generation (F₁) by counting the numbers of offspring of all treatments in a month and 14 days. To do this, the substrate, the powder, and the insects were separated using screens, and emerged insects were counted. Abbott's formula (1925) was used to correct mortalities.

Damage and loss estimated.

The percentage of damage is determined by taking the ratio of the number of damaged grains on the total number of grains:

$$\text{Damage (\%)} = \frac{\text{Number of damaged grains}}{\text{Total number of grains}} \times 100$$

Loss percentage is calculated using the Boxall formula (1986):

$$\% \text{Losses} = \frac{(E \times B) - (C \times D)}{(E \times A)} \times 100$$

Where A is the total number of grains; B, the number of infested grains; C, the

number of healthy grains; D, the weight of infested grains; and E, the weight of healthy grains.

Statistical analysis.

All data were reported as mean of 4 replicates for biological activities of siliceous sands. The data were subjected to variance analysis (ANOVA) on XL-STAT 6.1.9. The Tukey test was used for the separation medium significantly different treatments.

RESULTS

Dust composition.

Results show percentage differences between the components of the 2 silica sands Diobe1 and Diobe2 (Table 2). This table shows essentially siliceous nature of the sands with moderate contents of alumina, iron oxide, magnesium oxide and calcium oxide. Silica content varies according to the sand. Diobe2 with silica content 75.4% is richer than Diobe1 which contains 52.2% silica. But Diobe1 is richer in alumina (27.79%) and iron oxide (4.17%) than Diobe2.

Table 2. Chemical composition of siliceous sands for Diobe1 and Diobe2 (%)

Chemicals	Diobe 1	Diobe 2
SiO ₂	52,19	75,47
TiO ₂	0,70	1,35
Al ₂ O ₃	27,79	12,82
Fe ₂ O ₃	4,17	0,67
MnO	0,01	0,00
MgO	0,00	0,00
CaO	0,11	0,16
Na ₂ O	0,00	0,00
K ₂ O	0,09	0,01
P ₂ O ₅	0,27	0,02
LOI	12,11	7,70
SUM	97,44	98,20
Zr	0,1	0,2
Cl	-	0,1
Sr	0,3	-
Ce	0,1	-
S	0,1	-
La	0,1	-
Total	98,14	98,50

Assessing adult mortality.

Results are reported in Table 3. No mortality was observed in the untreated control, while treatments in Actellic® achieved 100% mortality after 24 hours of testing. For Diobe1 bioassays, mortalities in the treatments T₃, T₄ and T₅ were very low, below 10% for the tested insects except *C. maculatus* with T₄, where the mortality was 68.3%. Indeed, *C. maculatus* remains substantially affected

in the same way when the dose was increased to a maximum of mortality with T₁₀, 85%. The sensitivity of other insects was observed especially from T₆. For *S. zeamais*, mortality increased with the dosages and also depended on the particle size. For the same dose, the fineness of the particles was important on the level of mortality. *P. truncatus* and *T. castaneum* were more tolerant to treatments.

Mortalities between 10 and 20% are rated from T₈ with the rejection of 0.3mm only.

C. maculatus was as the most sensitive insect to treatment of Diobe2 sand. Mortalities were low between T₃ and T₆ with significantly better action with the 0.3mm diameter. T₇ to T₁₀, both particle sizes gave the same result, whether mortality rates were about 60%. *S. zeamais*

behaved in the same way as *C. maculatus* with greater tolerance. It is also more sensitive to fine sand. The T₁₀ treatment gave the best rate with almost 60% mortality. *P. truncatus* and *T. castaneum* are not affected by the silica sand Diobe 2. Mortality rates were below 5% and higher doses have acted as somewhat insignificantly.

Table 3. Effects of siliceous sands Diobe1 and 2 on adult insect's mortality (%)

Insect Treatment	<i>S. zeamais</i>		<i>C. maculatus</i>		<i>P. truncatus</i>		<i>T. castaneum</i>	
	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2
T ₁	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
T ₂	100 d	100 e	100 d	100 d	100 d	100 d	100 c	100 d
T ₃	5.51 a	4.8 ab	4.12 a	8.4 a	1.4 a	1.2 ab	1.8 a	0.7 ab
T ₄	5.42 a	5.1 ab	68.3 c	23.3 ab	4.5 a	2.7 ab	4.1 a	1.6 ab
T ₅	5.68 a	4.8 ab	8.2 a	8.2 a	1.4 a	2.2 ab	1.8 a	1.8 ab
T ₆	32.4 b	20.3 c	72.1 c	43.3 bc	5.5 ab	3.9 ab	5.9 a	2.4 ab
T ₇	23.8 b	5.2 ab	30.3 b	30.1 abc	3.0 a	2.4 ab	4.3 a	1.8 ab
T ₈	69c	45.6 d	72.4 c	61.5 c	19.5 c	3.4 ab	21.5 b	4 c
T ₉	38.3 b	18.3 bc	42.8 b	61.3 c	3.8 a	3 ab	3.9 a	2.3 bc
T ₁₀	72.6 c	58 d	87.1 cd	59.8 c	13.4 bc	7.1 c	22.4 b	3.9 c

Mortality percentages in same column followed by identical letters are not significantly different (P<0.05).

Effects of siliceous sands on emergences.

Table 4 reported results of the effects of siliceous sands on insect emergence. After one month, the emergence rates showed significant differences between insects. Indeed, *C. maculatus* gave the largest rate of progenies with 174 individuals, followed by *P. truncatus* and *S. zeamais* with respectively 97 and 78 individuals. Regarding *T. castaneum*, only 14 insects emerged in F₁. Treatment with Actellic® prevented the proliferation of insects. *P. truncatus* gave the highest number of

progenies (nearly 60 T₃ and T₅) with treatments Diobe1 sand. For a given dose and insect, emergences were not significantly different for a given particle size. Overall, the number of emerged insects was below 10 individuals. With regard to the treatments with sand Diobe 2, it appears that the analysis showed no statistical differences between Diobe2 sand for all insects (*S. zeamais*, *C. maculatus*, *P. truncatus* and *T. castaneum*). According to Wilks test, siliceous sands Diobe1 and Diobe2 have

effect on the rate of emergence of different insect ($F=1.35$ and $P = 0.0132$).

Indeed, for the same insect and at the same dose, the analysis showed no statistical differences. Moreover, in the tested range, higher doses do not induce significant differences in emergence rates.

P. truncatus and *S. zeamais* gave the greatest rate of emergence for all doses

with maxima of around 50 individuals. The number of *S. zeamais* emerged in F1 is statistically the same for all doses and the maximum is below 50 individuals. It is note worthy that *C. maculatus* does not emerge from T_6 to T_{10} except T_7 . As for *T. castaneum*, whatever the dose, emergence have been very low, most often less than 10 individuals.

Table 4. Rate of emergence (F_1 progenies) of insects on maize and cowpea grains treated with siliceous sands Diobe1 and 2 for different treatments

Insect Treatment	<i>S. zeamais</i>		<i>C. maculatus</i>		<i>P. truncatus</i>		<i>T. castaneum</i>	
	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2
T₁	78.2 c	78.2 c	173.5 b	173.5 b	96.5 b	96.5 b	13.7 b	13.7 c
T₂	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
T₃	14.7 b	48.2 bc	14.7 a	34.5 a	56.2 ab	52.5 ab	4.2 a	5 abc
T₄	7 ab	42 bc	3 a	4,2 a	38,5 ab	32,2 ab	1.5 a	9.2 bc
T₅	5.5 ab	27.2 ab	7.5 a	19 a	58,7 ab	48 ab	4.2 a	7 abc
T₆	3.7 a	24.5 ab	1.7 a	0 a	21.2 a	16.2 a	1 a	3.7 ab
T₇	6.7 ab	17.2 ab	14 a	18.5 a	42.7 ab	35.7 ab	5.2 ab	4.2 ab
T₈	0 a	23.7 ab	0 a	0 a	3.25 a	29.5 a	0.2 a	3.7 ab
T₉	8.2 ab	11.5 ab	2 a	0 a	21.7 a	41 ab	3.5 a	8 abc
T₁₀	0 a	12.7 ab	0 a	0 a	6 a	9.5 a	0 a	2 ab

Means emergences with the same letters in a column (between one sand and one insect) are not significantly different at $P < 0.05$.

Effects of siliceous sandson the damage caused by insects.

Results of siliceous sands on damage are shown in Table 5. In the control, after one month, the damage estimation showed that *P. truncatus* was the most active among the tested pests with 25.7%, followed by *C. maculatus* (15.6%), *S. zeamais* (10.6%) and *T. castaneum* (5.6%). However, with Actellic®, no damage was observed for any insect. As for the control, *P. truncatus* caused the

most damage (16.5%) on maize treated with Diobe1 sand. It also appears that beyond the dose of 1g and 0.3mm size (T_4), particle size appears important because a significant difference was noted between the treated groups with the refusal of 0.3mm and 1mm. The finest silica sand showed the lowest damage and the minimum was observed, with T_{10} (less than 5%). *S. zeamais* caused damage of around 5% between T_3 and T_9 given the size of the inert substance, and damage is

virtually zero to T₁₀. *T. castaneum* shows the same profile as *S. zeamais* with lower damage whose maxima were below 5%. *C. maculatus* caused damage of around 5% between T₃ and T₅ (between 1 and 3%). With Diobe2, *T. castaneum* and *C. maculatus* caused less damage in all treatments. Moreover, damage caused by *S. zeamais* varied between 6.6 and 1.8% in

T₃ to T₁₀. The difference in particle size had effect (F=1.48 and P = 0.0172). As Diobe1, higher damage percentages were recorded with *P. truncatus* 14% for T₃; 12.7 and 10.4% for T₅ to T₇. T₁₀ had a same efficacy than Actellic® and was significantly more effective in limiting the damage with 0.1% minimum damage caused by *S. zeamais* in Diobe1.

Table 5. Effects of siliceous sands Diobe1 and 2 on the damage (%) caused by insects according to treatments

Insect Treatment	<i>S. zeamais</i>		<i>C. maculatus</i>		<i>P. truncatus</i>		<i>T. castaneum</i>	
	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2	Diobe1	Diobe2
T ₁	10.6 e	10.6 d	16.1 e	16.1 c	25.7 d	25.7 f	5.2 d	5.2 e
T ₂	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
T ₃	5.7 d	6.6 c	5.2 cd	6 b	16.3 c	14 e	2.9 c	3.2 bcd
T ₄	4.1 bcd	5.9 c	4.2 bcd	1.4 a	12.0 bc	9.2 bcde	1.8 abc	3.2 cd
T ₅	3.6 bc	5.5 c	5.4 d	1.3 a	16.4 c	12.7 de	2.2 bc	3.5 d
T ₆	2.4 b	4.2 bc	1.2 ab	0.6 a	7.7 ab	8 bcd	0.7 ab	2.4 bcd
T ₇	4.2 bcd	3.7 bc	1.2 ab	2.4 a	14.6 bc	10.4 cde	2.2 bc	2.2 bcd
T ₈	0.4 a	3.9 bc	1.0 ab	0.3 a	2.5 a	6.2 bc	1.3 abc	1.6 abc
T ₉	4.9 cd	1.2 ab	1.9 abc	0.4 a	7.5 ab	10 cde	2.0 bc	3.2 cd
T ₁₀	0.1 a	1.8 ab	0.4 a	0.3 a	2.5 a	3.6 ab	1.2 abc	1.4 ab

Means damage (%) followed by identical letters percentages in a column (between one sand and one insect) are not significantly different (P < 0.05).

Effects of siliceous sands on the weight losses caused by insects.

Results related to sand treatment impacts on weight losses are reported in Table 6. Results of Diobe1 and 2 treatments showed that *P. truncatus* (6%) and *C. maculatus* (5.6%) caused the greatest losses. *S. zeamais* and *T. castaneum* have caused respectively 2.6 and 1% losses. No loss was registered with

Actellic® treatments. As for silica sand Diobe1, *P. truncatus* has caused the greatest losses in treated groups. Higher doses had a positive impact on reducing losses. The importance of fineness of the particle size appears beyond T₇ with losses less than 1% against more than 3% to the T₃ dose. *S. zeamais* and *C. maculatus* have the same profile with negligible losses especially to the particle size 0.3 mm

against losses between 0.5 and 1% for doses between T₃ and T₆. Losses caused by *T. castaneum* fall below 1% and vary significantly with low doses and sizes. Regarding Diobe2 treatments, the particle size of silica sand showed little significant effect of a given dose (*S. zeamais*, *C. maculatus*, *P. truncatus* and *T. castaneum* have caused respectively 0.36, 0.05, 0.61 and 0.22% losses to T₁₀). In addition,

increasing the dosage did not result in a significant decrease in losses. For *S. zeamais*, *C. maculatus* and *T. castaneum* induced losses below 1%, especially beyond T₄. The losses were insignificant with increasing doses. According to Wilks test, there is significant action on the particle size noted in these insect (F=1.26 and P = 0.0072).

Table 6. Effects of siliceous sands Diobe1 and 2 treatments on weight losses (%) caused by insects

Insect Treatment	<i>S. zeamais</i>		<i>C. maculatus</i>		<i>P. truncatus</i>		<i>T. castaneum</i>	
	Diobe1	Diobe2	Diobe1	Diobe 2	Diobe1	Diobe2	Diobe1	Diobe2
T ₁	2.6 e	2.6 f	5.6 c	5.6 c	6 e	6 e	1.02 d	1.02 e
T ₂	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
T ₃	1.46 d	1.43 e	0.92 ab	1.52 b	3.41 d	2.93 d	0.63 cd	0.76 de
T ₄	1.02 bcd	1.29 de	0.8 ab	0.29 a	2.01 bcd	1.69 bcd	0.36 abc	0.66 cd
T ₅	0.82 bc	1.08 cde	1.16 ab	0.24 a	3.14 d	2.77 cd	0.48 bc	0.75 de
T ₆	0.49 ab	0.8 bcde	0.25 ab	0.09 a	1.26 abc	1.36 abc	0.12 ab	0.42 bcd
T ₇	0.94 bcd	0.65abcd	0.19 ab	0.5 ab	2.77 cd	1.99 bcd	0.4 abc	0.43 bcd
T ₈	0.08 a	0.72abcde	0.15 a	0.04 a	0.49 ab	1.07 ab	0.19 ab	0.3 abc
T ₉	1.14 cd	0.22 ab	0.31 ab	0.06 a	1.45 abc	1.98 bcd	0.46 bc	0.7 cde
T ₁₀	0.01 a	0.36 abc	0.04 a	0.05 a	0.42 ab	0.61 ab	0.18 ab	0.22 ab

Means losses (%) followed by identical letters percentages in a column (between one sand and one insect) are not significantly different at P < 0.05.

DISCUSSION

The results presented in this study showed that both silica sands reveal interesting properties in protecting maize and cowpea against attacks caused by *S. zeamais*, *C. maculatus*, *T. castaneum* and to a lesser extent *P. truncatus*. High mortality of *C. maculatus* with silica sand Diobe1 was achieved. In light of the

results, it appears that with doses of more than 4% (w/w) and a particle size in the range of 0.3 mm, silica sand (Diobe1) could potentially be considered as a substitute for pesticides in the conservation of maize and cowpea seeds. The sands have given high levels of control depending on the insect. Similar performances are noted on *S. zeamais*

which is also particularly sensitive to the fineness of the particles where it is noted to cause higher mortality with the finer particle size. The silica sands have less effect on the survival of *P. truncatus* and *T. castaneum* adults. Indeed, the ability of silica sand to kill the adults of the first generation plays a key role in the infestation levels of stocks, especially for long term storage. Athanassiou et al. (2003) showed in rice, maize and wheat considerable variation in the efficacy of SilicoSec®, a freshwater diatomaceous earth composed of 92% silica against *S. oryzae* with doses ranging from 0.125 to 1.5 g/kg depending on grain, exposure time, and dose. It has revealed unsatisfactory efficacy on maize with non-significant emergence rates. Vayias et al. (2006) found that diatomaceous earth is much more effective when applied to wheat rather than maize. The wide spaces between the grains of maize could allow insects to move and avoid areas where the concentration of diatomaceous earth is high. Hertlein et al. (2011) also highlighted the sensitivity of *S. zeamais* with diatomaceous earth. According to Mulungu et al. (2010), *P. truncatus* adults penetrate grains most of the time, so are less exposed to the ground than adults of *S. zeamais* remaining on the surface of the grain. These observations are consistent with the higher damage caused by *P. truncatus*. Kavallieratos et al. (2010) showed that *T. confusum* was less sensitive than *Rhyzopertha dominica* and *S. oryzae* to three protectants diatomaceous formulations (SilicoSec® and Insecto®) and Spinosad. Arnaud et al. (2005) have meanwhile demonstrated a significant difference between sensitivity of several populations of *T. castaneum* towards four diatomaceous earth formulations. Based on these results, it appears that the greater sensitivity of *C. maculatus* could come from either better adhesion of the particles

of silica sands to cowpea, or a greater sensitivity of this insect compared to other tested pests. Moreover, *T. castaneum* has weak predatory action which is probably due to its status as secondary pest of maize. Its low ability to penetrate healthy grains lengthens its larval development according to low damage and loss.

Levels of emergences are opposed to the importance of mortality. The latter is significantly correlated with the fineness of particles. In many cases, lower doses with finer particle size gave higher mortality rates and correspondingly lower emergence. Losses have thus evolved in parallel with the severity of the infestation. *P. truncatus* caused 25% of damage and 6% of loss and confirmed its status as a major driller. Indeed, *P. truncatus* could generate loss of more than 3% at non-effective doses. However, it should be noted that with the insect multiplication potential and voracious nature of both larvae and adults, unprotected maize can be completely destroyed in a few months of storage. Its adaptation to cassava, sorghum, and possibilities to live in softwood silos makes it more dangerous insect. In this regard, Holst et al. (2000) reported that drilling caused by *P. truncatus* adults destroyed four times more than the larva consumption. Its presence in southern and eastern Senegal has been reported (Guèye et al. 2008). Therefore, increased monitoring is required to curb its spread in the country.

It is commonly accepted that the proportion of SiO₂ largely determines the efficacy of inert dusts such as diatomaceous earths. However, this is not the case for our siliceous sands where silica sand Diobe1 (despite its SiO₂ content being less than Diobe2) was more effective for the 4 insect species. This is probably due to the difference of physical properties such as a high percentage of fine

particles, a pH less than 8.5, and a density less than 300g/l (Korunic 1997). There is broad consensus on the mode of inert dusts action. They erode the layer of cuticular waxes of insects leading to death of the insect by drying (Korunic et al. 1996; Subramanyam and Roesli 2000). According to Mewis and Ulrichs (2001), contact between diatomaceous earth and *S. granarius*, *T. molitor* and *T. confusum* led to the loss of weight for adults and a reduction in body water content, which implies barrier disruption which retained water in the insect. However, some factors are known to adversely affect the efficacy of inert dusts such as water content of the grain, relative humidity, type of insect (morphology and shape) and temperature (Athanasios and Steenberg 2007). In contrast to moisture, temperature increase enhances the activity of diatomaceous earth (Vayias and Stephou 2009).

Siliceous sands applications against *S. zeamais*, *C. maculatus*, *P. truncatus*, and *T. castaneum* have shown convincing results. In some cases, the

efficacy of silica sands depends on the sensitivity of species, the fineness of particles, and the applied dose. Furthermore, Actellic® remains fully effective against insects of stored foodstuffs. The tested doses which did not exceed 4g/250g grains, gave a very good control with adult pests (1.6%). The matrix size has also proved very important. Indeed, with a diameter of silica sand particles smaller than 0.3mm, insect survival is strongly affected and subsequently the damage and loss tend to zero with increased doses. *P. truncatus* proved less sensitive responses to treatments. Thus, in a perspective of integrated pest management, higher doses of 2-3% should circumscribe the action of all pests and allow for a greater conservation of maize and cowpea. In the light of the obtained results, we can consider substituting synthetic pesticides in the conservation of stocks of maize and cowpea seeds especially by the application of silica sand.

RESUME

Cissokho P.S., Welle F., Gueye M.T, Diarra K., Sow E.H. et Lognay G. 2018. Effets insecticides des sables siliceux comme conservateurs pour le stockage du maïs et du niébé. Tunisian Journal of Plant Protection 13 (2): 229-241.

Des sables siliceux ont été testés en vue du stockage du maïs et du niébé contre les insectes ravageurs de stocks. Leur activité insecticide a été évaluée par application de doses croissantes de 1, 2, 3 et 4g/250g de denrées sur les adultes de *Sitophilus zeamais*, *Callosobruchus maculatus*, *Prostephanus truncatus* et *Tribolium castaneum*. Pour chaque sable siliceux, Diobel et 2, les refus des deux tamis de 0,3 mm et 1 mm ont été retenues. L'Actellic® et des lots non traités ont servi de témoins. Chaque dose est répétée 4 fois. Après un mois, les résultats ont montré une grande efficacité des sables siliceux à l'égard de ces 4 espèces d'insectes. Diobel s'est montré plus efficace avec des mortalités de 85% contre 100% pour l'Actellic® et 0% pour les lots non traités. Les émergences ont évolué inversement à la mortalité. Les dégâts et pertes pondérales ont atteint respectivement 25 et 6% en l'absence de traitement. *P. truncatus* a occasionné près de 16% de dégâts et 3% de pertes pondérales aux plus faibles doses. Cependant, avec la dose 4g/250g de denrées (1,6%, p/p), les pertes sont en dessous de 1%. Les insectes n'ont pas montré la même sensibilité et la finesse des particules des sables inhibe leur action quand les doses augmentent.

ملخص

سيّسوخو، باب ساينيو فاتو وال ومومار تالا وكاراموكو ديارا والحاجي سو وجورج لونيائي. 2018. تأثيرات مثل المبيدات الحشرية للرمال السيليزية الحافظة للذرة واللوبياء أثناء التخزين.

Tunisian Journal of Plant Protection 13 (2): 229-241.

تمت تجربة رمال سيليزية أثناء تخزين الذرة واللوبياء ضد حشرات الخزن. تم تقييم نشاط هذه الرمال المشابه لمبيدات الحشرية باستعمالها بجرعات متزايدة 1 و 2 و 3 و 4 غ/250 غ من المادة المخزنة على الحشرات البالغة *Sitophilus zeamais* و *Callosobruchus maculatus* و *Prostephanus truncatus* و *Tribolium castaneum*. لكل رمل سيليزي، ديوبال 1 و 2، تم اعتماد سواقي غربالين اثنين لـ 3 مم و 1 مم. تم استعمال المبيد الحشري أكتاليك® والحصى غير المعاملة كشاهد. أعيدت كل جرعة 4 مرات. بعد شهر واحد، بينت النتائج نجاعة كبيرة للرمال السيليزية تجاه الأنواع الأربعة للحشرات. تبين أن ديوبال 1 له أكثر نجاعة بنسبة وفيات 85% مقابل 100% للمبيد أكتاليك® و 0% للحصى غير المعاملة. وأظهر البروز تطور معاكس لنسبة الوفيات. وصلت الأضرار والخسائر في الوزن على التوالي 25 و 6% في غياب المعاملة. تسببت الحشرة *P. truncatus* في أضرار تقارب 16% وخسائر في الوزن 3% مع الجرعات الصغيرة. رغم ذلك، مع الجرعة 4 غ/250 غ من المادة المخزنة (1,6%، وزن/وزن)، كانت الخسائر أقل من 1%. لم تظهر الحشرات نفس الحساسية وكانت نعومة جسيمات الرمال تمنع نشاطها عندما ترتفع الجرعات.

كلمات مفتاحية: آفات حشرية، تخزين، ذرة، رمل سيليزي، لوبياء، نشاط مبيد حشري

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First diet survey in Niger River valley and acute risk assessment for consumers exposed to pesticide residues in vegetables

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ABSTRACT

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To control pests and crops diseases, small scale farmers in the Niger River valley use a wide range of plant protection products which could induce harmful impacts on human health and environment. Dietary exposure to plant protection products residues was assessed in Niger River valley using the 24-hour recall method. Portion sizes were estimated using a collection of pictures previously prepared according to the local usual diet. A total of 45 samples of eight types of vegetables, representative of the most consumed in the study area (Niamey) during the dry hot and the dry cold season were collected. Samples were analyzed using a multi-residue method (QuEChERS) by gas chromatography-mass spectrometry (GC-MS/MS) and liquid chromatography-mass spectrometry (LC-MS/MS) that can detect more than 540 active ingredients. Residues of insecticides were detected in 64.4% of the analyzed samples. Among them, 26.7% contained residues above maximum residue limits (MRLs), 35.5% below MRLs, 2.3% of samples had residue equal to the MRLs. Chili peppers, tomatoes, moringas, head cabbages, sorrel leaves and peppers were the most contaminated vegetables. Their residue levels were, respectively, 4.6 mg/kg of chlorpyrifos-ethyl, 0.29 mg/kg of dichlorvos, 1.8 mg/kg of cypermethrin, 1 mg/kg of chlorpyrifos-ethyl, 0.46 mg/kg of acetamiprid and 0.41 mg/kg of dichlorvos. To evaluate the intake and characterize the risk level for adults and children, the EFSA PRIMO model spreadsheet (Pesticide Residue Intake Model) was used. The exposure results based on consumptions at the 97.5th percentiles show that the highest predicted exposure values for a short-term intake (PSTI) was obtained in the case of consumption of head cabbages (532% of ARfD) for adults and tomatoes (1052% of ARfD) for children. Whatever the product, the risk of exposure was higher for children than for adults for all detected residues.

Keywords: acute exposure, consumers, Niger River valley, pesticide residues, risk assessment

Agriculture represents in Niger the main economic sector and participates

for approximately 40% in the country's Gross Domestic Product (GDP) (INS-Niger 2014). With an average population growth of 3.4%, one of the world's highest, the Niger population was estimated to 17,140,000 inhabitants. This average population growth largely exceeds

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the annual growth rate of agricultural production of the country estimated to 2.2% in recent years (INS-Niger 2010). With this significant food deficit, the Nigerien population is practicing increasingly horticulture in the Niger River valley and its tributaries.

In Niger River valley, horticulture is an important source of agricultural growth and poverty reduction in Niger. In fact, this activity is one of the sectors chosen by the country's policy to support food security and to fight against hunger. The horticulture sector is also an alternative proposed by the United Nations to face the challenges of urbanization (Perrin et al. 2014). The main vegetable crops are onion (*Allium cepa*), Chili pepper (*Capsicum annuum*), eggplant (*Solanum melongena*), head cabbage (*Brassica oleracea*), lettuce (*Lactuca sativa*), moringa (*Moringa oleifera*), pepper (*Capsicum annuum*), sorrel leaves (*Hibiscus sabdarifa*), tomato (*Lycopersion esculentum*) carrot (*Daucus carota*), cucumber (*Cucumis sativus*), okra (*Abelmoschus esculentus*), etc. However, they are subject to significant pest and disease pressure requiring the use of control methods. Chemical control often uses huge treatments of plant protection products (PPPs) (Andres et al. 2011; Illyassou et al. 2015).

PPPs contain at least one active ingredient and are mainly used to control pests and crop diseases (EU-database 2018). The active ingredients can be chemicals, plant extract, pheromone or micro-organisms including viruses (EC 2009). When they are correctly used, they have clearly shown their benefits in improving horticultural yields. Nevertheless, a possible consequence of their misuse can be the presence of residue levels in the treated products often exceeding Maximum Residue Limits (MRLs), and could induce negative

impacts on human health (Bhanti et al. 2007; Caldas et al. 2004; Nougadère et al. 2012). The presence of these residues in vegetables can be the consequence of poor dosage calculation, poor spraying technique, poor formulations, and repeated applications of the same active ingredient or the non-compliance to the pre-harvest interval (PHI). In recent years, chemical families of some active ingredient are known to have potential adverse health effects. Several diseases such as Parkinson disease, cancers or Alzheimer's could result from chronic exposure to some PPPs formulations (Chourasiya et al. 2015; Darko et al. 2008; Richard et al. 2014).

In order to protect consumer's health and assess the risks related to the use of PPPs, several methods and models have been used in current years in many countries over the world (Machera et al. 2003; Toumi et al. 2016). Risk assessment is a scientific process which consists in four steps: (i) hazard identification, (ii) hazard characterization, (iii) exposure assessment, and (iv) risk characterization. Risk assessment for consumers exposed to PPPs residues in beverages and food, is evaluated by comparing acute and chronic exposure values obtained from risk models to appropriate toxicological reference values (ARfD or ADI, respectively). Average concentrations of residues in food as well as usual and maximal consumptions are needed to estimate the risk level.

Unfortunately, few data are today available in Niger regarding the concentrations of residues in vegetables produced in the Niger River Valley. Therefore, a study was designed to monitor the levels of plant protection product residues in vegetables commonly consumed in this study area and to assess the risk level for different consumer groups, especially adults and children.

MATERIALS AND METHODS

Study area description.

This study was conducted in Niger River valley (Fig. 1), covering the entire study area (region of Niamey). The Niger River valley is the principal surface water resource of the country and therefore this region is one of the most populated. It covers the entire region of Niamey and located between 02°10' East longitude and 13°35' North latitude. It is the third largest river in Africa for its length (4,200 km) and its area of drainage basin (2,100,000 km²). It crosses the Niger territory over a length of 550 km. Entirely isolated in

Tillabery department, the river in Niamey is located in the West of the country where rainfall largely exceeds the national average. The banks of the river have a large capacity of agricultural production, especially horticulture.

Illyassou et al. (2017) recently performed a study on farmers' practices toward plant protection. They have identified the use of 25 active ingredients including 17 insecticides, 4 selective herbicides and 4 fungicides. The use of various counterfeit pesticides cocktails was also reported.

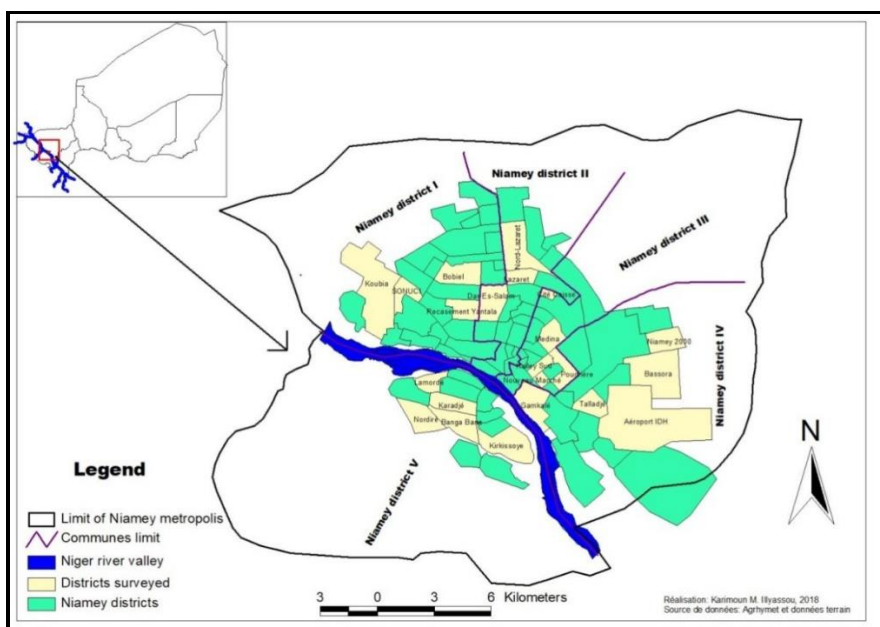


Fig. 1. Study area and municipal stratification of the Niamey region (framed in the figure shows the map of Niger)

Food consumption survey.

To collect reliable and representative food consumption data, a household survey based on European Food Safety Authority (EFSA) guidelines was conducted in Niamey metropolis (EFSA 2014). According to the National Statistics

Institute (NSI) of Niger, Niamey population was estimated to 1,026,848 inhabitants in 2012 and included 166,998 households. The population size for the consumption survey was determined according to the formula by Charan et al. (2013) using a 10% probability for cross

sectional studies about quantitative variables. A total of 100 households (twenty households in each district of Niamey) were interviewed (n = 571 persons). The formula is:

$$n = \frac{1.96^2 * N}{1.96^2 + (2e)^2 (N-1)}$$

where, 'N' is the household's number of Niamey city (166,998), 'e' is the margin of error (10%).

The choice of Niamey is explained by its position on the Niger River and high consumption of vegetables by its inhabitants, compared to the other urban inhabitants of the country (31.5 against 29.0 kg/person/year) (Andres et al. 2012). Using the 24-hour recall method (Gibson et al. 1999), the data collection was conducted with a questionnaire on vegetables and fruits locally produced and consumed in the Niger River valley. Portion sizes were estimated using a collection of pictures previously prepared according to the local usual diet, and percentiles were then calculated. According to the guidelines of Gibson et al. (1999), the list of the raw ingredients of each mixed dish was dressed and the total weight of the mixed dish was estimated. The proportions of each vegetable in all household mixed dishes were calculated and then used to estimate the consumption weight of each ingredient. A digital balance was used for the determination of the average body weight (bw) of the surveyed population.

Vegetables sampling.

In order to take into account the variability and the availability of vegetables produced in the Niger River valley, two sample collections were carried out during two different seasons of production, in Niamey and the surroundings. Thirty samples, representative of the most consumed

vegetables in Niamey were collected from 22 to 23 August 2017 during the dry hot season. Therefore, 15 samples of most consumed vegetables during the dry cold season were collected from 2 to 3 February 2018. Samples included Chili pepper, eggplant, head cabbage, lettuce, moringa, pepper, sorrel leaves and tomato. Vegetable samples (each sample consists of 4 subsamples) were collected randomly downstream of the watershed. According to the guidelines and principles of European Directive 2002/63/CE for the establishment of MRLs in foodstuffs, samples of 1 kg at the precise time of harvest were packed in papers, labeled and weighed. Fresh (unfrozen) samples were immediately sent by plane to a Belgian laboratory for analysis in the following hours. All useful information such as sample number, origin, sampling date, last treatment, pesticide applied, etc. were taken for each sample.

Pesticide residues analysis

Pesticide residues analysis was carried out by an accredited laboratory PRIMORIS (Technologiepark 2/3, 9052 Zwijnaarde-Gent, Belgium). It holds a BELAC accreditation to ISO/CEI 17025 for pesticide residues analysis on vegetables and herbal products in general. The method used for vegetables analysis was based on QuEChERS (Quick Easy Cheap Effective Rugged Safe) multi-residues method for pesticides in foodstuffs. QuEChERS is a separation method based on work done by the U.S. department of Agriculture research center in Wyndmoor (Anastassiades et al. 2003; Toumi et al. 2017). The QuEChERS method used by PRIMORIS helps to screen over 540 active ingredients and their metabolites. All active ingredients and their metabolites can be detected and quantified on harvested products. However, some ingredients which require

specific analytical methods, such as glyphosate or dithiocarbamates, were not investigated by this method but those chemicals are never used on vegetables in the study area.

The residues in extracted samples were identified and quantified by Gas Chromatography coupled with tandem Mass Spectrometry (GC-MS/MS) for small, thermally stable, volatile, non-polar molecules or by Liquid Chromatography coupled with tandem Mass Spectrometry (LC-MS/MS) for larger, thermolabile, non-volatile and polar molecules (Lehotay *et al.* 2007). The limit of quantification (LOQ) for all residues identified in extracted samples is 0.01 mg/kg.

Exposure Risk assessment.

To estimate the exposure level for different groups of consumers, the Predicted Short-Term Intake (PSTI) was used. The PSTI values were calculated with The EFSA PRIMO (Pesticide Residue Intake Model) model version 11 (RASFF 2016). The risk of each active ingredient detected in vegetable was characterized by comparing the PSTI value expressed in mg/kg bw/day to the Acute Reference Dose (ARfD) values obtained from the EU-Pesticides database (Table 1). There is a risk for consumers when the PSTI value of a given active ingredient is higher than the corresponding ARfD value.

Table 1. Toxicological reference values (TRVs) of active ingredients detected in the vegetable samples, the corresponding Acute Reference Dose (ARfD) values, the Acceptable Operator Exposure Level values (AOEL) and the Acceptable Daily Intake (ADI) values are expressed in mg/kg bw/day

Active ingredient	ARfD	AOEL	ADI	Source of TRVs
Acetamiprid	0.100	0.07	0.07	EC 2004
Allethrin	NA	NA	NA	NA
Chlorpyrifos-ethyl	0.005	0.001	0.001	EFSA 2014
Cypermethrin	0.040	0.06	0.02	JMPR 2006
Dichlorvos	0.002	0.0005	0.00008	EFSA 2006
Dimethoate	0.010	0.001	0.001	EFSA 2013
Emamectin	0.010	0.0003	0.0005	EFSA 2012
Fenitrothion	0.013	NA	0.005	EFSA 2006
Imidacloprid	0.080	0.08	0.06	EC 2008
Lambda-cyhalothrin	0.005	0.00063	0.0025	EU 2016

NA: Not Available

Statistical analysis.

All data collected during the survey (from the questionnaires) as well as the predictable exposure values were analyzed using Origin version 6.0 and/or Excel 2007 software.

RESULTS

Socio-demographic characteristics of the study population.

The 100 households interviewed were composed of 310 women and 261 men. People aged 18-39 were the most numerous with 46.4% of the population

(Fig. 2). According to the NIS of Niger (2016), the age distribution found in the population sample was estimated

sufficiently representative of the population of Niamey and therefore no quota method was applied.

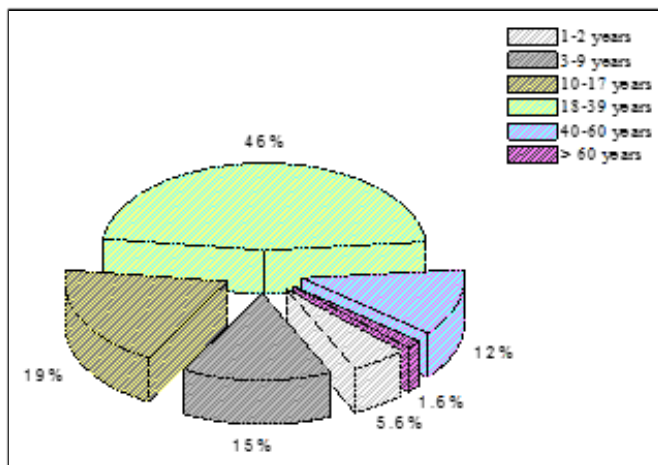


Fig. 2. Age classes of the surveyed population (n = 571) in the Niamey region

The average body weight (bw, used later in the PSTI calculation) of the population was 67 ± 10 kg and 30 ± 14 kg, respectively, for adults and children. The mean frequency of consumption for all the surveyed population was considered equal to two times per day. For the representativeness of the studied population, children aged 1-2 years are excluded for daily intake estimation.

Consumption survey results.

The 97.5th percentiles of consumption (or LP, used later in the PSTI calculation) for the studied population are shown in Table 2. This percentile was chosen as the most extreme value that can be estimated with any reasonable degree of certainty. Thus, are considered as big consumers both adults and children which

consume the 97.5th percentile of each vegetables analyzed. For children, daily consumption was estimated on the assumption that each member of the household participating in the family dish should consume a proportional share to his needs.

Plant Protection Product (PPPs) residues in analyzed vegetables.

PPPs residues were analyzed in 45 vegetable samples. Out of these samples, 29 (64.4%) have active ingredient residues while 16 (35.6%) have no residues ($< \text{LOQ} = 0.01 \text{ mg/kg}$). The percentage of the contaminated samples was important for all the analyzed vegetables except for eggplant and tomato samples (Table 3).

Table 2. The 97.5th percentiles of vegetables consumption for adults and children in Niamey metropolis

Vegetable	97.5 th percentile (g/people/day)	
	Adults	Children
Chili pepper	4.0	2.2
Eggplant	177.2	99.2
Head cabbage	356.5	199.7
Lettuce	221.9	124.3
Moringa	141.7	79.4
Pepper	8.6	4.9
Sorrel leaves	69.0	38.6
Tomato	310.9	174.1

Table 3. Residues detected in vegetables from Niger River valley

Vegetable	Average unit weight (g)	Number of analyzed samples	Samples with residues	
			Number	% *
Chili pepper	4 ± 1.7	5	4	8.9
Eggplant	101 ± 31.7	5	1	2.2
Head cabbage	524 ± 24.4	5	5	11.1
Lettuce	76 ± 28.2	6	5	11.1
Moringa	8 ± 14.4	6	4	8.8
Pepper	29 ± 14.4	6	5	11.1
Sorrel leaves	4 ± 1.9	6	4	8.8
Tomato	64 ± 32.0	6	1	2.2
Total		45	29	-

*: % of the total number of samples

Only insecticides residues were detected above the LOQ. Among the detected residues, chlorpyrifos-ethyl was found with the highest average concentration (1.8 mg/kg) in Chili pepper, followed by cypermethrin (1.6 mg/kg) in

moringa. λ -cyhalothrin and acetamiprid residues were the most predominant active ingredients found in the 45 vegetable analyzed samples (Table 4).

Table 4. Average concentrations of residues with variation and range, detected in each commodity; Limit of quantification (LOQ) = 0.01 mg/kg

Commodity	Active ingredient detected	n	Average concentration (mg/kg)	Range (min-max) (mg/kg)
Chili pepper	Emamectin-benzoate	2	0.012	0.011-0.013
	Chlorpyrifos-ethyl	3	1.799	0.017-4.6
	λ -Cyhalothrin	4	0.121	0.051-0.23
Eggplant	Chlorpyrifos-ethyl	1	0.400	<LOQ-0.40
Head cabbage	Fenitrothion	3	0.035	0.014-0.070
	Imidacloprid	1	0.014	<LOQ-0.014
	λ -Cyhalothrin	5	0.116	0.056-0.26
	Emamectin-benzoate	1	0.039	<LOQ-0.039
	Chlorpyrifos-ethyl	1	1.000	<LOQ-1.00
Lettuce	λ -Cyhalothrin	5	0.049	0.015-0.13
	Acetamiprid	1	0.100	<LOQ-0.10
Moringa	Allethrin	1	0.012	<LOQ-0.012
	Acetamiprid	4	0.093	0.010-0.21
	λ -Cyhalothrin	2	0.114	0.049-0.18
	Cypermethrin	2	1.600	1.4-1.8
Pepper	λ -Cyhalothrin	5	0.096	0.02-0.3
	Cypermethrin	2	0.029	0.019-0.039
	Dichlorvos	1	0.410	<LOQ-0.41
	Acetamiprid	4	0.063	0.014-0.2
	Imidacloprid	5	0.114	0.031-0.24
	Dimethoate	1	0.011	<LOQ-0.011
Sorrel leaves	Acetamiprid	3	0.313	0.12-0.46
	λ -Cyhalothrin	4	0.188	0.095-0.26
	Cypermethrin	1	0.024	<LOQ-0.024
	Dimethoate	1	0.013	<LOQ-0.013
Tomato	λ -Cyhalothrin	1	0.020	<LOQ-0.02
	Dichlorvos	1	0.290	<LOQ-0.29
	Acetamiprid	1	0.038	<LOQ-0.038

Out of the analyzed samples that had residues, more than a half (25 samples) was contaminated with λ -cyhalothrin residue followed by acetamipridone (Fig. 3). Residues levels of imidacloprid and cypermethrin were detected in six and five vegetable samples, respectively. A rate of 90% of the detected

active ingredients is authorized in Niger by the Sahelian Pesticides Committee (CSP 2017). Only dichlorvos active ingredient is banned for use in Niger. All detected residues (100%) are insecticides. Organophosphorus insecticides represent 40% of the residues followed by pyrethroids (30%) and neonicotinoids (20%).

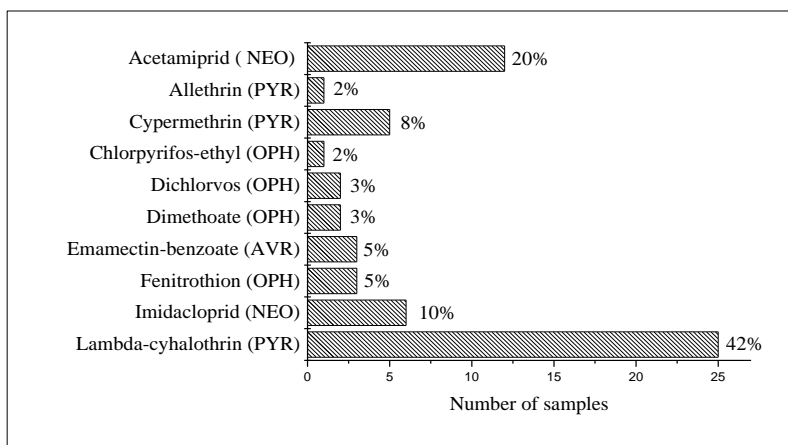


Fig. 3. Active ingredient residues detected in all vegetable samples in the Niamey region (OPH: organophosphorus, NEO: neonicotinoids, PYP: pyrethroids, AVR: avermectin).

In terms of the co-presence of the plant protection product residues, the combination of one or two and more than three insecticides from various chemical families was frequent. In fact, some samples contain multiples residues. Out of

positive samples, 49% contained more than one residue (Fig. 4). A rate of 27% of samples contains two residues and three residues were detected in 18% of the samples. The highest number of residue was found in pepper samples (up to 6).

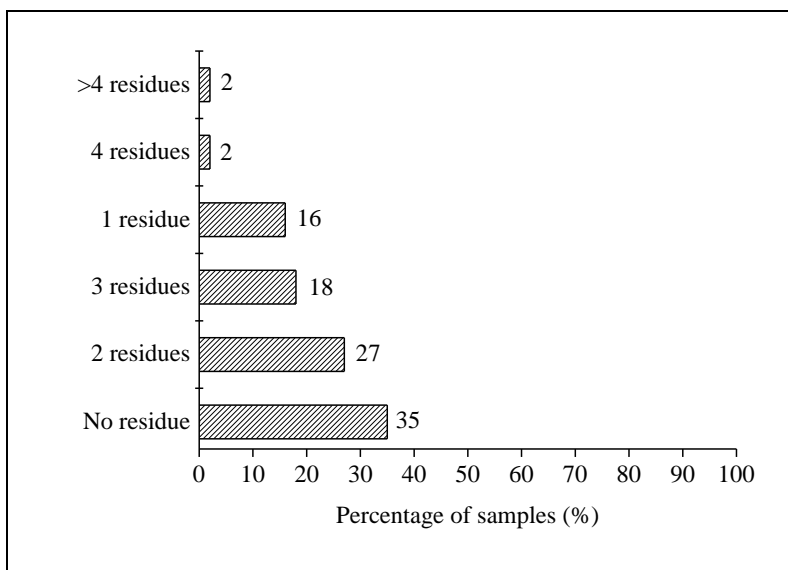


Fig. 4. Percentage of samples with multiple residues in the Niamey region.

Exceeding of maximum residue limits (MRLs).

Among the 45 analyzed samples, 12 contained residues exceeding the MRLs, while 16 contained residues below the MRLs set by European Union. As shown in Table 5, the MRL exceedances appeared particularly critical for chlorpyrifos-ethyl and dichlorvos, respectively, in Chili pepper and pepper.

Taking into account the multiple residues detected in several samples, the exceedances of MRLs per active ingredients were 16 and concern 6 active ingredients: acetamiprid (n = 3), chlorpyrifos-ethyl (n = 4), dichlorvos (n = 2), emamectin (n = 1), fenitrothion (n = 3) and λ -cyhalothrin (n = 3).

Table 5. Concentration of pesticide residues above the EU-MRLs in the vegetable samples

Vegetable	Active ingredient	Concentration (mg/kg)	EU-MRL (mg/kg)	Concentration (% of MRL)
Chili pepper	Chlorpyrifos-ethyl	0.017	0.01	170
Chili pepper	λ -Cyhalothrin	0.230	0.10	230
Chili pepper	Chlorpyrifos-ethyl	0.780	0.01	7800
Chili pepper	Chlorpyrifos-ethyl	4.600	0.01	46000
Chili pepper	λ -Cyhalothrin	0.110	0.10	110
Head cabbage	Emamectin	0.039	0.01	390
Head cabbage	Fenitrothion	0.022	0.01	220
Head cabbage	Fenitrothion	0.070	0.01	700
Head cabbage	Chlorpyrifos-ethyl	1.000	0.05	2000
Head cabbage	Fenitrothion	0.014	0.01	140
Pepper	λ -Cyhalothrin	0.300	0.10	300
Pepper	Dichlorvos	0.410	0.01	4100
Sorrel leaves	Acetamiprid	0.120	0.05	240
Sorrel leaves	Acetamiprid	0.460	0.05	920
Sorrel leaves	Acetamiprid	0.360	0.05	720
Tomato	Dichlorvos	0.290	0.01	2900

Acute dietary exposure and consumers risk assessment.

The exposure (predicted short-term intakes or PSTI) to residues of plant protection products was estimated with the EFSA PRIMO model version 11, an excel-based calculation spreadsheet used to perform the dietary risk assessment for pesticide residues at EU level (RASFF 2016) in which the unit weight of vegetables (U), the body weight of adults and children (bw) and consumptions (LP

of adults and children) were introduced (data collected thanks to the survey).

In this study, the exposure was estimated only for samples in which residues were above the limit of quantification (LOQ). Table 6 summarizes the nature of the detected active ingredients and their concentrations expressed in mg/kg in the vegetables, the calculated PSTI values expressed in mg/kg bw/day, the ArfD values and the PSTI values expressed as a percentage of ArfD

for adults and children consumers. The results based on consumption at the 97.5th percentile show that the most ARfD exceedance was found for children and only 4 exceedances were identified for adults. Among the residues detected, 4 active ingredients (organophosphorus (2) and pyrethroids (2)) were found to have an acute risk for adults and children. These active ingredients (dichlorvos, λ -cyhalothrin and chlorpyrifos-ethyl) have the lowest ARfD values. They are the most toxic in case of ingestion for adults and children. For all analyzed vegetables, the highest predicted exposure value for a short-term intake is obtained both for adults and children in the case of head cabbage consumption.

DISCUSSION

A total of 45 vegetables samples were collected at the fields in Niger River valley and analyzed. More than 64% of the samples contained plant protection product residues. The residues of 10 insecticides, belonging to 4 chemical families (organophosphorus, neonicotinoids, pyrethroids, and avermectins) were detected. The high frequency of detection pointed high levels of some pesticide residues detected in fruits, cereals and vegetables foodstuffs in West Africa (Bempah et al. 2011; Bempah et al. 2012; Kolani et al. 2016; Manirakiza et al. 2003; Ogbeide et al. 2016). It is partially in accordance with a study conducted in Brazil from 2001 to 2010 on pesticide residues in foodstuffs which highlighted that among 1,154 tomatoes and 1,007 lettuces analyzed samples, pesticide residues (\geq LOQ) were found in 59.8% and 33.9% of samples, respectively (Jardim et al. 2012). In another study,

among 724 analyzed samples (fruits and vegetables) collected from 8 South America countries, 72% of samples contained pesticide residues at or below MRL and 19% of the samples were found free of residues (Hjorth et al. 2011).

In the present study only 10 insecticides ($>$ LOQ) were identified. These results confirm that producers usually limit the number of pesticides used on vegetables, the dosage and number of sprays before harvest (Illyassou et al. 2017) to save money. Despite the sporadic use of 4 herbicides and 4 fungicides in the study area, no residues of these chemicals were detected in the harvested products and no residues of the other insecticides previously reported were detected.

Based on their toxicological properties, organophosphorus insecticides are known to disturb the nervous system due to an excessive accumulation of acetylcholine in synapse (Fulton et al. 2001). They act by an irreversible inhibition of the acetyl-cholinesterase enzyme in the synapse (Illyassou et al. 2018). Pyrethroids may also adversely affect the central nervous system, disrupting the function of axons by keeping open the sodium channels which causes a depolarization in the axon. Neonicotinoids act also on the central nervous system by blocking postsynaptic nicotinic acetylcholine receptors. Avermectin chemicals affect the ability of neurotransmitters such as glutamate and γ -amino-butyric acid (GABA) to stimulate an influx of chloride ions into nerve cells, resulting in loss of cell function (Roberts et al. 1998). Therefore, the potential hazard from human exposure to these chemicals is important.

Table 6. Adults and children predicted short-term intakes (PSTI in mg/kg bw/day) for egetables with residues; NA: Not Available

Vegetable	Sample	Active ingredient	Concentration (mg/kg)	ARfD (mg/kg bw)	Adults		Children	
					PSTI	%ARfD	PSTI	%ARfD
Chili pepper	Sample 1	λ-Cyhalothrin	0.230	0.005	9.61E-5	1.9	0.00021	4.3
		Chlorpyrifos-ethyl	0.017	0.005	7.10E-6	0.1	1.58E-5	0.3
	Sample 2	λ-Cyhalothrin	0.051	0.005	2.13E-5	0.4	0.00004	1.0
		Chlorpyrifos-ethyl	0.780	0.005	0.00032	6.5	0.00072	14.6
		Emamectin	0.013	0.010	5.43E-6	0.1	1.21E-5	0.1
	Sample 3	λ-Cyhalothrin	0.093	0.005	3.88E-5	0.8	0.00008	1.7
		Emamectin	0.011	0.010	4.59E-6	0.0	1.02E-5	0.1
	Sample 4	λ-Cyhalothrin	0.110	0.005	4.59E-5	0.9	0.00010	2.1
Eggplant	Sample 1	Chlorpyrifos-ethyl	4.600	0.005	0.00192	38.4	0.00429	85.9
		Chlorpyrifos-ethyl	0.400	0.005	0.00528	105.8	0.01181	236.3
Head cabbage	Sample 1	λ-Cyhalothrin	0.073	0.005	0.00194	38.8	0.00433	86.7
		Emamectin	0.039	0.010	0.00103	10.4	0.00231	23.2
	Sample 2	λ-Cyhalothrin	0.064	0.005	0.00170	34.1	0.00380	76.1
		Fenitrothion	0.022	0.013	0.00058	4.5	0.00130	10.1
		Imidacloprid	0.014	0.080	0.00037	2.9	0.00083	1.0
	Sample 3	λ-Cyhalothrin	0.130	0.005	0.00345	69.2	0.00772	154.5
		Fenitrothion	0.070	0.013	0.00186	14.3	0.00415	32.0
	Sample 4	λ-Cyhalothrin	0.260	0.005	0.00691	138.3	0.01544	309.0
	Sample 5	λ-Cyhalothrin	0.056	0.005	0.00148	29.8	0.00332	66.5
		Chlorpyrifos-ethyl	1.000	0.005	0.02660	532.1	0.05941	1188.3
		Fenitrothion	0.014	0.013	0.00037	0.5	0.00083	6.4
Lettuce	Sample 1	λ-Cyhalothrin	0.130	0.005	0.00215	43.1	0.00480	96.2
	Sample 2	λ-Cyhalothrin	0.067	0.005	0.00110	22.2	0.00247	49.6
	Sample 3	λ-Cyhalothrin	0.015	0.005	0.00024	5.0	0.00055	11.1
	Sample 4	λ-Cyhalothrin	0.015	0.005	0.00024	5.0	0.00055	11.1
	Sample 5	λ-Cyhalothrin	0.019	0.005	0.00031	6.3	0.00070	14.1
		Acetamiprid	0.100	0.100	0.00165	1.7	0.00369	3.7
Moringa	Sample 1	λ-cyhalothrin	0.049	0.005	0.00072	14.5	0.00162	32.4
	Sample 2	Allethrin	0.059	0.100	0.00087	0.9	0.00195	2.0
		λ-Cyhalothrin	0.012	NA	-	-	-	-
		λ-Cyhalothrin	0.180	0.005	0.00266	53.3	0.00595	119.0

	Sample 3	Cypermethrin	1.800	0.400	0.02664	66.6	0.0595	148.8
		Acetamiprid	0.210	0.100	0.00310	3.1	0.00694	6.9
	Sample 4	Acetamiprid	0.010	0.100	0.00014	0.1	0.00033	0.3
		Cypermethrin	1.400	0.400	0.02072	51.8	0.04628	115.7
Pepper	Sample 1	λ-Cyhalothrin	0.032	0.005	2.87E-5	0.6	0.00006	1.3
		Acetamiprid	0.014	0.100	1.25E-5	0.0	2.80E-5	0.0
		Imidacloprid	0.051	0.080	4.58E-5	0.1	0.00010	0.1
	Sample 3	λ-Cyhalothrin	0.300	0.005	0.00026	5.4	0.00060	12.0
		Cypermethrin	0.019	0.400	1.70E-5	0.0	3.81E-5	0.1
		Dichlorvos	0.410	0.002	0.00036	18.4	0.00082	41.1
		Dimethoate	0.011	0.010	9.88E-6	0.1	2.20E-5	0.2
		Acetamiprid	0.200	0.100	0.00017	0.2	0.00040	0.4
		Imidacloprid	0.098	0.080	8.80E-5	0.1	0.00019	0.2
	Sample 4	λ-Cyhalothrin	0.020	0.005	1.79E-5	0.4	0.00004	0.8
		Imidacloprid	0.031	0.080	2.78E-5	0.0	6.22E-5	0.1
	Sample 5	λ-Cyhalothrin	0.085	0.005	7.63E-5	1.5	0.00017	3.4
		Acetamiprid	0.017	0.100	1.52E-5	0.0	3.41E-5	0.0
		Imidacloprid	0.150	0.080	0.00013	0.2	0.00030	0.4
	Sample 6	λ-Cyhalothrin	0.046	0.005	4.13E-5	0.8	0.00009	1.8
		Acetamiprid	0.022	0.100	1.97E-5	0.0	4.41E-5	0.0
		Imidacloprid	0.240	0.080	0.00021	0.3	0.00048	0.6
		Cypermethrin	0.039	0.400	3.50E-5	0.1	0.00007	0.2
Sorrel leaves	Sample 1	λ-Cyhalothrin	0.140	0.005	0.00100	20.2	0.00225	45.1
		Acetamiprid	0.120	0.100	0.00086	0.9	0.00193	1.9
		λ-Cyhalothrin	0.095	0.005	0.00068	13.7	0.00152	30.6
	Sample 2	Cypermethrin	0.024	0.040	0.00017	0.4	0.00038	1.0
	Sample 3	λ-Cyhalothrin	0.260	0.005	0.00187	37.5	0.00418	83.7
		Acetamiprid	0.460	0.100	0.00331	3.3	0.00740	7.4
		Dimethoate	0.013	0.010	9.37E-5	0.9	0.00020	2.1
	Sample 4	λ-Cyhalothrin	0.260	0.005	0.00187	37.5	0.00418	83.7
		Acetamiprid	0.360	0.100	0.00259	2.6	0.00579	5.8
Tomato	Sample 1	λ-Cyhalothrin	0.020	0.005	0.00064	13.0	0.00145	29.0
		Dichlorvos	0.290	0.002	0.00941	471.0	0.02103	1051.9
		Acetamiprid	0.038	0.100	0.00123	1.2	0.00275	2.8

About 62% of the samples contain residues of λ -cyhalothrin, followed by residues of acetamiprid and chlorpyrifos-ethyl. The high detection in this study of these residues could in part due to the availability on the market in Niamey of pesticide formulations containing these active ingredients and their high persistence (Adamou et al. 2011; Illyassou et al. 2015). In fact, by comparing the active ingredients detected each other's, λ -cyhalothrin, dichlorvos and chlorpyrifos-ethyl have the lowest ARfD values. More than a half of the positive samples (75.9%) had multiple residues, with pepper being the vegetable with the highest number of residues in a single sample (up to 6). The presence of multiple residues in many vegetable samples could be explained by both the high frequency of treatments and the use of different formulations (Jallow et al. 2017). It could be also linked to non-compliance of the Pre-Harvest Intervals (PHI) highlighted in the study area (Illyassou et al. 2015). An average of three pesticide residues per vegetable sample was detected in gardening areas in Burkina Faso (Lehmann et al. 2017). Several studies have detected multiple residues in different foodstuffs including vegetables from many countries (Blankson et al. 2016; Dalvie et al. 2009; Jardim et al. 2012; Jallow et al. 2017; Keikotlhaile et al. 2010; Ngom et al. 2013; Son et al. 2017).

The highest concentrations of residues were found more in leafy vegetables than in fruiting vegetables. Nevertheless, the highest concentration of residue of all analyzed samples was detected for chlorpyrifos-ethyl in Chili pepper (4.6 mg/kg), which could be related to its lipophilic property. Cuticle analysis of some fruits revealed that the lipophilic composition of cuticle can help some lipophilic active ingredients to enter into the plant (Parsons et al. 2012; Trapp 2004).

A study conducted by Osman et al. (2010) in Saudi Arabia showed that the highest residue concentrations were found in cabbage (chlorpyrifos-ethyl, 6.207 mg/kg), tomato (tolclofos-methyl, 7.312 mg/kg), eggplant (carbaryl, 1.917 mg/kg), and pepper (carbaryl, 2.228 mg/kg). Nevertheless, the concentrations found were higher than those found in our study. In a previous study carried out in Burkina Faso, having similar agroclimatic conditions like Niger, authors reported the contamination of vegetables with pesticide residues (Lehmann et al. 2017). But, the concentration values were lower than those found in the present study.

Despite the high frequency of observed application (Illyassou et al. 2017 and 2018), 37% of samples collected in the dry hot season were free of residues. These findings could be linked to the degradation of active ingredients such as pyrethroids (the most used) by heat and sunlight (Adamou et al. 2011). All residues detected in lettuce samples were below the MRLs. However, 12 vegetable samples (26.7%) including 1 sample of tomato, 2 samples of pepper, 3 samples of sorrel leaves, 5 samples of head cabbage and 2 samples of Chili pepper had residues above the maximum residue limits for pyrethroids. The most exceedances of MRLs appeared for chlorpyrifos-ethyl and dichlorvos residues, respectively, in Chili pepper and head cabbage (46,00% and 2,00% of MRLs, respectively). The violation of MRLs in some vegetable samples could be a consequence of bad agrochemical practices and the non-respect of Good Phytosanitary Practices. In fact, the analysis of agrochemical practices in the Niger River valley has highlighted lack training of farmers for pesticide use. Farmers are not aware about the potential toxicity of pesticide formulations and/or active ingredient (Illyassou et al. 2017, 2018). These

findings corroborate the results of a study performed in Monze district (Zambia) where similar bad practices were highlighted (Mwanja et al. 2017). The results of this study are also in agreement with findings of a research performed in Ethiopia that showed lack knowledge among small farmers as one of the contributing factors of pesticide residues in vegetables (Mekonnen et al. 2002). In the study conducted by Jallow et al. (2017) on pesticide residues in Kuwait, 21% of the detected residues were found to exceed MRLs. A study conducted in Accra (Ghana) on pesticide residues in vegetables showed that okra and eggplant recorded the highest concentrations of allethrin (7.89 mg/kg) and permethrin (5.03 mg/kg). However, the detected concentrations were found to be higher than those detected in our study (Blankson et al. 2016).

PSTI expressed as a percentage of ARfD were in the range of 0.00-532.1% and 0.00-1188.3% for adults and children, respectively. Dichlorvos exposure in tomato consumption exhibited, respectively, acute risks for adults and children of 471 and 1,051%. Acute risk with λ -cyhalothrin and cypermethrin exposure was associated in the case of moringa consumption for children, but not confirmed for adults. An acute risk was identified for both adults and children in the case of eggplant consumption with chlorpyrifos-ethyl (105% and 236% ARfD, respectively). For λ -cyhalothrin, head cabbage consumption confirmed an acute risk exposure for children, but not confirmed for adults. However, the risk was observed both for adults and children in other samples of the same commodity (head cabbage) with residues of λ -cyhalothrin and chlorpyrifos-ethyl. In general, despite the high frequency of detected residues, consumers in Niger River valley do not face a significant acute

risk, except for dichlorvos, λ -cyhalothrin, cypermethrin and chlorpyrifos-ethyl.

These active ingredients present physicochemical and toxicological properties which induce an acute risk for general population. In fact, dichlorvos is expected to have a very high volatility in air (2.1 Pa, 25°C), but even if it is known to be a rather volatile compound, its octanol/water partitioning coefficient is high and its Log K_{ow} is positive ($K_{ow} = 7.94 \times 10^1$; Log $K_{ow} = 1.9$). This reflects its lipophilic property explaining its persistence on treated crops. Chlorpyrifos-ethyl is also expected to be a volatile compound (2.7 $\times 10^{-3}$ Pa, 25°C) (TOXNET-database 2018). But after treatment, it penetrates more deeply into the plant and becomes less available for volatilization thanks to its very high lipophilicity ($K_{ow} = 5.01 \times 10^4$; Log $K_{ow} = 4.7$) explaining also its persistency (Solomon et al. 2014). The dissipation rate (RL₅₀) on and in plant matrix range from 0.95-127.0 days (PPDB-database 2018).

λ -cyhalothrin and cypermethrin are pyrethroid active ingredients, having a low aqueous solubility (0.004 mg/kg, 20°C) are relatively volatiles (1. $\times 10^{-6}$ Pa and 2.3 $\times 10^{-7}$ Pa, 20°C, respectively). Like organophosphorous pesticides, the two pyrethroids have both a high K_{ow} (3.16 $\times 10^5$ and 3.55 $\times 10^5$, respectively), as they can also strongly penetrate into the treated crops. They are moderately persistent under field conditions on and in plant matrix (RL₅₀ range 0.5-15.3 days and RL₅₀ range 1.2-10.3 days, respectively) (PPDB-database 2018). Based on its chemical properties, all the active ingredients are not suggested to leach to groundwater except for chlorpyrifos-ethyl which has a high leaching potential index (GUS = 3.63).

Therefore, the presence of these residues could be explained not only by the high frequency of sprays, but also by their lipophilic properties in plant matrix. This

could be explained in part by the persistence of some residues in soils as well. By leaching or runoff, these active ingredients can reach surface or ground waters during a raining phenomenon very common in the Niger River valley. Contamination could also occur during crop irrigation with already contaminated waters. It should be noted that in Niger, vegetable crops are mostly grown in watershed with a steep slope (source of surface waters contamination). Finally, the presence of residues could be linked to non-compliance of the PHI observed at the field level (Illyassou et al. 2017).

In conclusion, the results of dietary exposure of plant protection product residues in vegetables in the Niger River valley show that from 45 vegetable samples, 26.7% exceeded the MRLs established by the European Union. More than a half of the positive samples (75.9%) had multiple residues. PSTI values appear to be relatively low compared to the corresponding ARfD values in all vegetable samples, except in 8 cases where PSTI values exceed several times ARfD. Therefore, to reduce the risk and safeguard the human health of the consumers in the Niger valley, producers need to replace persistent active ingredients and those with

low ARfD. Trainings should be initiated by agriculture agents to enhance farmer's knowledge on pesticide use and handling. More precautions must be taken to reduce dietary exposure especially for children (diversification of the diet). Processes of residue reduction (washing, boiling or cooking) must be taken by consumers to reduce significantly the risk level associated with active ingredient ingestion) (Bonnechère et al. 2012a, 2012b). Household processes like boiling can reduce some residues of pesticide from 20% to 100% in vegetables (Kumari 2008). There is also a need to more investigations on pesticide residues in all foodstuffs including cereals, meat, fish, milk products and water where other ingredients are often mentioned in many studies (Akoto et al. 2013; Darko et al. 2008; Ibgbami et al. 2015; Idowu et al. 2013; Topsoba et al. 2006). Regular close surveillance on potential introduction of herbicides and fungicides in the local agricultural practices and on house use insecticides is advisable. A chronic risk assessment in Niger in order to take overview of contamination levels and investigations on surface waters and sediments are also needed to get a large view on environmental impacts.

RESUME

Illyassou K.M., Adamou R. et Schiffers B. 2018. Première enquête alimentaire dans la vallée du fleuve du Niger et évaluation du risque d'exposition aiguë des consommateurs aux résidus des pesticides dans les légumes. Tunisian Journal of Plant protection 13 (2): 243-262.

Pour lutter contre les bioagresseurs des cultures, les agriculteurs de la vallée du fleuve du Niger utilisent une large gamme de produits phytopharmaceutiques. Particulièrement toxiques, les substances actives contenues dans ces produits peuvent induire des effets nocifs importants tant sur la santé humaine que sur l'environnement. Cette étude a porté sur l'exposition alimentaire aux résidus des produits phytopharmaceutiques dans la vallée du fleuve du Niger. L'enquête de consommation a été conduite en utilisant la méthode du rappel de 24 heures. Les tailles des portions sont estimées à partir d'une collection d'images préalablement préparées selon le régime alimentaire local habituel. Au total, 45 échantillons de huit types de légumes, les plus consommés dans la zone d'étude (Niamey) pendant la saison sèche froide et la saison sèche chaude ont été collectés. Les échantillons ont été extraits par la méthode QuEChERS multi-résidus puis analysés moyennant la chromatographie en phase gazeuse (GC-MS/MS) et la

chromatographie en phase liquide couplées à la spectrométrie de masse (LC-MS/MS); cette méthode concerne un jeu de plus de 500 substances actives. Les résultats montrent que des résidus d'insecticides ont été détectés dans 64,4% des échantillons parmi lesquels 26,7% des échantillons contiennent des résidus supérieurs aux limites maximales de résidus (LMR) et 2,3% des échantillons contiennent des résidus égaux aux LMR, 35,5% des échantillons ont des teneurs en résidus inférieures aux LMR. Les légumes les plus contaminés sont le piment, la tomate, le moringa, le chou pommé, les feuilles d'oseille et le poivron avec 4,6 mg/kg de chlorpyrifos-éthyl, 0,29 mg/kg de dichlorvos, 1,8 mg/kg de cyperméthrine, 1 mg/kg de chlorpyrifos-éthyl, 0,46 mg/kg d'acétamipride et 0,41 mg/kg de dichlorvos, respectivement. Pour évaluer l'apport journalier et caractériser le niveau de risque pour les adultes et les enfants, la feuille de calcul du modèle PRIMO de l'EFSA a été utilisée. Les résultats basés sur les consommations au percentile 97,5 montrent que les plus fortes valeurs d'exposition à court terme (PSTI) sont obtenues dans le cas de la consommation du chou-pommé pour les adultes (532% de la valeur de référence) et la consommation des tomates pour les enfants (1052% de la valeur de référence). Quel que soit le type de produit agricole, les enfants semblent être plus exposés que les adultes pour tous les résidus détectés.

Mots clés: évaluation des risques, exposition aiguë, résidus de pesticides, vallée du fleuve du Niger

ملخص

إلياسو، كريمون ماسالانثي ورياتي أدامو وبيرونو تشيفارس. 2018. أول مسح غذائي في وادي نهر النيجر وتقييم خطر التعرض الحاد للمستهلكين لمخلفات المبيدات الفلاحية في الخضروات.

Tunisian Journal of Plant protection13 (2): 243-262.

لمكافحة آفات وأمراض الزراعات، يستخدم المزارعون في وادي نهر النيجر مجموعة كبيرة من المبيدات الفلاحية. هذه المواد الفعالة، السامة بشكل خاص، يمكن أن تحدث تأثيرات سلبية خطيرة على صحة الإنسان وسلامة البيئة. أجريت هذه الدراسة حول تعرض الغذاء لبقايا مبيدات الآفات في وادي نهر النيجر. تم إجراء مسح للاستهلاك باستخدام طريقة الاستدعاء على مدار 24 ساعة. تم تقدير أحجام الأجزاء من مجموعة من الصور التي تم إعدادها مسبقاً وفقاً للنظام الغذائي المحلي المعتاد. في المجموع، تم جمع 45 عينة من ثمانية أنواع من الخضروات، الأكثر استهلاكاً في منطقة الدراسة (نيامي) خلال موسم الجفاف البارد وموسم الجفاف الحار. وقد تم تحليل العينات بطريقة متعددة البقايا (كوبيشن) وباستخدام الكروماتوغرافيا الغازية مقترنة بمطياف الكتلة (GC-MS/MS) والكروماتوغرافيا السائلة مقترنة بمطياف الكتلة (LC-MS/MS). وقد خصت هذه التحاليل 500 عينة. أظهرت النتائج أنه تم الكشف عن بقايا المبيدات الحشرية في 64.4% من العينات، منها 26.7% تحتوي على بقايا أعلى من الحد الأقصى للمادة المتبقية (MRL) و 2.3% ذات مستويات بقايا تتساوى مع الحد الأقصى للمادة المتبقية و 35.5% لديها مستويات متبقية تحت الحد الأقصى للمادة المتبقية. كانت الخضروات الأكثر تلوثاً هي الفلفل الحار والطماطم والمورينغا والملفوف وأوراق الحميض والفلفل الحلو، على التوالي 4.6 ملغ/كغ كلوربيريفوس-إيثيل و 0.29 ملغ/كغ ديكلوروفوس و 1.8 ملغ/كغ سيبرمثرين و 1 ملغ/كغ كلوربيريفوس-إيثيل و 0.46 ملغ/كغ أسيتامبريد و 0.41 ملغ/كغ من ديكلوروفوس. ولتقييم الإسهام اليومي وتحديد مستوى المخاطر للبالغين والأطفال، تم استخدام جدول بيانات نموذج PRIMO لـ EFSA. أظهرت النتائج على أساس استهلاك منوي يساوي 97.5، أنه يتم الحصول على أقوى قيم التعرض على المدى القصير (PSTI) في حالة استهلاك الملفوف للبالغين (532% من القيمة المرجعية) واستهلاك الطماطم للأطفال (1052% من القيمة المرجعية). يبدو أن الأطفال أكثر تعرضاً من الكبار لجميع البقايا التي تم الكشف عنها.

كلمات مفتاحية: بقايا مبيدات فلاحية، تقييم المخاطر، تعرض الحاد، مستهلكون، وادي نهر النيجر

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First Report of the Bark Beetle *Phloeosinus armatus* on the Mediterranean Cypress *Cupressus sempervirens* in Syria

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ABSTRACT

Aljouri, E., Gharib, M., Almanoufi, A., Daher-Hjai, N., and AlIssa-AlKharaba, H. 2018. First report of the bark beetle *Phloeosinus armatus* on the Mediterranean cypress *Cupressus sempervirens* in Syria. Tunisian Journal of Plant Protection 13 (2): 263-268.

The bark beetle *Phloeosinus armatus* is one of the most important pest that causes significant damages to the Mediterranean cypress *Cupressus sempervirens*. Adults of this insect were collected from the Mediterranean cypress trees from several sites located in the Eastern Ghouta near Damascus, South of Syria, in autumn 2014. The insect was morphologically described. Measurements were taken from different body parts. The adults are characterized by small sizes ranged between 3.60 to 3.88 mm for males and 3.88 to 4.08 mm for females, with a shiny chestnut color covered with short hair. The antennae are clavate (capitate) and consist of 5 flagella and their length ranged between 1.04 to 1.12 mm for males and 1.01 to 1.16 mm for females. They are characterized by chewing mouthpart type. The legs are similar in shape. The tarsus consists of 4 segments. The tip segment is prolonged and ends with a couple of claws. The leg length ranged between 1.26 to 1.49 mm for the males and 1.80 to 1.96 mm for females. This investigation, aiming mainly to describe *P. armatus*, is the first study in Syria.

Keywords: Bark beetle, cypress, morphological description, *Phloeosinus armatus*, Syria

The cypress trees *Cupressus sempervirens* are widely distributed in the Mediterranean region and in other areas with similar climate, including California, South Africa and southern Australia. How-

ever, its native distribution still unknown. *C. sempervirens* seems to be native from the Eastern Mediterranean region, including Libya, southern Greece, Turkey, Cyprus, Syria, Lebanon, and Iran (Govaerts 2015; USDA 2018).

Many cypress trees of *C. sempervirens* have died in forests, windbreaks, parks and gardens in Syria. However, they are in a state of decline due to many causal agents such as pathogens (*Seiridium cardinale*), insects (*Cinara*

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cupressi, *Phloeosinus* sp.) as well as abiotic agents, which are responsible for the most recurrent damages. In the Mediterranean region, the cypress bark beetle *Phloeosinus armatus* (Coleoptera, Curculionidae) is an important insect attacking particularly the Mediterranean cypress *C. sempervirens*. There are more than 70 species belonging to *Phloeosinus* genus and which are distributed in all continents (Wood and Bright 1992). Among them, six *Phloeosinus* species are native from Europe (Löbl and Smetana 2011). All of them are mainly associated with trees and shrubs from Cupressaceae family (Andrews 2002). *P. armatus* was described in 1887 based on specimens collected from *C. sempervirens*. Besides, the bark beetles of *Phloeosinus* genus are considered as an important factor of spreading of the fungus *S. cardinale* (Covassi et al. 1975; Pennacchio et al. 2013; Wägener 1939). Anderbrant et al. (1988) noted that beetles likely attack weakened trees under stress from fungi. In the last years, the neglecting and decreasing of irrigation of cypress trees in the Eastern Ghouta near Damascus caused the widespread and infestation by the bark beetle *P. armatus*.

The aim of this research was to collect information on this bark beetle species (*P. armatus*) in south of Syria as a preliminary report by the description of this pest morphologically and biometrically.

MATERIALS AND METHODS

Fifty samples of adults for bark beetles *P. armatus* were collected from several Mediterranean cypress *C.*

sempervirens in the autumn of 2014, from three locations: Qarahta (33.39 N, 36.42 E), Al Ghuzlanayah (33.39 E, 36.45 N) and Aqraba (33.44N, 36.38E); the three sites were located at altitude of 630 m, in the South of Syria. This region is characterized by a Mediterranean climate with hot and dry summer, cold winter and average rainfall about 250 mm. In the laboratory, samples were stored in 90% ethanol for the morphological analyses. The studied samples were classified according to key of *Phloeosinus* genus classification (Borror et al. 1976; Brues et al. 1954; Ciesla 2011; Reitter 1887; Wood and Bright 1992). The biometric measurements of the samples were taken by Olympus (SZ61-Japan-WD38) using an eyepiece reticle and with a different magnification scale; all readings were converted to millimeters.

RESULTS AND DISCUSSION

Damage and symptoms.

Adults often bore into twigs for feeding and then burrowing under the bark to lay their eggs. The result of feeding causes dying the branch tips which remained often hanged on the trees for a time before falling. The dead tips on the ground around the trees were symptomatic of bark beetle infestation.

Bits of very fine sawdust on the bark and wholes in the trees were observed. The cypress tree's crown turns first yellow and then reddish-brown (Fig. 1). Under the bark, tunnels in the wood have a pattern similar to the shape of the centipede (Fig. 2). The risk of this pest in this area is very considerable.

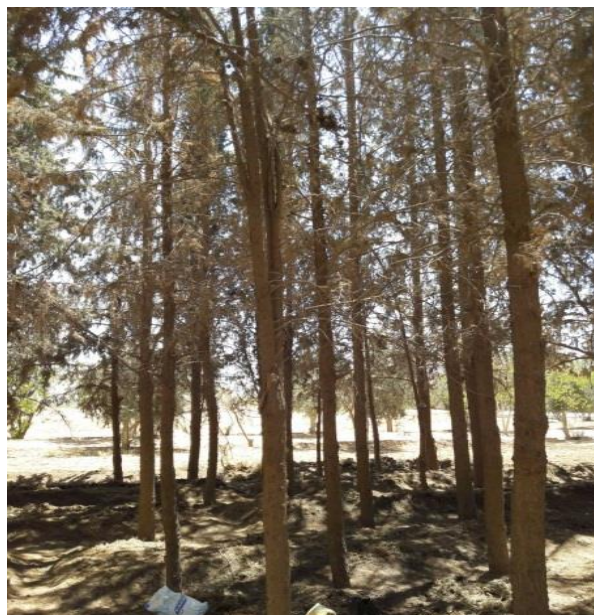


Fig. 1. Cypress branch dieback symptoms due to *Phloeosinus armatus*



Fig. 2. Cypress bark beetle gallery in cypress trunk

Adult morphology.

Body. Small, length 3-5 mm, with a chestnut color to a shiny dark brown, full-body covered with short hairs which are longer in front of the head and the last abdominal segments.

Head. Downward and forming an angle with the body. The antenna is clavate in form, its consist of five

segments and the last one enlarged as clavate shape. Compound eyes are sideling, elongate and front edge are concave. The three simple eyes take the shape of the inverted triangle (its head down). Mouthparts are chewing type, mandibles appear out of the mouth and head clearly, and form a black-coloured chitin spine.

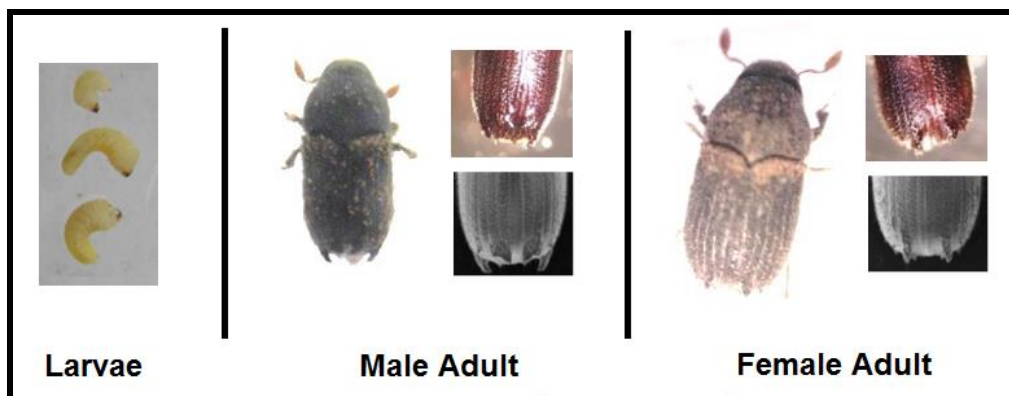


Fig. 3. *Phloeosinus armatus*: Larvae and adults (elytral declivity and dorsal view).

Thorax. Large, triangular pronotum, width scutellum doubler than sternum, and pleuron is diagonally with decorated small round pits.

Mesothorax. Horny wings (elytra wings) are decorated with longitudinal lines from pits. On each elytra, there are three pairs of symmetrical chitin enation which are present on the last third of them. These enations are wide and double in males compared to females. Enations vary in size so that the smaller ones will be at the end of the elytra and the larger toward the inside, it looks like a wide tooth form.

Legs. Each thorax segment has a pair of legs. These legs are similar in shape and size, however, female legs are longer than those of males with a wide femur covered by hairs on the back edge. Tibia is prolonged and enlarged from below and carrying the enlarged end several spines; there are on both front and rear borders hairs which are longer on rear border. Tarsus consists of 4 segments, the terminal segment is elongate and its length is equal to the other segments and has a pair of claw.

Abdomen. Short, less than a quarter of the body length and it consists of 9 segments, the first four segments are overlapping (compressed) and the last five ones are clear. There are three black lines separating the last four abdominal segments. The last abdominal segment is circular in shape, hiding underneath the reproduction system.

Biometric measurements.

The specimens collected in Southern Syria showed that the size of females was longer than that of males, about 3.97 ± 0.084 mm for females and 3.78 ± 0.011 mm for males; elytron length was 2.72 ± 0.034 mm and 2.40 ± 0.049 mm for females and males, respectively; antenna length was approximately similar for both; 1.07 ± 0.068 mm and 1.08 ± 0.029 mm for females and males respectively; while legs length was longer for females, about 1.87 ± 0.068 mm and about 1.40 ± 0.082 mm for males (Table 1). These values are similar to those reported by Pennacchio et al. (2013).

Table1. Morphometric parameters of adult bark beetle *Phloeosinus amateurs* on Mediterranean cypress *Cupressus sempervirens* in the Eastern Ghouta near Damascus, Syria

Body parts dimensions (mm)	Sex	Min	Max	Mean±SD
Overall Length	Male	3.60	3.88	3.78±0.011
	Female	3.88	4.08	3.97±0.084
Elytron Length	Male	2.36	2.48	2.40±0.049
	Female	2.68	2.76	2.72±0.034
Antenna Length	Male	1.04	1.12	1.08±0.029
	Female	1.01	1.16	1.07±0.068
Clavate Length	Male	0.48	0.52	0.51±0.018
	Female	0.48	0.56	0.52±0.033
Leg Length	Male	1.26	1.49	1.40±0.082
	Female	1.80	1.96	1.87±0.068

RESUME

Aljourri E., Gharib M., Almanoufi A., Daher-Hjai N. et AlIssa-AlKharaba, H. 2018. Premier signalement du scolyte *Phloeosinus armatus* sur le cyprès méditerranéen *Cupressus sempervirens* en Syrie. *Tunisian Journal of Plant Protection* 13 (2): 263-268.

Le scolyte *Phloeosinus armatus* est l'un des plus importants ravageurs qui cause des dégâts significatifs sur le cyprès méditerranéen *Cupressus sempervirens*. Des adultes de cet insecte ont été collectés d'arbres de cyprès dans plusieurs sites localisés à Ghouta-Est, près de Damas, au sud de la Syrie, en automne 2014. L'insecte a été décrit morphologiquement. Les mesures ont été prises de différentes parties du corps. Les adultes sont caractérisés par de petites dimensions allant de 3,60 à 3,88 mm pour les mâles et 3,88 à 4,08 mm pour les femelles, avec une couleur châtain-brillant couverte de courts poils. Les antennes sont clavées (capitées) et consistent en 5 flagelles et leurs longueurs varient de 1.04 à 1.12 mm pour les mâles et de 1,01 à 1,16 mm pour les femelles. Ils sont caractérisés par des pièces buccales du type broyeur. Les pattes ont la même forme. Le tarse est formé de 4 segments. L'extrémité du segment est prolongée et se termine par deux griffes. La longueur des pattes se situe entre 1,26 et 1.49 mm pour les mâles et 1,80 et 1,96 mm pour les femelles. Cette investigation, visant surtout de décrire *P. armatus*, est la première étude en Syrie.

Mots clés: Cyprès, description morphologique, *Phloeosinus armatus*, scolyte, Syrie

ملخص

الجوري، إبراهيم ومنيف غريب وعادل المنوفي ونورالدين ظاهر حجيح وهلا العيسى الخرابية. 2018. تسجيل أول لخنفساء القلف *Phloeosinus armatus* على السرو المتوسطي *Cupressus sempervirens* في سورية. *Tunisian Journal of Plant Protection* 13 (2): 263-268.

تُعد خنفساء القلف *Phloeosinus armatus*، واحدة من أخطر الحشرات التي تسبب ضرراً كبيراً على أشجار السرو المتوسطي *Cupressus sempervirens*. جُمعت بعض الحشرات البالغة في خريف 2014 من أشجار السرو من عدة

مواقع في الغوطة الشرقية قرب دمشق، جنوب سورية. لإجراء دراسة مورفولوجية عليها، أخذت القياسات الشكلية لعدة أجزاء من الجسم. تراوحت أطوال الجسم ما بين 3.60-3.88 مم للذكور و3.88-4.08 مم للإناث. يبدو الجسم ذو لون كستنائي لامع، تغطيه شعيرات صغيرة بينما قرون الاستشعار تبدو صولجانية ومؤلفة من خمس عقل. يتراوح طول قرن الاستشعار بين 1.04-1.12 مم للذكور و1.01-1.16 مم للإناث. ينتمي فم هذه الحشرة الى النوع القارض أم أرجلها فهي متشابهة في الشكل. تراوحت أطوال الساق ما بين 1.26-1.46 مم للذكور و1.80-1.96 مم للإناث. ويتألف الرسغ من أربع عقل مع العقلة الطرفية طويلة وتنتهي بزواج من المخالب. وتعد هذه الدراسة، المخصصة أساسا لتوصيف الحشرة، الأولى في سورية.

كلمات مفتاحية: خنفساء اللحاء، سرو متوسطي، سورية، وصف شكلي، *Phloeosinus armatus*

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First Report of *Carcina quercana* on the Strawberry Tree (*Arbutus unedo*) in North Western Tunisia

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ABSTRACT

Ezzine, O., Ben Yahia, K., Dhahri, S., Ammari, Y., and Ben Jamâa, M.L. 2018. First report of *Carcina quercana* on the strawberry tree (*Arbutus unedo*) in north western Tunisia. Tunisian Journal of Plant Protection 13 (2): 269-274.

Carcina quercana is a polyphagous insect. In April 2018, larvae of *C. quercana* were observed for the first time in Majen Essef (north western Tunisia) on the strawberry tree (*Arbutus unedo*). To estimate the percentage of tree infestation, the number of infested trees among 40 trees found in an area of one hectare was counted. Branches of about 30 cm in length were cut and examined in the laboratory to determine the percentage of the infested leaves. On infested leaves, shelters were counted and the number of larvae by shelter was determined. The percentage of infested tree was 20% and that of infested leaves was 7.16%. In each leaf, we found between 1 and 3 shelters, in which only one larva host it. Larvae of *C. quercana* build their shelters of about 1.91 cm in length and 0.73 cm in width by means of silk.

Keywords: *Arbutus unedo*, *Carcina quercana*, larvae, Tunisia

The Gelechioidea is a mega-diverse superfamily of Lepidoptera with 18000 described and more unnamed species and ranks among the most diverse Lepidopteran superfamilies (Kaila et al. 2011). The family of Peleopodidae includes 7 genera and 28 species (Van Nieukerken et al. 2011).

The species *Carcina quercana* is univoltine and a polyphagous insect (Luciano and Roversi 2001). Larvae attack several families: Fagaceae (*Quercus* (Luciano and Roversi 2001), *Fagus*

sylvatica, *Castanea sativa*), Rosaceae (*Crataegus monogyna*, *Prunus spinosa*, *Malus sylvestris*, *Sorbus*, *Rosa*), Caprifoliaceae (*Viburnum*), Hypericaceae (*Hypericum*), Aceraceae (*Acer pseudoplatanus*) (Emmet and Langmaid 2002) and Ericaceae (*Arbutus unedo*) (Luciano and Roversi 2001). *Carcina quercana* is widely distributed, particularly in central and southern Europe (Alford 2017), is common and widespread throughout the UK and Ireland (O'Reilly 2017) and an introduced pest in Canada (Alford 2017). It was reported in Algeria by Benia and Bounechada (2011) and in Morocco by Villemant and Fraval (1993).

The strawberry tree is one of 12 species, which belongs to the genus

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Arbutus of Ericaceae family (Ertekin and Kirdar 2010). It is an evergreen shrub species and typical of Mediterranean fringe and climate, but today it is also cultivated in many other regions such as the Near East and Transcaucasia (Seidemann 1995). In Tunisia, *A. unedo* is observed in the north west, in the forests of *Q. suber* and *Q. canariensis* (Mezghani 1992) and in the north east, in Jebel Abderrahmane (Ezzine 2016).

This work is a first report of *C. quercana* on *A. unedo* in northwestern Tunisia.

MATERIALS AND METHODS

Study area.

The investigations were carried out in the northwestern Tunisia (Majen Essef, Ain Drahem, 36°46'07.48" N.; 8°47'41.80" E.; alt.800 m) in April and May 2018. The plot was circular shaped (1 ha). The forest study area consists mainly of *Q. suber*, *Q. canariensis*, *Erica arborea*, *Myrtus communis*, *Halimium halimifolium*, *Daphne gnidium*, *A. unedo* and *Phillyrea angustifolia*.

Host plant infestation.

To study shrub species infestation of this site, investigations have focused on all existing shrub species. Infestation was observed only on *A. unedo*. To estimate the infested host plant, the number of infested trees was counted. The largest and smallest diameters of the crown of each infested plant were measured and the mean diameter per plant was calculated (Ezzine et al. 2015).

Collection of branches and examination of shelters.

Branches of about 30 cm in length were cut and examined in the

laboratory to determine the number of the infested leaves per branch. There were two types of leaves: non infested leaves and leaves hosting only shelters of *C. quercana*. Infested leaves were placed in Petri dish (9 cm diameter) and kept in the laboratory at 25°C. The length and the width of the infested leafves were measured and the number of shelters per leaf was counted. The number of larvae by shelter was determined (Ezzine et al. 2018). Identification of species was done using Luciano and Roversi (2001) key.

RESULTS

Infestation.

In total, 40 trees were investigated and among them, about 20% (n = 8) of *A. unedo* were infested. The number of infested leaves was 1 to 3 per branch (average 1.29). Mean crown diameter of the trees ranged from 37.5 to 170 cm (average 104.81 cm). The height of the trees ranged from 60 to 390 cm (average 199.37 cm).

Shelters density.

In a single leaf, we find between 1 and 3 shelters (average 1.42), in which only one larva was observed. The shelter length ranged from 1.2 to 2.7 cm (average 1.91 cm), while the shelter width ranged from 0.4 to 1.4 cm (average 0.73 cm).

Biological observations.

In Majen Essef, larvae of *C. quercana* (Fig. 1a,b) were observed between April and May 2018. The extremities of the shelter are open allowing the movement of larvae. When fully grown, larva pupates beneath an opaque, sheet-like web (Fig. 1c). Adult (Fig. 1d) was observed in June 2018.



Fig. 1. Different stages of *C. quercana* development. **a:** Shelter of *C. quercana* (with 1: Larva in the shelter; 2: Defoliated part of the leaf); **b:** larva; **c:** pupa; **d:** adult.

DISCUSSION

The Gelechioid larvae display a wide array adaptation (Powell et al. 1998). Most species are external feeders at the larval stage, but larvae stay in a shelter/a silken web or tube, or between leaves that have been tied together (Kaila et al. 2011). Some Gelechioid larvae spend most of their time below ground, and regularly resurface to consume leaf rosettes or other resources from there. Certain species-rich lineages, entirely or partly, consist of species with larvae as leaf miners during at least some part of their development. Many other species are leaf miners at least at some life stage (Emmet et al. 1996).

Carcina quercana lives sheltered behind a flat transparent silken web attached to the underside of the leaves and

feeds on. It folds the leaf very slightly by drawing the outer margins together (Luciano and Roversi 2001). It feeds singly on the underside of leaves (this part is more often in the shade and so it is cooler, and to flee predators). Young larvae bite out patches in the underside of leaves often leaving the upper surface intact. Later, complete holes are made in the lamina near the web, either in the center or at the margin of the leaf (Alford 2017) but without causing any serious damage (Alford 2017; Luciano and Roversi 2001). It is common for shelter-building microlepidopterans to spend the whole larval stage protected (Gaston et al. 1991) as observed for *Anacampsis scintillella* on *Q. coccifera* (Ezzine et al. 2018). Larvae of *C. quercana* are active in

May and June; each sheltering beneath a transparent web of transverse threads (Alford 2017). In Majen Essef, larvae were observed between April and May. Adult was observed in July and August (Alford 2017), but in our study site, it was observed in June.

Carcina quercana is polyphagous and mainly related to oak species: *Q. canariensis* (Mannai 2017) and *Q. suber* (Luciano and Roversi 2001). In this work, larvae were observed on *A.*

unedo, even though the vegetation of this plot is composed also by *Q. suber* and *Q. canariensis*. So, it will be interesting to make an investigation on *Q. canariensis* in this site (Majen Essef) to check whether it is infested by *C. quercana* or not, to eventually compare the infestation of the two host plants (*A. unedo* and *Q. canariensis*) in the same site and to study the life cycle and larvae development on each host species.

RESUME

Ezzine O., Ben Yahia K., Dhahri S., Ammari Y. et Ben Jamâa M.L. 2018. Premier signalement de *Carcina quercana* sur l'arbousier (*Arbutus unedo*) dans le nord-ouest de la Tunisie. Tunisian Journal of Plant Protection 13 (2): 269-274.

Carcina quercana est un insecte polyphage. En avril 2018, des larves de *C. quercana* ont été observées pour la première fois à Majen Essef (nord-ouest de la Tunisie) sur l'arbousier (*A. unedo*). Pour estimer le pourcentage d'infestation des arbres, nous avons compté le nombre d'arbres infestés parmi 40 arbres d'arbousier trouvés dans une zone d'un hectare. Des branches d'environ 30 cm de long ont été coupées et examinées au laboratoire pour déterminer le pourcentage de feuilles infestées. Sur ces feuilles infestées, les abris des chenilles ont été comptés et le nombre de larves par abri a été déterminé. Le pourcentage d'arbres infestés était de 20% et celui des feuilles infestées de 7,16%. Sur chaque feuille, nous avons trouvé entre 1 et 3 abris, et une seule larve par abri a été observée. Les larves de *C. quercana* construisent un abri en soie d'environ 1,91 cm de long et de 0,73 cm de large. Les extrémités de l'abri sont ouvertes pour assurer le déplacement de la larve.

Mots clés: *Arbutus unedo*, *Carcina quercana*, larve, Tunisie

ملخص

الزيتون، آفة وكوثر بن يحيى وسمير الظاهري ويوسف عماري ومحمد لحبيب بن جامع. 2018. أول تقرير حول تواجد العثة *Carcina quercana* على شجرة القطلب أونيدو/الشماري (*Arbutus unedo*) في شمال غرب تونس.

Tunisian Journal of Plant Protection 13 (2): 269-274.

إن العثة *Carcina quercana* هي حشرة متعددة العوائل النباتية. في أبريل 2018، شاهدنا يرقات *C. quercana* لأول مرة في ماجن الساف (شمال غرب تونس) على شجر القطلب أونيدو/الشماري (*Arbutus unedo*). لتقدير نسبة الإصابة بالأشجار، قمنا بحساب عدد الأشجار المتضررة على 40 شجرة قطلب أونيدو موجودة في مساحة هكتار واحد. تم قطع بعض فروع الشجرة بطول 30 سم وفحصها في المخبر لتحديد نسبة الأوراق المصابة. ثم على هذه الأوراق المصابة، تم إحصاء ملاجئ اليرقة وحساب عدد اليرقات في كل ملجأ. بلغت نسبة الأشجار المصابة 20% وكانت نسبة الأوراق الموبوءة 7.16%. على كل ورقة، وجدنا ما بين 1 و 3 ملاجئ، وقد لوحظت يرقة واحدة فقط في كل ملجأ. تقوم اليرقات ببناء ماوى من الحرير يبلغ طوله 1.91 سم وعرضه 0.73 سم. يكون طرفي الماوى مفتوحين لضمان حركة اليرقة.

كلمات مفتاحية: تونس، يرقة، *Carcina quercana*, *Arbutus unedo*

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First Report of three Tortricidae species on *Quercus suber* Forest in Northwestern Tunisia

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ABSTRACT

Mannai, Y., Ezzine, O., Hausmann, A., and Ben Jamâa, M.L. 2018. First Report of three Tortricidae species on *Quercus suber* Forest in Northwestern. Tunisian Journal of Plant Protection 13 (2): 275-280.

The Tunisian fauna of Tortricidae has been poorly investigated despite the great economic importance of this family. Sampling of Tortricidae insects was carried out in spring and summer 2010 in two cork oak (*Quercus suber*) forests in northwestern Tunisia. Three species are reported for the first time: *Archips xylosteana*, *Pammene splendidulana*, *Pammene giganteana*. Their identification was achieved using DNA barcodes.

Keywords: *Archips xylosteana*, DNA barcoding, *Pammene splendidulana*, *Pammene giganteana*, *Quercus suber*, Tortricidae, Tunisia

The Tortricidae family is one of the most diverse families in the so-called "Microlepidoptera" with more than 10,300 species described (Gilligan et al. 2012). The common name "leafrollers" refers to the behavior of their caterpillars that have the habit of winding or twisting the leaves of the plants on which they feed, using silk to fix the leaf pockets (Harrachi 1994). However, the larvae of those tortricids cover a wide range of feeding strategies, sometimes fairly divergent from the typical leaf-rolling habit (Horak and Brown 1991; Powell et al. 1998). There are

gall-makers, root-borers, fruit-borers, seed-predators, flower-feeders, and tip-tiers (Brown et al. 2008).

The Tortricidae family is of great economic importance; the larvae of many species cause major economic damage in agriculture, horticulture and forestry on a wide variety of crops including pome and stone fruits, citrus fruits, grapes, ornamental crops, tea, coffee, cereals and cotton. In forestry, many species of both coniferous and deciduous trees are attacked by the pest species belonging to this family (Meijerman and Ulenberg 2000). Mannai (2017) identified 82 species of Lepidoptera defoliators of *Quercus* species, containing eight species of the Tortricidae family. This work

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presents the first report of three Tortricidae species on *Q. suber* forests in northwestern Tunisia.

MATERIALS AND METHODS

Sampling of larvae.

Larvae of Tortricidae insects were collected on *Q. suber* from the cork oak forests Bellif (Long. 9°538'; Lat. 37°152'; alt. 70 m) and El Jouza (Long. 9°015'; Lat. 36°513'; alt. 550 m) in 2010 and were conserved into ethanol (96%) for the DNA analysis.

Molecular Identification.

DNA analysis of larvae was performed to identify the species. PCR amplification and DNA sequencing were conducted at the CCDB (Canadian Centre for DNA Barcoding, Guelph, Canada) following standard high-throughput protocols (Ivanova et al. 2006) that can be accessed under the website <<http://www.dnabarcoding.ca/pa/ge/research/protocols>>. PCR amplification with a single pair of primers consistently recovered a 658 bp region near the 5' terminus of the mitochondrial Cytochrome c Oxidase I (COI) gene that included the standard 658 bp barcode region for the animal kingdom (Hebert et al. 2003). DNA extracts are stored at the CCDB. All sequences are deposited also in GenBank according to the iBOL (international BARCODE OF LIFE) data release policy. Species identification and delineation were based on the system of Barcode Index Numbers (BINs: Hausmann et al. 2013; Ratnasingham and Hebert 2013).

RESULTS

Using PCR techniques, three Tortricidae species were identified: *Archips xylosteana*, and *Pammene splendidulana*, *Pammene giganteana* (Figs. 1-3).

Archips xylosteana.

BIN: BOLD: AAC0366; five DNA barcode sequences (INRGREF 0138; 0261; 0264; 0283; 0293; all with full length of 658 bp) of *A. xylosteana* were obtained from Tunisia; sequences and metadata are free accessible under the public dataset DS-TORTRITU on BOLD. Compared to 44 other DNA barcodes of *A. xylosteana* on BOLD from nine countries in Europe and north-eastern Canada, 99.1-99.7% similarity was found; all specimens are BIN-sharing.

Pammene splendidulana.

BIN: BOLD: ACS5540; one DNA barcode sequence (INRGREF 0073; with full length of 658 bp) of *P. splendidulana* was obtained from Tunisia; sequence and metadata are free accessible under the public dataset DS-TORTRITU on BOLD. Compared to seven other DNA barcodes of *P. splendidulana* on BOLD Bin-sharing and high similarity, 99.1-99.2% was found with two samples from Austria and Slovenia, but with other five samples from Germany and Finland belonging to another BIN (AAU2599), similarity was 97.7-98%. Additional taxonomic work is required to address potential cryptic diversity.

Pammene giganteana.

BIN: BOLD: AAI2598; one DNA barcode sequence (INRGREF 0065; with full length of 658 bp) of *P. splendidulana* was obtained from Tunisia, sequence and metadata are free accessible under the public dataset DS-TORTRITU on BOLD. Compared to twelve other DNA barcodes of *P. giganteana* on BOLD from eight European countries, 99.1-99.5% similarity was found; all specimens are BIN-sharing.



Fig. 1. Larva of *Archips xylosteana*

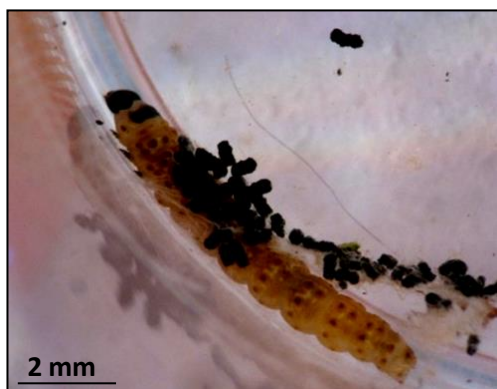


Fig. 2. Larva of *Pammene splendidulana*



Fig. 3. Larva of *Pammene giganteana*

DISCUSSION

Leaf-rollers.

A. xylosteana is a Palearctic species. In Europe, it has been observed in most European countries (Karsholt and Razowski 1996; Zhang 1994). In North Africa, this insect was reported in Algeria (Leraut et Luquet 1995). In Asia, *A. xylosteana* was detected in Korea (Razowski 1977), China, Japan and Asia Minor (Bradley et al. 1973; Hwang 1974;

Konno 2005; Zhang 1994). Schall (2006) reported the species in America, confirmed by a Canadian DNA barcode on BOLD.

Larvae of *A. xylosteana* are polyphagous (Dickler 1991) and may cause significant defoliation by feeding on new foliage and buds of ornamental trees and shrubs (Spears 2006). The larvae feed on the foliage of numerous trees and woody plants such as *Q. ilex*, *Q.*

pyrenaica, *Q. robur* (CAB 2006; Toimil 1987), *Acer* sp., *Corylus* sp., *Fraxinus* sp., (Bradley et al. 1973) and *Prunus* sp. (Luciano and Roversi 2001). *A. xylosteana* is univoltine (Dickler 1991). Adults are active from late June or early July to mid-August in Europe and Japan (Razowski 1977). The insect overwinters in the egg stage (Razowski 1977). Larval development requires 30-40 days. The pupal stage lasts 9-12 days (Razowski 1977). Adult moths rest in foliage during daytime and fly at night or when disturbed (Bradley et al. 1973).

The Genus *Pammene* is one of the genera related to *Grapholita* and belongs to the tribe *Grapholitini* of the subfamily *Olethreutinae* (Bae and Park 1998). *P. splendidulana* is widespread in the Western Palearctic zone. It is present in most European countries including the east to the Caucasus and Asia Minor (Karsholt and Razowski 1996). Their larvae feed on the underside of the leaves of *Quercus*, developing inside a small silky tube

interwoven between the leaves, within which they accumulate their own excrement (Ylla Ullastre and Marcia Vila 2005). This species shows two generations in the year in April and June (Razowski 2003). Larvae of *P. splendidulana* were observed on *Q. suber*.

Gall-makers.

Besides the leaf-roller *P. splendidulana*, the gall-maker *P. giganteana* was observed on *Quercus* species. *P. giganteana* is present in many countries of Europe such as Bulgaria (Toshova et al. 2016), Italy (Pinzari et al. 2013) and Hungary (Csóka and Szabóky 2005) and many others (Karsholt and Razowski 1996 under the synonymous name "*P. inquilana*"). This species is univoltine. Adults are active in spring (Kimber 2018). Their larvae feed on gall tissues inside the galls formed by gall wasps belonging to the hymenopteran family *Cynipidae* (Csóka and Szabóky 2005).

RESUME

Mannai Y., Ezzine O., Hausmann A., et Ben Jamâa M.L. 2018. Premier rapport de trois espèces de Tortricidae sur la forêt de *Quercus suber* dans le nord-ouest de la Tunisie. Tunisian Journal of Plant Protection 13 (2): 275-280.

La faune tunisienne des Tortricidae a été mal étudiée malgré la grande importance économique de cette famille. Un échantillonnage d'insectes *Tortricidae* a été effectué au printemps et en été 2010 dans deux forêts de chêne-liège (*Quercus suber*) du nord-ouest de la Tunisie. Trois espèces ont été signalées pour la première fois: *Archips xylosteana*, *Pammene splendidulana*, *Pammene giganteana*. Leurs identifications ont été réalisées moyennant l'ADN barcoding.

Mots clés : *Archips xylosteana*, ADN barcoding, *Pammene splendidulana*, *Pammene giganteana*, *Quercus suber*, Tortricidae, Tunisie

ملخص

مناعي، يسرى وألفة الزين وأكسال هوزمان ومحمد لحبيب بن جامع. 2018. أول تسجيل لثلاث أنواع من الفراشيات الفتالة في غابات بلوط الفلين (*Quercus suber*) في شمال غرب تونس. Tunisian Journal of Plant Protection 13 (2): 275-280.

إن الفراشيات الفتالة ليست مدروسة جيداً في تونس رغم أهميتها الاقتصادية الكبيرة. تم جمع عينات في ربيع وصيف 2010 من الفراشيات الفتالة في غابات بلوط الفلين (*Quercus suber*) في شمال غرب تونس. سجلت ثلاثة أنواع لأول مرة:

Pammene giganteana و *Pammene splendidulana* و *Archips xylosteana*. تم تشخيص أنواع الحشرات باعتماد ترميز الخطوط للحمض النووي ADN.

كلمات مفتاحية: ترميز الخطوط للحمض النووي ADN، تونس، فراشيات قتالة، *Pammene*، *Archips xylosteana*، *Quercus suber*، *Pammene giganteana*، *splendidulana*

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Plant Protection Events

Report

on

The Inter-African Phytosanitary Council Workshop: *“Strengthen cooperation on migratory pests between countries and RECs through workshops to discuss and solve the problems of main migratory and transboundary pests on crops”*

Hammamet, Tunisia, 29th November to 1st December 2018



This workshop was held at Hammamet, Tunisia, from 29 November to 1 December, 2018. It was realized

within the framework of the African Union, Inter-African Phytosanitary Council (AU-IAPSC) program aimed to

strengthen the capacity building of AU member states in plant protection from invasive and transboundary pests.

Objectives

The objectives of the workshop as presented by Prof. Abdelfattah Mabrouk Amer, AU-IAPSC, just after the opening ceremony, can be summarized in:

- (1) Improving national, regional and continental knowledge and information/data retrieval systems on pests' surveillance in support of early warning, plant production and related factors able to affect or threaten plant health;
- (2) Sharing, harmonizing and update of the pest lists especially in plant quarantine to control the entrance of transboundary pests;
- (3) Improvement of the diagnostic performance of laboratory at the national level and support of reference laboratories through technology transfer, and internal and external quality control;
- (4) Establishment of a technical guidance for national and regional initiatives ensuring collaboration across borders; and
- (5) Implementation of Good Emergency Management Practices that include early detection, reporting and pest control (national, regional and continental).

Expectations

It is expected to:

- * Understand the impact of the invasive and transboundary pests on

production, productivity, food security and trade;

- * Share the control of pests present in different areas with anticipation to form pest alert system for the continent; and
- * Acquire information on plant protection products, biological control agents and other management options.

Participants

There were 21 presentations from 19 African Countries, 1 Expert (from Egypt), 1 Pesticide National Organization for Central Africa (CPAC, Cameroon) in addition to the presentation of the Organizer. Participant countries were Algeria, Burkina Faso, Cameroon, Chad, Congo, Cote d'Ivoire, Egypt, Eritrea, Gambia, Niger, Nigeria, Senegal, South Africa, Sudan, Tanzania, Tunisia, Uganda, Zambia, and Zimbabwe.

Recommendations

The main recommendations taken during the workshop were:

- * Establish national, regional and continental emergency fund;
- * Training for trainers on Pest Risk Analysis;
- * Prepare national, regional and continental Alert Pest List;
- * Establish national, regional and continental Alert System;
- * Improve the pest list in NPPOs and renew the African Pest List; and
- * Establish a continental platform for Invasive Alien Species.

Report

on

The Inter-African Phytosanitary Council Workshop: *“Mainstream SPS in country CAADP investment plans and development of information systems and enhancing advocacy, awareness and communication to ensure sufficient safe biological control agents available in Africa”*

Hammamet, Tunisia, 3th December to 5th December 2018



This workshop was held at Hammamet, Tunisia, from 3 to 5 December, 2018. This workshop was realized within the framework of the African Union, Inter-African Phytosanitary Council (AU-IAPSC)

program aimed to strengthen the capacity building of AU member states in plant protection to reduce the using of chemicals and use the IPM especially biological control methods.

Objectives

The workshop as presented by Prof. Abdelfattah Mabrouk Amer, AU-IAPSC, just after the opening ceremony, aimed to:

- 1) Promote and improve continental plant protection and hold a workshop on biological control & Integrated Pest Management (IPM);
- 2) Improve and strengthen cooperation on migratory pests between countries and RECs by improving Pest Alert System;
- 3) Encourage the awareness of member states to compliance and implement International Standards for Phytosanitary Measures (ISPMs); and,
- 4) Organize policy meetings of international standard-setting bodies in support of building SPS capacities.

Expectations

It is expected to:

- * Understand the impact of the using of chemicals (pesticides, herbicides, fungicides ...) on the human and animal health in addition to their effects on environment, food safety, food security, trade,...;
- * Share the information about the biological agents used in Africa member states and their hosts and possibility of cooperation between member states for exchanging these agents together; and
- * Acquire information on plant protection products, biological control agents and other management options.

Participants

There are 23 presentations from 18 African Countries, 2 experts (from

Egypt), 1 Pesticide National Organization for Central Africa (CPAC, Cameroon), in addition to 2 interventions (expert from private agency in biological control and PhD Tunisian expert in biological control) and the presentation of the Organizer. Participant countries were Algeria, Burkina Faso, Cameroon, Chad, Congo, Cote d'Ivoire, Egypt, Eritrea, Gambia, Niger, Nigeria, Senegal, South Africa, Tanzania, Tunisia, Uganda, Zambia and Zimbabwe.

Recommendations

The main recommendations taken during the workshop were:

- * Create awareness about biological control of pests in national and regional and continental levels;
- * Advocate for specialized training in identification (morphological and molecular) of natural enemies and pests and capacity building of phytosanitary inspectors and improve human capacity in biological control;
- * Strengthen the collaboration between responsible ministries, the research institutions and academia in biological control;
- * Develop and harmonize guidelines for registration, introduction and production of biological control agents in regional and continental levels;
- * Develop an effective mechanism to coordinate and facilitate exchange of biological materials and expertise between NPPOs on biological control of pests; and
- * Encourage public-private partnership to produce, test and commercialize biological control agents.



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