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## Contents



**Photo of the cover page: *Sorghum bicolor*** (Courtesy *Tamara A. Al-Khateeb*)

# *Guest Editorial*

## *Allelopathic mechanisms and biochemical interaction and their impact on plant health*

*Allelopathy is a natural phenomenon described as various events and situations produced by higher plants, fungi, algae or other microorganisms that cause some effect, either inhibitory or stimulatory, on other members of the plant or microbial community. Unlike competition for a resource, the main principle in allelopathy arises from the fact that plants and microorganisms collectively produce several chemicals, and many of these chemicals are released from the producing organism by volatilization, leaching or decomposition processes and known as allelochemicals. The biosynthesis of allelochemicals, their release, environmental fate and action on other plant species can depend on genetic factors, the environment and their interaction. Because of these interactions, allelopathy is a complex phenomenon with limited field repeatability.*

*The bioavailability of allelochemicals in the soil is dependent on processes of transference (mainly adsorption and leaching) and degradation (abiotic and biotic). The characteristics of allelochemicals play an important role in their fate in the environment. For example, the water solubility of the compounds can affect their mobility within the soil; the chemical structure can influence their affinity with the soil surface and the vapor pressure can impact their volatilization. Allelochemicals are employed for growth regulation, weed infestation control and pest management, and are safer than synthetic chemicals and their use preclude the health defects and environmental pollution of synthetic chemicals.*

*Nowadays, the need for new herbicides should be emphasized for some reasons: (1) the number of herbicides launched onto the market is decreasing steadily, especially due to registration issues; (2) no new herbicides were released onto the market within the past two decades; and (3) limitations of current herbicides due to the increasing development of herbicide-resistant weeds. Thus, allelopathy offers a unique and eco-friendly solution of growth regulation, weed management and disease control at the same time to enhance the safe global per capita foodsupply. Compared to chemical-based herbicide discovery, further benefits of allelochemical-based herbicide discovery include reduced environmental impact, higher consumer acceptance and easier registration. Microbial producing toxins are also effective as herbicides, because they are selective and compared to actual pathogens used easier to formulate, less likely to spread disease to non-target species, and their activity is less dependent on environmental conditions. Microbial toxins may be produced by fermentation and used in the natural state, subjected to synthetic modification, or their chemistry used as a basis for producing synthetic herbicides.*

*Allelopathic inhibition is a complex process and involves the interaction of various classes of chemicals, such as terpenoids, phenolic*

*compounds, flavonoids, steroids, alkaloids, carbohydrates, and amino acids, with mixtures of different compounds that may have a greater allelopathic effect than individual compounds alone. Allelochemicals are active against higher plants through suppressing seed germination, causing injury to root growth and other meristems, inhibiting seedling growth, reducing ATP production, causing lipid peroxidation. Also, allelochemicals inhibit mineral uptake, chlorophyll content, photosynthesis, carbon flow, and phytohormone activity. Furthermore, allelochemicals disturb the metabolism of plants through production of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical and hydrogen peroxide which play a major role in the inhibition of the key enzymes of metabolism including glucose phosphate isomerase, glucose-6-phosphate dehydrogenase, nitrate reductase and glutamine synthetase. Major advancement in understanding allelopathy will be accomplished only by the combined effort of investigators from many disciplines.*

*Future research on allelopathy should focus on the following points: (1) establishing of practical ways of using allelochemicals in the field, (2) a continuous survey of effective allelochemicals from natural vegetation or microorganisms, (3) understanding the role of allelopathic chemicals in the biodiversity and ecosystem function, (4) understanding the modes of action of allelochemicals in receptor organisms, (5) to challenge the natural product chemists to develop a better methodology for isolating allelopathic compounds or their degraded compounds from the environment, particularly the soil environment and (6) to explore advanced biotechnology for allocating allelopathic chemical genes in plants or microorganisms for biological control.*

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# Differentiation of Allelopathic Potential of Sorghum (*Sorghum bicolor*) Cultivars Using Chemical and Molecular Techniques

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**ABSTRACT**

**Al-Khateeb, T.A., Alsaadawi, I.S., and Hadwan, H.A. 2017. Differentiation of allelopathic potential of sorghum (*Sorghum bicolor*) cultivars using chemical and molecular techniques. Tunisian Journal of Plant protection 12: 1-13.**

Laboratory tests were conducted to detect the differential allelopathic potential of two sorghum (*Sorghum bicolor*) cultivars (Enqath and Rabeh) residues. Chemical analysis of residues indicated that total phenolics were found to be higher in Enqath than in Rabeh plants suggesting the superiority of the allelopathic potential of the first cultivar over the second one against weeds. Results indicated that total phenolics were two folds in shoot than in root of both cultivars. These compounds appeared to be higher in Enqath shoot and root (1.60 and 0.80 mg/g, respectively) than in Rabeh shoot and root (1.2 and 0.50 mg/g, respectively). The increments in total phenolics in root and shoot of Enqath were clearly reflected on the increase of total phenolics in the whole plant reaching about 2.40 mg/g compared to Rabeh (1.70 mg/g). Chemical analysis by high performance liquid chromatography (HPLC) revealed the presence of seven allelochemicals namely ferulic, *p*-coumaric, gallic, vanillic, syringic, *p*-hydroxybenzoic, and sinapic acid in the residues of both cultivars. Total phenolic acids were found to be higher in Enqath than in Rabeh. Results of RAPD-PCR technique performed for sorghum genomic DNA revealed that all the 10 primers used in this study scored different amplification monomorphic and polymorphic bands in the tested genotypes with the exception of 3 RAPD primers which generated a unique amplification bands, one of them (125 bp) scored by OPN 16 primer, present in high allelopathic sorghum genotypes and absent in the low allelopathic ones (Rabeh). Further work is recommended to analyze the sequence of this band to find out whether it is related to allelopathic trait or not. These results recommend screening more sorghum cultivars in order to offer a potential source of allelopathic germplasm that could be manipulated to enhance weed suppression in an effective and environmentally sustainable approach.

*Keywords*: Allelopathy, RAPD-PCR, residues, sorghum cultivars, total phenolics

The allelopathy has existed for thousands of years but the intensive scientific research into the recognition and understanding of allelopathy has only occurred over the past few decades (Narwal et al. 2005). Allelopathy is an ecological term which was first introduced by Hans Molisch in 1937 and derived from two Greek words "Allelon" means mutual and "Pathos" means harm. Rice was the pioneer in the growing of this field. Allelochemicals are present in sizeable amounts in all parts of

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allelopathic plant including roots, stems, leaves, fruits, seeds, and flowers. They may be released from plants into the environment by volatilization, root exudation, leaching, and decomposition of plant residues (Kim et al. 1993; Narwal et al. 2005; Rice 1984). The crop allelopathic effects on weeds revolutionized the scientists to put much effort on this aspect with the aims of using this to reduce the dependence on chemical herbicides for weed control (Alsaadawi and Dayan 2009; Weston and Duke 2003). Several crops including *Sorghum bicolor* have been reported to have allelopathic potential against weeds and considerable variations have been reported among cultivars of the same crop species (Weston et al. 2013). Sorghum is a summer crop cultivated for grain and forage production in many countries such as Iraq (Al-Hashimi et al. 2010). The crop is included in a rotation system with several leguminous crops. The early work on weed suppression using sorghum residues was cited by many authors (Kim et al. 1993; Kohli et al. 2001; Netzly and Butler 1986; Weston 1996; Weston et al. 1989). These scientists found that phytotoxicity of residues varies with genotype, plant part, plant age, environmental factors, and the target weed species. Sorghum was explored as residues or as a water extract for weed control.

Field observations indicated that growth and population of companion weeds are variable within the stands of two locally cultivated sorghum cultivars (Enqath and Rabeh). This suggested that allelopathy might be the mechanism responsible for the variation in weed growth and population, and that the recorded differences among stands could be attributed to genetic diversity for allelopathic traits among the test cultivars. With this in mind, several experiments were conducted to i) isolate, identify and quantify the allelochemicals in residues of the tested sorghum cultivars, and ii) find out if there is marker(s) linked to allelopathic trait by using DNA fingerprinting techniques.

## MATERIALS AND METHODS

### Preparation of sorghum residues.

To prepare sorghum residues, at the end of the season, mature sorghum plants of both cultivars (Enqath and Rabeh) grown in field were harvested in July 2013. They were air-dried for several days under plastic shed during summer and chopped into pieces of about 2-3 cm pieces using electrical grinder (Abar Company, Syria) and kept in dry and dark place until use.

### Determination of total phenolics in sorghum residues.

Total phenolics in tested sorghum cultivars residues were extracted and quantified according to the Folin-Denis method (Helrich 1990). Sorghum plants, cvs. Enqath and Rabeh, were washed gently with distilled water and separated into two parts, shoots and roots, then chopped into 1-cm long pieces. All sorghum plant parts were dried at 70°C for 24 h. A quantity of 1.5 g of each plant part was placed in 25 ml distilled water in 100 ml flask and agitated on a rotary shaker for 24 h at 200 rpm. Extracts were filtered by Whatman No.2 filter paper under vacuum and centrifuged at 12000 rpm for 20 min at 8°C, prior to total phenolics analysis. A volume of 1 ml of sorghum extracts + 0.5 ml Folin-Denis reagent + 1 ml $Na_2CO_3$ reagents were mixed and the total volume was completed to 10 ml with distilled water.

Absorbance was determined at 750 nm and the content of total phenolics was obtained using the standard curve. Units of total phenolics were expressed in

milligrams of ferulic acid equivalents per milliliter extract. Ferulic acid equivalents were multiplied by 10 based on an extraction ratio 1:10 (w/w) as described by Ben-Hammouda et al. (1995).

**Identification of allelochemicals in sorghum cultivars residues by HPLC method.**

Phenolic acids in the residues of both cultivars (Rabeh and Enqath) was quantified by HPLC system (Shimadzo HPLC system Model LC-2010A HT) components, which includes a column of Hyper Clone 5u CN 120A (250 × 4.6 mm) phenomenex. Mobile phase is phosphoric acid 0.1% and acetonitrile (50:50 v/v), flow rate 0.5 ml/min. The injection volume for each sample was 1 µl lasted for 20 min. The amount of phenolic acids in each extract was quantified at 280 nm based on a calibration curve obtained with pure phenolic acids as standards.

**Determination of genetic diversity between tested sorghum cultivars by PCR technique.**

The present experiments were conducted to determine the genetic diversity between the tested cultivars and to find out if this technique may help to determine the allelopathic potential of the tested sorghum genotypes.

**Plant material.** Four sorghum cultivars were used in the present PCR experiments i.e. Rabeh, Enqath, Giza 15, and Giza 113. Sorghum seeds were provided by State Board for Agriculture Researches (SBAR), Ministry of Agriculture, Iraq. Previous work reported that cultivars Enqath, Giza 15 and Giza 113 were of high allelopathic potential compared to Rabeh (Alsaadawi et al. 2007). RAPD-DNA marker was used in this study to find out if there is any marker(s) link to gene(s) that control allelopathic traits.

**Sorghum genomic DNA extraction**. For DNA extraction, two grams of fresh leaf tissue (2-week-old) was taken from a single seedling plant of the four cultivars. Genomic DNA was extracted according to the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1990). Genomic DNA was dissolved in 250 µl 1X TE buffer using labeled Eppendorf tube (1.5 ml) and stored at 2-8ºC until use.

**Determination of DNA concentration and purity.** Ten microliters (10 µl) of each DNA sample were added to a tube containing 990 µl of sterile distilled water and mixed well. The optical density (OD) of the samples was measured according to Sambrook et al. (1989) by spectrophotometer at wave length of 260 nm and 280 nm. The OD of 1 corresponded to approximately 50 µg/ml for double stranded DNA (Maniatis et al. 1982; Sambrook et al. 1989). The concentration of DNA was calculated according to the formula DNA concentration (µg/ml) = OD 260 nm × 50 × Dilution factor, DNA purity ratio = OD 260 / OD 280. DNA fragments were separated using the following concentrations of agarose gel as follows i) 1% (w/v) used for detection of total genomic DNA, ii) 1.5% (w/v) used for separation of RAPD-PCR products.

Agarose gels were stained with ethidium bromide stain (Sigma-Aldrich C). The final concentration of stain solution was 1 µg/ml. DNA bands were visualized by UV transluminator at 366 nm wave length in a UV cabinet. A gel documentation system was used to document the observed bands.

**RAPD-PCR technique.** PCR reactions were performed using the following components:

*1- Primers*: Ten of random oligodecamers primers (10-mer) commercially available from Bioneer Company, Korea, were used for detection of polymorphism in this study. The lyophilized primers were dissolved in sterile distilled water to get a final concentration of 5 p mole/µl as recommended by provider. The sequences of the primers used in this study are listed in Table 1.

**Table 1.** The sequence of RAPD primers used in determining the genetic diversity of the tested sorghum cultivars

| No. | Primer's name | Sequence 5' - 3' |
|---|---|---|
| **1** | OPA-10 | G T G A T C G C A G |
| **2** | OPA - 11 | C A A T C G C C G T |
| **3** | OPA - 12 | TC G G C G A T A G |
| **4** | OPC - 16 | C A C A C T C C A G |
| **5** | OPN - 16 | A A G C G A C C T G |
| **6** | OPM - 14 | A G GG T C G T T C |
| **7** | OPD - 6 | AC C T G A A C G G |
| **8** | OPR - 17 | C C G T A C G T A G |
| **9** | OPT - 19 | G T C C G T A T G G |
| **10** | OPV - 14 | A G A T C CC G C C |

*2 - PCR pre master mix*: An AccuPower PCR pre-mix kit (Bioneer) was used for PCR amplification. The PCR pre master mix which contained the components of PCR reagent optimized for more accurate PCR amplifications is listed in Table 2.

**Table 2.** Bioneer pre-mix components of PCR reagent used in determining the genetic diversity of the tested sorghum cultivars

| Component | Concentration per reaction (in 25 µl) |
|---|---|
| *Taq* DNA polymerase | 1 U |
| Each: dNTP (dATP, dCTP, dGTP, dTTP) | 250 µM |
| Tris-HCl (pH 9.0) | 10 mM |
| KCl | 30 mM |
| $MgCl_2$ | 1.5 mM |
| Stabilizer and tracking blue dye | - |

For RAPD assay, PCR amplification was carried out on ice in a laminar air flow using 0.5, 0.2 µl tight cap Eppendorf tubes, by adding about 50 ng of genomic DNAs and 10 pmole of primer to PCR pre-mixture (Bioneer) containing buffer, dNTP and *Taq* DNA polymerase (Table 3). Final reaction volume was equal to 25 µl.

**Table 3**. Components of RAPD- PCR reaction

| Material | Final concentration | Volume for 1 tube |
|---|---|---|
| Bioneer pre- master mix | 1 X | 5 µl |
| Primer 5 p mol | 10 p mol | 2 µl |
| DNA samples | 50 ng/ µl | 2 µl |
| Sterile distilled water | - | 16 µl |

In each PCR experiment set up a negative control containing all components of the reaction except template DNA was prepared.

*3 - RAPD-PCR program*. The following steps were used for DNA amplification (Iqbal et al. 2010) as shown in Table 4.

**Table 4**. RAPD- PCR program used

| Step | | Temperature (°C ) | Time (min) |
|---|---|---|---|
| Initial denaturation | 1 cycle | 94 | 4 |
| Denaturation | 36 cycles | 94 | 1 |
| Annealing | | 36 | 1 |
| Extension | | 72 | 2 |
| Final extension | 1 cycle | 72 | 10 |

*4 - Gel electrophoresis*. Approximately 24 µl of PCR amplified products were loaded and separated by electrophoresis in 1.5% agarose gels (2 h, 5V/cm, 1X TBE buffer). After that, the gel was immersed for 20 min in 30 µl/100 ml ethidium bromide stain (stock concentration of 10 mg/ml, Sigma-Aldrich, USA). The gel was visualized under the ultraviolet light (UV) transilumination (366 nm) and then was imaged by gel documentation system using a Photo Gel System.

**Data analyses.**

**Molecular weight estimation**. Molecular weights for DNA bands were calculated by comparing the PCR products with the known size of standard DNA molecular weight marker (100 bp DNA Ladder: ranged from 100-2000 base pairs, or 1 Kb DNA Ladder: ranged from 0.5-10.2 kilo base pairs) provided by Bioneer Company was specially designed for determining the size of double strand DNA samples (Sambrook et al. 1989).

**Polymorphism estimation.** Data generated from the detection of polymorphic fragments were analyzed. The amplification profiles of all tested genotypes for any given primer were compared with each other and the absence of each band was scored as '0' while the presence of each band was scored as '1'. The intensity of bands was not taken into consideration. Only major bands consistently amplified were scored and faint bands were not considered. Monomorphic, unique, and polymorphic bands were scored for each primer. The polymorphism percentage of each primer was calculated according to Blair et al. (1999) and Ali (2003) methods and based on the formula: Polymorphism (%) = (Np/Nt) × 100, where Np = Number of polymorphic bands of the primer, Nt = Total number of bands of the same primer.

## RESULTS

### Quantification of total phenolics in sorghum residues.

Results indicated that total phenolics were two folds more in shoot than in root of both cultivars (Fig. 1). Total phenolics content was variable with the tested sorghum genotypes. These compounds appeared to be higher in Enqath shoot and root (1.60 and 0.80 mg/g, respectively) than in Rabeh shoot and root (1.20 and 0.50 mg/g, respectively). The increments in total phenolics in root and shoot of Enqath were clearly reflected on the increase of total phenolics in the whole plant reaching 2.40 mg/g compared to Rabeh (1.70 mg/g).

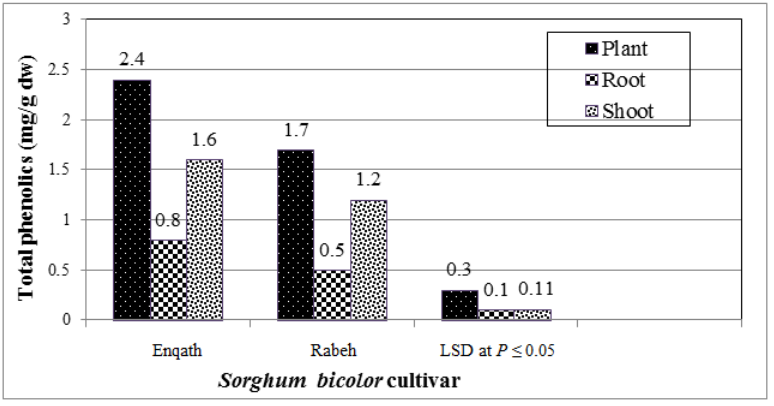


**Fig. 1.**Total phenolics in residues of *Sorghum bicolor* cultivars.

### Identification of allelochemicals in sorghum residues by HPLC method.

Chemical analysis by high performance liquid chromatography (HPLC) revealed the presence of several allelochemicals namely ferulic, *p*-coumaric, gallic, vanillic, syringic, *p*-hydroxybenzoic acid, and sinapic acid in the residues of Enqath and Rabeh cultivars. Total phenolic acids were found to be higher in Enqath cultivar than in Rabeh (Table 5).

**Table 5.** Isolation and quantification of phenolic acids in residues of two *Sorghum bicolor* cultivars

| Compound | Concentration µg/g dry weight | |
|---|---|---|
| | cv. Enqath | cv. Rabeh |
| Ferulic acid | 280.35 | 195.30 |
| *p*-coumaric acid | 195.00 | 100.70 |
| *p*-hydroxybenzoicacid | 270.20 | 190.48 |
| Protocatecheuic acid | 050.00 | 028.10 |
| Sinapic acid | 050.75 | 040.72 |
| Syringic acid | 250.64 | 200.81 |
| Vanillic acid | 078.00 | 044.81 |
| **Total** | 1175.00 | 800.92 |

**Molecular analysis of test *Sorghum bicolor* cultivars.**

**Genomic DNA isolation.** The genomic DNA isolated from the fresh leaf tissue of four test sorghum cultivars is presented in Fig. 2. Total DNA yield was approximately 60 µg/ml from 2 g of fresh leaf tissue with purity ranging between 1.6 and 1.8.

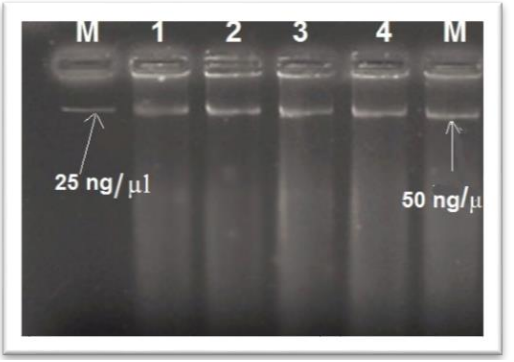


**Fig. 2.** Genomic DNA of sorghum cultivars visualized on 1% agarose gel. Lanes: 1. Rabeh; 2. Enqath; 3. Giza1; 4. Giza113. M: Standard size marker (λ DNA).

**RAPD- PCR analysis.** The tested sorghum genotypes were genetically differentiated by using the 10 RAPD primers. There were differences in the number of bands generated by each primer. The number of amplified bands produced per primer varied from 4 to 7, with a mean of 5.5 bands. These RAPD primers produced amplified fragments which varied from 125 to 1650 bp in size. The ten RAPD primers generated a total of 171 bands which appeared on agarosee gels. Of these bands, only 10 were polymorphic across the 4 sorghum genotypes tested. Several primers produced only monomorphic bands (share) with no polymorphism detected in the template DNA of the tested genotypes, such as bands scored using OPA 10, OPA 11, and OPR 17 primers. Other RAPD primers scored some polymorphic bands between the tested genotypes such as OPA 12, OPC 16, OPD 6, OPM 14, OPN 16, OPT 19, and OPV 14. Also, some primers generated unique bands (5 unique bands) that could be used as DNA marker to distinguish sorghum genotypes. A band was considered as polymorphic if the band differentiates at least any 2 of the 4 genotypes.

Out of ten RAPD primers used in this study, only 3 primers generated a unique amplified band which was present in only one sorghum genotype and absent in the others. The OPN 16 primer (Fig. 3; Table 6) produced 16 amplified bands, only one of them with molecular size 1250 bp present in 3 sorghum genotypes (with high allelopathic potential) namely Enqath, Giza 15, and Giza 113 and absent in Rabeh (with low allelopathic), and that may represent a first step towards the initial differentiation of genetic variation among four sorghum cultivars using RAPD markers. In addition, two unique bands of 1650 and 1400 bp produced by the same primer were present in Rabeh and absent in all the other studied genotypes.

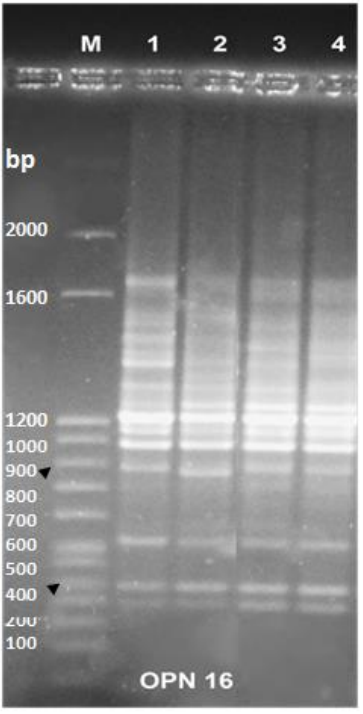


**Fig. 3.** RAPD banding profile of four sorghum cultivars visualized on1.5% agarose gel using primer OPN 16. Lanes: 1.Rabeh; 2.Enqath; 3.Giza15; 4.Giza 113. M: DNA Molecular weight marker (100 bp DNA Ladder).

**Table 6.** Bands scored and molecular weight of amplification products with OPN 16 primer

| Band number | DNA samples | | | | Molecular weight (bp) |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| **1** | 1 | 0 | 0 | 0 | 1650 |
| **2** | 1 | 0 | 0 | 0 | 1400 |
| **3** | **0** | **1** | **1** | **1** | **1250** |
| **4** | 1 | 1 | 1 | 1 | 1200 |
| **5** | 1 | 0 | 1 | 1 | 1150 |
| **6** | 1 | 1 | 1 | 1 | 1000 |

Total number of bands = 16

Polymorphism of each primer was calculated as the percentage of number of polymorphic bands to the number of total bands scored by the primer. Polymorphism ranged from 17 to 50%. Primer OPT 19 produced the highest percent of polymorphism 50% compared with the primer OPC 16. Table 7 shows the number of total bands, polymorphic, unique and the percentage of genetic polymorphism among tested genotypes.

**Table 7.** Band characteristics detected by the RAPD primers

| Primer name | Total number of bands | Monomorphic bands | Polymorphic bands | Unique bands | Polymorphism rate (%) |
|---|---|---|---|---|---|
| **OPA 10** | 7 | 7 | 0 | 0 | 0 |
| **OPA 11** | 4 | 4 | 0 | 0 | 0 |
| **OPA 12** | 5 | 3 | 1 | 1 | 20 |
| **OPC 16** | 6 | 3 | 1 | 2 | 17 |
| **OPD 6** | 4 | 3 | 1 | 0 | 25 |
| **OPM 14** | 6 | 4 | 2 | 0 | 33.3 |
| **OPN 16** | 6 | 2 | 2 | 2 | 33.3 |
| **OPR 17** | 4 | 4 | 0 | 0 | 0 |
| **OPT 19** | 4 | 2 | 2 | 0 | 50 |
| **OPV 14** | 5 | 4 | 1 | 0 | 20 |
| **Total number of bands** | 51 | 36 | 10 | 5 | - |

## DISCUSSION

The results of the present study indicated that the variation in allelopathic potential between the stands of Enqath and Rabeh sorghum cultivars observed in the fields depended not only on the differences in their root exudates as shown in the previous study (Alsaadawi et al. 2015) but also on their residues. Incorporation of residues of both cultivars into the field soil had significantly suppressed density and biomass of weeds grown in bean fields such as mung bean and broad bean with superiority of Enqath cultivar over Rabeh (Alkhateeb et al. 2013; Alsaadawi et al. 2013). The weed density and biomass reduction seems to be an outcome of inhibitory effects exerted by sorghum residues. Such a reduction is believed to be originated by the release of phytotoxic allelochemicals from sorghum residues in the immediate vicinity during their decomposition by soil microorganisms. Chou and Lin (1976) first reported that several microorganisms contribute in decomposition of rice residues in soil and liberate several allelopathic compounds including phenolics and short fatty acids.

Rice (1984) indicated that several species of soil microorganisms are involved in the decomposition of plant residues and in the release of the allelopathic compounds which were found to be toxic to several weed species. Chemical analysis revealed the presence

of sizeable amount of phenolic compounds in roots and shoot residues of Enqath cultivar compared to Rabeh. Chemical analysis by HPLC revealed the presence of seven allelochemicals as phenolic acids. Many attempts were made to identify the phenolic acids in the total phenolic content of residues. However, several investigators indicated that sorghum contains several putative allelochemicals of phenolic nature such as vanillic acid, syringicacid, ferulic acid, *p*-hydroxybenzoic acid, and coumaric acid (Al-Bedairy et al. 2013; Alsaadawi and Dayan 2009; Cheema et al. 2009; Weston et al. 1989).

The isolated phenolic acids are known to inhibit ion uptake (Olmsted and Rice 1970), chlorophyll biosynthesis (Weir et al. 2004), cell membrane stability (Bogatek et al. 2005; Keck and Hodges 1973), protein and hormone biosynthesis (Holappa and Blum 1991; Rice 1984), cell division and change of the ultra-structural components of cells (Sanchez-Moreiras et al. 2004). Inhibitory allelopathic activity of sorghum allelochemicals against grassy and broad-leaved weeds has been reported in previous studies (Alsaadawi et al. 2007; Cheema and Khaliq 2000). In most cases, greater weed inhibition was observed at higher residue incorporation rates. Khanh et al. (2005) and Khaliq et al. (2010, 2011) pointed out that suppression magnitude in allelopathic interactions is directly proportional to the applied dose of an allelopathic product. The positive effects of residues on growth and yield of some crops was not only due to the reduction of weed population and biomass but also due to improvement of physical, chemical and nutritional status of field soil, and thus enhance growth and yield of crops (Sangakkara et al. 2004, 2006).

The results obtained using DNA molecular markers especially RAPD technique revealed that all the 10 primers scored different amplification products, monomorphic, polymorphic and unique bands in the tested genotypes with the exception of one RAPD primer which generated a unique amplification band (1250 bp) present in highly allelopathic sorghum genotype and absent in the low allelopathic one (Rabeh). Further work is recommended to analyze this band to find out if it is related to allelopathic trait or not. If the responsible gene(s) for allelopathic effects are identified, allelopathic traits can be incorporated into improved cultivars through plant breeding programs. To achieve the goal of this allelopathic breeding program, a multidisciplinary team of a wide range of scientists, including weed scientists, ecologists, natural-product chemists, plant breeders and molecular biologists is required. Further studies on the identification of specific allelochemicals and genes responsible for their biosynthesis using other novel selection methods of allelochemicals fingerprinting will undoubtedly shed more light on breeding allelopathic crop cultivars (Weston et al. 2013).

The differential allelopathic potential between the tested cultivars was apparently due to different phenolic acids content of their residues. Chemical analysis revealed the presence of sizeable amount of phenolic compounds in roots and shoot residues of Enqath cultivar compared to Rabeh. Chemical analysis by HPLC revealed the presence of seven allelochemicals as phenolic acids. Incorporation of sorghum residues in field soil can be used as a tool for weed suppression in other field crops and thereby increasing crop yield. The molecular technique revealed the presence of one sharp band with high

intensity in highly allelopathic cultivars compared to low allelopathic cultivar (Rabeh). Screening of allelopathic potential in allelopathic crops needs to be recommended as an essential step for utilizing allelopathy in weed management. Finally, allelopathy can be considered as a useful agricultural practice for weed management in organic farming in order to reduce dependence on herbicides and to achieve an agroecosystem sustainability.


**RESUME**

**Al-Khateeb T.A., Alsaadawi I.S. et Hadwan, H.A. 2017. Différenciation du potentiel allélopathique de cultivars de sorgho (*Sorghum bicolor*) moyennant des techniques chimiques et moléculaires. Tunisian Journal of Plant Protection 12: 1-13.**

Des tests au laboratoire ont été conduits pour détecter le potentiel allélopathique différentiel des résidus de deux cultivars de sorgho (*Sorghum bicolor*), Enqath et Rabeh. L'analyse chimique des résidus a indiqué que les teneurs en composés phénoliques totaux sont plus élevés chez les plants d'Enqath que chez ceux de Rabeh suggérant la supériorité du potentiel allélopathique contre les adventices du premier cultivar par rapport à celui du second. Les résultats ont indiqué que les composes phénoliques totaux sont deux fois plus importants dans les tiges que dans les racines des deux cultivars. Ces composés semblent être plus élevés dans les tiges et les racines d'Enqath (1,60 et 0,80 mg/g, respectivement) que celles de Rabeh (1,2 et 0,50 mg/g, respectivement). Les augmentations des teneurs en composés phénoliques totaux dans les racines et les tiges d'Enqath ont été traduites par une augmentation des composés phénoliques totaux dans toute la plante en atteignant 2,40 mg/g comparé à Rabeh (1,70 mg/g). L'analyse chimique par HPLC a révélé la présence de sept composes allélochimiques à savoir les acides férulique, *p*-coumarique, gallique, vanillique, syringique, *p*-hydroxybenzoique et sinapique dans les résidus des deux cultivars. Les acides phénoliques totaux sont plus élevés chez Enqath que chez Rabeh. Les résultats de la technique RAPD-PCR menée sur l'ADN génomique du sorgho a révélé que les 10 amorces utilisées dans cette étude ont généré différentes bandes d'amplification monomorphique et polymorphique chez les génotypes testés excepté trois amorces RAPD qui ont généré des bandes uniques d'amplification, une parmi elles (125 bp) est générée par l'amorce OPN 16 et est présente dans les génotypes de sorgho hautement allélopathiques et absente chez les faiblement allélopathiques (Rabeh). Plus de travail est recommandé pour l'analyse de la séquence de cette bande pour voir si elle est liée au caractère allélopathique ou non. Ces résultats recommandent aussi le criblage de plus de cultivars de sorgho dans le but de trouver des sources potentielles de germoplasmes allélopathiques qui peuvent être utilisés pour la suppression des adventices moyennant une approche efficace et environnementalement durable.



*Mots clés*: Allélopathie, composés phénoliques totaux, cultivars de sorgho, RAPD-PCR, résidus



**ملخص**

**الخطيب،تمارا عدنان و إبراهيم شعبان السعداوي وحميد علي هدوان. 2017. تمييز قدرة المجاهضة لأصناف من الذرة البيضاء (*Sorghum bicolor*) باستخدام تقنيات كيميائية وجزيئية.**

**Tunisian Journal of Plant Protection 12: 1-13.**

نفذت تجارب مخبرية لتحديد الاختلافات في قدرة المجاهضة لمخلفات صنفين من الذرة البيضاء (*Sorghum bicolor*) إنقاذ ورابح. بينت نتائج التحاليل الكيميائية لمخلفات الذرة البيضاء تفوّق صنف إنقاذ في المحتوى الكلي للفينولات على الصنف رابح مما يشير إلى قدرة مجاهضة عالية لمخلفات هذا الصنف. أشارت النتائج إلى أن المحتوى الكلي للفينولات كان أعلى مرتين في المجموع الخضري من المجموع الجذري في كلا الصنفين. وتفوّق تركيزها في المجموع الخضري والجذري لصنف إنقاذ (1,6و 0,8 ملغ/غ، على التوالي) على تركيزها في المجموع الخضري والجذري لصنف رابح (1,2 و 0,5 ملغ/غ. على التوالي) وهذا يقود إلى تفوّق المحتوى الكلي للفينولات في نبات الذرة البيضاء صنف إنقاذ حوالي 2,4 ملغ/غ مقارنة بصنف رابح حوالي 1,7 ملغ/غ. أعطت نتائج التحليل الكيميائي بعمود الفصل السائل العالي

الأداء بوجود سبعة أنواع من الأحماض الفينولية في المخلفات النباتية لصنفي رابح وإنقاذ مع تفوّق المحتوى الكلي لهذه الأحماض في الصنف إنقاذ مقارنة بالصنف رابح. وهذه الأحماض هي الفيروليك والـب-كوماريك والغاليك والفانيليك والسيرانجيك والـب-هيدروبانزويك والسينابيك. كشفت نتائج استخدام تقنية التضخيم العشوائي متعدد الأشكال للحمض النووي (RAPD) عن طريق التفاعل المتسلسل للبوليميراز (PCR)للحمض النووي الجينومي للذرة البيضاء باستعمال عشرة بادئات عن وجود حزم متماثلة وأخرى متباينة من حيث العدد والوزن الجزيئي في الأصناف المدروسة، باستثناء 3 بادئات أعطت نتائج تضاعف أظهرت فيها حزم مميزة منفردة في صنف معين وفقدت في الأصناف المدروسة الأخرى. ظهرت الحزمة ذات الوزن الجزيئي 1250 زوج قاعدي عند التضاعف بوجود البادئ OPN 16، إذ تميزت هذه الحزمة بظهورها في الأصناف عالية القدرة على المجاهضة في حين فقدت في الصنف الأقل قدرة (رابح). يوصى بالعمل في تحليل هذه الحزمة المميزة ومعرفة تسلسلها من النيوكليوتيدات وذلك لمعرفة فيما إذا كان لها صلة بقدرة المجاهضة، وإذا ما تم تحديد الجينات المسؤولة عن صفات المجاهضة قد يمكن نقل هذه الصفات إلى أصناف الذرة المحسنة من خلال برامج التربية. كما يوصى بإجراء مسوحات على أصناف أخرى من الذرة البيضاء واستغلال الأصناف ذات قدرة مجاهضة عالية في برامج مكافحة الأعشاب الضارة.

*كلمات مفتاحية*: أصناف الذرة البيضاء، فينولات كلية، مجاهضة، مخلفات، RAPD-PCR

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# Use of Endophytic Bacteria Naturally Associated with *Cestrum nocturnum* for Fusarium Wilt Biocontrol and Enhancement of Tomato Growth

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**ABSTRACT**

**Aydi-Ben Abdallah, R., Mejdoub-Trabelsi, B., Nefzi, A., Jabnoun-Khiareddine, H., and Daami-Remadi, M. 2017. Use of endophytic bacteria naturally associated with *Cestrum nocturnum* for Fusarium wilt biocontrol and enhancement of tomato growth. Tunisian Journal of Plant Protection 12: 15-40.**

Three endophytic bacterial isolates, recovered from *Cestrum nocturnum* (night blooming jasmine) leaves and stems, were assessed for their ability to suppress tomato Fusarium wilt disease, caused by *Fusarium oxysporum* f. sp. *lycopersici* (FOL), and to improve growth of tomato plants. Isolates tested had significantly decreased disease severity by 46.6-97.7% compared to FOL-inoculated and untreated control. The isolate C4 was found to be the most effective in decreasing leaf damage by 86.6% and the vascular browning extent by 97.7% relative to control. A significant increment by 39-41.6%, compared to pathogen-inoculated and untreated control, was recorded in tomato growth parameters. Moreover, the isolate C4 had significantly enhanced plant growth by 24.5-53.3% over pathogen-free and untreated control. This isolate C4 was morphologically and biochemically characterized and identified using 16S rDNA sequencing genes as *Serratia* sp. (KX197201). Screened in vitro for its antifungal activity against FOL, *Serratia* sp. C4 led to 19.52% decrease in pathogen radial growth and to the formation of an inhibition zone of 8.62 mm in diameter. Cell-free culture filtrate of *Serratia* sp. C4, supplemented to PDA medium at 20% (v/v), had lowered pathogen growth by 23% as compared to 21.7 and 9.2% recorded after heating at 50 and 100°C, respectively. Chloroform and *n*-butanol extracts from *Serratia* sp. C4, applied at 5% (v/v), displayed antifungal potential against FOL expressed as growth inhibition by 54.6-66.5% compared to untreated control which was higher than that achieved using two commercial pesticides i.e. Bavistin® (50% carbendazim, chemical fungicide) and Bactospeine® (16000UI/mg, *Bacillus thuringiensis*-based biopesticide). *Serratia* sp. C4 was found to be a chitinase-, pectinase-, and protease-producing agent and was able to produce the indole-3-acetic acid and to solubilize phosphate.

*Keywords*: Antifungal activity, *Cestrum nocturnum*, endophytic bacteria, *Fusarium oxysporum* f. sp. *lycopersici*, growth promotion, secondary metabolites, tomato

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Tomato Fusarium wilt incited by *Fusarium oxysporum* f. sp. *lycopersici* (FOL) is a destructive disease infecting

tomato. Fusarium wilt causes important losses of tomato crops grown both in open field and under greenhouses (Ignjatov et al. 2012; Moretti et al. 2008). Diseased plants exhibit yellowing and wilting of the foliage, vascular discoloration, stunting and eventual death of the whole plant (Lim et al. 2006).

Control of tomato Fusarium wilt is difficult due to the pathogen survival structures (chlamydospores) in soil for many years without a host and due to its progress within vascular tissues (Ignjatov et al. 2012). Moreover, chemical and genetic control failed to successfully suppress disease due to fungicide resistance development and to emergence of new physiological races of FOL (Ge et al. 2004). Given the internal progress of the pathogen within vascular tissues, the use of endophytic fungi (Mahdi et al. 2014) and bacteria (Goudjal et al. 2014; Kalai-Grami et al. 2014) may be effective in biologically controlling disease.

These endophytic microorganisms are known to colonize plant tissues without causing any harmful effects on their host plants. They may remain at their entry points or spread throughout the plant (Hallmann et al. 1997). Endophytes are excellent plant growth promoters and/or sources of biocontrol agents (Strobel, 2006). Worldwide, endophytic bacteria have been used for the control of some pathogens causing vascular diseases on various plants such as *F. oxysporum* f. sp. *vasinfectum* on cotton (Chen et al. 1995) and *Verticillium dahliae* on rapeseed (Alström, 2001), eggplant, and potato (Eleftherios et al. 2004). Several previous studies have demonstrated the growth-promoting effect induced by endophytic bacteria on treated plants. Indeed, *Burkholderia caribensis*, *Kosakonia oryzae, Pectobacterium* sp., *Enterobacter asburiae*, *E. radicincitans*, *Pseudomonas fluorescens,* and *E. cloacae*, isolated from sugarcane roots and stems, have improved the development of this plant (Marcos et al. 2016). Four endophytic bacteria namely *Azospirillum brasilense*, *Burkholderia ambifaria*, *Gluconacetobacter diazotrophicus,* and *Herbaspirillum seropedicae* were shown able to colonize the internal tissues of roots, stems and leaves of *Solanum lycopersicum* var. *lycopersicum* and to stimulate its growth (Botta et al. 2013). Endophytic bacteria inhibit pathogen growth through the production of antibiotics, cell wall-degrading enzymes, competition for nutrients and minerals, and/or via the induction of systemic resistance (Lugtenberg et al. 2013). Plant growth promotion may be achieved through indole-3-acetic acid (IAA) and siderophore production, phosphate solubilization and nitrogen fixation (Rosenblueth and Martínez-Romero, 2006).

Several previous studies have shown that cultivated *Solanaceae* species may be useful as potential sources of bioactive molecules i.e. *Cestrum* spp. (*C. parqui*, *C. diurnum* and *C. sendtenerianum*) (Ahmad et al. 1993; Chaieb et al. 2007; Haraguchi et al. 2000) and as biocontrol agents, especially endophytic bacteria, i.e. *Capsicum annum* (Paul et al. 2013), *Solanum tuberosum* (Sturz et al. 2002), and *S. lycopersicum* (Ramyabharathi and Raguchander 2014).

*Cestrum nocturnum* is a cultivated *Solanaceae* species used as an ornamental plant; its flowers exude a special sweet fragrance at night, the main reason for its folk names night cestrum, lady of the night and night blooming jasmine (Sharif et al. 2009). Several previous studies have valorized this plant as natural source of bioactive metabolites with insecticidal (Jawale and Dama 2010; Patil et al. 2011; Yogalakshmi et al. 2014), antibacterial,

and antifungal potential (Khan et al. 2011; Prasad et al. 2013; Sharif et al. 2009). However, *C. nocturnum* was only reported as source of isolation of endophytic fungi without assessment of its antimicrobial activity (Huang et al. 2008). Moreover, to our knowledge, this ornamental species was not yet explored as natural source of isolation of endophytic bacteria.

In this study, three endophytic bacterial isolates, recovered from surface-sterilized stems and leaves of *C. nocturnum* plants were assessed for their antifungal potential toward FOL and for their growth-promoting traits on tomato plants.

## MATERIALS AND METHODS

**Tomato seedling preparation**.

Tomato cv. Rio Grande, known to be susceptible to FOL races 2 and 3 (Barker et al. 2005), was used in this study. Seedlings were kept under greenhouse with 16 h light and 8 h dark, 60-70% relative humidity and air temperatures ranging between 20 and 30°C, and grown until reaching the two-true-leaf growth stage. A sterilized peat® (Floragard Vertriebs GmbH für gartenbau, Oldenburg) was used as culture substrate.

**Pathogen culture**.

*F. oxysporum* f. sp. *lycopersici* isolate used in this study was originally recovered from tomato stems showing vascular discoloration (Aydi Ben Abdallah et al. 2016a) and maintained in the fungal culture collection at the Laboratory of Plant Pathology, *Centre Régional des Recherches en Horticulture et Agriculture Biologique* (CRRHAB), Chott-Mariem, Tunisia. It was cultured on Potato Dextrose Agar (PDA) and incubated at 25°C for 7 days before use.

***Cestrum nocturnum* sampling and isolation of endophytic bacteria.**

Endophytic bacteria, used in this study, were isolated from leaves (C1 and C2) and stems (C3 and C4) of healthy *C. nocturnum* plants sampled on April 2013 from Chott-Mariem (N35°56'20.451"; E10°33'32.028"), Tunisia.

Samples were individually disinfected by soaking in 70% ethanol for 1 min, immersion in 1% sodium hypochlorite for 10 min then in 70% ethanol for 30 s. They were rinsed three times in sterile distilled water (SDW) and air-dried on sterile filter papers. Each sample was checked for disinfection process efficiency based on Hallmann et al. (1997) protocol. In fact, 100 µl of the SDW used in the last rinse were injected onto Nutrient Agar (NA) medium. After 48 h of incubation at 25°C, if no microbial growth was observed on medium, the surface disinfection procedure was considered as succeeded.

Twenty surface-sterilized stem and leave pieces, of about 1 cm length, were cut longitudinally with a sterile scalpel and aseptically transferred onto NA medium with the longitudinal sectioning surface placed directly in contact with medium. Plates were incubated at 25°C for 48 h. For each sampled organ, bacterial colonies exhibiting morphological diversity were picked separately onto NA and purified.

Before being used in the different bioassays, bacterial stock cultures maintained at -20°C in Nutrient Broth (NB) supplemented with 40% glycerol were grown on NA medium and incubated at 25°C for 48 h.

**Test of endophytic colonization ability.**

The endohytic colonization ability of the four bacterial isolates collected was tested according to Chen et al. (1995) method. Isolates were grown onto NA

amended with streptomycin sulfate (100 µg/ml w/v) and rifampicin (100 µg/ml w/v). Only double-resistant isolates were selected in order to follow their presence in tomato stems after re-isolation in NA medium amended with these both antibiotics and the wild-type ones (the original isolates) were used for inoculation of tomato. Seedlings were dipped for 30 min into bacterial cell suspensions adjusted to $10^8$ cells/ml using a hemocytometer (Botta et al. 2013). SDW was used for treatment of control seedlings. Five seedlings were used for each individual treatment. Seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing commercialized peat and grown for 60 days under greenhouse conditions as previously described. Bacterial isolates were re-isolated from tomato stems onto NA medium supplemented with streptomycin sulfate and rifampicin (100 µg/ml (w/v) each) and incubated at 25°C for 48 h. Bacterial colonies similar to the wild-type ones were considered as endophytes (Hallmann et al. 1997) and subjected to further screening bioassays.

**Test of plant growth-promoting ability.**

Three endophytic isolates were tested for their potential to promote tomato growth under greenhouse conditions. Healthy tomato seedlings (cv. Rio Grande), at two-true-leaf stage, were carefully removed from alveolus plates and soaked for 30 min into water bacterial suspensions adjusted to $10^8$ cells/ml (Botta et al. 2013). Control seedlings were dipped into SDW only. Inoculated and control seedlings were transplanted into individual pots (12.5 × 14.5 cm) containing sterilized peat. Five replications were used for each individual treatment. At 60 days post-treatment, plants were carefully uprooted and their roots were washed under running water to remove peat. Growth parameters noted were plant height, fresh weight of the aerial parts and roots, and maximum root length.

**Test of disease-suppressive ability.**

Tomato cv. Rio Grande seedlings were treated with the three bacterial isolates separately by drenching 25 ml of bacterial suspensions into culture substrate near the collar level (Nejad and Johnson, 2000). Six days after bacterial treatment, 25 ml of FOL conidial suspension ($10^6$ conidia/ml) were applied as substrate drenching (Fakhouri and Buchenauer 2002). Negative control seedlings were not inoculated with FOL and treated with SDW only. Positive control seedlings were pathogen-inoculated and treated with SDW. Each individual treatment was replicated five times.

Assessment of Fusarium wilt severity was performed, 60 days post-inoculation (DPI), on tomato plants inoculated with FOL based on intensity of leaf yellowing and necrosis using the following 0-4 scale where 0 = no disease symptoms (healthy leaves in the whole plant) and 4 = 76-100% of leaves with yellowing and/or necrosis (Amini 2009). Furthermore, wilt severity was assessed based on the extent of the vascular browning (from collar) after performing longitudinal stem sectioning. Pathogen re-isolation frequency was calculated for five plants per individual treatment as the percentage of FOL colonization of stem sections on PDA (Moretti et al. 2008). Growth parameters such as plant height and fresh weight of whole plant were also noted for all tomato plants challenged or not with FOL.

The most effective isolate in suppressing Fusarium wilt severity and in promoting plant growth was further subjected to morphological and

biochemical characterization and molecular identification.

**Characterization of the most active endophytic isolate.**

**Morphological characterization.** Colonies of the most active isolate were morphologically characterized based on their size, shape, margin, elevation, texture, opacity, consistency and pigmentation on NA medium (Patel et al. 2012). Gram's staining was performed using light microscopy.

**Biochemical characterization**. The most bioactive isolate was also characterized using conventional biochemical tests according to Schaad et al. (2001) protocols. The biochemical tests performed in this study include catalase, Red of Methyl (RM), Vosges Proskauer (VP), mannitol, lecithinase, urease, indole, tryptophan deaminase, Simmons citrate, hydrogen sulfide, nitrate reductase, lysine decarboxylase, and pyocyanin on King A medium.

**Molecular characterization.** Molecular characterization of the selected isolate was performed after extraction of genomic DNA according to Chen and Kuo (1993) for the Gram negative bacteria. The 16S rDNA was amplified using the universal eubacterial primers 27f (5'-AGAGTTTGATC(A/C)TGGCTC AG-3') and 1492r (5'-TACGG(C/T)TAC CTTGTTACGACTT-3') (Moretti et al. 2008). The PCR conditions were as follows: one denaturing cycle at 94°C for 4 min, followed by 40 cycles of denaturing at 94°C for 30 s, annealing at 45°C for 30 s, and polymerization at 72°C for 45 s, then an extension cycle at 72°C for 7 min. Amplifications were carried out in Thermal Cycler® (CS Cleaver, Scientific Ltd., TC 32/80).

The homology of the 16S rDNA sequence of the given isolate was performed using BLAST-N program from GenBank database (http: www.ncbi.nlm.gov/BLAST/). Alignment of sequences was performed using the ClustalX (1.81). The phylogenetic analysis for the aligned sequences was performed using the Kimura two-parameter model (Kimura 1980). The phylogenetic tree was constructed based on neighbor joining (NJ) method with 1000 bootstrap sampling. The bioactive endophytic bacterium (isolate C4) sequence was submitted to GenBank and assigned the following accession number: KX197201.

**Hypersensitivity test.** Hypersensitivity test of the selected isolate was performed on tobacco plants. Ten microliters (10 µl) of water bacterial cell suspension (~$10^8$ cells/ml) were injected to tobacco leaves using a sterile microsyringe. Leaves injected with the same volume of SDW were used as negative control. All tobacco plants were incubated at room temperature for 24 h. After incubation, inoculated leaf areas were checked for the presence of chlorotic and/or necrotic zones indicating that the tested isolate is phytopathogenic and should be excluded from further biocontrol trials (Nawangsih et al. 2011).

**Hemolytic test**. Bacterial cell suspensions (~$10^8$ cells/ml, 100 µl) of the selected isolate were transferred on Blood Agar® (HiMedia, India) medium to test its ability to degrade hemoglobin. Bacterial cultures were incubated at 25°C for 48 h. Positive hemolytic activity is indicated by the formation of clear zones around bacterial colonies (Murray et al. 2003). Thus, the tested isolate will be considered to be pathogenic to humans and excluded from the following tests.

**Assessment of the antifungal activity of *Serratia* sp. C4.**

**Streak method**. Bacterial suspension of *Serratia* sp. C4 (~$10^8$ cells/ml) were streaked across the center and perpendicularly to the first streak on the surface of PDA poured in Petri plates (9 cm in diameter). Four agar plugs (6 mm in diameter), removed from 7 day-old cultures of FOL, were placed at each side of the streaked bacterial suspensions (Sadfi et al. 2001). Control plates were streaked with SDW only. Each individual treatment was repeated four times. After 4 days of incubation at 25°C, pathogen colony diameter was noted. The inhibition rate (IR) of FOL mycelial growth was calculated using Tiru et al. (2013) formula as follows: IR% = [(D2-D1) / D2] × 100 where D2: Diameter of pathogen colony in control plates and D1: Diameter of pathogen colony co-cultured with the tested bacterial isolate.

**Disc diffusion method.** The antifungal activity of the selected bacterial isolate was also evaluated on PDA using the disc diffusion method. FOL was incorporated into molten PDA and after medium solidification, 20 µl droplets of bacterial suspensions (~$10^8$ cells/ml) were deposited on Whatman No. 1. filter paper discs (6 mm in diameter). Four discs were used per plate (Vethavalli and Sudha 2012). For control plates, paper discs were treated with a same volume of SDW. Each individual treatment was repeated four times. After four days of incubation at 25°C, the diameter of the inhibition zone was noted.

**Activity of cell-free culture filtrates.** *Serratia* sp. C4 colonies were grown in Luria-Bertani broth (LB) at 28 ± 2°C for 3 days and under continuous shaking at 150 rpm. Two ml of the obtained liquid culture were centrifuged for 10 min at 10,000 rpm. The centrifugation was repeated three times. Cell-free culture filtrate was sterilized by filtration through a 0.22 µm pore size filter. To determine the stability of extracellular metabolites produced by this isolate, filtrate was incubated at 50 or 100°C for 15 min (Romero et al. 2007). Antifungal activity of cell-free cultures, untreated and heated, used at 20% (v/v) was assessed according to Karkachi et al. (2010). Control cultures contained LB filtrate only. Each individual treatment was replicated three times. After four days of incubation at 25°C, the diameter of FOL colony was measured and the mycelial growth inhibition rate was calculated (Tiru et al. 2013).

**Activity of *Serratia* sp. C4 organic extracts.**

*Extraction of secondary metabolites.* Two types of extraction were carried out to extract the antifungal metabolites produced by *Serratia* sp. C4. The first one was performed using chloroform (Bhoonobtong et al. 2012) and the second one with *n*-butanol (Romero et al. 2007). Sixty milliliters (60 ml) of cell-free culture filtrate of *Serratia* sp. C4, prepared as described above, were placed in a separating funnel. Then, 60 ml of the 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) were collected. The aqueous phase was replaced in the funnel and the extraction was repeated two other times as described. The solvent was evaporated in a rotary evaporator at 35°C for chloroform and at 75°C for *n*-butanol with a slight rotation at 150 rpm.

*Testing of antifungal activity of organic extracts.* Obtained organic extracts were assessed for their biological activity against FOL. Each extract was suspended in ethanol (1/1) (w/v) and added to Petri plates containing 10 ml of molten PDA amended with streptomycin sulfate (300 mg/l) (w/v) at two concentrations 2.5 and 5% (v/v). Control cultures were treated with ethanol also tested at 2.5 and 5% (v/v). The antifungal activity of secondary metabolites released by *Serratia* sp. C4 was compared to two commercial products i.e. Bavistin® (50%, chemical fungicide with carbendazim as active ingredient) and Bactospeine® (16000UI/mg, *Bacillus thringiensis*-based biopesticide). After solidification of the mixture, an agar plug (6 mm in diameter), removed from FOL culture previously grown at 25°C for 7 days, was placed at the center of each plate. After seven days of incubation at 25°C, FOL colony diameter was measured and the inhibition rate was calculated (Tiru et al. 2013).

**Assessment of *Serratia* sp. C4 enzymatic activity.**

**Chitinase production.** Chitinase production ability of *Serratia* sp. C4 was checked according to Tiru et al. (2013) on minimum-medium supplemented with chitin® (MP Biomedicals, LLC, IIIKrich, France) by streaking bacterial suspensions (~$10^8$ cells/ml) onto sterilized chitin-agar medium (0.5% w/v). Chitin-agar medium plates non-streaked with bacterial suspensions were used as control. Treatments were replicated thrice. After 72 h of incubation at 28 ± 2°C, the presence of clearing zones around bacterial colonies was noted.

**Protease production.** *Serratia* sp. C4 was assessed for its potential to release protease onto skim milk agar or SMA (3% v/v) medium (Tiru et al. 2013). Plates containing SMA only were used as control. 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.** Pectinase production ability of *Serratia* sp. C4 was detected according to Tiru et al. (2013) method. Water bacterial suspensions (~$10^8$ cells/ml) were streaked onto NA-pectin® (ICN Biomedicals, Inc, Germany) medium (0.5% w/v). Plates containing the NA-pectin medium only were used as control. Treatments were performed in triplicate. After 48 h of incubation at 28 ± 2°C, the presence or the absence of clear zones around bacterial colonies was noted.

**Phosphate solubilization.** Phosphate solubilization activity of the selected bacterial isolate was evaluated qualitatively according to Katzenlson and Bose (1959) method with some modifications. An agar plug (6 mm in diameter) containing *Serratia* sp. C4 colonies, previously grown on NA during 48 h, was putted onto Pikovskaya agar medium. Un-inoculated plates were used as control. Experiments were performed in triplicate. After seven days of incubation at 28 ± 2°C, clear zones formed around colonies, due to the utilization of tricalcium phosphate present in the medium, were measured.

**Production of indole-3-acetic acid (IAA).**The ability of *Serratia* sp. C4 to produce IAA was checked using the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. *Serratia* sp. C4 was cultivated into LB medium supplemented with L-tryptophan (50 µg/ml) under continuous shaking at 150 rpm for 2 days

in the dark. The bacterial suspension was centrifuged at 10,000 rpm for 10 min. One ml of the culture supernatant was mixed with 2 ml of Salkwoski's reagent and 2-3 drops of orthophosphoric acid. Un-inoculated LB medium was used as negative control. Absorbance was measured daily at 530 nm. The concentration of IAA was determined and compared to a standard curve prepared from IAA dilution series at 100 µg/ml in LB medium.

**Statistical 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. For all the in vitro antifungal potential bioassays and the in vitro tests of enzymes and IAA production and phosphate solubilization ability, each treatment was replicated three to four times. For cell-free culture filtrate tests, data were analyzed according to a completely randomized factorial design with two factors (Heating and bacterial treatment). The in vitro assay of organic extracts was analyzed according to a completely randomized factorial model with two factors (treatments and concentrations). For the remaining in vitro bioassays, data were analyzed according to a completely randomized design. All the in vivo bioassays were analyzed in a completely randomized model and each treatment was replicated five times. For the in vitro antifungal activity tests using the streak and the disc diffusion methods, means were separated using Student t test at $P \leq 0.05$. For the remaining bioassays, means were separated using LSD test for the in vitro antifungal activity test of cell-free cultures and organic extracts and using Duncan Multiple Range test for the others to identify significant pair-wise differences 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 ability of bacterial isolates recovered from *Cestrum nocturnum*.**

Four bacterial isolates exhibiting macro-morphological diversity on NA medium were selected among twenty others recovered from stems and leaves of *C. nocturnum* plants. The four selected isolates were found to be resistant to streptomycin and rifampicin (100 µg/ml) and only three were successfully re-isolated from the internal stem tissues of tomato cv. Rio Grande plants on NA medium amended with these antibiotics. These three endophytic isolates (namely C1, C3, and C4) were further assessed in vivo and in vitro for their antifungal potential toward FOL and for their growth-promoting effects on tomato seedlings.

**Plant growth-promoting ability displayed by the selected endophytic isolates.**

The three endophytic bacterial isolates (C1, C3 and C4) were screened for their growth-promoting potential onto pathogen-free tomato plants. ANOVA analysis revealed that all plant growth parameters (plant height, aerial part fresh weight, maximum root length, and root fresh weight), noted 60 days post-treatment, varied significantly (at $P \leq 0.05$) depending on bacterial treatments tested.

Data given in Table 1 revealed that, C1- and C4-based treatments led to significant ($P \leq 0.05$) increase in plant height by 18.7 and 31.9%, respectively, compared to the untreated control. The highest plant height improvement (by

31.9% over control) was achieved using C4 isolate.

The aerial part fresh weight was significantly enhanced by 50% using C4-based treatment compared to control (Table 1). As estimated by the maximum root length, a significant ($P \leq 0.05$) improvement (by 24.6% over control) was recorded on tomato plants treated with C4. Similar trend (53.3%) was noted based on root fresh weight (Table 1).

**Table 1.** Comparative plant growth-promoting ability of endophytic bacterial isolates recovered from *Cestrum nocturnum* on tomato cv. Rio Grande plants noted 60 days post-treatment

| Bacterial treatment* | Plant Height (cm) | Aerial part fresh weight (g) | Maximum root length (cm) | Root fresh weight (g) |
|---|---|---|---|---|
| **NIC** | 20 ± 0 c | 8 ± 0.1 b | 17.2 ± 0.6 b | 4.2 ± 0.1 b |
| **C1** | 24.6 ± 0.3 b | 6.8 ± 0.2 b | 19 ± 0.5 b | 5.8 ± 0.2 b |
| **C3** | 21.6 ± 0.5 c | 7 ± 0.5 b | 18.8 ± 0.8 b | 5.8 ± 0.2 b |
| **C4** | 29.4 ± 0.8 a | 16 ± 0.1 a | 22.8 ± 0.5 a | 9 ± 0.7 a |

*C1: Isolate from *C. nocturnum* stem; C3 and C4: Isolates from *C. nocturnum* leaves; NIC: Un-inoculated with the pathogen and untreated control. For each column, values followed by the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

**Fusarium wilt suppression by the selected endophytic isolates.**

The three selected endophytic bacterial isolates were tested on tomato cv. Rio Grande plants challenged with FOL. ANOVA analysis revealed that Fusarium wilt severity, noted on tomato plants 60 DPI, varied significantly ($P \leq 0.05$) depending on bacterial treatments tested. A significant ($P \leq 0.05$) decrease in leaf damage index (yellowing and/or necrosis), by 46.6 to 86.6% compared to pathogen-inoculated and untreated control, was noted on tomato plants already challenged with FOL and treated using the three tested isolates (Table 2). The reduction of the vascular browning extent was significant, by 55.5 to 97.7% compared to control, using C1-, C3-, and C4-based treatments. The isolate C4 was found to be the most effective in suppressing leaf yellowing and wilt symptoms (86.6%) and in reducing the vascular browning extent (97.7%) relative to FOL-inoculated and untreated control. Furthermore, C4-treated plants behaved significantly similar to the un-inoculated (disease-free) and untreated ones based on both disease severity parameters (Table 2).

Growth parameters of tomato plants (plant height and fresh weight), noted 60 DPI with FOL, varied significantly ($P \leq 0.05$) depending on treatments tested. The increment in plant height ranged significantly between 12.6 and 39% over FOL-inoculated and untreated control using C1-, C2-, and C4-based treatments and the highest enhancement (of about 39%) was achieved using C4 isolate. It should be also highlighted that tomato plants infected with FOL and treated with C4 exhibited a significantly higher (by 12.7%) plant height than disease-free and untreated ones.

All bacterial isolates tested had significantly ($P \leq 0.05$) increased plant fresh weight by 23.2 to 41.7% over FOL-inoculated and untreated control and the highest increment (41.7%) was induced by the isolate C4. The fresh weight of

FOL-inoculated tomato plants treated with C4 isolate was significantly similar to that of disease-free control ones (Table 2).

FOL re-isolation frequency from tomato stems varied depending on bacterial treatments tested. A decrease in pathogen isolation frequency, ranging between40 and 90% relative to the untreated control, was recorded from tomato plants already infected with FOL and treated with the three enpdophytic isolates tested. The highest decrease in FOL re-isolation frequency was achieved by using the isolate C4 (90%) (Table 2).

**Table 2.** Effects of endophytic bacterial isolates recovered from *Cestrum nocturnum* on Fusarium wilt severity, plant growth parameters and *Fusarium oxysporum* f. sp. *lycopersici* (FOL) re-isolation frequency from tomato cv. Rio Grande plants as compared to controls

| Bacterial treatment* | Disease severity (0-4) | Vascular browning extent (cm) | Plant height (cm) | Plant fresh weight (g) | FOL re-isolation** (%) |
|---|---|---|---|---|---|
| **NIC** | 0 ± 0 c | 0 ± 0 c | 24.8 ± 0.4 b | 7.05 ± 0.2 ab | 0 |
| **IC** | 3 ± 0.1 a | 9 ± 0.5 a | 17.3 ± 1 d | 4.2 ± 0.1 d | 100 |
| **C1** | 1.2 ± 0.1 b | 4 ± 0.1 b | 21 ± 0.5 c | 6.25 ± 0.1 bc | 50 |
| **C3** | 1.6 ± 0.2 b | 4 ± 0.1 b | 19.8 ± 0.4 c | 5.47 ± 0.3 c | 60 |
| **C4** | 0.4 ± 0.2 c | 0.2 ± 0.1 c | 28.4 ± 0.3 a | 7.21 ± 0.2 a | 10 |

*C1: Isolate from *C. nocturnum* stem, C3 and C4: Isolates from *C. nocturnum* leaves; NIC: Un-inoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control.
**The re-isolation of FOL was carried out from stems of five tomato plants cv. Rio Grande at 0-15 cm high from the collar. Ten (10) stem fragments were plated onto PDA medium and incubated at 25°C for 4 days. After incubation, the percentage of FOL colonization of stems sections was calculated.
For each column, values followed by the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

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

Pearson's analysis revealed that decreased Fusarium wilt severity as estimated by leaf damage index (and/or necrosis) and vascular browning extent led to increment in all plant growth parameters. In fact, plant height was significantly and negatively correlated to the leaf damage index (r = -0.874; $P$ = 0.053) (Fig. 1A) and to the vascular browning extent (r = -0.909; $P$ = 0.033) (Fig. 1B). Furthermore, the plant fresh weight was significantly and negatively correlated to leaf yellowing score (r = -0.981; $P$ =0.003) (Fig. 1C) and to the vascular browning extent (r = -0.973; $P$ = 0.005) (Fig. 1D).

Pearson's analysis demonstrated that lowered Fusarium wilt severity led to decrease in tomato stem colonization by FOL and consequently growth promotion where significant and negative correlations were also detected between FOL re-isolation frequency, plant height (r = -0.914; $P$ = 0.03) (Fig. 1E), and whole plant fresh weight (r = -0.975; $P$ = 0.005) (Fig. 1F). Moreover, pathogen re-isolation frequency was positively correlated to leaf damage index (r = 0.991; $P$ = 0.001) (Fig. 1G) and to the vascular browning extent (r = 0.987; $P$ = 0.002) (Fig. 1H).

The endophytic bacterial isolate C4 shown to be effective in suppressing Fusarium wilt severity and in promoting growth of tomato plants inoculated or not

with FOL (Fig. 2) was selected for further characterization, identification and elucidation of its mechanisms of action involved in those both effects.

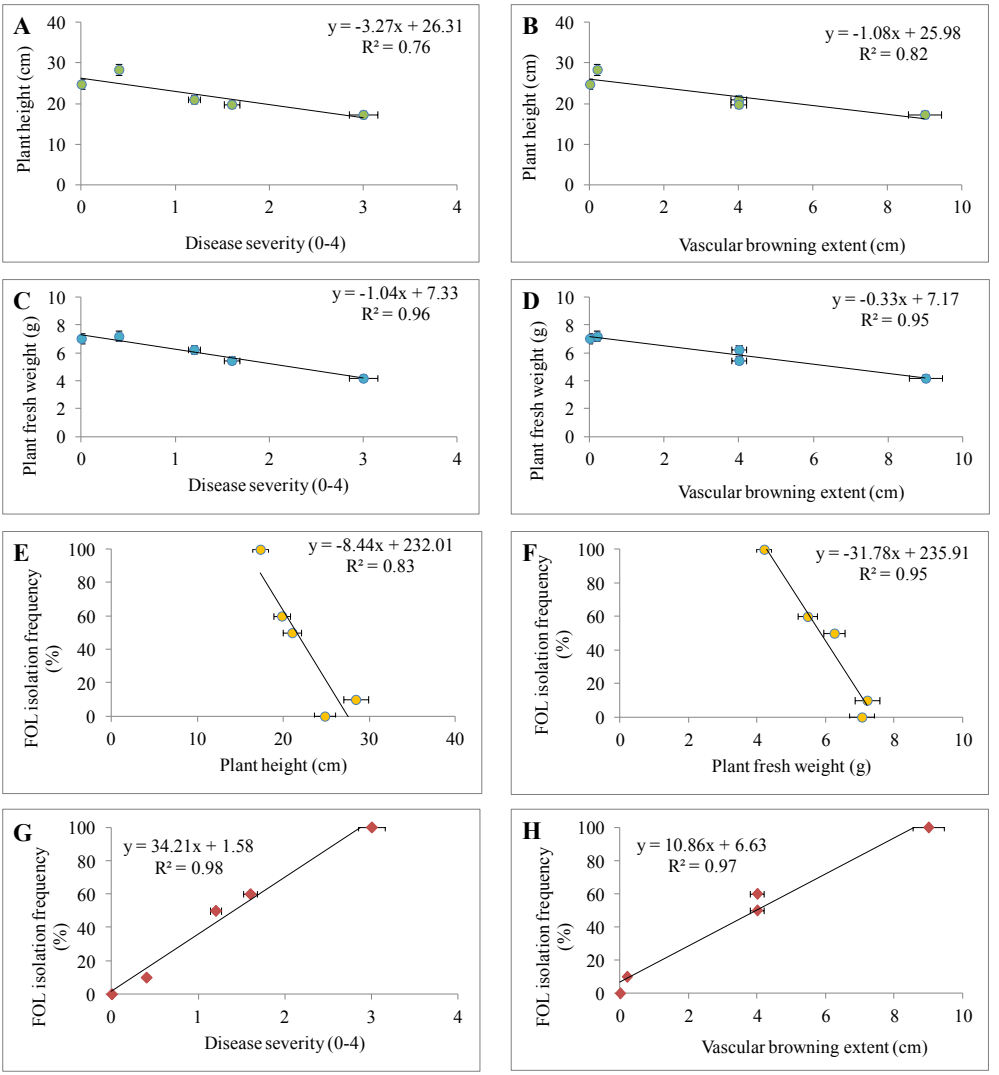


**Fig. 1.** Correlation between Fusarium wilt severity and plant growth parameters (A, B, C, D, E, F) and between FOL isolation frequency and Fusarium wilt severity parameters (G, H). Correlation analysis was performed using bivariate Pearson's test at $P \leq 0.01$.

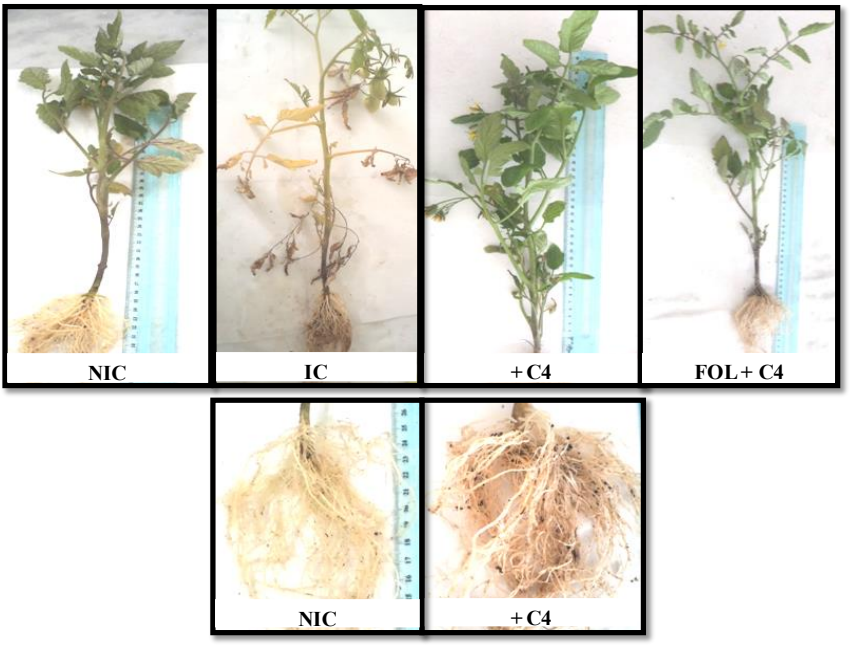


**Fig. 2.** Effect of endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* on Fusarium wilt severity and growth promotion of tomato cv. Rio Grande plants compared to the untreated controls. NIC: Un-inoculated with the pathogen and untreated control. IC: Inoculated with FOL and untreated control; C4: Isolate from *Cestrum nocturnum* leaves.

**Morphological, biochemical and molecular characterization of the selected bacterial isolate.**

The colony morphology of the selected isolate C4 showed a small size and translucent colonies with circular form, an entire margin and plane elevation, smooth surface and cream color on NA medium (Table 3). C4 was found to be a Gram negative strain.

C4 was able to produce catalase, lecithinase, lysine decarboxylase, nitrate reductase, and indole. The isolate C4 cannot produce urease, tryptophane desaminase, hydrogen sulfide, and pyocyanin on King A medium. Simmons citrate and mannitol were not used by C4 colonies as carbon sources. C4 cannot ferment glucose through the mixed acid (MR-) but by using the glycol butylene path (VP +) (Table 3).

Blast-N analysis of sequenced 16S rDNA gene homology and the phylogenetic analysis based on neighbor joining (NJ) method with 1000 bootstrap sampling revealed that the isolate C4 belonged with 100% of similarity to Uncultured *Serratia* sp. strain CTL-81 and *Serratia proteamaculans* strain AP-CMST (Table 3; Fig. 3). The accession number of *Serratia* sp. strain C4 deposed in GenBank was KX197201 (Table 3).

**Table 3.** Characterization and molecular identification of the selected endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* leaves

| **Morphological characterization** | |
|---|---|
| Size | Small |
| Form | Circular |
| Margin | Entire |
| Elevation | Plane |
| Surface | Smooth |
| Opacity | Translucent |
| Color | Cream |
| Gram staining | Negative |
| **Biochemical characterization** | |
| King A | - |
| Catalase | + |
| Urease | - |
| Lecithinase | + |
| Nitrate reductase | + |
| Tryptophane deaminase | - |
| Lysine decarboxylase | + |
| Mannitol | - |
| Simmons citrate | - |
| Indole | + |
| Red of Methyl | - |
| Voges-Proskauer | + |
| Hydrogen sulfide | - |
| **Molecular characterization** | |
| Most related species | CTL-81, Uncultured *Serratia* sp. (100) |
| | AP-CMST, *Serratia proteamaculans* (100) |
| Accession number GenBank | KX197201 |
| **Hypersensivity reaction** | |
| - | |
| **Hemolytic activity** | |
| - | |

+: Positive test; -: Negative test. Numbers in parenthesis indicate the percentage (in %) of sequence homology obtained from Blast-N analysis from GenBank database (http: www.ncbi.nlm.gov/BLAST/).

The nucleotide sequences used of representative strains were obtained from Genbank database under the following accession numbers: FJ752236 (*Serratia proteamaculans* AP-CMST), JQ798999 (Uncultured *Serratia* sp. CTL-81), KU682855 (*Serratia* sp. PKL:12), KJ922535 (*Shewanella* sp. XH39), JX162043 (*Enterobacteriaceae bacterium* Pokym2-b), NR_037112 (*Serratia quinivorans* 4364), KU999993 (*Serratia liquefaciens* strain ZMT-1), KM187235 (*Gluconacetobacter* sp. CC3H1), KM453915 (Uncultured bacterium D05 placa2), NR_113616 (*Serratia grimesii* NBRC 13537), and for the bacterial isolate tested: KX197201 (C4). The tree topology was constructed using ClustalX (1.81).

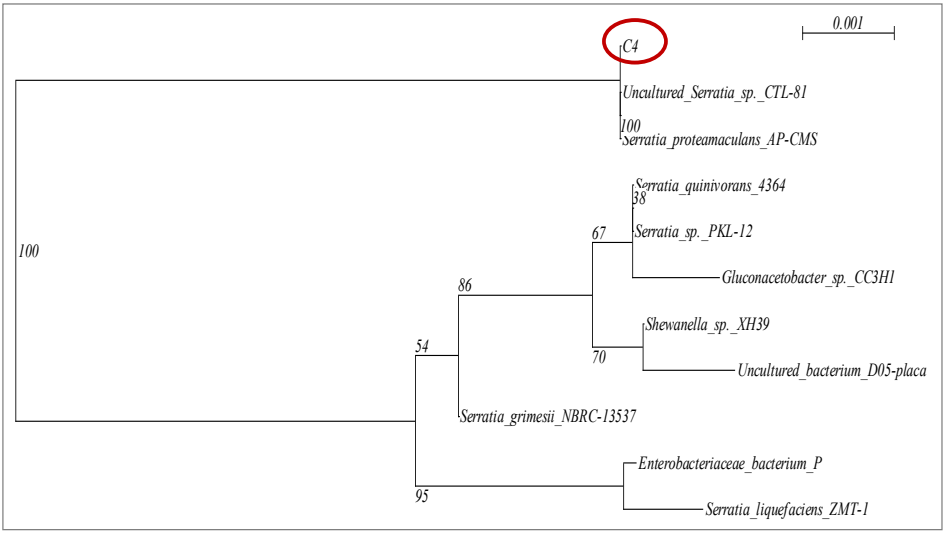


**Fig. 3.** Neighbor-joining phylogenetic tree of partial 16S rDNA sequences of the best antagonistic and plant growth promoting endophytic bacterial isolate C4 recovered from *Cestrum nocturnum* and their closest phylogenetic relatives.

**Hypersensivity reaction and hemolytic activity of *Serratia* sp. C4.**

No hypersensitive reaction (HR) (chlorotic or necrotic zone) was detected on inoculated tobacco leaf areas as compared to control ones after 24 h of incubation. Thus, the isolate C4 was found to be non phytopathogenic and was selected for further screenings.

No hemolytic activity was displayed by the isolate C4 expressed by the absence of clear zones around its colonies grown on Blood Agar medium after 48 h of incubation at 25°C. Thus, this isolate was nonpathogenic to humans and it can be used in the following tests (Table 3).

**Antifungal activity of *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici*.**

**Activity of whole culture**. The endophytic bacterial isolate *Serratia* sp. C4, tested using the streak method, induced a significant ($P \leq 0.05$) decrease, by 19.5% compared to control, in FOL mycelial growth noted after 4 days of incubation at 25°C (Table 4; Fig. 4 A).

Tested using the disc diffusion method on PDA medium, *Serratia* sp. C4 formed an inhibition zone of about 8.62 mm in size around FOL colony after 4 days of incubation at 25°C (Table 4; Fig. 4B).

**Table 4.** Antifungal activity of *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici* (FOL)

| Bacterial treatment | Diameter of FOL colony (cm)[a] | Inhibition zone (mm)[b] |
|---|---|---|
| Untreated control | 3.71 ± 0.08 | 0 ± 0 |
| *Serratia* sp. C4 (KX197201) | 2.98* ± 0.04 | 8.62* ± 0.5 |

Values with asterisk indicate a significant difference with the control (t-test at $P \leq 0.05$).
[a] Tested using the streak method on PDA medium and incubated at 25°C for 4 days.
[b] Tested using the disc diffusion method on PDA medium and incubated at 25°C for 4 days.

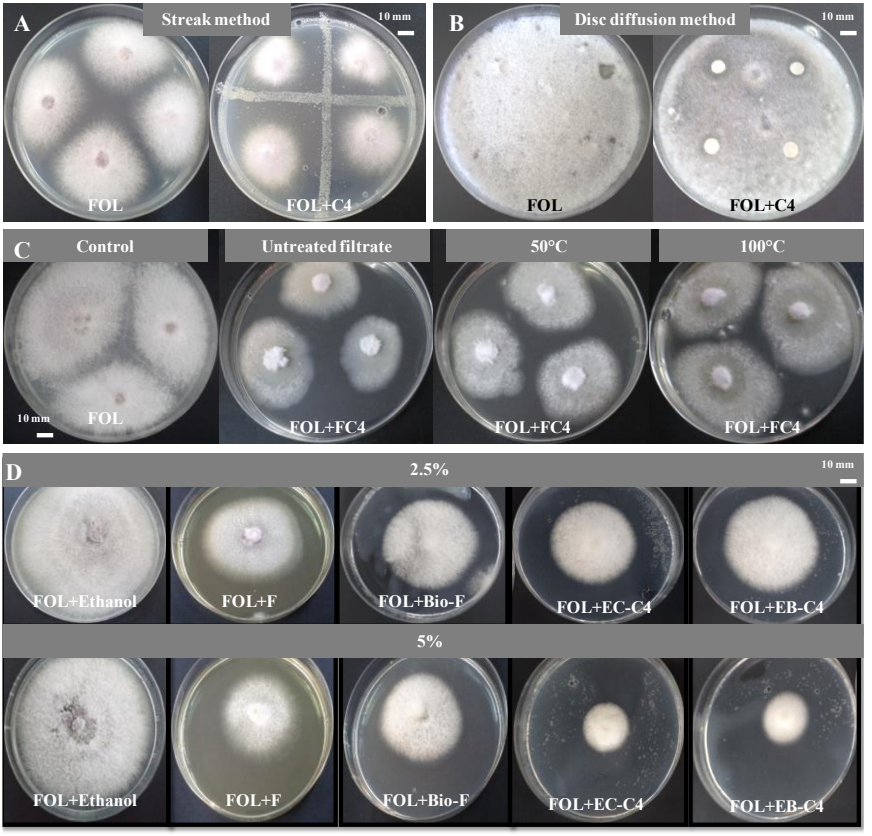


**Fig. 4.** Antifungal activity of endophytic *Serratia* sp. C4 against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) using whole culture (A and B), untreated cell-free culture and filtrates heated at 50 and 100°C (C) and organic extracts tested at two concentrations (D) as compared to controls.
C4: Whole culture of *Serratia* sp. C4; FC4: Cell-free culture filtrate from *Serratia* sp. C4; Ethanol: Negative control; F: Positive control (Bavistin®, chemical fungicide with carbendazim as active ingredient); Bio-F: Positive control (Bactospeine®, *Bacillus thuringiensis*-based biopesticide); EC-C4: Chloroform extract from *Serratia* sp. C4; EB-C4: n-butanol extract from *Serratia* sp. C4.

**Activity of cell-free culture filtrate.** Analysis of variance revealed significant ($P \leq 0.05$) variation in pathogen colony diameter depending on treatments tested and a significant interaction was recorded between cell-free treatments and heating. A significant decrease in FOL colony diameter, 23% versus the un-inoculated control, was induced by the untreated cell-free culture of *Serratia* sp. C4 (Fig. 5). The filtrate heated at 50°C for 15 min had also significantly ($P \leq 0.05$) reduced FOL growth by 21.7% (Fig. 5). However, heating treatment at 100°C for 15 min decreased the antifungal activity of the tested cell-free culture supernatant toward FOL where pathogen growth was inhibited by 9.3% relative to 21.7 and 23% recorded using filtrate heated at 50°C and unheated one, respectively (Figs. 4C and 5).

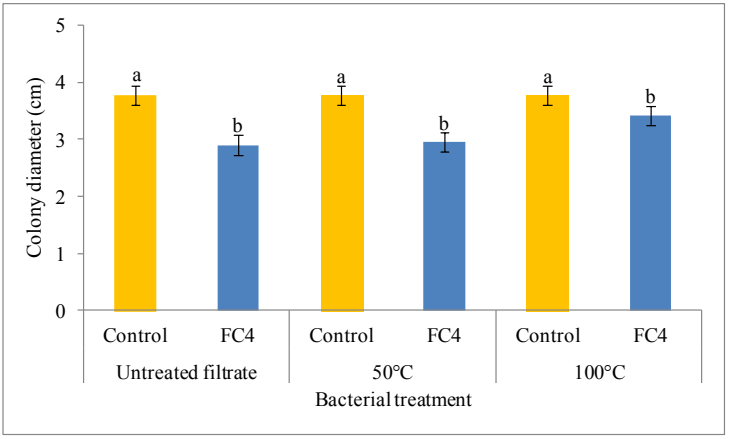


**Fig. 5.** Effect of heating of the cell-free culture filtrate of *Serratia* sp. C4 on its antifungal activity against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) noted after 4 days of incubation at 25°C as compared to controls.
FC4: Cell-free culture filtrate from *Serratia* sp. C4 (KX197201) isolated from surface sterilized *Cestrum nocturnum* leaves. Control: Luria-Bertani broth filtrate. LSD (Bacterial treatment × Heating): 0.33 cm at $P \leq 0.05$. For each heating treatment, bars with the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

**Activity of organic extracts**. ANOVA analysis revealed a significant ($P \leq 0.05$) variation in the mean colony diameter of FOL depending on organic extracts (chloroform and *n*-butanol extracts) tested, concentrations used, and their interactions. Chloroform and *n*-butanol extracts from *Serratia* sp. C4, applied at 1 mg/ml, had inhibited FOL growth by 27.6 to 66.5% as compared to ethanol control whatever the concentration tested (Fig. 6).

Both organic extracts from *Serratia* sp. C4 were found to be more active at the concentration 5% than at 2.5% (v/v) where pathogen growth was inhibited by 54.6-66.5% and 27.6-31.3%, respectively (Fig. 6). In fact, chloroform extract from *Serratia* sp. C4 decreased FOL growth by 54.6% when applied at

5% (v/v), compared to 31.3% recorded at 2.5% (v/v). In addition, *n*-butanol extract from this isolate, applied at 5% (v/v), had inhibited pathogen growth by 66.5% compared to 27.6% recorded at 2.5% (v/v) (Figs. 4D and 6).

It should be highlighted that the decrease in FOL growth was higher using *Serratia* sp. C4 organic extracts at 5% (v/v) (54.6-66.5%) than that achieved using Bavistin® (31.3-39.5%) and Bactospeine® (40.9-43.2%) whatever the concentration used (Figs. 4D and 6).

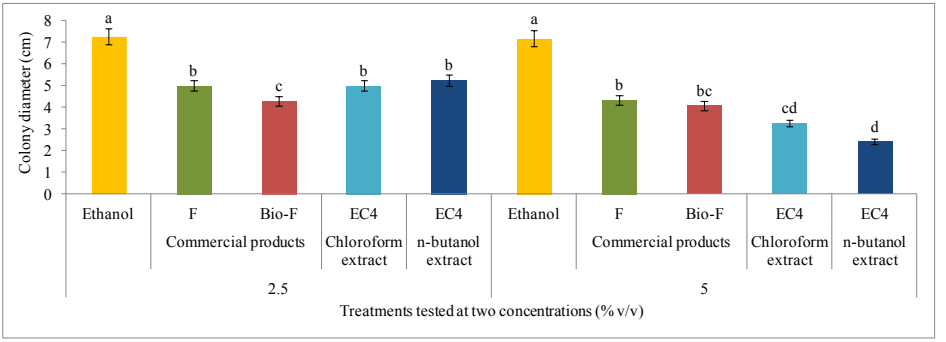


**Fig. 6.** Effect of chloroform and *n*-butanol extracts from endophytic *Serratia* sp. C4 tested at two concentrations against *Fusarium oxysporum* f. sp. *lycopersici* noted after 7 days of incubation at 25°C as compared to controls. EC4: Organic extract from *Serratia* sp. C4 (KX197201) isolated from surface sterilized *Cestrum nocturnum* leaves. Control: Ethanol control. F: Bavistin® (Chemical fungicide, carbendazim); Bio-F: Bactospeine® (*Bacillus thuringiensis*-based biopesticide). LSD (Treatments tested × Concentrations used): 0.86 cm at $P \leq 0.05$. For each concentration, bars sharing the same letter are not significantly different according to Duncan Multiple Range test at $P \leq 0.05$.

**Production of chitinase, protease and pectinase by *Serratia* sp. C4**

*Serratia* sp. C4 formed clear zones around its colonies when grown onto chitin-, pectin- and milk-agar media. This indicates that *Serratia* sp. C4 is able to produce cell-wall degrading enzymes i.e chitinase-, pectinase and protease, respectively (Table 6).

**Phosphate solubilization and IAA production ability of *Serratia* sp. C4**

*Serratia* sp. C4 was able to solubilize phosphate as indicated by the formation of a clear zone of about 10.33 mm width around its colonies when grown on Pikovskaya agar medium (Table 5).

The selected endophytic isolate, *Serratia* sp. C4, was able to produce the indole-3-acetic acid (IAA), involved in plant growth promotion, estimated at 29.52 µg/ml after 48 h of incubation compared to 0.3 µg/ml recorded after 24 h (Table 5).

**Table 5.** Production of cell-wall degrading enzymes and plant growth-promoting compounds by endophytic *Serratia* sp. C4 recovered from *Cestrum nocturnum* leaves

| Isolate | Cell-wall degrading enzymes | | | Plant growth-promoting compounds | | |
|---|---|---|---|---|---|---|
| | Chitinase[a] | Protease[b] | Pectinase[c] | Indole-3-acetic acid (µg/ml)[d] | | Phosphate solubilization[e] |
| | | | | 24 h | 48 h | |
| ***Serratia* sp. C4 ( KX197201)** | + | + | + | + | ++ | + |

[a] Tested on chitin-agar (0.5 % w/v) medium and incubated at 28 ± 2°C for 72 h; +: Presence of clear zone.
[b] Tested on skim milk agar (3% v/v) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone (14.5 ± 0.03 mm in diameter).
[c] Tested on pectin-agar (0.5 % w/v) medium and incubated at 28 ± 2°C for 48 h; +: Presence of clear zone.
[d] Indole-3-acetic acid (IAA) compounds production after 24 and 48 h of incubation at 28 ± 2°C in Luria-Bertani broth medium; +: Production of IAA compounds (0.3 ± 0 µg/ml) ++: Production of IAA compounds (29.52 ± 0.05 µg/ml).
[e] Tested on Pikovskaya agar medium and incubated at 28 ± 2°C for 7 days; +: Presence of clear zone (10.33 ± 0.01 mm in diameter).

## DISCUSSION

Endophytic bacteria isolated from cultivated plants i.e. tomato and colza were successfully used as biocontrol agents against Fusarium wilt of tomato (Nandhini et al. 2012; Nejad and Johnson 2000; Ramyabharathi and Raguchander 2014). In view of previous studies, the present investigation focused on potential use of endophytic bacteria recovered from *C. nocturnum* grown in Tunisia (Chott-Mariem, Sousse) for controlling tomato Fusarium wilt.

*C. nocturnum* was successfully used as a natural source of bioactive metabolites with antifungal activities. In fact, ethanol, methanol and butanol extracts from *C. nocturnum* were explored for their antifungal activity against *Trichoderma* sp. and *Aspergillus* sp. (Prasad et al. 2013). Furthermore, aqueous extracts and chloroform, ethyl acetate and *n*-butanol extracts from *C. nocturnum* showed an inhibitory effect against *Candida albicans*, *Microsporum canis*, *Candida glaberata* growth ranging between 30 and 65% (Khan et al. 2011). Few data were available on *C. nocturnum* use as potent source of isolation of microorganisms such as fungi i.e *Alternaria* spp., *Aspergillus* spp., *Colletotrichum* spp., *Fusarium* spp., *Gliocladium* sp., *Phoma* spp., *Phomopsis* spp., *Phyllosticta* sp., *Torula* sp. and Xylariales sp. (Huang et al. 2008). To our Knowledge, no data had previously valorized this species as natural source of isolation of bacterial biocontrol agents. Hence, this is the first study reporting on possible exploration of *C. nocturnum* as potential source of endophytic bacteria exhibiting antifungal activity against Fusarium wilt and growth-promoting potential onto tomato seedlings.

In the current study, four bacterial isolates were collected among many others based on macro-morphology diversity onto NA medium and were found to be resistant to streptomycin and rifampicin (100 µg/ml). Endophytic

progress within tomato stems was confirmed for three isolates among the four tested after re-isolation onto NA medium amended with these both antibiotics. These three bacterial isolates recovered from *C. nocturnum* and exhibiting endophytic behavior on tomato plants, were assessed for their ability to control Fusarium wilt disease and to enhance growth of tomato seedlings. In fact, assessed on seedlings un-inoculated with FOL, the current results clearly demonstrated that the bacterial isolate C4 had significantly stimulated the growth of the aerial plant parts by 31.9-50% and root development by 24.6 to 53.3% compared to the untreated control. Hence, the isolate C4, recovered from *C. nocturnum* leaves, exhibited bio-fertilizing properties. Similar results on plant growth-promoting potential (PGPB) were reported on wheat seedling treated with *Serratia marcescens* KR-4, *B. thuringiensis* KR-1 and *Enterobacter asburiae* KR-3, originally recovered from nodule of *Pueraria thunbergiana* (Selvakumar et al. 2008a). When assessed on tomato seedlings inoculated with FOL, the strongest suppressive effect of disease severity was also displayed by the isolate C4. This ability to suppress Fusarum wilt was expressed by more than 86.6% decrease in leaf damage index and 97.7% in vascular browning extent resulting in a significant decrease in FOL colonization of stem tissues. Nejad and Johnson (2000) also observed a decrease by at least 75% in tomato Fusarium wilt severity using two unidentified endophytic isolates PA and PF, issued from healthy cultivated oilseed rape plants. The selected C4 isolate was also able to enhance plant growth on tomato plants inoculated or not with FOL. Furthermore, Pearson correlation analysis indicates that Fusarium wilt severity decrease was found to be related to the registered increment in all plant growth parameters. Ramyabharathi and Raguchander (1994) also found that tomato Fusarium wilt-suppressive effect, by 68.4%, displayed by an endophytic bacterium *B. subtilis* str. EPC016, isolated from cotton plants, was associated to promotion of plant growth and fruit yield in tomato compared to control. In our recent studies, a strong decrease in Fusarium wilt severity was also achieved using various endophytic bacteria including *Stenotrophomonas* sp. S33, *Pseudomonas* sp. S85, *B. mojavensis* S40, *S. maltophilia* S37, *B. tequilensis* SV104, *Bacillus* sp. SV101, *Alcaligenes feacalis* subsp. *faecalis* S8, *B. cereus* S42 and *A. faecalis* S18, originally recovered from wild *Solanaceae* species namely *Datura metel, D. stramonium*, *Solanum elaeagnifolium*, *Withania somnifera*, and *Nicotiana glauca*, respectively. These endophytic isolates were also shown effective in enhancing tomato growth in plants inoculated or not with FOL (Aydi Ben Abdallah et al. 2016a-e).

The best antagonistic and plant growth-promoting isolate (C4) was macro-morphologically and biochemically characterized and molecularly identified by 16S rDNA gene sequencing as *Serratia* sp. C4 (KX197201).Gyaneshwar et al. (2011) used an isolate of *Serratia marcescens*, showing an endophytic ability on rice plants, which significantly stimulated plant growth as estimated by the length of roots and the dry weight of the whole plant. Similarly, plant height and fresh weight, leaf dry weight and the number of fruits per plant were also improved in cultivated tomato using various species of rhizospheric and/or plant-associated bacteria belonging to *Pseudomonas putida*, *P. fluorescens*, *S. marcescens*, *B. amyloliquefaciens*, *B. subtilis*, and *B. cereus* (Almaghrabi et al. 2013). Furthermore, *S. marcescens* B2, applied

to cyclamen plants inoculated with sclerotia of *Rhizoctonia solani* and/or conidia of *F. oxysporum* f. sp. *cyclaminis*, was found to be effective in suppressing both diseases induced by these pathogens (Someya et al. 2000).

In the current study, *Serratia* sp. C4, evaluated in vitro for its antagonistic potential toward FOL, had reduced pathogen mycelial growth and formed an inhibition zone. Similar effects were reported by Patel et al. (2012) using endophytic bacteria from cultivated tomato, identified as *Pseudomonas aeruginosa* str. HR7, for *F. oxysporum* biocontrol. The cell-free culture filtrate of *Serratia* sp. C4, from 3-day-old culture, tested in the current study had also significantly reduced FOL growth compared to control even if heated at 50, 100°C for 15 min and/or unheated. However, the antifungal potential of *Serratia* sp. C4 cell-free culture filtrate significantly declined, as compared to control filtrate, after heating at 100°C for 15 min. In the same way, antagonistic potential of culture filtrate from *Streptomyces hygroscopicus* toward *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* declined heating at 100°C for 45 min (Prapagdee et al. 2008). However, the antifungal activity of culture filtrate from *Bulkhorderia cepacia* toward *C. gloeosporioides* was similar to that expressed by the untreated filtrate even if heated at 50 or 100°C and autoclaved at 121°C for 20 min (Kadir et al. 2008).

In this study, *Serratia* sp. C4 cell-free culture filtrate was also subjected to an extraction with chloroform and *n*-butanol to elucidate the antifungal potential of its extracellular metabolites. Results showed that both organic extracts from *Serratia* sp. C4, used at 1 mg/ml, had reduced FOL growth by 27.6 to 66.5%, compared to ethanol control. Biologically active metabolites extracted with ethyl acetate, methanol and chloroform from *Serratia* sp., isolated from the coralline red algae *Amphiroa anceps*, inhibited the mycelial growth of several pathogenic bacteria and fungi (Karkachi et al. 2010). The chloroform-methanol and diethyl ether extracts from *S. marcescens* S10, isolated from the gut of the insect American cockroach showed antimicrobial potential toward various bacterial and fungal agents namely *Lesteria* spp., *Salmonella* spp., *Klebsiella* spp., *Staphylococcus aureus*, *C. albicans, Aspergillus niger*, and *Geotricum* spp. (Ahmed and Hassen 2013).

Chloroform and *n*-butanol extracts from *Serratia* sp. C4 were found to be more effective at the concentration of 5 than at 2.5% (v/v). The highest antifungal potential toward FOL growth (66.5%) was achieved using the *n*-butanol extract from the tested isolate used at 5% (v/v) versus the ethanol control and the two commercial products used i.e. Bavistin® (50%, carbendazim) and Bactospeine® (16000UI/mg, biopesticide). These results are in agreement, in part, with Aydi Ben Abdallah et al. (2015) findings using chloroform and *n*-butanol extracts from six endophytic *Bacillus* spp. isolates, recovered from wild Solanaceae plants (namely *D. metel*, *S. nigrum*, *S. elaeagnifolium* and *N. glauca*), where the two types of organic extracts from *Bacillus* spp. exhibited an interesting antifungal potential toward FOL which was higher than that induced by the same two commercial products used in the present study whatever the concentrations used. Furthermore, chloroform and *n*-butanol extracts from *Bacillus* spp. were found to be more effective at the concentration of 5 than at 2.5% (v/v) toward FOL (Aydi Ben Abdallah et al. 2015).

*Serratia* sp. C4 was elucidated in vitro for its properties deployed in the observed antifungal effect. In fact, this isolate was shown able to produce chitinase, protease and pectinase enzymes as shown on chitin-, milk-, and pectin-agar media, respectively. Several genera of endophytic bacteria such as *Serratia, Bacillus, Burkholderia*, *Acinetobacter*, *Pseudomonas*, *Enterobacter*, *Stenotrophomonas*, *Micrococcus,* and *Microbacteruim* were found able to produce chitinase, protease and β-1,3-glucanase involved in cell-wall degradation of various pathogens (Berg et al. 2005; Bibi et al. 2012). Moreover, pectinase and cellulase were previously reported by Hallmann et al. (1997) as essential enzymes for colonization of plant tissues. These enzymes may be also involved in the enhancement of tomato growth (Baldan et al. 2003). As metabolites involved in the recorded increment of tomato growth relative to the untreated control, in the present study, the selected isolate *Serratia* sp. C4 was found able to produce indole-3-acetic acid (IAA). The IAA amount released by our isolate *Serratia* sp. C4 (29.52 µg/ml, after 48 h of incubation) is interestingly higher when compared to 11.1 µg/ml produced by *S. marcescens* SRM, recovered from flowers of *Cucurbita pepo*, in Selvakumar et al. (2008b) study but lower than the amount produced by *Serratia nematodiphila* (58.9 µg/ml) isolated from forest soil (Dastager et al. 2011).

Phosphate solubilization ability was also assessed, in this study, and confirmed for *Serratia* sp. C4. Ngamau et al. (2012) study revealed that endophytic bacteria such as *Pseudomonas* spp., *Serratia* spp., *Enterobacter asburiae*, *Rahnella aquatilis*, *Ewingella americana*, and *Yokenella regensburgei* were able to solubilize the phosphate. Furthermore, plant growth promotion attributes such as phosphate solubilization, IAA production, hydrogen cyanide production and N-fixation were found in *S. marcescens* KR-4 recovered from *Pueraria thunbergiana* (Selvakumar et al. 2008a), *S. marcescens* KiSII isolated from the rhizosphere of coconut palms (George et al. 2013) and *S. nematodiphila* issued from forest soil (Dastager et al. 2011). The phosphatase activity of our endophytic isolate *Serratia* sp. C4 was indicated by the presence of clear zone of about 10.33 mm. Phosphate solubilization ability of unidentified endophytic bacterial isolates and *P. aeruginosa* HR7, recovered from tomato plants, was also expressed by the formation of a clear zone of about 8 to 31 mm around their colonies (Patel et al. 2012).

The chemical identification of *Serratia* sp. C4 extracellular metabolites separated in the different organic extracts tested will give additional information on the nature of bioactive molecules involved in the recorded disease-suppressive potential of this endophytic isolate.


**RESUME**

**Aydi Ben Abdallah R., Mejdoub-Trabelsi B., Nefzi A., Jabnoun-KhiareddineH. et Daami-Remadi, M. 2017. Utilisation de bactéries endophytes naturellement associées à *Cestrum nocturnum* pour la lutte biologique contre la flétrissure fusarienne et l'amélioration de la croissance de la tomate. Tunisian Journal of Plant Protection 12: 15-40.**

Trois isolats bactériens endophytes, isolés à partir des feuilles et des tiges de *Cestrum nocturnum* (jasmin de nuit), ont été évalués pour leur aptitude à supprimer la fusariose vasculaire, causée par

*Fusarium oxysporum* f. sp. *lycopersici* (FOL), et à améliorer la croissance des plants de tomate. Les isolats testés ont significativement diminué la sévérité de la maladie de 46,6 à 97,7% par rapport au témoin inoculé par FOL et non traité. L'isolat C4 s'est révélé le plus efficace dans la réduction des altérations foliaires de 86,6% et de la hauteur du brunissement vasculaire de 97,7% par rapport au témoin inoculé par FOL et non traité. Une augmentation significative, de 39 à 41,6% par rapport au témoin inoculé par le pathogène et non traité, a été enregistrée au niveau des paramètres de croissance de la tomate. De plus, l'isolat C4 a significativement augmenté les paramètres de croissance de 24,5-53,3% par rapport aux plants témoins non inoculés par le pathogène et non traités. Cet isolat a été morphologiquement et biochimiquement caractérisé et identifié en utilisant le séquençage du gène 16S ADNr comme *Serratia* sp. (KX197201). Criblé *in vitro* pour son activité antifongique contre FOL, *Serratia* sp.C4 a induit une diminution de 19,52% de la croissance radiale du pathogène et la formation d'une zone d'inhibition de 8,62 mm de diamètre. Le filtrat de culture de *Serratia* sp. C4, additionné au milieu PDA à raison de 20% (v/v), a réduit la croissance radiale du pathogène de 23% comparé aux 21,7 et 9,2% enregistrés après son chauffage à 50 et 100°C, respectivement. L'extrait *n*-butanolique de *Serratia* sp. C4, appliqué à 5% (v/v), a présenté un potentiel antifongique contre FOL traduit par une inhibition de la croissance de 66,5% par rapport au témoin non traité et qui a été supérieure à celle obtenue moyennant deux pesticides commerciaux, à savoir Bavistin® (carbendazime à 50%, fongicide chimique) et Bactospeine® (16000 UI/mg, biopesticide à base de *Bacillus thuringiensis*). *Serratia* sp. C4 s'est montré un agent producteur de chitinase, de pectinase et de protéase et capable de produire l'acide indole-3-acétique et de solubiliser le phosphate.

*Mots clés*: Activité antifongique, bactéries endophytes, *Cestrum nocturnum*, *Fusarium oxysporum* f. sp. *lycopersici*, métabolites secondaires, promotion de la croissance, tomate

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**ملخص**

**العايدي-بن عبد الله، رانية وبثينة مجدوب-طرابلسي وأحلام النفزي وهيفاء جبنون-خيار الدين، و ماجدة الدعمي-الرمادي. 2017. استخدام البكتيريا الداخلية المرتبطة طبيعيا بنبتة مسك الليل(*Cestrum nocturnum*) للمكافحة الحيوية للذبول الفوزاري وتحسين نمو الطماطم. Tunisian Journal of Plant Protection 12: 15-40.**

تمّ تقييم قدرة ثلاث عزلات بكتيرية داخلية نباتية، عزلت من أوراق وسيقان مسك الليل(*Cestrum nocturnum*)، على الحدّ من الذبول الفوزاري للطماطم الناتج عن الفطر *Fusarium oxysporum* f. sp. *lycopersici* (FOL) وعلى تحسين نموّ نباتات الطماطم. خفّضت العزلات بشكل ملحوظ من شدة المرض بنسبة تراوحت بين 46,6 و 97,7% مقارنة مع الشاهد الملقّح بـ FOL والغير معامل. تم انتقاء العزلة C4 بصفتها الأكثر فعالية في الحدّ من إتلاف الأوراق بـ 86.6% ومن ارتفاع اسمرار الأوعية بـ 97.7% مقارنة مع الشاهد الملقّح بـ FOL والغير معامل. سجّلت زيادة معتبرة، من 39 إلى41.6 %، مقارنة مع الشاهد الخالي من العنصر المسبب للمرض والغير معامل، في معالم نمو الطماطم. إضافة على ذلك، مكّنت العزلة C4 من الزيادة في معالم النمو بنسبة 24.5إلى53.3%مقارنة مع النباتات الشواهد الغير ملقّحة والغير معاملة. تمّ تشخيص هذه العزلة ماكرومورفولوجيا وبيوكيميائيا وتمّ تحديد انتمائها باستخدام تسلسل جينات الحمض النووي 16 S إلى نوع من الجنس البكتيري *Serratia* sp. (KX197201). عند اختبار الفعاليّة المضادة في المخبر ضد FOL، تمكّنت *Serratia* sp. C4 من التخفيض من نمو العنصر المسبب للمرض بـ 19.52% ومن تشكيل منطقة التثبيط بـ 8.62 مم من قبس القطر. وتمكنت الرّواشح الزراعية لـ *Serratia* sp. C4، المضافة للوسط الإنمائي PDA بنسبة 20% (سعة/سعة)، من التخفيض من نمو العنصر المسبب للمرض بنسبة 23% مقارنة مع 21.7و 9.2% المسجلة بعد التسخين في 50 و 100°س، على التوالي. أظهر مستخلص ن-بيوتانول لـ *Serratia* sp. C4، عند استعماله لجرعة بنسبة 5% (سعة/سعة)، فعالية مضادة ضد FOL بالحدّ من النمو بـ66.5%، مقارنة مع الشاهد الغير معامل، والتي كانت أعلى من تلك التي تم الحصول عليها باستخدام اثنين من المبيدات التجارية Bavistin® (Carbendazim 50%، مضاد فطري كيميائي) وBactospeine® (16000 UI/مغ، مبيد حيوي متكون من *Bacillus thuringiensis*). تميزت *Serratia* sp. C4 كذلك كمنتج للكيوتيناز والبيكتيناز والبروتاييناز والقادر على إنتاج حمض الأندول-3-أساتيك وعلى إذابة الفوسفور.

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# Impact of Aphids and Host Weeds Interaction on the Dissemination of *Potato Virus* $Y^N$ Strains

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**ABSTRACT**

**Boukhris-Bouhachem, S., Ben Fekih, I., Rouzé-Jouan, J., Souissi, R., and Hullé, M. 2017. Impact of aphids and host weeds interaction on the dissemination of *Potato virus* $Y^N$ strains. Tunisian Journal of Plant Protection 12: 41-48.**

Weeds and volunteer plants susceptible to *Potato virus Y* (PVY) infection in different seed potato production sites were investigated in this study. Aphids occurring within these plants and identified as *Aphis fabae, A. gossypii*, and *Myzus persicae* were studied for possible interaction occurring between vectors and plant reservoirs of PVY. Out of 772 plants belonging to 12 different families (Solanaceae, Amaranthaceae, Chenopodiaceae, Papaveraceae, Urticaceae, Convolvulaceae, Asteraceae, Polygonaceae, Euphorbiaceae, Brassicaceae, Portulacaceae, and Compositae), 337 were found to be infected by $PVY^N$ based on DAS-ELISA technique. Among these plants, *Solanum elaeagnifolium*, *Datura stramonium* and *Sonchus oleraceus* were found to be infected with the strain $PVY^{NTN}$. In addition to these reported weeds, *S. nigrum* seems to be an important host for $PVY^N$ since this plant hosts aphid vectors. This investigation provides basic information about weeds and volunteer plants infected with $PVY^N$ and aphid vectors. Such finding will increase knowledge of the $PVY^N$ epidemiology in potato fields and consequently the possible management of this viral disease.

*Keywords*: Aphids, potato, $PVY^N$, $PVY^{NTN}$, volunteer plants, weeds.

*Potato virus Y* (PVY) of the family Potyviridae (Potyvirus) is one of the most economically important viruses infecting various host plants of different families such as the Solanaceae, Chenopodiaceae, Amaranthaceae, Euphorbiaceae, Fabaceae, and Brassicaceae. Several studies reported PVY infection in weeds.

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For example, weeds within *Solanaceae* and *Portulaca oleracea* were found to be a reservoir for PVY in France (Marchoux et al. 1976). In Argentina, PVY infection was found on *Physalis viscose* and *Solanum atriplicifolium* (Pontis and Feldman 1963). Later, a study from Israel reported that both *S. villosum* and *Hyoscyamus desertorum* were infected with PVY (Ucko et al. 1998). In Tunisia, the occurrence of the perennial plant, the silver nightshade, *Solanum elaeagnifolium* was reported (Mekki 2005) and registered as PVY host plant

(Boukhris-Bouhachem et al. 2007; Edwardson and Christie 1997).

Aphids (Hemiptera: Aphididae) are serious insect pest playing a key role in the establishment and the dissemination of plant viruses. More than 73 species of aphids, including colonizer and non-colonizer of potato crops, are known to transmit PVY through a non-persistent manner (De Bokx and Piron, 1990; Nanayakkara et al. 2012; Perez et al. 1995; Varveri 2000). Among the reported aphids, *Myzus persicae* was recognized as the most efficient vector of $PVY^{NTN}$ the most abundant strain in Tunisia (Boukhris-Bouhachem et al. 2011; Mello et al. 2011; Verbeek et al. 2010). At spring season, winged aphids migrate to reach new secondary host plants such as potato. During this process, aphids may acquire PVY virions from infected weeds or volunteer plants which are considered as plant reservoirs. Such situation enhances the establishment and the increased dissemination of $PVY^{NTN}$ which is the most abundant strain in Tunisian potato seed-producing fields.

Tunisian potato seeds production is an important sector often threatened by virus contaminations leading sometimes to 12% declassification of potato seed-producing areas (Boukhris-Bouhachem et al. 2015). This occurs when the virus infection level in surveyed fields exceeds 5%. The aim of this study is to highlight the potential host plant, weeds or crops, which may play a role as sources of $PVY^{N}$. Such finding will enhance knowledge about the dissemination of $PVY^{N}$ in potato seed-producing fields.

## MATERIALS AND METHODS

### Plant sampling.

Sampling was carried out in four study areas (Fig. 1) namely Batan (Manouba), Sidi Mahmoud (Kairouan), Douala (Cap Bon), and Bousalem (Jendouba). Intensive sampling was performed during spring time from 2002 to 2007 in the site of Sidi Mahmoud and Douala. Occasionally, the investigation involved years 2013 to 2015. In all, 772 weed samples belonging to 12 different families (cultivated and volunteer Solanaceae, Amarantaceae, Chenopodiaceae, Papaveraceae, Urticaceae, Convolvulaceae, Asteraceae, Polygonaceae, Euphorbiaceae, Brassicaceae, Portulacaceae, and Asteraceae).

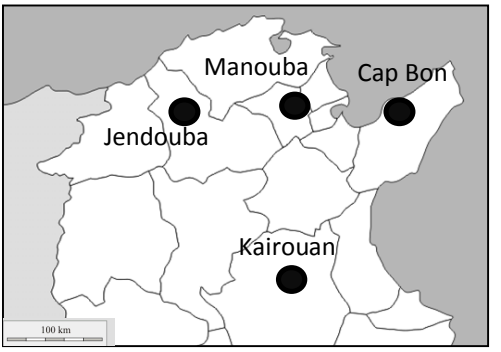


**Fig. 1.** Sites prospected for the survey of virus infections

**Aphid identification**

Aphids (alatae and aptera) were sampled randomly from weeds and volunteer plants occurring in potato fields. Aphid identification was done following both keys by Blackman and Eastop (2001) and Remaudière and Seco (1990).

**Detection and characterization of PVY**

All samples were tested by DAS-ELISA as described by Clark and Adams (1977), using a polyclonal anti-PVY (Bioreba) and anti-$PVY^N$ serotype (Bioreba and INRA/FNPPPT). Molecular tests were only conducted on $PVY^N$ positive samples from 11 *S. elaeagnifolium*, 6 *Datura stramonium* and 9 *Sonchus oleraceus* plants, considered as new host plants for $PVY^N$ in Tunisia. Samples were grinded in extracted buffer of the ELISA kit (Bioreba), 100 µl were placed in the microplate. $PVY^{NTN}$ strains detection was carried out by Immunocapture-RT-PCR. The reverse primer used for first-strand DNA synthesis (3'NTRC) was the same oligonucleotide used by Glais et al. (1998). The strain identity was based on the polymorphism in 5'NTR/P1 region amplified by specific primers (Glais et al. 2001). The Tunisian isolate $PVY^{NTN}$ C1-3 maintained in the laboratory was used as a positive control. Amplified products were separated by electrophoresis on 1.5 agarose gels, stained with ethidium bromide and observed under UV.

**RESULTS**

**Aphid identification.**

Overall, four aphid species were encountered in this study namely *M. persicae, Aphis gossypii, A.* fabae, and *Hyperomyzus lactucae*. They were encountered on various weeds where *M. persicae* was observed on *S. elaeagnifolium*, *D. stramonium* and *C. arvensis*; *A. gossypii* was detected on *S. elaeagnifolium*; *A. fabae* was associated to *S. elaeagnifolium*, *S. nigrum*, and *A. retroflexus*; and *H. lactucae* was noted on *S. oleranceus*.

**Virus detection.**

From a total of 772 sampled plants, 337 were recorded to be infected with $PVY^N$ (Table 1). A wide range of weeds from different families such as *S. elaeagnifolium*, *S. nigrum*, *S. oleraceus*, *Chenopodium album*, *Amaranthus hybridus*, *D. stramonium*, and *Convolvulus arvensis* have shown $PVY^N$ infections. From Solanaceae family, 43.65% of weeds and volunteer plants were registered to be infected. A rate of 39.82% of *S. elaeagnifolium*, 93.33% of *D. stramonium* and 41.86% of *S. nigrum* were positive to $PVY^N$ infection. *S. nigrum* and *D. stramonium* were the most frequently positive plants in Douala, while *S. elaeagnifolium* were mainly infected in Batan and Sidi Mahmoud. Two weeds belonging to Amaranthaceae were also positive for $PVY^N$ infection. In fact, *Amaranthus* sp. was found positive to virus infection in all prospected sites while *A. retroflexus* was reported only in Douala and Batan. For *S. oleraceus*, samples were positive to $PVY^N$ infection at the rates 13.63, 14.28 and 8.33% in the regions of Batan, Sidi Mahmoud, and Douala, respectively. It is necessary to highlight that this is the first report of $PVY^N$ infection on *S. oleraceus* in Tunisia. Potato volunteer plants were also infected with $PVY^N$ at the level of 74.2 and 85.44% in Batan and Douala, respectively.

Regarding the molecular investigations of the three tested weeds, *S. elaeagnifolium*, *D. stramonium* and *S. oleraceus*, the results showed that all samples were infected with $PVY^{NTN}$ (Fig. 1).

**Table 1.** List of weeds and plant volunteer showing a PVY infection according to DAS-ELISA, 2002-2015

| Sampling site | | Batan | Bousalem | Sidi Mahmoud | Douala | PVY infection[N] |
|---|---|---|---|---|---|---|
| **Weeds** | | N+/N | N+/N | N+/N | N+/N | |
| Amaranthaceae | *Amaranthus* sp. | 4+/19 | 3+/7 | 2+/6 | 2+/16 | 11+/48 |
| | *Amaranthus retroflexus* | 2+/17 | - | - | 1+/10 | 3+/27 |
| | *Amaranthus hybridus* | 1+/1 | - | - | - | 1+/1 |
| Chenopodiaceae | *Chenopodium album* | 7+/30 | 0+/1 | 8+/57 | 1+/5 | 16+/93 |
| | *Chenopodium amaranticolor* | 1+/5 | - | - | 0+/1 | 1+/6 |
| | *Beta vulgaris* | 4+/9 | 0+/1 | - | - | 4/+10 |
| Convolvulaceae | *Convolvulus arvensis* | 9+/14 | 3+/12 | 18+/29 | 6+/7 | 36+/62 |
| Portulacaceae | *Portulacaoleracea* | - | - | - | 1+/1 | 1+/1 |
| Solanaceae | *Solanum nigrum* | 6+/17 | -- | 2+/4 | 10+/22 | 18+/43 |
| | *Solanum elaeagnifolium* | 7+/13 | 1+/5 | 37+/95 | - | 45+/113 |
| | *Datura stramonium* | 1+/1 | - | - | 13+/14 | 14+/15 |
| | *Whithenia somnifera* | - | - | 3+/16 | - | 3+/16 |
| Compositae | *Sonchus oleraceus* | 9+/66 | 0+/7 | 1+/7 | 1+/12 | 11+/92 |
| Papaveraceae | *Papaver rhoeas* | - | 0+/1 | - | - | 0+/1 |
| Polygonaceae | *Emex spinosa* | 0+/2 | - | - | - | 0+/2 |
| Urticacae | *Urtica urens* | 1+/2 | - | 0+/3 | 1+/2 | 2+/7 |
| Asteraceae | *Calendula arvensis* | - | 0+/1 | - | - | 0+/1 |
| | *Erigeron* sp. | - | - | - | 1+/1 | 1+/1 |
| | *Picris echioides* | - | - | 0+/1 | - | 0+/1 |
| | *Xanthium* sp. | - | - | 1+/1 | - | 1+/1 |
| Brassicaceae | *Sinapis arvensis* | - | 0+/1 | - | - | 0+/1 |
| Euphorbiaceae | *Euphorbia* sp. | - | - | - | 1+/13 | 1+/13 |
| **Volunteer solanaceous plants** | | | | | | |
| Tomato | *Lycopersicum esculentum* | - | - | 4+/11 | 2+/5 | 6+/16 |
| Pepper | *Capsicum annuum* | - | - | 4+/9 | 1+/6 | 5+/15 |
| Potato | *Solanum tuberosum* | 69+/93 | - | - | 88+/103 | 157+/196 |
| **Total** | | **121+/279** | **7+/36** | **80+/239** | **129/218** | **337+/772** |

N: Number of positive plants (+); Nt: Total number of tested plants

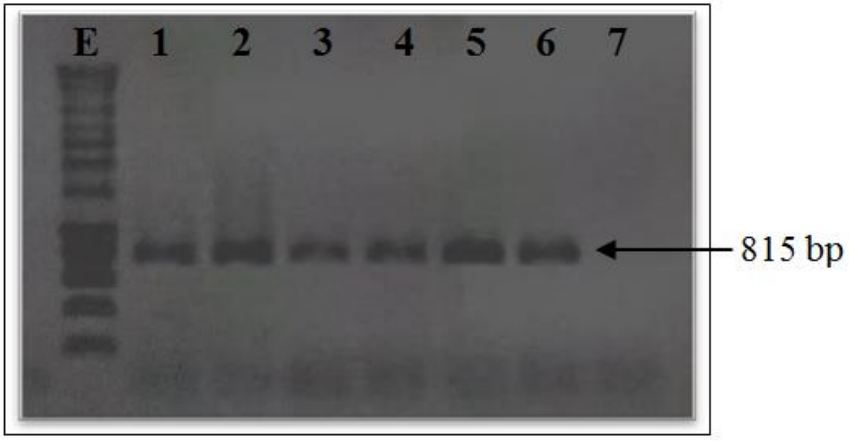


**Fig. 1.** Amplification of P1 region by RT-PCR.E: Smart Ladder (100 bp, EuroGentec); 1: PVY$^{NTN}$-C1-3, 2-3: *Solanum elaeagnifolium*; 4-5: *S. oleraceus*; 6: *Datura stramonium*; 7: Negative control.

## DISCUSSION

A screening of virus plant reservoirs was performed during 9 years in four Tunisian regions. Our study provides a list of weeds belonging to 25 species and 12 families that host $PVY^N$ infection. Aphid identification on infected weeds revealed the high occurrence of *M. persicae*, qualified as the most efficient vectors of $PVY^N$. All identified aphids on weeds were previously reported to be present on potato leaves (Boukhris-Bouhachem et al. 2011), and could be therefore involved in the secondary infection cycle.

Based on our study, 39.82% samples of *S. elaeagnifolium*, recently introduced in Tunisia, were infected with $PVY^N$. Except for Douala, *S. elaeagnifolium* infected with $PVY^N$ were registered in all prospected areas. The investigation of aphid species occurring on *S. elaeagnifolium* revealed the presence of *M. persicae*, *A. gossypii*, and *A. fabae*. These aphids have been previously reported to be vectors of $PVY^{NTN}$ in potato fields with a transmission rate estimated at 95, 82 and 43% for *M. persicae*, *A. gossypii* and *A. fabae*, respectively (Boukhris-Bouhachem et al. 2011). Our results showed that infection of the silver nightshade with $PVY^N$ was often detected from Sidi Mahmoudas reported by Boukhris-Bouhachem et al. (2007). Features such as the abundance of this weed, its ability to survive during the winter, the high infection rate within samples, and the presence of aphids suggest the silver nightshade as a plant reservoir for $PVY^N$.

Previous studies have reported that several aphid species, including *M. persicae* and *A. gossypii*, play an important role in the transmission of viruses such as *Alfalfa Mosaic Virus* (AMV), *Cucumber Mosaic Virus* (CMV) and PVY by feeding on these weeds (Graham et al. 1979; Harris et al. 2001). Another study reported the high incidence of $PVY^N$ in potato seed-producing fields has led to the decline of this crop (Boukhris-Bouhachem et al. 2015). In fact, despite the regular management of these plots (early removal of diseased plants, chemical treatments, haulm destruction ...), this situation occurred in Sidi Mahmoud where *S. elaeagnifolium* was relatively important (Boukhris-Bouhachem 2007). This observation may suggest that the occurrence of *S. elaeagnifolium* in the agricultural sites of the region of Sidi Mahmoud were the source of infection of potato fields by aphid vectors of $PVY^N$.

In Douala, the important frequency of $PVY^N$ infection revealed on both *S. nigrum* and *D. stramonium* and the occurrence of aphid vectors may increase more the impact of these plants as potential alternative hosts for virus dissemination. In comparison to the four studied regions, Bousalem showed the lowest $PVY^N$ infection on weeds.

The high levels of $PVY^N$ infection detected in plant reservoirs proved their important impact for the certified potato seed-producing crops. Agronomic and environmental context such as the proximity often observed in the same area of potato crops for commercial and seed production and the presence of other solanaceous crops such as pepper, commonly infected with PVY virus (Gorsane et al. 1999) and the occurrence of weeds provide an unfavorable environment for the production of potato seeds. Indeed, this situation preserves the virus inocula in the fields which may be a risk of PVY infection in potato crops. However, little is known about the possible cross transmission of $PVY^N$ strains between pepper, tomato, and potato. Some $PVY^N$ isolates from tomato are similar to $PVY^C$ strain infection on

potato or to isolates of $PVY^{NTN}$ variant, which may induce necrotic systemic infections under laboratory conditions (Moury et al. 2007). In this study, $PVY^{NTN}$ infection was reported for the first time on *S. elaeagnifolium*, *D. stramonium* and *S. oleraceus* in Tunisia. This finding is not a surprise and was already reported by Boukhris-Bouhachem et al. (2010) where $PVY^{NTN}$ was reported as the most abundant variant on potato seed-producing regions. This situation makes the eradication of these weeds, an important management to control $PVY^{NTN}$ propagation. However, it is imperative to highlight that not all infected weeds may contribute in $PVY^{N}$ dissemination. Therefore, additional assays should be performed to study the role of these infected weeds in $PVY^{N}$ dynamics.

The investigated weeds were shown to be a virus source since they may cumulate their effects and to be a risk for the sanitary quality of potato seeds. Under these conditions, it becomes important to inform and increase knowledge of farmers about these plant reservoirs and to build a strategy to discard them from the field. Treatment against aphids that are known to be $PVY^{N}$ vectors should be also conducted since they play important role in the virus infection establishment. As alternatives to chemical insecticides, the use of mineral oils (Boukhris-Bouhachem et al. 2015) or plant essential oils against insect pest seems to hold a promising future within integrated pest management concept (Isman 2000). In addition, it will be necessary as prevention measurements to search for new potato seed-producing areas far from the regions recognized to hold a risk for virus infection.

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**RESUME**

**Boukhris-Bouhachem S., Ben Fekih I., Rouzé-Jouan J., Souissi R. et Hullé, M. 2017. Impact de l'interaction pucerons-adventices hôtes dans la dissémination des souches du $PVY^{N}$. Tunisian Journal of Plant Protection 12: 41-48.**

Dans le contexte d'une étude épidémiologique du virus Y de la pomme de terre (PVY), une recherche des plantes adventices susceptibles d'héberger ce virus a été réalisée. Ces plantes peuvent être des sources d'infection par le PVY par le biais des pucerons vecteurs pour les plants prévus à la production de semences. En effet, trois espèces de pucerons ont été identifiées sur ces adventices dont *Aphis fabae, A. gossypii* et *Myzus persicae*. Ceci a permis d'étudier l'interaction possible entre les vecteurs et les réservoirs du $PVY^{N}$. Sur un total de 772 plantes appartenant à 12 familles différentes (*Solanaceae, Amaranthaceae, Chenopodiaceae, Papaveraceae, Urticaceae, Convolvulaceae, Asteraceae, Polygonaceae, Euphorbiaceae, Brassicaceae, Portulacaceae* et *Compositae*), 337 plantes se sont révélées infectées par le $PVY^{N}$ moyennant la technique DAS-ELISA. Parmi ces plantes, on cite *Solanum elaeagnifolium*, *Datura stramonium* et *Sonchus oleraceus* qui sont infectées par la souche $PVY^{NTN}$. En outre, ces trois adventices en plus de *S. nigrum* semblent être des plantes hôtes importantes du $PVY^{N}$ vu qu'elles sont aussi infestées par les pucerons vecteurs. Ces informations de base sur les mauvaises herbes et les repousses qui hébergent le $PVY^{N}$ et les pucerons vecteurs permettraient de mieux connaître l'épidémiologie du $PVY^{N}$ dans les champs de pomme de terre et de trouver les méthodes appropriées pour gérer cette maladie virale.

*Mots clés*: Adventices, pomme de terre, puceron, $PVY^{N}$, $PVY^{NTN}$, repousses

## ملخص

**بوخريص-بوهاشم، سنية وابتسام بن فقيه وجويل روزي-خوان ورابحة السويسي وموريس هولاي. 2017. تأثير تفاعل حشرات المن والأعشاب الضارة العائلة في انتشار سلالات فيروس $PVY^N$.**
**Tunisian Journal of Plant Protection 12: 41-48.**

في نطاق الأبحاث حول فيروس البطاطا (PVY) قمنا بدراسة حول الأعشاب الضارة العائلة والنبات المتطوع العائل اللذان يمكن أن يكونا مصدرا لإصابة بذور البطاطا وانتشار فيروس PVY بواسطة حشرات المن *Aphisfabae* و*Aphisgossypii* و*Myzuspersicae*. تبين أن من بين 772 عينة من الأعشاب الضارة التي تم اختبارها بالاعتماد على تقنية الأمصال (DAS-ELISA)، 337 عينة كانت تحمل الفيروس $PVY^N$. تنتمي النباتات المذكورة إلى 12 عائلة مختلفة هي Solanaceae, Amaranthaceae, Chenopodiaceae, Papaveraceae, Urticaceae, Convolvulaceae, Asteraceae Polygonaceae, Euphorbiaceae, Brassicaceae, Portulacaceae, Compositae. ومن أهم هذه النباتات نذكر *Solanum elaeagnifolium* و*Datura stramonium* و *Sonchus oleraceus* وهي تحمل سلالة الفيروس $PVY^{NTN}$. إذن، تلعب الأعشاب دورا هاما في انتشار الفيروس $PVY^N$ على بذور البطاطا خاصة وأن أنواع حشرات المن الناقلة للفيروس تتكاثر عليها. لذا، يتعين على مكثري البذور القضاء على هذه الأعشاب لتحسين الحالة الصحية للبذور ومقاومة هذا المرض.



*كلمات مفتاحية*:أعشاب الضارة،بطاطا، نبات متطوع،منّ، $PVY^{NTN}$، $PVY^N$


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# Insecticidal Activity Assessment of *Thymus capitatus* Essential Oils in Combination with Natural Abrasives against *Myzus persicae*

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**ABSTRACT**

**Khaled, W., Ben Fekih, I., Chaieb, I., Souissi, R., Harbaoui, I., and Boukhris-Bouhachem, S. 2017. Insecticidal activity assessment of *Thymus capitatus* essential oils in combination with natural abrasives against *Myzus persicae.* Tunisian Journal of Plant protection 12: 49-59.**

This study was performed to evaluate the insecticidal activity of *Thymus capitatus* essential oils and two natural abrasives to control *Myzus persicae*. The in vitro application of thyme essential oils showed a significant toxic effect by fumigation as well as by spraying. The $LC_{50}$ for both application methods recorded after 24 h were about 20.01 and 13.26 µl/l air, respectively. In addition, in vivo experiment based on bioinsecticide formulation ($LC_{50}$ thyme essential oils + kaolin or diatomaceous earth) were carried out. The mortality rates registered after 24 h were 74.19 and 97.84% for the combination with kaolin and diatomaceous earth, respectively. Meanwhile, emulsions with 1 µl of these oils have been tested on the target aphid. This treatment has lead after 24 h to a mortality rate of 55.55%. The mechanical effect of both abrasive powders has been highlighted through dehydration, shrinkage and deformation of the aphid cuticle. Interestingly, the combination of diatomaceous earth with the *T. capitatus* essential oils was significantly the most effective to control aphid populations.

*Keywords*: Diatomaceous earth, essential oils, kaolin, *Myzus persicae*, *Thymus capitatus*

Since 1940's, chemical pesticides have been excessively used within the green revolution concept for the development of an intensive agriculture (Davies 2003). Previous studies have shown that the intensive use of chemical insecticides has created several negative effects on non-targeted organisms and environment, which may affect human health (Davies 2003; Dich et al. 1997). In addition, resistance phenomena among pest insects have been severely increased to different chemical groups (Charaabi et al. 2008; 2016; Robert 2013). The use of

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essential oils as biopesticides has shown to hold a promising future as one of the alternatives to chemical insecticides (Pavela et al. 2010). Indeed, previous reports have highlighted the potential of the essential oils and their derivates as agents to control pest insects under the Integrated Pest Management (IPM) concept (Bachrouch et al. 2010; Chaieb 2011; Mediouni Ben Jemâa et al. 2012; Regnault-Roger 1997). The importance of the essential oil compounds was also shown by their ability to be more promptly degraded in the environment than synthetic chemicals (Kalita et al. 2013). Fumigant and contact toxic potential of essential oils have been reported against different biological stages of pest insects (adults, larvae and eggs) (Sahaf and Moharramipour 2008). In addition to their anti-feeding and repelling activities (Hernández-Lambraño et al. 2014), essential oils may inhibit reproduction, affect larvae development and adult emergence (Ayvaz et al. 2010). Despite the noteworthy potential of essential oils, their applications as biopesticides remain challenging in practice due to their volatility, poor water solubility, and aptitude for oxidation (Keita et al. 2001). Beside essential oils, mineral powders were used successfully to control insect populations (Nguemtchouin et al. 2010). Pure kaolin (i.e clay powder) is recognized by some traditional societies for its ability to control insects. Previous study showed the important insecticidal activity of the combined effect of these flavored powders with essential oils against *Callosbruchus maculatus* (Awojide and Fayemiwo 2010).

The green peach aphid, *Myzus persicae* (Hemiptera, Aphididae), is one of the most economically important aphids infesting a wide range of host plants (Charaabi et al. 2016). *M. persicae* is a cosmopolitan species causing direct damage as phloem feeders and indirect damage as vectors of several viruses (Boukhris-Bouhachem et al. 2007). The aim of this work is to investigate the insecticidal effects of *Thymus capitatus* essential oils against *M. persicae*. Moreover, this study inspects the biocidal activity of two natural abrasives (kaolin and diatomaceous earth) in combination with thyme essential oils.

## MATERIALS AND METHODS

### Plant material and essential oil extraction.

The spontaneous aromatic medicinal plant, *Thymus capitatus* (Lamiaceae), was used as plant material for the extraction of the essential oils. *T. capitatus* was gathered at the vegetative stage from the Kef region (N: 36°11′, E: 8°42′), in the north-west of Tunisia. Sampling was performed in June 2014. Freshly harvested leaves were left in the shade in a dry and aerated place for 4 weeks. The dried materials were grounded to a fine powder used for the extraction of essential oils. Then, 100 g dried-leaves were placed with 1000 ml of distilled water in a round bottom flask and submitted for hydrodistillation for 2 to 3 h in Clevenger-type apparatus. The essential oils were driven by water vapor. This gas mixture was condensed by the condenser and separated into two liquid phases; the higher organic phase was the essential oils, containing the majority of the odorous compounds and the lower aqueous phase, containing aromatic water. The essential oils samples were then stored in sterile glass bottles at 4°C. Essential oils yield was calculated using the following formula: $Y = (wo/wp) \times 100$, where Y: Essential oils yield, wo: Weight of essential oils, and wp: Dry weight of plant (Akrout 2004).

**Chemical composition.**

The chemical composition of the essential oils was analyzed by gas chromatography/mass spectrometry (GC/MS). The chromatographic analysis was carried out with a Hewlett-Packard type gas chromatograph (HP 6890) coupled to a mass spectrometer (HP 5973). The fragmentation was carried out by electronic impact at 70eV, by using an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness). The temperature of the column was set from 50 to 250°C at a rate of 4°C/min. The injected volume was 0.2 µl with a split ratio of 1:70. The device was connected to a computer system managing a NIST 98 mass spectrum library and piloted by "HP ChemStation" software to monitor the evolution of chromatographic analyses.

***M. persicae* rearing.**

Sampling of *M. persicae* was performed from potato crops in the Cap Bon region: site of Soliman (N: 36°42′, E: 10°29′). Rearing of *M. persicae* was conducted on tobacco plants in a controlled growth chamber at 23°C, 60% RH and LD 16:8 h photoperiod. Three individuals were collected to be placed on tobacco plants with height growth of 5 cm. The plants were placed in hermetic cages under the same condition as described previously.

**In vitro bioassays.**

**Fumigant toxicity**. Ten adults of *M. persicae* were placed on a peach leaf in a Petri plate and treated with different concentrations of thyme essential oils: 16.66, 33.33, and 66.66 µl/l air. Filter paper discs (1 $cm^2$) were impregnated with the appropriate oil concentration using a micropipette and glued to the lid. The Petri plate was hermetically closed and placed under the same conditions described above for mass rearing. For each concentration, the assay was repeated 5 times. Control insects were maintained under the same conditions without any essential oil. The mortality rate was determined 24 h post-treatment. Aphid was considered dead when it was completely immobilized under binocular.

**Contact toxicity**. As for the fumigant assay, 10 adults of *M. persicae* were placed on a peach leaf in a Petri plate of 60 ml of volume to be treated with the same concentrations. In this experiment, the aphids are sprayed with a solution of 100 µl of Tween (1%) combined with the essential oils at the appropriate concentration. Under the same conditions, control insects were treated only with Tween (1%). Each treatment was replicated five times and the mortality rate was determined 24 h post-treatment.

**In vivo essential oil formulations.**

This experiment was performed using 10 adults of *M. persicae* infesting pepper plants. Both fumigant and spraying assays were conducted. For fumigant toxicity, 6 different treatments based on the preparation of flavored powder with thyme essential oils were used:

(1) 0.1 g of kaolin: the powder was sprinkled on the infested plants. The plants were then placed in hermetically sealed bottles (1 liter volume).

(2) 0.1 g of diatomaceous earth: this treatment was carried out under the same conditions as the first treatment.

(3) 0.1 g of kaolin + $LC_{50}$ of thyme essential oils: the $LC_{50}$ of essential oils after 24 h was revealed by the in vitro fumigation test. The two products were mixed thoroughly by a vortex. The obtained powder was homogenized and sprinkled on the leaves of plants infested

by *M. persicae*. Afterwards, treated plants were placed in hermetically sealed bottles (1 liter volume).
(4) 0.1 g of diatomaceous earth + $LC_{50}$ of thyme essential oils: this treatment was carried out by the same methodology of the third treatment.
(5) Imidacloprid used at the homologated dose (35 ml/hl) as a reference insecticide**:** the purpose of this treatment was to compare the mortality rates observed following the application of a chemical insecticide to those of the abrasive + essential oil combinations.
(6) Under the same conditions, untreated plants were used as control.

Regarding the contact toxicity assay, 3 different treatments were conducted based on the preparation of an emulsion of thyme essential oils:
(1) Solution of Tween 1% + $LC_{50}$ of thyme essential oils: the $LC_{50}$ was revealed after 24 h by the in vitro contact test. Each plant infested with 10 aphids was sprayed with 1 ml of this product.
(2) Imidacloprid was used at the homologated dose as a reference insecticide.
(3) Controls were treated with 1 ml of a Tween solution (1%).

**Microscopic investigation.**

Investigation of the abrasive treatments effects was performed on 80 adults of *M. persicae* infesting tobacco leaves. The different treatments were applied by sprinkling 0.1 g of kaolin or diatomaceous earth powder on *M. persicae* placed in Petri plates. Control includes the same number of aphids with no treatment. Aphid mortality was recorded 30 min, 1 h, 90 min, 2 h, 4 h, 24 h, and 48 h post-treatment. From each treatment, 10 treated aphids were selected and stored directly in alcohol (70°) to be mounted in the Canada balsam according to Remaudière (1992). Observations were made under an "Olympus" optical microscope at 4× magnification.

**Statistical analyses.**

*M. persicae* mortality was corrected using Abbott formula (Abbott 1925). Data were analyzed using one-way ANOVA using Statistical Package for Social Sciences (version 20.0; SPSS). Means were separated using the Duncan Multiple Range test at $P \leq 0.05$. $LC_{50}$ and $LC_{90}$ at 24 h were calculated by probit analysis according to Finney's equation (Finney 1971).

## RESULTS

**Essential oils yield and chemical composition.**

Essential oils yield recorded from *T. capitatus* leaves was 2.03%. GC/MS analysis revealed the presence of 21 compounds (Table 1). Carvacrol was the major compound with 84.13%, followed by *p*-cymene (4.36%), caryophyllene (2.6%), gamma-terpinene (1.44%), β-caryophyllene (1.32%), and linalol (1.29%). Therefore, *T. capitatus* essential oils contained monoterpenes as well as sesquiterpene compounds.

**In vitro insecticidal activity.**

The fumigant activity was shown to be correlated to the concentrations of thyme essential oils. Mortality rates of 58.14 and 62.79% were obtained after 24 h of exposure using the concentrations 16.66 and 33.33 µl/l air, respectively. The analysis of the variance shows no significant difference between these two concentrations. However, a significant effect was recorded with the highest concentration used. Indeed, the maximum mortality (83.72%) was recorded with the concentration of 66.66 µl/l air.

Concerning the in vitro spraying test, mortality rates recorded after 24 h of exposure at the concentrations 16.66 and

33.33 µl/l air were estimated at 72.09 and 76.74%, respectively. The highest mortality rate (97.67%) was with the concentration of 66.66 µl/l air (Table 2).

**Table 1.** Chemical composition (%) of *Thymus capitatus* essential oils

| N° | RT (min) | Compound | Percentage (%) |
|---|---|---|---|
| **1** | 5.49 | α-pinene | 0.18 |
| **2** | 6.52 | β-pinene | 0.18 |
| **3** | 6.8 | β-myrcene | 0.19 |
| **4** | 6.93 | 3-octanol | 0.12 |
| **5** | 7.55 | α-terpinene | 0.35 |
| **6** | 7.78 | *p*-cymene | 4.36 |
| **7** | 7.9 | β-phellandrene | 0.06 |
| **8** | 8.76 | gamma terpinene | 1.44 |
| **9** | 9.04 | (Z)-sabinene hydrate | 0.11 |
| **10** | 10.02 | Linalol | 1.29 |
| **11** | 12.21 | Borneol | 0.72 |
| **12** | 12.56 | 4-carvomenthenol | 0.86 |
| **13** | 13.02 | β-fenchyl alcohol | 0.12 |
| **14** | 16.25 | o-cymen-5-ol | 0.14 |
| **15** | 16.48 | 2-isopropyl-5-methyl-phenol | 0.39 |
| **16** | 16.95 | Carvacrol | 84.13 |
| **17** | 17.26 | 5-isopropyl-2-methyl-phenol | 0.26 |
| **18** | 18.87 | 2-isopropyl-5-methylphenyl acetate | 0.93 |
| **19** | 20.4 | Caryophyllene | 2.6 |
| **20** | 25.21 | Spathulenol | 0.25 |
| **21** | 25.34 | (-)-β-caryophyllene epoxide | 1.32 |
| **Total** | - | - | 100 |

**Table 2.** Corrected mortality rates (%) of *Myzus persicae,* $LC_{50}$ and $LC_{90}$ values recorded 24 h post-treatment with *Thymus capitatus* essential oils

| Bioassay | Concentration (µl/l air) | Mortality (%) | $LC_{50}$ (µl/l air) | $LC_{90}$ (µl/l air) |
|---|---|---|---|---|
| **Fumigation** | 16.66 | 58.14 ±1.81a | 20.01 | 64.81 |
| | 33.33 | 62.79 ±0.83a | | |
| | 66.66 | 83.72 ±0.54b | | |
| **Spraying** | 16.66 | 72.09±0.54a | 13.26 | 39.55 |
| | 33.33 | 76.74±1.41a | | |
| | 66.66 | 97.67±0.44b | | |

For each essay, values followed by similar letters are not significantly different according to Duncan' Multiple Range test (at $P \leq 0.05$).

**In vivo essential oil formulations (powder and emulsion).**

The treatment with the abrasive kaolin or diatomaceous earth against *M. persicae* has led to a mortality rate of 26.88 and 19.35%, respectively. The combination of thyme essential oils with these abrasives induced a mortality rate of 74.19 and 97.84% for kaolin and diatomaceous earth, respectively. A mortality rate of 95.69% was noted after treatment with chemical insecticide (Fig. 1-A). The treatment with Tween (1%) combined to thyme essential oils led to a mortality rate of 55.55%. However, the effect of this bio-treatment was significantly lower than the chemical insecticide which causes more than 90% mortality (Fig. 1-B).

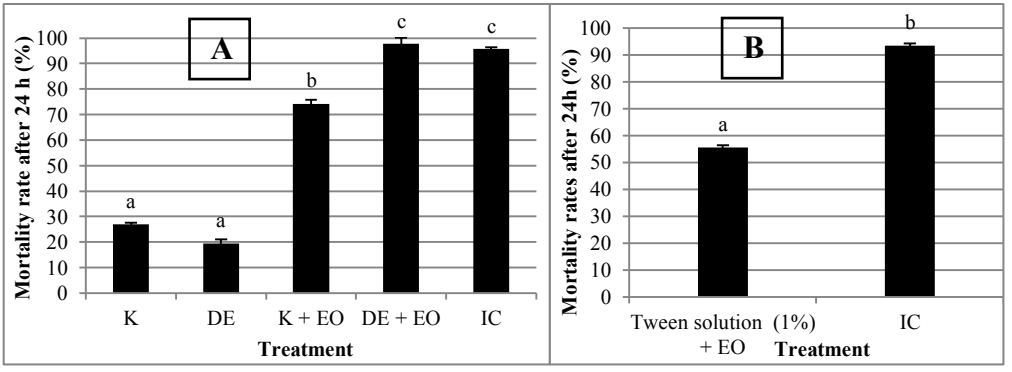


**Fig. 1.** Effect of *Thymus capitatus* essential oil (EO) on *Myzys persicae* mortality in vivo using fumigation assay (A) and spraying assay (B). K: kaolin; DE: diatomaceous earth; IC: Imidacloprid. Values with the same letter are not significantly different according to Duncan' Multiple Range test (at $P \leq 0.05$).

**Treatments effects of abrasives on *M. persicae***

Both kaolin and diatomaceous earth induced an interesting effect on size and color of treated *M. persicae* individuals. After treatment, aphid cadavers showed reduced size due to cuticle dehydration. These effects reflect the direct action of both treatments on the cuticle of *M. persicae*. In fact, the cuticle of control aphids was smooth and hydrated (Fig. 2-A), while the treated ones were distorted, rough and dry. *M. persicae* treated with diatomaceous earth were drier and more rigid (Fig. 2-B) than those treated with kaolin (Fig. 2-C). Therefore, the dehydrating action of diatomaceous earth was more important than that of kaolin.

**DISCUSSION**

This research study was performed to evaluate the biocidal activity of the thyme essential oils. The extraction process of the thyme has provided a yield of 2.03% which is similar to the one reported from a similar study performed in Morocco (Amarti et al. 2008; El Ajjouri et al. 2008). However, this reported yield was slightly less important than the one from *T. capitatus*, collected from Matmata region (South-East of Tunisia), evaluated at 2.75% (Akrout 2004). Regarding the biochemical composition, the results were similar to previous studies, showing that carvacrol was the main component of *T. capitatus* with 70.92% (El Ajjouri et al. 2008) and 66% (Akrout 2004).

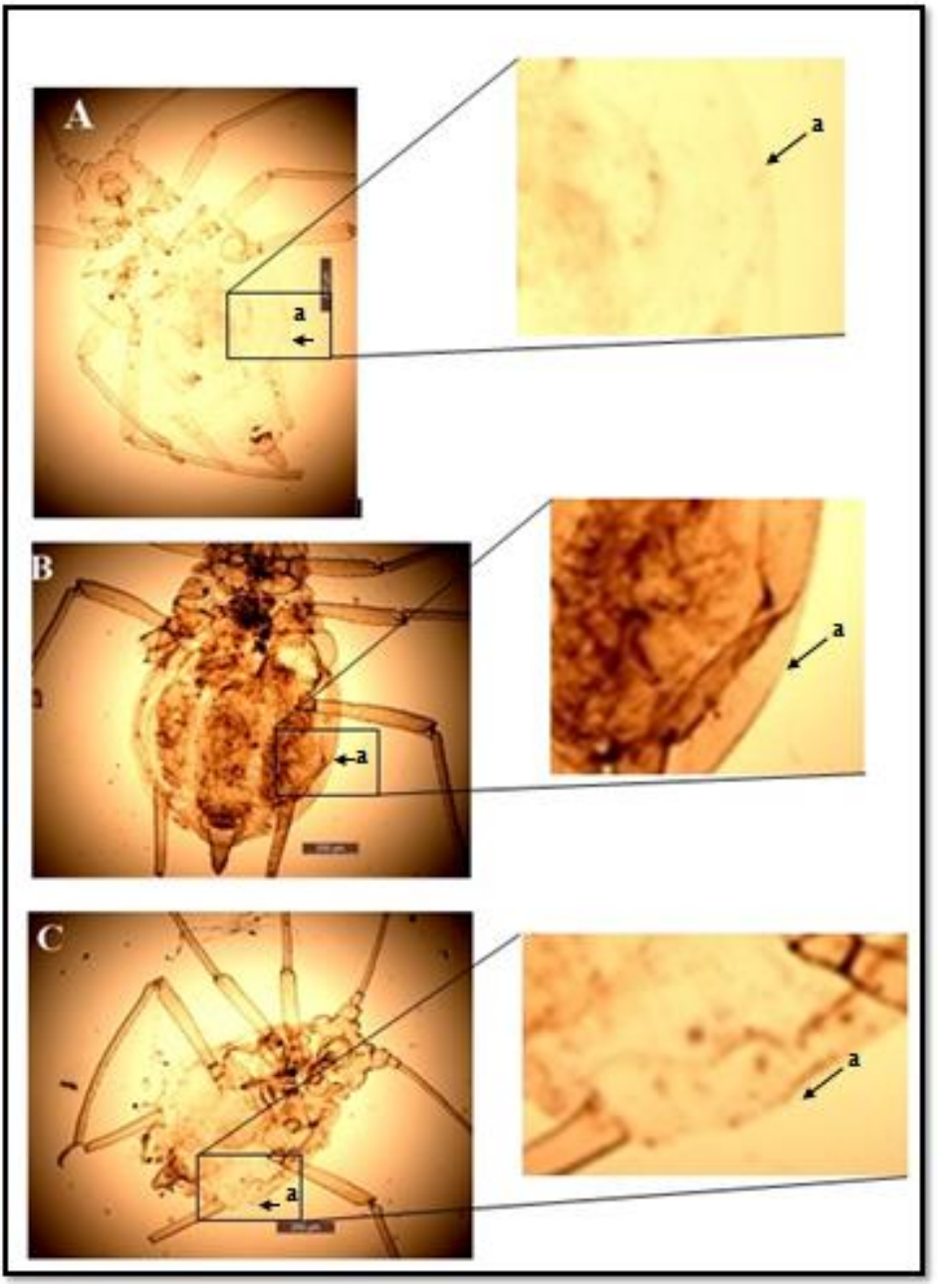


**Fig. 2.** Abrasive impact on *Myzus persicae* cuticle. Aa: Cuticle of the control aphid presenting smooth and hydrated texture; Ba: Cuticle of *M. persicae* treated with diatomaceous earth presenting a dry and rigid texture; Ca: Cuticle of *M. persicae* treated with kaolin showing a dry texture.

Our study reported also an important insecticidal activity of the thyme essential oils against *M. persicae* through both contact and fumigant experiments. The same activity was registered for both rosemary and thyme essential oils through an important repellent activity of 70% at the dose of 1 µl against *M. persicae* (Masatoshi 1998). Other studies showed the important toxic activities of thyme essential oils against several aphid species including *M. persicae* (Masatoshi 2008). The investigation of the insecticidal activity of the thyme essential oils has been also tested against other insect pests. In fact, a previous study has demonstrated the repulsive, contact and fumigant insecticidal activities of terpenes and phenols from thyme essential oils on insects (Isman 2000). The same author noted that components such as monoterpenes and phenols were responsible of the insecticidal activity against *Spodoptera litura* and *M. persicae*. Moreover, further investigation has also shown a higher biocidal activity of *T. capitatus* essential oils than those of *Tetraclinis articulata* against first instar larvae of *Tuta absoluta* (Ben yahia 2015). Beside the interesting toxic activity, *T. capitatus* essential oils has been also proved to reduce the egg production of *Tineola bisselliella* compared to the control (Bouchikhi-Tani et al. 2013).

Our research study also demonstrated the mechanical effect of kaolin and diatomaceous earth through the dehydration, shrinkage and deformation of aphid cuticle. The diatomaceous earth mechanical action has been also reported in a previous study (Ndiaye 2014). In fact, every insect that comes into contact with diatomaceous earth powder died within 24 to 72 h of dehydration (Fields et al. 2002). The mechanical action of kaolin was also reported against *M. persicae*.

The bio-insecticide formulation assays based on thyme essential oils, either by making emulsions or by combining them with natural substances such as diatomaceous earth and kaolin were significantly important against *M. persicae*. In the same context, it was reported that essential oils of some plant species have greater toxicity effects when combined with powdered products such as kaolin (Keita et al. 2001). The same study also showed that the direct sprinkler of the kaolin powder flavored in combination with *Ocimum basilicum* essential oils on *Callosobruchus maculates* had a significant effect on adult mortality (100%) recorded 48 h post-treatment (Keita et al. 2001). Furthermore, the use of 0.4 g of essential oils in the formulation (essential oils + kaolin) has led to 100% mortality in *C. maculates* within one hour post-treatment (Awojide and Fayemiwo 2010). The mechanical and chemical actions of the flavored powders once combined with the essential oils were previously reported by Ramaswamy et al. (1995). Concerning the mechanical action, the effect was mainly presented by blocking the insect articulation and filling the intergranular spaces at high dosages (Ramaswamy et al. 1995). However, the chemical effect was mainly acting on the insect glandular cells (Ramaswamy et al. 1995).

In conclusion, our study demonstrated the high effectiveness of the thyme essential oils in combination with the abrasive powder, diatomaceous earth, as bio-insecticide against *M. persicae*. Therefore, diatomaceous earth powder seems to improve the efficacy of thyme essential oils and make it more stable. Such findings may enhance knowledge about the use of essential oils and the

abrasives as one of the important feature of integrated pest management.


**RESUME**

**Khaled W., Ben Fekih I., Chaieb I., Souissi R., Harbaoui I. et Boukhris-Bouhachem S. 2017. Evaluation de l'activité insecticide des huiles essentielles de *Thymus capitatus* en combinaison avec des abrasifs naturels contre *Myzus persicae*. Tunisian Journal of Plant Protection 12: 49-59.**

Cette étude a pour objectif d'évaluer l'activité insecticide des huiles essentielles de *Thymus capitatus* et de deux abrasifs naturels pour le contrôle de *Myzus persicae*. L'application de ces huiles a montré un effet toxique et fumigant très important in vitro. La CL50 des essais de fumigation après 24 h est de l'ordre de 20,01 µl/l d'air et de 13,26 µl/l d'air pour les essais de pulvérisation. En outre, des essais in vivo de formulation de bio-insecticides ont été abordés. En effet, les huiles essentielles de thym ont été associées à une argile naturelle (le kaolin) et à un minerai naturel (la terre de diatomée). Les résultats ont montré qu'après 24 h, la poudre de kaolin ou de la terre de diatomée associés aux huiles essentielles de thym ont donné des taux de mortalité respectivement de 74,19 et 97,84%. Parallèlement, des émulsions de 1 µl de ces huiles ont été testées sur le puceron cible. Après 24 h, le taux de mortalité a été de l'ordre de 55,55%. L'observation microscopique a montré une action mécanique sur les aphides montés, notamment la déshydratation, le rétrécissement et la déformation de leur cuticule. Ainsi, la terre de diatomée additionnée aux huiles essentielles de *T. capitatus* a été l'association la plus efficace pour lutter contre les populations de pucerons.



*Mots clés*: Huiles essentielles, kaolin, *Myzus persicae*, terre de diatomée, *Thymus capitatus*



**ملخص**

**خالد، وفاء وابتسام بن فقيه وإقبال الشايب ورابحة السويسي وإيمان حرباوي وسنية بوخريص-بوهاشم. 2017. تقييم فاعلية الزيوت الأساسية لنبتة الزعتر (*Thymus capitatus*) ممزوجا بمواد طبيعية كاشطة، كمبيد حشري لمكافحة منّ الخوخ (*Myzus persicae*). Tunisian Journal of Plant Protection 12: 49-59.**

تهدف هذه الدراسة إلى تقييم فاعلية الزيوت الأساسية لنبتة الزعتر(*Thymus capitatus*) مع اثنين من المواد الكاشطة الطبيعية وذلك لمقاومة حشرة منّ الخوخ (*Myzus persicae*). أظهرت النتائج أن استعمال هذه الزيوت عن طريق التبخير أو الرش المباشر له تأثيرا سُمّيًا مهما جدا في المخبر حيث كانت درجة التركيز القاتلة لـ 50% من الحشرات التي وقع عليها الاختبار (LC50) بعد 24 ساعة في حدود 20,01 مكل/ل من الهواء بالتبخير وفي حدود 13,26 مكل/ل بالرش. تم اختبار هذه الزيوت على مجموعة نباتات تحتوي على حشرات المن حيث وقع مزج الزيوت الأساسية للزعتر مع الكاولين(kaolin) أو التربة الدياتومية (diatomaceous earth). أظهرت النتائج بعد 24 ساعة موت 97,84% من الحشرات المعاملة بالزيوت مع التربة الدياتومية و 74,19% بالزيوت مع الكاولين، في حين لم يسبب 1 مكل من الزيوت المستحلبة في الماء سوى 55,55% من السُّمّية لحشرات المنّ. قمنا بفحص مجهري للتغيرات النسيجية لحشرات المنّ المعاملة بالمواد الكاشطة فأثبتت أن لهذه المواد القدرة على تجفيف الأنسجة مع انكماش وتشوه في الجليدة عند الحشرات. هكذا، تظهر هذه الدراسة أن الجمع بين الزيوت الأساسية للزعتر مع المواد الكاشطة وخاصة التربة الدياتومية، يمكن أن يكون طريقة ناجعة لمكافحة حشرة من الخوخ.



*كلمات مفتاحية*: تربة دياتومية، زيوت أساسية، كاولين، *Thymus capitatus*، *Myzus persicae*


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# Effects of *Melia azedarach* Leaf Extracts on Nutritional Behavior and Growth of *Spodoptera littoralis*

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**ABSTRACT**

**Akacha, M., Chaieb, I., Laarif, A., Haouala, R., and Boughanmi, N. 2017. Effects of *Melia azedarach* leaf extracts on nutritional behavior and growth of *Spodoptera littoralis*. Tunisian Journal of Plant Protection 12: 61-70.**

Synthetic chemicals used nowadays as insecticides caused many negative effects (pollution, toxicity…) which led to an increasing interest in botanical insecticides because of their minimal costs and less ecological side effects. In this respect, the activity of *Melia azedarach* leaf extract against the third stage larvae of armyworm (*Spodoptera littoralis*) has been assessed in this study. The aqueous and ethanolic extracts exhibited an antifeedant activity against *S. littoralis* larvae according to the applied doses. Ethanolic extract reduced the food consumption and digestibility inducing growth rate decrease of the armyworm larvae. An increase in the larval stage duration was also observed as well as anomalies. Pupation stage was affected and occurred only for the lowest doses with a significant decrease in pupa weight. Consequently, ethanolic *M. azedarach* leaf extract may be used in the alternative control strategies against *S. littoralis* pest.

*Keywords*: Biopesticide, ethanolic extract, *Melia azedarach*, *Spodoptera littoralis*

Despite all the efforts exerted in protecting crops from noxious pests all over the world, insects cause several damages in crops varying from 10 to 30% for major crops (Ferry et al. 2004).

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Among the numerous insect pests, *Spodoptera littoralis* deserves to be well controlled since it infects more than 87 host plants belonging to 40 plant families which make it a model of serious polyphagous pests (Sadek 2003). It is considered as a major pest on cotton and different horticultural crops such as maize, sorghum, chick pea, pigeon pea, tobacco, okra, sunflower, and groundnut. However, the intensive use of chemicals to manage this pest has resulted in the

development of resistance to almost all classes of insecticides. In fact, *S. littoralis* developed resistance to organophosphorus and synthetic pyrethroidsamong others (Mosallanejad and Smagghe 2009). Therefore, it becomes necessary to search for other alternatives for its control to minimize the use of pesticides.

Plants are good candidates for developing chemical insecticides since they produced many secondary metabolites to protect themselves from insect attacks and herbivores. These phytochemicals can be widely used as an alternative to synthetic insecticides because when biosynthesized, they are easily degraded following enzymatic pathways, without any case of bio-amplification (Regnault-Roger et al. 2008). More interestingly, botanical pesticides tend to have broad-spectrum activity, and fewer environmental impacts stand out (Isman 2006).Unlike synthetic insecticides, they contain mixtures of biologically active compounds whose biological effectiveness can be additionally increased by a suitable synergic effect (Pavela 2008). Thanks to this fact, it is unlikely that the insect would acquire resistance to the entire biologically active complex (Koul 2005).

Among the numerous families with recognized botanical insecticide activity, those belonging to Meliaceae family merit mention. In fact, they are chemically characterized by polyterpenes such as limonoids able to affect insect growth and behavior (Senthil Nathan et al. 2006). *Melia azedarach*,an invasive deciduous tree, is one of species belonging to this botanical family. Although native to India and China, it is currently found in Africa, Australia, the Americas and other countries (Khan et al. 2011). This tree is widespread in Tunisia as an ornamental shade tree characterized by abundant foliage. It will be interesting if its abundant foliage will successfully be used for agricultural purposes against insect pests as demonstrated for its seeds which were used as source of allelochemicals with insecticidal properties (Coria et al. 2008) but leaves still less explored than seeds. Furthermore, chemical composition and bioactivity differences between species naturalized in different regions have been observed (Szewczuk et al. 2003). Therefore, the present research aimed to test the effectiveness of *M. azedarach* leaf extracts for the management of the armyworm *S. littoralis*.

## MATERIALS AND METHODS

### Plant material and extraction.

*M. azedarach* mature leaves were collected in July and August 2012 from Bizerte, North of Tunisia (Lat. 37.27 N, Long. 9.87 E). Shade dried leaves were powdered and 100 g of powder was extracted in 1000 ml of distilled water (aqueous extract) or absolute ethanol (ethalonic extract) using a Soxhlet apparatus for 12 h. Collected solutions were filtered through Whatman n°1 filter paper. The alcoholic extract was evaporated to dryness under reduced pressure in a rotary vacuum evaporator R-114 (Buchi, France). *M. azedarach* extracts are stored in dark at -4°C until future use.

### Insect rearing.

*S. littoralis* were obtained from a rearing of insects maintained under laboratory standard conditions of temperature (27 ± 1°C), photoperiod (L16:D8) and relative humidity (RH) (60-70%). Larvae were reared in Petri plates with an artificial diet cubes based on wheat germ according to Poitout and Bues (1974). Third instars larvae were used for bioassay.

**Feeding assay with leaf discs (no-choice test).**

Antifeedant activity of the ethanolic and aqueous *M. azedarach* extracts against third instars larvae was studied using the no-choice method with leaf disc (Isman et al. 1990). Extracts' solutions were prepared at concentrations of 0.5, 1 and 2% (w/v) dissolved in the adequate solvents (water for aqueous extract and ethanol for the ethanolic one).

Leaf discs (30 mm in diameter) were prepared from castor bean (*Ricinus communis*) leaves using a cork borer. Each disc was dipped in test solution for 1 min. Control leaf discs have been dipped in ethanol or water for the same period. All discs were left until the evaporation of the used solvent. Each arena contained two treated (or not for control) leaf disc (with the same treatment). Groups contained 30 arenas for each treatment as well as for control. After 24 h, antifeedant index (AFI) was calculated using the formula: AFI = (C-T)/(C+ T)×100, with C = Consumption of the control discs; T = Consumption of the treated discs.

The food consumed by 20 insects reared with the control discs was calculated and the mean was used as a parameter C for the calculation of AFI for each observed T. Consumption was recorded using a digitizing leaf area meter (Model LI-3000, LI-COR).

**Effects of ethanolic extract on food consumption and use.**

In this section, three concentrations of ethanolic *M. azedarach* extract were added to artificial diet of *S. littoralis* to investigate their eventual effects on food consumption and use by the third instars larvae.

*S. littoralis* larvae were weighted and individually placed in Petri plates. Then, they were fed with diets containing 0, 0.5, 1 and 2% (w/v) of *M. azedarach* ethanolic extract (n = 30 for each concentration) for 2 days, a period slightly shorter than instars duration. After 48 h, larvae and faeces were weighted and food consumption was determined. The nutritional indices, namely relative consumption rate (RCR), relative growth rate (RGR), efficiency of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD) and approximate digestibility (AD), metabolic cost (MC) were calculated as follows (Liu et al. 1990): RCR = I/BaT; RGR = ΔB/BaT; ECI = (ΔB/I)×100; AD = ((I)-F/I)×100; ECD = (ΔB/ (I)-F) ×100, where: I = Weight of food consumed; Ba = arithmetic mean of insect weight during the experiment = ((PF-PI)/log (PF/PI)); PF = larvae final weight (mg); PI = larvae starting weight (mg); T = feeding period (in h); ΔB = change in body weight; F = weight of faeces produced during the feeding period (Farrar et al. 1989).

**Effects of *M. azedarach* extract on larval development and survival.**

**Bioassay.** Thirty third instars larvae were separately placed in Petri plates (1 cm high and 9 cm in diameter) and fed with the appropriate artificial diet with 0 (control), 0.5, 1, and 2% (w/v) of *M. azedarach* ethanolic leaf extracts. Diet was changed every 24h and larval mortality was recorded until pupation. Then larval period was calculated and pupae were weighted.

The survived larvae were continuously fed with diet containing or not the extract until they became pupae. The experiment was conducted at laboratory condition (27 ± 2°C) with L14:D10 photoperiod and 75 ± 5% RH for 25 days.

**Larval and pupal durations.** Survived larvae were continuously fed with diet. The larval duration was calculated after treated larvae became pupae. The assay was carried out until reaching 100% of mortality. Larvae and pupae mortality were recorded and adjusted for control using Abbott's correction (Abbott 1925): Mc = ((Mo-Me)/(100-Me))×100, where Mo = mortality rate of treated insects (%), Me = mortality rate of control (%), Mc = corrected mortality rate (%).

**Statistical analysis.**

Each experiment was repeated three times (n=30). Data were analyzed using Statistica (version 8). Antifeedant index as well as nutritional indices were compared using analysis of variance ANOVA followed by Turkey's HSD test when significant differences were observed ($P < 0.05$). Data expressed as means ± SD of 30 replicates.

## RESULTS

**Antifeedant properties of *M. azedarach* extracts.**

*M. azedarach* leaf aqueous and ethanolic extracts showed significant antifeedant activity ($P < 0.05$) against *S. littoralis* (Fig. 1). The AFI values recorded using ethanolic and aqueous extracts were about 25 and 10% at the concentration of 1% (w/v) and 31 and 12% at the concentration of 2% (w/v), respectively, while no significant changes were noted for the lowest extract concentration of 0.5%.

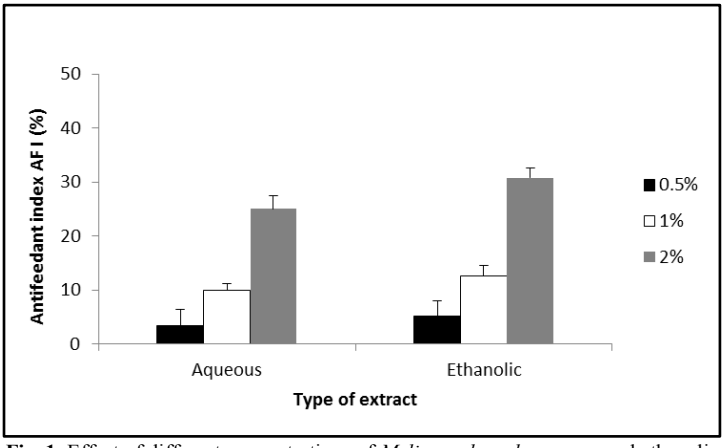


**Fig. 1.** Effect of different concentrations of *Melia azedarach* aqueous and ethanolic extracts on the third instar larvae of *Spodoptera littoralis* using the no-choice test.

**Food consumption and use.**

Nutritional analyses revealed that the ethanolic extract from *M. azedarach* leaves (EMLE) influenced all nutritional indices used when ingested by *S. littoralis* larvae (Table 1). When fed with artificial diet containing 0.5, 1, and 2% (w/v) of ethanolic extract an increase in RCR, AD and MC of larvae but a decrease in RGR, ECI and ECD were recorded and this generally for all doses tested ($P < 0.05$). The RCR rise was inversely proportional with extract concentration since its increase was not significant for 2% (w/v) concentration.

**Table 1.** Effect of different concentrations of *Melia azedarach* ethanolic extract on food consumption by *Spodoptera littoralis* larvae

| Extract concentration (%) | RCR (mg/mg/h) | RGR (mg/mg/h) | ECI (%) | AD (%) | ECD (%) | MC (%) |
|---|---|---|---|---|---|---|
| **0** | 0.067±0.003a | 0.0100±0.0008a | 14.94±1.9a | 90.57±0.85a | 16.50±2.17a | 83.49±2.17a |
| **0.5** | 0.108±0.009b | 0.0068±0.0008b | 6.34±1.21b | 96.09±0.55b | 6.61±1.29b | 93.38±1.29b |
| **1** | 0.094±0.013b | 0.0054±0.0013b | 5.93±2.02b | 96.24±0.92b | 6.18±2.15b | 93.81±2.15b |
| **2** | 0.081±0.006a | 0.0051±0.0012b | 6.42±1.76b | 96.14±0.97b | 6.69±1.88b | 93.30±1.88b |

Mean values with the same letter are not significantly different ($P < 0.05$). RCR = Relative consumption rate; RGR = Relative growth rate; ECI = Efficiency of conversion of ingested food; ECD = Efficiency of conversion of digested food; AD = Approximate digestibility; MC = Metabolic cost.

**Larval development and mortality under continuous exposure to *M. azedarach* ethanolic extract.**

After treatment with EMLE using all concentrations, the larval developmental period was longer than normal time for control ones (Table 2). The longest larval period (22.12± 2.83 days) was obtained in the case of larvae feeding on diet containing 0.5% of EMLE. The other concentrations (1 and 2%) caused early mortality after 20.75 ± 1.32 and 19.5 ± 2.08 days, respectively (Table 2).The larvae were not able to go into further instars; they were small in size and unable to continue normal physiological process since they consumed very low amount of diet. Molting was also delayed for all concentrations used. In fact, only 6% of larvae fed on diet containing 0.5% of EMLE can accomplish pupation. Nevertheless, the body weight of pupae (271.47 mg) was significantly reduced compared to the control (347 mg) (Table 2).

Moreover, EMLE caused larvae malformations (about 10%) at the concentration of 2% which was interesting to mention since it makes larvae enable to achieve their exuviation. EMLE caused a mortality of 66.67, 83.33 and 100% for 0.5, 1 and 2% of *M. azedarach* extract, respectively (Table 3).

**Table 2.** Effect of diets with different *M. azedarach* ethanolic extract concentrations on larval development of *Spodoptera littoralis*

| Extract concentration (%) | Duration of larval period (day) | Pupae weight (mg) |
|---|---|---|
| **0** | 08.89±1.07a | 347.35±4.15a |
| **0.50** | 22.12±2.83b | 271.47± 5.12b |
| **1** | 20.75±1.32b | - |
| **2** | 19.50 ±2.08b | - |

Mean values with the same letter are not significantly different ($P < 0.05$).

**Table 3.** Effect of different concentrations of *Melia azedarach* ethanolic extract incorporated in an artificial diet on larval mortality of *Spodoptera littoralis* larvae and pupae

| Extract concentration (%) | Days | Larval mortality (%) |
|---|---|---|
| **0.50** | 9 | 0 |
| | 14 | 38.889 |
| | 20 | 66.667 |
| **1** | 9 | 0 |
| | 14 | 44.445 |
| | 20 | 83.334 |
| **2** | 9 | 0 |
| | 14 | 50 |
| | 20 | 100 |

Values were adjusted by the control mortality using Abbott's correction (Abbott 1925)

## DISCUSSION

Ethanolic leaf extract of *M. azedarach* possess an antifeedant/insecticidal concentration-dependent activity against *S. littoralis* larvae. In fact, the study of nutritional indices after the ingestion of allelochemicals can help to determine if a chemical compound has antifeedant and/or any toxic post-ingestive effect.

Observed decrease in ECI indicates that more food is being metabolized for energy and less is being concerted to body substance (growth). ECD also decreased as the proportion of digested food metabolized for energy increased. Decreased ECI and ECD values indicate that ingested *M. azedarach* extract also exhibited some chronic toxicity. Similarly, this result corroborates with previous findings reporting on antifeedant and insecticidal activity of *M. azedarach* such as those of Coria et al. (2008) for *Aedesaegypti*, Bullangpoti et al. (2012) for *S. frugiperda* and Aouadia et al. (2012) for *Drosophila melanogaster*.

It was also observed that most of the treated larvae were not able to cross further developmental stages. In fact, elongation of larval durations was also observed probably due to interfering of toxic substances in the molting process (Carpinella et al. 2003). Low percentage of treated larvae *M. azedarach* extracts were able to rich pupation with a significantly reduction of pupa weights suggesting that the overall effects could be dramatic if accumulated along the insect's life cycle. Similar effects were reported in other studies (Carpinella et al. 2003; Chiffelle et al. 2009; Defago et al. 2006).

Furthermore, based on previous studies concerning *M. azedarach* phenolics and fatty acids profiles (Akacha et al. 2016), the observed insecticidal activities can be explained by the presence of flavonoids known to have high potential for controlling insect pests (Carpinella et al. 2002). Minor phenolic compounds should not be neglected since synergy between the different chemicals should be taken into consideration as they may be involved in the biological activity

(Djeridane et al. 2006). In fact, three flavonoids were detected in *M. azedarch* extracts: isoquercitrin, isorhamnetin and catechine hydrate (Akacha et al. 2016) and were effective against some insects. Catechin-based insecticidal product was reported to be efficient against *Tribolium castaneum*, *T. confusum*, *Callosobruchus chinensis* and *Sitophilus oryzae* (Khatun et al. 2011) while isoquercitrin, when added to the diet of the tobacco budworm *Heliothis virescens*, larval growth was reduced by 90% (Hedin et al. 1983). Similarly, Lattanzio et al. (2000) showed the direct involvement of quercetin or isorhamnetin, a metabolite of quercetin, in the plant resistance mechanism toward aphids. Moreover, gallic acid caused notable mortality in *Heliothis armigera* and *S. litura* individuals; the surviving larvae of *H. armigera* and *S. litura* treated with *Ocimum kilimandscharicum* showed significant growth retardation (Singh et al. 2014).

Moreover, the major phenolic compound i.e. protocatechuic acid (one of metabolites of quercetin) was one of the most active components against *S. frugiperda* (Murillo et al. 2014) and it might be one of the most components responsible for the insecticidal activity recorded against *S. littoralis* larvae. Furthermore, previous GC-MS analysis of the ethanolic extract of *M. azedarach* leaves (Akacha et al. 2016) revealed the presence of mainly palmitic, oleic, stearic, palmitoleic and linoleic fatty acids which are reported to possess insecticidal potentialities. In fact, many authors such as Ramos-Lopez et al. (2012) and Ramsewak et al. (2001) have demonstrated the insecticidal activity of linoleic acid, similar for oleic (Kannathasan et al. 2008; Rahuman et al. 2008), palmitic and stearic acids (Figueroa-Brito et al. 2002; Ramos-Lopez et al. 2012). Similarly, Khan and Usman (2012) found a moderate insecticidal activity of fatty acids against *Callosobruchus analis.* Besides, Abay et al. (2013) demonstrated toxicity activities of lauric, myristic and palmitic acids against the Turkish mosses: *Dicranum scoparium, Hypnum cupressiforme, Polytrichastrum formosum, Homalothecium lutescens* and the Turkish liverwort *Conocephalum conicum.*

Additionally, it is interesting to mention that *S. littoralis* treated larvae exhibited exuviation anomalies that might be caused by meliacarpine (Alché et al. 2003), a limonoid similar to azadirachtin from *Azadirachta indica* (Schmutterer, 2002). In fact, *S. littoralis* third instars larvae fed on 1 ppm of azadirachtin showed 75% of malformed adults (Martinez and Van Emden 2001) which can be attributed to the disturbance of insect hormones balance.


**RESUME**

**Akacha M., Chaieb I., Laarif A., Haouala R. et Boughanmi N.2017. Effets des extraits de feuilles de *Melia azedarach* sur le comportement nutritionnel et la croissance de *Spodoptera littoralis.* Tunisian Journal of Plant Protection 12: 61-70.**

Pour éviter les problèmes causés par l'utilisation des produits chimiques synthétiques comme les insecticides, une attention particulière a été attribuée aux plantes qui constituent une des alternatives de lutte qui préservent l'environnement. Dans ce cadre, l'activité des extraits de feuilles de *Melia azedarach*a été évaluée contre les larves de *Spodoptera littoralis*. Les extraits aqueux et éthanoliques ont eu une activité anti-appétante sur les larves de *S. littoralis* de plus en plus importante avec des doses croissantes. L'extrait éthanolique a réduit la consommation et la digestibilité des larves, induisant une diminution du taux de croissance des larves. Un allongement de la période larvaire ainsi que des

anomalies chez les larves ont été aussi observés. La pupaison n'a abouti que pour la dose la plus faible des extraits de *M. azedarach* et le poids de la pupe a été significativement réduit. Par conséquent, l'extrait éthanolique de *M. azedarach* peut être utilisé dans les stratégies alternatives de lutte contre *S. littoralis*.

*Mots clés*: Biopesticide, extrait éthanolique, *Melia azedarach*, *Spodoptera littoralis*

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## ملخص

**عكاشة،مروة وإقبال الشايب وأسماء العريف وربيعة حوالة ونزيهة بوغانمي.2017. تأثيرات مستخلص أوراق شجرة *Melia azedarach* على السلوك الغذائي ونمو دودة القطن *Spodoptera littoralis*.**
**Tunisian Journal of Plant Protection 12: 61-70.**

لتفادي التلوث الكيمياوي المنجر عن استعمال المواد الكيميائية الاصطناعية كمبيدات للحشرات تم توجيه الاهتمام إلي النباتات التي يمكن أن تعوض المبيدات الاصطناعية وتكون آمنة للمحيط. تمت خلال هذا البحث دراسة فعالية مستخلص أوراق شجرة *Melia azedarach* ضد يرقات دودة القطن *Spodoptera littoralis*. ظهرت لكلتا المستخلصين المائي والايثانولي تأثيرات مانعة للشهية عند يرقات دودة القطن وذلك حسب الجرعة. قلص المستخلص الايثانولي من استهلاك الغذاء والهضم مما خفض معدل النمو لدى اليرقات. كما لوحظ تمدد في المرحلة اليرقية وتشوهات لدى اليرقات. لم يحدث تشرنق إلا بالنسبة لأضعف جرعة مع انخفاض معنوي في وزن الشرنقات. بناء على ذلك فان المستخلص الايثانولي لأوراق *M. azedarach* ممكن أن يستعمل كبديل لمكافحة الآفات من نوع دودة القطن.

*كلمات مفتاحية*: مبيد بيولوجي، مستخلص ايثانولي، *Melia azedarach*، *Spodoptera littoralis*

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# Repellency and Toxicity of the Crude Ethanolic Extract of *Limoniastrum guyonianum* against *Tribolium castaneum*

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**ABSTRACT**

**Acheuk, F., Belaid, M., Lakhdari, W., Abdellaoui, K., Dehliz, A., and Mokrane, K. 2017. Repellency and toxicity of the crude ethanolic extract of *Limoniastrum guyonianum* against *Tribolium castaneum*. Tunisian Journal of Plant Protection 12: 71-81.**

In nature, the interaction between plants and insects has led to the production of a set of secondary compounds. Many plant secondary metabolites have significant insecticidal activity. The aim of this study is to evaluate the repellent and insecticidal effect of the crude ethanolic extract of *Limoniastrum guyonianum* against adults of the red flour beetle *Tribolium castaneum*. For the insecticidal activity, five doses (100, 200, 400, 600, and 800 µg/insect) were tested and were topically applied onto insect thorax. An area preference method was adopted to assess the repellent activity. A phytochemical study and measurement of two enzymatic biomarkers: acetylcholinesterase (AChE) and gluthatione S-transferase (GST) were made to understand the mechanisms of toxic action of the tested extract. Phytochemical study showed the presence of various groups of natural products. The plant is rich in flavonoids, tannins, alkaloids, and glycosides. Low amount of saponins was noted. The study also showed that this plant does not contain iridoids. For repellent activity, the results showed that the highest dose (800 µg/insect) exhibited obvious repellent effect against *T. castaneum*. The repellency percentage was 90.14 ± 2.5% after 4 h of exposure. The crude extract was found to be toxic to *T. castaneum* and the corresponding $LD_{50}$value was 218.3 µg/insect. Moreover, the extract inhibits the activity of the acetylcholinesterase ($IC_{50}$: 205.7 µg/insect).

*Keywords*: Crude ethanolic extract, enzymatic biomarkers, *Limoniastrum guyonianum*, repellency, toxicity, *Triboliumcastaneum*

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The red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), is one of the most serious secondary pests that feeds on a wide range of durable stored products including cereals, cereal

products and other high value produce such as cocoa beans and dried fruits (Adarkwah et al. 2010). In addition to direct consumption of the product, insect pests inflict their damage on stored products through excretion, exuviation, dead bodies and their own existence in the product, which is not commercially desirable (Sallam2003). In addition, the infestation of *T. castaneum* results in increase temperature and humidity of the storage environment which would eventually lead to an accelerated growth of molds, including toxigenic species (Morgan et al. 2003).

Fumigants and chemical insecticides are widely used to control of *T. castaneum* population (White and Leesch 1995). Application of these compounds has led to a number of problems including the development of insecticide resistance in stored grain insect pest (Lorini and Galley 1999), toxic residues in food and consequent health hazards (Park et al. 2003). Botanical insecticides have long been explored as attractive alternatives to synthetic chemical insecticides for pest management because botanicals are less harmful to the environment due to their biodegradation (Isman 2006). Algeria was characterized by a great ecological and floristic diversity, estimated at more than 3000 species belonging to several botanical families, of which 15% are endemic and which remain not very explored on both phytochemically and bioinsecticide levels (Hanifi 1991).

*Limoniastrum guyonianum* (Plumbaginaceae) is a 0.5-1.5 m tall perennial shrub, endemic to North Africa, growing on salty arid soils and sandy Saharan lands where rainfall is less than 150 mm/year. It has potential for use in dune stabilization and landscaping (Barhoumi et al. 2015). The plant is widely used by local people to cure dysentery, to treat gastric infections and as an anti-bacterial for bronchitis treatment (Le Floch 1983). This antimicrobial action has been demonstrated experimentally by Hammami et al. (2011). It appears that the volatile compounds possess potent antibacterial activity. Recent studies have also shown that *L. guyonianum* contains high amount of phenolic and is a potential source of new natural phenolic acid amides compounds and exhibit a potent antioxidant activity. This activity is related to the presence of three powerful flavonoids isolated from ethyl acetate fractions: gallocathechin, epigallocatechin and epigallocatechin-3-0-gallate (Trabelsi et al. 2012; 2014). Equally, Krifa et al. (2013; 2014) demonstrated that the aqueous gall extract of this plant exhibited an immune modutory and anti-tumor effects. Currently, no data concerning the insecticidal effect of this plant is available, only the work of Lakhdari et al. (2015) on the acaricidal effect of its aqueous extract. The aim of the current work is to screen the repellency and the toxicity of the crude ethanolic extract of *L. guyonianum* against adults of the red flour beetle, *T. castaneum*. On the other hand, to understand and to investigate the mechanisms of toxic action of the extract, phytochemical study and measurement of two enzymatic biomarkers: acetylcholinesterase (AChE) and gluthatione S-transferase (GST) were performed.

## MATERIALS AND METHODS

### Plant collection and preparation of crude ethanolic extract.

*L. guyonianum* was collected from Sidi Mehdi, Touggourt region (Southeastern part of Algeria) during autumn season 2015. The plant was taxonomically identified and confirmed

by Dr. Benhouhou from the National High College of Agriculture, Algiers, Algeria. Aerial parts of *L. guyonianum* were air-dried in the shade and grounded into fine powder using electrical blender. The powder was stored at room temperature in hermetically sealed plastic boxes until extraction.

**Insect rearing.**

Initial stock culture of *T. castaneum* was obtained from entomology laboratory of National Institute of Plant Protection, El-Harrach, Algiers. Beetles were reared, at the Laboratory of Zoology of Boumerdes University, in glass containers (0.5 l) containing wheat flour mixed with brewer's yeast (10/1 w/w). The culture was maintained in the dark in growth incubators at 28-30°C and 70-80% RH. Adults of 1-5 days post emergence were used in experiments.

**Preparation of crude ethanolic extract and phytochemical screening.**

The crude ethanolic extract of the aerials parts of *L. guyonianum* was prepared by macerating the powder for 3 days in ethanol, followed by filtration and evaporation at 40°C. The dried extract was kept at 4°C until further use. The ethanolic extract was tested for plant secondary metabolites, alkaloids, sugar, phenolic compounds, flavonoids, saponins, tannins, iridois, and coumarins. Phytochemical screening of the extract was carried out according to the standard method (Dohou et al. 2003). Visible color change or precipitate formation was taken into consideration for presence (+) or absence (-) of particular active constituents.

**Repellent activity.**

To assess the repellent activity of *L. guyonianum* crude ethanolic extract against *T. castaneum* adults, an area preference method of McDonald et al. (1970), with slight modifications, was adopted. The test was carried out under the same conditions described above for the mass rearing using glass Petri plates as containers. Filter paper (Whatman N°1, 9 mm in diameter) was cut in half. The extract was tested at the concentration 800 µg. This dose was selected on the basis of preliminary tests. The extract was dissolved in acetone and 500 µl of the test solution were applied uniformly to half filter paper disc. Another half was treated with acetone only. Treated and untreated halves were air-dried, carefully fixed and placed in Petri plates. For each test twenty adults (1-5 days post emergence) were introduced at the center of the Petri plates. Five replicates were performed and the number of insects on the two halves discs were counted after 2 and 4 h of exposure. The percentage of repellency was calculated as follows (McDonald et al. 1970): PR (%) = (Nc-Nt)/(Nc+Nt) × 100, with Nc: Number of insects on control part and Nt: Number of insects on treated part. The average values were then categorized according to the following scale: 0 = 0.0l to 0.1%, I = 0.1 to 20%, II = 20.1 to 40%, III = 40.1 to 60%, IV = 60.1 to 80%, V =80.1 to 100%.

**Contact toxicity test.**

The bioassay was carried out using five concentrations of the crude ethanolic extract: 100, 200, 400, 600, and 800 µg/insect. Acetone was used as solvent and served as control. Unsexed adults insects were immobilized with cold 15 min before the beginning of the test. Aliquots of 5 µl of each concentration were topically applied onto the thorax of insects using a micropipette. For each concentration, 20 insects were used in 5 replicates. After treatment, insects were transferred into glass Petri plates and

supplied with food (mixture of wheat flour and brewer's yeast). All treated and control insects were kept under the same conditions as described for the insect rearing. Insect's mortality was recorded daily and $LD_{50}$was calculated using Probit analysis according to Finney (1971).

**Inhibition of acetylcholinesterase and gluthatione S-transferase assays.**

Acethylcholinesterase (AChE) enzymatic activity was carried out following the method of Ellman et al. (1961) using acetylthiocholine as a substrate. Adults of *T. castaneum* were sampled from control and treated groups (100 and 800 µg/insect). Pools of 20 adults were homogenized in the solution containing 38.03 mg of ethylene glycol tetraacetic (EGTA), 1 ml Triton X-100, 5.845 g NaCl and 80 ml Tris buffer (10 Mm, pH 7). The homogenate was centrifuged (5000 g for 5 min at 4°C), and the resulting supernatant was used for enzymatic assay. The AChE activity was measured in aliquots (100 µl) of resulting supernatants added to 100 µl of 5-5' dithiobis-(2-nitrobenzoic acid) (DNTB) in Tris buffer (0.01 M, pH 8) and 1 ml Tris (0.1 M, pH 8). After 5 min, 100 µl of acetylthiocholine was added. Measurements were conducted at a wavelength of 412 nm with a run time of 20 min. The inhibition rate of each treatment was calculated using the following formula: Inhibition rate (%) = 100 - (Enzyme activity of treatment × 100/Enzyme activity of control).

On the other hand, gluthatione S-transferase **(**GST) activities were determined with the soluble fraction as enzyme source. GST activities toward 1-chloro-2, 4-dinitrobenzene (CDNB) were measured according to Habig et al. (1974). Treated (100 and 800 µg/insect) and control insects were homogenized in sodium phosphate buffer (0.1 M, pH 6) and centrifuged (14000 g, 30 min). Two hundred microliter of the resulting supernatant was added to 1.2 ml of reaction mixture containing 1 Mm of CDNB and 5 Mm of reduced glutathione (GST) in the homogenization buffer. Changes in absorbance were recorded at 340 nm. Total protein content was determined according to method of Bradford (1976) using bovine serum albumin as a standard. Enzyme activities were expressed as µmol/min/mg proteins. The percentage of activation was calculated using the following formula: Activation rate (%) = 100 - (Enzyme activity of control × 100/Enzyme activity of treatment).

**Statistical analysis.**

Results are expressed as means ± standard deviation (SD). To identify significant effects of the treatments on the variables measured, data were submitted to a monofactorial ANOVA using XLSTAT 7.5.2. Means were compared using Tukey's HSD test ($P < 0.05$).

## RESULTS

**Phytochemical screening.**

Phytochemical study of *L. guyonianum* showed the presence of various groups of natural products (Table 1). The plant is rich in flavonoids, tannins, alkaloids, coumarins, and glycosides. Low amount of saponins was noted. The study also shows that this plant does not contain anthocyanins and iridoids.

**Table 1.** Qualitative phytochemical screening of *Limoniastrum guyonianum* crude ethanolic extract

| Alkaloids | Anthocyanins | Comarins | Tannins | Saponins | Iridois | Flavonoids |
|---|---|---|---|---|---|---|
| ++ | - | ++ | +++ | + | - | +++ |

(-): Absent;(+): Low presence; (++): Moderate presence; (+++): Strong presence

**Repellent activity.**

For repellent activity, results given in Table 2 showed that at the concentration 800 µg/insect, the extract exhibited potent repellent effect against *T. castaneum* adults. The repellency percentage was 73.33 ± 22 and 90.14 ± 2.5%, respectively, after 2 and 4 h of exposure. The activity was increased when insects were exposed for a longer time. Extract belonged to repellency class V.

**Table 2.** Repellent activity of the crude ethanolic extract of *Limoniastrum guyonianum* against adults of *Tribolium castaneum* at different exposure times

| Time | 2 h | 4 h |
|---|---|---|
| **Repellency (%)** | 73.33 ± 22 b | 90.14 ± 2.5 a |
| **Class** | IV | V |

Within columns, values followed by different letters denote significant differences at $P < 0.05$ according to Tukey's test.

**Contact toxicity.**

*L. guyonianum* ethanolic extract was toxic to *T. castaneum* adults. Mortality was dependent upon doses and exposure time (Fig. 1). Of all the tested doses, 800 µg/insect resulted in the highest contact toxicity. This dose led to 83.33 ± 12.5% mortality after 6 h and 91.67 ± 7.5% after 3 days of exposure, respectively. $LD_{50}$ calculated after 6 h of exposure from the regression lines Probit $=f$(doses) was 218.3 µg/insect (Fig. 2).

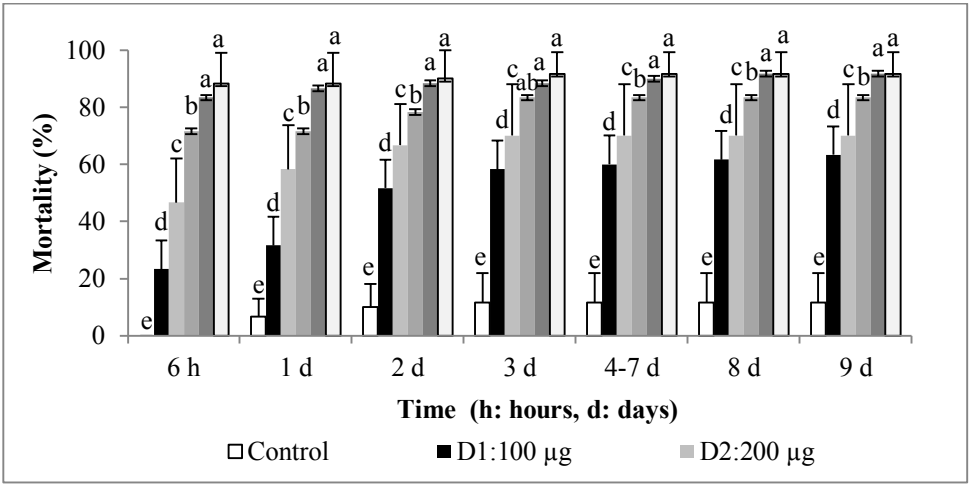


**Fig. 1.** Toxicity of the crude ethanolic extract of *Limoniastrum guyonianum* applied topically to the adults of *Tribolium castaneum* (Mean ± SD). N = 20 insects/replicate. Values followed by the same letter are not significantly different at $P < 0.05$according to Tukey's test.

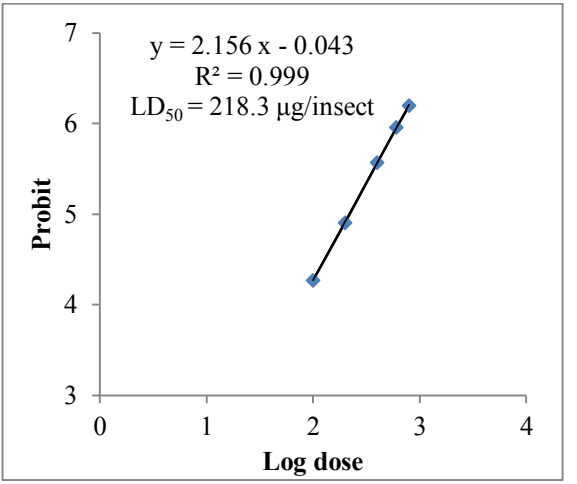


**Fig. 2.** Effect of crude ethanolic extract of *Limoniastrum guyonianum* applied topically on the adults of the red flour beetle *Tribolium castaneum* noted after 6 h of exposure (Mean ± SD). N = 20 insects/replicate.

**Enzymatic effects.**

The effect of the crude ethanolic extract of *L. guyonianum* on AChE and GST enzymatic activities is shown in Figs. 3 and 4. Results revealed that the extract significantly reduced the AChE activity. The highest AChE inhibition (89.6 ± 4.5%) was caused by the extract at dose 800 µg/insect, one day after treatment. A slight activation of GST (18.3 ± 2.5%) was observed with the highest dose 800 µg/insect, one day after treatment.

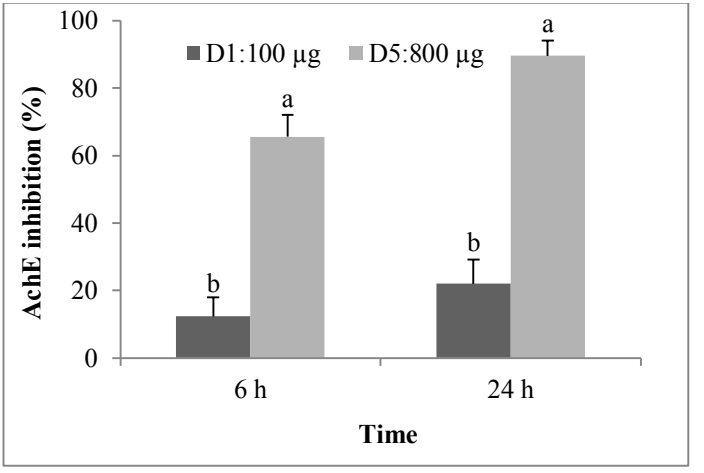


**Fig. 3.** Effect of crude ethanolic extract of *Limoniastrum guyonianum* on AChE activity against *Tribolium castaneum* adults (Mean ± SD). N = 20 insects. AChE activity reduction (as percentage of the control insects group). Means followed by the same letter do not differ significantly at $P < 0.05$according to Tukey's test.

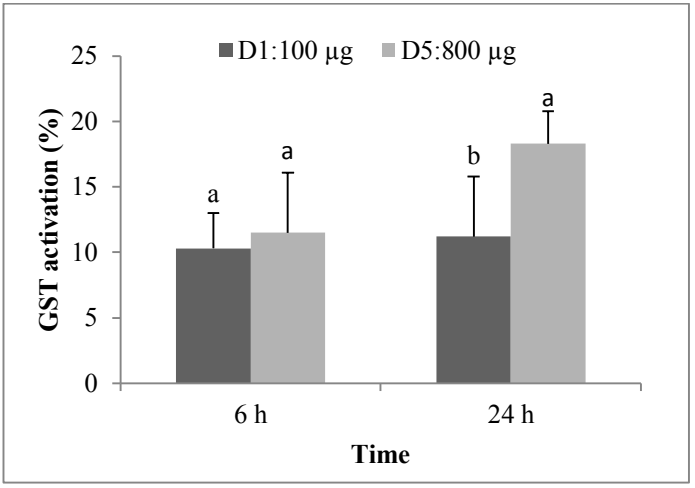


**Fig. 4.** Effect of crude ethanolic extract of *Limoniastrum guyonianum* on GST activity of the adults of the red flour beetle *Tribolium castaneum* (Mean ± SD). N = 20 insects. GST activity activation (as percentage of the control insects group). Means followed by the same letter do not differ significantly at $P < 0.05$ according to Tukey's test.

## DISCUSSION

Plants have evolved a plethora of different chemical defenses covering nearly all classes of (secondary) metabolites that represent a major barrier to herbivores; some are constitutive, others are induced after attack. Many compounds act directly on the herbivores, whereas others act indirectly via the attraction of organisms from other trophic levels that, in turn, protect the plant. An enormous diversity of plant (bio) chemicals are toxic, repellent, or anti-nutritive for herbivores of all types (Mithofer and Boland 2012). Botanical insecticides, such as nicotine and pyrethrum, once dominated crop protection and domestic pest control, before the discovery of the insecticidal properties of DDT and methylparathion during the late 1930s. However, a scientific 'renaissance' in interest in botanical insecticides was spurred by the Western 'discovery' of the profound anti-insect bioactivity of the triterpenoid azadirachtin, isolated from the seeds of the Indian neem tree (*Azadirachta indica*, Meliaceae) during the 1960s (Morgan et al. 2003).

In order to discover the biological properties of Algerian endemic plants, we screen, in this study the insecticidal activity of the crude ethanolic extract of the Saharan plant *L. guyonianum* on the red flour beetles *T. castaneum*. Phytochemical study of this plant showed the presence of various bioactive compounds. The plant is rich in flavonoids, tannins, alkaloids, and glycosides which have been documented to possess biological properties. The presence of these groups of secondary metabolites has already been reported by Hamidi (2012). It also indicates that acetone is the best extraction solvent for this plant. Previous studies carried out by Trabelsi et al. (2013) showed that *L. guyonianum* from Tunisia contained great total polyphenol and considerable amount of condensed tannins. The highest antioxidant activity, antibacterial effect and antitumor activity of the *L. guyonianum* extract were correlated to its contents in polyphenol and condensed

tannins (Bouzidi et al. 2016; Trabelsi et al. 2013; Ziani et al. 2015).

The insecticidal screening results indicate that the extract of *L. guyonianum* possess contact toxicity as well as repellent activity. At the highest dose 800 µg/insect, the extract exhibited obvious repellent effect against *T. castaneum* (91 ± 7.5% after 4 h of exposure) and potent insecticidal activity (91.67 ± 7.5%) after 3 days. This double action of the extract could be due to the presence of various bioactive compounds in the crude ethanolic extract. It is most likely that these chemicals interfere mainly with certain biological, ecological, and physiological aspects of the insect (Edriss et al. 2013). Plant extracts and essential oils of many species of plants caused similar mortality in *T. castaneum* adults. Insecticidal effects of methanol extracts from seven plant species on *T. castaneum* were investigated by Jbilou et al. (2006): *Centaurium erythraea*, *Peganum harmala*, *Ajuga iva*, *Aristolochia baetica*, *Pteridium aquilinum*, and *Raphanus raphanistrum* where all extracts inhibit growth of larvae. *C. erythraea* was the most toxic leading to 63% mortality, 10 days after treatment, followed by *P. harmala* with 58%. Twenty-two compounds were isolated from the leaves and stems of *Murraya alata* and tested for their repellent effect against *T. castaneum*. The tested compounds exhibited various repellent activities. Among them, three compounds, meranzin, phebalosin and muralatin K showed significant repellent activity (You et al. 2017).

Previous studies have shown that compounds extracted from diverse plants exhibited anti-insect properties by disturbing neuro-endocrine and growth regulatory processes (Xiao et al. 2012). Acetylcholinesterase has a very high catalytic activity, and it is a key enzyme that terminates nerve impulses by catalyzing hydrolysis of the neuro-transmitter acetylcholine in the nervous system(Wang et al. 2004; 2010). Our result revealed that the exposure of *T. castaneum* adults to *L.guyonianum* extract at a lethal dose (800 µg/insect) significantly reduced AChE activity. This acetylcholinesterase inhibition could be a possible mechanism of action of *L. guyonianum.* Kabir et al. (2013) indicate that the extract of *Seseli diffusum* exhibited a potent larvicidal activity and induced strong neurobehavioral toxicity against the 4$^{th}$ instar larvae of *Aedesa egypti.* Inhibition of AChE causes accumulation of acethylcholine at the synapses, which will lead to paralysis and death of the insect. In recent study, Gade et al. (2017) noted that the biological compounds stigmasterol and 1-hexacosanol from *Chromolaena odorata* were identified to be responsible of larvicidal activity against *Culex quinquefasciatus* and their mode of action has been observed to be neurotoxicity. At a molecular level, these compounds were found able to inhibit the acetylcholinesterase activity in *C. quinquefasciatus* and *A. aegypti*.

On the other hand, glutathione-S-transferases are a group of enzymes that catalyze the conjugation of reduced glutathione (GSH) with a wide range of lipophilic toxicants bearing electrophilic sites (Habiget al. 1974). Some plant allelochemicals have been reported as GST inducers (Yu 1982). These reports suggest that GST may participate in the primary detoxification, in phytophagous insects, of plant allelochemicals (Yu 1987). A slight activation of GST (18.3 ± 2.5%) was observed with the highest dose 800 µg/insect, one day after treatment. This result suggested that GST is not target of action of *L. guyonianum* extract and the insect could not completely

detoxify the bioactive compounds of this extract.

The findings of this investigation demonstrate that the ethanolic extract of *L. guyonianum* possess potent toxicity as well as repellent activity. The extract inhibits the activity of the AChE. The crude extracts have a mixture of different compounds of flavonoids, tannins, alkaloids and glycosides which may act synergically or separately. Fractions should be tested independently to separate the active compounds on insects.


**RESUME**

**Acheuk F., Belaid M., Lakhdari W., Abdellaoui K., DehlizA. et Mokrane K. 2017. Activité répulsive et toxicité de l'extrait éthanolique brut de *Limoniastrum guyonianum* à l'égard de *Tribolium castaneum*. Tunisian Journal of Plant Protection 12: 71-81.**

L'interaction entre les plantes et les insectes dans la nature a conduit les plantes à la production d'un ensemble de métabolites secondaires. De nombreux métabolites secondaires sont dotés d'activités insecticides. Le but de cette étude est d'évaluer l'effet répulsif et insecticide de l'extrait éthanolique brut de *Limoniastrum guyonianum* contre les adultes du tribolium rouge de la farine *Tribolium castaneum*. Pour l'activité insecticide, cinq doses ont été testées (100, 200, 400, 600 et 800 µg/insecte) et ont été appliquées topiquement sur le thorax des adultes. La méthode de zone préférentielle a été adoptée pour l'évaluation de l'activité répulsive. Une étude phytochimique et un dosage de deux biomarqueurs enzymatiques (acétylcholinestérase AChE etgluthatione S-transférase GST) ont été réalisés afin de comprendre le mécanisme d'action de l'extrait. L'étude phytochimique a montré la présence de divers groupes de métabolites secondaires. La plante est riche en flavonoïdes, tanins, alcaloïdes et glycosides. Une faible présence de saponines a été notée. L'étude a montré également que cette plante ne contient pas des iridoïdes. Pour l'activité répulsive, les résultats ont montré que la dose la plus élevée (800 µg/insecte) présentait un bon effet répulsif contre *T. castaneum*. Le pourcentage de répulsion était de 90,14 ± 2,5% après 4 h d'exposition. L'extrait brut s'est montré toxique pour les adultes de *T. castaneum* et la $DL_{50}$ correspondante était de 218,3 µg/insecte. En outre, l'extrait inhibe également l'activité de l'AChE ($CI_{50}$ : 205,7 µg/insecte).



*Mots clés*: Activité répulsive, biomarqueurs enzymatiques, extrait éthanolique brut, *Limoniastrumguyonianum*, toxicité, *Triboliumcastaneum*



**ملخص**

**عاشق، فاطمة ومسعودة بلعيد ووسيمة لخضاري وخميس عبداللاوي وعبد الرحمان دهليز وكهينة مقران. 2017.النشاط الطارد وسمية المستخلص الإيثانولي الخام للنبتة *Limoniastrum guyonianum* ضد سوسة الدقيق الحمراء *Tribolium castaneum*. Tunisian Journal of Plant Protection 12: 71-81.**

أدى التفاعل بين النباتات والحشرات في الطبيعة إلى إنتاج مجموعة من المركبات الثانوية. تملك هذه المركبات الثانوية فعالية ضد الحشرات. تهدف هذه الدراسة إلى تقييم النشاط الطارد الحشري للمستخلص الإيثانولي الخام لنبتة *Limoniastrum guyonianum* ضد سوسة الدقيق الحمراء *Tribolium castaneum*. تم اختبار خمس جرعات من المستخلص الكحولي الخام (100 و200 و400 و600 و800 مكغ/حشرة) وتم تطبيقه موضعيا على صدر الحشرة البالغة. اعتمدت طريقة المنطقة التفضيلية لتقييم النشاط الطارد للمستخلص. لفهم آلية عمل هذا المستخلص، تمت دراسة كيميائية للنبتة وتقدير اثنين من المؤشرات الحيوية الأنزيمية. أظهرت الدراسة الكيميائية وجود مجموعات مختلفة من المركبات الثانوية حيث أن النبتة غنية بمركبات الفلافونويد والعفص وقلويداتوجليكوسيدات كما تم ملاحظة وجود كميات قليلة من الصابونين. وبينت الدراسة أيضا أن هذه النبتة لا تحتوي على اردويداس. فيما يخص النشاط الطارد، أظهرت النتائج أن أعلى جرعة (800مكغ /حشرة)، كان لها تأثير طارد جيد ضد الحشرة حيث بلغت نسبة التنافر 90,14 ± 2,5% وذلك بعد 4 ساعات من التعرض. وقد ثبت أن مستخلص هذا النبات سام للحشرة البالغة حيث بلغت الجرعة القاتلة$LD_{50}$218,3 مكم/الحشرة. إضافة إلى ذلك، مكن المستخلص الإيثانولي الخام من منع النشاط الأنزيمي AChE.

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# Chemical Composition and Insecticidal Effects of *Citrus aurantium* of Essential Oil and its Powdery Formulation against *Tuta absoluta*

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**ABSTRACT**

**Zarrad, K., Chaieb, I., Ben Hamouda, A., Bouslama, T., and Laarif, A. 2017. Chemical composition and insecticidal effects of *Citrus aurantium* essential oiland its powdery formulation against *Tuta absoluta*. Tunisian Journal of Plant Protection 12: 83-94.**

The aim of this research was to investigate the chemical composition and to evaluate the insecticidal activities of the bitter orange *Citrus aurantium* essential oil and its major compound pure limonene against adults and larvae of the tomato miner *Tuta absoluta* using contact and fumigation bioassays. Results of chemical analysis of the essential oil using gas chromatography/mass spectrometry (GC-MS) revealed the presence of limonene (87.52%), β-myrcene (1.62%), α-pinene (0.56%) ß-ocimene (0.81%) and ß-pinene (0.61%) as major components. For bioassays, results indicated that both the oil and its major compound were found to be toxic to larvae and adults. In the fumigant assays, median lethal concentrations ($LC_{50}$) were 10.65 and 37.36 µl/l air respectively for *C. aurantium* essential oil and pure limonene. In contact toxicity assay, the tomato miner adults were more susceptible to the oil than to its major compound even at the lowest concentration: $LD_{50}$ values obtained after 48 h were respectively 0.21 and 0.73 µl. When insects were treated with the essential oil and its aromatized clay powder, significant differences in insect mortality were recorded depending on exposure time. The aromatized clay powder was more toxic ($LT_{50}$ = 101.8 h) than the pure essential oil ($LT_{50}$ = 146.32 h). Hence, bitter essential oil was found to be toxic for *T. absoluta*, and the clay powder could be used to stabilize the essential oil to increase its efficacy and possibly will be used as source of new eco-friendly insecticidal compounds.

*Keywords:* Aromatized powder, biopesticide, *Citrus aurantium*, essential oil, pure limonene, *Tuta absoluta*

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The tomato miner *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is one of the most devastating pests of tomato (Desneux et al. 2010). Since the time of its initial detection, the pest has caused serious damages to tomato in invaded areas (Germain et al. 2009) and it

is currently considered a key agricultural threat to European and North African tomato production. *T. absoluta* can cause 100% yield losses in tomato crops (Estay 2000). In 2008, it was detected for the first time in the region of Akouda in Coastal Tunisia and quickly spread in all Tunisian tomato-cropping areas (EPPO 2009) where it seriously threatened tomato production and trade of tomato fruits. In fact, Chermiti et al. (2009) reported that fruit loss recorded under greenhouses in the region of Bekalta ranged from 11 to 43% of production. In some cases, crops were totally destroyed. The use of synthetic insecticides has been the primary strategy for controlling *T. absoluta* resulting in environmental pollution and resistance in pest population (Desneux et al. 2007). Therefore, research on alternative methods for tomato miner control is needed.

In recent years, essential oils have been developed as potential alternatives for pest control because they are often of low mammalian toxicity and readily biodegradable (Maciel et al. 2010). Nevertheless, their high instability due to their extremely volatile nature handicaps the protection of the plants over a long duration (Ngamo et al. 2007). For that reason, it was necessary to develop new formulations of these active essential oils to improve their use in an integrated pest management approach (Nguemtchouin et al. 2010). Powdered suspensions of processed kaolin were found to be effective against a range of pest insects (Saour 2005; Valizadeh et al. 2013) and to retain the active compounds against insects for a long period (Ndomo et al. 2008).

The present work reported first investigations on insecticidal effects of *C. aurantium* essential oil, pure limonene, and the clay powder aromatized with this oil towards a Tunisian strain of *T. absoluta*.

## MATERIALS AND METHODS

### Insect rearing.

*T. absoluta* was provided by the Laboratory of Entomology at the *Centre Régional des Recherches en Horticulture et Agriculture Biologique,* Chott-Mariem, Tunisia. The tomato miner moth was reared on tomato plants placed in a rearing room. The rearing conditions were: 26 ± 2°C, 60 ± 5% RH, and photoperiod cycle of L: D 16 h: 8 h. Third instar larvae (best stage that endures manipulation) and adults were used for bioassays.

### Plant material.

Fruits of *C. aurantium* were collected at mature stage from trees located in the Chott-Mariem area (Sousse, Tunisia). They were transferred to the laboratory and rinsed with distilled water. Peels were excised from fruits using a razor blade, which left the white spongy portion (albedo) on the fruit.

### Essential oil extraction.

For the extraction of the essential oil, bitter orange peels were subjected to hydrodistillation for 3 h using a Clevenger type apparatus. The extracted oil was then stored at 4°C until future use.

### Gas chromatography/mass spectrometry analysis conditions.

The quantitative and qualitative analysis of the volatile profile in the essential oil was carried out using a model 5890 Series II plus gas chromatograph (GC) equipped with a 30 m × 0.25 mm HP-5 (cross-linked phenyl–methyl siloxane) column with 0.25 mm film thickness and an Agilent model 5972 inert mass spectrometry (MS) detector (Agilent, Palo Alto, CA). The initial oven

temperature was held at 60ºC for 4 min. It was then increased by 3ºC/min to 250ºC; the injection port and ionizing source were kept at 250 and 280ºC, respectively. Helium was used as carrier gas, the flow through the column was 1 ml/min, and the split ratio was 100:1 with 0.1 ml of injected sample. Mass spectra were scanned from m/z 35 to 300, generating 5.27 scan/s. The individual peaks were identified by retention times and the retention index (relative to C6–C17 n-alkanes), compared with those of corresponding reference standards and using the NIST98 library (NIST, Gaithersburg, MD). Percentage compositions of essential oil were calculated according to the area of the chromatographic peaks.

**Fumigant toxicity.**

To determine the fumigant toxicity of *C. aurantium* essential oil and its major compound pure limonene, Whatman N°1 filter papers (2 cm in diameter) were impregnated with oil at doses calculated to release fumigant concentrations of 5 to 50 µl/l air. Each impregnated filter paper was attached to the screw cap of a 40 ml Plexiglas bottle. Ten larvae on tomato miner were added to each bottle and caps were screwed on tightly. Each treatment and control was replicated five times. The number of dead and alive insects in each bottle was counted after 24 h of exposure.

**Contact toxicity.**

Essential oil contact toxicity towards newly emerged *T. absoluta* adults was measured according to Liu and Ho (1999). A serial dilution of the essential oil was prepared in acetone (0.1 ml). The doses of oil tested were 0.0125, 0.025, 0.05 and 0.1 µl. A volume of 0.5 ml of each essential oil and pure limonene in acetone solution was applied to the thorax of each adult. Control was treated with 0.5 ml of acetone. Each concentration was repeated five times. Mortality was observed each hour for 48h. The $LD_{50}$ and $LD_{90}$ values were estimated using probit analysis (Finney 1971).

**Aromatization of the clay powder with essential oil.**

The aim of this test was to resolve the volatilization constraint of the essential oil. For this purpose, the bio-efficacy of essential oil and aromatized clay powder based on the mixture of clay and essential oil extracted from *C. aurantium* were evaluated for their insecticidal activity against *T. absoluta* larvae.

Powdery formulation was prepared as described by Keita et al. (2001). For that, 10 g of kaolin were transferred in a 100 ml flask and 0.75 ml of *C. aurantium* essential oil diluted in 10 ml of acetone was added. Then, the mixture was placed in a water bath thermostated at 30°C for 90 min after 5 min of manual shaking, to total acetone evaporation. Aromatized powder was kept in vials tightly closed using aluminum foil under the cap.

**Effect of aromatized kaolin formulation on larval mortality.**

A 10 g-sample of powdery formulation previously prepared was introduced into a plastic jar containing a tomato plant infested with *T. absoluta*. All the plant is uniformly coated. Four different control treatments were applied. In the first one, the plants were treated with non-aromatized clay powder. In the second, the plants were treated with pure essential oil. In the third, the plants were treated with aromatized clay powder. For the last control jar, the plants were not treated. Each jar was tightly closed. Five replications were carried out for each set of treatment and dead insects were

counted daily. Percentage larvae mortality was calculated using the Abbott formula (Abbott 1925).

**Statistical analyses.**

Data are presented as means. One-way analysis of variance using Statistical Package for Social Sciences (version 11.0; SPSS, Chicago, III) was performed on the data. A Duncan Multiple Range test was applied to detect significant differences of mortality among concentrations at the 0.05 percent level. Probit analysis (Finney 1971) was used to estimate LC, LD, and LT values.

## RESULTS

**Chemical analysis.**

The oil yield *C. aurantium* was 0.67% on the basis of dry matter weight. Chemical composition of this essential oil is illustrated in Table 1. In fact, GC-MS analysis showed that 25 compounds were identified, which represent 97.69% of total constituents. Limonene (87.52%), β-myrcene (1.62%), α-pinene (0.56%), ß-Ocimene (0.81%), and ß-pinene (0.61%) were the main monoterpene hydrocarbons, whereas sesquiterpene hydrocarbons were represented by ß-caryophyllenne (0.35%) and ß-farnesene (0.12%). Alcohols (0.52%), aldehydes (1.26%) and esters (0.8%) constituted the non terpenic compounds.

**Table 1.** Chemical composition (%) of essential oil from *Citrus aurantium* peel

| N° | Compound | RI | RT | Percentage |
|---|---|---|---|---|
| 1 | α-pinene | 949 | 4.097 | 0.561 |
| 2 | Camphene | 969 | 4.420 | 0.007 |
| 3 | Sabinene | 1001 | 4.998 | 0.171 |
| 4 | ß-pinene | 1007 | 5.080 | 0.453 |
| 5 | ß-myrcene | 1026 | 5.448 | 1.628 |
| 6 | Octanal | 1034 | 5.775 | 0.381 |
| 7 | δ -3-carene | 1048 | 5.989 | 0.021 |
| 8 | Limonene | 1073 | 6.675 | 87.523 |
| 9 | (*E*)- ß -ocimene | 1086 | 7.224 | 0.325 |
| 10 | ɣ-terpinene | 1109 | 7.568 | 0.045 |
| 11 | Terpinolene | 1141 | 8.660 | 0.215 |
| 12 | Linalool | 1152 | 9.141 | 3.365 |
| 13 | Nonanal | 1157 | 9.309 | 0.066 |
| 14 | (*Z*)- limonene oxide | 1173 | 10.450 | 0.026 |
| 15 | (*E*)- limonene oxide | 1182 | 10.643 | 0.022 |
| 16 | Camphor | 1192 | 10.870 | 0.021 |
| 17 | Citronellal | 1201 | 11.368 | 0.013 |
| 18 | Terpinen -4-ol | 1220 | 12.353 | 0.171 |
| 19 | α - terpineol | 1231 | 12.982 | 0.928 |
| 20 | Neral | 1270 | 15.462 | 0.754 |
| 21 | Geraniol | 1280 | 16.157 | 0.222 |
| 22 | Geranial | 1291 | 16.896 | 0.430 |
| 23 | Neryl acetate | 1366 | 21.658 | 0.041 |
| 24 | Geranyl acetate | 1386 | 22.639 | 0.196 |
| 25 | Caryophyllene | 1420 | 23.988 | 0.111 |
| **Total** | | -- | 97.696 | |

**RT**: Retention Time. **RI**: Retention Indices calculated using an apolar column (HP-5).

**Fumigant toxicity.**

Both *C. aurantium* essential oil and pure limonene were toxic to the third instar larvae of *T. absoluta*. Mortality was directly related to the doses; it increased as the dose of essential oil increased (Table 2). At the highest concentration (50 µl/l air), the oil achieved 100% of mortality while pure limonene caused only 60% of mortality after 24 h of exposure. Results showed that *C. aurantium* essential oil was more toxic to *T. absoluta* larvae than pure limonene. These results were confirmed by lethal concentration values. The corresponding $LC_{50}$ and $LC_{90}$ were respectively 10.65 and 21.16 µl/l air for *C. aurantium* compared to 37.36 and 74.01 µl/l air for pure limonene. Statistical analysis showed significant differences between the oil and its major compound (Table 3).

**Table 2.** Percentage of mortality of *Tuta absoluta* larvae exposed for 24 h to *Citrus aurantium* essential oil and pure limonene.

| Dose (µl/l air) | Mortality (%) | |
|---|---|---|
| | ***C. aurantium* essential oil** | **Pure limonene** |
| **0** | 0 a, A | 0 a, A |
| **5** | 30 ± 0.13 b, B | 13.33 ± 0.04 b, A |
| **12.5** | 73.33 ± 0.08 c, B | 26.67 ± 0.04 c, A |
| **25** | 90 ± 0.06 cd, B | 43.33 ± 0.04 d, A |
| **50** | 100 d, B | 60 ± 0.06 e, A |

Values are means of five replications, each set-up with 10 insects. Within columns, means followed by the same letter (lowercase letters) were not statistically different based on Duncan Multiple Range test at $P < 0.05$. Within rows, means followed by the same letter (uppercase letters) were not statistically different based on Duncan Multiple Range test at $P < 0.05$.

**Table 3.** $LC_{50}$ and $LC_{90}$ values of fumigant toxicity of *Citrus aurantium* essential oil and pure limonene against *Tuta absoluta* larvae

| Fumigant | ***C. aurantium* essential oil** | **Pure limonene** |
|---|---|---|
| **$LC_{50}$ (µl/l air)** | 10.65 (0.96-24.72) | 37.36 (21.83-80.37) |
| **$LC_{90}$ (µl/l air)** | 21.16 (13.87-80.78) | 74.01 (47.43-103.01) |
| **df** | 3 | 3 |
| $\chi^2$ | 30.26 | 20.97 |
| ***P*** | 0 | 0 |

For $LC_{50}$ and $LC_{90}$ units (µl/l air), lower and upper confidence limits are given in parenthesis.

**Contact toxicity.**

The results depicted in Fig. 1 point out that *T. absoluta* adults were very susceptible to the contact toxicity of *C. aurantium* essential oil and pure limonene even at the lowest concentration (0.012 µl). Furthermore, the mortality was dose-dependent and increased with increasing concentrations. Indeed, results of the probit analysis revealed that adults were more sensitive to *C. aurantium* essential oil mostly to pure limonene with corresponding $LD_{50}$ and $LD_{90}$ values of 0.21 and 1.92 µl and 0.73 and 2.34 µl, respectively, for the oil and its major compound limonene (Table 4).

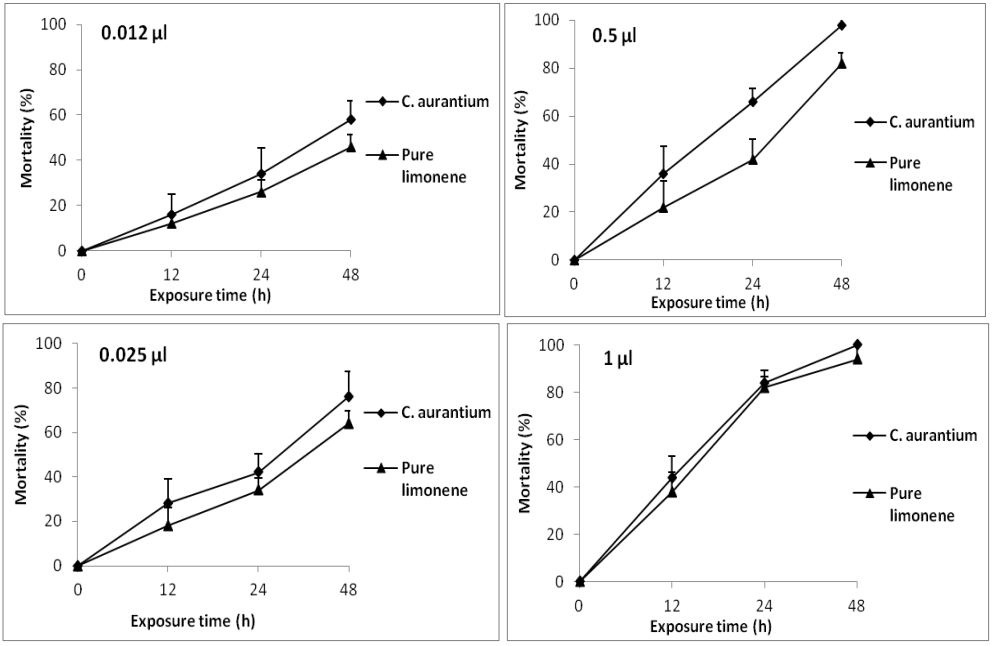


**Fig. 1.** Percentage of mortality of *Tuta absoluta* adults exposed for various durations to *Citrus aurantium* essential oil and pure limonene applied at different concentrations.

**Table 4.** $LD_{50}$ and $LD_{90}$ values (µl) of contact bioassay with *Citrus aurantium* essential oil and pure limonene

| Fumigant | *C. aurantium* essential oil | Pure limonene |
|---|---|---|
| **$LD_{50}$ (µl)** | 0.21 (0.09-1.7) | 0.73 (0.12-2.85) |
| **$LD_{90}$ (µl)** | 1.92 (0.79-4.82) | 2.34 (1.32-5.67) |
| **df** | 3 | 3 |
| $\chi^2$ | 8.96 | 11.61 |
| ***P*** | 0 | 0 |

For $LC_{50}$ and $LC_{90}$ units (µl/l air), lower and upper confidence limits are given in parenthesis.

**Effect of aromatized kaolin formulation on larval mortality.**

*T. absoluta* larvae were sensitive to the pure essential oil and its aromatized clay powder. The treatments showed time-response dependence, resulting in significant larvae' mortality when the time increased (Fig. 2). Indeed, mortality values ranging from 27 to 36% and from 57-75% were attained after 24 h and 3 days of exposure, respectively. The highest contact activity was achieved with kaolin powder aromatized at the three exposure durations (24, 48 and 72 h); mortality increased from 36% at 24 h to 75% at 72 h. The mortality observed in the controls and untreated plants was less than 4%. These results were proved by lethal time values (Table 5). The corresponding $LT_{50}$ for bitter orange oil and the powdery formulation were respectively 146.32 and 101.8 h.

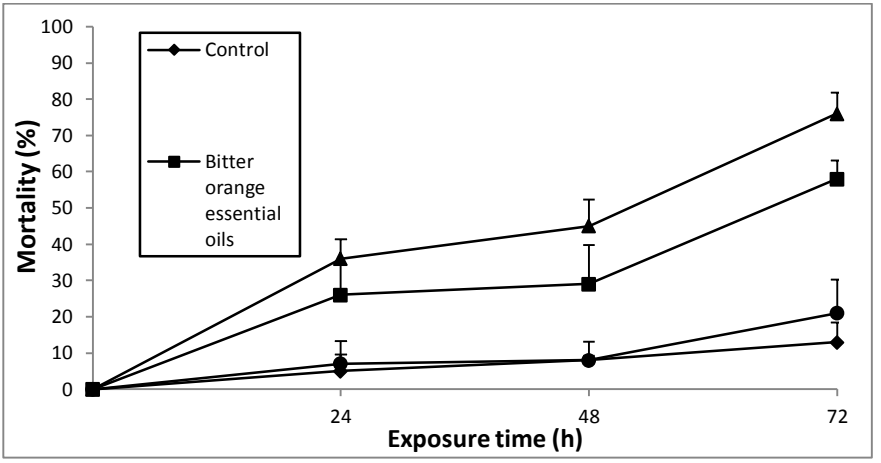


**Fig. 2.** Evolution of insect mortalities exposed to the essential oil and aromatized clay powder of bitter orange *Citrus aurantium*.

**Table 5.** $LT_{50}$ and $LT_{90}$ (h) values of *Citrus aurantium* essential oil and its powdery formulation

| Treatment | $LT_{50}$ | $LT_{90}$ | $\chi^2$ | df | *P* |
|---|---|---|---|---|---|
| ***C. aurantium* essential oil** | 146.32 | 328.29 | 25.23 | 3 | 0 |
| **Powdery formulation** | 101.8 | 250.7 | 17.32 | 3 | 0 |

## DISCUSSION

This study revealed that the essential oil of *C. aurantium* was rich in monoterpenoids, and its major compound was limonene (88.52%). Among various compounds of *Citrus* essential oil, limonene is the most important. This result is in agreement with previous investigations on bitter orange essential oil. Indeed, Bousaada and Chemli (2007) reported the predominance of limonene whose level varied from 87% to 92.2%. Additionally, Hosni et al. (2010) showed that bitter orange peel oil comprised mainly monoterpene hydrocarbons with limonene (96.86%), ß-pinene (1.37%), sabinene (0.28%), and α-pinene (0.27%) as major compounds. In the same context, Moraes et al. (2009) proved that Brazilian *C. aurantium* peel oil contained limonene (97.5-98%), myrcene (1.2-1.45%), and octanol (0.34-0.54%). Likewise, Caccioni et al. (1998) reported that *C. aurantium* oil from Italy was richer in limonene (94.3%) than myrcene (1.88%), linalool (0.78%) and α-pinene (0.4%). These qualitative and quantitative differences in compositions of essential oil depended on season, maturational stage, genotype and pedoclimatic factors (Boussaada et al. 2007).

The present study proved also the sensitivity of *T. absoluta* to bitter orange essential oil and pure limonene. In fact, the insecticidal activities of *C. aurantium* essential oil have previously been evaluated against several insect species (Rossi and Palacios 2013). Indeed, Pala and Pathipati (2010) reported that essential oil of *C. aurantium* were highly effective leading to 89 and 76% mortality against respectively *Sitophilus oryzae* and *Rhyzopertha dominica* when applied at 8.5 mg/cm$^2$. Besides, fumigant toxicity of *C. aurantium* essential oil was investigated on *Callosobruchus*

*maculatus* adults (Moravvej and Abbbar 2008). Siskos et al. (2007) suggested that *C. aurantium* contains secondary metabolites that are toxic to the adult olive fruitfly, *Bactrocera oleae*. Additionally, Siskos et al. (2008) studied the effect of the essential oil extracted from *C. aurantium* used at dose of 5 µg/cm$^2$ for the control of *B. oleae* and obtained 98% mortality after an exposure duration of 72 h. Palacios et al. (2009) reported that essential oil obtained from *C. aurantium* was effective in killing *Musca domestica* and showed that the $LC_{50}$ value was 4.8 mg/dm$^3$. Moreover, *C. aurantium* presented highly insecticidal toxicity against *Trialeurodes vaporariorum* adults, nymphs and eggs after 24 h of exposure.

The bioactivity of this oil could be mainly allocated to their constituents whose insecticidal activities have previously been proved against several insect species (Rossi and Palacios 2013). Certainly, the insecticidal mode of action of some monoterpene compounds is known (Koul et al. 2008). Indeed, Tripathi et al. (2003) reported the insecticidal activity of d-limonene against *Rhyzoperta domonica*, *Sitophilus oryzae*, and *Tribolium castaneum*. Furthermore, this compound showed promising fumigant toxicity against *S. zeamais* and *T. castaneum* (Rui et al. 2010). Additionally, Ezeonu et al. (2001) indicated the insecticidal properties of limonene against stored-product beetles and mosquitoes. Likewise, α-pinene was reported to be toxic on several insect species (Ceferino et al. 2006; Lucia et al. 2007). Moreover, terpinen-4-ol showed a relatively strong toxicity against the larvae and adults of *Leptinotarsa decemlineata* (Kordali et al. 2007). Also limonene, terpen-4-ol, 1,8- cineole and linalool acted by fumigant toxicity and presented a remarkable insecticide activity against eggs, larvae and adults of *Tribolium confusum* (Stamopoulos et al. 2007).

Our work related to contact toxicity bioassay showed that both bitter orange essential oil and pure limonene displayed an interesting insecticidal potential. As reported elsewhere, *C. aurantium* essential oil revealed a contact toxicity activity. In this context, Palacios et al. (2009) reported the contact toxicity of *C. aurantium* essential oil and pure limonene against *Musca Domestica* adults. Additionally, insecticidal activity of various *Citrus* species was demonstrated by contact toxicity using topical application to various insect pests. Indeed, Chungsamarnyart and Jansawan (1996) evaluated the acaricidial activity of *C. sinensis* oils and registered 49-99% mortality of *Boophilus microplus* after 24 h of exposure. Also, in contact toxicity assay, Kumar et al. (2012) reported that the $LC_{50}$ of *C. sinensis* essential oils against *M. domestica* larvae, varied between 3.93 and 0.71 µl/cm$^2$ while $LT_{50}$ varied between 5.8 to 2.3 days.

Furthermore, it has been established during the present study the effect of the aromatized clay powder on the mortality of *T. absoluta*. As previous study, kaolin was found to be effective against insects (Barker et al. 2006; Valizadeh et al. 2013) and could prevent damages of insect pests. In fact, our results are in accordance with those of Ndomo et al. (2008) who demonstrated that both aromatized clay powder and pure essential oils showed insecticidal activities against *Acanthoscelides obtectus*. After 2 days of exposure, the aromatized clay powder was more toxic ($LD_{50}$ = 0.069 µl/g grain) than the pure essential oil ($LD_{50}$ = 0.081 µl/g grain). There was, however, a highly significant loss of toxicity after 24 and 36 h following treatment with essential oil and

aromatized powder, respectively. Kèita et al. (2001) reported that the application of kaolin powder aromatized with *Thuja occidentalis* essential oils on *C. maculatus* lead to 95% mortality of females and 100% of males with no mortality in the control after 6 h of exposure. When insects were treated with pure oil the mortality rate was 39.1%. Besides, the formulation based on *Xylopia aethiopica* essential oils and powder clay produced mortality rates ranging from 22 to 100% for *Sitophilus zeamais* (Nguemtchouin et al. 2010).

The present work reported the insecticidal activity of the essential oil of *C. aurantium* and pure limonene against *T. absoluta*. Thus, the obtained results suggest the possibility of their use in the sustainable management of greenhouse pests. Although essential oil applied alone provides a good level of control of insect pests, they are very unstable because of their high volatility. The use of clay materials as a support for such oil as shown in the present study could not only increase their stability but also improve their insecticidal activities. Nevertheless, essential oil and clay could be an efficient alternative to synthetic products and therefore a method to reduce pollution.

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**RESUME**

**Zarrad K., Chaieb I., Ben Hamouda A., Bouslama T. et Laarif A. 2017. Composition chimique et effets insecticides de l'huile essentielle de *Citrus aurantium* et de sa formulation poudreuse contre *Tuta absoluta*. Tunisian Journal of Plant Protection 12: 83-94.**

Le but de ce travail a été d'étudier la composition chimique et d'évaluer les activités insecticides de l'huile essentielle du bigaradier *Citrus aurantium* et de son composant majoritaire le limonène pur contre les adultes et les larves de la mineuse de la tomate *Tuta absoluta* en utilisant des bioessais de contact et de fumigation. L'analyse chimique de l'huile essentielle, effectuée par chromatographie en phase gazeuse couplée à la spectrométrie de masse, a révélé le limonène (87,52%), le β-myrcène (1,62%), l'α-pinène (0,56%), le ß-Ocimène (0,81%) et le ß-pinène (0,61%) en tant que composants majeurs. Les résultats des bioessais ont montré que l'huile et son composé majoritaire se sont révélés toxiques vis-à-vis des larves et des adultes. Pour les essais de fumigation, les concentrations létales médianes $CL_{50}$ étaient respectivement de 10,65 et 37,36 µl/l air pour l'huile essentielle de *C. aurantium* et le limonène pur. Pour l'essai de toxicité par contact, les adultes de la mineuse de la tomate étaient plus sensibles à l'huile que son composé principal même à la plus faible concentration: les $DL_{50}$ obtenues au bout de 48 h étaient respectivement de 0,21 et 0,73 µl. Lorsque les insectes ont été traités avec l'huile essentielle et de la poudre d'argile aromatisée, des différences significatives de mortalité, en fonction du temps d'exposition, ont été enregistrées. La poudre d'argile aromatisée était plus toxique ($TL_{50}$ = 101,8 h) que l'huile essentielle pure ($TL_{50}$ = 146,32 h). Ainsi, l'huile essentielle du bigaradier a été trouvée toxique pour *T. absoluta* et la poudre d'argile pourrait être utilisée pour stabiliser cette huile essentielle afin d'augmenter leur efficacité et pourrait éventuellement être utilisée comme source de nouveaux composés insecticides respectueux de l'environnement.

*Mots clés*: Biopesticide, *Citrus aurantium*, huile essentielle, limonène, poudre aromatisée, *Tuta absoluta*

## ملخص

**الزراد، خولة وإقبال الشايب وأمال بن حمودة وثامر بوسلامة وأسماء لعريف. 2017.التركيبة الكيميائية والفاعلية الإبادية الحشرية للزيت الأساسي لنبتة *Citrus aurantium* وصياغته كمسحوق ضد حشرة *Tuta absoluta*.**
**Tunisian Journal of Plant Protection 12: 83-94.**

تهدف هذه الدراسة إلى تشخيص التركيبة الكيميائية وتقييم الفاعلية الإبادية الحشرية للزيت الأساسي للنارنج *Citrus aurantium* ومكونه الرئيسي الليمونين (limonene) ضد الطور البالغ ويرقات حافرة الطماطم *Tuta absoluta* باستخدام تجارب بيولوجية بالملامسة والتبخير. بينت دراسة التركيبة الكيميائية للزيت الأساسي باستعمال التحاليل الكروموتوغرفية أن limonene (87.52%) وβ-myrcene (1.62%) وß-Ocimene (0.81%) و ß-pinene (0.61%) وα-pinene (0.56%) عناصر أساسية. بينت النتائج نجاعة الزيت الأساسي ومكونه الأهم ضد اليرقات والطور البالغ. في اختبارات السمية بالتبخير بلغت الجرعة القاتلة 10,65 و37,36 مكل/لتر على التوالي بالنسبة للزيت *C. aurantium* والليمونين الصافي. في اختبار السمية بالملامسة، كانت الحشرات البالغة لحافرة الطماطم أكثر حساسية للزيت الأساسي من مكونه الأهم حتى بأدنى جرعة. كانت الجرعة القاتلة المتحصل عليها بعد 48 ساعة على التوالي 0,21 و0,73 مكل. عندما تمت معاملة الحشرات بالزيت الأساسي ومسحوق الطين المنكه، سجلت فوارق معنوية في نسبة الموت وذلك حسب مدة التعرض. وقد كان مسحوق الطين المنكه أكثر سمية حيث بلغ الزمن القاتل $LT_{50}$ = 101,8 ساعة مقارنة بالزيت الأساسي النقي (الزمن القاتل $LT_{50}$ = 146,32 ساعة). وبالتالي فإن الأساسي للنارنج كان ساما لحافرة الطماطم ويمكن استخدام مسحوق الطين لتحقيق استقرار الزيوت الأساسية لزيادة نجاعتها وربما يتم استخدامها مبدئيا كمصدر لمواد مبيدة للحشرات غير مضرة بالبيئة.



*كلمات مفتاحية*: حافرة الطماطم، زيت أساسي، ليمونين، مبيد حيوي، مسحوق منكه، *Tuta absoluta*، *Citrus aurantium*


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# Chemical Composition and Herbicidal Potent of Cauliflower (*Brassica oleracea* var. *botrytis*) and Cabbage Turnip (*Brassica oleracea* var. *gongylodes*)

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**ABSTRACT**

**Saad, I., Rinez, I., Ghezal, N., and Haouala, R. 2017. Chemical composition and herbicidal potent of cauliflower (*Brassica oleracea* var. *botrytis*) and cabbage turnip (*Brassica oleracea* var. *gongylodes*). Tunisian Journal of Plant Protection 12: 95-113.**

This study was conducted to evaluate the phytochemical content and allelopathic potential of two cabbages botanical varieties leaves, ie. cauliflower *(Brassica oleracea* var. *botrytis)* and cabbage turnip *(B. oleracea* var. *gongylodes)*. Their aqueous and organic extracts were evaluated on lettuce (*Lactuca sativa*) and one of the most dominant weeds in Tunisia, nettle-leaf goosefoot (*Chenopodium murale*). Field experiments were conducted to evaluate the smothering potential of the two varieties. The total phenolics, flavonoïds, flavonols and flavones, alkaloïds, and proanthocyanidins contents were higher in the aqueous extracts of both varieties. For organic extracts, petroleum ether and methanol cauliflower extracts and chloroform and methanol cabbage turnip extracts were the richest ones. All aqueous and organic extracts had significantly delayed germination, reduced its rate and affected seedling growth. Reduction of germination and growth were more important using the higher concentrations and in presence of cabbage turnip extract. The organic extracts of both varieties had significantly inhibited the seedling growth of target species, especially petroleum ether, and methanol cauliflower extracts and chloroform and methanol cabbage turnip ones. Field experiment highlighted the smothering potential of the two varieties and confirmed the higher allelopathic potential of cabbage turnip as compared to cauliflower.

*Keywords:* Cabbage turnip, cauliflower, *Chenopodium murale*, growth, phytochemical analysis, smothering potential, speed germination

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The current trend in agriculture production is to find a biological solution to reduce the perceived hazardous impacts from herbicides and insecticides (Khanh et al. 2005). Allelopathy is a natural and an environment-friendly phenomenon which was proved to be a

promising tool for weed control, increase of crop yields, decrease of reliance on both synthetic pesticides and for the improvement of the ecological environment (Hegab and Ghareib 2010). The allelopathic properties of plants may act as a biological weed control mechanism in agro-ecosystems and are effective tools to help resolve this critical issue (Xuan et al. 2005). Allelopathic crops release chemicals into the soil that can contribute to weed management through suppression of weed seed germination, seedling emergence and establishment and seedling growth (Haramoto and Gallandt 2004).

Plants may store these chemicals in cells in bound forms, for example as water-soluble glycosides of alkaloids, flavonoids and phenols (Javed 2011) and release them into the environment by special glands on stems, leaves and roots (Sugiyama and Yazaki 2012) by a variety of mechanisms, including decomposition of residues, volatilization, and root exudation (Cruz-Ortega et al. 2007). Once released into environment, these compounds may affect the neighboring receiver plants (Javed 2011) and play an important role on the pattern of vegetation, and on crop productivity.

*Brassica* species, including *B. oleracea*, are frequently cited as allelopathic crops (Haramoto and Gallandt 2004) and are a significant source of glucosinolates (Iori et al. 2004; Kusznierewicz et al. 2008; Windsor et al. 2005), polyphenols, flavonoids (Jaiswal et al. 2012), proanthocyanidins (Bahorun et al. 2004), and alkaloids (Guyria et al. 2015).

Many studies have shown fungicidal (Martinez et al. 2011), nematicidal (Wu et al. 2011), and insecticidal (Schramm et al. 2012) properties of *B. oleracea* secondary metabolites, but few have evidenced the role of phenolics, flavonoids and alkaloids compounds of Brassicaceae crops in inhibiting germination and seedling growth of weeds (Batish et al. 2008; Singh and Kaur 2014). Moreover, most previous works affirmed that glucosinolates and their breakdown products are the major chemical components responsible for the herbicidal activity in *Brassica* spp. (Petersen et al. 2001).

Therefore, the first aim of this study was to confirm richness of *B. oleracea* species in polyphenols, flavonoids, flavonones/flavonols, proanthocynins and alkaloids through a phytochemical screening of aqueous and organic extracts of external leaves of two varieties of this species namely cauliflower (*B. oleracea* var. *botrytis*) and cabbage turnip (*B. oleracea* var. *gongylodes*). The second aim is to evaluate the leaf aqueous and organic extracts of both cabbages varieties effects on germination and growth of lettuce (*Lactuca sativa*) and nettle-leaf goosefoot (*Chenopodium murale*), a weed accompanying cabbage crops. A field experiment will be also conducted in order to evaluate the smothering potential of these cabbage varieties on weed biomass, especially nettle-leaf goosefoot.

## MATERIALS AND METHODS

### Plant material.

Cauliflower and cabbage turnip leaves were collected after eliminating fruiting heads at the harvesting stage from organically grown crops at *Institut Supérieur Agronomique* of Chott-Mariem, Sousse, Tunisia (Latitude 35°55N, Longitude 9°28E, Altitude 15 m).

### Aqueous extraction.

Fresh cauliflower and cabbage turnip leaves were rinsed and then oven-

dried at 60°C for 72 h and grinded. Fifty grams of each dried material were soaked in 1 liter of distilled water at room temperature for 24 h to give a concentration of 50 g/l (Chon et al. 2005). The extracts were filtered several times and kept at 4°C in the dark until use.

**Organic extraction.**

Sequential extractions were carried out with organic solvents of increasing polarity: petroleum ether, chloroform, and methanol. A 100 g-sample of dried leaf powder was immersed in organic solvent for 7 days at room temperature. Organic extracts were evaporated to dryness under reduced pressure at 45-50°C, using rotavapor R-114 (Buchi, France). The residue was weighed and the yield was determined. Dry fractions were stored at 4°C until use. The extracts were tested at three concentrations (1, 3, and 6 mg/ml) in bioassays.

**Phytochemical screening.**

**Total phenolics (TP) content.** The TP content was measured using the modified Folin–Ciocalteu method (Velioglu et al. 1998). Sample extract (100 µl) was mixed with 500 µl of 1/10 diluted (in Milli-Q water) Folin–Ciocalteu phenol reagent and allowed to react for 5 min in the dark at room temperature. Then, 400 µl of sodium bicarbonate (7.5%) were added to the mixture. After 90 min of incubation in the dark at 30°C, the absorbance was read at 765 nm. TP was expressed as mg gallic acid equivalent/g dry matter (mg GAE/g dw) using gallic acid calibration curve ($R^2$ = 0.96).

**Total flavonoïds (TFd) content.** The TFd content was determined spectrophotometrically according to standard method (Quettier et al. 2000). Briefly, 0.5 ml of 2% solution of $AlCl_3$ in methanol was mixed with the same volume of extract. Absorption readings at 430 nm were taken after 30 min against a blank. TFd content was expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using quercetine calibration curve ($R^2$ = 0.999).

**Total flavonols and flavones (TFl) content.** The TFl content was determined using the method of Kumaran and Karunakaran (2007). To 2 ml of sample, 2 ml of 2% $AlCl_3$ methanol and 3 ml (50 g/l) sodium acetate solutions were added. The absorption at 440 nm was read after 2.5 h of incubation at 20°C. TFl content was expressed as mg quercetine equivalent/g dry weight (mg QE/g dw) using quercetine calibration curve ($R^2$ = 0.995).

**Total proanthocyanidins (TPA) (condensed tannins) content.** The TPA content determination was based on the procedure reported by Sun et al. (1998). A volume of 0.5 ml of extract was mixed with 3 ml of 4% vanillin–methanol (w/v) and 1.5 ml hydrochloric acid. The mixture was allowed to stand for 15 min, and then the absorbance was measured at 500 nm. TPA content was expressed as mg catechin equivalent/g dry weight (mg CE/g dw) using catechin calibration curve ($R^2$ = 0.998).

**Total precipitable alkaloïds (TA) content.** The TA content was determined by spectrophotometric method with Dragendorff reagent (Stumpf 1984). Principally, 300 µl of plant extract were mixed with 100 µl of Dragendorff reagent. After centrifugation at 7000 g for 1 min, the supernatant was removed and dissolved in 1 ml of 2.45 M NaI. An aliquot of 10 µl of each tube was added to 1 ml of 0.49 M NaI, after which the absorbance was read at 467 nm. TA

content was expressed as mg papaverine hydrochloride equivalent/g dry weight (mg PAHE/g dw) using papaverine hydrochloride calibration curve ($R^2$ = 0.99).

**Laboratory bioassays.**

**Tests with aqueous extracts.** Each cabbage extract was diluted appropriately with sterile distilled-water to give final concentrations of 10, 20, 30, 40 and 50 g/l. They were tested on lettuce known to be very sensitive to allelochemicals (Ervin and Wetzel 2003) and on *C. murale,* one of the most widespread weed species in Tunisia (Holmet al. 1991). Seeds were surface sterilized with 0.525 g/l sodium hypochlorite for 15 min, then rinsed four times with deionized water, imbibed in it at 22°C for 12 h and carefully blotted (Chon et al. 2005). Twenty imbibed seeds of target species were separately placed on the filter paper in 9 cm Petri plates, 5 ml of each extract were applied as per treatment. Seedlings watered with distilled water were used as control.

The Petri plates of lettuce and nettle-leaf goosefoot were then placed in a growth chamber at 21 ± 2°C and 25 ± 2°C temperature, respectively, and a 16/8 h light and dark photoperiod and relative humidity of around 75%.

Treatments were arranged in a completely randomized design with three replications. Germinated seeds were counted at 24 h intervals during 7 days. Data were transformed to percent of control for analysis.

The total germination G was determined using the formula (Anjum and Bajwa 2005): G = (Nt/N) × 100, where Nt: Proportion of germinated seeds in each treatment for the final measurement and N: Number of seeds used in bioassays. This index shows the final germination percentage.

The index of germination GI was determined using the formula (Chiapuso et al. 1997): GI = (N1) × 1 + (N2-N1) × 1/2 + (N3-N2) × 1/3 +.... + (Nn-Nn-1) × 1/n, where, N1, N2, N3,…., Nn: Proportion of germinated seeds observed afterwards 1, 2, 3,…., n-1, n days. This index shows the germination delay induced by the extract (Dorado and Lopez-Fando 2006).

For growth test, twenty pre-germinated seeds of target species were separately placed on the filter paper in 9 cm Petri plates and 5 ml of each extract were applied as per treatment. Seedlings watered with distilled water were used as control. The Petri plates were then placed in the same conditions as described above. Treatments were arranged in a completely randomized design with three replications. Shoot and root length of receiver species were measured 7 days after sowing. Data were transformed to percent of control for analysis.

The inhibitory or stimulatory percent was calculated using the equation given by Chung et al. (2001): Inhibition (-)/stimulation (+) % = [(extract – control)/control] × 100, where extract: Parameter measured in presence of leaf extract and control: Parameter measured in presence of distilled water.

**Tests with organic extracts.** For organic extracts, two residues concentrated from petroleum ether, chloroform and methanol were dissolved in methanol and three concentrations were prepared (1, 3 and 6 mg/ml) to estimate their effect on germination and growth of target species. Two controls were considered, distilled water and methanol, to eliminate the eventual organic solvent effect. Filter papers placed in Petri plates were soaked with distilled water, methanol or various organic extracts. Organic solvent was

evaporated for 24 h at 24°C, then 5 ml distilled water was added and 20 soaked seeds were put to germinate for 7 days. Germination index and total germination were estimated as before and expressed in percent of the control. Treatments were arranged in a completely randomized design with three replications.

For growth test, 20 pre-germinated seeds of target species were placed in Petri plates as described above for seven days, then shoot and root length of receiver species were measured 7 days after sowing. Data were transformed to percent of control for analysis. The inhibitory or stimulatory percent was calculated using the same equation as described above.

**Field experiment.**

Field study was conducted during 2013 and 2014 at *Institut Supérieur Agronomique* of Chott-Mariem, Sousse, Tunisia. In the study area, the climate is typically Mediterranean with hot-dry summers and mild-rainy winters. According to long term weather data (1973-2006), maximum monthly temperatures ranged between 16 and 31°C and minimum monthly temperature varied from 7 to 21°C. Mean relative humidity varied from 69 to 71%. Monthly rainfall ranged between 2 and 58 mm (Bhouri Khila et al. 2013).

The field essay was carried out to evaluate and compare the smothering effect of the two varieties of *B. oleracea* on total weed biomass and nettle-leaf goosefoot biomass. It was carried out in randomized complete block design and occupied a total area of 100 $m^2$ (10 m × 10 m). Blocks were replicated three times and each block contained three plots, each plot (3 m × 2 m) were occupied with one of both varieties, and one plot was left uncultivated (fallow) and considered as control. Varieties of *B. oleracea* (namely *B. oleracea* var. *botrytis* (cauliflower) and *B. oleracea* var. *gongylodes* (cabbage turnip)) were planted in four rows at a density of 0.5 m × 0.6 m and 0.5 m × 0.3 m, respectively.

Weeds were sampled at crop harvest, from 1 $m^2$ quadrate thrown at the middle of each plot. Aboveground weeds biomass were collected, identified and counted, then dried and weighed.

**Statistical analysis.**

All data were reported as mean ± standard deviation (SD) of three replicates for biological activities and of five replicates for phytochemical analysis. ANOVA and a post hoc Duncan Multiple Range tests were performed with IBM SPSS Statistics version 20, for Windows program, to analyze treatment differences. The means were separated on the basis of least significant differences (LSD) at the 0.05 probability level.

Weed biomass data were analyzed by ANOVA (IBM SPSS Statistics version 20) for randomized complete block design and performed for all treatments in the field experiment. The treatment and interaction LSD of the means were used to separate treatment means at 5% level of significance.

## RESULTS

**Phytochemical analysis.**

Cauliflower aqueous extract was shown to contain higher amounts of total polyphenols (7.17 mgGAE/g dw) and total alkaloids (2.27 mg PAHE/g dw) whereas cabbage turnip aqueous extract was richer in condensed tannins (19.28 mg CE/g dw) and flavonones (4.26 mg QE/g dw) (Table 1). Important amounts of alkaloïds were detected in cauliflower petroleum ether (2.38 mg PAHE/gdw) and chloroform extracts (2.25 mg PAHE/gdw) and at little amounts in methanol extract. Contrarily for cabbage

turnip, higher amounts of alkaloids were recorded in methanolic extract (1.71 mg PAHE/gdw) (Table 1), whereas condensed tannins were completely absent in both methanolic cabbage extracts.

**Table 1.** Total phenols (TP), flavonoïds (TFd), proanthocyanidins (TPA), flavonols and flavonones (TFl) and alkaloids (TA) contents recorded in petroleum ether (PE), chloroform (CH), methanol (MET) and water (W) cauliflower and cabbage turnip extracts

| Extract | TP (mg GAE/g dw) | TFd (mg QE/g dw) | TPA (mg CE/g dw) | TFl (mg QE/g dw) | TA (mg PAHE/g dw) |
|---|---|---|---|---|---|
| **Cauliflower** | | | | | |
| **PE** | 2.97±0.07 c | 1.3±0.01 b | 3.07±0.61 b | 1.37±0.05 c | 2.38±0.35 c |
| **CH** | 0.16±0.02 a | 0.23±0.01 a | 0.3±0.02 a | 0.11±0.01 b | 2.25±0.02 a |
| **MET** | 2.23±0.05 b | 1.92±0.11 c | 0.00a | 1.06±0.03 a | 0.77±0.06 b |
| **W** | 7.17±0.37 d | 3.45±0.26 d | 16.55±1.18 c | 2.95±0.07 d | 2.27±0.35 c |
| **Cabbage turnip** | | | | | |
| **PE** | 0.67±0.01 a | 0.73±0.03 a | 2.02±0.08 c | 0.38±0.04 b | 0.54±0.03 a |
| **CH** | 0.44±0.08 a | 2.2±0.07 c | 1.21±0.05 b | 0.76±0.01 a | 0.78±0.06 b |
| **MET** | 1.87±0.35 b | 1.13±0.12 b | 0.00a | 0.74±0.04 c | 1.71±0.29 c |
| **W** | 5.43±0.34 c | 3.5±0.05 d | 19.28±0.42 d | 4.26±0.18 d | 2.06±0.16 d |

GAE: Gallic acid equivalent; QE: Quercetine equivalent; CE: Catechin equivalent; PAHE: Papaverine hydrochloride equivalent; dw: Dry weight; Means within columns sharing the same letters are not significantly different at $P \leq 0.05$.

**Phytotoxicity of aqueous extracts .**

**Aqueous extracts effect on seed germination.** Water extracts from cauliflower and cabbage turnip leaves showed inhibitory effects on germination of lettuce and nettle-leaf goosefoot seeds. For lettuce, germination was suppressed from 40g/l with cauliflower extract and at 50 g/l with cabbage turnip extract (Table 2). However for nettle-leaf goosefoot seeds, the inhibitory effect was observed rather in delay of germination speed (GI) than in the final germination (G). GI reached 50 and 17.97%, respectively with cauliflower and cabbage turnip leaves extracts at 50 g/l (Table 2).

**Table 2.** Germination index (GI) and total germination (G), expressed in percentage over control (C), of target species lettuce and nettle-leaf goosefoot germinating in presence of water extracts from leaves of cauliflower and cabbage turnip tested at different concentrations

| Variety | Concentration (g/l) | Lettuce | | Nettle-leaf goosefoot | |
|---|---|---|---|---|---|
| | | G (% C) | GI (% C) | G (% C) | GI (% C) |
| Cauliflower | 10 | 100.00c | 93.52±12.65 d | 86.66±4.71 a | 78.35±6.59 c |
| | 20 | 96.66±2.35 c | 42.23±4.61 c | 90±4.08 a | 63.54±2.10 b |
| | 30 | 57.54±5.36 b | 19.35±4.35 b | 86.66±2.35 a | 57.60b±4.68a |
| | 40 | 1.66± 2.35 a | 0.47±0.67 a | 83.33±4.71 a | 52.10±2.63 a |
| | 50 | 0.00a | 0.00a | 86.66±2.35 a | 50.82±2.34 a |
| Cabbage turnip | 10 | 100.00a | 88.40±6.57 a | 60.00b | 34.27±0.80 bc |
| | 20 | 96.60±2.42 b | 79.80±6.12 a | 66.66±6.23 b | 36.61±2.40 c |
| | 30 | 96.40±2.35 b | 76.70±16.3 a | 70±4.08 b | 37.38±3.66 c |
| | 40 | 25.40±0.61 c | 13.20±2.70 b | 61.66±4.71 b | 27.56±2.15 b |
| | 50 | 0.00d | 0.00c | 41.66±12.47 a | 17.97±6.31 a |

For each tested Brassicaceae, means within columns sharing the same letters are not significantly different at $P \leq 0.05$.

**Aqueous extracts effect on seedling growth.** Both extracts reduced the root length of lettuce and nettle-leaf goosefoot seedlings (Fig. 1). At lower concentration (till 20 g/l), cauliflower and cabbage turnip extracts stimulated shoot growth of lettuce seedlings (63 and 30%, respectively), whereas for the weed seedlings, shoot growth was slightly stimulated (30%) only by cauliflower extract applied at 10 g/l. Radical length of lettuce and nettle-leaf goosefoot was totally inhibited by both extracts from concentrations of 30 and 20 g/l, respectively. Shoot length inhibition over control of target species exceeded 60% in presence of cabbage turnip extracts from concentration of 30 g/l.

**Phytotoxicity of organic extracts.**

**Yield of organic extracts.** Cabbage turnip leaves had the highest yield compared to cauliflower leaves (2.44% vs. 0.82% with petroleum ether and 3.39% vs. 1.18% with chloroform, respectively). Concerning methanol extract, leaves of both varieties showed the same extraction yield (4.4%). Compared with petroleum ether and chloroform, methanol extract gave the highest yield for both cabbage species (Table 3).

**Effect of organic extracts on germination.** Cauliflower extract in all solvents did not influence the germination rate (G) of lettuce except with petroleum ether extract used at 3 mg/ml, where a slight inhibition (15% over control) was recorded. However, the extract reduced the germination index (GI) of lettuce seeds with petroleum ether (GI reached means of 45% over control at all concentrations) and with methanol (GI ranged from 32% to 77.68%) extracts. However using the chloroform extract, the germination was slightly slowed (8% over control) (Table 4).

For nettle-leaf goosefoot, cauliflower extract was found to be very inhibitory for germination as estimated by rate and speed in all cases. Germination rate inhibition reached 76, 85, and 80%, respectively, with petroleum ether (at 6 mg/ml), chloroform (at 6 mg/ml) and methanol (at 1 mg/ml). Corresponding values of GI were 13.47, 7.7, and 16.18%, respectively.

The cabbage turnip extracts of petroleum ether and chloroform did not

affect G and GI of lettuce which was around 98%. However, weed germination rate was canceled in petroleum ether and decreased from 58.7% at 1 mg/ml to 21.74% at 6 mg/ml in chloroform (Table 4).

The methanol extract slightly affected the lettuce germination rate, at 6 mg/ml, which was reduced by 10% over control, but was very toxic for germination index which ranged from 65.77 to 29.63% over control at all concentrations. In presence of methanol extract, nettle-leaf goosefoot germination rate decreased from 28.26 to 10.86% when used at the concentrations 1 mg/ml and 3 mg/ml, respectively and was suppressed at 6 mg/ml (Table 4).

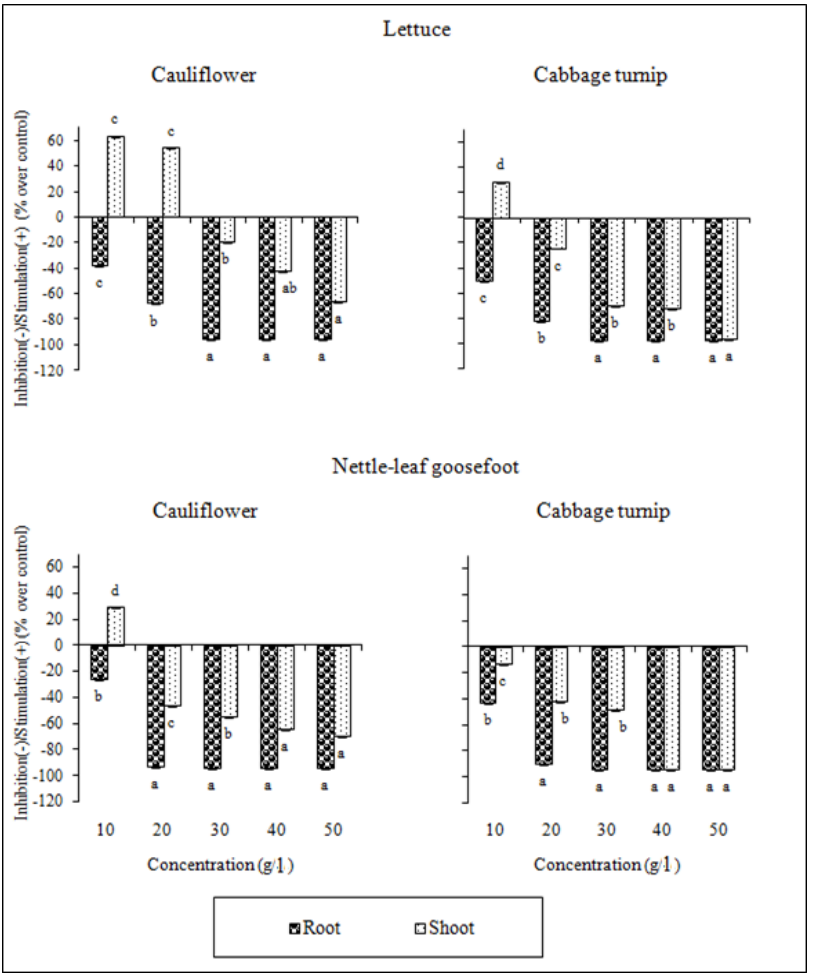


**Fig. 1.** Effect of aqueous extracts of leaves of cauliflower and cabbage turnip on root and shoot growth of lettuce and nettle-leaf goosefoot, 7 days after germination.
Value = Average ± SD, n=3. Different letters of bars indicate significant differences among treatments at $P \leq 0.05$ based on Duncan Multiple Range test.

**Table 3.** Residues yield (percentage of dry matter) after successive extraction in three organic solvents (petroleum ether, chloroform, methanol) of leaves of cauliflower (*Brassica oleracea* var. *botrytis*) and cabbage turnip (*Brassica oleracea* var. *gongylodes*)

| Extract solvent | Cauliflower yield (%) | Cabbage turnip yield (%) |
|---|---|---|
| **Petroleum ether** | 0.82 | 2.44 |
| **Chloroform** | 1.18 | 3.39 |
| **Methanol** | 4.43 | 4.4 |

**Table 4.** Germination index (GI) and total germination (G), expressed in percentage over control (C) of target species: Lettuce and nettle-leaf goosefoot germinating in presence of organic extracts from cauliflower and cabbage turnip leaves applied at different concentrations

| Extract/ Concentration (mg/ml) | | Lettuce | | Nettle-leaf goosefoot | |
|---|---|---|---|---|---|
| | | GI (%C) | G (%C) | GI (%C) | G (%C) |
| **Cauliflower** | | | | | |
| **Petroleum ether** | **1** | 48.66 bc | 96.66 b | 32.7 bc | 43.47 b |
| | **3** | 37.96 ab | 85 a | 28.57 abc | 41.30 ab |
| | **6** | 49.13 bc | 98.33 b | 13.47 ab | 23.91 ab |
| **Chloroform** | **1** | 100.71 d | 98.33 b | 39.62 c | 47.82 b |
| | **3** | 92.3 d | 98.33 b | 33.92 bc | 47.82 b |
| | **6** | 100.3d | 100 b | 7.70 a | 15.21 a |
| **Methanol** | **1** | 58.77 c | 100 b | 16.18 ab | 19.56 ab |
| | **3** | 77.68 bc | 98.33 b | 21.7 abc | 32.60 ab |
| | **6** | 32.01 a | 96.66 b | 20.12 abc | 32.60 ab |
| **Cabbage turnip** | | | | | |
| **Petroleum ether** | **1** | 98.58 c | 98.33 b | 10.47 ab | 13.04 ab |
| | **3** | 94.53 c | 96.67 b | 0.00 a | 0.00 a |
| | **6** | 93.71 c | 98.33 b | 0.00 a | 0.00 a |
| **Chloroform** | **1** | 98.45 c | 98.33 b | 48.53 d | 58.70 c |
| | **3** | 97.08 c | 98.33 b | 36.63 c | 52.18 c |
| | **6** | 90.84 c | 96.67 b | 16.08 b | 21.74 b |
| **Methanol** | **1** | 65.77 b | 100.00 b | 18.52 b | 28.26 b |
| | **3** | 62.21 b | 100.00 b | 5.62 ab | 10.86 ab |
| | **6** | 29.63 a | 90.00 a | 0.00 a | 0.00 a |

In each column, values with the same letter are not significantly different at $P \leq 0.05$

**Effect of organic extracts on seedling growth.** Using cauliflower extracts, the lettuce seedling growth was close to control or was slightly stimulated except in presence of petroleum ether extract used at 3 and 6 mg/ml which caused an average reduction of 60 and 51%, respectively, for roots and shoots. With cabbage turnip extract, lettuce seedlings growth was slightly stimulated, except the extracts of petroleum ether (3 and 6 mg/ml) and methanol (6 mg/ml) where maximum root and shoot growth inhibitions reached respectively, 40 and 33% with petroleum ether applied at 6 mg/ml. In other cases, the percentage stimulation varied between 4.26 and 22.78% for roots and between 1.07 and 36.86% for shoots (Fig. 2).

For nettle-leaf goosefoot seedlings, root growth was reduced by all organic extracts of cauliflower and

cabbage turnip, except in presence of cabbage turnip in chloroform extract (6 mg/ml) where a stimulation of 45.54% was recorded. Maximum inhibitions in root growth (72.28%) were registered with all organic extracts used at 6 mg/ml and with petroleum ether at 3 mg/ml. Maximum inhibition of shoot growth (a means of 64%) was recorded with petroleum ether extracts of the two varieties at 6 mg/ml (Fig. 2).

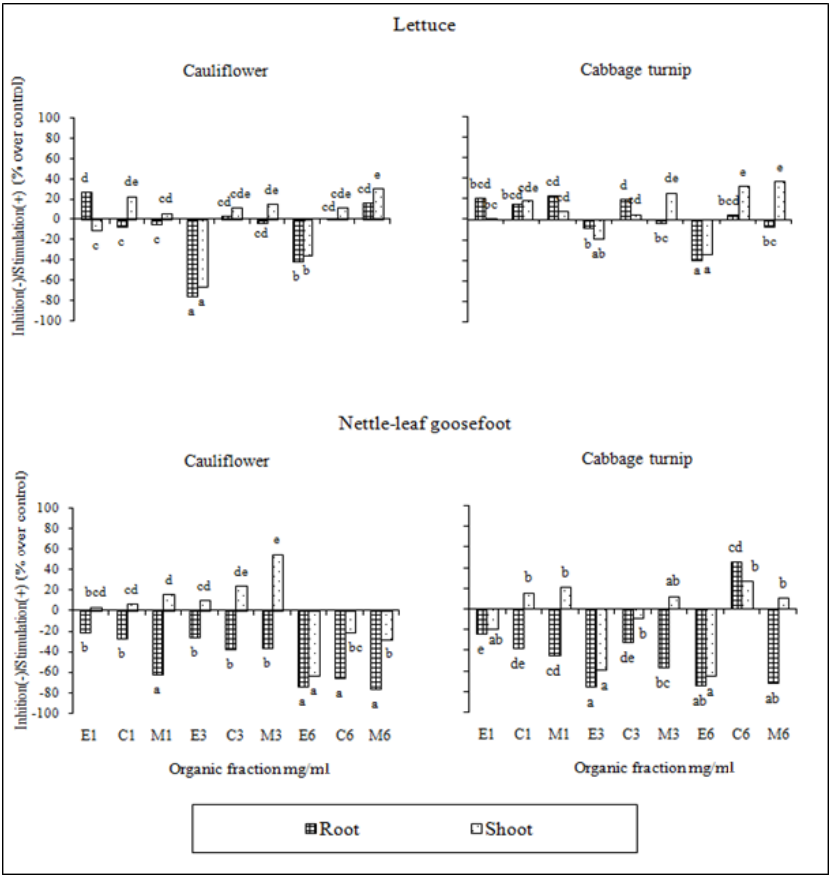


**Fig. 2.** Effect of organic extracts of cauliflower and cabbage turnip leaves prepared with Petroleum ether (E), Chloroform (C) and Methanol (M), applied at 1 (E1, C1, M1), 3 (E3, C3, M3) and 6 mg/ml (E6, C6, M6) on root and shoot growth of lettuce and nettle-leaf goosefoot, noted 7 days after germination. Value = Average ± SD, n=3. Different letters of bars indicate significant differences among treatments at $P \leq 0.05$ based on Duncan Multiple Range test.

**Evaluation of smothering potential of cabbages varieties**

There were great differences in total biomass of weed species between the plots cropped with test cabbage varieties and the fallow plots (uncultivated) in both years of experimentation (Table 5). Reduction

over fallow of total weed biomass was greater in cabbage turnip plots as compared with cauliflower plots in both years. In fact, total weed biomass was reduced over fallow plots by about 54 and 66% (means of two years), respectively in cauliflower and cabbage turnip plots. Cabbage turnip has also greater smothering potential of nettle-leaf goosefoot seedlings as compared with cauliflower.

**Table 5.** Dry weight of total weeds and nettle-leaf goosefoot (g/m$^2$), in cultivated plots by cauliflower and cabbage turnip and in fallow (not cultivated) and its reduction (% over fallow) during 2013 and 2014

| Cultivated plot | Total weed dry weight (g/m$^2$) | Reduction (% over Fallow) | Nettle-leaf goosefoot dry weight (g/m$^2$) | Reduction (% over Fallow) |
|---|---|---|---|---|
| **2013** | | | | |
| **Fallow** | 571.9±177.15 a | - | 58.8±30.37 a | - |
| **Cauliflower** | 266.27±26.8 b | 53.44 | 42.07±34.85 a | 28.45 |
| **Cabbage turnip** | 211.7±18.19 b | 63.00 | 6.11±4.06 a | 89.60 |
| **2014** | | | | |
| **Fallow** | 904.74 ±98.63 a | - | 292.13±69.10 a | - |
| **Cauliflower** | 405.7±69.66 b | 55.15 | 48.20±24.80 b | 83.50 |
| **Cabbage turnip** | 278.1±34.77 b | 69.27 | 20.87±34.01 b | 92.85 |

All values are average of three replicates ± SD. Means with the same letters in a column are not significantly different at $P \leq 0.05$.

Total biomass of nettle-leaf goosefoot was reduced over fallow by about 56% vs. 91% (means of two years), respectively, in cauliflower and cabbage turnip plots (Table 5). Total weed biomass in fallow plots was greater in the year 2014 as compared with year 2013 (904.74 g/m$^2$ vs. 571.9 g/m$^2$, respectively in 2014 and 2013). Similarly for nettle-leaf goosefoot biomass, it was greater in fallow plots in 2014 (292.13 g/m$^2$) than in 2013 (58.8 g/m$^2$). Moreover, in both cabbage varieties plots, total weed biomass decrease was most enounced in the second year of experimentation. In cauliflower plots, dry weight of nettle-leaf goosefoot decreased by 28.45% vs. 83.5% over fallow, respectively in 2013 and 2014. Similarly in cabbage turnip plots, nettle-leaf goosefoot weed dry weight was reduced over fallow by 89.6 and 92.85%, respectively in 2013 and 2014 (Table 5).

## DISCUSSION

Results revealed richness of *B. oleracea* species in phenolic compounds and alkaloïds which is in accordance with previous studies (Bahorun et al. 2004; Scalzo et al. 2008; Vallejo et al. 2003; Wu and Prior 2005). Results have also shown a variation in secondary metabolites amounts with cabbage varieties. Podsedek et al. (2006) found the highest content of phenolics in red cabbage and the lowest in white cabbage in a comparison of several varieties of red cabbage, white cabbage, savoy cabbage, and Brussels sprouts. The specific phenolic compounds also varied as anthocyanins dominated in red cabbage while hydroxycinnamic acids predominated in other *Brassica* cultivars. Comparing several varieties of broccoli, Brussels sprouts, cabbage, cauliflower and Chinese cabbage showed that broccoli generally had the highest levels of phenolics, vitamin C, β-carotene, lutein

and α-tocopherol, with Brussels sprouts a close second (Singh et al. 2007).

Phytochemical analysis of organic extracts revealed more contents in phytochemicals in methanol extracts as compared with petroleum ether and chloroform. In fact, extraction yield of phenolics and flavonoïds contents depended greatly on the solvent polarity (Turkmen et al. 2006). It may be due to higher polarity of methanolic solvent (Namuli et al. 2011) which is able to extract more phenolic and flavonoïd compounds (Cowan 1999) and to draw high variety of plant constituents than the other solvents (Paulsamy and Jeeshna 2011). In particular, methanol has been generally found to be more efficient in extraction of lower molecular weight polyphenols while the higher molecular weight flavanols are better extracted with aqueous acetone (Dai and Mumper 2010). In addition, phytochemicals like, polyphenols, flavonoïds, flavonones/flavonols, were found to be present in aqueous extracts and all tested organic extracts. However, condensed tannins were present in all the extracts but completely absent in methanolic ones. This could be due to its high molecular weight and its solubility in water (Khoddami et al. 2013). Similar results were observed by Guyria et al. (2015) who revealed that the methanolic extract of broccoli (*B. oleracea* var. *italica*) contained high quantities of alkaloids, tannins, flavonoïds, phenols and proteins whereas saponins were absent. Hexane extract of broccoli contains high amount of alkaloids, phenols and proteins. Acetone and water extracts of broccoli possess little amounts of sterols, alkaloids and tannins but are rich in phenols and proteins (Guyria et al. 2015).

For germination tests, often the phytotoxic effect is not observed in the final germination percentage, but rather in the speed of germination, which can provide important indications on the allelochemicals. Ahmed and Wardle (1994) affirmed that germination index is more sensitive indicator of allelopathic effects occurring during the germination process. Plants that germinate at slower rates are often smaller (Fallah Touzi and Baki 2012) and delays in seed germination of any species can have important biological implications, because this will affect the establishment of seedlings in natural conditions (Chaves et al. 2001) and their chances of competing for resources with neighboring species (Xingxinag et al. 2009). The germination rate (G) and speed inhibition (GI) increased with the extract concentration. This finding is supported by Turk and Tawaha (2003) who registered an increase germination inhibition of wild oat (*Avena fatua*) with the increase of black mustard (*Brassica nigra*) extract.

Effects of allelochemicals on seed germination appear to be mediated through a disruption of normal cellular metabolism rather than through damage of organelles. Reserve mobilization, a process which usually takes place rapidly during early stages of seed germination seems to be delayed or decreased under allelopathy stress conditions (Gniazdowska and Bogatek 2005). Alterations in germination patterns can be caused by changes in the cell membranes permeability, RNA transcription and translation, secondary messengers integrity, respiration, conformation of enzymes and receptors, or a combination of these changes (Ferreira and Aquila 2000). For example, 6-methoxy-2-benzoxalinone (MBOA) inhibits the germination of lettuce seeds by impeding inducement of α-amylase synthesis, which mobilizes the stored reserves and maintains seed respiratory activity (Kato-

Noguchi and Macias 2005). Baleroni et al. (2000) showed that *p*-coumaric and ferulic acids increased total lipid content in the cotyledons of canola seeds and suggested that this change is due to reduced mobilization of reserves during germination in the presence of these phenolic compounds.

Target species showed different responses to aqueous and organic cabbages extracts. These results agree with earlier studies reporting that allelochemicals, which inhibited the growth of some species at certain concentrations, might stimulate the growth of same or different species at lower concentrations (Narwal 1994). In growth tests, radical length was more affected by allelochemicals than hypocotyl length, for both target species. Many studies have reported the most sensitivity to allelochemicals of roots compared to aerial parts of seedlings (Ercoli et al. 2007; Oliveira and Campos 2006; Rahman 2006). This result was attributed to the fact that roots are the first to absorb allelochemicals from the environment (Turk and Tawaha 2003) and its growth is characterized by high metabolic rates and, for this reason, they are highly susceptible to environmental stresses such as allelochemicals in soils (Hussain and Reigosa 2011). Root growth inhibition by allelochemicals can be due to changes in DNA synthesis in cells of apical root meristem, alteration of the mitochondrial metabolism (Abrahim et al. 2000) or changes in cell mitotic indices (Iganci et al. 2006) and seedling length reduction may be attributed to the reduced rate of cell division and cell elongation due to the presence of allelochemicals in the aqueous extracts (Javaid and Anjum 2006).

Toxicity of cauliflower and cabbage turnip organic was most expressed for nettle-leaf goosefoot seeds. The difference in sensitivity of both varieties shows the specificity of allelochemicals which have no, or little, affected lettuce germination in this assay. Similar results were obtained with Saad et al. (2014) where white and red cabbage extracts were most toxic for *C. murale* seeds as compared with *L. sativa* seeds and germination inhibition of target species increased with concentrations. Seigler (1996) showed that allelochemicals can be selective in their actions and plants can be selective in their responses. The selectivity in allelopathic effects may be of considerable interest for the control of weeds in crops (Seigler 1996). Fallah Touzi and Baki (2012) demonstrated that germination rate of barnyard grass (*Echinochloa crus-galli)* decreased in presence of ethanol extract of *B. juncea* and such chemicals were both species-specific and concentration-dependent and these characteristics may influence the density and the composition of individual plant communities. In fact, germination and early seedling growth assay have been regarded as a basic experiment for figuring out the effect of any plant extract upon target plant development (Madany and Saleh 2015). Results revealed that both cabbage varieties extracts caused a noticeable reduction in both germination rate and early growth of lettuce and nettle-leaf goosefoot seedlings. Such depressive effect may be imputed to the adverse impact of phytochemicals in the extracts on enzymatic processes through some interactions with organic substances of the cell (Chethan et al. 2008). Indeed, earlier studies corroborated that phytochemicals, including phenolic acids (Batish et al. 2008; Singh and Kaur 2014), phenolics, flavonoïds and alkaloïds (Rice 1979), are potent germination and growth inhibitors. These phytotoxins are known to affect the cell

structure and physiological functions of the target species resulting in impaired germination and diminished growth (Duke and Dayan 2006). Inhibition in lipid mobilization in the presence of ferulic and *p*-coumaric acids was detected during canola (*B. napus)* seed germination (Baleroni et al. 2000), as well as in sunflower (*Heliantus annus*) seeds germinating in the presence of alkaloids from thorn apple (*Datura stramonium)* (Levitt et al. 1984). Phenolic allelochemicals can also lead to increased cell membrane permeability and increase lipid peroxidation followed by slow growth or death of plant tissue (Zhao et al. 2010).

Organic extracts of cauliflower and cabbage turnip were most toxic for nettle-leaf goosefoot growth as compared with lettuce and their toxicity increased with concentration. For this species, greater inhibitions of root growth and germination speed were recorded with all organic fractions of cauliflower extract. However, in cabbage turnip extract, the highest values of growth inhibition were recorded in petroleum ether and methanol extracts and those of germination index were recorded in petroleum ether extract. The phytotoxicity variation of cabbage varieties could be attributed to the differences in secondary metabolites richness of different organic fractions. Indeed, due to the different polarities of organic solvents, the organic extracts contain different allelochemicals groups, which explain their differential toxicity (Omezzine and Haouala 2013).

The difference in toxicity between aqueous and organic extracts could be attributed to the interactions between biologically active compounds that could act in synergy or antagonism (Omezzine and Haouala 2013). Anaya (2006) suggested that the combined effect varied, and in half of the cases, it followed the pattern expected under the assumption of independence; in the other, either synergistic or antagonistic interactions were found in both germination and elongation. Lyu et al. (1990) and Rasmussen and Einhellig (1997) reported that the combined actions of phenolic compounds could be additive, antagonistic or synergistic.

Recently, Saad et al. (2014) reported the smothering potential of white and red cabbage varieties on total weed biomass and density. Such effective suppression of total weed biomass may be attributed to several reasons. Crop allelopathy may play an important role in agrosystems as it could affect the germination and growth of neighboring plants. Roots are the site of greatest activity within the soil matrix during crop growth (Bertin et al. 2003) and crop plants have the capability to produce and exude allelochemicals into their surroundings to suppress the growth of weeds in their vicinity (Huang et al. 2003).

The allelopathic potential of *Brassica* species has been well documented (Haramoto and Gallandt 2004; Matthienssen and Kirkegaard 2006). The variation in weed smothering potential with cabbages varieties revealed in field assays may be explained by the differences in their allelochemicals levels, their glucosinolates profiles and their hydrolysis products which vary between *Brassica* species and cultivars (Castro et al. 2004; Chaplin-Kramer et al. 2011; Meyer and Adam 2008). It could also be due to differences in polyphenolic and alkaloids contents among these varieties, as found above (phytochemical analysis and germination and growth tests of aqueous and organic extracts). Singh et al. (2007) demonstrated a large variation in phenolic compounds between several cabbages varieties.

Total weed biomass and nettle-leaf goosefoot biomass were greater in fallow plots in the second year of field experiment as compared with the first one. Moreover, in plots cropped with both cabbages varieties, total weed biomass reduction was most enounced in the second year of experimentation. It is possible that allelopathy of these cabbages might have contributed to the greater suppression of nettle-leaf goosefoot. This may also partially explain why comparatively less nettle-leaf goosefoot biomass was observed during 2014 than 2013 in all plots (fallow and cultivated), probably due to the residual allelopathy from those crops carried over from the previous year 2013 (Narwal 2004). Allelopathy can affect many aspects of plant ecology including occurrence, growth, plant succession, structure of plant communities, dominance, diversity and plant productivity. Plants that germinate at slower rates are often smaller. This may seriously influence their chances of competing with neighboring plants for resources (Fallah Touzi and Baki 2012).

In line with these findings and similar to the results of the germination and growth bioassay, we can confirm allelopathic potential of cauliflower and cabbage turnip and their ability to smother weeds, especially the most abundant weed in Tunisia, nettle-leaf goosefoot. We can conclude also, that variation of both cabbages varieties in phenolic compounds, flavonoids and alkaloids amounts could be the principal raison of variation of its allelopathic potential.

The results of this study indicated that the phytochemicals content may contribute to the allelopathic activity of cabbage extracts, which is strongly dependent on the cabbage variety. Cabbage turnip extracts showed most important toxicity on target species as compared with cauliflower extracts. Similarly, in field experiment, smothering potential of this cabbage variety on weeds was more important. These results confirm the utility of introducing both cabbages varieties in a rotational crop system to improve biological weed control and decreasing herbicidal products use in agriculture.


**RESUME**

**Saad I., Rinez I., Ghezal N. et Haouala R. 2017. Composition chimique et potentialités herbicides du chou-fleur (*Brassica oleracea* var. *botrytis*) et du chou rave (*Brassica oleracea* var. *gongylodes*). Tunisian Journal of Plant Protection 12: 95-113.**

Cette étude a été réalisée pour évaluer la composition chimique et le potentiel allélopathique des feuilles de deux variétés botaniques de choux, le chou-fleur (*Brassica oleracea* var. *botrytis*) et le chou rave (*B. oleracea* var. *gongylodes*). Leurs extraits aqueux et organiques ont été évalués sur la laitue (*Lactuca sativa*) et l'une des adventices les plus dominantes en Tunisie, le chénopode des murs (*Chenopodium murale*). Des expériences sur le terrain ont été menées pour évaluer le potentiel étouffant des deux variétés. Les concentrations totales de phénols, de flavonoïdes, de flavonols et de flavones, d'alcaloïdes et de proanthocyanidines ont été élevées dans les extraits aqueux des deux variétés. Pour les extraits organiques, les extraits à l'éther de pétrole et au méthanol du chou-fleur et les extraits chloroformiques et méthanoliques du chou rave ont été les plus riches. Tous les extraits aqueux et organiques ont significativement retardé la germination, ont réduit son taux et ont affecté la croissance des plantules. La réduction de la germination et de la croissance ont été plus importantes aux plus fortes concentrations et en présence de l'extrait du chou rave. Les extraits organiques des deux variétés ont inhibé de manière significative la croissance des plantules cibles, en particulier ceux à l'éther de pétrole et au méthanol du chou-fleur et au chloroforme et au methanol du chou rave. L'essai

au champ a mis en évidence le potentiel étouffant des deux variétés et a confirmé le potentiel allélopathique plus élevé du chou rave comparé au chou-fleur.

*Mots clés*: Analyse phytochimique, *Chenopodium murale*, chou-fleur, chou rave, croissance, potentiel étouffant, vitesse de germination

## ملخص

**إيناس، سعد وإيمان ريناز ونادية الغزال وربيعة حوالة. 2017. التركيبة الكيميائية وقدرة إبادة الأعشاب للقرنبيط (*Brassica oleracea* var. *botrytis*) /للبروكلو والملفوف/للكرنب السلقي (*Brassica oleracea* var. *gongylodes*). Tunisian Journal of Plant Protection 12: 95-113.**

أجريت هذه الدراسة لتقييم التركيبة الكيميائية وقدرة المجاهضة لأوراق صنفين طبيعيين من الملفوف، القرنبيط (*Brassica oleracea* var. *botrytis*) والملفوف السلقي (*B. oleracea* var. *gongylodes*). تم تقييم مستخلصاتهما المائية والعضوية على نبتة الخس وعلى واحدة من أكثر الحشائش انتشارا في تونس، الخليمعة (*Chenopodium murale*). أجريت تجارب ميدانية لتقييم القدرة الخانقة للصنفين. كان المحتوى الكلي للفينول، الفلافونويد والفلافونول والفلافونون والالكالويد والاصباغ مرتفعا في المستخلصات المائية للصنفين. أما بالنسبة للمستخلصات العضوية، فقد كانت تلك المستخرجة بالايثير البترولي وبالميثانول للقرنبيط وبالكلوروفورم وبالميثانول للملفوف السلقي هي الأغنى. أدت جميع المستخلصات المائية والعضوية إلى تأخير إنبات الخس والخليمعة بشكل كبير وإلى تخفيض معدل إنبات ونمو البادرات. وسجلت أقوى التخفيضات في الإنبات والنمو مع التركيزات الأعلى لمستخلص الملفوف السلقي. وأدت المستخلصات العضوية لكل من الصنفين إلى الحد من نمو البادرات المستهدفة، وخاصة تلك المستخلصة بالايثير البترولي وبالميثانول للقرنبيط وبالكلوروفورم وبالميثانول للملفوف السلقي. أبرزت التجربة الميدانية القدرة الخانقة للصنفين والقدرة الأقوى للمجاهضة لدى الملفوف السلقي بالمقارنة مع القرنبيط.

*كلمات مفتاحية*: تحليل كيميائي نباتي، سرعة الإنبات، قدرة خانقة، قرنبيط، ملفوف سلقي، نمو، *Chenopodium murale*

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# Evaluation of Different Techniques for Economical Control of Weeds Associated to Chickpea

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**ABSTRACT**

**Khan, I.A., Waqas, M., Shah, S.M.A., Khan, N., and Khan, R. 2017. Evaluation of different techniques for economical control of weeds associated to chickpea. Tunisian Journal of Plant Protection 12: 115-122.**

A field experiment was carried out on farm at Peshawar, Pakistan, during spring 2012 for evaluating the efficacy of weed management strategies to control weeds associated to three chickpea cultivars and their cost benefit ratios. The design of the experiment was randomized complete block with split plot arrangement. Different chickpea cultivars (Karak-I, Sheenghar, and Karak-III) were assigned to main plot while weed control treatments i.e. black plastic mulch, white plastic mulch, saw dust mulch, wheat straw mulch, Stomp 330 EC, Dual Gold 960 EC, hand weeding and untreated control were assigned to subplots. The parameters recorded were the fresh weed biomass (kg/ha), the number of seeds/pod and the cost benefit ratio. The results revealed a relatively divergent response of various treatments and chickpea cultivars for all the studied parameters. The results showed that the lowest fresh weed biomass (655.33 kg/ha) was noticed in hand weeding followed by herbicides (Stomp 330EC and Dual Gold 960EC) while among chickpea cultivars the minimum fresh weed biomass (705.02 kg/ha) was recorded for Karak-III. Black plastic mulch and hand weeding positively affected the chickpea production where the maximum number of seeds/pod was of about 1.67 and 1.61, respectively. The cost benefit ratio results revealed that the highest net return to the farmer as a result of added cost to the crop was obtained by applying Stomp 330 EC (1:2.18) followed by Dual Gold 960 EC (1:1.94) and hand weeding (1:1.91). Hence, the present study recommends the sowing of chickpea cultivar Karak-III with hand weeding practice or herbicide application (Stomp 330 EC or Dual Gold 960 EC) at the recommended rate to obtain maximum weed control and high net income in the agro-ecological conditions of Peshawar.

*Keywords:* Chickpea, cost/benefit ratio, hand weeding, herbicide, Karak-III, mulch

Chickpea (*Cicer arietinum*) is a cool season pulse legume crop belonging to Fabaceae family. It is the third most important pulse in the world after dry beans and peas. Being a source of protein, it plays an important role in human nutrition for large population in the developing world and is considered to be a healthy food in many developed countries (Abbo et al. 2003). In Pakistan, chickpea is the most important pulse as well as a vegetable crop. The area under chickpea cultivation in Pakistan during 2011-12 was 1007.5 thousand ha with production of 284.4 thousand tons, while the average yield was 282 kg/ha. Similarly, in Khyber Pakhtunkhwa, the area under chickpea cultivation was 31.4 thousand ha with a production of 11 thousand tons, while the average yield

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was 350 kg/ha (MNFSR 2012). The average yield of chickpea in the developing countries is much higher as compared to Pakistan. Poor soil, inadequate moisture, harsh climatic conditions, weeds and inadequate or even no fertilizer supply constitutes the main factors responsible for low yield in Pakistan (Datta et al. 2009). Among these constraints, weeds strongly contribute in the yield reduction of chickpea because it is traditionally grown on residual soil moisture without any control of weeds in general. Yield losses due to weeds in chickpea ranged between 25 to 80% (Aslam et al. 2007). Control of weeds in various crops was accomplished using include manual, mechanical, cultural, biological, and chemical methods.

Herbicides are the most effective and quickest method of weed control. The judicious use of herbicides reduces yield losses caused by weeds and hence increase yields of various agronomical important crops including chickpea. When properly used, pre-emergence herbicides accomplish effective and economic weed control (Hassan et al. 2003). Among cultural methods, mulching is an effective non-chemical weed control method. Mulch is a material that covers the soil surface to protect and to improve the covered area (Ramakrishna et al. 2006). It lowers soil temperature, increases soil moisture, decreases weed density and enhances crop yield (Sinkeviciene et al. 2009). Khan et al. (2012) observed the effect of different herbicides (at pre- and post-emergence) and water extracts from two plants (*Parthenium* and *Eucalyptus*) on weeds and yield of chickpea. They concluded that Isoproturon 500 EW was very effective in suppressing chickpea-associated weeds by reducing weed density significantly as compared to control. In a recent research, Waqas et al. (2016) found that hand weeding followed by commercial herbicides depicted least density and biomass for weeds while among mulch treatments, the got superior weed control was under black plastic mulch. Keeping in view the importance of chickpea yield and its losses due to weeds, the present study was designed with the objective to assess the efficacy of different herbicides and mulches for controlling weeds and to select economically suitable control methods in chickpea.

## MATERIALS AND METHODS

### Trial site description

Field experiment was conducted during spring season 2012 at New Developmental Farm, The University of Agriculture Peshawar, Pakistan, situated about 1200 km in north of Indian oceans at an altitude of 350 m, latitude 34.01O N.

### Design ant treatments

The design of the experiment was randomized complete block (RCB) design with split-plot arrangement having three replications. The details of the main plots and sub-plots are given below.

**Main Plots: (Chickpea cultivars)**

1. Karak-I
2. Karak-III
3. Sheenghar

**Sub-plots: (weed management techniques)**

1. Black plastic mulch,
2. White plastic mulch,
3. Saw dust mulch,
4. Wheat straw mulch,
5. Stomp 330 EC2.5 l/ha,
6. Dual Gold 960 EC@ 2 l/ha,
7. Hand weeding (at 30, 60 and 90 days after sowing (DAS))
8. Control (untreated)

The size of each sub-plot was 4 × 1.5 $m^2$ with five rows and each row was 30 cm apart from other.

**Parameters**

The recorded parameters were fresh weed biomass (kg/ha), number of seeds/pod and cost-benefit ratio.

**Analysis**

All the recorded data for each trait was subjected individually to the ANOVA technique and means were separated through Fisher's protected LSD test at $P = 0.05$ using MSTATC computer software (Steel and Torrie 1980).

**RESULTS**

Analysis of the data revealed that control strategies, cultivars and their interactions had significant ($P = 0.05$) effect on fresh weed biomass. The treatment mean data in the given Table1 demonstrated that the minimum fresh weed biomasses (655.33 and 669.56 kg/ha) were obtained with hand weeding and Stomp 330 EC treatments, while the highest fresh weed biomass (1029.55 kg/ha) was recorded in weedy check treatments. Similarly, the chickpea cultivars mean data showed that the lowest fresh weed biomass (705.02 kg/ha) was calculated from Karak-III while the highest fresh weed biomass (785.71 kg/ha) was recorded in Sheenghar. The interaction data of control measures and cultivars showed that the lowest fresh weed biomass (608.69 kg/ha) was recorded in hand weeding × Karak-III whereas, the highest fresh weed biomass (1088.67 kg/ha) was noted in weedy check × Sheenghar.

The statistical analysis showed that differences among the various control strategies and cultivars were significant ($P = 0.05$) while the interaction of control strategies with cultivars showed no significant variation. The treatments mean data in given Table2 revealed that the lowest number of seeds/pod (1.30) was noticed in weedy check treatments while the highest number of seeds/pod (1.67 and 1.61) was calculated in black plastic mulch and hand weeding treatments, respectively. Significant differences ($P = 0.05$) were also recorded between cultivars with maximum number of seeds/pod (1.51) was given by cv. Karak-III while the lowest number (1.40) was recorded in Karak-1.

The calculated data given in Table 3 showed the cost benefit ratio for weed management strategies i.e. mulches, herbicides and hand weeding. The results showed that all the practiced techniques had significantly affected the crop yield and similarly varied in terms of costs. Highest cost benefit ratio (1:2.18) was resulted in treatments sprayed with Stomp 330EC followed by Dual Gold 960EC (1:1.94) and hand weeding (1:1.91) while the minimum cost benefit ratio (1:1.03) was recorded for saw dust.

**DISCUSSION**

The data revealed that both the weed control treatments and cultivars drastically affect fresh weed biomass. The lowest fresh weed biomass obtained in hand weeding treated plots were due to the three interventions during the cropping season in order to remove weeds from the plots contributing in reducing their number and biomass. The herbicides Stomp 330EC and Dual Gold 960EC controlled well weeds and produced low fresh biomass which might be due to selectivity of these herbicides to control weeds or delay their growth or disturb their internal functions resulting in poor development and therefore low fresh biomass. Similarly, among the chickpea cultivars, the minimum fresh weed

biomass in Karak-III was due to early growth and its smothering effect on weeds which led to lower weed biomass. The present results are in close proximity with the findings of Patel et al. (2006) who reported that hand weeding and Stomp 330 EC effectively reduced weed biomass. Many researchers also reported that hand weeding and herbicides doses have great influence on fresh weed biomass in chickpea crop (Khan et al. 2009). Other scientists stated that the lowest weed biomass in herbicides treatments were might be due to long persistence of herbicide in soil which negatively affects weed growth and biomass (Avola et al. 2008).

**Table 1.** Effect of weed control techniques on fresh weed biomass (kg/ha) in chickpea cultivars

| Treatment | Chickpea cultivar | | | Treatment means |
|---|---|---|---|---|
| | Karak-1 | Sheenghar | Karak-III | |
| **Black plastic** | 677.33 | 729.21 | 668.67 | 691.78 e |
| **White plastic** | 694.28 | 755.33 | 677.65 | 709.11 d |
| **Saw dust** | 721.31 | 767.15 | 697.67 | 728.78 c |
| **Wheat straw** | 741.33 | 778.24 | 724.66 | 748.11 b |
| **Stomp 330 EC** | 663.67 | 717.11 | 627.61 | 669.56 g |
| **Dual Gold 960 EC** | 675.25 | 740.67 | 647.71 | 687.89 f |
| **Hand weeding** | 647.67 | 709.63 | 608.69 | 655.33 h |
| **Weedy check** | 1012.11 | 1088.67 | 987.57 | 1029.55 a |
| **Cultivar means** | 729.11 b | 785.71 a | 705.02 b | - |

LSD for Cultivars (at $P = 0.05$) = 24.09 kg/ha; LSD for Treatments (at $P = 0.05$) = 2.13 kg/ha; LSD for Interaction (at $P = 0.05$) = 3.89 kg/ha. Means sharing the same letter in the respective category do not differ significantly based on LSD test at $P = 0.05$.

**Table 2.** Effect of weed control techniques on number of seeds/pod in chickpea cultivars

| Treatment | Chickpea cultivar | | | Treatment mean |
|---|---|---|---|---|
| | Karak-1 | Sheenghar | Karak-III | |
| **Black plastic** | 1.67 | 1.67 | 1.70 | 1.68 a |
| **White plastic** | 1.28 | 1.28 | 1.47 | 1.33 c |
| **Saw dust** | 1.30 | 1.27 | 1.30 | 1.29 c |
| **Wheat straw** | 1.47 | 1.47 | 1.47 | 1.47 b |
| **Stomp 330 EC** | 1.50 | 1.47 | 1.67 | 1.54 b |
| **Dual Gold 960 EC** | 1.30 | 1.27 | 1.47 | 1.34 c |
| **Hand weeding** | 1.47 | 1.67 | 1.70 | 1.61 a |
| **Weedy check** | 1.27 | 1.30 | 1.33 | 1.30 c |
| **Cultivar mean** | 1.40 b | 1.43 b | 1.51 a | - |

LSD for Cultivars (at $P = 0.05$) = 0.133 seed/pod; LSD for Treatments (at $P = 0.05$) = 0.032 seed/pod; LSD for Interaction (at $P = 0.05$) = Not significant. Means sharing the same letter in the respective category do not differ significantly based on LSD test at $P = 0.05$.

**Table 3.** Economic analysis of the different studied treatments

| Nature of expenditure | Treatment cost per ha in Rupees (Rs) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Black plastic** | **White plastic** | **Saw dust** | **Wheat straw** | **Stomp 330 EC** | **Dual Gold 960 EC** | **Hand weeding** | **Weedy check** |
| Ploughing (2 times) | 23826 | 23826 | 23826 | 23826 | 23826 | 23826 | 23826 | 23826 |
| DAP Fertilizer (2 bags/ha) | 8000 | 8000 | 8000 | 8000 | 8000 | 8000 | 8000 | 8000 |
| Seeds (60 kg/ha) | 3900 | 3900 | 3900 | 3900 | 3900 | 3900 | 3900 | 3900 |
| Black plastic mulch expenses for 1 ha | 16516 | - | - | - | - | - | - | - |
| White plastic mulch expenses for 1 ha | - | 22266 | - | - | - | - | - | - |
| Labors for mulch application | 600 | 600 | 600 | 600 | - | - | - | - |
| Saw dust mulch expenses for 1 ha | - | - | 34683 | - | - | - | - | - |
| Wheat straw mulch expenses for 1 ha | - | - | - | 30366 | - | - | - | - |
| Herbicides | - | - | - | - | 1625 | 1200 | - | - |
| Labors for spraying | - | - | - | - | 600 | 600 | - | - |
| Labors for weeding (3 times=12 days each) | - | - | - | - | - | - | 10800 | - |
| Labors for harvesting (8 working days) | 2400 | 2400 | 2400 | 2400 | 2400 | 2400 | 2400 | 2400 |
| Threshing | 1800 | 1800 | 1800 | 1800 | 1800 | 1800 | 1800 | 1800 |
| Total Expenditures | 57042 | 62792 | 75209 | 70892 | 42151 | 41726 | 50726 | 39926 |
| Grain yield (kg/ha) | 1473.1 | 1404.6 | 1191.9 | 1415.2 | 1413.8 | 1250.1 | 1491.6 | 979.8 |
| Gross Income : Grain Sale (1 kg=65 Rs) | 95751 | 91299 | 77473 | 91988 | 91897 | 81256 | 96954 | 63687 |
| Net Profit (Rs) | 38709 | 28507 | 2264 | 21096 | 49746 | 39530 | 46228 | 23761 |
| Cost Benefit Ratio | 1 : 1.68 | 1 : 1.45 | 1 : 1.03 | 1 : 1.29 | 1 : 2.18 | 1 : 1.94 | 1 : 1.91 | 1 : 1.59 |

*: **1 US $ = 104 Rs**

High value for seed/pod is desirable in chickpea (Ali et al. 2008). From the results, it is clear that hand weeding and mulches improves soil physical properties, effectively control weeds and make the availability of nutrients to chickpea crop at optimum level which influences the crop positively by enhancing the number of pods and seeds/pod. Chaudhary et al. (2005) also reported that both hand weeding and mulches had a significant effect on number of pods/plant, number of seeds/pod and grain yield of chickpea.

The results for the mulches effect on plant are in line with the findings of Kwabiah (2004) who stated that plastic mulch increased the total number of grains/pod in chickpea. Similarly among chickpea cultivars, the highest number of seeds/pod was observed in Karak-III for almost all treatments demonstrating that it was a genotypic trait.

Costs benefit ratio is the ratio between added income and added cost of the different packages of weed management tested. The cost benefit ratio results revealed that both the herbicides and hand weeding increased the farmer income. As among different mulches, black plastic positively enhanced the chickpea yield but due to high cost and lower net income as compared to herbicides and hand weeding, farmers could not afford the black plastic mulch. The economic analysis revealed that the application of herbicides seems to be economical in all treatments over mulches. Chaudhary et al. (2011) and Muhammad et al. (2011) also reported that the maximum net return was obtained by Stomp 330EC and hand weeding. Our results are also in line with the findings of Iqbal et al. (2010) who stated that hand weeding resulted in highest cost benefit ratio as compared to the other treatments.

In the light of foregoing results, it was concluded that all weed control strategies along with different chickpea cultivars had significantly influenced the fresh weed biomass and growth of chickpea. Hand weeding, Stomp 330 EC and Dual Gold 960 EC had effectively reduced the fresh weed biomass while among the different mulching practices, black plastic mulch gave satisfactory results by positively increasing the number of seeds/pod in chickpea. Similarly, the economic analysis revealed that the maximum net return to the farmers was obtained from herbicides and hand weeding as compared to mulches. Hence, the present study recommends growing chickpea cultivar Karak-III using either hand weeding or herbicides (i.e. Stomp 330 EC or Dual Gold 960 EC) at the recommended rate to obtain maximum weed control and high net income.


**RESUME**

**Khan I.A., Waqas M., Shah S.M.A., Khan N. et Khan R. 2017. Evaluation de différentes techniques pour la gestion économique des adventices associées au pois chiche. Tunisian Journal of Plant Protection 12: 115-122.**

Un essai de plein champ a été conduit à Peshawar, Pakistan, au printemps de 2012 pour l'évaluation de l'efficacité des stratégies de gestion des adventices associées à trois cultivars de pois chiche et leur rapport coût-bénéfice. Le dispositif expérimental a été mené en blocs aléatoires complets avec un arrangement en split plot. Les différents cultivars de pois chiche (Karak-I, Sheenghar et Karak- III) ont été répartis dans les parcelles principales alors que les traitements testés à savoir le paillage avec un plastique noir, le paillage avec un plastique blanc, le paillage avec de la sciure, le paillage avec la paille de blé, Stomp 330 EC, Dual Gold 960 EC, le désherbage à la main et le témoin non traité, ont été appliqués dans les sous-parcelles. Les paramètres enregistrés sont la biomasse fraîche des adventices (kg/ha), le nombre de graines/gousse et le rapport coût/bénéfice. Les résultats ont révélé une réponse relativement divergente des divers traitements et des cultivars de pois chiche et ce, pour tous les paramètres étudiés. Les résultats ont montré que la plus faible biomasse fraîche (655,33 kg/ha) a été notée avec le désherbage manuel suivi par les herbicides (Stomp 330EC et Dual Gold 960EC) alors que parmi les cultivars de pois chiche, la plus basse biomasse des adventices fraîches (705,02 kg/ha) a été enregistrée chez Karak-III. Le paillage plastique noir et le désherbage manuel ont affecté positivement la production du pois chiche où le nombre maximum de graines/gousse a été de 1,67 et 1,61,

respectivement. Les résultats du rapport coût-bénéfice ont révélé que le revenu net le plus élevé de l'agriculteur résultant de la valeur ajoutée de la culture a été obtenu suite à l'application de Stomp 330 EC (1:2,18) suivi par Dual Gold 960 EC (1:1,94) et le désherbage manuel (1:1,91). Ainsi, la présente étude recommande le semis du cultivar de pois chiche Karak-III et de pratiquer le désherbage manuel ou l'application des herbicides (Stomp 330 EC ou Dual Gold 960 EC) à la dose recommandée pour obtenir une gestion maximale des adventices et un revenu net élevé sous les conditions agro-écologiques de Peshawar.

*Mots clés*: Désherbage manuel, herbicide, Karak-III, paillage, pois chiche, rapport coût/bénéfice

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## ملخص

**خان، إجاز أحمد ومحمد وقاص وسيد ماهر علي شاه ونعيم خان ورحمداد خان. تقييم تقنيات مختلفة من أجل إدارة اقتصادية للأعشاب الضارة المرتبطة بالحمص. Tunisian Journal of Plant Protection 12: 115-122.**

تمت تجربة في الحقل بمنطقة بيشاور، الباكستان، خلال ربيع 2012 لتقييم نجاعة استراتيجيات إدارة الأعشاب الضارة المرتبطة بثلاثة أصناف من الحمص ونسبة الكلفة على الفائدة. كان تصميم التجربة من نوع المقاسم العشوائية الكاملة مع ترتيب قطعات مقسمة. تم توزيع مختلف أصناف الحمص(كراك 1 وشينغار وكراك 3) على أجزاء أساسية بينما نفذت المعاملات المجربة في أجزاء فرعية وهي غطاء بلاستيك أسود وغطاء بلاستيك أبيض وغطاء نشارة خشب وغطاء تبن قمح والمبيد العشبي Stomp 330 EC (ستومب) والمبيد العشبي Dual Gold 960 EC (دوال قولد) والإزالة اليدوية للأعشاب والشاهد غير المعامل. وكانت المعالم المسجلة هي الكتلة الحيوية الناضرة للأعشاب (كغ/هك) وعدد البذور/القرون ونسبة الكلفة على الفائدة. بينت نتائج المعالم المدروسة أجوبة نسبيا متباعدة لمختلف المعاملات لأصناف الحمص. بينت هذه النتائج أن أدنى كتلة حيوية ناضرة (655,33 كغ/هك) سجلت مع الإزالة اليدوية للأعشاب والمبيدان العشبيان ستومب ودوال قولد بينما بالنسبة إلى أصناف الحمص، سجلت أدنى كتلة حيوية ناضرة ( 705,02 كغ/هك) مع الصنف كراك 3. أثر غطاء البلاستيك الأسود والإزالة اليدوية للأعشابإيجابيا على الحمص حيث كانت أرفع أعداد البذور/القرون 1,67 و1,61 على التوالي. بينت نتائج نسبة الكلفة على الفائدة أن أعلى مدخول الفلاح الناتج عن القيمة المضافة للزراعة سجل مع المعاملة بالمبيد ستومب (2,18:1) يتبعه المبيد دوال قولد (1,94:1) والإزالة اليدوية للأعشاب (1,91:1). بذلك ينصح على ضوء هذه الدراسة بزراعة صنف الحمص كراك 3 وتطبيق الإزالة اليدوية للأعشاب أو أحد المبيدين ستومب أو دوال قولد بالجرعات المعتمدة للحصول على سيطرة قصوى على الأعشاب الضارة ومدخول صافي مرتفع تحت الظروف الفلاحية البيئية لمنطقة بيشاور.

*كلمات مفتاحية*: إزالة يدوية للأعشاب، حمص، غطاء، كراك 3، مبيد عشبي، نسبة الكلفة/الفائدة

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*Short Communication*

# Effect of Allelopathic Sorghum Mulch on Growth and Yield of Faba Bean (*Vicia faba*) and Companion Weeds

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**ABSTRACT**

**Alsaadawi, I.S., Tawfiq, A.A., and Malih, H.M. 2017. Effect of allelopathic sorghum mulch on growth and yield of faba bean (*Vicia faba*) and companion weeds. Tunisian Journal of Plant Protection 12: 123-127.**

Field experiment was conducted during the growing season of 2015/16 to test the allelopathic effect of sorghum mulch on yield of faba bean and companion weeds. Plots (1.5 m× 2 m) were covered by dry plant material of sorghum at 5 and 10 t/ha. Plots without sorghum mulch were used as control. Seeds of faba bean were sown in rows at the beginning of October keeping space 40 cm between rows and 20 cm between plants. Weed density and weed dry biomass were recorded at two months after sowing. Yield components of faba bean were measured at the end of the growing season using standard procedures. The experiment was conducted in randomized complete block design with four replications. Sorghum mulch at 5 and 10 t/ha had significantly inhibited weed density by 62 and 78% relative to control and weed biomass by 64 and 90% compared to control, respectively. Plots with sorghum mulch at 5 and 10 t/ha provided higher broad bean above ground biomass (2.71 and 3.05 t/ha, respectively) which were 43 and 61% higher than control. The seed yield was enhanced by 73 and 111% over control plots (0.721 t/ha), which would be attributed to the increase of number of pods per unit area.

*Keywords*: Allelpathic effect, companion weeds, faba bean, sorghum mulch

Weed invasion is one of the major factors affecting crop plants not only by competing with them for resources but sometime interfering with their growth by releasing toxic substances in the rhizosphere (Narwal et al. 2005). Weeds caused yield reductions nearly ranged from 45 to 95%, depending on crop species and ecological and climatic conditions (Ampong-Nyarko and De Datta 1991). Several strategies have been developed for weed control by allelopathy including the use of allelopathic cover crops.

Crop residues can be used as a potential source in agro-ecosystems such as weed control through their physical and chemical effects (allelopathy) (Alsaadawi et al. 2013). They are considered as a step towards sustainable weed management. Such approach could help in minimizing the deleterious effects of current agricultural practices (Mulvaney et al. 2011).

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Faba bean (*Viciafaba*) is one of the leguminous crops with high protein (21 to 34%), amino acids, fat and sugar contents (Aljubouri 2006; Hansen 1972). It fallows sorghum crop in crop rotation. The present study was conducted to evaluate the use of allelopathic sorghum mulch for weed control with the aim of getting cost effective and safe weed control in faba bean.

An experiment was performed in the Botanical Garden, College of Science, Baghdad University during the growing season of 2015. For preparation of sorghum mulch, seeds of sorghum cv. Enkath were grown in lines in plots of 4 m × 3.5 m with 10 cm between seeds and 75 cm between lines. All agricultural management including fertilization, irrigation and pest management were applied as recommended for sorghum crop. At physiological maturity the grains were harvested and the plants were left on the plots to dry under sun.

Based on field calculation, it was found that 13.3 mature plants of sorghum occupied 1 $m^2$ area which is equivalent to 10 tons (t) of air-dried tops per hectare (ha) of soil to the depth of 30 cm. Therefore, mulch rates at 5 and 10 t/ha were used in this experiment to test their effects on growth and yield of faba bean crop and companion weeds. Sorghum dried plant residues were add in to the plot soil at 5 t/ha (by removing 50% of plants) and 10 t/ha (by leaving all plant residues on the surface of plot soil). Seeds of faba bean were manually sown at 27of October in 40 cm spaced rows and 20 cm between plants in plots measuring 2 m × 1.5 m. Plots without sorghum mulch were used as control. Nitrogen (Urea 46% N) and phosphorus as triple super phosphate (46% $P_2O_5$) were used as recommended for faba bean crop. All plots received equal amount of irrigation during the growth period.

At physiological maturity of crop (130 days after sowing), weed density was measured, then weeds were cut from ground surface and dried at 70°C for 3 days. Weed density and biomass were measured as per $m^2$. Air-dried biomass, seed yield and yield components (number of pods per plant, number of seeds per pod and weight of 100 seeds) of faba bean were measured from randomly selected five samples following standard procedures.

The experiment was conducted in randomized complete block design with four replicates. The data were analyzed by analysis of variance (ANOVA) using GENSTAT computer software package. Mean values were measured using least significant difference (LSD) at $P \leq 0.05$ probability level.

Weed flora dominated the experimental site included several narrow and broad leaf weeds. Sorghum mulch at 5 and 10 t/ha had significantly inhibited weed density by 62 and 78% over weedy check and weed biomass by 64 and 90 % over weedy check, respectively (Table 1).

**Table 1.** Effects of sorghum residues mulch applied at 5 and 10 t/ha on density and biomass of weeds growing within faba bean under field conditions

| Treatment | Weed density (plants/$m^2$) | Weed dry biomass (g/$m^2$) |
|---|---|---|
| **Weedy check (Control)** | 63.8 | 150.8 |
| **Mulch at 5 t/ha** | 24.3 | 55.3 |
| **Mulch at 10 t/ha** | 14.3 | 15.2 |
| **LSD ≤ 0.05** | 6.8 | 19.9 |

Concerning their effects on crop yield, both treatments had significantly increased seed yield and dry weight biomass of bean crop over control (Table 2). Plots with sorghum mulch at 5 and 10t/ ha increased seed yield by 73 and 111% over control plots (0.721 t/ha), and dry biomass by 42 and 60% versus control. Maximum seed yield and dry weight biomass (1.53 and 3.05 t/ha) were recorded at high mulch concentration.

**Table 2.** Effects of sorghum residues mulch applied at 5 and 10 t/ha on seed yield and dry weight biomass of faba bean grown under field conditions

| Treatment | Seeds yield (t/ha) | Dry weight biomass (t/ha) |
|---|---|---|
| **Weedy check (Control)** | 0.72 | 1.90 |
| **Mulch at 5** T/ha | 1.25 | 2.71 |
| **Mulch at 10 t/ha** | 1.53 | 3.05 |
| **LSD ≤ 0.05** | 0.07 | NS |

Mulch of sorghum residues applied at 5 and 10 t/ha had significantly improved the number of pods per plant by 47 and 108% over control (Table 3). Maximum number of pods (of about 38.4) was scored in plants grown in plots covered by mulch residues at 10 t/ha. No significant differences were recorded among treatments for the number of seeds/pod and the 100-seed weight.

**Table 3.** Effects of sorghum residues mulch applied at 5 and 10 t/ha on number of pods/plant and seeds/pod and weight of 100 seeds of faba bean grown under field conditions

| Treatment | Yield components | | |
|---|---|---|---|
| | Number of pods/plant | Number of seeds/pod | 100-seed weight (g) |
| **Weedy check (control)** | 18.5 | 3.98 | 67.75 |
| **Mulch at 5 t/ha** | 27.2 | 4.98 | 72.75 |
| **Mulch at 10 t/ha** | 38.4 | 4.63 | 80.25 |
| **LSD ≤ 0.05** | 6.30 | NS | NS |

The data of the present study revealed that sorghum residues applied as a mulch to the field soil reduced weed density and dry biomass and that the reduction was increased with the increase of sorghum residue rate. The suppression of weed density and biomass could be attributed to different mechanisms such as change in soil physical environment and the release of chemicals (Ferreira and Reinhardt 2016). In fact, sorghum residue is reported to release different allelopathic compounds such as phenolics (Alkateeb 2014; Alsaadawi and Dayan 2009; Cheema et al. 2008; Weston 1996).

Application of sorghum residues as a mulch not only controlled weed but also enhanced yield of faba bean. The

improvement of yield may be attributed to the decrease in weed population and weed growth and to the enhancement of physical, chemical, biological, and nutritional properties of treated soil. It has been reported that weed control when using a cover crop is dependent upon the amount of biomass deposited at soil surface which affects soil chemical and physical properties and creates more favorable environment for root development and consequently, enhanced plant growth and yield (Alsaadawi et al. 2013; Hobbs et al. 2008; Mohammadi 2012;. Weston et al. 2013). More work is needed on other cover crops under different environmental conditions before definite conclusion can be made. However, such work would help farmers to use this easily available resource to manage weed and improve crop productivity and soil fertility in a sustainable manner.


**RESUME**

**Alsaadawi I.S., Tawfiq A.A. et Malih H.M. 2017. Effet allélopathique du mulch de sorgho sur la croissance et le rendement de la fève (*Vicia faba*) et les adventices associées. Tunisian Journal of Plant Protection 12: 123-127.**

Un essai au champ a été conduit durant la campagne agricole 2015/16 pour le test de l'effet allélopathique du mulch de sorgho sur le rendement de la fève et les adventices associées. Les parcelles (1.5 m × 2 m) ont été couvertes par un mulch de sorgho à raison de 5 et10 t/ha. Des parcelles sans mulch de sorgho ont été utilisées comme témoin. Les graines de fève ont été semées en ligne au début du mois d'octobre en laissant un espace de 40 cm entre les lignes et de 20 cm entre les plants. La densité et la biomasse sèche des adventices ont été enregistrées deux mois après le semis. Les composantes du rendement de la fève ont été mesurées à la fin de la culture en se basant sur des procédures standard. L'essai a été conduit selon un dispositif en blocs aléatoires complets avec quatre répétitions. Le mulch du sorgho appliqué à raison de 5 et 10 t/ha a significativement limité la densité des adventices de 62 et 78% par rapport au témoin et leur biomasse de 64 et90% comparé au témoin, respectivement. Les parcelles amendées avec le mulch de sorgho à 5 et 10 t/ha ont donné une culture de fève d'une plus grande biomasse aérienne (2,71 et 3,05 t/ha, respectivement) soit 43 et 61% plus élevées que les témoins. Le rendement en grains a été amélioré de 73 et 111% par rapport aux parcelles témoins (0,721 t/ha) et peut être attribué à l'augmentation du nombre de gousses par unité de surface.



*Mots clés*: Adventices associées, effet allélopathique, fève, mulch de sorgho



**ملخص**

**السعداوي، ابراهيم وأروى عبد الكريم توفيق وحسام مالح. 2017. تأثير التغطية بمخلفات نبات الذرة البيضاء في نمو ومحصول الفول (*Vicia faba*) وفي الأعشاب الضارة المرافقة.**

**Tunisian Journal of Plant Protection 12: 123-127.**

أجريت تجربة حقلية خلال موسم النمو 2015-2016 لاختبار تأثير التغطية بمخلفات الذرة البيضاء في نمو ومحصول الفول/الباقلاء وفي الأعشاب/الأدغال الضارة المرافقة. قسم الحقل إلى ألواح بأبعاد (1,5متر × 2 متر) وغطيت التربة بالمخلفات الجافة للذرة البيضاء بالتركيزين 5 و 10 طن/هك مع ترك معاملة الشاهد خالية من المخلفات للمقارنة. زرعت بذور الفول يدويا في بداية أكتوبر على خطوط المسافة بينها 40 سم والمسافة بين نبات وآخر20 سم. سجلت كثافة الأعشاب ووزنها الجاف، وكذلك مكونات محصول الفول عند نهاية موسم النمو باستخدام الطرق القياسية. أجريت التجربة باستخدام تصميم القطاعات الكاملة العشوائية وبأربعة مكررات. بينت نتائج التجربة أن التغطية بمخلفات الذرة البيضاء بالتركيزين 5 و 10 طن/هك اختزلت بشكل معنوي كثافة الأعشاب بنسبة 62 و 78% عن الشاهد والكتلة الجافة للأعشاب بنسبة 64 و 90% عن الشاهد، على التوالي. كما أظهرت الألواح بالتركيزين 5 و 10 طن/هك لمخلفات التغطية أعلى كتلة حيوية جافة للفول بلغت 2,71 و 3,05 طن/هك، على التوالي) والتي كانت أعلى 43 و 61% من معاملة الشاهد،

وكذلك زيادة في محصول البذور بلغت 73 و 111% أكثر من معاملة الشاهد(0,721 طن/هك) وأن الزيادة في محصول الفول تعود إلى الزيادة في عدد القرون/القرنات في وحدة المساحة.

*كلمات مفتاحية*: أعشاب مرافقة، تأثير المجاهضة، تغطية بمخلفات الذرة البيضاء، فول

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# *Plant Protection Events*

***Report on***

## The Second Africa-International Allelopathy Congress (AIAC-2016)

## *Sousse, Tunisia, November 16 - 19, 2016*

Tunisian Association for Sustainable Agriculture (ATAD)

Agronomic Higher Institute of Chott-Mariem (ISA-ChM)


*The Second Africa-International Allelopathy Congress (AIAC-2016) took place under the title "Allelopathy For Sustainability" on November 16-19, 2016 at Sousse, Tunisia. Known as the ability to continue a defined behavior indefinitely, the sustainability requires that the behavior we wish to continue indefinitely must be clear. A complete definition of sustainability is based on three aspects: environmental, economic, and social sustainability, which form its three pillars. Environmental sustainability is the ability to maintain rates of renewable resource harvest, pollution reduction, and non-renewable resource depletion that can be continued indefinitely. Economic sustainability is the ability to support a defined level of economic production indefinitely. Social sustainability is the ability of a social system, to function at a defined level of social well-being indefinitely. Moreover, by looking closely at the allelopathy definition, it appears that allelopathy is a safe alternative to sustain development in agriculture and forestry and maintenance of a clean environment for future generations. It aims to reduce the environmental pollution and maintain an ecological balance in fauna and flora through reduced use of nitrogenous*

*fertilizers and pesticides.*

*This congress was jointly organized by the Tunisian Association for Sustainable Agriculture* (Association Tunisienne pour une Agriculture Durable, ATAD) *and the Agronomic Higher Institute of Chott-Mariem* (Institut Supérieur Agronomique de Chott-Mariem, ISA-ChM).

*It was sponsored by the Ministry of Agriculture, the Institution of Agricultural Research and Higher Education* (Institution de la Recherche et de l'Enseignement Supérieur Agricoles, IRESA), *the Ministry of Higher Education and Scientific Research, the University of Sousse, ISA-ChM, The Doctoral School Agronomy and Environment of ISA-ChM, CRRHAB-ChM, the Research Unit "*Agrobiodiversité*" at ISA-ChM and the Unit "Integrated Horticultural Production in Tunisian Centre-East at the CRRHAB-ChM.*

*The opening ceremony was inaugurated by Prof. Rabiaa Haouala, the President of ATAD who welcomed all the participants and addressed a special welcome to the honorable guests namely Mrs Prof. Hichem Ben Salem, the Director General of IRESA, Prof. Mohsen Boubaker, the Director General of ISA-ChM, Prof. Messaoud Mars, the Director General of CRRHAB-ChM, and Prof. Hamed Mohamed Al-Shora, from Mansoura University, Egypt, and Prof Hisashi Kato-Noguchi from Kagawa University, Japan.*

*In the opening ceremony, three speakers have presented their special speeches devoted to this event namely Prof. Rabiaa Haouala who has introduced the congress, Prof. Hichem Ben Salem, who has highlighted the interest of organizing such events with this high number of partipants from different nationalities and Prof. H.M. Al-Shora who spoke of the importance of strengthening Tunisian-Egyptian collaboration in the allelopathy field.*

*About 115 participants, from abroad and Tunisia, have attended this second African congress: Tunisia (75 participants), Pakistan (5 participants), Algeria (17 participants), Iraq (4 participants), Egypt (13 participants), and Japan (1 participant).*

*The organizers succeeded in compiling an attractive scientific program which included 5 plenary lectures and conferences, presented by the most relevant scientists worldwide known by their original and reference work on allelopathy or allelopathy related topics namely Prof. H.M. Al-Shora (Egypt), Prof. Moncef Ben-Hammouda (Tunisia), Dr. Bouthaina Dridi Al Mohandes (Tunisia), Prof. Hisashi Kato-noguchi (Japan) and Prof. Rabiaa Haouala (Tunisia). These key lectures represented an update of the work carried out in the field of allelopathy relating to the various themes of the congress.*

*The scientific programme included 43 Oral Presentations and 70 Posters dealing with the five themes treated in the congress:*

- *Allelopathy in sustainable and organic agriculture*
- *Allelopathy in natural ecosystems*
- *Chemistry of allelochemicals*
- *Physiology biochemistry and molecular of allelopathy*
- *Allelopathy mechanisms and interactions*

*Summaries of all contributions have been compiled in an abstract book distributed to all participants and some of the most relevant participations were published in the Tunisian Journal of Plant Protection.*

*It was agreed during the meeting of the present African participants that*

*the next Africa-International Allelopathy congress will be organized in Egypt on 2018.*

*This symposium offered a great opportunity for all participants to promote the cooperation and the collaboration between Tunisian and foreign scientists working in the field of allelopathy, to share their experiences, findings and issue ideas on allelopathy research. The afternoon of the second day of the congress was dedicated to a marine trip.*

*Finally, it should be highlighted that at the opening ceremony of the congress, the Organizing Committee honored two people: namely Hamadi Boussetta, Professor at ISA-ChM, and Mr. Sami Mootamri, Secretary General of ISA-ChM. Both are retired, and have given so much of their time, energy and dedication to the institution for over 30 years.*

*At the closing ceremony, all the participants congratulated the organizing and the scientific staff and expressed their satisfaction with the content of the congress, the scientific organization and promised to participate to similar events that will be organized in this excellent welcoming country, Tunisia.*

***Prof. Rabiaa Haouala***
***President of ATAD (ISA-ChM),***
***Tunisia***

## *Sponsoring Structures*

| | |
|---|---|
| Tunisian Association for Sustainable Agriculture | Ministry of Agriculture |
| | Ministry of Higher Education and Scientific Research |
| | Institution of Agricultural Research and Higher Education |
| | University of Sousse |
| | Higher Agronomic Institute of Chott-Mariem |
| Tunisian Journal of Plant Protection | Regional Center of Research on Horticulture and Organic Agriculture of Chott-Mariem |
| | Research Unit "AGROBIO-DIVERSITE" |
| Bioprotection Company | Research Unit "Integrated Horticultural Production in Tunisian Centre-East" |
| | Technical Center of Organic Agriculture |

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Photo of the cover page: ***Sorghum bicolor*** (Courtesy *Tamara A. Al-Khateeb*)

*A Tunisian Journal of Plant Health Sciences Publications* (TJPP)

*Plantae Senae in Terra Sena*