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Bio-insecticidal effects of Oleaster *leaves aqueous extracts* against Psylla larvae (*Euphyllura olivina (Costa)*), a primary pest of *Olea europaea* L.

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Abstract: Many plant species produce phenolic compounds in their various organs and their use in crop protection. These plant secondary metabolites may serve as toxins against the insect pests. The objective of this study was to evaluate *in vitro* the bio-insecticidal effect of an aqueous extract of wild Olive leaves on Psylla larvae (*Euphyllura olivina*), a primary pest of the cultivated Olive tree (*Olea europaea* L. subsp *sativa*). Two concentrations of 0.05g/ml and 0.1g/ml leaves grinding powder in distilled water were sprayed on branches infested with Psylla larvae. The obtained results revealed a very significant mortality rate of the larvae 24 hours after spraying. The chemical composition of Oleaster *leaves aqueous extracts* is determined by HPLC-DAD. The results show in majority the presence of phenolic compounds represented by oleuropein and its metabolite hydroxytyrosol. The phenolic compounds of the crude extract were at the origin of this mortality. The Analysis of Variance revealed highly significant results both between the sampled trees and between the tested concentrations. The Principal Component Analysis (PCA) revealed a close relation between the physiological state of the studied trees and the degree of their infestation by the phytophagus. Taking into account, the physical and chemical characteristics of the sampled soils, data analysis showed that trees growing on nitrogen-rich soils were more infested than those growing on soils rich in organic carbon (C_{org}) and phosphorus (P_{org}).

Key words: *Olea europaea* subsp *europaea* var *sylvestris*; Bio insecticide; *Euphyllura olivina*; Aqueous extract; Polyphenols; Soil.

Introduction

Plants have evolved mechanisms to defend themselves against pathogens and herbivores (1). Secondary metabolites, in particular polyphenols, are an important family of antioxidants acting as real barriers of defense against pests attacks. However, these phenolic compounds are not essential to the individual plant survival but essential to the survival of the populations, since their function consists in regulating the interactions of the plant with its environment (2).

For these reasons, many woody and herbaceous plants have been studied for their biological activities including bio-insecticidal activity. Our study was focused on *Olea europeae* var *sylvestris*, a sclerophyllous and thermophilous ligneous species of Mediterranean shrub land (3).

The domestication process of the wild Olive tree required a great deal of time to give birth to the current cultivated Olive tree (*Olea europaea* subsp. *europaea* L var *sativa*) (4). Among the 35 most cultivated species in the world, the Olive tree is ranked 24th. The species is experiencing a renewed interest in Algeria and Olive growing currently occupies an important socio-economic place.

Unfortunately, the olive tree is prone to attack by several primary and secondary pests, such as olive fly (*Bactrocera oleae*), olive ringworm (*Prays oleae*) and olive thrip (Liothrips oleae). However, the olive psylla or Psylla (*Euphyllura olivina*) is considered the main pest in all olive orchards in the Mediterranean region (5).

Our investigations revealed the presence of a large number of Psylla larvae on leaves, inflorescences and fruits during the spring period. This phytophagous insect has a very rapid adaptability to changes in climatic conditions and a succession of two to four generations per year can be observed (6). Many studies have focused on the evaluation of the damage caused by the insect on the production and quality of Olive oil of infested Olive orchards (7, 8, 9). *Euphyllura olivina* is mostly harmful during the larval stage by drawing part of the sap that feeds the plant and by excreting a honeydew, which promotes the development of a fungus that affects photosynthesis (10).

This study is carried out with the aim of contributing

to the protection of olive trees against a very harmful pest by using a bio-insecticide to better protect the environment. During surveys of Olive orchards situated in the region of Tizi-Rached (Northern Algeria), a large infestation with the Psylla was observed. According to our investigations, the use of natural extracts against these attacks has not yet been attempted. The objective of this work was to test, under laboratory conditions, different doses of an aqueous leaves extract of *Olea europaea* subsp *europaea* var *sylvestris (MILL)* on Psylla larvae (*Euphyllura olivina (Costa)*), a primary pest of the cultivated Olive tree.

Materials and Methods

Study area

Five trees have been selected in a non-irrigated Olive orchard situated in Tizi-Rached (Northern Algeria) (N: 036. 41618. E: 004. 10016. Altitude: 295m). The region is characterized by a sub-humid to humid bio climate. The mean age of trees is 70 years and mean density is about 100-120 trees per hectare. In this orchard, the irregularity in Olive oil productivity mainly dedicated to local consumption was strictly due to a very low management *i.e*. no phytosanitary treatments, no fertilizers, no irrigation. Fructification and thinning cuts were done during the harvest, but it is not a systematic procedure. This kind of agro-ecosystem is currently still the dominant orchard management type in the region (11).

Soil sampling and characterization

Soil characteristics were determined adopting standard procedures proposed in Jackson (1967) (12). The soil is Cambisol (13). Soil samples were collected in March-April 2016 with a boring sampler (4 cm in diameter, 20 cm depth). Particle size distribution was measured according to the Robinson pipette method (organic matter oxidation by H_2O_2, shaking in a sodium hexametaphosphate solution). Soil pH was measured in a 1:5 soil distilled water suspension. $CaCO_3$ was determined only on bulk soil using the HCl 1 M volumetric method. Organic Carbon was evaluated by sulfochromic oxidation. Organic phosphorus was extracted by shaking soil samples in $NaHCO_4$ 0.5 M solution at pH = 8.5 (soil/solution ratio of 1/10) during 1 hour (12). The suspension was then filtered and phosphorus concentration in the solution was determined by colorometry.

Plant and Insect sampling

To carry out our experiment, from March to April 2016, 5 trees were selected and 18 infested Olive branches per tree were harvested. We sprayed our extracts on the infested branches and 24 hours later, we counted the dead larvae. Stored away from heat and light, the dried plant material used to test the insecticidal activity is composed of Olive leaves harvested in the same region.

Extraction method and experimental design

The aqueous extract was prepared according to a modified protocol elaborated by Ouguas et al (2010) (9). The phenolic compounds were obtained by grinding dry Oleaster leaves in the presence of distilled water using an electric mixer. The obtained mixture was centrifuged during 1 hour at a speed of 14000 rpm (brand: SIGMA 4-16K). Two concentrations (C1) and (C2) with respective values of 50 g/l and 100 g/l were tested on the larvae.

The aqueous extract was stored at 4°C for subsequent applications.The choice of these concentrations was based on effective tests carried out under laboratory conditions by Meftah et *al* (2011) (2) on Psylla of Moroccan Olive orchards. The infested branches were exposed to a 13 hours photoperiod at room temperature and were arranged in lots of 2 per crystallizer (20 cm in diameter, 05 cm depth). In each crystallizer, we placed a 20 cm diameter Whatman's N°.4 paper.

For all the samples, three crystallizers (each corresponding to a repetition) were used for the concentrations and the control. Since the extraction was performed with distilled water, the latter was also used as a negative control in our experimental design. To prevent the larvae from exiting, the crystallizers were completely covered with tulle. Thereafter, three sprays with a total volume of 2.5 ml were applied to each test at a distance of 20 cm.

Dead larvae were counted under a binocular microscope, 24 hours after spraying of both concentrations. The larvae were pricked with a fine needle to test their lifeless. The mortality rate was calculated as follows: % M = (number of dead larvae / total larvae number) * 100.

Chemical composition

Chemical analysis of different extracts is obtained by DAD liquid chromatography (Agilent Serial 1100) under the following conditions: the mobile phase corresponds to acidifed water at 0.2% with acetic acid at pH 3.1 and with acetonitrile by linear elution gradient during 30 minutes at 1.5ml/min, starting with 95% of water and with 100% of acetonitrile. The injected volume corresponds to 5µl of extract diluted in methanol. The identification of different compounds is made by comparing different time standards under the same conditions.

Statistical analysis

The histograms representing the distributions of larvae mortality rates, Microsoft Office Excel (version 2010) carried out number of tufts (cottony waxy secretions) and average length of branches per tree. An analysis of the variance (F-test) at a threshold of 5% for both selected concentrations and trees was carried out with the STATBOX software. A Principal Components Analysis (PCA) was carried out with the same software taking into account soil parameters. The aim of the multi-variable analysis was to target several factors *i.e*. larvae mortality rate (%), crude extracts concentrations, physical and chemical soil characteristics, plant material and to highlight the involved interactions.

Results and Discussion

Soil characteristics

Particle size analysis revealed a balanced texture for the studied Olive orchards soils with clay levels (A%) ranging from 14.25 to 17.49% “Table 1”. The pH values (pH_{H2O}) vary from one tree to another. They are slightly

Table 1. Soil characteristics.

A%	pH_{H2O}	CaCO3%	Corg%	N_T%	P_{org} ppm	P_T ppm	P_L
15,57±1,13	7,16±0,14	3,84±1,37	1,57±0,14	0,12±0,01	1,40±0,49	9,08±1,93	0,14±0,02

A%: Clay content, pH_{H2O}: Water pH, $CaCO_3$: Total limestone, C_{org}: Organic carbon, N_T: Total nitrogen, P_{org}: Organic phosphorus, P_T: Total phosphorus, P_L: Leaf phosphorus.

acidic to neutral. These variations can be assigned to the buffering capacity of soils and to the numerous reactions occurring inside these soils regulating thus the actual acidity (14). A low organic carbon (C_{org}) content was observed in all trees. The investigated soils are not calcareous with total nitrogen (N_T) below 0.15%. The enrichment of total nitrogen around the roots is explained by the exudation of root-derived amino acids, which are a source of total nitrogen.

The phosphate status of all the soils is limited. The values are below 5 ppm of organic phosphate. Since the critical threshold is 8 ppm for cultivated Olive trees, we can conclude that there is a deficiency of organic phosphorus (P_{org}) in the studied orchard (15). Knowing that the Olive orchard is not fertilized and the leaf phosphorus (P_L) concentration is greater than 0.07%, it can be concluded that the Olive trees do not suffer from phosphorus deficiency. Diseases, climatic accidents and insect pests can weaken trees. The interaction between biotic and abiotic factors is not well-understood (11). Boudiaf et al. (11) reported that the studied olive orchard has been leaching for years with nutrient losses leading to soil degradation. It is also important to take into account the pedoclimatic factors that inhibit the development of the root system.

Insecticidal effect of the extract on larvae

The crude extract showed positive results expressed by significant larvae mortality rates "Figure 1". 24 hours after the spraying of the concentrations on the infested branches, a large number of dead larvae was noted, in particular for the concentration C2 (0.1 g/ml).

The Analysis of Variance (ANOVA) revealed highly significant results (F_{obs} =11.56 > $F_{critical}$ =8.62) both between the trees and between the tested concentrations. The variation in larvae mortality rates (Figure 2) sampled per tree showed for (C2), values ranging from 55.5 to 100%, reaching the maximum rate for trees T1, T3 and T4. No values were recorded for the control (C). The obtained larvae mortality rates (48.3 to 100%) for C1 were also important. These results show the toxic effect of the crude extract on Psylla larvae. According to Larif et al. (2013) (17), the acidity of phenolic compounds may play a role in larval mortality of *Euphyllura olivina*. The measured pH values for C1, C2 and control (C) were 5.21, 5.17 and 6.85 respectively. These values demonstrate the difference in acidity between the control (C) and the extract explaining the high mortality rates for C2 which is the most acid.

These results support those obtained by Ouguas et al. (2010) (9) where the phenolic compounds of the tested Olive leaves were found to be toxic to Psylla adults. Studies conducted respectively by Dibou et al. (2010) (18); Meftah et al. (2011) (2) and Larif et al. (2013) (17) on aqueous crude extracts of *Melia azedarach, Capsicum frutescens, Peganum harmala* and used at the same concentrations showed a depressive effect on Psylla adults. For Meftah et al. (2011) (2), phenolic compounds namely flavonoids could be the cause of the toxicity that strongly inhibit or decrease adult food intake.

The studies of Feeny (1970) (19) and Bryant et al. (1983) (20) showed that the herbivor's attack induced high phenolic content in plant. By the results of Zouiten and El Hadrani (2001) (10), spraying raw extract containing phenolic compounds on young shoots and flower buds of cultivated olive tree reduce the reproductive potential of the females and affects negatively the survival of psylla especially at larval state

Ben Hamouda et al. (2015) (21) also reported in their work the toxic and insecticidal effect of olive leaf extracts on the peach aphid. Moreover, for a better explanation of the bio insecticidal action of the aqueous extract of *O. europaea* subsp *sylvestris*, an analysis by HPLC is carried out "Table 2".

The results obtained revealed a complex of phenolic compounds whose major constituents are oleuropein and its metabolite the hydroxytyrosol. These two molecules identified in various olive products such as, leaves, bark, roots and oil have shown several biological activities, among others, antimicrobial and bio insecticide (22-28).

Koudounas et al., (2015) (29) has shown that the species *Ligustrum obtusifolium* uses oleuropein as a chemical defense during an attack by herbivores, insects or pathogens. This potent compound, stored in the vacuoles or cytosol of foliar cells, is activated by an enzyme, the β-glucosidase, at the time of leaf tissue destruction by an attack. Oleuropein, strongly denatured in tissues damaged by cross-linking of pro-

Figure 1. Effect of bio-insecticide on the development of Psylla. **A**: Living larva surrounded by cottony tuft, **B**: Isolated living larva of its clump, **C**: Dead larva after spraying.

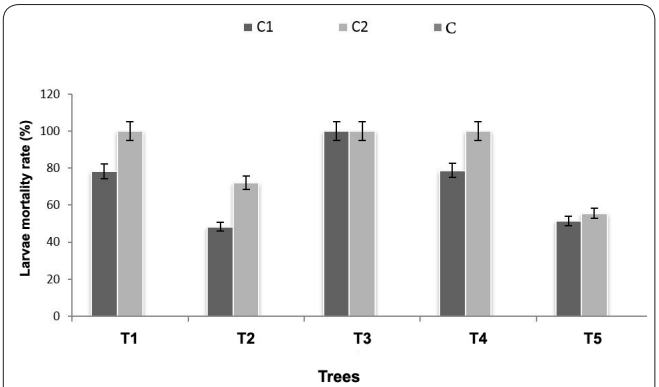

Figure 2. Distribution of Psylla larvae mortality rates under different treatments 24 h after spraying. T1, T2…:Tree one, two… C1, C2 : Concentration 1, 2. C : Control.

Table 2. Main chemical compounds identified in aqueous extract leaves of *Olea europaea* subsp. *europaea* L var *sylvestris (MILL).*

Chemical compounds	Aqueous extract	
	Retention time (min)	Area (%)
Hydroxytyrosol	4,898	1,24
Tyrosol	5,865	0,62
Caffeic acid	7,076	2,58
Hydroxyquinone	3,869	0,65
3 hydroxy 4 methoxycinamic acid	9, 788	3,23
Oleuropein	9,982	2,68
Naringenine 7 oglucoside	10,395	8,16
3, 4,5 trimethoxybenzoic acid	10,828	2,48
Glucose 7 Luteolin	9,236	20,69
Quercetin	12.827	5,45
Trimetoxy cinamic acid	12,827	5,45
Apigenin	14,383	1,12
Rutine	8,827	4,73

teins, causes a decrease in the nutritional value of these proteins and therefore a detrimental effect on the pest (30).

In this case, the toxic effect of the aqueous extract of the tested oyster on the larvae of *E. olivina*, would be the result of the action of oleuropein contained in the latter and which played the role of a powerful biopesticide causing 100% mortality rates.

For all types of treatments (C1, C2 and C), sampling during the spring season allowed us to observe a large number of tufts (cottony waxy secretions) produced by the larvae on the branches with a homogeneous mean length for all the individuals.

In Figure 3, we can evaluate the degree of infestation by examining the distribution of the number of tufts per tree. The observed values ranging from 3 to 10 tufts per branch indicate an inter-individual variability of infestation. Samples T3 and T5 appear to be the least infected, unlike T2 and T4.

Influence of soil characteristics on the degree of tree infestation

In order to explain the inter individual fluctuation observed in the studied Olive orchard and to raise any possible correlations between the different examined parameters, a Principal Components Analysis (PCA) was carried out taking into account soil parameters. Data analyzed revealed three discriminated groups along the axes F1 and F2 "Figure 4". PCA showed that

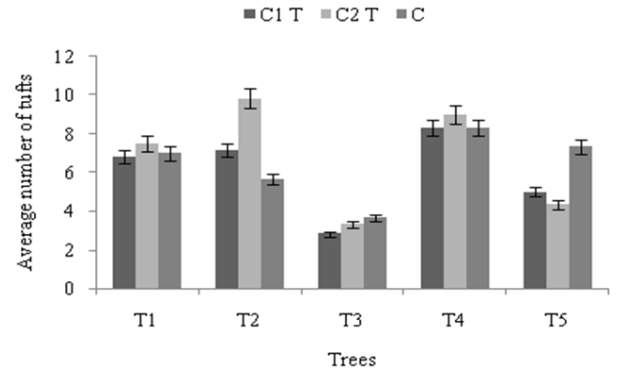

Figure 3. Distribution of average number of tufts per tree.

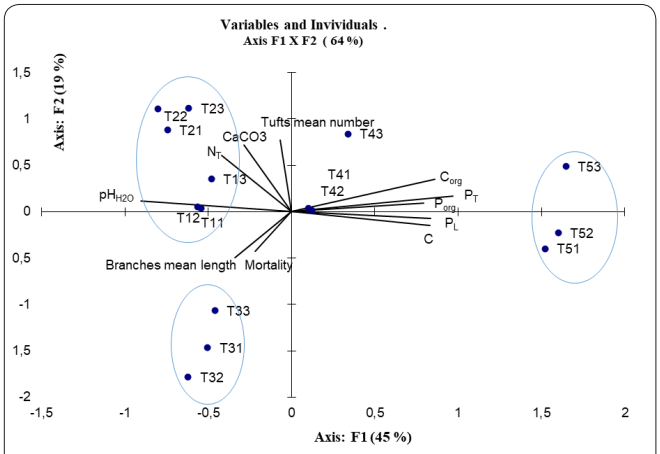

Figure 4. Principal Components Analysis. Representation of individuals (Trees) and variables on two factorial planes F1 × F2.

axis F1 discriminated distinct projections of individuals and soil parameters. This first axis identified two groups standing on the negative plan: group one (G1) comprising tree T3 with the different concentrations and group two (G2) containing tree T2. Group three (G3) standing on the positive plan is more remarkable by its insulated individual T5. The distribution of the projections of soil characteristic on the first axis are statistically significant. The PCA helped to reveal the existence of trends between soil characteristics and certain groups. The soil characteristics: Total limestone ($CaCO_3$), Total nitrogen (N_T) are oriented to group one (G1) while Organic carbon (C_{org}), Total phosphorus (P_T), Organic phosphorus. (P_{org}), Leaf phosphorus (P_L) and Clay (A) show an affinity to group three (G3). These first results of PCA reinforce the thesis of the prevalence of inter individual variability of the degree of infestation factor reported in the ANOVA.

The insulated individual T5 on the axis F1 is hypothetically explained by the ability of the plant to capture effectively more available mineral and organic elements from the soil making it more vigorous than other individuals. Mouas et al. (2013) (16) and Boudiaf et al. (2014) (11) reported that the presence of clays in soils promotes the fixation and formation of complexes with other constituents by releasing other chemical elements. Similarly, adequate organic and leaf phosphorus levels were noted for T5 reflecting a vigorous root and shoot growth, increased fertility and disease resistance.

Phosphorus is an essential element for all living organisms. It is present in nucleic acids, which are carriers of genetic information. Phosphorus plays an important role in the molecules responsible for the transfer of energy and the phospholipids that make up the cell membranes. It promotes the activation of bud growth (11). Interactions between low nitrogen amount and significant availability of organic carbon highlighted many aspects namely a readily synthesis of secondary metabolisms and a prevalence of a more balanced metabolism of the tree. A decrease of polyphenol concentration was observed in plants under nitrogen-fertilized soils (31, 32). Biotic and abiotic factors might affect plant chemical concentrations (33, 34)

The work of Radix et al. (1998) (35), support our findings, showing that soil nature has altered the composition of secondary metabolites in tissues, particularly the polyphenol composition in nut tissues making

the fruit more resistant to necrosis. Consequently, it is obvious that tree T5 that has more phenolic compounds is less exposed to Psylla attacks than tree T2. According to Estiarte et al. (1994) (36), plant resistance is attributed to the synthesis of phenolic compounds. Individualization of T2 can be explained by the inability of the plant to capture effectively some essential nutrients and a most likely blocked Phosphorus by the soil making the tree less vigorous and more exposed to attacks of various potential pests.

Axis F2 also contributes to the interpretation of some results where larvae mortality rates and group one (G1) with T3 are opposed to the tufts mean number parameter and group two (G2) with T2. This arrangement expresses the extent of the infestation and the low percentage of mortality observed in T2, in contrast to T3.

The importance of axis F1 reflected by its Inertia (45%), shows a significant correlation between the physiological state of the sampled trees and the examined soil parameters. The mean number of counted tufts expressing the degree of infestation of the studied samples is explained by the F2 axis representing 19% of the total inertia.

The results show clearly the effectiveness of the aqueous crude extract of the Oleaster leaves against Psylla larvae, a primary pest of the cultivated Olive tree. The mortality rates obtained for both concentrations are significant reaching 100% in some subjects especially in the case of C2. More experiments will be needed to verify the nature of the different molecules responsible for the toxicity against Psylla larvae. In the site of Tizi-Rached, we noted from one individual to another, a variation in tufts number (accumulation rates of waxy secretions) on the infested branches. The Principal Component Analysis (PCA) carried out on the data set led us to an understanding of the behavior of the Olive tree with regard to the pest attacks. The biosynthesis of secondary metabolites involved in the defense of the species against its various pests is more noticeable in trees with significant availability of phosphorus and organic carbon. These two edaphic parameters seem to give the species vitality and resistance by promoting better biosynthesis of secondary metabolites. As a sustainable alternative to chemicals, this extract can be used in an IPM program to control Psylla attacks especially during the proliferation period. Field applications are planned during the emergence of the first generation coinciding with the vegetative phase of the host plant and an important period of the egg-laying. Within an experimental orchard, it is important to report any possible interactions involving the extract, the pest and climatic factors such as wind, humidity, temperatures and photoperiod. Exposure to high temperatures and low relative humidity have a great influence on the phenological activity of Olive tree and high mortality of Psylla eggs and larvae. *Euphyllura olivina* has preferences for some Olive varieties compared to others. Due to the importance of the damage caused by the phytophagus, it is interesting to assess the degree of infestation of this species on several Algerian cultivars and to test the crude extract of the Oleaster leaves on these cultivars both against adults and larvae *of Euphyllura olivina*.

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