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EFFECTS OF *CITRUS SINENSIS* CRUDE PHENOL EXTRACT ON THE LARVAL DEVELOPMENT OF *PHYLLOCNISTIS CITRELLA* STAINT. (LEPIDOPTERA: GRACILLARIIDAE)

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Abstract

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Polyphenols are widely ranged in plants; some of them play a part in the chemical defense of plants and protect them against herbivorous insects. The absence of the damage on the spring growth seems to indicate a chemical action of *Citrus* against *Phyllocnistis citrella*. The aim of this work is to study the effect of crude phenol extract on the larval development of *Phyllocnistis citrella* Stainton. Young leaves were randomly collected, every week, from spring flushes of *Citrus sinensis*. A phenol solution was prepared to treat larvae of *P. citrella in vitro*. Two methods of treatment were adopted; pulverization and irrigation method. The results showed 54.66% of mortality by pulverization and 82.66% by irrigation method, 72h later. The antibiosis action was fast, especially by irrigation method (44% of mortality after 6h). The youngest larval instars ($L_1 – L_2$) seem to be more sensitive, when treated by irrigated method, with 97.37 – 97.14 – 51.02% of mortality for 1st, 2nd and 3rd instars larva, respectively. An anti-feeding effect on larvae treated by irrigation method were observed (on 1st, 2nd and 3rd instars larva were 94 - 88 and 68%, respectively). Phenols of orange leave present toxic and anti-feedant activity against *P. citrella.*

Key words: Phyllocnistis citrella, Citrus sinensis, polyphenols, larvae, treatments
Abbreviations: L_1: the first larvae instars - L_2: the second larvae instars - L_3: the third larvae instars

Introduction

The citrus leaf-miner *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae), native from south East Asia (Urbaneja et al., 2000) is considered among the most serious pest of citrus worldwide (Villanueva-Jimenez and Hoy, 1997). After its appearance announced in 1993 in Florida (Heppner, 1993), *P. citrella* was spread very quickly and infests the majority of citrus-growing regions of the world (Bermudez et al., 2004). In Algeria, it was first observed during summer 1994 in the western region (Berkani, 1995). The female leaf miner lays its eggs preferentially on the tender and early formed leaves (Urbaneja et al., 2001). Damage is caused by all four larval instars, which nourish themselves on the leaves sap between the parenchyma and the cuticle leaf, thus producing serpentine mines (Grafton-Cardwell et al., 2008).

Under Mediterranean climatic conditions, spring leaves escape usually at *P. citrella* damage (Argov et al., 1995; Berkani and Mouatz, 1996). The over wintering in adult stage (Lim and Hoy, 2006) and its early appearance in spring on the crops (Balachowsky, 1966) can be explained by seasonal variation in the contents of the foliage secondary metabolites, and thus converged towards the chemical defense of citrus species (Lotmani et al., 2008). Indeed, the antibiosis mode of resistance constitute is a strategy which plants can develop. Many

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studies showed that the production of allelochemical substances interfere with growth, development and the behavior of herbivores insects (Sener, 1998) and/or their fertility (Walling, 2000).

The objective of our study was to test the crude phenol extract of *Citrus sinensis* spring foliage against the leaf-miner *Phyllocnistis citrella* larvae. We report here the results of the experiment undertaken to assess the degree of mortality and the larvae behavior. However, there is no information about the effects of *Citrus* phenol extract against this lepidopteran insect; *Phyllocnistis citrella.*

Materials and Methods

Sampling site and experiment conditions

Samples of citrus leaves were collected randomly from the research station of Mostaganem University, in northern Algeria (35° 53' N, 0° 04' E), at an altitude of 147m above the sea level. The climate is Mediterranean, semi-arid with soft winter.

This experiment was done at the laboratory of plant protection, University of Mostaganem (Algeria) during summer 2007 at room temperature (27 ± 2°C).

Biological materials

The vegetable species used in this work is *Citrus sinensis* OSBECK. Its trees were implanted in 1988, with an interval of 7x7m and 2,5m height. Two-season foliage were collected: spring and summer foliage. Spring foliage was used for the preparation of the crude extract, and that of summer which was infested by the different larval stages, was intended for treatments.

Growths were excised in entire and only leaves that contained actively feeding larvae were used for bioassays. The first three evolutionary stages of the phytophagous *P. citrella* were used in this study: first larval stage (L_1), second (L_2) and third one (L_3).

In all bioassays, the number of larvae tested is about 50 per stage.

Methods

Extract preparation

Leaves collected during the first phenological stage of *Citrus* (spring) were used in this experiment. The operation was conducted using an organic solvent (methanol) through stirring (Macheix et al., 1990), as the following procedure: fresh materials were extracted with 80% aqueous methanol (15 ml) and 2,5ml of sodium metabisulfite (0.5%) under 15mn agitations. The extract was homogenized and rapidly vacuum-filtered through a sintered glass funnel and stored at 4°C in the dark. Before assays, the methanol solution was pooled, evaporated under vacuum at 40°C. Concentrated extract was then brought to initial volume with distilled water and mixed with a vortex prior to application.

Medium preparation

The Murashigue and Skoog (1962) culture medium, without micronutrients, was used in this experiment (Table 1). In this mineral medium 0.8% of Agar Agar and 0.2mg/ ml of Kinetin were added.

Laboratory assay

Laboratory assays were conducted, *in vitro*, to quantify the effects of crude phenol extract and treatment method on larval mortality and behavior. The numbers of dead larvae were counted after 6 - 18 - 24 - 48 and 72 hours and compared with an untreated test (control), prepared in the same way but extract application was excluded, using a stereo-microscope and binocular- microscope. After selecting the healthy larvae, their stage and position were determined. Two methods of treatment were utilized; spraying (pulverization) and irrigating.

Pulverization method

The infested leaves are immersed during 2 seconds in the extract (like an abundant pulverization), then mended immediately in test tube filled by the culture medium Agar to preserve and maintain the leaves intact three days minimum. Irrigating with the liquid culture medium is necessary after each 24h.

Table 1
Murashigue and Skoog (1962) composition and Macronutrients concentrations

Salts	Concentrations, mg/ml
KNO_3	1900
NH_4NO_3	1650
$MgSO_4$, $7H_2O$	370
$CaCl_2$, $2H_2O$	170
KH_2PO_4	440

Irrigation method

Leaves were directly put down on the culture medium Agar, only one leaf per tube. Irrigating with the prepared crude extract was preceded at 0.5ml/ tube.

Larvae sensitivity

To compare the larvae sensitivities, we calculated the effectiveness of the extract according to the formula of Schneider:

$$\left(\frac{B-K}{100-K}\right)x100 \quad ,$$

where *B* is the rate of dead larvae observed on treated essay, and *K* the rate of dead individuals counted on control test (Boulahia et al., 1996).

Feeding

Due to the life mode, strictly endophyte of *P. citrella* larvae, the investigation of antifeedant activity through choice and no choice tests is impracticable. So the evaluation of this activity was done according to the larvae movement just 6h later and thus justifying their food intake.

Statistical Analysis

The data was evaluated using the analysis of variance (ANOVA). Results were expressed as mean ± SD, and compared using Newman-Keuls' test. $P \leq 0.05$ were considered significant.

Results

A- Larval mortality

Table 2 presents results on *P. citrella* larva mortality. Leaves containing the three first evolutionary of leafminer larvae treated by pulverization method showed, after 6h, a little rate of larvae death. On the first larvae instars (L_1), from 6 to 18 hours, 10% of mortality was observed, and increased slightly to 14% after 24 hours (4% more than the first observations 6 and 18h). Higher mortality was recorded after 48 and 72h (46 and 78% respectively). Compared to the control, mortality was more important. Indeed, 72h after the solution application, the difference was about 54% (Figure 1a). However, the second larvae instars (L_2) did not show any mortality after 6h, whereas, 2% were observed on the bioassay control (Figure 1b). The results recorded after 18 – 24 and 48h of treatment 10 – 18 and 28% respectively, confirming the weak effect obtained on L_1. Mortality was more at 72h later, reaching 50% of total individuals used in this bioassay. The same diagram arises for the third larvae instars (L_3). A maximum death rate (36%) were recorded 72h after the extract treatment; lower value than those noted on L_1 and L_2 (Figure 1c).

In parallel, irrigation method seems to be more effective. When the extract was applied on orange leaves containing larvae L_1, toxic effect was shown only 6h after treatment with important mortality rate (42%), whereas no mortality was recorded on the control test (Figure 1a). A slight difference was observed 18h later; 6% more than the preceding observation. The toxic effect continued; more than the half of the L_1 population died 24h after treatment. The higher cumulated mortalities were visible 48 and 72h afterwards with 72 and 98% respectively. The extract effect is also observed, 60% of larvae L_2 died only 6h after its application, and confirm the data recorded on L_1 (Figure 1b.). After 18 - 24 - 48 and 72h, the observations recorded values of 78 - 78 - 88 and 98% of dead larvae respectively. For the third larvae instars (L_3), mortality was 30% after 6h (Figure 1c.), and only 38% was recorded 18 and 24h later. However, a resumption of the extract effect was noted afterwards 48 and 72h with 46 and 52% of mortality. These results seem to indicate the influence of larvae age.

Global mortalities (all stages) increase considerably on leaves irrigated by the crude phenol extract and record 44 - 54.66 - 57.33 and 68.66% after 6 – 18 - 24 and 48h respectively, and a maximum of 82.66% at the end of the experiment. Whereas with pulverization method, mortality larvae increase slightly (Figure 2): 6 - 12.66 -16.66 and 34% after 6 – 18 - 24 and 48h respectively, and 48% at the end. Whatever the treatment method used; died larvae were higher than those observed on

Table 2
Mortality of *P. citrella* larvae caused by application of crude phenol extract

Times after treatment	Mortality %								
	Control			Pulverization			Irrigation		
	L_1	L_2	L_3	L_1	L_2	L_3	L_1	L_2	L_3
6h	0	2	0	10	0	8	42	60	30
18h	4	6	0	10	10	18	48	78	38
24h	6	10	0	14	18	18	56	78	38
48h	16	24	0	46	28	28	72	88	46
72h	24	30	2	78	30	36	98	98	52

untreated leaves were. Indeed, results obtained are significantly different. Moreover, mortalities recorded on the irrigated essay were more effective.

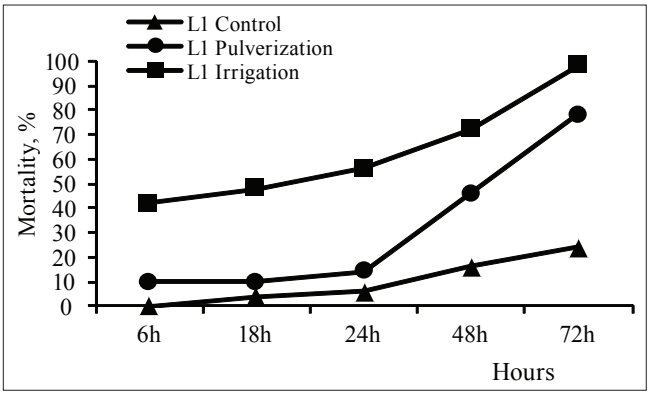

Fig. 1a. Mortality of the first larvae instars (L1) of P. citrella larvae treated with the crude phenol extract

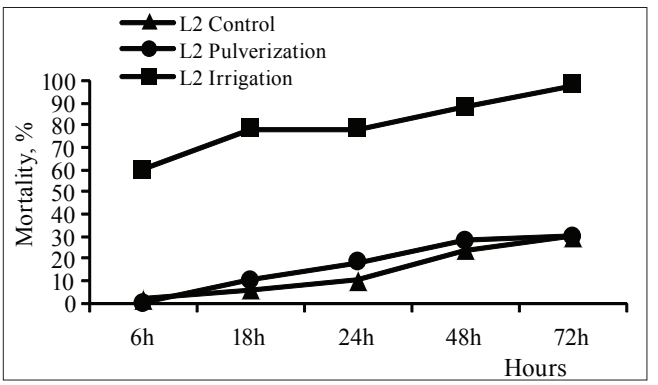

Fig. 1b. Mortality of the second larvae instars (L2) of P. citrella larvae treated with the crude phenol extract

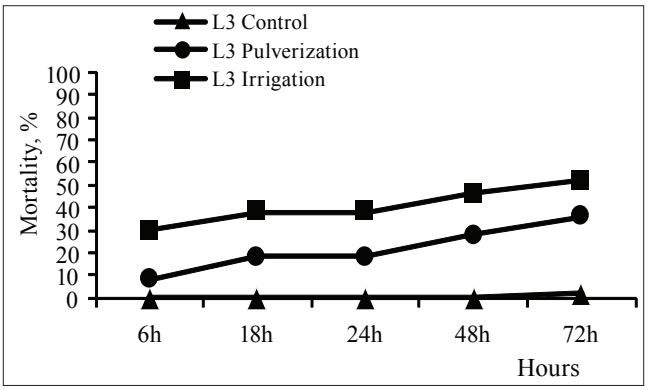

Fig. 1c. Mortality of the third larvae instars (L3) of P. citrella larvae treated with the crude phenol extract

The former recorded mortalities (Figure 3) were significantly affected by the treatment method used ($F_{2,8}$ = 93.74 and P<0.05). The total death rate obtained by irrigation method was more important than recorded by pulverization test and test control; 61.46 - 24.8 and 8.26%, respectively.

Larval sensitivity

The data in Figure 4 shows that the levels of extract effectiveness varied among larvae stage; the youngest larvae are most sensitive. Analysis of variance indicate stage effect ($F_{2,8}$ = 7.689 ; P<0.05); larvae of the first and second stage recorded practically equal averages (Newman-Keuls' test) with 43.612 and 41.102% respectively, while the solution generated a lower action on L_3 with 30.971% of mortality (Figure 5a.). Gener-

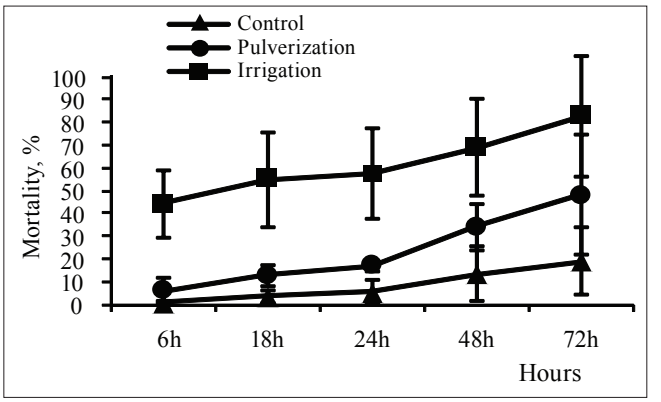

Fig. 2. Global mortality (all larval stages) observed 6, 18, 24, 48 and 72h after treatments

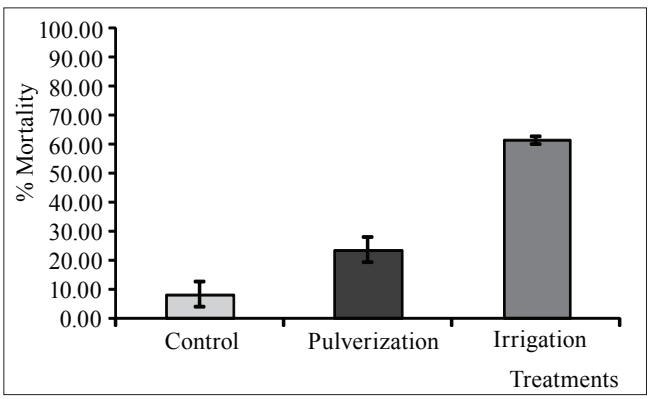

Fig. 3. Means of mortality per method of treatment (all larval stages). Means followed by the same letters are not significantly different (Newman-Keuls' test, 0.05 level)

ally, the sensitivities levels (Figure 5b) are significantly different 72h later ($F_{2,4}$ = 0.0319; P<0.05); L_1 was more sensitive with 84.21% of mortality, followed by L_2 48.57% and L_3 42.86%.

By treatment method, separately (Figure 5c), significant difference ($F_{2,4}$ =35.385; P<0.05) was observed in *P. citrella* larvae treated by irrigation; L_3 appeared the least sensitive to the crude phenol extracts (40.604%). At the same time, it caused 78.524% of larvae death on the second stage L_2, as well as the first with 60.882%. Unlike the irrigation method, L_1 and L_3 treated by pulverization method appeared least sensitive with 26.342 and 21.338% of mortality compared to L_2 (3.68%). Apparent differences were obtained at the end of the experimentation, especially by irrigation method; young larvae L_1 and L_2 are most susceptible with 97.14 and 97.37% of mortality respectively, in contrast to L_3 (51.02%).

B - Larvae behavior

The larvae behavior (Figure 6) varies according to the applied treatment method. On the irrigated test, the majority of larvae stop nourishing and moving immediately 6h after treatment; 94% of L_1 were immobilized under anti-feeding effect, in parallel, control recorded only 10% of larvae stopping feeding. Like the first larvae instars, the second one was also affected, thus 88% were reticent 6h after treatment. These results explain the capacity of the crude extract to prevent L_2 from food, whereas only 20% of larvae control presented the same reaction. For L_3, the extract disturbed feeding on

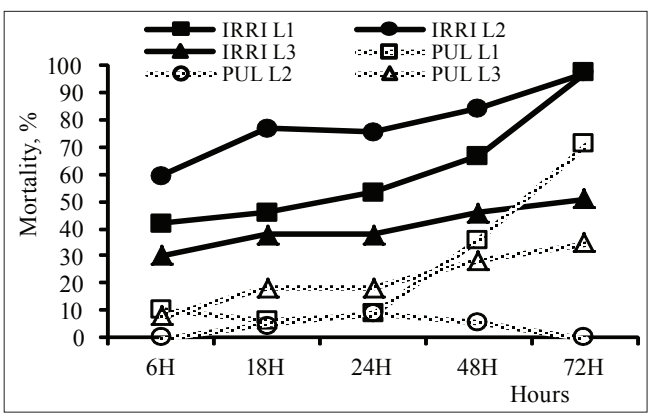

Fig. 4. Crude phenol extracts effectiveness on P. citrella larvae treated by pulverization and irrigation method

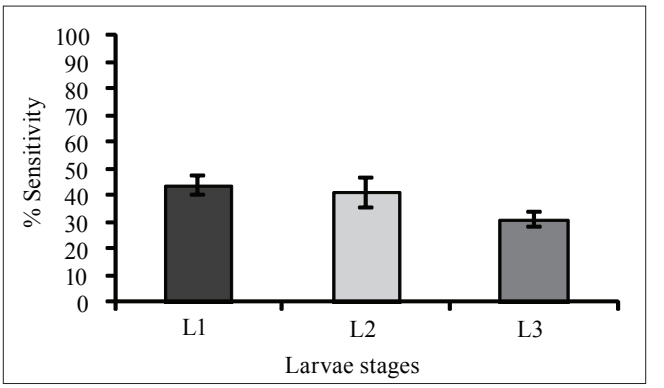

Fig. 5a. Sensitivity means of P. citrella larvae treated with the crude phenol extract, all treatment methods. Means followed by the same letters are not significantly different (Newman-Keuls' test, 0.05 level)

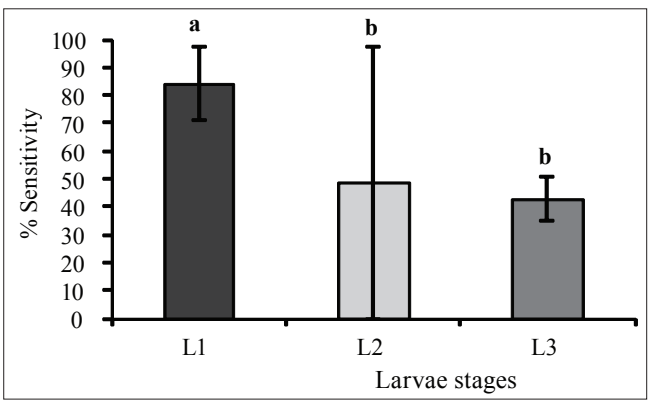

Fig. 5b. Sensitivity means of P. citrella larvae after 72h, all treatment methods. Means followed by the same letters are not significantly different (Newman-Keuls' test, 0.05 level)

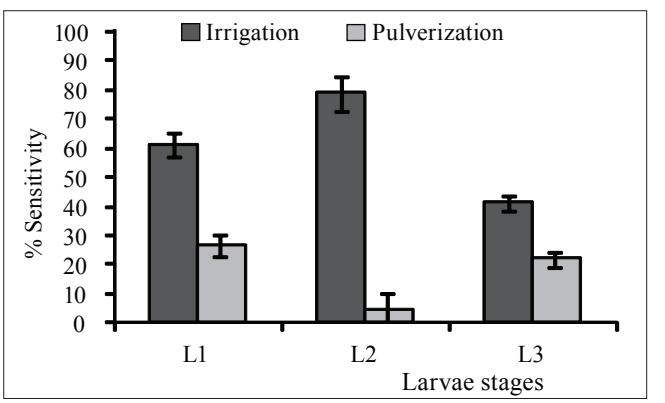

Fig. 5c. Sensitivity means of P. citrella larvae treated by irrigation and pulverization method, separately. Means followed by the same letters are not significantly different (Newman-Keuls' test, 0.05 level)

only 68% of larvae tested; however, the untreated did not record any action.

Anti-feeding effect is difficult to be distinguished, when the extract was applied by pulverization method, 6h later. The L_1 recorded 46%, value much lower than that obtained by the irrigation method. But no response was detected on the second stage larvae. The inhibited larvae were about 14% on the treated essay and 20% of L_2 on untreated test. The third larvae instars (L_3) recorded 22%, whereas in the control, no inhibition was observed.

It is important to announce that the impact of the crude phenol extracts was especially related to larvae physiology; a fast evolution of L_3 treated by irrigation method was observed, compared to control essay those change was not perceptible, the treated test records 6% of alive larvae evolved to the following stage (prepupa). This fact was also observed after 18 and 24h recording 16 and 24% evolved larvae, contrary to control (8 and 16%).

Discussion

Biology (Ba-angood, 1977; Berkani, 2003; Grafton-Cardwell et al., 2008) and control strategies (Boualem et al., 2007; Besheli, 2008) against *Phyllocnistis citrella* are well understood. Nevertheless, so far, few studies covered the subject of citrus resistance towards this insect (Sandhu and Batra, 1978; Bernet et al., 2005). Antibiosis is one of the plants resistance modes to phytophagous insects (Mauricio et al., 1998).

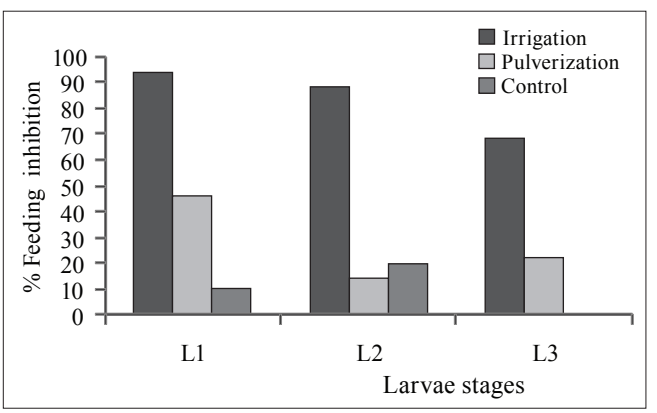

Fig. 6. Comparative effect of crude phenol extract on the larval feeding, 6h after treatments (for each age separately)

In this study, the antibiosis effect refers to the effect of total phenol compounds resulting from the extract of *citrus* spring leaves on *P. citrella* larva ($L_1 - L_2 - L_3$).

The results obtained in this experiment depend on the mode of used treatments (pulverization or irrigation). During the first hours, pulverization treatment gave weak mortality due to the slow penetration of phenol compounds, the effect is observed a long time later on. Knapp et al. (1994) report that chemical control by insecticides pulverization against *P. citrella* may be difficult to be achieved because of their slow penetrations; foliar cuticle and rolled leaf margins form obstacles and protect feeding larvae and pupae consequently. On the other hand, irrigation method showed all the effectiveness of *citrus* phenol extracts, generating important mortality, this is explained by leaves capacity to facilitate the rapid transition of the phenol compounds through cribro-vascular beams of stalks, which are dissolved in water, diluted and then quickly consumed with sap by feeding larvae. In *citrus*-growing orchards, with their systemic properties, insecticides showed very satisfactory results, with a short duration of action against *P. citrella* when they were involved with irrigation water (Mansanet et al., 1999). Contrary to the pulverization method, this quick action shows different sensitivities between larvae when they were treated by irrigation. The level of larvae sensitivity seems to be related to their ages; thus, the youngest larvae are most sensitive, and this result confirms their synthetic insecticidal sensitivities (Boulahia et al., 1996).

After detecting phenol compounds, citrus leaf-miner larvae stops nourishing themselves immediately and anti-feeding effects (Muakata, 1975) are very clear on irrigated leaves. Despite this action, the behavior of the third stage L_3 changed; a fast evolution to prepupa stage was noted, indeed, physiologically, those are not nourished (Chermiti et al., 2001).

Phenolic compounds were believed to be the most important plants chemical resistance (Jhonson et al., 2002). This point of view was based on several studies that showed the effect of flavonoides on the herbivorous insects. For examples, they are larval growths inhibitors of *Ostrinia nubilalis* Hubner (Abou-zaid et al., 1993) and *Trichoplusia ni* Hubner (Beninger et al., 2004). In addition, flavonoïdes showed antifeeding activity against *Ctenopsteustis obliquana* (Thoison et al.,

2004) and *Melicope subunifoliolata* (Ho et al., 2003). In addition, tannins play a considerable part in plants protection against several lepidopterous larvae (Salminen and Lempa, 2002); its activities reduced growth and feeding of *Helicoverpa armigera* (Hubner) larvae (Chan et al., 1978; Kathuria and Kaushik, 2005).

Conclusions

Substances present in the crude phenol extracts resulting from spring flushes and carried out on mining larvae led certainly a modification value of the sap. The results reported that the rapid cessation of feeding accompanied with a larvicide's effect, especially on larvae treated by irrigating method, should prove a valuable asset for botanical insecticide.

Chemical studies of the phenolics extract are currently in progress to isolate and identify the compounds responsible of these actions on the *citrus* leaf-miner *Phyllocnistis citrella* larvae.

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