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Investigations on *Tuta absoluta* (Lepidoptera: Gelechiidae): larval infestation on the tomato cultivated in open field and evaluation of five essential oils against larvae in laboratory

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Abstract
The tomato miner *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a Neotropical species, which cause serious damages to tomato in spread areas. It appeared in 2008 in Italy, where it seriously threatened tomato production both in greenhouse and open field cultivations. In this work a study on infestation by this leafminer on leaves was conducted in an organic cultivation of tomato (*Lycopersicon esculentum* Mill.) "Pizzutello" variety in Sicily (Italy), and the insecticidal activity of 5 essential oils (EOs) on *T. absoluta* was evaluated in laboratory. EOs of basil, cypress, laurel, peppermint and Spanish oregano were used against 3rd- and 4th-instar larvae to assess contact effects and, only for oregano and laurel, inhalation effects. Results showed that the infestation in field had a variable trend showing peaks in two periods, in the months of July and October. Larvae of *T. absoluta* developed on leaves for most of the growing season of tomato showing a peak of 1.5 larva per leaf in the first part of July decreasing in the subsequent months. Results on the use of essential oils against the leafminer showed a good larvicidal effects confirming their use as potential alternative for pest control. Basil, cypress and laurel oils showed major contact efficacy. Inhalation effects of the oils of oregano and laurel increased with increasing their doses with a positive correlation. Oregano oil was effective at lower doses than laurel.

Keywords: basil, cypress, laurel, larval toxicity, peppermint, Spanish oregano

1. Introduction

The leafminer *Tuta absoluta* Meyrick (Lepidoptera: Gelechiidae) is a species of Neotropical origin, and until the middle of the last decade its diffusion remained limited to South America [1, 2]. Outside the continent of origin it was reported in eastern Spain at the end of 2006 [3]. From that moment, the species began a rapid colonization of the entire Mediterranean basin, and today it is present in Africa, Europe, the Middle East and parts of Asia [4, 5, 2, 6, 7, 8, 9]. In Italy it was reported for the first time in 2008 in Calabria, Campania and Sardinia [10] while in Sicily it came in 2009 [11]. In new colonization areas, the Gelechide has revealed great ability to adapt to different agro-ecological conditions, with high reproductive potential and significant economic damage. *T. absoluta* is one of the most dangerous insects for tomato both in open fields and under greenhouses. Besides the tomato, the species can also be found on other cultivated species, such as potato (*Solanum tuberosum* L.), aubergine (*S. melongena* L.), black nightshade (*S. nigrum* L.) and bittersweet nightshade (*S. dulcamara* L.) [1]. However, attacks have also been reported to plants of other families, mallow (*Malva* spp.), cape gooseberry (*Physalis peruviana* L.), bean (*Phaseolus vulgaris* L.), cabbage (*Brassica oleracea* L.) and peanut (*Arachis hypogaea* L.) [11, 12, 1, 13, 14].
The life cycle of *T. absoluta* includes four stages of larva development that are well-defined in color and size. This pest, as other species of Gelechidae, does not present diapause. Environmental conditions influence significantly its biology: low temperatures extend the development time and reduce the number of cycles per year, indicating that the pest slowed down its life cycle but did not enter in diapause [15, 16]. On tomato leaves the life cycle can be completed in as few 22 days when temperatures are between 25 and 27 °C [17]. The larvae attack tomato plants during all growth stages, producing large galleries in their leaves. They feed only on mesophyll tissues leaving the epidermis intact. Damage is due feeding activity of the larvae on the whole aerial part of the plants (leaves, stems, fruits, buds and flowers) with losses of production up to 100% [1]. Following the moth attack, indirect damage may occur as a result of the development of pathogens (bacteria and fungi) [1, 17].
The control of *T. absoluta* is very difficult, because the moth has a marked tendency to rapidly acquire forms of resistance to active insecticidal substances, and many compounds have effects of onset on other phytophagous and / or impact negative on natural enemies [18, 19]. It would be suitable to use products against eggs and larval stages of the leafminer that result selective for the natural enemies. A control method of *T. absoluta* that shows low environmental impact is based on the use of phytochemical products, especially extracts or essential oils (EOs) from aromatic or officinal plants as bio-insecticides. Several studies are conducted on the effects of extracts from seeds, leaves, bark, bulbs, and fruits of the different plant species on *T. absoluta*: (i) rosemary (*Rosmarinus officinalis* L.), thyme (*Thymus vulgaris* L.), oregano (*Origanum vulgare* L.), cumin (*Carum carvi* L.), lemon balm (*Melissa officinalis* L.) [20] to reduce damage on the treated plants; (ii) wild tomato (*Lycopersicon hirsutum* f. *Glabratum*) [21], "Tossegn" Garden thyme (*Thymus vulgaris*) and "Gulo" Castor bean

(*Ricinus communis*) [22], wormwood (*Artemisia absinthium* L.) [23], toothache plant (*Acmella oleracea* L.) [24], European basil (*Ocimum basilicum* L.), chinaberry tree (*Melia azedarach* L.) [25], geranium (*Pelargonium zonale* L.), onion (*Allium cepa* L.), garlic (*Allium sativum* L.) [26], neem (*Azadirachta indica* L.), jatropha (*Jatropha curcus* L.) [27, 28], black cumin (*Nigella sativa* L.) [29], thyme (*Thymus capitatus* L.), pepper (*Piper amalago* var. *medium*) [30], juniper (*Tetraclinis articulate* L.) [31], jojoba (*Simmondsia chinsis*) [32], Clover (*Trifolium repens*), eucalyptus (*Eucalyptus camaldulensis*) [33], soap berry (*Phytolacca dodecandra*), Tobacco (*Nicotiana* spp.), lemon grass (*Cymbopogon citrates* L.) [34], *Turraea abyssinica* [35], cardamom (*Elettaria cardamomum*) [36], Shirazi thyme (*Zataria multiflora*) [37] and citrus fruit *Citrus* spp. [38, 39, 40] to cause insect mortality; (iii) African basil (*O. gratissimum* L.) and European basil (*O. basilicum*) to induce oviposition-deterring effects [41]; (iv) garlic (*A. sativum*) [33] as repellent for larvae.

Despite the large number of botanical extracts tested against the tomato borer only few have been tested against 3rd- and 4th-instar larvae of *T. absoluta*. In order to detect the most suitable periods for the potential use of EOs in open field against this moth, aim of this study was: (i) to investigate the larval infestation on leaves in an organic tomato cultivation (*Lycopersicon esculentum* Mill.) in Sicily (Italy); (ii) to evaluate in laboratory the insecticidal activity of EOs of peppermint (*Mentha piperita* L.), cypress (*Cupressus sempervires* L.), Spanish oregano (*Coridothymus capitatus* L. Reichemb. fil.) and laurel (*Laurus nobilis* L.) used for the first time against *T. absoluta,* and basil (*Ocimum basilicum* L.) used for the first time against 3rd- and 4th-instar larvae of this leafminer.

2. Materials and methods

The study was conducted in an organic cultivation of tomato (*Lycopersicon esculentum* Mill.), "Pizzutello" variety, located in the Naro territory (AG) (Sicily, Italy) (37°16'42.2" N, 13°45'13.5" E) at 400 meters above the sea level in 2015. An area of 2500 m^2 was used with 1300 plants with a planting distance of 1 m and within the row and 1.8 m between rows.

Weekly samples of 100 leaves randomly taken from the plants and brought to the laboratory of the SAAF Department of the University of Palermo (Italy). Leaves were observed under a stereomicroscope, in order to detect the leaf infestation. This parameter was estimated as mines with alive larvae (active infestation) and without alive larvae (intended as the number of abandoned mines + mines with dead or parasitized larvae) per leaf, which summed together resulted in the total infestation (total mines/leaf).

To evaluate the insecticidal activity of 5 essential oils (EOs) on larvae of *T. absoluta*, natural EOs 100% (industrial products standardized and marketed as food supplements, ERBAMEA) of basil (*Ocimum basilicum* L.), peppermint (*Mentha piperita* L.), cypress (*Cupressus sempervires* L.), Spanish oregano (*Coridothymus capitatus* L. Reichemb. f), and laurel (*Laurus nobilis* L.) were tested against 3rd- and 4th-instar larvae in laboratory (25±1 ˚C, 60-70% RH, and 16:8 L/D photoperiod). Two experiments were carried out: (i) tests (5 EOs) by contact and (ii) tests (oregano and laurel) by inhalation effects. Larvae used in the tests were collected from an infested greenhouse of Department SAAF and after observation under stereomicroscope, they carefully got out from their mines by using zero brush and then transferred in Petri dishes (9 cm diameter) or glass jars (600 ml) provided with a filter paper disk to be used in the bioassays.

Bioassays to evaluate contact insecticidal effects of 5 EOs (basil, peppermint, cypress, Spanish oregano, and laurel) were carried out using an emulsion of 200 µl of EO with 2% Tween® 20 (SigmaAldrich) in distilled water (2 ml) per each treatment as test, and 2% Tween® 20 in distilled water (2 ml) as control. Experiments were performed by means the Potter tower, where a Petri dish containing 8 larvae was exposed to each treatment that was replicated three times. After the treatment, larvae were transferred in new Petri dishes provided with filter paper disk (to limit the effects of any vapour released during the test) and maintained at the previously mentioned laboratory conditions. Number of live and dead larvae after each treatment and in the control was recorded at 2, 4, 6, 12, and 24 hours from the treatment. The percentage of mortality calculated at 24 hours from the treatment for each EO was assessed.

Bioassays to evaluate inhalation insecticidal effects of 2 EOs (Spanish oregano and laurel) were performed using 0.5, 1, 3, 5, 20 µl concentrations of oregano oil and 20, 100, 125, 150, 175, 200 µl concentrations of laurel oil in 1 ml acetone as test, and 1 ml acetone as control. Each treatment was replicated two times. Experiments were conducted using glass jars provided with an absorbent paper disk sprayed with 1 ml of solution and placed on the inside of the cork before introducing to larvae. 8 larvae were used per each treatment. A ring of vaseline oil was smeared onto the wall of the jar to avoid direct contact by larvae with the sprayed filter paper. Glass jars were maintained at the previously mentioned laboratory conditions.

2.1 Statistical analysis

To correlate percentage of infestation with temperature, linear regression was used. A logarithmic transformation of the larvae/leaf population and arcsine transformation of the percentage of infestation were used. However, data provided in figures are untransformed for interpretation. To compare data of mortality by contact of EOs, logistic regression was used with the "binomial" link function Post Hoc with the Tukey method. To compare data of mortality by inhalation of oregano and laurel oils, the general linear model (G.L.M.) was used with "Poisson" errors and link function "log" ($P<0.05$). Data were analysed with R statistical software [42]. Abbott's formula [43] was used to correct bioassay data for control response and for to determine insecticide efficacy (EA).

3. Results

3.1 Leaf infestation

During the tomato growing season, the percentage of infestation (total mines/leaf) of *T. absoluta* in the surveyed field was variable, ranging from a maximum of 25.2% in July to a minimum of 2.7% in November, with a peak of 18,7% in October (Figure 1). A significant correlation was found between infestation percentage and maximum temperature (SIAS data) ($F=6.60$, $df=0.11$, $R^2=0.34$, $P=0.02$).

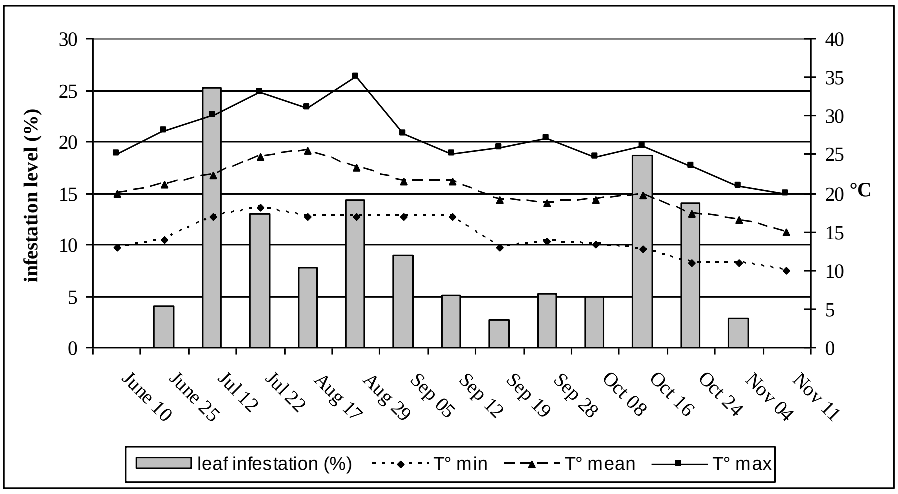

Fig 1: Percentage of infestation (total mines/leaf) of *T. absoluta* and trend of minimum, maximum and mean temperatures (°C) (SIAS data) detected in the surveyed field of tomato in Sicily (Italy) in 2015.

The larval density on leaves (active infestation) showed a peak of 1.5 larva per leaf in the first part of July with values gradually decreasing towards the end of the season and a sharp decrease until the first ten days of November (Figure 2).

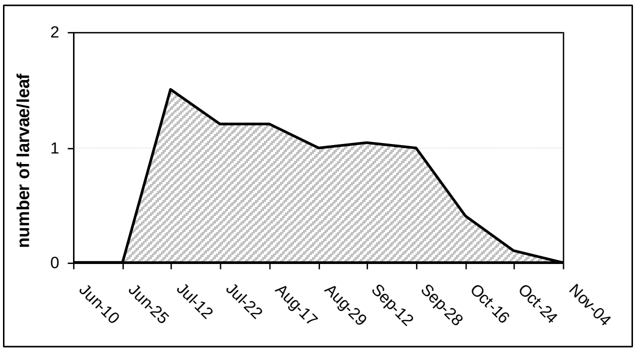

Fig 2: Larval density on leaves (active infestation) of *T. absoluta* detected in the surveyed field of tomato in Sicily (Italy) in 2015.

A significant correlation between average number of larvae per leaf and temperatures in the period of observations was found (Figure 3) (SIAS data) (min. T °C: F=35.5760, df=0.09, R^2=0.83, P=0.00; max. T °C: F=10.73, df=0.14, R^2=0.61, P=0.01; mean T °C: F=15.01, df=0.13, R^2=0.68, P=0.00), espite the low variability of temperatures.

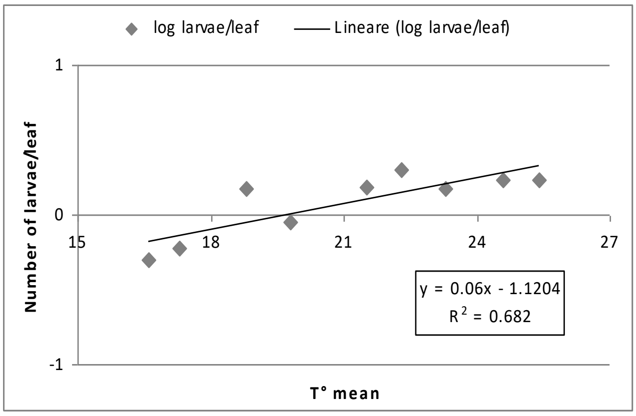

Fig 3: Linear regression between larva density and mean temperature (°C) (SIAS data) detected in the surveyed field of tomato in Sicily (Italy) in 2015

3.2. Insecticidal activity of EOs

EOs used in this work were investigated as bioinsecticides on several pests but not on *T. absoluta* except for basil (table 1).

Table 1: Toxic effects of EOs of basil (*Ocimum basilicum* L.), peppermint (*Mentha piperita* L.), cypress (*Cupressus sempervires* L.), Spanish oregano (*Coridothymus capitatus* L. Reichemb. f.) and laurel (*Laurus nobilis* L) on insect pests

Family	Species	Order	Species	References	activity
Lamiaceae	*Ocimum basilicum* L.	Hemiptera	*Aphis craccivora* Koch	[44]	IGR
		Lepidoptera	*Tuta absoluta* Meyrick	[26]	Insecticidal activity
			Spodoptera frugiperda (JE Smith)	[45]	
		Coleoptera	*Cryptolestes pusillus* Schönnher	[46]	
		Diptera	*Culex quinquefasciatus* Say	[47]	
			Musca domestica L.		
		Coleoptera	*Callosobruchus chinensis* L.	[48]	Oviposition deterrence
		Diptera	*Culex pipiens* L.	[49]	Repellence acivity
	Mentha piperita L	Coleoptera	*Callosobruchus maculatus* Fabricius	[50]	Antifeedant activity
		Diptera	*Culex quinquefasciatus* Say	[47]	Insecticidal activity
			Musca domestica L.		
	Coridothymus capitatus Reichemb. f.	Hemiptera	*Nezara viridula*	[51]	
		Coleoptera	*Letpinotarsa decemlineata* Say	[52]	Antifeedant activity

Lauraceae	*Laurus nobilis L.*	Coleoptera	*Lasioderma serricorne* Fabricius	[53]	Repellence acivity
			Tribolium castaneum Herbst	[54]	
		Diptera	*Culex pipiens* L.	[49]	
			Culex quinquefasciatus Say	[55]	Insecticidal activity
		Coleoptera	*Sitophilus granarius* L.	[56]	
Cupressaceae	*Cupressus sempervirens* L.	Isoptera	*Kalotermes flavicollis* Fabricius	[57]	Antimicrobial activity

Tests on the insecticide action by contact of 5 EOs in Potter tower showed that basil, cypress and laurel exhibited larvicidal effects than other oils (Figure 4). After 2 hours from the treatment larvae were all alive, at 4 hours mortality was 8.3% with both laurel and cypress and 4.2% with basil oils. At 6 hours mortality increased until 12.5% with laurel and cypress, and 16.6% with basil. Only basil oil caused an increase of mortality until 37.5% and 41.6% at 12 hours and 24 hours, respectively. No larval mortality was recorded with peppermint and oregano oils and in the control. Statistical analysis showed significant differences among treatments (χ^2= 31.69, df= 5, P=0.000) mostly at 12h and 24h between basil and other oils. The insecticide efficacy (EA) at 24 h from treatment was 71.4% for basil and 14.3% for both cypress and laurel oils.

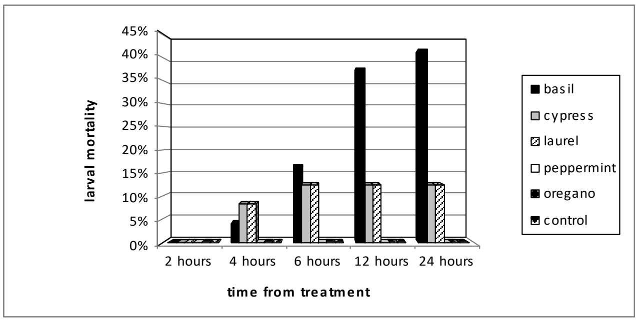

Fig 4: Mortality (%) of 3rd- and 4th-instar larvae of *T. absoluta* for contact toxicity after 2, 4, 6, 12, and 24 hours of exposure to treatments with 5 EOs in Potter tower

In relation to tests on larvicidal activity by inhalation, larval mortality occurred after 12 hours from the treatments with Spanish oregano and laurel EOs. The percentage of mortality increased with the oil doses highlighting a positive correlation between dose and determined mortality (χ^2= 46.75, df= 8, P=0.000). In particular, the essential oil of oregano used at the lowest dose, 0.5 µl/ml, caused 43.8% mortality that increased until 100% at dose of 3 µl/ml (Figure 5).

Fig 5: Mortality (%) of 3rd- and 4th-instar larvae of *T. absoluta* for inhalation toxicity after 12 hours of exposure to treatments with EO of Spanish oregano applied at different concentrations

The curve interpolating the percentage of mortality with oregano oil doses (Figure 6) showed that at 12 hours from the treatment the dose determining 100% mortality was 3.8 µl/ml.

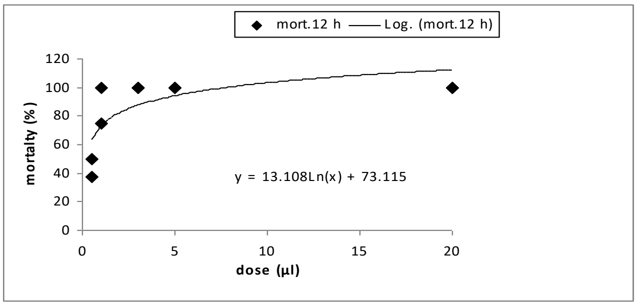

Fig 6: Curve interpolating the doses of EO of Spanish oregano and percentage of mortality of 3rd- and 4th-instar larvae of *T. absoluta* for inhalation toxicity after 12 hours of exposure to treatments

The essential oil of laurel at the lowest dose of 20 µl/ml did not cause mortality, at 100 µl/ml showed a mortality of 13%, that increased significantly up to 100% at dose of 175 µl/ml (Figure 7).

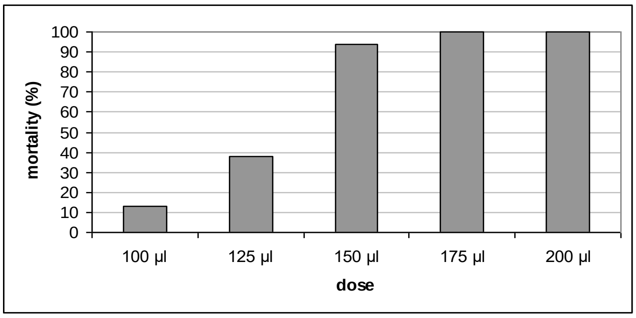

Fig 7: Mortality (%) of 3rd- and 4th-instar larvae of *T. absoluta* for inhalation toxicity after 12 hours of exposure to treatments with EO of laurel applied at different concentrations

The curve interpolating the percentage of mortality with laurel oil doses (Figure 8) showed a positive correlation between dose and determined mortality (χ^2= 10.88, df= 10, P=0.000). At 12 hours from the treatment the dose determining 100% mortality was 183 µl/ml.

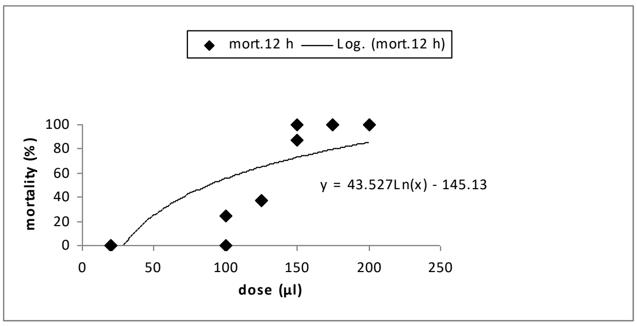

Fig 8: Curve interpolating the doses of EO of laurel and percentage of mortality of 3rd- and 4th-instar larvae of *T. absoluta* for inhalation toxicity after 12 hours of exposure to treatments

4. Discussion

Our results pointed out that the infestation (total mines/leaf) of *T. absoluta* was higher at the beginning of the season, becoming of concern for the tomato cultivation, but it decreased in following periods remaining below 15% with an increase in the middle of October and then decreasing again in November. This variability of the infestation levels likely was dependent on temperature or other natural factors as the absence of a natural control. In fact, a significant correlation was found between infestation percentage and maximum temperature. In addition, during observations very few natural enemies have been found, although a certain biological control is reported in the literature [58, 17]. The number of mines detected on leaves can be useful to have information on damage to the crop. Previous works reported a significant relationship between logarithmic estimation of the abundance of *T. absoluta* galleries in leaves of the tomato plant with the percentage of damaged young fruits. This relationship suggests that evaluation of the abundance of galleries may allow estimation of the level of damage on fruits [17].

In our results the major larval density on leaves (active infestation) was detected in the first part of July, but larvae developed on leaves for most of the growing season of tomato with a significant correlation with temperature, despite the low variability of temperatures. The abundance of larvae in the leaves detected during the season of tomato crop can give indications on pest targeted control. For example, in South America the economic infestation threshold is represented by 2 leaves with active mines, while in Colombia it is 26 larvae (mines) per plant, or 8% of defoliation [59, 1]. In this way, treatments should only be applied when the density of *T. absoluta* larvae exceeds a certain threshold. In our case applications with natural products could be made at the beginning of July to avoid population increase. However, further studies on population dynamics of *T. absoluta* are needed to better define the economic thresholds.

In the recent past many studies have investigated the insecticidal activity of EOs against dangerous insects and their potential use as alternatives to conventional pesticides [60]. EOs used in this work were investigated as bioinsecticides with bioactivity on numerous pests of agricultural importance, but they have never been used against *T. absoluta*, except EO of basil used against 2nd-instar larvae and as oviposition deterrent [26, 41]. In other works some EOs were tested against *T. absoluta* for their insecticidal activity by contact and fumigant toxicity [23, 31, 36, 37]. In this research we have highlighted that basil, cypress and laurel oils by contact application exhibited larvicidal effects than other oils. However, EO of basil is the only one that has determined a significant mortality of larvae respect to control and has shown a greater toxic effect compared to other EOs used. In contrast, previous researches report that basil extracts applied to the 2nd-instar larvae of *T. absoluta* showed a lower insecticidal action compared to other plant oils [26]. In addition, we detected a larvicidal effect after 4 hours from the treatments with all three oils (basil, cypress and laurel). Specially basil caused an increase of percentage of mortality until 24 hours from the treatment, highlighting its effectiveness over time.

In our results inhalation effects of the oils of oregano and laurel on larval mortality increased with increasing their doses showing a positive correlation. Larvicidal activity did not occur before 12 hours from the treatments. Oregano oil was effective at lower doses than laurel. In this experiment, oregano oil, unlike contact application tests, was effective on toxicity of larvae, causing 43% of mortality at dose of 0.5 µl/ml until 100% at dose of 3 µl/ml. Likely the method of topical application affected the insecticidal effect. Laurel was less effective than oregano showing a mortality of 13% at dose of 100 µl/ml.

In the integrated control programs against the Lepidoptera Gelechidae, this new control strategy could significantly reduce the costs of phytosanitary management and the harmful environmental effects found in conventional fighting methods. However, the efficacy of EOs on *T. absoluta* has been assessed mainly in the laboratory, but in the field it still needs to be fully investigated, also in the light of the authorization in Europe for EOs inclusion into IPM, favored or disadvantaged in depending of their environmental impact (e.g. the side effects on natural enemies and vertebrate animals, biodegradability, phytotoxicity etc.) [61].

5. Conclusions

The results obtained showed that the infestation of *T. absoluta* in the surveyed field of tomato was variable being higher at the beginning of the season, with a significant correlation with the temperature. This study showed a good larvicidal action of EOs of basil, oregano, laurel and cypress used for the first time against 3rd- and 4th-instar larvae of *T. absoluta* in laboratory confirming the usefulness of these for a control of the phytophagous with a low impact on the environment and man. We have highlighted that basil EO caused a mortality by contact over 40% and the EOs of laurel and oregano have insecticidal effects on the larvae by inhalation causing 100% of mortality with 3,8 µl dose and 183µl/ dose, respectively. On the basis of our results in the field, applications with natural products could be made at the beginning of July to avoid population increase. However, further studies in field are needed to develop new technologies to improve the effectiveness of EOs in open field and greenhouses, as well as to evaluate their impact on biological control agents.

6. Acknowledgments

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