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ORIGINAL ARTICLE

How do mentha plants induce resistance against *Tetranychus urticae* (Acari: Tetranychidae) in organic farming?

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Abstract

Tetranychus urticae (Acari: Tetranychidae) infesting many plants but *Mentha viridis* L., and *Mentha piperita* L., were low in number of infestation. Therefore the objective of this study was to identify the resistance of *M. viridis* and *M. piperita* plants against *T. urticae* by studying the external shape and internal contents of those plants. For morphological studies, dried leaves were covered with gold utilizing an Edwards Scan coat six sputter-coater. For histological studies, arrangements of Soft Tissue technique were used. For phytochemical studies, the plants were cut, dried and then high performance liquid chromatography (HPLC) was used. While feeding the mites were collected from the area between oily glands, trichomes and respiratory stomata in both mint species. The most important leaf structures in aromatic plants are the oily glands found on the external part of the leaves (both upper and lower epidermis). The number of oil glands in *M. viridis* leaves was greater than in *M. piperita*; the trichomes on the epidermis of *M. viridis* were greater in number than in *M. piperita*; the spongy mesophyll in *M. viridis* was much thicker than in *M. piperita*. The essential oils in the leaves of both mint species contained 71 compounds representing 99.61% of the total oil constituents identified from *M. viridis* before infestation, and 90.95% after infestation, and about 99.65% from *M. piperita* before infestation, and 99.98% after infestation.

Keywords: ecology, high performance liquid chromatography, *Mentha piperita*, *Mentha viridis*, organic farming, *Tetranychus urticae*

Introduction

The Lamiaceae family contains many genera and species. The vast majority of them have economical value due to their use as medicinal plants. A large number of its genera are considered to be a source of active substances especially essential oils. Among these is the genus *Mentha* which includes *Mentha viridis* (Chaker *et al.* 2011). Arthropods overlap between each other and are acclimatized to their location (Price *et al.* 2011). Positive and significant associations were found between glandular trichomes, oil yield, and essential oil constituents, and leaf morphology itself of *M. arvensis*, whereas morphological parameters of leaves showed positive and negative correlations to the average number of trichomes and essential oil constituents. The average number of glandular and non-glandular trichomes, their ratios, menthol content, and trichome numbers showed a good heritability (Mishra *et al.* 2016). It was demonstrated that these two plants contain high polyphenolic compounds and are potent antioxidant species (Najafian *et al.* 2016). This work was aimed at investigating the morphology and histology of leaves of both *M. piperita* and *M. viridis* as well as carrying out phytochemical analysis of their leaves, oils and phenolic acid. Essential oils were used in ancient Rome, Greece and Egypt. *Mentha* spp. possesses antioxidant properties owing to the presence of active constituents like menthone, menthol, flavonoids, rosmarinic acid and carvone (Meda *et al.* 2005). Also, essential oils are traditionally used in medicine for their antiseptic action. They constitute 1% of plant secondary metabolites and are mainly represented by terpenoids, phenypropanoids or benzenoids, fatty acid derivatives and amino-acid derivatives (Sokovic *et al.* 2009). The average number of glandular and non-glandular trichomes, their ratios, menthol content, and trichome number showed a good heritability 10. It has been demonstrated that these two plants contain a large number of polyphenolic compounds and are potent antioxidant species (Iscan *et al.* 2002). Development of resistance to pesticides has been extensive in its population primarily due to an irrational use of synthetic pesticides. Positive and significant associations were found between glandular trichomes, oil yield, and essential oil constituents, and leaf morphology itself in *M. viridis*, whereas morphological parameters of the leaf showed positive and negative correlations to the average number of trichomes and essential oil constituents. Medicinal plants have been used for centuries as remedies for human diseases because they contain chemical components of beneficial value (Mishra *et al.* 2016). This work was aimed at investigating the morphology and histology of the leaves of two *Mentha* species: *M. piperita* and *M. viridis*. Phytochemical analysis was carried out on the leaves and oils of two *Mentha* species: *M. piperita* and *M. viridis*.

Materials and Methods

Morphological studies

Dried leaves were covered with gold utilizing an Edwards Scancoat SIX sputter-coater and consequently analyzed under a Phillips XL 30 ESEM magnifying lens utilizing the HiVac modus (i.e. auxiliary electron identifier, increasing speed voltage 15 kV). Anatomical components were broken down on the lower surface of the leaf. The kind of stomata, epidermal cells, glandular trichomes, non-glandular trichomes and trichome thickness were recorded and the distances between the glandular trichomes were measured. For each of these subjective and quantitative characters, no less than 30 samples of every populace were examined. The same leaf pair was likewise utilized for anatomical investigations. This part of the investigation was conducted with the help of the Applied Centre of Entomonematodes, Faculty of Agriculture, Cairo University, Giza Governorate. This study was carried out from March to September 2016.

Histological studies

The Soft Tissue technique was carried out by washing the stems and leaves of both mint species under running water for two days, and then kept in still water, with the water temperature not surpassing 60°C.

Fixation

Leaves and stems were collected into formaline : acetic acid : alcohol (FAA)(5 : 5 : 90 v/v). The fixative was changed repeatedly till the solution looked transparent, and calmed in an oven of 60°C. The heating liquefies oil contents of gland, so that to insure complete leaching of the oil.

Lack of hydration

Leaves and stems were dehydrated through passing it into a series of concentrations of ethyl alcohol from 50 to 95%.

Clearing

The leaves and stems cleared through passing them in a mixture of solutes; ethyl alcohol : cedar oil (50 : : 50%) and cedar oil : xylene (50 : 50 v/v) for at least 6 h (incubation) and the third one was pure xylene for overnight respectively in 60°C.

Wax installing

The above specimens were transferred from xylene and parafine to pure wax to form blocks.

Separating

The waxed examples were separated utilizing a rotatory microtome.

Staining

Dewaxing of sectioned specimens by xylene and repeated to insure dewaxing. Then hydration of the dewaxed specimens by decreasing dilution of alcohol from 95 to 50% followed by hematoxylene for 60 min, then finally in running water for 15 min. Then again dehydration was conducted by passing the specimens through ammonified water (4%) for 30 s, followed by increasing concentrations of ethyl alcohol and finally to xylene.

Mounting

The prepared sections were mounted on Canada balsam, and then the sections were covered and placed immediately on a hot horizontal surface in the oven at 60°C for 72 h before microscopical examining.

Phytochemical studies

Two replicates (with zero and 10% infestation with *T. urticae* per each spearmint and peppermint potted plant were used to evaluate the changes before and after infestation to their compounds. The plants were cut and dried after two weeks of growing. Plant leaves were not small or large in order to unify factors on results and standardize conditions. To ensure that plants were completely free of any disease, injury, insects or physiological symptoms.

Extraction of essential oil content in different parts

Ten grams of the samples were subjected to water distillation (500 ml water) using a Clevenger's apparatus. The distillation was continued for 3 h after boiling. The volatile substances were isolated and dried over anhydrous Na_2SO_4 according to Gűnther (1953).

Identification of the chemical composition of oils

The components of chaste two essential oil were identified by gas chromatography mass spectrometry (GC/MS), using GLC Hewlett Packard model (5890) arrangement II additionally, furnished with a carbo wax 20 M slim segment (0.32 mm × 50 m, i.d.), fire ionization indicator (FID), helium as bearer gas at a stream rate of 1 ml · min^{-1}, starting segment temperature was 60°C and was expanded to 200°C at a rate of 3°C · min^{-1}.

Extraction of aggregate phenolic acid

Nine grams of air dried powdered mint was fractionated at room temperature with ether, chloroform and finally with MeOH 80% till complete extraction.

High performance liquid chromatography (HPLC) technique

HPLC analysis was carried out according to Abu-Zeid (1992) with slight modifications using an Agilent Technologies 1,100 series liquid chromatography equipped with an auto sampler and a diode-array detector. The analytical column was Agilent.

Results and Discussion

Morphological studies

In *M. viridis*, short, sharp non-glandular trichomes were dominant on the adaxial surface, whereas capitate glandular trichomes were commonly localized on the abaxial surface. In *M. piperita*, single non-glandular trichomes and peltate trichomes were present on the adaxial surface (Choi and Kim 2013). In Figure 1 the number of respiratory stomata in *M. piperita* was fewer than in *M. viridis* (9 and 11 per ml, respectively); the respiratory stomata diameters were very similar in both *M. viridis* and *M. piperita* (10.2 and 10.5 ml), respectively; the trichome length in *M. piperita* was shorter than in *M. viridis* (10.4 and 11.7 ml), respectively; the number of oil glands was 10 and 12 ml, respectively, in *M. piperita* and *M. viridis*; the diameter of oil glands on the upper and under surfaces of *M. piperita* (9.61 and 8.4 ml) was smaller than in *M. viridis,* 10.04 and 9.93 ml, respectively. The mites selected the same place on leaves (between oily glands, trichomes and respiratory stomata) of both mint species when feeding. Hair cover consisted of non-glandular and glandular hair types on the upper and lower leaf surfaces. The non-glandular hairs were unicellular or multi-cellular, some of which had cuticular micro papillae, and referred to two types of hairs: un-branched and dendroid hairs. Un-branched trichomes were found on both surfaces of the leaf of almost all the studied mints. In contrast, dendroid trichomes were observed on the abaxial leaf surface of only a few species. These mints can accordingly be distinguished from all other species by their possession of dendroid hairs. Based on the presence of the dendroid trachomas, there were defined to record the non-glandular hair cover of mints (Šarić-Kundalić *et al.* 2009). It is probable that most of these compounds are products of glands, but some may be produced by epidermal cells and then secreted through the cuticle as with surface waxes. In several studied cases, the response of microorganisms to exudate compounds have been compared in vivo and on plant tissue as in the case of the sesquiterpene lactone parthenolide from *Chrysanthemum parthenium* (Kelsey and Shafizadeh 1980). The morphological characters such as respiratory stomata, trichomes and oily gland (numbers and dimensions help the plant leaves in mechanical defense against *T. urticae* which is seen in population dynamics and biological control.

Histological studies

The most important leaf structures in aromatic plants are oil glands as found in the external parts (both upper and lower epidermis). In Figure 2 plates 3 and 4 show that the number of oil glands on *M. piperita* leaves was fewer than in *M. viridis*. Plates 5 and 6 show that the trichomes in *M. piperita*, were less present in the epidermis than in *M. viridis*. Plates 7 and 8 show that the spongy mesophyll, represented in *M. piperita* was much thinner than in *M. viridis*.

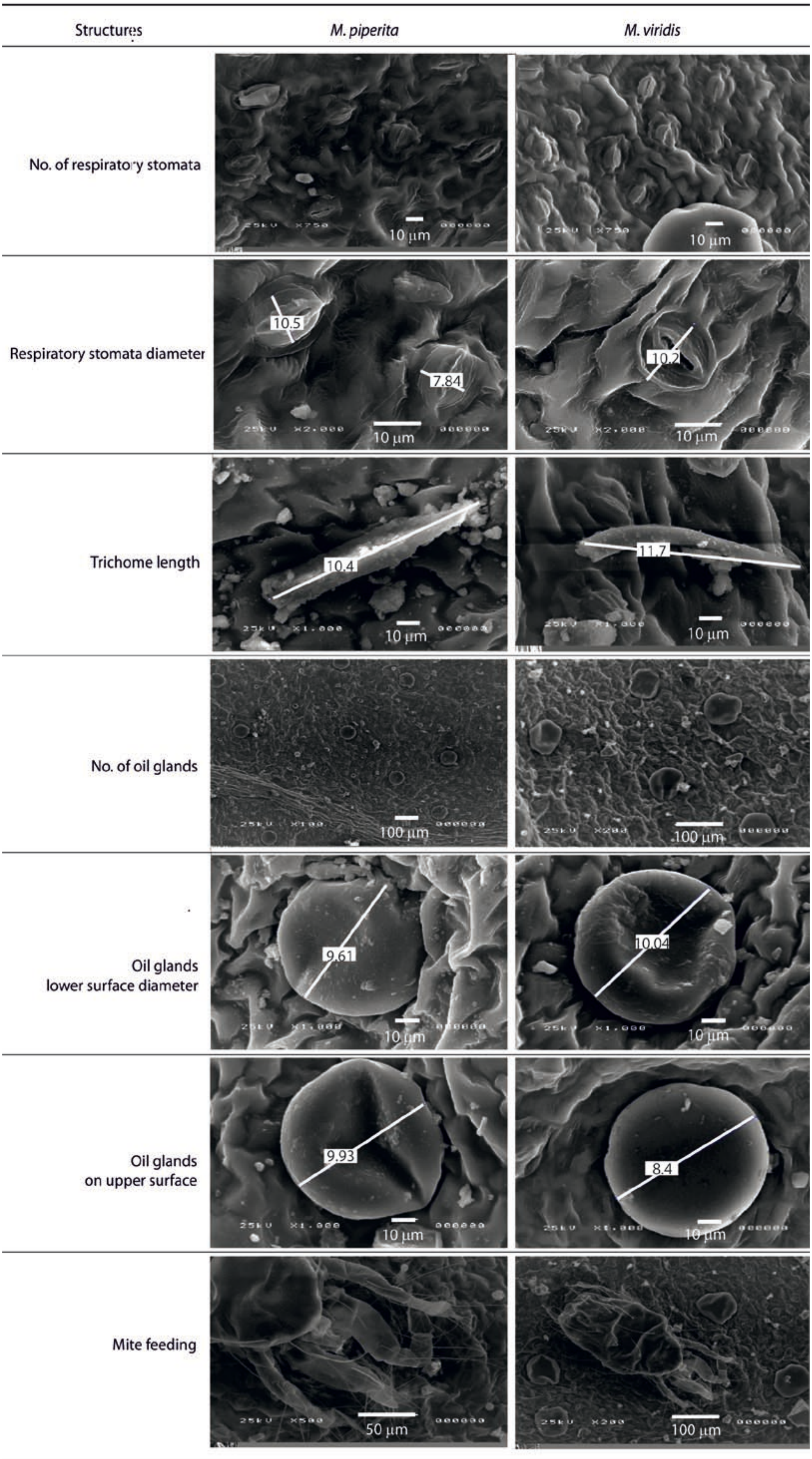

Fig. 1. Scanning Electron Microscopy comparison of two *Mentha* species leaf structures

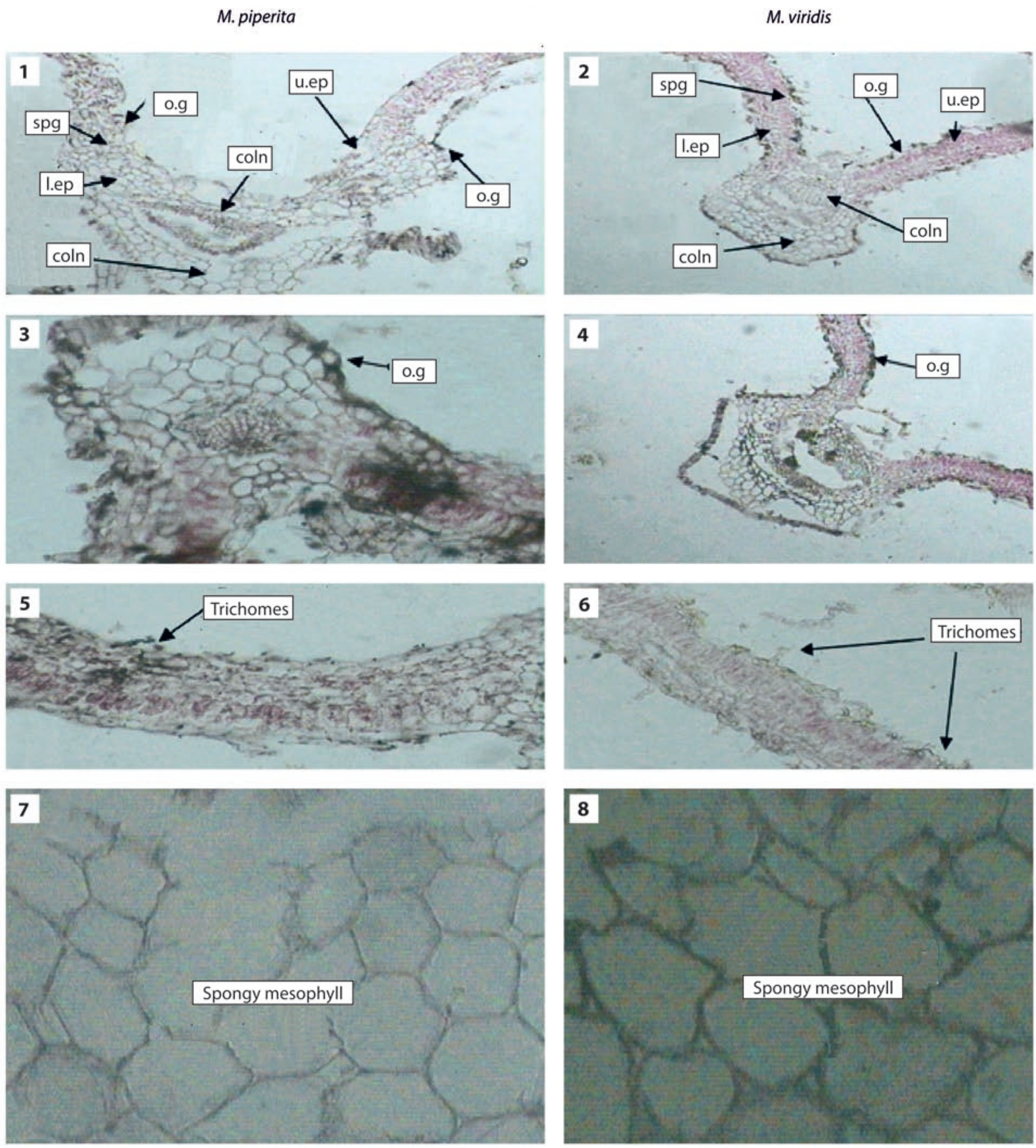

Fig. 2. Transverse section through the leaves (x100) of two *Mentha* species showing upper epidermis (u.ep), lower epidermis (l.ep), collenchyma (coln), oil gland (o.g), spongy mesophyll (spg)

These findings are in agreement with Abu-Zeid (1992) who said that the oil secretory structure of mint plants was found on an external structure known as gland hairs. It also coincides with Parry (1969) who illustrated the presence of oil glands on both the upper and lower epidermis of the leaves. The greater thickness of the cuticle epidermis especially that of the lower surface could be considered as a physical tolerance factor against spider mites (El-Sanady *et al.* 2008). Shakoor *et al.* (2010) reported that leaf thickness is a very important factor affecting the reproduction and development of mite populations. From the current histological results and previous studies, the layers and the cells were found to be thicker and more numerous in *M. viridis* than in *M. piperita* plants. This can be used as a genetic control against *T. urticae*. The number of cells and the thickness of layers influence mite feeding. When it is difficult to insert their mouth parts their population decreases.

Phytochemical studies

The effect of some phytochemical components on the infestation rates of *T. urticae* are presented as follows:

Volatile oil effect

The present investigation dealt with host plants' (*M. piperita* and *M. viridis*) reaction due to infestation in terms of the secondary metabolites emitted after infestation with the two-spotted spider mite, *T. urticae*. Different plant volatiles emitted from leaves of two mint species infested with the two-spotted spider mite, *T. urticae* are shown in Figures 3–6.

The essential oils of both mint species leaves contained many of compounds representing 99.65% (90.23% oxygenated compounds and 9.42% non-oxygenated compounds) of the total oil constituents identified from *M. piperita* before infestation (Fig. 3), and 99.98% (96.21% oxygenated compounds and 3.77% non-oxygenated compounds) after infestation (Fig. 4); 99.61% (65.21% oxygenated compounds and 34.4% non-oxygenated compounds) of the total oil constituents identified from *M. viridis* before infestation (Fig. 5), and 90.95% (76.47% oxygenated compounds and 14.48% non-oxygenated compounds) after infestation (Fig. 6).

The main constituents of the essential oil identified with GC/MS were carvone (48.18%), L(-)-menthol (30.76%), menthone (26.31%), dihydrocarvyl acetate (26.62%), cyclohexanol, 5-methyl-2-(1-methylethyl), acetate (CAS) (21.94%), menthofuran (20.12%), trans caryophyllene (10.20), cis-dihydrocarveol (10.12), eucalyptol (7.49) and D-limonene (3.10). The differences in oil content and composition may be attributed to factors related to infection with the two-spotted spider mites (*T. urticae*).

Based on GC/MS investigations, the oil of *M. viridis* was particularly rich in dihydrocarvyl acetate (26.62%) and trans caryophyllene (10.20%). The main components in *M. piperita* oil were L(-)-menthol (30.76%), and menthone (26.31%) presented in Figures 3–6.

It has been previously demonstrated that carvone is the main component of spearmint (*M. spicata* L.). This mono terpene is known for its effectiveness as an insect repellent (Lee *et al.* 1997). The current results revealed that *M. viridis* oils contain carvone (48.18%) after infestation with *T. urticae* as the major compound, while *M. piperita* contain menthofuran (20.12%) as the most abundant component after infestation.

Plants undergo a dynamic change in trans criptomes, proteomes, and meta bolomicsin response to herbivore-induced physical and chemical changes such as insect oral secretions (OS) and compounds in the oviposition fluids. It is generally believed that insect-induced plant responses are mediated by oral secretions and regurgitates of the herbivore van den Boom *et al.* (2003). The defenses generated by various elicitors differ according to the type of elicitor and the biological processes involved (Pauwels *et al.* 2009) and Villarroel *et al.* (2016). The essential oils are related to plant defense (Zhang *et al.* 2003). Like other secondary metabolite groups, these compounds play an important role in the plant's fitness and protection against herbivores under environmental variation. Monoterpenoids and sesquiterpenoids are the primary components of essential oils (Eteghad *et al.* 2009), which are highly volatile compounds that contribute to the essence of plants that produce them. Essential oils often function as insect toxins and many protect against fungal or bacterial attack. Mint plants (*Mentha* spp.) produce large quantities of the monoterpenoids menthol and menthone which are produced and stored in glandular trichomes on the epidermis (Paré and Tumlinson 1999).

Emitted volatiles can directly affect herbivore physiology and behavior due to their toxic, repelling, or deterring properties (Bernasconi *et al.* 1998; Kessler and Baldwin 2001; Moraes *et al.* 2001; van den Boom *et al.* 2003; Aharoni *et al.* 2003). They can also attract enemies of attacking herbivores, such as parasitic wasps, flies or predatory mites, which can protect the

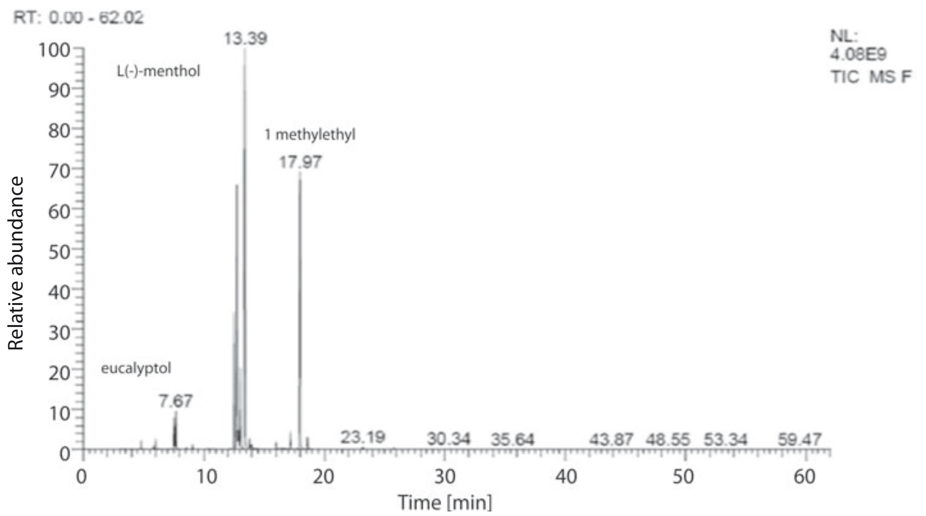

Fig. 3. GC/MS chromatogram of volatile oil extracted from undamaged *Mentha piperita* leaves before infestation

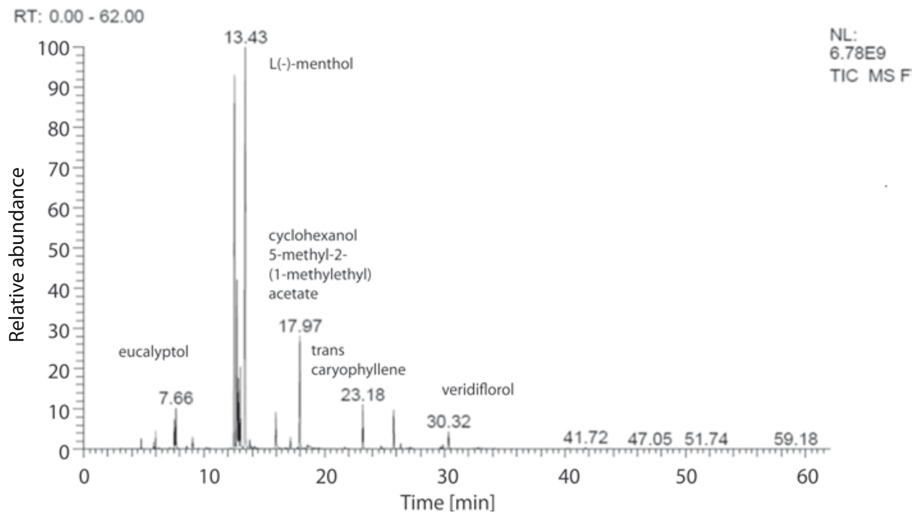

Fig. 4. GC/MS chromatogram of volatile oil extracted from undamaged *Mentha piperita* leaves after infestation

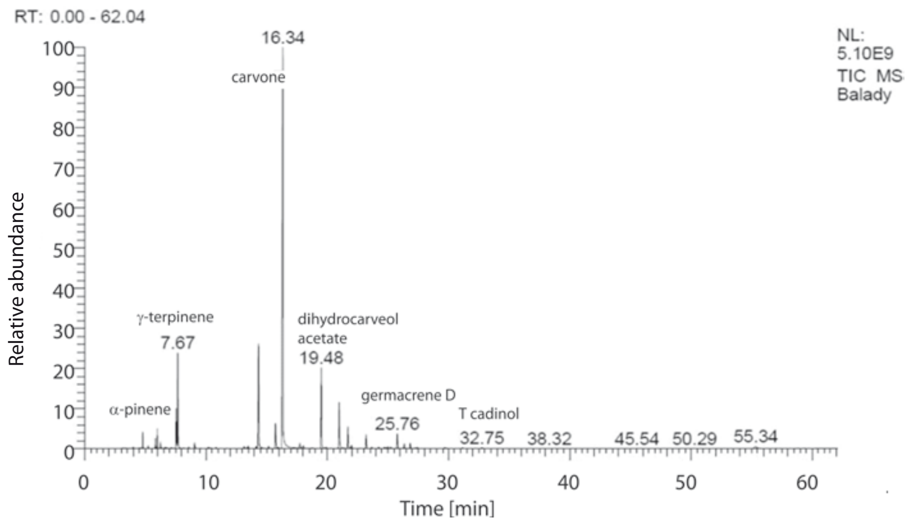

Fig. 5. GC/MS chromatogram of volatile oil extracted from undamaged *Mentha viridis* leaves before infestation

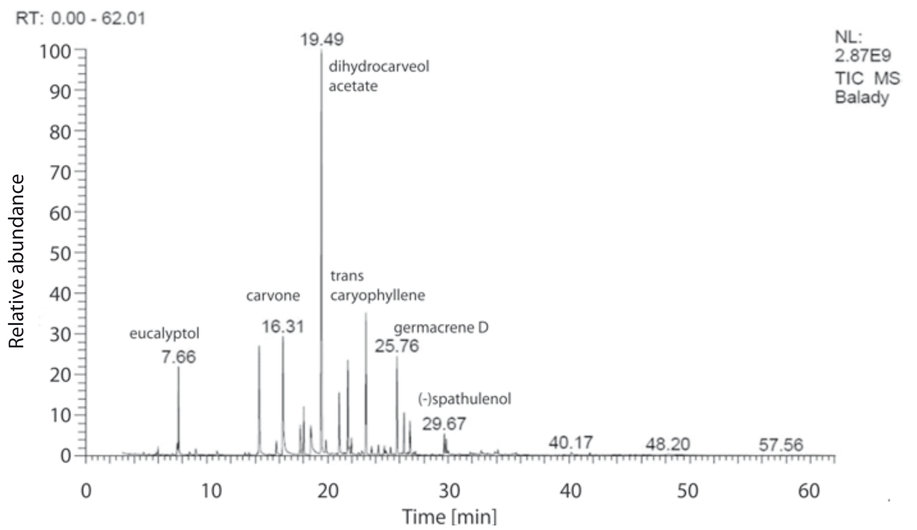

Fig. 6. GC/MS chromatogram of volatile oil extracted from undamaged *Mentha viridis* leaves after infestation

signaling plant from further damage (Turlings *et al.* 1990; Vet and Dicke 1992; Pare and Tumlinson 1997; Kessler and Baldwin 2001; Bosly 2013).

Certain essential plant oils have long been reputed to repel insects (Kim *et al.* 2013). Recent investigations in several countries confirm that some plant essential oils not only repel insects, but also have contact and fumigant insecticidal actions against specific pests. They also have fungicidal actions against some important plant pathogens of the most common and widespread group of defensive compounds (Seun-Ah *et al.* 2010), phenols act as a defensive mechanism not only against herbivores, but against microorganisms and competing plants as well (Padmini *et al.* 2010).

Phenolic compounds in defense against mites

Among the secondary metabolites, plant phenols constitute one of the most common and widespread groups of defensive compounds, which play a major role in host plant resistance against herbivores, including insects (Kanatt *et al.* 2008). Phenolics are present in all plants. Some classes are common to most plant species, while others are family or species specific. The wide occurrence of phenolics suggests an important role in the survival of plants and consequently their defense systems.

Phenolics which are produced in the shikimic acid of plants and pentose phosphate through phenylpropanoid metabolization, enzyme PAL (phenylalanine ammonia lyase) regulates the synthesis of whole groups of compounds including anthocyanins, flavonols, hydroxycinnamic acids liginin, condensed tannins, flavones, chalcones, and stilbenes (Sikora and Świeca 2018). They are probably synthesized in the endoplasmic reticulum and then translocated into the vacuoles, particularly in the epidermal cells or in the cell wall.

Phenolics may also be involved in the attraction of pollinators, seed distribution, pollen germination, and the regulation of auxin transport (Taylor and Grotewold 2005).

Table 1 includes different phenolics separated by the HPLC technique in the two mint species either infested or in the control, undamaged, leaves. In both mint species almost all the values of the different phenolics determined were higher in the leaves that had been attacked by mites.

Rosmarinic acid was the most abundant acid found in the two mint species, ranging from 109.48 to 407.96 μg · ml^{-1}, respectively, in *M. piperita* and *M. viridis* leaves in the control. The maximum content of rosmarinic acid accumulation was found in leaves infested with mites and ranged from 1,187.74 to 2,085.79 μg V · ml^{-1} in *M. piperita* and *M. viridis* leaves, respectively.

Secondly, came hisperidin acid, in both mint species. It ranged from 15.51 to 56.81 μg · ml^{-1}, respectively, in *M. piperita* and *M. viridis* leaves in the control. The maximum content of hisperidin acid accumulation was found in leaves infested with mites and ranged from 96.93 to 88.68 μg · ml^{-1}, respectively, in *M. piperita* and *M. viridis* leaves. Rutin acid, in both mint species, ranged from 43.35 to 6.50 μg · ml^{-1}, respectively, in *M. piperita* and *M. viridis* leaves in the control. The maximum content of rutin acid accumulation was found in leaves infested with mites and ranged from 167.06 to 74.86 μg · ml^{-1} in *M. piperita* and *M. viridis* leaves, respectively.

Lane *et al.* (1987) and Kondo *et al.* (1992) revealed that flavonoids which are considered to be one of the largest classes of plant phenolics, perform very different functions in plant systems including pigmentation and defense. A number of flavonoids such as flavones, flavonols, flavan 3-ols, proanthocyanidins, flavonones, isoflavonoids and flavans have been found to be not only feeding deterrents against many insect pests, but also have antifungal activity. Levin (1976), Swain (1977), Schlee (1986), and Wink (1988), Búfalo *et al.* (2016) reported the significant role of flavonoids (quercetine and rutin) and simple phenol (ferulic acid and caffeic acid), were toxicity and repellence against insects. The impact of the attack by mites was observed on the content of all phenolic compounds found in comparison with the control ones (two species of mint). Almost all of the leaves attacked by mites had greater amounts of all the phenolic acids by HPLC. Greater amounts of the phenolic acids were found in the infected mint species. Thus, it may be concluded that its induction had a crucial role in the defense strategy against mite. Our results revealed that the two mint species were resistant to *T. urticae* infestation by increasing their secondary metabolites which are considered to be involved in the plant defense. But *M. viridis* produces more secondary metabolites represented in phenolic acids with their toxic effect on mites. In contrast, gas liquid chromatography (GLC) showed that *M. piperita* had more monoterpinens than *M. viridis*, where its higher levels resulted in repellency against *T. urticae* and made it attractive for predatory mites. This agrees with Wuyts *et al.* (2006) who found that plants produce a large variety of secondary products which contain a phenol group. They could be an important part of the plants' defense system against pests and diseases including root parasitic nematodes. Any physiological defense which appeared after infestation with *T. urticae* on plant leaves of the genus *Mentha* was reflected on the population dynamics of *T. urticae* and control costs, especially in organic agriculture where the most common and widespread group of defensive compounds, phenols, act as a defensive mechanism

Table 1. High Performance Liquid Chromatography (HPLC) fractionation of phenolic acids [µg · ml^{-1}] extracted from two *Mentha* species leaves of check plots as mg · 5 g^{-1} of leaves

Compound	*M. piperita* before	*M. piperita* after	*M. viridis* before	*M. viridis* after
Gallic acid	ND	ND	ND	ND
Protocatechnic acid	5.84	ND	1.73	ND
Catechin	ND	ND	ND	ND
Syringic acid	2.41	2.09	2.41	1.79
Vanillic acid	ND	ND	ND	ND
Coumarin	ND	ND	ND	ND
Naringin	7.28	23.80	7.68	38.16
Hesperidin acid	15.51	96.93	56.81	88.68
Cinnamic acid	0.52	2.61	5.46	9.50
Chrysin	ND	ND	ND	ND
Gentisic acid	ND	ND	ND	ND
Chlorogenic acid	ND	ND	ND	ND
Caffeic acid	13.15	14.45	1.54	25.73
Ferulic acid	ND	0.51	ND	0.80
Sinapinic acid	ND	ND	ND	ND
Rosmarinic acid	109.48	1187.74	407.96	2085.79
Rutin acid	43.35	167.06	6.50	74.86
Qurcetin	ND	ND	ND	ND
Kaempferol	ND	ND	ND	ND

ND – non detected

not only against herbivores, but against microorganisms and competing plants as well. Qualitative and quantitative alterations in phenols and elevated activities of oxidative enzymes in response to insect attack are general phenomena. The impact of the attack by mites was observed on the content of all phenolic compounds. The wide occurrence of phenolics suggests an important role in the survival of plants and consequently their defense system.

Conclusions

The morphological studies show that the number of respiratory stomata and oil glands in *M. viridis* was more than in *M. piperita*. Also, the trichomes were longer. Histological studies showed that the epidermis of *M. viridis* had a greater presence in number of mites than in *M. piperita*. The spongy mesophyll was found in *M. viridis* much thicker than in *M. piperita*. Phytochemical studies found that there were more compounds and phenolic acids which lead to the process of resistance and expulsion of pests in *M. viridis* after infestation than in *M. piperita*. Finally, from these studies it can be said that the two species of mint resisted *T. urticae* infestation by increasing their secondary metabolites which are considered to be the plant defense, but *M. viridis* is more resistant against *T. urticae* than *M. piperita*.

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