**The modes of action of *Mentha arvensis* essential oilon the granary weevil *Sitophilus granarius* revealed by a label-free quantitative proteomic analysis**

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**Abstract**

Although synthetic pesticides are still used to control insect pests, greater efforts have been made to develop healthier and more environmentally friendly pesticides. Because of their insecticidal properties, botanical essential oils (EOs) are a promising alternative to the use of synthetic insecticides. However, little is known about mechanisms underlaying the insecticidal activity of these natural compounds. In the present study, we evaluated the contact toxicity and the modes of action of the EO from *Mentha arvensis* against the granary weevil *Sitophilus granarius* L. (Coleoptera: Curculionidae), a cosmopolitan insect pest that causes extensive damage to stored cereals. *M. arvensis* EO caused high contact toxicity in *S. granarius* adults, resulting in rapid paralysis and rapid alteration of walking behavior. Our label-free quantitative proteomics approach revealed that *M. arvensis* EO induced dramatic physiological changes in exposed insects. The majority of the differentially expressed proteins (DEPs) were upregulated and are related to the development and functioning of the muscular and nervous systems, cellular respiration, protein synthesis, and detoxification. These results suggest that *M. arvensis* essential oil is capable of affecting a variety of biological processes, and shed light on the repair mechanisms put in place in surviving insects to counter the damage inflicted. This work opens new perspectives on the mechanisms of insecticidal activity of a promising EO for controlling pests of stored cereals and is a first step in the development of new forms of insecticides.

**Keywords**: biopesticide, insecticidal activity, label-free quantitative proteomics, *Mentha*, *Sodalis*, weevil

**Declarations**

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**Conflicts of interest**

The authors declare that they have no conflicts of interest.

**Ethics approval**

Not applicable

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and material**

Not applicable

**Code availability**

The python code used for video tracking is publicly available from the following link: <https://gitlab.com/abaoula/codes-opencv>

**Authors’ contributions**

FR and TH conceived the study. SD, HD, OL, HM and FR performed the study. SD, HD, SCN, HM and FR analyzed the data; FR wrote the original draft. SD, HD, SCN, OL, HM, JLD, MLF, PM and TH contributed revisions to subsequent drafts. All authors read and approved the manuscript.

**Key message**

Studies on the use of essential oils (EOs) as insecticides are abundant, but little is known about their modes of action.

*Mentha arvensis* EO caused high contact toxicity in *Sitophilus granarius* adults resulting in rapid alteration of walking behavior.

By penetrating the insect cuticle, *M. arvensis* EO induced dramatic physiological changes.

This work shed light on the modes of action of a promising candidate in the development of EO-based insecticides against pests of stored cereals.

**Introduction**

Stored products of agricultural origin are attacked by many insect species, which can lead to a significant reduction in weight, quality and commercial value (Omkar 2018). More than 600 species of pest beetles and 70 species of moths are responsible for about 10–25% of the annual quantitative and qualitative losses of stored grain worldwide (Jayas et al. 1994; Pourya et al. 2018). Weevil species of the genus *Sitophilus* are among the most widespread and destructive insects of stored grain in the world (Tripathi 2018). Infestations do not only cause significant losses due to grain consumption, but also lead to high temperature and moisture conditions that accelerate growth of molds, including toxigenic species (Storey et al. 1984; Magan et al. 2003; Tripathi 2018). These storage pests are usually controlled with synthetic pesticides, which increases the risk of environmental pollution, insecticide residues, and pesticide resistance (Regev et al. 1983; Guedes et al. 1994; Kljajić and Perić 2007). In addition, legislation in many countries tends to limit the use of broad-spectrum contact insecticides. This corresponds to an increasing consumer demand for safe food (Handford et al. 2015). In this context, it has become crucial to develop alternative strategies for the control of these pests that are more respectful of the environment and human health.

Plant essential oils (EOs) are secondary metabolites of plants that act as communication and defense molecules (Regnault-Roger et al. 2012; Pavela and Benelli 2016). Because of their repellent, insecticidal, and growth-reducing effects on a wide variety of insects, they are increasingly being considered a credible alternative to synthetic/conventional insecticides, often neurotoxic to mammals (Ngamo and Hance 2007; Nerio et al. 2010; Regnault-Roger et al. 2012; Pavela and Benelli 2016). This is particularly the case for the protection of stored products whose confinement favors the action of naturally highly volatile molecules (Isman 2000; Isman and Grieneisen 2014). It has been reported that plant EOs may be effective against pests of stored grain including EOs from *Brassicaceae* (e.g. *Brassica juncea*), *Myrtaceae* (e. g. *Eucalyptus*), *Verbenaceae* (e. g. *Lippia sidoides*) and *Lamiaceae* (e. g. *Ocimum basilicum*) (Jembere et al. 1995; Lee et al. 2001; Kim et al. 2003; Popović et al. 2006; Cetin and Yanikoglu 2006; Oliveira et al. 2017, 2018). While the toxicity and biological activity of certain EOs on target insects have been demonstrated, our knowledge of the mechanisms of action of these natural substances is still very limited. Several studies have reported the neurotoxic mode of action of certain EOs that may inhibit the cholinergic and octopamenergic systems (Miyazawa et al. 1997; Kostyukovsky et al. 2002; Enan 2005), or block the GABA-gated chloride channels in insects (Bloomquist 2003). Other studies demonstrated that EOs can alter the functioning of the mitochondrial system, notably by disrupting ion exchange (Copping and Menn 2000). To cope with insecticides, insects may use a variety of detoxifying enzymes and other defense mechanisms that remain largely unknown (Zhang et al. 2013; Li et al. 2013). Despite the growing interest in EOs, few studies have focused on the mechanisms behind their insecticidal activity (Liao et al. 2016; Huang et al. 2019). Yet, deciphering the modes of action of EOs on target insects is a crucial step to highlight the synergies between molecules when designing new types of pesticides. In this context, “Omics” approaches are particularly well suited to better understand the mechanisms behind the insecticidal activity of EOs, as they can rapidly provide large-scale data and a systemic overview of the underlying mechanisms.

Previous research has reported the insecticidal properties of EOs from plant species of the genus *Mentha* (Tripathi et al. 2000; Ansari et al. 2000; Aggarwal et al. 2001; Cetin et al. 2006; Kumar et al. 2009, 2011; Koliopoulos et al. 2010; Govindarajan et al. 2012) that grow from tropical to temperate regions. In this study, we evaluated the contact toxicity of *Mentha arvensis* EO against the granary weevil *S. granarius*, a major pest of stored grain in temperate regions. Subsequently, we evaluated the *in vivo* cuticular penetration of the EO and its effects on the locomotor behavior of the insect. Finally, our label-free quantitative proteomics approach highlighted the physiological changes taking place in *S. granarius* after exposure to *M. arvensis* EO and shed light on the mechanisms behind its insecticidal activity.

**Material and methods**

**Insect mass rearing**

*S. granarius* used in this study was obtained from a laboratory colony kept for breeding in the insectariums at the catholic University of Louvain (Louvain-la-Neuve, Belgium). The insects were not exposed to insecticides and were reared in glass jars containing organic wheat grains placed in an incubator under the following conditions: 28 ± 1 °C and 75 ± 1% relative humidity (RH) in total darkness.

**Contact toxicity test of *M. arvensis* EO**

The organic *M. arvensis* EO was purchased from PRANARÔM (B‐7822 Ghislenghien, Belgium). The technical data sheet (GC-MS) relating to the specific batch of the EO used in this study showed that the main components are: menthol (73.72%), menthone (6.36%), Menthyl acetate (2.76%), isomenthone (3.67%), limonene (2.6%) and neomenthol (2.36%) (Table S1). *M. arvensis* EO was diluted with acetone to obtain the eight concentrations that were used to assess its toxicity and determine relevant toxicological endpoints: 6% (27.1 µg/insect), 9% (40.7 µg/insect), 10,5% (47.5 µg/insect), 12% (54.2 µg/insect), 14% (63.3 µg/insect), 16% (72.3 µg/insect), 20% (90.4 µg/insect) and 22% (99.4 µg/insect) (v/v). LD50 and LD90 values were determined using seven to fourteen days post-emergence adults. Aliquots of 0.5 μL of the dilutions were applied topically to the dorsal thorax of the insects using a 5 µl Hamilton® syringe (Model 75 RN SYR, Hamilton Company, Switzerland). Controls were determined using 0.5 μL of acetone. For each treatment, six replicates were performed. One replicate consisted of 20 randomly selected individuals treated individually and placed in glass Petri dishes (Ø 90 mm × 15 mm) with an absorbent paper and fed with about twenty grains of wheat and kept in the dark. The number of dead insects in each Petri dish was counted 24 h after exposure of the insects to the *M. arvensis* EO. Insects were considered dead when no reaction was observed by exposing them to a 100-watt cold lamp in front of their eyes for five seconds (Demeter et al. under review). The number of dead insects was confirmed 48 hours after treatment.

The dose-response curve of the toxicity assay against *S. granarius* showed a non-linear dependence of mortality as a function of the dose. This dependence follows an S-shape (Figure 1) that is reminiscent of a Hill’s function encountered in many molecular or cellular biology contexts (Likhoshvai and Ratushny 2007). This function allowed us to estimate the LD50 and is written

$$M= \frac{D^{n}}{D^{n} + K^{n}} (1)$$

where *M* is the proportion of mortality, *D* is the EO dose, *K* is a threshold dose above which the mortality is greater than 50% (corresponding to the LD50) and *n* is a cooperativity exponent. A value of *n* greater than 1 indicates the presence of cooperative processes between the dose level and the spread of mortality within the population. *n* and *K* were evaluated by fitting eq. (1) with the data.

**Testing locomotion behavior**

Adults of *S. granarius* (seven to fourteen days post-emergence) were treated with the *M. arvensis* EO LD50 (55.6 µg/insect) as described in the section “contact toxicity test”. To assess the impact of the treatment on locomotor behavior of *S. granarius*, each treated adult was individually placed in a glass Petri dish (Ø 90 mm × 15 mm) with an absorbent paper. Four Petri dishes were placed underneath a Logitech® HD Pro C920 webcam video camera, which recorded *S. granarius* activity for 24 hours at one frame per second. To avoid any external influence on the insects’ behavior, each Petri dish was surrounded by walls of 2 cm of height and the four Petri dishes were enclosed in a box illuminated by a red light. A video recording consisted of three treated individuals and one untreated individual (control) placed at random. A total of 54 replicates were performed for treatment with *M. arvensis* EO and 18 replicates for control. The experiments were conducted at room temperature (24 ± 1 °C). Video tracking was conducted with Python code using OpenCV4, which finds the contour of the individual and evaluates its centroid coordinates (*xt, yt*) at time *t*. The distance is then calculated using the formula $d=\sqrt{(x\_{t+1}-x\_{t})\^2+(y\_{t+1}-y\_{t})\^2}$ (code availability: <https://gitlab.com/abaoula/codes-opencv>). To compare the distribution of the distances and the space visited by treated and untreated individuals, we used a Mann-Whitney U test using SciPy library of Python3.8.1. The distributions were considered significantly different if *p* < 0.05.

***In vivo* cuticular penetration of *M. arvensis* EO**

The penetration of *M. arvensis* EO through the cuticle and the evolution of the internal quantity after topical application were monitored by GC-MS analyses. In practice, a group of 10 seven to fourteen days post-emergence adults were topically treated with the LC50 (as described in the section “contact toxicity test”). The insects were then left for four different incubation times: 0h, 6h, 18h and 24h. Five replicates were performed for each incubation time. After incubation, the 10 insects from each treatment were collected in glass tubes of 20 mL and were rinsed 3 times consecutively by 1 mL of ethanol (1st fraction), 1 mL of hexane (2nd fraction) and 1 mL of hexane (3rd fraction) before being crushed and extracted by 1 mL of hexane (4th fraction). Phenyl octane was used as an internal standard and added to each fraction. All four fractions were analyzed by GC-MS and quantification was performed using menthol standard calibration curves. The GC-MS used was an Agilent GC sytem 7890B (Agilent, Santa Clara, CA, USA) in splitless injection mode (with an injection volume of 1.0 µl) coupled with an Agilent MSD 5977B detector (Agilent, Santa Clara, CA, USA), with a VF-WAX column (Agilent, Santa Clara, CA, USA) of 30 meters, 0.25mm of diameter and 0.5µm of film thickness. The initial temperature of 50°C was maintained for 1 minute before a first progressive heating of 5°C per minute up to 155°C directly followed by a second one of 8°C per minute up to 250°C. Once the peak temperature of 250°C was reached, it was maintained for 5 minutes. The temperature of the injector and the interface was 250°C while that of the source was 260°C. The gas vector was helium at a pressure of 9,8 psi and the total ion chromatogram was recorded by using an electron-impact source at 70 eV of ion kinetic energy. Identification of the compound was made by comparison to National Institute of Standards and Technology (NIST) spectral library. To confirm identification, a mixture of homologue n-alkanes (C7-C30) was injected in GC-MS following the same chromatographic conditions to calculate the retention index (RI). In addition, the identification of the main compounds was confirmed by co-injection of pure references (Sigma, Darmstadt, Germany) in order to compare retention times and MS spectrum data. Finally, the results are expressed in µg of *M. arvensis* EO per insect.

**Proteomic analyses**

***Treatment and preparation of protein extracts***

For proteomic analyzes, the treatment procedure was essentially the same as for the contact toxicity test: the 50% lethal dose of *M. arvensis* EO (LD50 = 55.6 µg/insect) was applied topically to seven to fourteen days post-emergence adults and individuals treated only with acetone were used as a control. Treatments were carried out independently three times (for a total of 80 individuals per replicate). 24 h after treatment, fifteen living insects were collected for each sample and then immediately frozen in liquid hydrogen and stored at -80° C.

Proteins from the collected insects were extracted with the PrecellysTM 24 homogenizer (Bertin Instruments, Rockville, USA) using 2 mL cap Eppendorf tube with 500 µL of homogenization buffer (8 M Urea, 100 mM TEAB (Triethylammonium bicarbonate), pH 8.5 (HCl), 2 mM EDTA, 10 mM dithiothreitol (DTT), protease inhibitor mix (1 mM phenylmethylsulfonyl fluoride (PMSF)), 2 μg/mL each of leupeptin, aprotinin, antipain, pepstatin, and chymostatin, 0.6% w/v polyvinylpolypyrrolidone). The insects were ground three times at 500 Hz during 20 seconds using 15 ceramic beads of Zirconium oxide having an external diameter of 2.8 mm (Bertin Instruments). The homogenate was centrifuged for 5 min at 9000 rpm at 4°C and the supernatant was then centrifuged again at 54,000 rpm (TLAA55, Optima-Beckman, Indianapolis, USA) for 30 min at 4 °C to separate the soluble and membrane fractions. The protein concentration was determined according to Bradford method (1976) using the Bio-Rad Protein Assay kit with bovine gamma globulin as standard. 20 µg of each sample were transferred to 0.5 mL polypropylene Protein LoBind Eppendorf Tubes and precipitated with chloroform-methanol method (Wessel and Flügge 1984). 20µl of 100 mM TEAB, pH 8.5 (triethylammonium bicarbonate) containing 0.5% RapiGest surfactant (Waters, Milford, USA) were added to solubilize the proteins. The proteins were then reduced with 5mM DTT (dithiothreitol) and alkylated with 15 mM iodoacetamide. Samples were diluted five times with 20µl of 100 mM TEAB, pH 8.5. Proteolysis was performed with 0.5 µg of trypsin and was continued overnight at 37°C. Each sample was dried under vacuum with the Savant Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA) and stored at -80°C.

***Peptide separation using nanoUPLC***

Prior to peptide separation, the samples were dissolved in 20 µl of 0.1 % (v/v) formic acid and 2% (v/v) acetonitrile (ACN). The peptide mixture was separated by reverse phase chromatography on a NanoACQUITY UPLC M-Class system (Waters) working with the MassLynx V4.1 software (Waters). 200 ng of digested proteins were injected on a trap C18, 100Å 5 µm, 180 µm x 20 mm column (Waters) and desalted under isocratic conditions with a flow rate of 15 µL/min using a 99% formic acid and 1% (v/v) ACN buffer for 3 min. The peptide mixture was subjected to reverse phase chromatography on a C18, 100Å 1.8 µm, 75 µm × 150 mm column (Waters) PepMap for 120 min at 35°C with a flow rate of 300 nL/min using a two-part linear gradient from 1% (v/v) ACN, 0.1 % formic acid to 35 % (v/v) ACN, 0.1 % formic acid and from 35% (v/v) ACN, 0.1 % formic acid to 85 % (v/v) ACN, 0.1 % formic acid. The column was re-equilibrated at initial conditions after a 10 min wash at 85% (v/v) ACN, 0.1 % formic acid at a flow rate of 300 nL/min. For online LC-MS analysis, the nano UPLC was coupled to the mass spectrometer through a nano-electrospray ionization (nano ESI) source emitter.

***LC-IMS (Ion Mobility Separation)-QTOF-MS Analysis (HDMSE)***

The IMS-HDMSE (Ion Mobility Separation-High Definition Enhanced Mass Spectrometry) analyses were performed on an SYNAPT G2-Si high definition mass spectrometer (Waters) equipped with a NanoLockSpray dual electrospray ion source (Waters). Precut fused silica PicoTipR Emitters for nanoelectrospray, outer diameters: 360 µm; inner diameter: 20 µm; 10 µm tip; 2.5” length (Waters) were used to spray samples and Precut fused silica PicoTipR Emitters for nano-electrospray, outer diameters: 360 µm; inner diameter: 20 µm; 2.5” length (Waters) were used to spray the lock mass solution. The eluent was sprayed at a spray voltage of 2.4 kV with a sampling cone voltage of 25 V and a source offset of 30 V. The source temperature was set at 80°C. The HDMSE method in resolution mode was used to collect data from 15 min after injection to 106 min. This method acquires MSE in positive and resolution mode over the m/z range from 50 to 2000 with a scan time of 1 sec with a collision energy ramp starting from ion mobility bin 20 (20 eV) to 110 (45 eV). The collision energy in the transfer cell for low-energy MS mode was set to 4 eV. For the post-acquisition lock mass correction of the data in the MS method, the doubly charged monoisotopic ion of [Glu1]-fibrinopeptide B was used at 100 fmol/µl using the reference sprayer of the nano-ESI source with a frequency of 30 s at 0.5 µl/min into the mass spectrometer.

***Database search and protein quantification***

The HDMSE data were processed with the Progenesis QI software (Nonlinear Dynamics, Waters) using the proteome of *Dendroctonus ponderosae*, the closest insect species to *S. granarius* with a fully sequenced and annotated genome (Keeling et al. 2013). This database was supplemented by the protein sequences corresponding to *Sodalis pierantonius*, the obligate bacterial symbiont associated with all *Sitophilus* species that plays a key role in the metabolism of several amino acids and vitamins (Vigneron et al. 2014). A final database based on a total of 25986 entries was obtained. The following search parameters were selected: carbamidomethylation as the fixed cysteine modification, oxidation as the variable Methionine modification, trypsin as the digestion enzyme were selected and one miss cleavage allowed.

***Statistical analysis***

Three biological replicates and two technical replicates were used for each sample. Non-conflicting method was used as a relative quantification method. To identify statistically significant differentially expressed proteins, combined criteria of a minimum of three or greater unique peptides, a 1.2-fold change ratio or greater and an Anova *p*-value < 0.05 were adopted.

**Results**

**Contact toxicity of *M. arvensis* EO and effects on locomotor behavior**

The LD50 and LD90 of *M. arvensis* for *S. granarius* adults 24 h post-treatment was 55.6 µg/insect and 79.5 µg/insect, respectively (Figure 1).

For locomotion behavior tests, 24 of the 54 insects that were treated with *M. arvensis* EO died. Figure 2 shows the mean cumulative of the distance travelled over 24 hours by untreated (a), treated but surviving (b) and treated and dead (c) individuals. Untreated individuals had a constant speed for 24 hours, while treated individuals that survived the treatment were first knocked out (Movie S1), then travelled a negligible distance for the first 4 hours, and then travelled at a more or less constant but lower speed than untreated individuals. For individuals considered dead after the 24 treatments, the overall distance travelled was negligible: some individuals died quickly while others continued to move before they die. The mean speeds between untreated and treated individuals and their standard deviations are shown in Figure S1 (a). A distribution comparison using a Mann-Whitney U test reveals that the distributions (Figure S2 (a)) are different (U = 41, p < 0.001). Similarly, the mean proportions of space visited (Figure S1 (b)) by untreated and treated individuals shows that untreated individuals covered three to four times more space than treated individuals. Again, a comparison between the two distributions (Figure S2 (b)) with a Mann-Whitney U test shows a strong significative difference (U = 19, p < 0.001). A closer look at the distribution presented in Figure S2 (a) reveals a bimodality, expressing the fact that the mean speeds over 24h of the surviving treated individuals are either very low or close to those of the untreated individuals. However, this bimodality is not observed in the distribution of the proportion of spaces visited (Figure S2 (b)), which suggests that the locomotion of individuals is restricted in space.

Finally, Figure 3 presents on overview of the spatial behavior of untreated individuals (a), treated individuals that survived (b) and treated individuals that died (c). The three panels display an overlay of the positions for all experiments. It is interesting to note that untreated individuals moved rapidly towards the borders of the Petri® dish. Once they reached the border, they exhibited a strong thigmotactic behavior by following the border and rarely returning to the center. On the other hand, treated individuals that survived moved less and rarely reached the limit of the Petri dish, while treated individuals that did not ultimately survive appear to have remained in place for the duration of the experiment.

***In vivo* cuticular penetration of *M. arvensis* EO**

To examine the penetration of *M. arvensis* EO through the cuticle of *S. granarius* and the evolution of its amount in the 24 hours following the treatment, the internal amounts of the EO after topical application were examined via GC-MS analysis. We found that the amount in the insects treated with LC50 evolved over time: at about 2 µg/insect at the beginning of the experiment, it dropped to about 0.5 µg 24 hours after exposure (Figure S3).

**Identification of differentially expressed proteins**

We used a label-free quantitative proteomics approach to decipher the mechanisms behind the insecticidal activity of *M. arvensis* EO. Filtered with a cut-off of 1.2-fold change ratio, identification based on a minimum of three unique peptides, and *p*-value < 0.05, 55 proteins were found to be differentially expressed 24h after exposure to *M. arvensis* EO in *S. granarius* (Table S2). Of these DEPs, 54 were upregulated while only one protein was downregulated (Figure 4). The functional classification of DEPs produced by COG (Clusters of Orthologous Groups of proteins) (Tatusov et al. 2001) showed that they are involved in various types of biological processes: most DEPs are involved in the cytoskeleton network, signal transduction mechanisms, protein turnover, energy production and RNA processing and modification. Among the top upregulated proteins, our approach identified the 60S ribosomal protein L27 involved in protein biosynthesis, the electron flavoprotein subunit β involved in the respiratory electron transport chain, a S-formylglutathione hydrolase involved in the formaldehyde catabolic process and the flagellar transcriptional activator FlhD associated with *S. pierantonius*, the obligate bacterial symbiont of weevils of the genus *Sitophilus*.

Overall, DEPs include proteins involved in protein biosynthesis (e.g. ATP-dependent RNA helicase Ddx1, the elongation factor 1α, several chaperonins, etc.), cellular respiration (e.g. fumarate hydratatse, ATP synthase, etc.), the organization and function of the nervous system (e.g. glutamine synthetase 2 cytoplasmic, double SH3 domain protein 2, etc.) and the organization and function of the muscular system (troponin T, actin-5C, etc.). The flagellar transcriptional activator FlhD is the only detected DEP associated with *S. pierantonius*. The putative role of the identified DEPs is summarized in Figure 5 and discussed in detail in the following section.

**Discussion**

In this study, we found that *M. arvensis* EO caused high contact toxicity in adults of the grain weevil *S. granarius* with LD50 and LD90 values of 55.6 µg/insect and 79.5 µg/insect respectively 24 h after exposure. Previous studies have reported that *M. arvensis* EO exhibits antibacterial effects (Biswas et al. 2014; Horváth and Koščová 2017), antifungal effects (Varma and Dubey 2001; Kumar et al. 2007, 2009) and adulticidal activity on the storage pests *Tribolium castaneum* and *Sitophilus ozyzae*, both by contact and by fumigation (Tripathi et al. 2000; Aggarwal et al. 2001; Mishra et al. 2012). Our results confirmed the insecticidal activity of *M. arvensis* EO on *S. granarius* adults. However, it is important to keep in mind that the toxic properties of EOs strongly depend on their chemical composition, which itself can vary considerably depending on the extracted organ, the age of the plant, the geographical area and generally on the growing conditions (Noudjou et al. 2007; Schaneberg and Khan 2002; Gil et al. 2002; Ložienė and Venskutonis 2005; Olivero‐Verbel et al. 2010). This variability, but also the diversity of the insect models studied and the conditions of application of the EO, make it difficult to compare the toxicological data reported by the different studies. However, *M. arvensis* EO is a promising insecticidal compound not only because of its insecticidal properties, but also because *M. arvensis* can be easily cultivated in temperate regions (Tiwari 2016) and because its price is one of the lowest compared to other essential oils (Demeter et al. under review).

Our proteomic analyses reveal that *M. arvensis* EO induced dramatic physiological changes in *S. granarius* adults. They are a snapshot of the insect’s molecular response 24 h post-treatment and the differentially expressed proteins (DEPs) identified here should reflect how surviving insects reset their physiology after exposure to the EO. Our experimental approach resulted in the identification of a large number of DEPs reflecting the many systems affected by *M. arvensis* EO. The vast majority of these DEPs were upregulated, suggesting that protein biosynthesis took place in insects that survived treatment and that exposure to *M. arvensis* EO is associated with physiological costs. This hypothesis is supported by the upregulation of several proteins involved in the transcription and translation processes, including the ATP-dependent RNA helicases Ddx1 and p62, the elongation factor 1α, several 40S ribosomal proteins (S4 and S12) and several 60S ribosomal proteins (L27 and L27a) (Rafti et al. 1996; Herold et al. 2009; Sasikumar et al. 2012; Peña et al. 2017). In addition, several chaperonins, which are proteins that ensure the correct folding of other proteins, were found upregulated. This is the case of heat shock 10 kDa protein 1 (Hsp10) which is essential for the assembly of proteins imported into mitochondria (Cheng et al. 1989) and immunophilin 12 kDa FK506-binding protein which participates in diverse pathways (Preall et al. 2012; Bonner and Boulianne 2017). Interestingly, several of these chaperonins are involved in the development of the nervous system and the organization of cytoskeletal components. For example, T-complex protein 1 subunit delta is one of the components of a molecular chaperon complex playing a role in actin and tubulin folding (Leroux and Hartl 2000), and Heat shock 70 kDa protein cognate 4 is a folding proteins known to contribute to clathrin-mediated endocytosis, neurotransmitter exocytosis and axon guidance (Schmucker et al. 1997; Bronk et al. 2001). The upregulation of these chaperonins suggests that *M. arvensis* EO generated protein-damaging stress conditions which therefore require correct folding, but also that chaperonins are required for the correct folding of newly overexpressed proteins.

Previous studies have shown that insecticidal secondary metabolites of plant origin tend to inhibit the functioning of enzymes involved in carbohydrate and energy metabolism (Rattan 2010; Mansour et al. 2012; Liao et al. 2018; Huang et al. 2019). Damage to these metabolic pathways generally results in downregulation of the proteins involved (Liao et al. 2016, 2018; Huang et al. 2019). In our study, the DEPs involved in cellular respiration are upregulated. They include the enzyme fumarate hydratase involved in the TCA (tricarboxylic acid) cycle and several proteins involved in the electron transport chain including the cytochrome c oxidase subunit 5B, the electron transfer flavoprotein subunit beta, the ADP/ATP carrier protein 2 and the ATP synthase subunit b (Norden and Matanganyidze 1979; Klingenberg 1985; Fontanesi et al. 2008). Acyl-CoA dehydrogenase and enoyl-CoA hydratase were also upregulated. These two enzymes catalyze the initial steps of fatty acid β-oxidation, a process that takes place in the mitochondria and leads to the production of acetyl-CoA involved in the TCA cycle, and NADH and FADH2, whose electrons feed the electron transport chain (Thorpe and Kim 1995). The upregulation of all these proteins suggests that *M. arvensis* EO affected the mitochondrial system before it was rebuilt in insects that survived treatment. However, upregulation of these proteins may also reflect the need for metabolic energy for the biosynthesis of proteins involved in the many systems that have been damaged by *M. arvensis* EO, including the nervous and muscular systems. These results support the hypothesis that insects increase the abundance of the mitochondrial complex to improve energy efficiency and that mitochondria are involved in regulating the reprogramming of intracellular metabolism in EO-exposed insects (Liao et al. 2016).

Exposure to *M. arvensis* EO causes a significant alteration in the behavior of *S. granarius*: the application of the LD50 leads to a rapid paralysis of the insects, which, if they survive, suffer from locomotion disorders. Individuals which survived treatment tend to exhibit reduced locomotor activity, roaming shorter distance in the arena compared to control insects. However, our results suggest that the surviving insects are gradually recovering from the treatment. Indeed, after 4 hours of paralysis, the insects tend to be more and more mobile, which seems to be correlated with the evolution of the amount of *M. arvensis* EO found in the insects that must have evaporated and/or been partially metabolized within 24 hours after exposure. It is well known that EOs or some of their components can modify the behavior of exposed insects, in particular by influencing olfactory orientation and walking behavior (Deletre et al. 2016; Oliveira et al. 2018; Brügger et al. 2019). Certain EOs can cause hyperactivity in exposed insects, followed by hyperexcitation leading to rapid knock out and immobilization (Saroukolai et al. 2010; Rattan 2010; Plata-Rueda et al. 2018). This phase of hyperactivity is not associated with *M. arvensis* EO on *S. granarius*. However, the rapid alteration of insect behavior suggests that *M. arvensis* EO elicits physiological reactions, probably through neurotoxic effects (Bloomquist 2003; Enan 2005).

The neurotoxic properties of essential oils have been reported previously (Coats et al. 1991; López and Pascual-Villalobos 2010; Rattan 2010; Jankowska et al. 2018). In particular, EOs can inhibit the acetylcholinesterase (AChE) activity, interfere with the octopamenergic system by competing with octopamine in binding to its receptors and modulate GABA receptors (Enan 2001; López and Pascual-Villalobos 2010; Rattan 2010; Jankowska et al. 2018). In the present study, most upregulated proteins are linked to the organization and function of the nervous system, confirming that one of the modes of action of *M. arvensis* EO is to affect the nervous system. The upregulation of the related DEPs suggests that, within 24 h, mechanisms were established in surviving insects to repair the damage inflicted. Many upregulated proteins are key effectors involved in the development and functioning of the nervous system. Glutamine synthetase 2 cytoplasmic is vital for synaptogenesis, that is, the formation of synapses between neurons in the nervous system (Featherstone et al. 2002). RNA-binding proteins (RBPs) are involved in the regulation of neurogenesis (Olesnicky and Wright 2018). The tyrosine-protein phosphatase non-receptor type 9 is known to be involved in molecular logic of neocortical projection neuron specification in mammals (Zhang et al. 2016). Poorly described in insects, this protein probably plays a similar role in these animals. In *D. melanogaster*, F-BAR and double SH3 domains protein 2 (also called Protein nervous wreck) helps regulate the polymerization of actin filaments during synaptic growth (O’Connor-Giles et al. 2008; Rodal et al. 2008). The protein hu-li tai shao, the only downregulated protein in insects exposed to *M. arvensis* EO, is crucial for normal neuromotor function (Ohler et al. 2011) and is involved in various other biological processes, including oogenesis and cytoskeletal organization for the development of sarcomeres (Schnorrer et al. 2010; Pokrywka et al. 2014). In addition to this, there is the upregulation of key effectors involved in the development and functioning of the sensory nervous system. These include COP9 signalosome complex subunit 1 which is involved in axonogenesis and differentiation of photoreceptor cells (Suh et al. 2002; Wei and Deng 2003). The guanine nucleotide-binding protein G(o) subunit alpha was also upregulated. It is one of the most abundant G protein in nervous tissues and is involved in neurodevelopment, heart morphogenesis and the sensory perception of sweet taste (Yi et al. 2008; Bredendiek et al. 2011; Koon and Budnik 2012). Taken together, these results reveal that *M. arvensis* EO has severely damaged the insect nervous system and shed light on a range of effectors involved in the insect's response to the product.

Insects exposed to *M. arvensis* EO suffer from paralysis and behavioral disorders, which suggests that the muscular system is affected. This is confirmed by the upregulation of effector proteins involved in the development and functioning of this system. Troponin-C and troponin-T are parts of the troponin complex that regulates the Ca2+-activation of myofilaments during contraction and relaxation striated muscle, and plays a key role in contraction of flight muscles (Domingo et al. 1998; Cao et al. 2020). Tropomyosin plays a central role in the calcium dependent regulation of muscle contraction, in association with the troponin complex (Zot and Potter 1987). Tropomyosin is involved in myogenesis and muscle contraction, but also in other biological processes, including dendrite morphogenesis, oogenesis and response to hypoxia (Tetzlaff et al. 1996; Li and Gao 2003; Jan and Jan 2010; Zhao et al. 2010). Actin-5C is involved in muscle contraction (Mounier and Prudhomme 1991) as well as other biological processes including chromatin remodeling and mitotic cytokinesis (Echard et al. 2004; Klymenko et al. 2006). In *D. melanogaster*, the muscle LIM protein Mlp84B, here upregulated, helps maintain muscle integrity (Clark et al. 2007). Finally, PDZ and LIM domain protein Zasp and of Muscle-specific protein 20 are both involved in myogenesis (Benna et al. 2009; Bataillé et al. 2010).

The molecular upheaval due to *M. arvensis* EO does not stop at the nervous and muscular systems, since proteins which are at the crossroads of multiple biological processes were upregulated. For example, we found an upregulation of high mobility group protein DSP1, an HMG-like protein that plays multiple roles in insect development by regulating homeotic genes (Mosrin-Huaman et al. 1998; Decoville et al. 2001). The calcium transmembrane transporter calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type also performs multiple functions including regulation of adaptive thermogenesis, heart contraction, neuromuscular synaptic transmission, fatty acid beta-oxidation, flight behavior and many other biological processes (Sanyal et al. 2005; Banerjee et al. 2006; Abraham and Wolf 2013; Bi et al. 2014; Moraru et al. 2017). The alanine aminotransferase is involved in gluconeogenesis, amino acid metabolism, nitrogen use efficiency and detoxification of nitrogen waste (Wan et al. 2015). The coatomer subunit delta is a part of a complex involved in the morphogenesis of salivary glands, protein secretion and opening of the tracheal system (Jayaram et al. 2008). Dynein light chain 1 regulates dynamin-mediated F-actin assembly during oogenesis and spermatogenesis (Dick et al. 1996; Ghosh-Roy et al. 2005). Finally, polyubiquitin can perform various functions including DNA repair and the regulation of autophagy and programmed cell death (Li and Ye 2008; Zhou et al. 2013). Upregulation of all these proteins indicates that the response of *S. granarius* is systemic and involved many biological processes.

Compared to previous “omic” studies (Liao et al. 2016; Tak et al. 2017; Huang et al. 2019) we found relatively few DEPs associated with detoxification. Only S-formylgluthatione hydrolase, a serine hydrolase involved in the detoxification of formaldehyde, was upregulated. Formaldehyde is produced endogenously in all living organisms as a result of metabolism of amino acids and oxidative demethylation of DNA and RNA (Gonzalez et al. 2006). However, too high a concentration of formaldehyde leads to DNA and protein damage (Chen et al. 2016). The upregulation of S-formylgluthatione hydrolase suggests that *M. arvensis* EO induced an accumulation of formaldehyde that may have caused proteins and DNA damage.

Weevils are shielded with hard cuticle, which is the first and major barrier protecting the insect from penetration of external compounds (Balabanidou et al. 2018). Interestingly, *M. arvensis* EO has induced upregulation of Yellow protein, a protein essential for the pigmentation of the body wall cuticle of adult insects (Wittkopp et al. 2002; True 2003) and which is associated with an anti-dehydration function by rendering the cuticle waterproof (Noh et al. 2015). The flagellar transcriptional activator FlhD has also been shown to be upregulated. This protein is expressed by *Sodalis pierantonius*, the obligate endosymbiont of weevils. This bacterial associate provides the insect with many nutrients that are poorly present in wheat grains, including the amino acids tyrosine and phenylalanine which are precursors of dihydroxyphenylalanine (DOPA), itself a precursor in the synthesis of the cuticle (Andersen 2012; Vigneron et al. 2014; Hirota et al. 2017; Dale 2017). The proliferation of *S. pierantonius* after weevil metamorphosis corresponds to a drastic physiological need of the host for the amino acids tyrosine and phenylalanine, necessary for the development of its protective exoskeleton during the first two weeks of adulthood (Vigneron et al. 2014). The upregulation of FlhD suggests proliferation of the bacterial symbiont in surviving insects. The upregulation of yellow protein and FlhD suggests that *M. arvensis* EO altered the cuticular integrity of the insects and that the surviving individuals activated mechanisms that promote cuticular sclerotization in order to rebuild their exoskeleton.

Research to develop natural products for pest control is increasingly important as some synthetic pesticides are associated with environmental concerns or are withdrawn for economic and regulatory reasons. Essential oils show good potential in the control of insect pests, in particular because they can have diverse physiological targets within insects, and, therefore, can delay the evolution of insect resistance (Regnault-Roger et al. 2012). *M. arvensis* EO is a promising candidate in the development of EO-based insecticides against weevils because of its 1) high contact toxicity, 2) ability to penetrate a thick, hard exoskeleton, 3) ability to rapidly alters insect behavior and 4) because it is associated to a systemic physiological/molecular response. Our proteomic approach shed light on modes of action associated with EOs, some of which are presented here for the first time. Proteomic results indicate that insects that are able to recover from LD50 exposure do so at an apparently high energy cost, which could have consequences on their fitness. Further studies are needed to determine more finely how these compounds penetrate the insects, the kinetics of the induced physiological changes and the associated fitness costs. The use of insecticides of plant origin seem to be a promising alternative to the use of conventional insecticides as they are more biodegradable and probably safer for human health and the environment. However, like conventional insecticides, these new products can exert a wide range of effects on insects and other animals, including mammals and *M. arvensis* EO apparently has the potential to affect molecular pathways that are also found in mammals. Therefore, more attention needs to be paid to these aspects in the development of these new forms of biocidal products. Given the increasing use of natural compounds in chemical control practices (EOs are increasingly used as insecticides but also as herbicides, fungicides and bactericides), knowledge of the modes of action of these new biocides is essential to design new and efficient products, but also to determine the most appropriate way to use them in agriculture.

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**Figure 1.** Proportion of dead individuals (age: seven to fourteen days post-emergence) as a function of the dose of *M. arvensis* EO (markers) along with a nonlinear regression using eq. (1). Parameter values are: *n* = 6.175 ± 0.573, *K* = LD50 = 12.313 ± 0.192.

**Figure 2.** Cumulative distances (Mean ± SD) as a function of time for untreated insects (a), treated insects that survived (b) and treated insects that died (c).

**Figure 3.** Overlays of the positions for all the experiments for untreated individuals (a), treated ones that survived (b) and treated ones that died (c).

**Figure 4.** General annotation of the DEPs. (a) COG annotation of the DEPs. (b) Statistics of up- and downregulated DEPs in *S. granarius* on exposure to *M. arvensis* EO.

**Figure 5.** Summary of the DEPs in *S. granarius* responding to *M. arvensis* EO after filtration (identification based on a minimum of 3 unique peptides, cut‐off of 1.2‐fold change and *p*‐value < 0.05). Proteins are classified according to the biological processes in which they are involved (upregulated proteins are noted with an arrow highlighted in yellow and downregulated proteins are noted with an arrow highlighted in blue).