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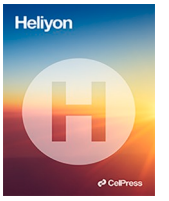

Research article

Examination of genetic lines of *Myrtus communis* as potential sources of organic agricultural pest control agents

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ABSTRACT

Myrtus communis is a Mediterranean shrub cultivated in Israel for traditional, ceremonial use only, with more than 98 % of the crop biomass, equivalent to 26–27 tons per ha per annum, considered agricultural waste. Therefore, potentially profitable use for this excess is being highly sought. As *Myrtus* is also known for its unique terpene and terpenoid content, this work evaluated the impact of essential oil (EO) extracted from several *M. communis* cultivars on storage insects, nematodes, fungi, and pathogens. In addition, the allelopathic effect of *M. communis* litter on the germination success of wheat seeds was evaluated. The EO extracts demonstrated an insecticidal effect on several storage insects in fumigation experiment and a potentially inhibiting effect on wheat development in allelopathy experiments. No significant impact of *M. communis* EOs on the examined fungi, pathogens, and nematodes was recorded. Additional uses of the *M. communis* biomass suggest supplying additional income to the farmer through the circular agriculture approach. In addition, the use of this local crop can contribute to sustainable intensification by increasing farming efficiency, providing nature-based substitutes for chemical pesticides, and possibly, improving the future design of agriculture through the integration of Myrtus in monoculture crops.

Key message

Myrtus communis crop imparts pesticidal effects that can contribute to the sustainable intensification of agriculture.

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1. Introduction

The only Myrtaceae species native to Israel is *Myrtus communis* L. (common Myrtle), a Mediterranean shrub (SI_1), which is listed as an endangered plant species [1]. At the same time, its shrubs are cultivated for traditional use or as ornamental garden shrubs [2] (SI_2). Since biblical times, young branches have been used in Judaism as one of the "Four Species" of the "Tabernacle" (Sukkot) feast [3], and its agricultural cultivation in Israel is oriented to its traditional use. Selection of genetic lines for breeding and agro-technical practices are driven to produce branches that qualify for the ceremonial "Four Species" market. A myrtle branch is considered kosher for the Sukkot holiday when it has a 'three-leaved' or a "tricussate leaf shoot" arranged with three leaves per node [2]. This requires that the predominant part of the plant biomass remains unused in the field (SI_3), resulting in inefficient agricultural production. Moreover, to facilitate the vegetative growth of tricussate shoots [4] for harvest in the fall, the plants are pruned in the spring, creating another cycle of wasted biomass.

Evidence suggests that chemical pest control negatively affects humans [5] and natural ecosystems [6]. Stored grain pests require special attention since the target for protection from insect pests is consumed by humans. Of particular interest is the identification of an alternative to toxins such as phosphine, a gas extremely toxic to humans [7], and the leading fumigant insecticide used today for the disinfestation of pests in stored products [8,9]. The intensive use of phosphine as a single fumigant over long periods, alongside poor fumigation techniques, has led to the selection of resistant insects and the development of immunity to strong toxins [10,11].

The essential oils (EOs) extracted from *M. communis* have demonstrated a broad scope of anti-pest activities [12,13], including insecticidal, anti-inflammatory [14], and antimicrobial activities. Other studies showed that *M. communis* EOs exhibit antioxidant and antimutagenic activities [15]. The EOs of *M. communis* are considered the active materials produced by the plant, containing mainly monoterpenes [15,16] and polyphenols [15]. Polyphenols have a hypothesized role in human health - protective effects in chronic and acute diseases [17]. Monoterpenes, herbivore-induced volatiles [18], are recognized as herbivory repellants, protecting the plant against herbivory damage [19]. In traditional medicine, Myrtle is used as an antiseptic agent against viruses, bacteria, fungi, and inflammation [20,21]. Giuliani et al. [22] found that the α-terpineol frequent in the EOs of *M. communis* has a detrimental effect on bark beetle pests. In addition, fumigation with *M. communis* EO showed toxic effects on storage pests [23], such as beetles [24] from the Tenebrionidae [25], Dermestidae [26], and other families. Application of myrtle leaves was found to impart a detrimental or repellant effect on additional storage pests [27,28], mosquito larvae [29], pest nematodes [30] and fungal plant pathogens [31–33]. In addition, *M. communis* has been shown to have a herbicidal effect on weeds [32] and improved quality of strawberries after their harvest [34].

In the face of climate change and growing pressures to implement sustainable agriculture – the use of surplus *M. communis* biomass can support several sustainable development goals (SDGs), as defined by the United Nations in 2015. If *M. communis* can be used as a biological substitute for chemical pest control, it will promote the third SDG – good health and well-being [35]. In addition, it may provide additional income to the Myrtle farmers – which has the potential to support decent economic growth (SDG8). A reduction in biomass loss may promote responsible production (SDG12). Furthermore, promoting the use of an endangered species in cultivated fields may support its protection (SDG15). The use of surplus biomass to develop bioactive material for pest control can facilitate sustainable intensification of agricultural production [36].

This research aimed to provide an alternative use for cultivated *M. communis* plant biomass that is currently wasted, as a biocide to control a broad range of agricultural pests. To evaluate the potential of *M. communis* as a bioactive pesticide, this work assessed the biomass production in a cultivated field and the phytochemical profiles of the EOs extracted from four genetic lines of *M. communis* which were selected for traditional use. EO production was measured over time to characterize the temporal chemical profile and quantity of the EOs.

2. Materials and methods

Plant material. A *M. communis* breeding plot at the Agricultural Research Organization (ARO), Rishon Le Tzion, Israel (31°59′31.7″N 34°48′59.9″ E) was used in this study. Established in 1990, the research plot includes genetic lines that were selected by breeders and growers for traits critical to their traditional use, i.e., three-leave branch features. The uniformity of genetic lines was achieved using vegetative propagation only, namely, by rooting plant cuttings. This uniformity is a unique character of the plant material, as vegetative propagation of Myrtus can be challenging, and seed propagation is more common [37]. The origins of these genetic lines were primarily wild populations in the Upper Galilee, and the Golan Heights, Israel. The genetic lines included 33 individuals of the cultivar (cv.) "Hadur", and 15 individuals of cv. "David", 22 individuals of cv. "Kfar Shamai", 29 individuals of cv. "Levi", and 20 individuals of cv. "Chaim". In addition, several populations were underrepresented, with fewer than 10 individuals each, i.e., cv. "White fruit" (4 individuals), an unknown population named "Mutant" (4), and another population from an "Unknown" source with 8 plants. To enhance vegetative growth in the fall, the plants were cut in February 2020, and an aerial image was taken by a drone in April 2020 to create an in-situ map (SI_4). Plants were harvested in October–November 2019, 2020, and 2021. The first harvest, in 2019, was taken from woody plants, which were not cut for several years before the harvest. The 2020 harvest was performed on fresh vegetative branches 6 months after the cutting. The harvest in December 2021 was performed 1.5 years after the last cut. *M. communis* plants from an agricultural field at Moshav Nov, the Golan Heights (32°49′30.7″N 35°46′59 0.9″E) served as a reference. In October 2019, plants at Nov that were cut 0.5 years and 1.5 years before sampling, served as a reference to study the impact of shoot age. For each sampling, five individual plants were used to represent the cultivar by biological replicates. Each EO sample is an extraction 500 g mixture of fresh shoot material from a single cultivar.

Plant biomass. To estimate the fresh plant biomass in the field, and the potential biomass available for EO extraction, ten mature *M. communis* (cv. David) in an agricultural field at Moshav Nov (see *Plant material* section for details) were pruned. The pruning took

place in October 2019, at the end of the harvest season. The total fresh biomass of each one of the cut mature plants was measured. In addition, 'three-leaved' branches were weighed before and after their ornamental pruning, to estimate the total surplus biomass produced in the field. Three branches were weighed at a time, before and after their final pruning. To complete the estimation of biomass production, information regarding the density of plants in the field, pruning time, irrigation, and pest control was acquired from the grower. This information was compared with agronomic details reported in the literature [2].

Essential oil extraction. A total of 500 g fresh shoot (leaves and branches) was collected, as described in the *Plant material* section. The fresh shoots were coarsely chopped and immersed in distilled water (approx. 2 L) in a 5-liter flask. The distillation was performed using a Clevenger's glass apparatus. The water in the flask was heated to boiling point, and cooling water was simultaneously pumped from a 20 L container and circulated by a simple water pump through the cooling apparatus. The extraction process was carried out for 3 h after the extraction of the first drop of distillate. The extracted EOs were recovered and weighed, and stored in a refrigerator at 4 °C in tightly closed vials, until further chemical analysis or the functional biological testing.

Essential oil phytochemical analysis. Five extractions per line were analyzed using a Gas Chromatography-Mass Spectrometer (GC-MS) by HP 6890 GCMS system with a 5973 Mass Selective Detector running GCD Plus ChemStation and an SPB-5 column. Experimental conditions: inlet, 250 °C; initial temp, 50 °C for 5.0 min, with 10 °C/min increments; final temp, 280 °C held for 10 min. Compounds were identified based on the Wiley Spectral Database and relative quantification was performed based on peak areas in the GC-MS TIC chromatogram.

Volatile organic compounds (VOCs). Five branches were collected at the ARO research plot in February 2020, one from each Myrtus genetic line - "David", "Levi", "Hadur" "Kfar Shamai" and "Chaim". The branches were closed in a sealed container immediately after cutting, then transferred to the Newe Ya'ar research station for the headspace analysis. VOCs were collected from each branch separately. The volatiles were adsorbed using a solid phase microextraction (SPME), HS-SPME MPS2 (Gerstel, Mülheim, Germany) by a 65 μm PDMS/DVB/CAR fiber (polydimethylsiloxane/divinylbenzene/carboxen; Supelco, PA, USA) for 1 h, at room temperature. Thereafter, the SPME syringe was introduced into the injector port of the GC-MS apparatus for 5 min. Volatile compounds were analyzed on a GC-MSD apparatus (6890N/5973N Agilent Technologies CA, USA) equipped with a Rxi-5 SIL MS (30 m * 0.25 mm * 0.25 μm) fused-silica capillary column (Restek). Helium (constant pressure 9.1 psi) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 300 °C at a rate of 5 °C/min. The detector temperature was 280 °C. The mass range was recorded from 41 *m/z* to 350 *m/z*, with an electron energy of 70 eV. A mixture of straight-chain alkanes (C7–C23) was injected into the column under the above-mentioned conditions for the determination of retention indices. The GC-MS spectrum profiles were analyzed using the chemstation software. Volatiles were identified by comparison of their retention indices with those reported in the literature and by comparison of spectral data with a standard or with the Wiley7N and HPCH2205 GC-MS libraries.

Chemical pesticide residuals. According to the Jewish tradition, the premier tricussate branches should be free of pests and diseases, and therefore, chemical pesticides are used in the field, and the harvested branches are manually washed in water to ensure their safe use. Therefore, for most of the plant biomass, the possibility that residuals of chemical pesticides biases the research had to be determined. In 2019, fresh plants from the harvested material were used. To identify the presence of insecticides, up to 5 g plant material was extracted with 10 mL acetonitrile, dried over MgSO4 and NaCl, and subsequently, 2 mL of the acetonitrile fraction was transferred to a new 15 mL Falcon tube and dried over MgSO4 and Bondesil DEA bulk sorbent. The dried organic solvent was transferred to a new tube, of which 1 mL was evaporated using a stream of N2 and the dried residual was reconstituted with 1 mL ethyl acetate and subjected to GC-MS analysis. The qualitative analysis was performed on a model 7890A gas chromatograph (Agilent Technologies, Santa Clara, USA) equipped with a single quadrupole 5975C VL-MSD, nitrogen phosphorus detector, and a J&W Megabore 5 % phenyl-95 % methyl silicone capillary column (0.25 μm × 15 m x 0.25 mm; Agilent Technologies, Santa Clara, USA). The temperature program for identifying organophosphorus pesticides was as follows: injector temperature, 220 °C; initial temperature, 80 °C for 0 min; gradient of 17 °C/min until 180 °C; gradient of 10 °C until 250 °C; gradient of 20 °C until 300 °C. The MS parameters were set as follows: source temperature, 230 °C; transfer line, 230 °C; positive ion monitoring; EI-MS (70 eV). For the identification of carbamate pesticides, the following temperature program was employed: injector temperature, 150 °C; initial temperature, 40 °C for 1 min; gradient of 15 °C/min until 150 °C; gradient of 20 °C until 280 °C; hold time, 5 min. The MS parameters were set as follows: source temperature, 250 °C; transfer line, 200 °C; positive ion monitoring; EI-MS (70 eV). Pesticides were identified by comparing the pure mass spectrum and retention time of each eluting compound with those in the NIST 05 mass spectral library.

Storage insect pests. Laboratory cultures of common insect pests in Israel were examined in this study. The following insects were examined at the adult stage (1-7-days-old, with undefined sex): *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), *Rhyzopertha dominica* (Fab.) (Coleoptera: Bostrichidae), *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae), and *Callosobruchus maculatus* Fab (Coleoptera: Chrysomelidae). The cultures were reared for over ten years under controlled conditions in a laboratory, without any contact with insecticides. *S. oryzae* and *R. dominica* have been reared on Israeli wheat grains, *T. castaneum* on crushed wheat, *C. maculatus* on chickpea grains, and *O. surinamensis* on an artificial substrate (ground wheat: glycerol: yeast, 8:1:1). All insects were maintained in 0.8 L glass jars with paper covers and bred at 30 ± 0.50°C and 65 ± 5 % relative humidity, in dark conditions. Two series of fumigation treatments were conducted: one with EO samples extracted in 2019 and another with the EOs produced in 2020. The procedure for both series was identical. Fumigation was conducted in chambers developed in our laboratory [38]. The chambers were comprised of 3 L glass flasks with a flat bottom, which were closed with a glass stopper fitted with a hook. The insects were placed in perforated cages (4 cm in length and 1.5 cm in diameter). A small amount of ground wheat was placed in each cage for food. The Myrtus oil samples were applied on filter paper (Whatman, No.1) at concentrations of 7.5, 10, or 30 μl/L air. Oil samples were suspended together with the test insects in the fumigation chamber for 24 h. Filter paper without EO served as control. Each concentration and control test were repeated three times. To ensure uniform distribution of the

volatiles in the flask, a magnetic stirrer was used during the entire treatment. Ten adult insects of each species were placed in each cage. Insect mortality was examined after 24 h and 7 days after the end of the treatment. In *C. maculatus*, the adult stage lasts seven days under laboratory conditions [39]. Therefore, the *C. maculatus* adults were exposed to EO one day after emergence, and after treatments, their mortality was monitored daily until the death of all individuals.

Fungi. The in vitro activities of the EOs against pathogenic yeast *Candida albicans* and mycotoxigenic fungus *Aspergillus flavus* were determined using the standardized CLSI M38-A2 broth microdilution method [40], with slight modification. Briefly, EOs extracted from the different *M. communis* cultivars were dispensed in 96-well microtiter plates with 2-fold serial dilutions of each oil/compound. The final oil/compound concentration was prepared from a stock solution in RPMI 1640 medium. The concentration of each EO in the wells ranged from 24.4 μg/ml to 12,500 μg/ml. The stock fungal conidial suspension (10^6 spores/ml) was diluted to a final inoculum concentration of 0.4×10^4 to 5×10^4 spores/ml and dispensed into the microdilution wells. The minimal inhibitory concentration (MIC) of the compounds was determined after a 48 h incubation at 28 °C. The MIC was defined as the lowest compound concentration that resulted in no visible pathogen growth. The data are presented as the means of three experiments, with three independent replicates per experiment.

Phytopathogens. Tomato plants cv. Sheeran at the physiological stage of four mature leaves, were planted in 1.6 L pots for a week, at 26 °C and 12:12 h dark:light cycle. Twenty-four hours before inoculation, each of the EO compounds was diluted to 0.5 %, and 50 ml was sprayed on the plant collar region until drainage. Then, a single toothpick infected with *Fusarium solani* hyphae was inserted into the base of the collar region and the plants were incubated for additional 21 days. The experiment was conducted in four pots, with three plants in each pot (n = 12) for each compound before lesion size was evaluated with a ruler. An additional 4 pots were inoculated with mock toothpicks.

Plant pathogenic nematode. Second-stage juveniles (J2) of the root-knot nematode *Meloidogyne javanica* were obtained from eggs extracted from infected tomato roots using a sodium hypochlorite solution [41]. Three concentrations of EO solutions (500, 1000, and 2000 μl per liter of 2 % DMSO) were prepared. EO solution (200 μl) was placed in wells of 24-well plates (Corning Glass Inc., Corning, NY), in addition to 200 μl of a nematode suspension containing ca. 100 J2. The final concentrations of EO solutions were 250, 500, and 1000 μL/L. The 24-well plates were incubated at 25 °C for 24 h in the dark, and the percentage of immobilized nematodes was calculated. This test was performed twice, with four replicates per treatment. Water containing 1 % DMSO was used as a control.

Evaluation of Nematocidal effect of M. communis EOs. To evaluate the repellency activity of EOs and of a methanolic extract of *M. communis* leaves (see SI_5), agar plates were prepared by pouring 8 ml of 1.7 % melted agar (Bacto Agar, Becton, Dickinson, and Company, NJ, USA) on 8.5 cm (ø) Petri dishes. The extract was prepared by agitating 2 g chopped fresh leaves in 6 ml methanol for 2 h and drying the supernatant. EO or the methanolic leaf extract was deposited on the agar plate using the method previously described for the one-compound attraction assay [42]. Briefly, 1.0 μl EO or 10 mg leaf extract (dry base) in 20 μl methanol was deposited on the test compound point, which was situated 3.0 cm from the center of the Petri dishes. An *M. javanica* J2 suspension (ca. 150 J2 in 10 μl) was dropped on each of three nematode inoculation points on the agar; one in the center of the dish, and the two were 2.5 cm from the center and 4 cm from the inoculation point. As a control, 20 μl methanol was used on a separate dish with the leaf extract, and no material was used for the experiment with EOs. The dishes were incubated at 25 °C for 96 h, in the dark, and then the number of J2 in the seven zones on the agar plate was counted, and the relative density (RD) of J2 in each zone was calculated after incubation for 24, 48 and 96 h [42]. Each experiment was performed twice, with four replicates per experiment.

Allelopathy. To evaluate the allelopathic potential of *M. communis*, the inhibition potential of its leaves and branches in the early stages of wheat development was assessed. In 2020, the impact of the following concentrations (by weight) of chopped leaves and branches in a garden growth medium (hereinafter "soil") was examined: 0 %, 1 %, 10 %, 20 %, and 30 %. Results of this preliminary experiment showed that concentrations of ≥10 % had a negative effect on the first stages of wheat growth. However, these results could not explain if the impact of *M. communis* was derived from its EO activity, or just the physical impact of leaves within the growth medium. Therefore, in 2021, we aimed to separate the chemical allelopathic impact and the physical impact of the chopped leaves and branches (myrtle litter). To separate chemical and physical impact of the litter on wheat development, we used fresh litter with EOs, leaves after extraction of EOs, and extracted leaves enriched with EOs. In other words, to avoid the possibility that leaf litter affects the water-holding capacity of the growth medium and to distinguish between chemical allelopathy and the physical impact of the leaf litter, the effect of EO addition to extracted and dried leaves was assessed. The following are the details of this conceptual research setup. Fresh branches from the ARO research plot, were cut in December 2021 and chopped manually into gross pieces (litter) for further processing. Commercial garden soil served as the control growth medium and was compared to two levels of soil + litter mixtures: 20 % myrtle litter, and 33 % litter. To summarize, the following treatments were examined: *M. communis* litter concentration (20 %, 33 %, and 0 % in garden soil); EO content (no EO = litter after extraction process, with EO = fresh leaves containing oil in their tissues, with artificial added EO = litter after extraction process + artificially added EO). The entire setup included four myrtle cultivars (David, Hadur, Haim, Levi). Altogether, 90 pots (0.5 L) with 5 repetitions per fully factorial treatment (litter concentration, EO, cultivar) were examined. The experiment was conducted in a growth room under controlled conditions (24 °C, 12 h daylight). In each pot, 10 wheat seeds were sown and germination success (%), individual height (cm), total fresh weight of all the seedlings in the pot (g), and total dry weight (dried in an oven, 65 °C, 48 h) were tracked 10 days after sowing. In addition, the average dry weight per individual in each treatment was calculated.

Data analysis. The data from each experiment were analyzed by different software, according to the preferences of the group that led the experiment. For storage insect experiments, standard errors and significance of variance were calculated by ANOVA (JMP® software). For the allelopathy experiment, data were analyzed by R software version 4.0.3 (R Core Team, 2020). First, visual plotting, examined by Shapiro-Wilk and Levene's tests, assessed the normality and homogeneity of residual variances. Variables that met these test assumptions, were analyzed by paired T-test or ANOVA tests, followed by Tukey HSD posthoc test, using *t.test* and *anova* functions,

multcomp [43] and *ggpubr* [44] packages. Variables that did not meet the above assumptions were treated as nonparametric, and were analyzed by Kruskal–Wallis test, followed by the Dunn posthoc test to assess treatment differences, using the *kruskal.test* and *dunn.test* function of the *FSA* package [45]. p-value <0.05 was considered statistically significant.

3. Results

Biomass production of M. communis. Intensive cultivation of *M. communis* plants yielded plants with an average height of 123 ± 16.4 cm (n = 10), average plant diameter of 146.5 ± 18.4 cm (n = 10), and average fresh weight per plant of 4.3 ± 1.7 kg. Plant density was 6000 individuals ha, the number of tricussate branches per plant was ~10, the fresh weight of pruned tricussate branches was 7.3 ± 1 g, the fresh weight of tricussate branches per ha was 440 kg, and surplus plant biomass at the harvest time was 98.3–98.4 %, equivalent to 26–27 tons per ha.

Essential oil production of M. communis. The different *M. communis* cultivars exhibited distinct oil content in Fall 2019–2020 and in Fall 2020–2021 (Fig. 1). In 2019–2020, cv. "David" carried the highest EO content, i.e., 1.37 % in comparison to 0.43–0.88 % in the other cultivars in the breeding plot. The commercial *M. communis* plants in Nov exhibited high EO content (2.04 %) in an agricultural plot that was pruned one year prior to their harvest. Nov II, which had the same genetic resource as Nov I, and which was pruned two years before harvest, showed an EO content of 1.4 %.

EOs profiles of M. communis cultivars. All of the *M. communis* lines contained large amounts (>5 %) of α-pinene, limonene, 1-8-cineole, and linalool (Table 1, SI_6). Significant amounts of myrtenol and myrtenyl acetate were only observed in "Levi," which also possessed much lower levels of α-pinene than the other lines. Limonene levels varied significantly, ranging from 3.61 % to 21.52 % in the different lines. SPME analysis of fresh plant tissue produced a somewhat different chemical profile. Several compounds including 1-8-cineole, linalool, and α-terpineol, observed in the essential oil analysis, were not identified in the SPME analysis. In addition, α-pinene, p-cymene, limonene, and γ-terpinene were found at much higher levels in the SPME analysis than in the essential oil analysis (SPME, Table 1, SI_6). With regards to the Chemical pesticide residuals - The GC-MS analysis found no residuals of any pesticide fungicide or herbicide, confirming that the bioactivity of the EOs could not be attributed to external chemical residuals, but to the EO *per se*.

M. communis showed a pesticidal effect on Storage insect pests. Fumigation with *M. communis* oils in a fumigation chamber resulted in various degrees of toxicity, depending on insect species and concentration. *S. oryzae* proved most sensitive to *M. communis* EOs, with complete mortality at a concentration of 30 μl/L air and high mortality (77–100 %) at 10 μl/L air (Fig. 2A); and various mortality levels (5–95 %) at 7.5 μl/L air. At a dose of 30 μl/L air, all *R. dominica* were killed, while 43–98 % mortality was documented at a dose of 10 μl/L air (Fig. 2B). Fumigation treatment at 30 μl/L air was found to be partially effective against *O. surinamensis* (Fig. 2C). In *C. maculatus*, complete mortality was observed 24 h after treatment with 10 μl/L air (Fig. 2D). *T. castaneum* was found insensitive to most of the treatments. Fumigation with 7.5 μl EO/l air by two *M. communis* cultivars, David and Kfar Shamai, was effective only against *S. oryzae*. Yet, in most treatments, no significant difference was found between the six myrtle lines.

High Minimal inhibitory concentration (MIC) of M. communis on Fungi. The MIC of EOs against tested fungal pathogens were rather high, falling in the range of 3.125–12.5 mg/ml. EOs from Kfar Shamai, Levi, and Hadur cultivars were more effective in comparison to EOs from the other cultivars. The EOs of Kfar Shamai, Levi, and Hadur inhibited the growth of *A. flavus*, with a MIC of 3.125 mg/ml, and *C. albicans*, with a MIC of 12.5 mg/ml. Oils from the other cultivars inhibited the growth of *A. flavus* starting at 6.25 mg/ml and did not show activity against *C. albicans*, even at a concentration of 12.5 mg/ml.

Effect of M. communis on Phytopathogens. EOs extracted from the David cultivar did not significantly affect the development of *F. solani* (Fig. 3). After 21 days, a black necrotic lesion was apparent on the lower stem of the plant of all treatments, including the control. The average lesion size of the infected control plants was 2.0 + 1.22 cm, while the average lesion size of the plants treated with the different cultivar EOs ranged between 1.15 (cv David 1, David2) and 4 cm (David2); namely, no treatment was significantly

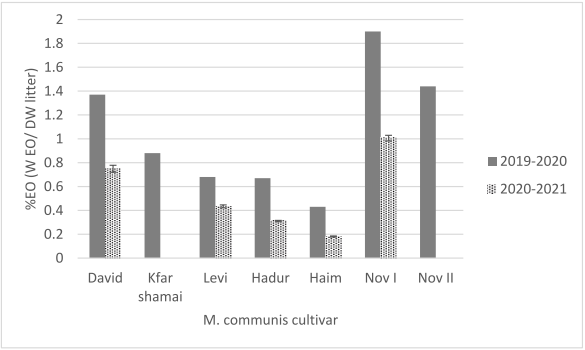

Fig. 1. The EO content of the studied *M. communis* cultivars was presented as EO weight divided by the total dry weight of the extracted litter (chopped branches and leaves). EO content in Fall 2019–2020 is denoted by grey bars, and EO content in Fall 2020–2021 is denoted by dotted bars. Error bars denote standard error in 2020–2021. "Nov I" denotes plants that were pruned one year before harvesting and "Nov II" denotes plants pruned two years before harvest.

Table 1
The phytochemical profile of five focal *M. communis* cultivars as identified by GC-MS analysis. SPME was conducted to measure volatile organic compounds (VOCs) in fresh branches of four *M. communis* cultivars (February 2020). GC (mg/g) was performed on 2021 samples. See SI_6 for a full table including analyses performed in 2020 and 2022. Values are presented in mg/g units.

Compound	David		Levi		Hadur		Haim		Nov
	GC	SPME	GC	SPME	GC	SPME	GC	SPME	GC
α-thujene	0.44 ±0.04	1.4	0.44 ±0.01	0.9	0.94 ±0.7	0.8	0.81 ±0.04	5.8	0.45 ±0.01
α-pinene	23.92 ±2.45	36.0	9.63 ±0.12	41.2	20.65 ±1.05		22.54 ±0.30		25.83 ±0.82
β-pinene	0.68 ±0.06	1.1	0.35 ±0.01	1.5	0.61 ±0.03	0.9	0.6 ±0.01		0.81 ±0.02
myrcene	0.85 ±0.06	0.3	0.63 ±0.02		0.56 ±0.08	0.1	0.28 ±0.01		0.85 ±0.03
δ-3-carene	0.51 ±0.04		0.95 ±0.13	2.4	1.95 ±0.07	0.3	1.91 ±0.08	0.2	
α-terpinene	0.17 ±0.03	0.3	0.28 ±0.01	2.5		26.1		18.3	
p-cymene		1.9	0.66 ±0.08	4.1		55.1		66.7	
limonene	6.07 ±0.65	28.4	3.61 ±0.07	29.2	14.25 ±1.82		21.52 ±0.48		6.49 ±0.12
1-8-cineole	21.82 ±1.97	4.3	12.4 ±0.21		20.49 ±1.51		14.82 ±0.35		23.65 ±0.27
β-ocimene	0.67 ±0.05		0.36 ±0.02		0.44 ±0.02	8.2	0.26 ±0.08	5.1	0.56 ±0.03
γ- terpinene	0.62 ±0.06	2.9	0.89 ±0.02		0.92 ±0.05		0.92 ±0.06		0.51 ±0.03
linalool oxide	0.33 ±0.03					1.2		1.0	0.21 ±0.01
terpinolene	0.66 ±0.15	0.6	0.69 ±0.03	0.3	0.77 ±0.08	1.3	0.45 ±0.07		0.54 ±0.29
p-cymenene					0.29 ±0.01		0.47 ±0.03		
linalool	15.4 ±0.79	17.9	12.15 ±0.34		12.7 ±1.59		4.27 ±0.29		14.03 ±0.49
pinocarveol	0.16 ±0.01		0.36 ±0.01				0.57 ±0.03		0.16 ±0.01
terpinen-4-ol	0.75 ±0.04		1.06 ±0.02		1.34 ±0.05		1.23 ±0.61		0.75 ±0.03
α-terpineol	8.7 ±1.09				6.83 ±0.93		4.64 ±0.14		8.27 ±0.38
myrtenol			19.02 ±0.55				0.56 ±0.07		
nerol	0.46 ±0.09		0.43 ±0.01		0.17 ±0.08		0.45 ±0.12		0.32 ±0.02
α-gurjunene				2.1					
linalyl acetate	2.52 ±0.38		2.82 ±0.29		0.52 ±0.74		0.26 ±0.09		2.57 ±0.07
geraniol	2.87 ±0.73		1.86 ±0.03		1.14 ±0.24		0.51 ±0.06		2.27 ±0.13
myrtenyl acetate			10.82 ±0.78				0.41 ±0.11		
α-terpinyl Acetate	2.8 ±0.44		1.45 ±0.03				0.18 ±0.15		2.79 ±0.12
allo-aromadendrene				10.7					
geranyl acetate	1.62 ±0.44		2.06 ±0.09		2.36 ±0.29		3.99 ±0.24		1.74 ±0.09
myrtanol acetate			0.13 ±0.02						
methyl eugenol	0.13 ±0.03		1.78 ±0.07		1.15 ±0.15	0.1	1.57 ±0.03		0.12 ±0.01
β-caryophyllene	0.96 ±0.16	0.3	1.14 ±0.05	2.7	0.97 ±0.07		1.27 ±0.16		0.76 ±0.05
α- humulene	0.64 ±0.14		2.28 ±0.09	0.8	1.11 ±0.10		1.51 ±0.15		0.5 ±0.04
caryophyllene oxide	0.23 ±0.04		0.3 ±0.02		0.32 ±0.03		0.95 ±0.05		0.2 ±0.01
Total	**93.54**	**95.6**	**88.11**	**98.3**	**89.54**	**94.1**	**86.14**	**97.2**	**93.93**

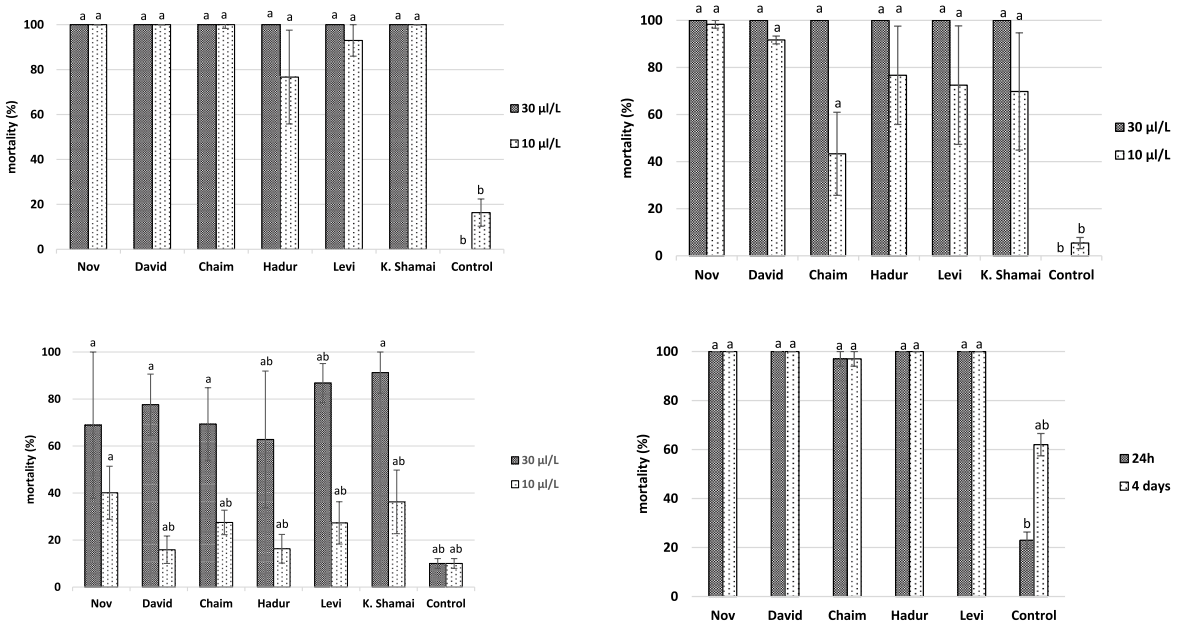

Fig. 2. A. Pesticidal effect of *M. communis* cultivars on *S. oryzae* adults. Mortality rates (%) are presented, seven days after a 24-h exposure to EOs from six strains of *M. communis*, at concentrations of 10 and 30 µl/L air. Means follow by the same letter are not significantly different (P⟩ 0.05). Fig. 2B. Pesticidal effect of *M. communis* cultivars on *R. dominica* adults. Mortality rates (%) are presented, seven days after a 24-h exposure to EOs from six strains of *M. communis*, at concentrations of 10 and 30 µl/L air. Means follow by the same letter are not significantly different (P⟩ 0.05). Fig. 2C. Pesticidal effect of *M. communis* cultivars on *O. surinamensis* adults. Mortality rates (%) are presented, seven days after a 24-h exposure to EOs from six strains of *M. communis*, at concentrations of 10 and 30 µl/L air. Means follow by the same letter are not significantly different (P⟩ 0.05). Fig. 2D. Pesticidal effect of *M. communis* cultivars on *C. maculatus* adults. Mortality rates (%) are presented, 24-h and four days after a 24-h exposure to EOs from six strains of *M. communis*, at concentrations of 10 µl/L air. Means follow by the same letter are not significantly different (P⟩ 0.05).

different from the control.

M. communis did not show a nematicidal effect. None of the myrtle crop EOs collected in 2019–2020 showed any nematicidal activity at any concentration, including the highest concentration tested (1000 µl/L) (Fig. 4A–C). The percentage of J2 paralyzed by the EO treatments was 0–2.0 %, which did not differ from the control. Data relating to the repellency effect of EOs from the seven tested sources were combined because no differences in the RDs of J2 were noted between them. In most cases, the RDs in the seven zones on the EO-treated agar plate were not different from those of the control after incubation for 24, 48, and 96 h, i.e., the EOs had no repellency or attraction effect (Fig. 4). The leaf extract imparted an attractive effect – RDs of zone 1 were higher than those of the control after 48 h and 96 h incubations. At the same time, the RDs of zones 2 and 3 were lower, and those of zones 6 and 7 were higher than the control (Fig. 4).

Allelopathy. Chopped leaves and branches of *M. communis* at concentrations of 20 % and 33 % of the growth medium, had similar effects on the germination of wheat seeds (Fig. 5A and D). No significant impact on germination success was observed with fresh leaves, extracted leaves, or with extracted leaves + EO. Similarly, *M. communis* leaf concentrates did not significantly affect fresh

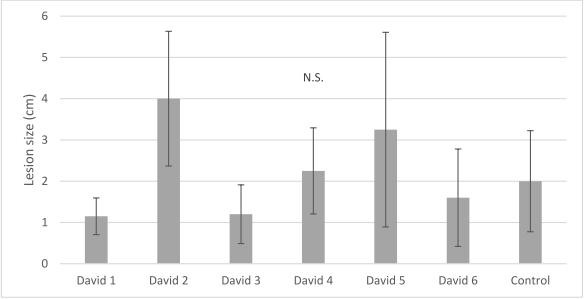

Fig. 3. Effect of *M. communis* essential oils extracted from 6 extracts of cv David plants, on the size of lesions created by *F. solani*. The experiment included three plants per each one of the four pots (n = 12). Lesion size was evaluated with a ruler. N.S. = no significant differences were detected between the treatment by using Tukey HSD test. Vertical line = S.D.

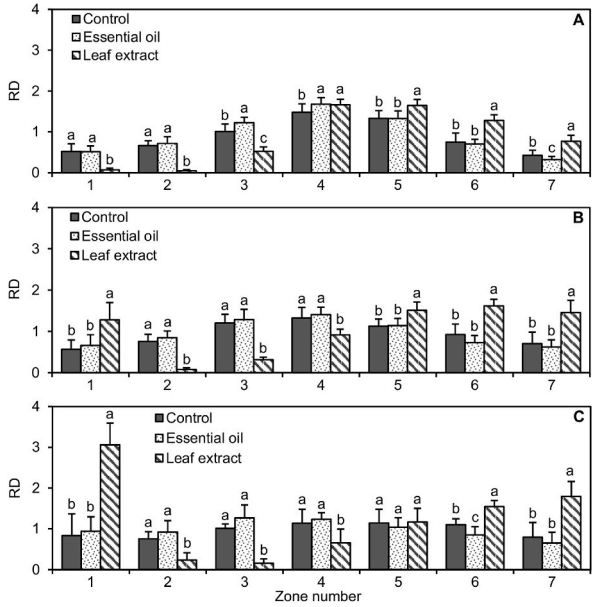

Fig. 4. Relative density (RD) of second-stage juveniles of *Meloidogyne javanica* in the seven zones (1–7) of an agar plate treated with 1 ml essential oils and 10 mg methanolic extract of *Myrtus communis* after incubation for 24 (A), 48 (B), and 96 h (C). Methanol was used for control. Values are presented as means +SD of eight replicates from two trials. Means in each zone were separated by the Tukey–Kramer HSD test (α = 0.05) and marked with different letters.

(Fig. 5B) or dry (Fig. 5C) wheat shoot weight. Treatments of soil growth medium with and without *M. communis* fresh leaves, extracted leaves, or extracted leaves with additional EO showed no significant effect on the germination of wheat seeds (Fig. 5D). In contrast, the addition of leaves to the soil significantly reduced wheat seedling height (Fig. 5E) and dry weight (Fig. 5F). More specifically, the fresh weight of wheat seedling grown with 33 % fresh *M. communis* leaves, extracted leaves and extracted leaves with EO was 42 %, 59 % and 64 % lower, respectively, when compared to wheat seedlings grown in untreated soil. The inhibiting chemical impact of the EO showed a similar reduction in wheat seedling development, which was 22 % shorter (Fig. 5E), and with a 24 % lower dry weight (Fig. 5F).

4. Discussion

This work aimed to determine whether *M. communis* can serve as a natural bio-pesticide and thereby address surplus biomass produced in its commercial cultivation. *M. communis* imparted an insecticidal impact on stored grain pests, as well as an allelopathic effect on wheat seeds. However, it did not exhibit nematocidal and fungicidal activities and its EOs did not have an anti-pathogenic effect on the studied phytopathogens. These findings suggest that with further research, *M. communis* growers can find additional use to the surplus production, either by producing EOs as a natural alternative to chemical pesticides or by adjusting the agronomic use of extra myrtle biomass to use its allelopathic effect, e.g. by using leaves and branches as mulch for weed control. This circular-agriculture approach can provide local, more sustainable solutions for agronomic needs, and valorize the roles of this wild, native, endangered plant. This approach aligns with that of Vanbergen et al. [46], who suggested essential phases toward sustainable intensification of agriculture. All steps suggested by Vanbergen were implemented in this research exploring the potential uses of *M. communis*, i.e., increasing agricultural effectiveness by finding alternative novel uses for excess biomass, substitutes of chemicals and other inputs by nature-based solutions such as bio-control, and, finally, re-design of agriculture, potentially by exploiting the allelopathic impact of *M. communis* shown here.

M. communis EOs did not show nematocidal activity even at a high concentration of 1000 ppm. This is in accordance with previous reports [47]. The methanolic extract of *M. communis* leaves both attracted and repelled *M. javanica* J2 on the agar plate. This dual effect was likely because attractants were more hydrophobic, while repellents were more hydrophilic, resulting in their separation within the agar plate during diffusion. To the best of our knowledge, the detection of both attractiveness and repellency against nematodes in an unfractionated plant extract has not been reported, probably due to bioassays that have been used for nematode response being incapable of detecting these two contrary nematode behaviors. However, the dual activity in an extract does not seem rare due to the variety of metabolites in it. An aqueous *M. communis* leaf extract did not attract *M. javanica* J2, whereas a leaf extract with acetone showed a high level of J2 attraction (*unpublished data*). Isolating and identification of J2 attractants and repellents may provide for sustainable nematode control of specific nematode behaviors, such as host-finding and infection processes. Further research may lead to the use of myrtle EOs against pest nematodes by expulsion/attraction that may be the base for push-pull use [48].

Chemical analysis found α-pinene, limonene, 1-8-cineole, and linalool to be the most dominant terpenes in the EO extracted from the commercial Nov and David cultivars (Table 1). All other cultivars had varying ratios of limonene and 1-8-cineole. These two

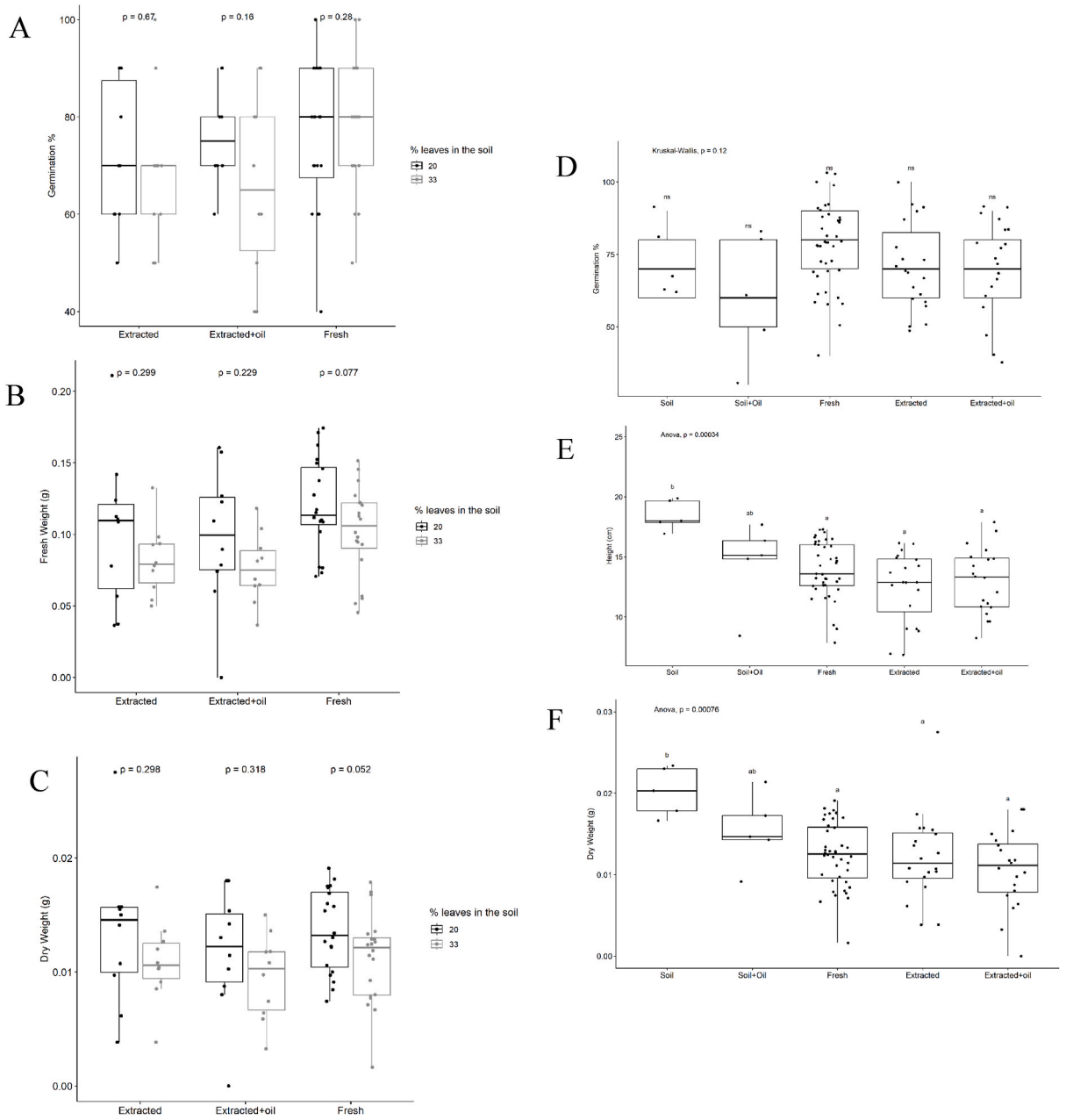

Fig. 5. Allelopathic effect of chopped *M. communis* leaves and branches on wheat in its first 10 days of growth. Figures A–C represent the development of wheat seeds grown in soil containing 20 % or 33 % *M. communis* leaves. The soil treatments included: extracted leaves without EO, extracted leaves supplemented with EO, or fresh leaves with their natural EOs. (A) Germination success, (B) fresh weight (C) dry weight of wheat seeds. Figures D–F represent the impact of soil treatment by *M. communis* leaves – on the development of wheat seeds, regardless of the concentration of leaves in soil: (D) Germination success, (E) sprout height, and (F) dry weight of wheat leaves. Different letters denote significant differences, and the statistical parameters are noted in the upper left corner. ANOVA was executed when data was distributed normally, and Kruskal Wallis for a-parametric data, both with the same threshold for statistical significance ($p < 0.05$).

monoterpenes are often confused due to similar chromatographic properties and care must be taken to accurately distinguish between them during chemical analyses. Earlier studies also found these terpenes to be most dominant in *M. communis* [49]. Limonene and α-pinene have been shown to impart antimicrobial and antifungal effects [50]. Yet, the present phytochemical analysis of the EOs found differences between cultivars, with possible effects of cultivation practice including pruning regime, time of harvest, irrigation, and nutrition of the Myrtles.

EOs are highly volatile and degrade rapidly and are therefore considered potential natural alternatives for chemical pesticides, with particular advantages in the pharmaceutical and food industries. In addition, locally available EOs may offer relative cost-

effectiveness. Some EOs extracted from aromatic plants belonging to the Myrtaceae families have proved effective against stored product pests, particularly against adult *S. oryzae* [51]. *R. dominica* and *S. oryzae* are common pests in grain warehouses in Israel. In the current study, *M. communis* exhibited high toxicity against *S. oryzae, R. dominica*, and *C. maculatus*, but was only partially effective against *O. surinamensis,* and had no impact on *T. castaneum.* Toudert-Taleb et al. [52] and Khani et al. [27] also reported on complete mortality of *C. maculatus* treated with 16 μl/L and 25 μl/L of Myrtle EOs/air, respectively. Koutsaviti et al. [53] measured high mortality of *S. oryzae* when treated with 49.4 μl/L Myrtle EO/air, while in the current study, complete mortality was recorded at a dose of 10 μl/L Myrtle EO/air. *T. castaneum* was found insensitive to all treatments that we performed; it fitted previous studies, which found the efficiency of *M. communis* against *T. castaneum* only at higher concentrations: 87–159 μl/L Myrtle EO/air [54,55].

Insect mortality was observed on treatment with most of the oils produced in the years 2019 versus 2020, from six different *M. communis* lines. This uniform effect may be explained by the dominance of α -pinene and limonene, which was identified in all the lines and both years (Table 1 and SI_6). These compounds are highly effective fumigants [56,57]. On the other hand, a conclusion that a few metabolites are responsible for any biological effect would ignore the complexity of synergistic and other effects expected when we study multi-molecule impacts [58,59].

M. communis leaves and branches demonstrated an inhibitory effect on the early development stage of wheat seedlings. This allelopathic impact observed on wheat seeds, is likely to be similar on some weeds, suggesting a new Myrtle cultivation design in which pruned branches can be used as a mulching cover to impart a herbicidal effect in the Myrtle field or neighboring agricultural fields.

In addition, the biomass analysis suggested that EO production may offer added income to the farmer. The crop density in an agriculture field is 10K–20K plants/ha [2]. *M. communis* crop fields were estimated in our study to produce approximately 25 T/ha excess biomass. This biomass can provide approximately 100 L EO per ha, which has the potential to be translated to an additional 100K ILS (27K USD) per ha. The stable composition of the main toxic compounds in the tested years and in different lines may support the development of pest control products. The results of this study promise to serve as a basis for continued evaluation of secondary uses of *M. communis* oils from local crops, as safe and environmentally friendly pesticides.

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CRediT authorship contribution statement

Elazar Quinn: Writing – review & editing, Resources, Investigation, Formal analysis, Data curation. **Eyal Ben-Simchon:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Jonathan Gorelick:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Yuji Oka:** Writing – review & editing, Investigation, Formal analysis. **Omer Frenkel:** Writing – review & editing, Investigation, Formal analysis, Conceptualization. **Edward Sionov:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Moshe Kostyukovsky:** Writing – review & editing, Validation, Supervision, Investigation. **Nativ Dudai:** Writing – review & editing, Methodology, Investigation. **Jakob Shimshoni:** Writing – review & editing, Investigation, Formal analysis. **Shmuel Zilkah:** Validation, Supervision. **Menashe Cohen:** Resources, Project administration. **Aviv Rapaport:** Investigation. **Oren Shelef:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Oren Shelef reports was provided by Agricultural Research Organization. Oren Shelef reports a relationship with Agricultural Research Organization that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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