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Potential of Octanol and Octanal from *Heracleum sosnowskyi* Fruits for the Control of *Fusarium oxysporum* f. sp. *lycopersici*

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Abstract: The antifungal activity of volatile compounds from the fruit, leaf, rhizome and root of 109 plant species was evaluated against *Fusarium oxysporum* f. sp. *lycopersici* (FOL) race 1—the tomato wilt pathogen—by using the modified dish pack method. Eighty-eight plant samples inhibited mycelial growth, including volatiles from fruits of *Heracleum sosnowskyi*, which exhibited the strongest antifungal activity, showing 67% inhibition. Two volatile compounds from the fruits of *H. sosnowskyi* (octanol and octanal) and *trans*-2-hexenal as a control were tested for their antifungal activities against FOL race 1 and race 2. In terms of half-maximal effective concentration (EC_{50}) values, octanol was found to be the most inhibitory compound for both pathogenic races, with the smallest EC_{50} values of 8.1 and 9.3 ng/mL for race 1 and race 2, respectively. In the biofumigation experiment, the lowest disease severity of tomato plants and smallest conidial population of race 1 and race 2 were found in *trans*-2-hexenal and octanol treated soil, while octanal had an inhibitory effect only on race 2. Therefore, our study demonstrated the effectiveness of volatile octanol and *trans*-2-hexenal on the control of the mycelial growth of two races of *Fusarium oxysporum* f. sp. *lycopersici* and may have potential for the future development of novel biofumigants.

Keywords: *Fusarium oxysporum* f. sp. *lycopersici*; antifungal activity; biofumigation; octanol; octanal; *Heracleum sosnowskyi*

1. Introduction

Plants produce a variety of secondary metabolites which act as a direct or indirect defense against fungal, microbial or insect attack [1–3]. Natural compounds are currently widely used in agriculture and the food industry to control plant pathogenic bacteria and fungi [4,5]. For instance, the extracts from adatoda (*Adhatoda vasica*), blue gum tree (*Eucalyptus globulus*), lantana (*Lantana camara*), oleander (*Nerium oleander*) and sweet basil (*Ocimum basilicum*) can inhibit the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* to some extent [6]. Essential oils of *Origanum heracleoticum*,

which are rich in phenols, are able to inhibit the growth of some post-harvest phytopathogenic fungi (*Botrytis cinerea*, *Penicillium expansum*, *Aspergillus niger* and *Monilinia fructicola*) [7]. Essential oils from aerial parts of oregano, thyme, lavender, rosemary, fennel and laurel inhibit the growth of *Phytophthora infestans* in a dose-dependent manner, and the effect of the volatile phase of essential oils is more effective than the contact phase effect [8]. The essential oil of laurel (*Laurus nobilis*) can inhibit the growth of some postharvest fungi of peaches and kiwi fruits. Furthermore, laurel oil can completely stop the growth of *Monilinia laxa* at a concentration of 200 µg/mL and *B. cinerea* at 1000 µg/mL [9]. The antifungal effects of volatile compounds from black zira and other herbs were investigated against *F. oxysporum*. Among the identified volatile compounds (gamma-terpinene, limonene, *p*-cymene, beta-pinene, alpha-pinene, cuminaldehyde and myrcene), cuminaldehyde was proposed as the main antifungal compound in black zira [10]. Neri et al. (2006) demonstrated in vitro and in vivo activities of nine plant volatiles against *P. expansum*, the cause of blue mold in pear, while *trans*-2-hexenal and carvacrol were the best inhibitors of conidial germination (ED_{50} (half maximal (50%) effective concentration) = 10·2 µL/L) and mycelial growth (ED_{50} = 9 µL/L) [11]. Volatile 2E-hexenal completely inhibited the growth of the potato blemish pathogens *Pectobacterium atrosepticum* (bacterial soft rot), *Colletotrichum coccodes* (black dot) and *Helminthosporium solani* (silver scurf) in an in vitro test [12].

Fusarium oxysporum f. sp. *lycopersici* (FOL) is a soil-borne plant pathogen that is the causal agent of fusariosis in the tomato plant, which is characterized by an initial yellowing of plants, leading to wilting and plant death. FOL can produce three types of asexual spores (macrospores, microspores and chlamydospores). FOL has caused production losses between 30% and 40% and has spread across many countries in North, Central and South America and Europe [13,14]. There are three known races of FOL (race 1, race 2, and race 3) distinguished by their pathogenicity to cultivars with specific dominant resistant genes [15]. The rapid identification of Fusarium strains to species and sub-species levels can be done using diverse molecular methods such as polymerase chain reaction (PCR) and esterase isozyme electrophoresis [16]. Race 1 is the most widely distributed and was initially reported in 1886, and race 2 was found in Ohio in 1945 [17–19]. In 1978, race 3 was reported in Australia [20]. However, examples of the same race may have a genetic diversity that is associated with difficulties in controlling the pathogen [21]. The control of the disease can be mainly done with the use of fungicides from the family of benzimidazoles and triazoles and soil fumigants [14]. Broad-spectrum fumigants such as methyl isothiocyanate not only control the disease but also increase crop yields [16]. However, despite their quick response and effectiveness, these chemicals pose a high risk to human health and environmental hazards. In this regard, alternatives to synthetic chemicals and soil fumigants are intensively being explored. One of the promising alternatives is chloropicrin, wich has shown consistent control of tomato diseases caused by *Verticillium dahliae*, FOL and *F. oxysporum* f. sp. *radicis-lycopersici* [22].

In the attempt to reduce the use of synthetic fungicides and risky fumigants, alternative methods of plant protection, especially by using natural products from plants which are non-toxic and biodegradable, should be considered [23]. Some proposed non-chemical methods involve the use of microorganisms such as *Pseudomonas, Trichoderma* and others [14].

Therefore, in the present study, we aim (i) to screen volatile compounds from 109 plant species with presumably high antifungal properties against FOL, (ii) to determine the antifungal activity of authentic volatile octanol, octanal and trans-2-hexenal on FOL, and (iii) to evaluate the effect of soil biofumigation with octanol, octanal and trans-2-hexenal on the mycelial growth of FOL and disease severity in tomato plants.

2. Materials and Methods

2.1. Plant Samples

Different parts (fruit, leaf, rhizome and root) of 109 plant species, including medicinal and endangered plants, were collected from Showa Pharmaceutical University, Tsukuba University and Tsukuba Botanical Garden, Japan from May to July 2014. Most plants were in their vegetative stage at

the time of sample collection. Among them, only one plant species, *Heracleum sosnowskyi* Manden., was collected from Minsk, Belarus in August 2014. All collected samples were dried inside a hot air circulation oven at 60 °C for 20 h and stored in paper bags until use. Plant samples were then cut into 2–3 cm pieces immediately before the experiment, and 500 mg of the samples was used in each treatment.

2.2. Fungal Isolates

Cultures of *Fusarium oxysporum* f. sp. *lycopersici* race 1 (MAFF 305121) and race 2 (JCM 12575) were obtained from the Plant Pathology Laboratory, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Japan. Fungal isolates were maintained on potato sucrose agar (PSA) media plates (potato: 200 g, agar: 20 g, sucrose: 20 g, water: 1 L). The plates were stored at 25 °C in an incubator before being used.

2.3. Screening of Antifungal Activity

The bioassay used to screen the antifungal activity of plant volatiles was performed by using a modified dish pack method [24]. Briefly, six-well multi dishes (Nunc, external dimensions: 128 × 86 mm, 35 mm-diameter wells) were prepared according to the protocol proposed in [10]. A five-day-old colony of FOL (race 1 and race 2) and 500 mg of each plant sample were used (Figure 1). The radial diameter of the fungal colony in each well was measured on the third day after incubation in an incubator (NTS Model MI-25S) at 25 °C. Mycelial growth inhibition was calculated according to Equation (1) [25] based on the average result from two wells at a distance of 41 mm (as two replications) from the source well with the dried sample:

$$\% \text{ inhibition of mycelial growth} = \frac{(C - T)}{C} \times 100 \quad (1)$$

where C is the colony diameter in the control multi dish without a sample and T is the colony diameter in the treatment.

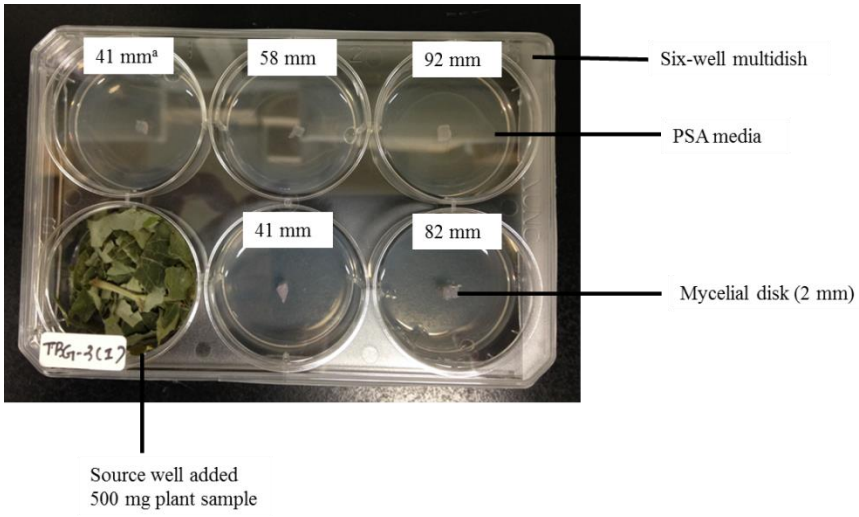


Figure 1. Modified dish pack method used to test the antifungal activities of plant samples. The value in mm indicates the distance from the sample. PSA: potato sucrose agar.

2.4. Antifungal Bioassay of Authentic Volatile Compounds

Based on the results of the antifungal activity screening, volatile compounds from fruits of *H. sosnowskyi*, which showed the highest antifungal activity, were chosen to evaluate their antifungal activities on race 1 and race 2 of FOL. Volatile compounds from fruits of *H. sosnowskyi* have already been described by Mishyna et al. (2015) [26]. Among these, two major volatile compounds (octanal and

octanol) were tested according to their previous reports in terms of their antifungal activities [27,28]. In addition, *trans*-2-hexenal (leaf aldehyde), which showed a strong inhibitory effect on postharvest fungus in [11], was used as a control. Authentic octanal (≥97%) and octanol (≥98%) were purchased from Wako Chemicals (Osaka, Japan), and *trans*-2-hexenal (≥95%) was purchased from Tokyo Chemical (Tokyo, Japan).

All authentic compounds were diluted with dimethyl sulfoxide (DMSO) to obtain six dilution ratios (1.0, 1.3, 1.7, 2.5, 5.0 and 10%, *v*/*v*). The antifungal bioassay was carried out following the same procedure as described in Section 2.3; however, 50 µL of diluted authentic volatile compounds was added into the source well instead of plant samples (Figure 2). The bioassay was replicated three times for each compound. Mycelial growth inhibition was measured and calculated as previously described.

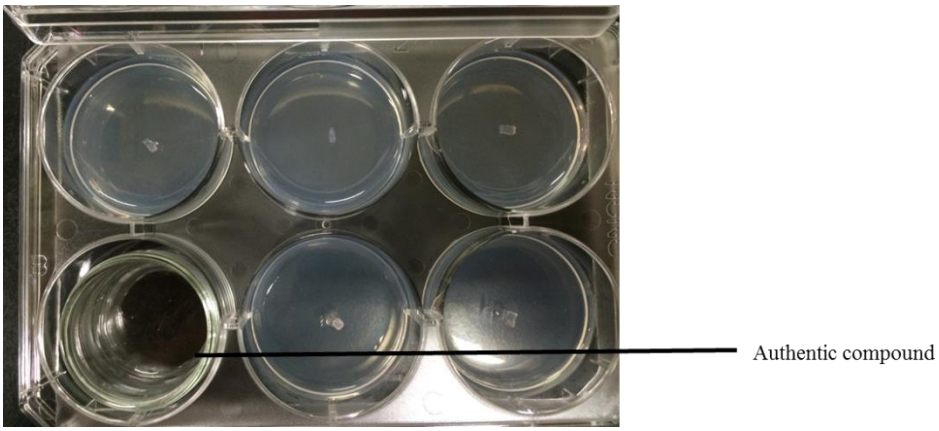
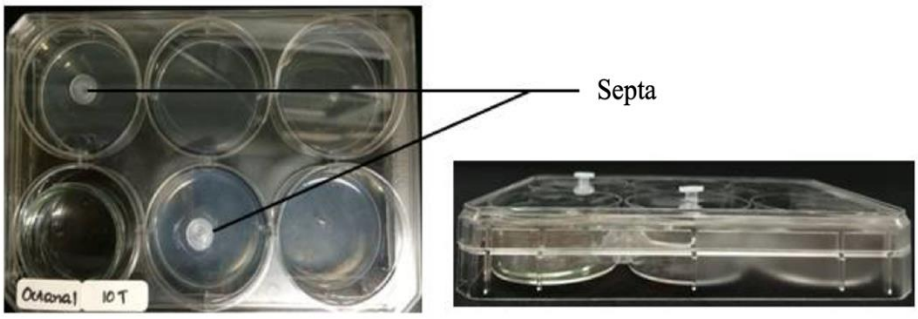


Figure 2. Dish pack method used to test the antifungal activities of authentic volatile compounds.

2.5. Measuring the Actual Volatile Concentration

To measure the actual volatile concentration of each authentic compound, septa were set up on top of the two 41 mm wells (Figure 3). After 24 h of incubation, headspace vapor (1 mL) was collected using a gas-tight syringe (Hamilton, Reno, NV, USA) and injected into the gas chromatograph–mass spectrometer (GC-MS-QP 2010 Plus system, Shimadzu, Japan). To draw a calibration curve, octanol, octanal, and *trans*-2-hexenal were diluted with hexane at different concentrations and were separately injected into the GC-MS. The actual volatile concentration of each compound was calculated based on the calibration curve of the authentic standards.



(**a**) (**b**)

Figure 3. Top (**a**) and side (**b**) view of the multi dish with septa attached for headspace vapor sampling.

2.6. Determination of the EC_{50} Values

The half-maximal effective concentration (EC_{50}) was calculated according to the linear relation between the actual volatile concentrations determined by GC-MS and the inhibition percentage of the mycelial growth of race 1 and race 2 of FOL.

2.7. Biofumigation Assay

2.7.1. Preparation of Conidial Suspension

Seven-day-old fungal colonies (2 mm^2) were inoculated into two sterilized conical flasks containing 50 mL of potato sucrose broth (PSB) medium. The flasks were shaken in a shaker at 120 rpm for 2–4 days. Each shaken medium was filtered by using a one-time folded cheesecloth and placed into a 50 mL tube. Then, the tubes were centrifuged in a swing rotator at 3000 rpm for 10 min. The supernatant was removed by decantation, and 15 mL of distilled water was added into the tubes. After making 100-fold or 1000-fold diluted solutions, the number of spores was measured by using a Thomas chamber.

2.7.2. Experimental Setup for Biofumigation Assay

Factorial arrangement (2 × 5) laid out in a completely randomized block design was used with three replications. Factor A included two fungal races (race 1 and race 2), factor B—five fumigation treatments including positive and negative control treatments. Fumigation treatments were as following: (I) Control "–" (soil treated with 300 µL of sterilized water), (II) Control "+" (soil treated with 50 mL of conidial suspension and 300 µL of sterilized water), (III) Octanal (soil treated with 50 mL of conidial suspension and 300 µL of authentic pure octanal), (IV) Octanol (soil treated with 50 mL of conidial suspension and 300 µL of authentic pure octanol), (V) *trans*-2-hexenal (soil treated with 50 mL of conidial suspension and 300 µL of authentic pure *trans*-2-hexenal).

Corn meal medium (50 mL) and potato sucrose broth (20 mL) were added into the plastic containers (17 × 11 × 5 cm) filled with 600 g of dry soils (JA Company, Japan) [N (220 mg kg^{-1}), NH_4^+ (177 mg kg^{-1}), NO_3^- (44 mg kg^{-1}), P (2775 mg kg^{-1}), K (220 mg kg^{-1}), MgO (220 mg kg^{-1}), pH (5.8–6.5)]. The conidial suspension (50 mL) was also inoculated into the containers and incubated for 24 h in the dark at 26 °C. After incubation, 300 µL of octanal, octanol, and *trans*-2-hexenal was injected into the container by using sterilized syringe. Five points were selected to inject the authentic compounds into the soil (Figure 4). Thus, 60 µL of a compound from 300 µL was injected into each point separately at 2.5 cm depth.

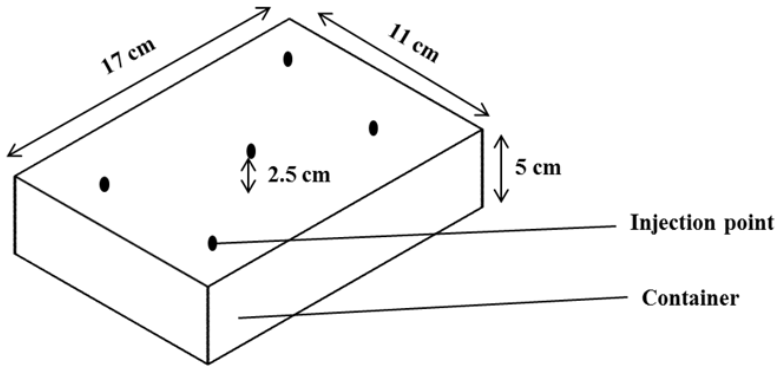


Figure 4. A container used for biofumigation showing five different injection points.

All containers were closed tightly and incubated for 7 days in the dark at 26 °C, and then fumigated soils from each container were transferred into the respective plastic pots (6 × 6 × 5 cm). A tomato cultivar "Ponderosa" (Tsurushin-shubyo seed company, Nagano, Japan), which is susceptible to all FOL races, was used as a test plant. Three-week-old seedlings were transplanted in each pot. The plants were kept inside a growth chamber at 25 °C with 12 h of fluorescent light for one month. A water

supply was provided every day. Data were collected one month after transplanting. Disease severity scores were judged according to the vascular discoloration, as described in [29]. All plants were uprooted, and the lower stem and tap root were longitudinally sectioned for the examination of internal tissues. Each plant was rated on a scale from 0 to 4 as follows: 0: healthy plants; 1: <25% vascular discoloration; 2: 26–50% vascular discoloration; 3: wilting with 51–75% vascular discoloration; and 4: 76–100% vascular discoloration or death [30]. The ratings were converted to a percentage disease severity index using the following Equation (2):

$$\mathrm{DSI} = \frac{\sum (\mathrm{n.\ v})}{\mathrm{N.\ X}} \times 100 \quad (2)$$

where DSI is the disease severity index, n is the infection class frequency, v is the number of each class, N is the number of observed plants and X is the highest value of the evaluation scale.

2.8. Counting the Conidial Density from Different Biofumigated Soil

The conidial density of FOL race 1 and race 2 from different fumigated soils was counted using the plate count method. The original diluted solution was made by suspending 1 g of fumigated soil into 9 mL of distilled water. In total, 100 µL of the diluted solution was transferred to a test tube which was originally filled with 900 µL of distilled water. Serial dilution was performed until a 10^{-6} solution was reached, and then 100 µL from each test tube was plated and incubated for 48 h. The conidial density was calculated using the following formula (3):

$$\text{Number of conidia} = \text{Number of colonies on plate} \times \text{reciprocal of the dilution of sample} \quad (3)$$

The experiment was conducted twice: after 7 days of fumigation, immediately before the tomato plants were transplanted, and 30 days after transplanting, at the time of judging the disease severity score. The number of conidia was expressed as the number of colony forming units (CFUs) $\times 10^7$/g of soil.

2.9. Data Analysis

The statistical evaluation of data was performed with SAS (the SAS System of Windows 9.0) software. The data were subjected to an analysis of variance (ANOVA) to detect significant differences between two pathogenic races of FOL and five fumigation treatments. The general linear model (GLM) was constructed to generate a two-way ANOVA. Mean differences among five fumigation treatments were separated using the least significant difference (LSD) test. Differences between two FOL races were tested using the Student's *t*-test. Statistical significance was assigned at a level of $p < 0.05$.

3. Results

3.1. Screening of Antifungal Activity

Table 1 represents the effect of volatiles from different parts of 109 plant species on the mycelial growth of *F. oxysporum* f. sp. *lycopersici*, which is expressed as an average result of the two 41 mm wells. Volatiles emitted from 88 plant samples exhibited an inhibitory effect on mycelial growth ranging from 2% to 67%. The strongest inhibition (67%) was observed for volatile compounds from *H. sosnowskyi* fruits, followed by leaves of *Matteuccia struthiopteris* (18%) and rhizomes of *Houttuynia cordata* (15%). Five plant species (leaves of *H. sosnowskyi*, *Pachysandra terminalis*, *Lonicera japonica*, *Allium sativum* var. *nipponicum* and *Bletilla striata*) showed no effect on mycelial growth, while a growth promotion effect (i.e., a negative value of inhibition percentage) was found in 19 plants species. Fruits of *H. sosnowskyi* were previously reported to release octanol and octanal [26], and these two compounds were selected for the further determination of their antifungal activity against FOL race 1 and race 2.

Table 1. Effect of volatiles from 109 plant species on the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* race 1.

Plant Species			Part Used	Growth Inhibition (%)
Common Name	**Scientific Name**	**Family Name**		
Sosnowskyi's hogweed	*Heracleum sosnowskyi* Manden.	Apiaceae	Fruit	67
Ostrich fern	*Matteuccia struthiopteris* (L.) Tod.	Onocleaceae	Leaf	18
Dokudami	*Houttuynia cordata* Thunb.	Saururaceae	Rhizome	15
Woad	*Isatis tinctoria* L.	Cruciferae	Leaf	14
Touki	*Angelica acutiloba* Kitagawa	Apiaceae	Leaf	12
Wadatsumi-no-ki	*Nothapodytes amamianus* Nagam. & Mak. Kato	Icacinaceae	Leaf	12
Black mangrove	*Lumnitzera racenosa* Willd.	Combretaceae	Leaf	11
Donan-koban-no-ki	*Phyllanthus oligospermus* Hayata subsp. *donanensis* T.Kuros.	Phyllanthaceae	Leaf	11
Cha-ran (Tea orchid)	*Chloranthus spicatus* (Thunb.) Makino	Chloranthaceae	Leaf	9
Peppermint	*Pelargonium tomentosum* Jacq.	Geraniaceae	Leaf	9
Macadamia	*Macadamia integrifolia* Maiden & Betche	Proteaceae	Leaf	9
Japanese yellow bark	*Phellodendron amurense* Rupr.	Rutaceae	Leaf	9
Turmeric	*Curcuma longa* L.	Zingiberaceae	Leaf	9
Indian mangrove	*Avicennia officinalis* L.	Acanthaceae	Leaf	8
Magic lilly	*Lycoris squamigera* Maxim.	Amaryllidaceae	Leaf	8
Mishima-saiko	*Bupleurum stenophyllum* (Nakai) Kitag.	Apiaceae	Leaf	8
Sanyo-aoi	*Asarum hexalobum* F.Maek.	Aristolochiaceae	Leaf	8
Pei lan	*Eupatorium fortunei* Turcz.	Asteraceae	Leaf	8
Yellow starwort	*Inula helenium* L.	Asteraceae	Leaf	8
Goat weed	*Epimedium grandiflorum* C.Morren var. *thunbergianum* (Miq.) Nakai	Berberidaceae	Leaf	8
Yellow ginger	*Dioscorea zingeberensis* C.H.Wright	Dioscoreaceae	Leaf	8
Rubber bark tree	*Eucommia ulmoides* Oliv.	Eucommiaceae	Leaf	8
Quaresmeira	*Tibouchina* sp.	Melastomataceae	Leaf	8
Taiyo-fuutou-kadzura	*Piper postelsianum* Maxim.	Piperaceae	Leaf	8
Ryukyu-suzukake	*Veronicastrum liukiuense* (Ohwi) T.Yamaz.	Plantaginaceae	Leaf	8
Winter rose	*Helleborus orientalis* Lam.	Ranunculaceae	Leaf	8
Aromatic ginger	*Kaempferia galanga* L.	Zingiberaceae	Leaf	8
Meik-thalin	*Zingiber barbatum* Wall.	Zingiberaceae	Leaf	8
Shell ginger	*Alpinia zerumbet* (Pers.) B.L.Burtt & R.M.Sm.	Zingiberaceae	Leaf	8
Bitter ginger	*Zingiber zerumbet* (L.) Roscoe ex Sm.	Zingiberaceae	Leaf	8
Wild turmeric	*Curcuma aromatica* Salisb.	Zingiberaceae	Leaf	8
Blue bush	*Maireana sedifolia* (F.Muell.) Paul G.Wilson	Amaranthaceae	Leaf	6
Sosnowskyi's hogweed	*Heracleum sosnowskyi* Manden	Apiaceae	Root	6
Goat horns	*Strophanthus divaricatus* (Lour.) Hook. & Arn.	Apocynaceae	Leaf	6
Urashima-sou	*Arisaema thunbergii* Blume subsp. *urashima* (H.Hara) H.Ohashi & J.Murata	Araceae	Leaf	6
Ohba-kan-aoi	*Asarum lutchuense* T.Itô	Aristolochiaceae	Leaf	6
Frosted jade	*Asarum kumageanum* Masam.	Aristolochiaceae	Leaf	6
Umano-suzukusa	*Aristolochia debilis* Siebold & Zucc.	Aristolochiaceae	Leaf	6
Maruyama-shuukaidou	*Begonia formosana* (Hayata) Masam.	Begoniaceae	Leaf	6
Cranberry	*Vaccinium wrightii* A.Gray	Ericaceae	Leaf	6
Sea derris	*Derris trifoliata* Lour.	Leguminosae	Leaf	6
Spear mint	*Mentha spicata* L.	Lamiaceae	Leaf	6
Creeping thyme	*Thymus quinquecostatus* Celak.	Lamiaceae	Leaf	6
Three-leafed akebia	*Akebia trifoliata* (Thunb.) Koidz.	Lardizabalaceae	Leaf	6
Tendai-uyaku	*Lindera strychnifolia* (Siebold & Zucc.) Fern.-Vill.	Lauraceae	Leaf	6
Cinnamon	*Cinnamomum zeylanicum* Blume	Lauraceae	Leaf	6
Bead tree	*Melia azedarach* L.	Meliaceae	Leaf	6
Okinawa-sokei	*Jasminum sinense* Hemsl.	Oleaceae	Leaf	6
Lacquered pepper-tree	*Piper magnificum* Trel.	Piperaceae	Leaf	6
Veronica	*Veronicastrum villosulum* (Miq.) T.Yamaz.	Plantaginaceae	Leaf	6
Yabu-kouji	*Ardisia japonica (Thunb.) Blume*	Primulaceae	Leaf	6
Silver dollar fern	*Adiantum peruvianum* Klotzsch	Pteridaceae	Leaf	6
Venus's hair fern	*Adiantum anceps* Maxon & C.V.Morton	Pteridaceae		6
Karatorikabuto	*Aconitum carmichaeli Debx.*	Ranunculaceae	Leaf	6
Japanese belladonna	*Scopolia japonica* Maxim.	Solanaceae	Leaf	6

Table 1. *Cont.*

Plant Species			Part Used	Growth Inhibition (%)
Common Name	**Scientific Name**	**Family Name**		
Giant Pelican Flower	*Aristolochia gigantea* Mart.	Aristolochiaceae	Leaf	5
Asparagus fern	*Asparagus densiflorus* (Kunth) Jessop	Asparagaceae	Leaf	5
Japanese silver	*Farfugium japonicum* (L.) Kitam.	Asteraceae	Leaf	5
Samoan cup fern	*Dennstaedtia samoensis* T.Moore	Dennstaedtiaceae	Leaf	5
Tamarind	*Tamarindus indica* L.	Leguminosae	Leaf	5
Lavender Moon	*Prostanthera baxteri* A.Cunn. ex Benth.	Lamiaceae	Leaf	5
Lily turf	*Liriope muscari* (Decne.) L.H.Bailey	Asparagaceae	Leaf	5
Ooba-ohyama-renge	*Magnolia sieboldii* K.Koch	Magnoliaceae	Leaf	5
Bilimbi	*Averrhoa bilimbi* L.	Oxalidaceae	Leaf	5
Grape Ivy	*Cissus rhombifolia* Vahl	Vitaceae	Leaf	5
Ginger	*Zingiber officinale* Roscoe	Zingiberaceae	Leaf	5
Mango-ginger	*Curcuma amada* Roxb.	Zingiberaceae	Leaf	5
Cassumunar ginger	*Zingiber cassumunar* Roxb.	Zingiberaceae	Leaf	5
Hanamyouga	*Alpinia japonica (Thunb.) Miq.*	Zingiberaceae	Leaf	5
River bell	*Mackaya bella* Harv.	Acanthaceae	Leaf	3
Sibirian yellow	*Achillea sibirica* Ledeb.	Asteraceae	Leaf	3
Silver mound	*Artemisia schmidtiana* Maxim.	Asteraceae	Leaf	3
Licorice plant	*Helichrysum petiolare* Hilliard & B.L.Burtt	Asteraceae	Leaf	3
May apple	*Podophyllum peltatum* L.	Berberidaceae	Leaf	3
Hop	*Humulus lupulus* L.	Cannabinaceae	Leaf	3
Celandine-leaved pelargonium	*Pelargonium fulgidum* L'Hér.	Geraniaceae	Leaf	3
Chocolate vine	*Akebia quinata* (Houtt.) Decne.	Lardizabalaceae	Leaf	3
Giant cigar plant	*Cuphea micropetala* Kunth	Lythraceae	Leaf	3
Mouse trap tree	*Uncarina grandidieri* (Baill.) Stapf	Pedaliaceae	Leaf	3
Dokudami	*Houttuynia cordata* Thunb.	Saururaceae	Leaf	3
Tar bush	*Eremophila glabra* (R.Br.) Ostenf.	Scrophulariaceae	Leaf	3
Ginger-lilies	*Alpinia oceanica* Burkill	Zingiberaceae	Leaf	3
Torch ginger	*Etlingera elatior* (Jack) R.M.Sm.	Zingiberaceae	Leaf	3
Ryuunou-giku	*Chrysanthemum makinoi* Matsum. & Nakai	Asteraceae	Leaf	2
Marian thistle	*Silybum marianum* (L.) Gaertn.	Asteraceae	Leaf	2
Ao-tsuzura-fuji (Kamiebi)	*Cocculus trilobus* (Thunb.) DC.	Menispermaceae	Leaf	2
Kusano-ou	*Chelidonium majus* L. var. *asiaticum* H.Hara	Papaveraceae	Leaf	2
Japanese umbrella pine	*Sciadopitys verticillata* (Thunb.) Siebold & Zucc.	Sciadopityaceae	Leaf	2
Sosnowskyi's hogweed	*Heracleum sosnowskyi* Manden	Apiaceae	Leaf	0
Japanese spurge	*Pachysandra terminalis* Siebold & Zucc.	Buxaceae	Leaf	0
Japanese honeysuckle	*Lonicera japonica* Thunb.	Caprifoliaceae	Leaf	0
Garlic	*Allium sativum* L.	Amaryllidaceae	Leaf	0
Urn orchid	*Bletilla striata* (Thunb.) Rchb. f.	Orchidaceae	Leaf	0
Chinese moonseed	*Sinomenium acutum* (Thunb.) Rehder & E.H.Wilson	Menispermaceae	Leaf	−2
Botan	*Paeonia suffruticosa* Andrews	Paeoniaceae	Leaf	−2
Wild betel	*Piper sarmentosum* Roxb.	Piperaceae	Leaf	−2
Lovage root	*Ligusticum sinense* Oliv.	Apiaceae	Leaf	−2
Beach carrot	*Glehnia littoralis* F.Schmidt ex Miq.	Apiaceae	Leaf	−3
Tatarian aster	*Aster tataricus* L.f.	Asteraceae	Leaf	−3
Joint-pine	*Ephedra gerardiana* Wall. ex Stapf	Ephedraceae	Leaf	−3
Kiwi	*Actinidia chinensis* Planch.	Actinidiaceae	Leaf	−5
Bittersweet	*Celastrus orbiculatus* Thunb.	Celastraceae	Leaf	−5
Tiger-lily	*Lilium lancifolium* Thumb.	Liliaceae	Leaf	−5
Victory onion	*Allium victorialis* L. var. *platyphyllum* (Hultén) Makino	Amaryllidaceae	Leaf	−6
Giant butterbur	*Petasites japonicus* (Siebold & Zucc.) Maxim.	Asteraceae	Leaf	−6
Sugar leaf	*Stevia rebaudiana* (Bertoni) Bertoni	Asteraceae	Leaf	−6
Japanese pagodatree	*Styphnolobium japonicum* (L.) Schott	Leguminosae	Leaf	−6
Red sage	*Salvia miltiorhiza* Bunge	Lamiaceae	Leaf	−6
Angular solomon's seal	*Polygonatum odoratum* (Mill.) Druce var. *pluriflorum* (Miq.) Ohwi	Asparagaceae	Leaf	−6
Chinese peony	*Paeonia lactiflora* Pall.	Paeoniaceae	Leaf	−6
Great burnet	*Sanguisorba officinalis* L.	Rosaceae	Leaf	−6
Eagle fern	*Pteridium aquilinum* Kuhn var. *latiusculum* (Desv.) Underw. ex A. Heller	Dennstaedtiaceae	Leaf	−9

3.2. Antifungal Bioassay of Authentic Volatile Compounds

The antifungal activities of octanol, octanal and *trans*-2-hexenal on the mycelial growth of both races of FOL were compared on the basis of their EC_{50} values, which were calculated based on the actual concentration of volatile compounds analyzed by headspace GC-MS (Tables 2 and 3).

Table 2. Actual volatile concentration (ng/cm^3) of authentic compounds in multi dishes at different dilution rates.

Authentic Compound	Concentration, %, *v/v*					
	10	**5.0**	**2.5**	**1.7**	**1.3**	**1**
Octanal	92.2	66.8	44.1	41.9	28.8	26.4
Octanol	8.6	8.0	7.7	5.1	2.9	2.3
Trans-2-hexenal	137.0	94.6	51.7	33.1	14.3	18.9

Table 3. The EC_{50} values (ng/cm^3) of octanal, octanol and *trans*-2-hexenal.

Authentic Compound	*Fusarium oxysporum* **f.sp.** *lycopersici*	
	Race 1	**Race 2**
Octanol	8.1	9.3
Octanal	57.0	51.0
Trans-2-hexenal	26.0	8.6

The actual concentration of all authentic volatile compounds decreased as the dilution rate increased and reached the minimum at 1%, *v/v*, making up a concentration in the headspace from 2.3 to 26.4 ng/cm^3 for octanol and octanal, respectively. The highest actual volatile concentrations of octanal, octanol and *trans*-2-hexenal in multi dishes were 99.2, 8.6 and 137 ng/cm^3, respectively, at a concentration of 10%, *v/v*. Although the actual volatile concentrations of octanol were much lower than octanal and *trans*-2-hexenal, it was observed that octanol could control both pathogenic races by more than 50% at the concentration of 10%, *v/v* (Table 3). The lowest EC_{50} values, indicating a stronger inhibitory potential, for race 1 were determined for octanol, with a value of 8.1 ng/cm^3, while race 2 was less resistant to both octanol and *trans*-2-hexenal (with an EC_{50} of 9.3 and 8.6 ng/cm^3, respectively).

The mycelial growth inhibition rates of FOL race 1 and race 2 varied as a function of the dilution rate and the distance from the source well with an authentic volatile compound (Figure 5). Among the tested compounds, octanal vapors did not change the inhibition of mycelial growth of both races as a function of the distance from the source well, but this effect significantly differed depending on the dilution rate (Figure 5a,b). Octanol (Figure 5c,d) and trans-2-hexenal (Figure 5e,f) showed the highest inhibition percentage at all dilution rates at the 41mm wells; unlike octanal, the inhibition percentage declined gradually when the distance from the source well increased.

3.3. Biofumigation Assay

One month after transplanting, the disease severity of tomato plants grown on soil fumigated with different compounds was evaluated.

3.3.1. Biofumigation Assay of FOL Race 1

Tomato plants grown on octanal-treated soil showed severe disease symptoms, with more than 70% of vascular discoloration (Figure 6) and with a 92% disease severity index (DSI), and they were not significantly different from plants grown on the soil of the positive control with 95% DSI; i.e., the soil without biofumigation (Figure 7). Plants on octanol-treated soil showed slight wilting symptoms, but the disease severity was very low, with less than 25% of vascular discoloration. The highest resistance to FOL race 1 was demonstrated by tomato plants grown on *trans*-2-hexenal-treated soil;

the plants were found to be healthy, and no external symptom was detected; i.e., the plants were similar to the negative control.

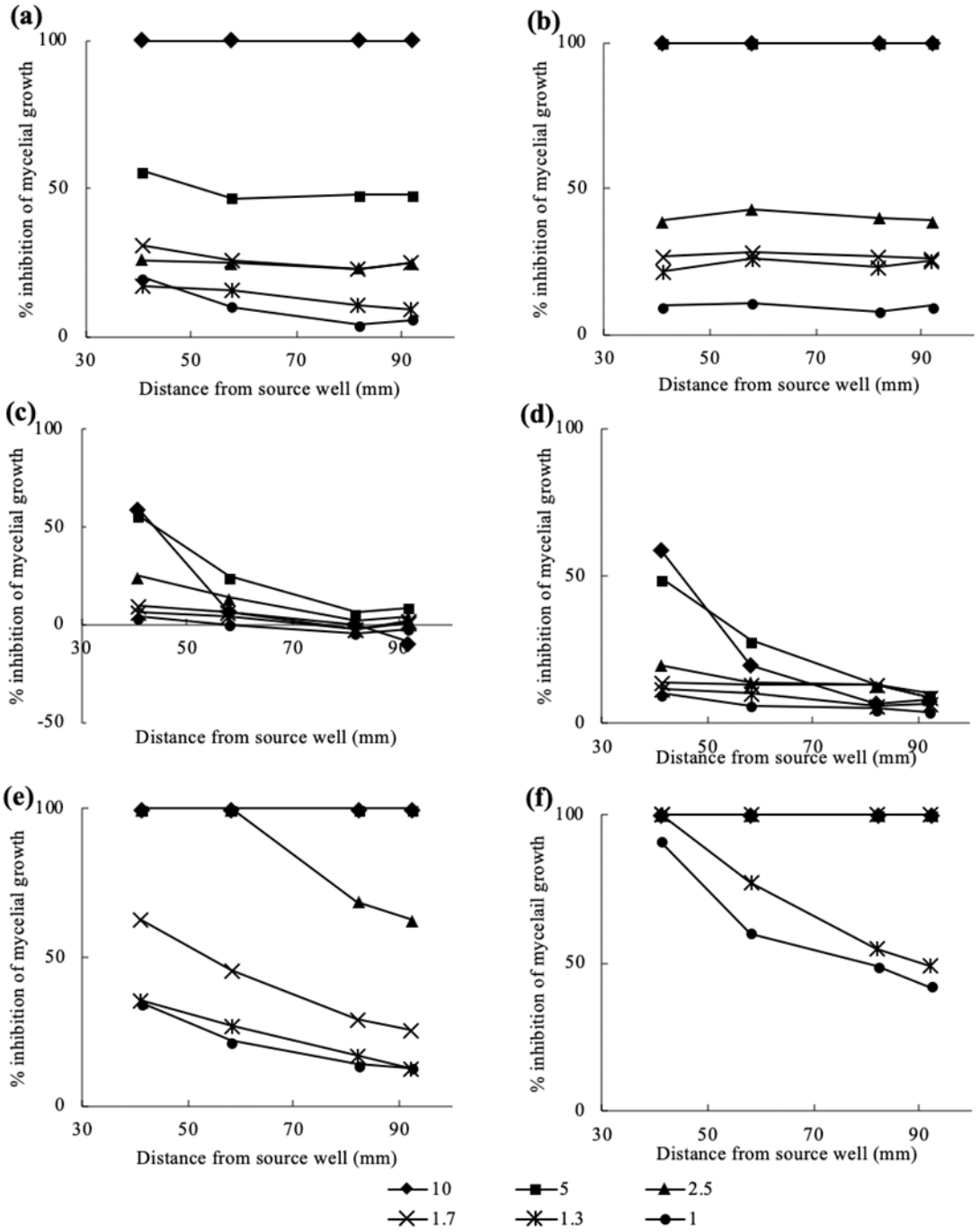


Figure 5. Mycelial growth inhibition of *Fusarium oxysporum* f. sp. *lycopersici* (FOL) race 1 (**a**,**c**,**e**) and FOL race 2 (**b**,**d**,**f**) as a function of distance from the source well with octanal (**a**,**b**), octanol (**c**,**d**) and *trans*-2-hexenal (**e**,**f**). Different lines represent the concentrations (%, *v*/*v*) of the compound added to the source wells. Each value represents the mean of three replicates.

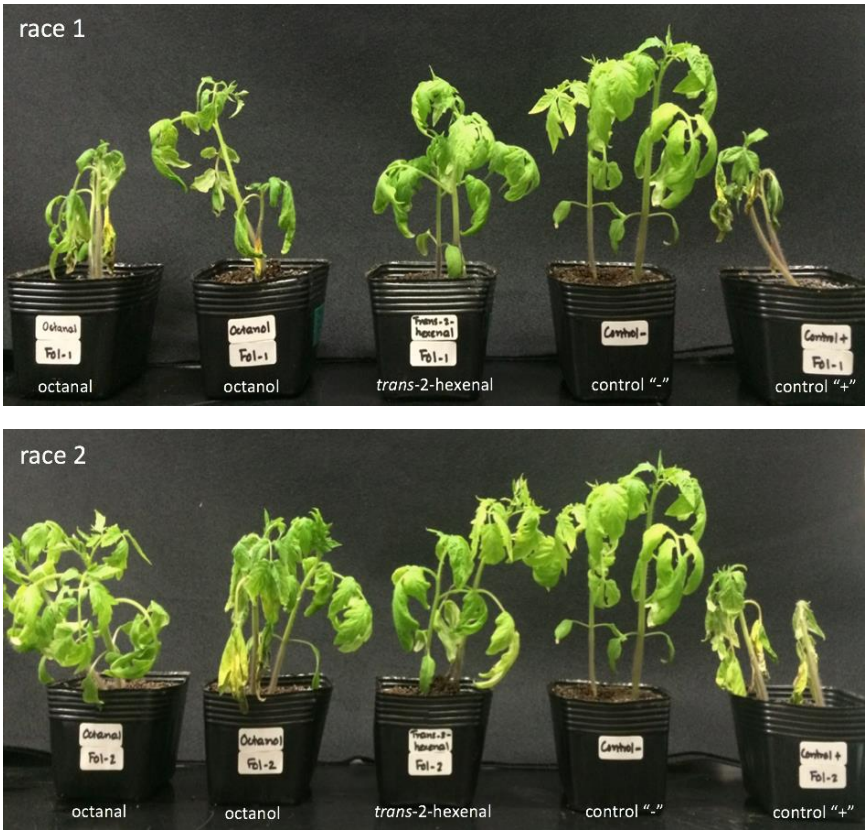


Figure 6. External disease symptom of FOL race 1 and race 2 on tomato plants grown in different biofumigated soils. The photo was taken one day before data collection: from left to right, octanal, octanol *trans*-2-hexenal, negative control and positive control.

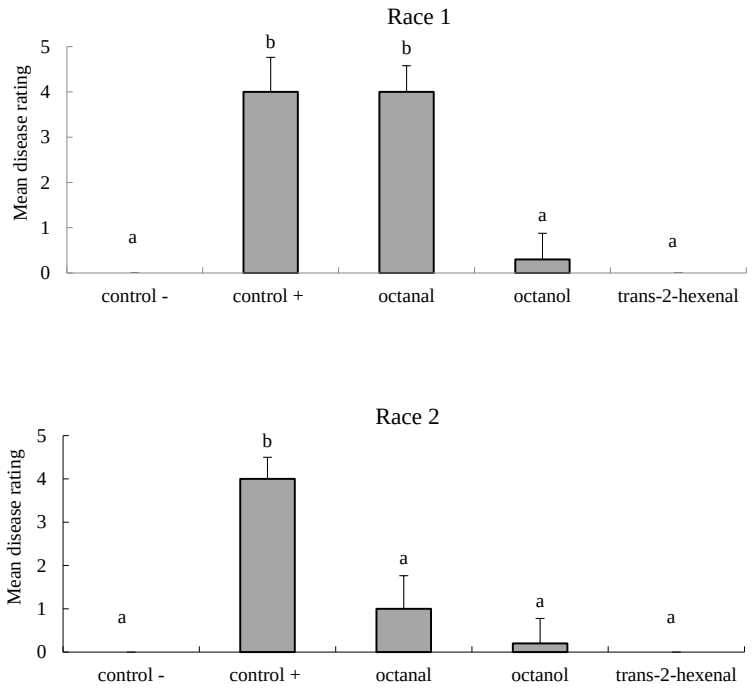


Figure 7. Disease severity of FOL race 1 and race 2 on tomato plants in octanal, octanol and *trans*-2-hexenal-treated soil. The same letters indicate that the values are not significantly different ($p < 0.05$) according to the Student's *t*-test. ND, No data.

In the soil conidial population assay (Figure 8), colonies of FOL race 1 in *trans*-2-hexenal-treated soil were absent, which was similar to the negative control, and visual disease symptoms were absent. In octanol-treated soil, a low number of conidial populations was detected 7 and 30 days after fumigation, while the highest amount of conidial populations was found in octanal-treated soil, which was comparable to the positive control.

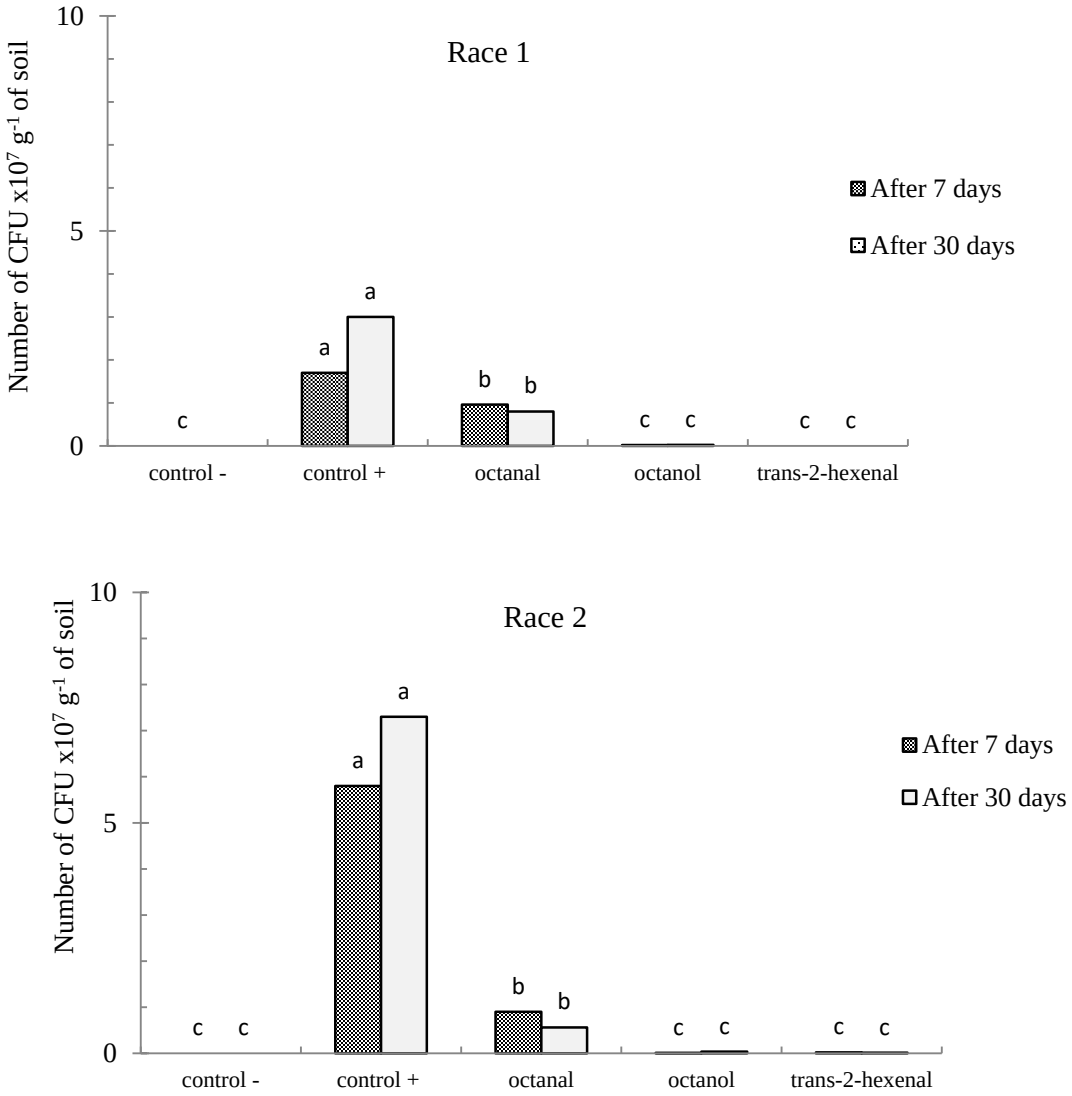


Figure 8. Number of CFUs (colony forming units) of FOL race 1 and race 2 after 7 days and 30 days of fumigation.

3.3.2. Biofumigation Assay on FOL Race 2

The development of disease symptoms in tomato plants caused by FOL race 2 was strongly inhibited by all tested volatile compounds (Figure 5). Thus, tomato plants grown octanal and octanol-treated soils both had a very low disease severity with less than 25% vascular discoloration (Figure 6) and scores of 16% and 8% on the disease severity index, respectively (Table 4). This demonstrated the potential of using of these compounds for the control of FOL race 2 to a larger extent. Similar to FOL race 1, *trans*-2-hexenal completely controlled FOL race 2 without showing any disease symptoms.

Table 4. Disease severity index (DSI) of FOL race 1 and race 2.

Treatment	DSI (%)	
	Race 1	Race 2
Control +	95	95
Control −	0	0
Octanal	92	16
Octanol	8	8
trans-2-hexenal	0	0

The conidial density was found to be significantly lower than in the positive control in all fumigated soils, both 7 and 30 days after fumigation (Figure 8), with the lowest value found in octanol-treated soil (1×10^5 CFU g^{-1} of soil). Thirty days after transplanting, even though conidial densities did not increase sharply, a few colonies of Fusarium were still detected in all fumigated soils.

4. Discussion

In the present study, fruit volatiles from *H. sosnowskyi* showed the strongest inhibition of mycelial growth of FOL race 1 (67%) of 109 tested plant species. *H. sosnowskyi*, or Sosnowskyi's hogweed, belongs to the Apiaceae family and is known as an invasive plant species which is widely distributed in European countries [31]. Antifungal and antibacterial activities of many *Heracleum* species have been previously reported. Thus, Dusko et al. [32] demonstrated the antibacterial properties of a number of Apiaceae species, including *Heracleum sphondyilium*, in relation to *Agrobacterium radiobacter* pv. *tumefaciens*, *Erwinia carotovora*, *Pseudomonas fluorescens*, and *Pseudomonas glycinea*. The essential oil of medicinal *Heracleum persicum* contains 30.2% of hexyl butyrate and exhibits strong anti-*Candida zeylanoides* activity [33], while its poor antifungal activity against phytopathogenic fungi, including *F. oxysporum* f. sp. *lentis*, *Sclerotinia sclerotiorum*, *Aspergillus flavus*, *Botrytis cinerea*, *Cladosporium cladosporioides* and others has been previously reported [34]. A previous study [26] showed that octyl acetate is the major volatile compound naturally emitted from *H. sosnowskyi* fruits; however, volatile octanal demonstrated strong plant growth inhibitory activity. Octanal has also exhibited antifungal activity against *Penicillium italicum* and *P. digitatum* in a dose-dependent manner [27] and strongly inhibited *Geotrichum citri-aurantii*, a postharvest pathogen in citrus, with a minimum inhibitory concentration of 0.50 µL/mL and minimum fungicidal concentration of 2.00 µL/mL [35]. Another 8-carbon compound, alcohol octanol, a volatile from *H. sosnowskyi* fruits [26], has been classified by the Environmental Protection Agency [36] as a biochemical pesticide and functions as a plant growth regulator by inhibiting sprout growth on stored potatoes when applied after harvesting. Therefore, in our experiment, octanal and octanol were selected as volatile compounds and were expected to exhibit antifungal activity. The volatile aldehyde *trans*-2-hexenal has been tested as a control because it is one of the most widespread volatile compounds naturally occurring in vegetables and fruit. It is called "green notes" and has been reported as a natural fungicidal compound on many postharvest pathogens, such as *Aspergillus flavus* [37] and the food pathogenic species *Escherichia coli*, *Salmonella enteritidis* and *Listeria monocytogenes* [38]. Volatile *trans*-2-hexenal exhibited strong antifungal activity against *Penicillium cyclopium*, one of the main tomato postharvest pathogens, with a minimum inhibitory concentration of 160 µL/L and minimum fungicidal concentration of 320 µL/L [39].

Besides the fact that the inhibitory activity of each volatile compound was dependent on its dilution rate, all tested volatiles showed a different degree of mycelial growth inhibition of both FOL races. The highest inhibitory activity was observed in the wells located at the closest distance of 41 mm to the source well. Similar effects of the diffusion of volatile compounds using a multidish bioassay and changes in the concentration of volatile compounds were previously described for volatiles from black zira tested to control *F. oxysporum* [10]. The diffusion rate might be important for the development of a biofumigation scheme for the treatment of plants to provide ecologically friendly antifungal control using low doses of biofumigants.

The actual concentrations of vapors of octanol were found to be lower than aldehydes (octanal and *trans*-2-hexenal), which can be explained by the difference in the volatilization rate of these compounds. The vapor pressure—i.e., the pressure of a vapor in thermodynamic equilibrium with its condensed phases in a closed system—of the 8-carbon compounds is 7.94×10^{-2} and 1.18 mm Hg at 25 °C for octanol and octanal, respectively [40], indicating the higher concentration of vapors of octanal in air, and this correlates with our results.

Specific activities of authentic compounds were evaluated based on their half-maximal effective concentration (EC_{50}). The lowest EC_{50} values were determined for octanol (8.1 and 9.3 ng mL^{-1} for FOL race 1 and race 2, respectively) compared to octanal and *trans*-2-hexenal, showing the high specific activity of octanol. Gueldner et al. have tested a number of volatile natural compounds and synthetic analogs in order to evaluate their inhibitory activity on *Aspergillus flavus* and reported that their activity followed the order aldehydes > ketones > alcohols [41]. Moleyar and Narasimham reported that unsaturated aldehydes, followed by geraniol, an unsaturated alcohol, were most inhibitory to *Aspergillus niger*, *F. oxysporum* and *Penicillium digitatum* [42]. However, the results of our bioassays disagree with these findings; i.e., the alcohol compound (octanol) was more effective than the aldehyde compounds (octanal and *trans*-2-hexenal) in controlling FOL race 1 and race 2 in terms of EC_{50} values. This can be attributed to the different sensitivity of *F. oxysporum* isolates to the particular volatile compounds and diffusion rate of volatiles.

The antifungal mechanism of action of volatile compounds may involve plasma membrane disruption and mitochondrial structure disorganization, as has been shown for the effect of *Cymbopogon citratus* essential oil on the mycelial growth of *Aspergillus niger* [43]. Zhou et al. showed that citral, octanal and α-terpineol caused a disruption of cell membrane integrity and leakage of cell components in *Geotrichum citri-aurantii* [35], while *trans*-2-hexenal disrupted cell membrane integrity, increased its permeability and caused a leakage of cell components in *Penicillium cyclopium* [39].

The biofumigation assay was conducted under controlled and standardized conditions and aimed to evaluate the disease incidence of two races of FOL on tomato plants after the application of octanal, octanol and *trans*-2-hexenal into the soil as fumigants. The results indicated that *trans*-2-hexenal was the strongest fumigant in soil without any observed disease incidence for both pathogenic races, evinced by the complete inhibition of the conidial germination of FOL race 1 by *trans*-2-hexenal during the incubation period. In octanol-treated soil, a very low disease incidence of FOL race 1 and race 2 was found in tomato plants; consequently, very low conidial density was also detected from the octanol-treated soil. This suggests that some conidia may survive after octanol treatment and can flourish in the mycelium to infect the tomato plants. However, the disease did not cause severe visual infection, which may be due to slow the volatilization of octanol, as has been shown in Section 3.2, between soil particles; this might effectively control the pathogen over a long period of time. However, higher concentrations of octanol would be required to achieve full inhibition of the conidial germination and the suppression of pathogen growth during the week after the treatment. The retreatment of soil during the growing period may not be possible; thus, adjusting the concentration or dosage of compounds for the first injection is crucial.

Safety concern for humans and the environment is paramount for any treatment in the management of soilborne pests and diseases, and this has led to an intensive search for new alternative buifumigants to, for example, methyl bromide. In this regards, the US Environmental Protection Agency has assessed the human health and ecological risks associated with the use of pesticide products which contain C6-C16 alcohols (in our case, octanol) and identified eye-irritation concerns and issues regarding the length of restricted-entry intervals after the of these aliphatic alcohols application for tobacco uses [44]. Therefore, the determination of the safety of octanol, octanal and *trans*-2-hexenal for buofumigation should be the objective of future studies.

5. Conclusions

In conclusion, among 109 plant species, fruit volatiles of *H. sosnowskyi* demonstrated the strongest antifungal activity against FOL race 1. Authentic volatile octanol demonstrated the lowest EC_{50} value against both FOL races, significantly inhibited the disease incidence of tomato plants and suppressed the growth of conidial density, similar to *trans*-2-hexenal and the negative control. In this regards, octanol and *trans*-2-hexenal may be considered to be prospective natural fungicides against *F. oxysporum* f. sp. *lycopersici*. It is still unclear if the tested compounds themselves can replace methyl bromide or other fumigants and fungicides; however, these compounds might be implemented into components of a new management strategy. However, further study is needed on the practical application and safety evaluation of these volatile compounds as novel biofumigants against *F. oxysporum* f. sp. *lycopersici*.

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