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Biochemical and Physiological Responses of *Cucumis sativus* L. to Application of Potential Bioinsecticides—Aqueous *Carum carvi* L. Seed Distillation By-Product Based Extracts

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Abstract: The extensive application of synthetic insecticides and herbicides over the past 50 years has led to a number of problems, including negative effects on non-target organisms and the evolution of pesticide-resistant pests. As an alternative means of pest control, plant-based biopesticides have emerged. While developing a new bioinsecticide, allelopathy on both target pests and target crops must be evaluated. We evaluated volatile organic compounds (VOCs), total phenolic content (TPC), total sugars and antiradical activity (ARA), as well as 18 photosynthetic apparatus characterizing functional parameters as stress signaling response to aqueous caraway seed distillation by-product-based extracts. VOCs were detected by headspace gas chromatography mass spectrometry (HS-GC-MS). The caraway extract application decreased "green leaf volatile" C4, C5 and C6 alcohol content in the cucumber leaves. Total phenolic content (TPC), total sugars and antiradical activity (ARA) using high-throughput 96-well plate spectrophotometric methods were tested in dried leaves. No significant changes in these parameters were detected in cucumber leaves after the application of extracts. The caraway extract application did not cause changes in the functioning of the photosynthesis apparatus. Aqueous caraway seed distillation by-product-based extracts can be considered non-phytotoxic to cucumber plants; however, they modify the VOC emissions even ten days after treatment.

Keywords: cucumber; caraway; allelopathy; stress signaling; leaf volatile; biopesticide

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1. Introduction

Synthetic insecticides and herbicides have been the major plant protection products for a long time [1]. The extensive use of synthetic products has resulted in effects on non-target organisms, including humans and pollinators, and a rise in target pest resistance to insecticides [2]. Throughout history, plant products have been successfully exploited as insecticides, insect repellents and insect antifeedants [1]. In recent years, plant-based bioinsecticides and bioherbicides have gradually increased their share in the crop protection product market, even though it is still less than 5% of the market share [3].

Plant-based biopesticides contain several secondary metabolites involved in plant-insect and plant-plant interactions [3], called allelopathy. While bioinsecticides must be effective against crop pests, they should have limited side effects on crop plants. Products containing multiple natural compounds, such as plant extracts, have diverse mechanisms of action on a plant's physiological processes [4]. The allelopathy can affect the target organism either positively or negatively. Plant-based products with a negative effect on plants or pests can be used for weed and insect elimination, but plant products' stimulating effect on other plant species can be used as growth enhancers [5,6].

Most studies focusing on bio-repellent development demonstrate the repellent activity of essential oils (volatile compounds); however, many bio-active ingredients (non-volatile

compounds) such as flavonoids, terpenoids, tannins, saponins, etc., with proven anti-repellent activity remain in essential oil production by-products [7].

The cultivation and processing of agricultural products is a time and resource-consuming process in which by-products with high potential for added value are sometimes discarded as waste. Agricultural waste mainly consists of cellulosic fiber and can be successfully used for alternative energy source processing (such as biodiesel) [8]. Waste streams containing high-value compounds should be first used for the extraction of phytochemicals and only then further processed. Previous research shows that high-value-added products, such as antioxidants, can be obtained from previously distilled biomass in the same concentrations or even higher than those compared to non-distilled biomass [9,10]. Even though some degradation and modification of phytochemicals occur during the steam distillation process, the modification of plant structure during the process facilitates the mass transfer of phytochemicals [10], and steam distillation by-product extracts are rich sources of biologically active substances.

Research in agricultural waste mainly focuses on the extraction of antioxidants [11], polyphenols and flavonoids for application in the food, cosmetic and pharmaceutical industries [12–14], but there are emerging studies combing the extraction of biopesticides from various agricultural wastes [15].

In this study, aqueous extracts produced from caraway (*Carum carvi* L.) seed distillation by-products have been studied. Caraway is widely cultivated all over the world for seed production, whose seeds mainly contain an essential oil that can be used in food, cosmetics, beverages and the pharmaceutical industry as a rich source of compounds [16]. Furthermore, caraway essential oil possesses insecticidal activity [17], and carvone, which is the main volatile compound of *C. carvi* oil, is approved as a pesticide in the European Union (EU Pesticide Database Regulation (EC) No 396/2005). Moreover, in aqueous seed-derived extracts, diverse flavonoids, flavonoid glycosides, monoterpenoid glucosides, lignins, alkaloids, tannins and other phenolic compounds have been found [18], which are among plant metabolite groups with insecticidal properties. Meanwhile, caraway essential oil also has herbicidal properties [19].

Plant extracts may have an allelopathic effect on target plants, influencing the growth, development and physiological parameters [20], therefore while developing new biopesticides, it is essential to determine their response on target plants, preferably by evaluating multiple plant physiological and morphological parameters by high-throughput methods [21]. In this study, we used the cucumber (*Cucumis sativus* L.) as a model plant, as it is a widely cultivated and consumed vegetable around the world [22].

Chlorophyll fluorescence imaging is a non-destructive and non-invasive method that can be used to assess photosynthetic dysfunctions in photosystem II (PS II) in plants caused by biotic and abiotic stress factors [23] even before visible symptoms on plants appear [24]. Furthermore, these techniques can be used on plants in both controlled and field conditions [25]. Several chlorophyll fluorescence measurement parameters have been reported for different plant stress assessments on cucumbers. (Jiang, et al., (2019) [26] reported a decrease in F_V/F_M and F_V/F_0 ratios and PSII as effective parameters in response to salinity stress. Estaji, et al., [27] described similar parameters for indicating salinity stress, including maximum dark-adapted fluorescence (F_M), maximum variable fluorescence (F_V), F_V/F_M ratio and the area above the OIJP curve. Applying the herbicide bensulfuron-methyl on cucumber decreased the F_V/F_M ratio, minimal fluorescence (F_0), the actual efficiency of PSII (ΦPSII) and electron transport rate (ETR) parameters [28]. The F_V/F_M ratio and ΦPSII are reported as stress indicators in response to insecticide application [29].

The application of pesticides, including biopesticides, influences the synthesis of primary and secondary metabolites. Cucumbers, among other plants, produce phytochemicals in the form of VOCs for direct and indirect defense against predators to attract pollinators or predatory insects feeding on pests. VOCs are also involved in the triggering of plant defense priming against biotic and abiotic stress and plant–plant communication [30]. VOCs, especially the C6 and C9 aldehydes [31], in cucumber fruit, contribute to

the flavor of the fruit [32]. Approximately 1700 VOCs have been characterized in various plant volatile blends, where they provide details about the identity and physiological state of the plant [33]. Among them, more than 160 VOCs have been identified in cucumber plants [34]. The application of chemical pesticides [35,36] and plant extracts [4,37] can affect the emissions of VOCs. As the plant extracts themselves contain VOCs with pest-repelling and deterring properties [30], the efficacy of a biopesticide depends on the mutual effect of the host plant and the sprayed VOCs. Generally, the detection of VOCs as chemically-signaling molecules in plants is performed by gas chromatography-mass spectrometry methods [34,38,39].

Plant sugars are the primary metabolites and energy sources of plants, but they also play many important roles in plant defense against stress [40]. They can act as signaling molecules, regulating the plant's response to stress by activating or inhibiting specific pathways in the plant. Furthermore, sugars can be converted into other compounds involved in plant stress responses, such as phenolic compounds [41].

Plant-based products used in agricultural settings must be biosafe. As the plant extracts affect plant physiological processes—including metabolism [37], growth and development [4]—elucidation of plant response to potential biopesticides is an essential part of biopesticide development. Our study aimed to investigate the physiological and biochemical response of cucumber plants to caraway extract application.

2. Materials and Methods

2.1. Characteristics of Plant Extracts and Commercial Agrochemicals

Aqueous extracts of *C. carvi* L. were supplied by Field and Forest, Ltd. (Priekuļi, Latvia). Extracts are produced by Field and Forest, Ltd. from discarded biomass and distillation container wastewater—by-products of essential oil steam distillation of organic caraway seeds. Extracts are characterized in Table 1. Four caraway extracts (E1–E4) were sprayed on plants, with a concentration range of 1.26 to 2.55 mg ml^{-1} and carvone contents ranging from 77.42 to 98.88% (see Table 1 for details). The chemical composition of the examined caraway extracts was characterized using the gas chromatography mass spectrometry method (GC-MS) according to the European Pharmacopoeia [European Pharmacopoeia, 10th Edition, Vol 1, Strasbourg: Caraway oil, 01/2008:1817, 1377–1378]. Treatments also included application of three controls: demineralized water (C1), 2% solution of herbicide Taifun® B (glyphosate, 360 g L^{-1}, S, Adama Registrations B.V.) (C2) and a 0.5% solution of insecticide NeemAzal®-T/S (azadirachtin A, 10 g L^{-1}, EC, Trifolio-M GmbH) (C3).

Table 1. Concentration and chemical composition of aqueous caraway seed distillation by-product-based extracts.

Code	Extract Concentration, mg mL^{-1}	Chemical Composition		
		Carvone, %	Limonene, %	Other Compounds, %
E1	1.26	77.42%	21.98%	0.60%
E2	2.37	97.66%	0%	2.34%
E3	1.87	98.88%	0%	1.12%
E4	2.55	98.62%	0%	1.38%

2.2. Plant Material and Plant Treatment

A pot experiment was conducted in August 2022 at the Institute for Environmental Solutions, Latvia. Seeds of cucumber (*Cucumis sativus* L.), cultivar 'Dirigent' (F1 hybrid) were sown into Root Riot plugs. Two-week-old seedlings were transplanted into fully soaked rockwool that was placed in 1 L plastic pots (11 × 11 × 12 cm) containing pressed on the bottom 350 mL soil mixture consisting of 60% Laflora KKS-1 peat substrate, 20% vermiculite and 20% perlite.

The seedlings were placed in a growing chamber with 16 h light and 8 h night regime. LED lighting modules from Heliospectra AB, Gothenburg, Sweden, provided full spectrum, 5700 K visible white light with peaks at approximately 446 nm, 534 nm and 625 nm, and photon flux density was 150 mol m^{-2} s^{-1} measured at canopy level. The temperature was kept at 24 ± 2 °C during the day and 21 ± 2 °C at night, with relative humidity (RH) of 69 ± 5% during the day and 86 ± 5% at night. Plants were grown in well-watered conditions and fertilized once a week with a nutrient solution containing 265 mg L^{-1} nitrogen, 364 mg L^{-1} potassium and 400 mg L^{-1} phosphorus.

The treatment occurred 30 days after sowing. For the experiments, uniformly developed plants with 5 unfolded true leaves were selected (BBCH 105) [42]. Before spraying, the plants were randomized. Each plant was uniformly sprayed with the planned treatment (3 mL per plant) and isolated for 4 h. Spraying was performed using laboratory reagent sprayer (DURAN®, Bensheim, Germany) with an air pressure of 0.20–0.30 kg cm^{-2}. Three replications were carried out for each treatment. Subsequently, all pots were placed in a completely randomized block design. Plant's location in the climatic chamber was randomly relocated on a daily basis.

2.3. Photosynthesis and Morphological Parameters

Five morphological parameters and 18 photosynthesis parameters were measured ten days after treatment.

Plant morphological parameters: plant height (PH10) and the number of true leaves were counted at the end of the trial (LN). The increase in number of leaves (ILN) was calculated by subtracting the total number of true leaves at the beginning of the trial from the total number of true leaves on the 10th day after spraying. The shoots were harvested at ground level and air-dried, and the shoot dry weight (PDW) was determined. The visual assessment (VA) of plant condition was assessed by determining the color of the unfolded leaves on a five-point scale: 5—no visible stress symptoms, 4—minor stress symptoms on some leaves, 3—medium stress symptoms, 2—visible stress symptoms on all leaves, 1—terminal senescence.

The photosynthesis parameters (Table 2) were determined by a portable fluorometer PAR-FluorPen FP 110-LM/D portable fluorometer (Photon Systems Instruments Ltd., Brno, Czech Republic), as described previously [23]. Measurements were taken on 30 min dark-adapted plants ten days after spraying at three locations on the third main leave of three plants for each treatment. The fluorescence measurement settings were the following: actinic pulse was set to 300 µE, super pulse—80% and flash pulse—30%.

Table 2. Abbreviations and definitions of the OJIP parameters [43,44].

Abbreviation	Measurement Specification
F_0	Minimal fluorescence with all PSII reaction centers (RC) assumed to be open
F_v	Maximal variable fluorescence
F_M	Maximal fluorescence, when all PSII RCs are closed
F_m/F_0	Ratio of maximal to minimal fluorescence
F_v/F_0	Ratio of variable to minimal fluorescence
F_v/F_M	Photosynthetic efficiency of dark-adapted reaction centers, i.e., which number of absorbed photons can be converted into electron transport
M_0	Approximated initial slope (in ms^{-1}) of fluorescence transient V = f(t)
QY	Quantum yield
Area	Total complimentary area between the fluorescence induction OJIP curve and F_m line
ΦP_0	Maximum quantum yield of primary PS II photochemistry
Ψ_0	Probability at t = 0 that a trapped excitation moves on electron into the electron transport chain beyond QA−

Table 2. *Cont.*

Abbreviation	Measurement Specification
ΦE_0	Maximum quantum yield of primary PS II photochemistry
ΦD_0	Quantum yield of energy dissipation
PI_{ABS}	Performance index on absorption basis
ABS/RC	Absorbed photon flux per reaction center (RC)
TR_0/RC	Maximum trapped exciton flux per PSII
ET_0/RC	Electron transport flux from Q_A to Q_B per reaction center
DI_0/RC	Heat dissipation per reaction center

2.4. Phytochemical Screening of Secondary Metabolites by 96-Wellplate Spectrophotometric Assays

Cucumber leaves were dried at 55 °C for 24 h and further crushed in a pestle. A 1:50 concentration ratio was used to make the extracts. All of the dried samples (0.05–0.19 g) were weighed in 15-mL plastic tubes, and an appropriate volume (according to concentration) of 70% ethanol was added. Samples were ultrasonically treated for 1 h at 70 °C. Then the samples were centrifuged for 10 min at 4400 rpm and filtered with a 0.45 µm filter. Extracts were diluted to 50% concentration. The amounts of total phenolic content (TPC), total sugars and antiradical activity in examined extracts were estimated as previously described by Nakurte, et al., [45] using an Epoch2 UV/VIS Microplate Spectrophotometer (BioTek, Agilent, Germany) in triplicates. A slightly changed version of the Folin-Ciocalteu method was used to measure TPC of the extracts studied at 765 nm, comparing the obtained results to a standard curve of gallic acid solutions (0.025–0.20 mg mL^{-1}). TPC content was estimated as mg of gallic acid equivalents (GAE). For total sugar estimation, a modified phenol-sulfuric acid colorimetric method was used at 490 nm and was proportional to the carbohydrate concentration initially present in the sample, comparing the obtained values to a standard curve of prepared glucose solutions (0.09–0.90 mg mL^{-1}). The total sugar content was expressed as mg of glucose equivalents (GLE). To assess the free radical scavenging properties of the tested extracts, a decrease in the absorbance of extracts at 517 nm was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH) compared with ascorbic acid standard solutions in the concentration range of 0.03–0.22 mg mL^{-1}. The antiradical activity (ARA) was measured in mg of ascorbic acid equivalents (ASE).

2.5. The Identification and Quantification of Volatile Compounds in Cucumber Plant Leaves

Fresh cucumber leaves were collected from experimental plants. The leaves were rolled into small rolls, and an average of 20.93 ± 0.90 g were placed into 20 mL HS vials, ranging from a minimum of 19.29 g to a maximum of 22.92 g, respectively, in the shortest possible time. The incubation temperature was set at 120 °C, and the incubation time was 40 min. The agitator (Gerstel, Mülheim, Germany) to provide orbital agitation during incubation was set to run at 250 rpm for 30 s on and 15 s off. The syringe temperature was 105 °C, and the volume of injection was 1700 µL. Analyses were performed on an Agilent Technologies 7820A gas chromatograph coupled to Agilent 5977B mass selective detector (MSD) equipment with a Gerstel MPS autosampler (Mülheim, Germany). A polar CP-Wax 52CB capillary column (50 m × 0.32 mm, 0.20 µm film thickness) with polyethylene glycol was used. The carrier gas was helium (He), with a split ratio of 1:50 and a flow rate of 1.0 mL min^{-1}. The temperature program was started at 60 °C, then increased at a rate of 10 °C min^{-1} to 265 °C. 265 °C was maintained for 3 min. The injector temperature was 250 °C. The mass spectra were recorded at 70 eV. The mass range was from m/z 50 to 650. The ion source temperature was maintained at 230 °C. The components were identified based on their retention indices (determined with reference to homologous series of C5–C24 n-alkanes) by comparison of their mass spectra with those stored in the NIST (National Institute of Standards and Technology) MS search 2.2 library. The Agilent MassHunter Qualitative Analysis 10.0 data acquisition software was applied to analyze GC-MS data.

The number of separated compounds was calculated in peak areas using the normalization method without correction factors.

2.6. Statistical Analysis

Principal component analysis (PCA) was carried out based on the 52 plant physiological status parameters described previously. One-way ANOVA with a post hoc Tuckey test was carried out to determine the effect of treatments on plant physiological status parameters. Principal component analysis (PCA) was performed using the *FactoMineR* (Lê, et al., 2008) and *factoextra* [46] packages. A heatmap was created with scaled data in the R package *pheatmap* [47]. For all statistical analyses, R version 4.0.4 was used.

3. Results

Ten days after the application of caraway extracts (E1–E4) and controls (water (C1), glyphosate (C2), azadirachtin (C3)), morphological growth parameters, photosynthesis parameters, as well as chemical composition, were determined.

None of the measured morphological parameters were affected by the application of water-based caraway extract compared to the application of water as the control. On the contrary, glyphosate (C2) and azadirachtin (C3) application caused pronounced yellow discoloration between the veins of leaves and a decrease in plant dry weight, plant height and the number of newly formed leaves (Table 3).

Table 3. Water-based caraway extract (E1–E4) and water (C1), glyphosate (C2) and azadirachtin (C3) effect on cucumber morphological growth parameters ten days after treatment.

Parameter	C2	C3	E1	E2	E3	E4	C1
PH10, cm	21.67 a	27.67 a	68.00 b	64.67 b	61.00 b	67.00 b	64.33 b
PDW, g	1.76 a	3.10 b	4.93 c	4.60 c	4.86 c	6.14 d	5.32 cd
LN	7.33 a	9.67 a	14.33 b	14.67 b	13.67 b	14.33 b	14.67 b
ILN	1.00 a	3.00 a	7.33 b	7.67 b	6.67 b	6.67 b	7.33 b
VA, scores	1.00 a	3.50 b	4.67 c	4.50 c	4.33 bc	4.83 c	4.50 c

Means in rows indicated with the same letter do not differ according to Tuckey test ($p > 0.05$).

Table 4 shows the VOCs identified by HS-GC-MS in the fresh cucumber leaves ten days after the spray application. In total, twenty-five VOCs were identified and classified into eight main chemical groups: alcohols, aldehydes, esters, ethers, sulfides, pyrroles, furans and sesquiterpenes, many of which are important contributors to the flavor of fruits, vegetables and green leaves [32].

Table 4. Main volatile organic compounds (VOCs) in fresh cucumber leaves.

No.	Compound	Class	Formula	Relative Amount Range, %
1	1-Deoxy-d-mannitol	Sugar alcohols	$C_6H_{14}O_5$	0–4.1
2	Dimethyl sulfide	Sulfides	C_2H_6S	6.5–70.6
3	Pentanal	Aldehydes	$C_5H_{10}O$	0–21.1
4	2,4-Hexadienal	Aldehydes	C_6H_8O	0–15.7
5	Ethyl-1-propenyl ether	Ethers	$C_5H_{10}O$	0–22.0
6	2,4-Dimethyl-3-hexanol	Alcohols	$C_8H_{18}O$	0–1.6
7	4-Methylhexan-3-ol	Alcohols	$C_7H_{16}O$	0–90.6
8	2-Methyl-1,3-butanediol	Alcohols	$C_5H_{12}O_2$	0–12.0
9	Hexanal	Aldehydes	$C_6H_{12}O$	0–6.2
10	1-Butanol	Alcohols	$C_4H_{10}O$	0–2.9
11	4-Methyl-1-penten-3-ol	Alcohols	$C_6H_{12}O$	0–2.7
12	1-Pentanol	Alcohols	$C_5H_{12}O$	0–1.2
13	4-Ethyl-2-methyl-1H-pyrrole	Pyrroles	$C_7H_{11}N$	0–2.1
14	2-Hexenal	Aldehydes	$C_6H_{10}O$	0–2.7

Table 4. *Cont.*

No.	Compound	Class	Formula	Relative Amount Range, %
15	n-Hexyl acetate	Esters	$C_8H_{16}O_2$	0–0.7
16	2-[(2E)-2-Pentenyl]furan	Furans	$C_9H_{12}O$	0–1.5
17	(4E)-4-Hexenyl acetate	Esters	$C_8H_{14}O_2$	0–26.8
18	1-Hexanol	Alcohols	$C_6H_{14}O$	0–2.0
19	Hex-3-en-1-ol	Alcohols	$C_6H_{12}O$	0–0.8
20	cis-3-Hexen-1-ol	Alcohols	$C_6H_{12}O$	0–23.4
21	Methyl salicylate	Esters	$C_8H_8O_3$	0–1.7
22	Benzaldehyde	Aldehydes	C_7H_6O	0–2.2
23	3-Hexanol	Alcohols	$C_6H_{14}O$	0–6.2
24	β-Cyclocitral	Aldehydes	$C_{10}H_{16}O$	0–1.6
25	Hexahydrofarnesyl acetone	Sesquiterpenes	$C_{18}H_{36}0$	0–4.6

Figure 1 shows the heatmap of the normalized average values of separated volatile organic compounds (VOCs), which separated all treatments into four clusters. One cluster comprised all the tested caraway extracts, and all the other clusters comprised each of the controls—water, glyphosate and azadirachtin. Caraway extract (E1–E4) application significantly increased the 4-methylhexan-3-ol amount in cucumber leaves compared to other treatments. Water (C1) sprayed cucumber leaves were characterized by significantly higher alcohols such as C6 (1-hexanol, hex-3-en-1-ol and cis-3-hexen-1-ol), C5 (2-methyl-1,3-butanediol) and C4 (1-butanol) compared to other treatments (Figure 1, Table S1). VOCs composition in plants treated with glyphosate (C2) and azadirachtin (C3) had the largest deviation from other treatments. Glyphosate-sprayed cucumber leaves were characterized by significantly higher levels of aldehydes (pentanal and β-cyclocitral), alcohols (2,4-dimethyl-3-hexanol-4), pyrroles (4-ethyl-2-methyl-1H-pyrrole) and sesquiterpenes (hexahydro-farnesyl acetone) emissions. All the sprays significantly reduced the cis-3-hexen-1-ol amount in fresh cucumber leaves compared to water application (Table S1).

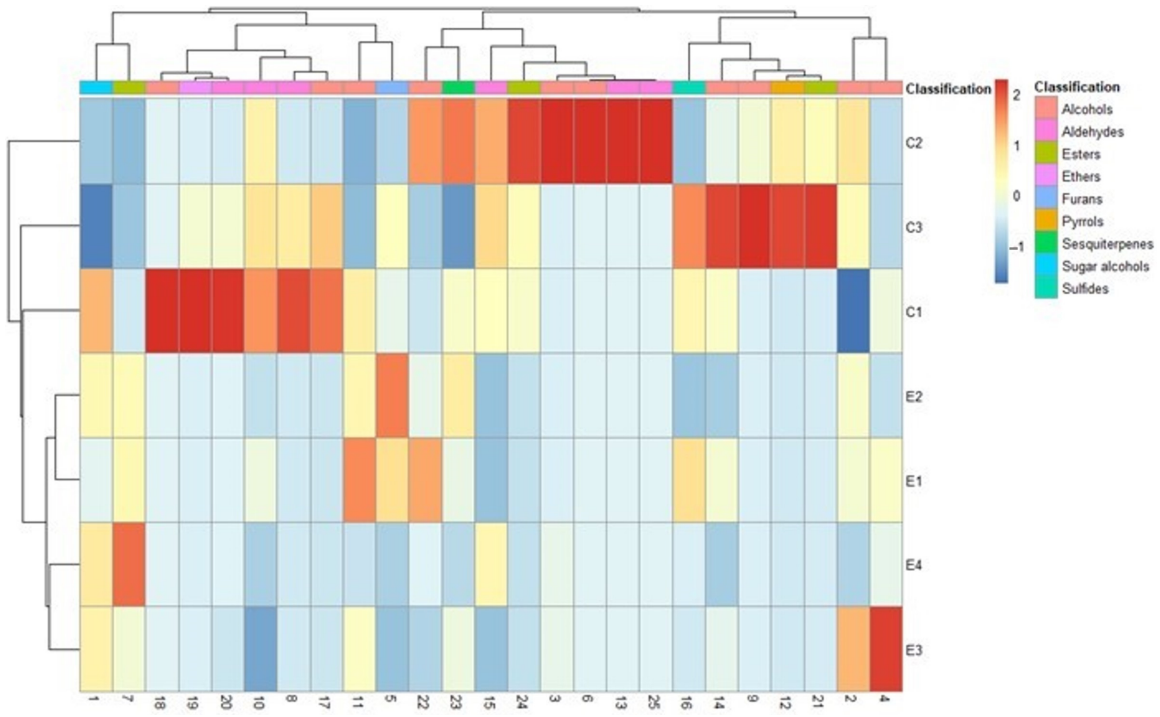

Figure 1. Heatmap of volatile organic compounds emitted from fresh cucumber leaves sprayed four caraway extracts (C1–C4) and controls water (C1), glyphosate (C2) and azadirachtin (C3). The legend denotes the scaled values of the volatile constituents, numbered and classified according to Table 4.

The levels of TPC, ARA, sugars, as well as antiradical activity (DDPH) were significantly elevated in glyphosate-treated leaves compared to other treatments (Table 5). However, caraway extract application did not change these parameters in comparison to water treatment.

Table 5. Total phenolic content, total sugar content, antiradical activity and DPPH free radical scavenging activity of water-based caraway extract (E1–E4) and water (C1), glyphosate (C2) and azadirachtin (C3) treated cucumber leaves ten days after treatment.

Parameter	C2	C3	E1	E2	E3	E4	C1
TPC [a] mg (GAE/g)	8.22 a *	4.64 b	6.26 ac	6.69 bc	6.97 ac	6.91 ac	6.42 c
Sugars [b], mg (GLE/g)	32.70 a	13.97 b	26.86 c	19.75 bde	23.57 acd	26.53 cde	17.37 be
ARA [c], mg (ASE /g)	6.95 a	2.86 b	4.19 ab	4.34 ab	4.49 ab	4.43 ab	4.80 ab
DPPH Quenched, %	40.67 a	17.39 b	25.03 ab	25.87 ab	25.74 ab	26.25 ab	28.74 ab

* Means in rows indicated with the same letter do not differ according to Tuckey test ($p > 0.05$). [a] TPC is calculated based on the gallic acid equivalents (GAE) in mg per 1 g of dry weight. [b] Sugars are calculated based on the glucose equivalents (GLE) in mg per 1 g of dry weight. [c] ARA radical scavenging activity is calculated based on ascorbic acid equivalents (ASE) in mg per 1 g of dry weight.

15 out of 18 photosynthetic apparatus functional parameters (Figure 2, Table S2) significantly differed between glyphosate and all the other treatments. Three parameters had no statistically significant differences among any treatments. Water-based caraway extract application did not reduce the functioning of the photosynthetic apparatus.

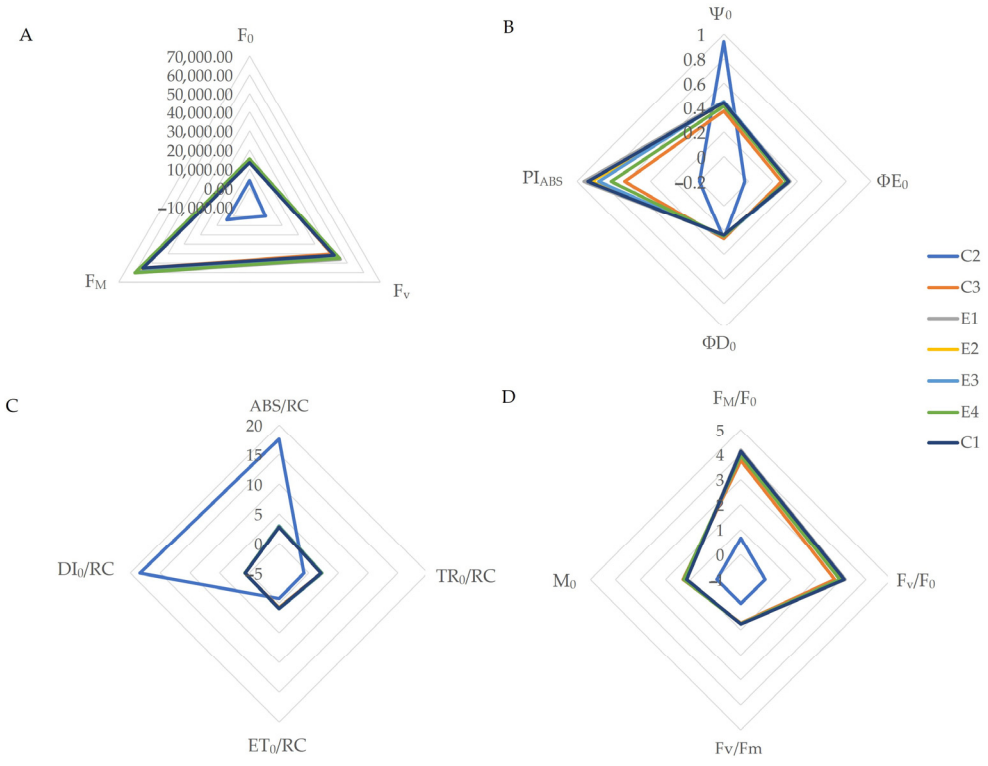

Figure 2. Chlorophyll fluorescence parameters in the fully expanded cucumber leaves ten days after spraying with caraway extracts (E1–E4), water (C1), glyphosate (C2) and azadirachtin (C3). (**A**) Values from the recorded fluorescence transient OJIP; (**B**) quantum yields, quantum efficiencies and performance index; (**C**) phenomenological energy fluxes; and (**D**) fluorescence parameters derived from the extracted fluorescence transitions OJIP.

Both the heatmap based on volatile emissions from sprayed leaves (Figure 1) and the PCA based on 52 parameters (VOCs, morphological and photosynthesis characterizing parameters) (Figure 3) separated treatments into four distinctive clusters. All caraway

extract treatments formed a single cluster, but water, azadirachtin and glyphosate formed separate clusters.

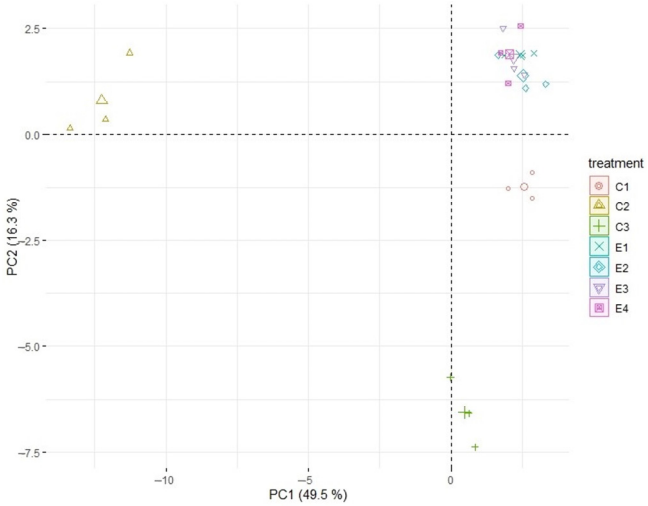

Figure 3. Principal component analysis of 52 parameters indicating plant physiological state in glyphosate, azadirachtin and caraway water extract sprayed cucumber plants. Centroids are represented by the largest point of the same color.

The parameters contributing the most to the stress indication of plants can be identified from the plots representing the PCA loading values of each variable. The maximum variation of PC1 originated from functional parameters of the photosynthetic apparatus: F_V/F_0, F_M/F_0, ΦD_0, F_V/F_M, ΦE_0 and QY (Figure 4). The methyl salicylate, hexanal, 1-pentanol, 2-hexenal and TPC were responsible for the maximum variation of PC2 (Figure 5).

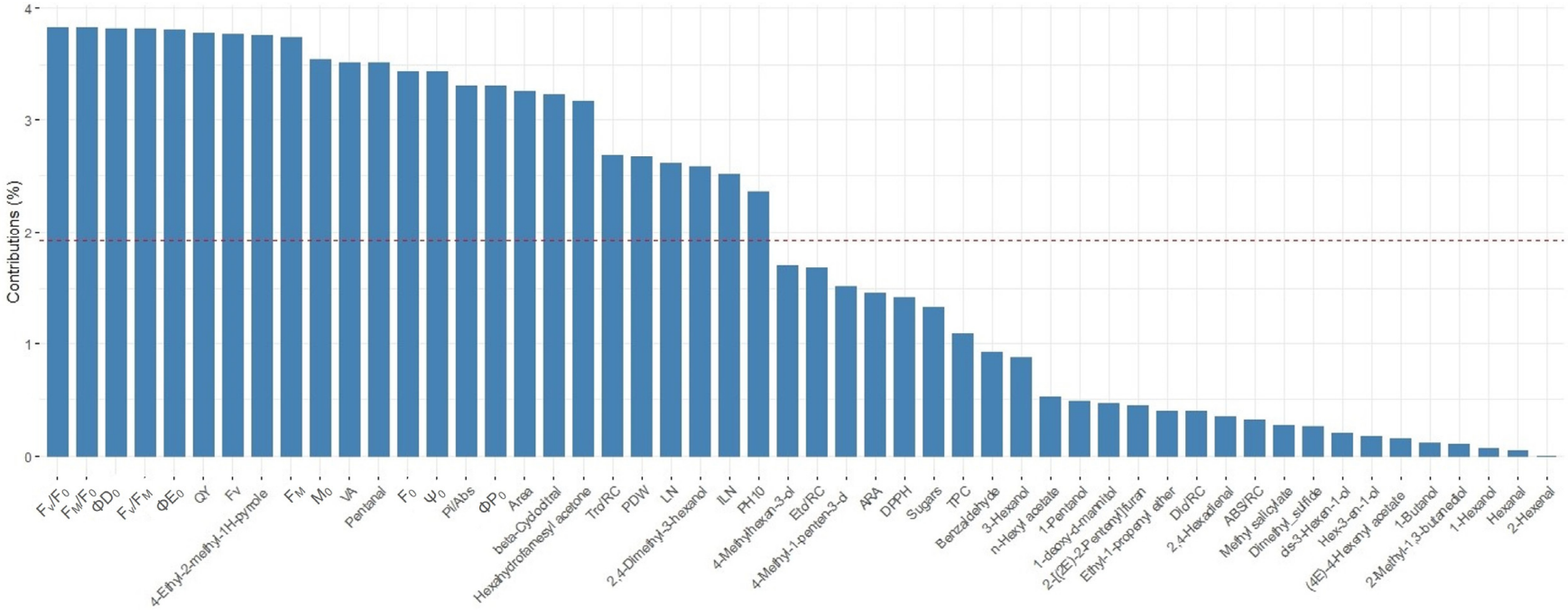

Figure 4. Loading values of PC1 from the principal component analysis of 52 parameters indicating plant physiological state in glyphosate, azadirachtin and caraway water extract sprayed cucumber plants.

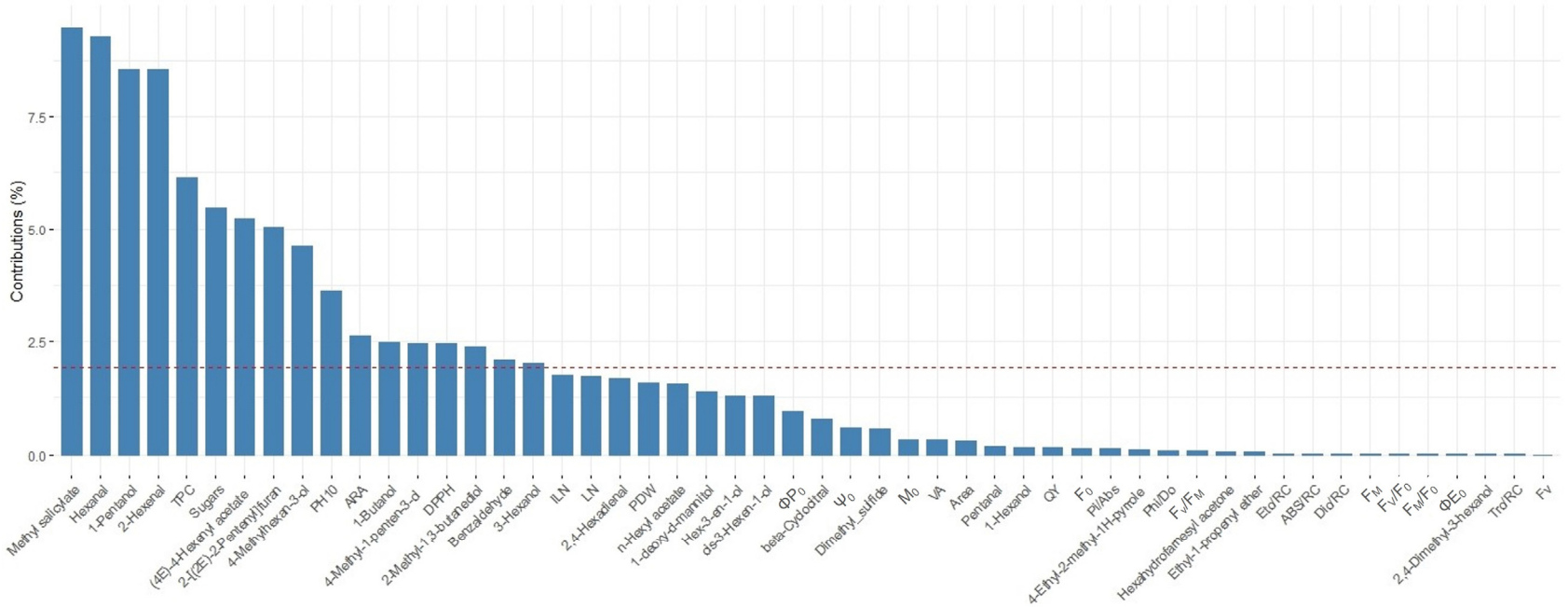

Figure 5. Loading values of PC2 from the principal component analysis of 52 parameters indicating plant physiological state in glyphosate, azadirachtin and caraway water extract sprayed cucumber plants.

4. Discussion

The current attempts to limit the overuse of synthetic pesticides create the necessity for alternative plant protection practices. One of the alternatives is the development of plant-based biopesticides. As the metabolites in plant extracts can have an allelopathic effect on other plants, reducing or stimulating growth and development, the phytotoxicity of potential products should be clarified. The plant extract application can also modify the primary and secondary metabolism of the host plant. Altered chemical composition can influence the host plant's direct and indirect response to biotic and abiotic stress. Therefore, changes in the chemical composition of plants and their emitted volatiles should be studied in response to biopesticide treatments to understand the effects on the host plant better.

This study demonstrated that the application of aqueous caraway seed distillation by-product-based extracts (E1–E4) on cucumber plants does not have phytotoxic effects on cucumber growth, thus outcompeting conventional herbicide glyphosate (C2) and biopesticide (C3). The caraway essential oil and leaf aqueous extract have previously been demonstrated to inhibit the germination and growth of canary grass and wheat [48]. In this study, the essential oil content of the extract was residual, and a water extract of seeds was used instead of leaves [48]. The host plants can have different reactions to extracts from different plant species. As expected, the conventional herbicide glyphosate (C2) and biopesticide (C3) significantly hindered cucumber growth in terms of plant height, dry weight and speed of leaf development. Additionally, a visible yellow discoloration related to senescence was detected in commercial biopesticide and herbicide-sprayed cucumber leaves. The active substance in the commercial biopesticide is azadirachtin, a chemical compound belonging to the limonoid group. It is the main compound in neem seed oil (*Azadirachta indica* A. Juss.). While limonoids are effective bioinsecticides, they also exhibit phytotoxic properties on germination, root and shoot growth in many plant species, e.g., *Lepidum sativum*, *Lactuca sativa*, *Lycopersicon esculentum* [49,50]. Neem leaf extract has been demonstrated to have a negative effect on cucumber germination and shoot and root growth [51]. Glyphosate is a synthetic chemical compound that belongs to the class of chemicals known as phosphonates. It works by blocking the production of a crucial enzyme, 5-enolpyruvylshikimate-3-phosphate synthase, that plants need for survival and growth [52]. As a result, glyphosate application causes different symptoms in plants, such as chlorosis, yellowing, reduced leaf area and shoot biomass [53]. As steady growth is a basis for the formation of yield in crop plants, the indifference of the growth pattern after the water and caraway extract applications is a positive outcome in the attempt to develop a bioinsecticide.

Another important step in developing a biopesticide is to make sure that the extract does not negatively affect the photosynthesis of the host plant [54]. Chlorophyll fluorescence emitted from the chloroplast thylakoid membrane is a sensitive indicator of the light absorption, conversion and transfer in photosystem II [55]. In this study, none of the 18 studied photosynthesis parameters were significantly affected by caraway extract or commercial biopesticide application in comparison to water treatment. Previously, it has been reported that caraway contains compounds with allelopathic properties, e.g., carvone, carvacrol and thymol [56–58]. However, the concentration of active substances and susceptibility of the host plant have to be taken into account when comparing these findings. The conventional herbicide, which was used as a control, significantly reduced photosynthesis, and the most pronounced reduction observed was in the ratios of variable to minimal fluorescence (F_V/F_0), the quantum yield of energy dissipation (ΦD_0), the maximum quantum efficiency of PSII (F_V/F_M), maximum quantum yield of primary PSII photochemistry (ΦE_0), chlorophyll fluorescence (F_M/F_0) and quantum yield (QY). The glyphosate effect on photosynthesis inhibition is well known and includes chlorophyll degradation (Baylis, 2000), indirectly decreasing chlorophyll synthesis [52], which reduces the minimal (F_0) and maximal (F_M) fluorescence values [59,60], as well as the electron transport rate and increasing photochemical quenching (qP) [61].

It is well known that living plants emit volatile organic compounds that, among many other purposes, serve as insect repellents and attractants. It is also known that plant extracts have repellant and attractant properties, and these extracts can modify the VOCs emitted by the plants they have been sprayed on [4]. Therefore, in the development of biopesticides, especially insect repellents, the interaction of host plant-emitted VOCs and VOCs in the applied extract are of utter importance as they can influence the effectiveness of the treatment. In this study, we focused on the identification of volatiles emitted from cucumber leaves after caraway extract application. The potential biopesticides, as well as commercial herbicides, decreased the C4, C5 and C6 alcohol content in the leaves, e.g., C6 (1-hexanol, hex-3-en-1-ol and cis-3-hexen-1-ol), C5 (2-methyl-1,3-butanediol) and C4 (1-butanol) compared to water (C1). These volatiles, usually called "Green Leaf Volatiles" (GLV), are derived by oxidative cleavage and decarboxylation of linoleic and linolenic fatty acids [62], which in cucumbers is only possible in the LOX (lipoxygenase) pathway [63]. In cucumbers, C6 and C9 volatiles make up most of the characteristic grassy, green fruit scent [38,63]. Therefore, we can conclude that caraway extract application affects the emission of cucumber-characteristic VOCs. GLVs have a dual function in plants—they can attract pests and pollinators, and on the other hand, they can induce plant indirect defense by attracting natural enemies of the pests [64]; the herbicide and biopesticide-induced decrease in GLVs can impact cucumber interaction with insects. A decrease in GLVs has previously been observed in response to the application of chemical herbicides and insecticides (imidacloprid) [64], as well as monoterpenes of plant origin [37].

The largest variation between cucumber VOCs after treatment with different caraway extracts (E1–E4) was in Ethyl-1-propenyl ether (absent in treatment E4), 2-[(2E)-2-pentenyl]furan (absent in treatment E1 and control C2) and n-hexyl acetate (present in E3 and all controls). These differences can be attributed to the concentration of carvone in the caraway extracts. Extract type and content also can impact the release rate of volatile organic compounds [65], which in turn can impact the emissions of plant VOCs due to their interactions [66]. Synergistic or antagonistic effects can also impact the plant VOC emissions, as the tested substances are natural extracts, not pure compounds. In order to evaluate the synergistic or antagonistic effect of aqueous caraway seed extracts, more experiments are needed.

As previously predicted, the glyphosate (C2) and azadirachtin (C3) treatments had the greatest influence on the amount and composition of VOCs in cucumber leaves. Glyphosate-sprayed cucumbers were characterized by significantly higher leaf aldehydes (pentanal and β-cyclocitral), alcohols (2,4-dimethyl-3-hexanol-4), pyrroles (4-ethyl-2-methyl-1H-pyrrole) and sesquiterpenes (hexahydro-farnesyl acetone). These observations can be explained by the fact that lipid peroxidation is impacted by glyphosate, and these changes affect jasmonic acid levels and green leaf volatiles [36]. Among the above-mentioned VOCs, β-cyclocitral emission was significantly increased by glyphosate application. β-cyclocitral is an apocarotenoid of β-carotene that increases plants' resistance against stresses [67]. Either an enzymatic mechanism or direct oxidation of β-carotene by reactive oxygen scavenging (ROS) results in the formation of β-cyclocitral. Azadirachtin application led to higher C6 aldehydes (hexanal and 2-hexanal), C5 alcohol (1-pentanol) and methyl salicylate synthesis in the leaves, pointing to its completely different effect on plant metabolism. According to Gondor, et al., [68], the salicylic acid pathway in plants is used to synthesize the methyl salicylate, which in turn plays an important role in plant signaling in the presence of biotic stressors. The presence of methyl salicylate also appeared when glyphosate was applied, although at lower concentrations.

In this study, we demonstrate that an integrative plant physiological state assessment and chemical analysis of nontargeted volatile organic compound profiles, total phenolic compound content (TPC), total sugars and antiradical activity (ARA) can be applied for rapid elucidation of biosynthetic pathways and disruption of photosystem II in plant extract treated cucumber plants. Therefore, it can be concluded that increased emission of VOCS, such as β-cyclocitral and methyl salicylate, increased synthesis of total sugars

and phenolic compounds, and a decrease in the ratio of variable to minimal fluorescence (F_V/F_0), quantum yield of energy dissipation (ΦD_0), maximum quantum efficiency of PSII (F_V/F_M), maximum quantum yield of primary PSII photochemistry (ΦE_0), chlorophyll fluorescence (F_V/F_0) and quantum yield (QY) can serve as stress response biomarkers in bioinsecticide testing on cucumber plants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13051019/s1, Table S1: Volatile organic compounds (according to Table 4) emitted from fresh cucumber leaves sprayed four caraway extracts (C1–C4) and controls water (C1), glyphosate (C2) and azadirachtin (C3); Table S2: Effect of four caraway extracts (C1–C4) and water (C1), glyphosate (C2) and azadirachtin application on cucumber photosynthetic apparatus characterizing functional parameters ten days after treatment.

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