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The Role of Volatile Substances Emitted by Cultivated Plant's Roots in Indirect Defense Against Soil Herbivores

Žiga Laznik and Stanislav Trdan

Additional information is available at the end of the chapter

Abstract

Plants in nature have developed many defense mechanisms to defend themselves against attacks by harmful organisms; these mechanisms are indirect and direct. When attacked by a harmful organism, many plant species release volatile substances that attract natural enemies of herbivores. Volatile substances have an important role in the tritrophic system consisting of a plant, a herbivore, and its natural enemy. They function as a kind of chemical signal (semiochemical) which directly influences both harmful pests and their natural enemy. Some of these substances appear on damaged as well as undamaged plants, while other substances are released in the case of mechanic damage or feeding of a particular herbivore species. Volatile substances may repel a herbivore. Harmful pests have an important role in attracting natural enemies, as they also emit chemical signals that function as kairomones for natural enemies. In order to increase our knowledge in the field of indirect plant defense we studied chemosensation of four entomopathogenic nematode species (*Steinernema*, *Heterorhabditis*) to compounds released by insect (wireworms and grubs) damaged and undamaged potato and carrot roots, and mechanically damaged maize roots. The aim of our research was (1) to study the effect of different EPN foraging strategies (ambush, intermediate, or cruise) toward the tested volatile compounds, (2) to determine whether chemotaxis is species-specific, and (3) to assess whether the volatile compounds from damaged and undamaged roots have any behavioral effects on the EPNs studied, and (4) if volatile compounds are a part of an indirect plant defense. Our results indicate that all of the tested EPN species exhibited attraction (or repulsion) to volatiles, irrespective of their foraging strategy, and suggest that responses to distinct volatile cues are a species-specific characteristic. These results expand our knowledge of volatile compounds as cues, which may be used by EPNs to find hosts and for other aspects of navigation in soil.

Keywords: Volatile compounds, indirect plant defense, potato, carrot, maize, entomopathogenic nematodes

1. Introduction

Plants have a unique role in food chains on the Earth. Like people and animals, plants also contract different diseases caused by fungi, bacteria, viruses, viroids, and phytoplasma [1]. They are also attacked by different animals (insects, mites, nematodes, snails, rodents, game) which feed on them and procreate on them [2]. Each animal species in nature has a unique role and significance. None of them is harmful per se. In natural biotopes we normally do not distinguish between harmful and useful species. This distinction is characteristic for agrarian biotopes, where animals multiply exceedingly and by feeding on cultivated plants causing economic damage [2]. Plant-damaging species are biotic factors which cause economic damage in agriculture and forestry. Useful organisms (biotic agents) are predators, parasitoids, entomopathogenic nematodes (EPNs), entomopathogenic fungi, bacteria, baculoviruses, which suppress harmful pests, and antagonistic microorganisms, which suppress disease agents [3, 4, 5].

Plants in nature have developed many defense mechanisms to defend themselves against attacks by harmful organisms. These mechanisms are indirect and direct [2, 6]. When attacked by a harmful organism, many plant species release volatile substances that attract natural enemies of herbivores [7, 8, 9, 10]. Volatile substances have an important role in the tritrophic system consisting of a plant, a herbivore, and its natural enemy [11]. They function as a kind of chemical signal (semiochemical) which directly influences both harmful pests and their natural enemy [8, 9, 10]. Some of these substances appear on damaged as well as undamaged plants, while other substances are released in the case of mechanic damage or feeding of a particular herbivore species [10]. Volatile substances may repel a herbivore. Harmful pests have an important role in attracting natural enemies, as they also emit chemical signals that function as kairomones for natural enemies [12].

2. The role of root exudates in rhizosphere

The soil furnishes a living environment to the extremely diverse communities of macro and microorganisms. Likewise, the rhizosphere is the zone of contact in soil surrounding a plant root where biological and chemical parameters of the soil are influenced by the roots. In these niches, complex biological and ecological processes occur [13]. The rhizosphere is a densely populated area in which plant roots must compete with invading root systems of neighboring plants for space, water, and mineral nutrients, and with other soilborne organisms, including insects, bacteria, and fungi [14]. Rhizosphere interactions are based on complex exchanges that evolve around plant roots. Root-based interactions between plants and organisms in the rhizosphere are influenced by edaphic factors [14]. The below-surface biological interactions that are driven by root exudates are more complex than those that occur above the soil surface [15]. These interactions include signal traffic between the roots of competing plants [16], roots, and soil microbes [17], and one-way signals that are dependent on the chemical and physical interactions of the soil with the roots [18].

Unseen part of the plant secretes chemical compounds, which acts as communication signal between the adjacent plant and microbial community present in the rhizosphere of the root. Root exudates correspond to an important source of nutrients for microorganisms in the rhizosphere and seem to participate in early colonization, inducing chemotactic responses of rhizospheric bacteria [19] and other organisms [10]. Root exudates play an active and relatively well-documented role in the regulation of symbiotic and protective interactions with microbes [20]. Through the exudation of a wide variety of compounds, it is suggested that roots can regulate the soil microbial community in their immediate vicinity, withstand herbivory, encourage beneficial symbioses, change the chemical and physical properties of the soil, and inhibit the growth of competing plant species [21].

A survey of the literature exposes an extensive range of compounds exuding from intact and healthy roots; these include sugars, amino acids, peptides, enzymes, vitamins, organic acids, nucleotides, fungal stimulators, inhibitors, and attractants [22]. Organic acids, sugars, amino acids, lipids, coumarins, flavonoids, proteins, enzymes, and aliphatic and aromatic compounds are examples of the primary substances found within the rhizosphere in root. Among these substances, the organic acids have received considerable attention due to their role in providing substrates for microbial metabolism and also for serving as intermediates for biogeochemical reactions in soil [23].

The field of rhizosphere biology has found the relative importance of root exudates in mediating interactions with neighboring plants and microbes [14]. Root exudation is an element of the rhizodeposition process, which is a major source of soil organic carbon released by plant roots [24]. Upon encountering a challenge, roots typically respond by secreting certain small molecules and proteins [25]. Root secretions may play a role in both positive and negative communication in the rhizosphere. The positive communication includes symbiotic associations with beneficial microbes, such as *mycorrhizae, rhizobia,* and plant growth promoting *rhizobacteria* (PGPR). Negative interactions include association with parasitic plants, pathogenic microbes, and invertebrate herbivores. The rhizospheric bacteria are responsible for the elimination of the contaminants, while the roots are responsible for providing nutrients (root exudates) used by the microorganisms to proliferate [26].

3. Factors affecting exudation

The exudation of organic compounds by roots are influenced by either biotic (for example, soil microbial uptake) [27] or abiotic processes [28]. In some instances, our knowledge is sufficient to explain why exudation is affected by the root environment, but often our ignorance of the physiological processes involved in exudation precludes a correct explanation. Some of the factors influencing exudation are listed below.

3.1. Plant species

The amount, range, and balance of compounds in root exudates differ for different plant species. [29] found differences between wheat and barley (*Hordeum vulgare* L.) root exudates

with respect to certain sugars (galactose, glucose, and rhamnose), whereas other sugars occurred in similar amounts in exudates of both plants. The specificity of root exudates from different plants in stimulating only certain groups of organisms is clearly demonstrated in the plant pathology literature, for example, the cysts of potato eelworm (*Heterodera rostochiensis*) hatched when supplied the root washings of potato (*Solanum tuberosum* L.), tomato, and some other solanaceous plants, but not the washings of beet (*Beta vulgaris* L.), rape (*Brassica napus* L.), lupin (*Lupinus lilosus* L.), mustard (*Brassica* sp.), or oats [30].

3.2. Root age

The research performed with peas and oats indicated that more number of amino acids and sugars exude during the first 10 days of growth than those during the second 10 days [31]. Another study [32] found 3-pyrazolylalanine in root exudate of cucumber (*Cucumis sativus* L.) only at the early seeding stage. In tomato and red pepper (*Capsicum anznumm* L.), they detected tyrosine in the exudate only at fruiting, but not at any other stages of growth.

3.3. Temperature

The release of amino acids, especially asparagine, from roots of tomato and subterranean clover (*Trifolium subterraneum L.*) increased with rise in temperature [31]. However, this effect is not universal, as some researchers reported more amino acids in exudates from strawberry plants (*Fragaria vesca* L.) grown at 5–10°C than that at 20–30°C; this markedly influenced the pathogenicity of pathogens that attack strawberries at low soil temperatures [33].

3.4. Microorganisms

Microorganisms may affect the permeability of root cells, metabolism of roots, and absorption and excretion of certain compounds in root exudates. It was reported that filtrates of cultures of some bacteria and fungi and also some antibiotics (penicillin), increased the exudation of scopoletin (6 methoxy -7 hydroxycoumarin) by oat roots [34]. It was found that certain polypeptide antibiotics, for example, polymyxin, produced by *Bacillus polymyxa* from soil, altered cell permeability and increased leakage [35]. There are two key factors in interpreting the significance of these results which show that culture filtrates or products increase the leakiness of plant roots. First, the conditions under which the organisms are grown are quite different both physically and nutritionally from those under which a rhizosphere population grows. Second, since it is not possible to calculate the concentration of biologically active substances in the rhizosphere, the concentrations used for "*in vitro*" experiments are selected rather arbitrarily. Moreover, any consideration of the significance of the rhizosphere population in altering exudation must involve the concept of microecology with a wide variety of organisms occupying different "niches" on the roots and only those plant cells in the immediate vicinity of "exudation-promoting" organisms are likely to be affected. Microorganisms also influenced the exudation of organic materials into soil. A supplementary study showed that the exudation from wheat roots into synthetic soil was increased at least fourfold by microorganisms [35]. The magnitude of the effects of microorganisms upon exudation no doubt will depend on the species colonizing the roots [36]. Some other plant biotic factors like develop-

mental status, shoot herbivory, photosynthesis, supply of carbon from shoot to root, evaporation, transpiration, nutrient deficiency, root architecture, cytosolic concentration, membrane permeability, membrane electrochemical potential, release of microbial signal, allelochemical release, mychorrhizas, nodulation, and some soil biotic factors are also influenced by the root exudation.

3.5. Light

The light intensity at which plants are growing affects the amounts and balance of compounds exuded into nutrient solution by tomato and subterranean clover roots [31]. Clover grown at full daylight intensity exuded more serine, glutamic acid, and c-alanine than plants grown in 60% shade. With tomato, the levels of aspartic acid, glutamic acids, phenylalanine, and leucine in exudate were reduced by shading. Beside these abiotic factors, few others such as moisture, humidity, wind speed and light intensity, elevated CO_2 pesticides, available space, atmosphereic nitrogen deposition, ozone, physical disturbance, fire, irrigation, erosion, altitude, and latitude also influence the exudation [37]. Some soil abiotic factors resembling compaction, soil type, salinity, soil pH, metal toxicity, water availability, organic matter, cation and anion exchange, drainage, aeration, rooting depth, soil texture, soil structure, and redox-potential influence the release of organic chemical from plant root [38].

3.6. Root-feeding insects

Plants in nature are exposed to attacks by insects which bite and suck plants' parts and thus diminish their vitality. Root-feeding insects play an important role in both agricultural and natural ecosystems [39]. In response to attacks by herbivores, plants excrete terpenes and monoterpenes [40]. So far it has not been established if excretion of volatile substances from damaged plants is due exclusively to attacks by insects or if these substances are stored in plant cells and are excreted only when a plant is in physiological stress [41]. Plants have the so-called morphological defense mechanisms (presence of prickles, thorns, hairs, enzymes, and secondary metabolites), whose presence is not conditioned by attacks of herbivore organisms. Besides morphological defense mechanisms, there are also induced defense mechanisms, which manifest as plants' reaction to attacks of herbivores. Induced defense mechanisms can be further divided into direct defense mechanisms (secretion of secondary metabolites as a response to attacks by insects) and indirect defense mechanisms (secretion of the [VOCs] VOCs, which attracts natural enemies of herbivore organisms) [6, 42].

Plants react to different types of injuries (mechanical, herbivorous) by excreting different volatile chemical substances, which can be specific also for the insect species attacking a plant [43]. Many studies have shown differences in excretion of volatile compounds from plants which were attacked by different insect species [8, 40, 44]. Simultaneous feeding of different herbivore organisms on a host plant is a very frequent phenomenon in nature [45], which can influence the success of natural enemies in finding their prey [46].

VOCs have been commonly identified as arthropod attractants belowground. [47] highlighted different compounds that are used by herbivores to locate the food source. One of the most

important signals in the soils are the emissions of CO_2 by roots [48]. [48] reported that detection of CO_2 seems to be dose-dependent, and soil insect are able to detect very small differences in the concentration of CO_2. Besides CO_2, plants emit various volatile compounds upon herbivore attack. The study of [49] investigated on-line VOC emissions by roots of *Brassica nigra* plants under attack by cabbage root fly larvae, *Delia radicum*. The investigation showed that several sulfur-containing compounds, such as methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl trisulfide and glucosinolate breakdown products such as thiocyanates and isothiocyanates, were emitted by the roots in response to infestation [49]. [50] reported that fatty acids in oaks (*Quercus* sp.) and monoterpenes in carrot (*Daucus carotta* ssp. *sativus*), and potato (*Solanum tuberosum*) plants triggered the attraction of forest cockchafer larvae (*Melolontha hippocastani*) and wireworms (*Agriotes* spp.). Volatiles of fresh perennial ryegrass roots attracted larvae of *Costelytra zealandica* [51], and roots of *Medicago sativa* and *Trifolium pratense* attracted larvae of *Sitona hispidulus* [52]. Furthermore, [8] reported that maize (*Zea mays*) roots release ß-caryophyllene in response to feeding by larvae of the beetle *Diabrotica virgifera virgifera*. In a related research, [10] reported that mechanically damaged maize roots release linalool, ß-caryophyllene, and α-caryophyllene.

4. The role of root exudates on beneficial soil organisms – Indirect defense against soil herbivores

4.1. Entomopathogenic Nematodes (EPNs)

Tritrophic interactions, which include a host plant, a harmful organism and its natural enemy, have been documented only recently for the underground parts of a plant. Some studies have shown that damaged roots of different plant species release into environment VOCs which can influence the movement of EPNs both as attractans [8, 9, 10, 44] and as repellents [53].

Soil is the natural habitat of EPNs (Steinernematidae and Heterorhabditidae) (Figure 1), and their application in pest management has been primarily used against soil-inhabiting insect pests [54]. EPNs are lethal pathogens of insects. These pathogens contribute to the regulation of natural populations of insects, but the main interest in them is an inundatively applied biocontrol agent [55]. Their success in this role can be attributed to the unique partnership between a host-seeking nematode and a lethal insect-pathogenic bacterium. Because of their biocontrol potential, considerable attention has been directed over the past few decades to genus, *Heterorhabditis* and *Steinernema* and their respective bacterial partners, *Photorhabdus* and *Xenorhabdus*.

Although heterorhabditids and steinernematids are not closely related [56], they share many features in common. These similarities, including their association with insect-pathogenic bacteria, are presumed to have arisen through convergent evolution [57]. In both *Steinernema* and *Heterorhabditis*, there is a single free-living stage, the infective juvenile (IJ) that carries in its gut, bacteria of the genus *Xenorhabdus* and *Photorhabdus* [58]. On encountering a suitable insect, the IJ enters through the mouth, anus, or spiracles and makes its way to the haemocoel [59]. Some species may also penetrate through the intersegmental membranes of the insect

Figure 1. Infective juveniles of entomopathogenic nematode *Steinernema feltiae* (photo: J. Rupnik)

cuticle [60]. In the haemocoel, the IJ releases cells of its bacterial symbiont from its intestine. Bacteria multiply rapidly in haemolymph and produce toxins and other secondary metabolites, which contribute to the weakening of the host's defense mechanism. The host attacked by EPNs usually dies because of poisoning or failure of certain organs in 24–72 hours after the infection [61]. Two developmental cycles thus occur in the host – one of nematodes and the other of bacteria. The first-generation nematodes pass into the second generation. After the larvae cast off the fourth sheath and enter into the adult period, nematodes pass into the third generation, which thrives in the host as long as there is availability of food. The host is by then already dead – being killed by the toxins secreted by bacteria. The third-generation nematodes are thus already saprophagic [62]. Bacteria also produce toxins, such as 3,5 *dihydroxy-4-isopropyl-stilben*, which deter other microorganisms from settling in the carcass [63]. When the developmental cycle is finished, nematodes leave the parts of carcasses that have not decomposed, and return to the ground. Nematodes cannot develop without a host (an insect) [64], without which they survive in the ground for only a very brief period of time [65].

The importance of EPNs and biological plant protection against harmful organisms was first established in the USA in the 1930s. In 1923, Glaser and Fox discovered a nematode which attacked and caused death of the beetle, *Popillia japonica* Newman [66]. Glaser introduced a method of growing EPNs *in vitro*. With such nematodes, he, in 1939, carried out the first field experiment in New Jersey to suppress the species, *P. japonica* [67].

When EPNs were first discovered, a hypothesis was proposed that nematodes alone cause death of the insects being attacked. In 1937, Bovien first hypothesized the possibility of the

existence of symbiotic bacteria that live with EPNs in a mutualistic relationship. His hypothesis was, in 1955, confirmed by Dutky and Weiser [68]. However in 1982, Boemare proved that nematodes from the genus *Steinernema* produce toxic substances which negatively influence the immune system of infected insects and can themselves alone – without the presence of symbiotic bacteria – cause death of the host. For EPNs from the genus *Heterorhabditis*, it has not yet been established that they can alone produce toxic substances that would diminish the vitality of infected insects [69].

The use of EPNs in biological plant protection was until some years ago still traditionally connected with suppressing soil-inhabiting insect pests [70]. The research results in the last two decades indicate they have also potential to suppress aboveground insect pests, but only in certain circumstances [71, 72]. Lesser efficiency of EPNs in suppression of aboveground insect pests is primarily due to inappropriate (insufficient) moisture [73], exposure to thermal extremes [74], and ultraviolet radiation [75]. These factors are of crucial importance for the survival of nematodes [65]. For this reason nematodes are less efficient against aboveground insect pests outdoors, though the previous laboratory tests showed much higher efficiency [76].

To lay nematodes on plants, equipments intended for spraying plant protection products, manuring, or irrigation can be used. *Backpack manual or tractor sprayers*, sprinklers, and also planes are suitable for this purpose. IJs can be passed through spray tubes with diameter of at least 500 μm, capable to withstand pressure up to 2000 kPa [77].

IJs can tolerate short-term exposure (2–24 hours) to many chemical and biological insecticides, fungicides, herbicides, fertilizers, and growth regulators and can thus be tank-mixed and applied together [78, 79, 80, 81]. Nematode–chemical combinations in tank-mixes could offer a cost-effective alternative to foliar integrated pest management (IPM) systems.

Due to the sensitivity of nematodes to ultraviolet radiation, nematodes have to be applied to plants in the evening, early in the morning, or during a cloudy weather, when the radiation is not so intense [73]. Nematode survival and efficacy on foliage has also been shown to be enhanced to varying degrees by addition of various adjuvants to the spray mixture, which have antidesiccant (e.g., glycerol, various polymers) or UV-protective (brighteners) properties [82], although additional measures are required to enhance post-application survival. The greatest potential for using EPNs against foliar pests is almost certain in IPM programs, in conjunction with other biocontrol agents [83] or selective chemicals [78, 84].

EPNs are considered exceptionally safe biological agents [85]. Because their activity is specific, their environmental risk is considerably lower than that of chemical agents for plant protection [86]. Since the first use of EPNs for suppressing beetles of the species *P. japonica* in the USA [66], until now, no case of environmental damage due to these biological agents has been documented. The use of nematodes is safe for users. EPNs and their bacteria are not harmful for mammals and plants [87].

4.2. Movement of EPNs

The ability of EPN IJs to disperse actively through soil and locate a host is a key element for the successful application of some EPN species in pest management [88]. When an EPN locates its host, it can enter it through natural openings. By excreting its symbiotic bacteria, which

release toxins into the host's body, it causes the death of the insect in 24–72 hours after the infection [55].

EPNs have through the evolution developed different ways of searching for hosts, which is a species-specific characteristic [89, 90, 91]. The species *Heterorhabditis bacteriophora* and *Steinernema kraussei* actively search for hosts (cruisers). Some species of EPNs wait for a host in an ambush (ambushers). The passive way of searching for a host is characteristic for the species *S. carpocapsae*. Some species (*S. feltiae*) combine both ways of searching for a host and are categorized as intermediates [89, 90, 91].

EPN species respond distinctly to cues associated with hosts (insects) or plants, depending primarily on their foraging strategy [89]. The cruisers spend most of their time searching for resource-associated cues as they move through their environment [89, 91]. In contrast, ambushers do not respond as strongly as cruisers and spend little time actively moving and searching for volatile cues. Ambushers are thought to wait for resources to come to them [10, 89]. Several EPN species adopt both (cruise and ambush) foraging strategies and are classified as intermediates [89].

EPNs uses chemosensation to find host, avoid noxious conditions, develop appropriately, and mate. Several authors report that IJs respond to CO_2 [89], temperature, changes in pH, bacterial symbionts [92], electrical field [93], and different plant VOCs [8, 9, 10, 44].

4.3. Indirect defense against soil herbivores

4.3.1. Potato

Here, we describe our study of the chemotactic behavior of *Steinernema feltiae* (Filipjev), *Steinernema carpocapsae* Weiser, *Steinernema kraussei* (Steiner), and *Heterorhabditis bacteriophora* Poinar toward Decanal; Nonanal; Octanal; Undecane; 1,2,4-trimethylbenzene; 2-ethyl-1-hexanol; and 6-methyl-5-hepten-2-one; compounds released from insect (*M. hippocastani* grubs) damaged and undamaged potato tubers (*S. tuberosum*) [50]. The aims of our research were (1) to study the effect of different EPN foraging strategies (ambush, intermediate, or cruise) toward the tested VOCs, (2) to determine whether chemotaxis is species-specific, (3) to assess whether the VOCs from damaged potato tubers have any behavioral effects on the EPNs studied, and (4) if VOCs are a part of an indirect plant defense.

The results of our research showed that the movement of EPNs was conditioned by the type of VOCs excreted by damaged/undamaged potato tubers (see Figures 2 and 3). VOCs Nonanal, Octanal, and Decanal proved to have a greater influence on the movement of EPNs as other tested volatiles in our investigation. Nonanal and Decanal are among other indicator substances for degradation processes [50]. Decanal is also described to be induced by mechanical and herbivore damage [46, 94, 95]. [50] reports that damaged potato tubers excrete the substances Nonanal, Octanal, and Decanal. The results of our research showed that the said substances acted as attractants in regard to the movement of EPNs. Decanal in our experiment proved as an attractant for the species *H. bacteriophora* and *S. kraussei* at both studied concentrations (pure concentration and 0.03 ppm concentration) (see Figures 2 and 3). Octanal proved an attractant

for the species *H. bacteriophora* and as a weak attractant for the species *S. carpocapsae*. Nonanal proved to be a weak attractant for the species *S. carpocapsae*. Thus we confirm the thesis that damaged plant roots release into the environment substances that influence the movement of beneficial organisms – indirect plant defense.

In our investigation two distinct VOC concentrations were used. A pure concentration, which does not reflect a concentration found near plant roots [96], had a bigger influence on IJ movement than a concentration of 0.03 ppm, which is the average concentration of volatile compounds found in soil, 10 cm away from the root system [12]. In our experiment, the difference in responsiveness of EPNs in regard to the concentration of VOC was most substantially expressed in the case of the chemical substance Undecane. At pure concentration, the said substance proved to be an attractant for the species *S. kraussei* and as a repellent for the species *S. feltiae*. At the concentration 0.03 ppm, the said substance did not have any influence on the movement of EPNs in our experiment (see Figures 2 and 3). We also found out that the duration of exposure of an EPN to VOCs is of key importance for perceiving chemical stimuli. After 24 hours we detected the movement of EPN in 32%, while the movement after 2 hours was detected only in 3% (see Figures 2 and 3). Similar findings were produced by our earlier research [10].

The results of our research showed that the movement of EPNs toward the selected VOC is substantially determined also by their foraging strategy. In regard to the way of searching the host EPNs fall into three types. Cruisers (*H. bacteriophora* and *S. kraussei*) actively move toward their prey by perceiving stimuli from the environment [97], while the so-called ambushers (*S. carpocapsae*) wait for their prey in an ambush [90]. Some species (*S. feltiae*) combine both ways of searching for the host and are classified as the so-called intermediates [89]. The VOC Decanal in our experiment proved to be an attractant for the species *H. bacteriophora* and *S. kraussei* (see Figures 2 and 3), which are classified as the so-called cruisers. We also found out that the movement of the nematodes classified as cruisers and intermediates was more pronounced than in the species *S. carpocapsae*, which proved to be the least mobile species of EPNs in our research. [98] says that the movement of cruisers at longer distances is conditioned by perceiving chemical stimuli, which, however, is not characteristic for the nematodes classified as ambushers. In some related studies the species *H. bacteriophora* proved to be very susceptible to perceive chemical stimuli from the environment [97, 99]. This was also confirmed in our research for the substances Decanal and Octanal, which affected the said species as attractants. The ambusher *S. carpocapsae* in comparison with other studied species in our experiment displayed a high degree of susceptibility to the VOC 6-Methyl-5-hepten-2-one. On the basis of some of our earlier research [10] and the current one, we conclude that the movement of EPNs toward the selected VOC is influenced primarily by the species and not so much by the way of searching the host. Our hypothesis is confirmed with the fact that Octanal acted as an attractant for the nematode *H. bacteriophora*, while the nematode *S. kraussei*, which is also classified as a cruiser, was not affected by it. Similar conclusions were reached also in the study by [91] who studied the reaction of EPNs on damaged citrus roots. Susceptibility to perceiving chemical stimuli from the environment is a species-specific characteristic prevailing over the foraging strategy [10].
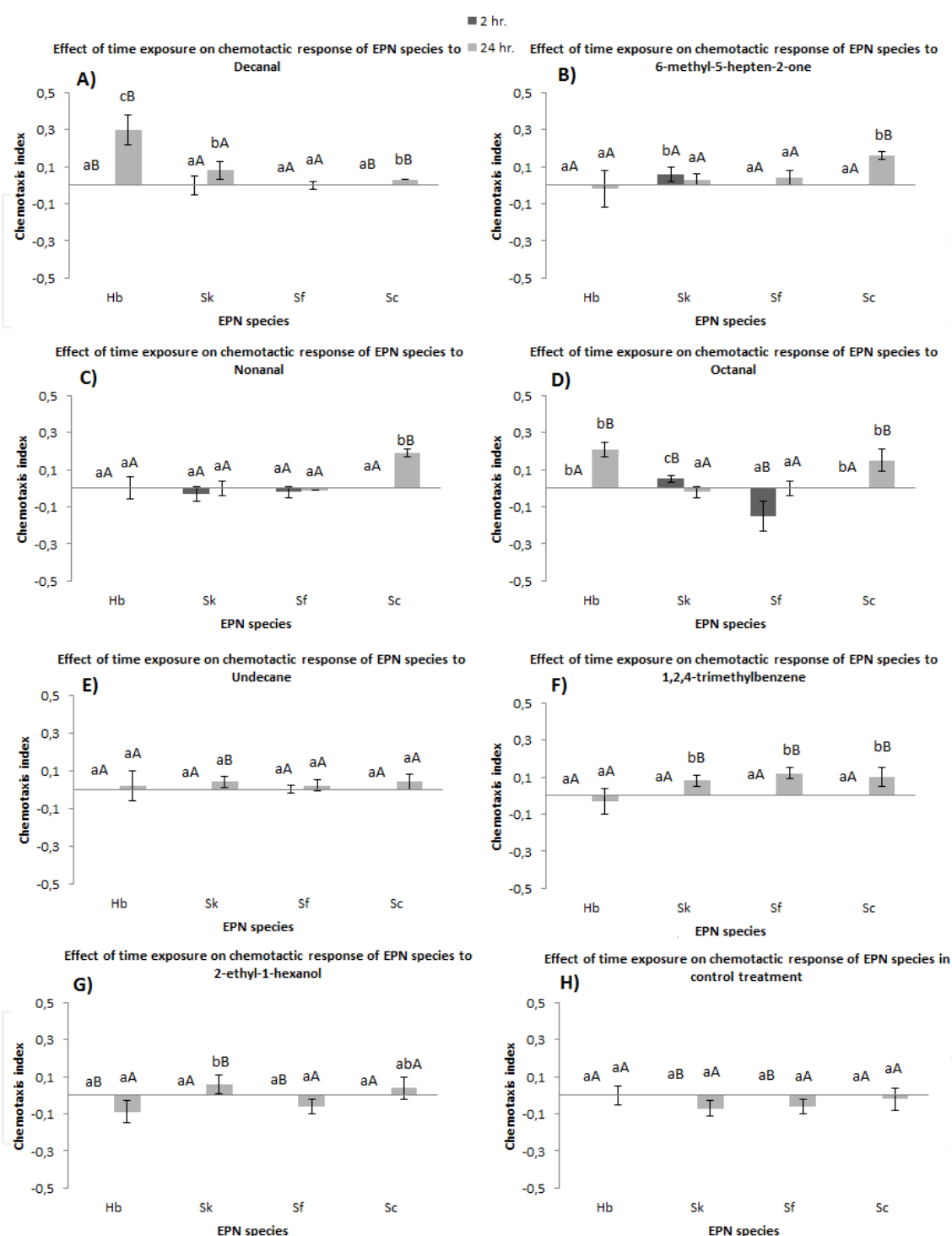
Figure 2. Effects of time of exposure to VOCs on the chemotactic response of EPN species (A-G), at a concentration of 0.03 ppm. Each data point represents the mean chemotaxis index ± S.E. Bars with the same letter are not significantly different (P>0.05). The small letters indicate statistically significant differences among different EPN species with the same time of exposure. The capital letters indicate statistically significant differences among different times of exposure within the same EPN species. Hb – *H. bacteriophora*; Sk – *S. kraussei*; Sf – *S. feltiae*; Sc – *S. carpocapsae*. The substances in our research were with the chemotaxis indexes divided into the following intervals: >0.2 (attractant); from 0.2 to 0.1 (weak attractant); from 0.1 to -0.1 (no effect); from -0.1 to -0.2 (weak repellent); < -0.2 (repellent) [10]
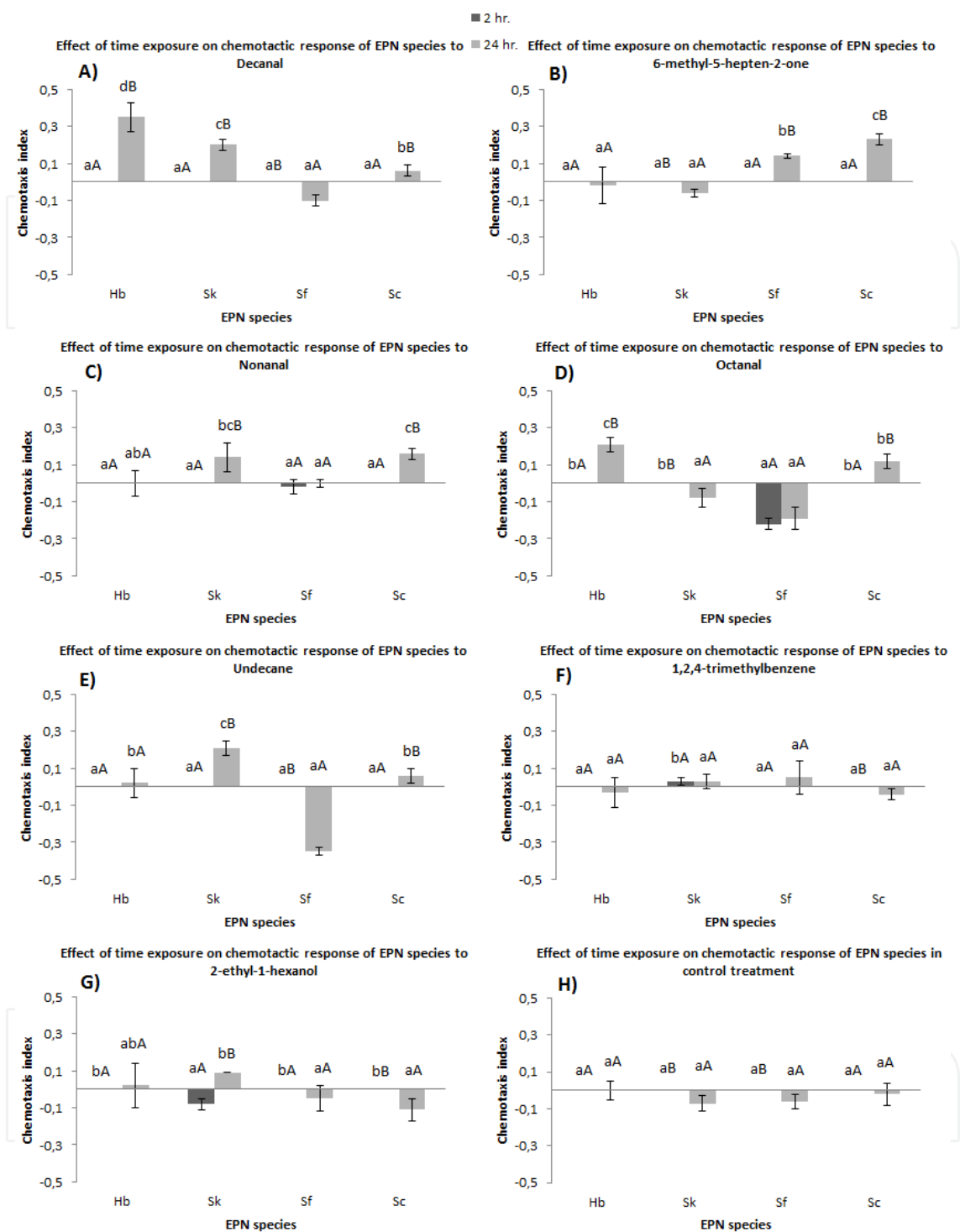
Figure 3. Effects of time of exposure to VOCs on the chemotactic response of the EPN species (A-G), at pure concentration. Each data point represents the mean chemotaxis index ± S.E. Bars with the same letter are not significantly different (P>0.05). The small letters indicate statistically significant differences among different EPN species at the same time of exposure. The capital letters indicate statistically significant differences among different times of exposure within the same EPN species. Hb – *H. bacteriophora*; Sk – *S. kraussei*; Sf – *S. feltiae*; Sc – *S. carpocapsae*. The substances in our research were with the chemotaxis indexes divided into the following intervals: >0.2 (attractant); from 0.2 to 0.1 (weak attractant); from 0.1 to -0.1 (no effect); from -0.1 to -0.2 (weak repellent); < -0.2 (repellent) [10]

4.3.2. Carrot

Here, we describe our study of the chemotactic behavior of *S. feltiae*, *S. carpocapsae*, *S. kraussei*, and *H. bacteriophora* toward α-Pinene, Bornyl acetate, Borneol, 2,4-Di-tetra-butylphenol, 2-Ethyl-hexanol, and Terpinolene; compounds released from insect (wireworms and grubs) damaged carrot (*Daucus carota* ssp. *sativus*) roots [50, 100]. The aims of our research were (1) to study the effect of different EPN foraging strategies (ambush, intermediate, or cruise) toward the tested VOCs, (2) to determine whether chemotaxis is species-specific (3) to assess whether the VOCs from damaged and undamaged carrot roots have any behavioral effects on the EPNs studied, and (4) if VOCs are a part of an indirect plant defense.

Our results show that the chemosensation of IJs toward and away from insect-induced carrot root volatile compounds [50, 100] varied depending on the EPN species, VOC, concentration of VOC, time of exposure and interaction between EPN species and time of exposure (Figures 4 and 5). Our results indicate that all tested EPN species exhibited attraction (or repulsion) to volatiles irrespective of their foraging strategy (in our investigation, terpinolene was a repellent for EPN species classified in all three foraging groups) (Figures 4 and 5). Similar conclusions were also reported in recent research from [91] in which a cruiser *H. indica* [89], ambusher *S. carpocapsae* [89], and two other species thought to exhibit an intermediate foraging strategy [89] were all attracted to root weevil *Diaprepes abbreviatus*-damaged roots of the Swingle rootstock. Furthermore, [10] reported that responses to different volatile cues are a strain-specific characteristic rather than a different host-searching strategy. Similar conclusions were also made by [9, 91]. Our current results suggest that responsiveness to different volatile cues is a species-specific characteristic.

In our investigation two distinct VOC concentrations were used. A pure concentration, which does not reflect a concentration found near plant roots [96], had a bigger influence on IJ movement than a concentration of 0.03 ppm, which is the average concentration of VOCs found in soil, 10 cm away from the root system) [12]. However, we are aware that such laboratory studies do not reflect a nematode's true behavior in nature because of exposure to different conflicting chemical signals [44, 101].

Plant roots emit an incredible variety of compounds, which are known to affect interactions between plants and other organisms [11]. The active role plants play in recruiting natural enemies, like belowground herbivores, has been recently demonstrated in a few plant species [8, 10, 88, 96, 102, 103]. EPN host finding is mediated by both long-range cues that facilitate root zone finding, as well as shorter-range cues that facilitate host localization within the root zone [8, 63, 91, 102]. Recently, [53] reported positive chemotaxis of the two EPN species *H. bacteriophora* and *S. carpocapsae* to several VOCs such as methyl salicylate, hexanol, heptanol, undecyl acetate, and 4,5-dimethylthiazole. Interestingly, they showed that several volatiles repelled the nematodes. Similar effects of VOCs on the behavior of EPNs were also observed in our investigation (see Figures 4 and 5). Terpinolene repelled both *Steinernema* and *Heterorhabditis* species in our investigation. [100] reported that terpinolene is a VOC released from the undamaged roots of cultivated carrots. Our results suggest that healthy plant roots release specific VOCs into the soil, which signal to natural insect enemies (EPNs) to keep away. Our findings could support the theory of [91]. [91] suggest that selection of a herbivore-induced signaling response should be directionally stronger toward channeling resources for produc-

tion of a distress signal only when necessary because a constant release would likely carry a high physiological cost [104, 105]. Our conclusion is also supported by the VOC α-pinene (released from undamaged carrot roots) [100], which was a weak repellent of *S. carpocapsae* and *S. kraussei*. The other tested VOCs in our investigation (Bornyl acetate, Borneol, 2,4-Di-tetra-butylphenol, and 2-Ethyl-hexanol) acted inconsistently (as a weak repellents or weak attractants) (see Figures 4 and 5).
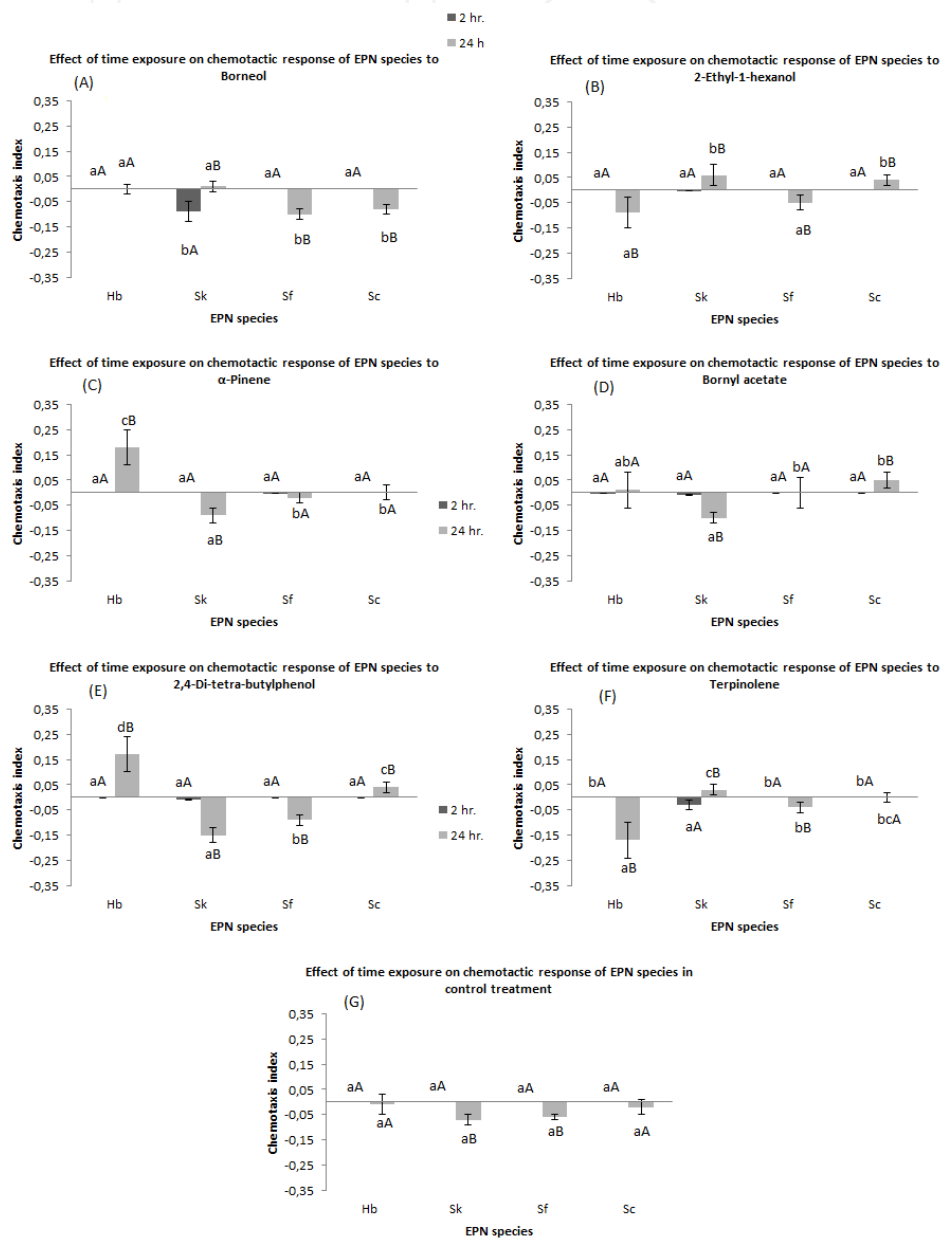

Figure 4. Effects of time of exposure to VOCs on the chemotactic response of EPN species (A-F), at a concentration of 0.03 ppm. Each data point represents the mean chemotaxis index ± S.E. Bars with the same letter are not significantly different (P>0.05). The small letters indicate statistically significant differences among different EPN species with the same time of exposure. The capital letters indicate statistically significant differences among different times of exposure within the same EPN species. Hb – *H. bacteriophora*; Sk – *S. kraussei*; Sf – *S. feltiae*; Sc – *S. carpocapsae*. The substances in our research were with the chemotaxis indexes divided into the following intervals: >0.2 (attractant); from 0.2 to 0.1 (weak attractant); from 0.1 to -0.1 (no effect); from -0.1 to -0.2 (weak repellent); < -0.2 (repellent) [10]
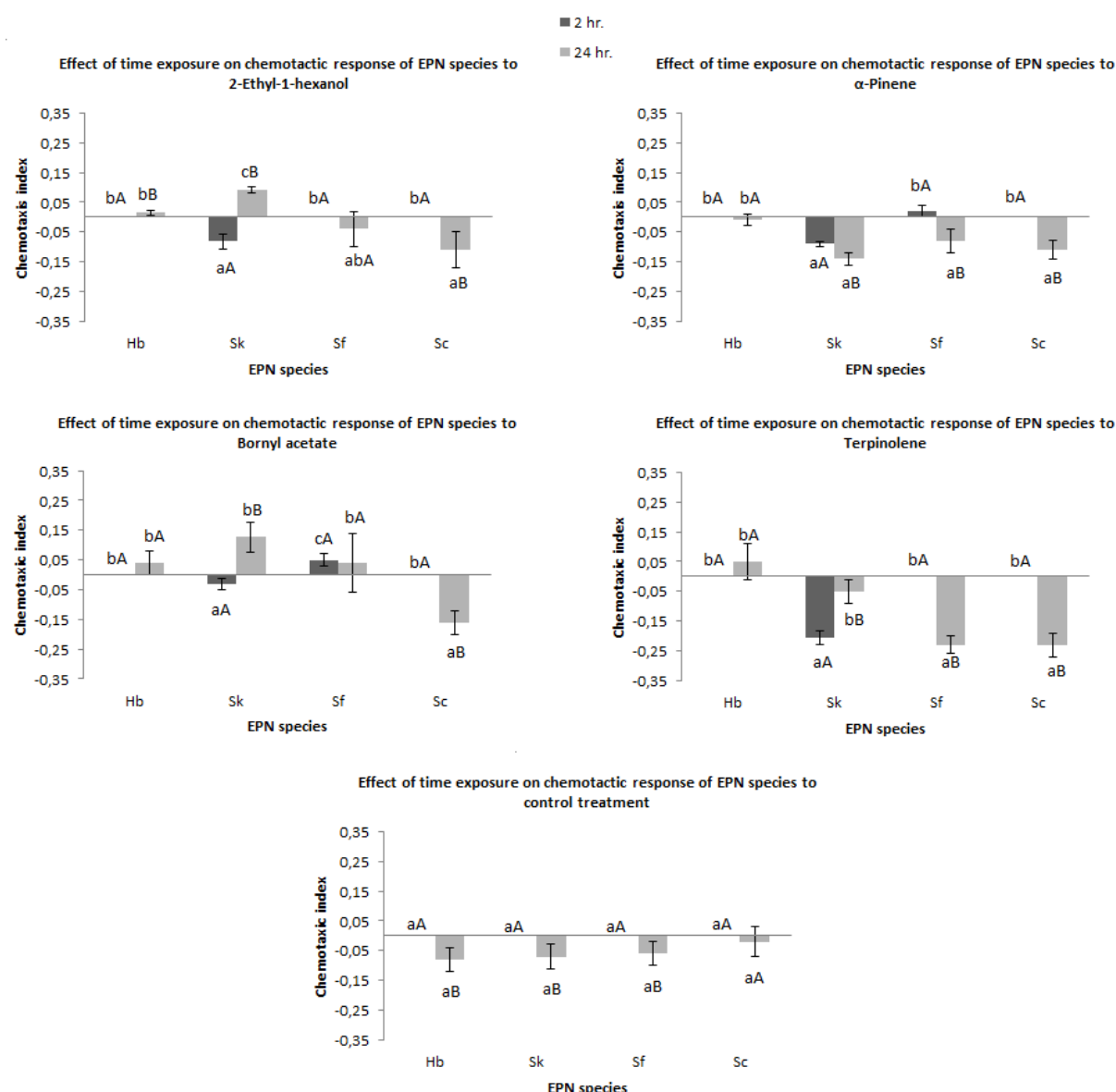
Figure 5. Effects of time of exposure to VOCs on the chemotactic response of the EPN species (A-F), at pure concentration. Each data point represents the mean chemotaxis index ± S.E. Bars with the same letter are not significantly different (P>0.05). The small letters indicate statistically significant differences among different EPN species at the same time of exposure. The capital letters indicate statistically significant differences among different times of exposure within the same EPN species. Hb – *H. bacteriophora*; Sk – *S. kraussei*; Sf – *S. feltiae*; Sc – *S. carpocapsae*. The substances in our research were with the chemotaxis indexes divided into the following intervals: >0.2 (attractant); from 0.2 to 0.1 (weak attractant); from 0.1 to -0.1 (no effect); from -0.1 to -0.2 (weak repellent); < -0.2 (repellent) [10]

4.3.3. Maize

Here, we describe our study [10] of the chemotactic behavior of *S. feltiae* (strain B30, strain C76, and strain 3162), *S. carpocapsae* (strain B49, strain C67, and strain C101), *S. kraussei* (strain C46), and *H. bacteriophora* (strain D54) toward linalool, α-caryophyllene, and ß-caryophyllene, compounds released from the mechanically damaged root systems of different *Zea mays*

hybrids [106]. In a related study, [9] reported that mechanically damaged citrus roots attracted less nematodes than insect-damaged roots. The aims of our research were (1) to study the effect of different foraging strategies (ambush, intermediate, or cruise) of EPNs to the tested VOCs, (2) to determine whether chemotaxis is species- and strain-specific, and (3) to assess whether the VOCs from mechanically damaged maize roots have any behavioral effect on the studied EPNs.

The results of our current laboratory investigation showed that the movement and chemo-sensation of IJs toward and away from damaged maize root VOCs [106] varied depending on the species, strain, foraging strategy, VOC, and interaction between the EPN strain and volatile compound (see Figure 6). The intermediate foragers (*S. feltiae*) proved to be less active in their movement toward the VOCs in comparison with the ambushers (*S. carpocapsae*) and cruisers (*S. kraussei* and *H. bacteriophora*); ß-caryophyllene proved to be the most attractive compound of the three substances tested in our experiment (see Figure 6). The results of our investigation showed that the cruisers were more attracted to β-caryophyllene than the ambushers and intermediates. The foraging strategy did not influence the IJ movement toward the other tested volatile compounds and the control (see Figure 6). Similar conclusions were also reported in the recent research of [91] in which the ambusher *S. carpocapsae* [89], the cruiser *H. indica* [89], and two species thought to exhibit an intermediate foraging strategy [89] were all attracted to *Diaprepes abbreviatus*-damaged roots of Swingle rootstock. Some related studies on the foraging strategies of EPNs have been conducted in nonsoil systems [107]; however, we are aware that such studies do not reflect the nematode's true behavior in nature, whereby they are exposed to a myriad of conflicting chemical signals [44, 63]. In our experiment, pure compounds were applied to agar [107], which does not reflect the concentration near the roots of plants [96]. [96] reported that the total sesquiterpene hydrocarbon content in the herbivore-damaged roots of *Zea mays* was 81 ng g^{-1}, whereas the control plants contained only 25 ng g^{-1}, and the relative amount of ß-caryophyllene among several other different terpenes in the maize roots was less than 5%. Moreover, [9] reported that roots damaged by insect larvae attracted more nematodes than mechanically damaged roots and sand controls. The speed of the nematode's response to the chemical stimuli in its natural environment largely depends on the diffusion rate of the chemical compound and on the soil structural heterogeneity [108]. When a foraging nematode is confronted with an array of signals originating from the same general area, the response may depend on the strength and exposure time and on the nature of the stimuli [63].

[89] reviewed the literature on foraging and host recognition in *Heterorhabditis* and *Steinerne-ma* IJs and proposed that ambusher nematodes respond to host (insect) cues in a hierarchical order, with the volatile cues only becoming important after the IJ had made contact with the insect cuticle, whereas remote volatile cues are more important for cruiser nematodes. Several related studies have also shown that IJs exhibit a preference for different volatile root com-pounds [8, 9, 63, 91]. ß-caryophyllene is a common compound and has been identified from various plant species [8, 44, 106]; however, its function, as for most plant volatiles, remains unclear. As [8] reported that ß-caryophyllene strongly attracted *H. megidis*, attraction has been confirmed for all of the tested species, with the exception of *S. feltiae* (see Figure 6).
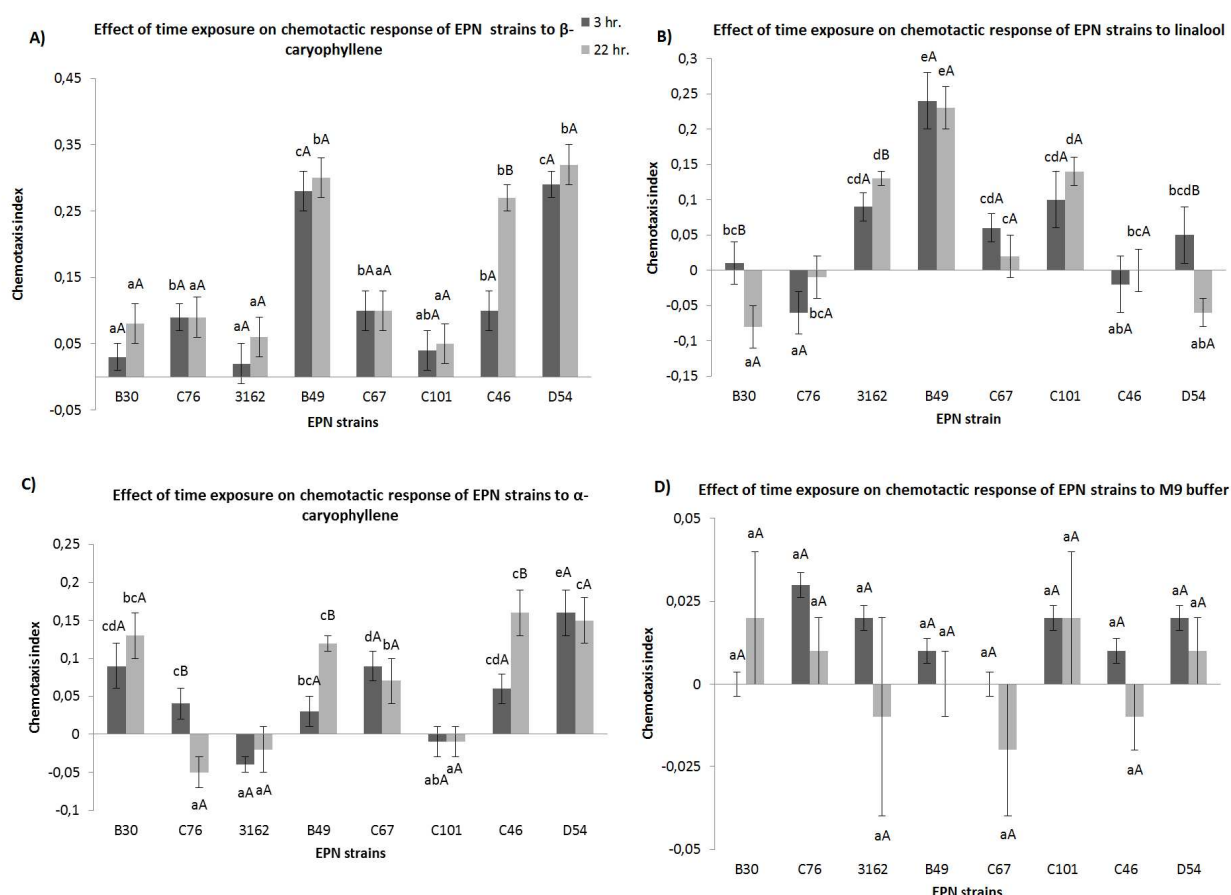
Figure 6. Effects of time of exposure to VOCs on the chemotactic response of the EPN species (A-C), at pure concentration. Each data point represents the mean chemotaxis index ± S.E. The bars with the same letter are not significantly different (P>0.05). The small letters indicate statistical significant differences among the different EPN strains at the same time of the exposure. The capital letters indicate statistically significant differences among the different times of exposure for the same EPN strain. B30, C76, and 3162 = *S.feltiae*; B49, C67, and C101 = *S. carpocapsae*; C46 = *S. kraussei*; D54 = *H. bacteriophora*. The substances in our research were with the chemotaxis indexes divided into the following intervals: >0.2 (attractant); from 0.2 to 0.1 (weak attractant); from 0.1 to -0.1 (no effect); from -0.1 to -0.2 (weak repellent); <-0.2 (repellent) [10]

Our results suggest that the response to different volatile cues is more a strain-specific characteristic than a different host-searching strategy. Similar conclusions were also made in the research of [9, 91]. Indeed, *H. bacteriophora* and *S. carpocapsae* strain B49 showed strong chemotaxis to ß-caryophyllene, whereas the other two isolates of *S. carpocapsae* hardly reacted (see Figure 6). A similar conclusion can be made with regard to linalool, with only *S. carpocapsae* strain B49 showing an attraction to this volatile compound from damaged maize roots (see Figure 6). One reason for the attraction of *S. carpocapsae* strain B49 to linalool and ß-caryophyllene may relate to its origin, as this strain was isolated in a grassland near a maize field [109], supporting the theory of [110] who concluded the possible genetic adaptation of EPNs to different biotic and abiotic factors. In related work, [111] reported that specialization rather than the foraging strategy may better explain the attraction of EPNs to different VOCs. The EPN strains in our experiment showed only a weak attraction to α-caryophyllene, suggesting that this compound could not have an important role in the orientation of IJs to the

damaged roots of maize plants (see Figure 6). *S. kraussei* showed a retarded reaction to both ß-caryophyllene and α-caryophyllene in our experiment, suggesting a different host (insect) cue hierarchical order than the other cruisers (*H. bacteriophora*), with the volatile cues only becoming important after a long exposure.

5. Conclusions

The research of VOCs related to plant biotic protection is unequivocally dependent on modern gas chromatography-mass spectrometry (GC-MS), since only this technique allows detection and identification of compounds at such low levels. Due to sample complexity (plant tissue, soil), the use of gas-sampling techniques, which allow sample enrichment, connected with GC-MS, is thus mandatory [8, 12].

The responsiveness of EPNs, the biotic agents which are used for biotic plant protection, was tested in some studies [8, 9, 10, 44]. The studies have shown that damaged plant roots release chemical substances (β-caryophyllene, linalool) which can act as attractants for EPNs. Recently, [53] reported positive chemotaxis of two EPN species to different VOCs such as heptanol, hexanol, methyl salcylate, etc. Interestingly, they showed that several volatiles repelled the EPNs. Similar effect of VOCs on the behavior of the EPNs was already observed by other researchers [99, 101].

Most VOCs that are involved in belowground tritrophic interactions remain unknown but an increasing effort is being made in this field of research. Understanding more of these complex interactions would not only allow a better understanding of the rhizosphere but could also offer ecologically sound alternatives in pest management of agricultural systems [44].

Biological control of plants is a way of controlling harmful organisms in agriculture and forestry by making use of live natural enemies (beneficial organisms). It aims to protect and stimulate useful organisms in nature, and to introduce targeted organisms outdoors or into places separated from nature. The application of biotic preparations requires the users to have considerable knowledge and greater ecological awareness. The preparations made biotically are ecologically more appropriate, their functioning is more specific, their formulation and application are important, as is the temporal precision of treatment. Their efficiency, on the other hand, is often lesser than that of chemical preparations, and such preparations are more expensive. The difference in price is due primarily to the relatively small market with biotic agents, which within the entire market of plant protection agents at the moment represent approximately 6% (according to the data of BCC Research), and is expected to rise at least to 8% until 2019.

The value of the global market of biotic agents was in 2013 assessed at approximately 1.8 billion $ and it is expected to reach approximately 4.4 billion $ until 2019. Globally, the USA is still the largest user of biotic agents; it is, however, expected that in the following years the fastest growing market with biotic agents will be Europe, whose strict legislation on plant protection products systematically stimulates ecologic production of food and fodder. Statistical data

have shown that the sales of biotic agents between 2005 and 2015 rose by 44%. The expansion of the market is undoubtedly also a consequence of the raised awareness of environmental issues, which are included in the EU directives.

Knowing the communication between plants, herbivores, and their natural enemies is crucial for more efficient implementation and optimization of biological control in food production systems. The European Union has set five ambitious goals – in the fields of employment, innovations, education, social inclusion, and climate/energy – to be reached until 2020. The market with new, improved biotic agents would contribute to environmental protection, as well as to the expansion of economic activities.

Acknowledgements

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