

Article

Signal, Not Poison—Screening Mint Essential Oils for Weed Control Leads to Horsemint

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Abstract: Weed control tries to suppress competitors for a crop and often relies on differential intoxication, making use of differences in uptake, development, or metabolism. We explored the possibility of using natural signals to shift competition in favour of the crop. Using the competitive horsemint (*Mentha longifolia*) as a paradigm, we showed that essential oils from certain mint species suppress the seedling development of different target species in a specific and efficient manner. The specificity concerned both the donor and the receptor. We demonstrated further that the effect of horsemint oil was specific for actin filaments, and not for microtubules. Since the elimination of actin will impair auxin transport, which is essential for root regeneration in vegetatively propagating weeds, we tested the efficacy of horsemint essential oil in combination with a slow-release carrier against field bindweed (*Convolvulus arvensis*), a pertinent weed in organic cereal production. We observed that the development of this weed can be specifically blocked, especially if the carrier is worked into the soil. We propose that allelopathic interactions, often relying on manipulative chemical signalling, harbour significant potential for organic weed control.

Keywords: *Mentha longifolia*; horsemint; allelopathy; actin; weed control; *Convolvulus*



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1. Introduction

The yield losses due to weeds are tremendous. For instance, according to estimates from 2021, winter wheat production in North America was impacted by weeds by more than 25% on average [1]. For Germany, maize yield was shown to be reduced by 70% when weeds were not controlled [2]. Thus, the efficient control of weeds is important for agricultural production to be economically successful. However, the use of herbicides is one of the most discussed issues in agricultural production practice. The toxicity and persistence of chemically synthesised substances pose a serious problem to the environment (for a review, see [3]). Moreover, the extensive use of herbicides has fostered the evolution of resistant weeds. A classic example is the R-type of goosegrass (*Eleusine indica*), which has acquired resistance to dinitroaniline herbicides due to a mutation in α -tubulin, affecting the binding site for these herbicides [4]. The ecological concerns along with the progressive loss of efficacy have eroded the lead structures available for the development of new herbicides, stimulating the search for alternative strategies, since mechanical weed control is energy- and labour intensive and, therefore, does not represent an attractive alternative. Total herbicides are usually confined to agricultural systems using GMO crops expressing a resistance marker, which is no option for organic agriculture.

In this context, natural products that can suppress weed growth have attracted considerable attention (for a review, see [5]). Many plant species use chemical interactions to ward off competitors, a phenomenon known as allelopathy. Such allelopathic activities might be useful for weed control (reviewed in [6,7]). This is possible by either growing plants in intercropping systems, or by identifying and isolating the compounds responsible for the allelopathic effect (for a review, see [8]). For instance, the bioherbicide Avenger[®], approved by the USDA, is based on limonene, extracted from *Citrus* peels and, thus, exploits the allelopathic inhibition of *Citrus* trees upon their neighbours. Likewise, pelargonic acid, a saturated C₉ fatty acid from *Pelargonium* species, is efficiently used against broadleaf weeds [9]. Although they derive from natural sources, essential oils as bioherbicides must be critically assessed with respect to toxicity in the same way as their synthetic counterparts (for reviews, see [10,11]). Following the principle of Paracelsus, the dosage is decisive to delineate undesired collateral damage on other life forms from the desired targeted inhibition of the weed. If the effect of the bioherbicide merely relied on more or less unspecific phytotoxicity, the line towards synthetic herbicides would be relatively arbitrary and just be based upon the mode of production (extraction from natural sources versus chemical synthesis by humans). However, there are indications that some of these bioherbicides do not exert a broadband toxicity, but rather act as specific signals. For instance, there is accumulating evidence that allelopathic interactions often evoke programmed cell death in the target species (recently reviewed in [12]). Signals differ from toxins with respect to their specificity, which is also reflected in far lower effective concentrations. Both specificity and low concentrations actually come from the target species itself—programmed cell death (PCD) is a central and necessary element of both normal plant development and resilience to stress (for a classical review, see [13]). Allelopathic signals hijack the underlying signalling, shifting PCD out of its physiological context and thus seriously damaging the target plant.

Mints (Lamiaceae) are interesting in this regard, since they are rich in essential oils (i.e., oils that can be extracted by water steam distillation), mainly volatile monoterpenes that are synthesised in glandular trichomes on the leaves and are widely used for medicinal and cosmetic purposes, but also as spices (for a recent review, see [14]). In addition to the genus *Mentha*, the neighbouring taxa, such as *Nepeta cataria* (cat mint) or *Agastache rugosa* (Korean mint), are also interesting for their highly active compounds. Since it is progressively clear that *Mentha* is not a monophyletic group and the taxonomic relationship with the neighbouring genera is currently under debate, in the current study, we will use the term “mint” in a pragmatic sense to describe the tribe of Menthaea. In their natural environment, mints are often very competitive, which is already indicative of their allelopathic power. In fact, essential oils from horsemint (*M. longifolia*) inhibited the germination and growth of wheat [15], as well as of chickpea [16], while aqueous extracts of peppermint inhibited germination and photosynthetic pigments in sunflower seedlings [17]. Likewise, the herbicidal action of mint essential oil has been shown for the pertinent weeds *Rumex* and *Convolvulus* upon treatment with oil from *M. longifolia* [18].

While the allelopathic activity as such has been well demonstrated, the cellular mode of action of mint essential oils has been addressed only rarely. In a previous study, we showed that the allelopathic activity of essential oil from Korean mint (*Agastache rugosa*) correlated with the abundance of menthone and isomenthone in this species [19] and targeted microtubules as central drivers of cell growth. The effect was dose-dependent and specific, since menthol, which differs from menthone only by a hydroxyl group instead of the carbonyl, required around ten times higher concentrations to exert the same effect in tobacco BY-2 cells. The rapid disassembly of microtubules was followed by cell death that was accentuated when microtubules were stabilised by the overexpression of the green fluorescent protein with tobacco tubulin $\alpha 3$. The microtubule effect of menthone was confirmed for seedlings of *Arabidopsis thaliana* as well [19].

Even though this study demonstrates that mint essential oil evokes a specific response that has the potential to be employed for a bioherbicidal effect, we did not explore this

potential, nor did we probe for differential sensitivity of different target species as a prerequisite for use as a bioherbicide. Therefore, we address these issues in the current study, comparing essential oils from three different mints and the lead compounds found in oils extracted from them with respect to germination inhibition in creess. The study was designed in an iterative manner, testing different mints (genus *Mentha*, in some experiments also cat mint, *Nepeta cataria*, and Korean mint, *Agastache rugosa*) that in preparatory research have been found to be endowed with allelopathic activity. Starting with a screening of oils and compounds, we funnelled down to the mode of action of *M. longifolia* (since the mode of action for *A. rugosa* has already been addressed) [19]. We confirm a dose-dependent inhibition of germination in a panel of different species, including the pertinent weed, *Rumex acerifolia*. We demonstrate further that the efficacy of the essential oils and the individual compounds therein depends on the target species, and that each target species has a specific profile of inhibitory oils and compounds. To obtain insight into the cellular mode of action, we test the effect of essential oil from *M. longifolia* in fluorescently labelled cytoskeletal marker lines in tobacco BY-2 and find that this oil specifically disrupts actin microfilaments, while microtubules are not sensitive. Since actin is crucial for auxin transport, which in turn is pivotal for the regeneration of vegetatively propagating weeds, we test the use of *M. longifolia* essential oil on bindweed (*Convolvulus arvensis*), a pertinent problem in organically grown cereals. Using a slow-release carrier and two different modes of application, we demonstrate that horsemint oil can be used as bioherbicide to contain bindweed. Our study connects phenomenologically assessed sensitivity patterns with a specific cellular mode of action, and a hypothesis-driven application derived from this mode of action, which can be experimentally confirmed. We propose that allelopathic signals can hijack biological signalling in the target species, which allows their valorisation for organic agriculture.

2. Materials and Methods

2.1. Extraction of Essential Oils

The plants, which were used for oil extraction, were raised in the greenhouses of the Botanical Garden of the Karlsruhe Institute of Technology, Karlsruhe, Germany. A few days prior to extraction, the plants were exposed to sunlight to stimulate the formation of essential oils. All source plants had been verified by taxonomic identification, as well as by genetic barcoding (Supplementary Table S1). In brief, essential oils were extracted from around 30–50 g of fresh leaf material by water steam distillation as described in [19] and the resulting essential oils were stored at 4 °C till experimental use. The lead compounds of these oils, identified by GC-MS [19], were used in parallel for comparison. The GC-MS data for the essential oils used in the current study are given in Supplementary Material S1. These included menthone/isomenthone, linalool, limonene, α -pinene and β -pinene, pulegone, and carvone, all of which were purchased from Sigma-Aldrich (Deisenhofen, Germany). With the exception of menthone, which was of 90% purity (with 10% isomenthone), these compounds were of analytical grade. The solvent, n-hexane, was of hypergrade for organic trace analysis (Sigma-Aldrich, Deisenhofen, Germany).

2.2. Assays for Germination Inhibition

To assess the inhibition pattern of the mint oils, we used a panel of different target species: *Arabidopsis thaliana* L. ecotype Columbia, *Triticum aestivum* L. cv. Wiwa (Sativa Rheinau, Switzerland), *Phacelia tanacetifolia* (UFA Samen, Winterthur, Switzerland), *Solanum lycopersicum* L. Marmande (Samen Mauser Eric Schweizer AG, Thun, Switzerland), *Avena sativa* L. cv. Ebners Nackthafer (Botanical Garden of the Karlsruhe Institute of Technology), *Rumex obtusifolia* L. (FiBL, field collection, Frick), and *Convolvulus arvensis* L. (FiBL, field collection). To analyse the bioactivity of the essential oils and the different compounds thereof, two different approaches were used, either through the gas phase without the direct contact of the target seeds with the active agent, or, alternatively, after preceding imbibition facilitated by a detergent.

For application through the gas phase, the target species was allowed to germinate in Plexiglass boxes with covers (that were sealed with Parafilm after sowing). The seeds were sown equidistantly on humidified tissue paper. For releasing the volatile substances, a glass slide carrying a filter paper doped with either 1 μL or 10 μL of the oil sample, the respective compound, or the respective solvent was placed in the centre of the box. Each combination was tested three times in technical triplicates. The seeds were incubated in the dark at 25 °C and germination was scored after two (*L. sativum*, *T. aestivum*) to five (*S. lycopersicum*, *Tanacetum*) days.

For detergent-facilitated imbibition, the probing solution was prepared from 10 mL deionised water with 1% *v/v* of Tween 20 and the respective test oil or compound (between 0.10 and 0.20% *v/v*) and vortexed vigorously. The seeds were then sown equidistantly on filter paper in Petri dishes and complemented with 2 mL of the probing solution (revortexed again immediately prior to application). The Petri dishes were sealed using Parafilm and the seeds allowed to germinate for 5 days at 20 °C in the dark. The experiments were repeated in triplicate.

2.3. Assay for Inhibition of Regeneration in *Convolvulus*

To test their effect on the regeneration of the pertinent weed *Convolvulus arvensis*, essential oils from *M. longifolia*, *M. spicata crispata*, and *A. rugosa* were mixed into a slow-release alginate carrier (proprietary formulation) in a concentration of 2.5% *v/v*. Segments of *Convolvulus* runners (3 cm) were exposed in two configurations in standard field soil sampled from the site of origin (see above). In the ‘in-soil’ configuration, the compound was in direct contact with the doped carrier and covered by 2 cm of soil. In the ‘application from top’ configuration, the compound was placed above the segments and separated by a thin layer of soil, while keeping the same overall depth (2 cm). In total, 2 g of the formulation was used per pot and the experiment was conducted with nine individual pots per configuration and type of essential oil. The entire experiment was repeated twice. The growth and development of the target rhizomes were scored after two weeks at 20 °C and a cycle of 16 h light and 8 h dark. As readouts, the shoot length, number of regenerated leaves, and fresh weight were monitored.

2.4. Live-Cell Imaging of the Cytoskeleton

To assess the cellular effects of the *M. longifolia* oil, we used two GFP-tagged tobacco BY-2 lines expressing GFP-tagged markers for the cytoskeleton. Microtubules could be followed by tobacco tubulin $\alpha 3$ with a N-terminal fusion of GFP under the control of a constitutive CaMV 35S promoter [20], while actin filaments were visualised by the second actin-binding domain of fimbrin (AtFIM1) fused to GFP, also under the control of the Cauliflower Mosaic Virus 35S promoter [21]. The cytoskeleton was followed in vivo by spinning disc confocal microscopy as described in [19].

2.5. Statistical Analysis

The data from the germination assays were tested by ANOVA. Post hoc comparisons of differences among means were compared by the Least Significant Difference (LSD) test, applying different numbers for a significance threshold of $p < 0.05$. For the *Convolvulus* experiment, significant differences from the control were probed using a two-tailed Student's *t*-test for significance thresholds of $p < 0.05$ and $p < 0.01$.

3. Results

3.1. Essential Horsemint Oil Has Allelopathic Activity

Circumstantial observations in Swiss mountain pastures (Figure 1) show that horsemint can efficiently outcompete other species, leading to extensive clusters where no other herbaceous species remain. These observations stimulated the question of whether horsemint exerts an allelopathic activity, and, if so, whether this activity is linked with its essential oils. We addressed this using the standard cress germination assay (Figure 2), administering

the essential oils or compounds found in different mint species through the gas phase. In fact, the essential oils from all three tested mint species (*M. spicata*, *M. spicata crispa*, *M. longifolia*) exerted a dose-dependent and strong inhibition of germination, especially for the essential oils from *M. spicata crispa* and *M. longifolia* (Figure 2A). The monoterpenes menthone/isomenthone (found in *M. longifolia*, accompanying high levels of menthol [19]) and linalool (found in small amounts in several mints) were highly active as well, while limonene (which is very abundant in *M. spicata crispa*, but not in *M. spicata*, [19]) produced only a weak inhibition. The same holds true for α - and β -pinene that were found in small amounts in several mints. Likewise, n-hexane, used as solvent, did not show any effect.

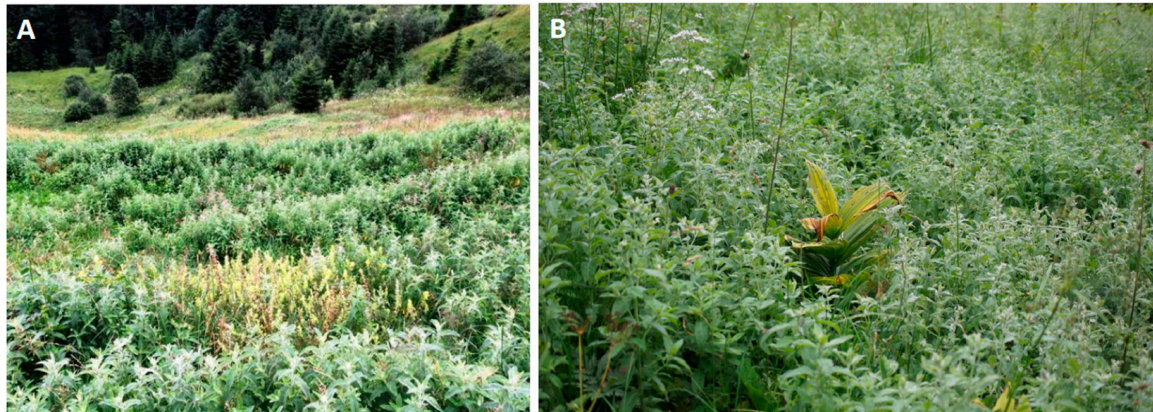


Figure 1. Horsemint can outcompete other species. (A) Overgrown mountain pasture at Alp Rossweid close to Lake Sihl, near Einsiedeln, Canton Schwyz, Switzerland. (B) Detail showing how *M. longifolia* has almost eliminated other species, with just one specimen of White Hellebore (*Veratrum album*) being able to remain. Images courtesy of Franz Steiner (Research Institute of Organic Agriculture, FiBL, Frick, Switzerland).

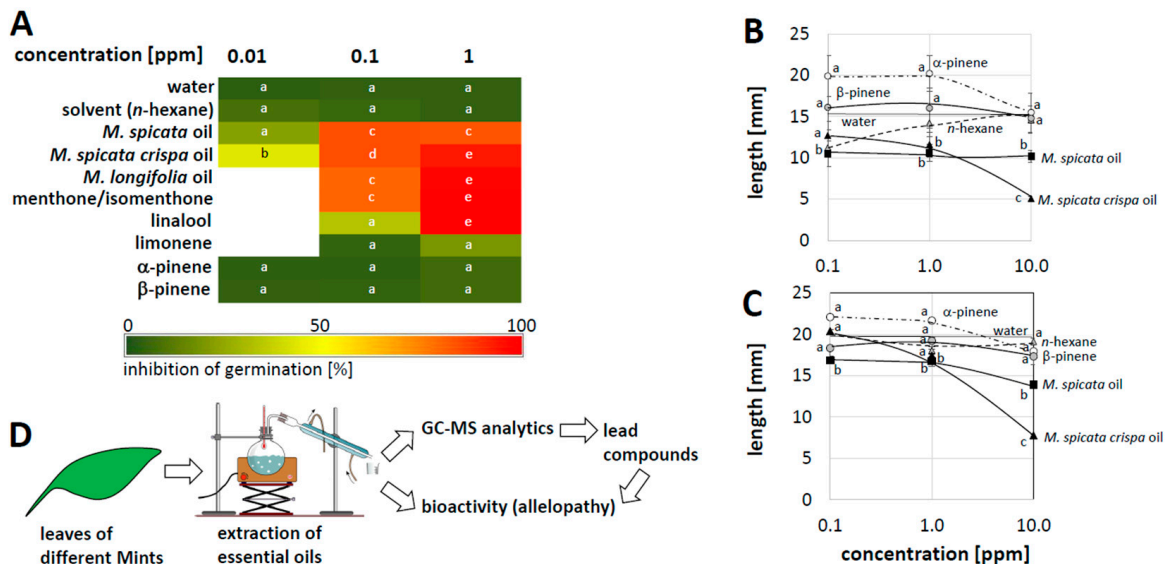


Figure 2. Response of cress seedlings to essential oils from different mints and of compounds found in these oils administered through the gas phase. (A) Inhibition of germination rate; mean values from five independent replications collected in two different laboratories with 100–297 individuals scored for each experiment. White fields have not been determined. (B,C) Modulation of seedling growth for hypocotyl (B) or root (C). Data show mean and SE over those seedlings that had germinated. Letters indicate groups that are different with respect to the Least Significant Difference (LSD) test for a significance threshold of $p < 0.05$. (D) Experimental design of the study.

Thus, there is a clear allelopathic inhibition of germination in cress. This inhibition is specific, since it differs depending on the source plant, and it is dependent on the dose. Among the individual compounds, menthone/isomenthone, which is abundant in horsemint, produced a strong inhibition, while limonene, which is abundant in *M. spicata crispa*, inhibited germination only mildly, contrasting with the strong inhibition by the essential oil of *M. spicata crispa*. Obviously, limonene cannot account for the strong effect of this oil, while the strong effect of horsemint oil correlates with a strong effect of its main compound menthone/isomenthone, which is present in this oil, albeit not as the main component. In addition, the development of the seedlings that managed to germinate was affected, as evidenced by the reduced root and hypocotyl elongation, as exemplarily shown for oil from *M. spicata* and *M. spicata crispa* (Figure 2B,C).

3.2. Effect of Horsemint Oil Depends on the Target Species

Since horsemint oil efficiently inhibited cress germination, even if just administered through the gas phase, we wondered whether this effect was general or whether it was dependent on the target species. We therefore compared the germination inhibition of *M. longifolia* and the individual active compounds thereof for cress (Figure 3A), *Phacelia tanacetifolia* as a representative of legumes (Figure 3B), tomato as a Solanaceous species (Figure 3C), and winter wheat as a representative of cereals (Figure 3D). While the overall pattern of inhibition was comparable at high concentrations, there were specific differences with respect to efficacy, especially at the lower concentration of 1 ppm. For instance, the essential oil of *M. longifolia*, while inhibiting all target species at 10 ppm, was less efficient in *Phacelia* and tomato than in winter wheat at 1 ppm, and the inhibition in winter wheat was, in turn, less pronounced than in cress. In contrast, for menthone, winter wheat was inhibited less than *Phacelia* or tomato at 1 ppm. Linalool was most efficient in winter wheat and tomato, but less in cress and in *Phacelia*, if administered at 1 ppm, while at 10 ppm, it inhibited all four targets. Limonene, as well as α - and β -pinene, did not show a particular effect in any of the four target species.

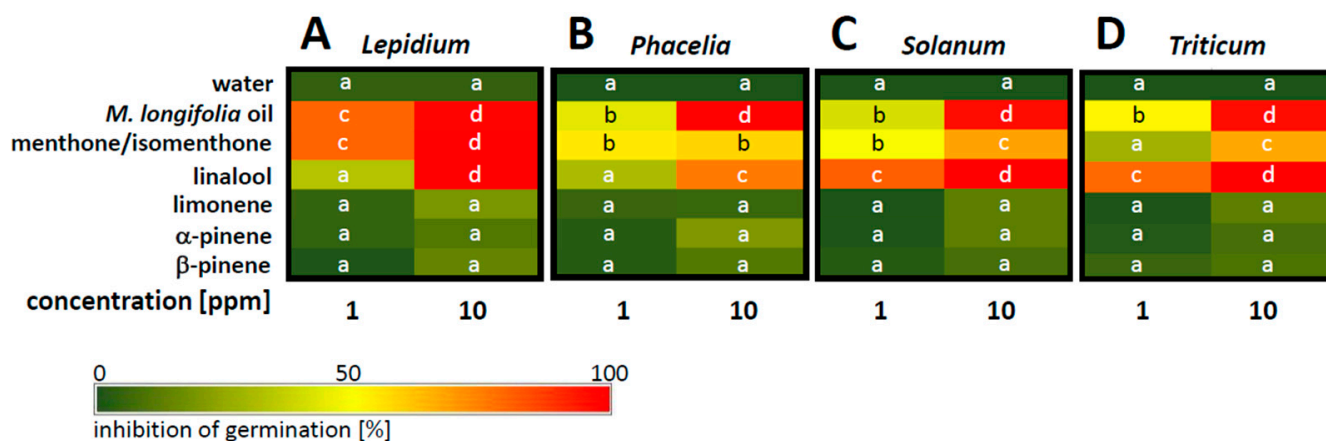


Figure 3. Species spectrum of sensitivity to essential oil from *M. longifolia* and of compounds found therein administered through the gas phase. Inhibition of germination rate; mean values from three independent replications with 100 individuals scored for each experiment. Responses are shown for *Lepidium sativum* (A), *Phacelia tanacetifolia* (B), *Solanum Lycopersicon* (C), and *Triticum aestivum* (D). Letters indicate groups that are different with respect to the Least Significant Difference (LSD) test for a significance threshold of $p < 0.05$.

In the next step, we expanded the species spectrum as well as the spectrum of compounds. Moreover, since the effective concentration for the application through the gas phase can only be estimated based on the assumption that the compounds are equally distributed, we changed the application to the detergent-supported imbibition of the target

seeds (Figure 4). While none of the solvents (*n*-hexane in the case of the pure compounds, Tween 20 for the essential oils) caused any inhibition, both the essential oils and the compounds found therein produced significant effects. These effects were not only dependent on concentration, but also on the donor species or the chemical nature of the compound, respectively. It was also dependent on the target species. These features indicate that the effect is specific and not caused by a general phytotoxicity.

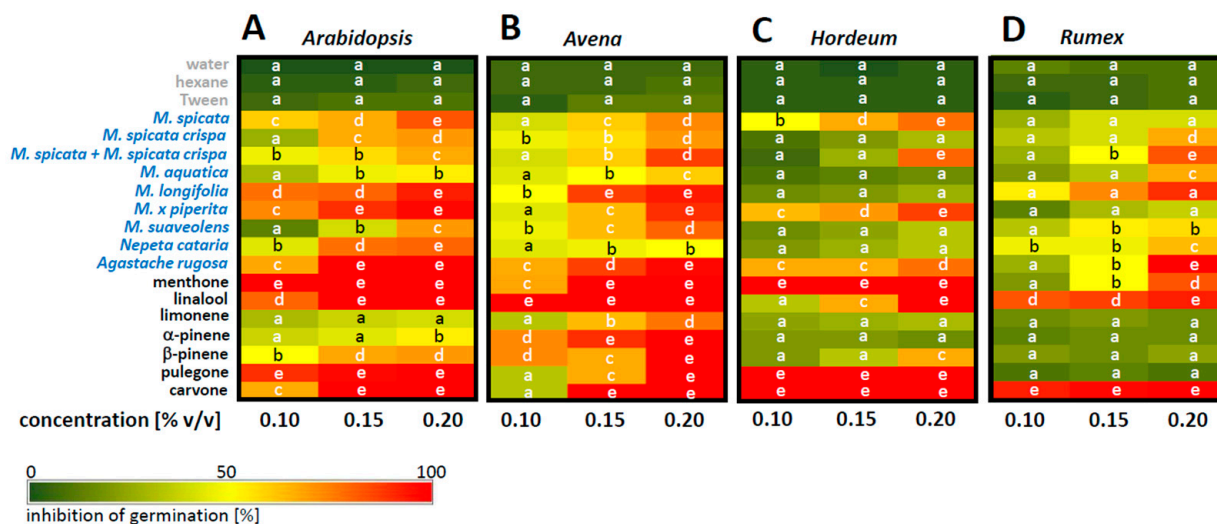


Figure 4. Species spectrum of sensitivity to essential oils from different mints and of compounds found therein administered through the liquid phase. Inhibition of germination rate; mean values from three independent replications with 30 individuals scored for each experiment. Responses are shown for *Arabidopsis thaliana* (A), *Avena sativa* (B), *Hordeum vulgare* (C), and *Rumex acetosella* (D). Solvent controls involve 0.15% hexane (as solvent for the pure compounds), 1% *v/v* Tween 20 (as solvent for the essential oils, blue), or water. Letters indicate groups that are different with respect to the Least Significant Difference (LSD) test for a significance threshold of $p < 0.05$.

With respect to the donor plants, the essential oils of *M. longifolia* and *Agastache rugosa* were generally the most potent inhibitors. *M. x piperita* was also effective, while *M. suaveolens* and *M. spicata crispata* only produced a weak effect. There were differences between the target species, though. For instance, the essential oil of *M. longifolia* efficiently suppressed the germination of *Arabidopsis*, *Avena*, and *Rumex*, while *Hordeum* remained largely unimpressed. On the other hand, *Avena* was blocked by the essential oil of *M. x piperita*, which in turn had only a low impact on *Rumex*. Thus, the sensitivity of a given target species is strongly dependent on the origin of the essential oil.

Similar conclusions can be drawn for the compounds found in these oils. While linalool is very potent against *Arabidopsis*, *Avena*, and *Rumex*, it is far less efficient in *Hordeum*. On the other hand, α - and β -pinene, which only produce weak inhibition in *Hordeum* and *Rumex*, are quite efficient in suppressing *Avena*. Menthone and carvone were the two compounds with a generally strong inhibitory effect, but even there, menthone was much less potent against *Rumex*, while (*R*)-carvone needed 0.15% *v/v* to efficiently suppress *Avena*.

To detect whether a particular oil or a particular compound targets one species over the others, we determined the efficacy spectra (Figure 5). For this purpose, we first determined the average inhibition for the compound of interest over the entire range of the four target species. In the next step, we related the inhibition for each individual target species to this average value. This approach revealed whether a given target species was specifically susceptible or resistant to this compound. Using this map, one can move along a column and see at a glance which compound generates a pronounced differential inhibition between two target species of interest.

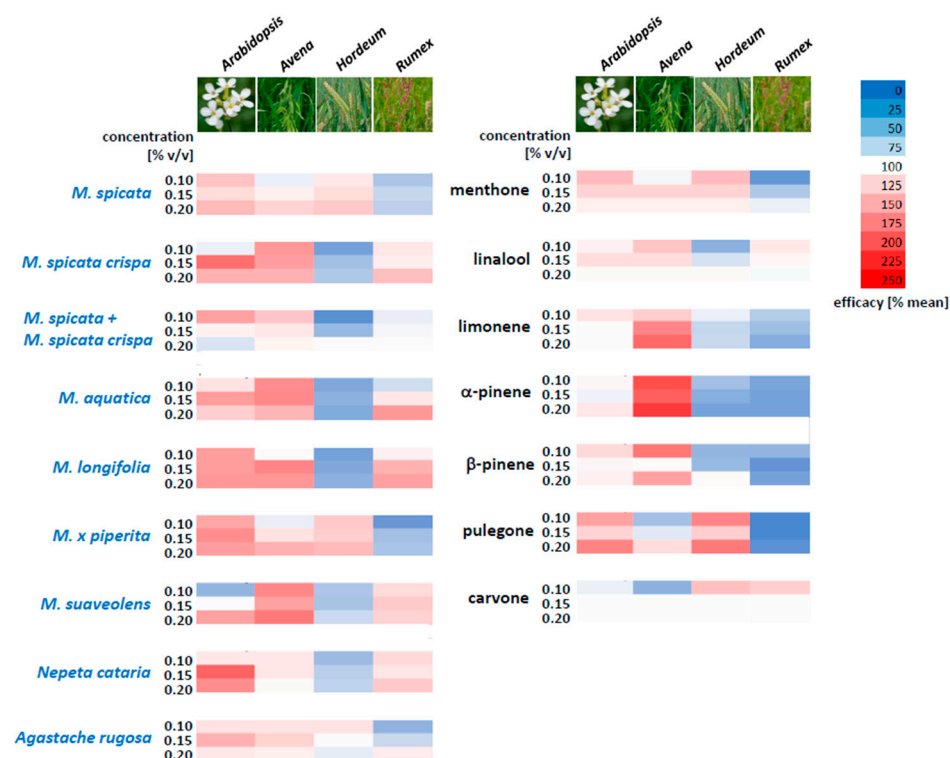


Figure 5. Efficacy spectrum for essential oils from different mints and of compounds found therein as percentage of the average inhibition over all four target species. Red colours indicate elevated efficacy while blue colours indicate reduced efficacy against the respective target species compared to the other species.

Using this approach, one sees that, for instance, *Arabidopsis* is especially sensitive to essential oils from *M. spicata crispa*, *M. longifolia*, *M. x piperita*, and *Nepeta cataria* when compared to the other species, while *Avena* is also sensitive to *M. suaveolens*. *Hordeum*, which is generally quite robust, shows sensitivity to *M. spicata* and *M. x piperita*, but is not sensitive to *M. longifolia*, which, instead, can be used to address *Rumex*. With the individual compounds, the sensitivity of *Avena* to α -pinene, limonene, and β -pinene is noteworthy, while pulegone is of little effect, but shows good activity against *Arabidopsis* and *Hordeum*. *Rumex* can be tackled by carvone and also, to some extent, by linalool, which does not affect *Hordeum*.

3.3. Horsemint Oil Disrupts Actin Filaments, but Not Microtubules

To obtain insight into the mode of action of horsemint oil, we probed for the response of the cytoskeleton. Microtubules are not only essential for cell division driving the development of meristems in germinating seedlings, but also sustain cell axis in elongating tissues such as the distal elongation zone of the root or the hypocotyl. Actin filaments are not only essential for the elongation of root hairs, but also support the polar flow of auxin through the plant. Therefore, we exposed tobacco BY-2 cells expressing GFP-tagged markers for actin filaments and microtubules [20,21] to horsemint oil acting through the gas phase as described in [19] and followed the responses using spinning disc confocal microscopy. While in cells exposed to the solvent control, *n*-hexane, the actin filaments displayed a well-developed network (Figure 6A,B) and the essential oil of *M. longifolia* caused the almost complete breakdown of the actin cytoskeleton (Figure 6C–E). The filaments were reduced to very short rod-like structures along with a soluble signal that often accumulates along the cross-walls (Figure 6D–E). In confocal z-stacks (Figure 6(F1–F4)), individual fluorescent *punctae* could be discerned on the surface of the vacuole, probably representing actin nucleation centres.

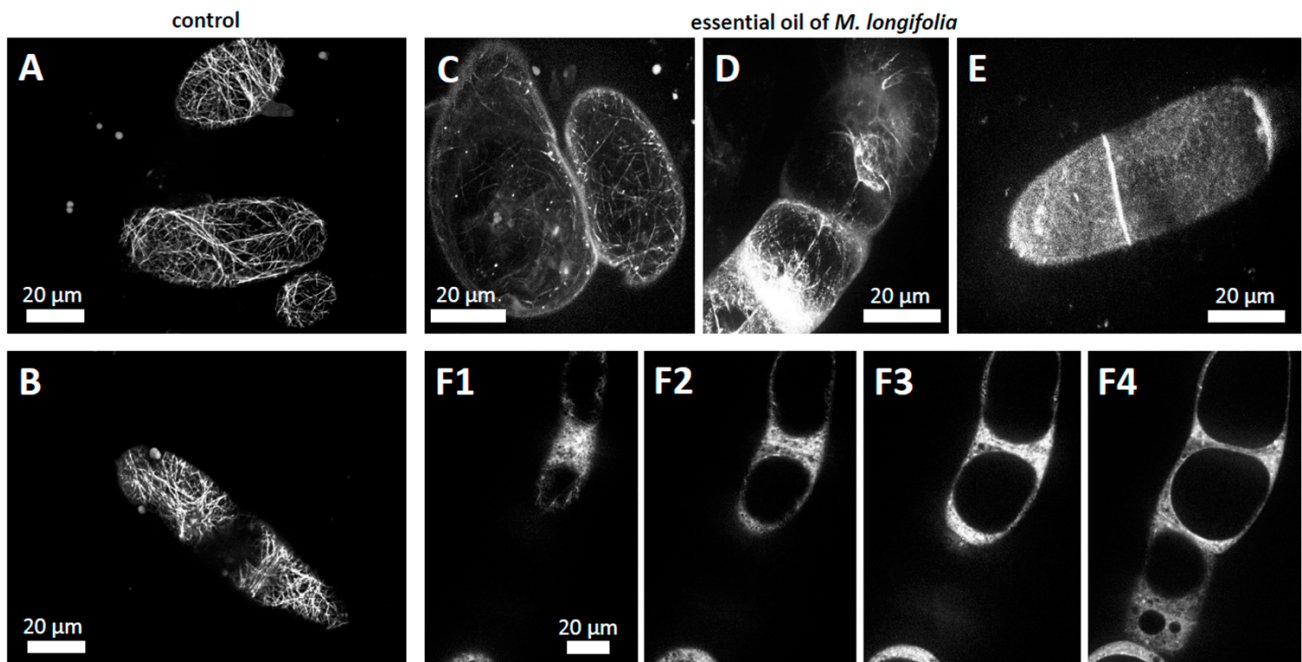


Figure 6. Effect of essential oil from *M. longifolia* on actin filaments in tobacco BY-2 cells visualised by the marker FABD2-GFP and spinning disc confocal microscopy. (A,B) Representative control cells after 30 min of treatment with the solvent control (0.1 v/v % *n*-hexane). (C–F) Representative cells after 30 min of treatment with essential oil of *M. longifolia* (0.1% v/v). (A–E) Geometric projection of z-stacks collected across the cell. (F1–F4) Individual sections collected from the cell periphery (F1) through the cell centre (F4). All treatments occurred through the gas phase, without physical contact between the cells and solvent or essential oil, respectively.

To assess whether this breakdown of the actin cytoskeleton was reflecting a general disruption of the cytoskeletal structure, we investigated the response of the microtubules in parallel. However, the microtubules that were organised in cortical arrays perpendicular to the axis of cell elongation persisted (Figure 7), contrasting the complete collapse of the actin filaments observed under the same conditions.

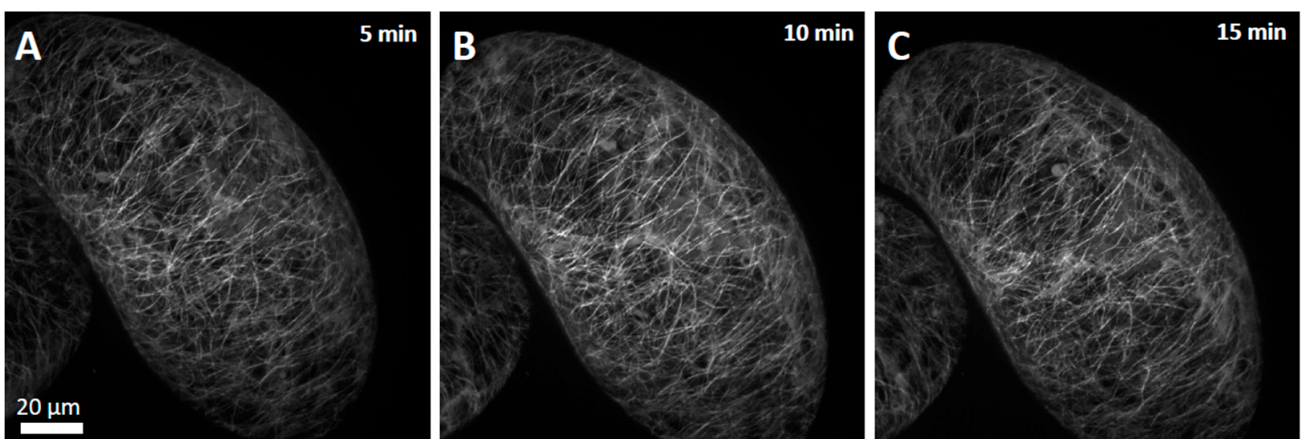


Figure 7. Effect of essential oil from *M. longifolia* on microtubules in tobacco BY-2 cells visualised by the marker NtTuA3-GFP and spinning disc confocal microscopy. Time course of a cell after 10 min (A), 20 min (B), and 30 min (C) of treatment with essential oil of *M. longifolia* (0.1% v/v).

3.4. Horsemint Oil Can Be Used to Control Bindweed

Since the effect of horsemint oil was specific for actin filaments, we wondered whether this could be exploited for specific weed control. Actin filaments are essential for polar auxin transport [22], which in turn is crucial to the regeneration of adventitious roots [23]. We wondered, therefore, whether a weed relying on vegetative regeneration could be targeted by this essential oil. For this proof-of-concept, we selected bindweed (*Convolvulus arvensis*), a pertinent problem in organic cereals. This weed mainly invades from rhizomes that can reach several metres in length and are difficult to tackle, even using herbicides. In organic cereal culture, it has to be controlled by intensive tilling, which is not only laborious, but also contributes to erosion. We tested two modes of application of horsemint oil mixed into a slow-release carrier in a pot experiment with rhizome segments of bindweed (Figure 8A): either the carrier was placed on top of the rhizomes and covered by soil, or, alternatively, the carrier was worked into the soil harbouring the rhizomes. The results were assessed after two weeks. To probe for specificity, we used oil from *M. spicata crispata* and of *Agastache rugosa* in parallel, along with a negative control, where the carrier was applied without doped with oil.

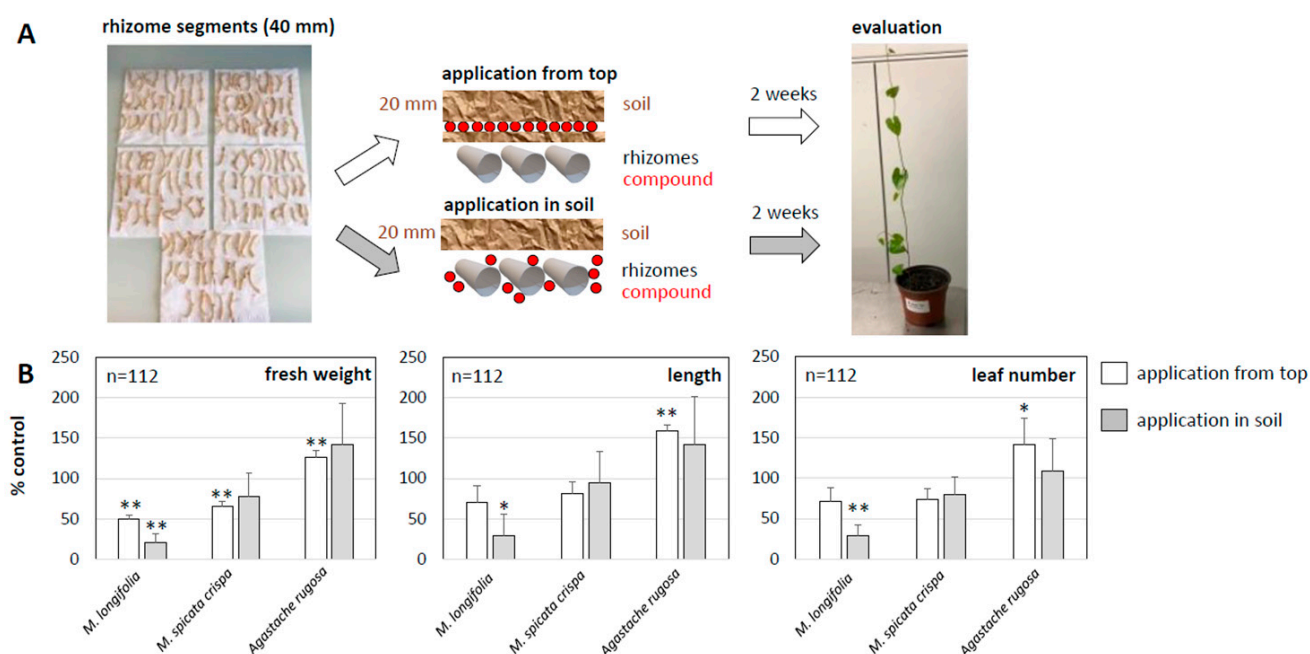


Figure 8. Effect from mint oil formulations on the development of regenerating rhizome sections of *Convolvulus arvensis*. (A) Design of the experiment: 50 μ L of essential oils from three different mints were formulated in 2 g of a slow-release carrier corresponding to 2.5% *v/v* and either placed on top of the rhizomes or worked into the soil where the rhizomes were cultivated. (B) Quantification of the effect after 2 weeks of treatment, measuring shoot fresh weight, shoot length, and number of leaves. Data points represent mean values and SE from two independent experimental series with nine individuals per data point and replication. Significant differences from the control are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

In fact, we observed a significant and substantial inhibition of bindweed development in response to the treatment, particularly in the setup where the doped carrier had been worked into the soil (Figure 8B). Compared to the control value, which was set to 100%, the fresh weight was reduced by a factor of four, and for the application from the top, the inhibition was weaker (a factor of two). A similarly strong inhibition was seen for the leaf number, although it was weaker for shoot length, indicating that bindweed was still partially sustaining cell elongation, but had problems forming new organs and, thus, accumulating biomass. The inhibitory effect was specific for horsemint oil, since oil from

M. spicata crispa, with exception of a reduction in fresh weight by around 40%, failed to evoke significant effects. For the essential oil of *Agastache rugosa*, we even obtained values that were larger than the control values (defined as 100%). In other words, this oil caused significant stimulation, especially with respect to elongation, such that the measured values turned out to be larger than in the controls.

4. Discussion

In the current work, we have conceptualised allelopathy using essential oils from different mints as inhibitory signals to the target plant rather than through general phytotoxicity. We demonstrate that the allelopathic effect depends on the dose as well as on the species of both the donor and recipient. We also demonstrate specificity with respect to cellular effect, since microtubules are not affected by horsemint oil, while actin filaments are severely disrupted. Using bindweed (*Convolvulus arvensis*), a weed pertinent in the organic production of cereals, as a paradigm, we demonstrate that horsemint oil can be used specifically to suppress this weed, while essential oils from two other mint species (spearmint and Korean mint) are not effective. These data stimulate two questions: first, does horsemint oil have the potential to develop bioherbicide strategies to contain weeds? Second, what can we infer for the mode of action of horsemint oil, how can we explain the specificity, and what is the potential for biological weed control?

4.1. Bioherbicides Based on Horsemint Oil: Potential and Challenges

To be considered as a candidate for a herbicide, a compound needs to meet several criteria. It must inhibit the target weed at low concentrations, while leaving the crop species unaffected at this concentration. It should be producible at a competitive cost, and it should not be toxic to other organisms. Are these criteria met by horsemint oil?

When administered through the gas phase, the inhibition of cress germination was noted at 0.1 ppm (Figure 2). It should be noted that the concentration was estimated based on the assumption that the oil was fully evaporated. In cases where this evaporation was incomplete, the effective concentration will be even lower. To put this into perspective in relation to commercial herbicides, a comparative dose–response study on *Brassica carinata* in different regions of the US (as a reference for *L. sativum* in the current study) can serve as a comparison. Using a spray of 187 L·ha⁻¹, around 500–1000 g·ha⁻¹ of diuron (corresponding to 2670 to 5340 ppm), 1000–2000 g·ha⁻¹ of simazine (corresponding to 2670 to 10,680 ppm), and >10,000 g·ha⁻¹ of napropamide (corresponding to 53,400 ppm) were needed to reach a 50% suppression [24] based on a nozzle setting of 187 L·ha⁻¹, which is several orders of magnitude higher than the concentrations required for the essential oils. Thus, the first criterion—activity at low concentration—is certainly met.

Likewise, the second criterion, specificity of action, also seems to be fulfilled. For a concentration of 1 ppm, cress was found to be more substantially suppressed than Phacelia, tomato, and winter wheat (Figure 3), indicating that cress, a Brassicaceae, which is a typical therophyte representing numerous ruderal species, is very sensitive to this oil. This conclusion is consistent with the observation that *Arabidopsis*, also belonging to the same family, is more sensitive than the other species (Figure 4). However, target species can differ in susceptibility to horsemint oil, as illustrated by the comparison between *Avena*, which is very sensitive to horsemint oil, and *Hordeum*, which is mostly resistant.

With respect to differential susceptibility, a combination of wheat and barley (both relatively resistant) and a brassicaceous weed would be a feasible candidate to develop a plant protection strategy. For instance, herbicide-resistant *Brassica carinata* has turned into a pertinent weed in Australia [24,25] and needs to be controlled with glyphosate, because chlorsulfuron, metosulam, and imazethapyr have lost their efficacy.

Compared to other mints, the essential oil of *M. longifolia* is commercially underrepresented. Bulk ware is currently traded for around USD 50 per kg [26]. Given the very low dose needed for the inhibition of germination, this would certainly be competitive compared to synthetic herbicides, especially in cases of herbicide resistance.

Concerning toxicity, the essential oil of horsemint was tested on Wistar rats using direct oral application and was found to cause kidney enlargement at the highest dose tested [27]. This dose (500 ppm) was almost three orders of magnitude above the concentrations used in the current study. For comparison, the toxicity of chlorsulfuron is in the range of 0.05 ppm per day [28]. In other words, compared to the currently used synthetic herbicides, horsemint oil is far less problematic.

Overall, horsemint oil meets all four criteria for a potential bioherbicide: low dosage, low cost, high specificity, and low toxicity. However, there are several challenges to be met. How can a compound that is volatile be administered in a manner whereby it is released over a long period without evaporating soon after application? This could be achieved by encapsulation for slow release using alginate, chitosan, or cyclodextrins (for a comprehensive review, see [29]).

4.2. Signal, Not Poison—What Is the Evidence?

The bioactivity of mint oils is endowed with a high degree of specificity. Not only do closely related donor plants differ considerably with respect to the inhibitory effect exerted by their oils (Figures 2, 4 and 5), but also different target species differ with respect to their response to a given essential oil (Figures 3–5). Moreover, the cellular mode of action differs—while the oil of Korean mint (*Agastache rugosa*) acts on microtubules [19], the oil of horsemint does not affect microtubules at all (Figure 7), but instead efficiently eliminates actin filaments (Figure 6).

Our findings on the effect of horsemint oil on plants and plant cells are strongly consistent with the published records demonstrating specific modes of action for monoterpenes. For instance, citral was shown to inhibit the growth of *Arabidopsis* seedlings in a dose-dependent manner by disrupting microtubules [30]. This disruption of the microtubules occurs with some monoterpenes as a permeabilisation of the membrane, while in others, the membrane remains intact [31], indicating that the microtubule response must derive from a physiological signal deployed at the membrane and cannot be a mere consequence of cell damage. The specificity was further supported by the finding that stereoisomers differed considerably in activity. In addition, our previous study [19] on the mode of action of isomenthone, a monoterpene underlying the allelopathic activity of Korean mint (*Agastache rugosa*), showed that the effect was strongly dependent on details of chemistry—if the aldehyde group of menthone was replaced by a hydroxyl group (menthol), the biological effects were eliminated. Both the stereospecificity [31] and the dependence on specific side groups [19] indicate that there must be a specific binding site for these compounds. This specific binding site might be a receptor.

In contrast to Korean mint, where the effect on microtubules can be attributed to menthone/isomenthone [19], the compounds responsible for the allelopathic effects of horsemint oil are not known. The essential oil of horsemint is rich in monoterpenes, whose composition can differ considerably depending on the respective chemotype (for a comprehensive review, see [32]). Some of these chemotypes (including the one used in the current study) contain menthone/isomenthone, but this seems not to be the active principle here, because the microtubules remain unaffected when treated with horsemint oil. On the other hand, neither the essential oil of Korean mint nor the lead compounds of menthone/isomenthone found therein caused any significant response in the actin filaments [19], which is the exact mirror-image of the effects we observed with horsemint oil. Thus, the effect of menthone/isomenthone found in both essential oils is qualitatively different, depending on unknown compounds present in horsemint oil.

How can something turn into a signal? This question was addressed almost a century ago by Karl Bühler (1934) in his *Organon Theory* [33]. He pointed out that any communication process means that a sender and a recipient exchange signals (*Zeichen*) that are proxies for a certain meaning. The communication is functional when both parties assign the same meaning to this symbol. Thus, whether a given molecule is a signal or not does not depend on the chemical nature of this molecule, but on the context of its use. One

hallmark of a signal is that its meaning will change when the context is changed. This is exactly what we see in the fundamentally different activity of menthone/isomenthone if given alone (eliminating microtubules, leaving actin filaments intact) or in the presence of the (unknown) second compound present in horsemint oil (leaving microtubules intact, eliminating actin filaments).

If volatile terpenoids act as signals, there must be a recipient for this signal: a receptor. There are two criteria for a molecule being a receptor. The binding of a ligand must be specific, and the binding needs to deploy or to modulate signalling events. The specificity of the binding site is supported by the stereospecificity [31] and the dependence of the biological effect upon small differences in the side groups [19]. However, is there any evidence that volatile terpenoids modulate signalling? A famous example for a monoterpene receptor is the menthol receptor TRPM8 that simultaneously acts as trigger for cold sensing [34]. However, this receptor seems to be confined to vertebrates and is absent in plants. However, there exist two examples of specific terpene receptors. Gossypol, a sesquiterpene from cotton, can induce apoptosis by binding to Bcl-XL, a protein in the outer membrane of mammalian mitochondria [35], but, again, this protein is confined to the metazoan and is thus absent in plants. Nevertheless, there is a plant-specific target, the transcriptional regulator TOPLESS, which was recently discovered as receptor for the sesquiterpene β -caryophyllene [36]. This protein is a transcriptional co-repressor that can be recruited by a protein called NINJA to a complex of the jasmonate receptor JAZ1 to inhibit the expression of jasmonate-responsive genes. In response to the active hormone jasmonate isoleucine, JAZ1 is doomed for degradation in the proteasome, such that TOPLESS is released and transcription can be initiated [37]. In fact, very recently, jasmonate signalling was shown to be regulated by the reversible acetylation of TOPLESS [38] and β -caryophyllene was demonstrated to activate the defence against herbivores in a jasmonate-dependent manner [39]. These findings link volatile terpenoids with a core pathway regulating the plant response to stress, including the initiation of programmed cell death. TOPLESS is a large protein with a modular structure composed of different domains that mediate the repression of cellular processes as diverse as jasmonate signalling, auxin signalling, embryo development, and flower development [40], which allows for versatile adaptation to achieve sensitivity or, likewise, insensitivity, depending on small changes in one of these modules. In other words, it would be rewarding to test whether different mint species have evolved versions of TOPLESS that render them insensitive to their own volatiles, which would explain why, in contrast to their competitors, they are not affected.

5. Conclusions

Using horsemint oil as a paradigm, we show that the allelopathic activity of mints can be exploited to design a bioherbicide. We demonstrate specificity for both the donor and recipient plant, and efficacy at very low concentrations. We show further that horsemint oil does not act through a general phytotoxicity, but targets the actin filaments, while the microtubules remain unaffected. Based on this mode of action and the sensitivity patterns, we develop the hypothesis that horsemint oil has the potential to suppress vegetatively propagating weeds in cereal crops. We then test this implication by using bindweed as a target making use of a slow-release carrier and demonstrate that this hypothesis-driven application is feasible. The novelty of this study is the proof-of-concept for the use of inter-organismic signalling for the design of novel plant-protection strategies.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture13030712/s1>: Supplementary Table S1: Source plants used for this study; Supplementary Material S1: GC-MS data for the essential oils used in the current study.

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