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ORIGINAL PAPER

Environmentally acquired chemical camouflage affects *Pieris brassicae* L. host plant selection and orientation behaviour of a larval parasitoid

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Abstract
Environmentally acquired chemical camouflage is a phenomenon, where a plant growing close to a strong volatile organic compound (VOC) emitter will adsorb and re-emit the VOCs produced by the neighbouring plant. The re-emitted volatile bouquet may resemble more the VOC composition of the neighbour than plant's own typical odour, and thus act as chemical camouflage against insect detection, potentially simultaneously providing associational resistance towards herbivory. We exposed a pest-sensitive horticultural crop, *Brassica oleracea* var. *italica* (broccoli) cv. Lucky, to the volatiles emitted by *Rhododendron tomentosum* [RT] twigs and assessed the host selection by ovipositing females and larval instars of the major caterpillar pest *Pieris brassicae* between RT-exposed and control plants. Potential impact of RT exposure on herbivore natural enemies was studied using behavioural tests with a parasitoid wasp *Cotesia glomerata*. *P. brassicae* females laid significantly less eggs and egg clusters were fewer on RT-exposed plants at both night-time (6 °C) and daytime (22 °C) temperatures. Larvae preferred leaves from control plants over RT-exposed plants at both temperatures. Preceding RT-exposure did not disturb orientation of parasitoid wasp *Cotesia glomerata* females towards *B. oleracea* plants damaged by its host *P. brassicae*. However, host-damaged control plants were favoured by the parasitoid over RT-exposed, host-damaged plants. Our results suggest that companion plant based chemical camouflage as a mechanism of pest suppression could be developed as an additional tool for the integrated pest management toolbox in agriculture.

Keywords Plant–plant volatile cues · Semivolatiles · Passive volatile adsorption · Orientation behaviour · Indirect defence · Associational resistance

Introduction

The high diversity of plant secondary metabolites (PSMs) with a current estimate of 200,000 specific chemical compounds (Kessler and Kalske 2018) is explained by the need of plants to protect themselves against various biotic and abiotic stressors (Blande et al. 2014; Julkunen-Tiitto et al. 2015; Kalske et al. 2019; Loreto and Schnitzler 2010; Moreira

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et al. 2018). In selection breeding of edible crop plants, the PSMs that are toxic or distasteful for humans have been reduced (Moreira et al. 2018), but genotypes rich with the specific PSMs that maintain the resistance against pests and pathogens, have been favoured (Wink 1988). In the Brassicaceae family, glucosinolates are the major PSMs that provide protection against polyphagous herbivores (Agut et al. 2018). Glucosinolate concentrations have been reduced in breeding of edible crop plants, but even low concentrations of those acts as attractants for numerous specialist Brassicaceae crop herbivores (Sontowski et al. 2019).

Since various secondary metabolites are known to be important for insect olfaction and host location, some PSMs might also be useful for protection against Brassicaceae specialist herbivores (Talekar and Shelton 1993). These could be e.g., higher concentration of flavonoid metabolites in host plant tissue (Onkokesung et al. 2014) or volatile terpenoids produced by neighbouring plant and adsorbed on the leaf surface of the host plant (Himanen et al. 2015). Furthermore,

the lowest energy cost for chemical defence could be in a case when the chemicals produced by other plants can be used to reduce the risk of attack by herbivores e.g., by adsorbing distinctive volatile organic compounds (VOCs) of neighbouring plant species (Himanen et al. 2010; Kessler and Kalske 2018).

In addition to plant–herbivore interactions, the plant VOCs play an important role in plant–plant interactions in ecosystems (Blande et al. 2010) and in plant's attraction of natural enemies of insect herbivores (Dicke et al. 2009; Stenberg et al. 2015; Turlings et al. 1990). Furthermore, specific VOCs emitted by a specific plant species are important signals that keep polyphagous herbivores and the specialist herbivores of other plant species away (Agut et al. 2018). Plants may have different strategies to avoid specialist herbivores to find the right host plant with the host-typical bouquet of VOCs. According to Kessler and Kalske (2018) these may include: (1) release of typical herbivore-induced VOCs which might indicate to other herbivores that plant is already occupied by herbivores; (2) reduce VOC emissions among other plants to become chemically less apparent; (3) release of a VOC which may disturb sensing of other host plant VOCs i.e. increase of chemical noise; and (4) releasing volatile cues that mimic other plant VOCs. There is still a lack of evidence of plant capacity to mimic VOC bouquet typical for other plant species (Kessler and Kalske 2018). However, mimicking another plant species VOC profile could be also based on adsorbed volatiles produced by another plant species (Himanen et al. 2010; Mofikoya et al. 2018a) or other exogenous source (Camacho-Coronel et al. 2020). Kessler and Kalske (2018) considered this as environmentally acquired chemical camouflage (EACC), i.e., a plant growing close to a strong VOC emitter will adsorb and reemit VOCs produced the neighbouring plant, might act as mimicking the chemical cues of a more defended or chemically differently defended plants.

Plant signalling via herbivore-induced volatiles to activate chemical defences in external parts of the damaged plant or in the intact receiver plant have been documented in various studies in recent years as summarised by Heil (2014). Less is known about biological effects accumulation of plant VOCs in surrounding soil (Barney et al. 2009) and on neighbouring plant surfaces (Himanen et al. 2010; Mofikoya et al. 2018b, 2019) or leaf surface vax (Camacho-Coronel et al. 2020; Li and Blande 2015). Accumulation of volatile monoterpenes released by a neighbouring plant in soil matrix in high concentration prevents seed germination (Santonja et al. 2019) and growth of other plants (Barney et al. 2009; Santonja et al. 2019). In addition, uptake of high atmospheric concentrations of isoprene (Ormeño et al. 2020) and monoterpenes (Copolovici et al. 2005; Noe et al. 2007) by growing plants via stomata and release back to atmosphere is reported (Copolovici et al. 2005; Mofikoya

et al. 2018b; Niinemets et al. 2014; Noe et al. 2007). Semi-volatile sesquiterpenes are sticky and can become adsorbed on various surfaces such as glass (Schaub et al. 2010) and plant wax layer (Camacho-Coronel et al. 2020; Joensuu et al. 2016; Li and Blande 2015) but also on the foliage of conspecific (Li and Blande 2015) and heterospecific (Himanen et al. 2010; Mofikoya et al. 2018b) neighbouring plants and could be released back to the atmosphere at higher temperatures (Himanen et al. 2010; Mofikoya et al. 2018b; Schaub et al. 2010).

Rhododendron tomentosum Harmaja (previously: *Ledum palustre* L.) is commonly known, highly recognizable aromatic plant species with English names such as wild rosemary, marsh tea or marsh rosemary, although it belongs to the Ericacea plant family. It is a subarctic and boreal fragrant shrub with circumpolar distribution and growing mostly in peatlands and forest understory (Dampc and Luczkiewicz 2013). Particularly European provenances (Raal et al. 2014; Zhao et al. 2016) of the species are rich with species specific volatiles and semi-volatile terpenoids in essential oil stored in glandular trichomes on surface of stems and leaves (Dampc and Luczkiewicz 2013; Mofikoya et al. 2018b; Zhao et al. 2016). Major volatile compounds in distilled essential oil and volatile emission are semi-volatile sesquiterpene alcohols palustrol and ledol, sesquiterpene ledene and monoterpene myrcene. Proportion of palustrol in total extracted essential oil (Raal et al. 2014) or in total emission (Himanen et al. 2010) can exceed 50%. The essential oil has shown repellent effects on blood feeding mosquitoes (Jaenson et al. 2006) and ticks (Jaenson et al. 2005) as well as herbivorous leaf beetles and bark-boring weevils (Egigu et al. 2011). The composition of *R. tomentosum* VOCs among Northern European plant species makes it easy to track the deposition of RT emissions on other plants (Himanen et al. 2010, 2015; Mofikoya et al. 2018a).

There are only few studies of environmentally acquired chemical camouflage yet, with very few plant and herbivore species tested. So far, and to our knowledge, the only studies that relate plant uptake and release of neighbouring plant VOCs to herbivory, i.e., showing EACC, have been conducted with VOCs of *R. tomentosum* (henceforth referred to as RT). Himanen et al. (2010) described the adherence of (RT) semi-volatile compounds and their release on the leaf surfaces of birch species (*Betula* spp.). In another study, adherence of RT volatiles from detached RT branches on white cabbage was shown (Himanen et al. 2015). Moreover, after a cold night, some of the *R. tomentosum* VOCs were more abundant in birch emissions than typical birch volatiles. During the day adhered compound were reemitted, and in the afternoon when temperature changed from 20 to 25 °C—RT volatiles were still detectable, but with low rates and not among dominant VOCs (Himanen et al. 2010). In the biotests with the diamondback moth, *Plutella xylostella*

(L.) (Lepidoptera: Plutellidae), RT-exposed white cabbage seedlings were less susceptible to moth oviposition at both night-time (12 °C) and daytime (22 °C) temperatures, and the moth larvae favoured unexposed control leaves and did more feeding damage on control than on RT-exposed leaves. More importantly, RT-exposure did not interfere with orientation of parasitoid *Cotesia vestalis* on *P. xylostella*-damaged foliage (Himanen et al. 2015).

Recent studies such as Himanen et al. (2010) have shown that, when RT and birch (*Betula* spp.) plants were grown in natural mixed field and peatland ecosystems, birch leaves could adsorb and re-release the specific arthropod-repelling signals such as ledene, ledol and palustrol produced by RT plants. When the distance between two plant species was < 5 m, birches neighbouring RT released a much higher ledene concentration. Similarly, *R. tomentosum* -neighbouring *B. pendula* had also increased emission of a sesquiterpene, alpha-humulene. Terpenes are the most important semi-volatile compounds in this mechanism.

This study is aimed to evaluate if exposure of *Brassica* plants on *R. tomentosum* volatiles will provide protection against the chewing foliar insect herbivore *P. brassicae* as shown earlier by Himanen et al. (2015) with *P. xylostella*. *Pieris* species are widely spread and their caterpillars damage various Brassicacea crops globally (Feber et al. 1997), so better knowledge of the possibilities for *Pieris* biocontrol with natural mechanisms interfering with its olfaction to host such as aromatic VOCs or companion planting will be valuable. Also, it is important to know, how a natural biocontrol agent of *Pieris* species endoparasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) (Driesche 2008; Takabayashi et al. 1998) is influenced by exogenously acquired plant volatiles on host plant surface. Its preferred hosts are young larvae of pierid butterfly species including *P. brassicae* (Brodeur et al. 1996; Harvey et al. 2003; Martijn Bezemer et al. 2010). Parasitoid *C. glomerata* in the fields can disperse quickly (Wanner et al. 2006) and can be found in meadow habitats (Benson et al. 2003). If a potential VOC-based biocontrol strategy would interfere with the olfactory host location of pest natural enemies, the strategy would be less effective, as being strongly compromised by the reduced natural biological control.

Thus, to understand the overall responses of a plant–herbivore–natural enemy complex to environmentally acquired chemical camouflage, we exposed *B. oleracea* var. *italica* (broccoli) plants to the volatiles of RT branches, followed by assessing host selection between RT-exposed and control plants by *P. brassicae* larvae, oviposition by *P. brassicae* females and orientation behaviour of the larval endoparasitoid *C. glomerata*. Younger 2nd instar and older 4th instar *P. brassicae* larvae were used in host selection, because additional terpenoids could be stronger repellent in early instar larvae than late in instar larvae (Rossiter et al. 1986).

We did not include VOC-analysis in this study, because recent studies (Himanen et al. 2015; Mofikoya et al. 2018a) in our laboratory have reported the major RT-emitted volatiles accumulated on the surface of *B. olearacae* plants. The major RT VOCs recovered from the foliage of *B. oleraecea* plants when exposed to the same RT provenance as used in this study were monoterpenes (myrcene and neoallo-ocimene) and sesquiterpenoids (aromadendrene, α-gurjunene, isoledene, ledene and palustrol) (Mofikoya et al. 2018b; Fig. 1).

Materials and methods

Plants and herbivores

Plant material. B. oleracea var. *italica* (broccoli) cv. *Lucky* seedlings were propagated at the Research Garden of the University of Eastern Finland, Kuopio campus. Seedlings were cultivated from seed in 0.66 l pots (four plants per each pot in a greenhouse). Pots filled with mixture 2:1:1 of fertilised compost, NPK 100:30:200 mg l^{-1}: Sphagnum peat (B2) peat NPK 110:40:220 mg l^{-1} and sand) were grown under greenhouse conditions (at temperature 20/16 °C day/night). Broccoli plants were utilized for experiments at around 3 weeks of age for oviposition experiments and 4 weeks of age for larval choice experiment (in order to have more leaf mass).

For VOC exposure experiments, *R. tomentosum* (RT) branches (about 25–30 cm high) were collected from a natural habitat of Suonenjoki in Finland (at 62°38′42.683″N, 27°3′55.383″E), and then kept at + 6 °C in the cold room before use in the *B. oleracea* exposure treatments in fume hoods and temperature-controlled plant exposure room.

Insects for experiments

Eggs of *P. brassicae* (Lepidoptera: Pieridae) were originally derived from the cultures at the Entomology Laboratory of Wageningen University, the Netherlands. Laboratory stocks of *P. brassicae* larvae and adults that were used for the experiments originated from laboratory populations reared from those eggs in Department of Environmental and Biological Sciences, University of Eastern Finland. Our stock larvae were an insect culture maintained on Brussels sprout plants (*B. oleracea* var. gemmifera L. cultivar Cyrus) in a room with controlled temperature at 20 ± 2 °C, with RH 50–70% (Khaling et al. 2015). Moreover, to establish a *P. brassicae* insect population for these experiments, females of this stock which were allowed to oviposit were maintained on broccoli (*B. oleracea* var. *italica*) plants (20–25 °C temperature and 16L: 8D photoperiod) in plant growth chambers.

Fig. 1 Relative proportion of RT VOCs recovered from *Brassica oleracea* foliage after exposure to the Suonenjoki RT provenance (data from Mofikoya et al. 2018a, b). The same RT provenance was used in the insect experiment reported this study. To better describe the type of compound detected. Sector fills: black = volatiles produce by *Brassica* plant; white = RT sesquiterpene alcohol; vertical dash = RT sesquiterpenes; horizontal dash = RT monoterpenes
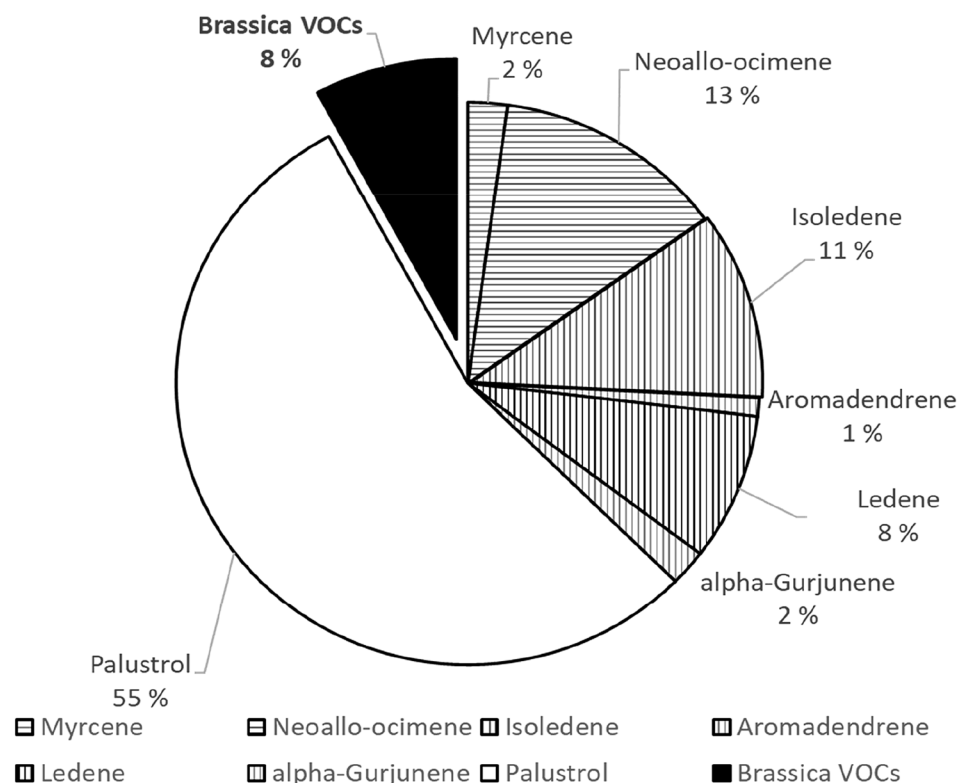
Parasitoids for experiments

Cocoons of parasitoid *C. glomerata* which were originally received from Wageningen University (Khaling et al. 2016) were reared on larvae of *P. brassicae* on broccoli (*B. oleracea*) plants in insect cages in an insect rearing room (conditions: temperature $23° \pm 2$ °C, ~60% RH, L 16 h: D 8 h photoperiod, light intensity of 300 µmol m^{-2} s^{-1}). Adult wasps were fed with 25% honey solution and tap water soaked in cotton wool. To synchronise the emergence of parasitoid *C. glomerata*, *P. brassicae* larvae were parasitized on the same day to be collected, and then the parasitized *P. brassicae* larvae by the wasps were kept on Brussels sprouts plants in cages. After that, the eggs of wasps were collected on the same day and maintained in the same cages to have adults of *C. glomerata* on the same day for these experiments. In addition, *C. glomerata* was reared on *P. brassicae* larvae grown on non-RT-exposed plants.

Exposure of *B. oleracea* to *R. tomentosum* volatiles

For RT exposure, the set-up was identical as used with *B. oleracea* plants in an earlier *P. xylostella* behavioural and volatile analysis study on *R. tomentosum* (Himanen et al. 2015). Here, the corresponding *B. oleracea* var. *italica* control plants with no RT exposure, as well as the equal plants used for RT-exposure were enclosed inside insect cages in fume hoods of laboratory for about 8 h at 22 °C

of the previous day, followed by 16 h at 6 °C (in the cold room temperature, representing typical early summer night-time minimum temperature) until the start of the oviposition, host choice and orientation behaviour of *C. glomerata* experiments (Fig. 2). Bases of 15 cut RT branches with 25–30 cm high (collected from the natural habitat right before the experiments) that had their stems in water-filled 250-ml decanter bottles were confined in insect cages ($33 \times 33 \times 60$ cm, with two sides of the cages covered with a fabric mesh) together with four pots of 21-days-old *B. oleracea* plants (with four plants per pot). The cages for control also had a similar set-up but without RT branches.

Exposure started at 10:00 a.m. at room temperature (+22 °C) in a fume hood, where air flow was from the bouquet of branch towards *B. oleracea* seedlings with the minimum distance of 10 cm between emitters plant and receiver plants. At 6 p.m. the cages were transferred to cold room at temperature of 6 °C until following morning when biotests at 6 °C or at 22 °C with *P. brassicae* larvae or adults were established. The colder temperature at night was used to mimic cold boreal early summer night temperatures. After 24 h, the plants were transferred into insect experiments at 6 °C and 22 °C, respectively.

Oviposition by *P. brassicae* females

The 6-h long oviposition choice experiments of *P. brassicae* adults with RT-exposed and control *B. oleracea* plants
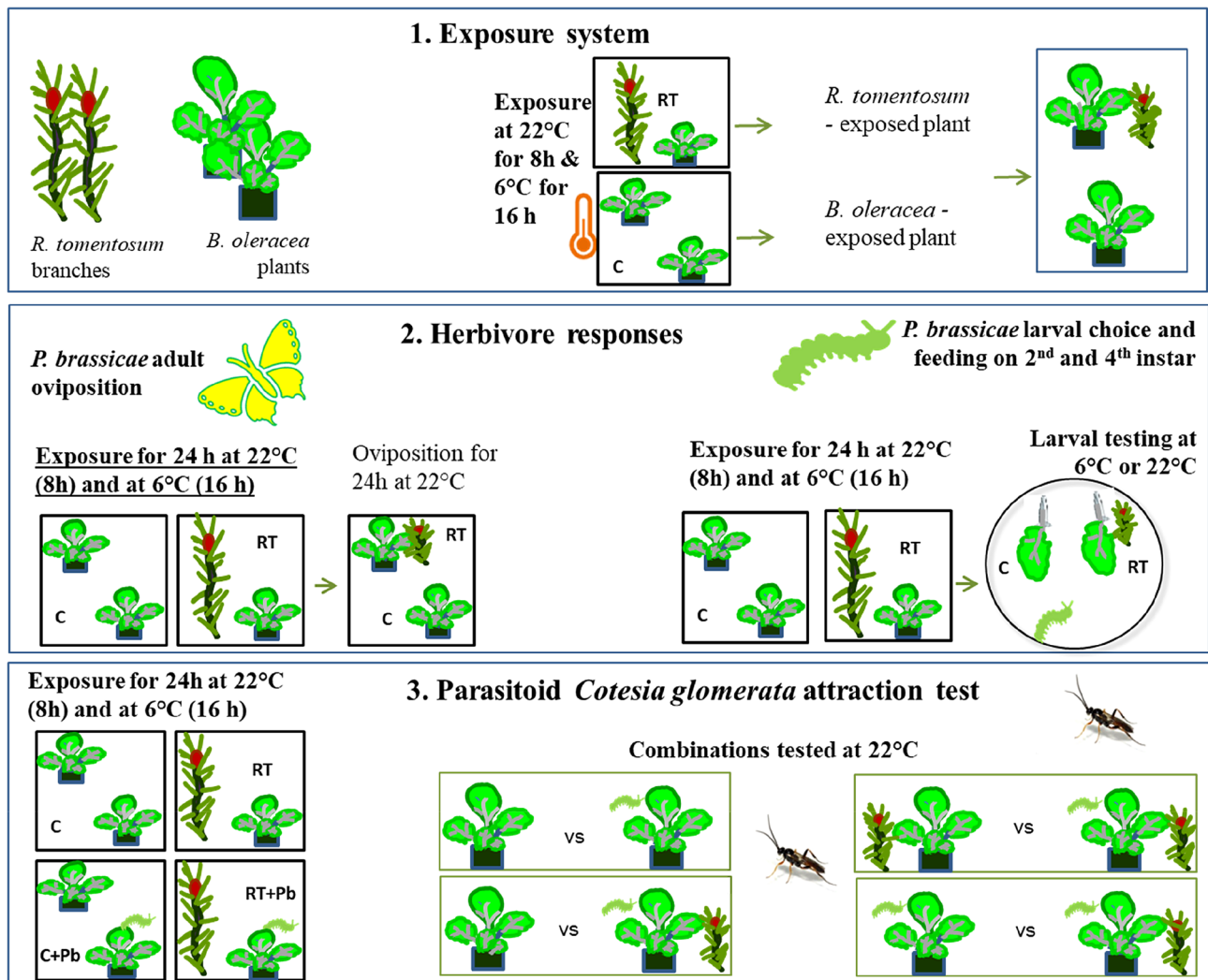
Fig. 2 Schematic presentation of the experimental set ups. There were three parts of testing plant *B. oleracea*, herbivore *P. brassicae* and parasitoid *C. glomerata* responses towards RT- exposure. The used temperatures varied in each part regarding the response to be tested and represented the variations of boreal environmental temperatures between early summer night-time and day-time temperatures. In Part 1, Exposure system (background of our research team), to reveal whether a temperature-dependent adsorption–desorption process of RT semivolatiles occurs on *B. oleracea*, the night-time minimum temperature was a 6 °C versus daytime was used of 22 °C. In Part 2, exposure of *B. oleracea* to RT was conducted and the *P. brassicae* ovipositions were compared at 22 °C (average day-time temperatures). Larval choice and feeding were tested at stabile 6 °C and 22 °C temperatures to allow comparison for the feeding between the night-time versus day-time temperatures along with the RT exposures of representative temperature. In Part 3, female *C. glomerata* parasitoids (day-active) were tested for olfactory orientation. The prior conducted RT exposure were tested at 22 °C because of the average daytime temperature (22 °C). Pb = *P. brassicae*. Photos of parasitoid: Professor Jarmo Holopainen

were conducted in an insect rearing room at temperature of 22 °C. Each experiment took place under approximately 250–300 µmol m^{-1} s^{-1} PAR from 9:00 a.m. until 03:00 p.m. Two similar oviposition experiments with 11 replicate cages were conducted and analyzed separately, because the butterflies used in these experiments originated from the different generations of the insect rearing. Four females and four males of 1–2 days old *P. brassicae* butterflies were randomly selected from the mixed-gender stock population and released into each cage. A vial with mix of honey and water was available for feeding of the butterflies. After a 6-h oviposition period, the *B. oleracea* plants were removed from cages and stored at + 6 °C until egg counting. After egg and egg cluster counting, the number of eggs and egg clusters per each pot (on all four plants) were calculated and used for the statistical analyses.

Host choice and feeding by *P. brassicae* larvae

At two temperatures, either at the cold room + 6 °C or at laboratory + 22 °C, two independent experiments with 48 replicate petri dishes (two similar tests with 24 dishes) were conducted on *P. brassicae* larvae (both 2nd and 4th instars). For herbivore choice experiment, one leaf of RT-exposed and one leaf of control *B. oleracea* var. *italica* plant was enclosed in each petri dish (a diameter of 13 cm) with their petioles in water- filled 1.5 ml Eppendorf tubes and set on a filter paper placed on the bottom of the dish. An equal light intensity (approximately 300 µmol m^{-1} s^{-1}) was created in all experiments by placing similar fluorescent lamps above the dishes.

Brassica oleracea leaves for the experiments were collected from plants at + 6 °C after their 24 h exposure to RT or *B. oleracea* plants (control treatment). Both leaves taken in one petri dish were approximately of the same size to eliminate size effect on larval preference test. Leaves of control and RT-exposed plants were handled with separate forceps to avoid any contamination of RT volatiles on control plant leaves. To test whether adhered VOCs could be stronger repellent in younger than older instar larvae (Rossiter et al. 1986), we selected second- and fourth instar *P. brassicae* larvae to represent younger and older instar larvae, respectively. We determined by head capsule slippage 1st and 3rd instar *P. brassicae* larvae beginning to moult and those were chosen for experiments. One newly molted second-instar (about 12 mm) or four-instar (around 32 mm) larva of *P. brassicae* was released between control leaf and RT-exposed leaf in the middle of each Petri dish. The glass lids of Petri disks were covered and sealed around the Petri dishes by sealing film (Parafilm®, Bemis Company, Inc, Neenah, WI, USA). Thus, the possibly released semi-volatile compounds from leaves could not leak outside.

In each of the 24 h larval choice behaviour test, larval position in the dish was monitored at 10 min intervals during the first 2 h of the test and after this, at 1 h or longer intervals. During the monitoring, the position of the larvae in each dish was recorded to indicate if they were on control leaf, on RT-exposed leaf or outside the leaves indicating "no selection". In total, the trial lasted approximately 24 h. The consumed leaf was not measured because of variable size of leaves between dishes, which each dish had equal sized leaves to be selected by one larva.

Orientation behaviour of *C. glomerata*

Olfactory orientation of the parasitoid *C. glomerata* was studied by using Y-tube olfactometer with four different comparisons (plant pairs) from respective broccoli plants originating from laboratorial treatments at + 22 °C. The comparisons were: (1) intact plant versus host-damaged plant (to verify the functioning of the system for testing orientation of the parasitoid to host damage); (2) intact plant versus host-damaged RT-exposed plant (to determine whether RT exposure disturbs the olfaction of induced volatiles); (3) host-damaged plant versus host-damaged RT-exposed plant (to determine whether *C. glomerata* is able to discriminate between these); and (4) RT-exposed plant versus host-damaged RT-exposed plant (to determine whether *C. glomerata* is intrinsically affected by neighbouring RT VOC exposure, and whether this disrupts host finding). Experimental set-ups of the study are described in Fig. 2.

We used both 2nd and 4th instar larvae to feed on plants to create sufficient fresh damage of diverse-aged larval population, but at same time to avoid total destruction of the seedlings. Control tests without exposing *B. oleracea* plants to RT volatiles were first conducted. For host-damage treatments, the larvae which had moulted to the 2nd and 4th instar, were starved for 2 h and 4 h respectively. Total of 16 both newly moulted 2nd and 4th instar larvae were placed on each damage-treatment *B. oleracea* plant pot (It means that each two of 2nd and 4th instar *P. brassicae* larvae were released on each *B. oleracea* plant. However, there were four *B. oleracea* plants per pot, accounted for 8 of 2nd instar and 8 of 4th instar larvae) to feed for 45 min in laboratory conditions at + 22 °C. The larvae were removed from the experimental plants before using the plants in olfactometer tests.

For host-damaged RT-exposed *B. oleracea* plant treatments, RT-exposed *B. oleracea* plants were kept in a temperature-controlled room (at 6 °C) before the tests were used. To ensure equal damage level along with adhesion and persistence of the exposure-originating VOCs in the test laboratory conditions (at + 22 °C), the larval feeding process to RT-exposed plants was performed for 45 min in the laboratory at + 22 °C. This took place right after those plants were taken from a cold room conditions and the newly molted 2nd and 4th instar larvae were starved for 2 and 4 h, respectively. After this, the larvae were removed and host-damaged RT-exposed *B. oleracea* plant pots were immediately transferred to the laboratory, where olfactometry tests were conducted (as previously described for the host-damage treatment).

Y-tube olfactometer (diameter = 1.6 cm, main arm = 10.5 cm, other arms 10 cm, and angle between them 90°) (Egigu et al. 2010; Pinto et al. 2007) was used to assess the orientation of the parasitoid *C. glomerata*. All the glass wares were sterilized at 121° before use. Moreover, after each tested insect, the Y-tube olfactometer was turned horizontally around, and after every third-tested insect it was rinsed with 96% ethanol.

Test plants in the pot covered with aluminum foil were enclosed inside 22-l glass vessel and both vessels were connected with Teflon tubes into the two arms of Y-tube to assess the orientation of parasitoids (Himanen et al. 2009, 2015). There was an equal and constant flow of

charcoal-filtered and ozone-scrubbed pressurized air in glass vessel, which was adjusted with pressure and needle valves.

The olfactometers was operated at an air-flow rate (500 ml), which was measured every time when plant or jar was changed. It was calibrated with M-5 mini-Buck calibrator between the olfactometer and those two glass vessels. The experiment was conducted in a laboratory with a constant temperature condition at 22 °C. An equal light level (approximately 300 μmol m^{-1} s^{-1}) was provided by with Osram Delux F, 24 W fluorescent lamps (Osram AG, Munich, Germany) by placing one lamp above each vessel and above the olfactometer.

Prior to the olfactory orientation testing, 2- to 4-days-old gravid females of *C. glomerata* wasps were chosen for the Y-tube olfactometer assays. For each trial for testing the orientation behaviour of the female parasitoids, one individual wasp was carefully introduced manually into downwind at the opening of a Y-tube. Each individual female wasp was used only once for the experiments and the behaviour was observed no longer than 5 min, or until they made their final choice earlier. A ''choice'' of parasitoid wasp in orientation behaviour testing was decided, when it walked about two-thirds along an arm of the olfactometer and remained there for at least 3 s. The results were recorded as the choices of the parasitoids which seemed clearly in the pair of the experimented odours. In each experiment, 102–116 wasps were tested for each comparison.

Statistical analyses

All tests were performed with IBM SPSS statistics 25.0 for Windows (IBM Corp., Armonk, NY, USA). All data were checked for normality and equality of variances before statistical analysis and log-transformed to meet the requirements when needed. For larval choice experiment, Chi square tests were used to test the selection frequency of larvae. In the oviposition experiment data of *P. brassicae* females, the egg-numbers per pot (four plants) were analysed by using the paired Student's *t*-test for the main effects of experimental treatment, temperature and their interaction, including the experiment as a random effect, using a mixed model for log-transformed values. The number of egg-clusters per pot were analysed using a non-parametric Wilcoxon Signed Ranks Test for two related samples. The binomial test was used to analyse the data from the orientation response assay of the parasitoid *C. glomerata.*

Results

Oviposition experiments

In the 1st experiment, *P. brassicae* females deposited marginally significantly lower numbers of eggs per pot (four plants) on RT-exposed *B. oleracea* plants than on control *B. oleracea* plants, but there was only a marginal statistical difference ($t_{10} = 2.223$, $P = 0.05$, the paired Student's *t*-test) (Fig. 3a). Furthermore, the number of egg cluster per pot on RT-exposed *B. oleracea* plants were significantly lower ($Z = -2.316$, $P = 0.021$, Wilcoxon Signed Ranks Test) than on control plants. (Fig. 3b).

In exp. 2, the number of laid eggs by *P. brassicae* females on plants in RT-exposed pots were significantly lower ($t_{10} = 2.467$, $P = 0.033$) than on control plants

Fig. 3 The oviposition by *P. brassica* females and the number of egg-clusters per pot on control and *R. tomentosum* (RT)-exposed *B. oleracea* plants in two experiments: **a–d**. "*" letters above charts indicate significant differences at $P < 0.05$ by Wilcoxon Signed Ranks Test
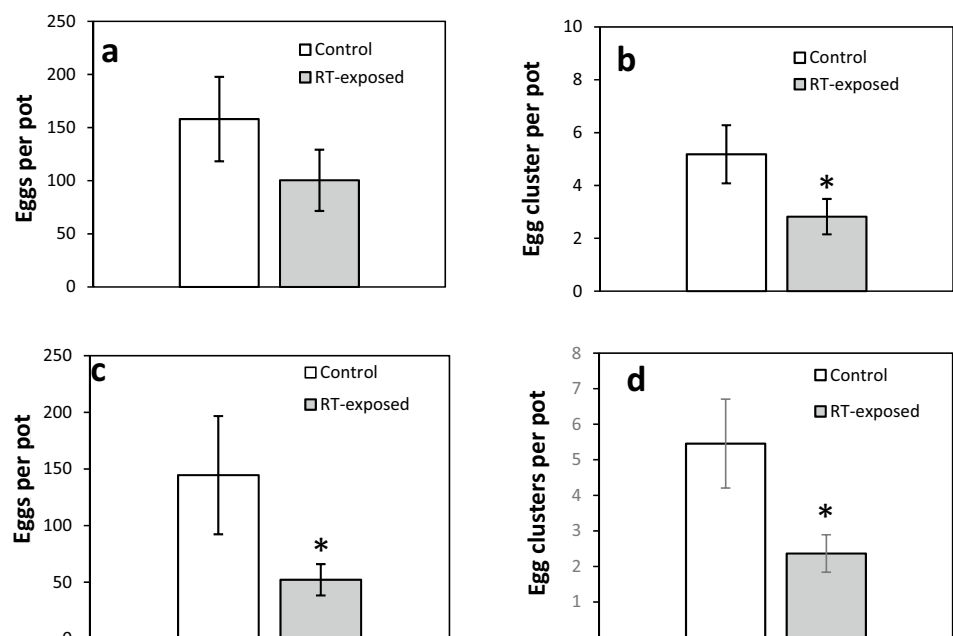
(Fig. 3c). Also, the number of egg clusters on RT-exposed plants were significantly lower ($Z = -2.144$, $P = 0.032$) than on control (Fig. 3d).

Larval choice and feeding preference experiments

Both the 2nd and 4th instar *P. brassicae* larvae at both tested temperatures showed significant and highly significant (2nd instar at 6 °C ($P = 0.041$) and 22 °C ($P < 0.001$), 4th instar at 6 °C ($P < 0.001$) and 22 °C ($P = 0.008$), Chi-square test) avoidance of RT-exposed leaves over the first test hours (tested timeframe varying from 1 to 10 h). However, except at one observation point (at 40 min), there were no significant differences in the 2nd instar *P. brassicae* larval choice behaviour between the control and the RT-exposed leaves (with $P = 0.102$). Additionally, the cold temperature inhibited the development rate of the 2nd and 4th instar *P. brassicae* compared to the warmer temperature at 22 °C. The larval choice activity at 6 °C (Fig. 4a, b) was slower than at 22 °C (Fig. 5a, b), and therefore the test was continued for a longer time at the colder than the higher temperature.

In the 2nd experiment (Fig. 5), the 4th instar *P. brassicae* larvae preferred control to RT-exposed *B. oleracea* leaves, with a statistically significant difference ($P < 0.05$; $P < 0.01$; $P < 0.001$. No asterisk as "–" was used in some cases where ratios are 100:0, Chi-square test). Statistical significance is given in Fig. 5a and b at both temperatures. Overall, RT-exposed *B. oleracea* leaves received less larval choices than equal distribution at 50%, compared with control leaves.

Orientation behaviour of parasitoid *C. glomerata*

The choosing rates of parasitoid *C. glomerata*, which highly significantly preferred volatiles from *P. brassicae*-damaged *B. oleracea* plants over intact plants as an odour source, were > 60% in all four orientation assays (Fig. 6).

RT-exposure of both control and *P. brassicae*-damaged plants did not disturb *C. glomerata* orientation towards damaged plant (test 3). However, the *P. brassicae*-damaged RT-exposed plants were highly significantly less preferred compared to the non-RT-exposed damaged plants (test 4).

Discussion

Our results showed that exposure of broccoli plants to *R. tomentosum* VOCs reduced the oviposition and larval preference of the major herbivore pest *P. brassicae* under laboratory conditions. We did not find clear reduced larval avoidance of leaf disk with adhered RT terpenoids in larger 4th instars larvae when compared to younger 2nd instar larvae as we expected on the basis of results of Rossiter et al. (1998). *Against the hypothesis*, in the beginning of the experiment at +6 °C, small 2nd instar larvae did not show preference to untreated control leaf disks. These observations are in agreement with earlier results indicating EACC and associational resistance by another Brassicaceae herbivore *P. xylostella* after host plant exposure to *R. tomentosum* volatiles (Himanen et al. 2015; Mofikoya et al. 2018a). However, the parasitoid *C. glomerata* preferred non-RT-exposed *P. brassicae* damaged plants compared to RT-exposed *P. brassicae* damaged plants. This
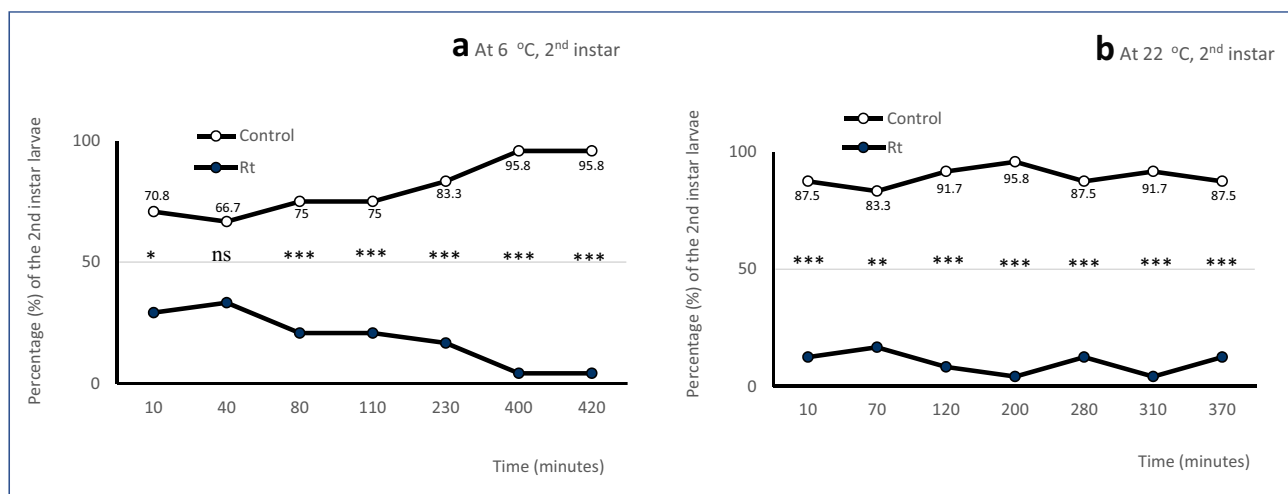

Fig. 4 The 2nd instar larval choice percentages of *P. brassicae* towards Control and *R. tomentosum* (RT)-exposed *B. oleracea* leaves. The choice tests were performed on 48 replicate Petri dishes. Statistically significant larval choice in the selection frequency of larvae were indicated by asterisks (*ns* not significant difference; "*" letters above charts indicate significant differences at $P < 0.05$; "**" at $P < 0.01$; "***" at $P < 0.001$ by Chi square test). The exposure and test temperature were in a) +6 °C, and in b) +22 °C
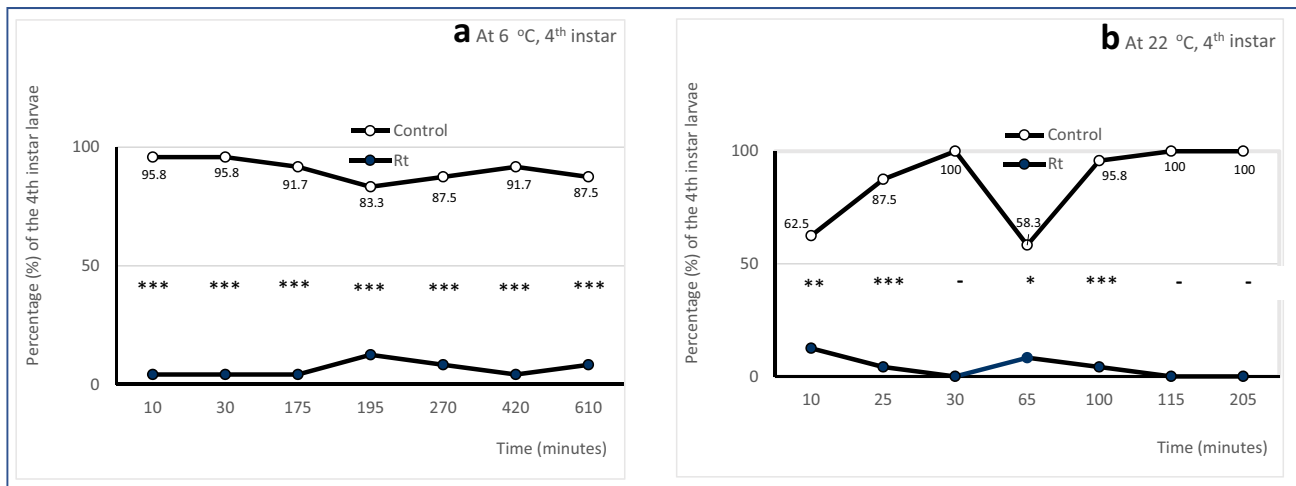
Fig. 5 The 4th instar larval choice percentages of *P. brassicae* towards Control and *R. tomentosum* (RT)-exposed *B. oleracea* leaves. The choice tests were performed on 48 replicate Petri dishes. Statistically significant larval choice in the selection frequency of larvae are indicated by asterisks (*ns* not significant difference; "*" letters above charts indicate significant differences at $P < 0.05$; "**" at $P < 0.01$; "***" at $P < 0.001$. No asterisk as "–" are used in some cases where ratios are 100:0, indicating extreme deviation from the expected 50:50 ratio, in Chi square test). The exposure and test temperature were in **a** + 6 °C, and in **b** + 22 °C
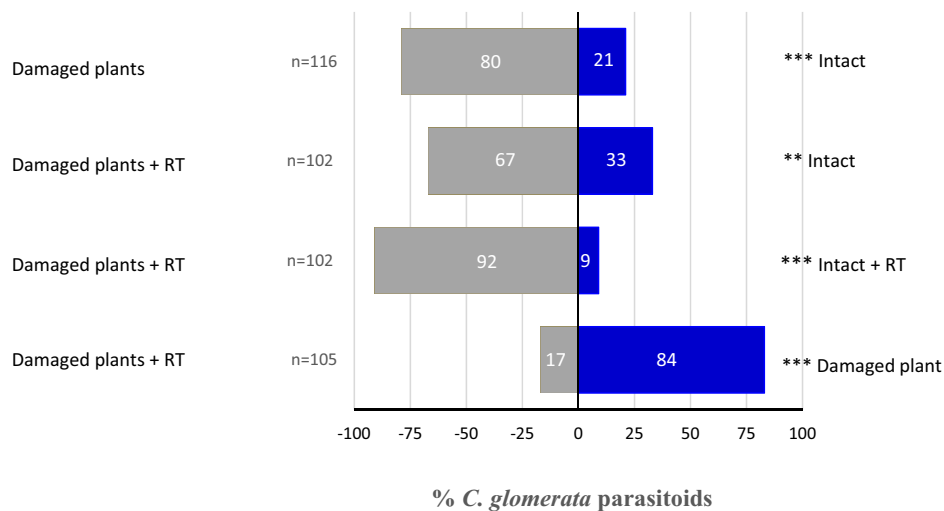

% *C. glomerata* parasitoids

Fig. 6 Orientation of the parasitoid *Cotesia glomerata* in at 22 °C temperature Y-tube olfactometer behavioural assays. The selection of the individually tested parasitoids, regarding both the percentage (%), total number (*n*) of tested parasitoids and number of individuals making a selection towards either of the odour sources in a two-arm olfactometer are reported. Female parasitoids were tested on intact, host-damaged and *R. tomentosum* (RT)-exposed *B. oleracea* plants. Host-damaged plants were subject to *P. brassicae* larval feeding for 45 min, whereas RT-exposed plants neighboured *R. tomentosum* branches for 24 h before testing. Statistically significant preferences in the selections are indicated by asterisks ("**"at $P < 0.01$ and "***" at $P < 0.001$, in binomial test)

was the main difference when compared to behaviour of *Cotesia vestalis* parasitoid, which did not respond to RT-exposure when RT-exposed and non-exposed host-damaged (*P. xylostella* feeding) plants were compared (Himanen et al. 2015). We did not have a possibility to analyse RT-emitted VOCs adsorbed by *B. olearacea* foliage in this experiment, but similar exposure systems have consistently led to notable RT fingerprints in receiver

plant emissions (Himanen et al. 2010, 2015; Mofikoya et al. 2018a). The repellent or camouflaging effects of RT exposure detected in our larval preference and oviposition experiments suggest that the major RT sesquiterpene alcohol palustrol and possibly some other RT-volatiles were successfully adsorbed on *B. oleracea* plants in the used exposure set-up.

Oviposition disturbed by RT exposure of host plant

Oviposition rates by *P. brassicae* females were significantly decreased in one experiment and only marginally significantly decreased in another experiment on RT-exposed *B. oleracea* plants. This suggests that *R. tomentosum* volatiles could regulate pest population pressure on broccoli by affecting egg deposition, but under high oviposition pressure with several females the repellent effect might not be significant. Gravid *P. brassicae* females are sensitive for the chemical quality and VOCs emitted by their host plant. Females can avoid landing on *Brassica* plants that already emit oviposition-induced volatiles indicating future herbivore pressure by larvae of another *P. brassicae* female, interspecific larval competition, and food shortage (Fatouros et al. 2012). However, *P. brassicae* females did not avoid landing on plants having eggs laid by the generalist herbivore species, cabbage moth *Mamestra brassicae* (Fatouros et al. 2012). Similarly, related *Pieris rapae* L. avoided laying eggs on plants damaged by conspecific larvae but did not avoid *P. xylostella* damaged plants (Shiojiri et al. 2015). Our both oviposition experiments indicated reduced egg-cluster number and reduced total egg number on plants exposed to RT-volatiles during the 6 h oviposition period, suggesting that *P. brassicae* females avoid laying eggs on plants that release volatiles atypical for undamaged *Brassica* volatile bouquet. However, we did not monitor, if occupation of control seedlings already by *P. brassicae* eggs led to further oviposition efforts preferably on RT-exposed plants. This should be tested in future more extensive behavioural tests with individually introduced female butterflies.

Gravid *P. xylostella* moths have also laid lower number of eggs on broccoli plants exposed to RT-volatiles than on plants exposed to undamaged broccoli plants (Himanen et al. 2015). Furthermore, Girón-Calva et al. (2016) showed that both *P. brassicae* and *P. xylostella* lay a lower number of eggs on cabbage plants exposed to volatiles of damaged conspecific neighbours than on plants exposed to undamaged conspecific neighbours under field conditions. It was suggested that VOCs from damaged neighbouring plants are passively adsorbed by undamaged plant leaves, altering the foliage quality less suitable for ovipositing females (Girón-Calva et al. 2016). It can be speculated that some of the adsorbed RT volatiles on *Brassica* plant may disturb the natural VOC composition (Conchou et al. 2019) of the host plant of *P. brassicae* so much that it will not anymore meet the sensory requirements for a suitable host (Conchou et al. 2019). Other possibility could be that some of the adsorbed RT VOCs may suppress the sensing of some key VOCs (Hatano et al. 2015) of the host plant by *P. brassicae*.

Pieris brassicae larvae avoid settling on RT-exposed leaves

Our results revealed that younger 2nd and older 4th instar *P. brassicae* larvae consistently avoided RT exposed leaves at both simulated night (+6 °C) and daytime (+22 °C) temperatures. This suggests that despite the different temperatures, the semi-volatile compound had been emitted by RT plants and absorbed by the broccoli leaves. An earlier study, with the diamond back moth *P. xylostella* (Himanen et al. 2015) with a similar laboratory exposure system showed that *P. xylostella* preferred control broccoli leaves over RT-exposed leaves at +12 °C but did not avoid RT-exposed leaves in daytime temperature (+22 °C). In our study *P. brassicae* avoided RT-exposed leaves at +22 °C. This possibly indicates a higher VOC emission rate of RT-branches and consequent higher adsorption rate and longer persistence of RT volatiles on the *B. oleracea* leaves than in study by Himanen et al. (2015). The intraspecific variation of plant VOC composition (Aartsma et al. 2019; Egigu et al. 2010; Himanen et al. 2015; Jankowska 2010; Khaling et al. 2015) such lack of sesquiterpene ledol and high proportion palustrol in RT provenance used in this study (Mofikoya et al. 2018a) could be another reason for variable response of *P. xylostella* and *P. brassicae* responses at +22 °C, but this needs further studies with parallel VOC measurement during behaviour tests.

At cold temperatures (+6 °C), the pest larvae (being Ectotherms) often have less movement, because the cold temperature can cause major disruptions in the central metabolism of cells, leading to the lower and slower metabolic rates and higher energy costs (Denlinger et al. 2010). Indeed, the movement of *P. brassicae* larvae at +6 °C was much slower and the larvae spent much more time for making their choice than at +22 °C. Our results suggest also that, for example, the smaller 2nd instar larvae were more strongly affected by the colder (+6 °C) temperature than the 4th instar larvae, because considerable proportion of the 2nd instar larvae had not selected host plant leaf within the first 230 min. This suggests that the cold temperature could inhibit some individuals of *P. brassicae* larvae, but we may conclude again that it had not become a decisive element, since there were no significantly different results gained from the experiments at +6 °C compared to the warmer temperature at +22 °C. *P. brassicae* larvae development and activity is strongly dependent on temperature. *P. brassicae* larval period last about 12 days at temperatures above 27.5 °C (Bisht et al. 2001; Das 2018) and could be 19 days or longer at 15 °C (Bisht et al. 2001; Das 2018). Therefore, it can be assumed that the carried experiments in the cold temperature condition (at +6 °C) had severely inhibited their host-choice and feeding activity of the 2nd and 4th instar *P. brassicae*.

Overall, the larval behaviour in both temperatures showed rather consistent avoidance of RT-exposed leaves, even if there is a possibility that RT volatiles adsorbed on leaf surface might have been released from RT-exposed leaves into the petri dish air space at +22 °C temperature. Previous studies with RT volatiles (Himanen et al. 2010, 2015; Mofikoya et al. 2018a, b) have not assessed how much emitter plant volatiles adhered on other plant foliage is adsorbed on leaf surface wax layer (Li and Blande 2015) and how much is taken up by stomata (Niinemets et al. 2014). It could be possible that substantial amount of RT volatiles during exposure has been taken up by actively growing *Brassica* leaves though stomata and stored in leaf mesophyll lipids (Noe et al. 2007). Therefore, more detailed studies of uptake and surface adsorbing mechanisms of neighbouring plant VOCs are needed to better understand the importance of bidirectional exchange of biogenic volatiles (Niinemets et al. 2014) for plant–herbivore and herbivore–parasitoid interactions.

We did not analyse volatile or semi-volatile compounds in this study. Following the exposure protocol used with RT exposure in an earlier study (Himanen et al. 2015), which showed adsorption of RT volatiles on *B. oleraceae* leaves, we assume that the RT volatiles were adhered on leaves of exposed plants and were most or at least partly responsible for the detected repellent effects on *P. brassicae* larvae. In natural field conditions, *Betula* spp. foliage was capable to adsorb RT VOCs when *R. tomentosum* was growing as a neighbouring plant and *Betula* seedlings showed some, while not universal, reduction in selected herbivore pressure tested on leaf weevils and aphids (Himanen et al. 2010).

Parasitoid wasps prefer damaged host plants over intact plants, even when exposed to *R. tomentosum* VOCs

RT-exposure did not prevent the Hymenopteran parasitoid *C. glomerata* to orientate towards *P. brassicae*-damaged plants when compared to intact RT exposed plants. However, among *P. brassicae*-damaged plants, this parasitoid preferred damaged plants which were not pre-exposed to RT-volatiles. Earlier study with a specialist larval parasitoid *C. vestalis* of *P. xylostella*, Himanen et al. (2015) found that *C. vestalis* preferred host-damaged plants despite of RT exposure of host plant, but among host-damaged plants RT-exposure did not affect parasitoid preference. In that sense, our results with *C. glomerata* were different and RT-exposure reduced parasitoid orientation to damaged host plants. In field scale, this could mean that a crop plant having higher concentration of adhered RT-volatiles may have lower parasitoid pressure on *P. brassicae* larvae and might face more larval damage. On the other hand, this will be compensated with lower oviposition pressure of RT-volatile-rich Brassica plant by *P. brassicae*.

Lack of response of parasitoids such as *C. vestalis* to exogenous non-host plant VOCs in host plant volatile bouquet (Himanen et al. 2015) can be a result of their high sensitivity to typical herbivore-induced VOCs from plants damaged by their host larvae and the specific key compounds in their composition (Khaling et al. 2016; Ponzio et al. 2014). *C. glomerata* preference to RT-exposed and host-damaged broccoli plant when compared to intact plant indicates that RT-volatiles do not block sensing of herbivore-induced Brassicaceae volatiles in this parasitoid wasp species, but the wasp reduces its preference if a similar bouquet is available without RT volatiles. The differences in *C. glomerata* and *C. vestalis* may indicate that the response to non-typical host plant volatiles could be species specific. Earlier behavioural tests with *Cotesia rubecula* wasps gave evidence that these wasps are not affected by transgenic isoprene-emitting *Arabidopsis* plants damaged by the host moth species. However, another parasitoid wasp species *Diadegma* sp. was repelled by these transgenic Brassicaceae plants emitting isoprene and the wasps preferred wild type plants (Loivamaki et al. 2008).

Potential of neighbouring plant VOCs to protect from herbivores

Companion cropping, for example by planting aromatic companion plants next to crop plants susceptible to major pest insects, is an old method for pest control in traditional and organic farming (Beizhou et al. 2012; Jankowska 2010; Ninkovic et al. 2013; Parolin et al. 2012; Sukegawa and Arimura 2019). The improved resistance of the main crop against herbivores is explained e.g., by neighbour-induced priming and production of chemical defence compounds in healthy crop plants (Sukegawa and Arimura 2019). These compounds act as typical herbivore-induced VOCs in crop plants, which prime plants to be better prepared against herbivore pressure (and competing plant) (Ninkovic et al. 2013). Exposure of *Brassica rapa* L. plants to volatiles of intact peppermint *Mentha × piperita* L. plants by growing them within 10–100 cm distances from peppermint plants, activated transcript accumulation levels of both JA- and SA-signalling-dependent defence genes in *B. rapa* and reduced feeding damage by the Lepidopterous larvae including *Pieris rapae* and *P. xylostella* (Sukekawa and Nakamoto 2018). Another experiment by Sukegawa and Arimura (2019) demonstrated that *P. xylostella* larval growth and oviposition rate were lower on *Mentha*-exposed *B. rapa*. Furthermore, production of herbivore-induced VOCs, reduced preference by stem-borer moths for egg deposition and enhanced parasitism by stem-borer parasitoid wasps in maize plants has been observed when plants were exposed to VOCs released

by intact molasses grass (*Melinis minutiflora*) (Tolosa et al. 2019). These examples demonstrate that there may be yet undiscovered potential in using plant–plant interactions for designing future pest control strategies. However, profound risk assessment of potential negative allochemical effects on crop plants from aromatic natural herbs might also be needed. This will also include the assessment of the capacity of major pest insect species to learn to avoid the chemical camouflage.

In a more mechanistic sense, it is important to note that the VOCs of a companion plant or an intercropped plant are known to affect the crop plant at least by two types of activity modes, as described above. First, companion plant VOCs can be passively adsorbed on crop plant surfaces, where they stay on leaf surface wax layer (Li and Blande 2015) or are taken up through stomata (Noe et al. 2007), but do not activate receiver plant's active chemical defense. The second mode is priming (Sukekawa and Nakamoto 2018) or activation of receiver plant active chemical defense by neighbouring plant volatiles leading e.g. emissions of typical herbivore-induced volatiles (Ninkovic et al. 2013; Tolosa et al. 2019). In our study, the discovered effects on *P. brassicae* behaviour by exposure of broccoli to atypical (RT) VOCs, seems to be more likely due to the first type of mechanisms, with VOCs passively adsorbed on broccoli foliage, although their existence was not measured in this particular study. However, in previous studies, no evidence of herbivore-induced VOCs such as homoterpenes or green leaf volatiles were detected in broccoli (Himanen et al. 2015) or white cabbage (Mofikoya et al. 2018a) emissions after exposure to RT branch emissions. This strongly suggests that our results are indicating EACC. For comparison, when essential oils extracted from plants are directly sprayed on the Brassicacea plants, the high concentration of terpenes might induce some cellular damage and production of typically herbivore-induced homoterpenes that attract e.g., parasitoid of moth larvae (Egigu et al. 2010).

Conclusions

Our results show that exposure of a Brassicaceae horticultural crop plant to the volatile bouquet of *R. tomentosum* will add protection against a specialist pest herbivore at the egg deposition stage and also partly at the larval developmental stage, and may thus reduce the resulting herbivore pressure at several stages of the pest attack. The exposure system we used is known to lead to adsorption and accumulation of *R. tomentosum* volatiles on receiver plants, mimicking environmentally acquired chemical camouflage. Reduced egg deposition and reduced larval preference of *P. rapae* on the sensitive receiver crop plant suggested that such mechanism relying on repellence or disguising of host VOCs by

plants emitting specific semivolatiles, may be useful for suppressing agricultural insect pests. Environmentally acquired chemical camouflage shows potential as one additional tool for an integrated pest management toolbox aiming to develop more sustainable crop protection for future agriculture and horticulture needs. However, the potential multiple mechanisms operating in plant–plant interactions mediated by VOCs, their potential synergies and trade-offs, and whether companion plants with EACC properties improve receiver plant resistance in practice should be assessed rigorously in field experiments.

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