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Potential of Scots Pine for a Push Strategy against the European Spruce Bark Beetle *Ips typographus*

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Abstract: *Ips typographus*, the European spruce bark beetle, is a destructive forest insect that attacks spruce trees. It poses a significant threat to the forestry industry, causing widespread tree mortality and economic losses, and its management through chemical ecology is a promising approach. Attraction relies on aggregation pheromones while repellency is achieved via antiaggregation pheromones combined with mixtures of nonhost volatiles (NHVs), but an effective push and pull strategy has not been established yet. In this study, we observed the beetle colonization dynamic and density comparing pure Norway spruce stacks and Norway spruce stacks with 20% Scots pine logs. We collected the spruce bark volatiles before the beetle colonization and at the end of the trial, and tested the behavioral effects on the beetles of both spruce and pine essential oils in olfactometer trials. In the stacks with Scots pine, we observed a delayed primary colonization and a 65% reduction of the secondary colonization, confirming the colonization disruption mediated by NHVs. Volatile analysis showed a significant change in the bark emission profiles, and the reaching of the antiaggregation phase was marked by the release of verbenone. Behavioral experiments confirmed the attractivity of spruce essential oil and the strong repellency of the pine essential oil. The study supports the use of complex NHVs blends for the beetle behavioral manipulation in the field. Further studies are needed to assess if the same results can be achieved by deploying essential oils in forest stands.

Keywords: bark volatiles; verbenone; Norway spruce; NHVs; behavioral assays

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1. Introduction

In the realm of forest ecology, the European spruce bark beetle *Ips typographus* L. (Coleoptera: Curculionidae) stands out as a significant insect pest that has garnered substantial attention due to its devastating impacts on Norway spruce (*Picea abies* (L.) Karst) forests. The ecological effects of *I. typographus* outbreaks extend beyond individual trees, affecting entire ecosystems and posing considerable challenges to forest management and conservation efforts [1]. *I. typographus* is a native bark beetle species widely distributed across Europe, from Scandinavia to the Mediterranean regions [2]. It primarily infests Norway spruce trees, which constitute a major component of European boreal and temperate forests. Outbreaks of *I. typographus* have been historically recorded and have displayed a cyclical pattern, with periodic population explosions occurring every few decades [3]. However, recent climate changes, including milder winters and prolonged droughts, have altered the dynamics of *I. typographus* populations, leading to an increased frequency and severity of outbreaks [4–6].

The ecology of *I. typographus* is tightly connected to its host tree. At low attack densities, pioneer beetles are more successful at infesting stressed or storm-felled trees than healthy, fully defended trees [7], thus enhancing the survival chances of the population. Pioneer beetles may be drawn to host trees' volatile emissions during the dispersal phase, which provide information about a tree's physiological state, such as its defense capacity [8]. Mass attack mediated by aggregation pheromones (−)-*cis*-verbenol and 2-methyl-3-buten-2-ol [9,10] follows the colonization by pioneers, stimulating the secondary colonization [11]. Adult beetles bore into the bark, where they construct systems of galleries, lay eggs, and introduce a symbiotic fungus, *Grosmannia* spp., which aids in the decomposition of the tree's defense mechanisms [12]. The larvae that emerge from these eggs feed on the inner bark and phloem tissues, disrupting the tree's nutrient transport system and ultimately leading to its decline and mortality [7,13]. Infested stands experience increased tree mortality, altered forest structure, and modified successional trajectories.

Forest managers and policymakers face significant challenges in mitigating the impacts of *I. typographus* outbreaks due to their scale and complexity. Integrated pest management strategies that combine silvicultural practices, pheromone-based trapping systems, and monitoring techniques have been employed to reduce beetle populations and limit damage [14]. Developing effective and sustainable management strategies to mitigate the impacts of *I. typographus* infestations is crucial for preserving forest health and ecosystem stability. In recent years, the use of antiattractant semiochemicals has emerged as a promising approach to reduce *I. typographus* attacks on standing trees. Antiattractant semiochemicals are volatile compounds that interfere with the communication system of bark beetles, reducing their attraction to host trees and disrupting their aggregation behavior [2]. By mimicking or altering the chemical signals released by attacked or suitable host trees, these semiochemicals can deter beetles from initiating attacks on healthy trees [15]. Several antiattractant compounds have been identified and tested for their efficacy against *I. typographus*. One prominent antiattractant compound used in the management of *I. typographus* is verbenone, a bicyclic ketone terpene converted from *cis*-verbenol by the beetles' gut microorganisms [16,17] that acts as an intraspecific pheromone inhibitor of attraction. Verbenone disrupts the aggregation behavior in bark beetles by signaling an already occupied or unsuitable habitat [18]. The application of verbenone-releasing devices in forest stands has demonstrated reduced *I. typographus* infestations and subsequent tree mortality [2]. An antiattractant effect is shown also by nonhost volatiles (NHVs), chemical compounds emitted by plant species that are not suitable or attractive for colonization by the bark beetles. The host searching by specialists such as *I. typographus* can be affected by the volatile diversity in the environment, thus masking the suitable hosts [19].

When combined with aggregation pheromones, the behavioral impact of NHVs on *I. typographus* has been studied, suggesting inhibitory effects on secondary attraction [20–24]. Studies when NHVs combined with the antiattractant verbenone were tested without aggregation pheromones revealed reduced attack rates in treated zones and attacks deflected to surrounding untreated zones [25,26]. However, since verbenone—which is not engaged in primary attraction—was tested alongside NHVs, its possible impact on *I. typographus* primary attraction is yet unknown.

In this work, we tested the potential of a nonhost tree species for a push strategy, starting from an empirical observation from the forest service: adding Scots pine (*Pinus sylvestris* L.) logs to spruce stacks repels *Ips typographus* pioneers and delays colonization even in epidemic conditions. We conducted field and laboratory trials to confirm this observation and gas-chromatographic analysis of the VOCs released by the bark. By doing so, we explored, for the first time, the impact of NHVs in the absence of aggregation and antiaggregation pheromones.

2. Materials and Methods

The study was conducted in South Tyrol, an alpine province in Northern Italy. On 26 October 2018, the Northeastern regions were impacted by the Vaia storm. The storm produced the worst forest loss ever recorded in Italy, consisting of around 8.5 million m^3 of growing stock felled over 41,000 hectares [27]. This dramatic windthrow allowed a huge supply of fell Norway spruce trees, and caused an impressive infestation of the European spruce bark beetle that affected the majority of the Norway spruce forests [28].

2.1. Field Trials

The field trial was established in Freienfeld/Campo di Trens (46°49′50.7″ N 11°31′59.4″ E), in a state-owned forest. On 12 April 2022, healthy Scots pines and Norway spruces were cut from the forest and, on the same day, arranged in stacks. These stacks were either composed of solely Norway spruce logs or mixed with Scots pine logs. They were situated 20 m apart, in a line beside a road along the forest edge (46°50′17″ N | 11°31′35″ O; 46°50′11″ N | 11°31′39″ O), for a total of 6 pure and 6 mixed wood stacks in an alternating pattern. Each wood stack comprised 15 logs; in the mixed wood stacks we had respectively 80% Norway spruce (12 logs) and 20% Scots pine (3 logs). The wood stacks were made when *Ips typographus* was still in diapause. Observations began on 2 May 2022 (T0) and were repeated weekly for three weeks: 9 May (T1), 16 May (T2), and 23 May (T3). In order to assess the bark beetle colonization, the number of bored holes in given area of bark was recorded weekly. Four square frames made of rope measuring 50 × 50 cm were attached to each stack, and the area within them considered. In mixed stacks, it was placed on Norway spruce logs only, avoiding the Scots pines. Within the rope frame area, the active holes producing frass were recorded for three weeks, and the last check was made on 23 May. During the preparation of the wood stacks, it was important to ensure that damage of the bark was minimized to avoid alteration of the released volatiles. The Scots pine stems were mostly placed on the outside of the stacks. Monitoring traps baited with the aggregation pheromones (Pheroprax, BASF, Ludwigshafen, Germany) were set up in the nearby area (Grasstein 46°51′21″ N | 11°30′44″ N, Gupp 46°50′8″ N | 11°27′18″ E, Trens 46°51′52″ N | 11°28′21″ E) to assess the bark beetle flight dynamics. As soon as the flight peak was completed, the trees were collected and debarked to prevent further attacks on standing trees.

2.2. Behavioral Trials

The behavioral studies were aimed at assessing the repellence on Scots pine volatiles and the attractivity of Norway spruce volatiles. To obtain a large amount of volatiles for the numerous replicates, we opted for the extraction of essential oils from the vegetable material of the two plant species. The vegetal material composed of twigs and leaves was collected at the field site and dried at 45 °C for 24 h. Once dried, the material was easily processed by a grinding mill (Grindomix GM300, Retsch GmbH, Germany). A final 750 g of dried material were used for the extraction. These were placed in a 6 L glass balloon connected to a Clevenger system (A.M.S., Trento, Italy) with 3 L of distilled water and 15 g of NaCl salt to enhance the extraction. After boiling, the material was extracted for 2 h, and yielded 2.05 mL of oil for the Norway spruce and 3.9 mL for the Scots pine. The oils were then diluted in paraffin oil (Merck KGaA, Darmstadt, Germany) at concentrations of 10^{-3}, 10^{-4}, and 10^{-5}, and the solutions were stored in a −80 °C freezer upon use. The behavioral trials tested attraction and repellence by using a Perspex four-arm olfactometer [29]. The olfactometer was cleaned prior to each experiment using Teepol® detergent (Teepol, Orpington, UK), 80% laboratory-grade ethanol (Merck KGaA, Darmstadt, Germany), and then distilled water. It was then allowed to dry naturally and lined with Whatman® filter paper (Cytiva, Buckinghamshire, UK) to give the insect a walking surface. The platform of the olfactometer was cross-shaped and separated into four arms, each of which has an area of 6.2 cm^2. Each arm of the olfactometer was fitted with a 2 mL pipette tip that was approximately 5 mm shortened at the tip. To test for the repellency, three of the olfactometer's arms were left as treated, with 30 µg of diluted essential oil loaded onto sterile cotton swab heads placed in the pipette tips, while the fourth arm was considered as a control, with 30 µg of pure paraffin oil. The attractivity tests were performed keeping three arms as control and one arm treated. To prevent outside visual stimulation, the olfactometer was kept in a box covered in black cardboard. A natural spectrum LED plate producing 500 lux of uniform overhead lighting illuminated the entire setup.

Adult *I. typographus* were collected from the traps employed for the monitoring (reported above) on 2 September 2022. The insects were placed in a 40 × 30 × 20 cm box with some fresh bark and stored in an incubator at 12 °C and 65% RH, and they were used within the next five days.

Single beetles were introduced into the olfactometer through a hole in the top. Air was drawn from the olfactometer through the center hole at a rate of 400 mL/min. Before the experiment continued for sixteen minutes, each insect was given two minutes to become used to being in the olfactometer. The beetle was free to move around the olfactometer during the experiment, and OLFA software version 1.1 (F. Nazzi, Udine, Italy) was used to track how much time was spent in each arm. To avoid spatial bias, the olfactometer was moved 90° every 4 min. The experiment was replicated ten times for each concentration of the essential oils, for a total of 60 beetles. Each beetle was only used once and was disposed of after the experiment. Both the time spent in each arm and the number of times the insect entered each arm was noted.

2.3. Bark VOCs Collection and Analysis

The headspace volatiles released by the bark in the field stacks were collected at two different times. The first time was on T0 (2 May), three weeks after the logging and when the stacks were without any sign of attack, while the second time was on T3 (23 May) after the colonization. Four Scots pine logs and four Norway spruce logs were selected from the mixed stacks. The analyzed area was always the bark of a single tree, with no mechanical injuries and protected from direct sun radiation. A BVOC-bag (Cuki, Torino, Italy) was open on the sides and attached to the bark with duct tape to create a confined headspace. A corner was cut and an adsorbent trap (glass tube, 6.5 × 0.55× 0.26 cm, loaded with 1.5 mg activated charcoal; CLSA filter LR-type; Brechbühler AG, Schlieren, Switzerland) was inserted in the headspace. The air was circulated through the trap and back in the headspace with a graphite pump. The collected VOC samples were eluted from the adsorbent traps with 100 µL GC-grade dichloromethane (Merck KGaA, Darmstadt, Germany) and stored at −80 °C prior to the GC–MS analysis. The collection time was 3 h, and both the collections were made from 11:00 to 14:00. The air temperature recorded at the collection site was 18–20 °C. The vials were transported in cold box and stored at −80 °C. As a negative control, volatiles were also collected from empty sampling bags attached to a glass panel with the same duct tape.

Two µL of the eluted bark volatiles were injected in a splitless mode in a GC system (7890A) coupled with an MS (5975C Network) (Agilent Technologies, Santa Clara, CA, USA) for the identification of the compounds. The GC column was a nonpolar HP-5MS (Agilent Technologies) with 0.25 µm coating, 30 m length, and 0.25 mm diameter. Helium at a flow rate of 1.2 mL/min was used as carrier gas. The oven method was programmed as follows: inject at 50 °C and hold for 1.5 min, then 7.5 °C/min until 250 °C and hold for 5 min. The temperature of the injector was 250 °C. The transfer line was set at 280 °C. The GC–MS data acquisition and analysis were performed using the MSD ChemStation software E.02.021431 (Agilent Technologies). Compounds were initially identified by mass spectra comparison with the databases NIST 14 (Gaithersburg, MD, USA) and Wiley 7N (Wiley, Hoboken, NJ, USA). Linear retention indices (LRI) of the peaks were calculated by using a mixture of n-alkane standards (nC9-nC20, Merck KGaA, Darmstadt, Germany). The obtained LRI values were compared with reference LRI values available in the literature. Finally, the identity of compounds was confirmed by comparing the mass spectra and the retention times with those of authentic standard compounds (Merck KGaA, Darmstadt, Germany).

2.4. Statistical Analysis

The data were analyzed using the software R version 4.3.1 [30].

- Stack volumes were compared with an analysis of variance test (ANOVA).
- The number of holes in the logs between pure and mixed stacks were analyzed for each time with an ANOVA test.
- The effect of stack position was analyzed with a generalized linear mixed effect model (glmer) from the lme4 package [31]. The number of holes was considered as response variable, while stack type, time and position were addressed as explanatory variables. The stack and the time were accounted as random variables to control for the variability.
- To test the attraction toward spruce essential oil, one arm was treated and tested against three blanks. The mean of the values from the three blank arms was considered for the statistical analysis. Reversely, the mean of the three treated arms was considered in the repellence from pine oil trials. Each concentration was tested separately. The number of entries in the treated arm vs. the control one was analyzed with a logistic regression. The time spent in the arms was tested with an ANOVA test.
- A dimension reduction principal component analysis (PCA) was performed using the R package "factoextra" [32]. The PCA calculated the combination of the compound area data by extracting eigenvalues and eigenvectors of a correlation matrix and then highlighting principal components. A two-dimensional score plot was created to compare the bark volatile profiles of spruce and pine logs at T0 and T3 of the field trial. Comparison of the point distances was achieved by analysis of variance using distance matrices (package Vegan [33]).

The package ggplot2 [34] was used for the graphics. All the values are reported as average ± standard deviation.

3. Results

3.1. Field Trials

The stack volumes did not differ between mixed and pure ones ($F_{1,10} = 1.06$, $p = 0.32$). Each log had a commercial length of 4.20 m and the average diameter was 25.2 ± 6.8 cm. The average volume in the mixed stacks was 3.52 ± 0.38 m^3 and 3.23 ± 0.49 m^3 in the pure spruce stack. However, the volumetric contribution of the Norway spruce logs was significantly higher in the pure stacks than in the mixed ones (2.66 ± 0.3 m^3) ($F_{1,10} = 5.838$, $p = 0.036$), as expected.

The difference in number of entry holes between mixed and pure wood stack confirmed the empirical observation. At T0, no entry hole was observed in any stack. At T1, when the colonization of *I. typographus* began but was still low, no entry hole was recorded in any of the six mixed stacks, but an average of 18 ± 7.64 holes/m^2 were recorded in the six pure stacks. This effectively worked until the colonization increased to the point that the Norway spruce in the mixed stacks was attacked, but a clear difference was still noticeable: at T2, the mixed stacks had an average of 20 ± 14.54 holes/m^2 and the pure ones an average of 76.66 ± 41.19 holes/m^2 ($F_{1,10} = 10.1$, $p = 0.009$), and at T3 the mixed stacks had an average of 37.16 ± 17.29 holes/m^2 against an average of 105.33 ± 46.65 holes/m^2 in the pure ones ($F_{1,10} = 11.26$, $p = 0.007$) (Figure 1). At T3, the summed number of holes for the six pure stacks (considering all the 24 frames, equal to 6 m^2) was 632, while in the mixed ones it was 223, with a difference between the two of 64.71%.

It was also noticed that the bark beetle attack was affected by the position of the stack in the row. The more toward the middle of the row the stack was, the higher was the colonization ($z = 4.40$, $p < 0.001$) (Figure 2).

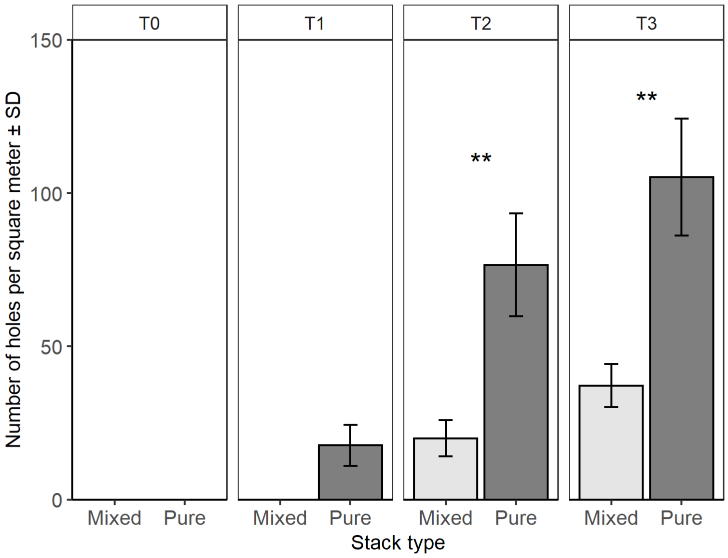

Figure 1. Number of holes counted on Norway spruce (*Picea abies* L.) bark within the four 50 × 50 cm frames positioned on the stacks (n = 6 + 6) comparing pure Norway spruce wood stacks with mixed wood stacks (80% Norway spruce + 20% Scots pine (*Pinus sylvestris* L.)). Times are reported as T0: 2 May 2022, T1: 9 May 2022, T2: 16 May 2022, and T3: 23 May 2022. Asterisks report statistical significance: ** $p < 0.01$.

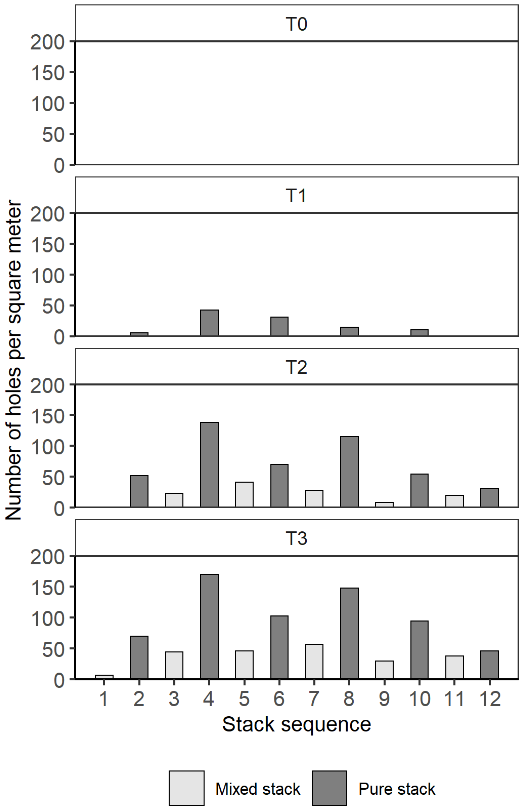

Figure 2. Sum of holes counted on Norway spruce (*Picea abies* L.) bark within the four 50 × 50 cm frames positioned on the stacks (n = 6 + 6) comparing pure Norway spruce wood stacks with mixed wood stacks (80% Norway spruce + 20% Scots pine (*Pinus sylvestris* L.)). Stacks are reported in the row order they were positioned in the field. Times are reported as T0: 2 May 2022, T1: 9 May 2022, T2: 16 May 2022, and T3: 23 May 2022.

The pheromone traps determined the *I. typographus* flight dynamic during the experimental weeks (Figure 3). The flight reached its maximum at T2 (16 May) and the trial was therefore considered concluded at T3 (23 May) when no more beetles were caught.

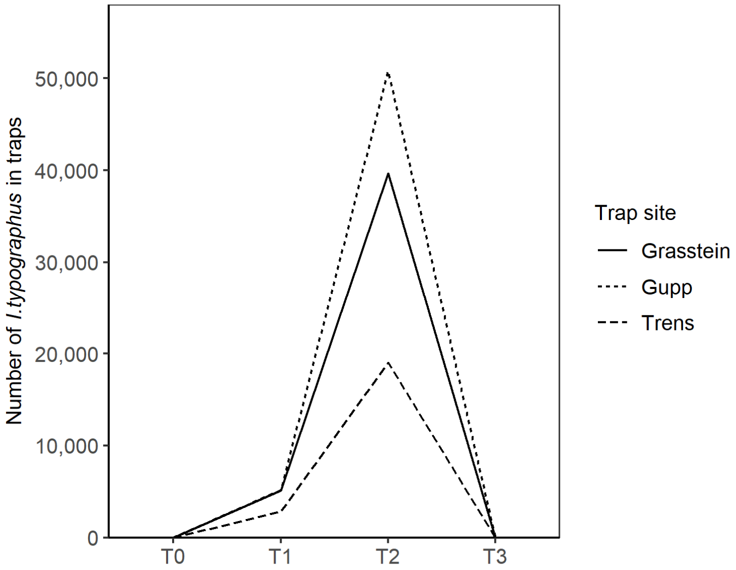

Figure 3. Weekly number of adult *Ips typographus* L. captured in traps loaded with pheromone lures (Pheroprax, BASF, Ludwigshafen, Germany) at three sites nearby the field trial location (Grasstein 46°51′21″ N | 11°30′44″ N, Gupp 46°50′8″ N | 11°27′18″ E, Trens 46°51′52″ N | 11°28′21″ E). Times are reported as T0: 2 May 2022, T1: 9 May 2022, T2: 16 May 2022, and T3: 23 May 2022.

3.2. Behavioural Trials

The Norway spruce showed an attractant effect on *I. typographus*. The number of entries in the treated arm was higher than the blank one at concentration 10^{-3} (7.90 ± 2.51 vs. 4.60 ± 2.06) ($z = -2.91$, $p = 0.003$) and 10^{-4} (6.70 ± 4.21 vs. 4.50 ± 2.06) ($z = -2.06$, $p = 0.038$), while no difference was detected at 10^{-5} (3.88 ± 0.60 vs. 3.44 ± 1.50) ($z = -0.49$, $p = 0.62$) (Figure 4A). The time spent in the arms was affected by the treatment at all concentrations. Interestingly, at the highest concentration, 10^{-3}, the difference was only almost significant ($F_{1,18} = 3.51$, $p = 0.077$) (3.61 ± 1.29 vs. 2.80 ± 0.43 for treated and blank, respectively), while it was significant at 10^{-4} (3.81 ± 1.21 vs. 2.73 ± 0.40 for treated and blank, respectively) ($F_{1,18} = 7.07$, $p = 0.015$) and at 10^{-5} (3.79 ± 0.87 vs. 2.78 ± 0.31) ($F_{1,18} = 10.67$, $p = 0.004$) (Figure 4B).

The Scots pine proved to be repellent. The beetles entered the blank control more often than the treated one (Figure 4C). The effect was significant at concentration 10^{-3} (6.4 ± 3.37 vs. 4 ± 1.69) ($z = -2.33$, $p = 0.019$) and concentration 10^{-4} (5 ± 2.05 vs. 3.2 ± 1.39) ($z = -1.97$, $p = 0.047$). The number of entries did not vary between blank and treated at concentration 10^{-5} (5.2 ± 2.29 vs. 4.5 ± 1.17) ($z = -0.71$, $p = 0.48$). Similarly, the time spent in the blank arm was higher than in the treated arm for all the concentrations: 10^{-3} 4.57 ± 0.41 vs. 2.48 ± 0.13 ($F_{1,18} = 232.2$, $p < 0.001$), 10^{-4} 4.06 ± 1.45 vs. 2.63 ± 0.46 ($F_{1,18} = 8.67$, $p = 0.008$), and 10^{-5} 3.83 ± 1.03 vs. 2.73 ± 0.35 ($F_{1,18} = 10.29$, $p = 0.004$) (Figure 4D).

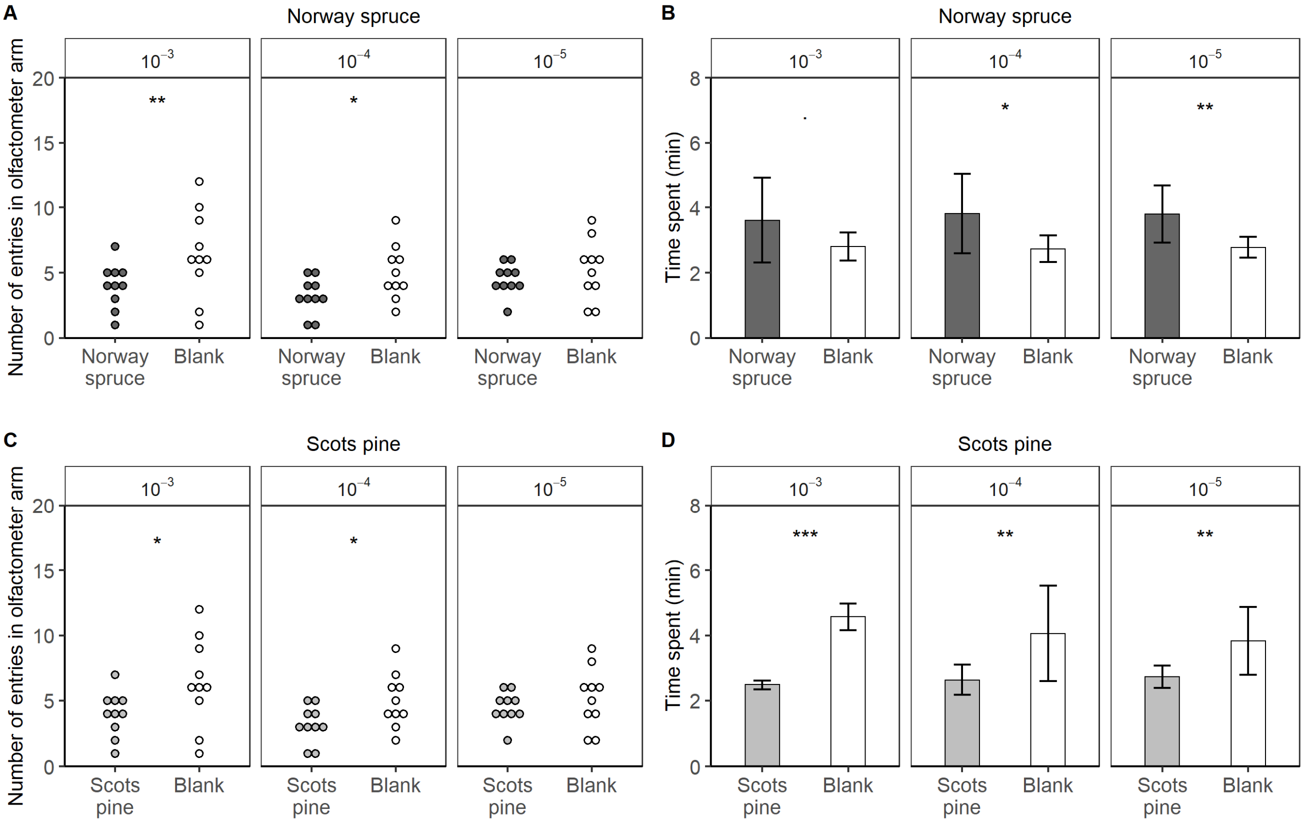

Figure 4. Behavioral responses of *I. typographus* L. in 4-way olfactometer essays (n = 10). For Norway spruce (*Picea abies* L.): (**A**) number of entries in the olfactometer arm and (**B**) time spent in the arm. For Scots pine (*Pinus sylvestris* L.): (**C**) number of entries in the olfactometer arm and (**D**) time spent in the arm. Norway spruce and Scots pine essential oils were diluted in paraffin oil at concentrations of 10^{-3}, 10^{-4}, and 10^{-5}. During a 16 min trial, the time spent in treated (darkened) or blank arms and the number of entries in each arm were considered. Asterisks report statistical significance: · $p \sim 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. VOCs Analysis

The gas-chromatography identified 31 compounds released by the Norway spruce logs bark and 28 by the Scots pine logs bark (Table 1). Spruce and pine logs were characterized by many aromatic hydrocarbons, both in the first (T0) and in the second (T3) collection, with some amount variations. In the Norway spruce, 1,3,5-trimethyl-benzene ($\chi^2 = 4.5$, $p = 0.033$) increased after three weeks, while many others decreased: 1-methyl-3-propyl-benzene ($\chi^2 = 3.7$, $p = 0.042$), 1-ethyl-2,5-dimethylbenzene ($\chi^2 = 5.6$, $p = 0.027$), 1-methyl-2-propyl-benzene ($\chi^2 = 4.7$, $p = 0.029$), 2-ethyl-1,3-dimethylbenzene ($\chi^2 = 3.4$, $p = 0.046$), 1-ethyl-2,4-dimethylbenzene ($\chi^2 = 4.1$, $p = 0.037$), 2-ethyl-1,4-dimethylbenzene ($\chi^2 = 4.5$, $p = 0.033$). In the Scots pine, the aromatic hydrocarbons varied little, with only 2-ethyl-1,4-dimethylbenzene decreasing at T3 ($\chi^2 = 4.3$, $p = 0.036$). Monoterpenoids were common in both tree species, and only in the Scots pine did α-pinene increase considerably after three weeks ($\chi^2 = 7.8$, $p = 0.025$). Verbenone, the *I. typographus* antiaggregation pheromone, was found to appear in Norway spruce samples at T3, thus confirming a successful mass colonization.

Table 1. Summary table of the volatile compounds identified by GC/MS analysis of the headspace of Norway spruce (*Picea abies* L.) and Scots pine (*Pinus sylvestris* L.) bark and their amounts (10^{-6} TIC ± SD) in field conditions (n = 4 + 3) at T0 (2 May 2022) and after three weeks (T3, 23 May 2022). The volatile compounds were collected by CLSA technique for 3 h. The compounds were identified by mass spectrometry and confirmed by linear retention indexes available from the literature or laboratory standards when available. The table also reports the *p*-value of the Kruskal–Wallis test of the TIC amount. The number of samples where the compound was found is reported between brackets. Asterisks indicate statistical significance between collection times (* = $p < 0.05$).

Compound	**LRIa**	**LRIb**	**Norway Spruce**		**Scots Pine**	
			T0	**T3**	**T0**	**T3**
Aromatic hydrocarbons						
toluene	<800	770[1]	6.3 ± 0.9 (3)		107 ± 165.2 (4)	
1,3,5-trimethyl-benzene	998	996[1]	15.9 ± 3.4 (3)	32.4 ± 10.8 (3) *	28.1 ± 18 (4)	37.1 ± 51.8 (3)
1,2,4-trimethyl-benzene	1001	1001[2]	17.2 ± 2.6 (3)		21.3 ± 18.7 (3)	
1,2,3-trimethyl-benzene	1029	1029[2]	76.8 ± 17.7 (4)			
1-methyl-3-propyl-benzene	1058	1059[2]	74.3 ± 20.1 (4) *	15.1 ± 7.1 (3)	47.2 ± 41.2 (4)	11.9 ± 14.1 (2)
1-ethyl-2,5-dimethylbenzene	1062	1063[2]	68.7 ± 30.7 (4) *	19.9 ± 9.9 (3)	48.6 ± 41.3 (4)	14.6 ± 17.1 (2)
1-methyl-2-propyl-benzene	1072	1073[2]	36.2 ± 13.9 (4) *	9.9 ± 5.8 (3)	17.6 ± 13.8 (4)	7.1 ± 8.4 (2)
2-ethyl-1,3-dimethylbenzene	1083	1082[2]	165.2 ± 69.7 (4) *	44.5 ± 25 (3)	79 ± 61.8 (4)	22.4 ± 32 (3)
1-ethyl-2,4-dimethylbenzene	1090	1089[2]	169.3 ± 64.4 (4) *	47 ± 25.4 (3)	85.4 ± 65.7 (4)	28.3 ± 42.6 (3)
2-ethyl-1,4-dimethylbenzene	1097	1090[2]	18.9 ± 8.8 (4) *	8.2 ± 5.2 (3)	7.7 ± 4.6 (4) *	2 ± 0.9 (2)
1,2,3,4-tetraisopropyl-5-methylene-1,3cyclopentadiene ‡	1111		48.5 ± 26.2 (4)	16.1 ± 10 (3)	21.6 ± 15.1 (4)	9.9 ± 14.1 (3)
1,2,4,5-tetramethylbenzene	1121	1121[2]	50 ± 29.6 (4)	18.4 ± 11.4 (3)	19.1 ± 12.7 (4)	15 ± 18 (2)
1,2,3,5-tetramethylbenzene	1125	1125[2]	73 ± 44.9 (4)	26.2 ± 15.8 (3)	29.2 ± 20.5 (4)	14 ± 20.1 (3)
4-methylindan	1144	1144[2]	14.9 ± 8.6 (4)	11 ± 5.8 (3)	7.4 ± 5.7 (4)	6 ± 7.3 (3)
1,2,3,4-tetramethylbenzene	1156	1155[2]	29.1 ± 18.6 (4)	12 ± 7.2 (3)	11.6 ± 7.1 (4)	10.3 ± 12.5 (2)
2,6,6-trimethyl-bicyclo-heptan-3-one ‡	1166		12.1 ± 6.9 (4)	7.3 ± 4.2 (3)		
Monoterpenoids						
α-pinene	941	941[2]	82.1 ± 145.6 (4)	165.6 ± 150.4 (3)	77.8 ± 47.8 (4)	801.6 ± 626.2 (3) *
β-pinene	979	983 †	113.9 ± 203.1 (4)	170.3 ± 153.9 (3)	123.5 ± 97.6 (3)	126.7 ± 163.8 (3)
β-myrcene	993	996 †	43.4 ± 74.2 (4) *	8 ± 1.3 (3)		
β-terpinene	1036	1036[2]		193.9 ± 40.4 (3)	100.6 ± 65.1 (4)	32.1 ± 24.6 (3)
isopinocamphone	1180	1180[1]	6.2 ± 3.1 (3)	11.3 ± 5.9 (3)	3.2 ± 1.8 (2)	
α-terpineol	1215	1218[1]		7 ± 4.3 (3)		
verbenone	1292	1285[1]		1.7 ± 0.3 (3)		
Sesquiterpenoids						
α-cubebene	1360	1360[2]				4.7 ± 4 (2)
cyclosativene	1379	1380[4]				8.2 ± 11 (3)
α-copaene	1388	1388[2]			1.8 ± 0.6 (2)	20 ± 26.3 (3) *
2-epi-α-funebrene	1395	1396[2]		9.8 ± 2.8 (2)		
β-bourbonene	1398	1391[4]			2.8 ± 2.1 (2)	10.4 ± 15.6 (3)
α-cedrene	1426	1423[2]		3.8 ± 2 (2)		
β-copaene	1443	1444[2]				18.5 ± 25.1 (3)
γ-muurolene	1458	1457[4]				7.7 ± 3.9 (2)
zonarene	1511	1518[2]		3 ± 2.4 (2)		
γ-cadinene	1525	1525[2]		6 ± 6.2 (2)		
δ-cadinene	1533	1539[3]		6.9 ± 7.4 (2)		
Esters						
bornyl acetate ‡	1362			5.7 ± 2.6 (3)		
Alcohols						
benzyl alcohol	1040	1039[2]			9.2 ± 2.4 (3)	
Alkenes						
1-tetradecene	1420	1418[2]		7.1 ± 3.8 (2)		
3-pentadecene	1494	1491[2]		2.7 ± 1.3 (2)		

Table 1. *Cont.*

Compound	LRIa	LRIb	Norway Spruce		Scots Pine	
			T0	T3	T0	T3
Alkanes						
2,2,4,6,6-pentamethyl-heptane	993	994 [2]			14.8 ± 9.6 (3)	16.8 ± 18.6 (2)
4-methyl-undecane	1167	1167 [2]			5.5 ± 2.4 (4)	5.5 ± 6.3 (3)
3,5-dimethylundecane	1215	1211 [2]			1.4 ± 0.3 (3)	
nC15	1502	1500 †		1.7 ± 0 (2)		
nC16	1601	1600 †		1.4 ± 0.3 (2)		
nC17	1702	1700 †		2.1 ± 0.4 (2)		

LRIa = linear retention index calculated in relation to n-alkanes. LRIb = linear retention index already published in peer-reviewed journals and listed in Pherobase [1], PubChem [2], and NIST [4]. The review of [35] was also considered [3]. When possible, the LRI was verified by a standard compound (†). ‡ = Putatively annotated compound.

A considerable amount of sesquiterpenoid was released by the Norway spruce bark only at T3, 41 days after the logging. Also in Scots pine, sesquiterpenoids appeared in high amounts in the second collection, having only a few of α-copaene and β-bourbonene emitted at T0, with α-copaene increasing (χ^2 = 5.3, p = 0.028). Linear alkanes and alkenes were found in the Norway spruce only, while Scots pine released some methylated alkane.

The volatilome of the tree bark changed according to the plant species and the colonization stage (Figure 5). PCA revealed a significant difference between T0 and T3 for both Norway spruce ($F_{(1,6)}$ = 6.2, p = 0.028) and Scots pine ($F_{(1,6)}$ = 3.22, p = 0.028).

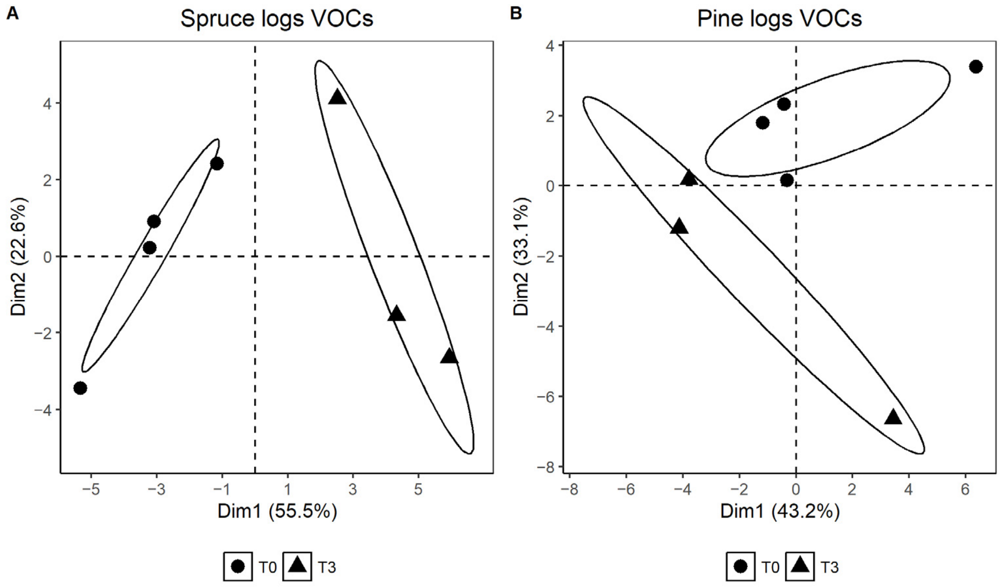

Figure 5. Principal component analysis score plot of the headspace bark volatiles of (**A**) Norway spruce (*Picea abies* L.) and (**B**) Scots pine (*Pinus sylvestris* L.) collected on T0 (2 May 2022) and T3 (23 May 2022) of the field trials. The trees were logged and the stacks prepared on 12 April 2022.

4. Discussion

This study demonstrates that a minor contribution of pine logs (nonhost) to the spruce stacks was enough to interfere with the host colonization process by the European spruce bark beetle *I. typographus.* The colonization, estimated by the number of active holes, was delayed at least one week and reduced at the end by 65%. Laboratory behavioral trials confirmed the repellency of the pine volatiles and the attractivity of spruce volatiles from the essential oils. Gas-chromatographic analysis highlighted the differences between the two plant species and showed a clear evolution of the volatilome during the field trial. We observed that the blend of volatiles released by the nonhost plant in absence of antiaggregation pheromone produces valuable information for pest behavioral manipulation in field conditions.

Nonhost volatiles from the leaves and bark of nonhost trees can be detected by conifer bark beetles with their antennae, and they also avoid the NHVs [17,32]. The repellency of NHVs to *I. typographus* deters beetles from boring into nonhost trees. Several studies have investigated the antennal [22,36–39] and behavioral [15,20,21] responses to volatiles from angiosperms or other nonhost plants. Others have evaluated the feasibility of NHVs application in field repellency trials [24,40]. To the best of our knowledge, none has tested the repellency of NHVs during the primary attraction, as they were always presented in combination with either aggregation (simulating a secondary attraction) or antiaggregation (enhancing the push effect) pheromones. Our results show the potential of Scots pine NHVs to partially disrupt the primary attraction by delaying host finding, and to reduce secondary attraction. Due to the fact that only verbenone was emitted by the Norway spruce bark and no aggregation pheromones (2-methyl-3-buten-2-ol, *cis*-verbenol, and ipsdienol) were detected by T3, we are confident that secondary attraction and the colonization phase was complete.

Despite the much lower density of entry holes in the mixed stacks, the release of the antiaggregation pheromone verbenone from the Norway spruce logs in these stacks at T3 confirms that the mass colonization was being achieved. Generally, verbenone marks reaching high colonization density, and this study suggests that the presence of pine lowers this limit compared to pure spruce stacks. It is unlikely that further colonization would have occurred in the mixed stacks if they remained in the field longer because verbenone was already being produced. It is then possible that in the presence of pine logs the infestation threshold for the initiation of verbenone production is lowered or that the production of verbenone is proportional to the population density, but supporting research is currently lacking. Had it been so, a higher amount of verbenone from the spruce logs of the pure stacks could have been detected. However, verbenone has often been found at the early stages of the lifecycle [16], and this contradicts its putative function as an indicator of mass colonization. Despite that the spruce logs in the mixed stacks might have not reached a high colonization density, it still remains unlikely that the logs could have been further colonized, and this circumstance is also supported by the fact that low pheromone trap captures by T3 indicated the flight peak was already over.

Lastly, it is worth considering that, since verbenol is an oxidation product of α-pinene, and verbenone can be spontaneously converted from verbenol by autoxidation [16], the presence of verbenone might be incidental. Similarly, [41] reported that oxygenated monoterpenes α-terpineol, terpinen-4-ol, bornyl acetate, (*Z*)-pinocarveol, and verbenone were released in the beginning of the attack, possibly due to spontaneous oxidation of monoterpene hydrocarbons from the tree. In our study, three monoterpenes (α-terpineol, bornyl acetate, and verbenone) were actually found in the Norway spruce bark emission, but only at T3, and not in the Scots pine. Despite a consistent amount of the precursor a-pinene, no verbenone was detected in the Scots pine VOCs, contrary to [18] who reported that noninfested control Scots pine logs released little verbenone at a constant rate. This could be due to low emissions beyond the detection threshold, but its presence and abundance in the Norway spruce logs seems likely to be related to the beetles' activity rather than chance. Verbenone is not the only antiaggregation pheromone, as ipsenol also plays a key role, but it has not been found among the collected VOCs, nor verbenol, the precursor of verbenone with aggregation function.

So far, NHVs were deployed in field trials as synthetic compounds in single or mixture formulations [21,23,42] and always combined with verbenone to enhance the antiattractant effect. Our 64.71% difference in the colonization density falls well within the estimated 60% effect magnitude in the meta-analysis by [40] that expressed it as a "% reduction" derived from an average effect size. The whole logs with their natural occurring volatile blends suggested a substantial reduction in the colonization success of the logged trees even without the employment of verbenone. However, the literature analyzed by [40] considered the number of trees attacked or killed and not with stacked logs, as in our study. Therefore, comparisons with our result must be considered carefully. Nonetheless, the situation offered to the beetle in our experimental setup was extremely favorable: a mass of cut stressed trees, with a compromised defense system, clustered together during a massive *I. typographus* outbreak. Whether the attractivity of this scenario is comparable to that of a suitable hosts stand is not our purpose to tell, but it must be just as tempting, and the results we obtained should be regarded as remarkable. The increasing colonization density converging towards the middle of the stacks line indicates an effect of the overall log mass.

A possible explanation for the colonization reduction we observed is the proximity of the host and the nonhost. Having *I. typographus* likely evolved in mixed forests landscapes [43], it is tuned to avoid nonhost trees [44], and the presence of pine log in the stacks might deflect the beetles away from the whole stack rather than to the neighboring logs. Further studies with nonhost logs located at increasing distance from spruce trap logs are required to assess this point.

Protection of the logged trees can be achieved in other ways (e.g., bark removal), as Scots pine or other nonhosts cannot always be available. Our results find, therefore, no applicable use in stacks protection but provide evidence for a strong repellency effect of NHVs alone without verbenone during both the primary and secondary attraction. The reason for such a repellence might rely on the use of the whole nonhost tree, rather than a blend of selected NHVs. [45] suggested that the perception of blends is crucial for host recognition and nonhost avoidance, and the components of the blend may not be recognized when perceived outside the context of that blend. The use of the nonhost tree as a volatile-emitting source might considerably enhance the repellency effect, but it is not applicable in the forest management. A viable alternative could be the use of nonhost essential oils instead. Our laboratory trials demonstrated that essential oils offer a potential solution as repellent (and attractant) substitutes. Both Scots pine and Norway spruce essential oils elicited significant behavioral responses in the beetles, and repellency was clearly achieved even at low concentration. These are encouraging results but the magnitude of these effect in the field must be evaluated with targeted trials. Essential oils have some advantages, as they can be produced locally with the available vegetable material, and nonhost plant essential oils are among the plant-derived semiochemicals that are frequently used as push stimuli in "push–pull" tactics against herbivores [46,47]. So far, application of NHVs in push and pull strategies against *I. typographus* have been proved controversial [23,24,26]. [24] suggested that using repellant to force beetles away from certain trees, while avoiding catching them will not give protection at the stand level, so its value for forest management is limited. Therefore, we propose an application of the Scots pine NHVs blend in situations at the forest margins in ongoing heavy *I. typographus* infestations. Moreover, the study findings can be extended to other bark beetle species, and further research into nonhost volatiles' repellency offers a practical path toward mitigating the impact of forest pests.

5. Conclusions

The European spruce bark beetle is a forest pest that causes significant damages to Norway spruce forests with losses expected to increase in the near future. Affordable, sustainable means to mitigate the impact of the beetle attacks are needed. Nonhost volatiles can be used as repellent for a push strategy. This study tested the repellency effect of Scots pine against the European spruce bark beetle in field and laboratory conditions. The study revealed a remarkable repellency of the Norway spruce stacks when mixed with few Scots pine logs, and the behavioral trials confirm the outcome. Gas-chromatographic analysis of the spruce bark volatiles showed a volatilome profile variation before and after beetle colonization, supporting the field results. The study provides encouraging findings for an application of Scots pine volatiles as repellent of the European spruce bark beetle.

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