Supplementary Information

Defense of Scots pine against sawfly eggs (*Diprion pini)* is primed by exposure to sawfly sex pheromones

Norbert Bittner[a], Janik Hundacker[a], Ander Achotegui-Castells[b,c] , Olle Anderbrant[d] and Monika Hilker[a,1]

[a]Freie Universität Berlin, Dahlem Centre of Plant Sciences, Institute of Biology, Haderslebener Str. 9, 12163 Berlin, Germany

[b]Centre de Recerca Ecològica i Aplicacions Forestals (CREAF), Cerdanyola del Valles, Barcelona, 08193, Catalonia, Spain

[c]Global Ecology Unit, CREAF-Consejo Superior de Investigaciones Científicas, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, 08193, Catalonia, Spain

[d]Lund University, Department of Biology, Sölvegatan 37, 223 62 Lund, Sweden

[1] **Email:** monika.hilker@fu-berlin.de

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Supplementary Material and Methods

Pine exposure to pheromones for 24 h. Male and female *D. pini* adults are spending their lives in the pine trees. Since no distinct mate calling behavior has been observed in *D. pini* females, no information is available on their active pheromone release. Interestingly, pheromonal components have also been detected in extracts of *D. pini* cuticle (1), suggesting some continuous, passive pheromone release. Regardless of the exposure to pheromones released from *D. pini* females sitting in a tree, the tree might also perceive pheromones *via* (gusts of) wind transferring diprionid pheromones over some distance, as indicated by studies showing that attraction of diprionids to traps baited with female sex pheromones are affected by wind conditions (2). Thus, depending on the distance of a tree from a pheromone source and on the speed of wind carrying a pheromone plume to a tree in a pine forest subjected to a mass outbreak of *D. pini,* an individual pine tree might be exposed to diprionid pheromones at any daytime. During a mass outbreak of *D. pini* with successive emergence of adults*,* high concentrations of pheromones might be around even for longer than 24 h.

Determination of pheromone release rate. To calculate the release rate of *D. pini* pheromones from cotton pads placed into the cylinders with the pine trees, we determined the initial quantity of pheromones applied to the pads and the remaining pheromone quantity after a 24 h exposure to pine trees. The pheromones were supplied by Olle Anderbrant from Lund University in Sweden; they were synthesized by Helen Edlund and Erik Hedenström at Mid Sweden University, with a GC purity of 99%.

More specifically, we applied 100 µl of a pheromone solution in hexane (50 ng μl^{-1} mixture of each of the pheromone esters (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate) to a cotton pad (as described in the main text). After pheromone application, the pads were placed for 30 min in a fume hood, thus allowing the hexane to evaporate. Thereafter, we extracted the pheromone with hexane from the dispenser cotton pads (n = 5). Analysis of the extracts by GC-MS (conditions as described in the main text, Material and Methods) provided data on the initial amount of pheromone per pad (and tree). After being used in the experiments (i.e. after the 24 h treatment of plants), we also extracted the cotton pads and analyzed the quantity of pheromone remaining on them (n = 48). Based on our data and assuming a continuous release rate, we calculated the proportion of the pheromone released and the release rate in ng h^{-1} (*SI Appendix,* Table S1). Approximately half of the propionate of (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanol, and two-thirds of the acetate were released during the 24 h incubation period.

The amounts of pheromone components per *D. pini* female were found to vary within a wide range (1). Small amounts of the acetate and propionate component were detected in a similar ratio, but the maximum amount of the acetate component detected in a female was 1000 pg, and of the propionate component 500 pg (1). Hence, the ratio of the two pheromone components may range from about 1:1 to 2:1. When comparing the release rate determined here in our study (see Table S1) with that of the maximum amount of pheromonal compounds determined by Anderbrant *et al.* per *D. pini* female (1), the quantity of pheromones released from a cotton pad in our study was equivalent to the possible emission by 270 to 450 females. This number of females is very similar to the numbers per tree that were previously observed during mass outbreaks of *D. pini* by us and others (3).

Pheromone residues on plants. To examine whether residues of the pheromone were left on pine needles, pine was exposed to both pheromone esters for 24 h following the method described in the main text. After exposure to the pheromones, pine was exposed to clean, charcoal-filtered air for additional 6 h. We exposed three *P. sylvestris* trees to the pheromones and harvested 1g needles of each tree. The three needle samples were extracted each with 1 ml hexane. The extracts were analyzed (i) directly and (ii) after concentration to 50 µl under N_2. A volume of 1 µl of the extracts was injected into a GC-MS (Agilent 7890 A GC model coupled to an Agilent 5975 C MS unit) in splitless mode (injector temperature 250 °C; Zebron ZB-5HT capillary column; 30 m x 0.25 mm i.d.; film thickness: 0.25 µm). Helium was used as carrier gas with a flow rate of 1 ml min^{-1}. The following program was used for analysis: 4 min hold at 40 °C, ramp of 10 °C min^{-1} to 180 °C, followed by a ramp of 20 °C min^{-1} to 280 °C and a 5 min hold of 280° C. A solvent delay of 4 min was added. The column effluent was exposed to electron impact ionization at 70 eV. We recorded a total ion current chromatogram (TIC) with a mass range of 25 to 300 m/z and additionally analyzed samples in the single ion mode (SIM) in search for characteristic ions of the pheromone esters: 87 *m/z*, 101 *m/z*, 210 *m/z.*

No (traces of) pheromone esters were detected in neither type of extract.

Supplementary Table S1. Determination of release rate of *Diprion pini* sex pheromones from cotton pads used in the experiments. Emission rate and percentage of emitted total proportion of the acetate and propionate esters of *D. pini* sex pheromone ((2*S*,3*R*,7*R*)-3,7-dimethyl 2-tridecanyl acetate and propionate) are given (means ± SE).

Pheromone ester	Emission rate in ng h^{-1}	Percentage emitted during 24 h
Acetate	270±16.3	64.7±3.9
Propionate	225±16.6	53.9±4.0

Supplementary Table S2. Details of evaluations by ANOVA. For data on egg survival and number of eggs laid: please compare Fig. 1, main text. For measurements of water content and H_2O_2 concentrations, which were conducted 2 and 12 days after pheromone exposure (i.e. 1 or 11 days after egg deposition): please compare Fig. 2, main text and Fig. S1, *SI Appendix*.

Analysis	Degrees of Freedom*	Sum of Squares*	Mean Square*	*F* value	*P value*
Egg survival	2 / 19	2015.47 / 4679.54	1007.74 / 246.29	4.092	= 0.033
Number of eggs laid	2 / 19	8977.36 / 28112.50	4488.68 / 1479.61	3.034	= 0.072
Water content, day 2	4 / 32	29.07 / 87.71	7.27 / 2.92	2.486	= 0.065
Water content, day 12	4 / 32	210.60 / 661.60	52.65 / 20.68	2.547	= 0.058
H_2O_2 conc., day 2	4 / 35	158.18 / 1159.24	39.54 / 33.12	1.194	= 0.331
H_2O_2 conc., day 12	4 / 35	544.93 / 90.12	136.23 / 2.58	52.911	< 0.001

* Source of variation: between groups / within groups

Supplementary Table S3. Details of paired *t*-test evaluations of comparison of numbers of laid eggs with numbers of hatched eggs per treatment. Compare Fig. 1, main text.

Treatment	Degrees of Freedom	*t* value	*P value*
Untreated	5	4.584	= 0.006
Hexane	7	6.200	< 0.001
Pheromone	7	8.233	< 0.001

Supplementary Table S4. Details of statistical evaluations of differences in gene expression by Kruskal-Wallis *H* test. Compare Table 1, main text.

Gene	Day*	Degrees of Freedom	*H* value	*P value*
		ROS mediating genes		
PsRboh	2	3	15.763	0.001
PsRboh	12	3	9.568	0.023
PsSOD	2	3	4.071	0.254
PsSOD	12	3	7.937	0.026
PsCAT	2	3	7.910	0.048
PsCAT	12	3	2.895	0.423
PsAPX	2	3	5.993	0.112
PsAPX	12	3	9.260	0.026
		Genes involved in SA- and JA-mediated responses		
PsLOX	2	3	4.588	0.205
PsLOX	12	3	10.513	0.015
PsPDF	2	3	2.065	0.559
PsPDF	12	3	1.215	0.729
PsPR-1	2	3	3.048	0.384
PsPR-1	12	3	16.682	0.001
PsPAL	2	3	1.622	0.654
PsPAL	12	3	10.177	0.017

* days after pheromone exposure

Supplementary Table S5. Sequences of primers used in this study for qPCR and related search information. Compare Table 1, main text.

Gene	Primer sequence (5‘ -> 3‘)	Pine template for primer design	Species for primer design	Species for BLAST search for pine primer template design
		Housekeeping genes		
PsUBIF	ACTTTACCAGAGTCATCAACC	HE629096	*Pinus sylvestris*	*Picea abies* (EF681766)
PsUBIR	GGTTCTTCGTCTGAGAGGTG			
PscATPF	GGGTCGGTCAAGTCGTCAGC	GW765967	*Pinus banksiana*	*Ginkgo biloba* (EU071049)
PscATPR	GCACGGAAATGGGTTCTTTGC			
PsPETBF	ACCATCATACTTGCCGACCATC	CV035597	*Pinus taeda*	*Populus euphratica* (XM011050173)
PsPETBR	TCGTCCGACCGTTACAGAAGC			
		ROS-mediating genes		
PsRbohF	GATGTACCTGGCAGTTCC	MF389973	*Pinus sylvestris*	*Picea abies* (KT192592)
PsRbohR	GCCACTCTTGTATCTGAACC			
PsSODF	GCTGATGTCAAGGGGGTTGT	X58578	*Pinus sylvestris*	-
PsSODR	ACCATGCTCCTTGCCTAACG			
PsCATF	AAGGGCTTTTTCGAGGTGAC	AL751103	*Pinus pinaster*	-
PsCATR	GGAATTACCTGCATGGCATC			
PsAPXF	TCTGGTTTTGAAGGACCATG	AY485994	*Pinus pinaster*	-
PsAPXR	AAACTAGGATCAGCCAGCAG			
		Genes involved in SA- and JA-mediated responses		
PsLOXF	TGGACTAATGATGGAAGAGCAC	DR169048	*Pinus taeda*	*Picea sitchensis* (CO218750)
PsLOXR	TGATGTTGGCAGCAATAACTCG			
PsPDFF	GGCAAGGGAGTTGGCAGTCG	EF455616	*Pinus sylvestris*	-
PsPDFR	TGGTGCTGTTCACACAATACCC			
PsPR-1F	TCGTCAACGTACACAGATGTTG	HE627106	*Pinus sylvestris*	*Arabidopsis thaliana* (NM127025)
PsPR-1R	ACTACGATCCGCCTGGGAAC			
PsPALF	CTGGCAGCGATCCACTGAAC	AF353967	*Pinus sylvestris*	-
PsPALR	CTTCGAGCAACGGCAGCAAC			

Supplementary Table S6. Nucleotide sequences of PCR products obtained from primers used in this study (if based on published sequences, the references are given here in the *SI Appendix,* section "References"). Compare Table 1, main text.

Name	Nucleotide sequence 5'-3'
	Housekeeping genes
PsUBI	ACTTTACCAGAGTCATCAACCTTGTAGTACTGCAGAACAGCCAATTTTACCTTCTTCTTCTTGT GCTTGAGCTTCTTAGGCTTAGTGTAAGTCTTCTTCTTTCTCTTCTTGGCACCACCTCTCAGACG AAGAACCAA
PscATP	GGGTCAAGTCGTCAGCAGGTACATAAACTGCTTGAATCGAGGTTATGGATCCCTTTTTTGTGG AGTAATTCTCGTGCCTTTACCCAAGAAACGTT
PsPETB	ACCATCGATGAATTGATCGGATTAACCAACCAAAGTTAACTTCGGTCATTAGGTATTGAACAG AGGCAAAAGCTTCTGTAACGGTCGGACGA
	ROS-mediating genes
PsRboh	GATGTACCTGGCAGTTCCCGTATTATTATATGGAGGAGAACGAACACTGAGAGCTTTCAG ATCAGGTTCAAAACCCGTGCAAATACTCAAGGTTTGTCTTTACATCAATTTTCATTTTTT GTGATTCTAGCTTTGCATCTGCAATCCTTGATGTGACCAATAGAATCTGTTGCATTTTTG GGGGATTTTGCTCTTAACATCTACACGGTCCACATTTGCAGGTAGCAATCTATCCTGGTA ATGTCTTGACATTTCACATGTCCAAACCTCAAGGGTTCAGATACAAGAGTGGC
PsSOD (4)	GCTGATGTCAAGGGGGTTGTTCAATTCACCCAGGAAGGAGATGGGCCAACAACTGTAACT GGGAAGATCAGTGGTCTGAGCCCTGGTCTCCATGGTTTCCATGTTCATGCACTAGGTGAC ACAACAAATGGGTGCATGTCAACTGGACCACATTTTAATCCGTTAGGCAAGGAGCATGGT
PsCAT (5)	TAAGGGCTTTTTCGAGGTGACCCACTATGTCTCCGATCTCACCTGTGCAGATTTCATGAG GGCACCTGGCGTTCAGACCCCAGTGATTGTTCGGTTTTCTACTGTCATACATGAACGTGG GAGCCCGGAGACTATGAGAGACCCCAGGGGTTTCGCTGTCAAGTTTTACACGAGAGAAGG GAACTTCGACATTGTTGGAAACAATATTCCCGTTTTCTTCACTCGTGATGCCATGCAGGT AATTCC
PsAPX (6)	TCTGGTTTTGAAGGACCATGGACCTCTAACCCTCTTATCTTTGACAACTCTTACTTCACA GAGCTTGTGACTGGAGAGAAGGAAGGCCTGCTTCAGCTGCCATCTGATAAGGCACTGCTG GCTGATCCTAGTTTA
	Genes involved in SA- and JA-mediated responses
PsLOX	TGGACTAATGATGGAAGAGCACTGGAGGCCTTTCAAAGGTTTTCTACCACAGTTCAGGGGGT AGAGGAAATCATACATCAGAGAAATGAAGATTCGAGTAAGAAGAACAGGAATGGGGCSGG CGTACTTCCTTACGAGTTATTGCTGCCAACATCAACC
PsPDF	GGCAAGGGAGTTGGCAGTCGACTCAGCACTCTTTTTCTGCTCGTGCTGCTTGTTATAACC ATTGGGATGATGCAGGTTCAAGTTGCAGAGGGCCGAATGTGCAAAACCCCGAGCGGCAAG TTCAAAGGGTATTGTGTGAACAGCACCA
PsPAL (7)	CTGGCAGCGATCCACTGAACTGGGTTCGAGCAGCCAAGGCCATGGAAGGAAGTCACTTTG AAGAAGTGAAAGCGATGGTGGATTCGTATTTGGGAGTCAAGGAGATTTTCATTGAAGGGA AATCTCTGACAATCTCAGACGTTGCTGCCGTTGCTCGAAG
PsPR-1	TCGTCAACGTACACAGATGTTGAAGATTTACAGTAACACGGAATATTAGAAGGAAATTAACG AAAACTAATACGATATGATAGGTCGGGATATCAGAATTCAGTATGGTTTCTGCCCTACATAGT TCCCAGGCGGATCGTAGT

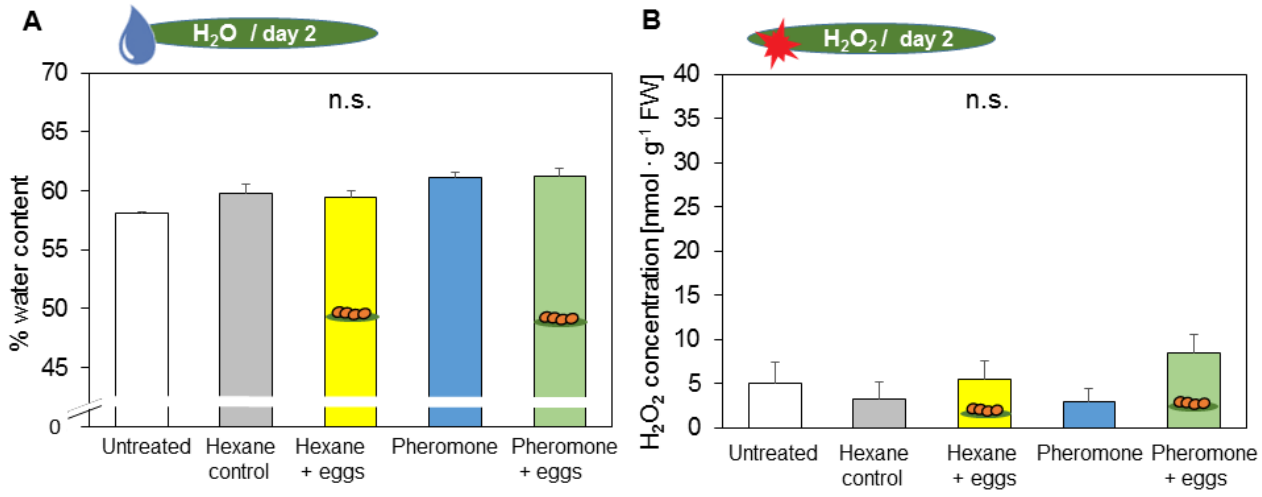
Supplementary Table S7. Transcript levels of genes in untreated pine trees and in trees exposed to hexane. Gene expression in untreated trees was normalized to the expression of the housekeeping genes (see main text, Material and Methods) and set to value 1. Gene expression in hexane-treated trees expressed as fold-change to expression levels in untreated controls. Data show means ± SE. *n* = 8 untreated and *n* = 5-8 hexane-treated trees. Expression levels were determined 2 and 12 days after treatment. *P* values: pairwise comparison of untreated and "hexane control" by **t*-test or †Mann-Whitney *U* test. Compare Table 1, main text.

Time	Hexane control	Untreated	*P* value
***PsRboh* - Respiratory burst oxidase homolog (plant NADPH oxidase)**			
2d*	0.76±0.09	1.00±0.20	0.311
12d†	2.06±0.28	1.00±0.16	0.126
***PsSOD* - Superoxide dismutase**			
2d*	1.21±0.24	1.00±0.17	0.498
12d*	1.46±0.15	1.00±0.13	0.720
***PsCAT* – Catalase**			
2d†	0.56±0.13	1.00±0.25	0.222
12d†	0.56±0.10	1.00±0.21	0.228
***PsAPX* - Ascorbate peroxidase**			
2d*	0.98±0.10	1.00±0.20	0.924
12d†	1.44±0.24	1.00±0.07	0.081
***PsLOX* - Lipoxygenase**			
2d†	1.59±0.46	1.00±0.17	0.442
12d*	0.68±0.12	1.00±0.09	0.055
***PsPDF* - Plant defensin**			
2d†	1.27±0.54	1.00±0.34	0.878
12d*	0.55±0.15	1.00±0.22	0.128
***PsPR-1* - Pathogenesis related 1**			
2d†	4.11±2.36	1.00±0.48	0.442
12d*	0.56±0.16	1.00±0.41	0.382
***PsPAL* - Phenylalanine ammonia lyase**			
2d*	1.48±0.44	1.00±0.18	0.335
12d*	0.75±0.17	1.00±0.16	0.294

Supplementary Table S8. Details of statistical evaluations of the EAG responses by *Diprion pini* to the acetate / propionate sex pheromonal components. Responses to test substance compared to responses to controls; Wilcoxon matched pairs test**.** Compare Fig. 3, main text.

Test substance	Sex	*Z* value	*P value*
Acetate pheromone component	male	2.521	0.008
Propionate pheromone component	male	2.521	0.008
Acetate + Propionate pheromone components	male	2.521	0.008
Acetate pheromone component	female	0.840	0.461
Propionate pheromone component	female	1.540	0.148
Acetate + Propionate pheromone components	female	1.183	0.297

Supplementary Fig. S1. (*A*) Water contents and (*B*) hydrogen peroxide concentrations of *Pinus sylvestris* after exposure to sawfly sex pheromones and subsequent egg deposition. Measurements were conducted 2 days after pheromone exposure, i.e. 1 day after egg deposition, and at equivalent time points in controls. Water concentrations and hydrogen peroxide concentrations were determined in pine needles from untreated trees, from trees exposed to the solvent hexane (without eggs: hexane control; with eggs: hexane + eggs), from trees exposed to the pheromones (dissolved in hexane) (without eggs: pheromone; with eggs: pheromone + eggs). Means + SE of water contents and hydrogen peroxide concentrations are given (n = 5 for water content untreated; n = 8 for all other treatments). All data evaluated by ANOVA (n.s., not significant) (compare *SI Appendix,* Table S2).



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