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Final Report

‘Chemical ecology of wood-borers associated to Cypress trees: an invasive jewel beetle rearranging olfactory communication channels’

‘Ciprusfélék kártevőinek kémiai ökológiája: egy inváziós díszbogár átrendezi az illatanyagokra épülő kommunikációs csatornákat’

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Why was the project scientifically novel, urgent for practice and what was the expected outcome?

The project aimed the uncover the chemical communication signals of two wood-boring pests of scale leaved conifers, in connection of their common/shared host range. The small cedar bark beetle (*Phloeosinus aubei*, Coleoptera, Scolytinae) and the cypress jewel beetle (*Ovalisia/Lamprodila festiv*a, Coleoptera, Buprestidae) are invasive species in Hungary and cause severe damage to *Thuja* and *Juniperus* species in ornamental tree nurseries, as well is urban green areas. Their chemical ecology and reliable control methods were unknown at the beginning of the project. Studies have been carried out to determine the pheromone and kairomone cues of the two species and to find monitoring and control methods against them.

Materials and methods in brief

To determine which specific compounds in the multi-component volatile of their preferred common host, the American arborvitae, *Thuja occidentalis* L. cultivar ‘Smaragd’, these pest species perceive respectively, we first collected headspace volatiles from twigs of living healthy trees. Intact twigs of *Thuja* were covered with a chemically inert baking bag, then headspace volatiles were collected using a charcoal CLSA filter (1.5 mg load, Brechbühler AG, Schlieren, Switzerland) with the flow of 2.6 L min 1 for 1 h 40 min, using a DC12 rotary vane pump (Fürgut GmbH, Tannheim, Germany). Volatiles from the filter were eluted three times with 25 µl of n-pentane (Merck, Darmstadt, Germany).

Adult specimens of *P. aubei* and *O. festiva* were collected from T. occidentalis ‘Smaragd’ trees at Tahi Tree Nursery (Tahi, Pest-county) and Prenor Tree Nursery (Szombathely, Vas-county).

Volatile compounds were analyzed by a gas chromatography coupled to electroantennographic detector (GC-EAD). A 6890N gas chromatograph (Agilent Technologies Inc., Santa Clara, California), equipped with a HP-5 column (J&W, 30 m 0.32 mm, 0.25 µm film thickness; Agilent Technologies Inc.); equipped with an electroantennographic detector connected to an IDAC2 amplifier (SYNTECH GmbH, Kirchzarten, Germany) was used. Electric signals from mounted insect antennae were simultaneously recorded to FID, using SYNTECH GC-EAD 2014 v. 1.2.5. software.

An Agilent 6890 GC (Agilent Technologies Inc.) coupled to an Agilent 5973 mass-selective gas detector (GC-MS) was used to identify compounds that elicited antennal responses. Synthetic samples of the identified compounds were purchased from Sigma-Aldrich (Merck KGaA,

Darmstadt, Germany) and abcr GmbH (Karlsruhe, Germany). Some compounds were obtained from the Max Planck Institute for Chemical Ecology (Jena, Germany).
For antennal test stimuli, 100 µg of the synthetic compounds were applied in 10 µl n-pentane solutions and analyzed individually with an electroantennographic detector. Mounted antennae of male and female *P. aubei* and *O. festiva*, respectively, were stimulated with the solution of synthetic host-plant components.

Identification of kairomone components from healthy and from declining host trees

We identified the chemical structure of 12 components, out of 16 antennaly active compounds. The results corroborated our previous results on *P. aubei* (Bozsik et. al 2016), however making a significant progress at the same time, as in addition four new compounds were identified at first time, such as α-pinene, sabinene, β-pinene, and limonene (some of which later proved to be important at behavioral level). The majority of compounds elicited antennal responses from both species and sexes, E-β-caryophyllene elicited response only from *O. festiva*, whereas borneol only from *P. aubei*.
The synthetic samples (–) and (+)-terpinen-4-ol, followed by (+)-fenchone and E-β-caryophyllene at a dose of 100 µg, elicited the strongest responses from antennae of both sexes of *O. festiva*. The compounds that elicited the strongest responses from both sexes of *P. aubei* were (+)-β-pinene and (–)-terpinen-4-ol.
When comparing the relative amplitudes of the responses between the corresponding sexes of the two species, *O. festiva* females reacted with significantly higher response to E-β-caryophyllene, (–)-terpinen-4-oland (+)-terpinen-4-ol, than *P. aubei* females, whereas *P. aubei* females gave significantly larger responses to (+)-α-pinene and to sabinene than *O. festiva* females. In males, E-β-caryophyllene and (+)-fenchone elicited significantly higher antennal responses from *O. festiva* than from *P. aubei*, while (+)-β-pinene, (–)-β-pinene, (+)-borneol (–)-borneol, sabinene and (+)-camphor elicited significantly higher antennal responses from *P. aubei* than from *O. festiva*.
Our results suggest that different kairomone cues play a role in the host finding of the two species. By other words, different specific compounds serve as key stimuli for the two pest species. This should be considered later, when composing synthetic blends for monitoring traps.

Following the identification of potential kairomone components, we related our findings to the life cycle and gallery types of *P. aubei*, revealed by us earlier (Bozsik and Szőcs, 2017). In short, freshly emerged *P. aubei* adults make their overwintering tunnels in the twigs of healthy trees at the second half of the season, while in the next spring for reproduction they prepare nuptial chambers in the trunks of decaying host trees during their mating season (Bozsik and Szőcs, 2017).
In order to reveal which odour compounds are involved in the host selection during the two flight periods of *P. aubei*, we made volatile collections from healthy, intact twigs of *T. occidentalis* ‘Smaragd’ trees, and from declining, semi-dried piece of trunks with the same method as we used in our previous studies. We also aimed to study the effect of *P. aubei* infestation on host volatiles, as the infested tree may become more attractive for further colonization. Adult female *P. aubei* were placed on a semi-dried trunk section of *T. occidentalis* ‘Smaragd’. When all the females prepared nuptial chambers under the bark of the trunk, volatile

collection was made. As an alternative method, volatiles were sampled directly from the lumen of nuptial chambers occupied by females. The tip of a Pasteur-pipette was inserted tightly into the tunnel. Sampling carried out in an open loop system, using the same type of absorbent within the pipette. Freshly produced frass from *P. aubei* female nuptial chambers in *T. occidentalis* 'Smaragd' was also sampled using volatile collection in a closed loop system.

From two other species of host trees, *T. occidentalis* 'Brabant' and *T. plicata* 'Excelsa', also preferred by *P. aubei*, headspace volatiles were collected from intact, healthy twigs with leaves using the same method as above.

Samples were analyzed by GC-EAD on a 30 m × 0.32 mm × 0.25 μm film thickness DB-WAX column (J&W, Scientific, Folsom, CA, USA), using *P. aubei* antennae as EAD detectors. Compounds that evoked antennal responses were identified by GC-MS, based on calculation of Kováts Retention Indices and comparison of spectra to the NIST Webbook database.

The predominant volatile compounds from intact *T. occidentalis* 'Smaragd' with pronounced antennal responses were fenchone, α-thujone and β-thujone. Among these compounds, α-thujone was the most abundant component. Two other components, 1-octen-3-ol and terpinene-4-ol, also evoked strong antennal responses, but they were present in trace amounts. These compounds were also present in the volatiles of decaying *T. occidentalis* 'Smaragd' trunks. Some additional early-eluting components with strong antennal responses were also detected in the samples of semi-dried trees. α-Pinene and α-thujene (eluting together) were the most abundant from these, followed by that of camphene, fenchone, β-pinene, myrcene, limonene, and p-cymene. In the volatile profile of female-infested trunk α-pinene and α-thujene were present in much more elevated levels with sharp increases of β-pinene, myrcene, limonene, and p-cymene. Volatiles collected from fresh frass showed a similar profile to those collected externally from a freshly made gallery in *T. occidentalis* trunks, but with a smaller amount of limonene.

The volatiles of healthy *T. occidentalis* 'Brabant' contained the same major, antennally active components, in similar ratios, as the healthy *T. occidentalis* 'Smaragd'. The composition of healthy *T. plicata* 'Excelsa' also looked almost identical to that, but did not contain fenchone.

In conclusion, the abundance of highly volatile, early eluting components, such as α-pinene increased dramatically in the volatiles of stressed trees when compared to samples collected from healthy trees. This pattern was also clearly observed in drought-stressed trees, free from *P. aubei* and fresh galleries, indicating that these compounds are of plant origin and stress-related.

Studying the effect of trap color and -type on captures of O. festiva

In order to develop a monitoring method for *O. festiva*, we conducted field studies in a *Thuja* plantation. Commercially available CSALOMON® transparent (PAL) and light green (PALz) sticky traps and multifunnel trap types (CSALOMON® transparent MULT, light green MULTz, and purple MULTp) were used in the trials. Experiments were conducted at TahiTree Nursery during the flight period of *O. festiva* (June to August) in two years. Sticky traps were attached to the branches of *T. occidentalis* trees at a height of 180 cm, stretched and tightly attached to the foliage. Multifunnel traps were suspended by a piece of wire attached to a horizontal bamboo stick and fixed to the trees at 200 cm. Reflectance of the traps were also determined using An Ocean Optics STS-VIS spectrometer equipped with a P400-010-UV-VIS

fiber (Ocean Optics, Largo, FL, USA) and a Spectralon® white diffuse reflectance standard (Edmund Optics Inc., Barrington, IL, USA) for the measurements.
A total of 623 *O. festiva* specimens were captured during the studies. Significantly more beetles were captured by sticky traps compared to non-sticky traps. There was no significant difference was between PAL and PALz.
The reflectance of non-sticky transparent surfaces was very low in the 350-700 nm range. A sticky surface (glittering glue) causes a 4-5% increase in reflectance at all wavelengths for transparent and green painted traps.

Revealing key components in the female-produced aggregation pheromone of P. aubei

A breakthrough in revealing intraspecific communication: Female *P. aubei* produced aggregation pheromones were identified in the framework of the project.
First, the courtship behaviour of *P. aubei* was observed. Feral-collected females, collected around the end of overwintering period were taken into the lab and used for the observations. To allow the females to continue their life cycle in the lab, a piece of cut *T. occidentalis* 'Smaragd' trunk was offered to them, in 4L glass jars. When they had completed their nuptial chambers, the trunk was placed under a stereomicroscope (Alpha STO-4 T zoom, Elektro-Optika Kft., Érd, Hungary), focusing on the opening. A single male was placed on the surface of the trunk, and the behaviour of the male and the appearance of the female were monitored. When the male reached the entrance of the female tunnel, they began to move the tip of the abdomen up and down, rubbing it against the inner surface of the edge of the elytra (stridulatory behaviour). This is followed by copulation in the opening of the chamber, or often, the male entered the chamber after stridulation.
For extraction the pheromone compounds, unmated females were placed on a cut *T. occidentalis* 'Smaragd' trunk after a maturation feeding, and allowed to complete their nuptial chambers. Females were removed from their chambers and their mid- and hindguts were extracted. Extracts were prepared from the two groups of females. In order to facilitate pheromone production, females in the first group were treated juvenile hormone III (JHIII), females in a control group were not. 10 mg of synthetic JHIII (CAS: 24198-95-6, Santa Cruz Biotechnology, 10410 Finnell Street, Dallas, TX 75220, US) was dissolved in 250 µL of acetone (analytical reagent grade, Reanal, Budapest, Hungary) to prepare a 40 µg/µL stock solution. The abdominal surface of females was treated with this solution using a bevel-tip Hamilton syringe. After the incubation period, the mid-and hindguts of JHIII-treated females were excised with sharp tweezers and combined, followed by extraction with n-hexane (reagent plus grade, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). In the first experiment, extracts were prepared from 14 untreated (dissolved in 25 µL n-hexane) and 7 treated females (dissolved in 25 µL n-hexane). The procedure was repeated in the subsequent year, with the same method. For the replication, 16 females were treated with JHIII, and their mid-and hindguts dissolved in 30 µL n-hexane. For untreated control, mid-and hindguts of 23 female were dissolved in 40 µL n-hexane.
The extracts were analyzed by GC-EAD, testing on DB-WAX and HP5 column, using male antennae as EAD detector.

Volatile collection from leafy twigs of *Thuja occidentalis* 'Smaragd' was also conducted with the same method as in our previous studies using n-pentane as solvent (Sigma-Aldrich, Merck KGaA).

Kairomonal and pheromonal communication: How are they connected?

P. aubei gut and *Thuja* headspace extracts were analyzed by gas chromatography-mass spectrometry (GC-MS) first in the laboratory of Plant Protection Institute, HUN-REN CAR and as a next step, to determine chirality of the natural compounds, in the Institute of Organic Chemistry, University of Hamburg, Germany with a GC 7890A gas chromatograph, coupled to a 5975C inert XL MSD mass spectrometer (Agilent Technologies - MSD ChemStation E.02.02.1431) equipped with a fused silica capillary column (VF-WAXms, 60 m x 0.25 mm, 0.25 μm film thickness; Agilent). The enantiomeric ratios of the antennally active compounds from *P. aubei* females and *T. occidentalis* leaves were determined using a GC 7890A gas chromatograph, coupled to a 5975C inert XL MSD mass spectrometer using a CP-Chirasil-Dex CB (25 m x 0.25 mm, 0.25 μm film) chiral capillary GC column (Varian, Lake Forest, USA).
Electroantennographic bioassays (EAG) using synthetic enantiomers of α-pinene and myrtenol were also conducted.
With the identified compounds, laboratory behavior tests were carried out during the mating season of *P. aubei* using a four-arm arena olfactometer (Sigma Scientific LLC, Micanopy, FL, United States). Forty males and 40 females were tested separately and individually in the various assays. The enantiomerically pure synthetics of the identified compounds, (−)-α-pinene, (+)-α-pinene, (−) myrtenol (Sigma-Aldrich, Merck KGaA), and (+)-myrtenol (synthetized in the laboratory of Institute of Organic Chemistry, Research Centre for Natural Sciences, Budapest) were dissolved in n-hexane and tested in different combinations.
Field trapping tests were carried out in a *T. occidentalis* 'Smaragd' plantation, in Szombathely (Prenor Tree Nursery). Four-unit, black Lindgren funnel traps (Contech Enterprises Inc., 19 Dallas Rd Unit 115, Victoria, BC V8V 5A6, Canada) were used for trapping. Based on the results of the behavioral test in the lab, traps were baited mixture of 250 μL (−)-α-pinene and 50 μL (−)-myrtenol dissolved in 1 mL of mineral oil (Sigma-Aldrich, Merck KGaA). Unbaited traps were used for controls. The experiment was conducted from the beginning of the mating season until the time when *P. aubei* adults were still flying (from mid-April to mid-June).
Two electrophysiologically active compounds were detected in the female gut extracts. Enantioselective GC-MS identification proved that the beetles produce the beetles produce (1S,5S)-(−)-α-pinene and (1R,5S)-(−)-myrtenol. α-Pinene and myrtenol, were detected in extracts from both JHIII-treated and untreated female beetles. However, the ratio in extracts from JHIII-treated females was 6:1 and 4:1, whereas extracts from untreated females it felt in quite different range (2:1 and 1:1).
(−)-Myrtenol was shown to be female-specific, while (−)-α-pinene was also found as a 2:1 mixture with the (+)-enantiomer in the volatiles of the host tree, *T. occidentalis*.
Both enantiomers of α-pinene and myrtenol elicited significantly stronger (in values several times superior) EAG responses in both male and female *P. aubei*, than the solvent control at each dose tested.
Four-arm olfactometer studies showed that females and males spent significantly more time in the arm containing the lures, such as racemic α-pinene, (−)-myrtenol, (+)-myrtenol, and racemic

myrtenol, than in the solvent control arm. In assays with males, (−)-myrtenol alone was only slightly more attractive than the control, whereas a mixture of (−)-myrtenol with racemic α-pinene was highly attractive. In assays with females, racemic α-pinene was more attractive than (−)-myrtenol or its mixture with racemic α-pinene.
Traps baited with a 5:1 binary mixture of (−)-α-pinene and (−)-myrtenol attracted *P. aubei* males and females in significantly higher numbers than unbaited control traps.
Our result in identifying *P. aubei* aggregation pheromone compounds is the first in the field of behaviourally active semiochemicals of this species that can be used in pest control.

Successful project structure – Successful publication policy

In summary, we revealed the key cues in the intra- and interspecific chemical communication of the invasive pest, *P. aubei*. Routinely using high-tech instrumentations in behavioral observations (Alpha STO-4 T zoom stereomicroscope), electrophysiology (GC-EAD), physiology of pheromone production (triggering by JHIII) stereochemical structure elucidation (chiral GC-MS – in international cooperation), new enantiomer-specific synthetic routes (in interdisciplinary national cooperation), laboratory behavioral test (four-arm olfactometer), in combination with intensive field trapping test were all necessary to achieve these results. Please, see our so-far publications below. Further evaluations of some not yet fully analyzed data sets will result in compiling some more manuscripts, which work is already under progress.

Outlook: Way from scientific results to practical tree protection

In one sentence, our results in *P. aubei* and *O. festiva* gives a significant breakthrough in the chemical ecology of both species and gives opportunities for establishing new, non-toxic, environmentally sound monitoring and control methods against the two pests.

PUBLICATIONS:

Bozsik G., Molnár B. P., Szőcs G. (2022) Comparison of antennal responses of *Ovalisia festiva* and *Phloeosinus aubei* to volatile compounds of their common host, *Thuja occidentalis*. Physiological Entomology 47(2): 136-146. https://doi.org/10.1111/phen.12383 (IF: 1.83; Q2)

Bozsik, G., Molnár, B. P., Domingue, M. J., Szőcs G. (2023) Changes to volatile profiles of arborvitae, *Thuja occidentalis*, from drought and insect infestation: olfactory cues for the cypress bark beetle, *Phloeosinus aubei*. Chemoecology 33: 113-124. https://doi.org/10.1007/s00049-023-00389-9 (IF: 1.6; Q2)

Matula, E., Bozsik, G., Muskovits, J., Ruszák, Cs., Jávorszky, L., Bonte, J., Paulin, M., Vuts, J., Fail, J., Tóth, Á., Egri, Á., Tóth, M., Imrei, Z. (2023) The optimal choice of trap type for the recently spreading jewel beetle pests *Lamprodila festiva* and *Agrilus sinuatus* (Coleoptera, Buprestidae). Insects 14(12): 961. https://doi.org/10.3390/insects14120961 (IF: 2.7; Q1)

Bozsik, G., Molnár, B. P., Hegedüs, K., Soós, T., Schulz, S., Tröger, A., Francke, W., Szőcs, G. (2024) (−)-myrtenol and (−)-α-pinene: Aggregation pheromone components of the cypress bark

beetle *Phloeosinus aubei*. Journal of Applied Entomology 148(4): 351-363. https://doi.org/10.1111/jen.13231 (IF: 1.7; Q2-2023)

Presentations in Hungarian conferences:

Bozsik G., Molnár B. P., Szőcs G. (2022) Ép tuja illatanyagainak elektrofiziológia vizsgálata borókaszú csápján: lehetséges ingerek a telelő bogár-populáció számára. 68. Növényvédelmi Tudományos Napok

Teski A., Kiss B., Bozsik G., Molnár B. P., Takács A., Szőcs G. (2023) Napszaki elkülönülés az inváziós boróka-tükrösmoly (Cydia interscindana) és az almamoly (*Cydia pomonella*) feromoncsapdás fogási adataiban. 69. Növényvédelmi Tudományos Napok

Bozsik, G., Molnár, B. P., Tröger, A., Schulz, S., Szőcs, G. (2024) Hogyan változik a tuja illatanyag komponenseinek összetétele a borókaszú kolonizációjának két szakasza során? 70. Növényvédelmi Tudományos Napok

Presentations in international conferences:

Bozsik, G., Molnár, B. P., Szőcs, G. (2023) Common host - same perception? Electrophysiological comparison of cypress bark beetle and cypress jewel beetle. Forest Protection Colloquium, Vienna, Austria

Bozsik, G., Tröger, A., Schulz, S., Molnár, B. P., Hegedüs, K., Soós, T., Teski, A., Szőcs, G. (2024) Chirality of α-pinene: Switch from plant kairomone to aggregation pheromone in the cypress bark beetle, *Phloeosinus aubei*. 39th Annual Meeting of the International Society of Chemical Ecology, Prague, Czechia

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