K124278
Final Report

„Cedar bark beetle: exploring chemical as well as acoustic communications and revealing potential of carrying pathogens of cypress canker in populations adapted to temperate region"

„Borókaszú: a kémiai- és akusztikus kommunikáció megfejtése, valamint a mérsékelt övhöz alkalmazkodott populációk szerepének tisztázása a ciprus-rák kórokozóinak terjesztésében"

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in collaboration of Senior Participants (SP)

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Studies aiming at revealing the intra- and interspecific communication of the invasive cypress bark beetle, CBB, *Phloeosinus aubei* (Coleoptera, Curculionidae, Solytinae) have been conducted in the frame of the present project along three research lines, chemical communication, acoustic communication and vectorology. Studying chemical communication was the leading part of the studies, however, the three lines interacted with each other in many ways.

Two main types of the chemical communication of CBB was studied, host plant finding (interspecific communication) and aggregation for mating (intraspecific communication). Again, these types are interconnected, as mating occurs on/in the host tree (see details later).

In order to reveal basic elements of these communications, first the life-cycle of CBB under temperate climate had to be clarified. CBB originally occurred in the Mediterranean region, and Asia minor, and adapted its life cycle to that climate. In its native range of occurrence CBB has several generations per year, while in „newly" invaded regions (in some places, close to the Mediterraneum, it appeared more than 50 years ago, while in other places, North from Hungary, first records were published only in course of this project), under temperate climate, as in Hungary, only two flights had been recorded. Just prior to the time of starting this project, it was believed that two generations per year develop in Hungary. However, we supposed that the second generation is actually just an autumn flight before overwintering. For starting pheromonal studies, it was important for us to see whether CBB populations in Hungary complete two generations, what means they have two breeding season per year, when their emit pheromone and mate, or just one generation with one breeding season (when pheromone could be collected for chemical identification). We started these life-cycle studies preceding this project (Bozsik and Szőcs, 2017, Phytoparasitica 45: 201-2010) and continued in the 1st and 2nd year of the project, for finely specifying the seasonal pattern of the breeding season. Results gained in course of this project corroborated our earlier observations, moreover, newly revealed that adults obligatorily feed on host plant foliage also in April-May, just before the breedig season (not only before overwintering, as thought before). We developed the methodology of our later pheromonal studies with consideration of these findings.

In order to collect all basic knowledge, which might be important for pheromone collection, first the key elements in the mating behavior of CBB had to be revealed. These studies were started right from the 1st year of the project.

Key elements in the close-range mating behavior of beetles were revealed for the first time, in course of our studies, with the help of video-recordings, taken by the newly purchased zoom stereomicroscope (Alpha STO-4T zoom stereo microscope with micro-camera- and video recordings, Elektro-Optika Kft., Érd, purchase covered from this project). For this, adults of the right stage were collected in tree nurseries (mainly at Szombathely and Tahi), sexed on the spot, then transferred to our lab, where they were allowed to continue their life-cycle under controlled, semi-natural conditions, in cages. Conditioning of beetles was made according to a protocol, established by us. Briefly, beetles were let to feed and start carving nuptial chambers on pieces of trunks of *T. occidentalis* 'Smaragd', being in transient level of declining, optimal for the attack of beetle. We revealed earlier that females are the singalling sex, and males are the responders, from a distance (Bozsik and Szőcs, 2017). We designed these lab tests accordingly. Recordings of mating behavior were made under semi-natural conditions, in the lab. Observed behavioral pattern strongly indicated that olfactory cues are involved in primary attraction of the opposite sex from a distance, while acoustic communication takes place later in the sequence, in close range interactions. Long range attraction to the host tree is governed by host plant kairomones, however, finding the openings of a nuptial chamber, on the surface of the trunk is controlled by female-emitted pheromone. We revealed that males were attracted to trunks with freshly made nuptial chambers with a female inside, over similar trunks with nuptial chambers, abandoned by (or taken out of) the female. At that first stage no acoustic signals were detected. Once male and female got in a close-range contact with each other, sex-specific acoustic signals were detected in a stereotype sequence. These observations and recordings were made in our lab, in a strong collaboration between PI and the SP charged for chemical ecology, as well as of the SP, expert in bio-acoustics. Ultrasound recordings were made using highly advanced technologies and expertise (Bruel&Kjaer microphone, type 4939; Nexus signal conditioning amplifier connected to a National Instruments data acquisition card NI USB 6122, at 200 kHz sampling rate; Avisoft Recorder 4.2.15 software). Regretfully enough, evaluations of sound-recordings are still just in progress.

Pheromone extraction methodology was developed by us, based on the above-mentioned results of mating behavior of CBB, using pre-conditioned, unmated females, being in the stage of building nuptial chamber. Preliminary trials with volatiles collections were unsuccessful, as the desired pheromone components could not be captured in enough amounts for chemical analysis, so we soon switched to direct extraction of mid- and hindguts.

In case of several other bark beetle species had been reported that the application of juvenile hormone III (JHIII) triggers pheromone production. Taking into accounts that in a gut extract the pheromone components can be expected to occur only in minute amounts, triggering their biosynthesis via JH application seemed to be a reliable assumption, as suggested to us by Steven J. Seybold (Univ. Calif. Davis / US Forest Service) (†2019 - for a commemoration see Szőcs and Tóth, 2020, Acta Phytopathologica et Entomologica Hungarica 55: 1-2.). So, we made two types of extracts, in parallel, one of using JHIII treated females, and another one, made of intact females. As mating period occurs once per season (preferably in April and May, depending on weather), this is the timed slot, when females produce pheromone, consequently the time for preparing extracts. It is known that synthetic JH is very sensitive, therefore freshly purchased sample should be used, and dilution should be prepared right before application. In the first year the JH sample, obtained from the USA, arrived safely under deep cooling (dry ice), which allowed us to prepare the first set of extracts.

We found two electrophysilogically active components in the extracts by gas chromatography linked to an electroantannographic detector (GC-EAD: 6890N GC, Agilent Technologies Inc., USA, DB-wax and HP5 columns, J&W USA, ChemStation software version Rev. A. 10.02 - EAD: MP 15 micromanipulator, IDAC2 amplifier, 2014 v. 1.2.5 sofware, SYNTECH Ockenfels, Germany, successor of SYNTECH, Hilversum, the Netherlands), using conspecific male antennae. Chemical identification was made in an informal, no-cost collaboration by Prof. W. Francke (Inst. Org. Chem., Univ. Hamburg, Germany), by means of GC-MS. However, extracts contained only limited amounts of the antennally active components, which were not decisive to see the possible effect of JH. Next year the freshly ordered synthetic JH arrived without cooling from another company, and looked dead, according to our measurements. In that season / year there was not enough time left from the breeding season, to complete another order for a new batch. Finally, our third purchase of a new JHIII batch was successful in the subsequent year (from Santa Cruz Biotechnology, USA), and so were the preparation of new extracts and GC-EAD measurements. Unexpectedly, Prof. W. Francke passed away (for a commemoration see Szőcs and Tóth, 2021, Acta Phytopathologica et Entomologica Hungarica 56: 3-4.), therefore, based on his identification, verification of structures in the extracts were attempted in our lab (GC-MS: 6890GC HP-5 MS UI column - 5973 MSD, Agilent, scan mode, EI 70 eV, NIST 11 and Wiley libraries). Our results were later confirmed by Prof. S. Schulz (Inst. Org. Chem., TU Braunschweig, Germany). The presence and structure of the two components were confirmed and it was also demonstrated that topical application of JHIII increased the amount of one of the pheromone components, while not that of the other component (statistically proven).

The behavioral effects on CBB adults (altogether 120 specimens/sex), evoked by the newly identified pheromone components, were verified in lab tests using a 4-arm arena (olfactometer) (Sigma Scientific LLC, Micanopy, FL, United States), connected to a Clean Air Delivery System (CADS) (Sigma Scientific LLC) and to a Stanley DST100/8/6 silent compressor. This instrument (which is to our knowledge unique to Hungary) was obtained from an independent project NKFIH "IF-1/2019", by G. Bozsik PhD and SP in the present project. In the field, the attractivity of a binary mixture of the pheromone components against unbaited control were proven by trapping tests, using four-unit Lindgren funnel traps (LFT) (Contech Enterprises Inc., Canada), conducted in a *T. occidentalis* 'Smaragd' plantation, at the Prenor Tree Nursery (Szombathely). Publication of these results are in the stage of circulating an advanced draft to foreign co-authors (Bozsik, Molnár, Soós, Schulz, Tröger, †Francke, Szőcs: Identifications of two compounds from female cypress bark beetle and evidence on their pheromonal behavioral role in laboratory and field tests. For J. Chemical Ecology).

A set of components in host plant volatiles, perceived by CBB, were identified by us, prior to this project (Bozsik et al., 2016, J. Appl. Entomol., 140: 434-443), however, sampling had been taken at that time from isolated (cut) *Thuja* twigs, mimicking injured trees. This matches to the situation during breeding season of CBB, when adults look for the trunks of declining trees to make their breeding galleries. However, freshly emerged adults look for healthy trees in autumn, for feeding on foliage and for boring their overwintering tunnels close to the edge of small twigs.

In a preliminary trial during the present project, volatile profiles of healthy and declining *T. occidentalis* ‚Smaragd' and that of *T. plicata* 'Atrovirens' were compared, and marked differences found (Bozsik et al., 2018, ISCE2018, p. 75).

Therefore, we continued and searched in the present project in more details for those components in the host plant volatiles, which are detected by CBB at autumn part of the season. As another upcoming xylophagous pest of scale-leaved conifers, the invasive cypress jewel beetle, *Ovalisia* (*Lamprodila* / *Palmar*) *festiva,* (CJB), (Coleoptera: Buprestidae, Chrysochroinae) turned to be a devastating pest practically in course of this project, we decided to include this pest as well into these studies. Odours were collected from intact *T. occidentalis* ‚Smaragd' trees, by means of volatile collection, using charcoal filter (Brechbühler AG, Schlieren, Switzerland, and DC12 rotary vane pump, Fürgut GmbH, Germany). GC-EAD of natural collections, GC-MS and electroantennographic (EAG) screening of synthetic compounds, all performed in our labs, were completed by basically the same methodolology, as described above. As a result, a set of odour components, eliciting antennal responses were chemically identified and differences in perception were verified using synthetic compounds, demonstrating that CBB and CJB „see" their common host tree differently (Bozsik et al., 2022, Physiol. Entomol., 47:136-146.). This finding should be of interest, when developing kaironone-based trap lures, for monitoring the flights of these pests.

In course of the project lot's of CBB in various larval stage, developing inside the breeding galleries, were collected and reared to adults. From these samples several hymenopterous parasitoids, some in significant numbers, emerged. Three parasitoid species were new to the Hungarian fauna, while for three parasitoid species CBB were documented as a new host. The dominant parasitoid species was *Leluthia transcaucasica* (Hymenoptera: Braconidae), which might become important in the future, as a biocontrol agent (Bozsik et al., 2019).

In the original range of distribution CBB is known as a vector of the phytopathogen fungus, *Seiridium cardinale* (Ascomyota, Sordariomycetes, Sporocadaceae), responsible of a pandemic disease of *Cupressaceae*, commonly referred to as cypress canker. We wanted to find out whether the CBB populations in Hungary carry this pathogen, and check whether the pathogen can be identified from tree tissues, showing virtual symptoms. Twigs of various trees (Cupressaceae), mainly that of *T. occidentalis* and *Cupressus x Leylandii*, with visual symptoms of cypress canker infection, were taken from various regions of Hungary (Budapest, Szombathely, Tahi, Velence), in course of the first year of the project. Freshly collected CBB adults (surface of cuticula and homogenisation) were also tested for the possible presence of *Seiridium cardinale.* The presence of *Seiridium cardinale* was not proven in any sample (DNA extraction, PCR, incl. nested PCR of the ITS region). With the involvement of a phytopathological team of our Institute, a new trial was carried out in the fifth year. Samples showing symptoms were taken also from *T. occidentalis* 'Malonyana', heavily infested by CBB, at various sites of Budapest and also from Tahi and Szombathely (yielding 207 isolates from 25 trees, checking 152 ITS sequences). No evidence on the presence of *S. cardinale* was found so far, however, some other fungi, highly pathogen in Cupressaceae, were identified to genus, some of them tentatively also to species. Upon unequivocal species identifications, these results are intended to publish in a reputed international phytopathological journal (submission scheduled later this year).

The above results on chemical communication provide basis for developing pest-specific lures for monitoring CBB. Future develoments should be directed on fine-tuning of the composition of baits, composed of selected set of host plant volatiles and pheromone components, as well as on formulating the optimized blend into dispersers, with strong field performance. Exploiting of the acoustic signals may, in theory, enhance the power of chemical lures, however, at present this possibility is still far away in the horizon. Later, the pheromonal / kairomonal baited traps offer ideal possibilities to easy sampling CBB populations for vectorological surveys.