Changing volatile profile of arborvitae, Thuja occidentalis, by drying up and infestation: selective olfactory cues for the cypress bark beetle, Phloeosinus aubei

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Abstract

The cypress bark beetle, *Phloeosinus aubei* (Coleoptera, Curculionidae, Scolytinae) prefers to build breeding galleries in decaying host trees. However, volatile cues, specific to trees suffering from drought stress or earlier infestation have not yet been reported. This knowledge would actually be most useful, as this invasive, wood-boring species established huge populations across the temperate zone of Europe and became a key pest of ornamental trees of Cupressaceae in tree nurseries and urban green areas. In order to reveal key components in host volatiles of stressed trees, which are perceived by the pest volatile profiles of intact arborvitae, *Thuja occidentalis* 'Smaragd' were compared to those suffering from drought, and fresh infestation of *P. aubei*. Analyses of volatiles by gas chromatography coupled to an electroantennographic detector revealed substantial differences between healthy, drying up and infested trees. Structure elucidation of the major antennally active components revealed that α-thujone was the major component in volatiles of healthy trees, while volatiles of infested trees were dominated by α-pinene and α-thujene. Besides that, trace amounts of camphene, fenchene, as well as substantial amounts of b-pinene, myrcene, limonene and p-cymene were also found in volatiles collected from trunks, housing fresh nuptial chambers of *P. aubei*. Fenchone was present in each type of volatiles, however, only in low amounts. Further studies should be directed to reveal the behavioral role of these components, which could be helpful in developing kairomone-based techniques for monitoring the flight of the pest in stands of scale-leafed trees.

1. Introduction

In host plant selection of most bark beetle species choosing a tree in proper heath status for colonization is just as important, as choosing the right tree species. Some bark beetle species are able to colonize healthy trees, others live in dead logs, while many others prefer trees, being in various degree of decline (Toffin et al. 2018; Schlyter and Birgersson 2000, and references therein). Drought is known to promote bark beetle attrack of pine-leafed associated species (Faiola and Taipale 2020). Little attention has been paid to long-range, kairomone-triggered attraction of bark beetles associated with scale-leafed conifers. A study in Oregon revealed that a bait composed of natural essential oils, acetone and ethanol attracted *Phloeosinus scopulorum* Swaine and *P. serratus* (LeConte), where ethanol, mimicking declining western juniper, *Juniperus occidentalis* var. *occidentalis* Hook odour, was primarily responsible for the attraction (Hayes et al. 2008). Although scarcely studied in this respect, this seems to be the case in the invasive cypress bark beetle, *Phloeosinus aubei* Perris, CBB, (Coleoptera, Curculionidae, Scolytinae). By revealing the life cycle of long time ago established populations, resulting by now economically alarming outbreaks in the temperate zone of Europe, it turned out that female beetles search for declining host trees for making breeding galleries in April-May, while adults of both sexes visit healthy trees for boring small overwintering tunnels, from August to October (Bozsik and Szőcs 2017).

CBB is confined to various tree species of the plant family Cupresseceae (Balachowsky 1949; Mendel 1984). While there are detailed phytochemical studies on the essential oil contents of scale-leafed conifers (Piovetti et al. 1981; Pauley et al. 1983; Adams 1998; Pierre-Leandri et al. 2003; Wang et al.

2022), only a few reports focused on the value of the volatile organic compounds, VOC, as potential host-plant kairomonal cues for CBB. Bozsik et al. (2016) reported more than twenty VOCs, from isolated (detached) twigs of American arborvitae, *Thuja occidentalis* L. cv. 'Smaragd' ('Emerald'), each of which elicited antennal response from CBB. In that study, however, the freshly cut twigs originated from otherwise healthy tree, therefore represented rather an immediate response to an acute physical stress factor, than that of an altered volatile profile of a declining tree, suffering from chronic stresses. In a more recent study, sixteen components were found in the VOC profile of healthy *T. occidentalis* 'Smaragd', which elicited antennal response from either males, or females of CBB, and of the cypress jewel beetle, *Ovalisia* (*Lamprodila/Palmar*) *festiva* L. (Coleoptera: Buprestidae, Chrysochroinae), respectively (Bozsik et al. 2022). The cypress jewel beetle is another upcoming, invasive wood-boring pest of Cupressaceae, which attacks both healthy and declining trees. Also, the question remained open: which are the specific components in the volatile profile of declining trees, which are picked up by CBB as cues for searching for suitable host for breeding galleries.

Answering this question is demanding also from practical point of view, because of the growing economic importance of CBB. Originally confined to the Mediterrean area and Asia Minor (Balachowsky 1949), CBB reached the Carpatian Basin probably along the so-called Illyr (West-Mediterranean) migration route (the route itself was described by Varga 2010) long time ago (see a historic record of Endrődi 1959). After several decades of latency period, which in precedented also in other invasive insect species (Kozár et al. 1995; Kozár 1997), CBB suddenly devastated young *T. occidentalis* 'Smaragd' stands, in ornamental tree nurseries around Szombathely, SW/W-Hungary (Rakk and Bürgés 1994; Reiderné-Saly and Podlussány 1994). Proceeding from South-West towards North-East, CBB spread all over Hungary, in the subsequent years (Both and Farkas 2005). On a European scale, the spread occurred along various directions, reaching for example Hessen, Germany (Dern 1976), East-Germany (Sobczyk and Lehmann 2007), the Netherlands (Mooral 2010), Romania (Olenici 2015) and United Kingdom (Winter 1998). The recent spread of CBB across the temperate zone of Europe, was followed by serious economic losses and ruining the aesthetic value of ornamental evergreen trees of Cupressaceae in urban green areas (Mooral 2010; Fiala and Holuša 2019).

At present, neither kairomone-, nor pheromone-baited traps are available for monitoring the flight of CBB, because of the insufficient knowledge of the chemical communication of the pest. In order to establish basic knowledge, which could support the development of kairomone-baited traps, this study is aiming to reveal the key olfactory signals for CBB in the volatile profile of stressed *T. occidentalis* trees.

2. Materials And Methods

2.1. The model system: an extrazonal host tree – bark beetle re-encounter

Our model system represents an example, resulted on man-made influence on vegetation in urban habitats, pre-adapted it susceptible for the settlement of invasive species, appearing in the doorway.

Notably in the present case, the Mediterranean cypress, *Cupressus sempervirens* L., which is the primary host plant of *P. aubei* in the Mediterranean, is not native to the temperate zone of Europe, moreover, only the common Juniper, *Juniperus communis* L. represents the family of Cupressaceae in Hungary (Soó 1964). However, cultivars of several scale-leafed conifers, such as various species of genera *Thuja, Chamaecyparis, X Cupressocyparis, Cupressus*, are frequently planted ornamental trees in gardens and city parks across the temperate zone of Europe. Also, horticultural activities had provided a spread table for *P. aubei*, well before it arrived this area. Interestingly, the arrival of the pest was not followed right then by an explosive invasion, instead it was preceded by a long latency period. When rapid population build-up, accompanied by alarming tree declines were detected in ornamental tree nurseries, actual plant protection techniques proved to be incapable to cope with the pest. Even proper monitoring systems were not available, as the chemical ecology of *P. aubei* had been largely unknown, consequently no pheromone- or kairomone-based traps were available, which is still the case todays.

For the development of kairomone-based monitoring, we revealed how *P. aubei* adapted to the temperate climate (Bozsik and Szőcs 2017) and began to study host plant kairomones intensively (Bozsik et al. 2016; Bozsik et al. 2022). Our present study fits to the latter line of research, by reporting on the effects of stress on the composition of volatiles emitted by declining trees, suffering from either draught, or infestation of *P. aubei*. In the present study, we focused on those components in stress-induced volatiles, which are perceived by *P. aubei*, rather than trying to chemically identify as many compounds as possible, regardless their role, they may play in a composed kairomone.

2.2. Selection of host trees for sampling volatiles

Two types of stressed *T. occidentalis* ‘Smaragd’ trees were chosen, representing the major stress factors. As irrigation poses a challenge in some tree nurseries, trees suffering from drought, but not infested by *P. aubei* were selected for volatile collection. On the other hand, well-maintained trees, infested by freshly made nuptial chambers of *P. aubei* were included in the studies. For comparison, volatiles were also collected from healthy, not infested trees, as well.

Tree specimens originated from Tahi Tree Nursery (GPS: 47.7653704536209 N, 19.06798750162125 E). Selected trees were approximately 4–5 years old, ca. 1.5 m height and ca. 5 cm diameter at 1 m above ground level. They were carefully taken out with ground ball and transported to the laboratory of Plant Protection Institute, Centre for Agricultural Research (Budapest, Hungary, GPS: 47.54803661427715 N, 18.93472731113434 E) and replanted into flowerpots. They were kept under natural conditions.

In order to have a preliminary insight into the differences in volatile profiles between cultivars of *T. occidentalis*, apart from cv. ‘Smaragd’, *T. occidentalis* ‘Brabant’ was also included in the studies. For similar reasons, just to have a first indication on the volatiles of another *Thuja* species, the closely related to *T. plicata* ‘Excelsa’ was involved too in the studies. These taxa are, in general, also heavily hit by the attack of *P. aubei*. However, the tree specimens, taken for the studies, were free from infestation, and looked healthy. Samples of trunks were isolated, as describe above for healthy *T. occidentalis* ‘Smaragd’.

2.3. Experimental insects

P. aubei adults were collected in Tahi Tree Nursery and in Prenor Tree Nursery (Szombathely, Vas-county, GPS: 47.26419949232103 N, 16.59957200288773 E) during their overwintering season, from *T. occidentalis* 'Smaragd' twigs. Specimens were isolated by sex and kept under ambient conditions in *T. occidentalis* 'Smaragd' twigs, in an open-wall greenhouse of the Plant Protection Institute.

2.4. Volatile collection from intact trees

Single healthy undamaged, intact twigs of *T. occidentalis* 'Smaragd' were covered right on the tree, by a chemically inert oven bag (Alufix GmbH, Wiener Neudorf, Austria), from the tip till ca. 25 cm length backwards. Headspace volatiles were collected by 1.5 mg charcoal CLSA filters (Brechbühler AG, Schlieren, Switzerland), with the flow of 2.6 L min^{-1} for 4 hours, under closed loop system, using a DC12 rotary vane pump (Fürgut GmbH, Tannheim, Germany). The procedure was carried out under ambient conditions, when the temperature was 23–24°C. Volatiles from the filter were washed by *n*-hexane (Sigmα-Aldrich, Merck KGaA, Darmstadt, Germany), for three times. The total volume of extracts was 50 µl. Extracts were kept in a -40°C freezer, until analyses.

2.5. Volatile collection from drying up trees

For mimicking drying up trees, a ca. 20 cm long, and 5 cm diameter part of *T. occidentalis* 'Smaragd' trunk was cut from living tree and kept under ambient conditions for 4 months (from December to April). Volatiles from the declining, semi-dried piece of trunk were collected using the same method as described above. Oven bag was used to isolate the undamaged bark of the trunk from the mechanically injured parts.

2.6. Volatile collection from trees, freshly infested by *P. aubei* females

Unmated female *P. aubei* adults (number of females: 32) were isolated with a ca. 20 cm long, ca 5 cm diameter part of *T. occidentalis* 'Smaragd' piece of trunk in a 4 L glass jar. All the females prepared nuptial chambers under the bark of the trunk. One month after the first females started to make their nuptial chambers, headspace volatiles of the trunk with *P. aubei* females in their nuptial chambers were collected by the same method as above.

2.7. Volatile collection from fresh chewing dust and frass of *P. aubei* females

320 mg of freshly produced chewing dust and frass of *P. aubei* females, gained from nuptial chambers in *T. occidentalis* 'Smaragd' were collected and isolated in a baking bag, then headspace volatile collection procedure was carried out.

2.8. Volatile collections from *T. occidentalis* 'Brabant' and *T. plicata* 'Excelsa'

For comparing the volatile constituents of two *Thuja* species, *T. occidentalis* 'Brabant' and *T. plicata* 'Excelsa', headspace volatiles of intact, healthy twigs with leaves were collected by the same method as described above, with the difference of the timespan of volatile collection, which were only 3 hours.

2.9. Analysis of volatile collections by gas chromatograph coupled to an electroantennographic detector

For comparing the volatile constituents of living, declining and female *P. aubei* infested *T. occidentalis* 'Smaragd', gas chromatographic analyses equipped with electroantennographic detector (6890 N gas chromatograph, Agilent Technologies Inc., Santa Clara, CA, USA) was used. A DB-WAX column was used for separation of the volatile components (30 m x 0.32 mm x 0.25 µm film thickness, J&W, Scientific, Folsom, CA, USA), which was linked to an electroantennographic detector (SYNTECH, Hilversum, the Netherlands) for simultaneous analyses with the flame ionization detector (FID) of the GC. Extracts were injected in spitless mode. The oven temperature was held at 60°C for 1 min, then set to 220°C at 10°C/min. The carrier gas was helium (4.0 ml/min).

Head and antennae of male *P. aubei* were prepared between two glass electrodes filled with Ringer solution and attached to silver/silver chloride electrodes and connected to an IDAC 2 amplifier (SYNTECH, Ockenfels SYNTECH GmbH, Kirchzarten, Germany).

For analyzing the extracts of *T. occidentalis* 'Brabant' and *T. plicata* 'Excelsa' the same column and GC settings were used as above, but instead of spiteless mode, injections were made in on-column mode.

Electroantennographic analyses were made with female and male antennae, too.

2.10. Analysis of volatile collections by gas chromatograph coupled to mass spectrometry

Chemical identification of components, which evoked antennal responses from *P. aubei*, were based on calculation of Kováts Retention Indices and comparison of spectra to NIST Webbook database (https://webbook.nist.gov/chemistry/). Injection of synthetic samples (source: camphene and fenchene are supplied from Thermo Fisher Scientific Inc., Waltham, MA USA; the others are supplied from Sigma Aldrich, Merck KGaA, Darmstadt, Germany) and comparison of retention times with the EAD active compounds in the GC-EAD setup, were performed to verify the results. Methodology and instrumentation were based on our related, previous MS identifications (Bozsik et al. 2016; Bozsik et al. 2022). Briefly, an Agilent 6890 GC (Agilent Technologies Inc.) coupled to an Agilent 5973 mass selective detector was used for GC-MS. The GC was equipped with a HP-5 MS UI (30 m x 0.32 mm x 0.25 µm, J&W, Agilent) column.

3. Results

The prevailing components with marked antennal responses in the volatiles of intact *T. occidentalis* 'Smaragd' trunk were fenchone (compound 9), α-thujone (compound 10) and b-thujone (compound 12), in the order of elution (Fig. 1A and Table 1 – numbering of compounds as in Figures and Tables). Out of

them α-thujone was the far most abundant component. Apart from these tree components, 1-octen-3-ol (compound 11) and terpinene-4-ol (compound 14) also evoked strong antennal responses, even though they were present only in subtle amounts in the collections.

Table 1

Identified components of volatile collections related to Figure 1.

A: Volatiles collected from trunk of healthy *T. occidentalis* 'Smaragd', B: from that of drying up trunks of *T. occidentalis* 'Smaragd', C: from that of infested by freshly carved nuptial chambers of *P. aubei*, and from chewing dust / frass of *P. aubei*, boring in trunks of *T. occidentalis* 'Smaragd'. "x" denotes that the compound was found, "-" that it was below the limit of the antennal detector. Compounds are uniformly numbered across figures and tables.

No.	Retention time (min)	Compound	A	B	C	D
1-2	2.00	**α-pinene; α-thujene**	-	x	x	x
3	2.20	**camphene**	-	x	x	x
4	2.28	**fenchene**	-	x	x	x
5	2.58	**b-pinene**	-	x	x	x
6	3.02	**myrcene**	-	x	x	x
7	3.41	**limonene**	-	x	x	x
8	3.48	**p-cymene**	-	x	x	x
9	5.52	**fenchone**	x	x	x	x
10	5.82	**α-thujone**	x	x	x	x
11	5.99	**1-octen-3-ol**	x	x	x	-
12	6.05	**b-thujone**	x	x	x	x
13	6.48	**camphor**	-	x	x	x
14	7.86	**terpinen-4-ol**	x	x	x	-
15	7.94	*beetle specific compound*	-	-	x	x
16	8.10	*unknown*	-	x	x	-
17	8.62	*unknown*	-	-	x	-
18	9.31	*unknown*	-	x	x	x

Each of the above-mentioned components were also present in volatiles of drying up *T. occidentalis* 'Smaragd' trunk, in rather similar ratios (Fig. 1B and Table 1). Accordingly, the pattern of antennal responses to this set of components was very similar. However, some early eluting components, such as α-pinene and α-thujene (compounds 1 and 2), which eluted right after that the *n*-hexane solvent, followed by camphene (compound 3), fenchene (compound 4), b-pinene (compound 5), myrcene (compound 6), limonene (compound 7), p-cymene (compound 8) also appeared in moderate amounts, however, abundant enough for evoking antennal responses. These compounds could be detected by the flame ionization detector (FID) in the collections from healthy trees as well, however, there were present only in trace amounts and did not evoke antennal responses.

A rather different volatile profile in respect of the relative amounts of components was found in *T. occidentalis* 'Smaragd' trunk, housing nuptial chambers, freshly bored by *P. aubei* females. Here α-pinene and α-thujene were the major components, which impressively outcompeted the rest of the components (Fig. 1C and Table 1). b-Pinene, myrcene, limonene and p-cymene stretched out also impressively, evoking significant antennal responses. Fenchone, α-thujone, 1-octen-3-ol and b-thujone were also present, however in low amounts only. Nevertheless, they evoked relatively strong antennal responses, likewise camphor (compound 13) did. A much later eluting trace compound 15, with strong antennal response, also showed up. This compound was absent from non-infested *T. occidentalis* collections, however present also in fresh chewing dust / frass samples. Evidence that this is a beetle-specific compound will be published elsewhere.

Volatiles collected from chewing dust / frass, taken out from newly carved nuptial chambers, showed a very similar profile to collections taken from newly infested *T. occidentalis*, with a living female in the nuptial chamber (Fig. 1D and Table 1).

Volatiles of healthy *T. occidentalis* 'Brabant' trunk contained the same major, antennally active components, in a rather similar ratios (Fig. 2A and Table 2), as healthy *T. occidentalis* 'Smaragd'. The composition of healthy *T. plicata* 'Excelsa' (Fig. 2B and Table 2) looked almost identical to that, with the only marked difference that it did not contain fenchone. EAD responses of male and female *P. aubei* antennae did not differ in this case.

Table 2

Identified components of volatile collections related to Figure 2.

For more explanation see Table 1.

No.	Retention time (min)	Compound	A	B
9	8.02	**fenchone**	x	-
10	8.36	**α-thujone**	x	x
12	8.59	**b-thujone**	x	x
14	10.72	**terpinen-4-ol**	x	x

Ratios of antennally active components in volatiles of intact, drought-stressed and CBB freshly infested *T. occidentalis*, as well as that of chewing dust / frass of CBB boring tunnels into trunk of *T. occidentalis* are displayed on Fig. 3. The dominant component in heathly tree volatile is α-thujone, while stressed trees emit mostly α-pinene and α-thujene.

4. Discussions

In this study the GC-EAD profiles of volatiles, collected from healthy, drying up and *P. aubei* attacked *T. occidentalis* trunks were compared to each other, by standard methodology. The aim was to search for differences between the patterns of those components which were perceived by CBB females, rather than pinpointing and identifying as many trace components, as possible, irrespective to their role, as potential kairomones for CBB. This was a complementary approach to that followed in earlier publications, where the composition of volatiles of either drying up (Bozsik et al. 2016), or intact trees (Bozsik et al. 2022) had been studied separately. The main emphasis in the present study was put on components appearing on early response to new infestation, caused by *P. aubei* females, which is, to our knowledge, a new approach.

The abundancy of some highly volatile, early-coming components, such as a-pinene, a-thujene, camphene, fenchene, b-pinene, myrcene, limonene and p-cymene drastically increased in volatiles of stressed trees, as compared to samples collected from healthy trees. At the same time, the amounts of fenchone, a-thujone and b-thujone, which compounds dominated the samples taken from healthy trees, significantly decreased in stressed trees. These shifts were especially spectacular in case of *P. aubei* infested trees and also on case of volatiles emanating from fresh chewing dust / frass, however, could clearly be observed in less pronounced manner also in draught-stressed trees. Fluctuations in abundancies of compounds strongly correlated to amplitudes of antennal responses of *P. aubei* in GC-EAD measurements. This indicates that *P. aubei* can distinguish between healthy and stressed host trees, at least at the level of peripheral perception.

The set of components found in the samples corroborated our earlier results in respect to antennally active components (Bozsik et al. 2016; Bozsik et al. 2022), and earlier publications, reporting barely on phytochemical analyses of volatile constituents of related scale-leafed conifer trees (Szolyga et al. 2014; Lis et al. 2019; Ni et al. 2019). As for minor and trace components, which were quite numerous in the present samples and reported also in the above publications, they felt aside from the attention of this

study, performed along the working assumption that the most abundant antennally active components, blended in some specific ratios, can be regarded as condidates for functioning as composed olfactory cues, responsible for long-range host seeking behavior of *P. aubei*. In this respect it is worth mentioning that a-thujene, camphene, fenchene, b-pinene, myrcene, limonene and p-cymene were found for the first time to elicit antennal response from CBB.

Also, further studies are needed to reveal the behavioral relevance of the antennally active components, specific to declining host trees. Seasonality of behavioral response of beetles should also be studied in this respect, because it has already been reported earlier that the preference for decaying trees is expressed only in the breeding season, when female beetles look for suitable host trees for preparing breeding galleries. Notably, in the breeding season, which occurs in spring and early summer under continental climate, females, having activated after overwintering, look for trunks of stressed trees for boring nuptial chambers to start preparing breeding galleries, while in autumn, freshly emerged adults search for heathy trees for maturation feeding and boring overwintering tunnels in tip of twigs (Bozsik and Szőcs 2017). Forming this latter type of tunnel (single-armed, short overwintering gallery) is probably resulted from an adaption process to the continental climate. This may indicate that invasions may alter not only the biodiversity of newly occupied regions and, in case of pests, pose new threatens to horticulture, agriculture and silviculture, but also could induce changes in the behavior of the invading insect species, itself. The fauna is under steady change, driven by either climate warming or human activities, therefore studying of insect migrations, ecological corridors and detecting newly arrived pests are of special importance, as Ferenc Kozár (1997) showed by examples on the Carpathian Basin, in his pioneering, conceptual review article. The recent piece of study may serve as a contribution to get closer to reveal olfactory cues for CBB, which could establish background knowledge for developing monitoring traps for trekking the spread of CBB.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Author Contributions

GB made the experimental design, carried out the experiments (volatile collections, GC-EAD), evaluated the datasets and created the figures and tables. BPM made the mass spectrometry (GC-MS) to identify the antennally active compounds. GSz managed the project. GSz and GB wrote the main manuscript text.

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Figures

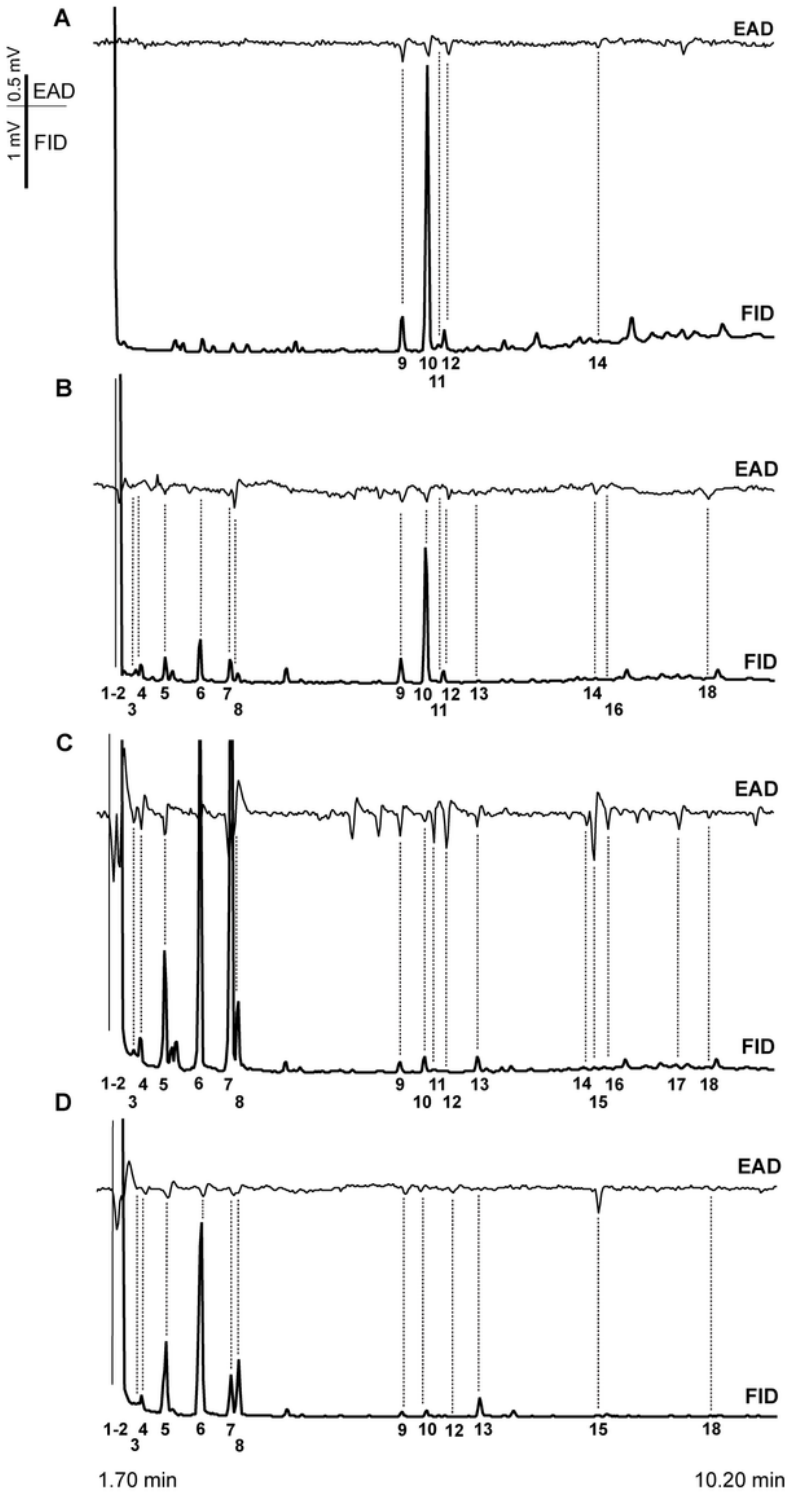

Figure 1

Electroantennographic responses of male *P. aubei* in GC-EAD measurements to volatiles of different collections from *T. occidentalis* 'Smaragd' trunks of various health status and from fresh chewing dust, /frass produced by *P. aubei* females, while boring in trunks of *T. occidentalis* 'Smaragd'.

A: healthy leaves and twigs

B: declining, semi-dried trunk

C: female *P. aubei* infested trunk

D: fresh chewing dust and frass of *P. aubei* females

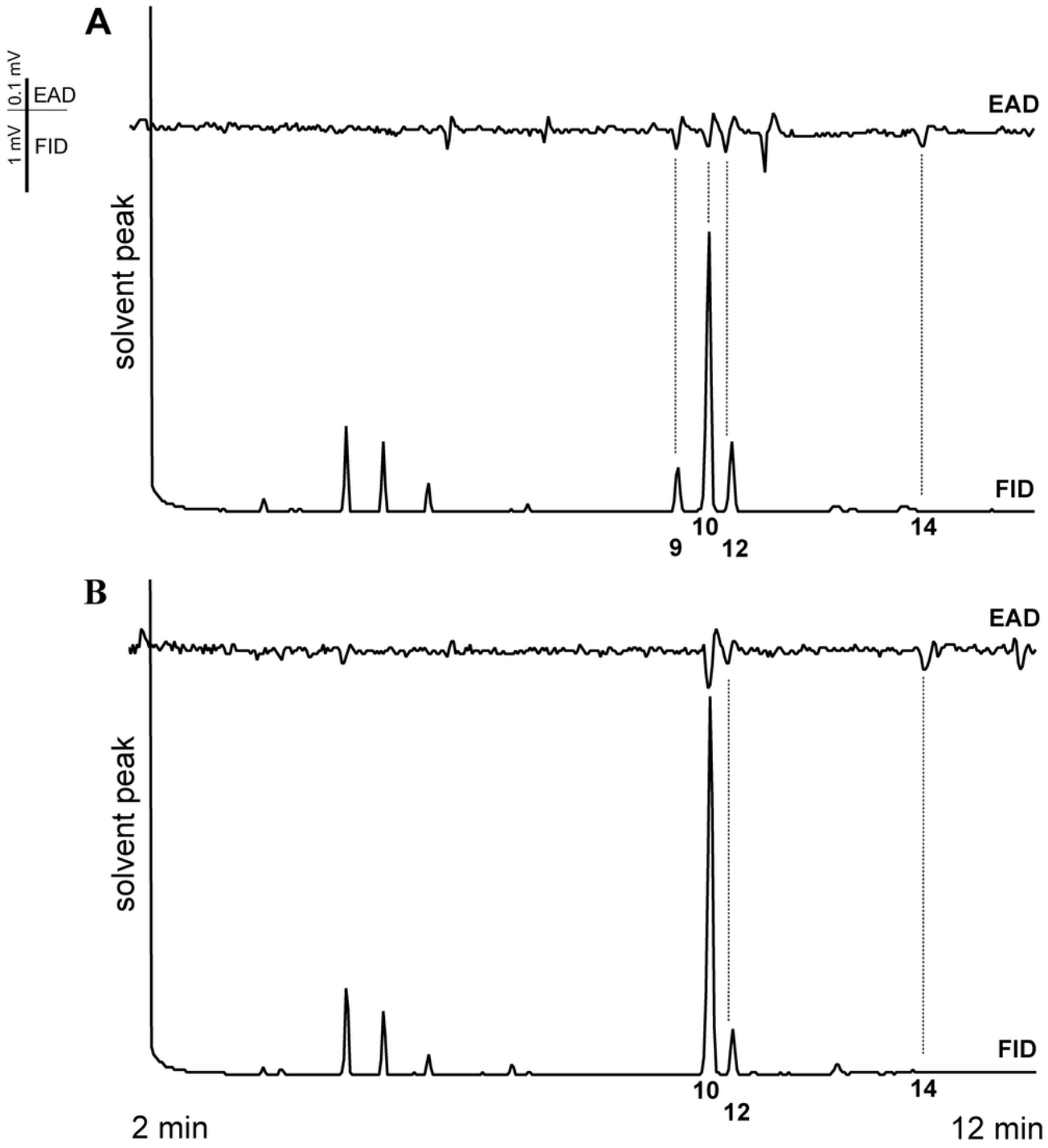

Figure 2

Electroantennographic responses of female *P. aubei* in GC-EAD measurements to volatiles of healthy twigs and leaves of *T. occidentalis* 'Brabant' (A.) and *T. plicata* 'Excelsa' (B.)

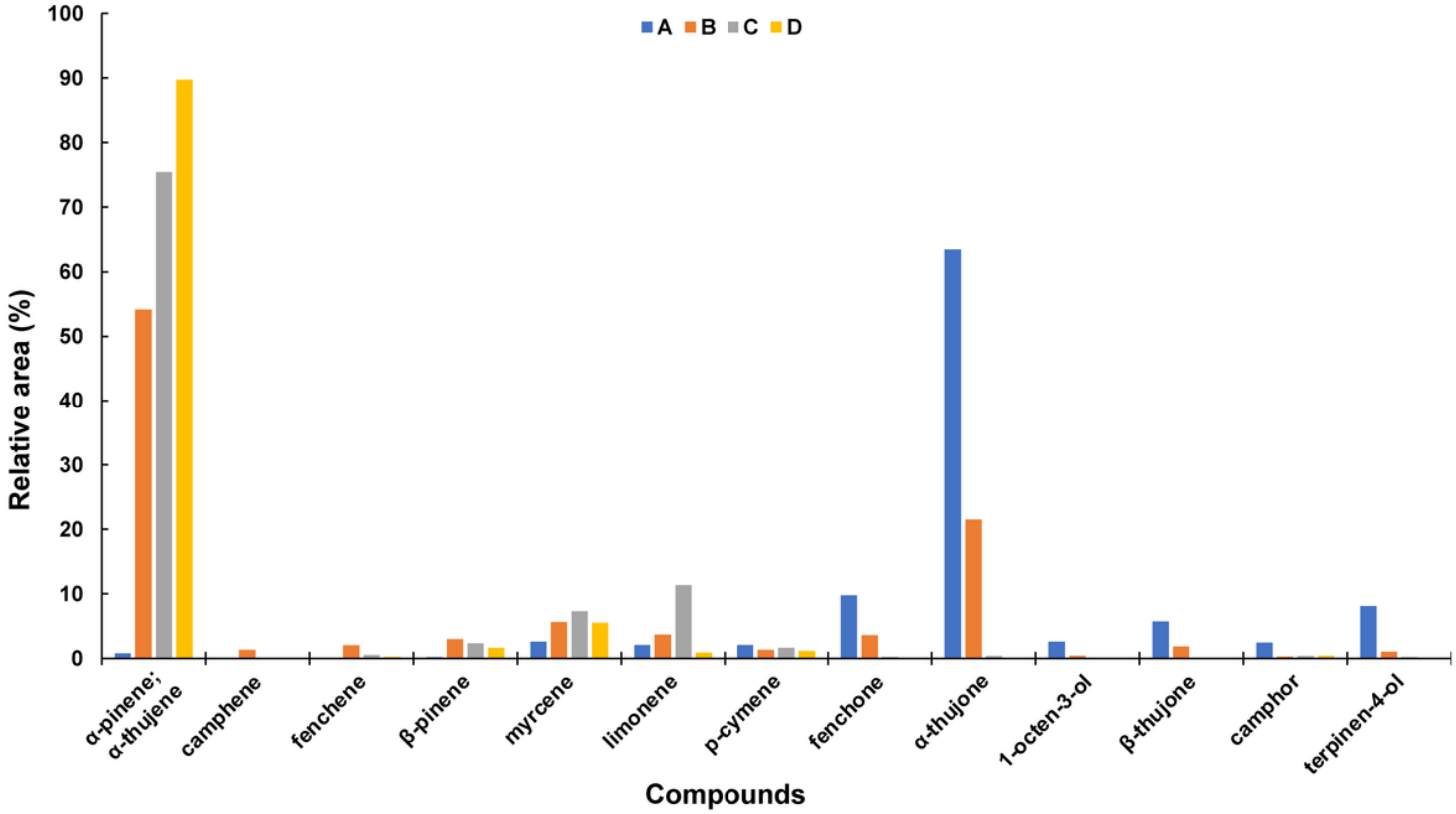

Figure 3

Relative area of the identified compounds

A: healthy leaves and twigs

B: declining, semi-dried trunk

C: female *P. aubei* infested trunk

D: fresh chewing dust and frass of *P. aubei* females