THESIS OF PhD DISSERTATION

HOST PLANT-RELATED AND PHEROMONAL CHEMICAL COMMUNICATION OF THE EUROPEAN FLEA BEETLE SPECIES (PHYLLOTRETA SPP., COLEOPTERA, CHRYSOMELIDAE)

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1. INTRODUCTION

Crucivorous flea beetles (*Phyllotreta* spp., Coleoptera, Chrysomelidae) rank among long-known, important pests of cruciferous plants both in Europe and in America. Their importance can be explained with the wide range of the cultivated cruciferous plants.

The most important damage of these univoltine species is caused by the overwintered adults, which become active in the early spring. Flea beetles cause sieve-shaped damage in the plant tissue which upsets the water balance. They cause high level of mortality of seedlings, growing in young plants is blocked, later they cause unequal ripening.

The importance of *Phyllotreta* species as pests is aggravated by the fact that several species are known to act as vectors of numerous plant pathogens as well.

In the control of flea beetles the application of a trap for detection would be very useful similarly to several other pest species, where such traps are applied successfully. With this method the appearance of the beetles in early spring, and the next generation in the second part of the summer could be detected and the population size of the beetles could be estimated.

The volatile isothiocyanates and thiocyanates are long-known food attractants for flea beetles. These drive as secondary metabolites from the decomposition of the non-volatile glucosinolates, which themselves evoke feeding, and egg-laying. Among isothiocyanates, allyl isothiocyanate (ALLYL ITCN) is the longest known chemical, which was found attractive to certain flea beetle species by several authors.

Beside ALLYL ITCN, other isothiocyanates and thiocyanates may have considerable attractive effect to flea beetles.

Recently US scientists showed out that pheromones also play a role in the chemical communication of the flea beetles. In the course of their research work it was found that males of *Phyllotreta cruciferae* Goeze produce an aggregation pheromone. Through the analysis of volatiles emitted by males of a North American population of *P. cruciferae* several pheromone candidate components were identified, as follows:

(A) \((+)-(6R, 7S)-2,2,6,10\text{-tetramethylbicyclo[5.4.0]-undeca-1(11),9\text{-diene}};\)
(B) \((+)-(1R,7R)-2,2,10\text{-trimethyl-6-methylene-bicyclo[5.4.0]undec-10\text{-ene}};\)
(C) \((+)-(6R,7S)-2,2,6\text{-trimethyl-10-methylene-bicyclo[5.4.0]-undec-1(11)-ene}};\)
(D) \((+)-\gamma\text{-cadinene};\)
(E) \((+)-(R)\text{-ar-himachalene};\)
(H) \((+)-(1S, 2R)-2,6,6\text{-trimethylbicyclo[5.4.0]undec-7-en-9-one}\).

In a previous North-American field test the mixture of the identified compounds was found active on \(Ph. cruciferae\). The mixture of the male-specific compounds showed synergism with the food attractant, ALLYL ITCN when they were presented together. Presumably these compounds can be used effectively as a more attractive lure, which may provide more sensitive detection in the future.

Based on the above, we selected the following lines of research in this study:

1.1. **Study of the species spectrum of flea beetles attracted to ALLYL ITCN in Central European countries**

The objective of this line of study was to investigate the range of \(Phyllotreta\) species responding to ALLYL ITCN, the longest known food attractant in Hungary, Slovenia and Bulgaria, so that the applicability of this attractant for plant protection purposes can be evaluated.

1.2. **Study of the relative attractive effect of different isothio- and thiocyanates to flea beetle species**

The different odour-preference of the different flea beetle species was investigated through testing several isothio- and thiocyanates, which can occur as food attractants.

Beside ALLYL ITCN, 2-butenyl-thiocyanate, butyl-thiocyanate, phenyl-thiocyanate, 3-butenyl- (3BUT ITCN), phenetyl-, 2-butenyl- and butyl-isothiocyanate were tested.

1.3. **Study of the pheromonal chemical communication of flea beetles**

In this line of study the behavioral effect of different combination and enantiomeric forms of the male-specific compounds from \(Ph. cruciferae\) were tested in the presence of ALLYL ITCN, or of 3BUT ITCN, in field trapping experiments.
The objective was to investigate, which male-specific compounds had field activity and could be considered as a pheromone component. In the course of the laboratory work, the identification of the pheromone structure of several flea beetle species was attempted.

1.4. Comparison of trap types suitable for capturing flea beetle species

For being able to start extended field trials, the selection of suitable sensitive trap types, with high catching capacity was needed, which capture flea beetle species effectively.

Subsequently the objective of this study was developing a nonsaturating trap type, which later could be applied for the detection of the flea beetles and for monitoring their flight pattern.

My research work was carried out in laboratories of the Department of Zoology, Plant Protection Institute, HAS, under supervision of Miklós Tóth. My contribution included planning, setting up and conducting several field experiments, the identification of the flea beetles (from 2002) and the statistical evaluation of the results. The extraction of the flea beetles’ pheromone I carried out myself, through the collection of the volatiles, emitted with the method of closed loop stripping analysis.

I took part of the gas chromatographic analysis of the extracts from the beetles. For these experiments the collection, keeping and identification of the beetles was my own work.

2. FIELD SITE DETAILS, MATERIALS AND METHODS

2.1. Field site details

Field experiments with different objectives were conducted in Hungary, in Pest county (Vecsés, Budakalász, Pusztazátor), in Fejér county (Agárd, Pusztaszabolcs, Nadap, Kápolnásnyék, Ercsi), in Győr-Moson-Sopron county (Dunasziget), in Tolna county (Dunaföldvár), in Zala county (Pusztaszentlélszló, Szentlélszló) and in Hajdú-Bihar county (Debrecen) from 2000-2006. Experimental sites included fields also from Slovenia (Ljubljana) and Bulgaria (Sofia). Experiments were set up in white mustard, maize, kale and cabbage plant cultures or bare soil on the weedy edge of the fields.
The traps set up at soil level were arranged as blocks so that each block contained one trap of each treatment. Traps within blocks were separated by 8-10 m, and blocks were sited at least 20-30 m apart. Block numbers varied between 2-10 depending on the experiment. In the field, old baits were replaced with new ones at 2-3 weeks intervals depending on the lure type. Traps were inspected at 2-3 days intervals.

The captured beetles were removed from the traps and placed in plastic bags with labels in order to their origin can be traced. Except for some experiments, which are marked in the dissertation, I performed the identification of the flea beetle specimen in the laboratory of Plant Protection Institute, HAS. The method of the identification was done according to training from Károly Vig (Savaria Museum, Szombathely).

### 2.2. Trap and baits

CSALOMON® sticky delta RAG and non-sticky VARL+, VARs+, KLP+ traps were used in the tests (Plant Protection Institute, HAS, Budapest, Hungary).

A small piece (1 cm x 1 cm) of household antimoth strip (Chemotox® active ingredient 15% dichlorvos) was placed into each catch container of the nonsticky traps as a killing agent for captured insects.

In the case of plant volatiles, for emitting the lures polyethylene bags or polyethylene vials were used. Baits of the different combination and chiral forms of the male-specific compounds identified from *Ph. cruciferae* were formulated on rubber septum and we got them ready made from the research group of Robert J. Bartelt (USDA, ARS, Natl. Ctr. Agric. Util. Res., Peoria, USA).

Dispensers were attached to 8 x 1 cm plastic handles for easy manipulation when assembling the traps. Baits were wrapped singly in pieces of aluminum foil and were stored at -18 °C until use.

### 2.3. Compounds

The plant volatiles tested were purchased from Sigma Aldrich Kft (Budapest) or resulted from syntheses of the late Prof. E. Möttus (Tartu University, Estonia) and István Ujváry (Chemical Research Center, HAS).
The racemic form of the compounds “A”, “C”, “E”, “H” identified from *Ph. cruciferae* were synthetized by the research group of Robert J. Bartelt (USDA, ARS, Natl. Ctr. Agric. Util. Res., Peoria, USA), the pure enantiomeric forms were synthetized by the research group of Kenji Mori (Tokio, Japan). The (+) enantiomeric form of compound “D” was gained from citronella oil.

### 2.4. Statistics

In the statistical analysis capture data were transformed to \((x + 0.5)^{1/2}\) and analyzed by Student *t* test (in the case of two treatments) or by ANOVA (in the case of more than two treatments) and then treatment means were separated by Games-Howell test. In the case when a treatment did not capture any beetles during the whole experiment, it was tested by Bonferroni-Dunn test if the mean capture of the other treatments differ significantly from zero capture.

In the case, when other statistics was used, it is noted on the given figures in the dissertation.

All statistical procedures were conducted using the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Inc., Berkeley, CA, USA).

### 2.5. Pheromone extraction of flea beetle species

For the pheromone assay of the flea beetle species, pheromone was collected from live beetles.

Beetles were collected in spring from experimental sites at Pusztazámor, Julianna-major and Kápolnásnyék, from different plant cultures. Collection was carried out using sweep net and live-catching KLP traps without antimoth strip.

Before the pheromone collection, collected beetles were kept on cabbage or kohlrabi plants under long-day conditions (light:dark period= 18:6 hours).

Identification of the live beetles was carried out with stereomicroscope on the basis of external marks. Determination of sexes was performed with the examination of the abdominal side of the tip of the abdomen. After the pheromone collection determination of sexes was checked subsequently through dissection of the beetles. During volatile collections flea beeeles
were kept under long-day conditions and were provided with food (chunks of cabbage, or kohlrabi).

Pheromone collection was carried out with the collection of emitted volatiles, in a closed loop stripping analysis system. For trapping the volatiles from the air, carbon filters were used (CLSA-Filter, Brechbühler AG, Schlieren, Switzerland), from which active compounds were extracted with dichlormethane.

Extracted volatiles were examined by gas chromatography (device type HP 5890 GC). As an internal standard tetradecanyl acetate was used in 10 ng injected amounts. The identification of the volatiles was performed by american researchers (Robert J. Bartelt and his research group) with GC-MS method.

3. RESULTS

According to each respective lines of research the results of the experiments were as follows:

3.1. Results of the study on the species spectrum of flea beetles attracted to ALLYL ITCN

In the course of this study we managed to clearly confirm several earlier reports on the activity of ALLYL ITCN for Ph. cruciferae.

Significant field attraction to allyl isothiocyanate was first described in the present study in field trapping experiments for Ph. nigripes Fabr., Ph. atra Fabr., Ph. vittula Redtenb., Ph. undulata Kutch, Ph. ochripes Curt., Ph. diademata Foudr., Ph. balcanica Heikert., Ph. procera Redtenb., a Ph. nodicornis Marsh. and the close relative species to Phyllotreta species, Psylliodes chrysocephala L.

3.2. Results of the study on the relative attractive effect of different isothio- and thiocyanates to flea beetle species

In this study remarkable differences were found in host-plant related volatile preference between Phyllotreta cruciferae and Ph. vittula. Ph. cruciferae responded better to ALLYL ITCN, while Ph. vittula to 3BUT ITCN.
For both species, the binary blend caught similar numbers as the respective single compounds.

Other isothiocyanates and thiocyanates tested did not show considerable activity.

### 3.3. Results of the study on the pheromonal chemical communication of the flea beetles

In these experiments we showed out, that the mixture of the male-specific compounds (identified by american chemists from *Ph. cruciferae*) had biological activity on the European population of this species.

The mixture of these compounds showed an attractive effect on *Ph. vittula* also. According to these results it was presumed, that the pheromone compounds of these two species were partly identical.

Similar tendency was shown in the catches of *Ph. nodicornis* és *Ph. ochripes*, *Ph. procerca*, *Ph. nemorum*, *Ph. nigripes*, and *Ph. undulata*.

In laboratory work, with the gas chromatographical analysis of the volatiles, collected in a closed system, it was shown out the first time, that males of the European populations of *Ph. cruciferae*, *Ph. vittula*, *Ph. undulata*, *Ph. nigripes* and *Ph. nigripes* produced compound “A”, “B”, “C”, “D” and “E”.

Males of these species emitted the compounds in a similar ratio, except for *Ph. cruciferae*, which emitted compound “D” and “E” in larger amount by tendency. Males produced compound “A” in the largest amount. Each examined species emitted compound “C” in the least amount.

From the examined species males of *Ph. nigripes* emitted compound “A” in the largest amount as compared the other species.

In the case of *Ph. cruciferae* it was confirmed, while in the case of *Ph. vittula*, *Ph. undulata* and *Ph. nigripes* it was firstly found, that females of these species did not produce compounds “A”, “B”, “C”, “D” and “E”.

In field experiments it was first shown out, that the plus (+) enantiomeric form of the compounds was active in the case of *Ph. cruciferae* and *Ph. vittula*.

In the case of both species compound “C”, “D”, “E” and “H” had no obvious behavioral activity.
Compound (+)-A [(6R,7S)-2,2,6,10-tetramethylbicyclo[5.4.0]undeca-9,11-diene] was the only male-specific compound for which pheromonal activity could be clearly established in our experiments. These results suggest that compound “A” is the key component and compound “E”, “H”, “C” have minimal or no role as a pheromone component. In the laboratory experiments all of the examined species emitted this compound in the largest amount.

Compound “B” was not examined in field experiments due to the difficulty of its synthesis.

In trapping experiments it was shown out, that volatiles emitted by males have a sufficient attractive effect only in the presence of host plant volatiles.

In the case of Ph. vittula 3BUT ITCN combined with compound “A” appeared to be the most active, while in Ph. cruciferae highest catches were observed in ALLYL ITCN combined with compound “A”.

3.4. Results of the comparison of trap types suitable for capturing flea beetles

In the first phase of the experiments, the sticky RAG trap and the funnel VARL+ and VARs+ traps examined were found sufficiently effective for capturing Ph. procera, Ph. cruciferae, Ph. vittula and Psyll. chrysocephala, which were present on the experimental site.

Although only by tendency, but both funnel designs captured more beetles and their catches were higher on each inspection date, then those of the sticky delta traps.

Most of our subsequent experiments were carried out with the VARL+ trap type.

On the latest phase of the experiments, when we were searching for a more sensitive trap type, it was found, that the KLP+ trap, which was developed for capturing the western corn rootworm (Diabrotica v. virgifera Le Conte) (Coleoptera, Chrysomelidae) recently, captured significantly better of Ph. cruciferae, Ph. vittula, Ph. nigripes and Psyll. chrysocephala than the VARL+ trap.

When the catches of the separate inspection dates were examined, it was found that captures of the KLP+ traps were larger almost on each date, than catches of the VARL+ traps.

These results showed, that the KLP+ traps are more suitable both in sensitivity and in amount of captured beetles than the VARL+ trap type.
4. DISCUSSIONS AND SUGGESTIONS

4.1. The species spectrum of flea beetles attracted to ALLYL ITCN

From the eleven Hungarian flea beetle species, which were attracted by ALLYL ITCN, six species are important pests.

*Ph. cruciferae* is one of the most important pest flea beetles. It causes damage on several cultured cruciferous plants such as cabbage (*Brassica oleracea* L. convar. *capitata* (L.) Alef.), kohlrabi (*Brassica rupestris* Raf. convar. *gongyloides* (L.) Janch.), kale (*Brassica bolla* DC.), broccoli (*Brassica cretica* Lam. convar. *cymosa* Plenck) rape varieties (*Brassica* spp.), white mustard (*Sinapis alba* L.), and this species is also able to propagate several plant pathogens.

*Ph. vittula*, unlike other pest cabbage flea beetles, causes damage not only on Cruciferae, but also on monocotyledonous plants such as winter and spring wheat (*Triticum aestivum* L.), spring barley (*Hordeum vulgare* L.), winter rye (*Secale cereale* L.), oat (*Avenna sativa* L.) and maize (*Zea mays* L.), but it does on millet species (*Setaria* spp.) also. It is a well-known vector of brome mosaic virus.

*Ph. undulata* is among the most important pests of cabbage flea beetles and is a known vector of turnip yellow mosaic virus.

*Ph. nigripes* is a frequent, serious pest of cabbage in Western Europe.

*Ph. atra* is also a very serious pest of cruciferous crops in Europe.

ALLYL ITCN also had an attractive effect to some species, which had narrower host plant specificity, or which were rare species.

*Ph. balcanica* is a rare species in the region. The host plant of this species is reported to be *Sinapis*, *Rorippa* and *Diplotaxis*.

*Ph. nodicornis* feeds on plants belonging to the genus *Rezeda*.

*Ph. diademata* is frequently collected at wet meadows and lives on *Nestia* and *Rorippa*, but has also been reported on Chinese cabbage (*Brassica pekinensis*, Lour.). The host plants of *Ph. ochripes* include *Alliaria officinalis* Andrz. and Chinese cabbage, while *Ph. procera* feeds mostly on *Rezeda* spp.

*Ps. chrysocephalus* is a serious pest of winter oilseed rape (*Brassica napus* L.), and mustard species.

Results showed, that ALLYL ITCN is a sufficient by effective and suitable bait and can be used in detection and monitoring of the flea beetles. Preliminary results in Hungary
indicate that the percentages of flea beetle species caught by ALLYL ITCN baited traps reflected well the percentage composition of flea beetle populations at the given site obtained by other sampling methods.

In practice, when detection is carried out by the grower, there is no need for the identification of the ALLYL ITCN-attracted flea beetle species, because species damage the cultivated cruciferous plants together, in mixed populations.

4.2. The relative attractive effect of different isothio- and thiocyanates to flea beetle species

The different preference to volatiles, which was detected between *Ph. vittula* and *Ph. cruciferae* is not surprising, because of the different host plant preferences of these two species. It is known that *Ph. vittula* has a larger host range as this species feeds not only on cruciferous plants, but also on monocotyledonous plant species.

Papers in the topic of the host–plant related chemical communication of the flea beetles are mostly deal with ALLYL ITCN-related studies, reports on the activity of other isothiocyanates and thiocyanates are not so frequent.

From the compounds which were examined in our study n-butil-isothiocyanate was tested in the experiments of other researcher. In our tests, this compound showed weak activity.

Based on knowledge of the different preference to volatiles, an optimal combination of isothiocyanate compounds may be developed, which attracts both flea beetle species with high efficiency.

4.3. The pheromonal chemical communication of flea beetle species

In *Ph. cruciferae* in trapping test in North America, which were carried out parallel with our experiments in Europe, the field activity of the mixture of male-specific compounds was shown out. Both in the American and the European experiments it was found that the mixture of the male-specific compounds synergisticaly increased the attractive effect of ALLYL ITCN, and their higher dose evoked larger response than the lower dose.
In our field and laboratory experiments it was found, that the same male-specific compounds occurred in a wide range of species in the *Phyllotreta* genus and the examined species produced compounds in similar ratios.

Field experiments suggested that in the case of *Ph. cruciferae* and *Ph. vittula*, compound "C", "D", "E", can be considered only to be different products of the biosynthetic way and they are not pheromone compounds according to their field activity. Further experiments are needed to study, whether their role in the activity is similar in the case of the other species examined.

According to our experiments it can be supposed that the pheromonal chemical communication of the flea beetles is very similar, and the different species can answer to the pheromone compounds of one another. This result is not surprisingly from neither the evolutionary, nor the functionality point of view, because aggregation pheromones serve the aggregation of a species on the host plant, and the host plant-preferences of the examined species is more or less similar, including many cruciferous plants.

Neither in the experiments carried out in America, nor in our own experiments it was shown, that females of the examined species produce compounds “A”, “B”, “C”, “D” and “E”.

Both in the American and in our own experiments it was shown out that photoperiod had an effect on the production of the male-specific compounds. Males, kept at short-day conditions (light:dark period= 10:14 hours) do not emit pheromone. The explanation of ceasing to emit the pheromone at the end of the summer, due to the effect of short-day conditions can be, that preparation for overwinterning and not for reproduction determines the behaviour of the adults at this period.

Based on the results of our studies it can be said, that flea beetles, probably are another example among insect species where the relative importance of pheromonal and host-derived cues are similar.

There are a number of examples known in the insect world, where the presence of the pheromones synergistically increase the attractive effect of host volatiles.

In the well-known example of *Melolontha* scarab beetles (Coleoptera, Scarabaeidae), males orient towards green leaf volatiles originating from damaged leaves from feeding female beetles. So green leaf volatiles in themselves are somewhat attractive. Other researchers found, that more beetles were attracted to blends of the green leaf volatiles with
the pheromone components benzoquinone or toluquinone. The quinones on their own showed no activity.

Similarly, with the scarab *Oryctes elegans* Prell. (Coleoptera, Scarabaeidae), the main aggregation pheromone component alone was minimally attractive, however, when presented together with host plant odor, it caught may beetles.

Similar cases of strong synergism between pheromone components and host volatiles have been described for several species of *Rhynchophorus* weevils (Coleoptera, Curculionidae) and *Carpophilus* spp. (Coleoptera, Nitidulidae) sap beetle species.

There are only few reports concerning the chemical communication in the Chrysomelidae insect family. This family includes several important pests species, so studies in this area are very valuable.

The first Chrysomelid pheromones to be characterized were sex pheromones from *Diabrotica* spp. rootworm beetles. These are produced by females and attract only males.

In the case of the colorado potato beetle (*Leptinotarsa decemlineata* Say) and cereal leaf beetle (*Oulema melanopus* L.) a male-produced aggregation pheromone is known, which attract both sexes.

Demonstration of the relationship between the host plant-related and pheromonal chemical communication of the flea beetles is also important from the practical point of view.

Synthetic compound "A" applied with ALLYL ITCN, or other isothiocyanates can be the basis of a lure combination, which is more attractive and so provides more sensitive monitoring of the flea beetles in the future. Because the (−) enantiomers were not inhibitory, racemic samples could be used as bait components in trapping studies.

### 4.4. Comparison of trap-types suitable for capturing flea beetle species

Until now, different types of sticky traps were the most frequently used trap types for trapping flea beetle species world-wide.

Instead of sticky trap types, the usage of nonsaturating trap types is more advantageous in plant protection monitoring of pests insect species. The reason of this can be explained with the common disadvantage of the sticky trap types, with the temporal changes in the capturing efficiency of the sticky surface, on the base of which they are not suitable for the study of quantitative aspects (inspecting changing population density, establishing threshold values, etc.).
From this point of view nonsaturating trap types, with larger catching capacity are more advantageous. The catching capacity of these traps are much larger than that of the sticky ones, and the leveling off of catches caused by the saturation of the traps can happen after much a longer time, than in sticky types which have a limited catching surface.

Identification of the captured beetles from nonsaturating traps is easier in contrast to catches from sticky trap types, because removing of the sticky material from their body is very difficult.

In the case of the flea beetles some examples can be found in the literature for the usage of water traps, which are difficult to manage, and for boll weevil traps, which was originally developed for the capturing of *Anthonomus grandis* (Boheman, Coleoptera, Curculionidae), as a nonsaturating trap.

Because of the above advantages of nonsaturating trap types, and because of the insufficient knowledge of these trap types in the trapping of the flea beetles, researches in this direction are very useful from the practical point of view.

In the course of our experiments the KLP+ trap type was found to be a sufficiently effective, nonsaturating trap type for capturing flea beetles. The yellow colour of the crawling up panel of the KLP+ trap is advantageous, because it is long known from several papers, that yellow colour is slightly attractive to flea beetles.

This trap type is one of the newest members of the CSALOMON® trap family and is excellent for the detection of the appearance of the flea beetles, for the estimation of their population size and for the following of their flight pattern. The usage of this trap gives help not only in the practice of the plant protection, but enlarges the knowledge about the life cycle of crucivorous flea beetle species with new details.

Because beetles overwinter near the damaged field, with the estimation of the summer population size the size of the overwintering population can be predicted, which will appear next spring. The detection of the summer population in adequate time can be important in the first phase of the plant protection of such cruciferous plants, which have late sowing-time planting technology.
### 5. PUBLICATION LIST GROUPED ACCORDING TO THE SPECIFIC RESEARCH LINES

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| COMPARISON OF TRAP-TYPES SUITABLE FOR CAPTURING FLEA BEETLES |
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