# Aggregation pheromone in *Sternechus subsignatus* (Coleoptera: Curculionidae): olfactory behaviour and temporal pattern of emission

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## Abstract

The Brazilian soybean stalk weevil, *Sternechus subsignatus* Boehman (Coleoptera: Curculionidae), is a pest of economic importance in many regions of Brazil. Volatiles from both sexes of *S. subsignatus* were collected by aeration and the behavioural response of males and females was evaluated using a Y-olfactometer. The results obtained demonstrate that the communication in *S. subsignatus* is mediated by aggregation pheromone as both sexes were attracted to host plant (HP) volatiles, and this attraction was increased by the addition of male volatiles. At least five male-specific compounds (1–5) were detected in the chromatographic analysis, providing chemical support to the behavioural data. Release of these volatiles is dependent on the presence of the HP as the amount of compounds differs significantly when volatiles are collected from weevils with or without access to food. The release takes place mainly during photophase, showing a peak between 4 and 6 h after its beginning, which is also a peak of the insect activity in the field. Studies are underway to elucidate the structures of these putative aggregation pheromone components, in order to evaluate their biological activity under laboratory and field conditions.

## Introduction

The Brazilian soybean stalk weevil, *Sternechus subsignatus* Boehman (Coleoptera: Curculionidae), is a pest of economic importance in many regions of Brazil. To oviposit, the adult female girdles around the main stem, cutting the epidermis and cortex of the soybean. In this region, the eggs are laid into small holes and covered with cut fibres and tissues. Soon after larvae hatch, they penetrate the stem and feed on the medulla, remaining in the oviposition site, and as they develop, a gall is formed in the girdled part. In the fifth instar, the larva hibernates in the soil, from February to November, remaining there until pupation and adult emergence (Hoffmann-Campo et al. 1991). These life history traits allow them to escape and survive from insecticide applications, rendering insecticidal control difficult (Silva et al. 1998). Therefore, complementary strategies for the management of this pest should be evaluated.

Aggregation pheromones have been elucidated and successfully applied in the field for several Curculionidade species (Tumlinson et al. 1969; Rochat et al. 1991; Budenberg et al. 1993; Ruiz-Montiel et al. 2003; Tafoya et al. 2003; Tinzaara et al. 2005). Hence, the objective of this study was to describe behavioural evidence for an aggregation pheromone in *S. subsignatus*, and to investigate the influence of host plant (HP) availability and photoperiod on pheromone release, as a foundation study for the identification, synthesis and field evaluation of this insect pheromone.



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## Materials and Methods

### Insects

*Sternechus subsignatus* adults of unknown age and mating status were collected from soybean crops located in Fazenda Rio Grande, Parana, Brazil. They were separated by sex based on the structure of the tibiae from the fore and mid legs (Rosado-Neto 1987), held separately in plastic boxes (20 × 20 × 20 cm), fed with freshly cut soybean stem, and maintained at 25 ± 2°C, 60 ± 5% relative humidity, and a photoperiod of 12 : 12 L : D.

### Collection of insect volatiles

To detect sex-specific compounds, separate groups of 30 males and 30 females, along with fresh cuttings of soybean stem (two pieces cut in 8-cm sections per chamber), were placed in different all-glass aeration chambers (33 cm height × 3.5 cm outside diameter) and the volatiles emitted were trapped on 0.8 g of Super Q (Alltech, Deerfield, Illinois) glass columns and collected daily for 30 days (n = 30), as previously reported (Zarbin et al. 2003). The soybean stem were replaced every 24 h and the insects were replaced only when it was needed, to keep always 30 adults. A pushed humidified and charcoal-filtered airstreams (1 l/min) was maintained through the aeration apparatus. Volatiles from each aeration were eluted from Super Q columns with distilled hexane, changing the adsorbent traps each 10 collections. The daily extracts were not combined and were concentrated to 300 $\mu$l (one insect per 10 $\mu$l) under an argon stream (Zarbin 2001). In order to investigate the effect of food-plant availability on the release of pheromones, the volatiles were collected as described earlier for 24 h during 3 days, either in the presence of food (soybean stems) in the aeration chamber or without it (n = 3 per treatment). The diel periodicity of release was investigated by collecting the volatiles in both photophase and scotophase (n = 3 per treatment), and subsequently every 2 h of the photophase, during three successive days (n = 3 per treatment).

### Analytical procedures

Gas chromatographic (GC) analyses were performed on a Varian 3800 GC equipped with flame ionization detector, electronic pressure control, and operated in splitless mode. A DB-5 (30m × 0.25 mm × 0.25 $\mu$m) (J&W Scientific, Folsom, California) capillary column was used under the following analytical conditions: initial temperature of 50°C for 1 min with an increase of 7°C/min until a final temperature of 250°C, which was maintained for 10 min. Upon finishing, the chromatograms were analysed for the presence of sex-specific candidate pheromone components. Quantitative data of released sex-specific volatiles are based on the production of the major component 4, because the profile of the other constituents showed a similar pattern, and its quantification was done based on an external standard curve of hydrocarbon (dodecane) at 10, 100 and 1000 ng.

### Laboratory bioassay

The behavioural response of males and females was tested in a binary choice Y-tube olfactometer, operated at an airflow of 4 l/min, previously humidified and filtered on active charcoal. Males and females were evaluated alternately with at least 20 individuals of each sex. Odour propagation simulation tests were performed to visualize the plume distribution inside the system. To accomplish this, hydrochloric acid and ammonium hydroxide were mixed, following the method described by Baker and Lin (1984). The olfactometer consisted of a Y-shaped glass tube of 4-cm diameter. The main tube of the olfactometer was 40 cm in length and the two arms were each 20 cm in length. One male or female was introduced into the base tube of the olfactometer and the behaviour was observed for 20 min. When a weevil crossed the choice line 5 cm after the division of the base tube and remained there for at least 2 min, it was recorded as a choice for the odour source in that arm. Age and mating status were not controlled during the bioassays because of the difficulty of rearing these insects in the laboratory. Odor sources used to stimulate the insects were: (i) soybean stem alone as HP × cleaned air as control; (ii) pieces (2 × 2 cm) of filter paper loaded with 3 $\mu$l of male extract plus HP; (iii) pieces (2 × 2 cm) of filter paper loaded with 3 $\mu$l of female extract plus HP. An equal-size piece of filter paper containing hexane plus HP, was used as control to treatments 2 and 3. The HP material was a 2-cm cutting of fresh soybean stem replaced after each replicate. After each test, all components of the olfactometer in contact with weevils or HP were washed in a detergent solution, rinsed with alcohol and dried at 100°C for 10 min to avoid contamination. The position of treatments was alternated after each replication, to avoid positional bias.

### Statistical analyses

Results of all olfactometer bioassays were analysed with a binomial test. Individuals that did not make a choice were excluded from the statistical analysis (R Development Core Team 2005). Data pertaining to pheromone emission in the presence and absence of the HP and in photophase and scotophase were analysed with *t*-test ($P < 0.05$) for comparison of means. The period of maximum pheromone release was determined using analysis of variance (ANOVA) followed by Bonferroni test ($P < 0.05$) to assess the statistical differences among levels (Ayres et al. 2003).

## Results and Discussion

The responses of *S. subsignatus* males and females in Y-tube olfactometer when stimulated with odour sources are shown in table 1. Significantly, more males ($P = 0.0003$) and females ($P = 0.0357$) were attracted to soybean stem than to the clean air control. The combination of male extract and soybean stem was more attractive to male ($P = 0.0003$) and female ($P = 0.0470$) weevils than soybean stem alone. On the other hand, males ($P = 0.7011$) and females ($P = 0.6636$) weevils were not attracted to the combination of female extract and soybean stem.

**Table 1** Responses of individual male and female *Sternechus subsignatus* adults to treatments in Y-tube olfactometer

| | Frequencies | |
|---|---|---|
| Odour sources | ♂ | ♀ |
| Host plant (HP) vs. air | | |
| Host plant | 21* | 20* |
| Air | 3 | 8 |
| Not decided | 4 | 6 |
| Total | 28 | 34 |
| Male extract + HP vs. control | | |
| Extract | 15* | 25* |
| Control | 0 | 12 |
| Not decided | 7 | 3 |
| Total | 22 | 40 |
| Female extract + HP vs. control | | |
| Extract | 12 | 12 |
| Control | 15 | 9 |
| Not decided | 3 | 2 |
| Total | 30 | 23 |

HP: soybean stem; control: hexane + HP.

*Statistically significant differences, binomial test, $P < 0.05$.

These results demonstrate that the communication among *S. subsignatus* conspecifics is mediated by pheromones. The attraction of both sexes to male extracts and not to female extracts indicates the presence of a male-produced aggregation pheromone in the species. Males were more attracted to the aggregation pheromone when compared with females, suggesting that these compounds could be used as chemical cues primarily by males of the species to find an appropriate site for mating. Besides, owing to the fact that the mating status of the insects was not controlled in our study, mated females could not be attracted to the aggregation pheromone, as found for *Metamasius hemipterus* (Ramírez-Lucas et al. 1996). Male and female aeration extracts were used as stimulus because, when using live insects, some of them may not release pheromone during the bioassays, contributing to variability in the results (Ruiz-Montiel et al. 2003).

Male-produced aggregation pheromones have been reported for many other species of weevils, and are generally used for both host-finding and bringing the sexes together (Bartelt 1999). It is likely that these are also the functions of this pheromone in *S. subsignatus*. The synergistic combination of species-specific pheromones and host-plant volatiles showed in the bioassay 2 has been reported in many studies (Giblin-Davis et al. 1994; Rochat et al. 2000).

The chromatographic profiles of volatiles from male and female *S. subsignatus* are shown in fig. 1, and clearly indicate the existence of, at least, five male-specific compounds (1–5), providing chemical support to the bioassay data.

The production of these components is dependent on the presence of the HP, as insects feeding on soybean significantly released more volatiles ($2.30 \pm 0.30$ μg/insect/day) than weevils alone ($0.09 \pm 0.01$ μg/insect/day) ($P = 0.0001$) (fig. 2a). Food has been shown to stimulate pheromone release in other beetles (Ruiz-Montiel et al. 2003). From this total amount, it was observed that release takes place mainly during photophase ($P = 0.0002$) (fig. 2b), with a maximum production between 4 to 6 h after its beginning ($P = 0.0004$) (fig. 2c).

The high pheromone release during photophase suggests that the activity of this insect is predominantly daytime. This is in accordance with our field observations, in which, the adults were found only during the daytime, with a peak of activity between 11 and 13 pm. The influence of the photoperiod in the diel rhythm of pheromone release was previsouly studied in *Anthonomus grandis* (Gueldner and



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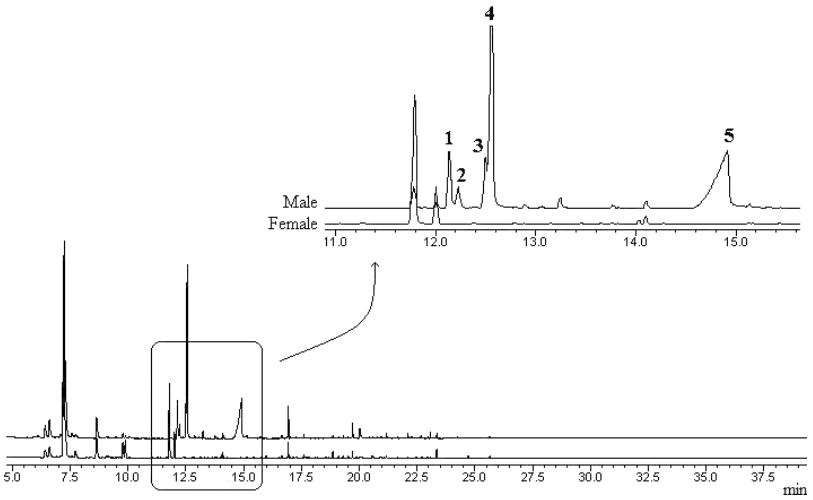


**Fig. 1** Gas chromatographic analysis of volatiles obtained from male and female *Sternechus subsignatus* adults, showing five male-specific compounds, peaks 1–5.

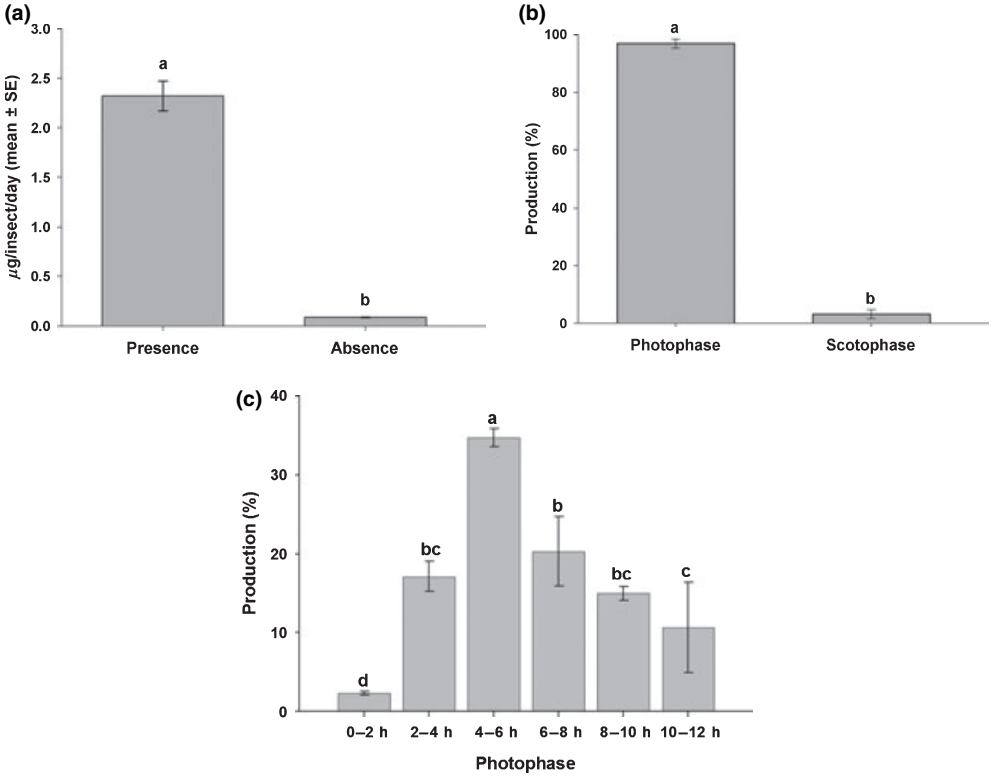


**Fig. 2** Amount of major component collected from the aeration of male *Sternechus subsignatus*: (a) in the presence and absence of soybean stem (n = 3 days per treatment); (b) during photo- and scotophase (n = 3 days per treatment); (c) during the 12 h of photophase (n = 3 days per treatment). Means followed by the same letter are not significantly different between them using *t*-test (a and b) and analysis of variance followed by Bonferroni test (c) ($P < 0.05$).

Wiygul 1978) and in some species of scarab beetles (Leal et al. 1993a,b).

In summary, behavioural and chemical evidence supports the occurrence of a male-produced aggregation pheromone in *S. subsignatus*, which largely increases the attraction of both sexes to HP. The five male-specific compounds detected are the putative pheromone components of the species, and their production is dependent on food and photoperiod. Studies are underway to chemically characterize all

the pheromones involved in the attraction, and their effect on laboratory bioassays and on trap catches will be addressed in future studies.

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