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Biosensor for volatiles released by damaged plants

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Abstract: One of the most crucial problems in biological or biotechnical plant protection is the timing of starting an application. The proposed biosensor measuring overall degree of plant damage is a useful tool for solving the problem.

If plants are damaged, for instance by cuts made artificially or by biting or chewing insects, volatile organic compounds (VOCs) will be released to a higher extent and in a different composition compared with undamaged plants. The biosensor based on an electroantennographic technique uses the sensitivity and selectivity of an insect's antenna to detect the changes in composition of the VOC.

This method is comparatively simple, inexpensive and sensitive (e.g. 1 ppbv *cis*-3-hexen-1-ol, dynamic range: 1 ppbv–100 ppmv) with respect to usual mass-spectrometric methods of VOC trace analysis. Moreover, the response time (10 ms) and the measurement cycle time (3 min) are short and the biosensor yields an overall parameter easier to deal with than complex mass-spectrometric data arrays. The lifetime of one antennal preparation, however, is 4 h and has to be improved. This paper presents results emerging from laboratory experiments with the Colorado potato beetle (*Leptinotarsa decemlineata* Say).

Keywords: biosensor, insect antenna, electroantennography, Colorado potato beetle, plant damage induced signals, VOC, trace analysis, chemical ecology

INTRODUCTION

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae) remains the most devastating defoliator of potato worldwide (Ferro, 1985; Gauthier *et al.*, 1981). Uncontrolled populations can completely defoliate potatoes and, at particular times during the plant growth cycle, can cause total loss of tuber production (Hare, 1980). In general, plants just beginning to bloom and to form and fill tubers

are most susceptible (Hare, 1980; Cranshaw & Radcliffe, 1980; Ferro *et al.*, 1983; Shields & Wyman, 1984; Wellik *et al.*, 1981; Zehnder & Evanylo, 1989). Most pest management programs therefore emphasize the need for early and midseason suppression, with higher potato beetle densities tolerated late in the season (Hare & Moore, 1988; Wright *et al.*, 1987).

Several preliminary models of potato growth have been developed in order to predict losses in yield due to defoliation at different times in

the growing season (Elkinton *et al.*, 1985; Johnson *et al.*, 1986; Logan & Casagrande, 1980). Most such models are closely tied to the cultivars and growing conditions used for their development, and they do not yet have sufficient flexibility or generality to be widely utilized in pest management programs.

The adequacy of degree-day models for predicting spring emergence and developmental rates of CPBs under field conditions varies with the form of the model and/or insect population (Tauber *et al.*, 1988) and is strongly tied to special regions.

A number of sampling procedures for the CPB have been proposed in different studies (Wright *et al.*, 1987; Logan & Casagrande, 1980; Martel *et al.*, 1986; Nyrop & Wright, 1985), but no single sampling scheme has yet been proven generally efficient and useful in all regions. However, most treatment guidelines are designed to limit total defoliation to 10–25% during the most critical periods of plant growth (Shields & Wyman, 1984; Wright *et al.*, 1987; Ferro *et al.*, 1985). Therefore, an additional tool is necessary to complement those sampling schemes by measuring plant damage by CPB directly. In this article a biosensor is presented. It can measure volatile organic compounds liberated from potato plants in response to mechanical damage by biting or chewing insects such as the CPB.

CHEMICALS

All solvents used, with the exception of water, were of analytical quality (p.a., Merck). Water was ion exchanged and membrane filtered with a Milli-Q-Plus-apparatus (Millipore). Inorganic chemicals used for the ringer-solutions were of analytical quality (p.a., Merck). *Cis*-3-hexen-1-ol, *trans*-2-hexen-1-ol, and *trans*-2-hexenal were of 98% purity (Merck).

APPARATUS AND PROCEDURE

Measurements were performed on an electro-antennographic setup consisting of an amplifier, an oscilloscope and a computer for data acquisition. The amplifier (constructed by Dr. Koch, University of Kaiserslautern) consists of a head-stage (input impedance: 100 MΩ) and a main amplifier, each providing amplification by a factor

of 10, resulting in a total amplification of 100. The EAG signal is low pass filtered with a corner frequency of 10 Hz. The oscilloscope (Hameg 205-2) has two channels and a sampling rate of 5 MHz in the digital storage mode. It is connected to the IEEE-488 bus of a computer (IBM 80286) by an interface (Hameg, HO79). The data acquisition is controlled by the program PRO-Scope (Hameg Software SP91).

For preparation of the recording electrode a glass capillary (inner diameter = 1.2 mm, outer diameter = 2 mm, tip diameter = 5 μm, WPI) was filled with receptor lymph Ringer solution (Kaissling & Thorson, 1980) and connected to a silver wire coated with AgCl at the tip. The reference electrode was prepared in the same way, except that hemolymph Ringer solution (Kaissling & Thorson, 1980) was used.

The CPBs used for the electroantennographic experiments were reared on 4 week old potato plants (*Solanum tuberosum* Desiree) at 25°C, 50% relative humidity, 16 h daylight with about 70 klux. The beetles used for measurement were males or females which had emerged from pupae 4 days earlier. The beetle was immobilized by dentist's wax (Reowachs, Candulor) on a longitudinal section of an Eppendorf pipet cap that could be inserted into a self-made holder.

The reference electrode was introduced into the ochrea through the borderline of the caput and thorax segment of the beetle and was connected to ground. After cutting off one-third of the last segment of the left antenna with a pair of micro-scissors, the measuring electrode was introduced into the last segment of the antenna (Fig. 1). The antenna was not excised because a whole beetle preparation results in a significantly increased lifetime (4 h). Artifacts caused by electrophysiological background signals in the immobilized beetle were greatly reduced by thoroughly designed mechanical restraints. If the resistance between the two electrodes was below 8 MΩ the contact of the electrodes was sufficient. The zero potential of a healthy antenna preparation was −35 mV.

Stimuli were delivered by puffing air in 5 ml samples over the antenna. In order to suppress a response of mechanoreceptors to an altered air velocity, the antenna was kept under a continuous airflow of 1 ms^{-1} velocity (Roelofs, 1984). To prevent desiccation of the antenna, the synthetic air (Messer Griesheim) was humidified to a
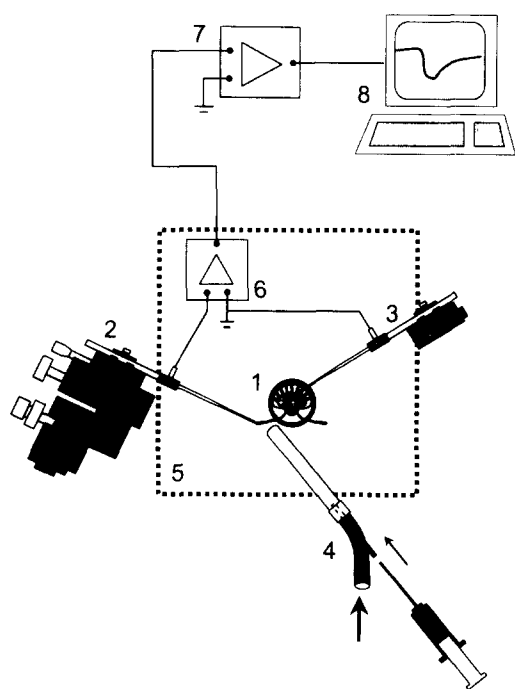
Fig. 1. Scematic of the electroantennographic system. (1) Immobilized beetle; (2) antenna contacting electrode; (3) reference electrode; (4) stimulus delivering tube; (5) Faraday cage; (6) headstage amplifier (*10); (7) external amplifier (*10); (8) EDV.

relative humidity of 100% by bubbling it through water at 20°C.

Filter paper leaflets (1 cm^2) with 200 μl standard solution in paraffin oil were used as standards in 10 ml glass syringes. Each compound and each dilution was prepared in a separate syringe. The concentration of the stimulating agent in the stimulating air was calculated according to Henry's law using fugacity coefficients calculated from the results of GC–FID (Ma & Visser, 1978) and GC–MS measurements (Schütz & Hummel, 1994).

Prior to puffing the standard over the antenna, the prepared standard samples were equilibrated for at least 2 min. These paraffin dilution standards are easy to handle but not very stable in time. In order to exclude the introduction of artifacts due to fading paraffin dilution standards, they were checked once per hour with the aid of a time constant permeation standard. The permeation standard was designed as a 5 cm piece of PFA-tubing filled with pure standard substance and sealed with tight fitting glass balls

(Anonymus, 1986). Permanent air flow under defined temperature and velocity results in constant and reproducible diffusion of the standard substance into the flowing air.

In order to rule out extremes of biological variance, each preparation was first tested with 10 ppmv cis-3-hexen-1-ol, and was discarded if it yielded a response less than 1.5 mV or more than 2 mV. The percentage of preparations discarded was about 10%. After 2–4 h measuring time the paraffin dilution standards had to be exchanged and new leaflets had to be prepared because of significantly reduced standard emission rate.

Every measurement of a sample includes a measurement cycle starting with a blank, proceeding with a standard and a further blank and ending with the sample measurement. The blank values preceding and following the sample measurements are averaged and subtracted from the sample value. This yields the EAG signal without responses of mechanoreceptors which are in the range of 0.2 mV. The standard value allows permanent control of the performance of the preparation. The time consumed by such a cycle was 3 min. Accordingly, 20 measurements could be performed within 1 h.

RESULTS AND DISCUSSION

CPBs are attracted to potato plants by an olfactory cue consisting of a mixture of volatile C$_6$ compounds called 'green leaf odor' (Visser & Ave, 1978; Visser, 1979; Visser et al., 1979; Visser & Thiery, 1985; Thiery & Visser, 1986, 1987). The selectivity and sensitivity of the olfactory system towards these compounds is high. For example, cis-3-hexen-1-ol was reported to be detected by the beetle's antenna down to 10^8 molecules cm^{-3} (Visser, 1979). Examining the quantitative response of the insect antenna and comparing it with that compound resulted in a stimulus-response curve function displayed in Fig. 2.

In order to test the suitability of the biosensor system for detection and quantification of plant damage, measurements of the correlation of damage intensity and EAG response were made. Young and old leaves were cut off from 4 week old potato plants; the cutting injury was sealed with wax. The single leaves received one or more cuts with a pair of micro-scissors of a certain
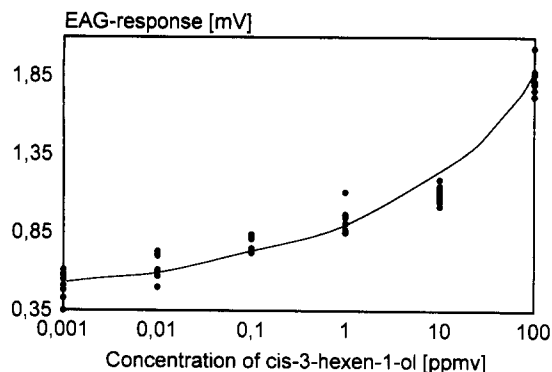
EAG-response [mV]

Concentration of cis-3-hexen-1-ol [ppmv]

Fig. 2. Dose-response curve of the EAG system for cis-3-hexen-1-ol: 23°C, 100% relative humidity, 1 ms⁻¹ flow rate (synthetic air).

length perpendicular to the leaf border. Then the prepared leaf was put into a 10 ml glass syringe.

After 5 min of equilibration, 5 ml of the air surrounding the damaged leaf was puffed over the antenna. Cutting injuries of 1–20 mm showed a strong dependence of EAG response on the severeness of injury. At higher levels of injury, saturation was observed (Fig. 3). Despite differing absolute response values towards damaged young and old leaves in relative terms, the EAG response shows a similar reaction pattern for each leaf. The EAG response differs in dependence of the time elapsed since the injury. Closer examination of the time dependence of the EAG response resulting from a 20 mm injury yields a nearly linear decline of the signal (Fig. 4). Because of the logarithmic dependence of the EAG signal on the green leaf odor concentration (Fig. 2), an exponential decrease of green leaf odor concentration with a half time of about 15
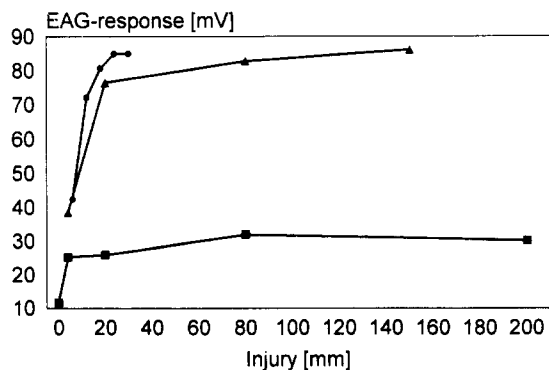
EAG-response [mV]

Injury [mm]

Fig. 3. Degree of mechanical injury vs. EAG response. The three curves correspond to leaves of different ages.
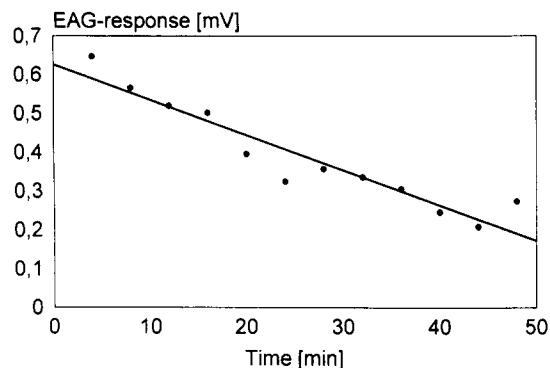
EAG-response [mV]

Time [min]

Fig. 4. Decline of the EAG response after a 20 mm injury: 23°C, 100% relative humidity, 1 ms⁻¹ flow rate (synthetic air).

min occurs as a result of mechanical injury to a potato leaf. Long cuts or furrow injuries of 1–20 mm on potato leaves within 15 min correspond very well to the feeding rates of most developmental stages of the CPB (Table 1). Therefore, the electroantennographic system should be suitable for the detection of potato plant damage caused by the CPB.

In order to examine the response pattern resulting from feeding damage by various developmental stages of the CPB, single animals were given the opportunity to feed for 15 min on young and old leaves of 4 week old potato plants; the leaves were cut off and the cutting injury was sealed with wax, then the prepared leaf was put into a 10 ml glass syringe. After 5 min equilibration, 5 ml of the air surrounding the damaged leaf was puffed over the antenna. Like the response pattern to mechanical damage, the response pattern resulting from feeding damage by various developmental stages of the CPB depends strongly on the age of the leaves. Young leaves damaged by feeding CPB elicit a stronger response than old ones.

Corresponding to the different plant damage caused by the various developmental stages of the CPB, the elicited EAG response increases strongly in the sequence L1–L2–L3 (Fig. 5). Thus, the electroantennographic system is able to distinguish between the different intensities of damage caused by different developmental stages of the CPB and is, therefore, a suitable biosensor for potato plant damage by chewing insects.

Besides being suitable for measurements in principle, such a biosensor has to fulfil further additional specifications in order to fit the needs

TABLE 1   Comparison of different measures of plant damage in laboratory and field trials.

Developmental stage	Line of injury within 15 min (mm)	Consumed leaf area within 15 min (mm²)	Relative plant damage in the field*
L1	3	1	3
L2	6	3	5
L3	15	10	15
Beetle	20	35	

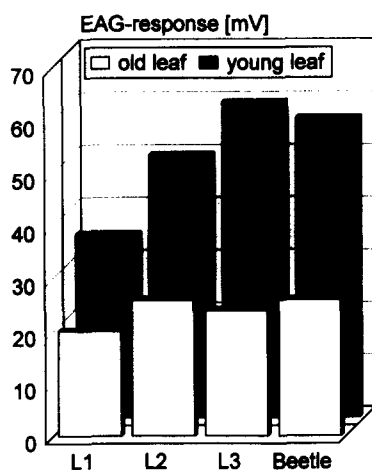*Ferro *et al.*, 1985; Logan *et al.*, 1985; Tamaki & Butt, 1978.

Fig. 5. Influence of the developmental stage of Leptinotarsa decemlineata *on the increase of EAG response caused by feeding damage on young and old leaves.*

of practical use (Vadgama, 1990; Rechnitz & Ho, 1990): reliability and sensitivity, a high dynamic range, a high selectivity for the important stimulus, short response times, dead times and adaptation times are required as well as a short preparation time, a sufficient lifetime and the possibility for storage and transport of the biosensor system (Table 2).

Both reliability and lifetime of the biosensor are strongly affected by abiotic parameters of the gas supply (relative humidity, temperature), therefore provisions for appropriate conditioning of the gas supply are necessary.

Complicating effects of biological variation of individual antennae can be reduced by an initial short test run. It uses a constant source of *cis*-3-hexen-1-ol contained in a gas permeation standard tube prepared according to Anonymus (1986). The preparations showing extreme response are discarded.

TABLE 2   Features of the biosensor.

Sensitivity	1 ppbv *cis*-3-hexen-1-ol
Dynamic range	1 ppbv–100 ppmv *cis*-3-hexen-1-ol
Selectivity	High (for green leaf odor)
Cycle of measurement	Blank–standard–blank–sample
Response time	10 ms
Dead time	4 s
Adaptation time	1 min
Cycle time	3 min
Preparation time	30 min
Lifetime	4 h

Sensitivity and dynamic range seem to be quite appropriate for short (0–2 m) and intermediate (2–20 m) distance measurements and can compete with the performance of GC–MS experiments. The response, dead and adaptation times are, like the cycle time, shorter by a factor of 10 than for a conventional GC–MS apparatus. Solid state sensors based on doped SnO_2 are inferior to the biosensor in terms of selectivity (general response to hydrocarbons, detection limit about 10 ppmv) (Heiland, 1990).

Compared with a lifetime of 4 h, a biosensor preparation time of half an hour seems to be quite long, but because of the short cycle time, 80 measurements can be performed within 4.5 h. Despite this convincing performance, the handling features should still be improved. A lifetime of 1–2 days should be reachable by optimized preparation in order to decrease the injury of antennal nerves, and a method for storage and reuse of previously prepared beetle preparations should also be developed.

A potentially tougher challenge is the transportability of the biosensor for measurements in the field. Gas sampling by glass bulbs seems to

be problematic because of adsorption on glass walls. Therefore the biosensor system itself has to be portable. Some approaches for developing portable electroantennographic systems have been made (Van der Pers, 1993; Sauer *et al.*, 1992) for applications in pheromone research. By adopting those experiences it should be possible to develop a portable biosensor for plant damage. Moreover, the selectivity of the biosensor has to be improved. The general detection of green leaf odors in a potato field can be caused by accidental mechanical damage of other plants in the vicinity and would cause a false positive result. In order to increase the selectivity for different plant species, a multi-sensor array (Gardner *et al.*, 1990; Nakamoto *et al.*, 1990) with antennae of different insects associated with different host plants could be useful.

CONCLUSIONS

An electroantennographic method was developed and tested for its suitability to operate as a biosensor for plant damage. In laboratory experiments we were able to confirm that the sensor has sufficient sensitivity and that a quantitative correlation between plant damage intensity and EAG response exists. Moreover, the different damaging potentials of the developmental stages of the CPB could be detected and correlated to literature data of sampling procedures in the field. In terms of sensitivity, dynamic range and time characteristics the biosensor is comparable with the conventional, expensive GC–MS experimental setup. In order to be comparable with conventional sampling procedures in the field, extended lifetimes, storage times and transportability of the electroantennographic system still have to be developed. Work in this direction is in progress.

As a conclusive assessment, insect antenna-based biosensors seem to be a promising development with potential applications in integrated pest management and food quality control.

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