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Electrophysiological and Behavioral Activity of (*E*)-2-Hexenal in the Granary Weevil and Its Application in Food Packaging

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ABSTRACT

The purpose of this work was to develop a biodegradable carrier material to control insect pests in cereal products. To this aim, (*E*)-2-hexenal was used, being a natural compound with antimicrobial activity that is also commonly adopted as a flavoring agent. Three coating layers of polycaprolactone (PCL) were spread onto the internal side of a paperboard carton, the first being the active coating containing (*E*)-2-hexenal. The antennal sensitivity of *Sitophilus granarius* to a broad range of doses of (*E*)-2-hexenal was first demonstrated. Next, the ability of different concentrations of this compound to disrupt the orientation of adult *S. granarius* beetles to odors of intact wheat kernels was established in a two-choice pitfall bioassay. In addition, invasion tests were carried out over an 8-week period to highlight the effects of the biobased repellent packaging and their potential persistence. The results demonstrated that during the entire monitoring period, the percentage of *S. granarius* adults found in cartons coated with (*E*)-2-hexenal–loaded multilayer PCL was about 10% of the total number of insects used in the bioassay, very low compared with the respective control samples, thus assessing both the effectiveness and persistence of the repellent system developed. Although the infestation level of treated packages was reduced relative to the infestation levels in the controls, any infestation of food packages is unacceptable to consumers, so further tests are required to determine whether infestation can be completely prevented using (*E*)-2-hexenal.

Insect infestation of packaged food can make a long-lasting negative impression on customers, resulting in considerable economic impact on food manufactures. Generally, insects enter packages through existing openings or flaws in damaged or inappropriately sealed packages and rarely through chewing *(24)*. Therefore, chemical barriers able to prevent insects from invading are more effective than the prevention of penetration *(15)*. The behavioral mechanisms by which stored-product insects invade packages have been little investigated, but there is evidence that volatiles emanating from holes in packaging materials play an important role in orienting both larval and adult stages of pests *(1, 23)*. Hence, the use in food packaging of insect repellents capable of disrupting insect orientation to packaged food has an important practical interest *(39)*.

In the last few decades, the intensive use of synthetic insecticides to control stored-product insect pests has caused many major concerns, such as the development of insect resistance, toxicity toward nontarget organisms, and adverse effects on the environment. Consequently, there is a need for new, effective, and environmentally friendly alternatives to reduce the use of synthetic pesticides in postharvest pest control. Several compounds, such as pyrethrins, methyl salicylate, silica gel, *N,N*-diethyl-*m*-toluamide (DEET), neem, citronella, protein-enriched pea flour, and propionic acid have shown repellent properties against insects when applied to packaging materials *(6, 14, 15, 22, 29, 36, 37, 39)*, even though they have not yet gained widespread commercial use *(23, 24)*. This evidence suggests that further work is necessary to develop biodegradable packaging systems based on the controlled release of insect-repellent compounds. Promising candidates could be edible or synthetic biodegradable polymers applied as coatings on paper materials *(6)*.

Short-chain aliphatic aldehydes are volatile compounds that occur widely in plants and in the aromas of natural and processed foods *(19, 32, 40)*. Being key components of food aroma, they are frequently used as additives to produce natural flavor notes in processed foods *(25, 26, 38)*. Among them, saturated and unsaturated C_6 aldehydes, along with their corresponding alcohols and esters, are produced by plant tissues in response to mechanical and herbivory damage through the hydroperoxide lyase pathway of oxylipin metabolism *(21)*. These compounds have been shown to possess fumigant and contact toxicity *(4, 12, 16)* and repellent effects *(7)* against various stored-product insect pests. Moreover, some of them were found to inhibit pathogenic fungi *(11, 35)* and bacteria *(3)*, thus indicating that not only might they be good alternatives to synthetic

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pesticides but also might find broad applications as food preservatives. The concentrations of volatile aldehydes eliciting repellence in insects are generally lower than those needed for toxicity; therefore, packaging materials incorporating insect-repellent aldehydes represent an intriguing approach for use in preventing packaged food infestation with minimal negative effect on sensory characteristics.

The granary weevil, *Sitophilus granarius* (L.) (Coleoptera, Dryophthoridae), is one of the most damaging pests of stored cereals and some processed foods throughout the world. The antennae of *S. granarius* adults detect a wide variety of cereal volatiles, including the aliphatic aldehyde (*E*)-2-hexenal *(10)*, and in olfactometer bioassays designed to evaluate the behavioral activity of increasing doses of pure volatile compounds, (*E*)-2-hexenal significantly repelled adults of this pest *(7)*.

In the present study, the olfactory sensitivity of *S. granarius* adults to (*E*)-2-hexenal was investigated in more detail by electroantennography (EAG), and the ability of the compound to disrupt the orientation of adult weevils towards odors of a feeding and oviposition substrate was evaluated by a pitfall bioassay. Finally, the effectiveness of a multilayer coating activated with (*E*)-2-hexenal for preventing the entry of insects into coated cartons containing wheat kernels was assessed at different time intervals after preparation by a multiple-choice bioassay.

MATERIALS AND METHODS

Insects. *S. granarius* insects were reared on whole wheat kernels for several generations. Colonies were maintained in the dark in a climatic chamber set at 23 ± 2°C and 60% ± 5% relative humidity. Adult beetles, of mixed sex and age, were used for the experiments.

EAG. The antennal sensitivity of both sexes of *S. granarius* to (*E*)-2-hexenal was assessed by using the EAG technique described in previous studies *(8, 9)*. The head of the insect was excised from the prothorax and mounted between two properly pulled (PC-10 puller, Narishige, Tokyo, Japan) glass capillary electrodes (Microglass, Naples, Italy) filled with Kaissling saline *(17)*. The recording electrode (diameter, ca. 100 μm) was placed in contact with the dorsal surface of the terminal antennal segment, while the neutral electrode was inserted into the base of the head. Electrical contact of the preparation with the amplifier (AC/DC UN-6, Syntech Laboratories, Hilversum, The Netherlands) was made with AgCl-coated silver wires. Stimuli were provided by 10-fold dilutions of (*E*)-2-hexenal (chemical purity of 99%) in mineral oil (Sigma-Aldrich, Milan, Italy). Test solutions (10 μl) were applied to filter paper (Whatman No. 1, Sigma-Aldrich) strips (1 cm^2) inserted in a Pasteur pipette (15 cm long). Vapor stimuli (3 cm^3) were blown for 1 s by a disposable syringe into a constant stream of charcoal-filtered humidified air (600 ml/min) flowing in a stainless steel delivery tube (inside diameter, 1 cm) with the outlet positioned at approximately 1 cm from the antenna. The intervals between stimuli were 1 min. Eight antennae of each sex were presented with seven (*E*)-2-hexenal doses ranging from 0.0001 to 20 mg. In order to correct for the possible reduction of antennal sensitivity during the experiment, a standard stimulus (10 μl of a 10-μg/μl dilution of hexanal in mineral oil) was applied before and after each (*E*)-2-hexenal dose *(34)*. Dose-response curves were calculated based on corrected EAG values. In the dose-response curves, the activation threshold was considered to be the lowest dose at which the lower limit of the standard error of the mean response was greater than the upper limit of the standard error for the lowest dilution tested *(30)*; the saturation level was taken as the lowest dose at which the mean response was equal to or less than the previous dose *(8)*. Male and female EAG responses to each dose of (*E*)-2-hexenal tested were compared using Student's *t* test (with significant differences corresponding to $P = 0.05$).

Pitfall bioassays. The ability of different doses of (*E*)-2-hexenal to disrupt the orientation of adult *S. granarius* beetles to odors of intact wheat kernels was evaluated by using a two-choice pitfall bioassay similar to that described in Germinara et al. *(9)*. The test arena was a steel container (32 cm in diameter by 7 cm high) with two diametrically opposed holes (3-cm diameter) located 3 cm from the side wall. Test or control (10-ml) stimuli were adsorbed onto a filter paper disc (0.7-cm diameter) suspended at the center of each hole by a cotton thread taped to the lower surface of the arena. Glass flasks (500 ml) were positioned under each hole to collect the responding insects. The inside necks of the collection flasks were coated with mineral oil to prevent insects from returning to the arena. The floor of the arena was covered in filter paper (Whatman No. 1) to facilitate insect movements. Twenty insects of mixed sex, left for at least 4 h without food, were placed under an inverted petri dish (3 cm in diameter by 1.2 cm high) at the center of the arena and allowed 30 min to acclimatize prior to release. The arena was covered with a steel lid to prevent insects from escaping. Tests lasted 3 h and were carried out in the dark at 25 ± 2°C and 60% ± 5% relative humidity. (*E*)-2-Hexenal was dissolved in mineral oil in decimal dilutions from 100 to 0.1 μg/μl. Insects were presented with a dose (1, 10, 100, 500, and 1,000 μg) of (*E*)-2-hexenal (10 μl of a test solution) plus the odors emitted by 200 g of uninfested wheat kernels (moisture content, 12.5%), left in the underlying collection flask, and mineral oil (control). Insects were used once. Five replicates of each test were performed. For each replicate, a response index (RI) was calculated according to the method of Phillips et al. *(27)*. Positive values of RIs indicate attraction to the treatment, and negative RIs indicate repellence. The significance of the mean RI to each treatment of the two-choice pitfall bioassay was evaluated by Student's *t* test for paired comparisons. The mean RIs to wheat odors alone and in the presence of increasing doses of (*E*)-2-hexenal were subjected to analysis of variance (ANOVA) and ranked according to Tukey's honestly significant difference test. Data were submitted to linear regression analysis to evaluate the effect of dose on insect response.

Carton coating. The carton board was obtained from a carton board mill, and it was 416 μm in thickness. The length:width:height dimensions of each assembled packaging carton were 7 cm by 3 cm by 10.5 cm, and the total surface area was 252 cm^2. A polycaprolactone (PCL) solution was applied to each carton to realize a multilayer biodegradable system. PCL (10.0 g; CAPA 6800, Solvay, Warrington, UK) was dissolved into 100 ml of dichloromethane (Baker, Milan, Italy) at room temperature. The solution was stirred for about 1 h using a magnetic stirrer hotplate. (*E*)-2-Hexenal was dissolved (0.52 g/g of dry polymer) in the PCL formulation. An amount of 25 ml of solution was then applied to the intended internal surface of the carton packaging with a casting knife (Warwick Massa S.p.a., Milan, Italy). The PCL monolayer-coated carton was allowed to dry at room temperature. To prepare the PCL multilayer system, two additional PCL coating solutions without (*E*)-2-hexenal were applied to the PCL monolayer-coated carton as described above. As controls, cartons without any coating and cartons coated with three layers of PCL solution without active compound were also prepared. Five replicates were produced for each type of carton. All of the prepared cartons were filled with 90 g of granary weevil food (uninfested wheat kernels) and stored at 18 ± 1°C for 1, 7, 14, 21, 28, 42, or 56 days.

Invasion tests. The method employed for invasion tests was similar to that described in Germinara et al. *(6)*. Experiments were carried out in plastic boxes (28 cm long by 28 cm wide by 22 cm high). The bottom of each box was covered with filter paper (Whatman No. 1) to provide a uniform surface and to facilitate insect movement. Before the start of the experiment, three circular (2-mm-diameter) holes spaced 1.5 cm apart were punched along the base of the two longer carton sides assigned to rest on the bottom of the box to simulate damaged or poorly sealed packages. Thirty insects of mixed sex, left for at least 4 h without food, were placed under an inverted petri dish (3 cm in diameter by 1.2 cm high) at the center of the bottom of the box and allowed 30 min to acclimatize prior to release. Invasion tests were carried out with packages aged for 1, 7, 14, 21, 28, 42, and 56 days. In each experiment, insects were presented with (i) one carton internally coated with multilayer PCL containing (*E*)-2-hexenal, (ii) one carton internally coated with multilayer PCL without (*E*)-2-hexenal, and (iii) one uncoated carton. Cartons were randomly and equidistantly distributed along the circumference of a circle around the center of the box and located 2 cm from the side walls. The test box was closed by a lid provided with two holes (2-cm diameter) screened with fine (0.1-mm) metallic mesh to prevent insects from escaping and allow air circulation. Tests were carried out in the dark at 25 ± 2°C and 60% ± 5% relative humidity. The number of insects in each carton was counted after 24 h. Insects were used once. Five replicates were performed for each test.

The ratio of beetles in a carton to the number of insects added to the test box was the percentage of insect infestation in each carton. A two-way ANOVA with carton treatment and aging period as the two main factors was performed. The mean percentages of insect infestation in different packages following the 1- to 56-day aging periods were analyzed by Wilcoxon test for separation of means.

RESULTS AND DISCUSSION

Antennal sensitivity of *S. granarius* to (*E*)-2-hexenal. An electroantennogram is the summation of receptor potentials evoked by an olfactory stimulus from various sensilla on the antenna that are tuned to the chemical tested. Compounds that are EAG active to an insect species are frequently of ecological significance *(34)* and, therefore, may act as insect behavior-modifying compounds. EAG tests showed that the antennal olfactory systems of *S. granarius* males and females are able to perceive (*E*)-2-hexenal over a broad range of doses and corroborate the results of a previous study indicating a repellent effect of increasing concentrations of compound against granary weevils *(7)*. A typical sigmoid-shaped dose-dependent EAG response was elicited by increasing doses of (*E*)-2-hexenal in both sexes (Fig. 1). The activation dose was 0.01 μM for both males and females. The saturation dose was 10 μM for females, while no saturation of male antennae was achieved in the dose range tested. Male and female EAG responses to each dose of (*E*)-2-hexenal were not significantly different ($t = 0.09$ to 1.6; df = 14; $P > 0.05$).

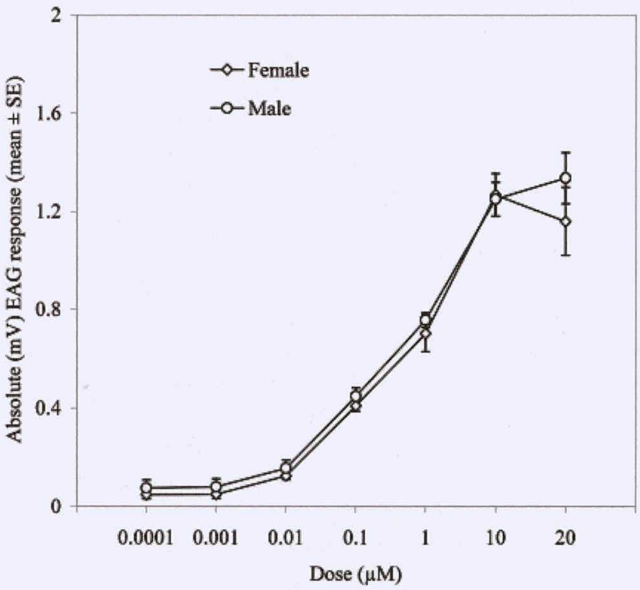

FIGURE 1. *EAG dose-response curves of female and male* Sitophilus granarius *antennae to ascending concentrations of* (E)-*2-hexenal.*

Pitfall bioassays. The olfactometer biossays demonstrated that (*E*)-2-hexenal may act as a repellent to *S. granarius* adults even in the presence of wheat kernel odors, which are highly attractive. (*E*)-2-Hexenal significantly reduced the attraction of insects to the volatiles of 200 g of wheat kernels at doses of 1 to 500 μg and repelled them at the 1,000-μg dose (Fig. 2). A significant negative effect of the dose of the compound on the behavioral response of granary weevils to wheat odors was confirmed by regression analysis ($R^2 = 0.85$; $P = 0.027$). Lipase and lipoxygenase activities increase with storage duration in cereals, enhancing the production of lipid oxidative products, including aliphatic aldehydes *(13, 28, 33)*. Therefore, from an ecological perspective, (*E*)-2-hexenal may serve as a reliable cue of habitat quality for the granary weevil to avoid suboptimal host habitats.

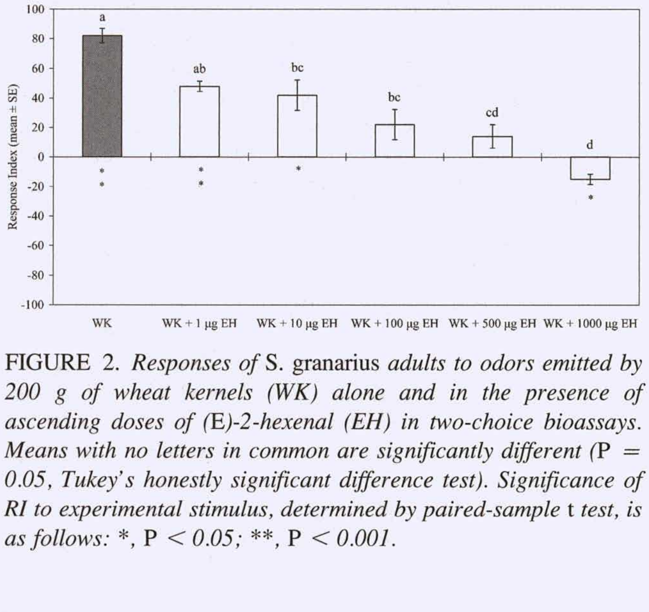

FIGURE 2. *Responses of* S. granarius *adults to odors emitted by 200 g of wheat kernels (WK) alone and in the presence of ascending doses of* (E)-*2-hexenal (EH) in two-choice bioassays. Means with no letters in common are significantly different* (P = *0.05, Tukey's honestly significant difference test). Significance of RI to experimental stimulus, determined by paired-sample* t *test, is as follows:* *, $P < 0.05$; **, $P < 0.001$.

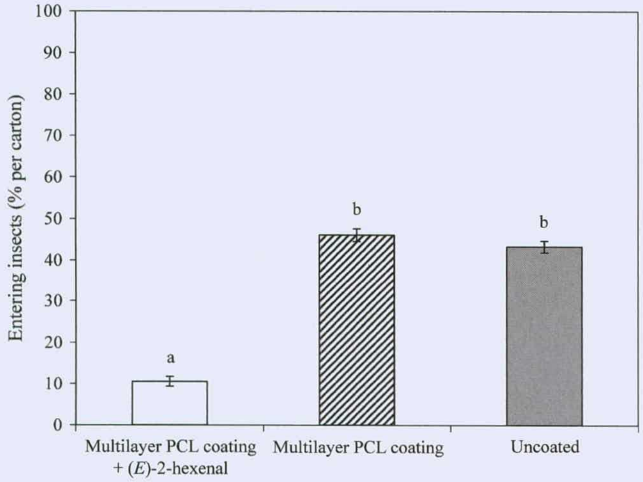

FIGURE 3. *Percentages of* S. granarius *adults (mean* ± *SE) that entered uncoated carton packages and cartons internally coated with a PCL multilayer, with or without* (E)-*2-hexenal, following 1- to 56-day aging periods at 18* ± *1°C* (n = *5). Bars with different letters are significantly different* (P = *0.05, Wilcoxon test).*

Influence of the active coating on repellent effects. The potential of (*E*)-2-hexenal for disrupting the beetles' orientation to host odors was evaluated in invasion tests. These experiments were carried out by using a large number of insects confined in a small space and damaged cartons in order to simulate the worst field conditions for packaged cereal goods. Despite the favorable experimental conditions for insect infestation, (*E*)-2-hexenal effectively disrupted the mechanism of food location by *S. granarius* adults. Two-way ANOVA showed a significant effect of carton treatment ($F = 193.4$; $P < 0.001$) but not of aging period ($F < 0.001$; $P = 1$) or interaction between these two main factors ($F = 0.79$; $P = 0.66$). The mean percentage of granary weevil adults that entered 1- to 56-day-aged cartons internally coated with the activated multilayer PCL was significantly lower than those recorded for the corresponding uncoated cartons and PCL-coated cartons without (*E*)-2-hexenal ($P = 0.05$, Wilcoxon test) (Fig. 3). These latter conditions showed percentages of insect infestation that were not significantly different.

In a previous study, a multilayer PCL system exhibited similar satisfactory performance in releasing propionic acid, another known repellent for the granary weevil *(6)*. A direct comparison between the two active multilayer systems cannot be made, due to the different sides on which coatings were applied to the carton and the different numbers of coating layers applied, because these factors affect the release mechanism of the active compound through the packaging material *(31)*. The solution previously investigated suggested the possibility of developing controlled release systems suitable for achieving long-lasting effects by modifying the number of outer coating layers. The results of the current work confirmed the ability of multilayer PCL to retain and then slowly release the repellent substance. Moreover, the application of more active biocoating layers to the inner side of the carton also demonstrated the persistence of the repellent effect. To this aim, invasion tests were performed during a period lasting 56 days (8 weeks).

As mentioned above, it is worth noting that the mean percentages of insects entering cartons with (*E*)-2-hexenal–activated PCL coating did not vary significantly during the 1- to 56-day aging period. This indicates a good ability of multilayer PCL to retain and then efficiently release (*E*)-2-hexenal. A number of factors point to good prospects for a practical application of (*E*)-2-hexenal in producing biobased packaging systems. The compound occurs naturally in several fruits and vegetables, including grains of some cereal species *(13, 20, 33)*. It has inhibitory effects toward fungi and bacteria, including pathogenic microorganisms frequently isolated in foods *(5, 18)*. The addition of (*E*)-2-hexenal to the storage atmosphere of freshly sliced apples resulted in a positive effect on shelf life, due to its antimicrobial activity against naturally occurring spoilage species *(2)*. Moreover, (*E*)-2-hexenal is generally recognized as safe by the U.S. Food and Drug Administration and is commonly used as a flavoring compound by food industries.

To summarize, this study showed that (*E*)-2-hexenal is repellent enough to reduce insect immigration into packages when formulated in biodegradable PCL coatings and, therefore, it has the potential to be used in the preparation of insect-resistant and environmentally friendly packaging materials. In addition, the differences in the behavioral responses of *S. granarius* adults to the stimuli tested demonstrated that the bioassay was suitable for assessing the biological activity of (*E*)-2-hexenal as an insect repellent when incorporated into biodegradable coatings. For widespread industrial acceptance of bioactive packaging solutions able to prevent insect infestation in cereal products, an effective repellent effect for an extended period of time is still necessary. To this end, long-lasting experiments involving different numbers or thicknesses of the outer coating layers are needed to define the optimal conditions for controlling the release of (*E*)-2-hexenal.

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