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Hydrolysis of Glycosidically Bound Volatiles from Apple Leaves (Cv. Anna) by *Aspergillus niger* β-Glucosidase Affects the Behavior of Codling Moth (*Cydia pomonella* L.)

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Glycosidically bound volatiles released from apple leaf extracts (cv. Anna) were analyzed by solid-phase microextraction−gas chromatography−mass spectrometry (SPME−GC−MS) and their behavioral effects on codling moth (CM) adults were evaluated in cage bioassays. The levels of 1-octanol, linalool, geraniol, benzyl alcohol, methyl salicylate, (2*R*,5*R*)-theaspirane, and (2*S*,5*R*)-theaspirane were significantly increased in the leaf extracts containing the *Aspergillus niger* β-glucosidase (BGL1) compared to the extracts containing the glucoimidazole. The attractiveness of individual compounds to CM adults was found in the following decreasing order: methyl salicylate and mixture of two theaspirane isomers, followed by linalool and benzyl alcohol. Geraniol was found to be repellent to CM adults. The addition of geraniol (39.4 ng mL^{-1}) to any of the individual volatiles or to a mixture of these attractants eliminated their attractiveness. Our data suggest the possible application of geraniol as a repellent and methyl salicylate or theaspiranes as attractants for the integrated control of CM in apple orchards.

KEYWORDS: β-Glucosidase; glycosides; volatiles; geraniol; codling moth; repellent

INTRODUCTION

The codling moth (CM) *Cydia pomonella* (L.) is the most economically important insect for apple cultivation worldwide. It has a close ecological association with apples as well as some other plants (*1*). Bengtsson et al. (*2*) observed the behavioral response of codling moth adults to apple branches with or without green fruit in the wind tunnel and found that both apple green fruit and leaves alone attract CM females. This association has been known to be largely mediated by some volatile attractants released from the host plants (*2*−*6*). (*E*,*E*)-α-Farnesene (*3*, *7*), linalool, and β-caryophyllene (*2*) have been identified as attractants for CM adults or neonate larvae (*5*, *6*). However, some of these compounds are also present in the volatile profiles of a wide variety of nonhost plants. It is therefore reasonable to assume that altering the volatile composition of the host plants may change their association with CM insects.

It is well-known that many plants contain significant amounts of glycosidically bound volatiles. Usually, plant leaves show the highest variability of aglycons, followed by flowers, stems, and roots (*8*). Some glycosidically bound volatile compounds in apple leaves and fruit have been isolated and identified (*9*, *10*). Furthermore, successful aroma enhancement of fruit juices and wines by exogenous application of β-glucosidase has been demonstrated (*11*−*13*). In this study, the recombinant *Aspergillus niger* β-glucosidase (BGL1) produced in *Pichia pastoris* was utilized to hydrolyze the glycosides of volatiles present in the leaves of apple (*Malus domestica* cv. Anna). The effect of enzymatically released volatile compounds on the behavior of CM adults was evaluated. The purpose of this study was to determine the potential to release plant volatiles from their nonvolatile glucosides in apple leaves by the broad specificity *A. niger* BGL1 and to determine potential interactions between the released volatiles and apple's most important insect, the codling moth. Furthermore, this study may open new horizons in metabolic engineering of plants via expression of β-glucosidase in different plant tissues that may introduce new strategies in biocontrol of pests in agricultural crops.

MATERIALS AND METHODS

Insects. Eggs of the CM *Cydia pomonella* L. were hatched at room temperature in a Petri dish. The larvae were fed an artificial diet (Manduca Premix−Heliothis Premix, Stonefly Inc., Bryan, TX), kept

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at 25 ± 0.5 °C, 60% ± 1% relative humidity, and a photoperiod of 16:8 h (L:D) until the fifth instar larvae, which were transferred to corrugated paper strips (2 cm^2). Upon emergence, adult insects of mixed sex and age (within 2 days) were used for the cage bioassays.

Apple Leaf Extract Treatments. Apple (cv. Anna) spurs with both leaves and fruit were excised from a commercial orchard in the suburb of Rehovot, Israel, in June.

The leaves were excised from the spurs, rinsed with double-distilled water (DDW), and then dried with filter paper. Dried leaves were homogenized in liquid nitrogen. Three times the weight of ice-cold extraction buffer, containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 4 mM dithiothreitol (DTT) in 50 mM citrate buffer, pH 4.3, was added to the leaf homogenate (4−5 g/treatment). The mixture was then rotated for 1 h at 4 °C and centrifuged at 16000*g* for 5 min. The supernatant (10 mL/treatment) was collected. In one treatment, 1 unit of recombinant *A. niger* BGL1 produced in *P. pastoris* (*14*) was added and the solution was kept at 37 °C for 4 h. In another treatment, 2 μM glucoimidazole was added to the leaf homogenate immediately after the addition of the extraction buffer in order to block any endogenous β-glucosidase activity. Quantitative β-glucosidase activity was assayed with *p*-nitrophenyl β-D-glucopyranoside (pNPG) as the substrate according to Shoseyov et al. (*12*).

Volatile Collection and GC−MS Analysis. Headspace volatiles released from the mixtures of leaf extracts were collected with a solid-phase microextraction (SPME) fiber coated with 100 μm of poly(dimethylsiloxane) (PDMS). The fiber was exposed to the headspace volatiles for 30 min. During SPME the leaf extract temperature inside the vial was 60 °C without stirring. The loaded SPME fiber was then desorbed for 3 min in the injection port of a Varian-3 GC−MS system equipped with a 30 m, 0.25 mm i.d. DB-5 capillary column (J&W, Folson, CA). Sampling and desorption times were precisely controlled by a Varian 8200 autosampler (Varian, Palo Alto, CA). 3-Octanol (0.1 parts per million, ppm) was added to every sample as an internal standard. Each sample was analyzed in independent triplicates. GC−MS parameters were set according to Shalit et al. (*15*). The injector temperature was 250 °C, set for splitless injection. The column was set to 50 °C for 1 min and then the temperature was increased to 200 °C at a rate of 4 °C min^{-1}. Mass range was recorded from 45 to 450 mass-to-charge ratio, with electron energy of 70 eV.

Most volatile chemicals were identified by comparing their mass spectra and retention data with those of authentic compounds, supplemented with the Wiley mass spectrum library (*16*) and literature data (*17*). The volatiles were quantified by calculating the concentrations relative to those of the internal standard. The areas of the volatile component peaks were normalized to the area of the internal standard peak.

Chemicals. Most synthetic standards were purchased from Sigma/Aldrich (St. Louis, MO). Purities ranged from 98% to 99.5%. Mixtures of (2*R*,5*R*)-theaspirane and (2*S*,5*R*)-theaspirane were from Fluka (Buchs SG, Switzerland).

Cage Bioassays. Trapping tests were conducted in screen cages (96 × 68 × 45 cm) in the laboratory at room temperature (23 ± 3 °C) based on the method of Zhu et al. (*18*). Sixty CM adults of mixed sex and ages (2 days after pupal emergence) were released into a cage containing the traps with different treatments. Traps were constructed from 100-mL beakers covered with a white paper lid that had a hole (5 mm diameter) at the center. A Whatman paper wick (10 cm long) was used as a dispenser. To examine the effects of adding recombinant BGL1 to the leaf extract, four traps were constructed and put into the same cage. The first trap contained 10 mL of leaf extract with the addition of recombinant BGL1. The second trap contained the same amount of leaf extract with the addition of glucoimidazole. The third trap contained only leaf extract, and the fourth trap contained water. The insects caught in the traps, or around the trap, were counted 12 h after their release. The insects around the trap were defined as follows: the space of the screen cage was divided equally into four parts. Each trap was put in the center of one part. The number of the insects present in each specific space, not including the ones inside the trap, was obtained in the experiments. To test the attractiveness of the synthetic compounds, traps were made with the individual chemicals that were first dissolved in ethanol at the concentrations determined by the SPME−GC−MS analyses in leaf extracts treated with BGL1. The final concentration of ethanol was 0.01%. Each trap was kept in the cage with only a control water trap including 0.01% ethanol. The experiments were conducted overnight (from 9 pm to 9 am) to cover their active time before the twilight in the early morning. Each experiment was replicated five times except with 1-octanol, which showed no behavioral activity to codling moth in the preliminary experiments. This experiment was replicated three times. The traps were randomly arranged in the replications to avoid possible position effect of the traps. The studies on sex ratios of trapped insects were skipped because of complicated involvement of insect pheromones.

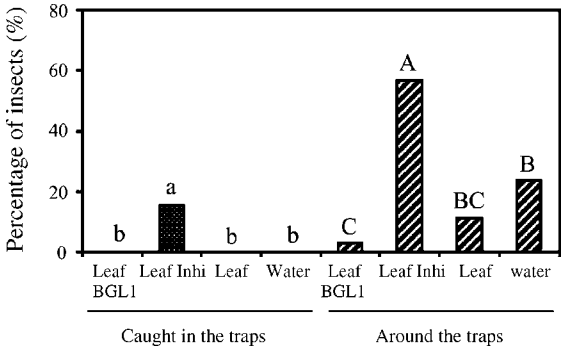

Figure 1. Attraction of apple leaf extracts treated with recombinant β-glucosidase (BGL1) and glucoimidazole (β-glucosidase inhibitor). Leaf BGL1, traps with leaf extract treated with β-glucosidase; Leaf Inhi, traps with the leaf extract treated with glucoimidazole (β-glucosidase inhibitor); Leaf–traps, with leaf extract; Water–trap, with water. The same lowercase and uppercase letter indicated no significant difference in the number of the insects caught in the different traps and around the traps respectively; $p = 0.05$, multiple Turkey test.

RESULTS AND DISCUSSION

Effect of β-Glucosidase Activity on the Attraction/Repulsion of Apple Leaf Extract to CM Adults. Quantitative β-glucosidase activity assay with *p*-nitrophenyl β-D-glucopyranoside as the substrate confirmed the residual activity of endogenous β-glucosidase in apple leaf extracts [0.24 unit·(g of fresh weight)$^{-1}$·min^{-1}]. Treatment with glucoimidazole (*19*) resulted in undetectable levels of β-glucosidase activity in the leaf extract.

Traps containing apple leaf extract and 2 μM glucoimidazole caught significantly more adult CMs inside the traps than did the traps containing leaf extract, leaf extract treated with BGL1, or water. Additionally, around the leaf plus inhibitor traps, there were significantly more insects (**Figure 1**) than any other traps. These well consistent results suggested that aglycons released by β-glucosidase activity have a repellent effect on CM insects. Furthermore, the complete inhibition of β-glucosidase activity by glucoimidazole and the significant attraction of leaf extract treated with this β-glucosidase inhibitor may suggest that these glycosides and the β-glucosidase are located in different compartments in the intact leaf.

Attraction or orientation responses of phytophagous insects to host plant odor may be enhanced or increased with injury to the plant. The increased response of CM larvae and adults to apple fruit infested with other CM larvae has been reported (*20*, *21*). In this study, we demonstrated that crushed apple leaves with glucoimidazole retain their attractiveness to the CM adults.

GC−MS Analysis of Apple Leaf Extracts Treated with BGL1 or β-Glucosidase Inhibitor. Headspace volatiles of the leaf extracts treated with BGL1, β-glucosidase inhibitor, or nothing were collected with SPME fiber and analyzed by GC−MS. Identification and quantification of the detected volatiles

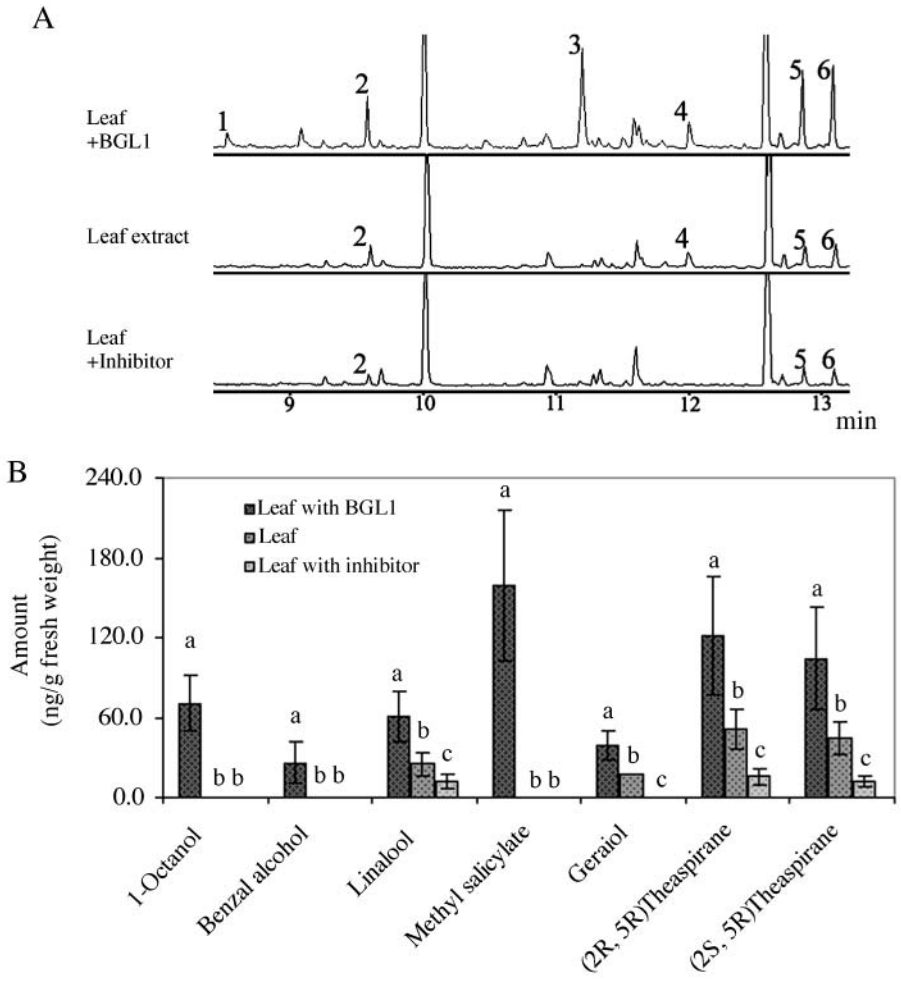

Figure 2. Increased levels of compounds released from apple (cv. Anna) leaf extracts added with external β-glucosidase. (A) GC chromatographs of apple leaf extracts added with β-glucosidase, glucoimidazole, or nothing. Peaks: 1, benzyl alcohol; 2, linalool; 3, methyl salicylate; 4, geraniol; 5, (2*R*,5*R*)-theaspirane; 6, (2*S*,5*R*)-theaspirane. (B) Increased levels of compounds in the leaf extracts with β-glucosidase BGL1. The same letter indicated no significant difference in the number of the insects caught in the different traps; $p = 0.05$, multiple Turkey test.

were carried out by comparison of their mass spectra and retention indices with authentic synthetic standards. The levels of 1-octanol (CV, coefficient of variation, 32.6%), linalool (28.6%), geraniol (22.4%), benzyl alcohol (32.1%), methyl salicylate (41.9%), (2*R*,5*R*)-theaspirane (38.7%), and (2*S*,5*R*)-theaspirane (32.4%) were significantly increased in the leaf extracts treated with BGL1 relative to leaf extracts treated with β-glucosidase inhibitor (**Figure 2**), indicating that these compounds are present mainly as glucosides in apple leaves. *A. niger* BGL1 released significantly more aglycons from leaf extract compared with leaf extract in the absence of exogenous β-glucosidase or β-glucosidase inhibitor. This may reflect either differences in the substrate specificity between *A. niger* BGL1 and apple β-glucosidase or simply higher activity of *A. niger* BGL1 in the reaction vial. Most of the identified volatiles are considered common aglycons in many plant species (*8*). The data on apple aglycons are quite limited. A substantial amount of C13-noriprenoids has been found present as glycosidically bound aromatic compounds in apple fruit and leaves (*9*, *10*). But there was no report about geraniol, methyl salicylate, and theaspirane isomers as apple aglycons before. The presence of certain aglycons may also vary between apple cultivars. Diastereomeric theaspiranes are found in nature (*22*). Two of them were identified as aglycons in purple passion fruit (*Passiflora edulis* Sims) (*23*). Schmidt et al. (*22*) synthesized four isomers of theaspiranes that differed distinctly in their sensory properties. (2*R*,5*R*)-Theaspirane was found to have a weak camphoraceous note, while (2*S*,5*R*)-theaspirane exhibited a strong camphoraceous, almost naphthalene-like note. In this study, the identification was based on these two synthetic theaspirane isomer standards. To the best of our knowledge, this is the first report of theaspirane isomers present as aglycons in apple leaves.

1-Octanol, benzyl alcohol, and methyl salicylate were detected only in the presence of *A. niger* BGL1. It might be because of the differences in substrate specificity of BGL1 and endogenous apple β-glucosidase or inefficient extraction of endogenous β-glucosidase. It is known that some plant glucosidases such as grape berry glucosidase are membrane-bound enzymes (*24*). Their extraction needs specific conditions. It is well documented that aglycone-moiety specificity of β-glucosidases varies considerably with the origin of the enzyme (*25*−*27*). In this study, low levels of linalool and theaspiranes were detected in leaf extracts treated with glucoimidazole, which completely inhibited β-glucosidase activity in the condition tested, suggesting that free forms of these compounds exist in apple leaves. Bengtsson et al. (*2*) also detected a small amount of free linalool in the headspace of Jonathan apple leaves. But they did not mention theaspiranes.

It should be kept in mind that, in addition to those above-mentioned compounds, there were many other volatiles released from apple leaf extract. Because of the limited sensitivity of

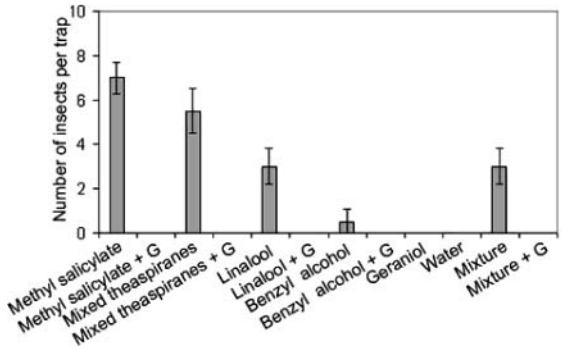

Figure 3. Attraction/repulsion effect of β-glucosidase-enhanced compounds on CM insects. Trapping tests were conducted in screen cages with paired traps and 60 adult insects. The numbers of insects caught in the traps were recorded and analyzed by *t*-test ($p < 0.05$). (+G) With addition of geraniol at a concentration of 39.4 ng mL^{-1}; (Mixed) mixture of all the attractants at the concentrations detected in leaf extracts.

the GC−MS, it is possible that there could be some other insect attractants or repellents present in the apple leaf headspace.

Attractive/Repulsive Effect of β-Glucosidase-Enhanced Compounds on CM Insects. In cage bioassays, traps with each of the individual compounds at the concentrations detected in the leaf extracts were paired with a control trap containing only water and ethanol. The attractiveness of the compounds to the adult insects followed the decreasing order: methyl salicylate and a mixture of two theaspirane isomers, followed by linalool and then benzyl alcohol. All these compounds were more attractive than water as blank (**Figure 3**). Interestingly, the trap with the mixture of all these compounds did not catch the most insects. To our knowledge, this is the first report of theaspirane isomers as a CM attractant. These compounds may be used as baits in mass-trapping of adult insects, in addition to the widely accepted technique of insect-mating disruption (*28*).

The trap with 70 ng mL^{-1} 1-octanol did not catch any insects and the addition of the same amount of 1-octanol to any other compounds did not significantly affect the number of trapped insects compared with these compounds alone (data not shown). This indicated that 1-octanol does not have a significant effect on the behavior of CM adults.

Remarkably, 39.4 ng mL^{-1} geraniol exhibited a repellent effect on CM adults. The traps with any of the aforementioned attractant compounds plus the same amount of geraniol did not capture any insects, suggesting that geraniol at that concentration eliminates the attractiveness of those compounds used in the experiments. Geraniol has been reported as a key ingredient in some commercial mosquito-repellent products (*29*) and as the main component of the natural essential oil of citrosa plants (*30*), which has been marketed as a biological repellent against mosquitoes.

Insect control focuses on both the protection of crops and animals and the maintenance of public health. One area of interest involves the development and production of environmentally safe and nontoxic insect repellents. Our results indicate that in the apple plant hosts of CM, geraniol exists as an inactivated glucoside/CM repellent. Only upon leaf injury does decompartmentation of the glucoside and β-glucosidase take place, resulting in the release of geraniol. More recently, we have shown that expression of the *A. niger* β-glucosidase gene (*BGL1*) in transgenic tobacco results in significant alteration of the volatiles in both intact and crushed leaves (*31*). The present study indicates that metabolic engineering of plants via expression of β-glucosidase may open new horizons in the biocontrol of pests in agricultural crops.

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