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Whole-cell bacterial biosensor with the capability to detect red palm weevil, *Rhynchophorus ferrugineus*, in date palm trees, *Phoenix dactylifera*: a proof of concept study

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ABSTRACT

The red palm weevil (RPW), *Rhynchophorus ferrugineus*, is considered a severe pest of palms. Usually, the early stages of infection are without visible signs. An attractive early sensing approach of non-visible infections is based on volatile organic compounds (VOCs). In this study, a whole-cell bacterial biosensor was used for the identification of RPW in date palm (*Phoenix dactylifera*). The cells are genetically modified to produce light in the presence of general stresses. The bioluminescent bacterial panel is based on three genetically engineered *Escherichia coli* strains that are sensitive to cytotoxicity (TV1061), genotoxicity (DPD2794), or quorum-sensing (K802NR). The bioluminescent bacterial panel detects the presence of VOCs and a change in the light signal is then generated, reflecting the health status of the date palm tree. The bioreporter bacteria cells are immobilized in calcium alginate tablets and placed in a sealed jar without direct contact with the tested sample, thereby exposing them only to the VOCs in the surrounding air. The immobilized bacteria cells were exposed to the air near infected by RPW or uninfected sugar canes, date palm tree pieces, and on date palm trees. Commercial plate reader was used for signal measurement. The findings show that quorum-sensing was induced by all the tested samples of infected sugar canes, date palm tree pieces, and date palm trees. While, cytotoxicity was induced only by infected date palm tree pieces, and genotoxicity was induced only by infected date palm trees. The bacterial monitoring results enable the identification of specific signatures that will allow a quick and accurate diagnosis.

1. Introduction

The red palm weevil (RPW), *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae), also known as the Asian palm weevil or sago palm weevil, is considered one of the most severe pests of palms. The beetle originated in Southeast Asia and from there spread to most areas of date and ornamental palms on the shores of the Mediterranean (Faleiro, 2006). It spread since the mid-1980 s to an expanded geographical range and is now reported from several Asian, African, European, and American countries (EPPO, 2020). The Canary Island date palm (*Phoenix canariensis*) is particularly sensitive to this pest (Ju et al., 2011); however, it also mainly attacks the date palm (*Phoenix dactylifera),* and coconut palm (*Cocos nucifera*) (Faleiro et al., 2002), attacking a total of 40 palm species worldwide. Surrogacy also includes agave and sugar cane (*Saccharum officinarum*) (Giblin-Davis et al., 2013). Usually, the early stages of infection are without visible signs of damage and therefore undetectable, making its identification problematic. The RPW infection begins with laying eggs and then the development of young larvae within the tree, reaching the pupa and adult stage. The RPW larvae are cryptic internal tissue-feeding pests of palm trees that are difficult to detect; consequently, infestations may remain hidden until they are widespread in the orchard. The internal larvae feeding in the palm trunk can irreparably damage its tissue and even kill it if left untreated. The signs of damage are noticeable only in relatively late

Abbreviations: GC–MS, Gas Chromatography-Mass Spectrometry; HS-SPME, Headspace Solid-Phase Micro-Extraction; IF, Induction Factor; RLU, Relative Light Units; RPW, Red Palm Weevil; VOCs, Volatile Organic Compounds.

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stages when the palms are beyond treatment and must be eradicated (Al-Dosary et al., 2016). The infestation starts either at the base of the trunk in the case of date palm or in the crown of the palm in the case of *P. canariensis*, making its identification more challenging (Dembilio and Jaques, 2012). Up to date, RPW is the most dangerous and deadly pest for coconut, oil, sago, and other palms, with severe economic loss. The savings from the treatment of palms in the early stages of infestation serves as the motivation for countries to strengthen their management schemes against this lethal pest (El-Sabea et al., 2009).

Conventionally, visual inspection is still mostly used for the detection of infested palms. Currently, advanced techniques are being examined, such as detection of chemical signatures, gas sensors, acoustic detection, infrared cameras, thermal imaging, satellite imaging/IoT, and sniffer dogs (Massimo et al., 2018; Soroker V et al., 2017). There is a need for the development of detection technologies that are quick, reliable, cost-effective, and easy-to-handle for early detection in order to increase the overall efficiency (EPPO, 2020). An attractive early sensing approach of non-visible infections is based on volatile organic compounds (VOCs) detection. The identification of volatiles in food or agricultural produce as a means of monitoring its chemical and microbial quality has been reported for meat, fruits, vegetables, oil, milk, beverages, and more (Plutowska and Wardencki, 2007). Plants emit various VOCs into their surrounding air, which serves essential functions in growth, survival, communication, defense, and signaling against pests and diseases. There are 1000 identified types of VOCs synthesized by plants (Pichersky et al., 2006). Furthermore, the profile of the emitted VOCs of healthy crops is altered after infection with pathogenic microorganisms (e.g., insects, fungi, bacteria, and viruses) (Holopainen and Gershenzon, 2010; Martinelli et al., 2015). Various VOCs were identified from palm tree tissues infested by RPW (Monzer and Al-Elimi, 2002). In addition, each type of insect emits its specific volatile substances (pheromones, etc.). In previous years, methods of isolating substances (Headspace Solid-Phase Micro-Extraction (HS-SPME)) and combining them with gas chromatography-mass spectrometry (GC–MS)) were used to detect the presence of insects (Seitz and Ram, 2004). In these cases, air samples are collected, and the identification is carried out by either pheromones and metabolites (Arnaud et al., 2002) or VOCs used for communication (Villaverde et al., 2007). The complexity of these methods, the requirement for expensive equipment, and the inability to monitor in real-time limit their use in the field. Electronic gas sensors and e-nose technologies were also examined for the detection of volatiles emitted by plants infested by insects (Cui et al., 2018) and the monitoring of crops quality (Peris and Escuder-Gilabert, 2009). However, these sensors are also sensitive to various compounds (alcohols, ketones, fatty acids, and esters), humidity, are costly, and incapable to identify volatiles concentrations. Thus, the use of e-nose technology in agriculture remains at the laboratory level and mostly for research purposes (Brezmes and Llobet, 2016). These significant shortcomings highlight the need for the development of alternative monitoring techniques that are sensitive and able to monitor full VOCs profiles.

There is great potential for methods that enable the continuous detection of VOCs in the air for the early detection of pests. Specifically, biosensors may be a preferable choice for the detection of RPW in date palm trees by VOCs profiling. A biosensor is a monitoring tool based on the specific responses of a biological component (i.e., DNA / RNA, antibodies, enzymes, or cells) or physical monitoring of these changes (i.e., optical, thermal, or electrochemical) and their conversion into measurable values in integrated systems (Rodriguez-Mozaz et al., 2005). Over the last two decades, biosensors have been developed for applications in various fields such as in health, the environment, agriculture, and food. Biosensor platforms offer advantages when compared to conventional analytical methods. They can be cheap, simple to use, and frequently measure the presence of the test target without sample preparation. Other advantages include the possibility of miniaturization and portability, which permits their use as on-site devices. Whole-cell bacterial biosensors are a type of monitoring tool that is based on the integration of microorganisms with an analytical format (e.g., optical or acoustic waveguide electrodes). The main advantages of whole-cell biosensors are their capability to identify the target analyte as well as to monitor its biological activity by analyzing the differences in the bacterial responses (e.g., gene expression, metabolic activity, viability). Moreover, the development of new tools in the field of genetic engineering has enabled the creation of biosensors based on microorganisms as a tool for monitoring various infections. These biosensors not only allow rapid detection (1–2 h) and monitoring of hazardous substances, but also provide a measure of how they damage cells (damage to genetic material, proteins, membranes, or damage from oxidants) (Eltzov et al., 2011). Modification of microorganisms allowed the expression of a measurable signal (such as discoloration, illumination, or electric current) in response to exposure to toxic substances. In addition to detecting hazardous substances in water (Axelrod et al., 2016; Eltzov and Marks, 2009), these cells have been also incorporated into optical sensors to create a device for continuous monitoring of air quality indoors to detect the presence of pollutants (Chalupowicz et al., 2020; E. Eltzov, A. Cohen, et al., 2015; Eltzov et al., 2011; Ma, Veltman et al., 2020). The cells are genetically modified to produce light in the presence of specific chemicals (Bakhrat et al., 2011; Bhuvaneshwari et al., 2019; Hakkila et al., 2004; Ma, Harpaz et al., 2020; Manivannan et al., 2020; Mazzai et al., 2017; Veltman et al., 2022) or general stresses (Axelrod et al., 2021; Eltzov et al., 2008; E. Eltzov, A. Yehuda, et al., 2015; Harpaz et al., 2021; Harpaz et al., 2018; Lior et al., 2018). Thanks to their simplicity of operation, good sensitivity and portability, these sensors can give a real solution for monitoring various substances in the air.

In this study, a bacterial panel was used to identify RPW in date palm trees (*Phoenix dactylifera*). The bioluminescent bacterial panel is based on three different genetically engineered *E. coli* strains that are sensitive to general stresses, i.e., cytotoxicity (TV1061), genotoxicity (DPD2794), or quorum-sensing stress (K802NR). The bioluminescent bacterial panel detects the presence of VOCs and a change in the light signal is then generated, reflecting the health status of the palm tree. The bioreporter bacteria cells are immobilized in calcium alginate tablets and placed in a sealed jar without direct contact with the tested sample, thereby exposing them only to the VOCs in the surrounding air (Fig. 1). The bacterial exposure to the VOCs occurs by their natural extraction from the sample into the jar and then diffusion from the headspace to the immobilization matrix. Firstly, the immobilized bacteria cells were exposed to the air near sugar canes, either infected by RPW or uninfected. Then, the bacteria cells were exposed to the air near date palm tree pieces that were cut either from infected or uninfected trees. Lastly, the immobilized bacteria cells were tested on the infected date palm trees in the orchard. The bacterial tablets were then placed into a 96-well microtiter plate for the measurement of their bioluminescent signal with a commercial plate reader. The bacterial monitoring results enable the identification of specific signatures that will allow a quick and accurate diagnosis, even before the appearance of visible disease symptoms.

2. Materials and methods

2.1. Materials

White opaque 96-well microtiter plates (30396) were purchased from SPL Life Sciences Co., Ltd. (26, Geumgang-ro 2047 beon-gil, Naechon-Myeon, Pocheon-si, Gyeonggi-do 487 835, Korea (11192)). Luria-Bertani broth (LB) (DF0446–17–3) was purchased from Becton Dickinson & Co., BD Diagnostic Systems, (7 Loveton Circle, Sparks MD 21152–0999, United States). Ampicillin sodium salt (BP1760–25) was purchased from Fisher BioReagents (300 Industry Dr. Pittsburgh, Pennsylvania 15275, United States). Alginic acid sodium salt (B25266) was purchased from Alfa Aesar (Shore Rd, Port of Heysham Industrial Park, Heysham LA3 2XY, United Kingdom). Calcium chloride (A119.2) was purchased from Carl Roth GmbH (Schoemperlenstraße 3–5, 76185

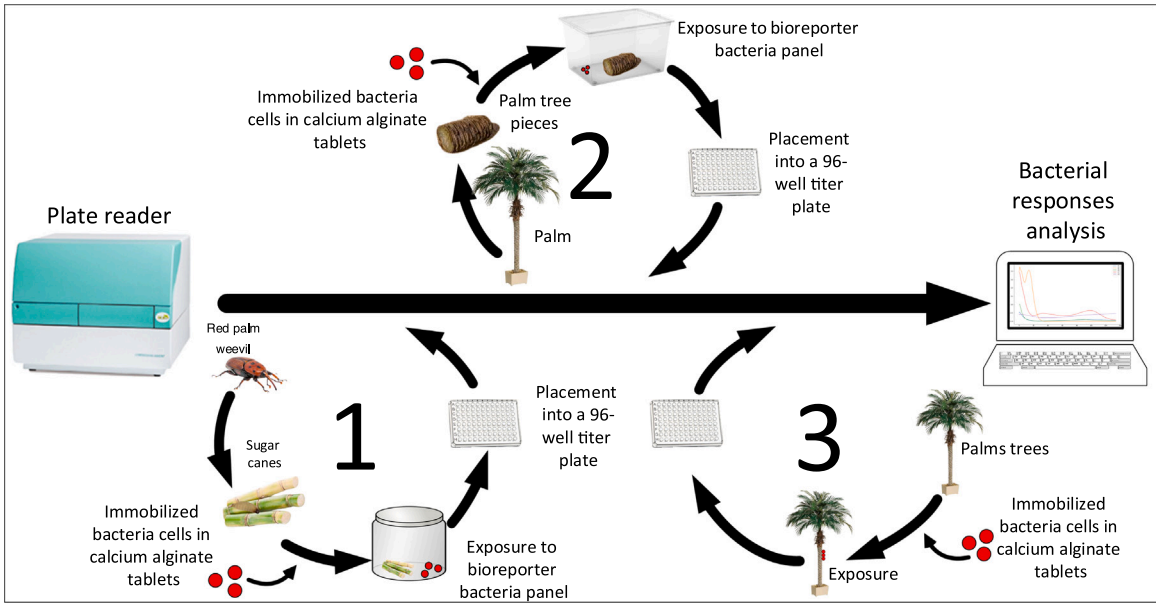

Fig. 1. Schematic presentation of the experiment flowchart. The immobilized bacteria cells were exposed to the air near infected by red palm weevil (RPW), *Rhynchophorus ferrugineus*, or uninfected (1) sugar canes, (2) date palm tree pieces, and on (3) date palm trees in the orchard. The bacterial tablets were then placed into a 96-well microtiter plate for the measurement of their bioluminescent signal with a commercial plate reader.

Karlsruhe, Germany). Cellulose filter sheets (BCS4040) were purchased from Romical (Ha-Khashmalai St 16, Be'er Sheva, Israel). A plastic syringe (7290010993659) was purchased from Kalir (Moshe Schwalb St 9, Petah Tikva, Israel). All stock solutions were diluted with double-distilled water and stored at the temperatures suggested by the manufacturers.

2.2. Equipment

Incubator (Binder (585 – 1D Johnson Ave, Bohemia, NY 11716 United States)), and rotary thermo-shaker MaxQ 4450 (Thermo scientific (401 Mill Creek Rd, Marietta, OH 45750, United States)) were used to grow the bacterial panel. Ultrospec 2100 pro spectrometer (Amersham Bioscience (Biochrom Ltd., Unit 7, Enterprise Zone, 3970 Cambridge Research Park, Beach Drive, Waterbeach, Cambridge, United Kingdom CB25 9PE)) was used to measure absorbance to adjust the optical density of the bacterial panel culture. SynergyHTX multi-mode reader (BioTek Instruments Inc., 100 Tigan St, Winooski, VT 05404, United States) was used to measure the bioluminescent signal of the bacterial panel.

2.3. Bioluminescent bioreporter bacteria

The bacterial panel consists of three different *E. coli* strains including DPD2794 (Feinstein et al., 1983; Gu et al., 2000), TV1061 (Van Dyk et al., 1994, 1995) (both obtained from Shimshon Belkin (Hebrew University, Jerusalem, Israel)), and K806NR (Goh et al., 2002; Shlaes et al., 1989) (obtained from Jan Davies (University of Calgary, Calgary, Canada)). Each bacterial strain harbors a plasmid-borne promoter fusion, by a multi-copy plasmid (mcp), to the lux CDABE reporter operon that is responsible for the synthesis of luciferase and its substrate. The *lux* operon contains 5 structural genes that are responsible for the synthesis of both the heterodimeric luciferase units (*luxA* and *B*) and its substrate tetradecanal, by an ATP-and NADPH-dependent multi-enzyme complex composed of fatty acid reductase, transferase, and synthetase (*luxC, D* and *E*) (Eltzov et al., 2015a, 2015c, 2015d). The different promotors include recA (DPD2794), grpE (TV1061), and lasI (K802NR). The promotor in each strain determines the translation of the plasmid upon the activation of a certain metabolic cycle countering cellular damage. The bacterial strains are sensitive to different types of stressors, according to the different promotor fusion. Each promotor is responsible for the different regulatory network in the bacterial cell, which includes DNA-repair mechanisms such as DNA degradation and cross-link mutations (recA), metabolic changes such as with cytotoxic substances (grpE), and bacterial communication as part of a positive feedback loop of *N*-3-oxo-dodecanoyl homoserine lactone ($3OC_{12}$-HSL) that is used for quorum sensing (lasI).

2.4. Bacteria growing conditions

The strains stocks were stored at − 80 °C with 20% (v/v) glycerol as a cell cryoprotectant additive. The bioreporter strains from the stocks solutions were placed on LB-agar plates (10 g/L Tryptone; 5 g/L Yeast Extract; 5 g/L NaCl) that were supplemented with 100 μg/mL ampicillin and incubated for two days at 37 °C in an incubator (Binder, USA). The bacteria cultures were then stored at 4 °C for future experiments. After, the bacterial strains were grown in 10 mL LB medium (Miller, 1972.) amended with 100 μg/mL ampicillin overnight at 37 °C on a rotary thermo-shaker at 120 rpm. The bacterial cultures were diluted to approximately 10^7 cells/mL and regrown in the same medium at 26 °C without shaking to the early exponential phase (OD_{600}nm = 0.2) as determined by a spectrometer.

2.5. Fixation of the bacterial cells in alginate tablets

The bacterial cells were immobilized in alginate tablets to then further test the bacterial panel responses to RPW in sugar canes, date palm tree pieces, and date palm trees. The calcium alginate tablets were prepared as previously reported (Ma, Veltman et al., 2020), by first cutting cellulose filter paper into rectangles (10 cm × 7 cm), which were then rolled into tubes (6 mm in diameter). Then, each tube was glued by using paper glue, so that the bottom of the tube was closed with the cap of a 500-μL centrifuge Eppendorf tube cup. The harvested bacterial cells were mixed 1:1 with a 2.5% (w/v) low-viscosity sodium alginate solution and then loaded into the cellulose tubes by using a 10-mL plastic syringe. After the addition of the mixture, the tubes were kept in place for 5 min for the removal of air bubbles, before the 20 min polymerization reaction by the calcium chloride solution at a

concentration of 0.25 M. The alginate hydrogel tubes with the immobilized bacteria cells were removed from the cellulose paper and then cut with a utility knife into 3 mm uniform tablets by using a customized 3D-printed alginate tube holder (100 mm length, 13 mm height, 13 mm width) with evenly distributed slots (3 mm apart) (Fig. 2).

2.6. Samples preparation of sugar canes, date palm tree pieces, and date palm trees

The evaluation of infestation level was conducted visually, either in the lab for sugar canes or in the field for date palm (*Phoenix dactylifera*) trees and pieces. Fresh sugar cane samples were cut into pieces of 10 cm in length. Then, the pieces were incubated in a tank with larvae and adult RPW for 2–4 weeks, which feeds off the sugar canes in all stages of adults and larvae. After, the pieces that were highly infested, eaten by adult and chewed or penetrated by large larvae, were further examined. Clean sugar canes were used as the control. Each group was kept separately in a concealed closed box for 24 h at room temperature before it was transferred to the lab for further testing. In addition, date palm tree pieces were collected from infested date palm trees (Fig. 3B) in the field and then highly infested pieces were further examined, while clean pieces were used as the control. The date palm tree pieces were also kept in the same conditions, separately in a concealed closed box for 24 h at room temperature before it was transferred to the lab for further testing.

2.7. Bioluminescent bioassay

The bacterial panel responses were examined with a high-throughput approach in white opaque 96-well microtiter plates. Firstly, the immobilized bacteria cells were exposed to the air near sugar canes, either infected by RPW or uninfected (Fig. 1). Then, the bacteria cells were exposed to the air near date palm tree pieces that were cut from either infected or uninfected trees (Fig. 1). Lastly, the immobilized bacteria cells were tested on the infected trees in the orchard (Fig. 1). The bacterial exposure to the VOCs occurs by their natural extraction from the sample into the container and then diffusion from the headspace to the immobilization matrix (Fig. 3). Control of exposure to only air in a sealed container was also tested in all these experiments. Then, the bacterial tablets were removed from the container and placed into a 96-well microtiter plate for the measurement of their bioluminescent signal with a commercial plate reader. The bioluminescence activity of the bacterial panel was measured in a SynergyHTX multi-mode reader. During the measurement, the temperature of the samples was maintained at 26 °C. The luminescence values are presented as relative light units (RLU).

2.8. Reproducibility and statistical analysis

The bioluminescence signal that is indicative of the bacterial panel responses is expressed as induction factor (IF), which was calculated using the formula: IF = Bs/Bc, where Bs is the maximum bioluminescence signal of the tested sample and Bc is the maximum bioluminescence signal of the control. For each parameter, a minimum of 30 separate and different setups was tested. A paired t-test was used in order to identify statistically significant differences ((*) $p < 0.05$ and (**) $p < 0.01$).

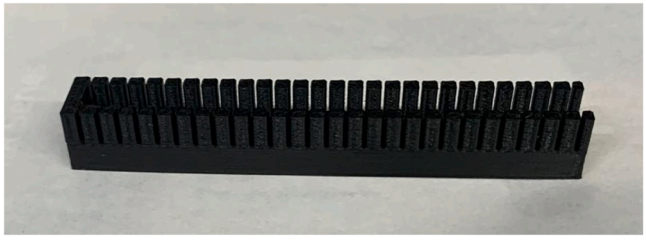

Fig. 2. Image of the customized 3D-printed alginate tube holder. The alginate hydrogel tube with the immobilized bacteria cells was removed from the cellulose paper and then cut with a utility knife into 3 mm uniform tablets by using a customized 3D-printed alginate tube holder (100 mm length, 13 mm height, 13 mm width) with evenly distributed slots (3 mm apart).

3. Results

The bacterial cells were immobilized in alginate tablets to then further test the bacterial panel responses to RPW in sugar canes, date palm tree pieces, and date palm trees. Alginate-based fixation processes have been successfully implemented in the field of biosensors for many years (Chai et al., 2004; Tombs and Harding, 1998). This fixation process is based on the spontaneous polymerization of alginate monomers in the presence of divalent ions (calcium or magnesium) to form a gel matrix that allows the fixation of microorganisms within it (Polyak et al., 2000). Nevertheless, disadvantages of the above fixation method (such as low resistance to deformation, degradation of the matrix in the presence of phosphate or additional calcium cultivators), high porosity, and a comfortable environment for fixed microorganisms make it preferable to living cell-based biosensors. Bioluminescent bacteria fixation in alginate has been developed as a method for continuous monitoring of hazardous substances in the air, where cells can be fixed both in beads (Polyak et al., 2004), in pads (Axelrod et al., 2016), and in the method of creating layers of alginate (E. Eltzov, V. Slobodnik, et al., 2015).

3.1. Bacterial responses to uninfected and infected sugar canes by Rhynchophorus ferrugineus

Firstly, the immobilized bacteria cells were exposed to the air near sugar canes, either infected by RPW or uninfected (Fig. 4). According to the literature, surrogacy also includes agave and sugar canes (Giblin-Davis et al., 2013). The IF values were compared between the uninfected and infected sugar canes while adjusting both values to the control. A statistically significant difference ($p < 0.01$) was seen between the uninfected (IF = 0.957) and infected (IF = 1.858) sugar canes only in the case of the K802NR strain, which is indicative of bacterial communication as part of a positive feedback loop of *N*-3-oxo-dodecanoyl homoserine lactone ($3OC_{12}$-HSL) that is used for quorum sensing (Goh et al., 2002; Shlaes et al., 1989). In this case of the K802NR strain, a substantial induction effect is seen in the infected sugar canes, with a higher IF value of 1.941-fold. Whereas, statistically non-significant differences were seen in the other two tested strains of DPD2794 (uninfected IF = 0.918; infected IF = 1.116) and TV1061 (uninfected IF = 1.148; infected IF = 1.390), which are indicative of DNA-repair mechanisms (Feinstein et al., 1983; Gu et al., 2000) and metabolic changes (cytotoxic substances) (Van Dyk et al., 1994, 1995), respectively. To conclude, sugar canes infected by RPW show an induction effect on the K802NR strain that is sensitive to interference in quorum sensing.

3.2. Bacterial responses to uninfected and infected date palm tree pieces by Rhynchophorus ferrugineus

The bacteria cells were then exposed to the air near date palm tree pieces that were cut either from infected by RPW or uninfected trees (Fig. 5). In this examination of the bacterial responses to the date palm tree pieces, all the tested bacteria strains (DPD2794, TV1061, and K802NR) showed statistically significant differences ($p < 0.01$) between the two tested groups of uninfected and infected date palm tree pieces, after the adjustment to the control. Both TV1061 and K802NR showed an induction effect of increased IF values in the infected group (TV1061 IF = 1.471; K802NR IF = 1.623) as compared to the uninfected group (TV1061 IF = 1.205; K802NR IF = 1.066). The elevated IF values were increased by 1.221-fold in the case of TV1061 and by 1.523-fold in the case of K802NR. This is strengthened by the sugar canes results that also

Fig. 3. Image of the experiment setup. (A) The immobilized bacteria cells were exposed to the air near infected by red palm weevil (RPW), *Rhynchophorus ferrugineus*, or uninfected date palm trees in the orchard. (B) Image of infected palm tree pieces collected from infested date palm trees. (C) Bacteria cells immobilized in alginate tablets. (D) Testing of bacterial cells response to VOCs in a closed container. The bacterial tablets were then placed into a 96-well microtiter plate for the measurement of their bioluminescent signal with a commercial plate reader.

showed a strong induction effect (higher by 1.941-fold) in the case of K802NR. Whereas, in the case of the DPD2794 strain, which is indicative of DNA-repair mechanisms (Feinstein et al., 1983; Gu et al., 2000), a statistically significant inhibition pattern was identified. The DPD2794 strain was inhibited by the infected date palm tree pieces (IF = 1.225), showing a 0.785-fold lower IF value as compared to the uninfected date palm tree pieces (IF = 1.561). To conclude, both TV1061 (cytotoxic) and K802NR (quorum sensing) were induced by the infected date palm tree pieces by RPW, while DPD2794 (genotoxic) was inhibited by them.

3.3. *Bacterial responses to uninfected and infected date palm trees by Rhynchophorus ferrugineus*

Lastly, the immobilized bacteria cells were tested on the infected date palm trees in the orchard (Fig. 6). The testing of the bacterial responses to the infected date palm trees is highly influenced by various environmental variables (e.g., temperature, moisture, air contaminants), as compared to the previous examinations of the sugar canes and date palm tree pieces, which were both conducted under controlled lab settings. Therefore, as expected, the detected standard deviation levels are higher in the cases of detection on date palm trees while adjusting for the control sample results accordingly. These results show that a statistically significant difference ($p < 0.01$) was seen between the infected and uninfected date palm trees in both DPD2794 and K802NR strains. Both DPD2794 and K802NR strains were induced by 3.272-fold and 2.741-fold, respectively, by the infected date palm trees (DPD2794 IF = 4.810; K802NR IF = 3.591) as compared to the uninfected palm trees (DPD2794 IF = 1.470; K802NR IF = 1.310). Whilst, the DPD2794 strain also demonstrated high standard deviation values as compared to the K802NR strain. In addition, the comparison between the uninfected and infected date palm trees showed statistically non-significant differences in the case of the TV1061 strain. To conclude, both DPD2794 (3.272-fold) and K802NR (2.741-fold) were induced by the infected date palm trees by RPW in the orchard.

4. Discussion

The responses of the bacterial panel were tested after exposure to sugar canes, date palm tree pieces, and date palm trees, either uninfected or infected by RPW (Fig. 7 and Table 1). The findings of this study can be categorized into three main observations. The first observation concerns the bacterial strains that were induced by the infected samples as compared to the uninfected. The K802NR strain (quorum sensing) was induced by all the tested samples of infected sugar canes, date palm tree pieces, and date palm trees, while the TV1061 strain (cytotoxic) was induced only by infected date palm tree pieces, and the DPD2794 strain

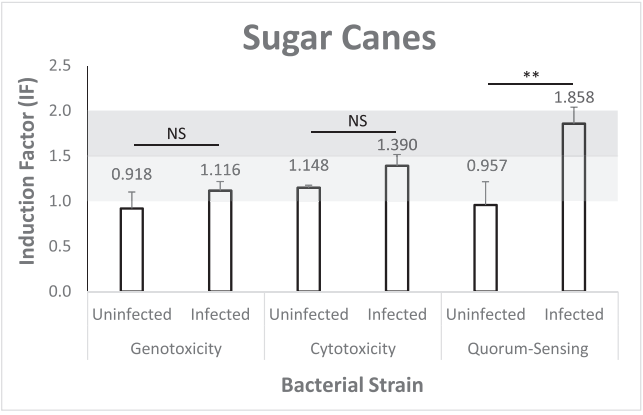

Fig. 4. Bacterial responses to uninfected and infected sugar canes by *Rhynchophorus ferrugineus*. A panel of three genetically engineered strains from *E. coli* that are sensitive to different stresses were tested: DPD2794 (genotoxic), TV1061 (cytotoxic), and K802NR (quorum sensing). The bioluminescence signal is reported as induction factor (IF = Bs/Bc, where Bs is the maximum bioluminescence signal of the tested sample and Bc is the maximum bioluminescence signal of the control). A paired t-test was used in order to identify statistically significant differences ((*) $p < 0.05$ and (**) $p < 0.01$).

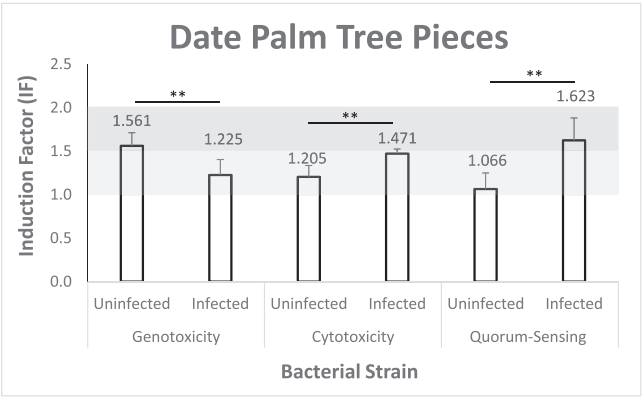

Fig. 5. Bacterial responses to uninfected and infected date palm tree pieces by *Rhynchophorus ferrugineus*. A panel of three genetically engineered strains from *E. coli* that are sensitive to different stresses were tested: DPD2794 (genotoxic), TV1061 (cytotoxic), and K802NR (quorum sensing). The bioluminescence signal is reported as induction factor (IF = Bs/Bc, where Bs is the maximum bioluminescence signal of the tested sample and Bc is the maximum bioluminescence signal of the control). A paired t-test was used in order to identify statistically significant differences ((*) $p < 0.05$ and (**) $p < 0.01$).

(genotoxic) was induced only by infected date palm trees. The light activity in the K802NR strain is induced by the expression of specific signaling molecules (e.g., *N*-acyl homoserine lactone) that are regulated by a bacterial density-dependent cell-cell communication process (Goh et al., 2002; Shlaes et al., 1989). This induction effect may occur also as a result of the insects sourced VOCs, which might interact with the bacteria reporter systems. It is clear that some VOCs are involved in microbial interactions, acting as signaling and quorum-sensing compounds (Arrarte et al., 2017; Effmert et al., 2012). Infected plants may produce structural analogs or mimics of *N*-acyl homoserine lactones or anti-quorum-sensing compounds as a defense response to the damages processes (Bastas and Kannan, 2015). Thus, the induction effect on the bioluminescent activity of the K802NR strain may be explained by the presence of these VOCs in the headspace due to the activities of the RPW in the infected sugar canes, date palm tree pieces, and date palm trees.

The second observational group contains bacterial strains that were inhibited by the infected samples. The bioluminescent activity of only

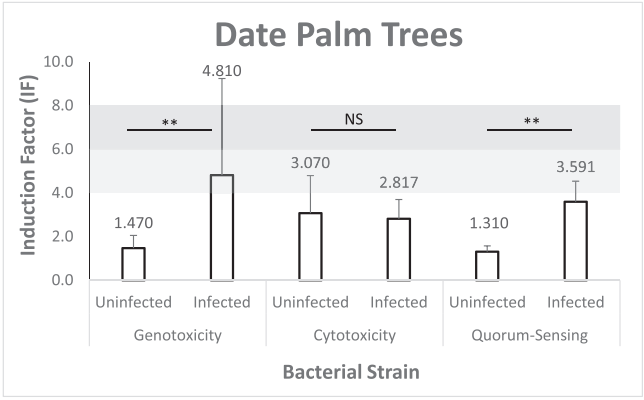

Fig. 6. Bacterial responses to uninfected and infected date palm trees by *Rhynchophorus ferrugineus*. A panel of three genetically engineered strains from *E. coli* that are sensitive to different stresses were tested: DPD2794 (genotoxic), TV1061 (cytotoxic), and K802NR (quorum sensing). The bioluminescence signal is reported as induction factor (IF = Bs/Bc, where Bs is the maximum bioluminescence signal of the tested sample and Bc is the maximum bioluminescence signal of the control). A paired t-test was used in order to identify statistically significant differences ((*) $p < 0.05$ and (**) $p < 0.01$).

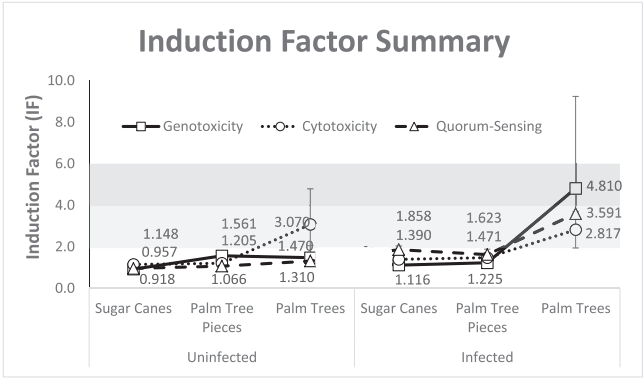

Fig. 7. Induction factor summarized analysis of the bacterial responses to uninfected and infected by *Rhynchophorus ferrugineus* sugar canes, date palm tree pieces, and date palm trees. A panel of three genetically engineered strains from *E. coli* that are sensitive to different stresses were tested: DPD2794 (genotoxic), TV1061 (cytotoxic), and K802NR (quorum sensing). The bioluminescence signal is reported as induction factor (IF = Bs/Bc, where Bs is the maximum bioluminescence signal of the tested sample and Bc is the maximum bioluminescence signal of the control).

Table 1
Induction and inhibition effects on the bacterial panel.

		Genotoxicity	Cytotoxicity	Quorum-Sensing
Sugar Canes	Uninfected	–	X	–
	Infected	X	X	+
	Ratio (fold)	X	X	++
Date Palm Tree Pieces	Uninfected	+	X	X
	Infected	X	X	+
	Ratio (fold)	–	X	++
Date Palm Trees	Uninfected	X	++	X
	Infected	+++	+	++
	Ratio (fold)	+++	X	++

*Induction Factor (IF): (-) inhibition effect; (x) no effect; (+) induction effect
* (—) $0 \leq IF \leq 0.49$; (-) $0.5 \leq IF \leq 0.99$; (X) $1 \leq IF \leq 1.49$; (+) $1.5 \leq IF \leq 2.99$; (++) $3 \leq IF \leq 3.99$; (+++) $IF \geq 4$
* Ratio (fold): (-) $= 0.5 \leq fold \leq 0.89$; (X) $0.9 \leq fold \leq 1.249$ (+) $1.25 \leq fold \leq 1.49$; (++) $1.5 \leq fold \leq 2.99$; (+++) $fold \geq 3$

the DPD2794 strain was slightly inhibited by the infected date palm tree pieces. The light activity in this strain is regulated by DNA-repair mechanisms such as DNA degradation and cross-link mutations (Feinstein et al., 1983; Gu et al., 2000). Previously it was reported that RPW demonstrated antimicrobial activity, mainly in Gram-positive strains but not in Gram-negative strains, including *E. coli* (Mazza et al., 2011). Lastly, the third observational group includes bacterial strains that were not affected by the tested samples. Meaning that the proposed bioreporters might only detect the presence of the insect at different infection stages. For example, the bacterial strain TV1061 showed a statistically non-significant difference between the infected and uninfected sugar canes and date palm trees. The TV1061 strain is sensitive to metabolic changes such as cytotoxic substances (Van Dyk et al., 1994, 1995), which might not occur in the tested infection stages. Currently, studies have not yet identified cytotoxic activity in RPW larval extracts (Hassan et al., 2018). Furthermore, the difference in the bacterial responses suggests the potential to create specific fingerprints for the different infection stages in future studies. Moreover, the response ratios were much higher in the cases of date palm trees as compared to date palm tree pieces. As well as, much higher response ratios were observed in the cases of date palm tree pieces as compared to the sugar canes. The possible reason for such an increase in the light activity and the differences in the bacterial response patterns may be the higher insects sourced VOCs concentrations in the surrounding air. Furthermore, the VOCs produced from the date palm trees and their decay processes may also be an explanation for these changes.

Lastly, in order to determine the capability of the proposed system to detect the presence of RPW larvae in the date palm trees, calcium alginate immobilized bacteria were placed on the infected and uninfected date palm trees in the orchard. In order to minimize the environmental effect on the bacterial strains, the bacterial tablets were placed on the date palm trees in a closed plastic box with small holes. High standard variation values may be explained by the effect of the environmental conditions on the bacterial cells. In this case, it is still possible to determine the presence of the infection, but with lower accuracy. The highest bacterial bioluminescence responses were detected when the bacterial strains were exposed to the infected trees in the orchard. While lower responses were observed when the bacteria cells were placed in sealed testing chambers near pieces of the infected trees in the laboratory. There are two possible reasons for such an increase in bacterial responses. The first is that infected trees change their VOCs patterns during the infection, or that even other palm volatiles may be detected by the bioreporter bacteria. A second possible reason is that the effect of the environmental conditions on the bacterial responses measurement process may enhance the influence of the infection-sourced VOCs in the air, as well as directly on the bacterial strains. An alternative explanation may revolve around the gut microbiota (Muhammad et al., 2017). A recent study reported evidence that alterations in the gut microbiota of RPW influenced its development by regulating the nutrition metabolism with a distinct impact on the host physiology (Habineza et al., 2019).

5. Conclusions

In this study, a bioluminescent bacterial panel of three different genetically engineered strains (cytotoxicity (TV1061), genotoxicity (DPD2794), or quorum-sensing stress (K802NR)) was used for the early identification of RPW in date palm trees. The immobilized bacteria cells were exposed to the air near infected by RPW or uninfected sugar canes, date palm tree pieces, and on the infected date palm trees in the orchard. The bioreporter bacteria cells are immobilized in calcium alginate tablets and placed in a sealed jar without direct contact with the tested sample, thereby exposing them only to the VOCs in the surrounding air. The findings show that K802NR that is sensitive to interference in quorum sensing was induced by all the tested samples of infected sugar canes (by 1.941-fold), date palm tree pieces (by 1.523-fold), and date palm trees (by 2.741-fold). While TV1061 was induced only by infected date palm tree pieces (by 1.221-fold) and showed a statistically non-significant difference between the infected and uninfected sugar canes and date palm trees. Moreover, DPD2794 was induced only by infected date palm trees (by 3.272-fold), and its bioluminescent activity was slightly inhibited by the infected date palm tree pieces (by 0.785-fold). Future examinations should focus on the design of a new exposure device that will eliminate most of the environmental effects. More experiments are needed to determine and eliminate the effects of the environment on the bacteria measurement processes. The bacterial monitoring results enable the identification of specific signatures that will allow a quick and accurate diagnosis, even before the appearance of visible disease symptoms.

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Author Contributions

Evgeni Eltzov: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Dorin Harpaz:** Data curation, Writing – original draft. **Boris Veltman:** Investigation, Validation. **Daniel Katz.:** Investigation. All authors have read and agreed to the published version of the manuscript.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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