Identification of Fusarium in Gerbera using Augmented Ruthenium Red Based Biosensor

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Abstract - Farmers often encounter significant financial losses due to pathogenic diseases. *Fusarium* is known for damaging plants by secreting polygalacturonase, which causes cell wall degradation. This paper reports the identification of polygalacturonase in *Fusarium* through the fabrication of the ruthenium red-based biosensor. The augmented ruthenium red-based biosensor uses the PGA-RR complex and allows physical experimentation in the actual field. Multiple polygalacturonase enzymes were tested with varying concentrations and the biosensor detected the reflected light from those enzymes using a spectral sensor. Two experiments were conducted: one with the use of chromatography paper and the other without it. The experiments suggested that the biosensors were capable of detecting the enzymes. As the concentration of the enzymes increased, the amount of reflected light at various wavelengths increased. This finding is significant because this new prototype offers possible ways of detecting these pathogenic diseases and, meanwhile, advancing the field of bioengineering and biotechnology.

Key Words – Polygalacturonase, Fusarium, Ruthenium Red, Polygalacturonic Acid, Polygalacturonase Inhibiting Proteins, Reflected Light, PGA-RR complex

INTRODUCTION

I. Gerbera

Gerbera Daisy (*Gerbera jamesonii*) is in the family of Asteraceae and originated from South Africa. It is known for its luminescent colors and is frequently used as ornamental flowers. In 2018, in Japan, the three largest producers and shipping amounts were Shizuoka-ken (59,000 k/flower), Fukuoka-ken (20, 9000 k/flower), and Chiba (8,530 k/flower) (Japan CROPs). Furthermore, Japan's popularity for Gerbera from 2004 to 2018 is in a declining trend with the planted acreage decreasing by 16.2% and the shipping amount by 21.4%. Although the economic impact of Gerbera daisies in Japan is not relatively significant, prefectures such as Shizuoka and Miyagi rely on these flowers for their major flower production. Moreover, flower shops in Japan typically sell these flowers due to their wide range of consumers.

FIGURE 1**:** Image of Gerbera flowers

II. Fusarium

Fusarium is a genus of multiple pathogenic, fungal diseases that are known for damaging seedlings and young shoots, causing leaf spots, leaf blights or leaf rots, sheath rots, and rots of the shoot tip, and also developing oval and dark brown necrotic spots (Srivastava et al. 2018). Its main hosts are over 150 species of fruits, vegetables, and flowers. Although there are nearly 20 species of *Fusarium*, most prefer sub-tropical environmental conditions. Still, there are variances in species regarding optimal humidity and temperature. According to William E. Nganje et al., from 1993 to 2001, *Fusarium* Head Blight had an economic loss of 2.492 billion dollars regarding hard red spring (HRS) wheat, SRW wheat, durum wheat, and barley. This cumulative economic impact continues until today due to the non-existence of effective detection methods. Although numerical evidence of *Fusarium* attacking Gerbera is still unknown, the existence of PGIPs in Gerbera implies the presence of pathogenic diseases such as *Fusarium*.

III. Plant Defense Against Fusarium

Plants, such as Gerbera, secrete polygalacturonase inhibiting proteins (PGIP) to defend against polygalacturonase (PG). To break through the cell wall, *Fusarium* produces PGs that cause cell wall degradation and breakdown of plant tissue. As the PGs slowly break down the cell wall, it also produces oligogalacturonides (OG) fragments, which triggers defense responses of the cell. When the flower detects these

fragments, it secretes PGIPs that inhibits the PGs by slowing down or stopping the spread of PGs.

IV. PGIP Expression in Other Flowers

Gerbera's expression of PGIP's is not well studied; however, researchers have studied other plants that are similar to Gerbera's biological system. *Arabidopsis thaliana* (Arabidopsis) encodes two PGIP's (*AtPGIP1* and *AtPGIP2*). This plant is frequently used in biological experiments because it acts as a model organism with a simple biological structure. Ferrari et al. conducted an experiment where they looked at the responses of two tandemly duplicated PGIP's when *Botrytis cinerea* infection's PG's were released. They discovered that both PGIP's encode functional inhibitors of PG from *Botrytis cinerea* and the overexpression of PGIP's in Arabidopsis slowed the infection and wounding (Ferrari et al. 2003). Sunflowers (*Helianthus annuus* L.), which is also part of the Asteraceae family, contain PGIPs that hinder the progression of PGs. Researchers identified four PGIP genes (*HaPGIP1, HaPGIP2, HaPGIP3,* and *HaPGIP4*) and discovered that sunflower's non-synonymous substitutions are genetically diverse in multiple species: *H. maximiliani*, *H. ciliaris*, *H. paradoxus*, *H. tuberosis*, and *H. petiolaris* (Livaja et al. 2016).

V. PGIP and PG Activity in Gerbera and Fusarium

Although PGIP and PG activity in Gerbera is not well understood, in 2015, Wen Fang discovered PGIP activity in Gerbera against PG's produced by *Botrytis cinerea* (Fang 2015). Fang discovered two distinct PGIPs and six endo-PGs in two Gerbera populations and the quantitative trait locus (QTL). They used gene sequencing and genotyping to identify Gerbera PGs and PGIPs. While the existence of PGIP activity in Gerbera is identified, the amount in which *Fusarium* produces PGs are relatively hard to quantify because of the variation in *Fusarium* species. Nevertheless, the production rate of PGs will not affect our results because the production rate does not directly affect the readings of the biosensor.

VI. Detection of PG activity using assays

Although our research was based on ruthenium red and the PGA-RR complex, the detection of polygalacturonase activity can be accomplished in numerous ways. Qian Li et al. (2015) introduced a method involving dilution of PGs and enzyme reactions with dinitrosalicylic acid (DNS). Although this method provided accuracy, it contained substances that were dangerous in a high school research environment. Another possibility was utilizing a color sensor to detect the presence of rot. However, this was impractical because the biosensor would detect any substance that has that specific absorbance wavelength. Finally, polymerase chain reaction (PCR) is another major detection method for numerous enzymes and substances. It allows researchers to rapidly create millions of copies of the gene to facilitate the observation. For example, the detection of COVID-19 (*Orthocoronavirinae*) utilizes PCR and Kovutunovych et al.'s (2003) research on the identification of *Klebsiella oxytoca* employs PCR to test for the presence of pathogenic DNA. However, our biosensor cannot easily incorporate a fully functioning PCR due to its technical requirements and compact size of the device. The biosensor fabricated in our experiment ensures safety, efficiency, applicability, and effectiveness. The biological idea and technology used in this biosensor are based on Choi et al.'s (2020). The Choi et al.'s research involved the creation of a paper-based biosensor that was able to detect polygalacturonase activity in Allium White rot disease. However, significant improvements are made to meet our research objectives and endeavors.

VII. Reasons to Conduct this Research

Often, farmers fail to detect *Fusarium* and its physical symptoms at an early stage. This causes their crops, flowers, and agricultural products to mold and rot. We hope that our research will allow farmers to detect *Fusarium* earlier and prevent financial and economic losses. Farmers will be able to use these machines without difficulty, and researchers will also gain a more concrete understanding of the relationship between *Fusarium's* PGs and Gerbera's PGIPs. This research puts a strong emphasis on the fabrication and improvement of newly designed biosensors and will possibly open doors for researchers now and in the future.

MATERIALS, METHODS, AND PROCEDURE

I. Reagents

The following reagents were used in this experiment: polygalacturonic acid (PGA) (Nacalai Cat. #: 26243-14), ruthenium red (RR) (Nacalai Cat. #: 30318-14), sodium acetate trihydrate (Nacalai Cat. #:31115-05), and commercially-available polygalacturonase enzyme Pectinex Ultra SP-L (Modernist Pantry Cat. #: 1043-250).

II. Materials for Biosensor and Experiment

The following components were used in the construction of the biosensor: SparkFun Spectral Sensor Breakout - AS7262 Visible, SparkFun 16x2 SerLCD - RGB on Black 3.3V, Plastic Container/Coating, SparkFun Arduino Uno R3, Rechargeable Lithium-Ion Batteries, Lithium-Ion Battery Holder, Switch, SparkFun High Precision Temperature Sensor - TMP117, Heating Pad - 5 x 10cm COM-11288, and Transistor.

III. Construction of PGA-RR Complex and Experiment

The PGA-RR complex requires the combination of polygalacturonic acid and ruthenium red. This substance is ultimately responsible for changing and indicating PG

activity. First, we created a 80 mM sodium acetate buffer. Although the optimum pH of the sodium acetate buffer is 5.5, the buffer that we created had a pH of 8. The change in pH does not directly affect the results but influences the size of the ultimate complex. Results of the chromatography paper, later discussed in the paper, showed that the biosensor worked fine with the pH out of the original range. Next, 120 ug of PGA and 80 ug of RR were combined with the most favorable ratio of 1.5 to 1. However, the amount of PGA-RR complex can vary if the ratio between the two substances and the pH of the sodium acetate buffer are maintained. Then, different enzyme concentrations were created and mixed with the PGA-RR complex. The initial values we attained are data before the heating and the final values we obtained were after being heated at 37 degrees celsius for 20 minutes. With the varying concentrations and reflected light at various wavelengths, a standard curve was created.

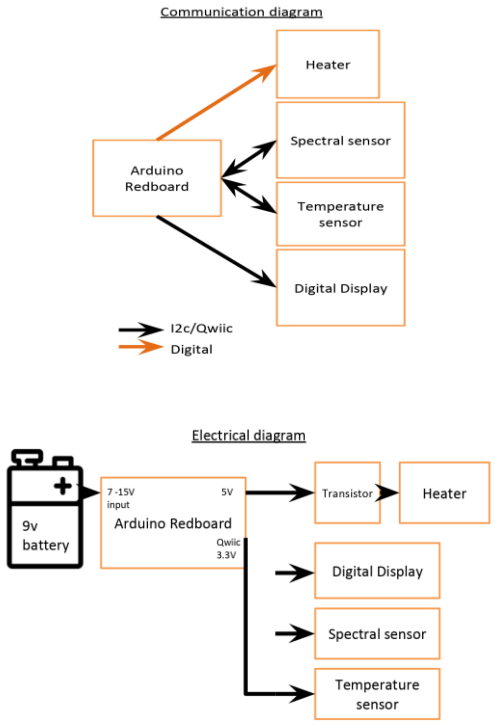

FIGURE 2: Visual diagrams for communication systems between the components and the electrical wiring between the components.

IV. Fabrication of the Biosensor

The overall biosensor mainly consisted of the PGA-RR complex (mentioned above), the heating system, the controller, loading and sensing area, chassis, and the display. To catalyze the chemical reactions, the PGA-RR complex needed to be heated at 37 degrees celsius. We attached *Spark Fun's* 5 x 10 cm Heating Pads, which are capable of producing constant heat similar to our body temperature. To maintain consistency, a temperature sensor was attached beneath it to act as a regulator for the heating pads. When the temperature of the heating pad is higher or lower than 37 degrees Celsius, the sensor sends a signal to the controller to regulate its voltage. Although our biosensor did not incorporate an insulation system to maintain the size and simplicity, an insulation system can be potentially added to keep the spectral sensor and other electronic parts to their optimal temperatures. The Arduino Uno R3 motherboard was placed on the bottom of the biosensor and connected to the display, spectral sensor, temperature sensor, switch, heating pads, and the batteries with wires. Computer programming was done for the biosensor to heat the solution, gather the amount of reflected light, display the results, and repeat this cycle. The loading and sensing area was designed so that the sample can be placed on the biosensor with ease. The chassis was constructed with 3D printing to incorporate simplicity and prevent cost inflation. Finally, the display was designed so that it would present the two wavelengths' (500 nm and 550 nm) reflected light (μW/cm²).

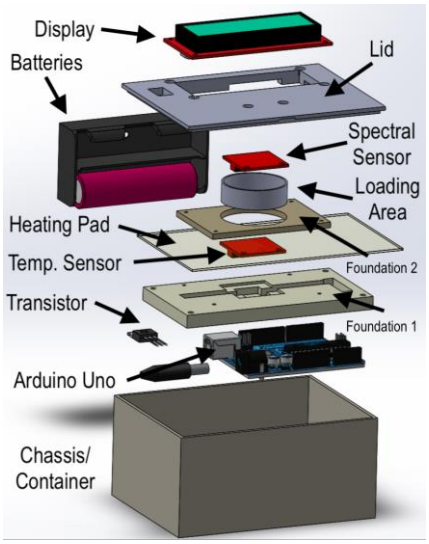

FIGURE 3: The prototype of the newly fabricated biosensor using CAD software. (Exploded View)

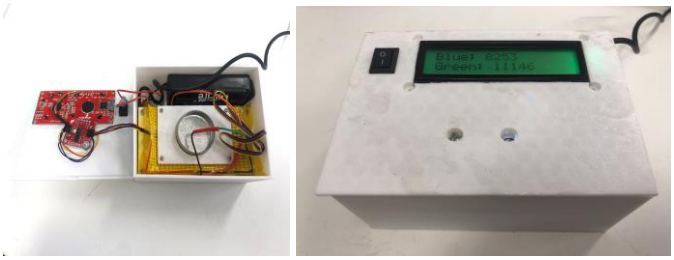

FIGURE 4: The biosensor prototype (Top: Outside view of the biosensor /Bottom Image: Inside view of the biosensor)

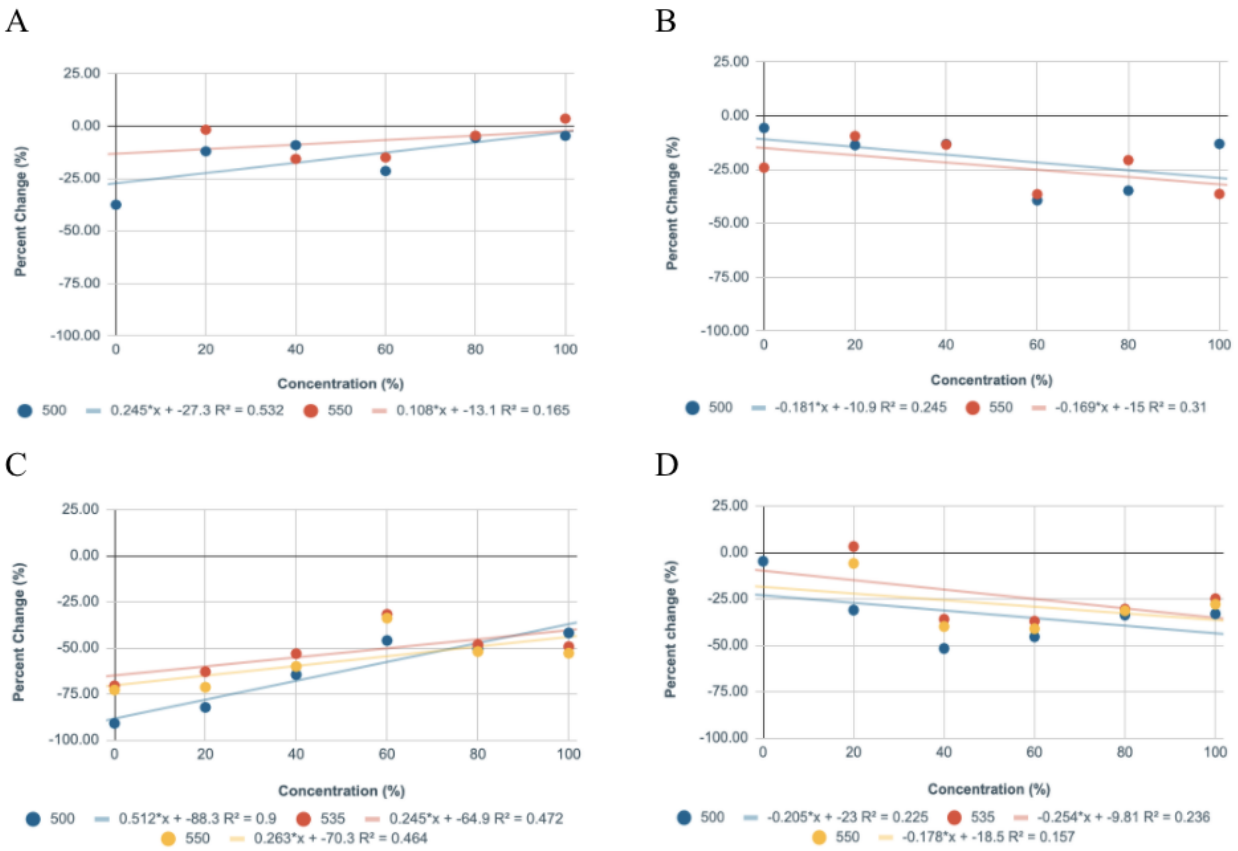

FIGURE 5: Graphs of the relationships/standard curves between the concentration level of polygalacturonase enzyme and the percentage change between the initial and final readings. (Graph A: Biosensor 2.5 uL / Graph B: Biosensor 5 uL / Graph C: Spectrophotometer 2.5 uL / Graph D: Spectrophotometer 5 uL) (Spectrophotometer acted as a control and was compared with the results of the biosensor) (100% Concentration means the solution solely consists of the polygalacturonase enzyme)

RESULTS

Important: (no significant differences between the two prototypes)

- The first prototype is the version where the biosensor lacked an overall chassis and consistent light source. Furthermore, manual programming was required.
- The second prototype is the version where the biosensor includes the overall container and consistent light source. In other terms, it is self-sustaining and no manual programming is required.

a. Analysis of Data of the Biosensor and Spectrophotometer Independently

In the experiment, the biosensor measured the reflected light from the liquid solution and the spectrophotometer measured the absorbance value. The optimum wavelength for PG and PGA-RR complex activity was 535 nm (Choi et al., 2020) but because the spectral sensor we incorporated in our biosensor can only measure 500 or 550 nm, results possibly have varied. In Graph A, it is evident that there is a positive correlation between the concentration and the percent change of reflected light. On the other hand, in Graph B, there is a negative relationship between the concentration and the percent change of reflected light. The positive relationship displayed in Graph A, possibly implies that that 2.5uL of PGA-RR dye was not enough for the biosensor to detect the certain wavelength. This resulted in the percent change, in other words, the amount of color the biosensor detected decreased. In Graph C, a positive relationship can be seen between the concentration and the percent change in absorbance. Furthermore, in Graph D, a negative correlation is evident. It can be theorized that similar to the biosensor's data, the 2.5 uL was probably not enough for the spectrophotometer to detect the absorbance.

b. Analysis of Data Combined

In both analyses, it is apparent that the standard curve's visible trends are consistent in terms of the overall positive and negative relationship between the biosensor and the spectrophotometer. After the heating, the reactions were catalyzed and the samples with greater enzyme concentrations displayed higher percent changes. Moreover, the spectrophotometer captured a greater percent change than the biosensor. In general, the consistency in the trends is highly ideal because the spectrophotometer is capable of detecting this form of activity in more detail due to its augmented sensors compared to the 3D printed and relatively cheap biosensor. As a result, it is reasonable that the spectrophotometer was capable of attaining a greater percent change than the biosensor. However, when contemplating the technology and the materials incorporated in the biosensor and the idea of portability, the augmented biosensor does a great job in achieving these goals with limited resources.

II. Prototype After Complete Fabrication and Design

Overall, the PGA-RR biosensor was fabricated for increased portability and effectiveness. Compared to the first biosensor, this new version can detect PG and PGIP activity in the field and on the spot with necessary materials. Although there is significant room for improvement, the biosensor is capable of conducting the whole process: inserting the plant sample, reacting it with the complex, detecting the reflected light through the spectral sensor, and displaying that data on the LED display. Moreover, the new version includes a concrete structure that holds all the components into one. Regarding results, the biosensor's output is the same as the past prototype (reflected light at wavelengths of 500 nm and 550 nm). However, for the results presented below, chromatography paper was used for improved visual data and results (FIGURE 4). After the reflected light from the solution was gathered, different concentrations of the solution reacted with the chromatography paper. Furthermore, for experimental purposes, we also tested with a wavelength of 650 nm.

a. Visual Data from Chromatography Papers

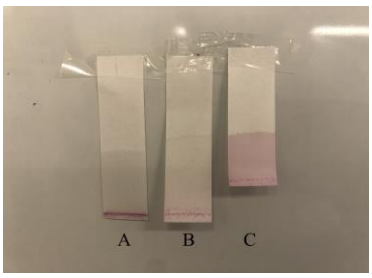

FIGURE 6: Image of different chromatography papers with varying enzyme concentrations and PGA-RR complex. (A: 0% enzyme concentration / B: 20% enzyme concentration / C: 50% enzyme concentration).

In FIGURE 6, it is evident that as the enzyme concentration increases, the color of the solution on the chromatography paper darkens. This darkening of the solution suggests that the biosensor is capable of detecting the increase in enzyme concentration, which means the existence of PG activity can be detected by this new biosensor prototype. Furthermore, because these results can be visually seen too, consumers can have a clear understanding of the existence of PG activity, which directly correlates to the existence of *Fusarium.*

b. Data from Chromatography Papers

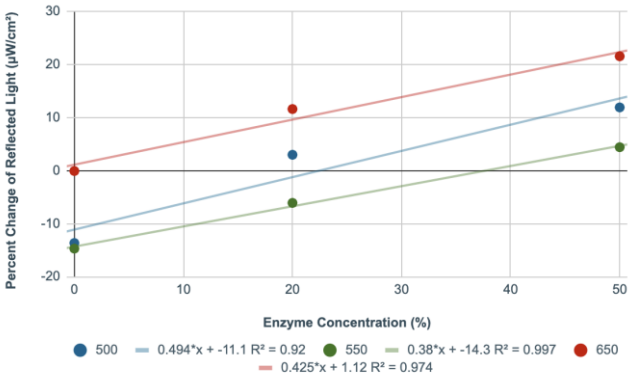

FIGURE 7: Graph of the relationship between the concentration level of polygalacturonase enzyme and the percent change of reflected light from the chromatography paper.

After using chromatography paper to visually see the difference in enzyme concentrations, the new biosensor was used to detect the reflected light from the papers. There is a positive correlation between the three different concentrations with most percent changes starting from the negative side and ending with positive values. The wavelength of 650 nm displayed a strong positive relationship comparable to the 500 and 550 nm. This may be the case because the solution's color itself was similar to the 650 nm's red color. The direct relationship visible in the graph suggests that the biosensor is capable of detecting the existence of PG activity and the breakdown between the complex and PG. These results perfectly match with the visual data from the chromatography paper and the percent change data from the first experiment, strengthening the effectivity of the new prototype.

CONCLUSION AND FUTURE OPTIMIZATION

Optimization of the Biosensor Prototype

With limited time and resources, creating a biosensor that is capable of detecting reflected light through ruthenium red was difficult. Although the final product was able to accomplish the established goals, optimizations and improvements can be made to enhance the biosensor as a

whole. First, better sensors and electronic signals with enhanced capabilities may have provided more accurate results. For example, the constant output from the spectral sensor involved drastic changes in the values, which made it difficult to attain accurate and precise data. In simpler terms, the spectral sensor was too sensitive that it made it difficult to attain constant readings. Second, it was difficult to claim with confidence that the values obtained from the spectral sensor were solely from the PGA-RR complex and the plant sample because the sensing area of the sensor is relatively unknown. In the future, the implementation of a sensor that has a clear indication of the sensing area may enhance the quality of future results.

Optimization of Both Experiments

The experiment itself involved a few errors and inconsistencies; therefore, optimizations can be made. First, an increased number of trials would have created more reliable data. When the number of trials increases, trends in data become more apparent, and numerical uncertainties can be created. Second, the inconsistent lighting environment in the first experiment with the early prototype may have negatively affected the data. Taking into consideration of the spectral sensor's extreme sensitivity and the output of the spectral sensor, consistent lighting is imperative. However, in the experiment, the distance from the emission of light to the sample varied every time. This can be fixed with a set distance from the light source to the sample, consistent light source, and constant lighting environment. Most of these attributes were tackled in the second version of the biosensor but improvements can still be made.

Conclusion and Discussion

This research involved the creation of a ruthenium-red based biosensor that detects fusarium in plant samples such as Gerbera. The biosensor we created mainly focused on portability, applicability, safety, and effectiveness, and taking into consideration the limited resources and time, the biosensor was relatively successful. From the first experiment, it is apparent that as the concentration of the PG enzyme increases, the percentage change between the initial and final readings increased. In the second experiment, as the concentration of PG enzyme increased, the solution darkened and the amount of reflected light at a certain wavelength increased drastically. These results suggest that the biosensor is capable of detecting more *Fusarium* and PG activity if the quantity of the sample is generally larger. Although we did not have the time to test the biosensor on Gerbera that contains *Fusarium* or PG in general, from the results that we have gained, this enhanced biosensor can accomplish this. The field of bioengineering and biotechnology is constantly advancing. Farmers need an efficient way of detecting *Fusarium* in their crops and they can possibly utilize these kinds of biosensors to solve this underlying problem.

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