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Root Phenotyping of Peptide-Treated Glycine max

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Root Phenotyping of Peptide-Treated *Glycine max*

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Abstract

Plant elicitor peptides (Peps) – endogenous chains of amino acids involved in natural plant defense – have been shown to decrease damage from herbivores and pathogens by inducing an immune response, increasing the emission of volatile organic compounds (VOCS), transcripts, and metabolites. Exogenous treatment of soybean seeds with plant elicitor peptide GmPep3 has been shown to induce these broad-spectrum defenses and offers a new method for increasing crop yield. However, the effects of GmPep3 on indicators of soybean health – root characteristics, growth stages, etc. – have not been fully realized.

Using the root-phenotyping platform RhizoVision Explorer, several root traits of soybean plants treated with GmPep3 were analyzed to determine whether there was a statistically significant difference between the roots of plants treated with peptide and without peptide. These root traits included total number of root tips, total root length (mm), and surface area (mm^2). Two concentrations of peptide were studied as well to determine whether increasing or decreasing the amount of GmPep3 would have an effect on chosen root characteristics.

Results indicated that there did not appear to be a statistically significant difference in the number of root tips between plants treated with GmPep3 and those not treated with GmPep3. There were, however, observed differences in total root length and surface area between treated and untreated seeds during one repetition of the experiment. However, these differences were no longer statistically significant by the end of the experimental period, indicating that although plant growth was initially impacted by the addition of the peptide treatment, these effects were no longer present by the end of the growing period. Increasing the concentration of GmPep3 did not appear to have a significant impact on root growth. Future studies with a larger sample size,

longer growing period, more varied root characteristics, and different concentrations of GmPep3 are necessary to uphold the hypothesis that GmPep3 does not negatively impact soybean root growth.

Introduction

The soybean plant, or *Glycine max,* is a fundamental crop worldwide, increasing in demand each year. According to the USDA, 86 million acres of soybean were harvested in the United States in 2021 alone, representing a 57-billion-dollar industry (USDA, 2021). Due to its high nutritional value and versatility, many experts believe it may be the key to alleviating world hunger. However, several biotic and abiotic factors threaten the staple crop each year, including temperature, access to nutrients, and diseases. One of the greatest threats are pests, leading to drastically decreased yields in the top three soybean-producing countries – USA, Argentina, and Brazil (Hartman, 2011). The soybean cyst nematode (SCN), for instance, accounts for approximately 30% of this annual yield loss, devastating roughly 20,000,000 bushels between 2010 and 2014 in Arkansas alone (Allen, 2017). Furthermore, SCNs can survive underground for extended periods of time, undermining the success of crop rotation once fully established (Jones, 2013). Root knot nematodes (RKN) are also of particular concern in the southern United States. In 2016, studies indicated that 82% of Arkansas soybean cultivars were susceptible to RKNs while only 18% had moderate to total resistance (Ross, 2016).

A combination of several approaches is typically used to counter pest-related yield losses. Pesticides are one of the most common management tools, increasing in use from the 1960s to the 1980s, and leading to increased yields worldwide. However, this approach has raised concerns regarding human and environmental health (Coupe, 2015). Integrated pest management (IPM) is another common approach, combining pesticide use with more sustainable practices to decrease negative impacts on human and environmental health. IPM allows for a certain level of plant damage so long as overall yields remain unaffected (Bueno, 2013). However, small-scale farms in rural areas often lack the appropriate technical support and knowledge to implement

these changes to the necessary degree (Grasswitz, 2019). In the 1990s, genetically modified (GM) crops came to the forefront of pest management, leading to the production of cultivars that are herbicide-resistant or internally protected against herbivory (Coupe, 2015). Concerns regarding this method include nutrient deficiencies in GM crops, although these concerns are not supported by scientific study. Clearly, the issue of pest control in crops has yet to be resolved and new technologies must be implemented to avoid decreased yields.

Induced plant defenses may be an additional tool in reducing yield loss. This term refers to the ways in which plants naturally defend them themselves against herbivory, without the use of pesticide. These defenses may include the accumulation of toxins, antidigestive proteins, and antifeedants on the surface of the plant after tissue has been damaged by feeding (Skibbe, 2008). For instance, the potato species *Solanum tuberosum* expresses cystine proteinase once feeding begins, which deters its thrip predator *F. occidentalis (*Steenbergen, 2018). This form of protection may also involve countering the negative effects of herbivory, including increased growth, photosynthetic rates, and nutrient uptake (Moreira, 2015). Finally, induced defenses may attract the predator of the specific herbivore, thereby indirectly reducing plant tissue damage (Skibbe, 2008). For example, the infestation of the tomato plant with spider mites leads to a volatile production that attracts the predatory mite, *Phytoseiulus persimilis* (Kant, 2004). Research has shown that the jasmonate (JA)-dependent signaling cascade is responsible for the execution of these broad-spectrum defenses (Skibbe, 2008).

Effectors released by the specific herbivore into the wound site activate these changes through transcriptional modification, phytohormone signaling, and posttranslational protein changes. Transcription factors and secondary metabolites are a common type of effector, and a

number have been identified. The parsnip, for example, produces a toxic secondary metabolite that reduces predatory webworm performance (Pappas, 2017).

Plant elicitor peptides (Peps) – endogenous chains of amino – are a type of signal involved in induced plant defense. Initially discovered in *Arabidopsis*, plant elicitor peptides correlated to an induced immune response, increasing the emission of volatile organic compounds (VOCS), transcripts, and metabolites – all involved in plant defense against pathogens and herbivory (Huffaker, 2015). Three soybean Peps (GmPep1, GmPep2, GmPep3) have since been isolated and developed into an exogenous seed treatment. When expressed, these genes have been shown to decrease nematode reproduction by approximately 40% to 70% (Lee, 2018).

Although previous research indicates the ability of soybean Peps to induce nematode resistance, the tradeoffs are not entirely understood. Although the biomass of soybean roots and shoots treated with GmPep have been studied, other indicators of soybean health – root characteristics, growth stages, etc. – have not been fully realized (Lee, 2018). There is a possibility that peptide treatments may have a negative impact on soybean root growth. A study in 2020 showed decreased *Arabidopsis* root growth due to interaction with receptor kinases PEPR; similar interactions may occur in soybean and reduce root growth (Shen, 2019).

Image-based phenotyping is a burgeoning field that attempts to standardize plant traits in an efficient and quantitative manner. In this method, several plant images are taken and run through a program that extracts the desired data. Image-based phenotyping is particularly useful because it allows for the possibility of non-destructive sampling and thus longitudinal data collection, as well as the ability to extract a large amount of data and increase statistical power. A study in 2013 used image-based phenotyping to study the relationship between phosphate

deficiency in soil and *Brassica* root architecture using the program ImageJ, showing a strong correlation between the two variables (Shi, 2013).

Rhizovision Explorer is a new software designed for image-based phenotyping of roots. It allows researchers to extract several root characteristics – length, diameter, volume, etc. – from images taken from a scanner. Rhizovision Explorer is unique due to the implementation of several techniques that allow for more accurate data extraction, including the ability to choose a precise region of interest (ROI), filter out non-root objects, and fill in holes in roots. The overall goal of the program is to standardize root data across fields of study and allow for increased data extraction from root images (Seethepali, 2021).

This study aimed to determine whether the addition of plant elicitor peptide GmPep3 to *Glycine max* seeds would result in a statistically significant difference in root growth using Rhizovision Explorer as the medium for root data extraction, focusing specifically on the total number of root tips, total root length (mm), and surface area (mm^2) as indicators of root growth. Decreased root growth in plants treated with GmPep3 would indicate the possibility that Peps involve trade-offs in plant health while protecting against herbivory and pathogen invasion.

Materials and Methods

Imbibition

General procedures regarding imbibing soybean seeds with GmPep3 were obtained from a previous study ("Plant elicitor peptides promote plant defense against nematodes in soybean," in *Molecular Pathology*, 2018*). In vitro* synthesis of the 23 amino-acid peptide GmPep3 (PSHGSVGGKRGSPISQGKGGQHN) was performed by Biomatik Corporation (Cambridge, ON, Canada), and purity was verified by C18 high-performance liquid chromatography (HPLC) and mass spectrometry. Sixty soybean seeds (*Glycine max*, cv Lee), twenty per treatment group, were imbibed in Petri dishes at room temperature (24° C) for eight hours in a solution of 0.1% Tween 20 and 1 µM or 4 µM of GmPep3. Control seeds were imbibed in water and Tween 20 only. Petri dishes were covered with aluminum foil during imbibition to simulate natural the germination process.

Plant Growth

To ensure the experimental results would be compatible with future nematode assays, procedures regarding plant growth were also obtained from the same study ("Plant elicitor peptides promote plant defense against nematodes in soybean," in *Molecular Pathology*, 2018). After imbibition, seeds were transferred to the greenhouse and grown under standard greenhouse conditions (16-h light/8-h dark photoperiod, 21–27 °C) in Sunshine Mix for approximately 72 hours until germination was complete. Seedlings were then transferred to autoclaved sandy loam in 8 oz Styrofoam pots with eight small punctures at the base to ensure proper drainage. Plants were watered daily by hand.

Root Scanning

Three days after transferring to sandy loam, 1/3 of the plants from each treatment group were removed from the Styrofoam cups and their roots were washed thoroughly to remove soil and debris. Plants were then placed on Epson scanner tray and their roots were manually spread to ensure adequate visualization could be achieved. The number of roots visualized at once varied based on the size of the root; it was ensured that no overlap occurred between different plant roots. JPEG images at 300 dpi resolution of roots were produced and roots were discarded after visualization. This process was repeated six days after germination and nine days after germination until all plant roots had been scanned.

Root Phenotyping

Guidance regarding root-phenotyping was obtained from a previous study ("RhizoVision Explorer: open-source software for root image analysis and measurement standardization" in *AoB Plants*, 2021). Root images were analyzed using open-source software RhizoVision Explorer. A Region of Interest (ROI) was drawn around each root, beginning at the soil line and ending at the root cap. Image pre-processing consisted of the following standardized parameters: whole-root analysis mode, converting pixels to physical units, image-thresholding level of 200, 'filter non-root objects' and 'fill holes in root objects' both set to 5. Color was inverted to ensure adequate visualization of the root system. Skeletonized versions of each root were then produced by the program. Forty quantitative traits were extracted from the skeletonized images. The three root traits of interest in this study were number of root tips, total length (mm), and surface area (mm^2).

Statistical Analysis and Graphing

All experiments were analyzed using JMP Genomics Pro 16 (SAS Institute, Cary, NC, USA). Data sets were first tested for equal variance and then one-way ANOVAs were performed to identify differences in the treatment groups between the three root traits of interest. Box plots were also created in JMP to display the total data collected. If statistically different at $\alpha = 0.05$, means separations were performed with a Tukey HSD test and displayed on the box plots.

Repetition

The procedures previously described were repeated three times, designated as Experiments 1-3 in the remainder of this study. For each repetition, root images were labeled as Day 3, Day 6, or Day 9 (days since germination) to indicate the time point the root image was taken.

Results

Images

Using RhizoVision Explorer, skeletonized images of each root were produced. An example of an output image is represented in **Figure 1**. A visual comparison of the skeletonized images from Experiment 1 are shown in **Figure 2** using the first root scanned in each treatment group on Day 3, Day 6, or Day 9.

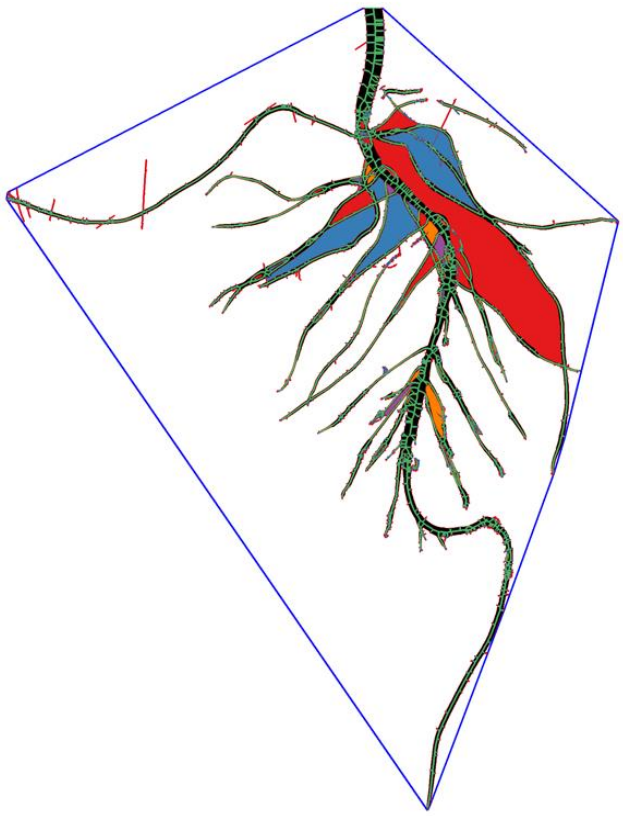


Figure 1. RhizoVision Explorer analysis of root image from Experiment 1, Day 3, Control group. Total root length (mm) and surface area (mm^2) are calculated using green lines; other colors correspond to different root characteristics not studied in this experiment.

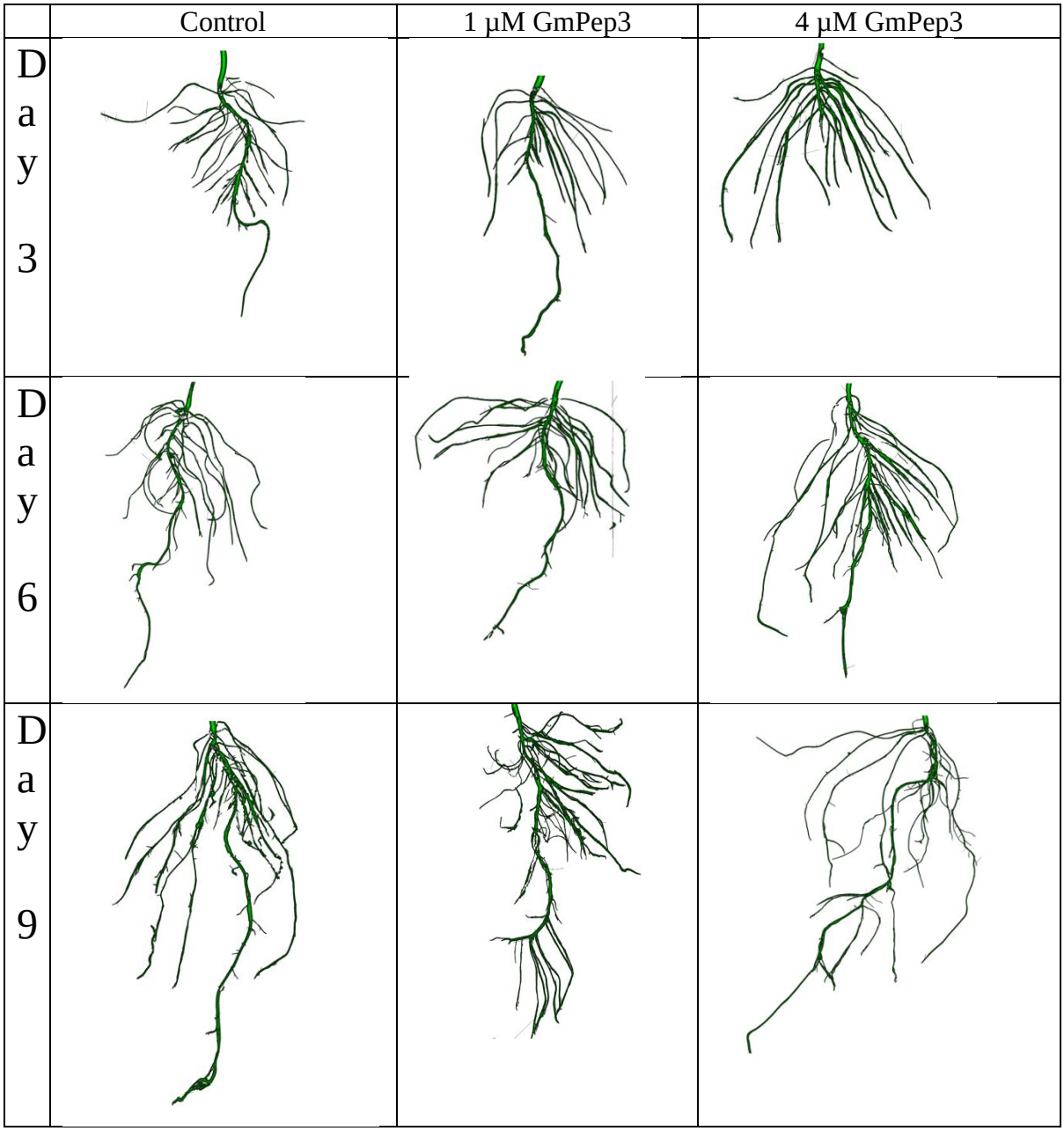
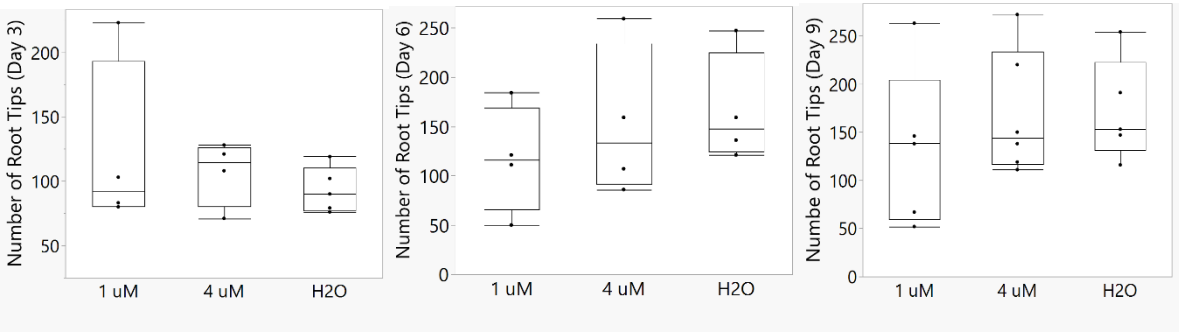


Figure 2. Output images from Rhizovision Explorer of plants treated with 1 µM GmPep3, 4 µM GmPep3, and no GmPep3 over the course of Experiment 1.

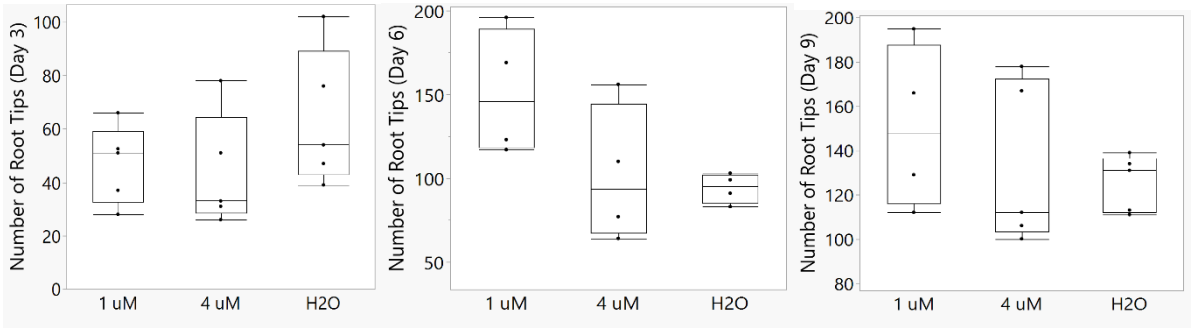
Root Tips

After three repetitions of the described experimental design, the number of root tips were not observed to significantly differ across the three treatment groups. One-way ANOVA testing demonstrated that there was not a statistically significant difference in the number of root tips on plants treated with water, 1 µM GmPep3, or 4 µM GmPep3 ($df = 2$, $p > 0.05$). Box plots were created to display the data (**Figure 3**).

Experiment 1



Experiment 2



Experiment 3

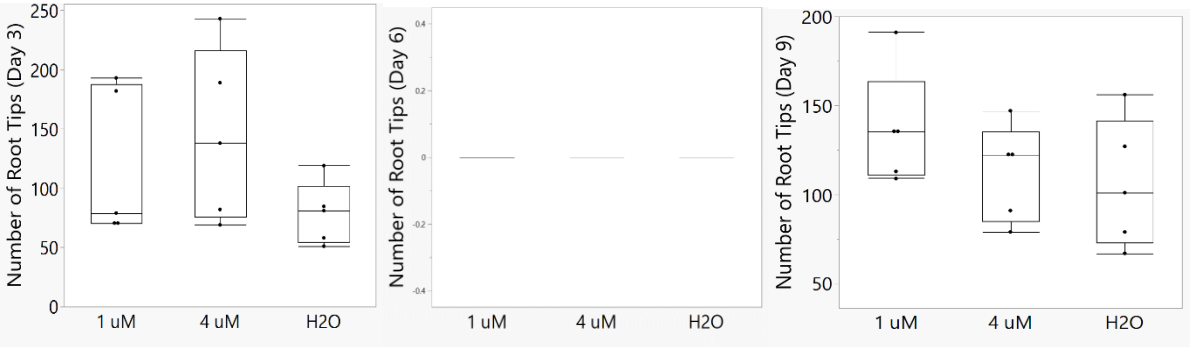
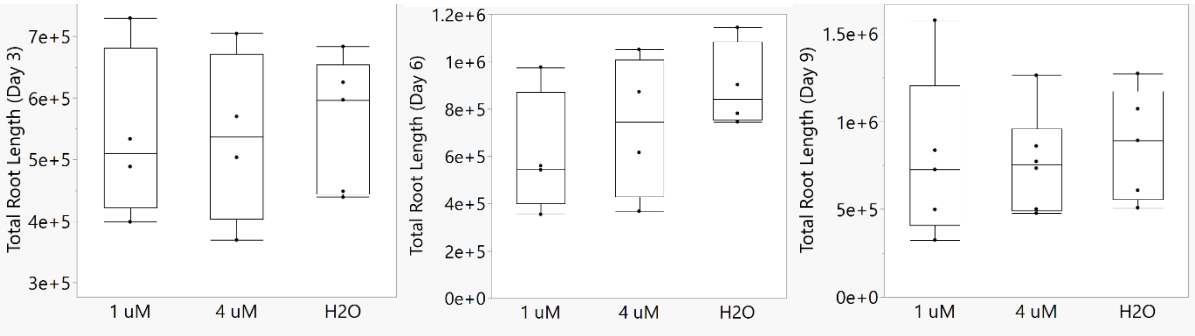


Figure 3. Effects of peptide treatment on number of root tips for Experiments 1-3

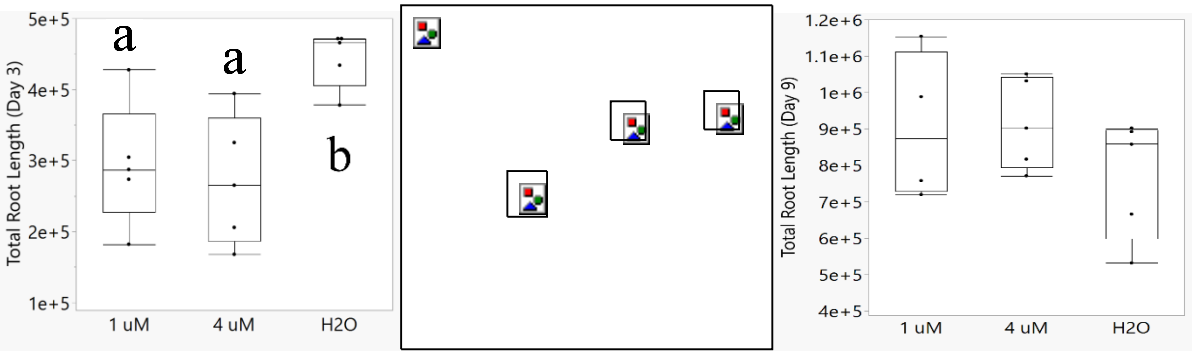
Root Length

For experiments 1 and 3, one-way ANOVA testing demonstrated that there was not a statistically significant difference in the total root length between the treatment groups ($df = 2$, $p > 0.05$). For experiment 2, ANOVA testing indicated a significant difference in root length on Day 3 ($df = 2$, $F = 7.4393$; $p > 0.0079$) and Day 6 ($df = 2$, $F = 9.6703$, $p > 0.0057$). If statistically different at $\alpha = 0.05$, means separations were performed with a Tukey HSD test. By Day 9, no statistically significant difference was detected ($df = 2$, $p > 0.05$). Box plots were created the display the data (**Figure 4**). On the graphical display, **a** and **b** are used to indicate significant difference between treatment groups. If no letters are present, no significant difference is assumed.

Experiment 1



Experiment 2



Experiment 3

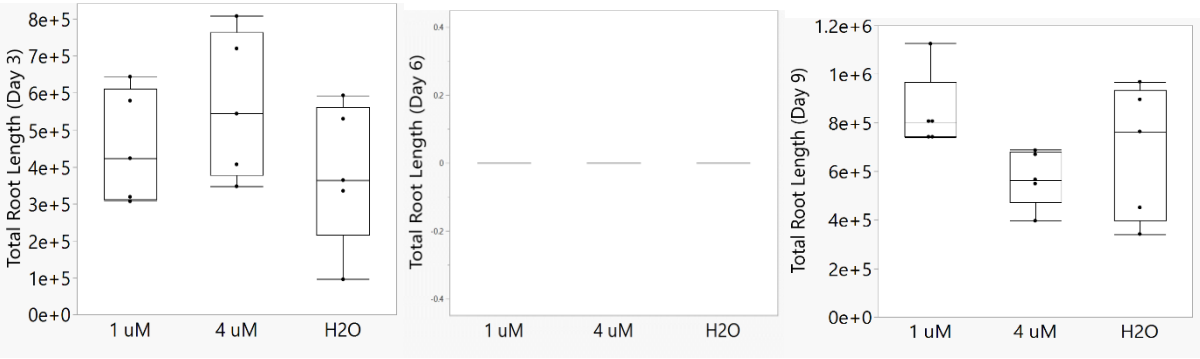
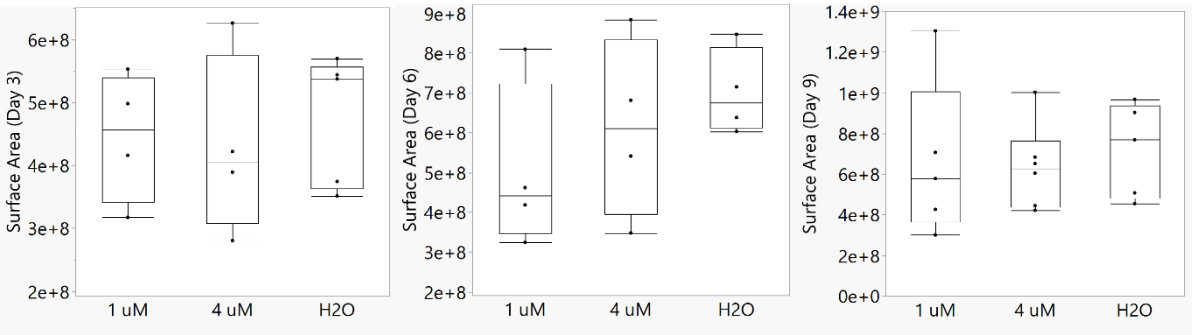


Figure 4. Effects of peptide treatment on total root length (mm) for Experiments 1-3.

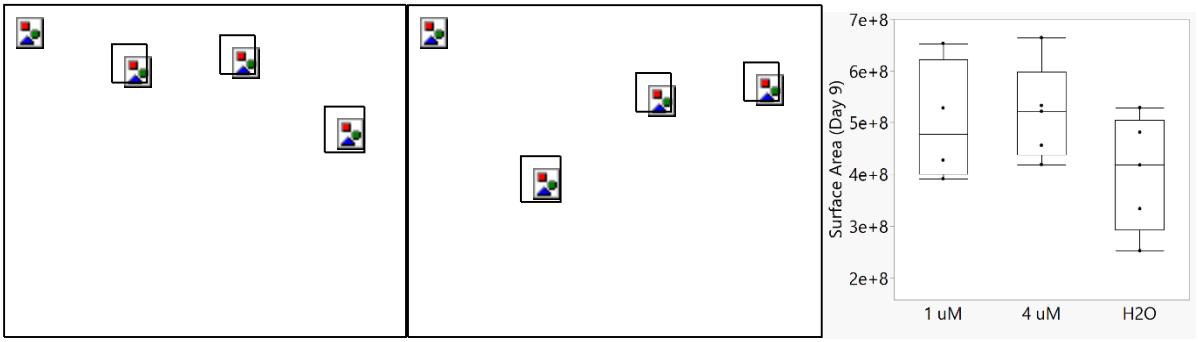
Surface Area

For experiments 1 and 3, one-way ANOVA testing demonstrated that there was not a statistically significant difference in the total surface area (mm^2) between the treatment groups ($df = 2$, $p > 0.05$). For experiment 2, ANOVA testing indicated a significant difference in surface area on Day 3 ($df = 2$; $F = 9.3066$; $p > 0.0036$) and Day 6 ($df = 2$, $F = 7.7622$, $p > 0.0110$). If statistically different at $\alpha = 0.05$, means separations were performed with a Tukey HSD test. By Day 9, no statistically significant difference was detected ($df = 2$, $p > 0.05$). Box plots were created the display the data (Fig. 5). On the graphical display, **a** and **b** are used to indicate significant difference between treatment groups. If no letters are present, assume no significant difference.

Experiment 1



Experiment 2



Experiment 3

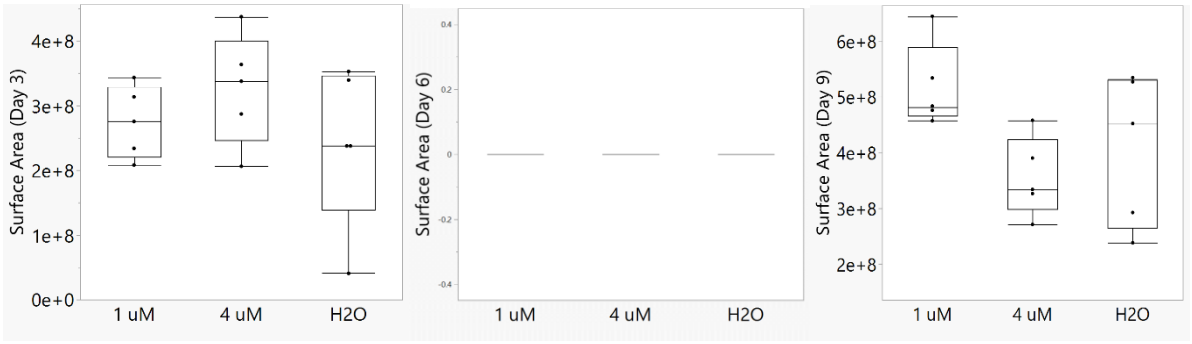


Figure 5. Effects of peptide treatment on surface area (mm^2) for Experiments 1-3.

Areas to Note

Data from Experiment 3, Day 6 was unable to be obtained due to scanner error and thus statistical analysis and graphical display are not present. It was also noted that small scratches were present on the scanner tray that were unable to be removed during image processing. These scratches can be seen in both **Figure 1** and **Figure 2** as unnaturally straight lines that do not correspond to the actual root.

Discussion

The aim of this research project was to determine whether soybean seeds treated with GmPep3 would display differences in root growth - quantified by the number of roots tips, total root length (mm), and surface area (mm^2) - compared to plants that were not treated with GmPep3. An additional question raised was whether increasing the concentration of GmPep3 would correlate to differences in root growth.

Based on the statistical tests done, there does not appear to be a significant difference in the number of root tips between treatment groups. This indicates that GmPep3 does not increase or decrease the number of root tips in soybean up to nine days after germination. This finding is consistent with **Figure 2**, which does not show a visually apparent difference in the number of root tips between the treatment groups.

Looking at total length (mm) and surface area (mm^2), two out of the three experiments indicate no statistically significant difference at any time point, although Experiment 2 indicated statistical difference at the first two time points. However, by the third time point no significant difference was observed in either total root length or surface area. This may indicate that although plant growth was initially impacted by the addition of the peptide treatment, these effects were no longer present by the end of the growing period. This is not an unexpected result, as the addition of bio activators such as peptide treatments are known to involve a metabolic cost due to the energy needed to defend against herbivory, (Gatehouse, 2002). This metabolic cost has the potential to vary expected growth in a variety of ways and could explain the leveling-out that was observed in Experiment 2. **Figure 2** does not appear to display a clear difference in total root length or surface area between treatment groups, supporting the general indication by

Experiments 1 and 3 that treating soybean seeds with GmPep3 does not significantly alter these root traits over the course of nine days.

Increasing the concentration of GmPep3 from 1 µM to 4 µM did not appear to display differences in the chosen root characteristics. Only in Experiment 2, Day 6 was there a statistically significant difference between total length and surface area between seeds treated with 1 µM peptide and 4 µM peptide. By Day 9, this difference was no longer observed. These findings are somewhat consistent with **Figure 2**, although visual comparison between 1 µM and 4 µM treated seeds on Day 9 shows a marked difference in root tips and general root fullness. This visual difference, however, does not appear to represent the data according to statistical analysis.

Unfortunately, data was unable to be obtained from Experiment 3, Day 6 due to scanner error. Due to the nature of root scanning which involved destructive sampling, these plants had to be discarded and could not be visualized. However, data from Day 3 and Day 9 of this experiment can still be compared to the other repetitions.

An area to note are the small scratches that were present on the scanner tray and could not be removed from the processed images. Although these scratches likely affected the extracted data to some degree, they appeared to be relatively uniform across the tray rather than being concentrated in specific areas. A base-level of error was thus assumed, and images were still compared to one another with the expectation that they would all be affected by the scratches to a similar degree. A new scanner tray or research into digital removal of these scratches would be beneficial in future repetitions of this experiment.

Although this study suggests that GmPep3 does not significantly alter root growth in soybean, there were several limitations. The sample size was relatively small and signals more experimentation is necessary to determine whether the data can be generalized to a larger population of soybeans treated with GmPep3. Due to time constraints and scanner size, the ability to study root growth over a long period of time was also not feasible and therefore the data cannot be used to make assumptions regarding root growth after a certain period. Furthermore, only three root parameters were chosen to represent root growth. Other key parameters – convex area to quantify the spread of the roots, average root orientation to determine the direction of growth – would also be meaningful factors to consider when studying root growth. Because total root length and surface area data appeared to display a strong positive correlation, choosing another parameter like convex area or root orientation may have provided a more wholistic view of how root growth was affected by the GmPep3 treatment.

There are several avenues this area of study could take in the future. For example, studying how roots of plants other than *Glycine max* are affected by peptide treatments would broaden the scope of the study and allow for comparison between plant species. Furthermore, it would be beneficial to better understand how concentrations of GmPep3 correlate to root growth by creating a peptide concentration gradient rather than choosing only two concentrations. Additionally, indicators of induced plant defenses could be studied in more detail. The presence of reactive oxygen species (ROS) can signify induced plant defenses and could be an avenue for determining more exact differences between plants treated with peptide and those not treated with peptide (Chen, 2020).

In conclusion, findings indicate that imbibing soybean seeds with GmPep3 does not significantly alter the total number of root tips, root length, or surface area during the initial

growth stage. This further supports the use of peptide treatments in agriculture as a tool to increase natural plant defenses and thus increase crop yield.

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