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Using Bromelain Application to Inhibit the Spread of the Tobacco Mosaic Virus and Promote Tissue Regeneration in Red Bell Pepper Plants

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Abstract

Plant viruses are a prevalent issue as they pose detrimental effects on agriculture, the food economy, and numerous individuals worldwide. One major effect of these viruses is tissue necrosis (breakdown of tissue), which plays a significant role in the decrease of crop yields. This study focused on the Tobacco Mosaic Virus (TMV), which infects over 350 plant species, causes necrotic symptoms, and exhibits a high transmission rate. Presently, various pesticides are in use that cause health issues in humans and are ineffective as some plants exhibit resistance. Moreover, this research aims to inhibit the spread of TMV in red bell pepper plants and promote tissue regeneration using an enzymatic approach since some enzymes have defense capabilities. A protease derived from pineapple, bromelain, was implemented due to its antimicrobial and anti-inflammatory properties. Bromelain was introduced to the plants as a pre-treatment before infection of TMV to analyze potential antiviral effects. Additionally, bromelain appeared to have correlations with a defensive enzyme located within plants known as phenylalanine ammonia lyase (PAL), which initiates the production of secondary metabolites. Using ImageJ software, the amount of plant tissue damage was highlighted and calculated, and a PAL assay was later conducted in conjunction with UV-Vis Spectroscopy to determine the level of PAL within the infected plants. Based on the experimental results, higher concentrations of bromelain contributed to lower levels of PAL, while lower concentrations of bromelain contributed to higher levels of PAL, indicating that higher treatment concentrations produced more antiviral activity.

Keywords Bromelain, PAL, Bell pepper plants

1. INTRODUCTION

Tissue necrosis is a common result of most diseases and occurs in numerous living organisms during or after disease introduction. Particular diseases that cause tissue damage are viral infections which overtake an organism and replicate within the host, weakening defense mechanisms and promoting conditions detrimental to overall health. A major field that experiences these negative effects is agriculture, suffering losses of approximately 30 billion dollars each year globally due to plant viruses [1] which are also responsible for about half of crop losses [2]. Additionally, plant viruses are connected to the issue of starvation in various areas of the world as they lead to lower crop yields and poorer quality of the crops produced [3]. The Tobacco Mosaic Virus (TMV) was first discovered in the late 1800s and is known to infect over 350 plant species [4]. The ability of this virus to remain active even when the plant tissue dies creates a major issue as this causes higher rates of transmission and increased levels of crop loss. In response, a variety of pesticides are constantly in use, which have been known to cause gastrointestinal, respiratory, carcinogenic, and other health issues in humans and harm from an environmental standpoint [5]. With such issues, identifying a safe, effective, and efficient way to reduce viral diseases and alleviate symptoms of tissue necrosis in plants is crucial. A possible direction is the application of enzymes to enhance defense systems within a plant during infection, which can lower the effect of disease through various counteractions [6]. The ability of enzymes to catalyze chemical reactions is necessary for most life processes, and in a situation of tissue damage due to disease, enhancing enzyme activity to optimal levels is essential for proper recovery [7].

Moreover, this research investigates the ability of bromelain, a protease derived from pineapple, to inhibit TMV within red bell pepper plants. Bromelain is currently used as a dietary supplement and anti-inflammatory treatment for humans, however, it possesses numerous antimicrobial properties that have been primarily studied against bacteria and fungi, but rarely viruses [8]. This study aims to identify the efficacy of bromelain as a pre-treatment to combat TMV and exhibit antiviral activity within the infected plants.

Furthermore, this research could reveal the antiviral capacities of bromelain, which could be beneficial in enhancing agricultural practices and serving as a potential method of biocontrol.

A particular enzyme that is being investigated in this research is phenylalanine ammonia lyase (PAL) because this compound is essential for the initiation of the phenylpropanoid pathway in plants and is responsible for the production of secondary metabolites such as lignin and phytoalexin during plant infection [9]. A major focus of this study is to identify the relationship between bromelain and the compound PAL to observe how bromelain affects PAL activity in plants during infection with TMV. Additionally, when defense-related enzymes are activated early on in plant-defense pathways, the prevention of microbial accumulations is likely [10]. Therefore, using a pre-treatment is an effective approach as plants tend to acquire disease later on, and increasing defense prior to infection is essential [6]. To test the efficacy of the bromelain pre-treatment, varying concentrations of bromelain are used to ensure which is the most optimal in producing antiviral effects. Based on the antiviral, antimicrobial, anti-inflammatory, and protease properties of bromelain, the plants will exhibit antiviral activity, and tissue will be preserved when red bell pepper plants infected with TMV are pre-treated with bromelain.

2. MATERIALS AND METHODS

Seed Planting and Germination Process Before being pre-treated with bromelain, red bell pepper plants were grown using a Jiffy Seed Starting Greenhouse kit which contains peat pellets for seed germination [Figure 1](#). To initiate the germination process, the pellets were watered with about 35 mL of water and left to rise before seed planting. In total, 24 peat pellets were used with 3 seeds in each pellet, resulting in 72 red bell pepper plants. These pellets were watered with approximately 10 mL of water as needed over two weeks.

2.1. Preliminary Bromelain Treatment

To test the effects of bromelain on red bell pepper plants before infection, a preliminary treatment was conducted in which two plants from the sample size were treated with different concentrations of bromelain. The first plant received 110 mg of



Figure 1. Beginning stages of plants in Jiffy Seed Starting kit

bromelain in a 20 mL solution of water and the second plant received 140 mg of bromelain in a 20 mL solution of water. Each concentration was administered using spray bottles and the plants were consistently watered and received adequate sunlight.

2.2. Transplanting and Plant Upkeep

After reaching adequate size, the plants were transplanted into smaller pots containing garden soil; Each plant received approximately 20-100 mL of water over a span of 7 weeks as needed and a sufficient amount of sunlight per day.

2.3. Bromelain Pre-Treatment Application

For preparation of the pre-treatment, approximately 40 mg, 80 mg, 200 mg, and 340 mg of bromelain were weighed out and placed in respective beakers containing 20 mL of spring water (these concentrations were based on previous studies that tested bromelain against bacteria and fungi). Each solution was then transferred to the corresponding spray bottles, which were swirled to ensure even distribution of the enzyme. Following this, each concentration was sprayed on the corresponding group of plants.

2.4. Inoculation

After two days, all the pre-treated plants were infected with TMV. To inoculate the plant leaves with TMV, a foam applicator was dipped in a vial of diatomaceous earth (abrasive) and dabbed on the leaf epidermis with support under the leaf using a paper towel pad [Figure 2](#). The virus was then painted over the surface of the leaf with another foam applicator and left to air-dry before rinsing with sprays of water for about 3 seconds. This process was repeated for 4 leaves on each of the 22 red bell pepper plants, including a control group that did not receive pre-treatment.

2.5. Microscope Imaging

Infected leaves from the treatment groups and the control group were collected and placed between glass plates for clear imaging. The respective samples were then aligned with the microscope camera for imaging [Figure 3](#). Next, the images were uploaded into the Las X microscope software and acquired for further analysis [Figure 4](#).

2.6. ImageJ Software Analysis



Figure 2. Treating the leaves



Figure 4. Collection of leaf samples

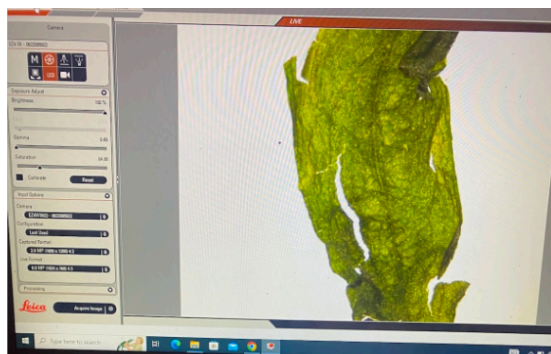


Figure 4. Alignment of leaf samples with microscope

To obtain numerical data regarding the amount of leaf damage produced, the software ImageJ was utilized. The images of the damaged leaves were first inputted into the software and were outlined to calculate the total leaf area. The color threshold settings were then changed to adjust the hue, saturation, and brightness levels to highlight the diseased regions of the leaves **Figure 5**. Lastly, the total leaf area and diseased area values were obtained in pixels and used to determine the percent tissue damage.

2.7. Phenylalanine Ammonia Lyase (PAL) Assay

The infected plant tissue was collected (approximately 0.03 g) from each treatment group along with the control group and tested to determine PAL concentrations. To begin, each sample was

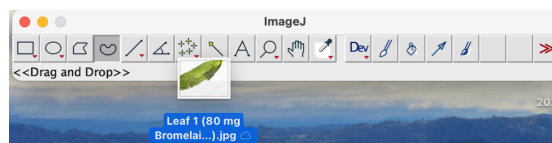


Figure 5. Importing image file into ImageJ Software

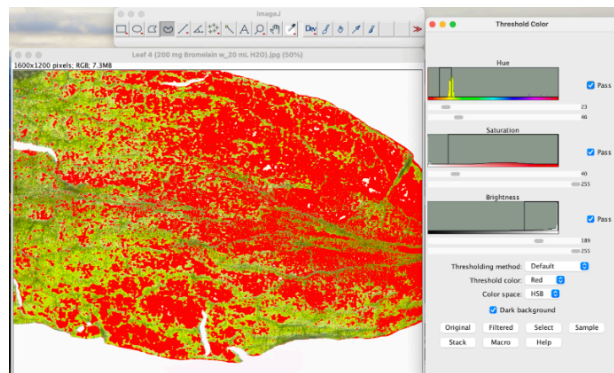


Figure 6. Adjustment of color threshold settings (i.e. hue, saturation, and brightness) to highlight tissue damage

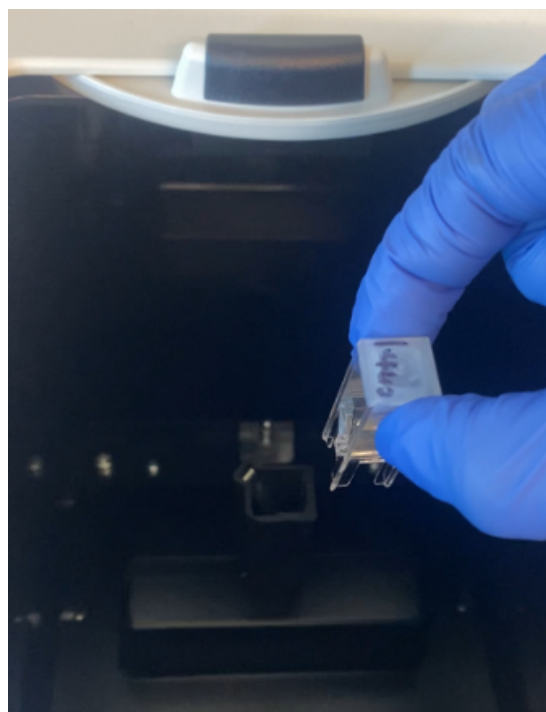


Figure 7. Transfer of sample cuvette into UV-Vis spectrophotometer

homogenized in an ice water bath and centrifuged for 10 minutes. Next, supernatant was obtained from each tube and placed in new tubes. Appropriate reagents were then added to the samples and the tubes were vortexed.

2.8. UV-Visible Spectroscopy

A 1 cm optical path along with a 290 nm wavelength was set for UV-Vis spectroscopy to calculate absorbance values. Using the samples from the PAL assay, 3 mL of each sample was transferred into cuvettes and placed in the spectrophotometer (**Figure 7**). Finally, the concentrations of PAL in the control and treatment samples were calculated.

3. RESULTS AND DISCUSSION

3.1. Preliminary Bromelain Treatment

Based on the bromelain concentrations administered to the plants (110 mg and 140 mg), it could be understood qualitatively that bromelain had no adverse effects on the plants and seemed to enhance the growing process. The treated plants had a stronger structure and were less flimsy compared to the untreated plants, indicating that bromelain most likely contributes to stronger cell walls within plant cells. In addition, the bromelain-treated plants could go longer periods without water based on increased posture that was observed, highlighting possible capacities of bromelain to promote water retention. It was also observed that the higher concentration of bromelain (140 mg) contributed to healthier outcomes qualitatively compared to the lower concentration (110 mg).

3.2. Leaf Damage Calculations

After the ImageJ processing of the infected leaves for the rest of the sample size, the total and diseased area values were obtained in

pixels. Based on the data, higher concentrations of bromelain were associated with greater leaf surface areas, while lower concentrations of bromelain contributed to lower leaf surface areas. The percent tissue damage values amongst the leaves treated with higher bromelain concentrations had larger ranges than those treated with lower bromelain concentrations, highlighting the efficacy of bromelain against TMV in higher dosages. In addition, the average percent damage was higher amongst leaves with greater bromelain concentrations (28.55% for 340 mg & 33.26% for 200 mg) and lower amongst leaves with smaller bromelain concentrations (19.11% for 80 mg & 8.46% for 40 mg), which could be attributed to uncontrolled conditions such as the varying surface areas, the amount of sunlight each plant received, the varying sizes of the leaves, and potential errors in outlining the leaves using the ImageJ software.

3.3. Phenylalanine Ammonia Lyase (PAL) Assay

Using the absorbance values from the UV-Vis Spectroscopy and the equation provided by the Elabscience PAL Assay Kit, PAL activity was calculated for each treatment group and the control. From the calculation performed, the results were directly correlated with UV-Vis spectroscopy results as there was an inverse relationship between PAL activity and bromelain concentrations; PAL activity was higher in plants treated with lower concentrations of bromelain and lower in plants treated with higher concentrations of bromelain. With this information, it could clearly be understood that bromelain exhibited antiviral activity and was effective as a pre-treatment against TMV in the infected plants, especially with higher dosages.

3.4. UV-Vis Spectroscopy

According to the results obtained by UV-Vis spectroscopy, leaves with higher concentrations of bromelain (340 mg and 200 mg) had lower PAL activity compared to leaves treated with lower bromelain concentrations based on the absorbance values.

This most likely occurred because higher bromelain concentrations contribute to increased levels of antiviral activity, decreasing the need for PAL within the plant, while lower bromelain concentrations contribute to antiviral activity on a lower scale, indicating a higher need for PAL activity. Moreover, higher

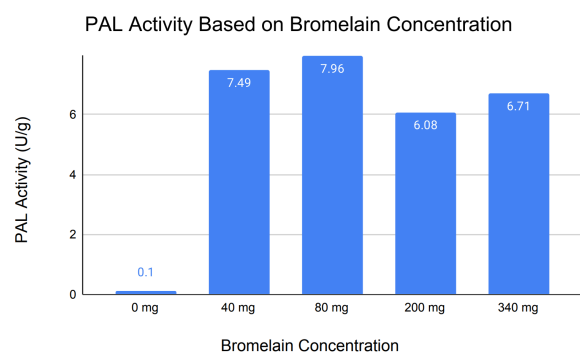


Figure 8. Based on the graph, PAL activity was higher in plants treated with lower bromelain concentrations and lower in plants treated with higher bromelain concentrations. This suggests that bromelain in higher dosages contributes to increased antiviral activity as PAL enzyme activity is not as high, while lower dosages contribute to antiviral activity on a lower scale. With little to no PAL activity in the control, bromelain overall appeared to have antiviral effects.

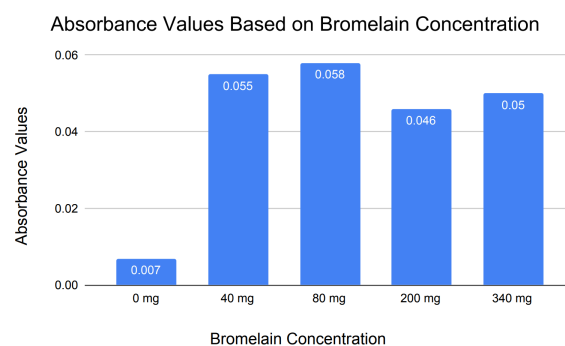


Figure 9. From the UV-Vis results, absorbance values were higher in plants treated with lower bromelain concentrations and lower in plants treated with higher bromelain concentrations. In the control, there was no significant absorbance value, which suggests bromelain's efficacy as an antiviral plant treatment.

concentrations of bromelain appeared to be more optimal in assisting plant defense mechanisms and produced greater antiviral activity as PAL levels were lower.

4. LIMITATIONS

In this experiment, there were also uncontrolled circumstances that could have affected the results. To begin, the growing conditions of the pepper plants posed variations in the leaf sizes as some plants were closer to sunlight than others, which could have affected plant tissue damage calculations. Additionally, when applying the bromelain pre-treatment, it was not ensured that all the solution reached the plants as the liquid also absorbed into the soil and touched external surfaces. Another aspect that impacted this experiment was the lack of repeated trials due to time constraints, which could have affected the conclusiveness of the results.

5. CONCLUSION

From the data obtained, the use of bromelain as a pre-treatment was effective against TMV as antiviral activity was produced within the infected plants. Moreover, this research highlights the potential of implementing defense enzymes as a mechanism against plant viruses and the capacity of natural substances to combat plant disease effectively with minimal adverse effects. With higher bromelain concentrations, lower PAL values were reflected, which indicated that these concentrations were more optimal as more antiviral activity was exhibited. This was also confirmed with the qualitative data as lower tissue damage was observed in treatment groups with higher bromelain concentrations, and higher tissue damage in treatment groups containing lower bromelain concentrations. Furthermore, this research is groundbreaking in the agricultural field as it could change the perspective of farming and positively impact many individuals worldwide since the use of this enzymatic approach offers newer potential for crop yield and presents a solution to numerous plant diseases.

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