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The Effect of Green Tea Catechins on *Solarum lycopersicum* Infected with *Agrobacterium tumefaciens*

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ABSTRACT

Agrobacterium tumefaciens is a soil-borne pathogen that causes crown-gall disease and growth stunting. Current preventative treatments are not feasible for organic or GMO-free farms. A potential natural alternative to such control methods is the use of green tea (containing epigallocatechin-3-gallate (EGCG). In this study, the effect of green tea catechins on tumor progression in *Solanum lycopersicum* (tomato plants) infected with *A. tumfaciens* was investigated. It was hypothesized that plants treated with green tea catechins would have fewer signs of oxidative stress, and smaller tumors. Tomato plants, grown from seed were watered with green tea or tap water for 9 weeks before inoculation. The control groups included: 1) plants watered with 100ml of water and inoculated with *A. tumefaciens*; 2) plants watered with 100ml water only; 3) plants watered with 100ml of green tea with an EGCG content of 119 mg. The experimental group included plants watered with green tea and inoculated with *A. tumefaciens*. After 14 weeks, a leaf Spectrometer was used to measure signs of oxidative stress and tumor size was measured. The p-value from Anthocyanin Reflectance (0.038) and Chlorophyll Content (0.020) showed a significant relationship between treatments and the interaction of treatment and infection cohorts respectively. There was no observed significance between tumor size (p= 0.13). However, these significant relationships suggest that pre-treatment with Green Tea Catechins (EGCG) is an effective Non-GMO option for preventing profit loss from *A.tumefaciens* infection. EGCG can also be considered a compound of interest for further research in human cancer treatment.

Introduction

Agrobacterium tumefaciens is a soil-borne pathogen causing the formation of “crown galls” on the stems of infected plants. It infects plants by transferring its DNA into the nucleus of the plant cell and is responsible for crown-gall disease. These tumor-like growths on the stems of plants restrict water flow and essential nutrients to the upper parts of the plant through the xylem, which stunts growth. While *A.tumefaciens* affects many woody plants, the most significant financial impact has occurred in the citrus and almond industries. For example, in 1976, Californian almond farmers lost 23 million dollars due to growth stunting because of *A.tumefaciens* infections (Kawaguchi, 2014).

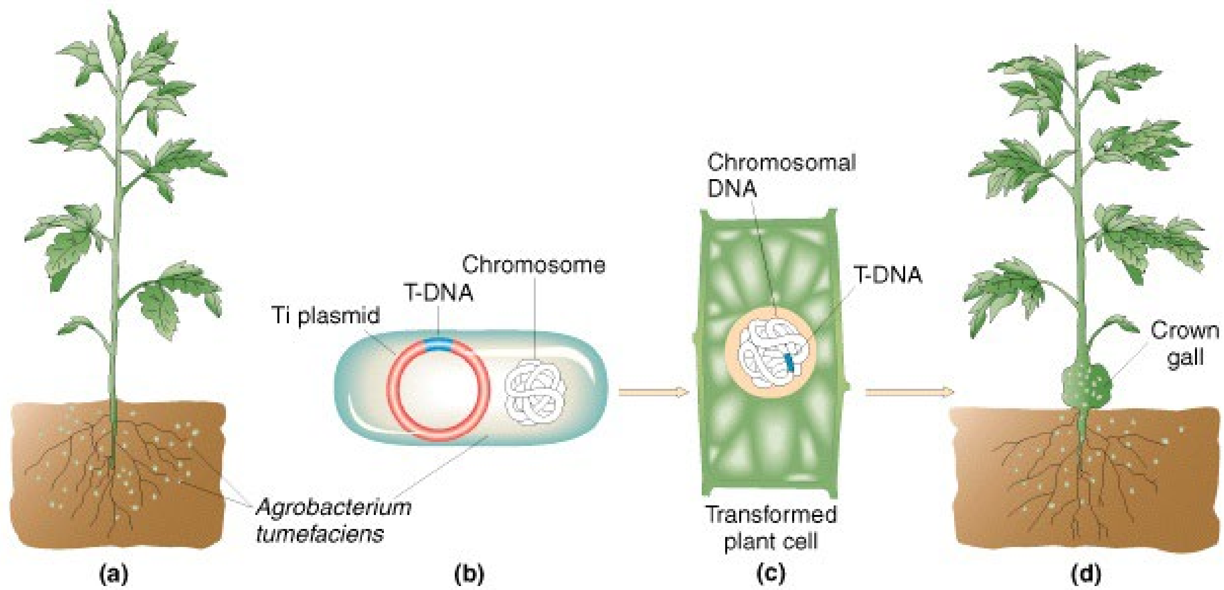

Figure 1: Pathogenic process of *A.tumefaciens* (Strong, J., & Le Blanc, Z.)

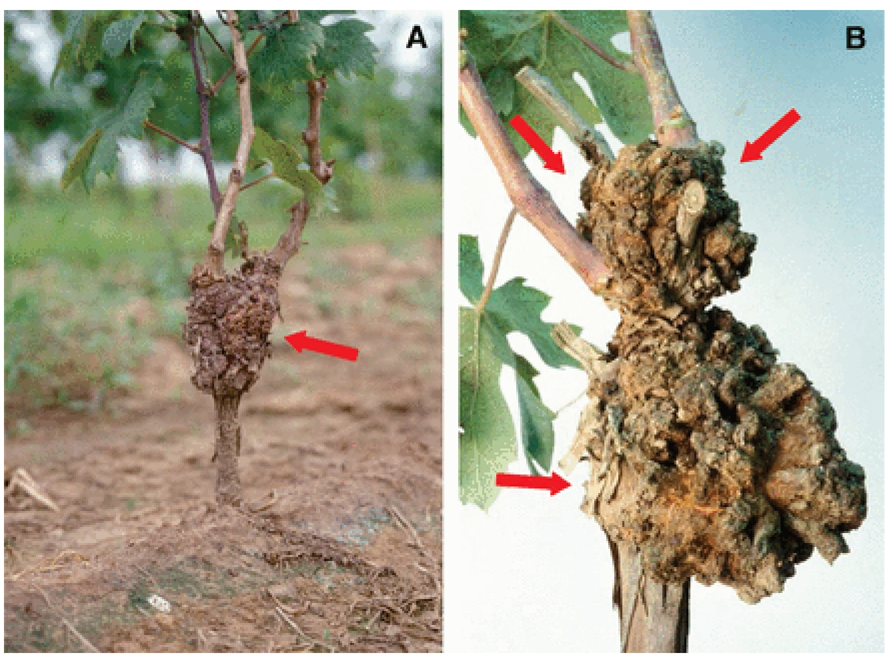

Figure 2: Crown gall formations on grapevines due to infection with *A.tumefaciens* (Filo et al., 2012)

A. tumefaciens has a distinct pathogenic process which highlights it as a useful tool in gene-environment interaction studies (Bourras et al., 2015). *A. tumefaciens* pathogenesis is prompted by the presence of phenolic compounds, which are produced by plants due to physical injury, or disease (Dai & Mumper, 2010, p.1). Virulence or "vir" proteins are part of the mechanism within *A.tumefaciens* that begins the pathogenic process. The phenolic compounds bind to the protein virA, causing the activation of protein virG. The virulence genes responsible for the production of virA and virG are constantly phosphorylated (activated), but the activation of virulence genes in the Ti plasmid of *A.tumefaciens* requires the activation of virG to be activated. This process results in the production of virD1 and virD2, which serve to cleave the T-DNA from the TI plasmid, guide this sequence into the plant cell nucleus, and integrate it into the genome. The T-DNA disrupts the regulation of the cell cycle at the G1 phase, causing increased cell division due to increased production of cytokinin and auxin. As the cells divide rapidly, they form a mass at the site of infection. This is the hallmark of *A.tumefaciens* infection, also known as crown gall disease (Bourras et al., 2015, p. 659).

Epichinagallete-3-catechin

In the agricultural industry, there are products that prevent *A.tumefaciens* infections, such as NOGALL, however, these biological control treatments work by genetically modifying the plant, ruling it out as a treatment option for organic or GMO-free farms (Kawaguchi, 2014).

The impact of green tea consumption on cancer incidence is still inconclusive, but some studies have found that among participants with no other risk factors (ie. smoking), green tea consumption was found to decrease the incidence of lung cancer (OR= 0.65, 95%, CI= 0.45-0.93 (Zhong, et.al, 2001). This highlighted green tea polyphenol as a compound of interest. According to previous research studies, green tea catechins, such as epigallocatechin gallate (EGCG), changes the epigenome of exposed cells and increase histone acetylation in tumor suppressor genes to increase their expression, and allow for reduced risk of mutations in proto-oncogenes ((Bimonte et al., 2020).

There are several polyphenolic compounds that are thought to reduce the risk of human cancers. Among these is Epigallocatechin-3-gallate (EGCG). EGCG is a component of green tea polyphenols (GTP). Both compounds' antitumorigenic properties were examined in the 2007 study "Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo". This study found that both compounds were effective both in-vitro, and in-vivo at inhibiting the growth of cancer cells, promoting apoptosis, and regulating the cell cycle at the G1 phase. These anti-tumor effects highlighted GTP and EGCG as compounds of interest in this study (Thangapazham et al., 2007). Since *A.tumefaciens* disrupts the cell cycle at the G1 phase, these compounds are of interest in possibly inhibiting this damage.

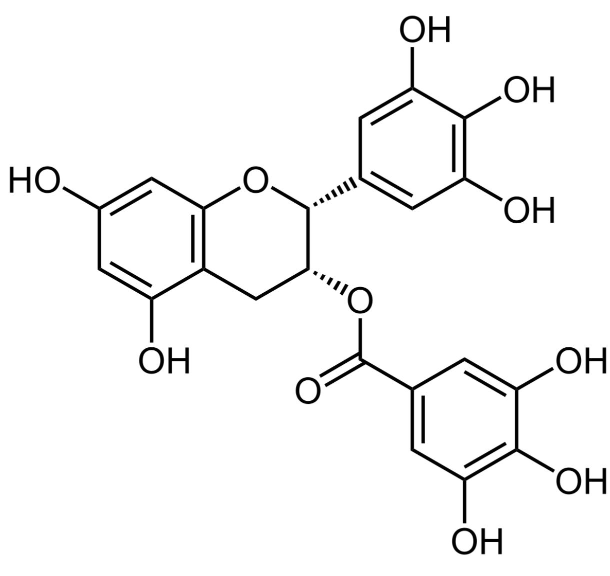

Figure 3: Chemical structure of Epigallocatechin-3-gallate or EGCG (Dettlaff et al., 2017).

In the present research study, the following question was investigated: Does chronic exposure to green tea impact the development of crown-gall tumors and associated plant stress in *Solanum lycopersicum* (tomato plants) infected with *A.tumefaciens*? It was hypothesized that plants with chronic exposure to green tea polyphenols would exhibit fewer signs of chemical stress associated with *A.tumefaciens* infection because they would limit the pathogenic process and tumor progression, therefore limiting the degree of constriction. This experiment was carried out to determine the cell regulatory capabilities of EGCG and GTP when faced with *A.tumefaciens* infection and to determine the effectiveness of pre-treatment for crops at risk from *A.tumefaciens*. Additionally, this experiment serves to model the effects of EGCG and GTP on tumor cell proliferation in humans without the use of animal or cell models. This may highlight this experiment as a preliminary study before advancing to live subjects.

Materials and Methods

Epichinagallete-3-catechin (EGCG) and Green Tea Polyphenol (GTP) derivation

The GTP was delivered to plants via Pukka® Supreme Matcha Green Tea for this experiment. This tea was chosen due to its high EGCG and GTP concentration. According to the Pukka website, this tea contains, "Sencha Green Tea (34%), Pin Ho Wild Jade green tea (32%), Indian green tea (32%), Matcha (Jeju Island Emerald) (2%)". These ingredients are high in GTP and EGCG. Korean matcha (Jeju Island Emerald Matcha) contains an EGCG of 111 mg/100 ml of tea. Indian Green Tea has an EGCG concentration of 113 mg/ 100 ml of tea. Pin Ho Wild Jade green tea contains an EGCG concentration of 123 mg/100ml of tea. Sencha Green Tea contains an EGCG of 124 mg/ 100 ml of tea (Koch et al., 2018). With these concentrations, and the ingredient percentages, it can be concluded that EGCG content of 100ml of brewed tea is 119.9 mg.

Plant Assessment Methods

As xylem restriction occurs, the plants exhibit chemical signs of stress which may be measured by a leaf spectrometer. Two of the most significant indices for measuring oxidative stress are Anthocyanin Reflectance and Chlorophyll content. Anthocyanin Reflectance Index 1 (ARI1) is used to measure stress due to a lack of resources (Moran & Moran, 1998). In plants with decreased access to nutrients and water, ARI1 will increase (Asner, G. et.al, 2004). This is valuable in the measure of the degree of severity of *A. tumefaciens* infection, as a plant's access to nutrients is restricted due to crown-gall tumors. Chlorophyll Content Index (CCI) is also a useful spectrographic tool in the analysis of oxidative plant stress. As stress increases, again due to lack of access to nutrients, the chlorophyll content also decreases. This will prove to be a useful indicator of the severity of *A. tumefaciens* infection (Mafakheri et al., 2010).

Experimental Design

Four cohorts of *Solanum lycopersicum* plants, each with 7 specimens, were created. Two cohorts were watered with 100ml of Pukka® Supreme Matcha Green Tea tri-weekly from seed. The other two cohorts were watered with 100ml of H_20 tri-weekly from seed. The 100ml dose was selected because this is the recommended water content per tea bag. Additionally, all brewing instructions were followed as per Pukka® Supreme Matcha Green Tea packaging. The plants were placed in a greenhouse with identical growing conditions, and light availability. After a growth period of 9 weeks, one green tea cohort, and one water cohort were nicked with a scalpel and inoculated with *A. tumefaciens* from Carolina® Biological via an inoculation needle. To inoculate, the inoculation needle was scraped inside the bacterial sample vial and then touched to the plant wound site for 10 seconds. This was done to replicate the infection process in orchards or farms, in which the plants undergo physical damage, and come into contact with *A. tumefaciens* at these wound sites.

The tumors were allowed to grow for 14 weeks before measurements were taken. A CI-710s SpectraVue Leaf Spectrometer was used to measure chemical signs of stress in the plants. Anthocyanin Reflectance 1, and Chlorophyll Content were the selected indices. Additionally, tumor size was measured from the two farthest points of each tumor.

CI-710s SpectraVue Leaf Spectrometer

A CI-710s SpectraVue Leaf Spectrometer was used to screen for chemical stress indicators in the plants. The Spectrometer was first calibrated using the white and black calibration strips. The spectrometer was kept on a standard wavelength setting, and calibrated to reflectance. Anthocyanin Reflectance and Chlorophyll Content were both added to the experiment "queue", and then leaves, which remained attached to each plant were evaluated.

Data Analysis

The Anthocyanin Reflectance and Chlorophyll content were both analyzed using a two-factor analysis of variance (ANOVA) using the program Excel 2016Ⓡ. Tumor size was analyzed using a single-factor ANOVA. A p-value of 0.05 was used as a cutoff to determine statistical significance. A single-factor analysis of variance (ANOVA) was used to analyze tumor size between the infected cohorts. Again, a p-value of 0.05 was used as a cutoff to determine statistical significance.

Results

Anthocyanin Reflectance

Table 1: Table organizing Anthocyanin Reflectance by treatment group and Infection Cohort

Anthocyanin Reflectance

	Infected							**Avg.**
Green Tea	0.0011	0.0038	0.0006	0.0006	0.0006	0.0031	0.0001	**0.0014**
Water	0.0006	0.0010	0.0011	0.0234	0.0235	0.0236	0.0233	**0.0138**

	Not Infected							**Avg.**
Green Tea	0.0023	0.0044	0.0011	0.0001	0.0019	0.0029	0.0011	**0.0020**
Water	0.0002	0.0008	0.0450	0.0005	0.0003	0.0004	0.0006	**0.0478**

Table 2: ANOVA results for Anthocyanin Reflectance

Source of Variation	SS	*df*	*MS*	*F*	*P-value*	*F-crit*
Sample	7.17E-05	1	7.17E-05	0.662608	0.423644	4.259677
Columns	0.000519	1	0.000519	4.801704	0.038376	4.259677
Interaction	9.88E-05	1	9.88E-05	0.913424	0.348736	4.259677
Within	0.002596	24	0.00018			
Total	0.003286	27				

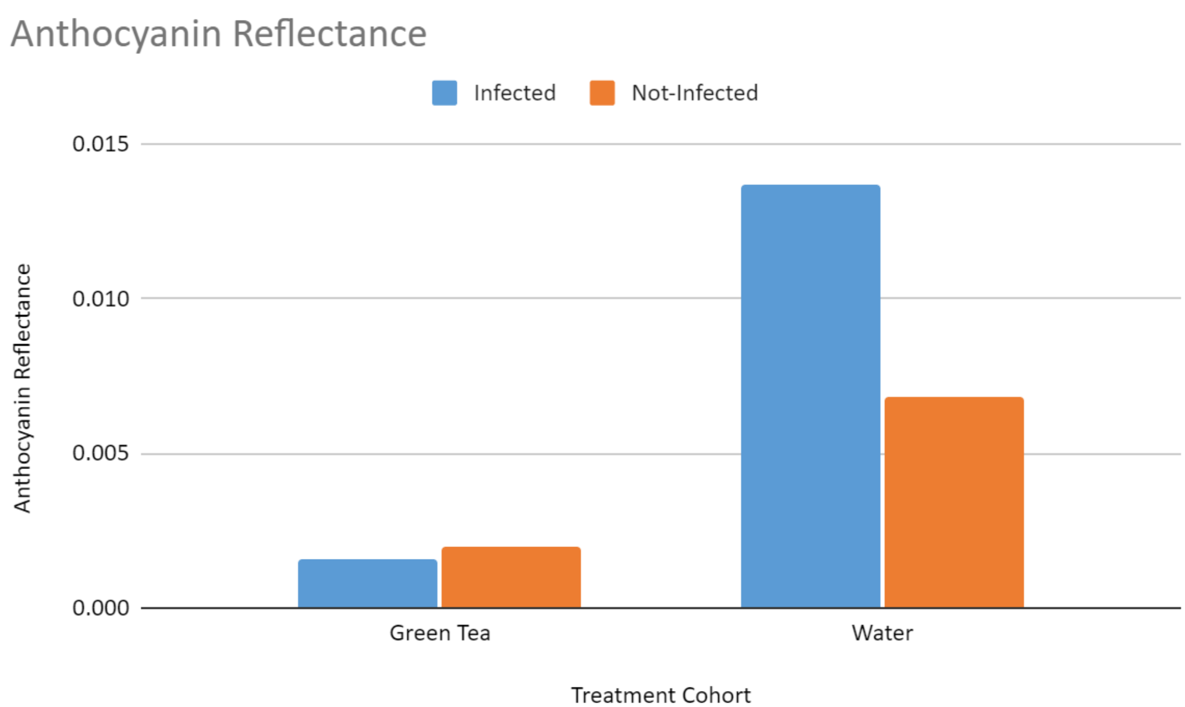

Figure 4: Bar graph showing Anthocyanin Reflectance by Treatment group and Infection Cohort

Using the Anthocyanin Reflectance values obtained via the CI-710s SpectraVue Leaf Spectrometer, a two-factor analysis of variance was performed. There was an observed statistical significance between treatment with H_2O, and PukkaⓇ Supreme Matcha Green Tea in the infected plant group (**p**=0.038). There was no empirical significance between infected and non-infected groups in the cohort that received green-tea treatment. This may be because the treatment was effective, as demonstrated by the p-value at preventing chemical stress associated with *A. tumefaciens* infection. The significance between treatment groups in the cohort infected with *A. tumefaciens* was further verified with a T-test. The resulting p-value was 0.012371, which falls under the cutoff of statistical significance of p= 0.05.

Chlorophyll Content Index

Table 3: Table organizing Chlorophyll Content by Treatment and Infection Cohort

Chlorophyll Content

	Infected							Avg	Not Infected							Avg
Green Tea Treatment	65.3	59.8	66.0	68.2	60.3	63.2	74.9	**65.4**	52.2	53.3	66.9	65.3	76.3	85.8	56.3	**65.2**
Water Treatment	73.5	57.7	68.3	5.3	5.4	5.2	5.1	**31.5**	80.1	68.6	63.4	79.9	47.2	64.8	73.7	**68.2**

Table 4: ANOVA results for Chlorophyll Content Index.

Source of Variation	SS	*df*	*MS*	*F*	*P-value*	*F-crit*
Sample	2315.041	1	2315.0414	6.5955865	0.0168725	4.2596773
Columns	1675.556	1	1675.5557	4.7736824	0.038896	4.2596773
Interaction	2373.601	1	2373.6014	6.7624248	0.0156879	4.2596773
Within	8423.966	24	350.99857			
Total	14788.16	27				

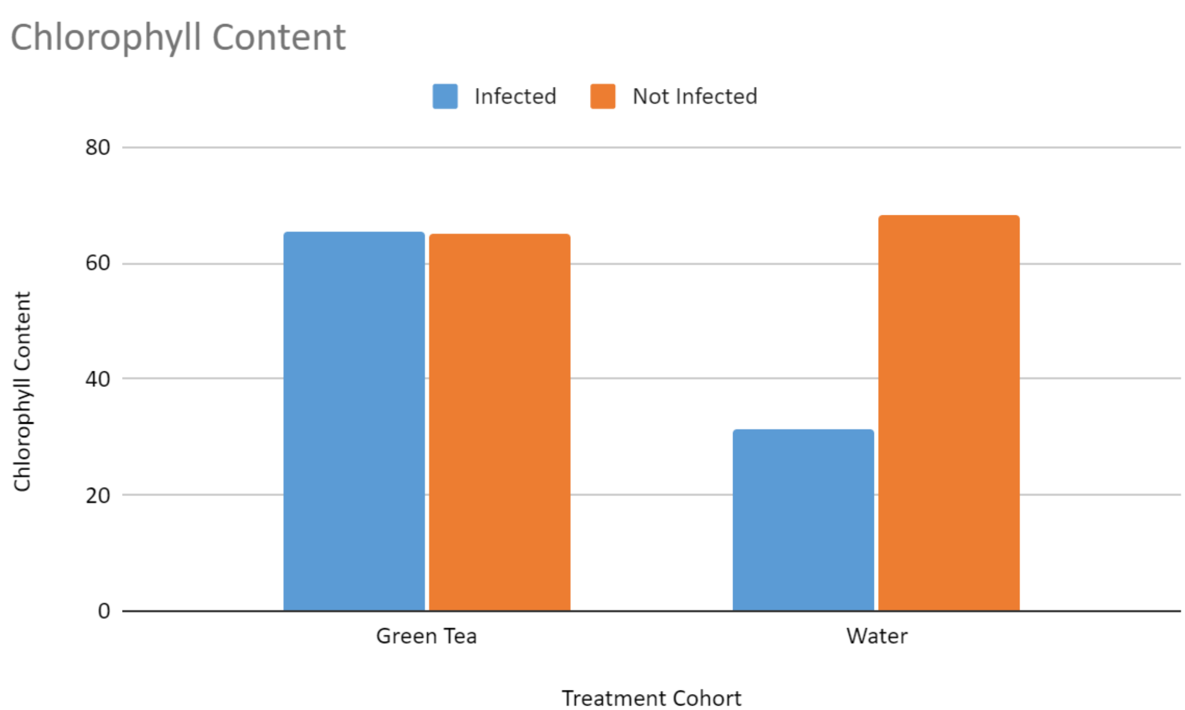

Figure 5: Bar graph showing Chlorophyll Content by Treatment group and Infection Cohort

Using Chlorophyll Content values obtained via the CI-710s SpectraVue Leaf Spectrometer, a two-factor analysis of variance was performed. There was observed significance in the interaction between infection cohorts ("Sample" p=0.017) and treatment group ("Column" p=0.039). Additionally, there was an observed significance of the interaction between the treatment cohort and the infection cohort (p=0.016). This may result from the significant effect water availability has on Chlorophyll content. The significance between treatment groups was further verified significance between treatment groups in the cohort infected with *A. tumefaciens* (p= 0.020).

Tumor Size (meters)

Table 5: Table Organizing tumor size by treatment cohort

	Infected							Avg
Green Tea	0.2100	0.5300	0.2600	0.0300	0.2657	0.2800	0.0100	**0.2265**
Water	0.6400	0.3593	0.2656	0.2656	0.2500	0.2656	0.5600	**0.3723**

Table 6: Single-factor ANOVA for tumor size

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.074387	1	0.074387	2.628848	0.1309	4.747225
Within Groups	0.339558	12	0.028296			
Total	0.413945	13				

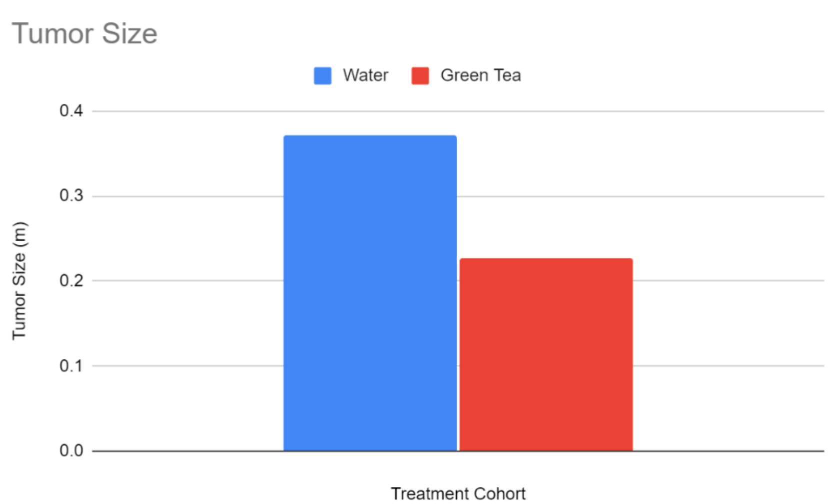

Figure 6: Bar graph demonstrating average tumor size by treatment cohort

The tumor size was measured using calipers, and a single-factor ANOVA was performed. Although the average tumor sizes were different, they ANOVA determined the difference was not significant (p= 0.13)

Discussion

This investigation was designed to determine whether chronic exposure to green tea impacts the development of crown-gall tumors and associated plant stress in *Solanum lycopersicum* plants infected with *A. tumefaciens*. The results of the two-factor ANOVA demonstrate statistical significance between both Anthocyanin content and chlorophyll content as measures of oxidative stress in the cohort infected with *A. tumefaciens*. A statistically significant t-test confirms that the difference between the content of oxidative stress, as determined by Anthocyanin and Chlorophyll content in the infected cohort is driven by treatment with either H_2O or Pukka® Supreme Matcha Green Tea. From this, it can be concluded that treatment with EGCG via Pukka® Supreme Matcha Green Tea has a significant effect on the content of oxidative stress pigments in the infected cohort. The result of the two-factor ANOVA performed found statistical significance between treatment with Pukka® Supreme Matcha Green Tea (Anthocyanin: p= 0.038, Chlorophyll Content: p= 0.039). There was no observed significance in Anthocyanin reflectance between infected and non-infected groups in the treated cohort. This may result in the effectiveness of treatment with Pukka® Supreme Matcha Green Tea in preventing oxidative stress as a result of *A. tumefaciens*. An overall limitation of this experiment was the small sample size. Additionally, the tumor size was determined to be insignificant (p=0.1309), however, due to the differences in oxidative stress associated with xylem constriction between treatment cohorts, it is possible that treated plants had similar tumor sizes, with less internal involvement, which might lead to less xylem constriction. Due to the degree of expected variability when working with plants, the small sample size of this experiment may have skewed the results.

Figure 7: Example of xylem constriction as a result of crown-gall tumor formation. The right sample demonstrates healthy xylem tissues, while the left sample demonstrates xylem constriction (Image taken by author).

Anthocyanin Reflectance

The lack of significance between infected and non-infected cohorts can further be discussed concerning Anthocyanin reflectance. Anthocyanin Reflectance values are affected by oxidative stress pigment, anthocyanin in the plant which is usually caused by nutrient deficiency. However, anthocyanin pigment concentration in plant leaves can also be affected by leaf age. To minimize error, measurements were taken on relatively similarly aged leaves, though some level of error can be expected from this method. Additionally, the normal range for Anthocyanin Reflectance in leafy green plants is 0.001 to 0.1 (Gamon, J. A., et.al, 1999). All of the Anthocyanin values in all cohorts fall within this range. This suggests that a longer period of time should be considered before data collection to promote further differentiation between cohorts.

Chlorophyll Content

The lack of significance between infected and non-infected cohorts can be further discussed concerning chlorophyll content. There was little source information regarding normal Chlorophyll content ranges, however, before inoculation, the average chlorophyll content was 87.54. After infection, most values fell below this. A decrease in chlorophyll content is a sign of plant stress. According to previous studies, a decrease in chlorophyll is most often associated with drought stress. The significant interaction between the treatment cohort and the infection cohort demonstrates that treatment with EGCG via Pukka® Supreme Matcha Green Tea is effective at limiting the effects of *A. tumefaciens*, including xylem constriction which is the most significant effect of infection.

Human Parallels

A. tumefaciens is unique in its pathogenic method and in its clinical effects. The study of crown gall tumors in plants can be an alternative method of studying uncontrolled cell proliferation that might be associated with human cancers. For example, the pathogenic method of *A. tumefaciens* is similar to that of Human Papillomavirus (HPV). Both pathogens involve the genome of host cells to cause proliferation (Reinson et al., 2015). Due to this fact, *A. tumefaciens* may serve as a useful tool in studying the effects of certain treatments or pre-treatments on the pathogenic processes of pathogens that may cause human cancers without using human cell lines.

Conclusion

Since there is a statistically significant relationship between treatment with either H_2O or Pukka® Supreme Matcha Green Tea in the cohort infected with *A. tumefaciens*, it can be concluded that the hypothesis that plants with chronic exposure to green tea polyphenols would exhibit fewer signs of chemical stress associated with *A. tumefaciens* infection is correct. Pre-treatment of crops with EGCG via Pukka® Supreme Matcha Green Tea is effective at preventing damage and profit loss. Additionally, this result furthers EGCG as a compound of interest in preventative medicine related to human cancers, especially those with pathogenic origins, such as HPV.

Further Research

This research framework can be implemented to study other dietary polyphenolic compounds, such as Quercetin, which already has a connection to anti-cancer properties in preliminary studies (Madhuri, S. et al. 2009). Additionally, the impact of chronic EGCG exposure on the spread of *A. tumefaciens* infection between plants in the same soil can be investigated.

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