Journal of
Emerging Investigators

Green Tea Extract As an Environmentally Friendly Antibacterial Agent Against *Pseudomonas syringae* pv. *tomato* on Plants

Terence Lo[1] and Bingyu Zhao[2]

[1]Veritas Home Academy

[2]Department of Horticulture, Virginia Tech, Blacksburg, VA, 24061

Summary

Green tea contains polyphenols, such as tannins and catechins, that are effective against bacteria. As it is naturally produced by plants, green tea has been explored as an environmentally friendly antibacterial product for use in human health. However, there are very few reports of green tea extract being used as an antibacterial product to protect plants from various bacterial diseases. In this study, we demonstrated that green tea extract could inhibit the growth of *Pseudomonas syringae* pv. *tomato*, the causal agent of tomato bacterial leaf spot disease, on agar plates. We also demonstrated that spraying green tea extract on *Nicotiana benthamiana* plants could inhibit the growth of *P. syringae* on plant leaves. Therefore, we conclude that green tea extract has the potential to be used as a 'green' antibacterial product in agriculture production.

Received: November 11, 2014; **Accepted:** June 9, 2015; **Published:** October 27, 2015

Introduction

Green tea originated in China many centuries ago and has been an integral part of Chinese society. Many health benefits have been attributed to the consumption of green tea, specifically the consumption of the compounds that are abundant in green tea (1,2). These are commonly known as polyphenols, which are characterized by a large amount of phenol groups attached to a group of benzene rings. Polyphenols have a wide range of uses, particularly because of their antimicrobial properties (3–5). Recently, much research has been done on green tea polyphenols and their effect on a wide range of human pathogens. They are effective growth inhibitors of bacteria and fungi (6,7). They also significantly reduce the efficacy of viruses penetrating a cell (8).

Traditionally, green tea leaves are brewed in hot water and consumed (9). However, much of the current research done on green tea compounds examines the effect of compounds identified and extracted using organic solvents (10,11). The compounds that are extracted are therefore not water-soluble. Thus far, only a few studies have used the crude water-soluble tea extract for testing its antimicrobial activity (12). Additionally, most research done on green tea polyphenols has been done on human applicants or directly on bacteria.

Although previous research has suggested that tea extract can effectively inhibit the growth of human pathogens, not much research has been done specifically on plant pathogens. Currently, up to 30% of the world's crop yields are lost due to the damage caused by pathogens and insects (17,18). Various bacterial diseases have been identified from almost all plant species. Although some of them can be controlled by bactericides or antibiotics, antibacterial agents are still not commonly used to control the pest damage because of high cost, varied effectiveness, and environmental concerns. Many effective antibacterial agents are costly to produce, costly to use, and less effective on crop plants. Many of the most effective antibacterial chemicals are also harmful to natural ecosystems (19).

The plant bacterial pathogen *Pseudomonas syringae* pv. *tomato* (strain DC3000) (*P. syringae*) can infect tomato plants, causing bacterial leaf spot disease, which is one of most problematic bacterial diseases in tomato fields worldwide (13,14). Recently, it was also shown that the *P. syringae* pv. *tomato* strain DC3000 can cause weak disease in the wild tobacco species *Nicotiana benthamiana* (*N. benthamiana*) when inoculated in growth chamber condition (14).

N. benthamiana is a common model plant because of its relatively small genome, quick growth, and ease of infection. In this study, *N. benthamiana* was used because of its conveniently large leaf size, which eases data quantification. In this study, we explored the viability of green tea as an environmentally friendly bactericide to control the bacterial leaf spot disease caused by *P. syringae* pv. *tomato*.

Based on previous research, we hypothesized that the green tea extract would be effective in controlling *P. syringae*. To test this hypothesis, we designed two experiments. One tested whether the crude water-soluble green tea compounds were able to effectively inhibit growth of *P. syringae* on agar plates. The second

experiment tested whether the green tea extract would be able to inhibit the growth of *P. syringae* on *N. benthamiana* plants. Our results suggest that green tea extract can indeed effectively inhibit the growth of *P. syringae* on both agar plates and on living plants.

Results

Extraction of Water-Soluble Phenolic Compounds from Dried Longjing Tea Leaves

To produce a green tea extract that could be tested as an antibiotic, we steeped loose tea leaves in water at 80°C for 30 minutes. We used a spectrophotometer to measure the resulting concentration from each new sample of green tea extract. We found that 2 g of Longjing tea leaves steeped in 200 mL of ddH_2O at 80°C for 30 minutes would extract water soluble phenolic compounds to a concentration of OD260 = 0.6 ± 0.02. The pH was measured to ensure that any effects on *P. syringae* were not due to a highly acidic or basic solution. We found that the pH value of the undiluted tea extract was 6.3.

Treatments/Controls	Tea extract (ml)	ddH_2O (ml)	Total Pseudomonas Agar volume (ml)
Control	0	100	100
Treatment-1	5	95	100
Treatment-2	10	90	100
Treatment-3	20	80	100

Table 1: Tea extract concentrations used to make agar plates.

Inhibition of Bacteria on Agar with Green Tea Extract

To test the inhibition effect of tea extract, we used three concentrations of tea extract to make the agar plates (**Table 1**). The tea extract was mixed with the agar medium, and the bacterium was grown on the plates. As shown in **Figure 1A & 1B**, the green tea extract could completely inhibit the growth of the bacteria even at the lowest concentration being tested (1% of tea extract). The bacterial colony formation was quantified (**Table 2**) and suggests that all three concentrations are equally effective at inhibiting bacterial growth.

In each trial of the experiment, the antibiotic rifampin was used to control the growth of other bacteria. The strain of *P. syringae* used in the experiment is resistant to rifampin, and so *P. syringae* is able to grow in the presence of rifampin. To further test if the tea extract had any additive effect with the antibiotic rifampin, the experiment was repeated, but the bacterial solution was spread onto agar plates without rifampin. A similar result was obtained, which suggests the tea extract does not have an additive effect with rifampin (**Figure 1C & 1D**). Therefore, this experiment affirmed t that green tea extract can effectively inhibit *P. syringae* on agar plates.

Inhibition of Bacteria on Plants with Green Tea Extract

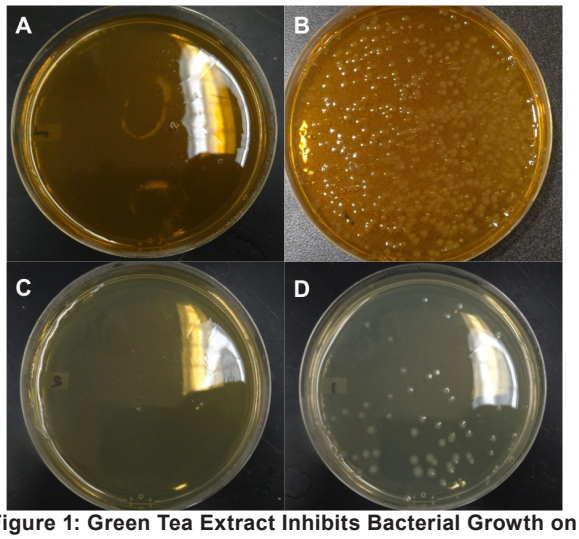

Figure 1: Green Tea Extract Inhibits Bacterial Growth on Agar Plates. Comparison of bacterial growth on agar plates with (A) and without (B) green tea extract. Comparison of bacterial growth on agar plates without rifampin with (C) and without (D) green tea extract. The color difference between plates A and B versus C and D is due to the absence of rifampin.

To test if green tea extract is also effective at inhibiting bacterial growth on plants, an experiment simulating airborne infection was performed by spraying the tobacco plants with the tea extract and a bacterial solution or water as a control. The green tea extract was tested as a preventive measure against the infection of *P. syringae* by spraying the first fully expanded leaf of five-week old *N. benthamiana* plants with green tea first (**Figure 2**). After one day in the incubation chamber, the five-week old *N. benthamiana* plants were spray-inoculated with *P. syringae* strain DC3000 (**Figure 2**). The bacterial proliferation was measured by a growth curve assay, as detailed in the materials and methods section. The bacterial population in the inoculated leaf tissue was calculated based on the number of Colony Forming Units (CFUs) on the agar plates spread with the grounded leaf samples. As displayed in **Figure 3**, the experiment showed that spraying green tea extract on *N. benthamiana* plants could effectively inhibit bacterial growth (**Figure 3**). The treatment that contained the bacterial solution and the green tea extract resulted in fewer bacterial colonies than the treatment that

Control	5 ml Tea Extract	10 ml Tea Extract	20 ml Tea Extract
8.099±0.025	0	0	0

Table 2: Inhibition of the growth of *P. syringae* on agar plates with green tea extract. Both control and treatments had three replicates. Results are in $\log_{10}$ CFU's. $p = 0.003$. The p-value is same when comparing the control to each treatment since all the treatments have same values. The whole experiment was repeated three times and consistent results were obtained.

Figure 2: *N. benthamiana* Plant. Used for this experiment at five weeks old.

contained bacterial solution and water. The application of green tea extract resulted in small, but still significant, effect on controlling bacterial proliferation. Therefore, the hypothesis that green tea extract can inhibit bacterial growth on plants was again affirmed.

Discussion

In this experiment, we showed that green tea extract can inhibit *P. syringae* growth on both agar plates and living *N. benthamiana* plants. The tea extract was first tested *in vitro*. To test the efficacy of different concentrations of tea extract, different amounts of green tea were mixed into the Pseudomonas Agar. An additional experiment was added to examine whether green tea had an additive effect when combined with rifampin. The results of the first experiment were surprising, since even the lowest concentration of tea extract being tested could completely inhibit bacterial growth on the plates. In multiple trials, even at low concentrations, the tea was able to effectively eliminate the bacteria.

In the second experiment, the green tea extract was tested on *N. benthamiana*. To simulate airborne infection, the plants were inoculated by spraying a bacterial solution onto the plants. The green tea extract was sprayed onto the plants first to examine whether the green tea extract could prevent the bacteria from infecting the plant. The bacteria were not directly exposed to the green tea extract, like it was in the agar plates. The green tea extract had less of an antibacterial effect when applied *in vivo*. *In vivo*, there are many more variables than on an agar plate where the researcher has greater control of the environment. The normal functions of the plant, such as metabolism, may interfere with the green tea extract. The bacteria were also able to penetrate inside the plant, whereas the green tea extract remained on the outside. It is unknown whether applying higher doses of green tea extract would have a greater effect on the efficacy *in vivo*.

The results of this experiment are significant. The agent or combination of agents present in the tea were able to completely inhibit bacterial growth. Many studies have tested polyphenols; however, the compounds tested were already isolated and identified. Additionally, the results of these studies did not show the level of inhibition this study did.

There are a few important variables that could have influenced the results. The most important is that the compounds present in the tea extract were never isolated and identified. The green tea extract was composed of all the water-soluble compounds present in the tea leaves. Therefore, there is no way to conclude whether a particular polyphenol was the active agent or a whether it was a combination of several compounds.

A variable that could have quite a large impact on the results was the varying pH between agar plates with different concentrations of green tea. Additionally, it is not known whether the pH of the green tea extract is one of the reasons the green tea extract was able to effectively inhibit bacterial growth.

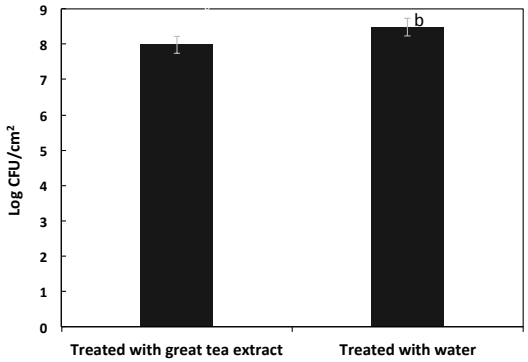

Figure 3: Inhibition of *P. syringae* on *N. benthamiana*. The bacterial populations of plants inoculated with bacterial solution only or tea extract and bacterial solution combined were compared using a t-test. Different letters represent significant statistical differences. p = 0.01, n = 4. The error bars represent the Standard Error of the Mean (SEM).

Because we did not measure the effects of the green tea extract on other species of bacteria, we cannot extrapolate our results. *P. syringae* pv. *tomato* is a model bacterial pathogen that has been widely used in molecular plant pathology research (13,15,16) because of its reactiveness, ease of cultivation, and its similarity to many other bacteria. In this study, we are not sure if the compounds that have been shown to inhibit the growth of *P. syringae* also inhibit the growth of other plant-associated bacterial species. It is possible that different bacteria may have different sensitivities to the green tea extract. Therefore, the bacteria itself may be

a variable.

Similarly, an additional limitation of this experiment is that we did not test the efficacy of green tea extract on other plants. *Nicotiana benthamiana* has particularly soft leaves, which can efficiently absorb bacterial solution and green tea extract. Other species of plants, however, may have different anatomies, and therefore, using a different plant species could completely change the results of the experiment. Unfortunately, there is no previous research report to which the second objective can be compared.

In the future, we could test whether we could soak the plant seeds in tea extract to eliminate contaminated bacteria during the seed germination stage. Many plant bacterial diseases are seed borne, meaning the seeds were contaminated with bacterial pathogens and spread during the seed germination stage (20). Various methods have been tried to eliminate contaminated bacteria from commercial seeds (20). If this method works, it could have very significant applications in plant seed treatment.

The next logical experiment would be to test whether green tea extract is able to inhibit *P. syringae* on other plants, such as tomato plants, since these experiments focused only on *N. benthamiana*, a well-known model organism for plant pathology (21). Although *N. benthamiana* is useful for research, success in this experiment does not necessarily mean green tea extract will be effective on other plants. To further the results of this experiment, it would require testing different plant species.

Much further research is still needed to understand green tea extract's mechanisms of action in inhibiting bacterial growth on plants. Future research should focus on isolating and identifying the active compounds in green tea. Because the green tea extract was so effective on agar plates, experimenting with different concentrations of tea extract is still needed to learn the lowest concentration of tea extract with anti-bacterial activity. One way would be to use less dry tea leaves per 200 mL of water, as opposed to 2 g of tea for 200 mL of water. Another way would be to continue to lower the ratio of agar to Green Tea extract in the plates.

Finally, a broader experiment would test the effectiveness of different teas on different bacteria. Longjing, the tea used in this experiment, is one of many green teas that perhaps share the same properties. Black teas, Oolong, or White teas may also have similar or more potent effects. Even broader would be to test tea extracts on many different species of bacteria. Testing efficacy on different bacteria would make tea extracts more applicable to the agricultural world.

In conclusion, this experiment has suggested that green tea extract can be an effective antibacterial agent on plants. The active compounds in green tea, which have been shown to be effective bacterial growth inhibitors, are natural and normally consumed by humans without ill effect; thus, they are less likely to be toxic to the environment. Additionally, the cost would be low as the extract is made simply with water and tea leaves. It will be interesting to test if the crude green tea extract can be used as an environmentally friendly bactericide.

The significance of the results of this experiment is great. Harmful bactericides simply cannot be used in many cases because of their cost and environmental impact. However, with green tea extract, there is potential for an effective and cost efficient preventive measure for controlling bacterial growth.

Methods

Plant Material, Bacterial Pathogen, and Green Tea

The *P. syringae* v. *tomato* DC3000 strain was originally obtained from Dr. Boris A. Vinatzer's lab (22) and maintained as glycerol stock in -80°C. Before the experiment, the bacterial strain was streaked on Pseudomonas Agar medium (Thermo Scientific, Pittsburg, PA) supplemented with rifampin (100 µg/mL) and cultured in 28°C for 48 hours. *N. benthamiana* plants were planted in small pots filled with Sunshine #1 soil and maintained in a growth chamber for five weeks before infection (**Figure 2**). The growth chamber was programed for 12 hours with light at 25°C and 12 hours in dark at 22°C. The plants were watered every other day. Longjing, also known as Dragon Wellness tea, was used (Hangzhou Meijiawu Travelling Service Co., Ltd, Hangzhou, China).

In Vitro Growth Assay

Objective 1 was to test the green tea extract on agar plates. The green tea extract was prepared by steeping two grams of tea leaves in 200 mL of double detailed water heated to 80°C. The tea leaves were steeped for 30 minutes. The original green tea extract was sterilized by filtering through a 0.22 µm filter unit (Thermo Scientific, Pittsburg, PA) and stored at 4°C before use. The concentration of the tea extract was determined using a Spectrophotometer set at 260 nm wavelength of UV light. Pseudomonas Agar powder was prepared in multiple bottles and sterilized by autoclave. Before pouring the agar into the plates, the different volumes of green tea extract were mixed into each bottle. The total volume of each bottle was adjusted with sterilized ddH_2O. Four tea extract concentrations were used to prepare the agar plates (**Table 1**).

Fresh bacterial cells grown on Pseudomonas Agar were collected and re-suspended in 10 mM $MgCl_2$. The concentration of bacterial suspension was measured using a spectrophotometer set at 600 nm. The bacterial

concentration was adjusted to OD600 = 0.1x10-6 by serial dilutions. One milliliter of bacterial solution was then spread onto the agar plates. Each treatment and control (without tea extract) had at least three replicates. The inoculated plates were incubated at 28°C for two days before examination. The entire experiment was repeated two times.

In Vivo Growth Assay

Objective 2 tested green tea extract on plants. Airborne infection and treatment was simulated by spraying the green tea extract and the bacterial solution. The green tea extract was made using the same procedures as described previously. The bacterial solution was also prepared as described previously; however, the bacterial solution was adjusted to OD600 = 0.1 by serial dilutions instead. The plants were prepared by removing all flowers, wilted leaves, and leaf buds so that the remaining leaves were all mature and healthy leaves. The green tea extract and the bacterial solution were applied by spraying until each leaf was covered. The green tea extract was first sprayed onto the plants and then the plants were placed in a growth chamber for one day. The plants were then sprayed with the bacterial solution that was diluted to OD600 = 0.1. The sprayed plants were incubated for three more days before leaf disks for measuring the bacterial populations were collected. Small lead disks were sampled with a corer borer (size 3, 0.33 cm^2) into 1.5 mL Eppendorf tubes containing 10 mM $MgCl_2$. The leaf disks were ground using a small pestle. This solution was then serially diluted from the original solution and 25 µl of the solution was spotted onto Pseudomonas Agar plates. The bacterial population was measured using Colony Forming Units (CFU), which is done by counting the colonies formed on agar plates that were spread with various dilutions of the grounded leaf samples. Then the colony numbers were used to calculate the total bacterial numbers in the leaf tissue per square centimeter. The data was then converted to log CFU/cm^2. Three biological and three technical repeats were used to calculate the mean value and graphed using Microsoft Excel.

Data Analysis

Data analysis was performed by counting bacterial colonies visually. After the plates were taken out from incubation, pictures were taken of the plates. The pictures were printed out and the colonies on each plate or section of the plate were counted. Averaged CFUs were used for graphical presentation and statistical analysis. Because the data collected was similar, a two-tailed t-test was performed on two data sets to determine whether there was a significant difference in the results. The null hypothesis was that there would be no difference between the bacterial growth in the treated and non-treated bacterial populations. Significant difference was determined based on the calculated *p*-value. If the *p*-value was less than or equal to 0.05, then the results were considered significant and the null hypothesis would be rejected. The t-test calculation was run using Microsoft Excel, and the results were recorded. This procedure was used for counting colonies in both assays.

Acknowledgments

We would like to thank Virginia Tech for its resources and National Science Foundation (IOS-0845283 to BZ) and the Virginia Agricultural Experiment Station (VA135872) for funding this study. We would also like to thank Danyu Kong for supervising the experimental methods.

References

1. Ho CT, Chen Q, Shi H, Zhang KQ, Rosen RT. (1992). Antioxidative effect of polyphenol extract prepared from various Chinese teas. *Preventive Medicine* 21(4): 520-5.
2. Clement Y. (2009). Can green tea do that? A literature review of the clinical evidence. *Preventive Medicine* 49(2): 83-7.
3. Hamilton-Miller JM. (1995). Antimicrobial properties of tea (*Camellia sinensis L.*). *Antimicrobial Agents and Chemotherapy* 39(11): 2375.
4. Stoicov C, Saffari R, Houghton J. (2009). Green tea inhibits Helicobacter growth *in vivo* and *in vitro*." *Int J Antimicrob Agents* 33(5): 473-8.
5. Daglia M. (2012). Polyphenols as antimicrobial agents. *Current Opinion in Biotechnology* 23(2): 174-181.
6. Friedman M. (2007). Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Molecular Nutrition & Food Research* 51(1): 116-34.
7. Gordon NC, Wareham DW. (2010). Antimicrobial activity of the green tea polyphenol (−)-epigallocatechin-3-gallate (EGCG) against clinical isolates of *Stenotrophomonas maltophilia*. *Int J Antimicrob Agents* 36(2): 129-31.
8. Ciesek S, von Hahn T, Colpitts CC, Schang LM, Friesland M, Steinmann J, Manns MP, Ott M, Wedemeyer H, Meuleman P, Pietschmann T, Steinmann E. (2011). The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. *Hepatology* 54(6): 1947-55.
9. Komes D, Horžić D, Belščak A, Kovačević Ganić K, Vulić I. (2010). Green tea preparation and its influence on the content of bioactive compounds. *Food Research International* 43(1): 167-76.
10. Perva-Uzunalić A, Škerget M, Knez Ž, Weinreich

B, Otto F, Grüner S. (2006). Extraction of active ingredients from green tea (*Camellia sinensis*): extraction efficiency of major catechins and caffeine. *Food Chemistry* 96(4): 597-605.

11. Turkmen N, Sari F, Velioglu YS. (2006). Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. *Food Chemistry* 99(4): 835-41.
12. Yam TS, Shah S, Hamilton-Miller JM. (1997). Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea components. *FEMS Microbiol Lett* 152(1): 169-74.
13. Dong X, Mindrinos M, Davis KR, Ausubel FM. (1991). Induction of Arabidopsis defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. *Plant Cell* 3(1): 61-72.
14. Wei CF, Kvitko BH, Shimizu R, Crabill E, Alfano JR, Lin NC, Martin GB, Huang HC, Collmer A. (2007). A *Pseudomonas syringae* pv. *tomato* DC3000 mutant lacking the type III effector HopQ1-1 is able to cause disease in the model plant *Nicotiana benthamiana*. *Plant Journal* 51(1): 32-46.
15. Whalen MC, Innes RW, Bent AF, Staskawicz BJ. (1991). Identification of *Pseudomonas syringae* pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell* 3(1): 49-59.
16. Dangl JL, Ritter C, Gibbon MC, Mur LA, Wood JR, Goss S, Mansfield J, Taylor JD, Vivian A. (1992). Functional homologs of the Arabidopsis RPM1 disease resistance gene in bean and pea." *Plant Cell* 4(11): 1359-69.
17. Somerville C, Briscoe J. (2001). Genetic engineering and water. *Science* 292(5525): 2217.
18. Oerke EC. (2006). Crop losses to pests. *Journal of Agricultural Science* 144(1): 31-43.
19. Cha J, Cupples AM. (2009). Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. *Water Research* 43(9): 2522-30.
20. Gitaitis R, Walcott R. (2007). The epidemiology and management of seedborne bacterial diseases. *Annu. Rev. Phytopathol*. 45: 371-97.
21. Goodin MM, Zaitlin D, Naidu RA Lommel SA. (2008). *Nicotiana benthamiana*: its history and future as a model for plant-pathogen interactions. *Molecular Plant-Microbe Interactions* 21(8): 1015-26.
22. Cai R, Lewis J, Yan S, Liu H, Clarke CR, Campanile F, Almeida NF, Studholme DJ, Lindeberg M, Schneider D, Zaccardelli M, Setubal JC, Morales-Lizcano NP, Bernal A, Coaker G, Baker C, Bender CL, Leman S, Vinatzer BA. (2011). The plant pathogen *Pseudomonas syringae* pv. *tomato* is genetically monomorphic and under strong selection to evade tomato immunity. *PLoS Pathog* 7(8): e1002130.