

In Vitro Studies on Some Natural Beverages as Botanical Pesticides against *Erwinia amylovora* and *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*

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Abstract: Several tannin-rich beverages were tested for their antibacterial activity against 2 important phytopathogenic bacteria, *Erwinia amylovora* and *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*. Black tea (9.5, 19 and 38 g l⁻¹), green tea (9.5, 19 and 38 g l⁻¹) and tannic acid (0.2, 0.4 and 0.8 g l⁻¹) inhibited the growth of *E. amylovora* and *C. f.* subsp. *poinsettiae*. Coffee (8.75, 17.5 and 35 g l⁻¹) and cocoa (8.75, 17.5 and 35 g l⁻¹) did not display any inhibitory effect on the growth of bacterial cultures. The numbers of colony forming units (CFUs) of *E. amylovora* in the presence of black tea, and of *C. f.* subsp. *poinsettiae* in the presence of tannic acid were lower than their control counterparts. Over prolonged incubation the inhibitory effect of extracts on the number of CFUs was diminished. The results suggest that tea extract might be a safe agricultural chemical against some important plant diseases, play a vital role in meeting the demand for organically produced plants and alleviate some environmental problems associated with the use of synthetic chemicals.

Key Words: biological control, botanical pesticide, environmental pollution, health risk, organic farming

Bazı Doğal İçeceklerin *Erwinia amylovora* ve *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*'ye Karşı Botanik Pestisit Olarak Kullanımı Üzerine İn Vitro Çalışmalar

Özet: Bu çalışmada tannince-zengin bazı doğal içeceklerin iki önemli bitki hastalık etmeni, *Erwinia amylovora* ve *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*, üzerindeki antibakteriyal aktiviteleri araştırılmıştır. Siyah çay (9.5, 19 ve 38 g l⁻¹), yeşil çay (9.5, 19 ve 38 g l⁻¹), ve tannic asit (0.2, 0.4 ve 0.8 g l⁻¹) *Erwinia amylovora* ve *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*'nin çoğalmalarını engellemiştir. Kahve (8.75, 17.5 ve 35 g l⁻¹) ve kakaonun (8.75, 17.5 ve 35 g l⁻¹) bakteri kültürlerinin büyümesi üzerinde engelleyici herhangi bir etkisi görülmemiştir. Siyah çay ile muamele edilen *Erwinia amylovora* ve tannic asit ile muamele edilen *Curtobacterium flaccumfaciensis* subsp. *poinsettiae*'nin koloni oluşturan ünite sayısı (colony forming units) kontrolün koloni oluşturan ünite sayısından daha düşük bulunmuştur. İnkübasyon süresi uzadıkça ekstraktların koloni oluşturan ünite sayıları üzerindeki engelleyici etkilerinin azaldığı görülmüştür. Bulgular çay ekstraktının bazı önemli bitki hastalıklarına karşı etkili bir potansiyel kimyasal olarak kullanılabileceğini göstermiştir. Ayrıca, bulgular sentetik pestisitlerin yerine çay ekstraktının kullanılmasının organik yetiştirilen ürün talebini karşılamada önemli bir rol oynayabileceğini ve sentetik kimyasal kullanımının sebep olduğu bazı çevresel problemleri ortadan kaldırdılabileceğini göstermiştir.

Anahtar Sözcükler: biyolojik kontrol, botanik pestisit, çevre kirliliği, sağlık riski, organik yetiştiricilik

Introduction

As a part of their defense systems, many plants produce antimicrobial and pest repellent chemicals. These natural plant products, known as botanical pesticides or herbal medicines, have long been used in the control of microorganisms causing plant and human diseases. However, with the employment of synthetic pesticides in agriculture, the use of botanical pesticides has significantly diminished. Although highly effective, synthetic pesticides often have undesirable side effects

such as toxicity to mammals and causing environmental pollution.

Nowadays, people are more aware of the potential health risks and environmental hazards of synthetic chemicals. The tendency toward consuming organically produced plants has increased dramatically. The use of plant-derived alternative pesticides seems to be regaining popularity and could play a vital role in meeting the demand for organically produced plants.

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Of the herbal medicine and botanical pesticides, much attention has been given to the use of phenolic-rich plant extracts. Tannins are important water soluble plant phenolics. Tannin-rich plant extracts have traditionally been used as medicines to treat infectious human diseases (Toda et al., 1989; Djipa et al., 2000) and they exhibit antimicrobial activity against phytopathogenic fungi and bacteria (Scalbert, 1991; Alstrom, 1992). The toxicity of tannins on microorganisms operates either by their direct action on the microbial membrane or by metal ion depletion. In general, tannin-chelated metal ions are not bioavailable. The low decomposition of tannin-rich plant materials (walnut, chestnut and oak) has been in part attributed to the low levels of biologically available metal ions (Vitousek et al., 1994; McDonald et al., 1996).

Tea and coffee, 2 tannin-rich beverages consumed in large quantities, have been reported to have antibacterial activity against human (Toda et al., 1989) and plant (Alstrom, 1992) pathogens. Unlike synthetic chemicals, most tannin-rich natural beverages are health-promoting and inexpensive. In organic farming, if found effective against important phytopathogens, they will be safe for human use and environmentally friendly.

The objective of this study was to explore whether some natural beverages (black tea, green tea, coffee and cocoa) could potentially be used to control *Erwinia amylovora* (the cause of fire blight in apple, pear, quince, etc) and *Curtobacterium flaccumfaciensis* subsp. *poinsettiae* (the cause of stem cancer and leaf spot in poinsettia (*Euphorbia pulcherrima*)), 2 important phytopathogenic bacteria.

Materials and Methods

Bacterial strains

Bacterial strains were provided by Dr. Anne K. Vidaver (University of Nebraska, Lincoln, USA). *C. f. subsp. poinsettiae* and *E. amylovora* were cultured overnight at 27 °C and shaken at 250 rpm. Bacterial suspensions (O.D. ~ 0.8) were subcultured by adding 0.2 ml of overnight culture to 10 ml of nutrient broth. After 2 h, 10 µl of fresh bacterial cultures were added to 3 ml of warm (soft) Tryptic Soy Agar (TSA; 10 g l⁻¹ tryptic soy broth and 10 g l⁻¹ of bacteriological agar) medium. The soft TSA containing bacterial cultures was evenly spread on TSA medium in petri plates.

Preparation and application of extracts

Samples of shredded black and green tea leaves and fine cocoa and coffee bean powder were taken from commercial products. Tannic acid (0.2, 0.4 and 0.8 g l⁻¹) was used as the positive control, and polyvinylpyrrolidone (0.2, 0.4 and 0.8 g l⁻¹) and distilled-deionized water were used as the negative controls (containing no tannin). Green and black tea leaves (9.5, 19 and 38 g l⁻¹), cocoa powder (8.75, 17.5 and 35 g l⁻¹) and coffee powder (8.75, 17.5 and 35 g l⁻¹) were added to hot water and left for 30 min, and then were filtered through cheesecloth. After the pH was adjusted to 7.0, all extracts and control solutions were filter sterilized (0.2 µm).

The inhibitory effect of extracts was tested by slightly modifying the agar diffusion method (Toda et al., 1989; Alstrom, 1992). Each concentration of an extract was spotted 3 times (each 10 µL) on the surface of a TSA medium plate loaded with bacterial suspension. Each treatment was applied to 2 plates (replications). After 1 week, the diameters of the inhibition zones were measured and the experiment was repeated. For statistical analysis, the means of 3 spots in 1 plate (replication) were used. Analysis of variance (ANOVA) was done using SAS GLM (SAS Institute Inc. 1996). Separation of treatment means was done by Fisher's least significant difference (LSD) test at P = 0.05.

Determination of the purity of cultures and the viable cell count

The purity of cultures was determined by the streaking agar plate method. To determine whether the inhibition effects were bactericidal or bacteriostatic, the viable cell count method was used (Toda et al., 1989; Alstrom, 1992). To do this, equal volumes of overnight cultured bacterial suspension (O.D. ~ 0.8) and black tea (38 g l⁻¹), green tea (38 g l⁻¹) or tannic acid (0.8 g l⁻¹) were mixed and incubated for 5 min, 30 min, 2 h, or 12 h, and then the mixtures were diluted from 10⁻² to 10⁻⁶. Ten microliters of dilutions were spread on TSA medium plates (2 replications) and the mean number of colonies was compared with that of the control (water-treated) plates. The experiment was conducted twice, and data analysis was the same as for the growth inhibition zones.

Results and Discussion

Two independent experiments gave similar results, and the data were therefore combined and are discussed together. Two negative controls, water and polyvinylpyrrolidone, did not display any inhibitory effect on the growth of bacterial cultures. Of the natural extracts, coffee and cocoa did not inhibit the growth of bacteria, and the surface of TSA plates that were treated with coffee and cocoa were completely covered by bacterial colonies (data not shown). However, tannic acid (Figure 1), black tea (Figure 2) and green tea (Figure 3) inhibited the growth of both *E. amylovora* and *C. f. subsp. poinsettiae*. All growth inhibition zones were clear and had smooth round edges, although some isolated colonies were able to grow in the inhibition zones. As the concentration of extracts was increased, greater inhibition zone diameters were observed ($P = 0.001$). The inhibitory effect of extracts was strain dependent ($P = 0.001$). In general, the growth of *C. f. subsp. poinsettiae* was inhibited more than that of *E. amylovora*. The mean differences among the inhibition zones obtained with 3 inhibitory extracts were not significantly different ($P = 0.3$).

In some studies higher amounts of tea and cocoa were used against pathogenic bacteria. For instance, Toda et al. (1989) used tea and coffee at the levels of 200 g l^{-1} and 250 g l^{-1} , respectively. They reported that tea and coffee inhibited the growth of human bacterial pathogens causing diarrhea and that tea was a more effective inhibitor than coffee. On the other hand, Alstrom (1992)

reported that both tea and coffee extracts exhibited antibacterial activity against phytopathogenic bacteria (*Pseudomonas syringae* strains) but that their effects varied depending on bacterial strain and the method of application. Our results, at least partially, are in agreement with those reported above. The fact that coffee and cocoa did not inhibit the growth of *E. amylovora* and *C. f. subsp. poinsettiae* could be due to the bacterial strains. Another explanation might be that the amounts of coffee and cocoa we used in the current study were insufficient, compared to those used by Toda et al. (1989), to exhibit any inhibitory effect. This is also suggested by the greater growth inhibition zones observed when higher levels of tea and tannic acid were used (Figures 1, 2 and 3).

Although tannic acid, black tea and green tea inhibited the growth of *E. amylovora* and *C. f. subsp. poinsettiae*, some isolated colonies were able to grow in the inhibition zones. The growth of isolated colonies in the inhibition zones could be due to contamination of the stocks from which the cultures were initiated. In such cases, although the growth of targeted strains may be totally inhibited, resistant colonies (contaminants) may still be able to grow. The results from streaking agar plates indicated that both original cultures were pure. Therefore, the possibility that the original stocks from which cultures were initiated might have been contaminated was dismissed.

Alternatively, the growth of isolated colonies in the inhibition zone could be explained if an extract has

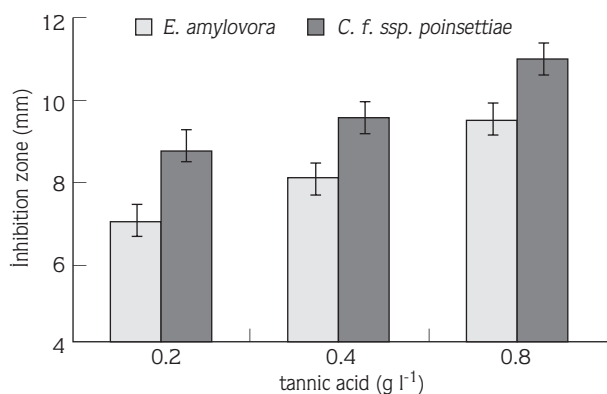


Figure 1. Inhibition effects of tannic acid on growth of *Erwinia amylovora* (□) and *C. f. subsp. poinsettiae* (■). Means represent combined data of 2 independent experiments. Bars represent standard errors ($n = 4$).

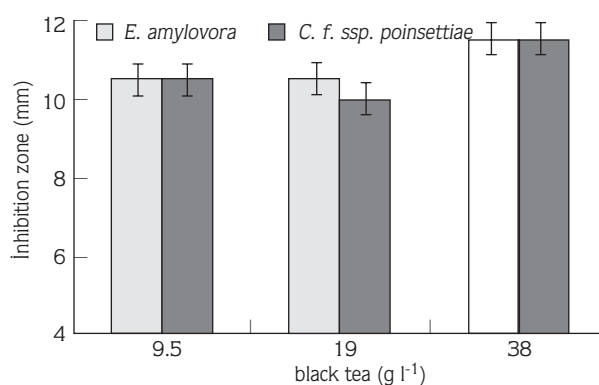


Figure 2. Inhibition effects of black tea on growth of *Erwinia amylovora* (□) and *C. f. subsp. poinsettiae* (■). Means represent combined data of 2 independent experiments. Bars represent standard errors ($n = 4$).

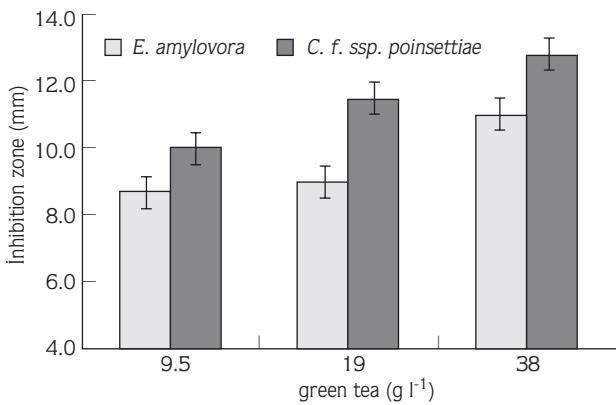


Figure 3. Inhibition effects of green tea on growth of *Erwinia amylovora* (□) and *C. f. subsp. poinsettiae* (■). Means represent combined data of 2 independent experiments. Bars represent standard errors (n = 4).

bacteriastatic but not bacteriacidal activity. Viable cell counts indicated that only colonies formed from bacteria-extract mixtures diluted to 10⁻⁶, 10⁻⁵ and 10⁻⁴ and cultured at 5 min and 30 min intervals were comparable. The numbers of colonies formed from higher bacterial suspensions or bacteria-extract mixtures cultured at longer intervals were too great and therefore could not readily be counted. The numbers of *E. amylovora* colonies formed from 10⁻⁶ and 10⁻⁵ dilutions treated with black tea, and the numbers of *C. f. subsp. poinsettiae* colonies formed from 10⁻⁶ to 10⁻⁴ dilutions treated with tannic acid were lower than those of their control counterparts (Table 1). However, in longer incubation times the

inhibitory effects of extracts on the number of CFUs were diminished or disappeared. The inhibitory effect of green tea on culture growth was not observed with the viable cell count.

The fact that the numbers of colonies formed in the presence and absence of black tea and tannic acid in short incubation times were different, but that in longer incubation times they were similar indicates that these extracts have bacteriastatic activity against *E. amylovora* and *C. f. subsp. poinsettiae*. Alternatively, the fact that no inhibitory effects of the extracts were observed in higher bacterial suspensions or mixtures cultured at longer intervals could be due to insufficient concentrations of inhibitory compounds (i.e. molecules/cell) found in the amount of extracts tested.

Conclusion

Based on the results presented here and reported by other researchers (Fukai et al., 1991, Alstrom, 1992), we conclude that tea may be a safe agricultural chemical against some important plant diseases. It may alleviate the spread of phytopathogenic bacteria. The employment of such an inexpensive daily beverage as a botanical pesticide could play a vital role in meeting the demand for annual/seasonal organically produced plants in particular and in alleviating some environmental problems associated with the use of synthetic chemicals. However, our conclusion is based on data from in vitro studies and should only be considered as a reference for future field studies.

Table 1. Number* of colonies formed in the presence of black tea, green tea, tannic acid and water (control).

extracts	strain	mixture incubated for 5 min then diluted to			mixture incubated for 30 min then diluted to		
		10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶
black tea	<i>E. amylovora</i>	tmc	0.5 ^d	0.1 ^a	tmc	12.7 ^c	0.1 ^b
black tea	<i>C. f. subsp. poinsettiae</i>	tmc	1.5 ^d	0.1 ^a	tmc	2.1 ^d	0.6 ^{ab}
green tea	<i>E. amylovora</i>	tmc	37.6 ^a	2.1 ^a	tmc	42.3 ^a	2.6 ^{ab}
green tea	<i>C. f. subsp. poinsettiae</i>	21.9 ^b	2.2 ^d	0.1 ^a	44.4 ^a	2.6 ^d	0.6 ^{ab}
tannic acid	<i>E. amylovora</i>	tmc	35.0 ^b	1.0 ^a	tmc	33.4 ^b	2.7 ^a
tannic acid	<i>C. f. subsp. poinsettiae</i>	7.9 ^c	0.1 ^d	0.0 ^a	11.4 ^c	0.1 ^d	0.0 ^b
water	<i>E. amylovora</i>	tmc	32.6 ^c	1.6 ^a	tmc	33.0 ^b	0.3 ^b
water	<i>C. f. subsp. poinsettiae</i>	30.9 ^a	1.3 ^d	0.1 ^a	33.5 ^b	0.5 ^d	0.0 ^b

Means represent combined data of 2 independent experiments (n = 4). * Means in the same column followed by the same letter are not significantly different at P = 0.05. tmc: too many colonies to count.

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