Antimicrobial Activity of Chestnut Extracts for Potential Use in Managing Soilborne Plant Pathogens

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Abstract

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Chestnut extracts were studied for antimicrobial activity against selected microorganisms, including plant pathogens. Chestnut extract on paper discs was applied to an agar medium to evaluate the inhibition to multiple microorganisms or the extract was added at various concentrations to a culture medium to evaluate the growth of target microorganisms. Chestnut type, tissue of plants (shell, pellicle, and leaf), extraction methods, and physical characteristics were studied to determine antimicrobial activity. Most test microorganisms were inhibited by the extracts at different effective concentrations for 50% growth inhibition (EC_{50}). *Pseudomonas fluorescens* was the most sensitive (EC_{50} = 4.4 μ g/ μ l), *Phytophthora cambivora* was one of the least inhibited (EC_{50} =

185 μ g/ μ l), and *Cryphonectria parasitica* was not inhibited. Extracts of the Japanese $\times$ European chestnut (*Castanea crenata* $\times$ *C. sativa*) 'Colossal' showed a greater inhibition than those of wild trees of the Chinese species (*C. mollissima*). High temperature did not affect the inhibitory effect. Extracts from chestnut pellicle had the highest concentration of antimicrobial compound, compared with leaf and shell. The active fraction contained several substances with molecular masses consistent with one flavonol glycoside and several terpenoid substances. Pellicle and shell tissue reduced radish scab disease caused by *Streptomyces scabies* in the greenhouse.

Species of *Castanea* are perennial plants varying from small shrubs and large trees in forests and orchards of Europe, Asia, and North America (6,9,11,22). Despite the worldwide dissemination of several devastating pathogens and pests, such as chestnut blight (*Cryphonectria parasitica* (Murrill) M.E. Barr) (2) and Asian gall wasp (*Dryocosmus kuriphilus*) (10,34), chestnut remains one of the most popular nut-bearing trees throughout Eurasia (5,6). Today, South Korea, China, Italy, and Turkey account for more than 70% of worldwide chestnut production (4,7). Chestnut farming and production are increasing in Australia, New Zealand (19), Chile (17), and the United States (15). A large research program to restore chestnut blight-resistant American chestnut (*Castanea dentata*) to the forest environment is currently being pursued in North America (12).

The popularity of many *Castanea* spp. is based on several attributes of various species, including the production of nutritious, low-fat nuts and decay-resistant, high-quality wood; the ability of roots to sprout after harvest, eliminating the need to replant; and the role of several species in forest ecosystems. The chestnut fruit is known for its flavor, nutritional value, and potential uses as nutritional supplements or for medical purposes (1,7,8,32).

In addition to these attributes, tissues of *Castanea* spp. have been found to contain compounds with inhibitory effects on microorganisms and plants (3). Tannins, flavonoids, and antioxidants in shell extracts of Chinese chestnut (*C. mollissima*) (27) appeared inhibitory to bacteria and fungi, including *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (23,24). Recently, Donis-Gonzalez (13) reported that the greatest antimicrobial activity was found in the pellicle, a thin fibrous tissue found between the shell and kernel. When chestnut pellicle, shell, and nut kernel were directly placed on agar media, the pellicle

tissue was the only tissue found to inhibit fungi (including yeast) and bacteria. Acetone-extracted pellicle and shell had inhibitory activity against the test organisms, with the pellicle showing a higher level of inhibition. Chestnut kernel tissue extract showed no inhibitory activity (13).

The objectives of this work were to determine the level of the inhibitory effect on a range of microorganisms, the tissues producing the effect, and potential uses for chestnut shell material as a soil amendment in managing soilborne diseases.

Materials and Methods

Microorganisms and media. Strains of bacteria, fungi, and oomycetes were collected from various sources (Table 1). The microorganisms were grown in the laboratory on appropriate culture media, including potato dextrose agar (PDA; EMD Chemicals, Inc., Gibbstown, NJ) for fungi and some oomycetes, rye seed agar medium (29) for *Phytophthora infestans* Leonian, yeast malt extract agar (YME) (28) for *Streptomyces* spp., and Lysogeny broth (LB)-Miller agar medium (EMD Chemicals, Inc.) for other bacteria.

Chestnut extracts. Compounds were extracted from two different chestnut sources: wild, ungrafted Chinese chestnut trees (*C. mollissima*) growing in established Michigan orchards and commercially grafted 'Colossal' chestnut (*C. sativa* $\times$ *C. crenata*), also found on various farms in Michigan (15). The extract from shell and pellicle used in this study was obtained from hand-peeled chestnuts, unless otherwise stated. Hand-peeling was done by cutting the chestnut in half with a knife and then manually separating the pellicle from the shell, which were stored at -20°C until use.

Some shell and pellicle were obtained by processing chestnut through a commercial brulage peeling line (Boema S. P. A., Neive, Italy; (18) located at the Michigan State University Roger's Reserve, Jackson, MI. The peeling line uses propane burners coupled with hot water (70°C) to separate chestnut shell material from kernels. Shell material from the peeling line, which is often contaminated with pellicle tissue, was collected, placed in plastic bags, and stored at 4°C . This mixed material was used for greenhouse studies. For laboratory studies, the shell material was separated by removing as much attached pellicle tissue as possible. Pure pellicle

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tissue was also collected from the peeling line during the same process. Both shell and pellicle were stored at -20°C until use. Chestnut leaves were collected on 10 September 2010 from wild Chinese and Colossal trees.

Chestnut tissues were extracted using an appropriate solvent. A 10-g subsample (shell, pellicle, and leaf) was dried (100°C) in the oven for 24 h to calculate the tissue dry weight prior to extraction. The extract concentration was calculated as tissue dry weight per volume of solvent. Pellicle, leaf, or shell tissue (1 g) was placed in liquid nitrogen in a mortar and ground into powder with a pestle. Chestnut shells were pre-ground to small particles using a coffee grinder (80365 Custom Grind; Hamilton Beach, Glen Allen, VA) before grinding them with a mortar and pestle. The powder was transferred to a 15-ml centrifuge tube with 5 ml of solvent (75% ethanol, acetone [ACS grade; J. T. Baker, Phillipsburg, NJ], or sterile distilled water), and placed on a shaker at 200 rpm at 25°C for 24 h. The tube was centrifuged (Sorvall RC6+ centrifuge; Thermo Scientific, Asheville, NC) at $12,000 \times g$ for 15 min, and the supernatant was transferred to a new centrifuge tube and placed in an oven at 50°C until the solvent was completely evaporated. The dried residue was redissolved with 1 ml of 30% ethanol and filtered through a $0.22\text{-}\mu\text{m}$ filter (Millipore Corporation, Billerica, MA) using a syringe. The extract was stored at 4°C in a refrigerator. Throughout the study, ethanol was used as a standard solvent for extraction. The only exception was that water and acetone were used as a solvent for comparison with ethanol.

Assessing chestnut tissue extracts for antimicrobial activity.

Paper disc diffusion method. Discs (5 mm in diameter) of filter paper (Number 1; Whatman International Ltd., Maidstone, UK) were cut, autoclaved for 15 min, and placed at the center of a petri dish (9 cm in diameter) on the surface of the appropriate solidified medium for the target microorganism used in each assay. For oomycete and fungal (*Phytophthora capsici*, and *Rhizoctonia solani*) inhibition assays, two mycelial plugs (5 mm in diameter) were cut from a fresh culture using a cork borer and transferred to the culture medium containing the filter disc. The mycelial plugs were placed 2.5 cm apart with the paper disc between them. A $100\text{-}\mu\text{l}$ ($854\text{ }\mu\text{g}/\mu\text{l}$) aliquot of pellicle extract from Chinese and Colossal chestnut was transferred onto the filter paper disc using a pipette. Then, $100\text{ }\mu\text{l}$ of a 30% ethanol solution was applied to the disc as a control. Plates were incubated at 22°C in the dark. After 3 days of incubation, the radius of the mycelial growth from each mycelial plug was measured at two locations: the first measurement was taken from the center of the mycelial plug to the edge of the colony, away from the paper disc, and the second measurement was taken from the mycelial plug to the edge of the colony toward to the paper disc. The inhibition was calculated as $[100 \times (\text{radius } 1 - \text{radius } 2)/\text{radius } 1]$. For *Streptomyces scabies* inhibition, $20\text{ }\mu\text{l}$ of cell suspension of *S. scabies* (10^4 CFU/ml) was spread on an agar plate, followed by placing a filter paper disc with the chestnut extract added. A paper disc with ethanol was used as a control. The diameter of inhibition zones (zone 1 for chestnut extract treatment

Table 1. Effective concentrations of chestnut pellicle extract for 50% inhibition on the growth of selected microorganisms

Microorganism name	Type ^w	Sources ^x	EC ₅₀ ($\mu\text{g}/\mu\text{l}$) ^y	Media ^z
<i>Verticillium dahliae</i>	Fungi	Hao	13.37	PDA
<i>Trichoderma harzianum</i>	Fungi	SipcamAdvan	15.96	PDA
<i>Cladosporium cucumerinum</i>	Fungi	Hammerschmidt	16.37	PDA
<i>Penicillium citrinum</i>	Fungi	Hao	43.61	PDA
<i>Fusarium solani</i>	Fungi	Hao	45.57	PDA
<i>Sclerotinia sclerotiorum</i>	Fungi	Hao	45.57	PDA
<i>Rhizoctonia solani</i>	Fungi	Hao	56.42	PDA
<i>Botrytis cinerea</i>	Fungi	Hao	86.16	PDA
<i>Alternaria solani</i>	Fungi	Hao	110.99	PDA
<i>Cryphonectria parasitica</i>	Fungi	Fulbright	NI	PDA
<i>Pseudomonas fluorescens</i>	G– bacteria	Kirk	4.39	LB
<i>Bacillus</i> sp. strain BAC03	G+ bacteria	Hao	5.02	LB
<i>Bacillus subtilis</i>	G+ bacteria	AgraQuest	5.21	LB
<i>Streptomyces aureofaciens</i>	G+ bacteria	NRRL	5.55	YME
<i>B. mycoides</i>	G+ bacteria	Hao	5.69	LB
<i>Pseudomonas syringae</i>	G– bacteria	Kirk, MI	6.05	LB
<i>Streptomyces acidiscabies</i>	G+ bacteria	ATCC	9.24	YME
<i>S. lydicus</i>	G+ bacteria	SipcamAdvan	9.46	YME
<i>Streptomyces</i> sp. strain DS3024	G+ bacteria	Hao, MI	10.38	YME
<i>S. scabies</i> strain B-1231	G+ bacteria	NRRL	11.96	YME
<i>S. scabies</i> strain 1019	G+ bacteria	NRRL	12.50	YME
<i>S. europaeiscabiei</i>	G+ bacteria	NRRL	12.97	YME
<i>S. bototropensis</i> strain 5262	G+ bacteria	NRRL	13.28	YME
<i>S. scabies</i> strain 49173	G+ bacteria	ATCC	13.59	YME
<i>S. laceyi</i>	G+ bacteria	Hao	13.97	YME
<i>S. stelliscabiei</i>	G+ bacteria	Hao	14.35	YME
<i>Xanthomonas phaseoli</i>	G– bacteria	Kirk	15.40	LB
<i>Erwinia carotovora</i>	G– bacteria	Kirk	20.45	LB
<i>Burkholderia cepacia</i>	G– bacteria	Kirk	21.07	LB
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	G+ bacteria	Kirk	21.49	523
<i>Agrobacterium tumefaciens</i>	G– bacteria	Day	22.12	LB
<i>Pythium irregulare</i>	Oomycete	Hao	18.71	PDA
<i>Phytophthora infestans</i>	Oomycete	Kirk	28.45	RSA
<i>P. capsici</i>	Oomycete	Hao	32.60	PDA
<i>P. cinnamomi</i>	Oomycete	Fulbright	62.28	PDA
<i>P. cambivora</i>	Oomycete	Fulbright	184.65	PDA

^w G– and G+ = Gram-negative and Gram-positive bacteria, respectively.

^x Laboratories of Hao, Hammerschmidt, Fulbright, Kirk, and Day are all located at Michigan State University; ATCC: American Type Culture Collection (<http://www.atcc.org/>), VA; NRRL: United States Department of Agriculture–Agricultural Research Service Agriculture Collection or Northern Regional Research Laboratory (<http://nrri.ncaur.usda.gov/>), IL. Other resources included SipcamAdvan, Roswell, GA and AgraQuest, Davis, CA.

^y Effective concentration for 50% growth inhibition (EC₅₀) either for mycelial growth (fungi) or colony counting (bacteria), which was calculated with data combined from two trials. NI = pathogen was not inhibited at any concentration.

^z PDA: potato dextrose agar; LB: Lysogeny broth medium; YME: yeast malt extract agar; 523: bacterial screening medium (Sigma-Aldrich, Inc., St Louis); and RSA: rye seed agar medium.

and zone 2 for ethanol treatment) was measured, and relative inhibition was calculated as $[(100 \times (\text{diameter of zone 1} - \text{diameter of zone 2}) / \text{diameter of zone 1})]$. The experiment was conducted twice.

For *S. scabies* inhibition assays comparing extracts, a bacterial suspension was made by removing the cells from a single bacterial colony and suspending them in 500 μl of sterile water. The bacterial suspension was transferred to a petri dish (9 cm in diameter) containing YME solidified with agar and the suspension was uniformly dispersed with a sterile glass rod. A sterile filter paper disc was placed at the center of the plate. A 100- μl (854 $\mu\text{g}/\mu\text{l}$) aliquot of chestnut pellicle extract was added to the filter paper, and the plate was incubated at 28°C in the dark for 3 days. The zone of growth inhibition (the diameter of the area without bacterial growth subtracted by the diameter of paper disc) was measured after 3 days of incubation. The inhibition effect $[100 \times (\text{inhibition zone of treated plate} - \text{inhibition zone of control}) / \text{inhibition zone of treated plate}]$ was also evaluated with extracts using acetone as a solvent to compare with ethanol from Chinese and Colossal chestnut. Each treatment was replicated three times. The experiment was done twice.

Culture dilution method. Spore suspensions of *Streptomyces* sp. strain DS3024, *S. scabies* strain ATCC49173, and *S. bottropensis* were used to assay the inhibitory effect of pellicle extracts from Colossal. Aliquots of 0, 10, 20, 40, 60, 80, 100, 300, and 500 μl of pellicle extract (854 $\mu\text{g}/\mu\text{l}$) were added to a 1-ml bacterial spore suspension (10^8 CFU/ml). Additional sterile water and ethanol were added to bring the total volume up to 1.5 ml/tube with same amount of ethanol. After 6, 12, and 24 h, a 100- μl bacterial spore suspension was transferred to 900 μl of sterile water in a 1.5-ml centrifuge tube, with five replications. Aliquots of 20- μl extract-spore mixtures were applied to YME solidified medium using an Autoplate 4000 (Advanced Instruments, Inc., Norwood, MA) in the Uniform mode. The plates were incubated at 28°C in the dark, and colonies were counted after 3 days. Each concentration was replicated three times. The experiment was done twice.

To determine whether the antimicrobial effect of chestnut extract was lethal or inhibitory, an additional experiment was conducted. Chestnut (Colossal) extract (1 mg/ml) with volumes of 0 (control), 50, 250, or 400 μl was mixed with 600- μl spore suspensions of *S. bottropensis* (10^7 CFU/ml) in a 1.8-ml tube. Additional sterile water and ethanol were added to bring the total volume up to 1 ml/tube with the same concentration of ethanol (12%). After 12, 24, and 36 h of incubation, the extract was centrifuged at $6,000 \times g$ for 10 min. The supernatant was discarded and sediment was suspended with 1 ml of sterile distilled water and centrifuged at $6,000 \times g$ for 10 min. This step was repeated three times to assure that the bacteria were free of chestnut extract. An aliquot of 50 μl of the cell suspension was spread on YME plates. The plates were incubated at 28°C in the dark, and colonies were counted after 3 days. Each treatment was replicated three times and the experiment was conducted twice.

Medium plate method to determine effective concentration for 50% inhibition. To determine the effective concentration for 50% growth inhibition (EC_{50}) of chestnut pellicle, leaf, or shell extract on various microorganisms, protocols of Duffy and Power (14) were followed. Because the effective concentration varied depending on the microorganism used in the assay, a pilot test was conducted to determine the range of effective extract concentrations for each microorganism. This pilot test provided the highest and lowest concentration for 90 and 10% growth inhibition, respectively. Final concentration of solvents used to dissolve chestnut extract was adjusted to the same level (1% by volume) in each plate. Each treatment was replicated three times.

For fungal testing, a plug (5 mm in diameter) of fungal or oomycete (Table 1) mycelium was cut using a cork borer, placed at the center of a PDA plate, and incubated at 22°C. The colony diameter was measured perpendicularly every 24 h, and the two measurements were averaged. For bacterial testing, colonies were transferred to 1 ml of phosphate-buffered saline and shaken with a vor-

tex mixer. The bacterial suspension was 10-fold serially diluted. An aliquot of 20 μl of the various dilutions was placed on a chestnut-extract-amended YME plate using an Autoplate 4000 in the Uniform mode. Colonies were observed on the third day after incubation at 28°C in the dark. EC_{50} of the extract was determined using a regression equation. Each treatment was replicated three times. Additionally, factors including chestnut species, tissue, and high temperature were examined to determine activity of the inhibitory effect. This was done by evaluating EC_{50} s for the extract using *Pythium irregulare*, which grows rapidly, as a test organism. The experiments were all conducted twice.

Effect of chestnut tissues on *S. scabies* in soil. In a growth chamber, radish seed were sown in high-porosity professional potting soil (BACCTO Michigan Peat Company, Houston, TX) mixed with chestnut tissues (shell/pellicle complex of both chestnut species collected from peeling processing line) at 0:1, 1:8, 1:4, 1:2, 1:1, and 1:0 (chestnut tissue/potting soil = vol/vol), with four replications each. Prior to this soil mix, the chestnut tissue was ground using a Romer Series II mill (Romer Labs, Union, MO). In the case of a 1:0 ratio, only chestnut tissues were used without adding any potting soil. Growth chamber settings were 16 h of light and 8 h of dark, with light intensity of 200 $\mu\text{mol}/\text{m}^2/\text{s}$, 90% relative humidity, and temperature of 13 and 18°C for night and day, respectively. One week after seedling emergence, a spore suspension of *S. scabies* strain ATCC49173 was mixed with soil to a final concentration of 10^6 CFU/g soil. Six weeks after planting, when radish plants were mature, the roots were evaluated for scab symptoms according to established rating scales (33). The experiment was conducted twice.

Bioautography of antimicrobial compounds in chestnut pellicle extract. Thin-layer chromatography (TLC) was conducted on a 20-by-20-cm cellulose TLC plate (AnelTech, Inc., Newark, DE). In total, 10 μl of chestnut extract (851 $\mu\text{g}/\mu\text{l}$) (Colossal) of leaf, pellicle, and shell was spotted on the line of the TLC plate, 2 cm from the lateral edges. Ethanol (10 μl) was used for control. Samples were spotted at 2-cm spaced intervals. The TLC plate with chestnut extract was placed in a developing tank containing a solvent composed of ethylacetate/formic acid/glacial acetic acid/water at a ratio of 100:11:11:26 (by volume). The plate was air dried completely. Two TLC plates were simultaneously developed and each was divided with a pencil into 10 sections (2 cm/section).

One of the TLC plates evaluated the different tissue samples (leaf, pellicle, and shell) and was sprayed with a cell suspension of *B. mycoides* using a sterilized hand sprayer (Kemtech Glassware, 125 ml; VWR International, Inc. Batavia, IL) in a laminar flow hood. The TLC plate was incubated at 28°C in a moist plastic chamber. After 24 h of incubation, the plate was air dried to stop further growth of the bacteria. Cellulose powder of each section of the first TLC plate was removed using a metal blade. The powder was collected and placed into a 1.5-ml centrifuge tube, corresponding to the section. The cellulose powder was dissolved in 300 μl of sterile water and 20 μl of the suspension was placed on LB medium and spread with a sterile glass rod. Bacterial colonies were counted after 24 h of incubation at 28°C. The ratio of distance from sample spot to the center of each section and distance between sample spot to the front of the solvent (R_f values) were calculated.

The second TLC plate was developed without spraying bacteria. The section corresponding to the highest antimicrobial activity (shown with the first TLC plate above) was identified, and the cellulose powder in those areas were collected and placed into a 2-ml vial. The material was analyzed with a high-performance liquid chromatography-mass spectrometry (HPLC-MS) system at the Michigan State University Mass Spectrometry Facility. Chestnut pellicle extracts were dissolved in HPLC-grade methanol and filtered (0.22 μm , MILLEX-GS; Millipore Corporation). The final concentration was 1 mg/ml for subsequent MS injection and analyses. The sample was analyzed by LC/MS using a QToF Ultima API mass spectrometer coupled to a 2795 HPLC (Waters, Milford, MA), using electrospray ionization in both positive and negative

ion modes. The chemical separation was done with an Ascentis Express C18 column (2.1 by 50 mm, 2.7- μ m particle), at a flow rate of 0.3 ml/min and a binary linear gradient consisting of solvent A (0.1% formic acid in H₂O) and solvent B (methanol), programming from 95 to 10% A from 0 to 7 min and a hold at 10% A until 15.0 min; column temperature was 50°C.

Statistical analysis. SAS statistical software (version 9.4; SAS Institute Inc., Cary, NC) was used to analyze the data. Mixed model was used for variance analysis. Values of means were compared by an unpaired Student's *t* test or Fisher's least significance difference at significance level $\alpha = 0.05$. In determining EC₅₀, data of spore concentrations were transformed to log10 before analysis. If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

Results

Antimicrobial activity of chestnut tissues on microorganisms. To determine the best solvent for extracting potential inhibitors from chestnut tissues, acetone and ethanol extracts from the pellicle of wild Chinese and Colossal chestnut were compared in

Fig. 1. Effect of chestnut extracts on the growth of *Phytophthora capsici*, *Rhizoctonia solani*, and *Streptomyces scabies*, where extract is added to paper discs and placed on culture plates with each microorganism. Extracts were derived from pellicles of Chinese and 'Colossal' chestnut. Acetone and ethanol were used as extraction solvents. Bars on each column are standard deviation. Relative inhibition was calculated as $[100 \times (R_1 - R_2)/R_1]$. For *S. scabies* inhibition, R1 and R2 were the inhibition zones of chestnut extract treatment and ethanol control, respectively; for *R. solani* and *P. capsici*, R1 and R2 were the growth radius away and near the extract-treated filter paper disc, respectively. Data were analyzed on combined trials.

Table 2. Analysis of variance on the effect of chestnut extract against *Phytophthora capsici*, *Rhizoctonia solani*, and *Streptomyces scabies*

Organism, source	df	Mean square	F	P > F
<i>P. capsici</i>				
Cultivar	1	1.6875	7.28	0.010
Solvent	1	0.2408	1.04	0.314
Cultivar $\times$ solvent	1	0.5208	2.25	0.142
<i>R. solani</i>				
Cultivar	1	0.0408	0.20	0.653
Solvent	1	0.3008	1.51	0.226
Cultivar $\times$ solvent	1	1.8408	9.24	0.004
<i>S. scabies</i>				
Cultivar	1	0.1200	8.00	0.022
Solvent	1	0.0533	3.56	0.096
Cultivar $\times$ solvent	1	0.0533	3.56	0.096

disc assays against three microorganisms. Zones of inhibition appeared around discs of all three tested species. No consistent and significant difference was observed between acetone and ethanol pellicle extracts in this assay, except ethanol extraction of Colossal had higher inhibition levels than acetone extraction, tested against *R. solani* ($P = 0.005$) and *S. scabies* ($P = 0.001$). Accordingly, ethanol was used as the solvent in future experiments (Fig. 1; Table 2).

To determine whether the ethanol extract from pellicle tissue simply inhibited growth or if the extract was lethal to bacteria, Colossal pellicle extract was added to liquid cultures of *S. bottropensis* containing 10^8 spores/ml of similar age. After the extract was added, the bacterial spore population was negatively correlated with incubation time and extract dosage (*S. scabies* population $[\log \text{CFU/ml}] = 0.15 \times \text{incubation time [hours]} + 0.0189 \times \text{extract concentration}$ [$P < 0.0001$]; Fig. 2); as the concentration of pellicle extract increased and as the time of exposure increased, the bacterial populations decreased when compared with controls without pellicle extract (Fig. 2). Furthermore, when the treated bacterial cells were washed free of chestnut extract, the majority was not viable; nearly 100% of cells were dead when treated with the extract at 250 mg/ml or higher for both 24 and 36 h (Table 3). These results strongly suggest that the pellicle extract is lethal to preexisting cells. A similar trend was observed when *S. scabies* and *Streptomyces* sp. DS3024 were used as tester strains (data not shown).

Due to the nature of the disk assay, where inhibition of fungal mycelial growth was necessarily measured differently than bacterial inhibition, it was not possible to quantitatively compare effects of extracts on these microorganisms. Therefore, to standardize the

Fig. 2. Effect of chestnut ('Colossal') pellicle extracts on *Streptomyces bottropensis* population in yeast malt extract (YME) liquid medium. Chestnut pellicle extract was added into YME at various concentrations, and *S. bottropensis* was incubated for different periods of incubation. After incubation, 20 μ l of bacterial culture was plated on YME agar plates, and colonies were counted after 3 days of incubation and converted to CFU/ml.

Table 3. Growth inhibition of *Streptomyces bottropensis* in chestnut ('Colossal') pellicle extracts after 24 and 36 h of incubation

Conc. (mg/ml) ^y	24 h		36 h	
	Inhibition (%)	SD ^z	Inhibition (%)	SD ^z
0	0	0.00	0	0.00
50	67.5	1.10	97.4	1.65
250	99.9	0.03	99.7	0.70
400	100	0.00	100	0.00

^y Concentration of chestnut extract.

^z Standard deviation.

effect of pellicle extract on a wide array of microorganisms, the EC₅₀ assay was used. A variety of microorganisms, most considered soilborne saprophytes, and plant pathogens were used as test strains (Table 1). The calculated EC₅₀ of the fungi and bacteria made it possible to directly compare inhibition among fungi, oomycetes, and bacteria. In this assay, bacteria were more sensitive to chestnut extract than fungi and oomycetes. The streptomycetes, pseudomonads, and bacilli were some of the microorganisms most sensitive to the pellicle inhibitor. *Verticillium dahliae*, *Cladosporium cucumerinum*, and *Trichoderma harzianum* were the three fungal species most sensitive to the inhibitor but were less sensitive than the most sensitive bacterial strains tested. *Phytophthora cambivora* was less sensitive to the pellicle extract than *P. cinnamomi*, and was the least sensitive microorganism tested other than the chestnut blight pathogen, *Cryphonectria parasitica*. Interestingly, the *P. cambivora* strain tested in Table 1 was isolated from roots of Colossal trees in Indiana (D. W. Fulbright, unpublished).

To learn more about the inhibitor found in tissues of *Castanea* spp., a series of experiments were conducted to determine activity of the inhibitory effect using *P. irregulare*. When leaf extract from wild Chinese chestnut was compared with leaf extract activity of Colossal, the extract from Colossal inhibited *P. irregulare* significantly more than leaf extract from Chinese trees. Similarly, extract from Colossal pellicle significantly inhibited *P. irregulare* more than extract from pellicle of wild Chinese chestnut, although the difference appears to be biologically negligible (Table 4).

To compare leaf, shell, and pellicle tissue extracts for inhibitory activity against *P. irregulare*, data of wild Chinese and Colossal extracts from the respective tissues were combined for EC₅₀ because there was no interaction between cultivar and tissue. In this assay, all tissues showed inhibition to *P. irregulare*, and pellicle extract showed significantly more inhibition than either shell or leaf extract. However, when leaves were extracted with water instead of ethanol, a large portion of the inhibitory activity was lost (Table 4).

Because extreme heat is often used in the process of peeling chestnuts, it was important to determine whether high temperatures could reduce the inhibitory effect of extracts from chestnut shells and pellicle. Pellicle tissue was autoclaved and then extracted with ethanol. When compared with the nonautoclaved pellicle tissue, the autoclaved extract maintained its inhibitory activity against *P. irregulare*. However, when hand-peeled chestnuts were compared with machine-peeled chestnuts, the machine-peeled chestnuts had significantly less activity than hand-peeled chestnuts, indicating that some but not all activity was lost (Table 4).

Inhibitory effect of chestnut tissue on *S. scabiei* in soil. To determine whether mixtures of *Castanea* spp. shells and pellicles could affect the outcome of disease in a soilborne pathogen environment, a disease assay using radish seedlings and *S. scabiei*

was conducted in the greenhouse. Data were combined from the trials because there was no interaction between trials and inhibition detected. Disease severity on radish roots was greatly reduced when chestnut shell mixtures were incorporated in the soil and was negatively correlated with the amount of chestnut shell material applied (Fig. 3).

Bioautography of antimicrobial compounds in chestnut pellicle extract. To isolate the compound or compounds involved in inhibition, *B. mycoides* was sprayed over the developed plates to locate zones inhibitory to this bacterium. The plates were divided into 10 sections (2 cm/section) from top to the bottom and *B. mycoides* was recovered from most of the areas on the TLC plates. An inhibitory zone, with Rf value 0.74, was observed when *B. mycoides* samples were cultured, indicating the presence of antimicrobial compounds (Table 5).

Not surprisingly, when the TLC plates of *Castanea* sp. extracts from pellicle, shell, and leaf tissues were compared by position, *B. mycoides* was inhibited in the same relative zone with Rf 0.74, regardless of the tissue extract that was spotted on the plates (Table 5). This strongly suggests that pellicle, shell, and leaf tissues produce similar antimicrobial compounds.

To follow up this possibility, the compounds from the inhibitory zone were collected for further identification using HPLC/high-resolution MS. The active fraction was found to have about 12

Fig. 3. Inhibitory effect of chestnut tissue on radish scab disease in greenhouse assays. Data were combined from Chinese and 'Colossal' chestnut (three replications per chestnut type). Chestnut tissue was incorporated in potting soil at various ratios and was infested with *Streptomyces scabiei* before planting radish seed in pots. Data represent two combined trials.

Table 4. Factors that impact effectiveness of chestnut extracts in inhibiting *Pythium irregulare*

Source of chestnut tissue ^x	N ^y	Factors for comparison	EC ₅₀ (μg/μl) ^z
Leaf extract of Chinese and 'Colossal' chestnut	6	Water as solvent	216.0 a
		Ethanol as solvent	36.5 b
Leaf	6	Chinese chestnut	53.3 a
		Colossal chestnut	36.5 b
Pellicle	12	Chinese chestnut	29.3 a
		Colossal chestnut	25.5 b
Chinese and Colossal chestnut	24	Leaf	44.9 a
		Shell	42.8 a
		Pellicle	26.4 b
Colossal chestnut shell and pellicle	15	Hand peeling	24.9 b
		Machine peeling	43.7 a
Colossal chestnut pellicle	6	Autoclaved	24.7
		Nonautoclaved	24.1

^x Chestnut tissues from Chinese species (*Castanea mollissima*) and hybrid of Japanese × European (*C. crenata* × *C. sativa*) ('Colossal') were used.

^y Number of samples examined.

^z Effective concentration for 50% growth inhibition (EC₅₀) based on two trials with three replications. Fisher's least significant difference was performed to compare the effect in each group. The nut was hand peeled except in comparison of peeling method. Extraction was done with solvent ethanol except in comparison of solvent. Means followed by same letters are not significantly different at level α = 0.05.

peaks in the negative-mode LC/MS chromatogram, with molecular masses of 350 to 750 Da. After excluding substances present in the blank TLC control, two substances with molecular masses and relative mass defects (30) consistent with flavonol glycosides (m/z 459.16 and 577.06) were observed. Several additional substances (m/z 403.27, 397.25, and 735.39) consistent with terpenoid conjugates were also present.

Discussion

This study corroborates previous work demonstrating the presence of antimicrobial compounds from tissues of *Castanea* spp. (24); however, the extracts caused differential reactions from the various microorganisms tested. We have found that inhibition to the test microbe occurred where a flavonol glycoside and several terpenoid substances were detected, indicating that this group of compounds is probably responsible for the inhibition. Flavonol glycosides, and tannins and flavonoids that Li and Song detected (24), are all polyphenol compounds, and may have similar function for antimicrobial activities. Terpenoids have been associated with antimicrobial and plant defense-enhancing activities (16).

Because different media were required for growth by different microorganisms, the comparison of the sensitivity of the microorganisms to chestnut extract was difficult to statistically evaluate; however, by comparing the EC_{50} values, there was a trend that bacterial species were more susceptible than fungal species and both gram-positive and -negative bacteria were inhibited.

This report verifies the presence of antimicrobial activity in extracts of pellicle, the thin fibrous tissue surrounding the kernel (13), and demonstrated that this tissue has more activity per unit of mass than the shell or leaves. This study also extends the type and number of microorganisms that are inhibited because earlier work focused on food-borne microorganisms (24). Our study focused on fungal and bacterial species frequently isolated from the soil environment, including microorganisms known to cause plant diseases. This is also the first report to determine that inhibitors found in the pellicle, shell, and leaves could be extracted from two different *Castanea* spp., Chinese chestnut and the Japanese/European chestnut hybrid Colossal. Colossal demonstrated higher antimicrobial activity than the Chinese chestnut species.

It is plausible that preformed antimicrobial compounds in the shell and pellicle of chestnut seed play a role in seed survival in the environment. For example, preformed, defense-related antimicrobial compounds such as flavonoids and antioxidants have been shown to play a role in protecting fruit from premature decay (14,20,21,25,26,35). To compete with other species in nature, the American chestnut tree (*Castanea dentata*) produces allelopathic substances demonstrated in the Appalachian Mountain ecosystem (31); however, it is not known whether the compounds causing allelopathy are the same as those inhibiting plant pathogens in this study.

Our interests in understanding more about the microbial inhibitory effects and the compounds responsible for them are based on our interest in utilizing chestnut shell material from the chestnut peeler as a biomaterial for compost. Because the peeling unit re-

quires extreme heat from a propane burner, followed by a hot water treatment up to 80°C, it was important to determine whether inhibitory activity in the shell and pellicle would be lost after brulage peeling. Based on our results, this should not be a problem because it appears that the peeling machine reduced the antimicrobial activity of the shell but did not entirely eliminate it. This reduction could be due to the partially burned nature of the chestnut tissues affecting extraction efficiency. Autoclaving the extract did not reduce the activity; therefore, it is likely the 80°C water treatment would have little or no effect on the inhibitory activity of shell and pellicle. It is possible that the reduction of antimicrobial activity resulted from the intense heat of the propane burner.

Because *S. scabies*, causal agent of potato and radish scab, was shown to be sensitive to the chestnut shell or pellicle extracts, we attempted to determine whether the chestnut tissue could be used as a soil-improving biomaterial. Our data showed that this amendment suppresses radish scab in greenhouse assays, and use of this material for future studies on other scab diseases will be pursued.

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Table 5. Bioautography using thin-layer chromatography (TLC), with developing solvent of ethylacetate/formic acid/glacial acetic acid/water at a ratio of 100:11:11:26 (by volume) for identifying the spot with highest antimicrobial activity

Pathogen	Extract of chestnut	Inhibition (%) ^y	Rf value ^z
<i>Bacillus mycoides</i>	Leaf	52.8	0.74
	Pellicle	88.6	0.74
	Shell	80.6	0.74
<i>Streptomyces bottropensis</i>	Pellicle	96.4	0.74

^y Inhibition was calculated as percentage of CFU of test organism on a designated zone relative to a zone without sample.

^z Rf = ratio of distance from sample spot to center of the section over the distance from sample spot to front line of solvent on TLC plate.

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