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Phytotoxic and Antimicrobial Activities of Catechin Derivatives

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(±)-Catechin is a potent phytotoxin, with the phytotoxicity due entirely to the (−)-catechin enantiomer. (+)-Catechin, but not the (−)-enantiomer, has antibacterial and antifungal activities. Tetramethoxy, pentaacetoxy, and cyclic derivatives of (±)-catechin retained phytotoxicity. The results indicate that antioxidant properties of catechins are not a determining factor for phytotoxicity. A similar conclusion was reached for the antimicrobial properties. *Centaurea maculosa* (spotted knapweed) exudes (±)-catechin from its roots, but the flavanol is not re-absorbed and hence the weed is not affected. The much less polar tetramethoxy derivative may, however, be absorbed and hence be able to cause toxicity. Because of the combination of phytotoxicity and antimicrobial activity, (±)-catechin could be a useful natural herbicide and antimicrobial.

KEYWORDS: Catechin derivatives; herbicide; phytotoxicity; antimicrobial activity

INTRODUCTION

Natural products have become increasingly more sought after as ecologically safer alternatives to herbicides and pesticides, as well as a source of new medicines. However, the biological activity of these compounds is often affected by their stereochemistry, lipophilicity, and other factors. Slight modifications can alter the bioactivity either positively or negatively. For example, (−)-boscialin, which is a natural product found in a number of medicinal plants, was less active against various bacterial and fungal pathogens than its (+) isomer, whereas (−)-epiboscialin was more active than (+)-epiboscialin (*1*). This structure-dependent activity has also been noted in a number of synthetic molecules used as herbicides and insecticides (*2*−*4*).

Recently, we have reported that (±)-catechin is secreted into the soil rhizosphere from the roots of an invasive weed, *Centaurea maculosa* L. (*5*). Bioassays revealed that (−)-catechin, (−)-**1**, but not (+)-catechin, (+)-**1**, is potently phytotoxic (*5*, *6*). (−)-Catechin inhibits seed germination in addition to killing plant seedlings via the roots and is as potent as the herbicide 2,4-D when applied to broad-leaf plants. When naturally secreted by the roots of *C. maculosa*, (−)-catechin undoubtedly plays a role in the invasive behavior of the plant. Because catechins are present in many plants, including food plants, the use of either (±)- or (−)-catechin as a herbicide could be ecologically benign.

Alternatively, (+)-catechin produced by the plant, but not (−)-catechin, was found to have strong antimicrobial properties (*5*, *6*). (+)-Catechin is also a well-known antioxidant free radical scavenger, and antifungal, antitumor, and insect repellant properties have been attributed to it as well. Previous papers have speculated that the antioxidant properties of (+)-catechin are increased by converting the freely rotating, flexible catechin to a rigid, planar molecule (*7*).

We have begun a series of studies to explore relative phytotoxicity and antimicrobial potencies among these and other common flavonoids. The flavones kaempferol and dihydroquercetin and the flavanol (+)-epicatechin were phytotoxic, but less so than (−)-catechin, whereas quercetin, naringenin, and (−)-epicatechin were devoid of such activity (*6*). To investigate structure−activity relationships for root phytotoxicity and antimicrobial activity among the catechins, we compared several synthetic derivatives of (+)- and (±)-catechin (**Figure 1**) for these properties.

MATERIALS AND METHODS

Plant Culture Conditions. Seeds of *C. maculosa* were obtained from natural populations in Missoula, MT, and seeds of *Potentilla arguta* were obtained from Dr. Ruth Hufbauer (Colorado State University). Seeds of *Gaillardia aristata*, *Arabidobsis thaliana* (Col-0), *Lycopersicum lycopersicon*, and *Ocimum basilicum* were purchased from Wind River Seed (Manderson, WY), Lehle Seeds (Round Rock, TX), The Rocky Mountain Seed Co. (Denver, CO), and Shepherd's Garden Seeds (Torrington, CT). Seeds were washed in running tap water and surface sterilized using sodium hypochlorite (0.3% v v^{-1}) for 30 min, followed by three or four washes in sterile distilled water. Surface-sterilized seeds were inoculated on static Murashige and Skoog (MS) (*8*) basal medium in Petri dishes for germination. Seeds were allowed to germinate for 10 days after roots and shoots emerged. The temperature within the growth chamber was 27 ± 2 °C, and the light intensity was 4.41 J m^{-2} s^{-1}. Ten-day-old seedlings were transferred to sterile 50 mL culture tubes with 5 mL of liquid MS basal medium.

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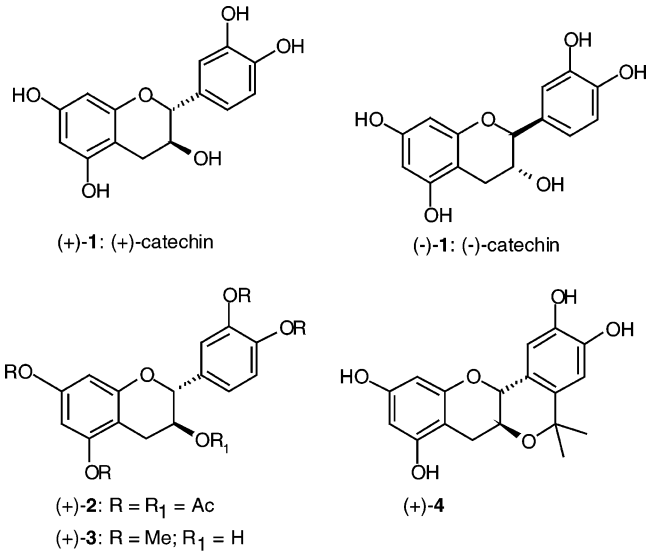

Figure 1. Structures of catechins and derivatives.

Plants were then allowed to stabilize in the liquid medium for 5 days prior to treatment with (+)/(±)-catechin derivatives.

Plant Growth Inhibitory Bioassay. A stock solution of 10 mg mL^{-1} of each catechin (Sigma, St. Louis, MO) and catechin derivative was prepared in methanol. To ensure that maximum activity was retained, fresh stock was prepared prior to each experiment. The solutions were filter sterilized using 0.22 μm filters (Millipore, Cork County, Ireland) and added to the liquid media containing the various plant species at concentrations of 10, 50, 100, and 200 μg mL^{-1}. Control plants were treated with a filter-sterilized solution of methanol added at the same volume as the highest concentration of treated plants. Plant cultures were maintained at 27 ± 2 °C on an orbital platform shaker set at 90 rpm and exposed to 24 μmol m^{-2} s^{-1} light intensity for 7 days. After 7 days, root length, shoot number, and fresh weight were recorded, and all plants were scored for mortality.

Antibacterial Assays. Bacterial isolates from a broad phylogenetic range were tested for inhibition of growth with catechin and synthesized catechin derivatives. Initial stock solutions of (1 mg mL^{-1}) of each compound were prepared in dimethyl sulfoxide (DMSO). Bacterial suspension cultures of *Erwinia carotovora*, *Erwinia amylovora*, *Xanthomonas campestris* pv. *vesicatoria*, and *Pseudomonas fluorescens* were grown overnight at 37 °C to $OD_{600} = 0.2$. Assays were performed in 96-well, sterile, flat-bottom microtiter plates (Nalge Nunc International, Roskilde, Denmark). Test wells contained 5 μL of the bacterial suspension and serial dilutions of the tested derivatives ranging from 100 to 0.05 μg. Control wells contained 5 μL of bacterial isolates alone with the highest volume of DMSO used or serial dilutions of a 1 mg mL^{-1} stock solution of (+)-catechin, which has known antifungal activity (*2*). Bacterial isolates were incubated at 37 °C for 24 h. The absorbance of each well was determined at OD_{600} nm with an Opsys MR, microtiter plate reader (Dynex Technologies). Each isolate was tested against all compounds at the total range of concentrations (100−0.05 μg) in two separate replicates.

Fungal Bioassay Procedures. Five fungal isolates, *Aspergillus niger*, *Trichoderma reesi*, *Trichoderma viridens*, *Pennicillium* sp., and *Fusarium oxysporum*, were tested for inhibition of spore germination with synthetic derivatives of catechin. Initial stock solutions of (1 mg mL^{-1}) of each derivative were prepared in DMSO. Fungal isolates were maintained on potato dextrose agar (PDA) in the dark at 24 °C until sporulation occurred. Spore suspensions were prepared from 4-week-old fungal cultures by rinsing the plates with 5 mL of sterile distilled water. Spore suspension concentration was estimated using an Ultra Plane Improved Neubauer cell counting chamber (Scientific Products, West Sussex, U.K.) and adjusted to 1×10^5 spores mL^{-1} with sterile distilled water. Fungal spore germination assays were performed in 96-well, sterile, flat-bottom microtiter plates (Nalge Nunc International). One hundred microliters of the 1 mg mL^{-1} stock solutions of catechin derivatives was added to the liquid PDB media and serially diluted to provide tested concentrations ranging from 0.05 to 100 μL. One hundred microliters of the spore suspensions was then added to the wells to determine the minimum concentration of each derivative required to inhibit fungal germination. Control wells contained 100 μL of fungal spores alone with the highest volume of DMSO used or serial dilutions of a 1 mg mL^{-1} stock solution of (+)-catechin. The plates were covered with sterile lids and placed in a polystyrene box lined with moistened filter paper to maintain high humidity and incubated at 37 °C. The absorbance of each well was determined at OD_{630} nm with an Opsys MR, microtiter plate reader (Dynex Technologies) after 96 h of incubation.

Microscopy. To check activity against bacterial plant pathogens, bacterial cells in microtiter plates treated with the catechin derivatives were stained with Molecular Probes *Bac*Light bacterial viability kit (Eugene, OR) by incubation at room temperature in the dark for 20 min, according to the manufacturer's manual. The samples were mounted with Citifluor antifading (Sigma) and observed for fluorescence with a fluorescent microscope (Fluroview LGPS-2, Olympus).

Synthesis of Pentaacetylcatechins, (+)- and (±)-2. (+)-Catechin (Sigma, 100 mg) was dissolved in 0.5 mL of pyridine, and 0.5 mL of acetic anhydride was added; the solution was allowed to stand overnight at room temperature. The solution was then poured into 15 mL of water and extracted with EtOAc (10 mL × 3). The extract was evaporated to yield (+)-**2** (104 mg), the ^1H and ^{13}C NMR and mass spectra of which were identical to those in the literature (*9*). Acetylation of (±)-catechin (Sigma) was similarly carried out to yield (±)-**2**.

Synthesis of Tetramethoxycatechins, (+)- and (±)-3. A mixture of (+)-catechin (100 mg) and trimethylsilyldiazomethane (2.0 M solution in *n*-hexane, 5 mL) in methanol (5.0 mL) was stirred at room temperature overnight. After the excess of trimethylsilyldiazomethane was decomposed with acetic acid, the reaction mixture was evaporated to dryness. The residue was purified by column chromatography using silica gel with hexane/ethyl acetate (80:20) to yield (+)-**3** (45 mg), the ^1H and ^{13}C NMR spectra of which were identical with those in the literature (*10*). Similarly, methylation of (±)-catechin yielded (±)-**3**.

Synthesis of 6*a*,12*a*-*trans*-2,3,8,10-Tetrahydroxy-5,5-dimethyl-5,6*a*,7,12*a*-tetrahydro[1]benzopyrano[3,2-*c*][2]benzopyrans (+)- and (±)-4. These were prepared similarly to literature methods (*7*, *11*, *12*). (+)-Catechin (100 mg) was dissolved in moist acetone. *p*-Toluenesulfonic acid (60 mg) was added and the solution stirred for 3 days at room temperature. The solvent was evaporated (N_2 gas) and the mixture subjected to preparative layer chromatography in $CHCl_3$/MeOH (90:10) to yield (+)-**4** (18 mg), 0.35 R_f, the ^1H and ^{13}C NMR and mass spectra of which were identical to those in the literature (*7*, *11*, *12*). Similarly, (±)-catechin was converted to (±)-**4**.

Statistical Analysis. Due to the incremental nature of the treatment concentrations, standard deviations for bacterial minimum inhibitory concentrations (MICs) are expressed as ±5% of the mean MIC, and all other standard deviations are calculated on the basis of the square root of the variance from the mean.

RESULTS AND DISCUSSION

Comparative phytotoxicities at the 200 μg mL^{-1} level, as measured by inhibition of shoot and root differentiation, varied depending on both derivative structure and test plant (**Figure 2**). The MICs for some derivatives against some plants were as low as 50 μg mL^{-1}; however, the results at 200 μg mL^{-1} allowed presentation of all data on a single graph (**Figure 2**). All of the synthesized derivatives were screened for phytotoxicity. The general results were that only (±)-**3** and (±)-**4** were active phytotoxins, usually equal to the potency of (−)-catechin (**Figure 2**). Because the same derivatives of (+)-catechin were inactive, the inhibitions observed for the (±)-derivatives underestimate the true activity of (−)-catechin by about half. One of the more striking results was the inhibition of root differentiation of *C. maculosa* by the (±)-**3** and (±)-**4** derivatives (**Figure 2B**). *C. maculosa* is very resistant to its own exuded

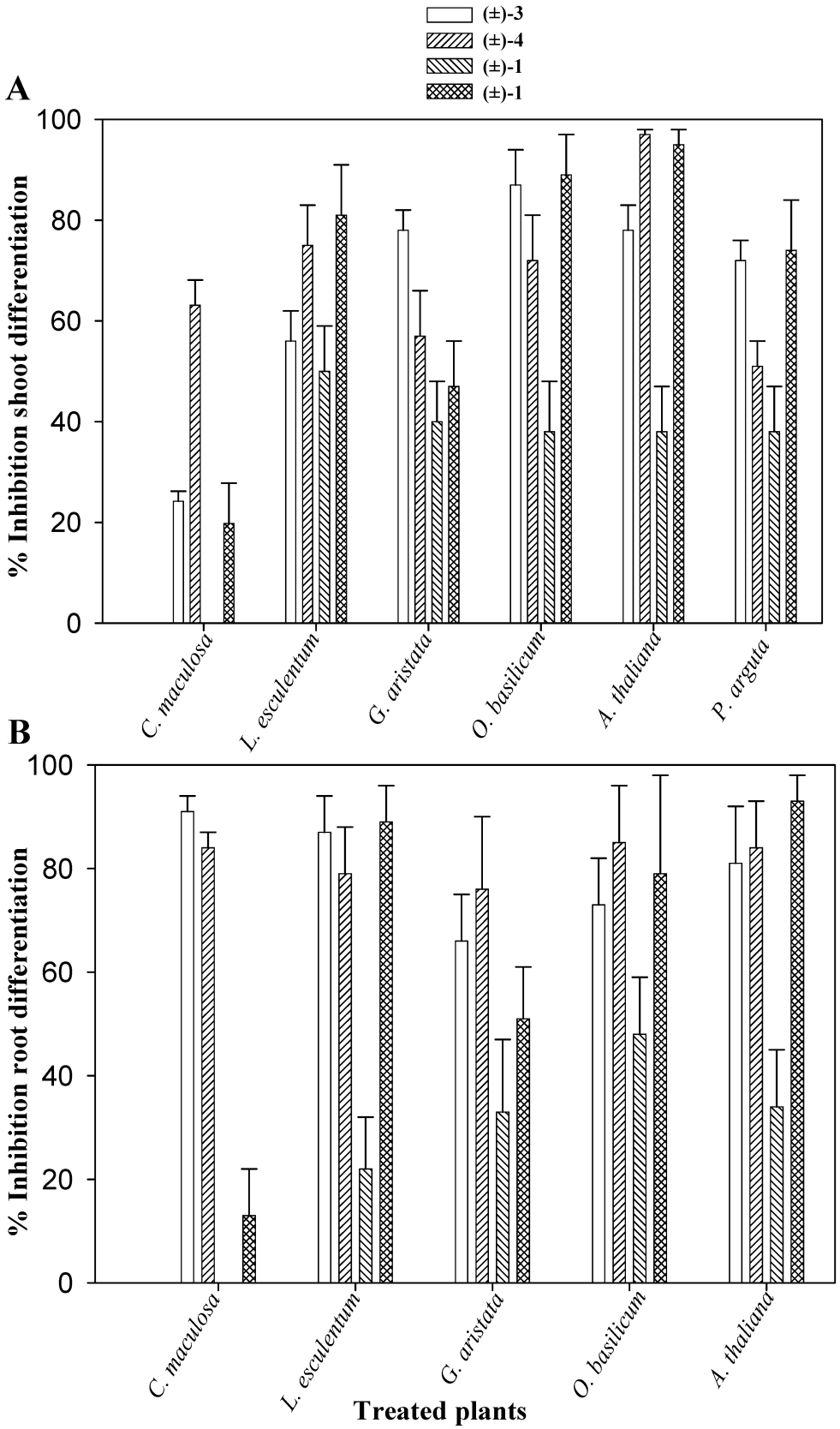

Figure 2. Effect of 200 μg mL^{-1} of (–)-catechin (**1**), (±)-**1**, tetramethoxycatechin [(±)-**3**], and the isopropylidylcatechin [(±)-**4**] on shoot (**A**) and root (**B**) differentiation of selected plant species on the seventh day after treatment. Data represent the percent inhibition relative to the untreated control in shooting and rooting efficiency response in various tested seedlings. Values are mean ± SD, $n = 5$.

(−)-catechin (*5*), but here we find that root differentiation is potently inhibited (**Figure 2B**) by both (±)-**3** and (±)-**4**. Although *C. maculosa* is not susceptible to exogenous (−)-catechin in the growth medium, if the flavanol is directly injected into plant cells, mortality occurs (*13*). Thus, it is possible that conversion of (−)-catechin to the much less polar methoxy and acetyl derivatives now allows penetration of the phytotoxin into the root, thus inhibiting growth at the cellular level.

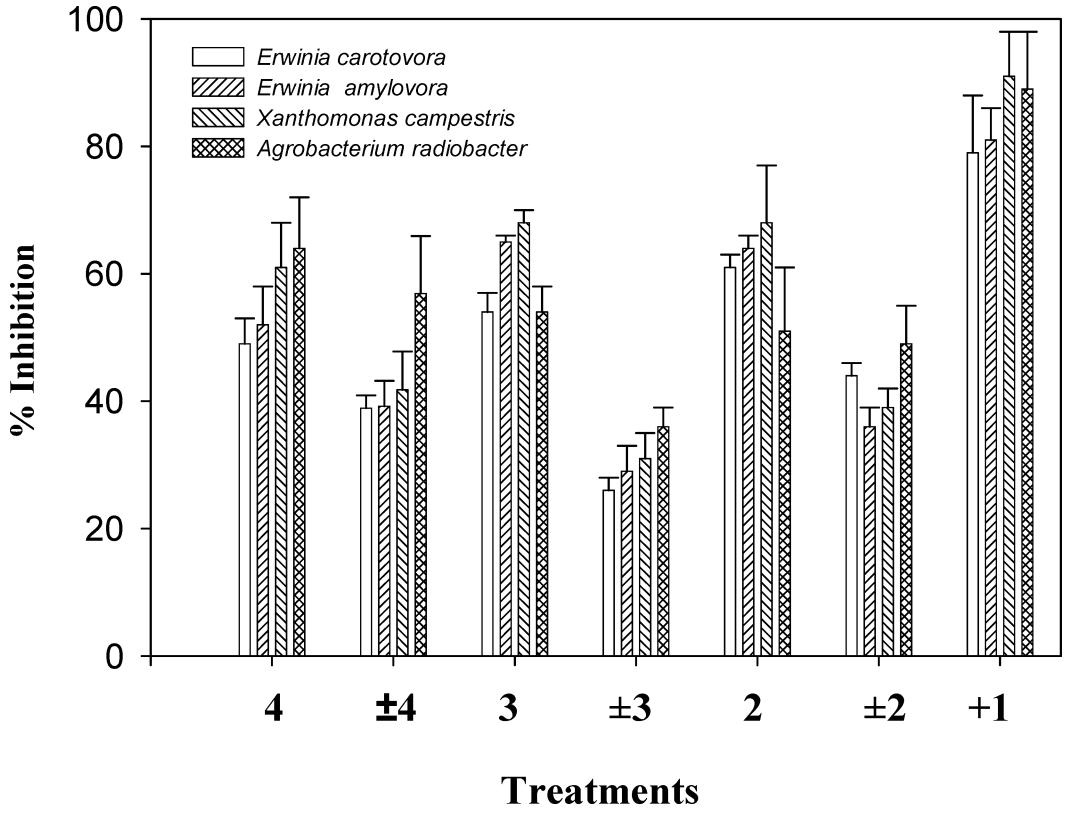

Figure 3. Antibacterial activities of (+)-catechin (**1**), pentaacetylcatechin -(+)- and (±)-**2**], tetramethoxycatechin [(+)-**3** and (±)-**3**], and the isopropylidylcatechin [(+)-**4** and (±)-**4**] on different bacteria. The concentration of compounds was administered at one dose below their respective MIC levels (see **Table 1**). Net bacterial growth was calculated by subtracting the OD_{600} at the beginning from the OD_{600} after 24 h of incubation. Percent inhibition (%I) was calculated using net bacterial growth based on OD_{600} readings with the following formula: [(untreated − treated)/untreated] × 100. Values are mean ± SD, $n = 5$.

The catechins are widely known as strong antioxidants, with those properties being derived from the ortho-disubstituted phenolic groups (*14* and references cited therein). Blocking these functions with methyl groups [as in (±)-**3**] removes the antioxidant properties. Because (±)-**3** was still potently phytotoxic, this property must not stem from antioxidant activity.

That bioactivity of catechins, in some cases, is not dependent on antioxidant power has also been noted by others (*15*, *16*).

The enantiospecificity of phytotoxicity suggested an important role for the C-2/C-3 stereochemistry and/or the presence of a free 3-OH group. Compound (±)-**4** has a blocked 3-OH group and, because it is planar (*13*), has an altered spatial arrangement between the B and C rings. Because (±)-**4** was active in all systems (**Figure 2**), it is clear that a free 3-OH is not necessary and that some modification of spatial relationships in the C-2/C-2′ region (through cyclization) is also not detrimental.

(+)-Catechin had significant antibacterial activity against all four organisms and was similarly potent against each (**Figure 3**). All of the (+)-derivatives were also active, but none were quite as active as (+)-catechin. The activities of (+)-**2**, (+)-**3**, and (+)-**4** were essentially equal to each other. As compared to (+)-catechin, the absence of phenolic character as in (+)-**2** and (+)-**3** or lack of a free C-3 OH as in (+)-**2** and (+)-**4** had some detrimental effect on activity but did not eliminate it. In each case the (+)-derivative was, as expected, more active than the (±)-derivative. Fluorescence microscopy of bacterial cultures treated with the synthetic catechin derivatives and stained with the *Bac*Light bacterial viability kit revealed that all of the tested derivatives had bactericidal activity. **Figure 4** shows an example for compound (+)-**4**.

The potent antibacterial activity of (+)-catechin does not appear to have been previously recognized. This may stem from a general focus on Gram-positive bacteria of direct importance in human health and the fact that (+)-catechin has little or no effect on those organisms previously tested. Thus, (+)-catechin was reported to be completely inactive against *Bacillus cereus*, *Staphylococcus aureus*, *Staphylococcus coagulans*, and *Citrobacter freundii* and showed only minimal activity against *Escherichia coli* (*17*). Very weak activity was reported against *Lactobacillus rhamnosus*, but none against other *Lactobacillus* species, *E. coli*, *Salmonella enterica*, *Enterococcus faecalis*, or *Bifidobacterium lactis* (*18*). These results are to be contrasted with those of **Table 1**, where potent activity was observed against a group of Gram-negative plant pathogens. It seems likely that part of the ecological imperative (*13*) for *C. maculosa* invasiveness is its root exudation of a potent inhibitor of such pathogens.

(+)-Catechin and the derivatives were also tested for antimicrobial activity against root-colonizing fungi: *Trichoderma reesi*, *Trichoderma viridins*, *Fusarium oxysporum*, *Aspergillus niger*, and *Penicillium* sp. (+)-**1** was active against all five fungi at 12.5−25 μg mL^{-1}, (+)-**2** was active only against *F. oxysporum* (25 μg mL^{-1}), and (+)-**3** showed activity against *Penicillium* sp. and *A. niger* (25 μg mL^{-1}). None of the other derivatives showed antifungal activity. (+)-Catechin was previously reported to be inactive against the crop plant fungi *Botrytis cinerea*, *Cladosporium echinulatum*, and *Penicillium griseofulvum* (*17*). Our results extend the view that (+)-catechin exudation can be an ecologically important plant defense.

Both (±)- and (−)-catechin are commercial products and hence could be available as herbicides. As a very rare plant

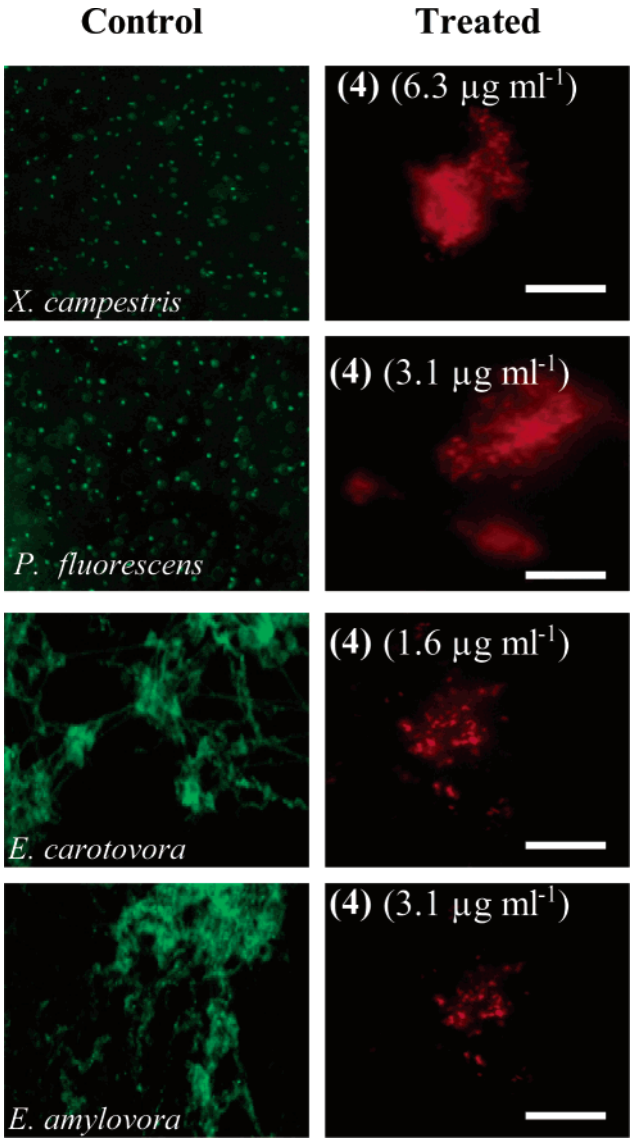

Figure 4. Fluorescence microscopic visualization of live–dead staining to show bactericidal activity of the isopropylidylcatechin, (+)-**4**, against different bacteria in a titer plate assay. Bacterial suspensions were treated with the MIC of (+)-**4** against each organism and stained with propidium iodide and SYTO9 for visualizing the polysaccharides and nuclei, respectively. Green fluorescence depicts live material surrounding/inside the bacterial colony. Red fluorescence shows dead cells. The scale bars indicate 20 μm.

Table 1. Minimum Inhibitory Concentration (MIC), Expressed in Micrograms per Milliliter, of (+)-**1** and Synthetic Derivatives against Various Plant Pathogenic Bacteria (Standard Deviations Are ±5% of the Mean)

tested compd	*E. carotovora*	*E. amylovora*	*X. campestris*	*P. fluorescens*
(+)-**4**	1.6 ± 0.08	3.1 ± 0.16	6.3 ± 0.32	3.1 ± 0.16
(±)-**4**	12.5 ± 0.63	12.5 ± 0.63	12.5 ± 0.63	12.5 ± 0.63
(+)-**3**	6.3 ± 0.32	25 ± 1.25	25 ± 1.25	6.3 ± 0.32
(±)-**3**	6.3 ± 0.32	12.5 ± 0.63	12.5 ± 0.63	12.5 ± 0.63
(+)-**2**	6.3 ± 0.32	6.3 ± 0.32	6.3 ± 0.32	6.3 ± 0.32
(±)-**2**	25 ± 1.25	25 ± 1.25	25 ± 1.25	12.5 ± 0.63
(+)-**1**	1.6 ± 0.08	1.6 ± 0.08	1.6 ± 0.08	1.6 ± 0.08

compound, the price of (−)-catechin is currently very high, but it could be made semisynthetically by epimerization of the more common and cheaper (−)-epicatechin (*19*). On the other hand, commercial development of (±)-catechin would provide a compound with both potent herbicidal and antimicrobial activities.

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