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ARTICLE

Study on the Anthraquinones Separated from the Cultivation of *Trichoderma harzianum* Strain Th-R16 and Their Biological Activity

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The biocontrol fungal species of *Trichoderma*, which colonizes plant roots, are well-known for their potential to control plant pathogens. Six anthraquinones, of which four have been identified for the first time from *Trichoderma* and two have already been reported in other strains, were purified from *Trichoderma harzianum* strain Th-R16 to evaluate their biological activities. The structures of the compounds were determined by one- and two-dimensional NMR. The compounds were shown to exhibit stronger antifungal activity than antibacterial activity. Low yield compounds, like 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, were found to be more active against fungal pathogens than pachybasin and crysophanol, which were found to be the major extracellular metabolites. Test anthraquinones with higher oxidation numbers had better antifungal activity, and their activities were concentration-dependent.

KEYWORDS: *Trichoderma*; anthraquinones; structure activity relation

INTRODUCTION

The deuteromycetes fungi *Trichoderma* are proven to be a potential source of bioactive molecules (*1*−*5*). The secondary metabolites of *Trichoderma* confer the biocontrol activity of *Trichoderma* either by directly inhibiting pathogens (direct antagonism) of the host or by inducing host plant resistance (*6*). Anthraquinones, pyrones, viridins, butenolides, diketopiperazines, ergosterol derivatives, and peptaibols, few among the vast group of secondary metabolites isolated from various *Trichoderma* spp., are reported to possess antimicrobial activities (*7*).

Anthraquinones represent an important group of natural products occurring in bacteria, fungi, lichens, and higher plants. Since the first isolation of pachybasin, chrysophanol, and emodin from *Trichoderma*, several other anthraquinones have been subsequently isolated from *Trichoderma*, and their biological activities have been investigated. Anthraquinones exhibit a variety of biological functions, such as serving as laxatives, diuretics, phytoestrogens, immune stimulators, and antifungal, antiviral, and anticancer agents (*8*−*17*).

Although anthraquinones have been isolated from various strains of *Trichoderma*, their biological functions are poorly understood. Meanwhile, the potential for discovery of more novel bioactive compounds from *Trichoderma* still remains large. *Trichoderma harzianum* strain Th-R16 has been reported as a strong biological agent capable of controlling several soil-borne diseases and enhancing plant growth (*18*, *19*). In this investigation, six anthraquinones from *T. harzianum* strain Th-R16 were isolated, and their roles in pathogen control were evaluated. The results suggest that some but not all of the six anthraquinones are involved in the direct inhibition of plant pathogens. The antimicrobial activity of the anthraquinones isolated was dependent on their oxidation levels. Anthraquinones with higher oxidation levels had higher bioactivities.

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MATERIALS AND METHODS

General Experimental Procedures. The structures of the compounds were determined on a Bruker AM-400 spectrometer with $CDCl_3$/acetone-d_6. Chemical shifts are shown in δ values with tetramethylsilane as the internal reference. Spectra of pure compounds were processed using Bruker one-dimensional WIN-NMR or two-dimensional WIN-NMR software. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^{1}H, ^{13}C, distortionless enhancement by polarization transfer, ^{1}H−^{1}H correlation spectroscopy, 23 ^{1}H−^{13}C direct correlation (heteronuclear multiple quantum coherence), or ^{1}H−^{13}C long-range correlation (heteronuclear multiple bond correlation). EI-mass spectra (electron impact ionization) were recorded on a Finnigan/Thermo Quest MAT 95XL mass spectrometer at the Department of Chemistry, National Chung Hsing University. The IR spectrum was recorded on a Jasco IR Report-100 spectrometer by KBr pellet press and calibrated by polystyrene film. UV/vis spectra were recorded, using methanol as the solvent, on a Hitachi UV-3100 spectrophotometer. Thin-layer chromatography (TLC) analysis was performed on silica gel Kieselgel 60G F_{254} (Macherey Negal). *Staphylococcus aureus* ATCC 10781, *Bacillus cereus* F4810, *Pseudomonas aeruginosa* ATCC 10145, and *Escherichia coli* ATCC 29522 were provided by

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Dr. Lin Shih-Bin, Department of Food Science, National Ilan University (Taiwan). All of the chemicals used were purchased from Sigma (St. Louis, MO).

***T. harzianum* Cultivation.** *T. harzianum* strain Th-R16 was maintained on potato dextrose agar (PDA) until sporulation. The conidia were flushed with 10 mL of deionized water and then poured (10^6 spores/mL of water) into a mixture containing 100 g of dehydrated, pulverized sugar cane bagasse and 600 mL of liquid culture medium [$CaCl_2 \cdot 2H_2O$ (0.12 g/L), Na_2HPO_4 (2 g/L), $MgSO_4 \cdot 7H_2O$ (1.5 g/L), $FeCl_3$ (2 g/L), $(NH_4)_2SO_4$ (0.5 g/L), KH_2PO_4 (7 g/L), and $ZnSO_4 \cdot 7H_2O$ (1 mg/L)]. The mixture was incubated for 14 days at 28 °C in the dark for mass production of fungal metabolites.

Extraction and Fractionation of the Sample. The whole culture material was sequentially extracted with *n*-hexane (4 × 1300 mL) and ethyl acetate (EtOAc). The extraction was completed in four cycles with a 12 h static period. The extracts were concentrated in a rotary evaporator (Eyela, Tokyo, Japan) at 37 °C that gave 7.93 g (dry weight) of hexane extract and 26.10 g of EtOAc extract. By TLC, the hexane extract was analyzed using hexane:EtOAc (19:1) as the mobile phase, and the EtOAc extract was analyzed using hexane:EtOAc (9:1) as a mobile phase. The spots were visualized in UV light at 254 and 365 nm.

The hexane extract was fractionated by silica column (41 mm × 300 mm, Kieselgel 60, 70−230 mesh) chromatography and eluted with hexane:EtOAc (19:1 to 1−9). A single fraction (5 × 200 mL) was collected and developed on TLC (4 cm × 2 cm or 4 cm × 1 cm) with hexane:EtOAc (1:9, v/v) as the mobile phase. Similarly, the EtOAc extract was fractionated and eluted with hexane:EtOAc (19:1 to 10−0) at a flow rate of 8.0 mL/min. Four fractions were collected and analyzed by TLC using hexane:EtOAc (4:1) as the mobile phase.

Purification of the Compounds. Fraction 1 of the hexane extract and the EtOAc extract showed similar TLC profiles and were hence pooled together. So, a total of four fractions were selected for further purification on a silica gel column (31 mm × 300 mm) presaturated overnight and eluted at a flow rate of 5 mL/min at room temperature (27 °C) with a suitable solvent system as described below.

Fraction 1. Fraction 1 (5.84 g), in a concentration of 20 mg/mL in dichloromethane, was further separated in a silica gel column presaturated with hexane:EtOAc:triethylamine (Et_3N) (19:0.5, v/v) and was eluted with hexane:EtOAc (19:1, v/v).

Fraction 2. On a silica gel column (31 mm × 300 mm) presaturated with hexane:EtOAc:Et_3N = 20:1 (19:1:0.5, v/v/v) fraction 2 (0.60 g), diluted to 10 mg/mL with EtOAc, was eluted with EtOAc:MeOH (20:1, v/v).

Fraction 3. Using hexane:EtOAc (7:3, v/v), 0.2 g of fraction 3 (10 mg/mL) in EtOAc was further separated on a silica gel column saturated with hexane:EtOAc:Et_3N (16:4:0.5, v/v/v).

Fraction 4. Fraction 4 (0.69 g) diluted to 5 mg/mL in chloroform was further separated on a silica gel column saturated with chloroform:Et_3N (20:0.5, v/v) by eluting with chloroform:MeOH (10:1, v/v).

Two major compounds were separated from both fraction 1 and fraction 3 where as the other fractions yielded one compound each. The compounds were named 1A, 1B, 2A, 3A, 3B, and 4A, respectively, before characterizing their antibacterial and antifungal properties.

Antibacterial Test. The gar disk diffusion method was performed to analyze the antibacterial activity (*20*). One mL of culture with 1×10^7 colony-forming units (CFUs) of *S. aureus* and *E. coli* was plated on tryptic soy agar (TSA), whereas the same number of *B. cereus* and *P. aeruginosa* cultures were plated on nutrient agar (NA). Separate filter papers of 8 mm diameter, blotted with 50 μL of each test sample of 500 μg/mL concentration, were evenly placed on the cultured plates. Kanamycin sulfate 50 μg/mL discs served as the positive control. After incubation at 37 °C for 24 h, the zones of inhibition around each disk were measured. Experiments were done in triplicate. The IC_{50} for the compounds against the test bacterial strain was determined by growing 200 μL of test bacterial strains (1×10^7 CFU) in a 96 well plate, containing 0, 12.5, 25, 50, and 100 μg/mL of the test compound dissolved in 1% dimethyl sulfoxide (DMSO), at 37 °C for 24 h.

Antifungal Assay. The antifungal activity of the compounds was tested against *R. solani* and *B. cinerea* as described by Saiman et al. (*21*). Briefly, pathogen plugs were placed at the center of PDA Petri dishes containing 10 mL of the test sample at concentrations ranging from 25 to 500 μg/mL. The pathogen growth was determined by measuring the diameter (mm) of the mycelial plug after 2 days for *R. solani* and after 4 days for *B. cinerea*. Experiments were done in triplicate.

RESULTS AND DISCUSSION

The biocontrol mechanisms of *Trichoderma* are complex processes mediated by the secretion of secondary metabolites. Anthraquinones are well-known metabolites of *Trichoderma* species. Pachybasin and chrysophanol have been isolated from *T. harzianum* ETS 323 (*6*). 1,7-Dihydroxy-3-hydroxymethyl-9,10-anthraquinone was first isolated in 2006 (*22*), and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone was first reported in 1976 (*23*). However, the biological activities of these compounds have not been well characterized.

Six major anthraquinones were purified from four fractions obtained from hexane and EtOAc extractions. The compounds were also detected under UV light at 254 nm. The NMR spectra of compounds **1A**, **1B**, **2A**, **3A**, **3B**, and **4A** were consistent for those of pachybasin, crysophanol, emodin, ω-hydroxypachybasin, 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, respectively, as previously reported (*22*−*28*) (**Figure 1**).

The purified compounds have the basic aromatic structure of anthraquinone with various levels of oxygenation. Among the six compounds, pachybasin has the lowest oxidation number. ω-Hydroxypachybasin is an oxidized form of pachybasin, with an oxidized methyl group in C-3. 1,5-Dihydroxy-3-hydroxymethyl-9,10-anthraquinone and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone are forms of ω-hydroxypachybasin that are oxidized at C-5 and C-7, respectively. Crysophanol is another form of pachybasin, oxidized at C-8. Emodin is an oxidized form of crysophanol, oxidized at C-6 and C-8.

Fraction 1, which yielded 3370 mg of pachybasin and 430 mg of crysophenol, was the highest yielding fraction. Pachybasin that was purified comparatively in a higher concentration is the major metabolite among the six anthraquinones. The yields of chrysophanol and pachybasin from *T. harzianum* strain Th-R16 are much higher to those previously reported from a different strain, *T. harzianum* ETS 323 (*7*). Emodin was less than 1% (16.3 mg) of the total EtOAc. ω-Hydroxypachybasin and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone were 28 and 7% of the total EtOAc, respectively. Among the six compounds, 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone from fraction 4 had the lowest yield with 4.7 mg dry weight.

Antibacterial Activity. Each compound showed distinct antibacterial activity against different pathogens. All of the compounds except crysophanol were active against the growth of *S. aureus* but were inactive against both of the Gram negative bacterial species (*P. aeruginosa* and *E. coli*) tested (**Table 1**). ω-Hydroxypachybasin and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, the two most effective compounds against *S. aureus*, were also active against *B. cereus*, whereas 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone was much effective against *S. aureus* but had no activity against *B. cereus*. While pachybasin was least active against *S. aureus*, crysophenol was inactive against all of the bacterial strains tested. ω-Hydroxypachybasin followed by 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone showed larger inhibition zones in *S. aureus* cultures with diameters of 20 ± 2 and 17.67 ± 2.08 mm, respectively. The compounds that showed inhibition zones larger than 10 mm diameter were considered active. Although emodin, ω-hydroxypachybasin, and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone inhibited *B. cereus*, their activity appears considerably low. Although 50 μL of emodin was comparatively not much active against *S. aureus*, they were more active at lower

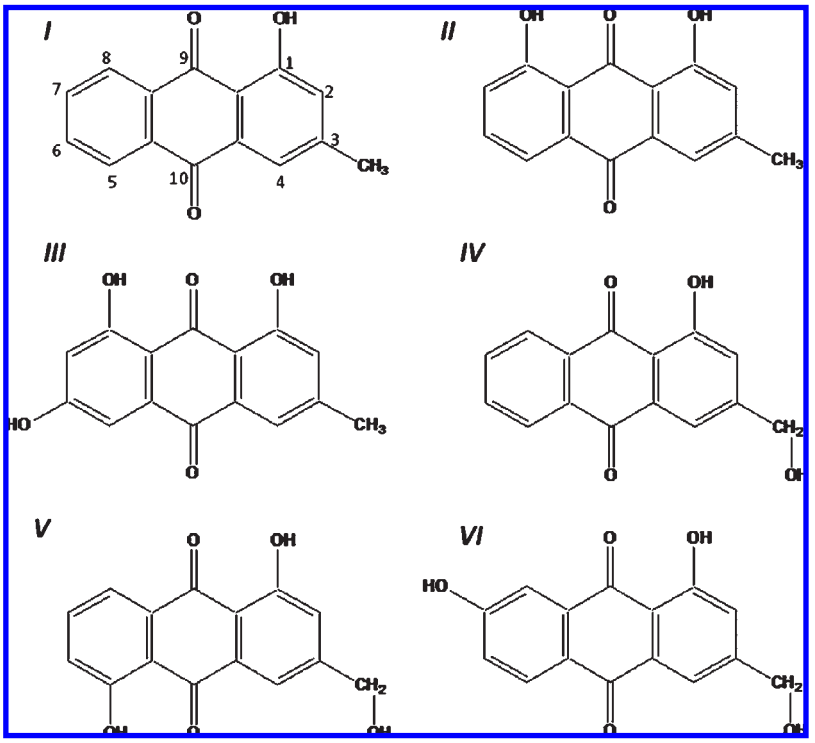

Figure 1. Tentative structures of the six anthraquinones isolated from *T. harzianum* SL-NBR1-6: (**I**) pachybasin, (**II**) crysophanol, (**III**) emodin, (**IV**) ω-hydroxypachybasin, (**V**) 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and (**VI**) 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone.

Table 1. Inhibition Efficacy of Each Compound against *P. aeruginosa*, *E. coli*, *S. aureus*, and *B. cereus*[a]

	diameter of inhibition zone (mm)			
compounds (500 μg/mL)	*P. aeruginosa*	*E. coli*	*S. aureus*	*B. cereus*
pachybasin	−	−	10.33 ± 0.57	−
crysophanol	−	−	−	−
emodin	−	−	11 ± 0	10 ± 0
ω-hydroxypachybasin	−	−	20 ± 2	11 ± 0
1,5-DHA	−	−	17.67 ± 2.08	10.66 ± 0.57
1,7-DHA	−	−	14.33 ± 2.08	−
kanamycin	13 ± 0	12 ± 0	13 ± 0	15 ± 0

[a] The values represent the diameters of the inhibition zones. − denotes no inhibition activity (diameter of the inhibition zone less than 10 mm).

concentrations. The IC_{50} data reveal that the IC_{50} of 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone (220.7 μM) was the highest and that of emodin (46.3 μM) was the lowest. The IC_{50} values of ω-hydroxypachybasin and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone were 122.8 and 117.4 μM, respectively.

Antifungal Activity. The compounds showed effective antifungal activity against the pathogens belonging to taxonomically unrelated groups, *B. cinerea* (Ascomycete) and *R. solani* (Basidiomycete). The results showed that all six compounds were active against the test pathogens, which contrasts with an earlier report, for pachybasin and chrysophanol from *T. harzianum* A6, by Vinale et al.(*29*). At a concentration of 500 μg/mL, 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone showed comparatively higher activity against *R. solani* and *B. cinerea* (67.3 and 92.8% inhibition with respect to control), whereas pachybasin and chrysophanol were the two least effective compounds (**Figure 2**). ω-Hydroxypachybasin and 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone showed a higher activity against both pathogens. Emodin and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone also showed a similar activity. When compared with *R. solani*, *B. cinerea* was more susceptible to all of the compounds except pachybasin. 1,7-Dihydroxy-3-hydroxymethyl-9,10-anthraquinone inhibited 53.7% growth of *B. cinerea* but just 25.4% of *R. solani*. ω-Hydroxypachybasin inhibited the growth of *R. solani* and *B. cinerea* in a similar level of 41.5 and 47.3%, respectively. The antifungal activity of 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone against both pathogens was concentration dependent (**Figure 3**). Meanwhile, the activity of emodin and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, which showed comparatively higher activity against *B. cinereai* than *R. solani*, was also concentration dependent (**Figure 4**).

Assuming equal extraction efficiency of the six compounds isolated, pachybasin and crysophanol had a higher percentage of the total extract but were found to have lower inhibitory activity. In contrast, emodin, ω-hydroxypachybasin, 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, which were present in lower absolute levels, had higher activities. This suggests that the pachybasin and crysophanol may primarily be involved in functions such as root colonization and that *Trichoderma* may produce other compounds with different functions in response to interaction with the host plant or pathogen or both (*29*).

The 3-methyl anthroquinones (pachybasin, crysophanol, and emodin) have lower levels of antibacterial activity, while the 3-hydroxy methyl anthroquinones (ω-hydroxypachybasin, 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone) were more efficient. The difference in position and degree of methoxylation has been correlated with the difference in activity among several other classes of compounds (*30*−*32*). Therefore, the oxidation of the methyl group may increase the antimicrobial activity of the compound.

A hierarchy of the compounds based on their increasing oxidation level is given as pachybasin > ω-hydroxypachybasin

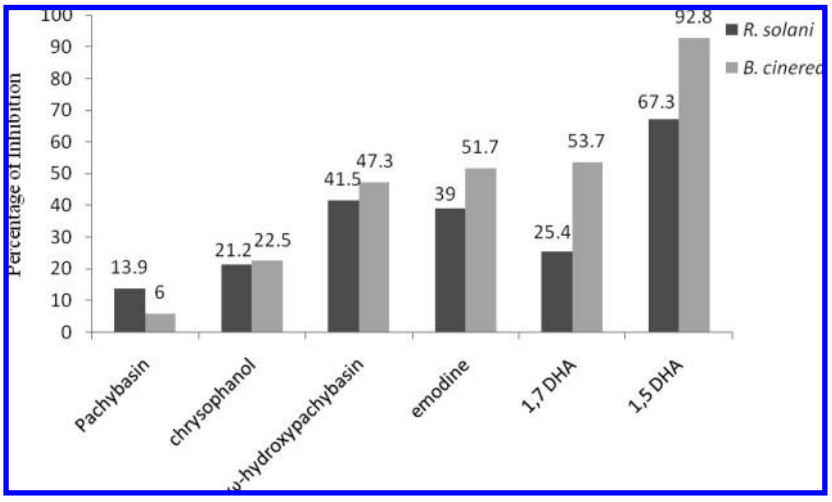

Figure 2. Antifungal activities of six compounds (500 μg/mL) against *R. solani* and *B. cinerea* shown as a percentage of inhibition as compared to the control.

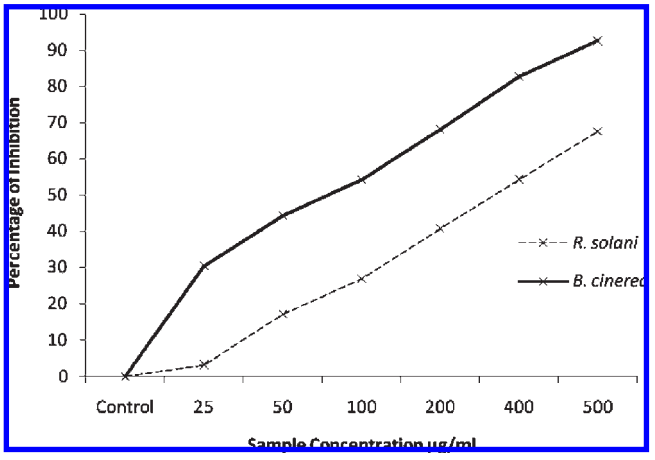

Figure 3. Effects of various concentrations of 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone on the growth of *R. solani* and *B. cinerea*. The diameter (mm) of the mycelia plug was measured.

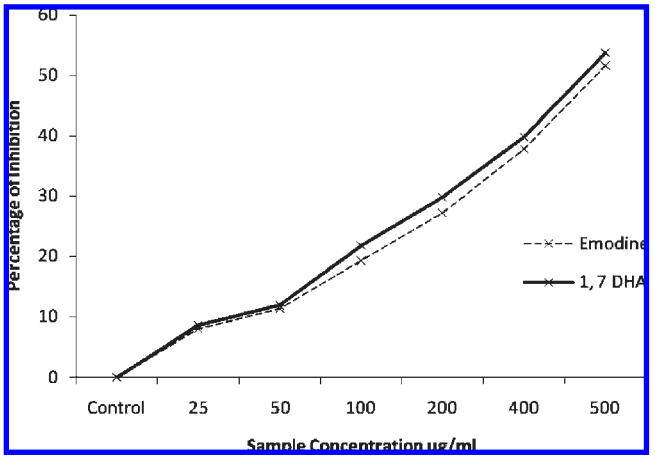

Figure 4. Effects of various concentrations of emodin and 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone on the growth of *B. cinerea*. The diameter (mm) of the mycelia plug was measured.

> crysophanol > 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone > 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone > emodin. The level of antifungal activity depends on the degree of oxidation of a compound. Among the six compounds, pachybasin has the lowest oxidation level and is the least active antifungal compound, whereas 1,5-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and emodin with a higher degree of oxidation possess more antifungal activity than the rest of the test anthraquinones. Thus, we conclude that the higher the degree of oxidation, highest is the antimicrobial activity. However, the stability of the compounds may decrease with an increase in the number of hydroxyl groups.

In response to pathogenic attack, plants undergo a "hypersensitive reaction" leading to specialized programmed cell death (PCD). Usually, PCD is accompanied by an increase in production of reactive oxygen species (ROS), which helps to contain or kill the pathogen (*11*). When *Trichoderma* symbiotically inhabits a plants roots system, the less oxidized secondary metabolites, chrysophanol and pachybasin, secreted by *Trichoderma*, have the potential to form a reservoir that can be oxidized to become 1,5- dihydroxy-3-hydroxymethyl-9,10-anthraquinone, 1,7-dihydroxy-3-hydroxymethyl-9,10-anthraquinone, and emodin by the host ROS that are released in response to attack by a microbial pathogen. These highly oxidized secondary metabolites are more potent antimicrobial agents and may act to increase *Trichoderma* competitive efficiency and host resistance to other pathogens.

Furthermore, in acidic conditions, anthraquinones, by a process known as autoxidation, release hydrogen peroxides (*33*), which may have one or more of the following functions, such as oxidation of other anthraquinones, reaction against the fungal cell wall, and induction and enhancement of the plant host immune system (*24*). The same effect would be expected during increased ROS levels due to other pathogen infection. Furthermore, it is reported that *Trichoderma* produces metabolites against plant pathogens more actively during the three way interaction between the plant, the pathogen, and *Trichoderma* (*29*). The report stands true for metabolites as well, as the amount of efficient antimicrobial metabolites that are produced at low levels without the three way interaction will increase during pathogen attack, due to the oxidation of weak antimicrobial compounds by the host ROS .

The results suggest that the role of the test anthraquinones from *Trichoderma* is not limited to direct inhibition of pathogen. The higher yield compounds have a lower degree of oxidation and possess less antimicrobial activity. We propose that in the presence of other microorganisms, higher degrees of oxidized anthraquiones are generated, either by direct synthesis or due to transformation, to eliminate microorganisms.

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