© 2016
Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas 15 (1): 1 - 17
ISSN 0717 7917
**www.blacpma.usach.cl**

**Artículo Original | Original Article**

# Metabolism and antifungal activity of safrole, dillapiole, and derivatives against *Botryodiplodia theobromae* and *Colletotrichum acutatum*

[Metabolismo y actividad antifúngica de safrol, dilapiol, y derivados contra *Botryodiplodia theobromae* y *Colletotrichum acutatum*]

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**Abstract:** The direct in vitro fungitoxicity and metabolism of safrole and dillapiole (isolated from *Piper auritum* and *Piper holtonii*, respectively) by *Botryodiplodia theobromae* and *Colletotrichum acutatum* were investigated. Higher values of mycelial growth inhibition for both fungi were obtained for dillapiole, as compared with safrole. *B. theobromae* was able to metabolize both compounds to their respective vicinal diols, reaching 65% relative abundance during the biotransformation of dillapiole; while *C. acutatum* only transformed safrole to various metabolites with relative abundances under 5%. According to the low antifungal activity of the major metabolic products (< 5% for vicinal diols), a detoxification process was implied. Studies on the influence of some substituents in the aromatic ring of safrole and dillapiole on the antifungal activity against *B. theobromae* were also carried out. As result, the safrole nitrated derivative, 6-nitrosafrole, showed a fungitoxicity level similar to that displayed by the commercial fungicide Carbendazim® under the conditions used. In light of this, safrole and dillapiole could be suggested as feasible structural templates for developing new antifungal agents.

**Keywords:** Biotransformation, plant pathogenic fungi, safrole, *Botryodiplodia theobromae*, *Colletotrichum acutatum*

Resumen: Se investigó la fungitoxicidad directa in vitro y el metabolismo de safrol y dilapiol (obtenidos desde *Piper auritum* and *Piper holtonii*, respectivamente) por *Botryodiplodia theobromae* y *Colletotrichum acutatum*. Los valores mayores de inhibición del crecimiento micelial de ambos hongos se obtuvieron para dilapiol, en comparación con safrol. *B. theobromae* metabolizó ambos compuestos a sus respectivos dioles vecinales, alcanzando abundancias relativas del 65% durante la biotransformación del dilapiol; mientras que *C. acutatum* solo transformó safrol en varios metabolitos con abundancias relativas menores al 5%. De acuerdo con la baja actividad antifúngica de los productos metabólicos mayoritarios (< 5% para los dioles vecinales), se sugiere un proceso de desintoxicación. Adicionalmente, se evaluó la influencia de algunos sustituyentes en el anillo aromático de safrol y dilapiol sobre la actividad antifúngica contra B. theobromae. Como resultado, el derivado nitrado del safrol, el 6–nitro safrol, presentó un nivel de fungitoxicidad similar al exhibido por el fungicida comercial Carbendazim® bajo las condiciones usadas. A la luz de lo anterior, safrol y dilapiol podrían ser sugeridos como plantillas estructurales adecuadas para el desarrollo de nuevos agentes antifúngicos.

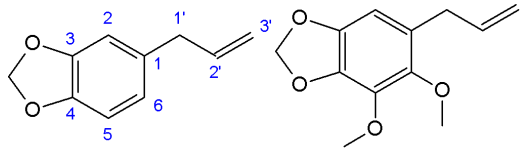
**Palabras clave**: Biotransformación, hongos fitopatogénicos, safrol, *Botryodiplodia theobromae*, *Colletotrichum acutatum*.

**Recibido | Received:** November 13, 2014
**Aceptado | Accepted:** March 15, 2015
**Aceptado en versión corregida | Accepted in revised form:** October 21, 2015
**Publicado en línea | Published online:** January 31, 2016
**Declaración de intereses | Declaration of interests:** This work was supported by a grant (Vicerrectoría de Investigación, Nº 20101007957, Universidad Nacional de Colombia).
**Este artículo puede ser citado como / This article must be cited as:** S Vizcaíno-Páez, R Pineda, C García, J Gil, D Durango. 2016. Metabolism and antifungal activity of safrole, dillapiole, and derivatives against *Botryodiplodia theobromae* and *Colletotrichum acutatum*. **Bol Latinoam Caribe Plant Med Aromat** 15 (1): 1 – 17.

## INTRODUCTION

Anthracnose and stem-end rot (caused by fungi *Colletotrichum acutatum* and *Botryodiplodia theobromae*, respectively) are two severe diseases that contribute considerably to the pre- and postharvest losses of economically important fruits and vegetables around the world (Damm *et al.*, 2012; Twumasi *et al.*, 2014). Conventionally, both diseases have been successfully controlled through the application of non-selective fungicides (Gaviria-Hernández *et al.*, 2013; Syed *et al.*, 2014). Nonetheless, phytopathogenic microorganisms have been progressively developing resistance against commonly used fungicides, which has reduced the effectiveness of chemical treatments during recent years (Gutiérrez *et al.*, 2003; Hahn, 2014). Furthermore, the use of non-selective fungicides to control deterioration of fruits and vegetables has been restricted as a result of negative public perception of their possible undesirable side effects on human beings. Therefore, researchers are actively working on alternative methods to combat fungal diseases. In this way, the spraying of essential oils, plant extracts, and their main constituents may be an attractive method for controlling pre- and postharvest fruit diseases. Particularly, the discovery of novel antifungal agents representing new chemical classes with different toxicities and modes of action is highly desirable (Lopéz-García *et al.*, 2012). Hence, evaluating antifungal activity of natural products offers an interesting approach to identify potential new fungicides and discover valuable structural templates that may be transformed on antifungal agents. In addition, the study of the microbial metabolism of potential antifungal agents using phytopathogenic fungi could provide information on the detoxification mechanism used by these microorganisms and give an indication of the structural modifications that may be necessary if certain substrates are to be further developed as selective fungal control agents (Daoubi *et al.*, 2005a; Daoubi *et al.*, 2005b). Safrole (**S**) and dillapiole (**dA**) (Figure 1) are the main components of the essential oils of *Sassafras albidum*, and *Piper spp.* (Guerrini *et al.*, 2009). This kind of compounds can reduce the mycelial growth of some plant pathogens, acting as phytoanticipins (Iriti & Faoro, 2009). Nevertheless, knowledge on the direct antifungal effect and microbial metabolism of these compounds by phytopathogenic fungi is still limited.

**Figure 1**
**Chemical structures of safrole (S) and dillapiole (dA)**




(**S**) (**dA**)

The aim of the current work was to evaluate the metabolism and antifungal activity of **S**, **dA**, and some amine and nitro derivatives against *C. acutatum* and *B. theobromae*.

## MATERIALS AND METHODS

### Biological and chemical materials

*C. acutatum* and *B. theobromae* were isolated from infected tamarillo (*Solanum betaceum* Cav. Sendt) and avocado (*Persea americana* Mill.) fruits, respectively. The fungi were characterized and kindly provided by the Laboratory of Phytopathology (Universidad Nacional de Colombia-Medellín). The fungi were maintained in a Potato Dextrose Agar (PDA) medium at 24 ± 2º C, and subcultured monthly in Petri dishes. In order to evaluate the antifungal activity, 15 cm-diameter, sterile Petri dishes were inoculated with 1 mL of a mycelial suspension of the fungi. The suspension was uniformly spread over the medium using a bent glass rod. Then, the inoculated medium was incubated at 25º C for 48 h. A 8 mm-diameter mycelial disc was used for the fungitoxicity test.

The compounds **S** and **dA** were isolated from *Piper auritum* and *Piper holtonii*, respectively, and their structures confirmed with spectroscopic methods (FTIR, MS and 1D and 2D NMR), as described elsewhere (Pineda *et al.*, 2012). A yeast extract was obtained from Oxoid Ltd (Basingstoke, UK). Piperonal was purchased from Sigma-Aldrich (St. Louis, MO, USA). Piperonyl alcohol was obtained by reduction of piperonal using $NaBH_4$

(87% yield) (Smith, 2013). Nitro- and amine derivatives were prepared through nitration ($HNO_3/H_2SO_4$) and further reduction (Sn/HCl) of nitro-derivatives (Olah *et al.*, 1989; Singh *et al.*, 2006). The compounds 1-allyl - 5 , 6 - dimethoxy - 2 - nitro - 3, 4 -methylenedioxybenzene (2-nitro dillapiole, 43% yield) and 1-allyl-5,6-dimethoxy - 2- amino -3,4 - methylenedioxy benzene (2-amino dillapiole, 80% yield) were obtained from the dillapiole, whereas, 1-allyl-6-nitro-3,4-methylene-dioxy benzene (6-nitro safrole, 92% yield) and 1-allyl-6-amino-3,4-methylenedioxy benzene (6-amino safrole, 83% yield) were synthesized from safrole.

**Fungitoxicity bioassays of dA and S**

In order to investigate the toxicity of **dA** and **S** against *C. acutatum* and *B. theobromae*, the poisoned food technique was used (Grover & Moore, 1962; Velasco *et al.,* 2010). Different concentrations of **dA** (50, 75, 100, 300 and 600 µg $mL^{-1}$) and **S** (250, 500, 750, 1000, 1250, 1500 and 1750 µg $mL^{-1}$) dissolved in ethanol (0.2%, v/v) were diluted in Petri dishes with PDA. All of the concentrations were tested in triplicate, and the results are shown as mean values of three replications of colony diameters [± standard deviation (SD)]. Petri dishes without compounds were used as negative control, which only contained ethanol (0.2%, v/v) in the PDA medium. The common fungicide Carbendazim (methyl benzimidazol-2-yl carbamate) was used as positive control at 50 µg $mL^{-1}$. The Petri dishes were incubated at room temperature and the diameter of the mycelial growth was measured every 12 (for *B. theobromae*) and 24 hours (for *C. acutatum*). The incubation was stopped when the mycelial mass of control Petri dishes almost filled them (ca. 10 and 4 days for *C. acutatum* and *B. theobromae* respectively). The fungitoxicity of **S** and **dA** in terms of inhibition percentage of the radial growth was calculated by using the formula:

$$Inhibition\,(\%) = 1 - \frac{radial\ growth\ of\ treatment\,(mm)}{radial\ growth\ of\ control\,(mm)}\,x100$$

The statistical analysis of least significant difference between means was carried out employing the ANOVA and LSD test in Statgraphics (Statgraphics Centurion XV, Version 15.2.06).

**Metabolism of dA and S**

***Preculture of C. acutatum and B. theobromae***

Mycelia of *C. acutatum* or *B. theobromae* from a 2-day-old culture were employed, following a methodology described in a previous work (Numpaque *et al.,* 2011).

***Preparative-Scale Metabolism***

The mycelia of *C. acutatum* and *B. theobromae* were transferred into six 1.0 L Erlenmeyer flasks containing 0.5 L of a sterilized broth and **dA** or **S** dissolved in 96% ethanol (final concentration of 0.2%, v/v). When *C. acutatum* was used as a biocatalyst, **dA** and **S** were incorporated into the culture medium at 50 and 400 µg $mL^{-1}$, respectively. In the case of *B. theobromae*, 100 and 800 µg $mL^{-1}$ concentrations of **dA** and **S**, respectively, were employed. The cultivation was developed at room temperature with stirring at 120 rpm for 360 h. After the incubation period, the culture medium and mycelia were separated by filtration. Controls (without substrate) were performed in order to verify the presence of similar compounds in the fungi culture (Correa *et al.,* 2009).

***Isolation and identification of metabolic products***

Crude extract from culture medium was obtained as described by Velasco *et al.* (2012). Then, the extract was chromatographed on a silica gel column. Elution was performed with an *n*-hexane-EtOAc gradient system. Extracts from the metabolism of **dA** and **S** by *B. theobromae* were separately fractionated to afford ten fractions, grouped according to TLC profiles. The seventh (*n*-hexane:EtOAc, 4:6) fraction from **S** and the eighth (*n*-hexane:EtOAc, 3:7) fraction from **dA** were re-chromatographed over silica gel column using *n*-hexane-EtOAc as eluent to yield two metabolic compounds (**1** and **2**). The identification of these metabolites was based on the interpretation of their nuclear magnetic resonance (NMR), infrared, and mass spectra, and by contrast with the NIST 2002 Mass Spectral Library. In addition, the GC-MS analysis detected the metabolite (**3**) from the biotransformation of **S**.

Furthermore, six metabolites were detected in the transformation of **S** by *C. acutatum* (including **1** and **3**), which were identified according to the mass spectral data and comparison with authentic samples. Unfortunately, the very low abundance of some of

the compounds in the biotransformation excluded isolation, enrichment and further direct spectroscopic characterization through NMR. Metabolic products were not detected by GC-MS in the metabolism of **dA** using *C. acutatum*.

***Time-course experiments***

Precultured *B. theobromae* and *C. acutatum* were separately transferred into fifteen 250 mL Erlenmeyer flasks containing 125 mL of the Czapek-Dox medium and the compounds **dA** and **S**. The flasks were stirred under the same conditions as the preculture. The concentrations of **dA** and **S** were the same as described for the preparative-scale metabolism. The plant pathogenic fungi were cultivated at 120 rpm for 216 and 360 h for *B. theobromae* and *C. acutatum*, respectively. The culture medium was taken from each flask daily for *C. acutatum* and every 12 h (during the first 5 days) and 48 h (during the last 4 days) for *B. theobromae*. Then, the crude extract was obtained as described by Velasco *et al.* (2012), re-dissolved in 5 mL of chloroform and analyzed by TLC and GC-MS. The ratios among the substrate and products were determined based on the GC peak area; the results were expressed as relative abundances. Additionally, control cultivations without **dA** or **S** were performed.

**Antifungal activity of some metabolic products and derivatives of dA and S**

Major metabolites resulting from the metabolism of **S** and **dA** by *C. acutatum* and *B. theobromae* were tested for antifungal activity against *C. acutatum* at a 200 μg $mL^{-1}$ concentration in triplicate. Finally, in order to evaluate the effects of electron-releasing and electron-withdrawing groups on the antifungal activity, both nitro and amino derivatives of **S** and **dA** were also analyzed. These derivatives were prepared by conventional nitration and amination procedures (Smith, 2013). Structural elucidation was performed by NMR.

**Analytical methods**

Thin layer chromatography (TLC) was carried out on Merck Kiesegel 60 $F_{254}$ (0.25 mm thick). Mixtures of *n*-hexane:EtOAc were used as the mobile phase. Column chromatography (CC) was performed using silica gel 60 (0.040-0.063 mm; Merck). GC–MS analysis was performed using a Hewlett-Packard 6890 (Agilent Technologies) gas chromatograph coupled with a HP 5973 MSD (Mass selective detector-Quadrupole type). A Zebron ZB 35 column (30 m x 0.25 mm i.d.; coating thickness 0.25 μm) was employed. The chromatographic conditions were: column temperature, 50-250° C at 10° C $min^{-1}$ and maintained for five minutes; injector temperature, 150° C; detector temperature, 280° C; and carrier gas, helium at 1.0 mL $min^{-1}$. The relative composition of each constituent was established from the average peak area obtained in the GC. The nuclear magnetic resonance spectra were measured with a Bruker AMX 300 NMR spectrometer. The proton chemical shifts (δ) and coupling constants (*J*) are given in ppm and hertz, respectively. The attribution of $^{13}C$ NMR signals for some compounds has been done using JMOD experiments. FTIR spectra were carried out using $CHCl_3$ on a Perkin-Elmer RXI. Optical rotations were measured in a $CHCl_3$ solution at 25° C with a JASCO P-2000 digital polarimeter.

**RESULTS AND DISCUSSION**

**Antifungal activity of safrole S and dillapiole dA**

The inhibitory effects of **dA** and **S** against *C. acutatum* and *B. theobromae* are shown in Figures 2 and 3. For both fungi, the mycelial growth was only completely inhibited during the evaluation time by **S** at 1500 μg $mL^{-1}$ and above. After the first 24 h, **S** exhibited a total inhibition of radial growth in *C. acutatum* at all evaluated concentrations; at 1250 μg $mL^{-1}$ and above, however, this effect remained for 72 h. Then, the inhibitory effects strongly decreased at all the evaluated concentrations. Particularly at 250 μg $mL^{-1}$, **S** presented radial growth inhibitions for *C. acutatum* of from 100 to 25%, after 240 h. Only at 1250 μg $mL^{-1}$ and above did **S** exhibit inhibitions higher than 70% after 240 h.

For *B. theobromae*, **S** reduced the radial growth of *B. theobromae* completely during the first 48 h at 1250 μg $mL^{-1}$; then, the inhibitory effect decreased by almost 80% after 96 h. The antifungal activity of **S** found in the present study is in agreement with previous reports. Simic *et al.* (2004) reported that **S** is the major constituent of the essential oil (85%) of *Sassafras albidum* (Lauraceae), which has shown an antifungal activity similar to that of the commercial drug bifonazol. In addition, some authors (Kubo *et al.*, 1993; Fujita & Kubo, 2004) have reported that **S** possesses moderate activity against *S. cerevisiae* and *Candida utilis*, with MICs (Minimum inhibitory concentration) of 200 μg $mL^{-1}$. They suggested that the allyl moiety in **S** is a minimum structural requirement necessary to display its antifungal activity.

**Figure 2**
**Effect of safrole (S, up) and dillapiole (dA, down) on the radial mycelial growth of *C. acutatum*. Results are shown as mean values of three replicates of the mycelium diameter; bar = ± SD. Columns that share at least one letter do not have statistical difference between them.**

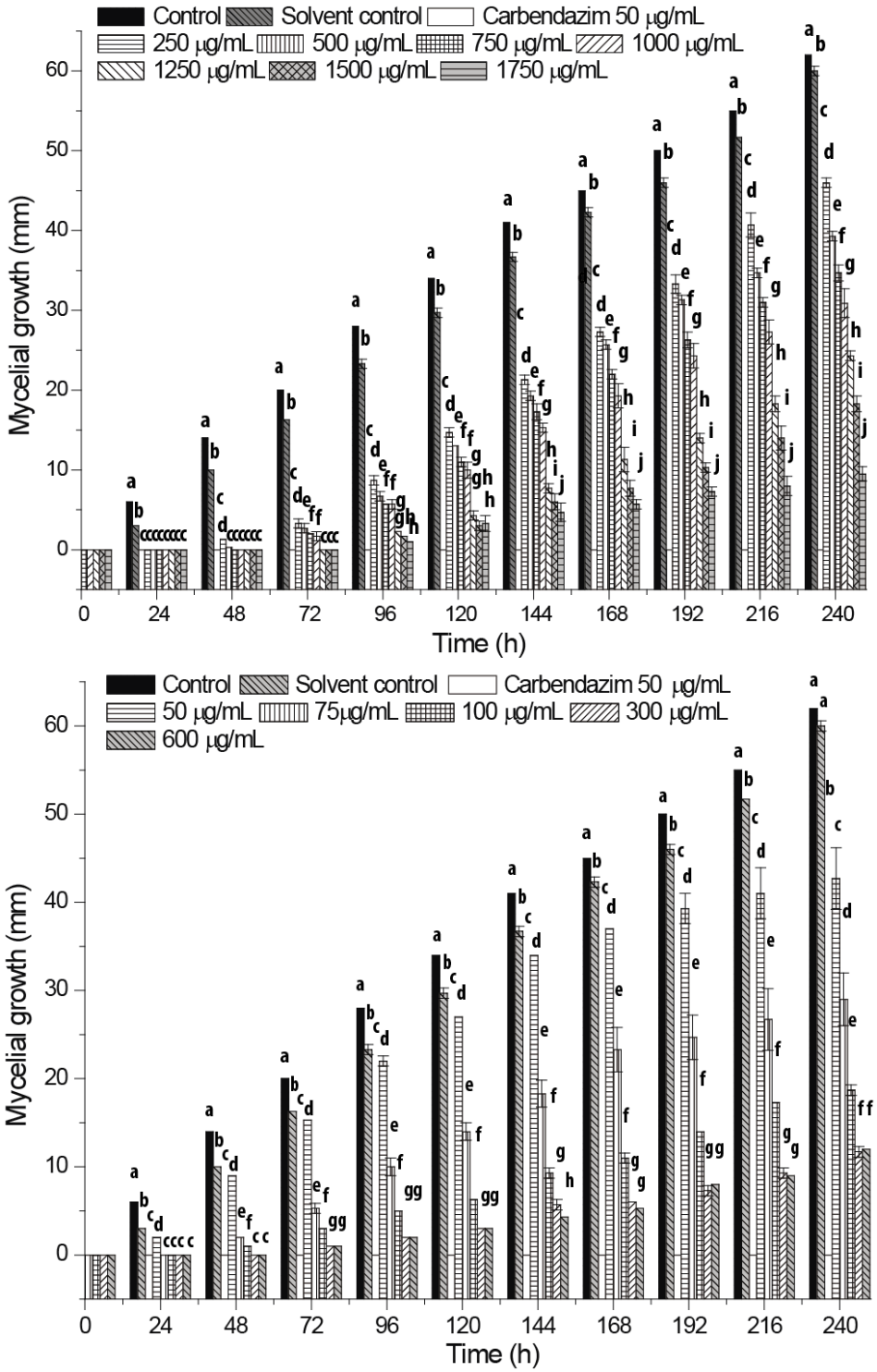
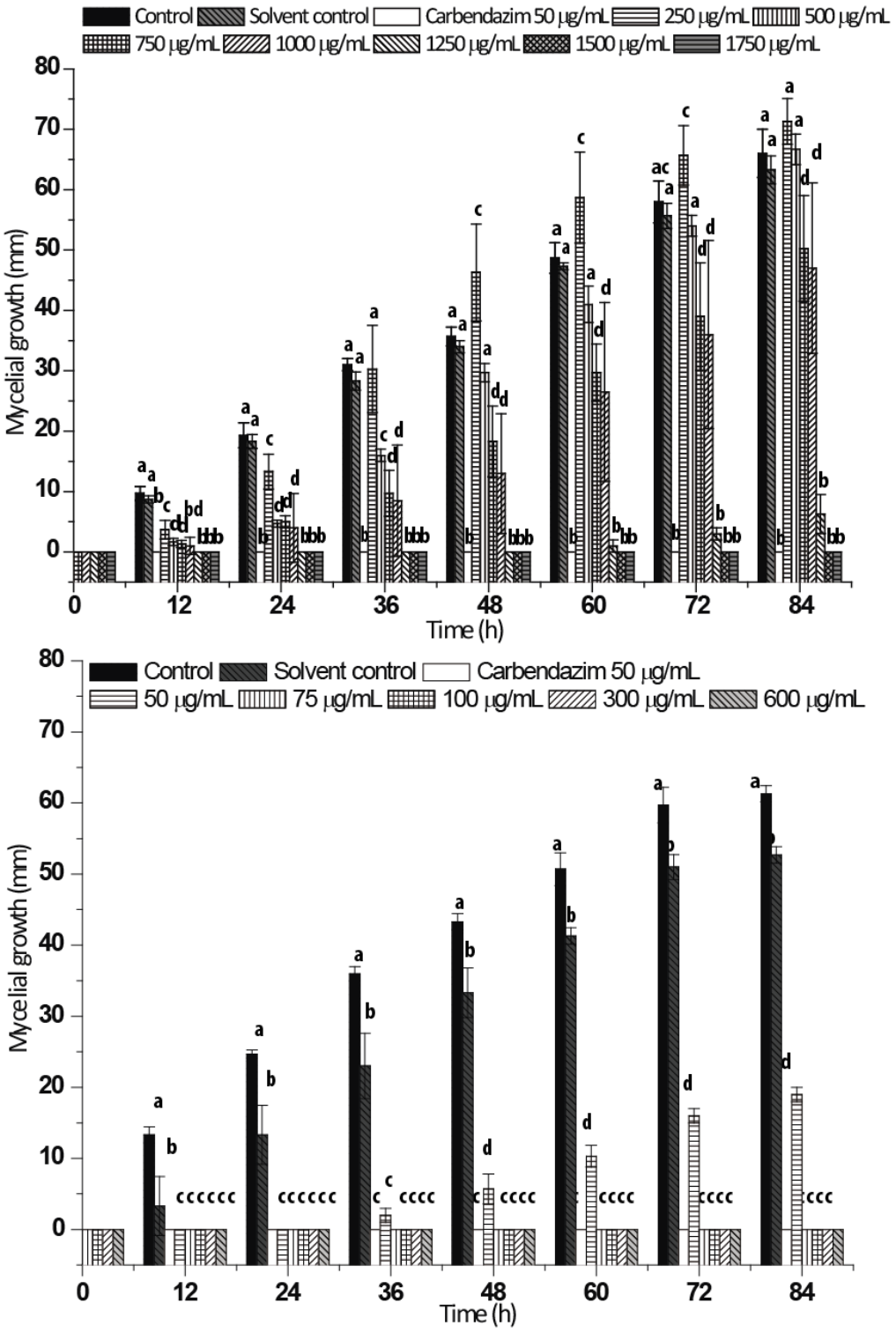


**Figure 3**
**Effect of safrole (S, up) and dillapiole (dA, down) on the radial mycelial growth of *B. theobromae*. Results are shown as mean values of three replicates of the mycelium diameter; bar = ±SD. Columns that share at least one letter do not have statistical difference between them.**



Meanwhile, **dA** exhibited a fungistatic effect against both pathogens, reaching inhibitions of 81 and 100% for *C. acutatum* and *B. theobromae* respectively. In the case of *B. theobromae*, its growth was completely inhibited during the entirety of the assay when a concentration of 75 to 500 μg $mL^{-1}$ of **dA** was used. Moreover, the fungal colony development only initiated after 24 h of inoculation when *C. acutatum* was treated with **dA** at 50 μg $mL^{-1}$. The growth inhibition percentages of *C. acutatum* and *B. theobromae* achieved by 75 μg $mL^{-1}$ **dA** varied between 100-70% (after 96 h) and 100-50% (after 240 h), respectively. Our results for the good antifungal activity of **dA** are in accordance with previous studies (Singh *et al.,* 2005; de Almeida *et al.,* 2009; Maxia *et al.,* 2012). In an earlier study on the essential oil from *Apium nodiflorum,* which contains a high proportion of **dA**, the fungi *Aspergillus flavus*, *A. niger* and *A. fumigatus* were highly inhibited with MIC values from 0.32 to 2.5 μg $mL^{-1}$, as tested by macrodilution assays (Maxia *et al.,* 2012). In others assays, the mycelial growth inhibition for different fungi, such as *Penicillium* sp., *Acrophialophora* sp., and *Aspergillus* sp.; was obtained by the addition of the essential oil from *Carum nigrum* (seeds), which contains 29.9% **dA** (Singh *et al.,* 2005). In addition, the basidiospores of *Clinipellis perniciosa* were completely inhibited by **dA** at 0.6 μg $mL^{-1}$ (de Almeida *et al.,* 2009). However, information on the antifungal activity of **dA** against plant pathogenic fungi is still quite limited.

Similar to **S**, the compound **dA** has an allyl side chain bonded to the aromatic ring which confers a lipophilic characteristic to both phenylpropenes. Although not much is known about the mechanism of antifungal action of these phenylpropenes, it has been reported that their lipophilic characteristic enables them to enter between the fatty-acid-chain constituents of the membrane lipid bilayers and alter the fluidity and permeability of the cell membranes (de Almeida *et al.,* 2009). As can also be seen, the inhibitory effect of **dA** and **S** decreased with time, a fact which suggests that both fungi possess a detoxification mechanism. In order to study this mechanism, *B. theobromae* and *C. acutatum* were incubated with **S** and **dA** at different concentrations.

## Metabolism of dA and S

### *Identification of metabolic products*

The main metabolic products were isolated from a preparative-scale incubation of **dA** and **S** using *C. acutatum* and *B. theobromae*. The **S** and **dA** concentrations for the metabolism studies were based on the antifungal activity of each compound. A comparison between extracts obtained from the biotransformation and control showed that *B. theobromae* transformed **S** and **dA** into two compounds (**1** and **2**, respectively), which were identified by spectroscopic analysis (Table 1). Metabolite (**1**) had a molecular formula of $C_{10}H_{12}O_4$ based on its mass spectrum, $M^+$ = 196 amu. The molecular ion was 34 mass units (2OH) higher than that of the parent compound **S**, indicating a dihydroxylation of the double bond. Also, (**1**) showed a fragment ion at *m/z* 135 (base peak) signifying the formation of 3,4-methylenedioxybenzyl cation. The $^1$H NMR spectrum of the product showed primary alcohol resonance at δ 3.65-3.77 ppm (*m*, 2H), and secondary alcohol resonance at 3.85–3.97 ppm (*m*, 1H), suggesting the introduction of two new hydroxyl groups at the double bond to yield a vicinal diol (glycol). From the spectral data, (**1**) was elucidated to be 2',3'-dihydroxydihydrosafrole (safrole glycol). Additionally, metabolic compound, (**3**) was tentatively identified as 3-(3,4-methylenedioxy-phenyl)-propan-1-ol by EI-MS.

Meanwhile, the biotransformation of **dA** by *B. theobromae* allowed for the isolation and identification of (**2**). It presented a molecular formula of $C_{12}H_{16}O_6$, consistent with the molecular ion $M^+$ = 256 amu. Similarly, metabolic product (**2**) gave a molecular ion that was 34 mass units higher than that of parent compound **dA**, indicating a dihydroxylation of the double bond. This was further confirmed by the signals on $^1$H NMR at δ 3.86 (*m*, 1H) and 3.59-3.63 ppm (*m*, 2H), corresponding to the protons attached to the carbinol carbon of secondary and primary alcohol, respectively. In addition, the mass spectrum showed a fragment ion at *m/z* 195, corresponding to a 2,5-dimethoxy-3,4-methylenedioxybenzyl cation. Therefore, it metabolite was identified as 2',3'-dihydroxydihydro dillapiole (dillapiole glycol).

On incubation of **S** with *C. acutatum*, six metabolites (including **1** and **3**) were detected by the GC-MS analysis; the low concentration of these metabolites prevented their isolation. These metabolites were not found in the control (the culture of *C. acutatum* without **S**). In general, the structure of these metabolites was derived from their EI-MS and comparison with the NIST 2002 computerized mass spectral library and authentic standards (Table 1). The identified metabolic compounds included 2',3'-

dihydroxydihydrosafrole (**1**), 3-(3,4-methylenedioxy-phenyl)-propan-1-ol (**3**), 3,4-methylenedioxy cinna-mylalcohol (**4**), 1-(3,4-methylenedioxyphenyl)-propan-1-one (**5**), piperonyl alcohol (**6**), and piperonal (**7**).

**Table 1**
**Metabolic compounds identified from the biotransformation of S and dA by *C. acutatum* and *B. theobromae***

| Metabolite | Rt (min) | Isolated from | Spectroscopic data |
|---|---|---|---|
| **(1)** | 20.2 | *Both fungi*/**S** | **$^1$H-NMR (CDCl$_3$, 300 MHz):** δ 6.77-6.75 (d, 1H, $H_6$), 6.73 (s, 1H, $H_2$), 6.68-6.66 (d, 1H, $H_5$), 5.94 (s, 2H, –$OCH_2O$–), 3.85-3.97 (m, 1H, $H_{2'}$), 3.65-3.77 (m, 2H, $H_{3'}$), 3.49-3.59 (m, 2H, $H_{1'}$). **MS-EI m/z, (fragment) [% rel. int.]:** 196 ($M^+$) [18], 177 [6], 166 [12], 165 (M-$H_2CO$-H) [6], 136 [31], 135 (M-$C_2H_5O_2$; $C_8H_7O_2^+$) [100], 122 [35], 107 [10], 79 [13], 77 [20], 51 [10], 43 [7]. $[\alpha]_D^{25}$ **(CHCl$_3$):** +8.59°. |
| **(3)** | 18.6 | | **MS-EI m/z (fragment) [% rel. int.]:** 180 ($M^+$) [40], 162 (M–$H_2O$) [7], 161 (M-$H_2O$-H) [7], 149 (M-$H_2CO$-H; α-cleavage of primary alcohol group) [7], 136 [71], 135 (base peak, formation of 3,4-methylenedioxybenzyl cation: $C_8H_7O_2^+$) [100], 106 [20], 105 [7], 91 ($C_7H_7^+$) [10], 78 [18], 77 ($C_6H_5^+$) [35], 65 ($C_5H_5^+$) [7], 51 [29], 45 [27]. Molecular formula: $C_{10}H_{12}O_3$. Tentatively identified as 3-(3,4-methylenedioxyphenyl)-propan-1-ol. |
| **(2)** | 25.5 | *B. theobromae*/ **dA** | **$^1$H-NMR (CDCl$_3$, 300 MHz):** δ 6.33 (s, 1H, $H_6$), 5.97 (s, 2H, –$OCH_2O$–), 3.92 (s, 3H, –OMe), 3.86 (s, 3H, –OMe), 3.82-3.89 (m, 1H, $H_{2'}$), 3.60-3.64 (dd, 1H, *J*=11.4, 3.5, $H_{3'a}$), 3.47-3.51 (dd, 1H, *J*=11.4, 6.0, $H_{3'b}$), 2.76-2.81 (dd, 1H, *J*=13.6, 5.7, $H_{1'a}$), 2.70-2.75 (dd, 1H, *J*=13.6, 7.3, H-1'b). **MS-EI m/z, (fragment) [% rel. int.]:** 256 ($M^+$) [39], 225 (M–$H_2CO$–H) [67], 195 ($C_{10}H_{11}O_4^+$) [100], 180 [17], 165 [7], 135 [7]. **IR ῡ (cm$^{-1}$):** 3289 (OH), 3206 (OH), 3015, 1611, 1503, 1435. $[\alpha]_D^{25}$**(CHCl$_3$)**: -7.13°. |
| **(4)** | 17.9 | *C. acutatum*/**S** | **MS-EI m/z, (fragment) [% rel. int.]:** 178 ($M^+$) [21], 161 (M–OH) [6], 149 (M–CO–H; $C_9H_9O_2^+$) [38], 135 (base peak, formation of 3,4-methylendioxybenzyl cation, M–CO–$CH_3$; $C_8H_7O_2^+$) [100], 123 [50], 121 ($C_7H_5O_2^+$) [28], 103 [25], 93 [75], 91 ($C_7H_7^+$) [25], 77 ($C_6H_5^+$) [16], 65 ($C_5H_5^+$) [27]. Metabolite (**4**) was elucidated to be 3,4-methylenedioxy cinnamyl alcohol. |
| **(5)** | 19.8 | | **MS-EI m/z, (fragment) [% rel. int.]:** 178 ($M^+$) [21], 149 (base peak, formation of 3,4-methylenedioxybenzoyl cation -acylium ion-, due to α-cleavage of the benzyl bond: $C_8H_5O_3^+$) [100], 135 [50], 121 (formation of 3,4-methylenedioxyphenyl cation, $C_7H_5O_2^+$) [30], 91 ($C_7H_7^+$) [9], 77 ($C_6H_5^+$) [7], 65 ($C_5H_5^+$) [31], 63 [25], 62 [11], 29 [16], 27 [13]. Metabolite (**5**) was tentatively identified as 1-(3,4-methylenedioxyphenyl)-propan-1-one |
| **(6)** | 15.3 | | **MS-EI m/z, (fragment) [% rel. int.]:** 152 ($M^+$) [100], 151 (M–H) [37], 135 (base peak, formation of 3,4-methylendioxybenzyl cation: $C_8H_7O_2^+$) [53], 123 [30], 122 [27], 93 [59], 91 ($C_7H_7^+$) [30], 77 ($C_6H_5^+$) [18], 65 ($C_5H_5^+$) [32]. Molecular formula: $C_8H_8O_3$. Metabolite **(6)** was elucidated as piperonyl alcohol. |
| (**7**) | 14.2 | | **MS-EI m/z, (fragment) [% rel. int.]:** 150 ($M^+$) [86], 149 (M–H, base peak, formation of 3,4-methylenedioxybenzoyl cation -acylium ion-, due to α-cleavage of the benzyl bond) [100], 121 ($M^+$–CO–H, formation of 3,4-methylenedioxyphenyl cation, $C_7H_5O_2^+$) [34], 91 ($C_7H_7^+$) [13], 65 ($C_5H_5^+$) [21], 63 [34], 61 [10]. Metabolite (**7**) was identified as piperonal. |

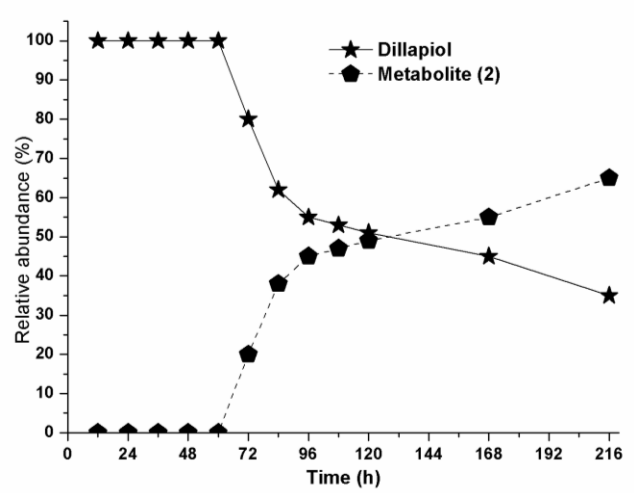
To the best of our knowledge, the metabolism of **S** and **dA** by plant fungal pathogens was evaluated for the first time in this study. On the other hand, *C. acutatum* was not able to transform **dA**.

Thus, the high antifungal activity of **dA** and the low level of microbial transformation, makes **dA** a promising candidate to control *C. acutatum*. It is important to note that the biotransformation effected by *C. acutatum* and *B. theobromae* on **S** and **dA** did not affect the dioxole moiety. Earlier works have reported that microbial conversion of **S** by an *Arthrobacter* strain and its *t*-anethole blocked mutants yield a dihydroxybenzene derivative, i.e. hydroxichavicol (Shimoni *et al.*, 2003). Similarly, in mammalians, the $P_{450}$ type enzyme converts **S** to hydroxychavicol in the liver (Scheline, 1991). Unlike the *Arthrobacter* strain and its *t*-anethole blocked mutants, the transformation of **S** by *C. acutatum* and *B. theobromae* involves the oxidation of the allyl side chain.

***Time-course experiments***

Compounds **S** and **dA** were incubated with the microorganisms during 216 and 360 h for *B. theobromae* and *C. acutatum*, respectively. The time course of relative concentration changes of **S** and **dA** and metabolites were monitored by TLC and relatively quantified through a GC analysis (Figures 4 and 5). When **dA** was metabolized by *B. theobromae*, only metabolite (**2**) was produced. As shown in Figure 4, compound **dA** started transformation after 60 h of incubation, and about 50% was consumed in 120 h.

**Figure 4**
**Time-course study for microbial transformation of dillapiole (dA) by *B. theobromae*. Metabolite (2) corresponds to dillapiole glycol.**



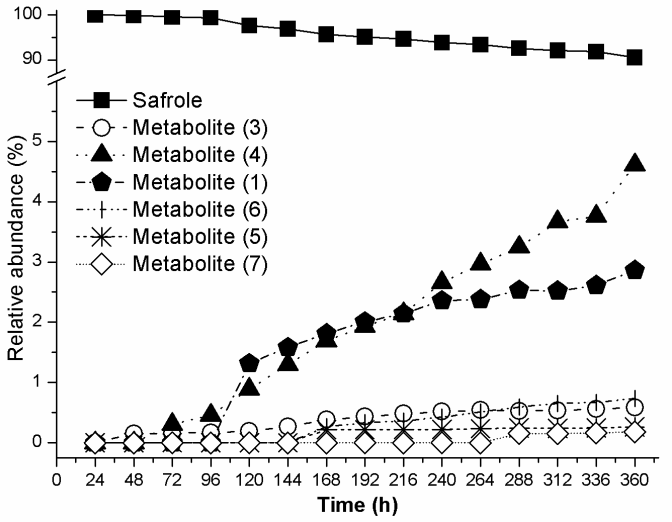

Metabolite (**2**) increased during entire assay, reaching the greatest abundance after 216 h (~65%). Additionally, *B. theobromae* mainly transformed **S** to metabolites (**1**) and (**3**), reaching the highest relative abundance after 72 h (~15%), and 48 h (~25%), respectively (*Data not shown*). As can be seen in Figure 5, the fungus *C. acutatum* only transformed about 10% of **S** after 360 h, principally converted to (**4**) and (**1**). However, the relative abundance of both metabolites was always lower than 5%. The other metabolites reached even lower levels. These results suggested that under the studied conditions, **S** and **dA** are slightly converted by the plant pathogenic fungus, *C. acutatum*. This could possibly be associated with the high toxicity of both compounds, which possess a methylenedioxyl group. Substances with that structural characteristic act as potent inhibitors of cytochrome-$P_{450}$ monoxygenases, dramatically diminishing the aerobic respiration (Santos *et al.*, 2004; Barreiro & Fraga, 2008). In addition, both compounds have an allyl side chain bonded to the aromatic ring that confers a high fungitoxicity (Fujita & Kubo, 2004).

Based on the structure of the identified metabolites, a metabolic pathway for the biotransformation of **S** and **dA** by *C. acutatum* and *B. theobromae* was proposed (Figure 6). The transformation of **S** and **dA** may resemble that of eugenol due to the similarity in its 2-propenyl side chain. Many bacteria and fungi, such as *Corynebacterium*, *Pseudomonas*, *Streptomyces*, *Byssochlamys*, *Penicillium* and *Rhodococcus* can transform eugenol (Plaggenborg *et al.,* 2006; Jin *et al.,* 2007). Studies have revealed that microorganisms follow different metabolic pathways for eugenol degradation. Recently, six possible biotransformation routes were presented (Mishra *et al.,* 2013). Thus, the formation of eugenol epoxide, followed by hydrolysis to yield a diol (eugenol-diol), have been proposed as the initial reactions of degradation in *Pseudomonas* sp. (Tadasa & Kayahara, 1983). Epoxides and dihydrodioles were also found in urine and liver of rats pretreated with of eugenol (and analogs such as safrole, estragole, and eugenol methyl ether) (Delaforge *et al.*, 1980). However, eugenol epoxide and eugenol-diol have not been identified from microbial metabolism (Xu *et al.,* 2007). Additionally, it has been suggested that the microbial metabolic pathway of eugenol involves the intermediates coniferyl alcohol (4-hydroxy-3-methoxy cinnamyl alcohol), coniferyl aldehyde and ferulic acid (Mishra *et al.,* 2013). Some of the genes that are essential to the degradation of eugenol by *Pseudomonas* sp. strain HR199 have already been identified (Overhage *et al.*, 1999; Priefert *et al.,* 1999).

According to our results, *B. theobromae* was able to dihydroxylate the double bond of **S** and **dA**, generating the major products: the glycols (**1**) and (**2**) respectively. Metabolite (**1**) was also detected in the biotransformation of **S** by *C. acutatum* (only 3% after 360 h). The identification of (**1**) and (**2**) implies that both are intermediates in the metabolic pathway from **S** and **dA**. These major metabolites are presumed to be formed via epoxidation at the double bond and subsequent hydrolysis of the epoxides. A similar epoxide intermediate was suggested in the degradation pathway of eugenol by a *Corynebacterium* sp. strain, but its existence has not been confirmed (Tadasa, 1977; Xu *et al.,* 2007). Safrole and dillapiole epoxide could be rapidly detoxicated by the formation of diols, possibly by an enzyme, since epoxide was not detected at any appreciable concentration (Guenthner & Luo, 2001). On the other hand, the opening of the epoxides could also be performed during the acidic extraction (Mohan *et al.*, 2000). The epoxidation of C-C double bonds, and further hydrolysis to yield vicinal diols, have been widely reported for the *Colletotrichum* genus (García-Pajón *et al.,* 2003). To the best of our knowledge, this is the first report on the degradation of 2-propenylbenzenes through an epoxide-diol route in both fungi.

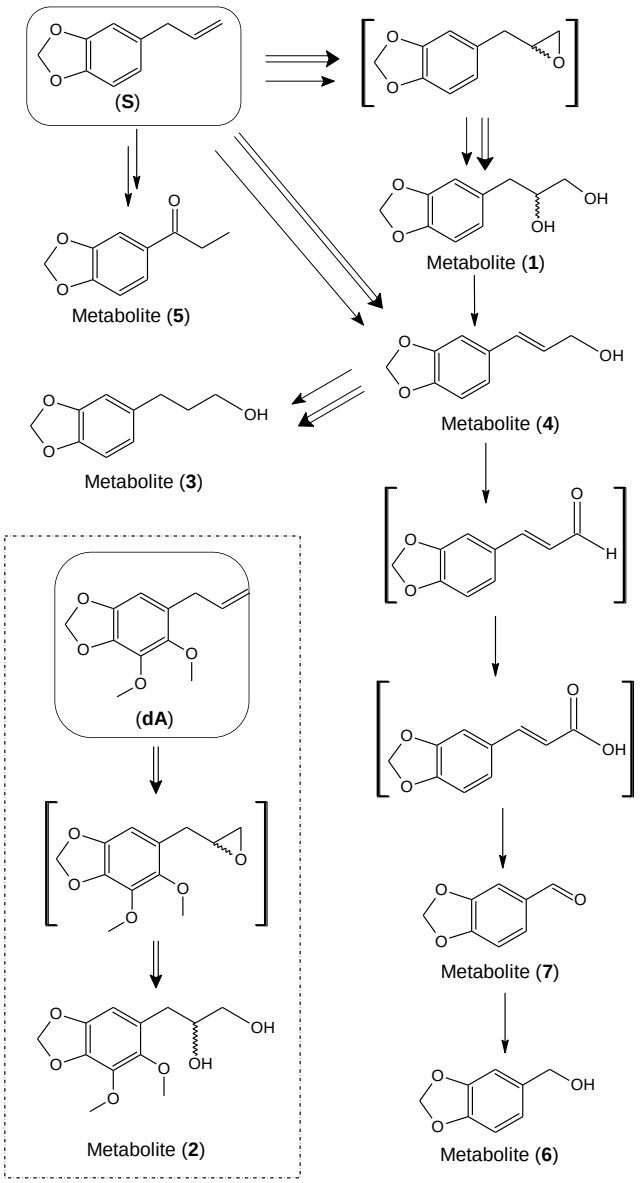
**Figure 5**

**Time-course study for microbial transformation of safrole (S) by *C. acutatum*. Metabolites (1), (3), (4), (5), (6) and (7) correspond to safrole glycol; 3-(3,4-methylenedioxyphenyl)-propan-1-ol; 3,4-methylenedioxy cinnamyl alcohol; 1-(3,4-methylenedioxyphenyl)-propan-1-one; piperonyl alcohol; and piperonal, respectively.**



Metabolite (**3**) might be formed via two pathways: possibly **S**→(**1**)→(**4**)→(**3**), or **S**→(**4**)→(**3**). The former pathway involves the dihydroxylation of the propenyl side chain of **S**, followed by dehydration to afford (**4**), and finally reduction of the double bond to yield (**3**). A similar metabolic pathway from eugenol to coniferyl alcohol by *Corynebacterium* sp. and *Pseudomonas* sp. was proposed by Tadasa and Kayahara (1983) and Mishra *et al.* (2013), respectively. It was found that eugenol hydroxylase/vanillyl alcohol oxidase (*ehyA*, *ehyB*, and *vaoA*) catalyzes the formation of eugenol epoxide and further transformation into coniferyl alcohol (analog of **4**) (Overhage *et al.*, 1999; Priefert *et al.*, 1999).

**Figure 6**
**Possible metabolic pathways of dillapiole (dA) and safrole (S) by *C. acutatum* and *B. theobromae*. The arrows indicate the transformations carried out by: (→) *C. acutatum* and (⇨) *B. theobromae.* Structures within brackets indicate hypothetical intermediates.**



Metabolite (**4**) may also be formed directly from **S** by a safrole hydroxylase. This analogous route, with a reaction mechanism including a quinone propenide intermediate, has been suggested for the catabolism of eugenol in *Pseudomonas* sp. strain HR199 (Overhage *et al.*, 1999). However, the exact side-chain oxidation mechanism of eugenol to coniferyl alcohol is still unknown. Finally, the reduction of (**4**) leads to (**3**). The ability of *C. acutatum* to reduce allylic alcohols was previously reported by Correa *et al.* (2009) and Velasco *et al.* (2012).

Furthermore, *C. acutatum* was able to transform **S** to (**5**); therefore, two pathways could be considered on the formation of (**5**). The substrate **S** could be reduced at the double bond, hydroxylated in the benzylic carbon and subsequently oxidized to produce the metabolite (**5**). Nevertheless, the reduction of the double bond may proceed before or after the hydroxylation. Thus, two different intermediates could be suggested: 1'-hydroxysafrole or 3,4-methylenedioxyphenylpropane. This oxidation of the propenyl side chain is unusual and has not been reported before in the microbial metabolism of 2-propenylbenzene compounds.

Metabolic product (**7**) is hypothesized as possible being derived from (**1**) via dehydration of the secondary alcohol function to afford (**4**), which is then oxidized to 3,4-methylenedioxycinnamyl aldehyde and further oxidized to the corresponding carboxylic acid (3,4-methylenedioxy cinnamic acid). A similar route has been proposed to the metabolism of eugenol by bacteria and fungi (Xu *et al.*, 2007; Mishra *et al.*, 2013; Han *et al.*, 2013). In this type of pathway, coniferyl alcohol is oxidized to coniferyl aldehyde by coniferyl alcohol dehydrogenase (*CaIA*) and further oxidized to ferulic acid by coniferyl aldehyde dehydrogenase (*CaIB*).

Then, the elimination of an acetate moiety from the unsaturated side-chain of 3,4-methylenedioxy cinnamic acid directly yields (**7**). The intermediate carboxylic acid, however, was not detected in the medium. A similar deacetylation has been proposed in the conversion of ferulic acid to vanillin (employing 4-hydroxycinnamate CoA ligase and 4-hydroxycinnamate CoA hydratase/ligase) for different bacteria and some fungi, such as *Aspergillus* sp., *Fomes fomentarius*, *Fusarium solani*, *Polyporus versicolor*, and *Rhodotorula rubra* (Huang *et al.*, 1993; Gasson *et al.*, 1998; Overhage *et al.*, 2003;). Moreover, a coenzyme-A-dependent mechanism has been proposed for the conversion of substituted cinnamic acids in *Pseudomonas putida*, and ferulic acid in *Rhodotorula rubra* (Huang *et al.*, 1993). However, the precise mechanism of such deacetylation has not been fully established. In a previous work, Santos *et al.* (2003) reported that metabolite (**7**) was formed on the biotransformation of isosafrole by *Cladosporium sphaerospermum*. Subsequently, the reduction of the carbonyl group from (**7**) affords (**6**). These results indicate that *C. acutatum* and *B. theobromae* were able to modify the allyl chain side of **S** and **dA**. Additionally, our results support the fact that *Colletotrichum* sp. species have considerable oxido-reductase activity (García-Pajón *et al.*, 2003).

## Antifungal activity of some metabolic products and derivatives of dA and S

### *Antifungal activity of metabolic products*

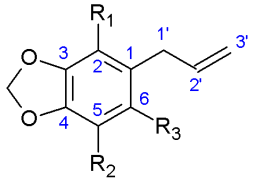
To determine if **S** and **dA** were metabolized by *C. acutatum* and *B. theobromae* through detoxification pathways, the inhibitory effects of metabolites (**1**) and (**2**) against *B. theobromae*, and (**6**) and (**7**) against *C. acutatum* were evaluated. Metabolites (**1**) and (**2**) were analyzed at 50 μg $mL^{-1}$ whereas (**6**) and (**7**) were evaluated at 200 μg $mL^{-1}$ concentration. All assays were carried out in triplicate. For *C. acutatum*, the mycelial growth inhibitions achieved by (**7**) were between 80.0 (after 24 h) and 30.8% (after 240 h). Meanwhile, metabolite (**6**) showed only about 36% growth inhibition after 24 h of incubation. Then, the antifungal activity steadily decreased, reaching only 25% inhibition after 240 h. Overall, the radial growth inhibition displayed by metabolites (**6**) and (**7**) decreased progressively in accordance with the incubation time. At a similar concentration, **S** only exhibited a mycelial growth inhibition of *C. acutatum* between 50 (at 24 h) and 19% (after 240 h). Therefore, the results of the antifungal bioassays showed that metabolic product (**6**) was less potent at inhibiting the fungal growth of *C. acutatum* than its precursor **S**. Since metabolite (**6**) was less fungistatic than **S**, it may suggest that the metabolism of **S** by *C. acutatum* was a detoxification process. Interestingly, metabolite (**7**) displayed a higher fungistatic effect against *C. acutatum* than **S**. So, it is possible to think that the low conversion of **S** to (**7**) by *C. acutatum* seeks to ensure no-inhibitory levels of (**7**) in the medium. In fact, the amount of (**7**) produced during the biotransformation of **S** by this plant pathogenic fungus (the minor metabolite) was almost negligible in comparison to that used in the bioassays.

Otherwise, the inhibitory effect of metabolites (**1**) and (**2**) against *B. theobromae* was weak. Mycelial growth inhibitions exhibited by both glycols were lesser than 5%. The higher fungistatic effect of **S** and **dA** against *B. theobromae*, as compared to metabolic products (**1**) and (**2**), suggests that its metabolism is a detoxification process. As mentioned above, the allyl side chain bonded to the aromatic ring confers a lipophilic characteristic and the ability to disrupt fungal membranes to **S** and **dA**. However, the dihydroxylation of the allyl chain to afford (**1**) and (**2**) reduced the lipophilic characteristic and abolished the antifungal activity, as compared to the parent compounds. Previously, it was reported that the relatively high antifungal activity of compounds that contain the methylendioxyl group is due to its capacity to act as a cytochrome $P_{450}$ inhibitor (Santos *et al.*, 2004). However, our results indicated that the allyl substituent is even more important in displaying such an inhibitory effect. Thus, our results are in agreement with a previous report that 1-allyl moiety plays a positive role in the antifungal behavior (Carrasco *et al.*, 2012). Indeed, these authors found that the replacement of the allyl radical resulted in the disappearance of the antifungal activity. Glycols (**1**) and (**2**) were not further metabolized under the conditions studied by the fungus *B. theobromae*.

***Antifungal activity of derivatives from S and dA***

It is noteworthy that, although the structural difference between **dA** and **S** is only seen in the presence of two methoxyl groups on the aromatic ring, **dA** was significantly more active than **S** against both fungi. These results indicate that electronic and/or steric factors in phenylpropenes might be important for antifungal activity. In order to investigate the relationship between the ring substitution and the antifungal activity of these compounds, the nitro and amino derivatives were also tested against the fungus *B. theobromae*. The compounds nitro safrole (1-allyl-6-nitro-3,4-methylenedioxybenzene) and (amino safrole) 1-allyl-6-amino-3,4-methylenedioxybenzene were prepared from **S**; whereas, nitro dillapiole (1-allyl-5,6-dimethoxy - 2 - nitro - 3, 4 - methylenedioxy benzene) and amino dillapiole (1-allyl-5,6-dimethoxy- 2 -amino - 3,4-methylenedioxy benzene) (Figure 7) were synthesized from **dA**.

**Figure 7**
**Chemical structure amino and nitro derivatives of S and dA.**




$R_1$= $R_2$= $R_3$= H; Safrole (**S**)
$R_1$= $R_2$= H; $R_3$= $NO_2$; Nitro safrole
$R_1$= $R_2$= H; $R_3$= $NH_2$; Amino safrole
$R_1$= H; $R_2$= $R_3$= -$OCH_3$; Dillapiole (**dA**)
$R_1$= $NO_2$; $R_2$= $R_3$= -$OCH_3$; Nitro dillapiole
$R_1$= $NH_2$; $R_2$= $R_3$= -$OCH_3$; Amino dillapiole

Confirmation of the structures of the derivatives was carried out using spectroscopic data (Table 2).

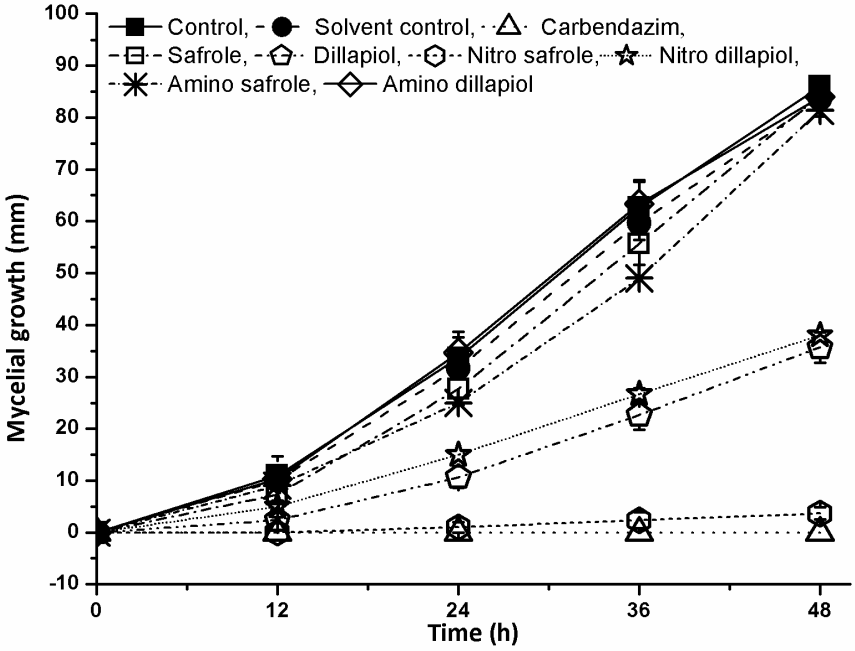
Nitro- and amino derivatives of **S** and **dA** were screened for their antifungal activity against *B. theobromae*. The results showed that the inclusion of an electron-releasing group in the aromatic ring, such as the amino group, on **S** or **dA** produced no significant contribution to the antifungal activity (Figure 8). On the contrary, this group extinguished the previously observed high fungistatic effect for **dA** against *B. theobromae* at the same concentration (ca. 25 times less active than **dA** at 48 h of assay). On other hand, the insertion of an electron-withdrawing group, such as the nitro group, in both phenylpropenes produced different effects on the antifungal activity of the two derivatives. The inhibitory effect on the mycelial growth of *B. theobromae* from the nitro dillapiole was comparable to that of **dA**. Otherwise, nitro safrole

was found to be the most active compound against *B. theobromae*, being almost 49 times higher than **S** at the same concentration and after 48 h of the assay.

**Table 2**
**Spectroscopic data of safrole and dillapiole derivatives**

| Compound | Spectroscopic data |
|---|---|
| **Nitro safrole** | **$^{1}$H-NMR ($CDCl_3$, 300 MHz):** δ 7.51 (s, 1H, $H_3$), 6.78 (s, 1H, $H_6$), 6.12 (s, 2H, –$OCH_2O$–), 6.06-5.92 (ddt, 1H, *J* = 16.8, 10.2, 6.6, $H_{2'}$), 5.09-4.99 (dd, 2H, *J* = 13.6, 1.8, $H_{3'a,b}$), 3.69-3.66 (d, 2H, *J* = 6.6, $H_{1'a,b}$). **$^{13}$C-NMR ($CDCl_3$, 75 MHz):** 151.7 ($C_1$), 146.5 ($C_2$), 110.4 ($C_3$), 142.8 ($C_4$), 132.2 ($C_5$), 105.7 ($C_6$), 37.62 ($C_{1'}$), 135.2 ($C_{2'}$), 117.0 ($C_{3'}$), 102.8 (–$OCH_2O$–). |
| **Amino safrole** | **$^{1}$H-NMR ($CDCl_3$, 300 MHz):** δ 6.57 (s, 1H, $H_3$), 6.29 (s, 1H, $H_6$), 5.96-5.85 (ddt, 1H, *J* = 16.8, 10.2, 6.3, $H_{2'}$), 5.85 (s, 2H, –$OCH_2O$–), 5.13-5.09 (dd, 2H, *J* = 6.0, 1.8, $H_{3'a}$), 5.06-5.05 (d, *J* = 1.5, $H_{3'b}$), 3.23-3.21 (d, 2H, *J* = 6.0, $H_{1'a,b}$). **$^{13}$C-(JMOD) NMR ($CDCl_3$, 75 MHz):** 135.6 (CH, $C_{2'}$), 115.5 ($CH_2$, $C_{3'}$), 109.6 (CH, $C_5$), 100.2 (–$OCH_2O$–), 97.9 (CH, $C_2$), 35.8 ($CH_2$, $C_{1'}$). |
| **Nitro dillapiole** | **$^{1}$H-NMR ($CDCl_3$, 300 MHz):** δ 6.03 (s, 2H, –$OCH_2O$–), 5.91-5.77 (ddt, 1H, *J* = 15.7, 9.6, 6.6, $H_{2'}$), 5.05-5.01 (dd, 2H, *J* = 12.4, 1.1, $H_{3'a,b}$), 3.98 (–OMe), 3.93 (–OMe), 3.28-3.26 (d, 2H, *J* = 6.6, $H_{1'a,b}$). **$^{13}$C-(JMOD) NMR ($CDCl_3$, 75 MHz):** 135.2 (CH, $C_{2'}$), 116.7 ($CH_2$, $C_{3'}$), 102.8 (–$OCH_2O$–), 61.5 (-OMe), 60.8 (-OMe), 30.2 ($CH_2$, $C_{1'}$). |
| **Amino dillapiole** | **$^{1}$H-NMR ($CDCl_3$, 300 MHz):** δ 6.00 (s, 2H, –$OCH_2O$–), 5.99-5.86 (m, 1H, $H_{2'}$), 5.08-4.96 (m, 2H, $H_{3'a,b}$), 3.81 (–OMe), 3.70 (–OMe), 3.21-3.18 (m, 2H, $H_{1'a,b}$). The **$^{13}$C-NMR** analysis was not possible due to insufficient quantity. |

**Figure 8**
**Effect of safrole (S), dillapiole (dA), and their nitro and amino derivatives on the radial mycelial growth of *B. theobromae*. Results are shown as mean values of three replicates of the mycelium diameter; bar = ± SD.**



The data showed that the antifungal activity of nitro safrole was comparable to that exerted by the commercial synthetic fungicide Carbendazim at the same concentration. In contrast to a previous report (Carrasco *et al.*, 2012), our results indicated that the introduction of a $NO_2$ group led to an increase in the antifungal activity of **S**. Thus, it is possible to believe that the presence of the two methoxyl groups in **dA** or the nitro group in C6 in nitro safrole may lead to a correct balance of hydrophilicity-lipophilicity, which is an essential factor for the antifungal properties seen in these compounds.

## CONCLUSIONS

The present paper reports on the ability of *B. theobromae* to convert **S** and **dA** to the corresponding vicinal diols (glycols), which could be used in a wide variety of applications. Time-course studies indicated that **S** is converted to safrole glycol at a rapid rate. Also, it was demonstrated that *C. acutatum* transformed **S** into various metabolites at a low proportion, including safrole glycol, piperonal, piperonyl alcohol, among others. However, *C. acutatum* was ineffective in transforming **dA**. Additionally, the fungitoxicity of **S** and **dA**, their metabolic products, and nitro- and amino-derivatives were evaluated against both fungi. **S** and **dA** may effectively inhibit the mycelial growth of the evaluated pathogenic fungi. However, **dA** was the most active. Also, the products from the metabolism of **S** and **dA** displayed lower antifungal activities than the parent compounds. So, it was concluded that the metabolism of **S** and **dA** is a detoxification process. It can also be concluded that allyl moiety plays a positive role in the inhibitory effect of **S** and **dA** against *C. acutatum* and *B. theobromae*. Finally, the most active derivative against the fungus *B. theobromae* was nitro safrole, 49 times more active than **S** at the same concentration and assay time. Nitro safrole displayed an activity against *B. theobromae* that was comparable to that of the commercial fungicide Carbendazim®. Therefore, it is possible to believe that **S** and **dA** offer interesting structural templates that may be transformed for developing new antifungal agents.

## ACKNOWLEDGEMENTS

This work was supported by a grant (Vicerrectoría de Investigación, Nº 20101007957, Universidad Nacional de Colombia). We thank to Dr. Afanador-Kafuri for providing cultures of the plant pathogen fungi.

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