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RESEARCH ARTICLE / *ARTIGO*

Thymol and carvacrol: biotransformation and antifungal activity against the plant pathogenic fungi *Colletotrichum acutatum* and *Botryodiplodia theobromae*

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ABSTRACT

Fungal growth in fruits may cause spoilage and result in a reduction of their quality and quantity. The aim of the current study was to investigate the antifungal activity and the metabolism of thymol and carvacrol by *Colletotrichum acutatum* and *Botryodiplodia theobromae*. The results showed that both compounds provided relatively good control against these plant pathogenic fungi. Mycelial growth of *C. acutatum* and *B. theobromae* was inhibited at 50 µg/mL and above. At 150 µg/mL, thymol and carvacrol inhibited the radial growth of fungi completely and this effect remained for 240 h. Furthermore, thymol and carvacrol were metabolized by the plant pathogenic fungi in low proportion to several compounds, including thymoquinone, thymohydroquinone, thymyl- and carvacryl acetate, thymyl- and carvacryl methyl ether. The transformations affect the structural requirements of thymol and carvacrol related to their antimicrobial activity and mode of action. The relatively high antifungal activity of thymol and carvacrol against *C. acutatum* and *B. theobromae* and the low levels of microbial transformation indicate that both compounds could be an alternative to traditional chemical fungicides for control of pre- and postharvest phytopathogenic fungi on fruits or vegetables.
Key words: Alternative control, arylpropanoids, fungal detoxification, metabolic pathways, thymoquinone.

RESUMEN

Timol y carvacrol: biotransformación y actividad antifúngica contra los hongos fitopatogénicos *Colletotrichum acutatum* y *Botryodiplodia theobromae*

El crecimiento de los hongos en frutas puede causar el deterioro de los alimentos y resultar en una reducción de la calidad y la productividad. El objetivo del presente estudio fue investigar la actividad antifúngica, y el metabolismo de timol y carvacrol por *Colletotrichum acutatum* y *Botryodiplodia theobromae*. Los resultados muestran que ambos compuestos despliegan un relativo buen control contra los hongos fitopatogénicos; el crecimiento micelial de *C. acutatum* y *B. theobromae* se inhibió a concentraciones de 50 µg/mL y mayores. A 150 µg/mL, timol y carvacrol inhibieron el crecimiento radial de los hongos completamente y este efecto permaneció durante 240 h. Además, los hongos fitopatogénicos metabolizaron timol y carvacrol a varios compuestos en una baja proporción, incluyendo timoquinona, timohidroquinona, timil- y carvacril acetato, timil- y carvacril metil eter, entre otros. Las transformaciones afectan los requerimientos estructurales del timol y carvacrol relacionados con su actividad antimicrobiana y modo de acción. La actividad antifúngica relativamente alta de timol y carvacrol contra *C. acutatum* and *B. theobromae* y los niveles de transformación microbiana bajos, indican que ambos compuestos pueden ser una alternativa a los fungicidas químicos tradicionales para el control de los hongos fitopatogénicos en pre- y poscosecha de frutas y vegetales.
Palabras-clave: Arilpropanóides, control alternativo, desintoxicación fúngica, rutas metabólicas, timoquinona.

INTRODUCTION

Stem-end rot (*Botryodiplodia theobromae*) and anthracnose (*Colletotrichum acutatum*) are two serious diseases that contribute significantly to harvest and postharvest loss of tamarillo, avocado, mango, papaya, and citrus in Colombia (Afanador-Kafuri et al., 2003; Martínez et al., 2009). The use of synthetic chemicals as fungicides is the primary method of control of postharvest fungal decay caused by both diseases. However, the rapid development of tolerance to commercial fungicides by *C. acutatum* and *B. theobromae* has led to an increase in the quantities of these compounds that have to be used. Consequently, the presence of fungicide residues on the fruits decreases their quality and can prevent their export to some foreign markets (Tripathi & Dubey, 2004). Furthermore, the use of synthetic chemicals to control pre- and postharvest deterioration of food commodities is restricted, due to their possible carcinogenicity, teratogenicity, acute toxicity, environmental pollution and side effects on human beings (Tripathi & Shukla, 2007). Therefore, the fruit industry urgently demands alternative pre- and postharvest treatments that are

free of synthetic fungicides and acceptable to consumers. Given these facts, the use of essential oils and some of their constituents can be a very attractive method for pre- and postharvest disease control of fruits, due to their relative safety and wide acceptance by consumers (Ormancey et al., 2001).

Carvacrol (5-isopropyl-2-methylphenol; - "C") and thymol (2-isopropyl-5-methylphenol; - "T") are the main components of the essential oils of some Laminaceae members like oregano, thyme, and savory. They are produced by these plant species as a chemical defense mechanism against phytopathogenic microorganisms (Vázquez et al., 2001). Accordingly, the potent antimicrobial and fungitoxic properties of (C) and (T) against various plant pathogens have been previously documented (Sokovic et al., 2002; Falcone et al., 2005). It has been found that these agents cause alterations in the hyphal morphology and hyphal aggregates, resulting in reduced hyphal diameters and lyses of hyphal wall, as these interact with the cell membrane of the pathogen (Soylu et al., 2007). In addition, chemical modification of these phenolic compounds to various ether and ester derivatives has been reported to result in change in biological activity (Mathela et al., 2010).

Despite their antimicrobial characteristics, Chamberlain and Dagley (1968) found a *Pseudomonas* strain able to degrade (T) completely and (C) partially. The authors proposed a metabolic pathway for (T) that involves *meta*-ring opening of a trihydric phenol, 3-hydroxythymo-1,4-quinol to 3,7-di-methyl-2,4,6-trioxo-octanoate. Hydrolysis of the latter, catalyzed by β-ketolase, yields acetate, 2-ketobutyrate and isobutyrate. Thus, biotransformation experiments provide information on the detoxification mechanism used by phytopathogenic microorganisms and give an indication of the structural modifications that may be necessary if substrates of this type are to be further developed as selective fungal control agents (Daoubi et al., 2005). The aim of the present study was to evaluate the antifungal properties against *C. acutatum* and *B. theobromae* of (T) and (C), along with their metabolism.

MATERIALS AND METHODS

Biological and chemical materials

C. acutatum and *B. theobromae* were isolated from infected tamarillo (*Solanum betacea*) and avocado (*Persea americana*) fruits, respectively. Fungi were characterized and kindly provided by the Laboratory of Phytopathology (Universidad Nacional de Colombia-Medellín). Pure cultures were maintained on potato dextrose agar (PDA) at 24°C, and freshly subcultured before using in the transformation experiment. The substrates for biotransformation, (T) and (C), were purchased from Abaquim Ltda. (Medellín, Colombia) and Merck KGaA (Darmstadt, Germany), respectively. Thymoquinone was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Thymohydroquinone was prepared by reduction of thymoquinone, as described by El-Dakhakhny (1963). Methylated and acetylated derivatives of (T) and (C) were obtained according to Mathela et al. (2010), and Smith & March (2007). The purified synthetic compounds were analyzed by ^{1}H- and ^{13}C-Nuclear magnetic resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS) to confirm their identity and purity.

Antifungal bioassay

In order to investigate the antifungal activity of (T) and (C), against the microorganisms, the poisoned food technique described by Grover and Moore (1962) with slight modifications, was used. In poisoned food technique, both fungi were inoculated on Potato dextrose agar (PDA) plates and incubated at 25°C for 48 h to obtain young, actively growing colonies. A total of 30 µL of ethanolic solutions of (T) and (C) at concentrations of 0.0250, 0.0375, 0.0500, 0.0625, 0.0750 and 0.100 mg/µL were taken and mixed with 15 mL of cooled molten PDA medium (final concentrations 50, 75, 100, 125, 150 and 200 µg/mL), and allowed to solidify at room temperature for thirty minutes. Concentration at 125 µg/mL of (T) and (**C**) was not evaluated for *B. theobromae*. Subsequently, a mycelial disc (5 mm in diameter) of *C. acutatum* or *B. theobromae*, cut with a sterile cork borer from the periphery of 48 h old cultures, was aseptically inoculated onto the agar plates containing (T) or (C). Petri dishes with PDA plus ethanol (2 µL/mL) were used as a control. The plates were incubated at room temperature and the diameter of the mycelial growth was measured every 24 hours. Incubation was ceased when the mycelial mass of control Petri dishes had almost filled it (ca. 240 and 144 hours for *C. acutatum* and *B. theobromae*, respectively). All concentrations were tested in triplicate. The relative growth inhibition of the treatment compared to the control was calculated as percentage, using the following formula:

Inhibition (%) = {1 – [radial growth of treatment (mm)/ radial growth of control (mm)]}x100

Pre-culture of *C. acutatum* and *B. theobromae*

Fungi were transferred into 1.0 L Erlenmeyer flasks containing 500 mL of Czapeck-Dox liquid medium (Solution A: Glucose 5%, yeast extract 0.1%; Solution B: K_2HPO_4 0.5%, $NaNO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%). Erlenmeyer flasks were agitated in a reciprocating shaker (Centricol series 0239) at 120 rpm and room temperature for 8 days. Mycelia were harvested by filtration, washed with sterile H_2O and immediately employed in the biotransformation and time course experiments.

Biotransformation, isolation and identification of metabolic products

Mycelia of *C. acutatum* and *B. theobromae* were transplanted into four 1.0 L Erlenmeyer flasks containing 500 mL of sterilized Czapeck-Dox culture medium and substrate (at 80 µg/mL). Flasks were incubated on a reciprocating shaker (120 rpm) at room temperature for 14 days. A substrate-free control was included in all

experiments. After incubation, culture medium and mycelia were separated by filtration. Mycelia were discarded and culture medium was saturated with NaCl and extracted with ethyl acetate (EtOAc, 3x2.0 L). Afterwards, the medium was acidified to pH 2 with 1M HCl, and extracted again with CH_2Cl_2 (2x2.0 L). Both organic extracts were mixed, dried over anhydrous Na_2SO_4 and concentrated in vacuum. Some metabolites were partially purified by column chromatography (CC), passing the extract through a silica gel 60 (mobile phase: mixtures of *n*-hexane: EtOAc) column, and then through a Sephadex LH-20 column (mobile phase: mixture of *n*-hexane:CH_2Cl_2: methanol, 2:1:1 v/v). The structures of the products were determined by mass spectrometry (MS), ^1H and ^{13}C NMR analysis, and comparison with the NIST 2002 computerized mass spectral library and authentic standards (Table 1).

Time-course experiment

Pre-cultured *C. acutatum* or *B. theobromae* was transferred into fourteen 500 mL Erlenmeyer flasks, each containing 125 mL of Czapeck-Dox liquid medium and substrate (T) or (C), and stirred for 14 days under the same conditions as described for preculture. Daily, the content of one flask was removed for each of 14 days, saturated with NaCl, and extracted with EtOAc (3x150 mL). The extracts obtained after solvent evaporation were analyzed by thin layer chromatography (TLC) and GC-MS. The ratios between the substrate and metabolic products were determined on the basis of GC peak areas. Control cultures in which the microorganism was grown under identical conditions, but without substrate, were carried out.

Analytical methods

TLC was performed on precoated plates (Si 60 F_{254}, 0.25 mm, Merk) using mixtures of *n*-hexane:EtOAc as mobile phase. Column chromatography (CC) used silica gel 60 (0.040-0.063 mm; Merck) and Sephadex LH-20 (Sigma-Aldrich Co., St. Louis, MO, USA). Gas chromatography (GC) was performed on a Hewlett-Packard 6890 (Agilent Technologies) gas chromatograph equipped with a HP 5973 MSD (Mass selective detector-Quadrupole type). A HP-5 column (30 m x 0.25 mm i.d.; coating thickness 0.25 µm) was used. Chromatographic conditions were as follows: column temperature, 50-250°C at 10°C/min and keep for five minutes; injector temperature, 150°C; detector temperature, 280°C; carrier gas, N_2 at 1 mL/min. Relative composition of the individual constituents was determined from the average area of the peaks. Electron ionization mass spectrometry (EI-MS) measurements were obtained using GC-MS. Chromatographic conditions were the same as described earlier. Substances were identified by comparison of their mass spectra with those of reference substances and by comparison with the NIST 2002 Mass Spectral Library. NMR spectra were measured on a Bruker AMX 300 NMR spectrometer (^1H NMR, 300.12 MHz; ^{13}C NMR, 75.42 MHz). Chemical shifts, δ, are expressed in ppm units downfield from TMS and coupling constant *J* is in Hertz (Hz).

RESULTS AND DISCUSSION

Antifungal activity

The inhibitory effects of (T) and (C) against *C. acutatum* and *B. theobromae* are shown in Figures 1 and 2. As can be seen, mycelial growth of these fungi was dependent on the concentration of (C) and (T) in the culture medium. At 150 µg/mL and above, (C) and (T) inhibited the radial growth of *C. acutatum* completely and this effect remained during the 240 h duration of the experiment (Figure 1). Furthermore, fungal colony development initiated only 48 and 96 hours after inoculation when *C. acutatum* was treated with (C) and (T) at 100 µg/mL, respectively. In addition, inhibition percentages of *C. acutatum* at 75 µg/mL ranged from 25-30% for (C) and 50-60% for (T). This shows a slightly higher antifungal activity of (T) in comparison to (C) against *C. acutatum*. In the case of *B. theobromae*, its growth was completely inhibited when a concentration of 150 and 200 µg/mL of (C) or (T) was used. In general, results revealed that (C) and (T) exhibited a relatively high antifungal action toward *C. acutatum* and *B. theobromae*. Some of these results are in agreement with those obtained earlier by Alzate et al. (2009). The antifungal activity of (T) and (C) against other plant pathogenic fungi has also been investigated (Tsao & Zhou, 2000; Holley & Patel, 2005). In particular, (T) and (C) showed a very strong antifungal activity against *Phytophthora cactorum* and *Cryphonectria parasitica* at 28 x 10^{-3} mg/mL air concentration (Kim et al., 2008), and a complete growth inhibition of *Alternaria arborescens* and *Rhizopus stolonifer* (Plotto et al., 2003). Consequently, the potent antimicrobial properties of (T) and (C) against spoilage bacteria and food borne pathogens have been also reported in the literature (Valero et al., 2006).

The mode of action for (C) and (T) is uncertain; however, activity may be linked to the hydrophobicity and consequently, the ability of both compounds to pass through the fungal cell membrane (Knobloch et al., 1988), which further affects pH homeostasis and equilibrium of inorganic ions (Ultee et al., 2000; Cristani et al., 2007) disturbing the cell structures. Furthermore, the involvement of the hydroxyl group in the formation of hydrogen bonds and the acidity of these phenolic compounds may be another important factor involved in the bioactivity. Ultee et al. (2000) additionally hypothesized that (C) acts as a proton exchanger, thereby reducing the pH gradient across the cytoplasmic membrane. This leads to the collapse of the proton motive force and depletion of the ATP pool, causing cell death. Moreover, it seems possible that both compounds may interfere with cell

TABLE 1 - GC retention time (*Rt*) and EI mass spectral properties and identification of products detected in incubation of *C. acutatum* and *B. theobromae* with (T) and (C)

Compound No.[a]	*Rt* (min)	*m/z* of fragment ions [% relative intensity]	Identification
(*CC1*),(*TC2*), (*CB2*), (*TB2*)	18.94	166 (M^+) [47], 152 [14], 151 (M^+- CH_3) [100], 137, 123 (M^+ - C_3H_7) [10], 95 [10], 83 [10], 77, 71, 69, 57, 55, 43 ($C_3H_7^+$) [14], 41 [10].	*Thymohydroquinone**
(*CC2*),(*CB4*)	16.96	166 (M^+) [24], 151 (M^+ - CH_3) [41], 138 [21], 107 (M^+ - C_3H_6O- H) [71], 83, 77, 57, 43 ($C_3H_7^+$) [100].	*5-(1-Hydroxy-1-methyl-ethyl)-2-methyl-phenol*‡
(*CC3*)	19.34	164 (M^+) [100], 149 (M^+- CH_3) [30], 147 (M^+- OH) [12], 137 [11], 121 (M^+- C_3H_7) [26], 107, 91 ($C_7H_7^+$) [19], 77 ($C_5H_5^+$) [11], 57, 55, 39.	*2-Isopropenyl-5-methyl-benzene-1,4-diol*‡
(*CC4*)	17.50	164 (M^+) [100], 149 (M^+- CH_3) [40], 121 (M^+- C_3H_7) [27], 103, 91 ($C_7H_7^+$) [16], 77 ($C_5H_5^+$) [11], 43.	*Carvacryl methyl ether*†,*
(*CC5*),(*CB5*)	14.05	164 (M^+) [63], 149 (M^+- CH_3) [70], 136 (M^+- CO) [40], 135 (M^+- HCO) [51], 121 (M^+- C_3H_7) [81], 108 (M^+- CO- C_2H_4) [29], 107 [25], 93 [100], 91 ($C_7H_7^+$) [61], 79 [35], 77 ($C_5H_5^+$) [47], 68 [45], 39 [85].	*Thymoquinone*†,*
(*CC6*), (*CB7*)	13.28	192 (M^+) [7], 150 (M^+- H_2C_2O) [58], 135 (M^+- H_2C_2O- CH_3) [100], 91 ($C_7H_7^+$) [14], 83, 77 ($C_5H_5^+$), 44.	*Carvacryl acetate*†,*
(*TC1*),(*TB1*)	19.36	166 $(M)^+$ [40], 151 (M^+- CH_3) [100], 133 (M^+- H_2O- CH_3) [9], 121 (M^+- H_2CO- CH_3) [20], 107 (M^+- H_2CO- C_2H_4- H) [8], 105 [8], 103 [8], 95 [10], 91 [10], 77 ($C_6H_5^+$) [18].	*2-(1-hydroxypropan-2-yl)-5-methylphenol*‡
(*TC3*)	18.33	164 $(M)^+$ [41], 149 (M^+- CH_3) [100], 135 [29], 121 (M^+- C_3H_7) [29], 105 [38], 103 [24], 91 ($C_7H_7^+$) [46], 77 ($C_6H_5^+$) [36], 43 ($C_3H_7^+$) [81].	*Thymyl methyl ether**
(*CB1*)	19.25	166 (M^+) [28], 135 (M^+- H_2CO- H) [100], 115 [11], 105 (M^+- H_2O- C_2H_4- CH_3) [32], 91 ($C_7H_7^+$) [22], 79 [8], 77 ($C_6H_5^+$) [10].	*5-(2-hydroxy-1-methyl-ethyl)-2-methyl-phenol*‡
(*CB3*)	18.35	166 (M^+) [29], 148 (M^+- H_2O) [56], 133 (M^+- H_2O- CH_3) [21], 105 [100], 91 $(C_7H_7)^+$ [19], 83, 77 ($C_6H_5^+$) [25], 57 [14], 43($C_3H_7^+$) [15].	*2-hydroxymethyl-5-isopropyl-phenol*‡
(*CB6*)	14.15	148 (M^+) [100], 133 (M^+- CH_3) [44], 108 [29], 107 (M^+- C_3H_5) [22], 105 [30], 91 ($C_7H_7^+$) [22], 77, 57 [20], 43, 41 [14].	*5-Isopropenyl-2-methyl-phenol*‡
(*TB3*)	14.08	168 $(M)^+$ [7], 153 (M^+- CH_3) [13], 150 (M^+- H_2O) [47], 139 [9], 135 (M^+- H_2O- CH_3) [41], 126 (M^+-C_3H_6) [100], 111 [42], 104 [55], 97 [37], 91($C_7H_7^+$) [25], 83 [65], 69 [30], 55 [31], 43 [26], 41 [31].	*4-Hydroxy-3-isopropyl-6-methyl-cyclohex-2-enone or position isomer*‡
(*TB4*)	13.28	192 $(M)^+$ [6], 150 (M^+- H_2C_2O) [34], 135 (M^+- H_2C_2O- CH_3) [100], 115 [10], 105, 91 ($C_7H_7^+$) [15], 77 ($C_6H_5^+$) [7].	*Thymyl acetate**,†
(*TB5*)	13.88	168 $(M)^+$ [6], 153(M^+- CH_3) [12], 150 (M^+- H_2O) [49], 139 [8], 135 (M^+- H_2O- CH_3) [31], 126 (M^+-C_3H_6) [100], 111 [41], 107 [24], 97 [37], 83 [55], 69 [32], 55 [34], 43 [43].	*4-Hydroxy-6-isopropyl-3-methyl-cyclohex-2-enone or position isomer*‡
(*TB6*)	13.04	182 $(M)^+$ [7], 140 (M^+- H_2C_2O) [20], 139 (M^+- H_2C_2O- H) [15], 122 [19], 112 [25], 111 (M^+- H_2C_2O- C_2H_4- H) [100], 96 [39], 83 [19], 96 [39], 83 [18].	*2-Hydroxymethyl-5-isopropyl-benzene-1,4-diol* ‡

Identified by: *, proved with authentic standard and NMR; †, mass spectrum identical with literature spectrum; ‡, structure derived from the interpretation of the mass spectrum. [a]Notation indicates the substrate and microorganism used: (C)-*C. acutatum*, *CC*; (C)-*B. theobromae*, *CB*; (T)-*C.acutatum*, *TC*; (T)-*B. theobromae*, *TB*. (*CC1*) is the same compound that (*TC2*), (*CB2*), (*TB2*). Similarly, (*CC2*) = (*CB4*); (*CC5*) = (*CB5*); (*CC6*) = (*CB7*); and (*TC1*) = (*TB1*).

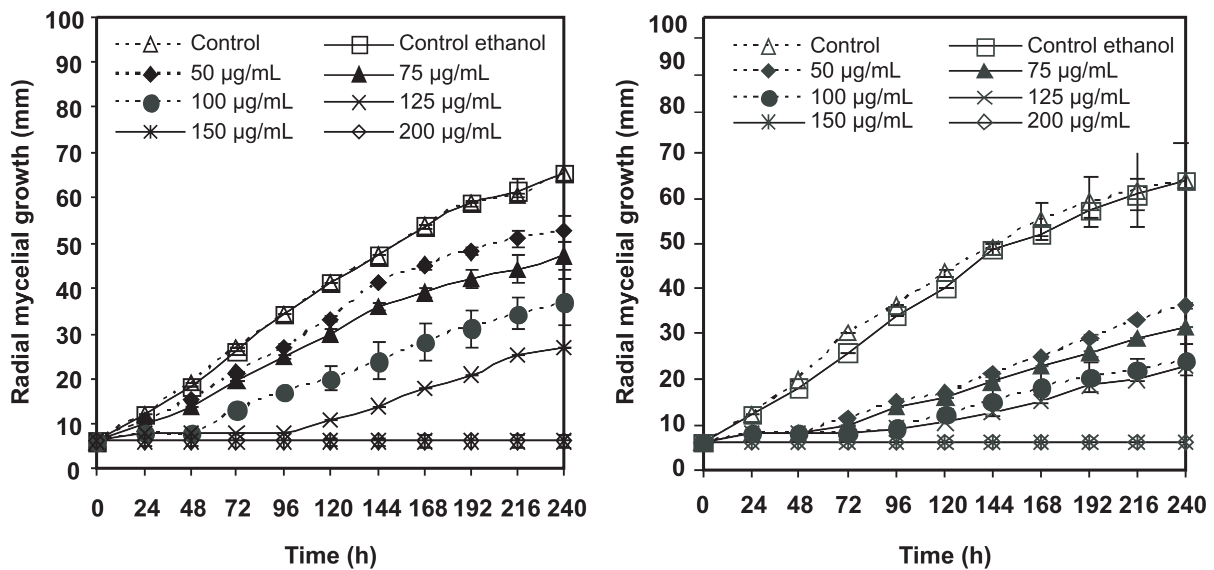

FIGURE 1 - Effect of (C) (left) and (T) (right) on radial mycelial growth of *C. acutatum*. Results are shown as average values of three replicates of mycelium diameter; bar = ±SD.

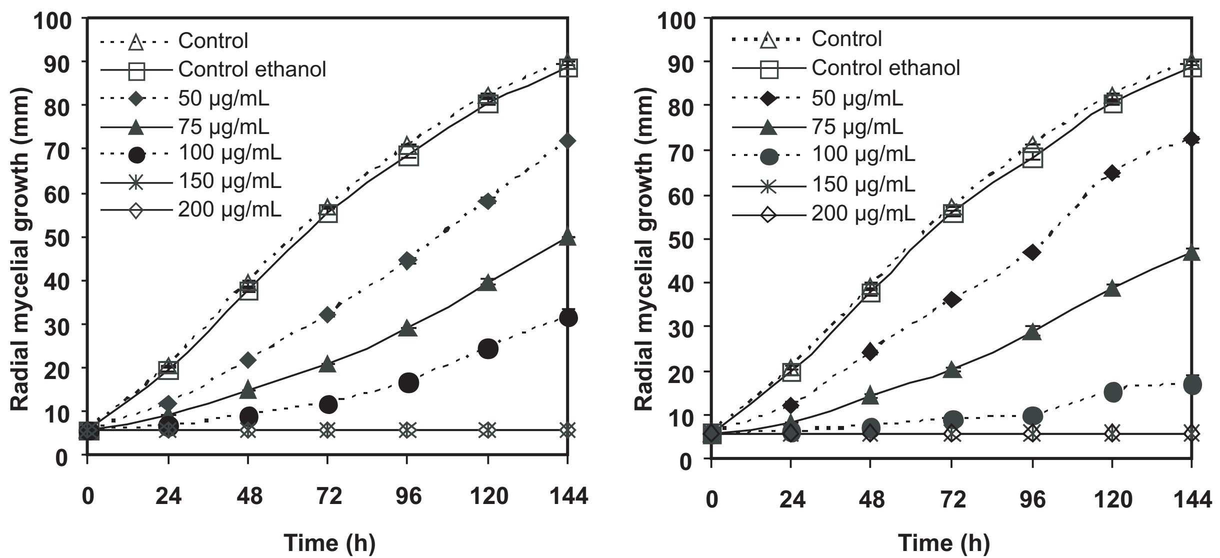

FIGURE 2 - Effect of (C) (left) and (T) (right) on radial mycelial growth of *B. theobromae*. Results are shown as average values of three replicates of mycelium diameter; bar = ±SD.

wall enzymes like chitin synthase/chitinase as well as with the α- and β-glucanases of the fungus (Adams et al., 1996).

Although the difference between (T) and (C) is only in the position of the hydroxyl group, (T) was more active than (C) against *C. acutatum*. Growth inhibition percentages of *C. acutatum* achieved by (T) and (C) at 100 µg/mL for 10 days were from 86.0 (day 1) to 55.0 (day 10), and from 63.0 (day 1) to 30.5% (day 10), respectively. Different studies indicate that the position of the hydroxyl group is very important in antifungal and insecticidal activity (Kim et al., 2008; Park et al., 2008).

For *B. theobromae*, only slight differences in the percentage of inhibition among (T) and (C) were found. Inhibition of *B. theobromae* growth by (T) at 100 µg/mL and for 6 days reached values between 97.7 (day 1) and 86.5% (day 6), whereas for (C) the values were between 93.2 (day 1) and 69.1% (day 6). As can be seen, growth inhibition percentages of *C. acutatum* and *B. theobromae* achieved by (T) and (C) at 100 µg/mL decreased with time, a fact which suggests that both fungi possess a detoxification mechanism. Similar behavior occurred when (T) and (C) were evaluated at concentrations below 150 µg/mL (data not shown).

Metabolic compounds

A comparison of the TLC profiles and GC chromatograms obtained from biotransformation and the control, showed that *C. acutatum* transformed (C) into six major metabolites and a small amount of minor compounds not found in the control (data not shown). The structures of these metabolites were determined from the interpretation of spectral data (MS and NMR), and comparison with authentic samples (Table 1).

Two compounds (*CC1* and *CC2*) had a molecular formula of $C_{10}H_{14}O_2$ based on its mass spectrum, $(M)^+$ = 166 amu. The molecular ion was 16 mass units higher than that of the parent compound (C), indicating the addition of a hydroxyl group to (C). Metabolite (*CC1*) had a fragment ion at *m*/*z* 151 (base peak) attributed to the loss of methyl group. Also, peaks of low intensity appear at *m*/*z* 123 and 43, corresponding to loss of isopropyl group and formation of isopropyl cation, respectively. From the spectral data, (*CC1*) was elucidated to be 2-methyl-5-isopropylhydroquinone (thymohydroquinone). Unfortunately, the very low abundance of (*CC1*) from the biotransformation excluded purification, enrichment and further spectroscopic characterization, and the full structural confirmation required the synthesis of this compound. The mass spectrum and retention time for (*CC1*) were consistent with those of synthetic thymohydroquinone obtained according to literature (El-Dakhakhny, 1963). It is noteworthy that thymohydroquinone was previously reported to be produced during biotransformation of (T) and (C) by a *Pseudomonas* strain (Chamberlain & Dagley, 1968). Similarly, metabolic product (*CC2*) gave fragment ions at *m*/*z* 43 (base peak) and 151 indicating formation of isopropyl cation and loss of methyl group. Also, (*CC2*) gave a peak at *m*/*z* 107 (absent in *CC1*) corresponding to loss of $(CH_3)_2CO$ plus the radical hydrogen. This metabolite was tentatively identified as 5-(1-Hydroxy-1-methyl-ethyl)-2-methyl-phenol.

Three compounds (*CC3*, *CC4* and *CC5*) presented a molecular formula $C_{10}H_{12}O_2$ consistent with the molecular ion, M^+ = 164 amu. Metabolite (*CC3*) had fragment ions at *m*/*z* 149, 147, and 121 attributed to the loss of methyl, hydroxyl and isopropyl groups, respectively. From the spectral data, (*CC3*) was tentatively elucidated to be 2-Isopropenyl-5-methyl-benzene-1,4-diol. (*CC4*) corresponded to methyl ether derivative of carvacrol. Fragments at *m*/*z* 149 ($M^+ - CH_3$), 121 ($M^+ - C_3H_7$), 91 ($C_7H_7^+$) and 77 confirmed this conclusion. Also, compound *CC4* was identified by comparing the retention time of GC and GC-MS with an authentic sample, and NMR data: ^{1}H NMR: δ 1.14 (d, 6H, *J* = 7.0), 2.24 (s, 3H), 2.85 (sept., 1H, 7.0), 3.90 (s, 3H), 6.88 (d, 1H, *J* = 2.0), 7.13 (dd, 1H, *J* = 7.8, 2.0), 7.27 (d, 1H, *J* = 7.8). Mass spectrum of product (*CC5*) was characterized primarily by the fragment ions at *m*/*z* 149, 136 and 121, corresponding to the loss of a methyl group, carbon monoxide, and isopropyl group, respectively. Comparison of the mass spectrum with that reported in the NIST 2002 Mass Spectral Library indicated that (*CC5*) was thymoquinone. Also, compound *CC5* was identified by comparing the retention time of GC and GC-MS with an authentic sample, and NMR data: ^{1}H NMR: δ 1.10-1.30 (d, 6H), 2.24 (s, 3H), 3.10-3.20 (m, 1H), 6.55 (s, 1H), 6.64 (s, 1H). ^{13}C NMR: 15.4, 21.4, 26.5, 77.2, 130.4, 133.9, 145.5, 155.3, 190.1, 191.6. Thymoquinone was previously reported to be produced upon biotransformation of (T) by immobilized microalgae for 24 h (Rasoul-Amini, 2010). The authors found that (T) was hardly converted into thymoquinone and suggested that this low level of transformation may be due to (T) toxicity. The molecular formula of compound (*CC6*) was shown to be $C_{12}H_{16}O_2$ on the basis of its mass spectrum (M^+ 192 amu). MS analyses gave the fragment ion (*m*/*z* 150), which corresponded to loss of the neutral molecule of ketene ($M^+ - CH_2$=C=O) and suggests the presence of the acetyl group. The metabolite (*CC6*) was identified as carvacryl acetate. The structure of (*CC6*) was confirmed by co-injection with authentic carvacryl acetate and NMR data: ^{1}H NMR: δ 1.32 (d, 6H, *J* = 7.0), 2.22 (s, 3H), 2.37 (s, 3H), 2.95 (sept., 1H, *J* = 7.0), 6.94 (d, 1H, *J* = 2.0), 7.10 (dd, 1H, *J* = 8.0, 2.0), 7.23 (d, 1H, *J* = 8.0); ^{13}C NMR: 16.2, 21.3, 24.4, 34.0, 120.2, 124.6, 127.6, 131.4, 148.5, 149.8, 169.7.

Using (T) (80 µg/mL) as substrate and *C. acutatum* as biocatalyst, three major metabolites (*TC1*, *TC2*, and *TC3*), and many minor compounds were detected by GC analysis. The MS spectrum of metabolite (*TC1*) exhibited a molecular ion at *m/z* = 166 $(M)^+$ and a fragmentation pattern consistent with the loss of methyl group ($M^+ - 15$; base peak), the methyl group plus water ($M^+ - 18 - 15$), and the acetaldehyde plus the hydrogen radical at *m/z* = 121 amu. As a result, metabolite (*TC1*) was tentatively identified as 2-(1-Hydroxy-1-methyl-ethyl)-5-methyl-phenol. Otherwise, compound (*TC2*) showed a retention time (*Rt*) and mass spectrum indistinguishable from that obtained for an authentic sample of thymohidroquinone. In addition, metabolite (*TC3*) was identified by comparing the retention time of GC and GC-MS with an authentic sample of thymyl methyl ether, and NMR data: ^{1}H NMR: δ: 1.22 (d, 6H, *J* = 7.0), 2.33 (s, 3H), 3.37 (sept., 1H, *J* = 7.0), 3.80 (s, 3H), 6.79 (d, 1H, *J* = 8.0), 6.92 (dd, 1H, *J* = 8.0, 2.0), 7.05 (d, *J* = 8.0). ^{13}C NMR: δ 21.3, 23.5, 27.6, 56.3, 112.3, 125.9, 126.6, 137.0, 137.5, 158.4. Some of these metabolites detected for *C. acutatum* were also found when an incubation of (C) and (T) using *B. theobromae* was carried out (Table 1). In fact, retention times (*Rts*) and mass spectra of (*CC1*) = (*TC2*), (*CC2*), (*CC*5), (*CC6*), and (*TC1*) were similar to those obtained for (*CB2*) = (*TB2*), (*CB4*), (*CB5*), (*CB7*), and (*TB1*), respectively.

Likewise, microbial transformation of (C) by *B. theobromae* afforded seven major metabolites not detected in a fungal culture medium lacking the substrate. Metabolic products (*CB2*), (*CB4*), (*CB5*) and (*CB7*) showed retention times and mass spectra (Table 1) identical to those observed for the thymohydroquinone, 5-(1-Hydroxy-1-methyl-ethyl)-2-methyl-phenol, thymoquinone, and carvacryl acetate,

respectively. In addition, the structure of (*CB1*), (*CB3*), and (*CB6*) was derived from their EI mass spectra. The fragmentation pattern of (*CB1*), as listed in the experimental section, was in good agreement with the proposed structure. Metabolite (*CB1*) had a molecular formula $C_{10}H_{14}O_2$ consistent with the molecular ion, M^+ = 166 amu. In addition, this compound showed fragment ions to *m/z* = 135 and 105 consistent with the loss of H_2CO and hydrogen radica,l and with water, ethylene and methyl radical. Thus, (*CB1*) was suggested to be 5-(2-hydroxy-1-methyl-ethyl)-2-methyl-phenol. Metabolite (*CB3*) had a molecular formula ($C_{10}H_{14}O_2$; M^+ = 166) and fragment ions at *m/z* 148 (M^+−18), 133 (M^+−18−15), and 105 (M^+−18−28−15) attributed to the loss of water, water + methyl radical, and water + ethylene + methyl radical, respectively. From the spectral data, (*CB3*) was tentatively elucidated to be 2-hydroxymethyl-5-isopropyl-phenol. Metabolic compound (*CB6*) had a molecular formula of $C_{10}H_{12}O$ based on its mass spectrum, $(M)^+$ = 148 amu. The molecular ion was 2 mass units (2H) lower than that of parent compound (C), indicating the elimination of hydrogen from (C). Metabolite (*CB6*) had fragment ions at *m/z* 133 and 107 attributed, in that order, to the loss of methyl and isopropylene groups. From the spectral data, (*CB6*) was elucidated to be 5-Isopropenyl-2-methyl-phenol.

Upon incubation of (T) with *B. theobromae*, six major metabolites were detected by GC-MS analysis. The structures of (*TB3*), (*TB5*), and (*TB6*) were derived from their EI mass spectra. It should be emphasized that the data only tentatively suggest the identity of these metabolites. The molecular formula of compounds (*TB3*) and (*TB5*) was shown to be $C_{10}H_{16}O_2$ on the basis of its mass spectrum (M^+ = 168 amu). These metabolic products gave fragment ions at *m/z* 153, 150, and 135 indicating the loss of methyl group, water, and both, respectively. Also, there was a prominent peak at *m/z* = 126 (base peak), corresponding to a ring fission fragment. Although these data do not allow a definitive identification, they suggest a hydroxy-cyclohexenone substituted-like structure for metabolites (*TB3*) and (*TB5*). Furthermore, metabolite (*TB6*) had a molecular formula $C_{10}H_{14}O_3$ consistent with the molecular ion, M = 182 amu. The mass spectrum showed fragment ions at *m/z* 140, 139, 111 (base peak), and 96 among others, corresponding to the loss of the ketene (M^+−CH_2=C=O), ketene plus hydrogen radical, ketene + ethylene + hydrogen radical, and M^+−$C_5H_{10}O$, respectively. (*TB6*) was tentatively elucidated to be 2-Hydroxymethyl-5-isopropyl-benzene-1,4-diol.

Finally, (*TB4*) was identified by comparing the retention times of GC-MS with those of authentic samples, and NMR data. NMR data for (*TB4*) were: ^{1}H NMR: δ 1.22 (d, 6H, *J* = 7.0), 2.38 (s, 3H), 2.39 (s, 3H), 3.05 (sept., 1H, *J* = 7.0), 6.89 (d, 1H, *J* = 2.0), 7.11 (dd, 1H, *J* = 8.0, 2.0), 7.29 (d, 1H, *J* = 8.0); ^{13}C NMR: 21.2, 21.3, 23.5, 27.6, 123.2, 126.9, 127.6, 137.1, 137.5, 148.4, 170.2. Also, the mass spectrum of (*TB4*) was consistent with that reported in the NIST 2002 Mass Spectral Library for thymyl acetate.

Time course experiment

The time course of relative concentration changes of (T) and (C) and metabolites is represented in Figures 3A-D. The starting substrate (C) was consumed slowly by both fungi. The fungus *B. theobromae* only transformed about 50% of (C) after 14 days, being mainly converted to (*CB1*) and (*CB4*). However, the relative abundance of both metabolites was always lower than 20%. As is shown in Figure 3A, the other metabolites reached very low concentrations.

Furthermore, *C. acutatum* metabolized (C) more quickly than *B. theobromae*. Interestingly, some metabolites produced from (C) by *C. acutatum* were not detected in a fungal culture of *B. theobromae* incubated with the same substrate. According to Figure 3B, the substrate (C) was mainly transformed by *C. acutatum* to (*CC4*) (~12%) and about 55% was consumed in 14 days. These results suggest that under the conditions used, (C) can be only slowly detoxified by the plant pathogenic fungi. Otherwise, (C) was metabolized at a faster rate than (T) by *C. acutatum* (Figures 3B and 3D).

As can be seen from Figure 3C, the substrate (T) was only transformed by about 55% after 14 days, using *B. theobromae* as biocatalyst. The major metabolic product (*TB1*) reached a relative abundance of ~20% after 11 days. In addition, metabolites (*TB2*), (*TB3*), (*TB4*), (*TB5*), and (*TB6*) presented relative abundances below 10% during the time of analysis. It seems noteworthy that (T) was consumed by *B. theobromae* to a greater extent than *C. acutatum*. In fact, analysis of extracts obtained from fungal cultures of *C. acutatum* incubated with (T) showed it to be weakly metabolized (<10 %) (Figure 3D). In addition, some metabolites previously detected on incubation of (T) with *B. theobromae* were absent in the biotransformation using *C. acutatum*.

The marked difference between the metabolism of (C) and (T) by *C. acutatum* may reflect the higher toxicity (antifungal activity) of (T) against this pathogenic fungus. In the same way, small differences between the metabolisms of both compounds by *B. theobromae* are consistent with the observed similarities in the antifungal activity against this fungus. Based on the structures of the metabolites, metabolic pathways for the biotransformation of (C) and (T) by *C. acutatum* and *B. theobromae* were proposed (Figure 4).

Overall, it becomes apparent that aromatic and aliphatic hydroxylation, and the methylation and acetylation of hydroxyl group over aromatic ring, represents the main metabolic pathway from (C) and (T) by both phytopathogenic fungi. These transformations may be the result of a process of detoxification of both phenolic compounds by *C. acutatum* and *B. theobromae*. Recent studies have demonstrated the necessity of the phenolic hydroxyl group of (C) for antimicrobial activity (Ultee et al., 2002). The elimination of the hydroxyl group by replacing it with the methyl ether or methyl ester group affects the hydrophobicity of the molecule and the size of the ring

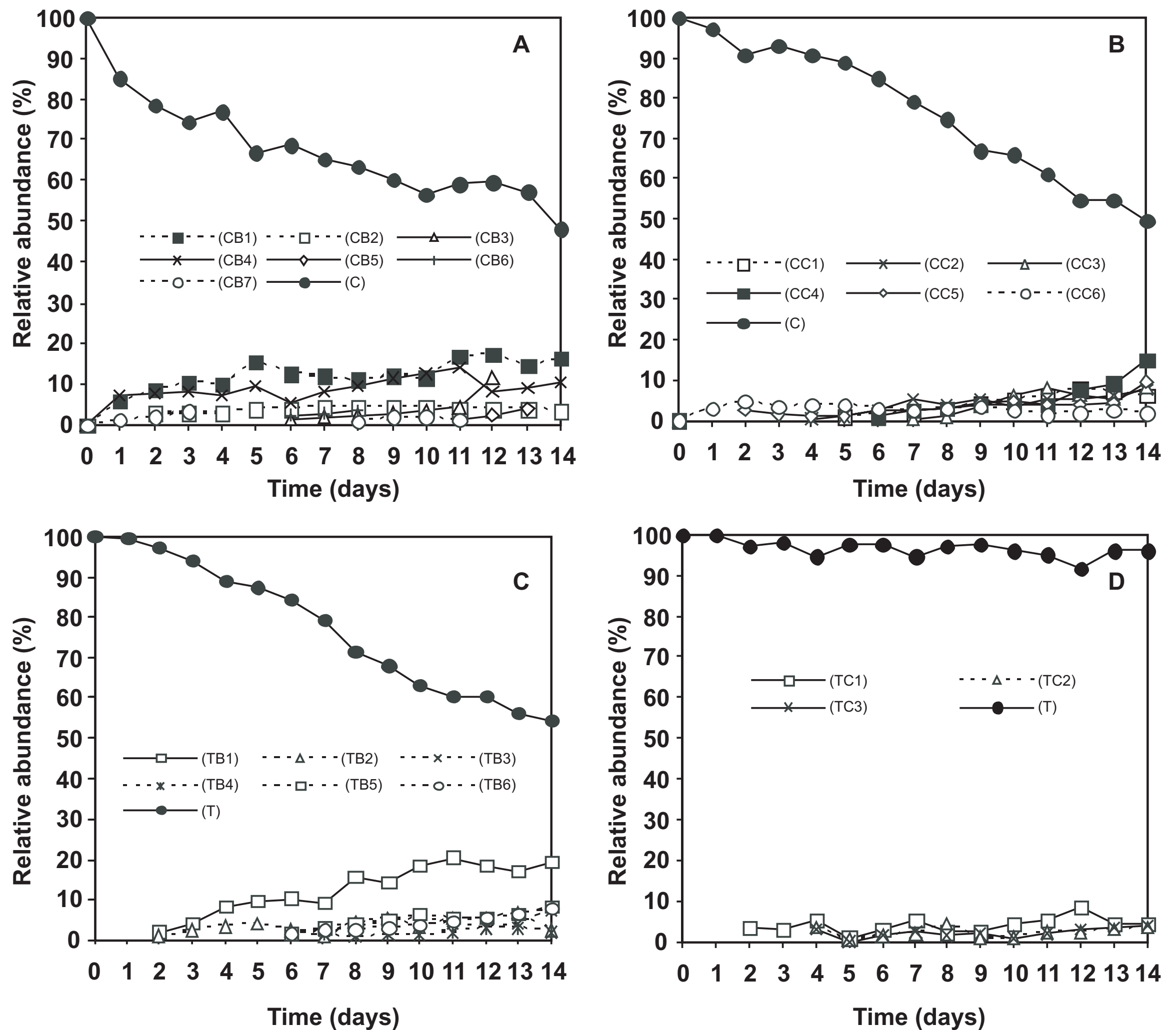

FIGURE 3 - Time course for the microbial transformation of: (C) by *B. theobromae* (3a) and *C. acutatum* (3b); and (T) by *B. theobromae* (3c) and *C. acutatum* (3d). (*CC1*) is the same compound that (*TC2*), (*CB2*), (*TB2*). Similarly, (*CC2*) = (*CB4*); (*CC5*) = (*CB5*); (*CC6*) = (*CB7*); and (*TC1*) = (*TB1*).

substituent, both of which may have a major influence on specific interactions with microbial cells. Veldhuizen et al. (2006) found that carvacrol methyl ether, containing a methyl ether group instead of a hydroxyl group, did not inhibit the growth of *B. cereus* at the concentrations tested (0 to 10 mM), whereas no growth was observed with (C) at concentrations of 0.75 mM and above. Furthermore, (*TC3*), (*TB4*), and (*CC6*) = (*CB7*) do not have the ability to release a proton and behave as a proton exchanger.

In addition, the aliphatic side chains have a direct influence on the antimicrobial activity of (C) and (T), as has been described by several researchers (Ultee et al., 2002; Veldhuizen et al., 2006). They have observed that the removal of ring substituents from (C) (using 3-isopropylphenol and *o*-cresol) leads to a reduction in antimicrobial activity. Therefore, the hydroxylation of the aliphatic chains of (C) and (T) may be a mechanism used by *C. acutatum* and *B. theobromae* to reduce the antifungal activity. These microbial transformations can reduce the hydrophobic features of these molecules, which may affect the initial interaction with the membrane. Consequently, we suggest that these hydroxylation reactions emerge as potential metabolic targets to control *C. acutatum* and *B. theobromae.* Another important structural feature associated with the antimicrobial activity of (C) and (T) is the presence of a system of delocalized electrons. It was described earlier that the hydroxyl group bound to a benzenic ring is important for the activities of some antimicrobial compounds (Ultee et al., 2002; Veldhuizen et al., 2006; Xu et al., 2008). This feature allows compounds to destabilize the cytoplasmic membrane and act as a proton exchanger, reducing the pH gradient across the membrane. Hence, we suggested that the formation of (*CC5*) = (*CB5*), (*TB5*), and (*TB3*) as previous intermediaries reflects the need of *C. acutatum* and *B. theobromae* to reduce the toxicity of (C) and (T) by eliminating the system of delocalized electrons.

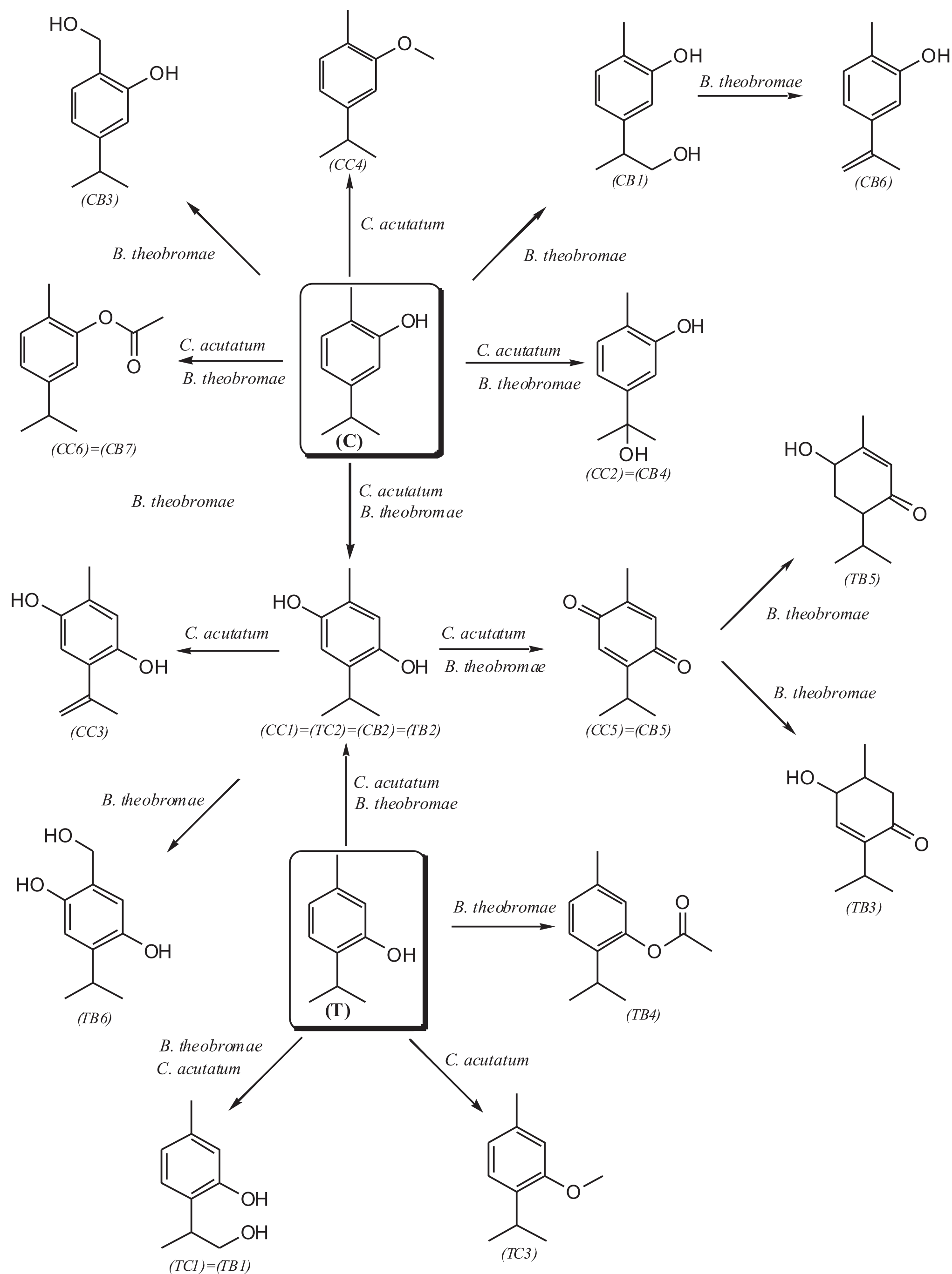

FIGURE 4 - Possible metabolic pathways of (C) and (T) by *C. acutatum* and *B. theobromae*

We are currently evaluating the antifungal effect of the obtained metabolic compounds, and some synthetic derivatives of (T) and (C), against *C. acutatum* and *B. theobromae*. Synthetic derivatives present modifications in the aliphatic chains and benzenic ring of (T) and (C), in the same positions that were hydroxylated according to the metabolic studies.

Arylpropanoids, specifically thymol and carvacrol, which are strongly antifungal against the phytopathogenic fungi *C. acutatum and B. theobromae*, are mainly metabolized via aromatic and aliphatic hydroxylation, and the methylation and acetylation of hydroxyl group over the aromatic ring. Antifungal activity of thymol was slightly higher than that observed for carvacrol against *C. acutatum*.

In conclusion, the arylpropanoids thymol and carvacrol, which were highly active against the phytopathogenic fungi *C. acutatum* and *B. theobromae*,

were mainly metabolized via aromatic and aliphatic hydroxylation, and through methylation and acetylation of hydroxyl group. These transformations affect the structural features of thymol and carvacrol associated with their antimicrobial activity and mode of action. Overall, the antifungal activity of thymol was higher than that observed for carvacrol against *C. acutatum*. Consequently, compared to the transformation of carvacrol, the metabolism of thymol by *C. acutatum* was a slower process. Similarly, the small differences in the metabolism of both compounds by *B. theobromae* were consistent with the observed similarities in the antifungal activity against this fungus. High antifungal activity coupled with a low level of microbial transformation makes thymol and carvacrol promising candidates to control *C. acutatum* and *B. theobromae*.

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