METABOLIC RESPONSE INDUCED BY ENDOPHYTIC FUNGI AND BACTERIA IN *H. marrubioides* Epling *IN VITRO* MICROPLANTS

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Hyptis marrubioides Epling is a native plant from Brazilian Cerrado. In this paper, the response of *in vitro* microplants of this species to inoculation with bacterial and fungal endophytic isolates is evaluated. HPLC-DAD analysis showed the presence of 3,4-*O*-(Z)-dicaffeoylquinic acid and quercetin-7-*O*-glucoside as the main components. GC/MS analysis demonstrated that the sesquiterpenes τ-cadinol and caryophyllene oxide were only produced in microplants inoculated with endophytic bacteria, while methyl hexadecanoate, methyl heptadecanoate and methyl (Z,Z,Z) 9,12,15-octadecatrienoate and the triterpene methyl 3β-hydroxy-urs-12-en-28-oate were overexpressed only when the microplant was treated with endophytic fungi.

Keywords: *in vitro* culture; *Hyptis marrubioides*; Lamiaceae.

INTRODUCTION

Currently, many mechanisms are employed for the induction of resistance responses in plants. These methods involve the activation of latent defense mechanisms, which increase the ability of the plant to either prevent or delay the establishment of pathogenic microorganisms.[1] Post-formed defenses can be triggered by exposure to inducing agents, which act as elicitors of the defense response.[2] Resistance induction often involves the accumulation of secondary metabolites in plant tissues and the production of phytoalexins with antimicrobial properties by the plant itself.[3]

Elicitors could have a chemical or biological nature, such as non-pathogenic microorganisms,[4] avirulent forms or incompatible strains of a pathogen, or even virulent pathogens. Fungal culture filtrate,[5] yeast extract,[6] and polysaccharides from microorganism walls[7] can also be utilized. The major bacterial determinants for systemic resistance induction elicitors are the lipopolysaccharides (LPS) present in their membrane, siderophores, and salicylic and jasmonic acids.[8]

Plant-endophyte association is a type of symbiotic relationship with a similar or higher degree of complexity than a phytopathogenic microorganism. Plant–endophytic association involves fungi and bacteria living inside plant tissues without causing any visible disease symptoms and sometimes interacting mutualistically with their hosts.[9] Endophyte-host interactions involve a balance of antagonism and exhibit stronger phenotypic plasticity compared to the interactions of plant pathogens.[10] Currently, the signals released by the two partners and their roles remain largely unknown. Researchers have tried to clarify the molecular mechanisms involved during the establishment of endophyte colonization.[11] Yet there is little literature about the secondary metabolism of plants induced by its endophytes.[12]

Tissue culturing is a useful tool for the reproduction of explants with desirable characteristics, such as resistance to pests and other stress conditions, high productivity, and high yield of active substances of interest.[13] *In vitro* elicitation has been proven as an efficient biotechnological process for the attainment of secondary metabolites from plant cells or tissues. Chong and coworkers have used yeast extract, jasmonic acid, a mycelia homogenate of *Aspergillus niger* and *Aspergillus flavus*, chitosan, and glucan in cell suspensions of *Morinda elliptica* (Hook. f.) Ridl.[6] These elicitors had different effects on anthraquinone production. Naoumkina and coworkers have reported that the use of yeast extract in cell cultures of *Medicago truncatula* induced the accumulation of flavonoids, triterpenes, and other defense-related molecules.[14]

Several species of the genus (Lamiaceae) occur in the Brazilian savannah (Cerrado biome), some of which have several medicinal properties and great pharmaceutical relevance.[15] The species *Hyptis marrubioides* Epling is a medicinal plant native to this region,[16] with no scientific data on its *in vitro* elicitation, even though this plant has drawn researchers' attention due to the remarkable concentration of terpenoids in its essential oils (e.g., cariofila-4(14),8(15)-dien-5β-ol, eudesma-4(15),7-dien-1β-ol, caryophyllene oxide, and β-caryophyllene).[17] The roots of *H. marrubioides* are colonized by bacteria and endophytic fungi[18] with different functional traits important to promoting plant growth.[19]

In this study, we have evaluated the phytochemical profile of *H. marrubioides* microplants exposed to inoculation with endophytic isolates.

EXPERIMENTAL

Plant material selection, *in vitro* establishment, and maintenance

Previously established seedlings were used via in vitro germination of seeds suitable for inoculation obtained from the Três Barras farm in Lavras, MG, Brazil. The seeds were taken to the Laboratory of Plant Tissue Culture at the Instituto Federal de Educação, Ciência e Tecnologia Goiano – Campus Rio Verde. They were disinfected

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with ethanol 70% (v/v) for 30 sec and sodium hypochlorite (2.5% of active chlorine) for 20 min, and inoculated in Murashige & Skoog (MS) medium (50% salt concentration).[20] Seedlings were cut into small pieces, using stalk segments with a height of approximately 1 cm as explants, and kept in 50% MS. The cultures were incubated in a growth chamber for 45 days at 0.5 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR), with an average temperature of 25 °C and a photoperiod of 16 hours.

Inoculum preparation

The endophytic microorganism lineages were obtained from H. marrubioides roots used in a previously mentioned study.[18]

The following isolates were selected as bacterial inoculants: RG8 (simple coccus, Gram-positive, catalase negative); RG12 (coccobacilli, Gram-positive, catalase negative); RF10 (coccobacilli, Gram-positive, catalase negative); and RF11 (large bacillus, Gram-positive, catalase negative). These isolates were grown in nutrient broth (3 g beef extract, 5 g peptone, and H$_2$O qs 1 L) overnight at 30 °C, under 150 rpm agitation. Subsequently, 15 µL aliquots of cultures were inoculated near the roots of *H. marrubioides* microplants growing in 50% MS medium (T2). Axenic nutrient broth inoculum (15 µL) was inoculated into microplant control (T3).

The fungal lineages used were RG29 (*Trichoderma* sp.), RG31 (*Papulaspora* sp.), and RG32 (*Fusarium* sp.). These fungi were grown on solid PDA (infusion of 200 g potato, 20 g dextrose, 15 g agar, and H$_2$O qs 1 L) and their colonies were fragmented using the bottom of 1000 µL tips. This fragmentation generated disks with a diameter of 5 mm, which were then inoculated near the base of *H. marrubioides* Epling microplants grown in 50% MS medium. PDA discs without the presence of mycelium were inoculated in the microplants control (T8).

Culture conditions

The *Hyptis marrubioides* microplants were inoculated with the elicitors after 45 days of establishment in 50% MS. After inoculation, the flasks containing the cultures were kept in a BOD incubator with 15 µmol m^{-2} s^{-1} PAR, at an average temperature of 30 °C, and with a photoperiod of 16 hours. The plants were evaluated on a daily basis for the appearance of disease symptoms, and the ones subjected to inoculation with the bacterial isolates had their shoots collected on the tenth day after inoculation. The plants subjected to treatment with fungal isolates were collected on the fifth day after inoculation. These shoots, as well as the *H. marrubioides* plants grown *ex vitro* (T1), were dried in a forced-air oven at 35 °C until a constant weight was reached.

Chemical analysis of plants grown *ex vitro* to obtain standards

Extraction and purification

Hyptis marrubioides shoots grown *ex vitro* were collected in the Seedling Nursery of the Laboratory Tissue Culture Instituto Federal de Educação, Ciência e Tecnologia Goiano in Rio Verde, Goiás (S17°48'15.9" W50°54'19,5"), on April 20th, 2011. A voucher specimen (HRV71) has been deposited in the Herbarium of that Institute (Herbarium HRV).

The air-dried *H. marrubioides* shoots (502 g) were powdered and exhaustively extracted by maceration with 96% ethanol. After concentration in a rotary evaporator, an ethanolic crude extract (26.10 g) was obtained. A sample (20 g) of this extract was dissolved in a MeOH:H$_2$O mixture (2:8 v/v) and partitioned successively with *n*-hexane, ethyl acetate, and *n*-butanol (*n*-BuOH). After removal of

the solvent under reduced pressure, the following fractions were obtained: *n*-hexane (H1, 4.01 g), ethyl acetate (H2, 3.67 g), *n*-BuOH (H3, 6.28 g), and hydroalcoholic (H4, 2.16 g).

The ethyl acetate fraction (H2, 3.67 g) was initially chromatographed on a silica gel column (Merck silica 100 g, 30 cmx3.5 cm) using an *n*-hexane/ethyl acetate (EtOAc) and EtOAc/MeOH gradient solvent system as eluent, which yielded 66 fractions. Fraction 65 (1.61 g, MeOH) was purified over Sephadex LH-20® (Sigma-Aldrich, 75 g, 37.5x3.0 cm) eluted with a MeOH and MeOH/H$_2$O (1:1 v/v) mixture, from which 74 fractions were obtained. Fraction 33 (46 mg, MeOH) was subsequently submitted to Preparative Thin Layer Chromatography (Sigma-Aldrich PTLC, 20x20x0,5 cm) using CHCl$_3$/MeOH/H$_2$O (43:37:20 v/v/v) as the eluting system. This procedure afforded results **1** (8 mg) and **2** (10 mg), which were used as standards for the quantitative analysis performed by the High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) on the microplants inoculated with endophytic microorganisms as well as the control microplants. These results were compared with those of *ex vitro H. marrubioides* plants.

Chemical general experimental procedures

The Nuclear Magnetic Resonance (^{1}H NMR and ^{13}C NMR) spectra were registered on a Varian 500 instrument using tetramethylsilane (TMS) as the internal standard (1%). The spectra of **1** were obtained in CD$_3$OD (Sigma-Aldrich®), and the spectra of **2** were acquired in DMSO-d$_6$ (Sigma-Aldrich®).

The quantitative analysis by HPLC-DAD was carried out on a Shimadzu LC-6AD system equipped with a degasser DGU-20-A5, a diode array detector Prominence SPD-M20A series, and a communication bus mode CBM-20A. The analyses were conducted on a Phenomenex ODS column (250x4.6 mm, 5 µm) equipped with a pre-column of the same material and with the following parameters: injection volume: 20 µL; flow rate: 1.0 mL min^{-1}; mobile phase: CH$_3$OH/H$_2$O/HOAc (40:59.9:0.1, v/v/v) linear gradient until 100% CH$_3$OH in 30 min, 10 min at 100% CH$_3$OH, and 15 min to return to the initial condition; UV detection at 230, 254, and 325 nm.

Quantitative HPLC-DAD analysis

Pulverized air-dried *H. marrubioides* microplants (50 mg) were extracted with 3 mL HPLC grade methanol, using an ultrasound bath (20 min). The samples had been previously filtered through a filter with 0.2 µm pore size prior to the injection. Extractions were accomplished in triplicate.

The chlorogenic acid (**1**) and flavonoid (**2**) solutions were prepared in HPLC grade MeOH (2 mg mL^{-1}). Stock solution dilutions were done in HPLC grade MeOH, to obtain solutions containing 0.102, 0.203, 0.406, 0.813, and 1.626 mg mL^{-1} of **1**, and 0.05, 0.109, 0.218, 0.436, and 0.872 mg mL^{-1} of **2**. Each standard solution was injected in triplicate. A four-point calibration curve was constructed to determine the linearity of the method. The calibration curve was determined by plotting the peak area versus the concentration of the substance in mg mL^{-1}.

Gas chromatography mass spectrometry (GC/MS) analysis

Sample preparation for GC/MS analysis

The extracts inoculated with the elicitor microorganisms and the control microplants were prepared by mixing 25 mg of the powder obtained from the *H. marrubioides* microplants in 3 mL ethyl acetate, p.a. (Synth). After 20 min in an ultrasonic bath, the samples were filtered, in order to achieve their crude extracts. Prior to the GC/MS analysis, these extracts were methylated by treatment with diazomethane using previously described conditions.[21]

GC/MS operating conditions

The methylated extracts of *H. marrubioides* microplants by means of different treatments were analyzed by GC/MS (Shimadzu model QP-2010) (low resolution). A capillary column Rtx-5MS (30 m × 0.25 mm i.d. × 0.25 μm of film) was employed and a He carrier gas flow of 1.1 mLmin^{-1}, split 1:10, was utilized. The injector temperature was set at 250 °C, and the oven was heated from 100 to 290 °C at 3 °C per minute; the temperature was maintained at 290 °C for an additional 30 min. The mass spectra obtained at 70 eV were compared with the Wiley 7, NIST 08, and FFNSC 1.2 libraries using GCMS Solution software (Shimadzu), which furnished the similarity index (SI) expressed as a percentage. The retention indices relative to a homologous series of *n*-alkanes (C$_8$-C$_{40}$) measured on a Rtx-5MS capillary column under the same operating conditions were also provided. Acid compounds present in the extracts were identified as their corresponding methyl esters.

Experimental design

The experiment was carried out with a completely randomized design involving 11 treatments (T1 to T11) T1 was the *ex vitro* plant control; T2 the microplant control; T3 the bacterial elicitation control; T4 to T7 microplants with bacterial inoculation; T8 fungal elicitation control and T9 to T11 microplants with fungal inoculation (Table 1). Each culture bottle was considered a sampling unit, and each flask contained four microplants. The samples were analyzed in triplicate. Averages for the emergence of symptoms, as well as for the 3,4-*O*-(*Z*)-dicaffeoylquinic acid (**1**) and flavonoid quercetin-7-*O*-glucoside (**2**) quantification were obtained by HPLC-DAD analysis and subjected to the Tukey test (5%) with the support of the SISVAR statistical software.

RESULTS AND DISCUSSION

The interaction between plant and endophyte was evaluated on a daily basis. For the statistical analysis, we included the last ten and five evaluations performed on plants inoculated with bacteria and fungi, respectively. Despite strains of fungi and bacteria being obtained from healthy roots of *H. marrubioides*, the effect of inoculation with endophyte in aseptic *in vitro* plants varied according to the strain used. These factors disturbed the equilibrium between microorganism virulence and plant defense that is important for asymptomatic colonization.[11] The same effect was reported for *Arabidopsis thaliana* where only three of eight strains of fungi colonized the root system asymptomatically *in vitro*.[22] The bacteria RF11 and RG8 were pathogenic in this work, leading to wilting, leaf-descending death, and darkness in the roots. More specifically, RF11 resulted in wilting of 100% of the inoculated microplants (Table 1). The incidence of disease symptoms has been previously described as typical bacterial symptoms.[23] In the presence of the bacteria RF10 and RG12, the plants exhibited no obvious pathology signs (Figure 1). These observations demonstrated that those endophytic bacteria had the same endophytic behavior in *in vitro* conditions. Although the inner tissues of the *H. marrubioides* have not been evaluated, the penetration of these lineages into the root's host may have occurred.

On the other hand, in the same context, bacteria RF11 and RG8, as well as the fungi *Trichoderma* sp., *Papulaspora* sp., and *Fusarium* sp. (isolated from the roots of *H. marrubioides* in the *in vitro* system) started to act as pathogens despite being originally endophytic strains. This behavior was evidenced by the appearance of visual symptoms in the affected plants. The wilting symptom is often related to vascular diseases caused by the colonization of xylem vessels by pathogenic microorganisms and presents with associated leaf fall. It is possible that bacteria RF11, RG8 as well as the fungi, *Trichoderma* sp., *Papulaspora* sp., and *Fusarium* sp. colonized the xylem conductor system in the *H. marrubioides roots* causing the observed *in vitro* wilting.[24]

Although the fungus *Papulaspora* sp. caused widespread wilting in the microplants, it did not trigger leaf drop, as in the case of the other tested fungal species. The successful symbiosis of endophytic organisms is directly related to environmental conditions and the physiological state of the host, and under adverse conditions, they may manifest themselves as pathogens. In this case, the endophytic organism can be seen as a pathogen under *in vitro* conditions.[25] The experimental conditions used were possibly a stress factor for the plants; additionally, the MS agar culture medium improved the conditions for growth of the endophyte.

Trichoderma, *Papulaspora*, and *Fusarium* are common genera in endophytic symbiosis.[26] However, there are few reports on the

Table 1. Symptoms of pathogenicity caused by endophytic bacteria (at the 10th inoculation day) and fungi (at the 5th inoculation day) used as elicitors in *H. marrubioides* microplants

	Treatment	Plants with Wilting (%)	Plants with Falling Leaves (%)	Color of the Roots
	Plant			
T1	E*x vitro* plant (control)	-	-	-
T2	Microplant (control)	0d[a]	0b	Light
	Bacterial Elicitation			
T3	Microplant + nutritive broth (control)	0d	0b	Light
T4	Microplant + RF11	100a	80a	Dark
T5	Microplant + RG8	80b	80a	Dark
T6	Microplant + RF10	0d	0b	Light
T7	Microplant + RG12	0d	0b	Light
	Fungal Elicitation			
T8	Microplant + PDA (control)	0d	0b	Light
T9	Microplant + *Trichoderma sp.*	30c	0b	Light
T10	Microplant + *Papulaspora sp.*	100a	0b	Light
T11	Microplant + *Fusarium sp.*	30c	0b	Light

[a] Letters compare rows. Means followed by the same letter do not differ from each other by the Tukey test (5%).

pathogenicity of species from these genera in *in vitro* systems.[27] *Trichoderma* species have been used in the biological control of plant pathogens, and this control can be manifested through mycoparasitism or antibiosis.[28] The genus *Papulaspora* has also been shown to display antimicrobial activity[29] and potential for secondary metabolites production.[30] On the other hand, the genus *Fusarium* is known to trigger wilting, or Fusariosis, characterized by xylem hypertrophy[31] and wilting followed by death in affected plants.[32] These symptoms were detected in plants submitted to treatment T7 (RG32), which underwent inoculation with *Fusarium* (Figure 1). These data demonstrated that this endophytic organism had pathogenic behavior in the *in vitro* systems.
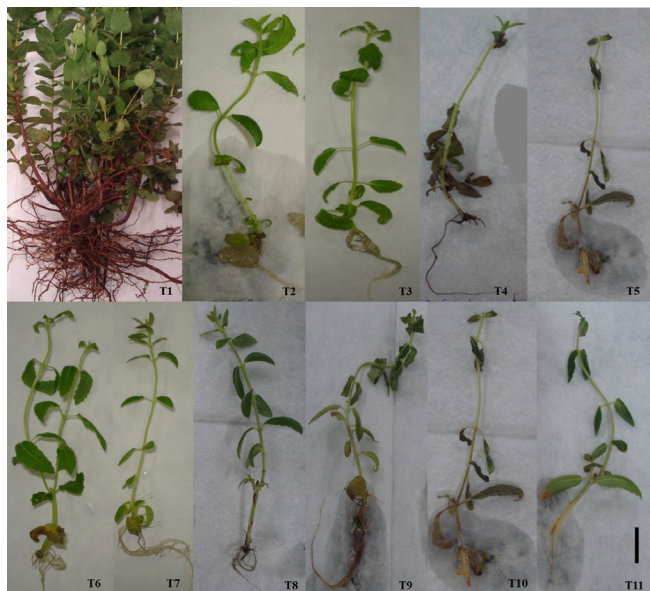

Figure 1. H. marrubioides microplants used in the control experiment and in the elicitation by endophytic bacteria (RF11, RG8, RF10, and RG12) and fungi (RG29, RG31, and RG32). Symptoms of wilting and leaf fall (bacterial inoculation, treatments T4 and T5); symptoms of wilting (fungal inoculation, treatments T9, T10, and T11) and symptomless plants (bacterial inoculation, treatments T6 and T7). Bar in T1 = 15 cm, others = 1.5 cm

The chromatographic purification of the ethyl acetate fraction led to the isolation of compounds **1** and **2**. Compound **1** presented three absorption maxima at 194, 220, and 329 nm. The ^{1}H and ^{13}C NMR spectra, together with the two-dimensional HMBC experiment, revealed the presence of two caffeoyl units, as well as signals related to quinic acid. The positions of the caffeoyl groups were determined on the basis of the chemical shifts and coupling constants obtained for the hydrogens H-3 (δ 5.62, m), H-4 (δ 5.13, dd, 8.6 Hz; 2.9 Hz) and H-5 (δ 4.42, m). The coupling constant value for H-4 indicated the axial-equatorial relationship between H-4 and H-5, according to the 3,4-diequatorial conformation proposal for the caffeoyl groups. Comparing our results with literature data for the isomeric 3,4-; 3,5- and 4,5-*O*-dicaffeoylquinic acids, the identity of compound **1** was confirmed as 3,4-*O*-(*Z*)-dicaffeoylquinic acid (Figure 2).[33]

The response of the UV detector at 325 nm was linear from 0.102 to 1.626 mg mL^{-1} for **1** and from 0.05 to 0.872 mg mL^{-1} for **2**. The obtained regression equations were y = $4.0 \times 10^7 \times$ –217572 and y = $3.0 \times 10^7 \times$ –409470 with a correlation coefficient (r²) of 0.9995 and 0.9989, respectively.

In this study, the quantification of chlorogenic acid evidenced that T11 (i.e., in which *Fusarium* sp. was employed as elicitor) doubled the amount of acid that was produced (128.74 mg gDW[-1], as compared to the control treatment without fungi (T8, 60.57 mg gDW[-1]) (Table 2).

The endophyte–plant interaction leads to qualitative and quantitative alterations in the phytochemical profile of the host. [34] *Neotyphodium lolii*, endophyte fungus, establishes a symbiotic relationship with their host *Lolium perenne* and this relationship alters the metabolic profiles of *L. perenne* dramatically.[35] This alteration is characterized by the production of a range of chemically unrelated alkaloids such as ergovaline, lolitrem B, and peramine that have never been detected in endophyte-free *L. perenne*. *Neotyphodium lolii* infection is found to enhance the level of chlorogenic acid in *L. perenne*, and also increases the concentrations of polyphenol biosynthesis precursors, quinic and shikimic acids.[35,36]

The production of 3,4-*O*-(*Z*)-dicaffeoylquinic (**1**) by elicited microplants can be considered a defensive response to the pathogenic behavior that this endophyte assumed in the *in vitro* system. Chlorogenic acid has been described as being responsible for plant resistance against attack by pests such as caterpillars, beetles, leafhoppers, aphids, fungi, and bacteria.[37] Baptista and coworkers have inoculated *Eucalyptus urophylla* plants *in vitro* with two *Pisolithus tinctorius* ectomycorrhizal isolates and they found that the roots had a significantly higher chlorogenic acid level 96 hours after inoculation as compared to a control.[38]

On the other hand, the UV spectrum of compound **2** displayed absorption bands at 197, 255, and 354 nm, typical of flavonoids. The ^{1}H and ^{13}C NMR spectra exhibited the characteristic signals of a flavonoid skeleton, whose ring B coupling pattern suggested a quercetin core. Diagnostic signals for a monoglycoside unit were also observed in this spectrum. The downfield-shift of *ortho* carbon signals C-6 (δ 98.9) and C-8 (δ 94.9) and *para* carbon signal C-10 (δ 104.7), together with upfield-shift of C-7 (δ 162.8) suggesting the 7-*O*-glycosilation of the aglycone quercetin. The structure of compound **2** is proposed on the basis of the set of ^{1}H and ^{13}C NMR spectral data and on the heteronuclear, multiple-bond correlation spectroscopy (HMBC), which were consistent with those reported in the literature for the flavonoid quercetin-7-*O*-glucoside.[39]

The quercetin-7-*O*-glucoside (**2**) quantification results obtained for *H. marrubioides* microplants inoculated with endophytic isolates, both bacterial and fungal, showed that all the microplants, elicited or control, contained low concentrations of this compound as compared to the amount found in the *ex vitro* plant (19.02 mg g^{-1} dry weight) (Table 2). In contrast, other studies have shown increased synthesis of flavonoids *in vitro* with or without elicitors, for example, quercetin-3-*O*-glucoside has been identified as a secondary metabolite obtained from the ethanol extract of *in vitro Drosera binata* cultures,[40] while Xu and coworkers have verified increased flavone synthesis in *Scutellaria baicalensis* Georgii suspension cultures using methyl jasmonate as an elicitor.[41]

GC/MS analysis of the extracts obtained from the 10 treatments undergone by *H. marrubioides* revealed the presence of one phenolic compound (**3**, $C_{15}H_{24}O$), three sesquiterpenes (τ-cadinol, **4**, $C_{15}H_{26}O$; caryophyllene oxide, **5**, $C_{15}H_{24}O$; and eudesm-4(14)-en-11-ol, **6**, $C_{15}H_{26}O$), one diterpene (kaur-16-ene, **11**, $C_{20}H_{32}$), one triterpene (methyl 3β-hydroxy-urs-12-en-28-oate, **12**, $C_{31}H_{50}O_3$), one unsaturated hydrocarbon (heptadec-1-ene, **7**, $C_{17}H_{26}$), and three fatty acid methyl esters (methyl hexadecanoate, **8**, $C_{17}H_{34}O_2$; methyl (*Z,Z,Z*)9,12,15-octadecatrienoate, **9**, $C_{19}H_{32}O_2$; and methyl heptadecanoate, **10**, $C_{18}H_{36}O_2$), as well as two unknown compounds (Figure 2). The diterpene kaur-16-ene was the major biosynthesized constituent in all the treatments, with the best results being attained in the case of treatments T3 and T7 (Table 3).

A significant amount of the fatty acid methyl esters **9** (13.1% and 2.8%) and **8** (10.1% and 2.9%) were detected in treatments T2 and T3, respectively. Concerning microplant inoculation with bacteria RF11, RG8, RG12, and RF10, the sesquiterpenes caryophyllene
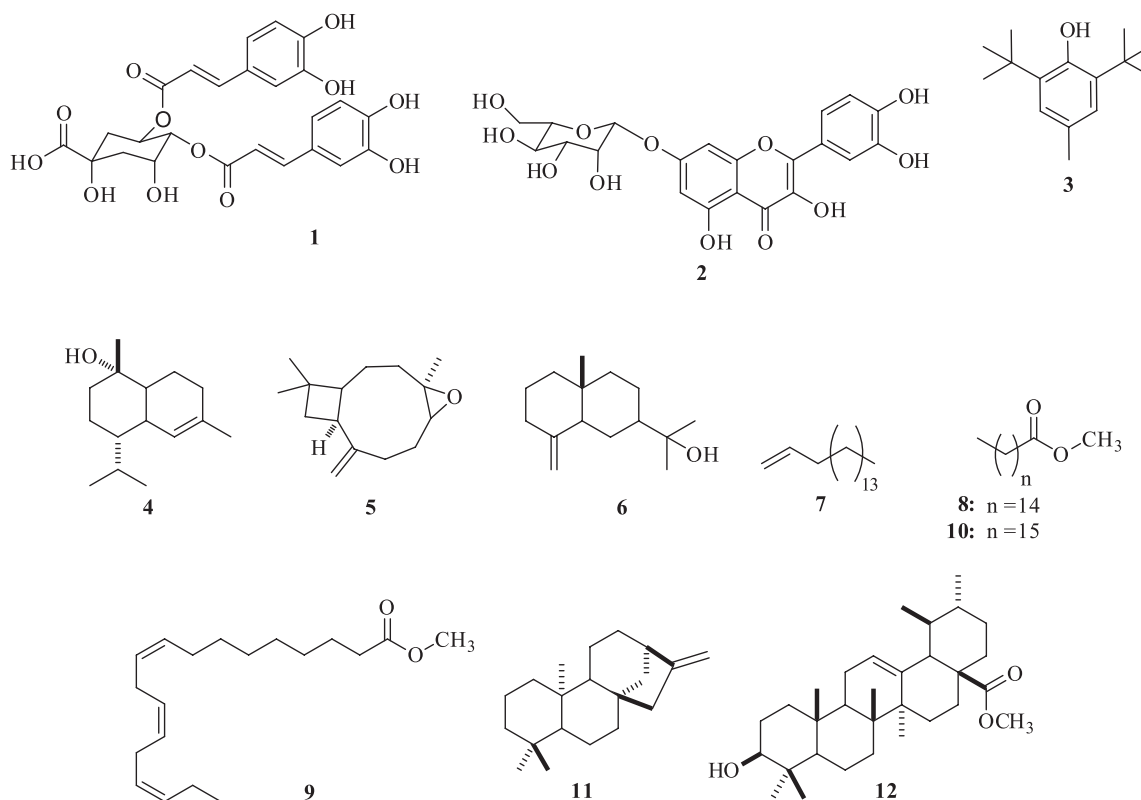
Figure 2. Chemical structures of 3,4-O-(Z)-dicaffeoylquinic (**1**), and acid quercetin-7-O-glycoside (**2**) other compounds identified in *H. marrubioides* microplants extracts subjected or not to elicitation by endophytic bacterial and fungal isolates

Table 2. Chlorogenic acid (**1**) and flavonoid (**2**) present in *H. marrubioides*

	Treatment	1 (mg gDW^{-1})	2 (mg gDW^{-1})
Control Plants	T1[b]	1.91b ± 0.31	19.02a[c] ± 0.47[d]
	T2	32.84b ± 8.08	1.26b ± 0.20
Bacterial Elicitation	T3	34.50b ± 7.50	1.74b ± 0.44
	T4	16.32b ± 4.56	0.92b ± 0.06
	T5	19.13b ± 6.70	0.85b ± 0.01
	T6	38.13b ± 2.23	0.94b ± 0.03
	T7	29.71b ± 8.16	1.27b ± 0.17
Fungal Elicitation	T8	60.57ab ± 10.8	1.59b ± 0.36
	T9	58.54ab ± 8.6	2.36b ± 1.15
	T10	16.57b ± 10.5	2.19b ± 0.20
	T11	128.74a ± 35.20	1.00b ± 0.04

[b] Treatments T1 to T11 were described in table 1. [c] Letters compare rows Means followed by the same letter do not differ from each other by the Tukey test (5%). [d] Mean standard deviation.

oxide (**5**) and eudesm-4(14)-en-11-ol (**6**) appeared in treatments T4 and T5 while the τ-cadinol was found only in T5. The presence of the triterpene methyl 3β-hydroxy-urs-12-en-28-oate (**12**) was observed in higher relative proportion in treatments T4 (8.5%) and T5 (6.9%), contrasting with the intensified hydrocarbons and fatty acids contents verified for treatments T6 and T7.

Terpenoid biosynthesis induction has been investigated in many plant species. Accumulation of the terpenoid essential oil in *Ocimum basilicum* L. following elicitation with methyl jasmonate gave rise to a total percentage increase of the terpenoids β-caryophyllene, 1,8-cineole, linalool and limonene.[42] Suspension-cultured cells treated with fungal elicitors have been found to increase the terpenoids content, especially in the case of sesquiterpenoids. This is because some of them have been classified as phytoalexins, due to their induced biosynthesis and their potential role in plant resistance to phytopathogens.[43]

Comparing treatments T9, T10, and T11 with endophytic fungi, to the control T8, it was possible to note that the sesquiterpene eudesm-4(14)-en-11-ol (**6**) was present only in T9 (0.1%). The highest relative concentration of the methyl 3β-hydroxy-urs-12-en-28-oate (**12**) was obtained for T9 (10.1%) followed by treatment T11 (6.42%). The strains employed in T9 and T11 also increased the diterpene **11** content (67.3% and 65.9%, respectively), compared with the control T8 (59.1%). On the other hand, treatment T10 prompted a significant rise in the production of the fatty acid methyl esters **8** (29.0%), **9** (11.5%), and **10** (7.9%).

The effect of yeast extract on the accumulation of different types of triterpenoid acids has been previously described. Significant enhancement of oleanolic acid accumulation has been observed upon elicitation with the fungus *Trichoderma viride* homogenate in *Calendula officinais* L. cell suspension cultures.[44]

The induced accumulation of oleanolic and ursolic acids by yeast extract in *Uncaria tomentosa* cell suspension cultures has also been verified by Feria-Romero and coworkers.[45] Triterpene production in the presence of yeast extract might be promoted as a response to cell wall damage involving plant defense mechanisms mediated by endogenous jasmonate induction.[46] Another study has revealed that ursolic acid inhibits the synthesis of jasmonate-induced proteins.[47]

Interestingly, comparison of fatty acids production in the experiments conducted in the presence of bacteria and fungi showed that the predominant fatty acids were **8**, **9** and **10** in all cases. Ribeiro and coworkers have demonstrated that sesame (*Sesamum indicum* L. - Pedaliaceae) leaf fractions contained a mixture of fatty acids, for which the major components were tetradecanoic, hexadecanoic,

Table 3. GC/MS identification of the main chemical constituents of *H. marrubioides* microplants extracts subjected or not to elicitation by endophytic bacterial and fungal isolates

Compound	MW	Rt (min)	RI	Area (RSD)%									
				T2[e]	T3	T4	T5	T6	T7	T8	T9	T10	T11
3	220	16.02	1225	4.1±0.3	6.5±1.0	0.7±0.3	0.4±0.1	0.5±0.2	t[d]	0.5±0.1	t	0.6±0.2	0.8±0.3
4	222	16.20	1230	-----	------	------	0.4±0.2	-----	-----	0.2±0.1	-----	-----	-----
5	220	18.69	1293	-----	------	1.1±0.7	0.4±0.1	-----	-----	0.2±0.1	----	-----	-----
6	222	21.09	1354	-----	------	0.2±0.1	0.3±0.1	-----	-----	t	0.1±0.1	-----	-----
Unknown	222	22.38	1386	0.2±0.1	0.5±0.3	2.6±0.9	1.6±0.7	1.3±0.4	1.4±0.3	1.3±0.2	1.2±0.3	0.1±0.1	1.4±0.3
7	238	27.50	1523	-----	1.0±0.4	0.5±0.2	1.5±0.4	2.1±0.6	1.9±0.6	0.1±0.1	1.1±0.4	0.2±0.1	2.8±0.5
8	270	30.50	1605	10.1±0.4	2.9±05	2.8±0.5	3.3±0.7	4.5±0.6	4.7±1.0	2.6±0.5	5.5±0.3	29.0±.3.2	4.0±0.2
Unknown	314	35.93	1766	4.8±0.7	1.8±0.3	0.2±0.1	0.7±0.3	1.9±0.5	1.8±0.4	0.5±0.2	2.6±0.5	3.4±0.6	2.7±0.6
9	292	36.14	1772	13.1±1.2	2.8±0.8	0.6±0.2	1.9±0.4	3.5±0.4	4.6±0.7	0.7±0.2	4.7±1.0	11.5±1.0	5.8±0.9
10	284	36.92	1796	2.1±0.6	0.4±0.2	0.5±0.2	0.7±0.2	1.2±0.4	1.2±0.3	0.6±0.1	1.5±0.3	7.9±1.4	0.8±0.1
11	204	47.94	2156	65.0±4.5	77.8±5.4	64.1±4.3	62.2±5.1	67.8±4.7	77.3±3.6	59.1±2.4	67.3±4.1	35.2±3.7	65.9±2.6
Unknown	448	57.81	2541	0.5±0.1	4.3±1.0	4.4±0.9	5.0±0.9	1.5±0.3	1.7±0.4	4.3±0.5	1.6±0.3	0.7±0.2	2.9±0.4
12	470	77.76	n.c.	t	t	8.5±1.0	6.9±1.0	2.0±0.4	1.7±0.4	15.5±2.0	10.1±2.0	3.4±0.6	6.4±0.7

MW: molecular weight; **Rt**: retention time; **RI**: retention index relative to a mixture of homologous series of *n*-alkanes (C_8-C_{40}); **RSD**: Relative Standard Deviation; **n.c.**: not calculated; t: traces (relative area lower than 0.1 %). [e] Treatments T1 to T11 were described in table 1.

octadecanoic, eicosanoic, docosanoic, and 9,12,15-octadecatrienoic acids.[48] These constituents were able to strongly inhibit the symbiotic fungus *Leucoagaricus gongylophorus* (Möller) Singer (syn. of *Rozites gongylophora* Möller) cultivated by the leafcutter ant *Atta sexdens* L. Because the *in vitro* culture, is a highly stressful system,[49] it may have stimulated the synthesis of fatty acids by *H. marrubioides* Epling microplants. However, the production of these acids was higher for most of the fungi and bacteria tested as elicitors, as compared to control treatments. This may represent a plant response to the presence of the microorganism.

The obtained data suggested that, in the case of the bacteria, T5 followed by T4 were the treatments that afforded greater structural diversity and a larger number of produced metabolites. As for the fungal species, T9 provided more diverse data.

CONCLUSIONS

The *in vitro* elicitation technique has been an important strategy for the production of active compounds from medicinal plants. The *in vitro* inoculation response of *H. marrubioides* with different strains of bacteria and fungi ranged from asymptomatic symbiotic relationships characterized by the possible endophytism of the bacteria RF10 and RG12, to symptoms of parasitic associations with bacteria RF11 and RG8 and also the fungi *Trichoderma sp. Papulaspora sp. and Fusarium sp*. In this study, some metabolites were only synthesized in treatments where there was elicitation, suggesting that this method could be better designed to increase the metabolic diversity of *H. marrubioides* elicited *in vitro*. Indeed, the sesquiterpenes τ-cadinol (**4**) and caryophyllene oxide (**5**) were only produced in treatments with bacteria, while the fatty acids methyl esters **8** (methyl hexadecanoate) and **9** (methyl (*Z,Z,Z*)9,12,15-octadecatrienoate) and the triterpene methyl 3β-hydroxy-urs-12-en-28-oate (**12**) were overexpressed only in treatments with fungi. These results indicated that the bacterial and fungal endophytic isolates employed as elicitors induced different metabolic responses from *H. marrubioides* microplants. However, other biotic, or even abiotic, elicitors should be tested, in order to obtain more metabolites of interest.

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