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Ecofriendly chemical diversification of *Eupatorium buniifolium* essential oil by endophytic fungi

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ABSTRACT

Essential oils (EOs) are complex chemical matrices on which whole cell biotransformations have not had much success due to their well-known antimicrobial activities. The approach in this work is to use endophytic microorganisms to increase the chemical diversification of *Eupatorium buniifolium* EO by biocatalysis. Three endophytic fungal strains showing ability to biotransform the EO and four of the EO main pure components, *R*-(+)-limonene, α-(−)-pinene, α-(+)-pinene and sabinene, were isolated from *E. buniifolium* plants. Two strains were characterized up to species level as *Fusarium solani* Eb01 and *Alternaria alternata* Eb03 and the third strain at genus level as *Neofusicoccum* sp. Eb04. The three fungi, as resting cell systems, allowed to access to three new complex volatile matrixes from *E. buniifolium* EO by increasing its degree of oxyfunctionalization. The robustness and biocatalytic skills of these microorganisms make them worth their inclusion within the tool-box for the generation of new valuable bioproducts from hydrocarbon monoterpene rich feedstocks.

1. Introduction

Essential oils (EOs) are complex mixtures of low molecular weight compounds derived from plant secondary metabolism [1] that carry in themselves the smell and taste footprint of the plant material from which they come from. They are well-known for their bioactivity, mainly their antimicrobial properties [2,3].

The interest in EOs has increased in many areas such as organic agriculture [4] as well as in the pharmaceutical, cosmetic [1,2,5] and food industries [6,7]. The steady growth of consumer interest in a wider repertory of bioproducts obtained directly from nature and/or processed by certified sustainable methods makes natural sources become insufficient to cover the market demand. This leads to the increase of bioproduct prices, which can reach up to 100 times that of their synthetic analogues [6].

The development of new tools to obtain valuable biocompounds from abundant and renewable natural resources has thus become imperative. Furthermore, it has been reported that an increase in the degree of oxyfunctionalization of natural flavors, fragances and volatile bioactive compounds correlates with an increment in their bioactivities [8–10].

Biocatalysis is a suitable natural instrument for the transformation of natural products. This technology is not only ecofriendly but also socially accepted. Biocatalytic transformations can be carried out in plain water or green solvents and under mild conditions as room temperature and atmospheric pressure, with the subsequent benefits in terms of decreasing energy demands and costs [11]. Moreover, biocatalysts possess the intrinsic ability to add stereo- and/or enantioselectivity to the bioprocesses, thereby reducing or avoiding the complicated and costly enantiomer separation steps [12]. These properties become particularly relevant in the case of fragrances, flavors and drugs, since enantiomers usually show different aromatic or pharmacological properties [13].

Biotransformation mediated by microorganism whole cells is a mature, green and cost-effective technology that, once optimized, can be transferable to different productive sectors. A comparative advantage of this type of biotransformation, which mainly involves redox reactions, is the natural regeneration of the necessary cofactors [14]. However, the well-known antimicrobial activity of EOs [2,15] has limited the possibility of using microbial whole cell systems to biotransform them, although there are a couple reports dealing with the modification of the composition of two EOs by immobilize-lipase-catalyzed acylation of their alcoholic components [16,17].

Endophytic organisms are bacteria and fungi that live within the plant tissues [18]. They do not cause apparent diseases to the hosts, and they can act as opportunistic pathogens, mutualists, commensalists and/or saprophytes [19]. Although they have developed interesting metabolic pathways to synthesize original *de novo* secondary

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metabolites [20], more attention has been drawn to the fact that some endophyte fungi produce metabolites considered exclusive to their host plants. Many impressive demonstrations of the production of "plant compounds" by endophytic organisms have been reported [21,22], the best-known example being the synthesis of the anti-neoplastic paclitaxel by *Taxomyces andreanae*, an endophyte fungus of the Pacific yew [23]. In fact, many authors have supported the hypothesis proposed by Stierle et al. [24] that endophytes and their hosts might have coevolved through the exchange of genetic information.

Although the literature reports some interesting examples of successful biotransformation processes mediated by endophytes [12,25–28], their remarkable biochemical capabilities have not been vastly used in biocatalysis.

Eupatorium buniifolium is an Argentinean endemic plant that belongs to the Asteraceae family. Several species of this genus have been used in folk medicine because of their antimalarial, antimicrobial and anti-inflammatory activities [29]. Additionally, some of their EOs and ethanolic extracts possess insecticide, nematicide and phytotoxic properties [30,31].

Lancelle et al. [32] has reported that *E. buniifolium* EO is mainly constituted by mono- and bicyclic monoterpene hydrocarbons. This profile is appropriate to study chemical diversification by increasing the oxyfunctionalization level employing endophytic fungal whole cell biotransformations. These authors have also informed that sabinene, α-pinene and limonene are among the main *E. buniifolium* EO components. These three hydrocarbon moterpenes are ubiquitous; therefore, they constitute interesting feedstocks for obtaining other bioproducts. For example, α-pinene, the main constituent of turpentine, is used as precursor for the synthesis of oxygenated counterparts and as raw material and solvent in the manufacturing of fragrances, flavors and antibacterial and antitumor agents [33–35]. The bicyclic monoterpene sabinene, which has also been isolated from EOs of other plants, including oak (*Quercus ilex*), spruce (*Picea abies*) [36] and nutmeg (*Myristica fragrans*) [37], is one of the chemicals that contribute to the pungent taste of black pepper (*Piper nigrum*) [38] and is also present in carrot seed oil (*Daucus carota*) [39]. Its anti-inflammatory and antimicrobial activities have also been studied [36,40]. As regards limonene, it is present in the volatile fraction of more than 300 plants and is considered the most abundant monoterpene in nature [41]. In addition to its well-known reputation as flavor and fragrance, it is widely used as solvent for resins, rubber, paints, and oils and in the synthesis of other compounds such as menthol [42].

The present work is based on the hypothesis that endophytic microorganisms possess the ability to survive in the presence of the EOs of the host plants of which they come from because they have developed metabolic skills to biotransform their main antimicrobial components. Therefore, the purpose of this work is to recover endophytic fungi from *E. buniifolium* specimens and assess their abilities to biotransform both *E. buniifolium* EO and pure monoterpenes.

2. Materials and methods

2.1. Plant material

Aerial parts of *Eupatorium buniifolium* were collected in Potrero de los Funes (27° 97 'S, 66° 15′ W) in the Province of San Luis, Argentina, in February 2015. One specimen was deposited in the Herbarium of the National University of San Luis (UNSL voucher number # 495-Del Vitto).

2.2. Chemicals

Substrates and standards of sabinene (CAS Number: 3387-41-5), α-(+)-pinene (CAS Number: 7785-70-8), α-(−)-pinene (CAS Number 7785-26-4), *R*-(+)-limonene (CAS Number: 5989-27-5) purity over 98%, α-ciano-4-hydroxicinamic acid, acetonitrile and triflouroacetic acid HPLC grade were purchased to Sigma-Aldrich Argentina.

2.3. Extraction and characterization of E. buniifolium EO

Fresh aerial parts of *E. buniifolium* (5 kg) were chopped and subjected to stem distillation at 96 °C for 3 h using a Clevenger type apparatus. The recovered EO was dried over Na_2SO_4 anhydrous and stored in sealed vials at 4 °C in the dark.

Characterizations of both the EO and the biotransformation products were performed by GC–MS using a Thermo Trace 1300 gas chromatograph coupled to an ITQ900 ion trap mass spectrometer (GC/MS-ITD). The analysis was performed using a DB-5 fused silica capillary column Ohio Valley (5% phenyl, 95% dimethylpolysiloxane, 30 m × 0.25 i.d., film thickness 0.25 μm). Mass transfer line and injector temperature were set at 240 °C and 220 °C, respectively. The oven temperature was programmed from 60 to 246 °C at rate of 3 °C min^{-1} and finally raised to 300 °C at 15 °C min^{-1} carrier gas was He (10 psi). Samples (0.1 μL of the EO and 1.0 μL of biotransformation extracts) were injected in split mode (1:50). Spectra were acquired in full scan positive mode. The EO components were identified by comparison of their linear Retention Indices (IRLs) in relation to the homologous series of *n*-alkanes (C9–C26) according to Adams [43]. MS fragmentation patterns were compared with those stored in the NIST 2.0 spectra library [43,44]. A match factor ≥800 was considered when compare MS spectra with those from NIST according to [45].

The quantification of the EO components and the biotransformation samples was performed using a GC-FID Perkin Elmer Clarus 500, equipped with the same column described above. N_2 (31.9 cm s^{-1}) was used as carrier gas and FID detector was set at 300 °C. The temperature program used for the GC-FID analysis was the same already described for GC–MS. Peak identification was carried out by comparing the GC-FID retention indices with those from GC–MS. Relative amounts of each individual component amounts were expressed as percentages of each peak area relative to the total chromatogram peak area. Meanwhile, chiral chromatographic analyses of both α-pinene isomers in the EO was performed by GC-FID using a β-DEX™ SUPELCO (20% permethylated β-cyclodextrin in SPB-35 poly(35% phenyl/65% dimethylsiloxane), 30 m × 0.25 i.d., film thickness 0.25 μm) column. The initial oven temperature was set at 60 °C and increased to 200 °C at a rate of 5 °C min^{-1}. Samples of 0.1 μL were injected in split mode (1:50). N_2 (31.9 cm s^{-1}) was used as carrier gas and the detector was set at 300 °C. *E. buniifolium* EO samples doped with optically pure standards of each enantiomer were analyzed as controls.

2.4. Isolation of endophytic microorganisms from E. buniifolium fresh plant material

Fresh plant material of *E. buniifolium* harvested at the same time that the material used to obtain the EO was washed with abundant water and separated into roots, stems and leaves (5 g each). Endophytic microorganism isolation was carried out according to the methodology described by Rodríguez et al. [46]. Briefly, surface disinfection of each lot was carried out by immersion in a 70% ethanol aqueous solution for 5 min and then in a 30% NaClO solution for 20 min. Finally, 8 rinses with sterile distilled water were performed in aseptic conditions under laminar flow. As disinfection controls, 50 μL of the last rinsing water were inoculated on PDA plates and incubated at 28 °C for a week in order to discharge epiphytic microorganisms and/or external contaminants. The disinfected material was placed into a sterile mortar under sterile conditions and macerated to isolate microorganism from the inner tissues. Then, each lot was incubated in liquid Murashige-Skoog medium (MS) [47] with and without the addition of the antimicrobial agents chloramphenicol (Cam) and cycloheximide (Chx) (see SM for details), at 28 °C and 150 r.p.m. in an orbital shaker for 4 days. Serial dilutions were performed on physiological serum, and 100 μL of the 10^{-1}, 10^{-2} and 10^{-3} dilutions were spread onto agar plates of

potato dextrose agar (PDA), and onto plant-agar (AP). The former was prepared by adding autoclaved and chopped *E. buniifolium* fresh aerial material to an agar solution (Tables S1 and S2). Dishes were incubated at 28 °C and checked every 8 h for 14 days, and single colonies were subcultured in the same media. Isolated fungal strains were kept at 4 °C and subcultured monthly.

2.5. Screening and biotransformation assays with endophytic fungal strains

Screening assays were performed with all the isolated strains in order to detect those capable to biotransform the selected substrates *(E. buniifolium* EO, sabinene, α-(−)- and α-(+)-pinene and *R*-(+)-limonene). Fungal precultures were grown in Czapeck liquid medium for 72 h at 28 °C at 150 r.p.m on a rotary shaker. After centrifugation (20 min at 8000*g*) 2 g of fungal biomass (wet weight) were transferred to 50 mL Erlenmeyer flasks and resuspended in 20 mL of 100 mM potassium phosphate buffer pH 7.0. Substrates dissolved in ethanol (1:4) were added at a final concentrations of 0.25 and 0.125% v/v. Incubation was carried out under orbital agitation at 150 r.p.m., for 72 h at 28 °C in the dark. After incubation cells were separated by centrifugation (10 min at 3000*g*) and biotransformation media extracted 3 times with 5 mL of EtOAc. Organic layers were dried over Na_2SO_4 (anh.), and stored at 4 °C for further GC analysis. Biotransformation assays were performed under the same experimental conditions above described but using only the strains that previously showed the ability to biotransform any of the selected substrates. Resting cell systems and a substrate concentration fixed at 0.25% v/v were used. Additionally, control batches without biocatalyst and without substrate were included (controls). Experiments were done in duplicate.

2.6. Identification of isolated endophytic fungi

Only strains showing positive results in the biotransformation screening were identified. Phylogenetic analysis of the ITS1-5.8SrDNA-ITS2 and D1-D2 regions was performed as previously described by Rodriguez et al. [48]. Fungal DNA genomic was extracted by standard procedures [49]. The ITS1-5.8SrDNA-ITS2 region was amplified using primer pair ITS1–ITS4 [50]. Amplification of D1/D2 domain of the LSU rRNA gene was performed with primers ITS1-F (5′-TCCGTAGGTGAACCTGCGG-3′) and NL-4 (5′-TCCTCCGCTTATTGATATGC-3′). The reaction mixture contained 1 μg/μL DNA sample, 200 μMdNTPs (Promega®), 1.5 mM $MgCl_2$, reaction buffer 1×, 1.25 U Taq polymerase (Sigma-Aldrich) and 0.4 μM of each primer in 50 μL of total volume. PCR conditions for the amplification of ITS1-5.8SrDNA-ITS2 region consisted of initial denaturation at 94 °C (5 min), 30 cycles of amplification at 94 °C (30 s), 55 °C (30 s), and 72 °C (1 min), plus one final extension step 5 min at 72 °C. PCR was carried out using an automatic thermocycler, (Techne TC 3000). The amplified bands were observed under UV illumination (Benchtop UVP) after electrophoresis on 1% (w/v) agarose gels and staining with ethidium bromide. PCR products were sequenced with an Applied Biosystems automatic sequencer ABI 3730XL at Macrogen Corp., Seoul, Korea, and analyzed with the program Vector NTI 10.3 Advance™. Fungal taxonomic affiliations were assigned based on the closest match to sequences available at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using the BLAST algorithm [51]. Sequences of fungi obtained were deposited in the GenBank nucleotide sequence database.

In addition, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) was carried out up to species-level identification. Protein extraction was carried out according to Lau et al. [52] using *Aspergillus ustus* (CBS 261.67T) as positive quality control organism in each run (see details in Supplementary document). Supernatant from each endophytic isolate (1.0 μL) was spotted onto a clean MALDI-TOF BigAnchorChip target plate on a 45 °C slide warmer (Premiere slide warmer XH-2002; Daigger, Vernon Hills, IL), and 1 μL of *Escherichia coli* Bruker bacterial Test Standard was dropped on the calibration spot. Then, 2 μL of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) were poured into each spot of dried sample. Spectra were acquired in a MALDI-TOF MicroFlex LT mass spectrometer (Bruker Daltonics, Inc.) over a mass/charge (*m/z*) ratio ranging from 2000 to 20,000. Each spot was measured using 250 laser shots at 60 Hz and each sample was processed in duplicate. The acquired data were compared toward Bruker databases using Biotyper software (version 3.0; Bruker Daltonics, Inc.), which assigns a logarithmic score ranging from 0 to 3 (cutoff scores of 2.0 for species-level identification and 1.7 for genus-level identification are recommended). After preliminary identification, the samples were again tested toward *F. solani* 103811 and *A. alternata* 62899 reference strains from the Instituto Malbran collection (see details in Supplementary document).

3. Results and discussion

3.1. E. buniifolium EO characterization

Hydrodistillation in a Clevenger apparatus of *E. buniifolium* aerial parts yielded 2.67 g of oil/kg of fresh plant material. GC–MS and GC-FID analysis on a DB-5 column allowed the identification of 63 compounds representing 97% of the EO total area (Table S3, Fig. S1). The major compound group was constituted by monoterpene hydrocarbons (82.4%), while sesquiterpene hydrocarbons only reached 9.6%. Oxygenated terpenes also constituted a minority group accounting for

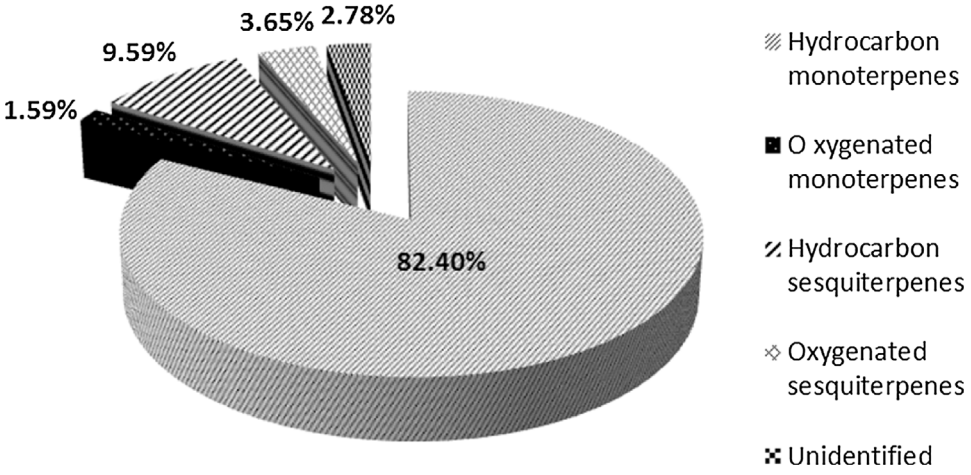

Fig. 1. Percentage distribution of the different compound groups in EO obtained by hydrodistillation from *E. buniifolium* aerial parts. Compounds were characterized by GC–MS and quantified by GC-FID.

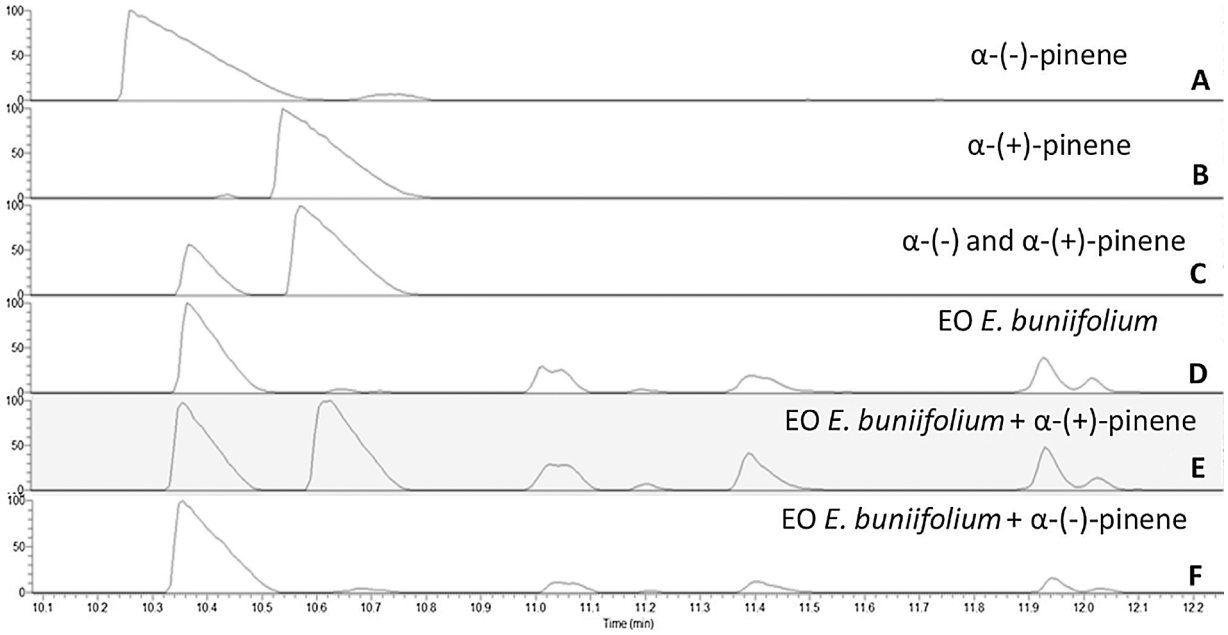

Fig. 2. Chiral chromatographic analyses of both α-pinene isomers in the *E. buniifolium* EO performed by GC-FID. (A) α-(−)-pinene standard, (B) α-(+)-pinene standard, (C) α-(−) and α-(+)-pinene standard mixture, (D) *E. buniifolium* EO, (E) *E. buniifolium* EO doped whit α-(+)-pinene standard and (F) *E. buniifolium* EO doped whit α-(−)-pinene standard.

only 5.3% of the *E. buniifolium* EO (Fig. 1). These results agree with those reported by Lancelle et al. [32], although only 19 compounds were identified in this seminal work. Other authors, who studied E. *buniifolium* EOs from plants collected in Uruguay, a different global ecological zone [53], reported dissimilar compositions, mainly regarding to the monoterpene/sesquiterpene ratios [54,55]. Moreover, we observed that α-(−)-pinene (95.6% *ee*) (Fig. 2) and *R*-(+)-limonene were the main α-pinene and limonene isomers when *E. buniifolium* EO was analyzed by chiral chromatography, in line with the reports of Lorenzo et al. [55] who worked with one of the *E. buniifolium* EO of Uruguayan origin above alluded.

3.2. Endophytic fungi isolation and biotransformation screening

Endophytic fungi were recovered from roots, stems and leaves of *E. buniifolium* incubated in liquid MS media, with and without the presence of antimicrobials. Since no development of epiphytic microorganisms was observed in the disinfection control assays, we considered that all the isolates corresponded to endophytic strains. Two culture media were used for isolation, a general one (PDA) and the specially designed AP media, which tries to mimic plant host internal environment. A total of four different filamentous fungi (Eb01–Eb04) and one yeast (Eb05) were recovered from all the treatments as shown in Table 1. Yeast growth was observed after 48 h of incubation, while the four filamentous strains evidenced development after 5 days to (Fig. S2). Based on preliminary morphological observations it was possible to determine that, except for the ubiquitous strain Eb03, each isolated microorganism was located in a particular tissue. Roots and stems provided the greatest diversity of endophytes.

The biotransformation abilities of the five fungi were evaluated by screening assays toward *E. buniifolium* EO, α-(−)- and α-(+)-pinene, *R*-(+)-limonene and sabinene as substrates (Table 1). Three of the five strains, namely Eb01, Eb03 and Eb04, were able to transform any of the substrates. Although there are a few reports of the biotransformation abilities of non-conventional yeasts (NCYs) [56] toward monoterpenes, such as pinene and limonene, unfortunately our Eb05 did not show this capacity. Two substrate concentrations (0.25 and 0.125% v/v) were tested and no remarkable differences were observed in the biotransformation profiles.

Subsequently, the active fungi were classified and only positive biotransformation processes were studied in detail using the highest substrate concentration.

3.3. Endophytic fungi identification

By 5.8S-ITS analysis, fungal strain Eb01 was identified as *Fusarium solani* since it exhibited 99% identity with strain *F. solani* AS 240 (KU 382597.1) (Table S4). Further comparisons toward the specific database of the genus, *Fusarium* MLST (http://www.cbs.knaw.nl/fusarium/), confirmed this classification. Additionally, MALDI-TOF MS analysis of Eb01 protein profile revealed a high-degree match with *Fusarium solani* according to Bruker database (Fig. S3, Table S5). The Eb01 sequence was deposited in the GenBank under the number KX831941.

On the other hand, strain Eb03 showed 99% identity with *Alternaria alternata* strain funbio26 (MF029625.1) and *Alternaria brassicae* isolate HG3 (KX099621.1) (Table S4). However, due to its morphological characteristics, and mainly to its protein profile obtained by MALDI-TOF MS analysis, it was classified as *A. alternata* Eb03 (KY968699) (Fig. S4, Table S5).

Strain Eb04 presented identity with several reference strains (CBS), such as *Neofusicoccum vitifusiforme* (KX464533.1), *Neofusicoccum* sp. 2 JZG-2016 (KX464494.1) and *Neofusicoccum pistaciarum* (KX464468.1) (Table S4), so the species level could not be determined. Neither the morphological nor the MALDI-TOF MS analysis enabled to better the identification degree for this strain. Other authors have reported on the complexity of the taxonomy of the Botryosphaeriaceae family since its teleomorph is rarely seen in nature, thus forcing the use of anamorphic characteristics [57]. Moreover, the 5.8S rRNA gene is highly conserved in Botryosphaeriaceae family and its sequence data cannot resolve a large clade. Consequently, recent phylogenetic analyses of this family are based on ITS together with 28S rRNA gene (LSU), translation elongation factor 1-alpha (EF1-α) and RNA polymerase second largest subunit (RPB2) sequences [58]. The Eb04 sequence was deposited in the GenBank under the number MF276906.

3.4. Biotransformations

Among the monoterpene hydrocarbons of *E. buniifolium* EO, α-(−)- and (+)-pinene, *R*-(+)-limonene and sabinene were selected as substrates for biotransformation studies due to both their availability as

Table 1
Recuperation, isolation and screening of endophytic fungi. The strains were recovered from different *E. buniifolium* organs with and without antimicrobial agents and isolated in specific media. Screening of their abilities to biotransform *E. buniifolium* EO and four of its components was checked. The substrate consumption and the appearance of new products were checked by GC-FID.

	Endophytic Isolation			Biotrasnformation screening				
				Substrate				
Strain.	Plant Organ	Recuperation media	Isolation media	α-(−)- pinene	α-(+)- pinene	R-(+)-limonene	sabinene	EO *E. buniifolium*
Eb01	R	MS	AP	+	+	−	+	+
Eb02	R	MS	PDA	−	−	−	−	−
Eb03	R	MS + CAM	PDA	−	−	+	+	+
	S	MS	AP					
	S	MS + CAM	PDA, AP					
	L	MS	AP					
Eb04	S	MS + CAM	PDA	+	+	+	+	+
Eb05	S	MS	PDA	−	−	−	−	−

S = stems; R = roots; L = leaves; MS = Murashige Skoog; CAM = Chloramphenicol; AP = Agar Plant; PDA = Potato dextrose agar; (+) represents substrate biotransformation; (−) represents no substrate biotransformation.

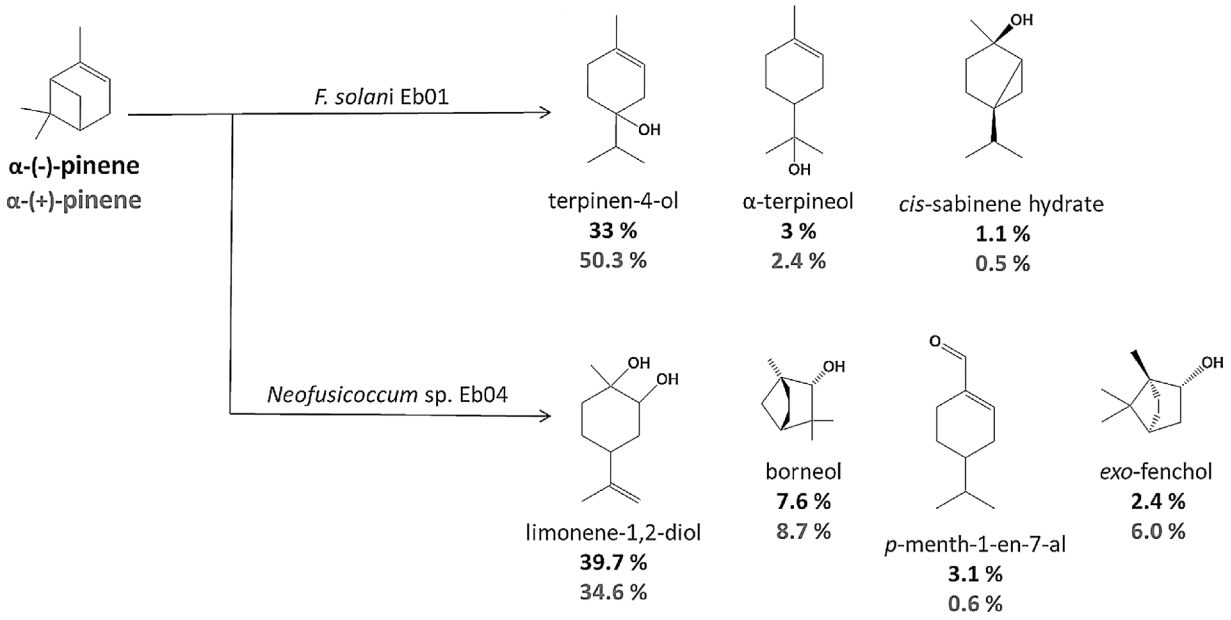

Fig. 3. Main oxygenated bioproducts obtained from the biotransformation of α-(−)- and α-(+)-pinene by *F. solani* Eb01 and *Neofusicoccum* sp. Eb04 in resting cell systems. Compounds were characterized by GC–MS and quantified by GC-FID. Relative amount of each individual product is expressed as percentage of each peak area relative to the total chromatogram peak area.

pure compounds and their frequent occurrence in essential oils of different plant species.

3.4.1. Biotransformation of α-pinene isomers

Numerous pinene biotransformation attempts have been carried out using fungi [59–61] and bacteria [59,61] as whole cell biocatalysts. In agreement with the screening shown above, both *F. solani* Eb01 and *Neofusicoccum* sp. EB04 were selected to transform this substrate.

When resting cells of *F. solani* Eb01 were used utilizando α-(−)-pinene, 13 biotransformation products were identified after 72 h of incubation. Only 12% of the total peak area of the biotransformation product volatile fraction corresponded to monoterpene hydrocarbons, while 88% were oxygenated monoterpenes, mainly alcohols. Interestingly, 33% of the total peak area of the biotransformation extract was accounted for by a single oxygenated metabolite, terpinen-4-ol. The bioconversion of α-(+)-pinene isomer yielded the same main products although bioconversion percentages were higher. Also five new terpene products at trace level were evident. Terpinen-4-ol represented 50% of the total peak area of the biotransformation extract (Table S6, Figs. S5–S7).

The bioconversion of α-pinene to limonene, α-terpineol and terpinolene has already been described [59]. However, at the best of our knowledge, the appearance of terpinen-4-ol as a minor metabolite of the pinene biotransformation has been reported only by using a strain of *Pseudomonas* [62]. The present result becomes relevant considering that terpinen-4-ol is bioactive toward viruses, bacteria, fungi, insects and acari [63–65]. Besides, its usefulness in the treatment of certain types of psoriasis [66] and its potentiality as an anticancer agent have also been reported [67].

Furthermore, *Neofusicoccum* sp. Eb04 biotransformed α-(−)-pinene mainly into oxygenated monoterpenes, which correspond to 95% of the total area of the biotransformed products, being limonene-1,2-diol, borneol, *p*-menth-1-en-7-al and *exo*-fenchol the main ones. When the substrate was α-(+)-pinene the biotransformation profile was similar. The main products were limonene-1,2-diol, borneol and *exo*-fenchol, but *p*-menth-1-en-7-al was present as a minor compound. Terpinen-4-ol was observed as a minor product in the α-(+)-pinene biotransformation (Fig. 3, Table S6, Figs. S8–S10).

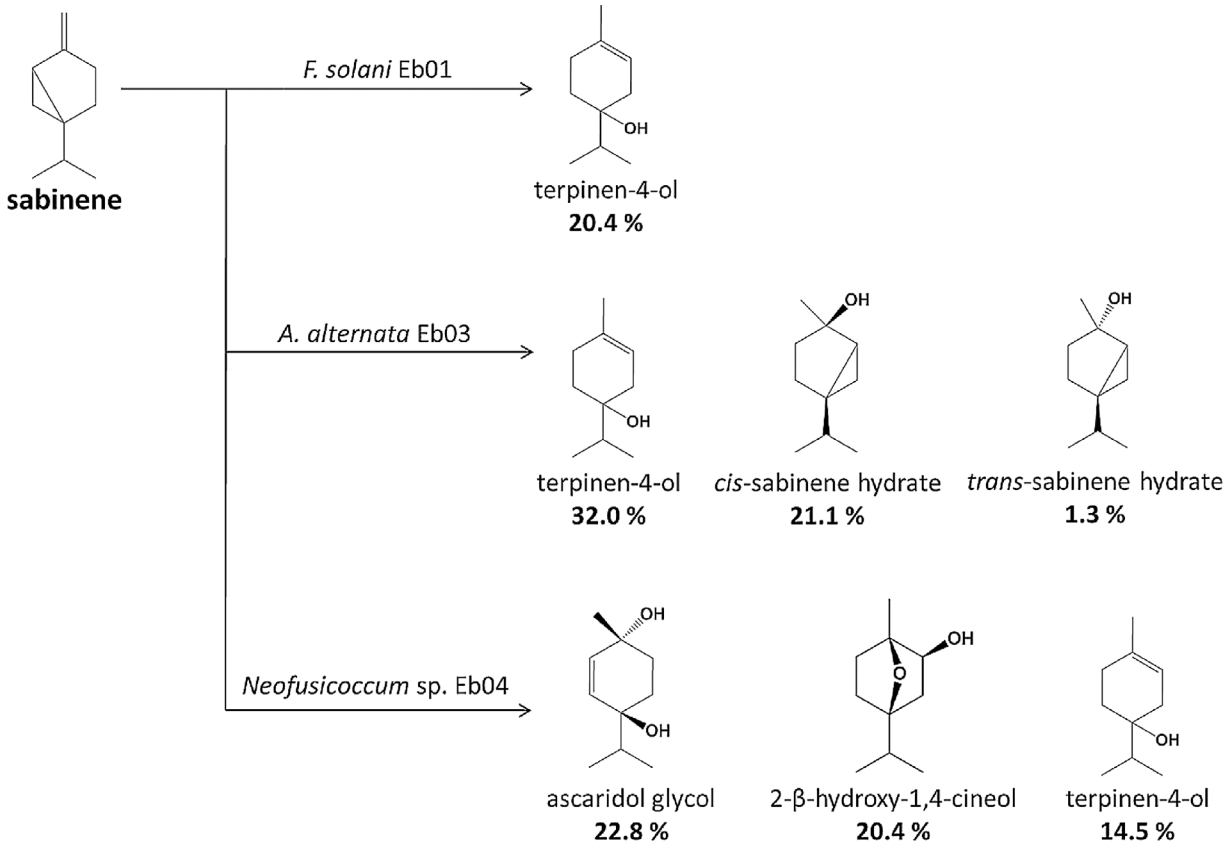

Fig. 4. Main oxygenated bioproducts obtained from the biotransformation of sabinene by *F. solani* Eb01, *A. alternata* Eb03 and *Neofusicoccum* sp. EB04 in resting cell systems. Compounds were characterized by GC–MS and quantified by GC-FID. Relative amount of each individual product is expressed as percentage of each peak area relative to the total chromatogram peak area.

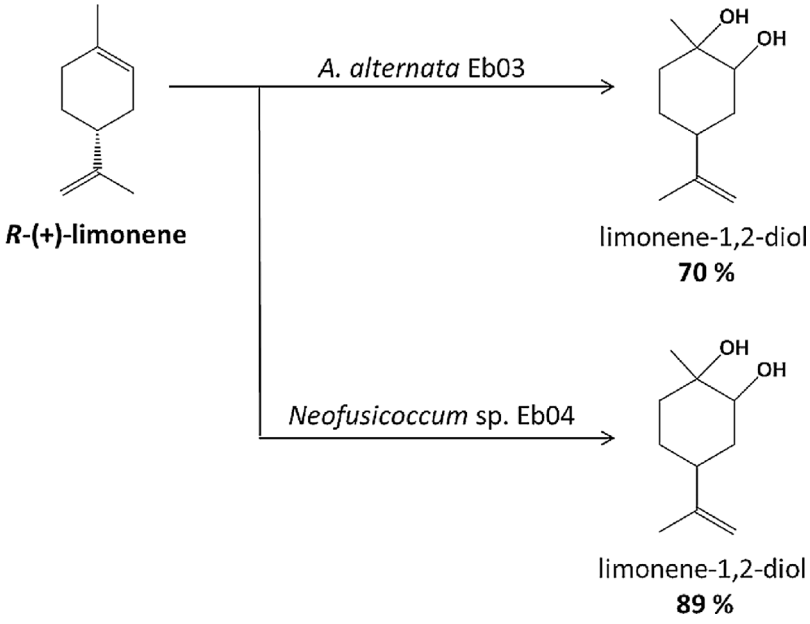

Fig. 5. Production of limonene-1,2-diol by biotransformation of *R*-(+)-limonene with *A. alternata* Eb03 and *Neofusicoccum* sp. EB04 in resting cell systems. The product was characterized by GC–MS and quantified by GC-FID. Product relative amount is expressed as percentage of the total chromatogram peak area.

3.4.2. Biotransformation of sabinene

The bioconversion of sabinene to terpinen-4-ol, *cis*- and *trans*-sabinene hydrate using fungal strains has already been described [68]. In agreement with this, in the present research, the three selected endophytic strains were able to biotransform this substrate. *F. solani* Eb01 produced terpinen-4-ol as the major biotransformation product, although in much lower amounts than those obtained from pinene (20% of the total peak area) (Table S7, Figs. S11–S12). With *A. alternata* Eb03 as biocatalyst, terpinen-4-ol, *cis*-sabinene hydrate and γ-terpinene were obtained as major compounds (Table S7, Figs. S13–S14). Finally, the bioconversion with *Neofusicoccum* sp. Eb04 yielded ascaridol glycol, 2-β-hydroxy-1,4-cineol and terpinen-4-ol as major metabolites and *cis*- and *trans*-sabinene hydrate as minor products (Table S7, Figs. S15–S16). Fig. 4 shows the main oxygenated monoterpenes obtained by

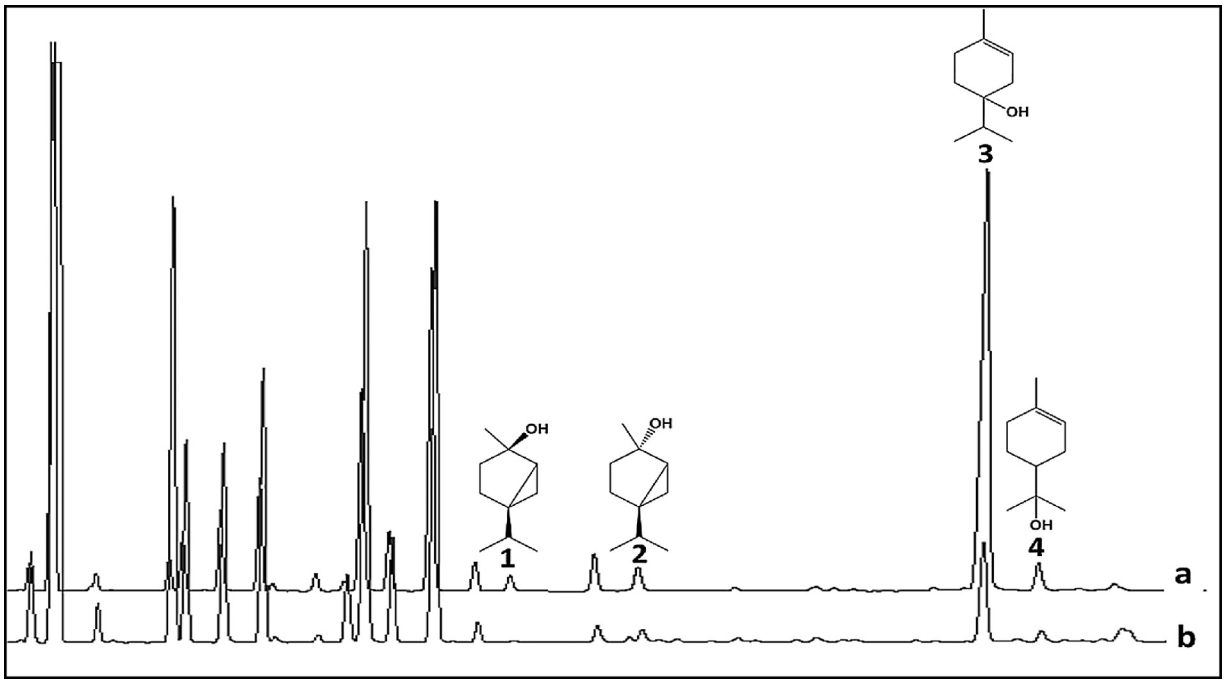

Fig. 6. Comparison between the GC-FID profile of *E. buniifolium* EO (b) and its biotransformation by *F. solani* Eb01 (a). Main oxygenated biotransformation structures are shown on the corresponding peaks.

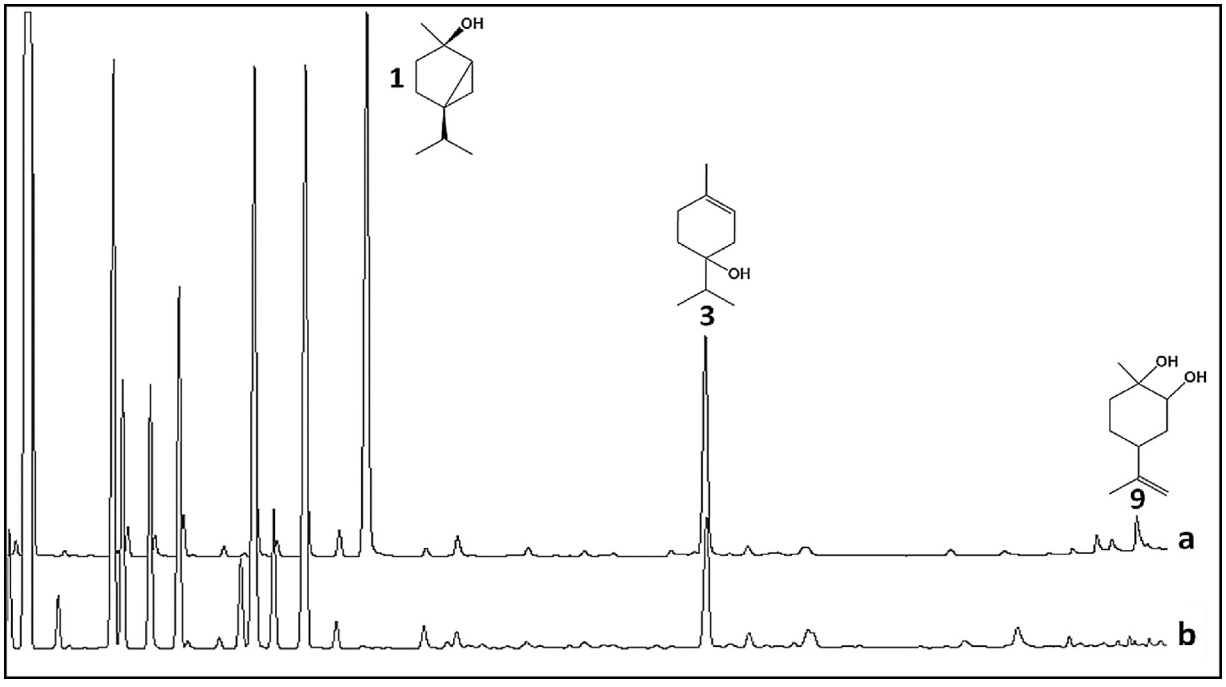

Fig. 7. Comparison between the GC-FID profile of *E. bunifolium* EO (b) and its biotransformation by *A. alternata* Eb03 (a). Main oxygenated biotransformation structures are shown on the corresponding peaks.

biotransformation of sabinene using the three endophytic fungal strains.

3.4.3. Biotransformation of R-(+)-limonene

When *R*-(+)-limonene was used as substrate, both active strains, *A. alternata* Eb03 and *Neofusicoccum* sp. Eb04, produced limonene-1,2-diol as the only biotransformation product, with 70% and 89% yield, respectively (Table S8, Figs. S17–S20). Limonene-1,2-diol is an important precursor in the synthesis of several molecules. It is also used in the preparation of beverages, chewing gums and other foodstuffs due to its fresh mint aroma [43]. There are many reports dealing with its production from limonene by using fungi, bacteria and plant cells as biocatalysts [59,69–71]. In fact, the pathway from limonene to limonene-1,2-diol in the bacteria *Rhodococcus erythropolis* was postulated many years ago [72]. Fig. 5 shows the bioconversion of *R*-(+)-limonene into limonene 1,2-diol.

3.4.4. Biotransformation of E. buniifolium EO

The biotransformation of *E. buniifolium* EO with *F. solani* Eb01 shows a profile that agrees with the bioconversion of its major

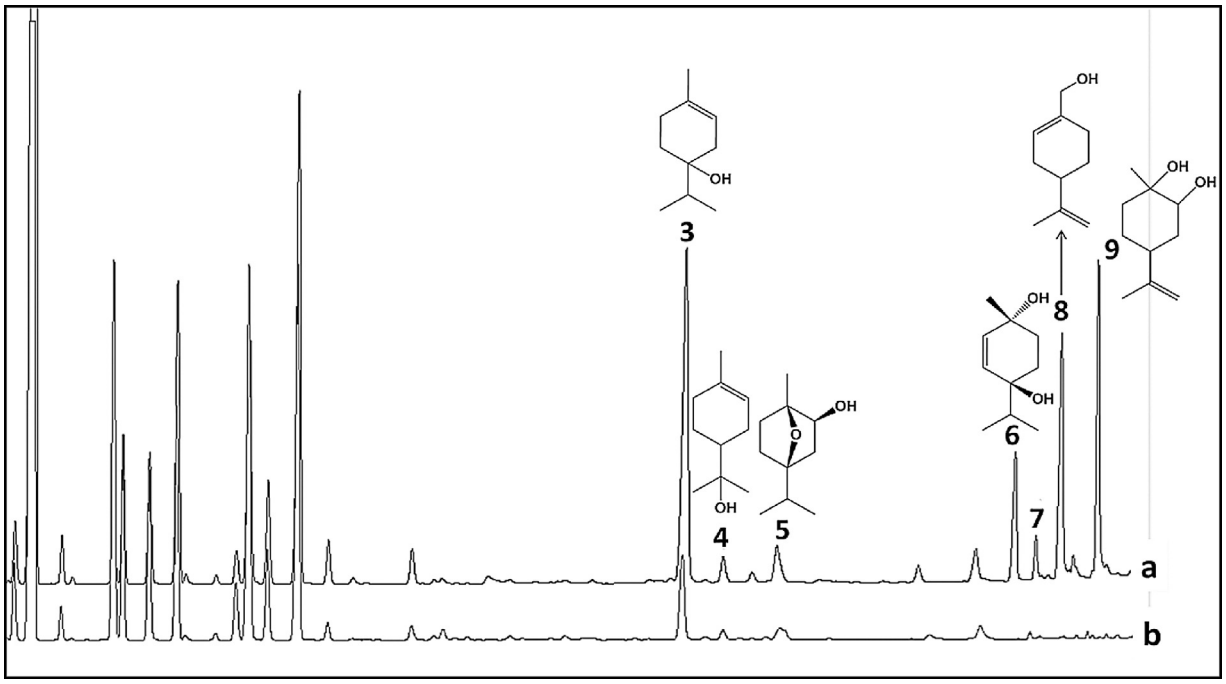

Fig. 8. Comparison between the GC-FID profile of *E. bunifolium* EO (b) and its biotransformation by the fungus Eb04 (a). Main oxygenated biotransformation structures are shown on the corresponding peaks.

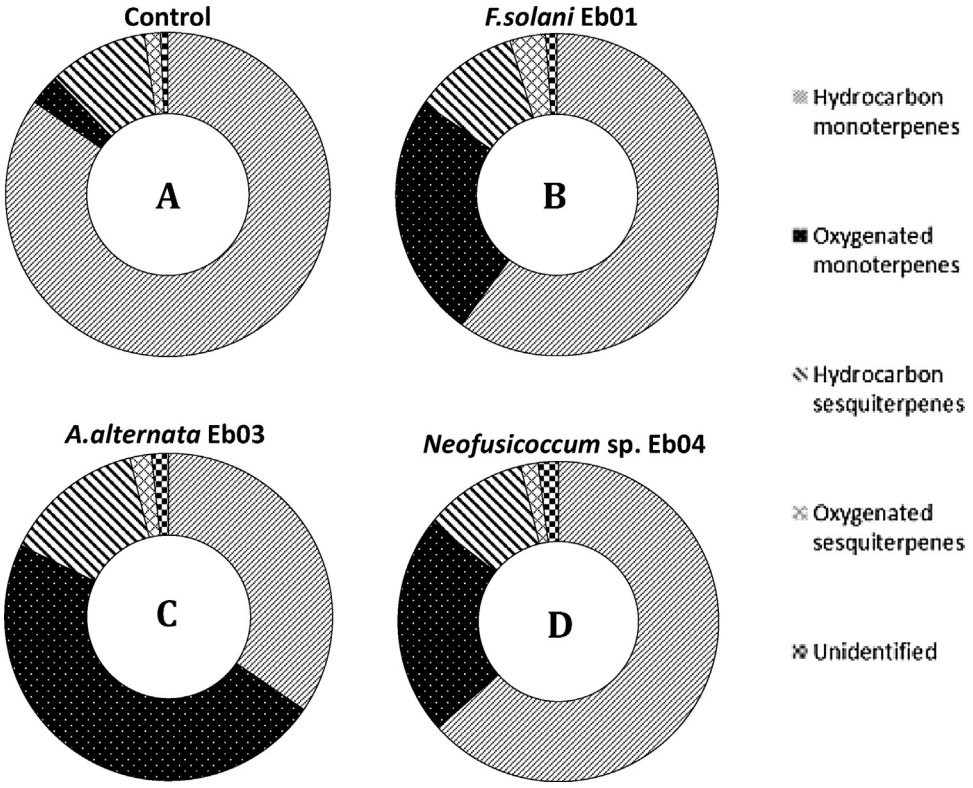

Fig. 9. Comparison between the percentage distribution of different compound groups in *E. buniifolium* EO (A) and its biotransformation products obtained by resting cell systems with: (B) *F. solani* Eb01, (C) *A. alternata* Eb03 and (D) *Neofusicoccum* sp. Eb04. Compounds were characterized by GC–MS and quantified by GC-FID.

compound, α-pinene, yielding an oil notably enriched in terpinen-4-ol (3). Other oxygenated monoterpenes observed in lower percentages were α-terpineol (4), *cis*-sabinene hydrate (1) and *trans*-sabinene hydrate (2). In addition, the sesquiterpene alcohol junenol (10), not present in the original EO, was identified (Table S9, Fig. 6).

The bioconversion of the EO with *A. alternata* Eb03 also showed a considerable increase in the relative concentration of terpinen-4-ol (3) although the major product was *cis*-sabinene hydrate (1). Limonene-1,2-diol (9) was also identified among the new oxygenated molecules, as a minor compound (Table S9, Fig. 7).

Fig. 8 shows the main compounds obtained by bioconversion of *E. buniifolium* EO by *Neofusicoccum* sp. Eb04, where it is worth noting the presence of compounds 5–9, which were not detected in the original EO composition (Table S9).

In summary, the three selected endophytes demonstrated the ability to increase the oxyfunctionalization degree of the *E. buniifolium* EO by a

sustainable method based on whole cell biotransformations, as it is depicted in Fig. S12, Fig. 9.

4. Conclusion

At the best of our knowledge, this is the first report of the biotransformation of complex chemical matrices of plant origin such as EOs by the use of endophytic fungal strains. We assessed the chemical diversification of *E. buniifolium* EO composition by using endophytic fungal strains isolated from the same plant. It was observed that three endophytic fungi were able to tolerate, and therefore biotransform, both the EO and its main monoterpene components, α-pinene, sabinene and limonene. Using these endophytic fungi as biocatalysts in resting cell systems, variants of the original EO with a higher degree of oxyfunctionalization were obtained. This kind of research lays the basis for the development of sustainable and transferable biotechnologies based on the use microorganisms that allow obtaining bioproducts that are more diverse, more effective and better accepted by the market.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2017.09.028.

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