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Metabolic divergence between island populations of the Canarian plant Bethencourtia palmensis and their endophytic fungi: a potential new source of biopesticides

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Abstract

This study explores the metabolic and biopesticide potential of endophytic culturable fungi isolated from two island populations of *Bethencourtia palmensis* (Tenerife, T, and La Palma, P), an endemic plant from the Canary Islands. The two populations showed distinct chemical profiles: 11β-acetoxy-5α-(angeloyloxy)silphinen-3-one and hexatriacontane were major components in T, while 5α-(angeloyloxy)silphinen-3-one, tetratriacontane and jacaranone were predominant in P. From PDA medium, 21 and 16 fungal isolates were obtained from T and P, respectively. Ethyl acetate extracts were tested against *Myzus persicae*, *Meloidogyne javanica*, *Botrytis cinerea* and *Fusarium verticillioides*. Among the isolates, 14% from T showed bioactivity and belonged to *Aspergillus*and *Penicillium* genera, while 81% from the P population were bioactive and belonged to *Alternaria*, *Aureobasidium*, *Aspergillus*, *Penicillium*, and *Stemphylium*. Additional isolation from P using YES medium yielded new isolates, with 65% being bioactive, including genera not recovered on PDA. GC/MS-based metabolite fingerprinting revealed distinctive patterns associated with specific genera. The results highlight significant differences in the plant chemistry and bioactive endophytes between two island populations of *B. palmensis*. Furthermore, these strains are a promising source of bioactive compounds. The diverse range of bioactivities observed suggests high potential for the discovery of novel biopesticides with applications in sustainable agriculture.

Introduction

Endophytic fungi are microorganisms that live within plant tissues without causing harm to their host [1]. In many cases, they confer beneficial effects, such as enhancing plant fitness or promoting resistance to biotic and abiotic stresses. These fungi are emerging as a major source of new bioproducts with promising applications in medicine, agriculture, and the food industry[2]. Despite this potential, most fungal endophytes remain understudied. Estimates of their global diversity range between 1 and 3 million species[3]. However, to date, only 203,556 species have been cataloged (https://www.speciesfungorum.org; accessed 28th July 2025). This gap between what is estimated to exist and what has been really described highlights the urgency of continued exploration, particularly in environments associated with high biodiversity, ecological isolation, and chemical novelty, especially as the discovery of truly novel natural products is becoming increasingly rare[4].

Among potential reservoirs for novel endophytic fungi, endemic plants are especially attractive targets. Their unique phytochemistry and restricted distribution make them likely to harbor distinct and underexplored microbial communities. Targeted exploration of these systems can lead to the discovery of new species or strains with specialized bioactivities, as demonstrated in recent studies focused on agricultural[5] or medical[6] applications.

Bethencourtia palmensis is an endemic plant species present in the islands of Tenerife and La Palma (Canarian Archipelago, Spain). A study based on molecular analyses revealed significant genetic divergence between the Tenerife and La Palma populations of *B. palmensis*, which are morphologically similar. This was explained by the ocean acting as a natural barrier, leading to lower levels of gene flow between islands[7]. Although morphological traits are similar between both plant populations, their chemistry and the composition of their endophytic communities could be affected by environmental effects[8,9]. The chemistry of

B. palmensis (Tenerife population) has been extensively studied due to its unique content in silphinene sesquiterpenes with strong insect antifeedant effects[10,11], but the chemistry of *B. palmensis* from La Palma has not been studied. The endophytic mycobiota of *B. palmensis* from Tenerife has been partially explored, and the metabolic potential of two isolates, both of them *Aspergillus* strains, was demonstrated by the isolation of antifungal, acaricidal[5,12] and nematicidal (Patent No. ES2920198 B2) compounds. However, a more complete description of the mycobiota from *B. palmensis* has not been yet reported.

In this study, the qualitative and quantitative differences in silphinene sesquiterpene content between two populations of *B. palmensis* from Tenerife and La Palma were analyzed. A screening of culturable fungal endophytes from the two populations was conducted, and their ability to produce bioactive extracts (insecticidal, nematicidal, or antifungal activity) evaluated. The bioactive isolates were identified taxonomically via ITS sequencing, and comparative metabolite fingerprinting was performed to assess endophytic chemical diversity.

Material and methods

All procedures that required it were carried out under sterile conditions.

Biological material

Bethencourtia palmensis aerial plant parts and seeds were collected from their natural habitats in Tenerife and La Palma (Canary Islands, Spain). Voucher specimens have been deposited at the herbarium of the Botanical Garden, La Orotava, Tenerife, Spain (voucher number ORT 36393). In Tenerife, *B. palmensis* was collected in Barranco del Río (Arico, Tenerife) (28°11'26"N, 16°29'52"W), 1,400 m altitude. In La Palma, two specimens of *B. palmensis* were collected in Caldera de Taburiente National Park (28°45'38.62"N, 17°52'24.51"W and 28°44'56"N, 17°50'54.18"W at 2285m and 2271m altitude, respectively). La Palma specimens were collected and identified by Ángel Palomares (Park Director), and Tenerife specimens were identified by Dr. A. Santos.

Endophyte isolation

Stem, flowers and leaves were used for the isolation. Plant surfaces were washed and then surface sterilized by immersion in EtOH 75% for 1 minute, followed by immersion in NaClO 1% for 2 min. Samples were then thoroughly rinsed in three successive washes of sterile distilled water to remove residual sterilizing agents. The effectiveness of surface sterilization was verified by plating the final rinse water on PDA to confirm the absence of microbial growth.

Equal pieces of each organ were cultured on PDA (Difco) or YES (2% yeast extract; 15% sucrose; 1.5% agar) plates, both media containing 50 $mg.L^{-1}$ of chloramphenicol to inhibit bacteria. Additionally, in order to obtain more isolates, stem samples were also crushed in sterile water with a mortar and pestle, and both solid and liquid fractions were cultured as well.

As soon as fungi started to spread on the plates, they were serially subcultured onto new plates for purification. Isolates from T populations had been obtained previously and were given a “SP” identifier.

Isolates from the P populations were given a “BP” identifier, followed by “H,” “F,” “T, “M,” or “G,” for leaf, flower, stem, crushed stem, or crushing supernatant, respectively, to indicate their origin.

Molecular identification of isolates

Molecular identification of selected bioactive isolates was carried out through PCR amplification of the ITS2 region and Sanger sequencing.

To that end, DNA was extracted using a modified CTAB method[13] as follows: mycelium was incubated for 3 days in PDB at 25°C, washed with distilled water, and centrifuged at 16,000 x g for 1 minute. Portions of approximately 100 mg mycelium were ground to powder in liquid nitrogen. Ground mycelia were suspended in 500 µL of CTAB buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris-HCl, 20 mM EDTA, pH 8), 1.2 µL 2-mercaptoethanol and 2 µL RNase A (10 $mg.mL^{-1}$) per extraction tube, and incubated at 65°C for 30 min, with periodic inversion every 5 min. Proteins and lipids were removed with 500 µL chloroform-isoamyl alcohol (24:1), mixed gently, and centrifuged at 17,300 x g for 10 min. The aqueous phase was transferred to a clean tube and this step was repeated once again. DNA was precipitated with 550 µL cold isopropanol, mixed gently, and incubated at -20°C for 15 min. Following centrifugation at 9,500 x g for 10 min at 4°C, the pellet was washed with 500 µL 100% ethanol, centrifuged again, and the process was repeated with 70% ethanol. Residual ethanol was removed and the pellet dried at 40°C for 15 min. The DNA pellet was resuspended in 100 µL of milli-Q water and stored at -20°C.

PCR amplification was carried out using 1 µL of the extracted genomic DNA solution as template and DreamTaq polymerase (Thermo Fisher Scientific, USA) in a thermocycler. with an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. A final extension step was carried out at 72°C for 5 minutes to ensure complete termination of amplified sequences. The primers used for this reaction were, for the SP isolates, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5’-TCCTCCGCTTATTGATATGC-3’), and, for the BP isolates, ITS3 (5’-GCATCGATGAAGAACGCAGC-3’) and ITS4[14].

Correct amplification was confirmed by agarose gel electrophoresis; bands were excised from gels using NucleoSpin® Gel and PCR Clean-up kit (Takara Bio, USA) and outsourced for Sanger sequencing. Forward and reverse sequences were aligned with BioEdit (v. 7.2.5), trimming unaligned ends. Base calls were manually curated against chromatograms and consensus sequences derived for BLAST (https://blast.ncbi.nlm.nih.gov) comparison to publicly available databases for taxonomic identification of isolates. Finally, basic morphological properties of isolates were compared to those described for their assigned taxonomic entity.

ITS sequences were deposited in GenBank. Accession numbers are listed in Supplementary Table S2.

Extract preparation

Plant extracts

B. palmensis aerial parts were dried in an air-forced oven at 30°C and ground. Dry materials were extracted with EtOH by maceration for three days at room temperature and the solvent was evaporated at reduced pressure to give crude extracts.

Fungal extracts

For each fungal isolate, mycelia were scrapped from fresh cultures on PDA plates and transferred to three 100 mL flasks containing 50 mL of PDB (Difco) for La Palma isolates, or modified Czapek Dox (per liter: Glucose 6 g, Yeast extract 0.1 g, KH_2PO_4 0.5 g, $NaNO_3$ 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $FeSO_4 \cdot 7H_2O$ 1 mg, $ZnSO_4 \cdot 7H_2O$ 3 mg) for Tenerife isolates for batch fermentation (120 RPM at 25°C) for 21 days or 14 days, respectively. After this period, all flasks were checked for contamination and culture media were separated from mycelia by Büchner vacuum filtration.

Filtrates were liquid/liquid extracted with ethyl acetate (1:1) three times to maximize metabolite extraction. For each isolate, pooled organic phases were mixed with sodium sulphate in order to remove water traces, filtered, and dried in a rotary evaporator at 40°C under a vacuum of 240 mbar. Dried extracts were weighed and kept dry at 4°C.

Chromatographic analysis

Gas chromatography-mass spectrometry (GC–MS) was carried out on a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector with a single quadrupole analyzer, electron impact ionization source at 70 eV and helium as the carrier gas and equipped with a 30 m x 0.25 mm ID and 0.25 µm phase thickness capillary column (Teknokroma TRB-5, 5% phenyl, 95% dimethyl polysiloxane for plant extracts, or MEGA-5 MS 5% phenyl, 95% methyl polysiloxane for fungal extracts).

Working conditions: Dried extracts were dissolved in dichloromethane (4 mg/mL, DCM), filtered and analyzed by GC–MS. Portions of 1 µL were injected in split mode (split ratio 20/1) using a Shimadzu AOC-20i automatic injector, injector temperature 300°C, temperature of the transfer line connected to the mass spectrometer 250°C, and temperature of the ionization source 220°C. For plant extracts, the initial column temperature was 70°C, ramping up to 290°C at 6°C.min^{-1}, and running at 290°C for 20 min. For fungal extracts, the initial column temperature was 110°C, ramping up to 290°C at 7°C.min^{-1}, and running at 290°C for 20 min.

A full scan with *m/z* range 35–450 was performed. Relative amounts of individual components were calculated based on the peak area without a correction factor. Retention data were used to assess compound identity by comparing them with those of standards (silphinenes previously isolated in our laboratory from *B. palmensis* and incorporated into our in-house library) or found in the Wiley 229 Mass Spectral Database.

Chemical profiles of each isolate were established based on their GC/MS analysis, considering the relative percentage of peak area for each compound. Compounds with a peak area above 1% were included, as well as those below 1% if they appeared above that threshold in any other isolate. Based on these data, isolates were grouped by hierarchical clustering according to their chemical profiles. Clustering was performed in R (v. 4.3.1) using Euclidean distance and the Ward.D2 method. The graphical representations of clustering results, including dendrograms and heatmaps, were generated using the R packages dendextend, heatmaply, and plotly.

Bioassays

Antifeedant activity

The *Myzus persicae* colony was reared on bell pepper (*Capsicum annuum*) and maintained at 22 ± 1°C, > 70% relative humidity with a photoperiod of 16:8 h (L:D) in a growth chamber.

Bioassays were conducted with 1.0 cm^2 leaf fragments of *C. annuum*. Test solutions (10 µL) were applied at initial doses of 10 $mg.mL^{-1}$ to the upper surface of leaf fragments[15]. *M. persicae* apterous adults (24–48 h old) were placed in 20 (2x2 cm) ventilated plastic boxes (10 per box) and kept in a growth chamber for 24 hours. Settling was measured by counting the number of aphids on each leaf fragment. Settling inhibition (%SI) was calculated as [1 - (T/C) x 100], where T and C represent treated and control leaf fragments, respectively. The effects (%SI) were analyzed by the non-parametric Wilcoxon Signed-Rank Test. Extracts with an effect > 70% were tested in dose-response experiments (3–5 serial dilutions) to calculate their EC_{50} (effective dose to give a 50% settling) with linear regression models (%SI on Log-dose). The positive control for *M. persicae* was Thymol (Sigma-Aldrich, USA) with an EC_{50} of 7.6 $g.cm^{-2}$.

Nematicidal assay

A population of the root-knot nematode *Meloidogyne javanica* was maintained on tomato plants (*Lycopersicon esculentum* var. Marmande) cultivated in pots under controlled conditions (25 ± 1°C and relative humidity above 70%). Second-stage juveniles (J2) that emerged within 24 hours from egg masses manually extracted from infected tomato roots were selected for use in the assays. Bioassays were conducted in 96-well microplates (Becton-Dickinson, NJ, USA), following the procedure described by Andrés et al.[16]. Organic extracts were tested at 1 $mg.mL^{-1}$ concentration in each well. Juvenile mortality was assessed after 72 hours. Each treatment was performed in quadruplicate. Mortality percentages were calculated and corrected using the Schneider-Orelli formula.

Antifungal assays

Botrytis cinerea was obtained from the fungal collection at Instituto de Productos Naturales y Agrobiologia-CSIC. The spore stock was frozen and maintained at -80°C in 20% glycerol.

For “BP” isolates, spore germination inhibition assays were conducted using 96-well plates as described[17]. Each well (4 replicates) contained 100 µL RPMI medium, 20 µL of spores (10^7 $spores.mL^{-1}$), and 80 µL water with 2.5% of a DMSO solution (80 $mg.mL^{-1}$ of extract in DMSO). Plates were then incubated at 25°C for 24 hours.

After incubation, 25 µL of a solution containing MTT (5 $mg.mL^{-1}$) and menadione (1mM) were added to each well and incubated for three hours. After removal of the supernatant, 200 µL acid isopropanol (containing 5% HCl) were added to each well and incubated for 30 minutes. Changes in medium absorbance (490 nm) were recorded with an ELx800 absorbance microplate reader (Biotek, USA) using Biotek Gen5 2.01 software to assess fungal growth inhibition.

For “SP” isolates, extracts were tested against *Fusarium verticillioides* following the protocol described in Morales-Sánchez et al.[5] Assays were carried out in 12-well cell plates. Extract solutions dissolved in EtOH

were mixed with PDA medium (reaching a final concentration of 1 mg.ml^{-1}) and 0.05 mg. ml^{-1} of MTT and added to each well of the plate. Subsequently, a 1 cm diameter disk of *F. verticillioides* was placed at the center of the well. All treatments had four replicates, and EtOH was used as a negative control. After an incubation period of 48 h in the dark at 28°C, or until the mycelium reached a diameter of 2 cm, mycelial growth was measured using ImageJ software (http://imagej.nih.gov/ij/). Bioactivity was expressed as a percentage of mycelium growth inhibition.

Results

Chemical analysis (GC-MS) of plant extracts from both Tenrife (T) and La Palma (P) populations of *B. palmensis* showed significant differences in their chemical composition (Fig. 1). The T population had 11β-acetoxy-5α-(angeloyloxy)silphinen-3-one (**3**) and hexatriacontane (**5**) as the major components, while the P population contained jacaranone (**1**), 5α-(angeloyloxy)silphinen-3-one (**2**), tetratriacontane (**4**).

Endophytic fungal isolates

Endophytic fungi from *B. palmensis* were isolated in PDA plates. From the T population, a total of 21 “SP” isolates were obtained, and only three of them (14%) showed relevant bioactivity. These were characterized as *Aspergillus* sp. (SPH2 and SPH9, both isolated from stems) and *Penicillium* sp. (SPH17, isolated from leaves). From the P population, a total of 16 “BP” isolates were obtained, and 13 were active against at least one target (81% bioactive strains). They belonged to 5 genera: *Alternaria, Aureobasidium, Aspergillus, Penicillium* and *Stemphylium*). *Aspergillus* and *Penicillium* species appeared in both populations, while *Alternaria, Aureobasidium* and *Stemphylium* were only found in the P population (Fig. 2).

The striking abundance and diversity of bioactive isolates found in the P population prompted us to undertake an additional isolating effort for this population. Using YES medium for isolations, 31 new isolates were obtained, out of which twenty (65%) were bioactive (Table 1). Overall, slightly more isolates with bioactivity were obtained on YES than on PDA medium (16 PDA, 20 YES), but with a clear media selectivity for certain taxa, such as *Alternaria* (6 PDA/2 YES) and *Aureobasidium* (3 PDA/1 YES), preferentially isolated on PDA, *Aspergillus* (3 PDA/9 YES), or *Stemphyllium* (1 PDA/2 YES), preferentially isolated on YES. *Penicillium* did not show any difference (2 PDA/2 YES), while strains from *Cladosporium, Epicoccum, Phoma*, and *Mucor* (the only isolate outside phylum Ascomycota) were only isolated on YES. Fermentation yields (Table 1) were strain dependent, but some genera, such as *Aspergillus*, showed consistently higher yields with two strains, BPT13 and SPH9 showing the highest yields.

Table 1

Bioactive fungal isolates from *B. palmensis*. Extract yield (mg extract.100 mL^{-1} medium), ITS-based identification at genus level, and bioactivity (Mean ± Standard Error) against *M. persicae* (% settling inhibition), *M. javanica* (% mortality), and pathogenic fungi (*Botrytis cinerea* / *Fusarium verticillioides*. P and T isolates were fermented in PDB and modified Czapek-Dox, respectively. Extracts were considered bioactive above a 70% threshold.

Population[a]	Isolate	Culture medium	Yield (mg in 100 mL)	ITS Identification	Antifeedant activity (%)	Nematicidal activity (% mortality)	Antifungal activity[b] (%)
					100 $\mu g.cm^{-2}$	1 $mg.mL^{-1}$	800 $\mu g.mL^{-1}$
P	BPF4.1	PDA	5.9	*Alternaria* sp.	88.2 ± 8.3	99.7 ± 0.3	79.5 ± 1.3
P	BPF5	PDA	4.5	*Alternaria* sp.	64.9 ± 9.7	100 ± 0	77.1 ± 2.4
P	BPF6	PDA	3.5	*Alternaria* sp.	70.9 ± 8.2	0 ± 0	86.3 ± 0.4
P	BPT2	PDA	2.9	*Alternaria* sp.	92.9 ± 1.9	94.9 ± 0.6	46.4 ± 7.5
P	BPT3	PDA	5.6	*Alternaria* sp.	60.8 ± 9.6	15.0 ± 3.0	70.1 ± 2.3
P	BPT4	PDA	5.0	*Alternaria* sp.	49.2 ± 9.0	100 ± 0	79.8 ± 1.7
P	BPF7	YES	4.6	*Alternaria* sp.	43.3 ± 7.8	16.8 ± 1.1	73.6 ± 2.0
P	BPH1	YES	4.3	*Alternaria* sp.	57.5 ± 9.5	8.6 ± 1.9	77.6 ± 1.2
T	SPH2	PDA	33.1	*Aspergillus* sp.	52.2 ± 8.0	25.0 ± 5.0	81.8 ± 1.6
T	SPH9	PDA	55.4	*Aspergillus* sp.	59.5 ± 7.1	100 ± 0	53.9 ± 4.5
P	BPT13	PDA	47.0	*Aspergillus* sp.	73.1 ± 8.6	100 ± 0	29.9 ± 3.0
P	BPG5	YES	7.2	*Aspergillus* sp.	80.3 ± 4.9	96.8 ± 1.1	48.9 ± 3.2
P	BPG6.2	YES	24.5	*Aspergillus* sp.	90.8 ± 3.5	100 ± 0	25.7 ± 4.2
P	BPH6	YES	25.9	*Aspergillus* sp.	78.2 ± 7.8	100 ± 0	33.3 ± 4.3
P	BPM1	YES	24.4	*Aspergillus* sp.	94.9 ± 2.2	100 ± 0	19.2 ± 15.2
P	BPM2	YES	19.9	*Aspergillus* sp.	79.0 ± 4.5	100 ± 0	23.2 ± 4.2
P	BPM3	YES	24.7	*Aspergillus* sp.	88.1 ± 7.0	91.6 ± 4.7	53.6 ± 3.6
P	BPM5	YES	22.7	*Aspergillus* sp.	96.6 ± 1.6	100 ± 0	0 ± 0
P	BPM7.1	YES	17.8	*Aspergillus* sp.	48.3 ± 12.1	100 ± 0	37.8 ± 4.5
P	BPM9.2	YES	24.1	*Aspergillus* sp.	86.0 ± 4.4	100 ± 0	23.9 ± 4.4
P	BPH3	PDA	15.2	*Aureobasidium* sp.	78.2 ± 6.3	10.1 ± 2.0	64.6 ± 3.7
P	BPH5	PDA	10.7	*Aureobasidium* sp.	73.1 ± 10.9	14.4 ± 1.9	75.3 ± 1.5

Population[a]	Isolate	Culture medium	Yield (mg in 100 mL)	ITS Identification	Antifeedant activity (%)	Nematicidal activity (% mortality)	Antifungal activity[b] (%)
					100 $\mu g.cm^{-2}$	1 $mg.mL^{-1}$	800 $\mu g.mL^{-1}$
P	BPT1	PDA	10.8	*Aureobasidium* sp.	72.5 ± 7.9	6.6 ± 3.3	45.5 ± 1.6
P	BPT15	YES	6.3	*Aureobasidium* sp.	66.5 ± 9.6	0.5 ± 0.8	74.5 ± 1.9
P	BPM13	YES	22.1	*Cladosporium* sp.	72.1 ± 9.0	1.9 ± 1.2	78.4 ± 1.0
P	BPF3	YES	6.0	*Epicoccum* sp.	95.8 ± 1.1	13.8 ± 4.5	0 ± 0
P	BPG3	YES	7.7	*Mucor* sp.	90.7 ± 5.6	8.0 ± 1.8	57.2 ± 4.7
T	SPH17	PDA	32.2	*Penicillium* sp.	78.8 ± 8.8	52.4 ± 7.3	16.6 ± 9.5
P	BPH4	PDA	2.8	*Penicillium* sp.	79.6 ± 7.9	4.2 ± 0.6	79.1 ± 1.1
P	BPG2	YES	3.5	*Penicillium* sp.	88.9 ± 6.1	10.6 ± 0.9	61.6 ± 1.7
P	BPG6.1	YES	5.1	*Penicillium* sp.	46.6 ± 8.6	18.5 ± 2.7	80.2 ± 0.5
P	BPT11	YES	2.8	*Phoma* sp.	29.8 ± 8.5	15.0 ± 2.1	77.9 ± 0.4
P	BPF8	PDA	9.7	*Stemphylium* sp.	14.8 ± 6.2	100 ± 0	38.1 ± 3.2
P	BPF9	YES	5.0	*Stemphylium* sp.	78.7 ± 9.3	17.6 ± 2.7	23.4 ± 8.5
P	BPT9	YES	2.8	*Stemphylium* sp.	74.6 ± 6.8	97.6 ± 0.6	73.3 ± 1.6

[a]P, La Palma, T, Tenerife. [b]Fungal targets: *Botrytis cinerea* spore germination for La Palma isolates, *Fusarium verticillioides* mycelial growth for Tenerife isolates.

Chemical profiling of endophytic isolates

Isolates displaying bioactivity (Table 1) were analyzed by GC/MS and clustered based on their chemical profiles (Fig. 3 and Supplementary Table S1). Additionally, activity data were categorized as active (bioactivity > 70%), moderately active (40–70%), or not active (< 40%). Bioactive extracts were grouped into five major clusters (A-E) and 2 subclusters (Ef, Eg) with 2 outgroups (Ef1 and Ef2; Eg1 and Eg2). A total of 192 different compounds were detected, with 87 remaining unidentified (< 80% similarity with the database) indicating a high number (45%) of potentially undiscovered compounds. The presence of unidentified compounds varied among clusters: A (0%), B (36.6%), C (21.4%), D (40.7%), and E (33.6%).

Cluster A: formed exclusively by *Penicillium* strain SPH17, characterized by the exclusive production of compound **15** (RT: 7.676; penicillic acid), that showed antifeedant activity.

Cluster B: Cladosporium BPM13 and *Aspergillus* SPH2 strains characteristically produced compound **25** (RT: 9.093; mellein, 66.6% and 32% abundance, respectively), with antifungal (both strains) and antifeedant (BPM13) activities.

Cluster C: composed exclusively of *Aureobasidium* strains (BPT1, BPH3 and BPH5), it is characterized by compounds **15** (RT: 7.940; massoia lactone, 27.5% average abundance) and **42** (RT: 11.622; 3,5-dihydroxydecanoic acid δ-lactone, 31.5% average abundance), with activity against *M. persicae* (all) and *B. cinerea* (BPH5).

Cluster D: In this cluster, *Alternaria* is the predominant genus (BPF6, BPH1, BPT4, BPF4.1, BPF7, BPF5, BPT2), followed by *Aspergillus* (BPG5, BPM7.2, BPM2), *Stemphylium* (BPF9, BPF8) and *Penicillium* (BPH4, BPG2). It is characterized by compounds **39** (RT: 11.434, acrylic acid tetradecanyl ester; 6.64% average abundance) and **129** (RT: 20.856; oleamide, 27.63% average abundance), and includes antifungal (all the *Alternaria* strains, except BPT2, and the *Penicillium* BPH4), nematicidal (*Alternaria strains* BPT4, BPF4.1, BPF5 and BPT2; *Aspergillus* strains BPG5, BPM7.2 and BPM2; *Stemphylium* sp. BPF8) and antifeedant (*Alternaria* strains BPF6, BPF4.1 and BPT2; *Aspergillus* strains BPG5, BPM7.2 and BPM2; *Penicillium* strains BPH4 and BPG2; Mucor sp. BPG3; *Stemphylium* BPF9).

Cluster E:

Ef: composed exclusively of *Aspergillus* isolates (Ef1: BPM5, BPM1, BPM9.2) and (Ef2: BPG6.2, BPM3, BPM7.1) is separated into Ef1 and Ef2 by differences in compound **17** (RT: 8.026 min; 2-(4-hexyl-2,5-dioxofuran-3-yl)acetic acid, 3.11% average in Ef1 vs 17.56% average in Ef2), and characterized by **161** (RT: 25.807 min; 3,6-dibenzylpiperazine-2,5-dione, 17.38% average). The strains were mostly active on *M persicae* (all of them, except BPM5) and *M. javanica* (all of them, except BPM7.1).

Eg: Composed of Eg1 (BPH6, BPT9, SPH9, BPT13, BPT11, BPT3, BPT15) and Eg2 (BPF3, BPM4), is a heterogeneous group in both chemical profile and bioactivity.

Eg1: BPH6 is an *Aspergillus* isolate and characterized by **192** (RT: 37.597 min; stearyl palmitate 28.79%). *Stemphylium* sp. BPT9, is characterized by **92 (**RT: 16.989 min; oleyl alcohol, 21.0%) and **67** (14.401 min; C15-C19 alkane, 15.0%). *Aspergillus* strains SPH9 and BPT13 have **38** (11.341 min; 9-hydroxyhexyl-itaconic acid methyl ester, 14.9 and 8.0% respectively). Additionally, BPT13 is characterized by **10** (RT 6.167 min, succinic acid, butyl ethyl ester, 20.1%). BPT3 with **67** (RT: 14.401 min; C15-C19 alkane, 6.18%), **92** (RT: 16.989 min; oleyl alcohol, 13.5%), **93** (RT: 17.073; 2,4-ditert-butyl-6-(1-phenylethyl)phenol, 8.0%). *Aureobasidium* sp. strain BPT15 is characterized by **15** (RT: 7.940; massoia lactone, 7.4%) and **42** (RT: 11.622; 3,5-dihydroxydecanoic acid δ-lactone, 5.4%). The most active strains were *Stemphylium* sp. BPT9 (on all targets), *Aspergillus* strains BPH6 and SPH9 (nematicidal and antifeedant), followed by *Aspergillus* sp. BPT13, *Phoma* sp. BPT11, *Aureobasidium* sp. BPT15 (antifungal) and *Aspergillus* sp. BPT13 (antifeedant).

Eg2: composed of *Epicoccum* strain BPF3 and *Penicillium* strain BPM4, with **125** as common differential compound (RT: 19.608; tributyl acetlycitrate, 22.15% average), with individual major compounds **163** (RT: 26.171; squalene, 32.05%) in *Epicoccum* BPF3 and **23** (RT: 9.009, 4-hydroxyphenylacetic acid, 8.19%) in *Penicillium* BPM4. These strains were antifeedant (BPF3) or antifungal (BPM4).

Based on the ITS identification and the chemical profiles obtained for the two dominant genera, *Aspergillus* and *Alternaria*, as well as other less prevalent ones, such as *Aureobasidium* and *Stemphylium*, the results suggest that not all isolates represent distinct strains. It is likely that some of them correspond to the same organism isolated multiple times during the process. For example, most *Aspergillus* isolates—except SPH2, SPH9, and BPM7.1—share the same bioactivity pattern (antifeedant and nematicidal), which could indicate repeated isolation of a single strain. In contrast, *Alternaria* isolates do not display such a consistent pattern, which may reflect the presence of different species or subspecies within the genus.

Discussion

Clear metabolic differences between two *B. palmensis* populations from La Palma (P) and Tenerife (T) islands were observed. The T extract was dominated by the presence of 11β-acetoxy-5α-(angeloyloxy)silphinen-3-one, a compound with reported antifeedant effects against *Leptinotarsa decemlineata,* and previously isolated from this population as the major component[18]. The P extract showed a different profile, characterized by the presence of the 5α-(angeloyloxy)silphinen-3-one, lacking antifeedant effects[11,19], along with jacaranone, previously described in another *Bethencourtia* species (*B. hermosae*) as an aphid antifeedant and cytotoxic[20], and the alkane tetratriacontane, a kairomone-like compound that favored parasitic/predatory activity of the biological control agents *Trichogramma chilonis* and *Chrysoperla zastrowi sillemi*[21]. The reported genetic divergence between the Tenerife and La Palma *B. palmensis* populations, explained by a low gene flow across islands due to the ocean barrier[7], could explain these chemical differences.

Higher biodiversity and bioactivity was found among endophytic fungal isolates from La Palma *B. palmensis* population. Tenerife is a more ecologically diverse island, with steeper altitudinal gradients and older, more weathered soils. La Palma Island is geologically younger, with more recent lava flows, potentially hosting different microbial pools[22]. In general, altitudinal and climatic variation leads to functional shifts in plant microbiota, with implications for nutrient cycling, stress tolerance, and defensive traits[23]. The fact that plant populations across different islands harbor distinct fungal assemblages, even when grown under similar conditions, suggests that there is a host-genotype-driven component to microbial divergence[24]. Other plant-related factors, such as the host plant metabolome and host genotype/environment interactions can also affect fungal colonization[25,26] and microbiota composition[25,27,28]. In this case, besides environmental differences, the metabolic differences observed between both populations could play a role in the composition of plant endophytic community.

This work underscores the potential importance of endophyte screenings for plant protection traits. Among others, strains BPF3, BPF8, and BPG3, from La Palma, and SPH2, SPH9 from Tenerife, showed high potential for biocontrol applications. Our group has previously reported on metabolites from fungi isolated from endemic plants of the Canary Islands with potential applications in agriculture[29], such as *Phyllostictia capitalensis* (formerly *Guignardia magniferae*) isolated from *Persea indica*, with remarkable nematicidal activity[30], and two *Aspergillus* strains producing antifungal, acaricidal[5,12] and nematicidal (Patent No. ES2920198 B2) compounds.

The identification of bioactive fungal isolates has been carried out at the genus level on the basis of ITS sequence comparisons. Fungal taxonomic identification at species-level resolution remains challenging due to the limited availability of high-quality fungal genome assemblies and the inconsistent curation of public databases. Additionally, the internal transcribed spacer, although widely used as a fungal barcode, often lacks the resolution needed to distinguish among closely related species. This limitation, coupled with potential annotation errors in repositories such as GenBank, has been shown to complicate accurate species assignment, particularly in metabarcoding[31]. As a result, identifications based solely on ITS frequently remain at the genus level in order to avoid misidentifications. Our findings show that some isolates, especially the most abundant ones (i.e *Aspergillus* with most isolates displaying a similar chemical and bioactivity profile), are likely the same species based on genetic, morphologic, and metabolic criteria. However, this hypothesis, based on distinct chemotypes and bioactivity profiles, should be confirmed through more comprehensive genetic analyses beyond ITS sequencing and morphological assessment. Deeper studies using multiple molecular markers, including protein-coding genes[32], or even whole genome sequencing[33], would be necessary to determine the true genetic divergence among the isolated strains.

Some of our isolates were assigned to genera that are traditionally considered as pathogenic. However, our findings are consistent with endophyte screenings and strains reported in other studies. Members of *Alternaria*[34,35] and *Aspergillus*[36,37] are commonly identified as prevalent culturable species and have been previously reported as major producers of secondary metabolites[38,39]. *Aureobasidium*, another highly prevalent taxon, also appears frequently as an endophyte of various plants[40,41], and some these strains have yielded interesting bioproducts[42]. Among the less abundant endophytes found here, there are notable examples in the literature of species with bioactive compounds that have potential applications in agriculture: *Epicoccum*[43], *Penicillium*[44], *Stemphylium*[45], and *Phoma*[46].

Metabolic fingerprinting represents a complementary approach to help distinguish chemotypes within similar species and to identify fungal strains producing novel bioactive metabolites[47]. Beyond taxonomic resolution, metabolome-based analyses also offer a valuable strategy to pre-select strains with distinctive chemical profiles for downstream analysis[48]. In our study, metabolic fingerprinting revealed clear chemical divergence among several fungal isolates that appeared taxonomically related. These differences suggest the presence of distinct chemotypes within the same genus, with some isolates producing compounds not detected in closely related strains, even finding some strains able to produce potentially new compounds among them. This has been documented, especially in bacteria such as *Bacillus* species showing distinct patterns of compound production across different strains[49]. In another example, the analysis of 60 bacterial strains from diverse families highlighted one particularly promising isolate that exhibited 107 unique MS features, including a cluster of 28 potentially novel compounds inferred from MS/MS similarity[48].

Among the compounds identified in the fermentation extracts (Table S1), some have been described as bioactive or having industrial interest.

Cluster A: Penicillium SPH17 strain was characterized by the exclusive production of penicillic acid (**14**) with antifungal effects. This metabolite has been reported to have antimicrobial[50] and antifungal[51] activities, and also against the plant pathogenic oomycete *Pythium aphanidermatum*[52]

Cluster B: Cladosporium BPM13 and *Aspergillus* SPH2, with antifungal and antifeedant activities, were characterized by mellein (**25**),reported as having acaricidal and antifungal activities [5].

Cluster C: composed of *Aureobasidium* strains BPT1, BPH3 and BPH5 and with activity against *M. persicae* and *B. cinerea*, was characterized by massoia lactone (**15**)and 3,5-dihydroxydecanoic acid δ-lactone (**42**). Both compounds have been previously reported in this genus, and might account for the observed antifungal activity, as the first one has been described as antifungal[53], and the second one is structurally, and likely biosynthetically, related to the first one[54].

Cluster D: dominated by *Alternaria* strains, several of them with antifungal activity, was characterized by acrylic acid tetradecanyl ester (**39**) and oleamide (**129**), none of them reported as bioactive to date. Strains BPF6 and BPH1 are separated from the other *Alternaria* isolates and were characterized by the presence of unidentified compound **88** (RT 16.328; base peak at m/z 181), a compound chemically exclusive to these two isolates within the genus, and the second most abundant metabolite in both strains, which makes it a potentially promising new compound.

Cluster E:

Ef: composed exclusively of *Aspergillus* isolates, it is characterized by 3,6-dibenzylpiperazine-2,5-dione (**161**),and separated by differences in 2-(4-hexyl-2,5-dioxofuran-3-yl)acetic acid (**17**). Compound **161** has been reported to have antiproliferative activity against cancer cells[55]. However, compounds derived from piperazines have been used as pesticides against fungi, insects or bacteria, due to their low toxicity in mammals[56]. On the other hand, compound **17** has been reported to have growth promotion effects in plants[57], antifungal effects against *Neurospora crassa*[58] and weak antibacterial activity[59]. Interestingly, these extracts were not antifungal against the targets used in this work.

Eg: Eg1: Several of the compounds found in isolates from this cluster have potential interest as biopesticides. 9-Hydroxyhexyl-itaconic acid methyl ester (**38**),present in *Stremphyllium* BPT9 and *Aspergillus* SPH9, is a nematicidal agent (Patent No. ES2920198 B2), and succinic acid, butyl ethyl ester (**10**)has beenreported as antibacterial[60]. 2,4-Ditert-butyl-6-(1-phenylethyl)phenol (**93**; reported with the code A 13-70736)had antifertility effects on the fly of *Chochliomyia hominivorax*[61]. BPT3 was chemically separated from the rest of the *Alternaria* strains. The presence of compounds **96** (not identified, RT 17.232; base peak at m/z 57), C34-C36 alkane (**121**), and β-sitosterol (**185**)suggests that BPT3may represent a different chemotype or species. The *Aureobasidium* strain BPT15 differs from other strains in cluster C, showing a lower content of massoia lactone (**15**) and 3,5-dihydroxydecanoic acid δ-lactone (**42**)**,** compoundspresent in all *Aureobasidium* strains,while producing higher amounts of a C22-C36 alkane (**145**; 3.33% vs 0.26% average area), and also produces 2,4-bis(1-phenylethyl)phenol (**146**) and 2,4-bis(dimethylbenzyl)-6-t-butylphenol (**148**). This supports the idea that BPT15 may represent a different strain/chemotype within *Aureobasidium*. Compound **148** has been reported to exhibit both insecticidal and antifungal activity[62].

Eg2:composed of *Epicoccum* strain BPF3and *Penicillium* strain BPM4, with tributyl acetlycitrate (**125**)as common differential compound, described as antifungal against *Curvularia verruculosa*[63]. The individual major compounds were squalene (**163**) in BPF3, and 4-hydroxyphenylacetic acid (**23**)in BPM4. Squalene is the

central precursor for all phytosterols and a basic intermediate in the biosynthesis of sterols, hopanoids and triterpenes as well as a key precursor in cholesterol production[64] and known to have antimicrobial activity against Gram-positive and Gram-negative bacteria, and *Candida* species[65]. Squalene also has potential as a bio-based acaricide[66]. 4-Hydroxyphenylacetic acid has been reported as nematicidal[67] and aphid antifeedant, similarly to the structurally related 2-hydroxyphenylacetic acid [68].

Conclusions

This study revealed significant chemical and microbiological differences between the two *B. palmensis* island populations studied. The La Palma population accumulated the non-active 5α-(angeloyloxy)silphinen-3-one, while the Tenerife population produced the bioactive 11β-acetoxy-5α-(angeloyloxy)silphinen-3-one. These findings support the existence of a metabolic divergence between populations, in line with previous evidence of genetic differentiation between island populations.

Culturable endophytic fungi also differed notably between populations. PDA cultures from La Palma yielded a broader diversity of mostly bioactive isolates across more genera than those from Tenerife. Moreover, additional isolations using YES medium from the La Palma population increased both the number of bioactive strains and the recovery of new genera. This highlights that employing diverse isolation conditions could be relevant to maximize the retrieval of fungi with biotechnological potential.

Chemical profiling of fermentation extracts from the isolates showed distinct metabolite patterns associated with specific phylogenetic groups, supporting the presence of different strains or chemotypes within the dominant genera. These results reinforce the value of endophyte screening for the discovery of novel bioactive compounds and suggest a promising potential for their development as biopesticides in sustainable agriculture. Future optimization of fermentation conditions for the most promising strains will be essential to improve yields for scale-up production and to identify specific molecules responsible for the observed bioactivities.

Declarations

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Author contributions

Conceptualization, AGC, JI, CED and MFA; data curation, JRLM, AGC and JI; formal analysis, JRLM, AGC and JI; funding acquisition, AGC and CED; investigation, JRLM, AGC, JI and MFA; methodology, JRLM, AGC, JI, MFA and CED; project administration, AGC and CED; resources, AGC, CED and MFA; supervision, AGC and JI;

validation, JRLM, AGC, JI, MFA and CED; visualization, JRLM and AGC; writing—original draft preparation, JRLM and AGC; writing—review and editing, JRLM, AGC, JI, CED and MFA. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Plant material statements

Plant sample collection and experimental research in this study followed all institutional, national, and international guidelines and regulations, with proper authorization obtained for sampling.

Data availability

Datasets generated in the current study are available from the corresponding author upon reasonable request. GenBank accession numbers for all strains isolated in this study are included in Supplementary Table S2.

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Figures

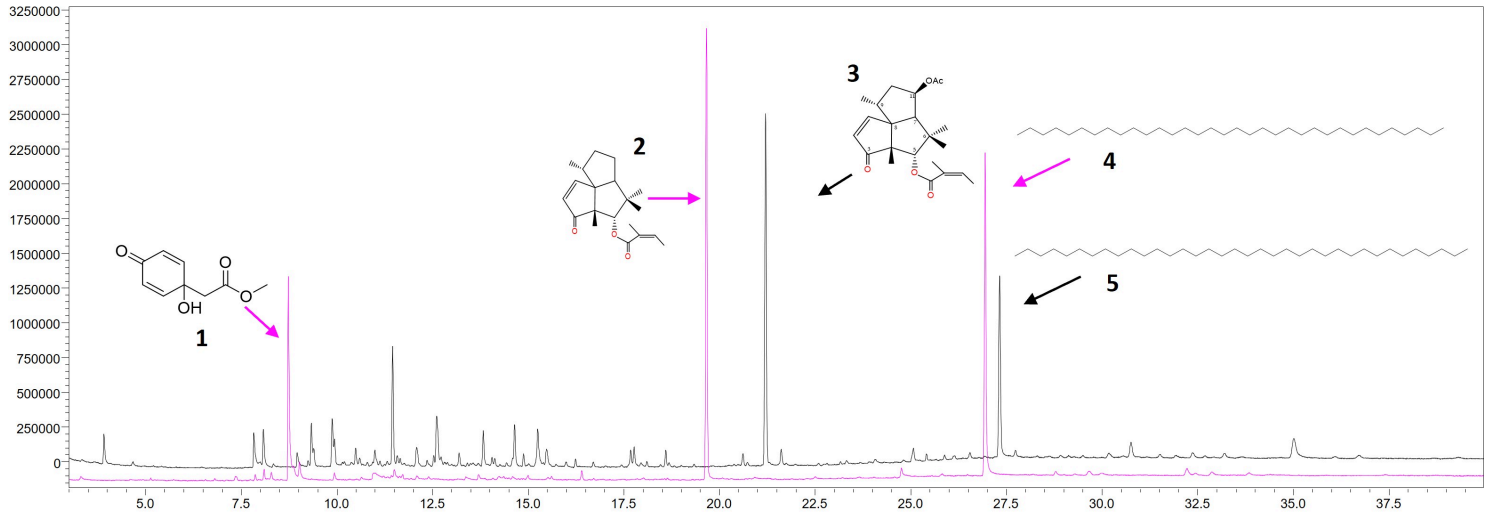


Figure 1

GC-MS chromatograms showing the chemical profiles of La Palma (**pink)** and Tenerife (black) extracts. Compounds: jacaranone (**1**), 11β-acetoxy-5α (angeloyloxy)silphinen-3-one (**2**), 5α-(angeloyloxy)silphinen-3-one (**3**), tetratriacontane (**4**), hexatriacontane (**5**).

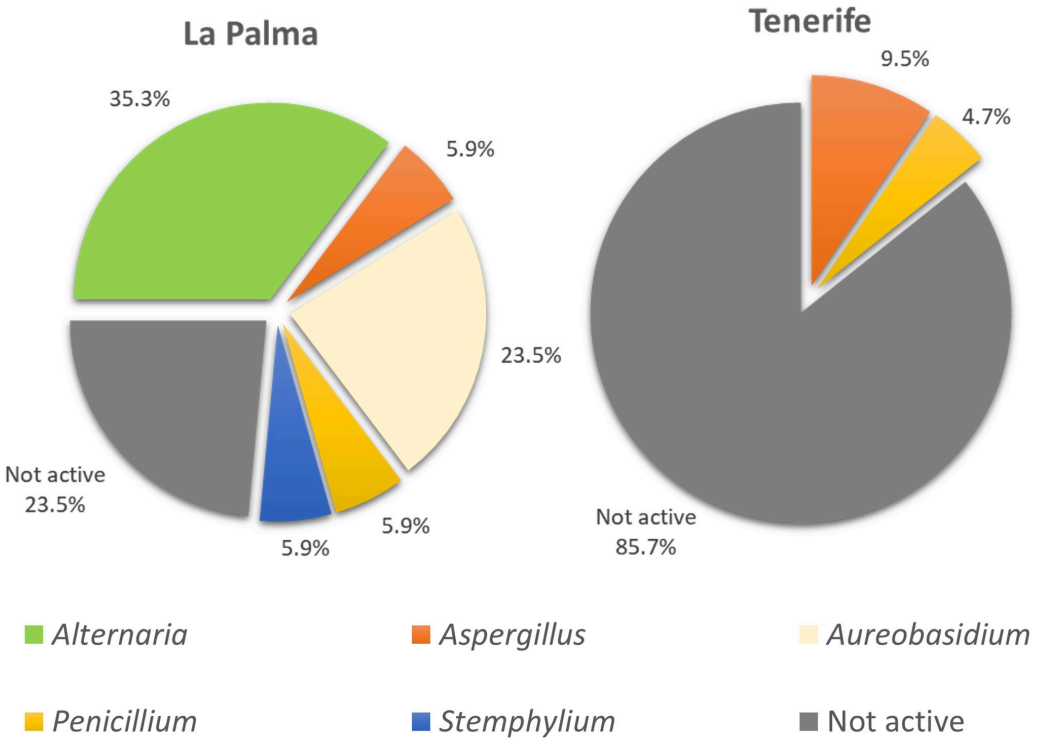


Figure 2

Comparison of PDA fungal isolates from La Palma and Tenerife *B. palmensis* populations.

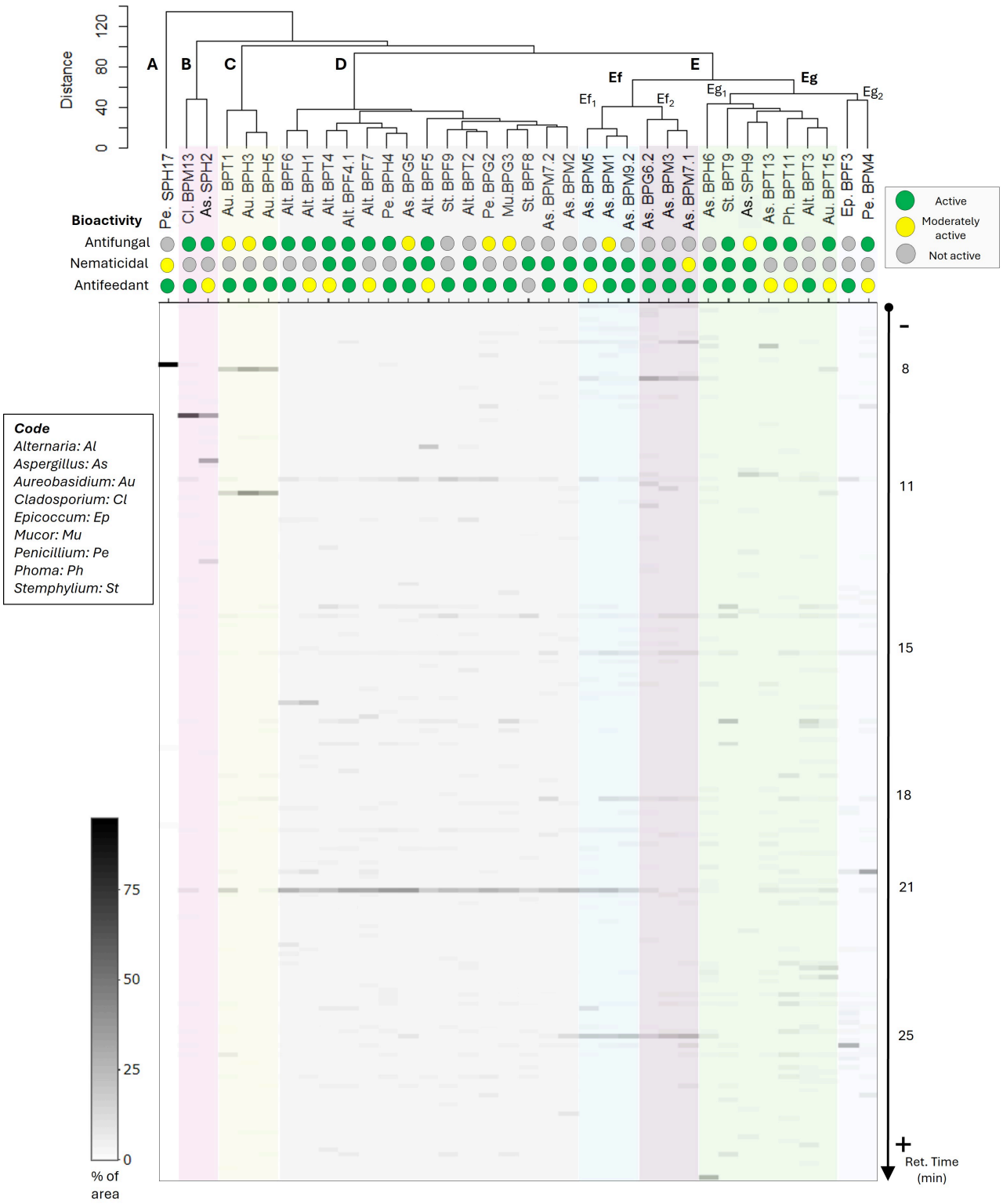


Figure 3

Hierarchical clustering of extract chemical profiles from *B. palmensis* fungal isolates determined by GC/MS. Bioactivities against different targets of each extract are represented circles colored in green (active) yellow (moderately active) and grey (not active). Relative abundances of compounds are represented by a greyscale heatmap and arranged unidimensionally as bands along a retention time scale. Heatmap displays Compounds 1-192 (numbers not shown in the y-axis) between retention times 3.406 to 37.597 min.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTableS1.xlsx
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