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Optimization of Fungicidal and Acaricidal Metabolite Production by *Aspergillus* sp. SPH2

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Abstract: The endophytic fungus *Aspergillus* sp. SPH2 was isolated from the stems of the endemic plant *Bethencourtia palmensis* and its extracts were found to have strong fungicidal effects against *Botrytis cinerea* and ixodicidal effects against *Hyalomma lusitanicum* at different fermentation times. In this study, the fungus was grown using three different culture media and two methodologies, Microparticulate Enhancement Cultivation (MPEC) and Semi-Solid Fermentation (SSF), to increase the production of secondary metabolites in submerged fermentation. The addition of an inert support to the culture medium (SSF) resulted in a significant increase in extract production. However, when talcum powder was added to different culture media, unexpected results were observed, with a decrease in the production of the biocompounds of interest. Metabolomic analyses showed that the production of aspergillic, neoaspergillic, and neohydroxyaspergillic acids peaked in the first few days of fermentation, with notable differences observed among the methodologies and culture media. Mellein production was particularly affected by the addition of an inert support to the culture medium. These results highlight the importance of surface properties and morphology of spores and mycelia during fermentation by this fungal species.

Keywords: endophyte; *Aspergillus*; antifungal; ixodicidal; fermentation; mellein

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1. Introduction

Current agriculture is facing the global challenge of being productive, efficient, sustainable, and environmentally friendly [1]. Food production is affected by plant diseases and insect pests, causing millions of losses and jeopardizing food security [2]. To date, plant protection has depended on synthetic pesticides, resulting in a number of direct negative effects on farmer and consumer health, soil erosion, water quality, and a number of associated problems, such as the emergence of pest resistance. In this context, there has been an increase in research, development, and application of biopesticides because of their efficacy, harmlessness to the population and environment, and target-specific properties [3,4]. Additionally, there is an increase in the demand for food safety, product quality, and stricter pesticide regulations, making the search for new biopesticides a priority [4].

Microorganisms, including endophytes, which live within the internal tissues of plants and their secondary metabolites, are a source of biopesticides [5]. Endophytes from plant species of the genus *Bethencourtia* have been reported to exhibit insect antifeedant activities [6]. *Bethencourtia*, endemic to the Canary Islands, comprises three species: *B. hermosae* (Pit), *B. palmensis* (Nees) Choisy, and *B. rupicola* (B. Nord) B. Nord. [7]. *B. palmensis* contains silphinene sesquiterpenes with potent insect anti-feedant effects [6]. Based on these results, this plant was selected for the isolation of endophytic fungi

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to identify secondary metabolites with biopesticidal properties. In this context, the endophyte *Aspergillus sp.* SPH2 was isolated from *B. palmensis* [8]. The production of secondary compounds with biopesticidal potential is grounded in the recognition of endophytic fungi as prolific producers of compounds effective against pathogens and herbivores, also some studies have report production of compounds with antiviral, antifungal, antibacterial and insect action [9], these compounds comprises a wide range of chemical classes, including alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, chlorinated compounds, and volatile organic compounds (VOCs) [10]. Notably, the endophytic fungal isolate *Aspergillus sp*. SPH2 has recently been shown to produce mellein and neoaspergillic acid during different stages of fermentation [8]. Mellein is a subgroup of 3,4-dihydroisocoumarins; usually, secondary metabolites belonging to the polyketide group; with antimicrobial and phytotoxic activities [11] and efficacy against the disease vector *Hyalomma lusitanicum* ticks [8]. Neoaspergillic acid is a siderophore [12] with reported fungicidal activity against *Botrytis cinerea* [8]. Therefore, optimization of the fermentation conditions for the production of these metabolites could increase the potential for the development of bio-based pesticides.

Considering that the morphology of the fungus in submerged media affects its productivity, semi-solid-state fermentation, in which the fungus grows in a biofilm, allows the production of secondary metabolites in high quantities [13,14]. One optimization approach includes techniques that interfere with the formation of fungal conglomerates, causing the pellets to be smaller, less dense, or even the mycelium to be dispersed in the culture medium [15], leading to more efficient substrate consumption, much greater oxygen transfer, and increased productivity of the fungus. The most widely used technique is microparticle-enhanced cultivation (MPEC) using talcum powder or aluminum oxide [16]. The media may be rich in nutrients and optimally aerated, but the cells inside the sphere are stressed by limitations in the exchange of oxygen and nutrients [17] because effective growth occurs exclusively on the surface of the mycelium aggregate.

In this work, the application of talcum powder as an MPEC method as well as the addition of a metallic mesh to the culture medium as an SSF method have been evaluated to observe changes in the production of mellein, aspergillic, neohydroxyaspergillic, and neoaspergillic acids by the endophyte *Aspergillus sp.* SPH2.

2. Materials and Methods

2.1. Plant Material

Bethencourtia palmensis were collected from Barranco del Rio, Abona (Tenerife, Spain) (28°34′10″ N, 16°18′48″ W). Within 48 h of collection, samples were placed in sterile polybags and transported in a box container under refrigeration until isolation.

2.2. Cultivation of SPH2 for Extract Preparation

SPH2 was cultivated on PDA solid medium for eight days at 25 °C. Sterile water (10 mL) was added to each Petri dish to obtain a spore suspension for subsequent counting in a modified Neubauer chamber and then, 216 Erlenmeyer Flasks (100 mL) were prepared (72 primary each with their respective 2 replicates), in which 72 flasks were prepared with 50 mL of Czapek-Dox liquid media ([CZD: $NaNO_3$ (2 g/L), KH_2PO_4 (5 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.01 g/L), $ZnSO_4$ (0. Three g/L), and glucose (30 g/L)], 72 with 50 mL of modified Czapek-Dox-Yeast liquid medium ([CZDM: $NaNO_3$ (2 g/L), KH_2PO_4 (5 g/L), $MgSO_4$ (0. 5 g/L), $FeSO_4$ (0.01 g/L), $ZnSO_4$ (0.003 g/L), yeast extract (1 g/L) and glucose (60 g/L)] and 72 others with 50 mL with Potato Dextrose Broth (Sigma-Aldrich). Inoculation with 1×10^6 spores/mL was then performed. Three flasks were sampled on days 1, 2, 5, 9, 13, 16, 19, and 21 of the incubation. The culture medium was separated from the mycelium, dried in an oven at 40°C for 24 h, and weighed.

2.3. Modification of the Fermentation Conditions

Talcum powder (10 g/L) with a particle size of 350 MESH (Fisher Chemical) was added to 72 of the 216 Erlenmeyer Flasks prepared (24 for each medium) according to the specifications of the

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optimal value evaluated and presented by Antecka et. al. [18]. Furthermore, a stainless steel (INOXIA) metal mesh 304 L with a pore size of 40 MESH and a surface of 0.044 m^2 was added to the bottom of 72 Erlenmeyer's following a design similar to that described by Francis et. al. [14].

2.4. Extract Preparation

The culture media was filtered through a paper filter using a Buchner funnel to separate the mycelium, submitted to exhaustive liquid/liquid extraction with ethyl acetate (3x EtOAc), dried over SO_4Na_2, and concentrated under reduced pressure to obtain crude SPH2 extracts.

2.5. Compound Identification and Quantification

Qualitative and quantitative determination of the ethyl acetate extract was performed by gas chromatography-mass spectrometry (GCMS) using a Shimadzu GC-2010 gas chromatograph coupled to a Shimadzu GCMS-QP2010 Ultra mass detector (electron ionization, 70 eV). Sample injections (1 µL) were carried out using an AOC-20i instrument equipped with a 30 m × 0.25 mm i.e., capillary column (0.25 µm film thickness) Teknokroma TRB-5 (95%) Dimethyl- (5%) diphenylpolisiloxane. The working conditions were as follows: split ratio (20:1); injector temperature, 300 °C; temperature of the transfer line connected to the mass spectrometer, 250 °C; initial column temperature, 70 °C; and heating to 290 °C at 6 °C/min. Electron ionization mass spectra and retention data were used to assess the identity of the compounds by comparing them with those found in the Wiley 229 and NIST (version 17) mass spectral databases. All the extracts (4 µg/µL) were dissolved in 100% DCM for injection.

2.6. Bioassays

2.6.1. Antifungal activity

Botrytis cinerea was obtained from the fungal collection at the Instituto de Productos Naturales and Agrobiologia-CSIC (Santa Cruz de Tenerife, Spain). The mycelial growth inhibition test was performed in 12-well Falcon plates using a modified agar-dilution method with 0.05 mg/mL methyltetrazolium salts (MTT). Extracts and pure compounds dissolved in ethanol (EtOH) were tested at various concentrations (extracts at 1, 0.5, 0.25, and 0.1 mg/mL; compounds at 0.5, 0.1, 0.25, and 0.05 mg/mL) before being incorporated into the culture medium and poured into the plates. For each concentration tested, a series of test solutions was prepared in potato dextrose agar (PDA) and MTT, and 300 µL was added to each well. EtOH was used as a negative control and all treatments were replicated four times. After 48 h of incubation in the dark at 27 °C, fungal colonies were digitalized and measured using ImageJ (http://imagej.nih.gov/ij/). Percent inhibition (%I) was calculated as %I = (C T/C) 100, where C represents the diameter of the control colonies and T represents the diameter of the test colonies [19]. Data were analyzed using STATGRAPHICS statistical analysis software (Centurion XVIII), and EC50 values (effective dose to achieve 50% inhibition) were calculated using a regression curve of mycelial growth inhibition versus log dose.

2.6.2. Ixodicidal activity

Female *Hyalomma lusitanicum* ticks were collected from their hosts (deer) in central Spain (Finca La Garganta, Ciudad Real) and kept at 22-24 °C and 70% relative humidity until oviposition and egg hatching. The resulting larvae (4-6 weeks old) were used in bioassays [8]. Briefly, 50 L of the test solution was added to 25 mg of powdered cellulose at different concentrations, before the solvent was evaporated. Three replicates of 20 larvae were used for each test. Under the conditions described above, dead ticks were counted using a binocular magnifying glass 24 h after contact with treated cellulose. Larvicidal activity data are presented as percent mortality corrected using the Orelli-Schneider formula. Probit Analysis was used to calculate effective lethal doses (LC50) (5 serial dilutions, STATGRAPHICS Centurion XVI, version 16.1.02).

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3. Results

3.1. Appearance and Coloring of SPH2 mycelium

The modifications carried out in the different fermentations resulted in changes in the coloration and aggregation patterns of the fungal pellets as well as their size and general transformation over time (Table 1).

Table 1. Changes in SPH2 pellet formation and coloring during fermentation.

Culture Media	Modification	Characteristics
PDB	None	Homogeneous pellets, reddish color
	Talcum Powder	Pellet agglomeration, red-brown color
	Metallic Mesh	Mycelium on mesh, light brown color
CZD	None	Homogeneous pellets, no change in color
	Talcum Powder	Pellet agglomeration, light red color
	Metallic Mesh	Mycelium clumps on mesh, reddish color
CZDM	None	Non-homogeneous pellet agglomeration, yellowish red
	Talcum Powder	variable-sized pellets, light red color
	Metallic Mesh	pellet clustering on mesh, strong orange color

3.2. pH

The time course of fermentation of the SPH2 fungus showed a minimum pH of 4 and 3.52, on days 5 and 16 in the CZDM culture medium, respectively. With the addition of an inert support (metallic mesh), the maximum pH of 8.41. was observed on day 21 of fermentation in PDB and talcum powder was added (Figure 1).

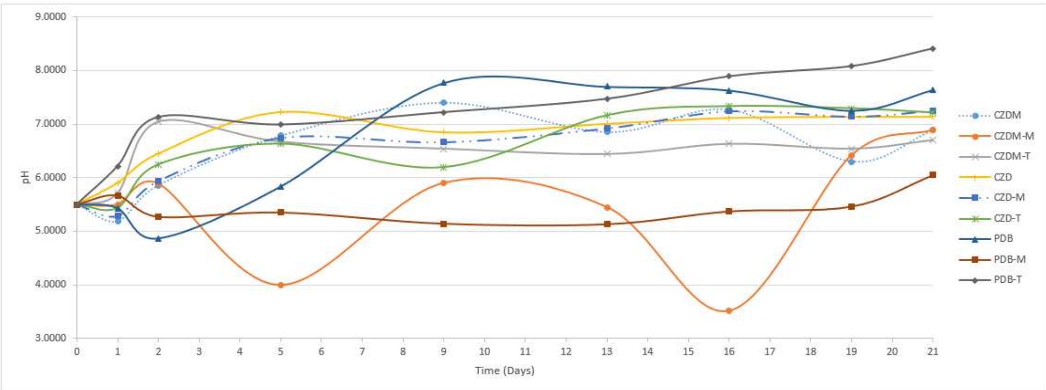

Figure 1. Time course of SPH2 pH.

3.3. Mycelium yield

The lowest mycelium dry weight values were found for PDB-M, whereas CZDM, CZDM-T, and CZDM-M showed significant increases (2.1234 g, 1.7529 g, and 1.6968 g, respectively). The general trend observed for the dry weight yield of the remaining fermentations was similar over time, with PDB-T yielding the lowest yield (0.6195 g) and CZD the highest (1.4199 g) (Figure 2).

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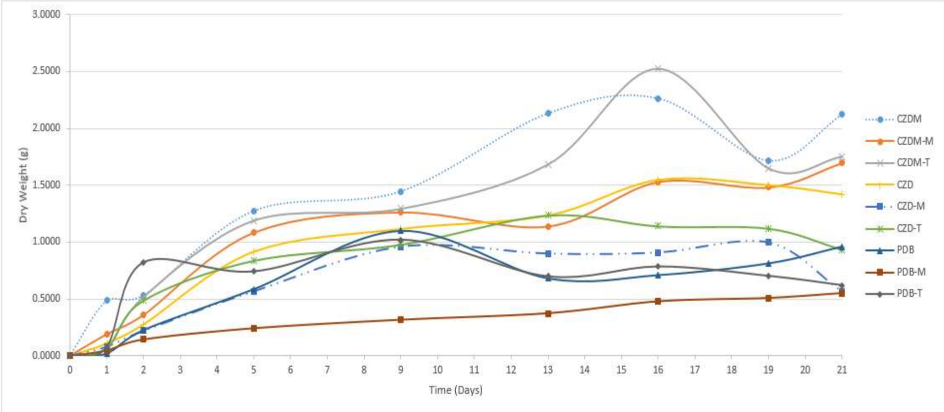

Figure 2. Time course of SPH2 mycelial yield.

3.4. Extract yield

The CZD-M medium showed superior performance for all samples in the first 9 days of measurement, while CZDM-M gave the highest yield value on day 16 (1.1280 g/L); however, on day 21 of fermentation, the yield dropped to 0.4120 g/L, while CZDM and CZDM-M yielded 0.6360 g/L and 0.6000 g/L, respectively. The performance of PDB medium in stationary fermentation was prolonged over time, although the yield was not the highest.

The rest of the fermentations had similar performance, not exceeding 0.2040 g/L (maximum value achieved at 21 days for PDB-M) throughout the incubation period (Figure 3).

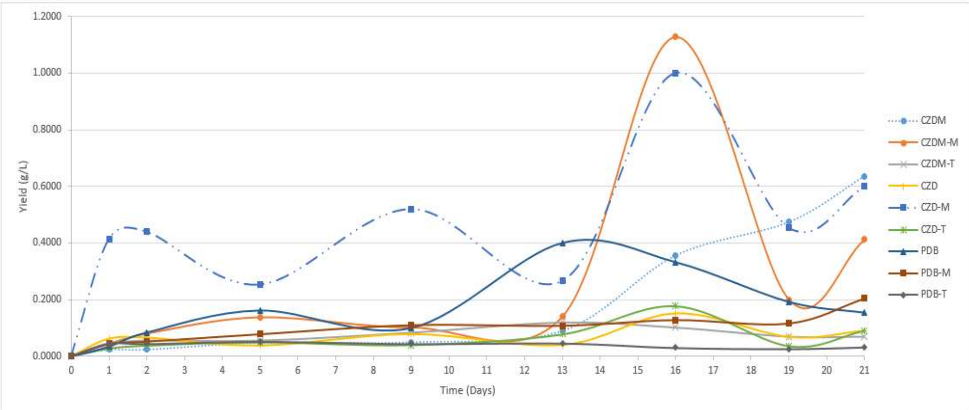

Figure 3. Time course of SPH2 extract yield.

3.5. Secondary Metabolite Analysis

Fungi are the most important source of melleins, with (*R*)-(-)-mellein (**1**) being the most common among these groups [11]. Mellein showed ixodicidal activity, and aspergillic (**2**), neoaspergillic (**3**), and neohydroxyaspergillic (**4**) acids showed antifungal activity [8]. Therefore, these compounds were selected to monitor the fermentation optimization process. Figure 4 shows the chemical structures of these compounds.

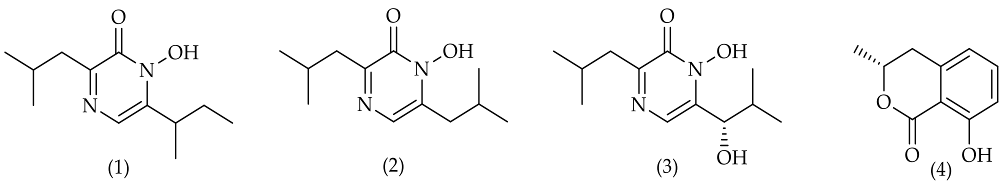

Figure 4. Compounds of interest: (**1**) Aspergillic acid, (**2**) neoaspergillic acid, (**3**) neohydroxyaspergillic acid, and (**4**) mellein.

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The selected secondary metabolites were analyzed in various culture media using GC-MS at specific time points during the fungal growth curve. The chosen time points included the onset of the exponential phase (day 5), the transition from the exponential phase to the stationary phase (day 9), the transition from the stationary phase to the senescent phase (day 16), and the fermentation endpoint (day 21). The production of aspergillic acid (1) showed two peaks on days 5 and 9 in the CZD and PDB (Figure 5). Incubation with CZD increased the production by 1 to 30% on day 5 compared to PDB in the absence of metallic mesh. It's also worth noting that 1 was absent in the CZDM medium. Furthermore, consistent production of this compound appeared to be concentrated within the initial 9-day period.

Figure 5. Quantification of aspergillic acid (1) over time (days 5, 9, 16, and 21) for different culture media (W=without any modification, T=with talcum powder, and M=with metal mesh).

Figure 6 shows the production of neoaspergillic acid (2), with a maximum value on day 5 instead of day 8, as previously reported [9]. The addition of talcum to the PDB culture medium reduced production by 2-to 10 times. Similarly, the addition of talcum powder to CZD medium resulted in poor yields. In the PDB medium, the synthesis of 2 increased by 20% in the presence of metallic mesh on day 16, compared to a previous study [9], and also increased on day 5 in CZD and CZDM media.

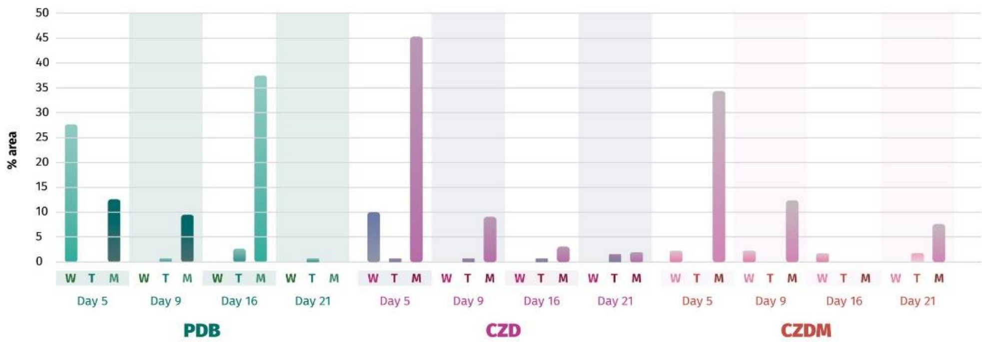

Figure 6. Quantification of neoaspergillic acid (2) over time (days 5, 9, 16, 21) in different culture media (W=without any modification, T=with talcum powder, and M=with metal mesh).

Figure 7 shows the production of neohydroxyaspergillic (3), which was notably enhanced in the PDB culture medium. Without any modifications to the medium, a production yield of approximately 5-8% was achieved between days 9 and 21. To match this yield, the CZD culture medium required the incorporation of a metallic mesh, which was observed on day 21 of fermentation. In the CZDM culture medium, the addition of a metallic mesh triggered the production of 3 in trace amounts.

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Figure 7. GC-MS quantification of neohydroxyaspergillic acid (3) with time (days 5, 9, 16, 21) for different culture media (W=without any modification, T=with talcum powder and M=with metal mesh).

The production of mellein (4) (Figure 8) ranged between 36-2% between days 9-21; while a range of 4-18% between days 7-13 was found previously [9], indicating that the production of this targeted compound starts between days 5 and 9 in fermentation with PDB. Observations from Figure 8 indicated that the production of compound 4 was altered by the introduction of inert supports. The effects of talcum and metallic mesh are different. A yield of approximately 35% was recorded between days 9 and 16 in the PDB culture medium without any support. In contrast, when metallic mesh was used, similar yields were observed on day 9 with CZD and on day 21 with CZDM (60% yield).

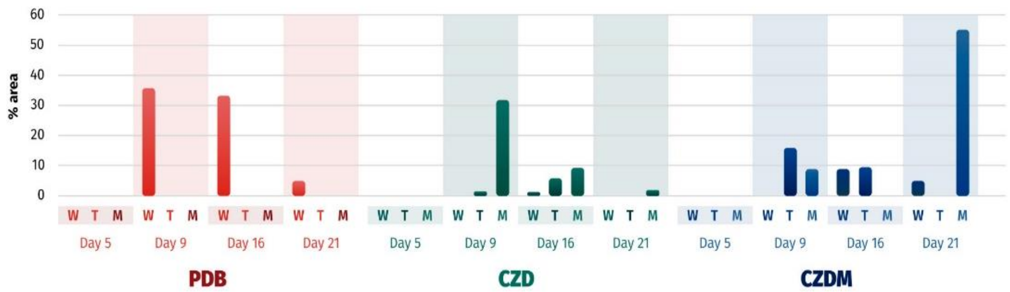

Figure 8. GC-MS quantification of mellein (4) with time (days 5, 9, 16, and 21) for different culture media (W=without any modification, T=with talcum powder, and M=with metal mesh).

3.5.1. Cluster Analysis for Secondary Metabolites. Extract selection

The extracts were grouped into discrete categories based on their relative percentage areas as determined by GC-MS for compounds **1**, **2**, **3**, and **4**. The objective of this classification was to identify homogeneous clusters for future bioassay-based studies.

Figure 9 shows a dendrogram constructed based on the proportions of antifungals **1**, **2**, and **3**. The clustering algorithm considers the relative percentage area of each compound, thereby defining the selection parameters for their prospective application as antifungal agents. Following this clustering, three representative branches from each cluster were chosen for comprehensive analysis.

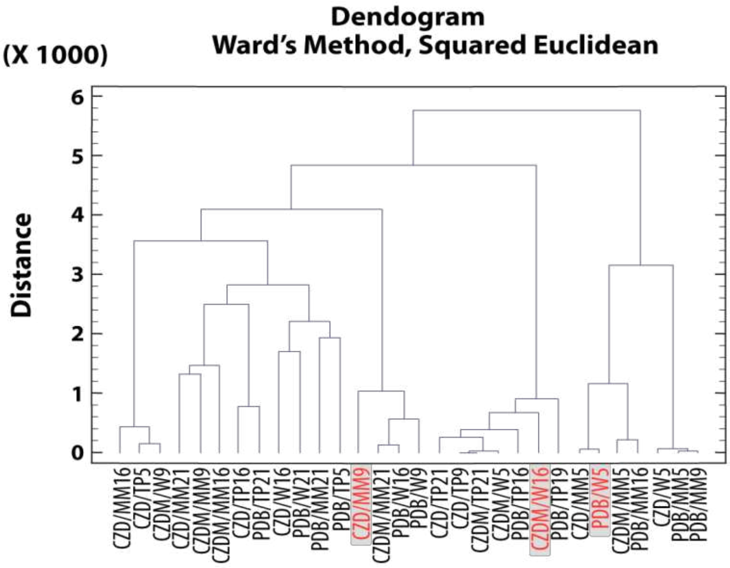

Figure 9. Dendrograms of compounds 1, 2, and 3.

Based on this analysis, it was possible to identify compounds that exhibited higher yields in production, considering the sum of compounds **1**, **2**, and **3** in relation to the extract production. This determination was made according to Eq. (1), as outlined below:

$$\left(\sum_{Compound\ 1}^{Compound\ 3} \%\ Area\right) * Extract\ production\ of\ each\ fermentation \quad (1)$$

Considering these results, the possible antifungal candidates are PDB/W5, CZDM/W16, and CZD/MM9, which are highlighted in red in Figure 9. Subsequently, the same clustering process was performed for compound 4 (Figure 10).

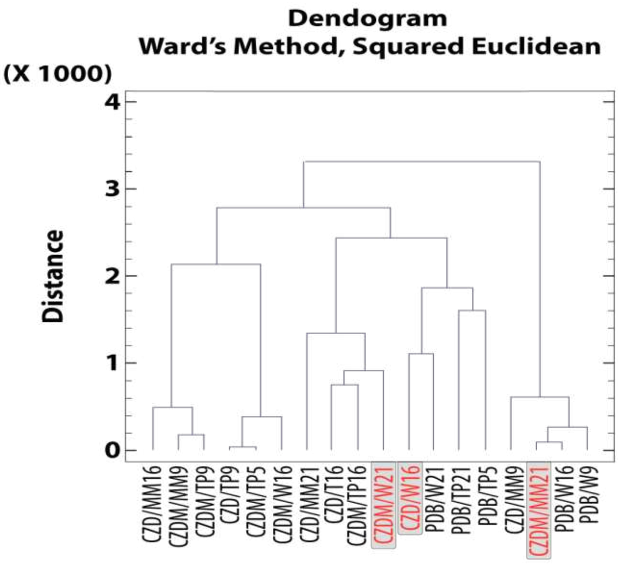

Figure 10. Dendrogram based on compound 4.

Based on the analysis conducted using the formula outlined below (2), the extracts to be evaluated were correctly established.

$$\left(\sum \%\ area\ compound\ 4\right) * Extract\ production\ of\ each\ fermentation \quad (2)$$

From this indicator, as depicted in the dendrogram in Figure 10, extracts delineated in red, such as CZDM/MM21, CZDM/W21, and CZD/W21, emerged as prospective candidates exhibiting ixodicide activity attributable to compound **4**.

3.6. Bioassays

Compounds **1**, **2**, and **3** were tested against *Botrytis cinerea* Pers. (1794), whereas the **4**-based cluster were evaluated against *Hyalomma lusitanicum* Koch. (1844).

3.6.1. Spore germination inhibition bioassay

Figure 11 shows the percentage inhibition of spore germination at different doses of the selected extracts. The CZD/MM9 extract was the most active (inhibition values of 80.98, 81.17, 77.46 and 69.71 at 800, 400, 200 and 100 µg/mL), followed by PDB/W5 (inhibition values of 69.81, 72.56, and 64.48 at 800, 400, and 200 µg/mL) with EC_{50} values of 0.0027 and 0.0150 µg/mL respectively (Table 2).

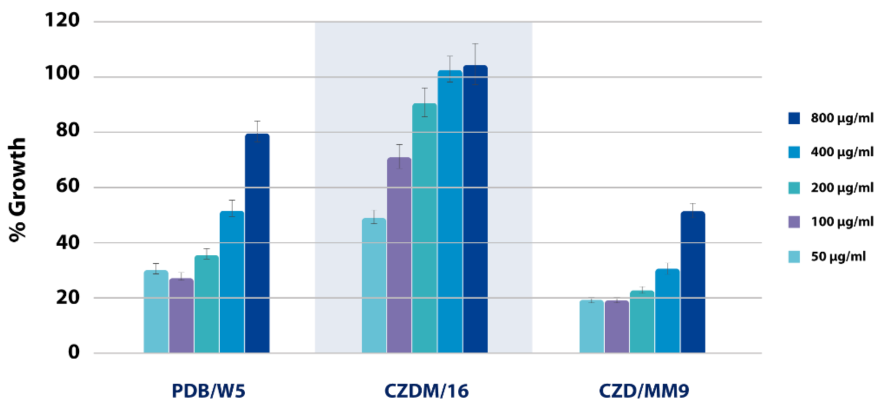

Figure 11. Growth rates in *the Botrytis cinerea* germination inhibition bioassay.

Table 2. Efficient doses of the antifungal SPH2 extract against *Botrytis cinerea* spore germination.

Extract	EC_{50} (µg/mg)	95% Confidence Limits
CZD/MM9	27.17	14.84 – 49.86
PDB/W5	149.75	116.49 – 192.38
CZDM/W16	>40	>40
SPH2 [8]	22.00	19.00 – 26.00

3.6.2. Ixodicidal bioassay

Table 3 shows the ixodicidal effects of **4**-based selected extracts (CZDM/MM21, CZDM/W21, CZD/W16). The most active extracts were CZDM/MM21, followed by CZDM/W21, with calculated lethal doses (LD_{50}) of 5 and 28 µg/mg, respectively.

Table 3. *Hyalomma lusitanicum* larval mortality of selected SPH2 extracts.

Extract	Mortality Rate (%)	LD_{50} (µg/mg)	95% Confidence Limits
CZDM/MM21	100.00	5.00	4.4 - 5.66
CZDM/W21	100.00	28.08	25.16 - 31.26
CZD/W16	12.10	>40	>40
***Aspergillus spp.* SPH2** [8]	100.00	7.18	6.67 – 7.78
Mellein (4) [8]	100.00	0.48	0.44 – 0.51

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4. Discussion

In the present study, different fermentation parameters were tested for *Aspergillus* sp. SPH2 (isolated from *B. palmensis*) has been shown to produce bioactive metabolites [8]. The fungal media PDB, CZD, and CZDM were modified using two physical methods (microparticle enhancement culture with talcum powder, MPEC, and the addition of a metallic mesh to the culture medium, SSF), resulting in changes in both pH and dry mycelium weight.

The results with talcum powder (MPEC) showed the formation of pellets of regular and homogeneous size in the first growth stage and a more pronounced agglomeration in the second stage (around day 13 of fermentation), which could be an indication of changes in production, as studies suggest that at a smaller particle size, the yield increases considerably [20]. The generation of different oxygen and mass transfer profiles that occur with the addition of talcum powder generally leads to the production of higher amounts of secondary metabolites [21]. In this study, secondary metabolites were produced, but not in the expected amounts. Therefore, the microparticle enhancement culture (MPEC) should guarantee a pellet size that is sufficiently small to avoid agglomeration over time to ensure the correct transfer of oxygen and mass in the surface layer of the fungus.

The addition of metallic mesh (SSF) resulted in the formation of pellet agglomerations during both stages of growth. In the first stage, irregularly sized and shaped pellets aggregate around the metallic mesh. In the second stage, all pellets agglomerated, resulting in the formation of round, hairy pellets. These observations are consistent with those of previous studies of *A. niger* using the MPEC method [12]. However, during the second stage, we observed changes in the viscosity of the medium between days 13 and 21, which coincided with a peak in the production of the target compounds in CZD and CZDM media modified with metallic mesh. These results suggest that the viscosity of the culture medium is the primary factor influencing the production of the target compounds is the viscosity of the culture medium [22].

Significant improvements in the extract production were observed when various culture media and inert supports were used. Specifically, CZD and CZDM culture media demonstrated superior performance, yielding higher extract production than that reported in previous studies [8]. Fermentation in PDB and Czapek-Dox culture media without any modification favored the production of 1 and 3, respectively. The addition of talcum powder to different culture media yielded unexpected results regarding the production of the biocompounds of interest, possibly due to a consequence associated with oxygen and mass transfer limitations. The addition of an inert support (Metallic Mesh) led to the production of 2 and 4 in Czapek-Dox and the Modified Czapek-Dox, respectively.

The antifungal extract CZD/MM9 showed results comparable to those of the reported SPH2 extract [8]. However, the performance of the PDB/W5 extract was within the range previously reported. The extract from day 21 with the modified Czapek Dox and the addition of the metallic mesh (inert support) was promising against the tick, with an increase in performance of approximately 12% compared to the described SPH2 extracts [8]. This means that owing to the stimulation by fungal nucleation and subsequent generation of a biofilm around the metallic mesh, there was an increase in the production of 4.

5. Conclusions

In conclusion, this study highlights the potential of improving the cultivation conditions of *Aspergillus* sp. SPH2, with implications extending beyond this specific fungus to other endophytic species. The methodological approach introduced in this study provides a valuable framework for advancing the techniques under investigation. Notably, modifications to the culture conditions for *Aspergillus* sp. SPH2 exerts profound effects on the production and biosynthesis of secondary metabolites, resulting in improved biocidal properties against *Botrytis cinerea* and *Hyalomma lusitanicum*. These findings underscore the versatility and promise of fungal biotechnology for both biocompound production and potential biocontrol applications, offering sustainable solutions in agricultural and broader ecological contexts.

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