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Advanced fermentation techniques enhance dioxolanone type biopesticide production from *Phyllosticta capitalensis*

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In the current work, the production of dioxolanone and meroterpene type secondary metabolites under different fermentation conditions by the endophytic fungus *Phyllosticta capitalensis* (isolate YCC4) isolated from the leaves of the endemic plant *Persea indica* has been evaluated. Different techniques, such as microparticle-enhanced culture (MPEC: bentonite, talcum powder) and surface adhesion fermentation (SAF: metallic mesh and glass wool) were applied to the culture medium. Ethyl acetate extracts from different fermentations of the isolate YCC4 were analyzed for their content and revealed the following dioxolanone derivatives: metguignardic acid (1), guignardianone C (2), ethyl guignardate (3), guignardianone D (4) and phenguignardic acid methyl ester (5), and meroterpenes: guignardone A (6) and B (7), guignarenone C (8), guignarenone B (9) and guignardone I (10). Additionally, the biological activity of extracts was tested against the aphid *Myzus persicae* and the root-knot nematode *Meloidogyne javanica*. Our study revealed significant variations in the production of the target metabolites as well as notable differences in biopesticide activity influenced by the presence of inert supports. Overall, the findings indicate that glass wool (GW) is a high-performance material to improve the production of dioxolanone derivatives. These findings underscore the significance of innovative cultivation methods such MPEC and SAF as drivers to produce valuable secondary metabolites from fungal organisms.

Keywords Fungal endophyte, Biopesticides, Fermentation, Microparticle-enhanced culture (MPEC), Surface adhesion fermentation (SAF), Dioxolanones

Endophytic fungi, strongly associated with plants, have emerged as a keystone in the field of plant protection. Residing within plant tissues without causing any detectable harm. Some of these fungi play a crucial role in support plant resilience against biotic and abiotic stresses. For example, research on *Hordeum bogdanii* has demonstrated that endophytic fungi can modulate secondary metabolites under alkaline stress, thereby enhancing the resilience of the host plant[1]. Beyond their protective role, endophytic fungi are known for their high production of secondary metabolites[2]. Some of these compounds are also those synthesized by their host plants and show a great potential for protecting plants against pathogens, pests and environmental challenges[3–5].

Endophytic fungi have emerged as potential solutions to crop protection problems, with certain strains demonstrating the capability to suppress widespread and harmful plant pathogens[6]. For instance, certain fungal endophytes associated with the *Rubiaceae* family have the ability to produce potent antimicrobial compounds, presenting a novel strategy to combat plant diseases[7]. Furthermore, the biopesticide efficacy of various endophytic fungi has been reported against insect pests, such as *Duponchelia fovealis* (Zeller)[8] or *Myzus persicae*[9] phytoparasitic nematodes[9] and economically significant fungal plant pathogens like *Sclerotinia sclerotiorum*[10], *Botrytis mediterranea* and *Diplodia corticola*[11].

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Phyllosticta (Ascomycetes) species can colonize a variety of plant hosts, and are potential fungal or nematicidal biocontrol agents[10,12]. *Phyllosticta capitalensis*, widely distributed worldwide[13], has been also isolated as an endophyte and can produce of a wide variety of secondary metabolites[14] including meroterpene and dioxolanones derivatives[15] with antifungal activity against *Rhizoctonia solani*, *Fusarium graminearum* and *Botrytis cinerea*[16]. A recent study of *P. capitalensis* (YCC4) isolated from the Macaronesian paleoendemism *Persea indica*, showed the ability to produce insect antifeedant and nematicidal compounds[9]. The chemical study of the isolate YCC4 resulted in the identification of bioactive dioxolanones including guignardic acid (aphid antifeedant), guignardianone C (aphid antifeedant) and metguignardic acid (aphid antifeedant, nematicidal), guignardianone D (nematicidal) and phenguignardic acid methyl ester (nematicidal), along with ethyl guignardate, guignardianones D and E, and the meroterpenes guignardone A and B and guignarenone C, (-)-guignardone I, (-)-epi-guignardone I and phyllomeroterpenoid B[9]. Since the insect antifeedant and nematicidal activity of the *P. capitalensis* extract depended on the presence of dioxolanone components, fermentation optimizations are needed to promote the biosynthesis of these compounds instead of the meroterpenes.

Studies on microbial production have focused on microparticle enhanced cultivation (MPEC) as a useful technique to improve microbial secondary metabolite production in vitro[17,18], in particular of fungal metabolites[19]. This technique leverages micro- and macro-particles to influence microbial morphology and physiology by changing the pattern of metabolite production. This effect is affected in different extents depending on fungal morphology and growth (pellet formation or mycelia dispersion). For instance, the addition of microparticles affects the formation of pellets and some features such as roughness, size or even pellet aspect. This changes on the pelletization produce variations in the metabolism due to the change of the nutrient consumption rates or oxygenation among other factors[20,21]. For example, the addition of talc powder to the medium, reduced the size of the pellets of *Aspergillus terreus* and improved the production of lovastatin[22], a drug used for its cholesterol-lowering ability[1]. It has also been observed that in filamentous fungi[23] such as *Aspergillus niger*, the addition of silicates[24] as well as the addition of silicon titanium oxide[25] promoted enzyme formation.

Fungal morphology in submerged medium has a significant impact on metabolite production. Fungi are more frequently adapted to grow on surfaces than in liquid cultures, and on surfaces they are able to reach higher fermentation yields[26]. Surface adhesion fermentation is a hybrid technique between liquid and solid culture, promoting formation of filamentous fungal biofilms, in which the fungus grows attached to a support. This technique enhances the production of secondary metabolites in larger quantities[27–29]. Normally, the optimization approach involves techniques that disrupt the formation of fungal conglomerates, resulting in the production of smaller pellets, reduced density, or even dispersion of the mycelium within the culture medium[20]. This, in turn, enhances substrate utilization efficiency, facilitates improved oxygen transfer, and ultimately leads to increased fungal productivity[30]. For instance, *Trichoderma* spp. increased their biomass and hydrophobin production when cultured in some solid supports in the medium such as glass, rubber or Teflon[31]. The roughness of the materials can affect the attachment by the fungi and subsequent metabolite production; similarly, carbon source can modulate biofilm formation[31,32].

In this study, the production of dioxolanones and meroterpenes by the endophyte *Phyllosticta capitalensis* (isolate YCC4) has been studied after the application of talcum powder and bentonite as MPEC methods, as well as the addition of a metallic mesh and glass wool to the culture medium as a SAF method. The extracts produced were analyzed for metabolite content using high-performance liquid chromatography coupled with mass spectrometry (HPLC–MS) has been used for the identification and quantification of the previously isolated meroterpene and dioxolanone-type compounds produced by *P. capitalensis*[9]. Their biological activity was tested against the insect pest *Myzus persicae* and the plant parasitic nematode *Meloidogyne javanica*. Detailed findings are presented in this article.

Results

Variations in dioxolanone production

Among the dioxolanones described in *Phyllosticta capitalensis* (Isolate YCC4)[9], metguignardic acid (**1**), guignardianone C (**2**), ethyl guignardate (**3**), guignardianone D (**4**) and phenguignardic acid methyl ester (**5**), (Fig. 1), were detected in the different fermentations carried out in this study by HPLC–MS analysis.

Figure 2 presents the peak area measurements of five dioxolanone derivatives produced under different fermentation modifications, including bentonite (B), talcum powder (TP), glass wool (GW), and metallic mesh (MM), evaluated at four time points (6, 10, 15, and 21 days); all measurements are expressed relative to the control fermentation (C), which serves as the reference for comparison. Compound production varied significantly depending on the fermentation method and time. For compound 1, an improvement was observed with GW on day 6. Compound 2 showed higher yields with GW on days 6, 10, and 21, as well as with B on days 6 and 10, with the highest production recorded for GW on day 10. Compound 3 exhibited increased production with B on days 6 and 21, TP on days 6, 10, and 21, and GW on days 6, 10, and 21, reaching its maximum with GW on day 10. For compound 4, production was enhanced with B on days 6 and 21, TP on day 10, GW on day 10, and MM on day 10, with the most significant increase occurring with B on day 6. Finally, compound 5 responded positively to TP on days 6 and 10, GW on days 6, 10, and 15, and MM on days 10 and 15, achieving its highest production with MM on day 15.

Overall, the highest production was observed for guignardianone C (2). Furthermore, GW was the most effective modifier, improving the production of dioxolanone derivatives.

Dynamics of meroterpene production

Among the meroterpenes described in *P. capitalensis* (Isolate YCC4)[9], guignardone A (**6**) and B (**7**), guignarenone C (**8**), guignarenone B (**9**) and guignardone I (**10**), (Fig. 3), were detected in the extracts from the different fermentations.

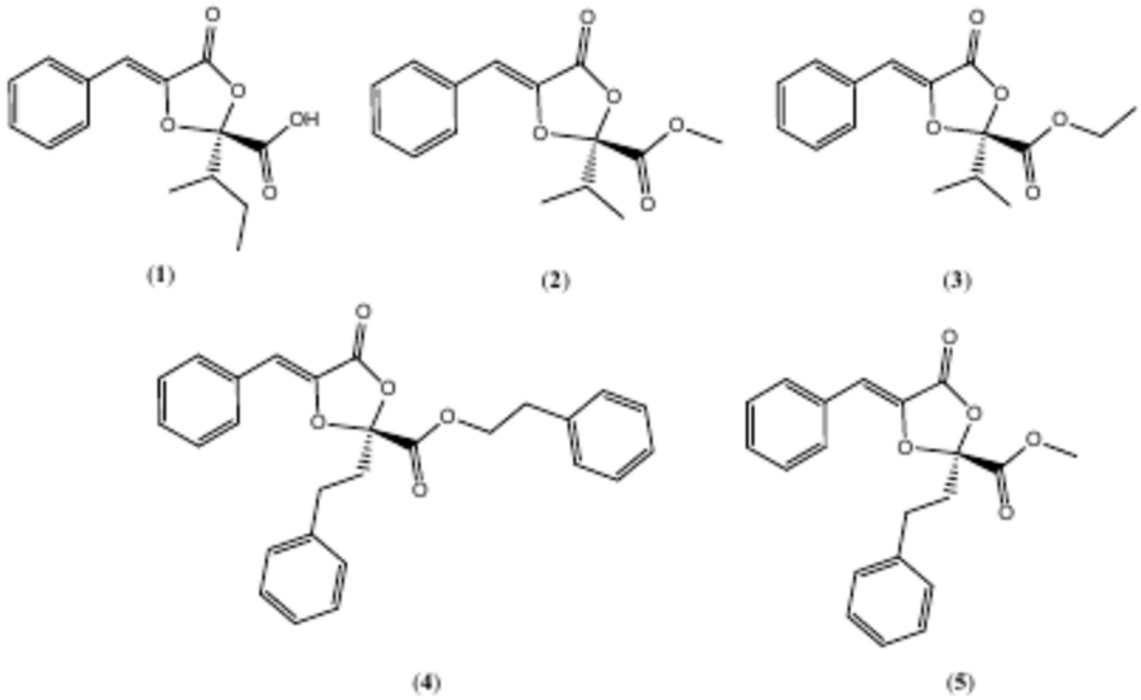

Fig. 1. Dioxolanones identified. Metguignardic acid (**1**), guignardianone C (**2**), ethyl guignardate (**3**), guignardianone D (**4**) and the methyl ester of phenguignardic acid (**5**).

Figure 4 shows the peak area measurements of five meroterpene derivatives produced using different fermentation modifications bentonite (B), talcum powder (TP), glass wool (GW), and metallic mesh (MM) at different time periods (6, 10, 15, and 21 days); all measurements are expressed relative to the control fermentation (C), which serves as the reference for comparison. Overall, the meroterpenes analyzed decreased or were not affected by the fermentation conditions except for: Compound **6** with B, days 6 and 21; TP Day 6 and GW day 6; Compound **8**, MM Day 21; compound **9**, with a moderate increase with B, TP, and GW at day 6, and MM Day 21; Compound **10** with TP Day 6.

Hierarchical clustering

Clustering was performed using Ward's method; the proximity between objects was measured by the criteria of dissimilarity based on Euclidian distance, this can be appreciated in Fig. 5.

In the dendrogram with heat map, a relationship is observed between the production of all compounds (identified and not identified), and the addition of inert supports to the fermentation. The analysis of compound production during the fermentation of various materials revealed six principal clusters. There are no clear patterns in the distribution of the extracts along the clusters although some clusters contain extracts from the first fermentation type. In general, main clusters are formed by different methods at different fermentation days, indicating that each fermentation modifier alters metabolic pathways in unique ways, resulting in distinct metabolic profiles for each fermentation day without a consistent pattern related to fermentation type and duration. Notably, bentonite fermented for 21 days (B 21) and metallic mesh fermented for the same interval (MM 21) exhibited high production levels of compounds 5 and 10 suggesting that both bentonite and metallic mesh are highly efficient in producing these compounds when fermented for extended periods.

In contrast, talcum powder fermented for 6 and 21 days (TP 6 and TP 21), along with the control fermented for 6 days (C 6), were found in isolated clusters, highlighting unique production profiles that do not align with other treatments. C 6 is found out of all clusters, which could be explained by its short production of dioxolanones and meroterpenes, highlighting the general fermentation improvement of most methods at early stages of fermentation. This could indicate that the interaction between the material and the fermentation duration generates distinctive results in these cases. Additionally, the fermentation of glass wool and metallic mesh for shorter periods (6 and 10 days) resulted in low compound production, underscoring the importance of longer fermentation times to achieve more robust production profiles.

Variability in the production of compounds: a PCA approach

The Principal Components Analysis (PCA) groups the different extracts on the basis of their content in secondary metabolites of interest (**1–10**) (Fig. 6). The x-axis represents component 1 with a variance of 40.3%, while the y-axis represents component 2 with a variance of 18.6%.

Analysis of the production of the compounds of interest in relation to the inert support used reveals distinct trends. Positive projections on the PC1 axis indicate an increase in the production of compounds **4, 5, 6, 7** and **9** with the addition of talcum powder. On the other hand, negative projections on the PC2 axis show that

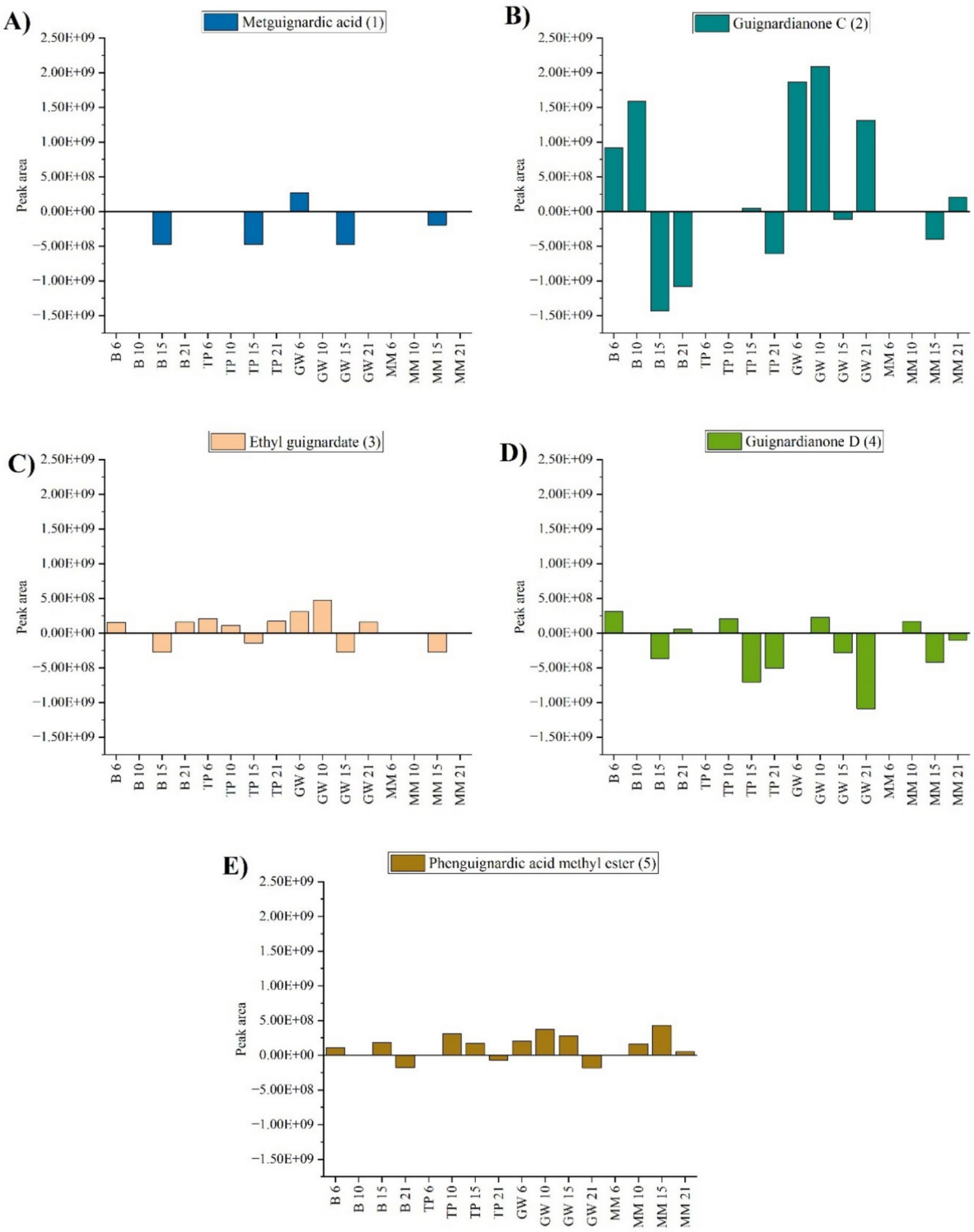

Fig. 2. Dioxolanone production across different fermentation techniques and time point relative to the control. **(Panel A)** production of Metguignardic acid (**1**), (**Panel B)** production of guignardianone C (**2**), (**Panel C)** production of ethyl guignardate (**3**), (**Panel D)** production of guignardianone D (**4**) and (**Panel E)** Production of the methyl ester of phenguignardic acid (**5**).

compounds **1, 2, 3, 8** and **10** could be affected by longer fermentations. These observations are supported by the analysis of the eigenvalues of these compounds. Compounds **1**, **2** and **3** exhibit significantly higher eigenvalues (4.02, 1.86 and 1.35, respectively), suggesting a higher sensitivity to variations in fermentation. In contrast, the

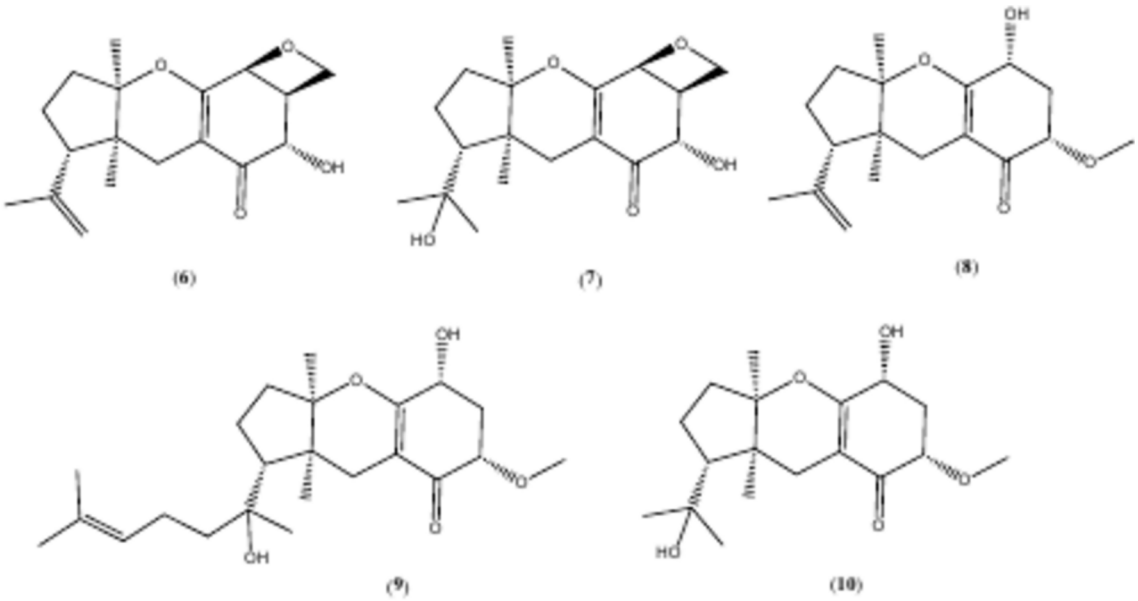

Fig. 3. Meroterpenes identified: Guignardone A (**6**), Guignardone B (**7**), Guignarenone C (**8**), Guignarenone B (**9**), Guignardone I (**10**).

eigenvalues of the other compounds are less than 1, implying higher stability in their production in spite of the variations in the fermentation process.

The proximity of the red points to the arrows suggests a direct correlation between specific fermentation days and the chemical compounds analyzed, allowing for a precise interpretation of the relationships between variables. Notably, the points representing fermentations B6, MM15 and GW21 are clustered near the origin, indicating that these experiments share a similar chemical composition or are not strongly associated with any of the 10 evaluated compounds. In contrast, compounds **6**, **7**, and **9** appear to be highly correlated with samples located towards the right side of the graph. The close proximity and similar direction of these arrows suggest that these compounds share a similar pattern of concentration across the samples in this area. This implies that the specific fermentations located on the right side of the plot likely have higher concentrations of compounds **6**, **7**, and **9**, contributing significantly to the observed variation along the PC1 axis. This cluster of compounds highlights a distinct chemical profile associated with these fermentation samples.

Bioactivity of the extracts

Figure 7A and B shows the bioactivity of the different extracts against the selected targets based on previous data and the content in dioxolanones and meroterpenes of each extract. Bioactivity against *M. persicae* was tested at a dose of 100 µg/cm^2 and against the nematode *M. javanica* at a dose of 1 mg/ml[9].

For the antifeedant assay (Panel A), a 70% inhibition threshold (red dotted line) indicates when an extract is considered active. Bentonite (B) extracts displayed the highest antifeedant activity overall, reaching about 95% at day 6 and ~ 90% at day 10 (both exceeding the 70% mark), then around 85% at days 15 and 21. Glass wool (GW) extracts showed similarly strong inhibition—roughly 80–90% at day 10 before decreasing slightly thereafter. Talcum powder (TP) extracts showed slightly activity on day 6 (~ 80%). Meanwhile, Metallic Mesh (MM) extracts achieved their best antifeedant performance (~ 90%) at day 21, whereas Control (C) extracts peaked at ~ 70% at day 15. Finally, the concise summary (GW extracts > B > MM > TP) aligns with the reported data.

Against M. javanica (Panel B), a 90% mortality threshold was used to classify extracts as active. GW formulations were the most effective, surpassing 90% mortality at days 15 and 21 (100%), while day 10 was slightly upper that threshold (~ 90%). B and TP extracts each showed nematicidal activity (100%) on the 6 day, however TP at day 21 showed an increase of activity (100%). In contrast, MM evidence of activity between days 6 and 15 (100%) with a small decline on day 10 (~ 90%). The overall summary (GW = MM extracts > TP > B) is consistent with the reported data. Therefore, the presence of GW resulted in strong antifeedant effects while GW or MM enhanced the nematicidal activity.

Notably, despite these strong performances, no direct correlation emerged between bioactivity and the ratio of dioxolanones/meroterpenes, suggesting that additional components or synergies may be influencing the final biopesticidal efficacy.

Discussion

The endophyte *P. capitalensis*, isolated from *Persea indica*, produced dioxolanones guignardic acid, ethyl guignardate, metguignardic acid, guignardianones A, C, D and E, and phenguignardic acid methyl ester plus the meroterpenes guignardone A, B, I, the C-14 epimer of (−)-guignardone I, guignarenone C, and the

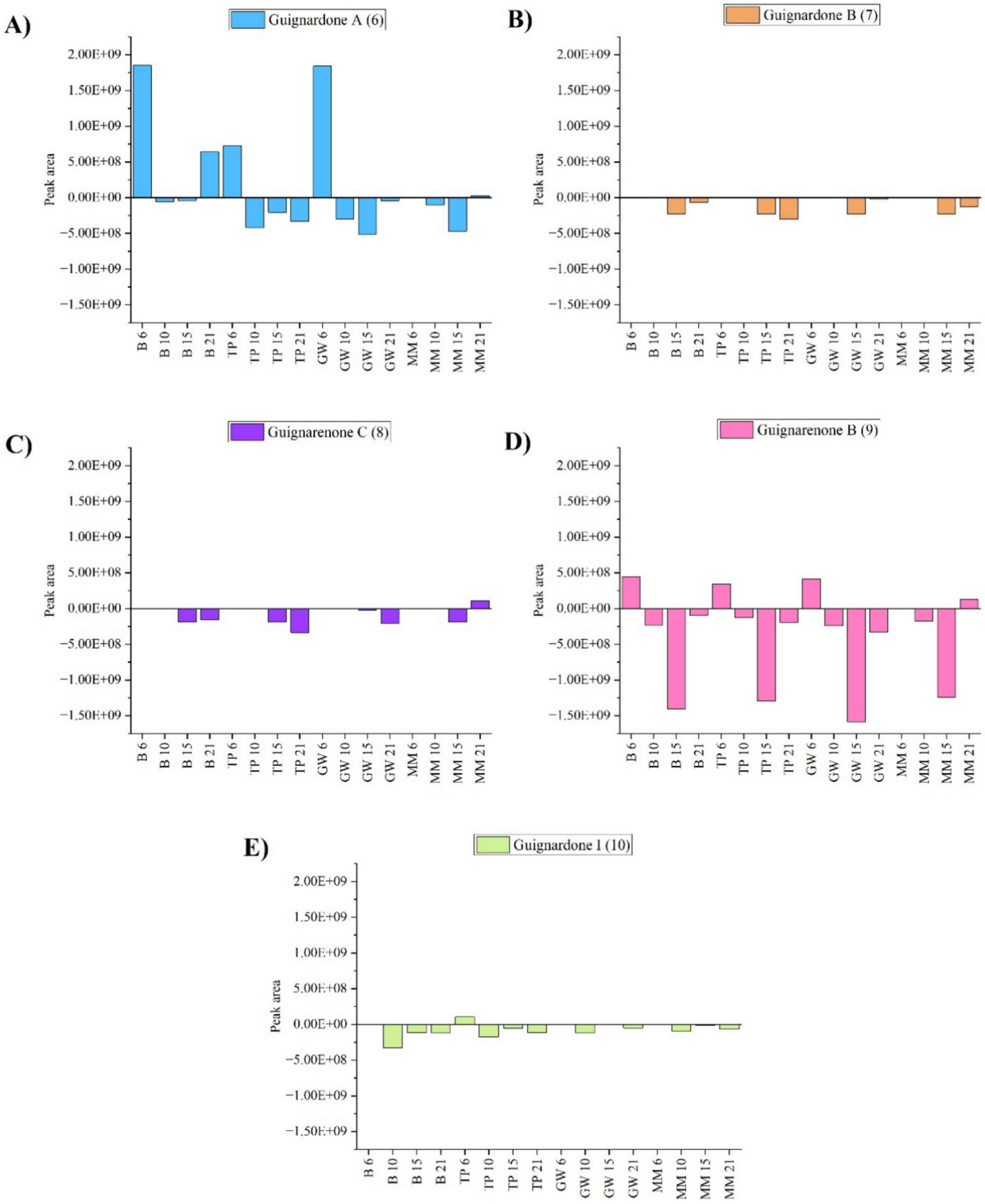

Fig. 4. Meroterpenes production on different fermentation techniques over time with relative comparison. **(Panel A)** Production of Guignardone A (**6**), **(Panel B)** production of Guignardone B (**7**), **(Panel C)** production of Guignarenone C (**8**), **(Panel D)** production of Guignarenone B (**9**) and **(Panel E)** Production of Guignardone I (**10**).

phyllomeroterpenoid B[9]. In this work we have fermented this isolate YCC4 under different conditions and analyzed the production of these compounds to compare the metabolite production among the different fermentation techniques used (MPEC and SAF). The dioxolanones, metguignardic acid (**1**), guignardianone C (**2**), ethyl guignardate (**3**), guignardianone D (**4**) and phenguignardic acid methyl ester (**5**) and the meroterpenes,

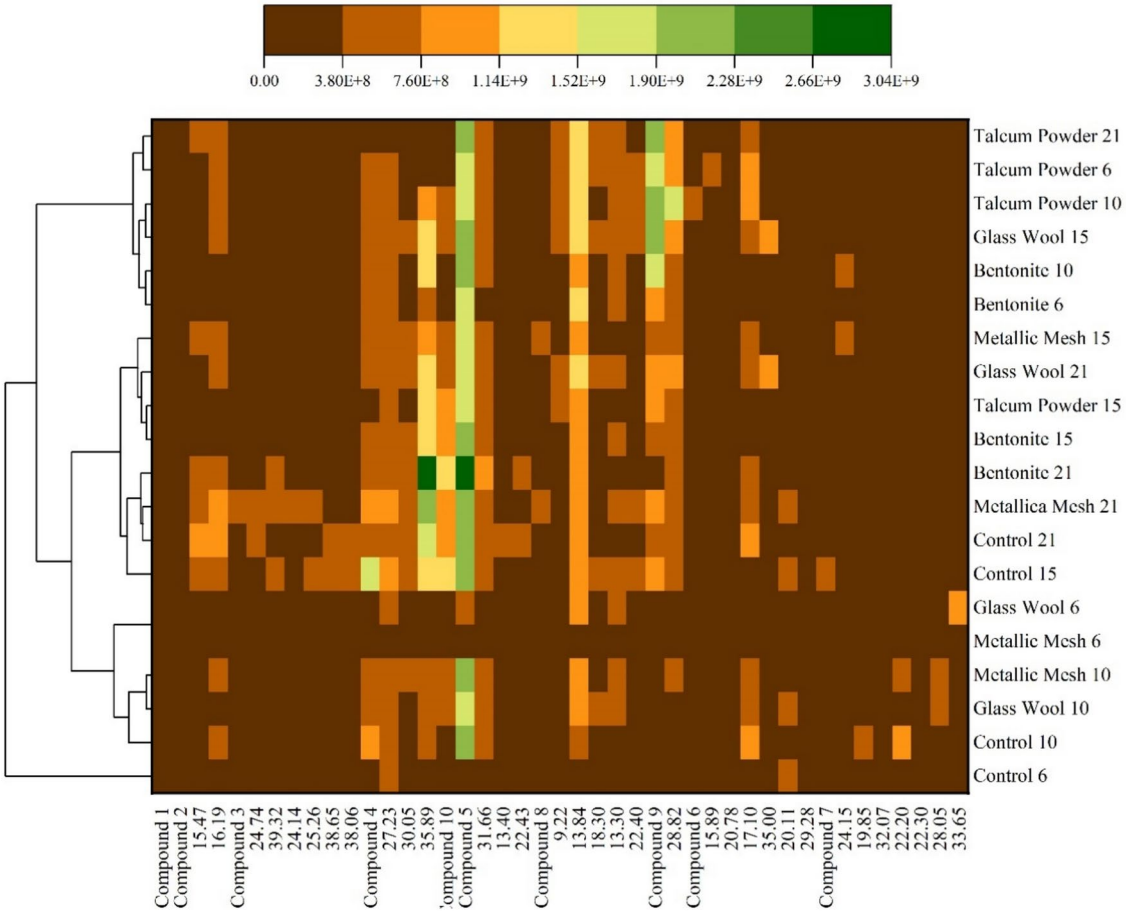

Fig. 5. Hierarchical clustering dendrogram. This shows the relative patterns of each of the 20 fermentation modifications, including controls (y-axis), and each of the 43 compounds reported in all fermentations, including those of interest (x-axis), in clusters. The colors correspond to the production of each compound related to the area found in the chromatographic analyses (HPLC–MS) performed with brown values up to 3.8E8 and maximum green values up to 3.04E9.

guignardone A (**6**) and B (**7**), guignarenone C (**8**), guignarenone B (**9**) and guignardone I (**10**) were detected in the extracts from the different fermentations.

The use of bentonite as MPEC has been barely described in literature. Bentonite is a clay composed of hydrated aluminum silicate minerals. Traditionally, it has been used in industry for wine clarification due to its high porosity and negative charge helping to reduce the turbidity in wines caused by polysaccharides and proteins[33,34]. In this context, bentonite was chosen due to its low impact on the culture medium, particles size and affordable price. Other silicates had been used in similar processes. *Mortierella isabellina* increased its rate of lipids production for biofuel production when applying magnesium silicate microparticles to promote the dispersion of the mycelia[35]. Magnesium silicate was also applied to enhance the enzymatic activity of *Aspergillus sojae* for β-mannanase production[36]. The presence of bentonite could have a positive effect on the production of these compounds, possibly by providing conditions that favor the growth of the fungus on bentonite microparticles or by stabilizing physicochemical conditions that enhance its production. This work emphasizes the potential use of bentonite in future MPEC experiments as biocatalyst for secondary metabolites production. Although none of the peaks of production of any of the compounds studied in this work have been achieved with this method, a stability in the production of certain compounds have been reported.

The surface adhesion fermentation (SAF) carriers have several advantages derived from the biofilm formation. For instance, higher biomass densities or increased productivity[37,38] take place due to an increased protection against the environmental stresses[39] or an easier removal from bioreactors, reducing the operational problems caused by free pellets in the medium[40]. Furthermore, the fungi can be easily reused in other fermentation processes. The surface adhesion methods used in this work (glass wool, metallic mesh) showed different outcomes depending on the material.

Glass wool yielded the highest production of **1** (2.82-fold of the control), the metallic mesh increased **5** (1.92-fold higher yield). The increase of the yields as well as the differences found in the materials can be explained by their roughness and shape, modulating the metabolism of the fungi in different ways[31,41]. After cells attach to a surface, signaling pathways trigger various molecular and physiological changes, leading to tissue formation, specialized cell functions, or biofilm development, which explains the metabolically changes during

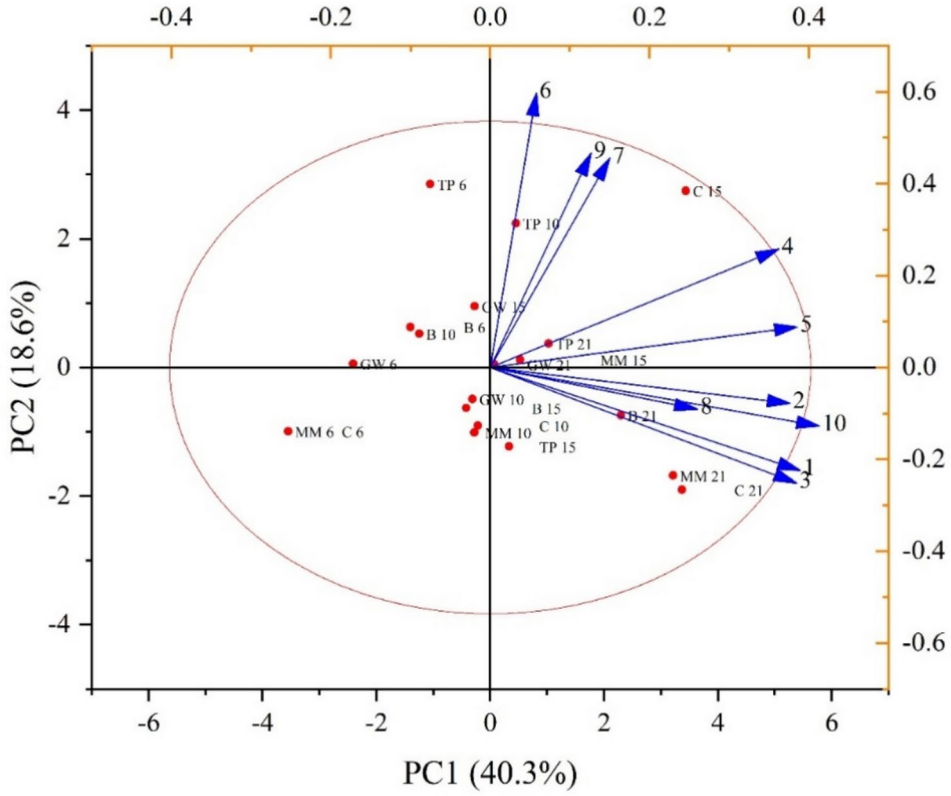

Fig. 6. PCA analysis. Analysis considering the different modifications to the fermentation.

fermentation and its increased yield when compared to the control[42]. This observation highlights morphology in the fermentation as a key element at the time of the production of compounds since the production of dioxolanones increased with this method.

Metallic materials have been used widely at lab scale and in bioreactors to induce the formation of biofilms during fermentation[43–46], reporting improvements in enzymatic reactions as well as in secondary metabolites production. Previous works have shown enhanced adhesion and biomass production when growing on steel than when growing on glass[31] probably due to the smoothness and the lack of angles in glass to ease the adhesion of the mycelium. Nevertheless, the irregular shapes of glass wool can favor the adhesion of the fungi to the carrier, improving the yield of the control with no carrier and also to other carriers in some conditions.

Previous reports have shown that only dioxolanone derivatives, guignardic acid, metguignardic acid (**1**), guignardianone C (**2**), ethyl guignardate (**3**), guignardianone D (**4**) and phenguignardic acid methyl ester (**5**), exhibited activity against the insect and nematode targets[9]. In this work, the overall activity did not correlate with the expected dioxolanone/meroterpene ratio of the extracts, suggesting the presence of additional bioactive compounds other than the ones isolated previously[9] and/or synergistic effects. It is also possible that talcum powder provides a growth surface or physical conditions that stimulate the production of compound **9** by the fungi. These changes may reflect a complex interaction between fungi, substrates and inert supports, which underlines the importance of considering the whole system in the optimization of fermentation processes of fungal extracts. This is supported by the lack of clear compound-based grouping of the different extracts shown in the cluster.

Table 1 summarizes the best performing fermentation parameters for each compound. As shown in the table, GW notably enhances the production of compounds **1**, **2**, and **3** at early fermentation stages (with optimal results for compound **2** at 10 days), while MM yields the highest production of compound 5 at 15 days. This indicates that selecting the appropriate inert support is essential for optimizing the production of certain target metabolites in *P. capitalensis*.

In summary, diverse outcomes have emerged with the utilization of distinct methodologies. Establishing a clear trend remains challenging, given the varied enhancement of metabolite production across different carriers and conditions. However, our findings underscore the efficacy of both MPEC and SAF methods. Across all studied products and the bioactivity of fermentation crude extracts, the control method exhibited comparatively inferior performance.

Conclusion

This research provides important information regarding the improvement of cultivation conditions for *Phyllosticta capitalensis* (isolate YCC4), leading to the potential enhancement of bioactive compound production. Furthermore, our findings provide methodological approaches for the development and refinement

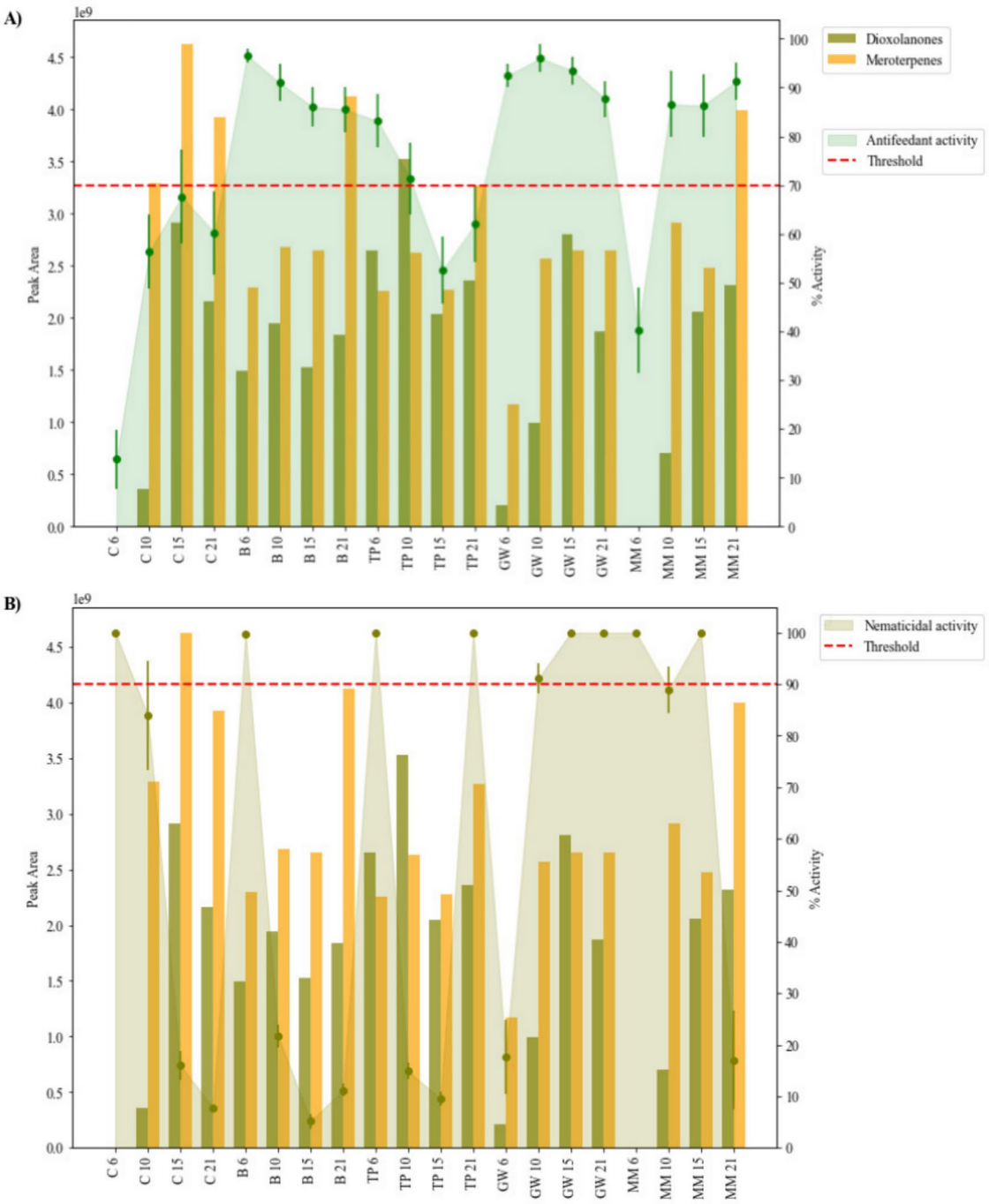

Fig. 7. Biopesticide results. **(Panel A)** Antifeedant results against *Myzus Persicae*. The red dotted line represents the 70% activity, at which the extracts are considered active. (**Panel B)** Nematicide bioassay against *Meloidogyne javanica.* The red dotted line represents the 90% activity, at which the extracts are considered active. The area shows the antifeedant activity and the bars show the production of dioxolanones and meroterpenes in terms of area.

of Microparticle Enhanced Culture (MPEC) and Surface Adhesion Fermentation (SAF), two promising techniques for fungal research. This study highlights the potential of these techniques for sustainable and effective production of bioactive compounds in agriculture. These methods also offer valuable additions to the existing techniques used for this purpose, and have been shown to be effective tools for the production of bioactive compounds. The results of this study revealed a robust correlation between culture conditions and the production of secondary metabolites in the endophytic fungus *P. capitalensis.* Our findings demonstrate that under certain conditions, MPEC and SAF have overperformed traditional submerged fermentations in production of bioactive dioxolanones as for the case of the use of bentonite, glass wool and talcum powder

Compound	Optimal fermentation technique	Optimal time of fermentation (Day)
1	GW	6
2	GW	10
3	GW	10
4	B	6
5	MM	15

Table 1. Optimal fermentation conditions for dioxolanone production in *P. capitalensis*.

in the 6th day of fermentation, and specifically glass wool for the production of guignardianone C on day 10. Moreover, some of these methods have boosted bioactivity of the extracts against pests and plant pathogens These findings not only highlight a clear link between culture conditions and enhanced biopesticide efficacy but also provide a robust, standardized protocol that can be adapted for large-scale production. In the future, this protocol can be scaled up and integrated into industrial bioprocesses, thereby enabling the consistent manufacture of high-quality secondary metabolites. Such large-scale production will support the development of eco-friendly biopesticide formulations, ultimately contributing to more sustainable and effective pest and disease management strategies under field conditions.

Material and methods

Plant material

The endophytic fungus *P. capitalensis,* strain YCC4 (formerly *Guignardia mangiferae*, isolate YCC4) was originally isolated from the leaves of *Persea indica* (L.), an endemic tree in Macaronesian. Samples were collected from Las Mercedes, Parque Rural de Anaga (Tenerife, Spain). This fungus has been officially deposited in the Spanish Type Culture Collection under accession number CECT 20914[9].

Molecular characterization of Phyllosticta capitalensis

The molecular identification of the strain was conducted via PCR amplification and sequencing of its ribosomal ITS1-5.8S-ITS2 using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-GCTGCG TTCTTCATCGATGC-3'). The amplified sequence of the rDNA was aligned with BLAST with sequences in the NCBI database (National Center for Biotechnology Information) The strain was determined to be *P. capitalensis* by comparing the sequence region with those available in the NCBI database using nBLAST (GenBank accession MT649668). A sample of this fungus was deposited in CECT (Valencia, Spain) under the number 20914[9].

Pre-inoculum of P. capitalensis for extract preparation

P. capitalensis was cultivated in PDA medium in Petri dishes for 15 days at 28 °C. Sterile distilled water (10 mL) was then added and the surface of the mycelium was gently scraped with a spatula. This mycelial pre-cultivation suspension was poured into a baffled Erlenmeyer flask (250 mL) containing 125 mL of Czapek-Dox-Yeast liquid medium modified with amino acids [CZD-YA: $NaNO_3$ (2 g/L), KH_2PO_4 (5 g/L), $MgSO_4$ (0.5 g/L), $FeSO_4$ (0.01 g/L), $ZnSO_4$ (0.03 g/L), yeast extract (1 g/L), glucose (60 g/L), leucine (0.08 g/L), and phenylalanine (0.08 g/L)], and cultivated at 26 °C with continuous stirring (120 rpm) for 4 days.

Modifications to the culture medium

Talcum powder (10 g/L) with a particle size of 350 MESH (Fisher Scientific; Waltham, USA) was incorporated into Erlenmeyer flasks prepared following the optimal conditions as evaluated and presented by Antecka et al.[21]. In addition, the conditions reported by Kurdish et al.[47] for the addition of 1 g/L bentonite (Fisher Scientific; Waltham, USA) were considered. Additionally, a stainless-steel mesh (Inoxia Ltd; Cranleigh, UK) made of 304 L stainless steel with a pore size of 40 MESH and a surface area of 0.044 m2 was placed at the bottom of the Erlenmeyer flasks following a design similar to that described by Francis et al.[28]. In the case of glass wool (Merck; New Jersey, USA) the amount described by Ellaiah et al.[48] as adsorbent support was modified to use a concentration of 10 g/L.

Time-course fermentation

A total of 60 baffled 250 mL Erlenmeyer flasks containing Czapek-Dox-Yeast liquid medium modified with amino acids (125 mL) were prepared containing different inert supports (Three replicates/sample) including the control, and inoculated with 10% v/v from the pre-inoculum suspension. Triplicates were sampled on days 6, 10, 15, and 21 of the incubation.

Extract preparation

The culture medium was filtered through a 25 µm pore diameter filter paper using a Buchner funnel to separate the mycelium, submitted to exhaustive liquid/liquid extraction with ethyl acetate (EtOAc), dried over Na_2SO_4, and concentrated under reduced pressure in a rotary evaporator to obtain crude *P. capitalensis* extracts.

Bioassays

Antifeedant bioassay

Colonies of *Myzus persicae* were cultivated on the host plants *Capsicum annuum* L. (bell pepper). The host plants were germinated from seeds in pots filled with a standardized commercial substrate. The insect colonies and their respective host plants were housed in a controlled growth chamber, maintained at a temperature of 22 ± 1 °C and relative humidity exceeding 70%, and subjected to a 16:8 h light–dark photoperiod. Leaf fragments (1.0 cm^2) of *C. annuum* were treated on their adaxial surfaces with 10 µl of the test solution. The testing dose for the crude extracts was 100 µg/cm^2. For each assay, 20 boxes (2 × 2 cm) containing ten apterous aphid adults (aged 24–48 h) were allowed to feed under the aforementioned growth chamber conditions for a 24-h duration. Aphid settling was quantified by counting the individuals that settled on each leaf fragment. The settling inhibition (%SI) was calculated with the formula: %SI = [1 – (T/C) × 100], where 'T' and 'C' denote the settling on treated and control leaf fragments, respectively. The statistical significance of the antifeedant effects (%SI) was determined using a nonparametric Wilcoxon signed-rank test[49].

Nematicidal bioassay

The root-knot nematode (*Meloidogyne javanica*) population was maintained on tomato (*Lycopersicon esculentum* var. Marmande) plants in pots at 25 ± 1 °C and > 70% relative humidity was used for the experiments. Second-stage juveniles (J2) that hatched within 24 h (from egg masses handpicked from infected tomato roots) were used. The experiments were carried out in 96- well microplates (Becton, Dickinson; New Jersey, USA) as described[9]. The organic extracts were tested at initial concentration of 1.0 mg/mL (final concentration in the well) and diluted serially, if needed. The number of dead juveniles was recorded after 72 h. All treatments were replicated four times. The data were determined as percentage mortality and corrected according to Scheider-Orelli's (1947) formula[9].

Compound quantification

The organic extracts were analyzed by liquid chromatography coupled to mass spectrometry (HPLC–MS) using a Shimadzu apparatus equipped with an LC- 20AD pump and a CTO-10AS VP column oven coupled to a mass spectrometer with triple quadrupole as the analyzer (LCMS-8040) with an electrospray ionization source (ESI). An ACE 3 C18 column (150 mm × 4.6 mm, 3 µm particle size) with an ACE3 C18 analytical pre-column was used for separation. The compounds were eluted with methanol (LC–MS grade, Merck; New Jersey, USA) with 0,1% acetic acid (MeOH): Mili-Q water with 0,1% acetic acid starting in isocratic method 20% MeOH during 5 min to continue with a gradient 20:67% MeOH during 20 min, 67:100% during 10 min, 100% MeOH during 10 min and then 100:20% MeOH during 8 min at before the next injection, with a flow rate of 0.5 mL/min. The nitrogen flow (drying gas for solvent evaporation) was 15 L/min. The potential for the electrospray capillary was + 4.50 kV and a Full Scan was used in positive mode (m/z 100–850) used the Q3 quadrupole with a potential of 1.66 kV and a capillary temperature of 250ºC. The heat-block temperature was 400 °C. The stock solutions of the extracts were injected at 0.25 mg/ml with a 5 µl injection through an automatic injector (SIL-20A XR). All extracts (0.5 µg/µl) were dissolved in 100% MeOH for injection.

Statistical methods

The dendrogram and principal component analysis (PCA) were obtained using Origin 2023 software (OriginLab, Northampton, USA).

Data availability

The obtained ITS1-5.8S-ITS2 sequence data was compared with these published in the NCBI (National Center for Biotechnology Information, https://www.ncbi.nlm.nih.gov/) database using Basic Local Alignment Search Tool (nBLAST). This strain was identified by similarity with another P. capitalensis strain (GenBank accession MT649668). A sample of this fungus was deposited in CECT (Valencia, Spain) under the number 20914 in compliance with the Budapest Treaty. The datasets used in the current study are available from the corresponding author on reasonable request.

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Author contributions
Conceptualization, AAGC and NRC; methodology, NRC, JRLM and WAUP; validation, NRC and JRLM; formal analysis, NRC; investigation, NRC, JRLM, WAUP, AAGC and CED; resources, MFY, CED and AAGC; data curation, NRC and AAGC; writing—original draft preparation, NRC, JRLM and AAGC; writing—review and editing, NRC, JRLM and AAGC; visualization, CED and MFY.; supervision, AAGC and CED; project administration, AAGC and MFY.; funding acquisition, AAGC, MFY and CED. All authors have read and agreed to the published version of the manuscript.

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Declarations

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.

Additional information
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