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Original Article

Endophytic fungi as enhancers of secondary metabolite production in *Duroia saccifera* cell suspension

Fungos endofíticos como potencializadores da produção de metabólitos secundários na suspensão celular de *Duroia saccifera*

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Abstract

The chemical response of callogenic suspension cultures of the plant *Duroia saccifera* was evaluated through elicitation by the endophytes *Colletotrichum dianesei* and *Xylaria* sp. Dried mycelia and methanolic extracts of dried mycelia from both fungi were used as elicitators. *D. saccifera* calli were multiplied in Murashige and Skoog medium with growth regulators, while endophytic fungi were cultivated in three different media: potato-dextrose (PD), czapek (CZ) or sabouraud (SB). Part of mycelia cultured in PD medium were dried and autoclaved. The remaining mycelia from the PD, and those from SB and CZ media were filtered, dried, weighed, and extracted with methanol (MeOH). The methanol fungal extracts used as elicitors were chemically analized by ^{1}H nuclear magnetic resonance (^{1}H-NMR) and thin layer chromatography (TLC) and their toxicity was evaluated using *Artemia salina* as a bioindicator. For elicitation, 100 mg of mycelium or 20 mg of fungal extract were used for 2 grams of friable callus. After 30 days, the elicited callogenic suspensions were filtered, weighed, dried, and extracted with methanol. The culture medium obtained from filtration was lyophilized and partitioned with ethyl acetate (EtOAc). Analyzes were performed using TLC and ^{1}H-NMR. As results, it was observed that fungal extracts of *C. dianesei* and *Xylaria* sp. intensified the classes of metabolites already produced by the callus culture, with exception of the extract from *Xylaria* sp. grown in PD medium, which yielded the production of different aromatic substances did not present in the callus control extract. This fungal extract also exhibited high toxicity against *Artemia salina*. This suggests a correlation between the level of extract toxicity and the callus elicitation response.

Keywords: bioprospection, amazonian plant species, plant tissue culture, elicitation.

Resumo

A resposta química da cultura em suspensão calogênica da planta *Duroia saccifera* foi avaliada através da eliciação dos endófitos *Colletotrichum dianesei* e *Xylaria* sp. Como eliciadores foram utilizados tanto os micélios secos quanto os extratos metanólicos dos micélios secos de ambos os fungos. Calos de *D. saccifera* foram multiplicados em meios Murashige e Skoog com reguladores de crescimento, enquanto os fungos endofíticos foram cultivados em três meios diferentes: batata-dextrose (PD), czapek (CZ) ou sabouraud (SB). Parte dos micélios cultivados em meio PD foram secos e autoclavados. Os micélios restantes do PD e os cultivados nos meios SB e CZ foram filtrados, secos, pesados e extraídos com metanol (MeOH). Os extratos metanólicos fúngicos utilizados como eliciadores foram analisados quimicamente por ressonância magnética nuclear de ^{1}H (RMN de ^{1}H) e cromatografia em camada delgada (CCD) e sua toxicidade foi avaliada utilizando *Artemia salina* como bioindicador. Para eliciação, foram utilizados 100 mg de micélio ou 20 mg de extrato fúngico para 2 gramas de calo friável. Após 30 dias, as suspensões calogênicas eliciadas foram filtradas, pesadas, secas e extraídas com metanol. O meio de cultura obtido da filtração foi liofilizado e particionado com acetato de etila (AcOEt). As análises foram realizadas utilizando CCD e RMN de ^{1}H. Como resultados, foi observado que os extratos fúngicos de *C. dianesei* e *Xylaria* sp. intensificaram as classes de metabólitos já produzidos pela cultura dos calos, com exceção do extrato de *Xylaria* sp. cultivado em meio PD, que resultou na produção de substâncias aromáticas diferentes que não estavam presentes no extrato dos calos (controle). Este extrato fúngico também exibiu alta toxicidade contra *Artemia salina*. Isso sugere uma correlação entre o nível de toxicidade do extrato e a resposta de eliciação dos calos.

Palavras-chave: bioprospecção, espécies vegetais amazônicas, cultura de tecidos vegetais, eliciação.

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1. Introduction

Plants produce a variety of substances essential for their development, including secondary metabolites, which, although not directly linked to primary needs, play a crucial role in the survival and perpetuation of species by providing ecological advantages (Dewick, 2011; Setyorini and Antarlina, 2022).

To mitigate the predatory exploitation of plant species for bioprospecting purposes, the technique of *in vitro* plant tissue cultivation has been developed. This technique allows for the cultivation of cells and entire plants *in vitro*, with the aim of optimizing the production and extraction of substances of interest (Souza et al., 2018; Wawrosch and Zotchev, 2021). However, some species cultivated *in vitro* do not produce the same metabolites as their parent plant, or when they do, the metabolites are present in low quantities. Therefore, to enhance the synthesis of secondary metabolites *in vitro*, elicitation techniques have been applied to the *in vitro* culture of several plant species, such as *Catharanthus roseus* L. G. Don, *Mitragyna speciosa* Korth, *Thevetia peruviana* (Pers.) K. Schum, and other species of economic and medicinal interest (Mohamad Zuldin et al., 2013; Liang et al., 2018; Durán et al., 2021).

One of the agents used to elicit *in vitro* cultures are endophytic fungi. These organisms live symbiotically with plant species without causing harm and can induce the production of both primary and secondary metabolites by the host plant (Bogas et al., 2022; Gurgel et al., 2023). This symbiotic relationship offers several advantages to the host plant, such as increased tolerance to herbivore and insect attacks, greater resistance to environmental variations, and even the attraction of pollinating agents. Additionally, fungi benefit by obtaining the necessary nutrients for survival and a higher probability of species perpetuating through transfer to subsequent generations (Rajamanikyam et al., 2017).

Some studies suggest that endophytes, during the period of co-evolution, may have acquired genes from the host plant, enabling the associated fungi to produce the same chemical substances as the parent plant (Tan and Zou, 2001; Saikkonen et al., 2004). This finding has encouraged the cultivation of these organisms as an alternative source of active substances, thereby contributing to the reduction of plant collection, particulary for species that are threatened with extinction, difficult to locate, and/or have restricted distribution (Tsipinana et al., 2023).

Co-cultivation of a viable fungal strain into *in vitro* plant tissue culture can impair plant development and potentially lead to the death of plants or calluses due to competition for nutrients available in the culture medium. Therefore, alternative methods can be employed to incorporate these organisms into the culture, such as using mycelia and/or concentrated extracts (Zepeda et al., 2009; Tonk et al., 2023).

Previous studies have demonstrated the enhanced production of secondary metabolites due to the presence of the elicitor endophyte. For instance, the production of mitragynine was significantly increased in the suspension of *Mitragyna speciosa* callus after elicitation with yeast extract; and the concentration of the alkaloids vindoline, catharanthine and ajmaline increased when the culture of *Catharanthus roseus* was elicited with the fungus *Aspergillus flavus* (Mohamad Zuldin et al., 2013; Liang et al., 2018). In this context, the use of endophytic fungi emerges as a promising technique for stimulating the production of secondary metabolites in *in vitro* cultures, especially for species with a promising phytochemical history.

Duroia saccifera (Mart. Ex Roem. and Schult.) K. Schum. (Rubiaceae) is a plant known for producing triterpenoids, flavonoids, which exhibit significant biological properties, including antibacterial and antioxidant activities, attributed to its chemical composition (Mesquita et al., 2015; Contreras-Mejia, 2017). Alzate-Lozano et al. (2020) successfully established seedlings and calli *in vitro*, and their chemical analysis revealed the presence of steroids, terpenes, and aromatic substances. These findings confirmed the antibacterial activity observed in the wild species, particularly highlighting the antimycobacterial activity of the ethyl acetate extract from the calli.

Since studies regarding the elicitation of *in vitro* culture of *D. saccifera* have not yet been carried out, and since in nature the association of this species with endophytic fungi may be directly related to its chemical response, the objective of this research was to analyze the production of metabolites by cell suspension of *D. saccifera* elicited with mycelia and extracts of *Colletotrichum dianesei* and *Xylaria* sp., in order to verify whether there is a change in the chemical profile of the calli, and/or an increase in the production of classes of metabolites already reported in the species.

2. Materials and Methods

2.1. In vitro callus multiplication of Duroia saccifera and obtaintion of suspension cells

Duroia saccifera calluses pre-established by Alzate-Lozano et al. (2020), were multiplied in MS semisolid medium (Murashige and Skoog, 1962) containing 4 $mg.L^{-1}$ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 $mg.L^{-1}$ of Kinetin (KIN), with the pH adjusted to 5.7 (± 0.1). Subcultures were conducted out at the Bioprospecting and Biotechnology Laboratory (LABB) of the National Institute for Amazonian Research (INPA) every 30 days, following the protocol by Alzate-Lozano et al. (2020).

To obtain the cell suspension, 2 grams of friable callus were added to 250 mL Erlenmeyer flasks containing 100 mL of MS liquid medium supplemented with 4 $mg.L^{-1}$ of 2,4-D and 2 $mg.L^{-1}$ of KIN.

2.2. Isolation and identification of the fungi Xylaria sp. and Colletotrichum dianesei

The fungi *Xylaria* sp. and *C. dianesei* were isolated from *Palicourea corymbifera* (Rubiaceae) leaves, following the standard laboratory procedure: the leaves were subjected to pre-disinfestation by being washed with mild soap in running water. Subsequently, the disinfestation stage began in a biological safety cabinet, in which the leaves were immersed in 70% ethyl alcohol (1 minute), 2.5% sodium

hypochlorite (4 minutes), 70% ethyl alcohol (1 minute), and finally, the leaves were washed 3 times in sterile distilled water. The leaf fragments were inoculated in potato dextrose agar medium and subsequently incubated in a microbial growth oven at 30 °C, and the strain purification process was carried out (Da Silva et al., 2020). The taxonomic identification of the fungus was performed at the ILMD/Fiocruz Amazônia, using DNA amplification of the ITS (ITS1-5.8s-ITS2) rDNA region.

2.3. Reactivation of the fungi Xylaria sp. and Colletotrichum dianesei

The fungi *Xylaria* sp. and *C. dianesei* were reactivated in potato dextrose (PD) agar culture medium and maintained in a BOD-type microbial growth chamber for 7 days at 30 ºC. After reactivation, fragments of approximately 0.5 cm were made, and three fragments/Erlenmeyer flasks of 500 mL were inoculated with 200 mL of PD culture medium (n. 6), sabouraud (SB, n. 3), and czapek broth with 0.2% yeast extract (CZ, n. 3). The Erlenmeyer flasks were kept for 10 days on an orbital shaking table at 120 rpm at a temperature of 30 ºC to obtain the mycelial mass.

2.4. Obtaining mycelia and methanolic extracts from Xylaria sp. and C. dianesei

After 10 days of growth, the mycelium suspension from 3 Erlenmeyer flasks containing *Xylaria* sp. and *C. dianesei* in PD medium was filtered using a vacuum pump to separate the broth and mycelium. The mycelia were dried in an oven at a temperature of 40 ºC for 36 h, then weighed and separated into 100 mg per vial (3 vials of dried mycelia of *Xylaria* sp. and 3 vials of dried mycelia of *C. dianesei*). These were autoclaved (20 minutes at 1 ATM) and kept in an ultrafreezer at -80 ºC for later use in elicitation.

Filtration of the other mycelia from the PD, SB and CZ media was also carried out using a vacuum pump to separate the broth and the mycelium. The mycelia were dried in an oven at a temperature of 40 ºC for 36 h, then weighed and extracted six times (extraction ratio 1 g: 3 mL) with methanol in an ultrasonic bath (Unique®, model USC-2800: US Frequency, 40 kHz, São Paulo), and then concentrated in a rotary evaporator (Fisatom, model 550, São Paulo). The final yield of the extracts was calculated using the formula: % yield = (mass of extract obtained x 100) / mass of dry mycelia.

2.5. Chemical and toxicity evaluation of callus elicited extracts

The callus elicited extracts were analyzed by comparative thin layer chromatography (TLC - Macherey-Nagel, Model Aluminum chromatosheet for TLC Alugram) and ^{1}H nuclear magnetic resonance (NMR) to verify similarities and/or differences between them.

The callus elicited extracts were also analyzed for toxicity against the bioindicator *Artemia salina*. For the toxicity test, concentrations of 1000, 500, 250 and 125 $\mu g.mL^{-1}$ were analyzed. A saline solution containing 38 g of sea salt/L of distilled water was prepared and 20 mg of *A. salina* cysts were added to this solution. These were kept under artificial light for 48 hours at 28 ± 2 ºC. After hatching, the microcrustaceans were subjected to the extract.

The test was performed in a 24 well culture plate (10 individuals per well, in triplicate for each concentration). 5% DMSO was used as a negative control and potassium dichromate (100 $\mu g.mL^{-1}$) was used as a positive control. The plates were stored at 28 ± 2 ºC under artificial light for 24 hours. The LD_{50} was calculated using the dose-response curve in the statistical software GraphPad Prism (GraphPad Prism, 1994).

2.6. Elicitation of cell suspension of D. saccifera with mycelia of Xylaria sp. and C. dianesei

To elicit the cell suspension of *D. saccifera* with the mycelia, the methodology adapted from Zepeda et al. (2009) was used. 100 mg of sterilized mycelium were used for 2 grams of friable callus, which were added to 250 mL Erlenmeyer flasks with 100 mL of MS medium supplemented with 4 $mg.L^{-1}$ of 2,4-D and 2 $mg.L^{-1}$ of KIN. The evaluated treatments consisted of: 1. Supplemented MS medium + *Xylaria* sp. mycelium (control); 2. Supplemented MS medium + *C. dianesei* mycelium (control); 3. Supplemented MS medium + friable callus (control); 4. Supplemented MS medium + friable callus + *Xylaria* sp. mycelium (elicitation test); 5. Supplemented MS medium + friable callus + *C. dianesei* mycelium (elicitation test) and 6. MS medium (control).

After 30 days of cultivation, the treatments were removed from the shaker (120 rpm, 30 ºC), filtration was performed using a vacuum pump, and the cells were separated from the culture medium, weighed, and dried in a freeze dryer. Subsequently, the material weighed and extracted an ultrasonic bath using methanol (MeOH) in the proportion of 1 g of material for 10 mL of solvent (6 times).

The culture medium obtained from the filtration of control suspensions and elicitation suspensions was lyophilized until reaching a final volume of 5% of the initial volume, then partitioned with the medium polarity solvent ethyl acetate (AcOEt) in proportion 1: 1, for 3 times.

Finally, the extracts and phases were concentrated and weighed, and the final yield of the methanolic extracts was calculated using the formula: % yield = (mass of extract obtained x 100) / mass of dry cells.

2.7. Elicitation of D. saccifera cell suspension with fungal extracts from Xylaria sp. and C. dianesei

20 mg of methanolic extract of *Xylaria* sp. and *C. dianesei* were used for 2 grams of friable callus. The extracts were solubilized in 2 mL of sterile distilled water, filtered using a 0.22 µm millipore membrane and added to Erlenmeyer flasks of 250 mL with 100 mL of MS medium with 4 $mg.L^{-1}$ of 2,4-D and $mg.L^{-1}$ of KIN. Similarly, for the control, 2 mL of sterile distilled water were filtered using a 0.22 µm millipore membrane and added to Erlenmeyer flasks with the same hormonal composition. The suspensions were kept in a shaker at a temperature of 30 ºC. The elicitation test was carried out in triplicate, with the treatments presented in Table 1.

After 30 days of cultivation, the suspensions were removed from the shaker and filtered using a vacuum

Table 1. Elicitation treatments with methanolic extracts of *Xylaria* sp. and *C. dianesei*.

	***Xylaria* sp.**
T1	2 g of friable callus + 20 mg of methanolic extract of *Xylaria* sp. grown in PD medium
T2	2 g of friable callus + 20 mg of methanolic extract of *Xylaria* sp. grown in CZ medium
T3	2 g of friable callus + 20 mg of methanolic extract of *Xylaria* sp. grown in SB medium
T4	2 g of friable callus without added elicitor (friable callus control)
	Colletotrichum dianesei
T1	2 g of friable callus + 20 mg of methanolic extract of *C. dianesei* grown in PD medium
T2	2 g of friable callus + 20 mg of methanolic extract of *C. dianesei* grown in CZ medium
T3	2 g of friable callus + 20 mg of methanolic extract of *C. dianesei* grown in SB medium
T4	2 g of friable callus without added elicitor (friable callus control)

pump, the calluses were weighed, dried in a freeze dryer, weighed again, and extracted six times with methanol in an ultrasonic bath, for a period of 20 minutes each (proportion of extraction 1 g: 10 mL).

Since cells can release metabolites into the extracellular environment, suspension culture media elicited with *Xylaria* sp. and *C. dianesei* were lyophilized until reaching a final volume of 5% of the initial volume, then partitioning was performed with EtOAc in a 1:1 ratio, 3 times. Finally, the extracts and phases were concentrated and weighed, the final yield of the methanolic extracts was calculated using the formula: % yield = mass of extract obtained x 100 / mass of dry cells.

2.8. Chemical analysis of methanolic extracts obtained from D. saccifera callus elicitations

To obtain information about the chemical classes present in extracts from elicitations of *D. saccifera* callus, carried out with the endophytes *Xylaria* sp. and *C. dianesei*, were realized analysis by comparative thin layer chromatography (TLC) and hydrogen nuclear magnetic resonance (^{1}H-NMR).

For TLC analysis, 5 µL aliquots of the methanolic extracts (solubilized in a proportion of 20 mg.mL^{-1}) were applied to silica chromatoplates (Alugram® Xtra SIL G/UV254), eluted with DCM/MeOH 6:4 or DCM/MeOH 9:1. To obtain evidence of the chemical classes present in the samples, the plates were revealed with physical developers: UV 254 and 365 nm; and chemical developers: iodine resublimed, anisaldehyde sulfuric, sulfate ceric, ferric chloride, potassium hydroxide (KOH), NP-PEG and Dragendorff reagent.

^{1}H-NMR analysis was performed on a Bruker Fourier 300 spectrometer, operating at 300 MHz, using 15 mg of sample for 500 µL of DMSO-d_6. Tetramethylsilane (TMS) was used as an internal standard.

3. Results

3.1. Chemical and toxic characteristics of eliciting extracts

The analysis of the elicitated extracts using comparative thin layer chromatography (TLC) demonstrated a similarity among the extracts. Signs of double bonds were observed when revealed in UV light 254 and 365 nm and iodine, and signs of terpenes when revealed in anisaldehyde sulfuric. The samples did not reveal with ferric chloride, KOH and Dragendorff reagent, thus not indicating the presence of aromatics, quinones and nitrogenous substances, respectively.

The ^{1}H-NMR analysis of the elicited methanolic extracts corroborated the TLC analysis, demonstrating similarities between the extracts of both *Xylaria* sp. and *C. dianesei*, regardless of the culture medium used. However, extracts of *Xylaria* sp. presented a greater diversity of signals in the aromatic region (between 6 and 8.5 ppm) compared to *C. dianesei* (Figure 1).

Regarding the toxicity of the eliciting extracts against the bioindicator *Artemia salina*, the extracts of *Xylaria* sp. caused a higher percentage of microcrustacean mortality compared to *C. dianesei* extracts. The lethal doses are presented in Table 2, with an LD_{50} below 100 µg.mL^{-1} considered indicative of high activity (Meyer et al., 1982; McLaughlin et al., 1991).

3.2. Analysis of D. saccifera suspension culture after elicitation

From the experiment, it was noted that the cells in the suspensions elicited with mycelia of *Xylaria* sp. and *C. dianesei* showed a lower percentage of water retention compared to the control. Additionally, the yield of MeOH extracts obtained from elicitation was lower compared to the control (Table 3).

Contrary to what was observed with mycelia, when using the methanolic extract of the endophytes *Xylaria* sp. (cultivated in PD and CZ medium) and *C. dianesei* (cultivated in PD medium) for elicitation, the yield of the methanolic extract from *D. saccifera* callogenic cells was higher than that observed for the control (Table 3). The water content of the cells in the elicited suspensions was not affected by the eliciting extracts.

3.3. TLC analysis of methanolic extracts from D. saccifera suspension after elicitation with Xylaria sp. mycelia and C. dianesei

The TLC analysis indicated similarities between the methanolic extracts from the elicitation with the mycelia (Table 4), suggesting the presence of terpenes and steroids, as verified in the control. The analysis of the EtOAc phase

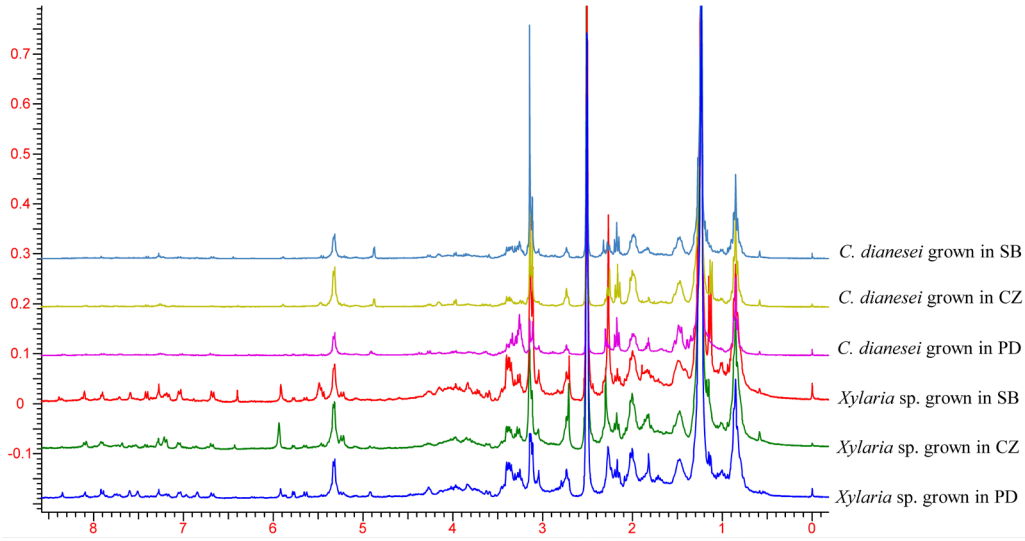


Figure 1. ^{1}H-NMR spectra of endophytic fungi methanolic extracts (300 MHz, DMSO-d_6).

Table 2. Average lethal dose (LD_{50}) of eliciting fungal extracts against *Artemia salina*.

Endophytic fungus	Cultivation culture medium	DL_{50} (μg.mL^{-1})	Toxicity
C. dianesei	PD	> 500	low
	SB	> 1000	low
	CZ	> 1000	low
Xylaria sp.	PD	< 100	high
	SB	> 500	low
	CZ	< 125	high
Control +		< 100	high
Control -		-	-

Table 3. Water content and yield of methanolic extracts of elicited suspensions.

Treatments			Fresh weight (g)	Dry weight (g)	Water content (%)	Mass of MeOH extract (g)	MeOH extract yield (%)	Mass of the medium EtOAc phase (g)
Elicitation with mycelium	*Xylaria* sp.	**T1**	0.281	0.223	20.64	0.033	14.79	0.020
		T2	3.888	0.463	88.09	0.131	28.29	0.024
		T3	2.758	0.599	78.28	0.118	19.69	0.110
	C. dianesei	**T1**	0.348	0.150	56.89	0.001	0.66	0.008
		T2	3.326	0.392	88.21	0.048	12.24	0.019
		T3	2.978	0.625	79.01	0.003	0.48	0.117
Elicitation with MeOH extract	*Xylaria* sp.	**T-PD**	4.020	0.334	91.69	0.161	48.20	0.021
		T-CZ	3.888	0.337	91.33	0.161	47.70	0.039
		T-SB	3.762	0.306	91.86	0.109	35.62	0.070
	C. dianesei	**T-PD**	8.740	0.552	93.68	0.256	46.47	0.022
		T-CZ	4.155	0.330	92.05	0.108	32.98	0.039
		T-SB	7.213	0.553	92.33	0.195	35.34	0.039
Callus suspension without elicitor extract			13.915	1.114	91.99	0.438	39.30	0.058
Medium			-	-		-	-	0.003

Legend: material obtained from cultivation in MS medium with: T1 = mycelium, T2 = friable callus, T3= friable callus + mycelium, T–PD= friable callus + MeOH extract of the endophyte cultivated in PD medium, T–CZ = friable callus + MeOH extract from endophyte cultured in CZ medium, T-SB= friable callus + MeOH extract from endophyte cultured in SB medium.

Table 4. TLC comparison of methanolic extracts and ethyl acetate methanolic phase from the suspension of callus of *D. saccifera* elicited with mycelia of *Colletotrichum dianesei* and *Xylaria* sp.

Revelator	Fungus mycelia					
	MeOH extract			EtOAc phase		
	Eliciation with *C. dianesei*	Eliciation with *Xylaria* sp.	Control	Eliciation with C. dianesei	Eliciation with *Xylaria* sp.	Control
UV light 254 nm	+	+	+	-	+	+
UV light 365 nm	+	+	+	+	+	+
Dragendorff (alkaloids)	-	-	-	-	-	-
Ferric chloride (aromatic substances)	-	-	-	-	-	-
Anisaldehyde (general/terpenes)	+	+	+	+	+	+
Ceric sulfate (terpenes)	+	+	+	+	+	+
NP-PEG (flavonoids)	-	-	-	-	+	+
KOH (quinones)	-	-	-	-	-	-

Legend: (-) = absent, (+) = present, Control: sample without the presence of elicitor.

Table 5. TLC comparison of methanolic extracts from the suspension of callus of *D. saccifera* elicited with extract of *Colletotrichum dianesei* and *Xylaria* sp.

Revelator	Methanolic extract						
	C. dianesei			*Xylaria* sp.			Control
	PD	CZ	SB	PD	CZ	SB	
UV light 254 nm	+	+	+	+	+	+	+
UV light 365 nm	+	+	+	+	+	+	+
Draggendorff (alkaloids)	-	-	-	-	-	-	-
Ferric chloride (aromatic substances)	-	-	-	-	-	-	-
Anisaldehyde (general/terpenes)	+	+	+	+	+	+	+
Ceric sulfate (terpenes)	+	+	+	+	+	+	+
NP-PEG (flavonoids)	-	-	-	-	-	-	-
KOH (quinones)	-	-	-	-	-	-	-

Legend: (-) = absent, (+) = present, Control: sample without the presence of elicitor.

showed similar results to those presented in the analysis of the methanolic extract, displaying bands that suggest the presence of the same chemical classes, such as terpenes and steroids. Therefore, there was no chemical change in the callogenic culture in suspension due to the presence of the eliciting mycelium.

Due to the low diversity of bands observed in the TLC and the limited available mass of the extracts, ^{1}H-NMR analysis was not performed for the methanolic extracts and EtOAc phases, obtained from elicitation with *Xylaria* sp. and *C. dianesei* mycelia.

3.4. TLC analysis of methanolic extracts from D. saccifera suspension after elicitation with methanolic extracts of Xylaria sp. and C. dianesei

To determine whether the higher yield of the extracts obtained from the elicitation was related to an increased production of secondary metabolites, or merely a growth response based on the use of the extracts for primary metabolism, the extracts and the EtOAc phases in the suspension medium were analyzed and compared chemically by TLC and ^{1}H-NMR.

The methanolic extracts of the elicitations showed similarities among themselves and with the control, based on TLC analysis (Table 5). However, when analyzed by ^{1}H-NMR, some differences could be found (Figures 2 and 3). Despites the greater amount of sugar signals in all samples (between 3 and 5 ppm, with the sugar anomeric signals very intense in 4.2 and 4.8 ppm), there are some minor signals visible between 6.0 and 8.0 ppm. This region is characteristic of substances with double bonds, and specially of aromatic substances. It can be seen two signals in 6.2 and 6.6 ppm present in the methanolic extract from callus elicited with *Xylaria* sp. cultured in PD medium and also present in less amount in the methanolic extract from callus elicited with *Xylaria* sp. cultured in SB medium (Figure 2). The rest of the spectra were very similar (Figure 2 and Figure 3).

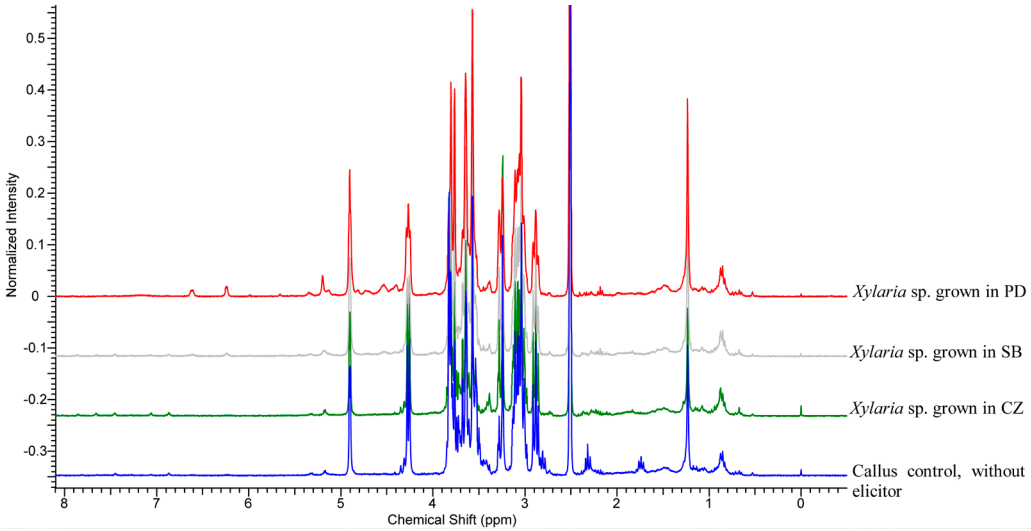


Figure 2. ^{1}H-NMR spectra of methanolic extracts from callus elicited with *Xylaria* sp. grown in different culture media (300 MHz, DMSO-d_6).

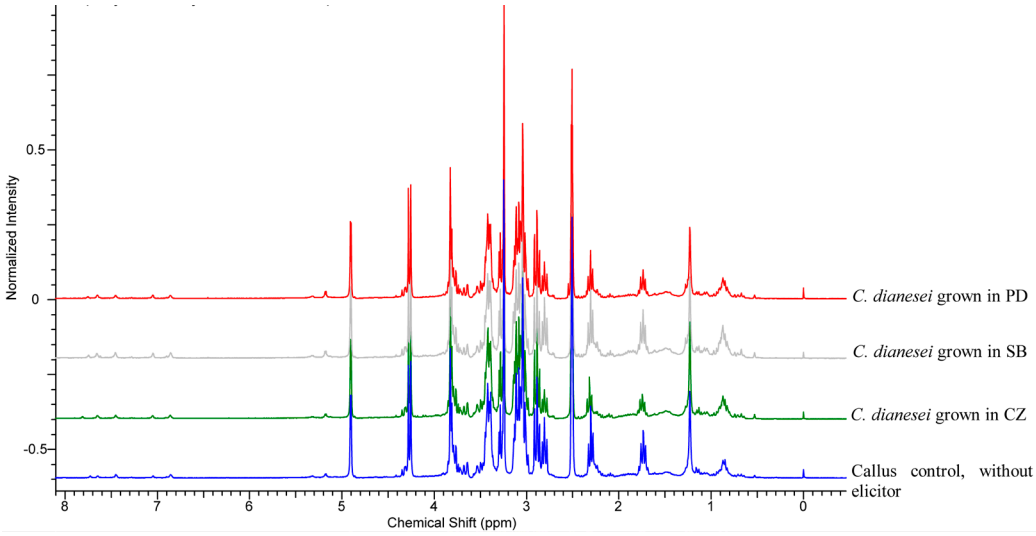


Figure 3. ^{1}H-NMR spectra of methanolic extracts from callus elicited with *C. dianesei* grown in different culture media (300 MHz, DMSO-d_6).

Since metabolites can be excreted into the culture medium, the ethyl acetate phase of the suspension culture medium was also analyzed by TLC. Similar to what was observed in the methanolic extract of the cells, bands were evident that suggest the presence of the same chemical classes, such as terpenes and steroids, although the intensity of production varied. A yellow band (UV light 365 nm) was observed only in the control and in the elicitation with *Xylaria* sp. (rf = 0.47, system: DCM/MeOH 9:1). The EtOAc phase of the elicitation with *Xylaria* sp. grown in PD medium showed more intense bands than the other elicitations and the control, suggesting that the presence of this extract in the culture intensifies the production of metabolites previously produced by *D. saccifera* callus.

4. Discussion

Several studies evaluated elicitation techniques focus on cell suspension culture, observing faster cell growth compared to whole plants. However, certain biochemical characteristics develop only in specific organs or during particular developmental stages, with the accumulation of some metabolites depending on the presence of specific cells and tissues (Wawrosch and Zotchev, 2021).

Although numerous studies demonstrate the efficiency of fungal elicitors, factors such as fungal species, elicitor components, dosage, exposure time, and the characteristics of the elicited culture must be considered (Zhao et al., 2001; Salehi et al., 2020). These findings are corroborated by the

data obtained in this research, which indicate that mycelium caused an inhibition of plant cell growth, whereas the eliciting extracts promoted greater cell growth. Xie et al. (2023) also observed this relationship, noting that the use of the methanolic extract of *Schizophyllum commune* promoted better synthesis of ginsenoside in the hairy roots of *Panax ginseng* than the direct addition of mycelia.

Eliciting fungal extracts can induce gene expression and activate metabolic pathways, thereby increasing the accumulation of metabolites (Hahn, 1996). Furthermore, they can complement the culture medium, serving as a source of nutrients for the development of the cell suspension.

However, a factor that must be considered is the toxicity of the eliciting extracts, which can cause greater stress to the culture and is directly related to the chemical response. Our data suggest this relationship, indicating that the eliciting extract of *Xylaria* sp. cultivated in PD and CZ promoted a better elicitation response, and these were also the most toxic extracts among those evaluated.

It is inferred that the greater toxicity of *Xylaria* sp. extracts may be related to the presence of substances that interfere at the cellular level, such as cytochalasin, an alkaloid present in high concentrations in extracts of this genus, widely known for its cellular toxicity (Cooper, 1987). The toxicity analysis of *C. dianesei* extracts was performed for the first time in this research, and the evaluated extracts did not demonstrate any signs of toxicity.

The chemical classes verified in the elicited cultures have already been described in other studies involving *Duroia saccifera*. In the research conducted by Alzate-Lozano et al. (2020), two steroids were isolated: β-sitosterol and stigmasterol. Additionally, the presence of terpenoids, steroids, and aromatic substances was observed, the same classes also being verified by Souza (2016) and Sousa (2018).

The elicitation of plant cell and tissue culture has become an effective alternative to intensify the production and increase the yield of substances of interest (Souza et al., 2018). In cambial meristematic cells of *Catharanthus roseus*, the presence of the elicitor *Aspergillus flavus* intensified the production of ventoline and catharanthine by up to 1.45 times, and the synthesis of ajmaline by approximately 2.14 times (Liang et al., 2018). When the culture of *Corylus avellana* cells was elicited with extracts and filtrates of *Camarosporomyces flavigenus*, there was an increase in the synthesis of paclitaxel, an alkaloid of significant chemotherapeutic importance (Salehi et al., 2020).

However, this relationship cannot be generalized to all species elicited *in vitro*. In addition to the type of elicitor, Cai et al. (2012) point out that the interference of the elicitor on the culture is primarily affected by the biochemical characteristics of the target plant species, the growth stage of the culture, and experimental conditions. Another factor that can influence the success of the elicitation is the sterilization of the elicitor. Kumar et al. (2012) confirmed this relationship, verifying that the filtrate of the *Piriformospora indica* culture sterilized with a filter presented greater elicitation potential compared to the autoclaved filtrate of the culture.

These results allow us to infer that, similar to *Tinospora cordifolia*, the portions of the mycelial elicitor that would act on the *D. saccifera* culture were sensitive to heat and lost their elicitation capacity during the autoclaving process (Kumar et al., 2017). Additionally, prolonged exposure of cultures to the elicitor may lead to the conversion or degradation of substances (Kumar et al., 2012). Therefore, for the *in vitro* production of metabolites from the elicitation of the cell suspension of *D. saccifera*, new approaches must be considered, such as evaluating different doses of the elicitor, exposure time, and the growth phase of the culture.

5. Conclusion

Corroborating the literature on fungal elicitation, this research found that the presence of the fungal elicitor did not significantly alter the chemical production of the culture but intensified the chemical classes previously produced by the species. Only the callus elicited with *Xylaria* sp. cultured in PD medium yielded to the production of different aromatic substances did not present in callus control, also produced in less amount by the callus elicited with *Xylaria* sp. cultured in SB medium.

Among the fungal elicitors evaluated, the methanolic extract of *Xylaria* sp., regardless of the growth medium, promoted greater diversity and signal intensities in the region of aromatic hydrogens. It was also the most toxic extract in relation to the bioindicator *Artemia salina*, suggesting a possible relationship between the toxicity of the elicitor and the elicitation response.

Therefore, the data obtained provide new information about the cell culture of *D. saccifera* and its response to one of the commonly used elicitation techniques. Further investigations should be carried out to establish the ideal elicitation period and to isolate the produced substances.

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