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Agroindustrial residues exploration to maximize metabolites production in fungal biocontrol co-culture

Exploración de residuos agroindustriales para maximizar la producción de metabolitos bio-controladores en un co-cultivo fúngico

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ABSTRACT

Agroindustrial residues are generated during several manufacturing procedures, representing an environmental concern, nonetheless, also is an ideal material for microbial support, and biotechnological molecule production. Four fungi: *Trichoderma asperellum* (TA), *Trichoderma harzianum* (TH), *Aspergillus niger* GH1 (A.GH1), and *Aspergillus niger* PSH (PSH), were investigated in terms of growth rate, co-culture compatibility, and

antagonistic assays against *Fusarium spp* and *Colletotrichum spp.,* for co-culture design. The viability of peanut shells (PS), orange (OP), and melon peels (MP), for Solid State Fermentation was analyzed by proximate, physicochemical characterization, and growth kinetics. SSF extracts were kinetically obtained and their polyphenolic content and *in vitro* activity against *Colletotrichum spp* were tested. TA-TH and TH-A.GH1 co-cultures enhance the biocontrol character of single strains through higher growth speeds (0.85-1.57 mm/h) and phytopathogen inhibition for volatile (39.5-67.9%) and nonvolatile (66.3-76.7%) compounds. MP and PS promote higher growth speeds. The 36 h PS TH-TA extract had higher antifungal effects (88.8%). MP and TH-TA co-culture promote the higher polyphenolic content of 1.32 ± 0.02 mg GAE/g at 72 h. SSF rise polyphenol content 85.9% in PS, and 11.1% in MP. The fungal co-culture improves the antifungal activities through multiple biocontrol tools. Agro-wastes represent a green solution for BCA propagation.

Keywords: Agro-wastes, biocontrol, co-culture, secondary metabolites, solid state fermentation, *Trichoderma* sp.

RESUMEN

Los residuos agroindustriales se generan a lo largo de diferentes procesos de manufactura, generando una preocupación ambiental, sin embargo, también son un material ideal para el desarrollo microbiano y la producción biotecnológica de moléculas. Cuatro cepas de hongos: *Trichoderma asperellum* (TA), *Trichoderma harzianum* (TH), *Aspergillus niger* GH1 (A.GH1) y *Aspergillus* PSH (PSH), se investigaron en términos de velocidad de crecimiento, analisis de compatibilidad y confrontaciones duales en contra de *Fusarium* spp y *Colletotrichum* spp., para el desarrollo de un co-cultivo. Se caracterizaron tres residuos: cáscaras de cacahuate (PS), naranja (OP) y melón (MP), para su uso en la fermentación en estado sólido, a través de análisis fisicoquímicos y cinéticas de crecimiento. Se obtuvieron extractos a partir de la SSF y se cuantificaron los polifenoles totales y la actividad *in vitro* contra *Colletotrichum* spp. Los co-cultivos TA-TH y TH-A.GH1 mejoran el potencial biocontrolador de los monocultivos, presentando tasas de crecimiento más altas (0.85-1.57 mm/h) y porcentajes de inhibición por compuestos volátiles (39.5-67.9%) y no volátiles (66,3-76.7%). MP y PS promueven velocidades de crecimiento más altas. El extracto TH-TA de PS de 36 h tuvo mayores efectos antifúngicos (88.8%). El co-cultivo de MP y TH-TA libera una mayor cantidad de polifenoles 1.32 ± 0,02 mg GAE/g) a las 72 h. SSF aumentó el contenido de polifenoles en un 85.9% en PS y en un 11,1% en MP. El co-cultivo mejora el potencial antifúngico de las cepas a través de múltiples herramientas de biocontrol. Los desechos agrícolas representan una solución ecológica para la propagación de BCA.

Palabras clave: Biocontrol, co-cultivo, fermentación en estado sólido, metabolitos secundarios, residuos agro-industriales, *Trichoderma* sp.

1. INTRODUCTION

Worldwide food demand promotes exponential growth and agro-industrial residues generation. These residues perform a sustainable strategy to develop green processes

(Freitas *et al*., 2021). Mexico constitutes a country with an elevated number of raw materials employed in multiple industries, generating numerous by-products (Torres-León *et al*., 2018). The food residue generation rises to millions of tons, and their mismanagement provokes soil, water, and air pollution (Ramírez-Guzmán *et al.*, 2018). Nevertheless, they are normally employed in biomaterials elaboration, natural oils and antioxidant extraction, food manufacturing, renewable energy generation, but also fermentable metabolites production (Torres-León *et al.,* 2018). Biotechnology represents an alternative to solve the environmental problems caused by agro-industrial residues (Buenrostro-Figueroa *et al*., 2017; Bloem & Salimi, 2022), allowing the cost reduction by bio-conversion of cheap materials into add-valued components (Yaashikaa *et al*., 2022).

Among biotechnology tools, Solid State Fermentation (SSF), remains an excellent alternative to biotransformation and extraction of interesting metabolites (De la Cruz-Quiroz *et al*., 2018b). SSF involves substrates with low available water which supports a wide range of microorganism growth, where filamentous fungi are strongly adapted (Bibi *et al*., 2023), besides, generates low environmental impact and low costs input, allowing the direct and total employment of agro-industrial residues during proceedings (Leite *et al*., 2021b). SSF enables a wide variety of bio-molecule production and could be potentialized through novel techniques such as co-culture (Leite *et al.,* 2021a).

Trichoderma is a fungal genus with biological controller properties. A biological control agent suppresses plant diseases and also enhances growth and productivity (Joo & Hussein, 2022). *Trichoderma* species employ multiple mechanisms to perform these activities as well as nutritional competition, and secondary metabolites production, nevertheless, lytic activity, mycoparasitism, and antibiosis remain as main mechanisms (Sánchez-Montesinos *et al*., 2021). The secondary metabolites produced by *Trichoderma* include terpenes, pyrones, gliotoxin, gliovirine, and peptaibol, all strongly involved in the antifungal properties against phytopathogenic fungi (Khan *et al*., 2020), while the enzymatic complex produced reaches chitinases, β-glucanases, cellulases and protease which primary help to dissolves pathogenic strains cellular wall, facilitating the mycoparasitism of this species (Maruyama *et al*., 2020).

Further, the genera *Aspergillus* is responsible for producing a wide variety of enzymes, and also it develops in a broad range of temperatures and pH values (Bhavsar *et al*., 2010; Paz-Arteaga *et al*., 2023), making this a key attribute for the biotechnological and biocontrol approach. Both genera could be employed as biocontrol agents, and the SSF represents not only a choice but also a requirement in biopesticide product formulation, and has a positive effect on sustainable agriculture and environmental care (De la Cruz-Quiroz *et al*., 2018a). Nevertheless, emerging researches point to the co-culture application, due to shows a significative impact on metabolism in involved microbial strains, resulting in increased yields, bioactive compounds induction and mechanisms merge, among other activities (Mace & Mills, 2017). *Trichoderma* and *Aspergillus* are two of the most studied fungal genera employed in co-culture works, they are extremely versatile strains. The co-culture allows a decrease in downstream processing and enzymatic mixtures employed, promoting enhancement in bioactive compounds yield, compared with their individual production (Sperandio & Ferreira Filho, 2019). Fungal co-culture employment is easier due to the

immense fungal kingdom diversity and their symbiotic association in natural solid substrates (Lodha *et al*., 2020).
The present work aims the revalorization of orange, melon, and peanut peels, three highly important agro-industrial residues in Mexico by their bio-transformation into added value biotechnological products and their possible effectivity as biocontrol agents employing *Trichoderma* and *Aspergillus* strains co-cultures in SSF.

2. MATERIALS AND METHODS

2.1. Agro-industrial residues sampling

Peanut, orange, and melon were sourced locally from “Mercado de Abastos Benito Juárez” a regional market located in Saltillo, Coahuila (Mexico) PC 25020. The shell/peels were separated and cut into smaller pieces, and then dried at 60°C for 48 h in a convection oven. Samples were ground using a cutting mill SM-100 RETSCH. The particles were sieved into 0.84-0.42 mm and stored in sealed bags protected from light until their usage.

2.2. Agro-industrial residues proximate composition

The proximate analysis for all residues includes crude ashes (A) (AOAC 942.05), determined by incineration at 500 °C per 8 h. Crude protein (CP) (AOAC 991.20 940.25) was analyzed by the Kjeldahl method employing a 6.25 conversion factor. Crude fat (CF) (AOAC 920.39) was calculated by a gravimetric method after fat extraction using the Soxhlet system (AOAC, 1990), and, the neutral detergent fiber method (NDF) was employed to determine cellulose, hemicellulose, and lignin, following the reported by (Van Soest *et al*., 1991). The Total carbohydrates were calculated by de following formula: TC= 100 – (%A + % CP + %CF).

2.3. Tests of support in solid-state fermentation

The water absorption index (WAI), Crític Humidity Point (CHP), and Water activity (A_w) were determined to know if residues are able for a fermentative procedure (Torres-León *et al*., 2019).
For WAI, 2.5 g of sample was dissolved in 30 mL of distilled water and heated at 70 °C for 30 min in a water bath. The obtained paste was cooled at room temperature, transferred to previously weighted tubes, and centrifugated at 3000 rpm for 20 min. The supernatant was decanted and sediments were weighted. WAI was calculated with the following formula:

$$WAI = \frac{Sediment\ weight\ \ (g)}{Dry\ susbtrate\ weight\ (g)}$$

The CHP was calculated in thermobalance (Ohaus MB 23), employing 1 g of WAI-sediment at 60°C, for 120 min.

2.4. Microorganisms

The fungal antagonistic strains: *T. harzianum, T. asperellum, A. niger GH1, and A. niger PSH*, and pathogenic strains: *Fusarium spp.* and *Colletotrichum spp.,* belong to the fungal collection from the Food Research Department of the Autonomous University of Coahuila. All strains were re-activated in potato dextrose agar (PDA).

2.5. PDA radial growth kinetic studies

For the four strains, firstly, kinetic assays were conducted in PDA plates. The center of all agar plates was inoculated with an 8 mm PDA disc of five days of growth fungal mycelium and incubated at 28 °C. The radial growth was measured in mm, each 12 h, employing a vernier caliper, to estimate growth rate (mm/h) a linear growth function was employed. The growth rate is represented by the slope during the exponential microbial growth curve. The experiments were conducted in triplicate and stopped until the agar plate was completely invaded (Ramírez-Guzmán *et al*., 2024).

2.6. Compatibility test

The interactions of four antagonistic strains were analyzed by a dual confrontation assay in Petri dishes with PDA. The plates were inoculated with two 8 mm PDA discs of different fungal strains of five-day growth mycelia. Discs were cultured 5 cm of distance from each other and incubated at 28 °C. The growth rate was calculated.

2.7. Agro-industrial radial growth Kinetic studies

Another kinetic study was conducted over the three agro-industrial residues. The Petri dishes were filled with 8 g of the substrate at 70 % humidity and also inoculated with 8 mm PDA discs, incubated, and measured under the same parameters of previous PDA-Kinetic assays.

2.8. Volatile compounds penetrability assays

The volatile compounds interactions of individually antagonistic fungi and their co-cultures against phytopathogenic fungi *Fusarium sp.* and *Colletotrichum sp*. were studied employing sandwich plate assay (Voloshchuk *et al*., 2024). The studied fungal co-cultures were *T*. *asperellum*- GH1, *T*. *asperellum*- *T*. *harzianum* and *T*. *harzianum*-GH1.The center of the PDA plate was inoculated with 10 µL of fungal spore suspension at 1 x 10^{7} spores/mL, using half of each one for co-culture groups. After, the antagonistic fungi and the selected co-cultures were overlaid with a sterile cellophane membrane. The plate with pathogenic fungi was placed on top of antagonistic plates, sealed with parafilm, and incubated at 28 °C for five days. The controls were plates inoculated with 10 µL of sterile water sandwiched against all phytopathogenic strains. The fungal colony diameter was measured every day, and the inhibition percentage (IP) was calculated.

$$\mathrm{IP} = \frac{\mathrm{C} - \mathrm{T}}{\mathrm{C}} X\ 100$$

Where C is the measurement of the phytopathogenic-control colony and T is the measurement of the phytopathogenic-test colony.

2.9. Co-culture antagonism potential

The antagonistic effect was determined by dual culture assay among individual antagonistic fungal and antagonistic co-cultures against phytopathogenic fungi. PDA plates were inoculated with 1 mL of fungal spore suspension at $1x10^7$ spore/ mL. The inoculum was obtained from five days of PDA culture at 28 °C. The fungal-antagonic inoculum was placed at the end of the plate, avoiding the petri dish edge. In the same plate, the opposite extreme (perpendicular direction) was inoculated with the pathogenic strain with the same inoculum size. The fungal colony diameter was measured every day, and the inhibition percentage was calculated.

2.10. Co-culture solid state fermentation

SSF was carried out in Petri dishes containing 6 g of peanut shell, orange and melon, and peels as substrate. The employed co-cultures were *T*. *harzianum*-*T*. *asperellum* and *T*. *harzianum*-GH1. The inoculum size was $1x10^7$ spore/g substrate, the moisture content was adjusted to 70 % and properly mixed. SSF incubation was carried out at 28 °C and kinetically monitored for 84 h. The extracts were obtained by substrate press.

2.11. *In Vitro* antimicrobial activity assay

The antimicrobial effect of SSF bioactive extracts against *Colletrotrichum sp*. was determined by a microplate test. Briefly, a 96-well microtiter plate was filled with 100 µL of Potato Dextrose Broth and mixed with 100 µL of bioactive extracts, each well was inoculated with 10 µL of pathogen spore suspension at $1x10^6$ spore/mL. The microplate was incubated at 30 °C for 48 h. The extract-pathogen broth was inoculated in PDA plates by microdrop technique (10 µL) and finally incubated at 30 °C for 72 h.

2.12. Determination of total phenolic content

The total phenolic content (TPC) was determined by the Folin-Ciocalteu technique. 20 µL of the extract was mixed with 20 µL of Folin-Coicalteu in a 96-well microplate. After 5 minutes, 20 µL of 0.01 M sodium carbonate was added, and 5 minutes later samples were diluted at 125 µL with distilled water. Absorbance was measured at 790 nm employing a microplate reader. All samples were realized in triplicate and reported as gallic acid equivalents per each 100 g of substrate (Torres-León *et al*., 2019).

2.13. Statistical analysis

All data were performed in triplicate assays with independent samples. The statistical analysis was conducted using GraphPad Software version 8.0.1 (Boston, USA). The results were analyzed using ANOVA, and a post-hoc comparison was conducted with Dunett´s test.

3. RESULTS

Table 1 shows the proximate composition of agro-industrial residues. Peanut shells show higher dry matter and crude fat (4.8%). Orange peels possess elevated carbohydrate percentages (88.6%). Compared with the other residues, melon peels show elevated protein (16.8 %) and ash content (12.2%). The fiber contents are higher in peanut shells (65.2%), followed by melon (23.7%) and orange peels (11.8%).

Table 1. Proximate composition of orange, melon, and peanut peels.

Analysis (%)	Orange peel	Melon peel	Peanut shell
Total dry matter	91.47 ± 1.55^{a}	88.68 ± 1.15^{a}	96.14± 1.37^{a}
Total Carbohydrates	88.63 ± 2.63^{a}	69.37 ± 3.52^{b}	84.50 ± 0.09^{a}
Protein	5.3 ± 2.20^{a}	16.83 ± 2.89^{b}	7.72 ± 0.47^{a}
Crude Fat	1.52 ± 0.50 ^{a}	1.58 ± 0.27^{a}	4.77 ± 0.53^{b}
Ashes	4.52 ± 0.93^{a}	12.21 ± 0.90^{b}	3.01 ± 0.14^{a}
Hemicellulose, cellulose, and lignin	11.76 ± 2.11^{a}	23.7 ± 3.06^{b}	65.20 ± 0.20^{c}

The physicochemical characterization for agro industrial residues is reported in Table 2. Melon peel presents the most elevated WAI (7.99 ± 0.14), while for CHP orange possesses de major percentage.

Table 2. Support-test results.

	WAI	CHP (%)	C/N
Orange peel	5.77 ± 0.24^{a}	59.83 ± 0.93^{c}	16.72
Melon peel	7.99 ± 0.14^{b}	57.73 ± 0.74^{b}	4.12
Peanut shell	5.05 ±0. 24^{a}	48.25 ± 0.67^{a}	10.95

WAI: Water Absorption Index, CHP: Critic Humidity Point, Aw: Water Activity,C/N: Carbon-Nitrogen ratio

The results of the dual culture assay are shown in Fig.1. The assay was performed to analyze the growth speed among the four antagonistic fungi in co-culture, and their possible behavior during their inoculation in the same media.

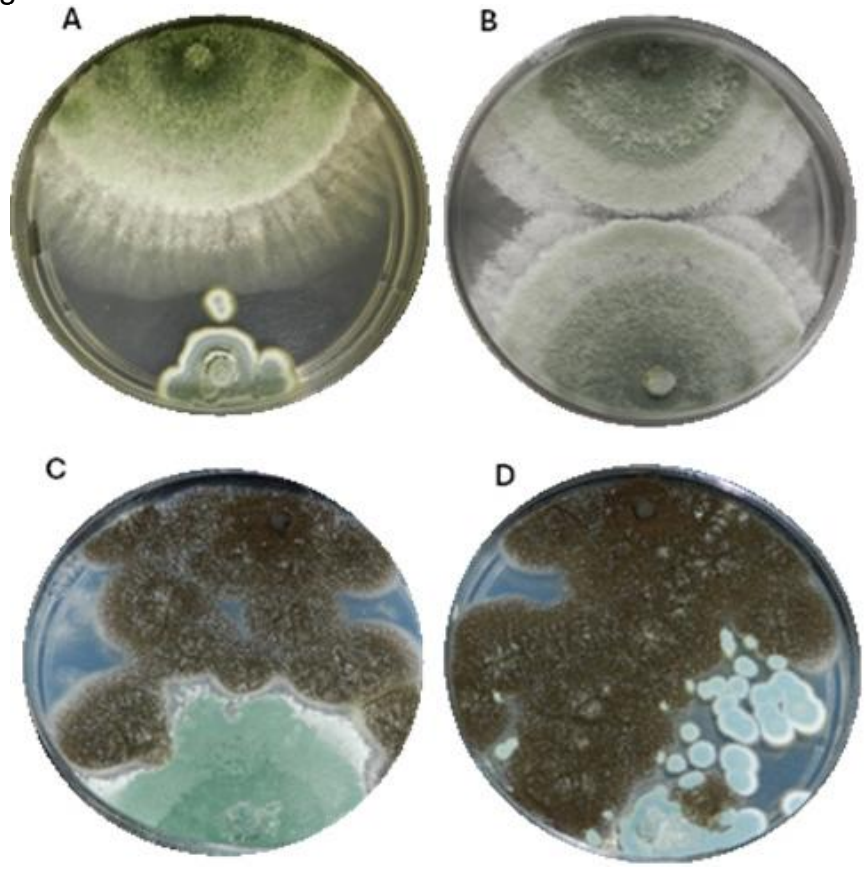

Fig. 1. Compatibility assay in PDA. A= *Trichoderma*-PSH; B= *T. harzianum*-*T*. *asperellum*; C= *T*. *harzianum*-GH1; D= GH1-PSH.

The PDA kinetic study results are reported in Table 3 and employed as a control in the compatibility test. The kinetic study was carried out during 72 h for *Trichoderma* strains and 48 h for *Aspergillus*. All strains start their visible invasion at 12 hours. Among *Thricoderma* species the faster strain was *T*. *asperellum* (0.77 mm/h) and for the *Aspergillus* group, the PSH strain was better (0.98 mm/h). Table 3 shows the growth rate for combined strains. *Trichoderma* strain co-cultures show similar values (0.85-0.86 mm/h), compared with the values obtained by single culture (0.65-0.77). Nevertheless, when confronting other strains the growth rate is higher in all cultures for *T. harzianum* and when *T. asperellum* confronts *A*. *niger GH1*. Also, when the *Aspergillus* PSH strain is confronted with *T*. *asperellum* promotes a speed decrease (0.32 mm/h), compared with the mono-culture velocity (0.79 mm/h). The strains *T.harzianum*, *T*. *asperellum,* and *A. niger* GH1, were better adapted during dual culture assay and selected for co-culture development.

Table 3. PDA compatibility test.

Confronted strains	Growth Rate (mm/h)			
	TH	TA	A.GH1	PSH
TH	0.65 ± 0.01^{a*}	0.86 ± 0.05^{a}	1.57 ± 0.01^{a}	0.60 ± 0.07^{a}
TA	0.85 ± 0.04^{b}	0.77 ± 0.03^{a*}	2.15 ± 0.12^{b}	0.32 ± 0.04^{b}
A.GH1	0.88 ± 0.02^{c}	1.57 ± 0.45 ^{b}	0.83 ± 0.11^{a*}	0.79 ± 0.08^{a}
PSH	1.13 ± 0.05^{c}	0.92 ± 0.03^{a}	2.50 ± 0.62^{c}	0.98 ± 0.20^{a*}

*Control, TH= *T*. *harzianum*; TA= *T*. *asperellum*, A. GH1= *A*. *niger* GH1; PSH= *Aspergillus* PSH.
Means with different small letters (a,b,c) within a column were significantly different compared to the control*($p < 0.05$).

The growth potential over agro-industrial residues for the three selected strains was evaluated, and its results are shown in Table 4. The employed residues were invaded at different speeds. For *Trichoderma* strains, the residues were easier to invade than *A*. *niger* GH1. Peanut shells result in a faster invasion, especially by *T*. *harzianum* and *T*. *asperellum*, with similar growing rates(2.55-2.56 mm/h), being totally invaded at 36 h. Melon peels were the second proficient substrate with higher speeds for *T*. *asperellum* (1.13 mm/h) followed by *T*. *harzianum* (0.89 mm/h), totally invading their plates at 66 and 84 h respectively. Finally, orange peels provoke poor growing conditions and lower speeds, without presenting total invasion.

Table 4. Agro-industrial residues kinetic study results.

Residue	Fungal strains	Growth Speed (mm/h)
Orange peel	*T. harzianum*	0.33 ± 0.01^{a}
	T. asperellum	0.35 ± 0.03^{a}
	A. niger GH1	0.11 ± 0.03^{b}
Melon peel	*T. harzianum*	0.89 ± 0.04^{b}
	T. asperellum	1.13 ± 0.04^{a}
	A. niger GH1	0.13 ± 0.00^{c}
Peanut Sheell	*T. harzianum*	2.56 ± 0.02^{a}
	T. asperellum	2.55 ± 0.02^{a}
	A. niger GH1	0.18 ± 0.00^{b}

Means with different small letters (a,b,c) within a column were significantly different ($p < 0.05$).

The volatile compounds produced by Antagonistic fungi were tested. The assay includes a group of mono-culture samples: *T.asperellum*, T. harzianum, *A. niger* GH1 and a co-culture group: *T.asperellum -T.harzianum*, *T.harzianum-A. niger* GH1 and *T.asperellum- A. niger* GH1. Table 5 shows the inhibition percentages generated by the assay.

Table 5. Inhibition percentage in volatile compound penetrability assay.

Fungal strains	*Fusarium sp*	*Colletotrichum sp*
TA	21.1± 1.4 [a]	18.5 ± 4.2[a]
TH	18.1 ± 4.8[a]	55.0 ± 4.2[b]
A.GH1	20.2 ± 4.5[a]	24.8 ± 0.4[a]
TA- TH	67.9 ± 0.3[b]	39.5± 0.29[b]
TH - GH1	56.4 ± 2.8[b]	55.8 ± 2.1[b]
TA- GH1	51.6 ± 2.9[b]	26.3 ± 3.3[a]

TH= *T. harzianum*; TA= *T. asperellum*, A. GH1= *A. niger* GH1

In respect to *Fusarium sp.,* the co-cultures promote major antifungal volatile compounds production, reaching 51.6-67.9 % of inhibition percentage. For *Colletotrichum sp.,* the higher inhibition percentage reached 39.5-55.8 % of inhibition, the statistical comparison shows that mainly *T. harzianum* is responsible for volatile compounds production.

The assay shows that co-cultures promote a major inhibition against both pathogens. The co-culture TA-TH generates the major inhibition rates (67 %) against *Fusarium sp.*, while TH-A.GH1 maintains elevated inhibition rates after co-culture (55.8%).

Table 6 shows the effect of multiple co-cultures in a confrontation assay during 60 hours. Mono-culture generates above 50 % of pathogen inhibition except for *A. niger* GH1, inclusive pathogen strains growth without effort. During TA-GH1 co-culture antagonistic fungi do not associate, nevertheless the pathogen is inhibited. All co-cultures promote a pathogen inhibition being more than 60 %. In this assay, other classes of metabolites (Non-volatile) are tested.

Table 6. Inhibition percentage in confrontation assay against pathogens.

Fungal strains	*Fusarium sp*	*Colletotrichum sp*
T. asperellum	66.82± 0.28^{b}	60.35 ± 1.13^{b}
T. harzianum	62.47 ± 1.56^{b}	65.77 ± 0.99^{c}
A. niger GH1	28.00 ± 0.28^{a}	29.06 ± 2.40^{a}
T. harzianum - GH1	66.29 ± 0.85^{b}	76.59± 0.07^{d}
T. asperellum- T. harzianum	75.47 ± 0.21^{c}	66.59 ± 2.83^{c}
T. asperellum- GH1	79.71 ± 1.20^{c}	68.94 ± 0.49^{c}

Fungal behavior is variable. The co-culture among both *Trichoderma* strains is a most homogeneous system, growing faster and with major sporulation, despite this, TH-A.GH1 co-culture grows well together but slower, regarding other cultures. TA-GH1 is possibly growing in competition. Some of these results are shown in Figure 2. For this reason, the selected co-cultures were TA-TH and TH-A.GH1, again *T. harzianum* result a key fungal for antagonistic behavivor.

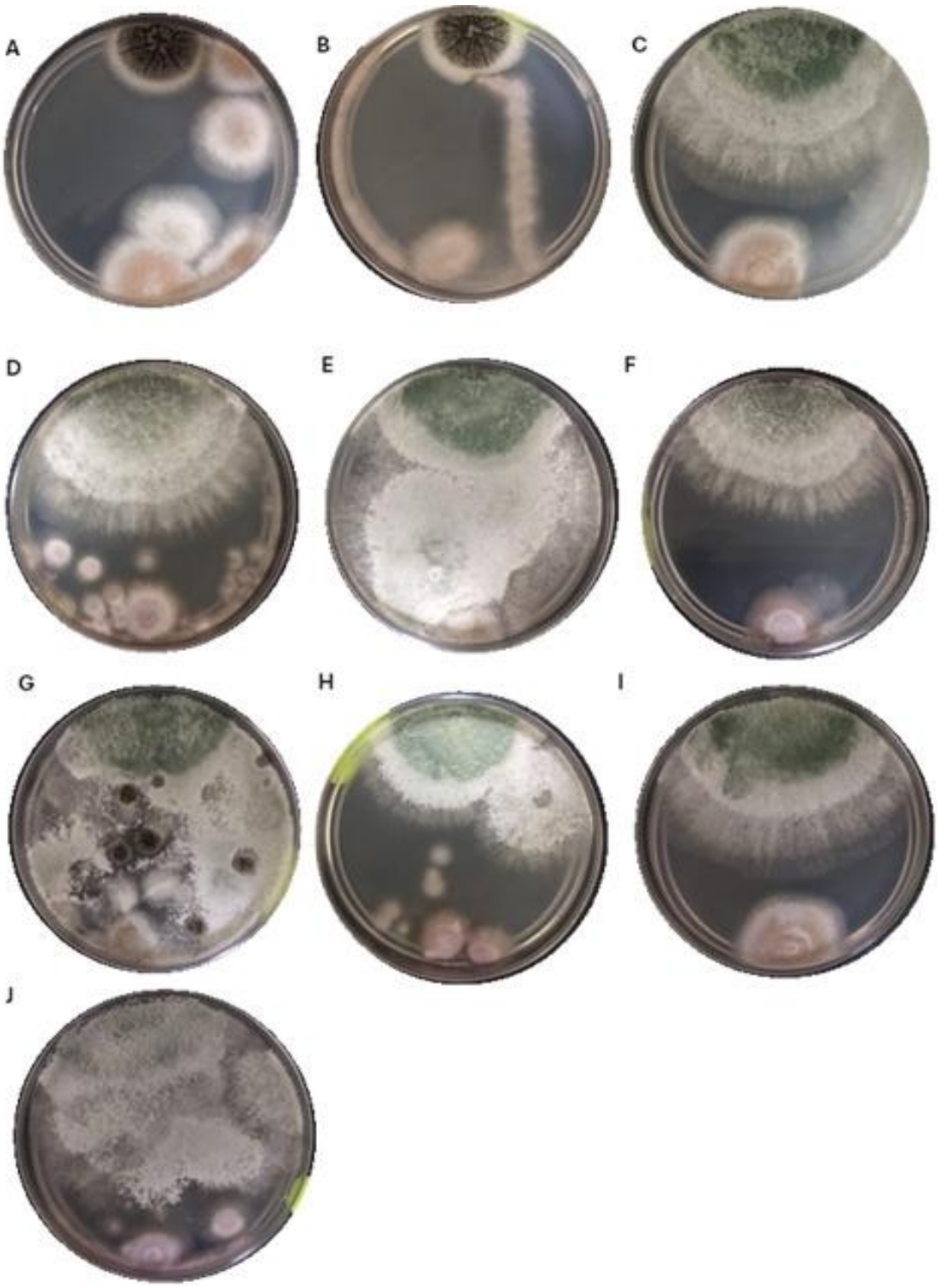

Fig. 2. Pathogen confrontation assay in PDA. A= A.GH1vs*Colletotrichum sp.*, B= A.GH1vs *Fusarium sp*, C= *T*. *harzianum*vs*Colletotrichum sp*, D= *T*. *harzianum*vs *Fusarium sp*, E= *T*. *harzianum-GH1* vs *Colletotrichum sp*, F=*T*. *harzianum*-GH1 vs *Fusarium sp*, G= *T. asperellum*-GH1 vs *Colletotrichum*, H= *T. asperellum*-GH1 vs *Fusarium sp*, I= *T. asperellum*-*T. harzianum* vs *Colletotrichum*, J= *T. asperellum*- *T. harzianum* vs *Fusarium sp*.

The bioactive extracts generated during SSF were tested against *Colletotrichum sp*. and Table 7 shows the results. Major inhibition percentages (88.84 %) are generated in Peanut shell Fermentation at 36 h by TH-TA co-culture. In all fermentative procedures during 36 and 48 h, the inhibition percentages are higher with better results using TH-TA co-cultures in both residues.

Table 7. Inhibition percentages for SSF bioactive extracts against *Colletotrichum sp.*

Time (h)	Melon peel		Peanut Shell	
	TH-TA	*TH-A.GH1*	*TH-TA*	*TH-A.GH1*
0	22.28 ± 0.32	7.65 ± 0.06	20.86 ± 0.68	14.31 ± 0.51
24	60.24 ± 0.18	57.02 ± 0.21	59.56 ± 0.26	62.74 ± 0.15
36	78.62± 0.65	76.47 ± 0.70	88.84± 0.31	74.84± 0.50
48	68.90± 0.79	67.51 ± 0.25	72.22 ± 0.84	81.66 ± 0.15
60	60.90 ± 0.63	59.12 ± 0.27	75.26 ± 0.20	59.64 ± 0.32
72	51.26 ± 0.50	51.26 ± 0.27	63.31 ± 0.15	57.02 ± 0.35
84	54.05 ± 0.46	46.28 ± 0.50	53.09 ± 0.12	39.02 ± 0.31

TH (*T.harzianum)*, TA (*T.asperellum*), A.GH1 (*Aspergillus GH1*)

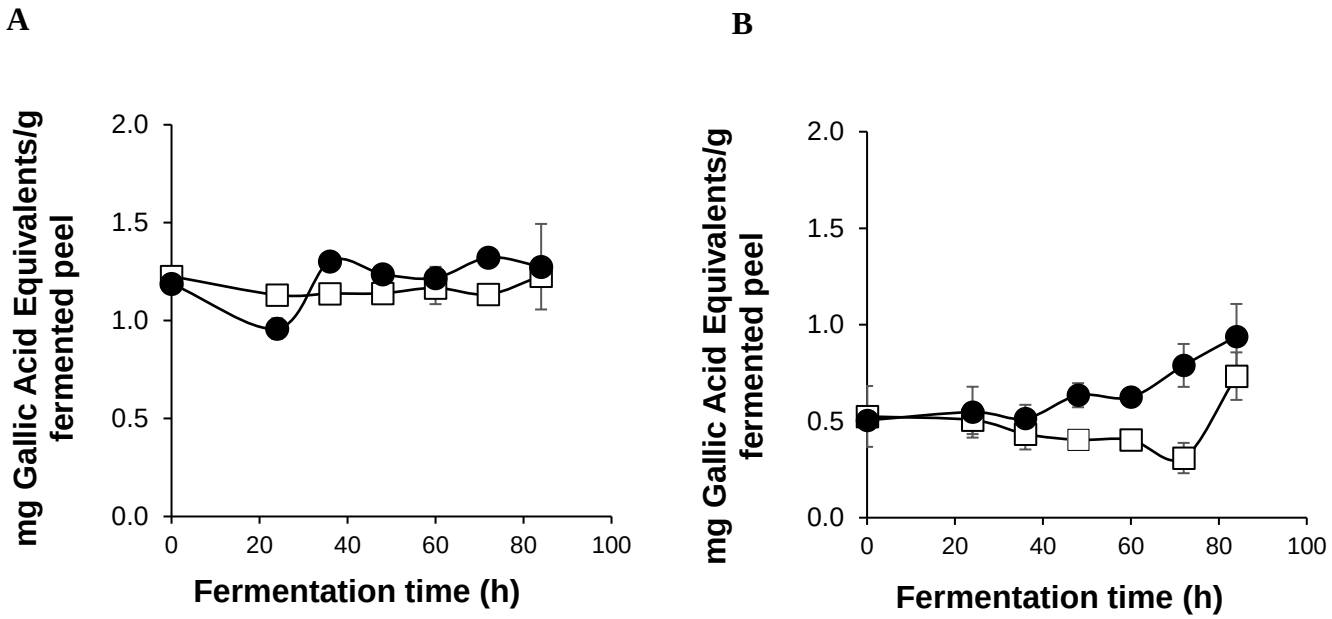

Fig. 3. Polyphenolic content during *SSF.* (A) Melon peel SSF and (B) Peanut shell SSF. *T. harzianum-T. asperellum* (full circle), *T. harzianum-A. niger* GH1 (empty square).

The polyphenolic content determined by the Folin-Ciocalteu method is shown in Figure 3. Melon peel shows higher polyphenolic content than peanut shell. SSF enhances the polyphenolic content through fermentation time. In both cases, *T.asperellum*-*T.harzianum* co-culture allows major polyphenol-release. In melon peel, extracts show higher polyphenolic contents at 72 h, with 1.322 ± 0.021 mg GAE/g fermented substrate, followed by 38 h extracts with1.303 ± 0.045 mg GAE/g. For Peanut shell, at 88 h the extracts present 0.939 ± 0.168 mg mg GAE/g. Peanut peel SSF rises 85.9% polyphenol content, while melon peel increment 11.1%.

4. DISCUSSION

The chemical composition in agro-industrial wastes employed during SSF is very important, due to its constituents supporting the microbial nutritional requirements. Generally, agro-industrial residues composition includes soluble and complex sugars, proteins, lipids or mineral compounds, which are employing by microbial as carbon, nitrogen and mineral support for their metabolism (Šelo *et al.*, 2021), and also act as physical support (Angel-Cuapio and Loera, 2016).

Other authors report similar total carbohydrates in Peanut shells (87.3%) (Abid et al., 2024), Orange (86.53 %) (Ohara *et al*., 2015), and melon (54.5-66.0%) peels (Hussain *et al*., 2024). Melon peels show higher protein (4.1-5.0%) and ash (6.1-8.4%) content, than in other reports (Hussain *et al*., 2024). Peanut shells also show elevated crude fat amounts (1.2-1.7%) (Zuo *et al*., 2018; Abid *et al*., 2024).
For NDFs (Cellulose, hemicellulose, and lignin) content, in Peanut shells is elevated and close to previous analysis (68%) (Abid *et al*., 2024), while in orange peel the values are reported highly variable (7.4-16.3%) (Ricci *et al*., 2019; Olowu & Firincioglu, 2023), and near to other melon wastes (15.4-44.7%) *(*Rodríguez-Luna *et al*., 2022; Kazemi, 2023). Proximate composition differences must be regarded to vegetal variety, ripening stage, and climatic, and agronomic factors *(*Torres-León *et al*., 2016).

The composition of the substrate is directly correlated with its potential for SSF employment. Despite the quantity of main nutrients as carbon or nitrogen source, the relation between the two main nutrient sources also influences microbial growth, being represented by the C/N ratio.
The C/N ratio determines the availability of carbon relative to the availability of nitrogen in organic materials (Grunert *et al.*, 2016). The C/N ratio during fermentative procedures normally influences the effectiveness of materials biotransformation by microorganisms, and is dependent on the class of the searched bio-product and microbial dependent (Rousk & Bååth, 2007, Gao *et al*., 2007), including sporulation, biomass, enzymes or secondary metabolites production.

Some authors mention that C/N among 10 to 20 supports the fungal growth (Gao *et al*., 2007), and close to 15 promote enzyme production (Leite & Salgado, 2021), but also dependes in the class of enzyme, for example proteases or lipases are better produced in lower C/N (4-12) ratios (Oliveira *et al.*, 2017, Qazi *et al*., 2008), the C/N ratio could vary especially when the product is associated or partially associated to to fungal growth

(Krishna, 2005). The involved substrates satisfy this important nutritional parameter, taking into account that a biocontrol class of substrates could improve different biocontrol strategies as competency, secondary metabolites production or enzymes production.
One concern during Agro-industrial wastes use in SSF is the variability generated by multiple agronomic factors, which also could be compensated by C/N adjustment using substrates mixtures (Leite and Salgado, 2021).

The SSF allows microbial growth under low free water values. All support tests provide a measure of possible water physicochemical behavior in employed residues. WAI values represent the estimation of water that can be absorbed by agro-industrial waste (Torres-León *et al*., 2019). Elevated WAI values are recommended during SSF-substrate selection, making moisture control feasible over the process (Robledo *et al*., 2008). (Orzua *et al*., 2009) select the agro-industrial residues with WAI-values under 4 for SSF. WAI values depend on hydrophilic compounds and water-holding capacity molecules in substrates (Buenrostro-Figueroa et al., 2014). A fiber-rich residue sugar cane bagasse (WAI= 9.46) generates higher biomass content than candelilla stalks a wax-rich plant (WAI= 3.14), under SSF, the inverse behavior is expected for CHP (sugarcane bagasse=12 and candelilla stalks=29.5) (Buenrostro-Figueroa *et al.*, 2014), being lowest CHP values a properly contribution to microbial develop, indicating the quantity of water bonded to material and that is not available for microorganisms (Torres-León *et al*., 2019). The physicochemical character in wastes strongly influences microbial successful invasion and behavior during SSF. For residues employed in SSF, it is recommended WAI values higher than 4.00 (Orzua *et al*., 2009), with the lower CHP % possible. Some authors use substrates with CHP values near to 55.6 % (pineapple residues) (Paz Arteaga *et al*., 2023) and 56.5% (mango seed residues) (Torres Leon *et al*., 2019), being successfully employed by fungal strains during SSF procedure. The peanut shell, the orange and melon peels satisfy these parameters being suitable for SSF processing.

During Mendarte-Alquisira *et al.*, 2024 work, PDA-growth kinetics of diverse *Trichoderma* strains were analyzed, during the study the strain Trich CP038 (*T*. *harzianum*) showed a growth rate ∽0.5 mm/h, in the other hand, the reports made by Umaña-Castro *et al*., 2019, reveals 0.87 mm/h rate for *T*. *asperellum*, for *A*. *niger* (Ubogu et al., 2015) reports 0.29 mm/h and 0.39 mm/h for *A*. *fumigatus* during (Gabiatti *et al*., 2006) experiments. The growth rates in PDA for multiple strains are reported with highly variable values, Guigón-López *et al*., 2010 research point out that this measure is a good estimate for antagonistic capacity, during the study multiple *Trichoderma* strains are evaluated, and observed that the warm-weather isolated fungi exhibit a faster growth, data also supported by calorimetric analysis, and showing a higher optimal temperature for growth.

The growth speed in fungal species is closely related to the media components (Acosta-Urdapilleta *et al*., 2016), but also the elevated growth rates of co-cultures during the compatibility test are related to synergism among strains, especially a complementary enzyme or metabolite production, derived from a chemical signal exchange among microbials through the media (Copete-Pertuz *et al*., 2019), in the contrary case, the *Aspergillus* PSH dual cultures, presents lower growth, conduct linked to nutritional competition or secondary metabolites production.

During radial growth kinetic in corn-cob, *T. harzianum* show 0.281 mm/h, 1.526 mm/h, and 1.622 mm/h for *A*. *niger* NRRL3 and GH1 respectively, nevertheless, during that study the residue was impregned with Mandel's medium, and 75 % humidity (Boggione *et al*., 2020), other *A. nige*r strain show 0.224 mm/h in green tea residues, 0.067 mm/h for coffee pulp, for *T. asperellum* no growth was measured in tea and 0.156 mm/h in coffee pulp, all experiments were conducted under same conditions in present work (Saldaña-Mendoza *et al*., 2021).

Agro-industrial wastes are not only physical support, nonetheless, the composition of the residues is closely related to faster microbial growth, orange peel shows, lower hemicellulose, cellulose, lignin, and protein content, and also presents elevated CHP contents which do not promote microbial development. Great quantities of agro-industrial waste are rich in lignocellulosic materials, and this character makes it feasible for SSF, also *Trichoderma* and *Aspergillus* species are reported as important hydrolytic enzyme producers which could help to generate an optimal substrate invasion at higher velocities, mainly cellulases, xylanases, exo-polygalacturonases, β-glucosidases, and laccases (Diaz *et al*., 2016, Carrillo-Nieves *et al*., 2020, Leite and Salgado, 2021), but depending on the residue nature also proteases, lipases, or even tannases, eligatannases, and arabinofuranosidases could be produced (de Castro and Sato, 2015, Amaya-Chantaca *et al*., 2022).

The antagonistic effect in Biocontrol Agents (BCAs) is mediated by different strategies, including volatile compounds (VCs). Among *Trichoderma* species diverse VCs have been reported as tools during the pathogens control. The study of (Chávez-Avilés *et al*., 2024) reports *Trichoderma atroviride* IMI206040 and *T*. *asperellum*, VC-mediated inhibition of 47.4 % and 42.1 % against *Colletotricum acutatum* in PDA media, the assay also has been carried out in Luria Bertani media (LBm) integrated by peptone, yeast extract, and NaCl, analysis shows that LBm promotes higher inhibition percentages (56.9 %) by other *Trichoderma* sp. strain. The BCAs activities are strain- and medium-specific. In the same study the VCs were identified, and for individual *Trichoderma* species terpenes, ketones, and heterocyclic compounds are abundant, nevertheless, other classes are also present (alcohols, aromatic compounds, carboxylic acids, esters, ethers, thiocyanates, thiols, and non-identified compounds). For *C. acutatum*, the most abundant VCs are organo-sulfurous (56.5%) and unknown compounds (35.3%). During confrontation analysis, the VCs are mainly ketones, but their variability is reduced. Authors suggest that *Trichoderma* species modulate their metabolism to the larger group of antimicrobial molecules and mainly to 6-Pentyl-2H-pyran-2-one (the most abundant molecule). Multiple authors mention that during the non-controlled environment, the microbial community is complex, and the production of this class of metabolite can help to shape their own environment, nevertheless, these interactions remain low-explored. The VCs are especially effective due to their low molecular weight which easily could cross the environment and affect fungal pathogens, being the endophytes a vital source of this class of compounds (Rangel *et al.*, 2021).

The Voloshchuk *et al*., 2024 work shows single-analyzed *Trichoderma* strains inhibition percentages against *Aspergillus parasiticus* (38-40 %), and *Aspergillus flavus* (40-45 %) in dual confrontation tests, while (Rajani *et al*., 2021) reports values > 80% inhibition in *Fusariun oxysporum* from *Trichoderma longibrachiatum*, *Trichoderma pleuroti,* and *T. harzianum*., under the same class of studies. During dual confrontation assay, several BCA-

pathogen interactions are developed including mycoparasitism, nutritional stress, or antibiosis, the pattern in microbial growth could help to obtain information about it. Voloshchuk *et al*., 2024 points that *Trichoderma* and *A. parasiticus* dual confrontation shows a parasitism behavior, whereas an antibiosis mechanism is driven by *Trichoderma* sp. and *A. flavus* interaction; on the other hand, for *F. oxysporum* during dual confrontation in Rajani *et al.*, 2021 experiments for *Trichoderma spp.* the interactions with pathogens were predominantly mycoparasitic. The authors link the antibiosis effect to the possible aflatoxin production by *A. flavus*. Both mechanisms could be emphasized especially during developed co-culture, taking advantage of the antibiosis effect from *Trichoderma sp.* species and broadly enzymatic tools from *A. niger* GH1, observable behavior in Fig.2 (*A. niger* GH1 overgrowth), raising the inhibition average. Different reports suggest that Co-cultures enhance the antimicrobial molecule production in microbial species, Nicault et al., 2021 research demonstrates that the compounds diffused in PDA-medium by the dual- culture of different *Streptomyces* sp. strains (S1D4-11 and S1D4-23) with basidiomycete *Schizophyllum commune* 6601-A, generates > 80 % of inhibition in *Bacillus subtilis* ATCC6633 growth, compared with the 0-20 % of inhibition of mono-cultures. If well, co-cultures could be designed with different classes of microorganisms, but fungal-fungal co-cultures remain low explored (Tironi *et al*., 2024).

The fermentative process allows the exploitation of BCAs benefits previously studied. Li *et al.*, 2024 employs a casein-glycerol liquid co-culture between *T*. *harzianum* and *Burkholderia vietnamiensis*, and reveals that the fermentation filtrate generates a 91.4% inhibition rate against *Colletotrichum siamense* in *PDA*, compared with 11.9 % and 43.0% from bacteria and fungi mono-culture. The same filtrate was directly sprayed into *C. siamese*-infected and detached strawberry leaves and the anthracnose symptoms were significantly reduced <2 mm radio, compared with ∽ 8 mm (water-control)< ∽7 mm (fermentation broth-control)< ∽6 mm (*B*. *vietnamiensis* mono-culture)<∽4mm (*T. harzianum* mono-culture). The authors also carried out green-house experiments and co-culture broth (including biomass) enabling longer roots and major chlorophyll content in leaves. The incorporation of biomass and co-culture extracts also promotes plant- responses. Yang *et al.*, 2018 employs a rice-based media with a co-culture among *Aspergillus sydowii* and *Penicillium citrinum* promoting novel citrinin-analogs production, with antimicrobial effect.

Co-culturing interactions promote a “turn-on” in alternative metabolomics pathway, being identified until 30-40 biosynthetic gene clusters for unknown metabolites only for *Aspergillus* sp., and achieving a wide and novel chemical diversity to explore (Knowles *et al*., 2022), some studies show that co-culturing in *T. asperellum* lead to activate genes specially involved in secondary metabolism, sporulation, and enzymes related with myco-parasitism and plant growth (Karuppiah *et al*., 2019).

The antifungal potential in SSF-extracts against *Colletotrichum sp*. Is derived from different strategies, mainly antibiosis, and enzyme secretome, but released antioxidants also can take part due to the substrate nature.
The search for novel microbial components exploring fungal-fungal co-culturing is an interesting and emerging approach, being fermentative procedures as a primary tool, nevertheless, the achieved studies in SSF are reduced, and commonly realized in PDA

media or rice. Xu *et al*., 2023, despite that secondary metabolite production is highly influenced by culture media.

Hussain *et al.*, 2024 show a TPC in non-fermented peels of 1.68 mg GAE/g in muskmelon and 0.99 mg GAE/g in canary melon. Also, for non-fermented Peanut shells, Abid *et al*., 2024 reports 0.8 mg GAE/g. The initial values obtained during the present work are different from previous reports, and the variability could related to fruit characteristics (crop or climate conditions, ripening stage, and fruit varieties), but also by extraction conditions (solvent and solids proportion). Regardless of that, polyphenolic content presents changes during SSF. Torres-León *et al*., 2019 Reports 32. 9 mg GAE/g of TPC at 24 h during SSF of mango seed using *A. niger* GH1, compared to 9.9 mg GAE/g from the un-fermented substrate, increasing 3.23 times the polyphenolic content, Paz-Arteaga *et al*., 2023 reports ∽3.5 mg GAE/g TPC in pineapple peels at 32 h in SSF using *A. niger* GH1, compared with ∽2.0 GAE/g from non-fermented peels. Xue *et al*., 2022 realize a mixed fermentation employing *Trichoderma reesei* and *A. niger*, using orange peels as substrate, they observe that at 168 h in SSF, TPC rise at 7.0 mg GAE/g compared with 1.2 mg GAE/g from the initial system.

Polyphenols are plant secondary metabolites distributed in entire vegetal biomass and could be found free, esterified, or in insoluble-bounded forms. The insoluble forms are found in great proportions (20-60 %) and are covalently bound to cell wall components including pectin, cellulose, arabinoxylan, or structural proteins through ester, ether, or carbon-carbon bonds (Shahidi & Yeo, 2016). During SSF, microbial enzymes such as xylanases, cellulases, β-glucosidases, polyphenol oxidases, tannases, and α-L-arabinofuranosidases, help cell wall degradation, and subsequent polyphenolic content release at the same time, microorganism could degrade this class of compounds and use them as a carbon source for its development (Torres-León *et al*., 2019; Amaya-Chantaca *et al.*, 2022; Paz-Arteaga *et al*., 2023), during present work the peanut shells shows important polyphenolic enrichment trough fermentative proccedure, being *Aspergillus sp.* and *Trichoderma sp.* co-culture the main reason of it. Both, are groups of microorganisms with prominent enzymatic tools and their cooperation during SSF enhances an extended and synergic cell wall hydrolysis and polyphenolic release.

5. CONCLUSION

The study helps to design a co-culture employing *Trichoderma sp*.- *Aspergillus sp.* strains. The co-culture stimulates through different biocontrol mechanisms a clear effect against phytopathogenic fungi, and could successfully develop over melon peels and peanut shells by solid-state fermentation, additionally improving a polyphenolic content release and maintaining strong antifungal activities. The above-employed strategies could help to explore the novelty fungal-fungal interactions and develop an eco-friendly alternative for biocontrol agent exploitation, directly employed or in biopesticide formulation. Besides this, the benefits under greenhouse and field conditions would generate key contributions to the present study.

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AUTHOR CONTRIBUTION

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this work.

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