UNIVERSIDAD REGIONAL AMAZONIA IKIAM

FACULTAD DE CIENCIAS DE LA VIDA

CARRERA INGENIERÍA EN BIOTECNOLOGÍA

EVALUACIÓN ANTIFÚNGICA DE *Trichoderma* spp. AISLADAS DE CULTIVOS DE PITAHAYA AMARILLA (*Selenicereus megalanthus*) PARA EL CONTROL DEL FITOPATÓGENO *Alternaria* spp.

Proyecto de investigación previo a la obtención del Título de:

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AUTOR

ODALIS MAHOLI JIMENEZ GUAMAN

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2023

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0920360898

DEDICATORIA

A los docentes que me guiaron y proporcionaron sus conocimientos durante mi trayectoria académica.

A mis padres, hermanos y familiares cercanos quienes me motivaron con su ejemplo de perseverancia y trabajo constante.

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RESUMEN

La pitahaya amarilla es un cultivo de importancia nutricional y económica; sin embargo, este puede ser susceptible a hongos fitopatógenos. El género *Alternaria* spp. es uno de los principales patógenos de este cultivo y controlarlo sin emplear fungicidas químicos representa un reto para la agricultura. Se ha propuesto el uso de cepas de *Trichoderma* spp. como una alternativa sostenible para control de este patógeno en la pitahaya amarilla. Los objetivos de este estudio son evaluar In vitro la capacidad antifúngica de seis cepas de *Trichoderma* spp. frente a dos cepas de *Alternaria* spp. ambas nativas de cultivos de pitahaya amarilla ubicados en la provincia de Morona Santiago, Ecuador e identificarlas molecularmente. Para la identificación molecular a nivel de especie se amplificaron 5 regiones distintas. Los ensayos de antibiosis se realizaron empleando extractos de *Trichoderma* spp. en concentraciones del 5%, 10% y 15% y los de antagonismo mediante cultivo dual. Mediante identificación molecular se determinó que dos cepas correspondían *a Trichoderma asperellum,* cuatro a *Trichoderma koningiopsis* y los dos restantes a *Alternaria burnsii* y *Alternaria alternata.* En la evaluación antifúngica se observaron diferencias significativas ($P<0.0001$) entre los tratamientos realizados. El mayor porcentaje de inhibición en antibiosis lo tuvo la cepa MS-P-03 con un 48% y en antagonismo la cepa MS-P-07-1 con el 79,4%. En conclusión *T. asperellum* y *T. koningiopsis* tienen la capacidad de controlar el crecimiento de *A. burnsii* y *A. alternata.*

Palabras clave: Antagonismo, Controlador biológico, Antibiosis, *Trichoderma* spp., *Alternaria* spp.

ABSTRACT

Yellow pitahaya has a high nutritional value and economic importance but can be susceptible to pathogenic plants. The genus *Alternaria* spp. is one of the primary pathogens for this crop, and its management without agrochemicals represents a challenge to agriculture. The use of *Trichoderma* strains has been proposed as a sustainable alternative for the control of this pathogen in yellow pitahaya. This study aims to evaluate In vitro the antifungal capacity of six strains of native *Trichoderma* spp. against two strains of *Alternaria* spp. native to yellow pitahaya cultivars located in Morona Santiago province, Ecuador and to identify them molecularly. For molecular identification at the species level, 5 different regions were amplified. Antibiosis assays were performed at 5%, 10%, and 15% concentrations. Antagonism assays were performed by dual culture. According to molecular identification, it was determined that two strains corresponded to *Trichoderma asperellum,* four to *Trichoderma koningiopsis,* and the remaining two to *Alternaria burnsii* and *Alternaria alternata.* Significant differences ($P<0.0001$) were observed in the antifungal evaluation between the treatments. The highest percentage of inhibition in antibiosis was obtained by strain MS-P-03 with 48% and in antagonism by strain MS-P-07-1 with 79.4%. In conclusion, *T. asperellum* and *T. koningiopsis* species can control or reduce the growth of *A. burnsii* and *A. alternata.*

Keywords: Antagonism, Biological controller, Antibiosis, *Trichoderma* spp., *Alternaria* spp.

1. INTRODUCTION

In Ecuador, the yellow pitahaya (*Selenicereus megalanthus*), is known originally as Pitahaya Amazónica de Palora, thus acquiring a unique sense of identity and belonging(Yadira et al. 2020). It is a tropical fruit with great acceptance in national and international markets. It is considered a functional food for its excellent flavor, appearance, quality, and nutraceutical properties (Verona-Ruiz et al. 2020). Ecuador has approximately 1 528 hectares of pitahaya with an average yield of 7.6 t/ha (Yadira et al. 2020); however, like all crops, it is susceptible to diseases caused by various pathogenic plants(Gupta et al. 2015).

Currently, 17 genera and 25 species of plant pathogenic fungi are known to produce diseases in the stem, flowers, and fruits, causing damage to crops (Balendres & Bengoa, 2019). One of the typical diseases of *S. megalanthus* is brown rot, caused by the genus *Alternaria* (Vilaplana and Valencia 2017), which is characterized by causing spots on cladodes, as well as rotting and discoloration in many parts of the plant, causing up to 80% of damage to the crop, decreasing its commercial value and generating significant economic losses (Companioni, González Barbarita, Domínguez Arizmendi and García 2019).

The control of this phytopathogenic fungus is a highly relevant issue because of the low efficiency and little specificity of agrochemicals, causing health issues and environmental damage (Rey et al. 2000). For this reason, it is necessary to address alternatives to ensure sustainable agriculture (Companioni, González Barbarita, Domínguez Arizmendi and García 2019).

One alternative is the use of biocontrol microorganisms, which are characterized by improving plant tolerance to abiotic stress caused by abrupt variations in temperature, pH, salinity, water stress, and nutrient shortages in soils, or to biotic stress caused by phytopathogenic organisms such as pests, bacteria, and fungi (Abiala et al. 2013; Olawuyi et al. 2014; Asemoloye et al. 2019).

Its application decreases genetic variations associated with plant pathogen resistance due to inappropriate pesticide use (Arthurs and Dara 2019). Microorganisms categorized as biocontrol employ different mechanisms such as competition, parasitism, antibiosis,

and systemic resistance (Kuć 2001; Benítez et al. 2004; Haggag and Mohamed 2007; Viterbo et al. 2007; Purin and Rillig 2008). Some microscopic fungi are commonly used for the biological control of plant pathogens. There are various species or genera, such as *Paecilomyces variotii*, *Penicillium chrysogenum*, *Penicillium spinolosum, Penicillium oxalicum, Cunninghamella sp., Absidia* spp.*, Thermoascus aurantiacus, Thermoascus aurantiacus, Fusarium sp., Rhizopus sp., Beauveria* spp. *and Metarhizium* spp.(Rajkumar et al. 2010; Diánez et al. 2018). Among them, the genus *Trichoderma* stands out, as it is the most relevant alternative in the agricultural sector for biologically controlling pathogenic plants (Gupta et al., 2015).

The *Trichoderma* genus has the capacity to function as a biocontrol organism due to its ability to parasitize fungi and inhibit the development of diverse pathogenic plants (Hernández-Melchor et al. 2019). Secondary metabolites of *Trichoderma* spp. are excreted at the rhizosphere level, generating chemical variations in the soil and changes in the rest of the rhizosphere microbiota (Zhang et al. 2018). *Trichoderma* spp. can also increase the availability of Mg and Fe nutrients and stimulate plant growth by releasing phytohormones such as indoleacetic acid (IAA) (Tucci et al. 2011). Indoleacetic acid (IAA) is essential for the plant to carry out various physiological processes such as cell elongation and division, tissue differentiation and responses to external stimuli. This technology is emerging as an eco-friendly strategy of great potential (Molla et al. 2012). Therefore, this research aims to evaluate *In vitro* the antifungal potential of six indigenous *Trichoderma* spp. strains. against two strains of *Alternaria* spp. native, which were analyzed on yellow pitahaya (*S. megalanthus*) located in Palora city, Morona Santiago province, Ecuador and identified molecularly.

1.1. METHODS

The strains were provided by the Instituto Nacional de Investigaciones Agropecuarias (INIAP), obtained from cultures of *S. megalanthus* located in Morona Santiago province, Palora city, as displayed in the (**Figure No. 1**).

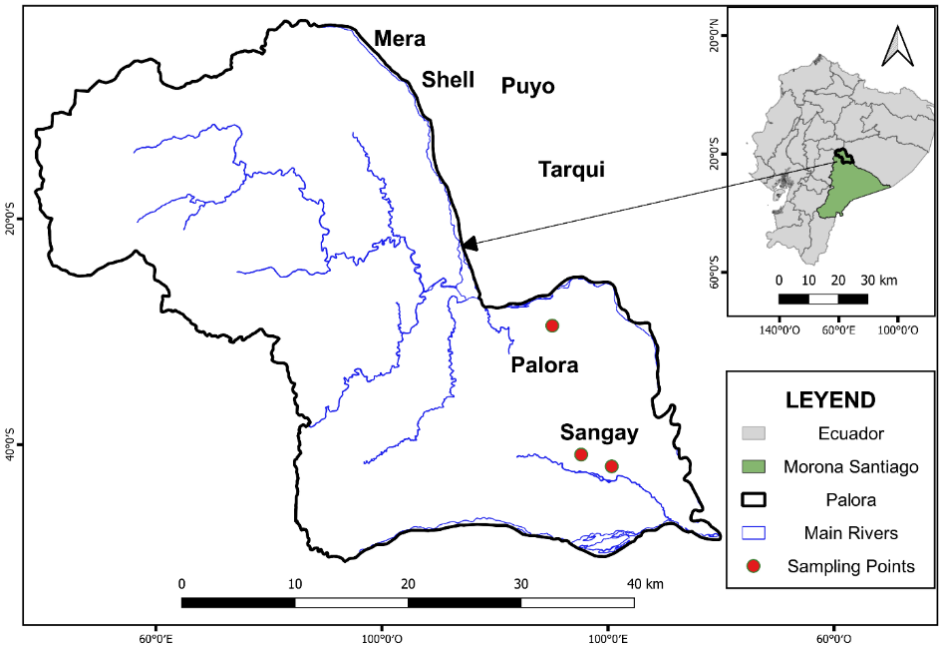

Figure 1: Sampling location of native *Trichoderma* spp. and *Alternaria* strains.
Made by: Jimenez, 2023.

1.2. DNA extraction, amplification and purification

The DNA extraction of the isolates of *Trichoderma* spp. and *Alternaria* spp. were performed using 200 mg of the mycelium growth in Potato-Dextrose-Agar (PDA) for four days. It was collected in a tube of 1.5 ml following the instructions described by Fischer *et al*. (2014). The DNA obtained was quantified with a spectrophotometer (Eppendorf BioPhotometer D30). For *Trichoderma* spp. the sequences of (ITS), tef1, and rpb2 genes were amplified by polymerase chain reaction (PCR) with primer pairs: ITS1 and ITS4, EF1 and EF2, rpb2_FRPB2-7cr and rpb2_FRPB2-5F (SAMUELS et al. 2000; Chaverria et al. 2003; Samuels et al. 2006; Cai and Druzhinina 2021) respectively (**Annex A**). In the case of *Alternaria* spp. ITS, tef1, LSU and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) region genes were amplified with primer pairs: ITS1F and ITS4, EF1-728F and tef1-EF1-986R, LR0R and LR5, gpd1 and gpd2, respectively (**Annex B**) (Aloi et al. 2021; Nichea et al. 2022). The amplified products were visually confirmed by agarose gel electrophoresis. Electrophoresis was subjected to 100 V, 30 mA for 20 min. The amplified reactions were sequenced by Macrogen, Seoul - South Korea.

1.3. Species identification

Once obtained the sequences of interest from the strains were, these were processed with the MEGA11 software®. Then in order to determine the reference similarity percentage among strains; each of the sequences of *Trichoderma* spp. and *Alternaria* spp. strains were submitted to the NCBI BLAST (Aloi et al. 2021). *Trichoderma* spp. identification was made using the pairwise comparison protocol detailed in **Figure No. 2.** This protocol is used with the *Trichoderma* genus due to its great diversity and the exponential expansion of its taxonomy, with up to 50 new species recognized per year, constituting up to July 2020 a total of 375 validated species (Cai and Druzhinina 2021). On the other hand, for the analysis of the strains belonging to the genus *Alternaria,* the Bayesian phylogenetic method was used (Aloi et al. 2021).

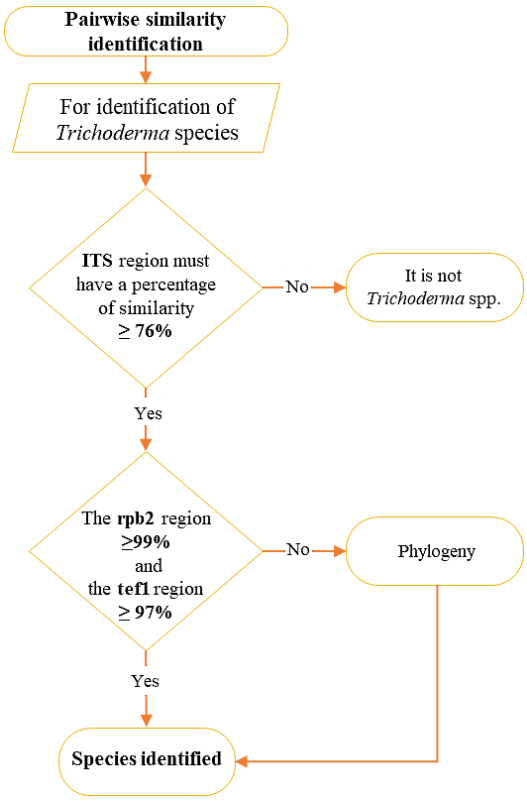

Figure 2: Diagram of Pairwise Similarity Identification based on three DNA sequences according to (Cai and Druzhinina 2021)**.**

Made by: Jimenez, 2023

The first requirement for pairwise similarity identification is that the ITS sequence reaches a similarity value greater than or equal to ≥ 76 % with the sequences of reference strains. Second, the amplified sequences with tef1 and rpb2 are similar to the sequences of the reference strains, fulfilling the following condition: rpb2 ≥ 99% and tef1 ≥ 97%.

On the other hand, in *Alternaria* spp., the sequences amplified with the five pairs of primers were concatenated and loaded into the Blast. The sequences with a similarity percentage greater than 99% were chosen.

1.4. Obtaining extracts from isolated strains of native *Trichoderma*.

Trichoderma strains were cultured in Petri dishes containing 20 ml of PDA and incubated at 27 °C for five days. The six strains were placed separately into 500 ml-capacity flasks, with 250 ml of potato dextrose broth (PDB) medium, which were kept under agitation at 180 rpm for 14 days at 27 °C. The mixture was filtered, and the liquid phase was preserved and mixed by a separatory funnel in a 1:1 ratio with ethyl acetate (Acosta et al. 2011). It was shaken several times, and the remaining PDB phase was discarded. Lastly, a rotary evaporator removed the organic solvent from the extract until a small extract volume was recovered.

1.5. Antibiosis of *Trichoderma* spp. on *Alternaria* spp.

The effectiveness of the extracts was tested using the modified microculture protocol (Tortora et al. 2007). Firstly, 2.5 ml of PDA culture medium was placed on a slide, with three different concentrations (5%, 10%, and 15% (Pun et al. 2020) of each *Trichoderma* spp. extract, which was previously diluted in acetone. For each concentration, three replicates were done with their respective control obtaining a configuration of 36 treatments (**Table No.1**); which consisted of PDA medium and acetone without extract. Then *Alternaria* strains were inoculated; these assays were performed at 27±1 °C for ten days, with daily records of *Alternaria* spp. growth using ImageJ software ®. The data were processed and statistically analyzed using the Tukey and Shapiro Wilks tests in the Infostat software ®.

Table 1: Configuration of the treatments carried out by antibiosis tests with extracts obtained from *Trichoderma* strains against *Alternaria* strains**.**

Treatment	*Trichoderma* spp.	*Alternaria* spp.	C (%)	Treatment	*Trichoderma* spp.	*Alternaria* spp.	C (%)	Treatment	*Trichoderma* spp.	*Alternaria* spp.	C (%)
T1	MS-P-07-1	Negra	15	**T13**	MS-P-03-1	Negra	15	**T25**	MS-P-05	Blanca	15
T2	MS-P-07-1	Negra	10	**T14**	MS-P-03-1	Negra	10	**T26**	MS-P-05	Blanca	10
T3	MS-P-07-1	Negra	5	**T15**	MS-P-03-1	Negra	5	**T27**	MS-P-05	Blanca	5
T4	MS-P-07	Negra	15	**T16**	MS-P-03	Negra	15	**T28**	MS-P-03-2	Blanca	15
T5	MS-P-07	Negra	10	**T17**	MS-P-03	Negra	10	**T29**	MS-P-03-2	Blanca	10
T6	MS-P-07	Negra	5	**T18**	MS-P-03	Negra	5	**T30**	MS-P-03-2	Blanca	5
T7	MS-P-05	Negra	15	**T19**	MS-P-07-1	Blanca	15	**T31**	MS-P-03-1	Blanca	15
T8	MS-P-05	Negra	10	**T20**	MS-P-07-1	Blanca	10	**T32**	MS-P-03-1	Blanca	10
T9	MS-P-05	Negra	5	**T21**	MS-P-07-1	Blanca	5	**T33**	MS-P-03-1	Blanca	5
T10	MS-P-03-2	Negra	15	**T22**	MS-P-07	Blanca	15	**T34**	MS-P-03	Blanca	15
T11	MS-P-03-2	Negra	10	**T23**	MS-P-07	Blanca	10	**T35**	MS-P-03	Blanca	10
T12	MS-P-03-2	Negra	5	**T24**	MS-P-07	Blanca	5	**T36**	MS-P-03	Blanca	5

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1.6. *In vitro* antagonism tests

This evaluation was performed following the dual culture technique (Cabrera et al. 2020). *Alternaria* species that were 10 days old were cut into 8 mm circles and placed on the edge of a Petri dish with PDA medium. The inoculated plates were incubated for 4 days at 27±1 °C to allow colony establishment. Subsequently, a circular diameter (8 mm) of a 4-day-old *Trichoderma* spp. colony was placed on the opposite side to *Alternaria* spp. (Corrêa et al. 2007), then inoculated Petri dishes were incubated at 27±1 °C. The inoculated Petri dishes were then incubated at 27±1 °C. Nine confrontations of *Trichoderma* strains against *Alternaria* and five control Petri dishes were established; obtaining a configuration of 12 treatments (**Table No.2**). the control plates consisted of non-facing *Alternaria* spp. *Alternaria* growth was recorded daily for 10 days, every 24 hours using the ImagenJ software®.

Table 2: Configuration of the treatments carried out through dual cultures of *Trichoderma* spp. against *Alternaria* spp.

Treatment	*Trichoderma* spp	*Alternaria* spp.
T1	P-07	Negra
T2	P-07-1	Negra
T3	P-03	Negra
T4	P-03-1	Negra
T5	P-03-2	Negra
T6	P-05	Negra
T7	P-07	Blanca
T8	P-07-1	Blanca
T9	P-03	Blanca
T10	P-03-1	Blanca
T11	P-03-2	Blanca
T12	P-05	Blanca

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The percentage inhibition of mycelial growth (IGP) was calculated using the **Eq. No. 1** given by (Vincent 1947), cited in (Kantwa & Tetarwal, 2014; Meza et al., 2008; Roopa et al., 2014). The data were processed and statistically analyzed using the Tukey and Shapiro Wilks tests in the Infostat software ®.

$$IGP = \left(\frac{C-T}{C}\right) * 100 \qquad \textbf{(Eq.1)}$$

Where, C represents the measurement of the pathogen radius as a control.
T represents the measurement of pathogen radius against the biocontroller.

2. RESULTS AND DISCUSSION

2.1. Molecular identification of fungi

The strain codes belonging to the genus *Trichoderma* spp. represented the six species and showed the similarity percentage by pair (Table No.3). The results showed all the strains with similarity to ITS belong to the genus *Trichoderma* spp. with values higher than 97%, which are supported by Chaverri and Samuels (2003), Gams and Meyer (1998), since the basic morphological characteristics, both macroscopic and microscopic for each isolate in this research are alike (Annex C), being able to observe typical features for the genus as the coloration of the colony, the presence of phialides and conidia, among other**.**

Table 3: Molecular identification of *Trichoderma* strains isolated from the agroecosystem of *S. megalanthus* native from Palora canton, based on pairwise similarity comparison of ITS, rpb2 and tef1.

N°	Code/ Strain	Similarity standard with reference strain						Phylogenetic concordance of rpb2 and tef1	Species ID
		Gender/ ITS (≥76%)		Specie/ rpb2 (≥99%)		Specie/tef1 (≥97%)			
		Accession	% Ident	Accession	% Ident	Accession	% Ident		
1	MS-P-07	CP072832	99.84%	FJ150788	99.78%	FJ211381	99.71%	Yes	*T. asperellum*
2	MS-P-07-1	MT889994	99.19%	MK044190	99.56%	MN580171	97.81%	Yes	*T. koningiopsis*
3	MS-P-05	MT529923	100.00%	MN557371	99.47%	MK411020	98.90%	Yes	*T. koningiopsis*
4	MS-P-03	KY225592	99.68%	FJ150788	99.67%	MN307415	100.00%	Yes	*T. asperellum*
5	MS-P-03-1	MK396898	100.00%	MZ361069	98.48%	KJ871202	97.28%	No	Unidentified
6	MS-P-03-2	EU280131	97.33%	FJ442784	99.14%	KJ871202	97.54%	Yes	*T. koningiopsis*

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In relation to the percentage of similarity of tef1 between the strains of reference and the 6 strains, all of them complied with a top percentage to 97%. However, compared with the accessions of reference for rpb2, only 5 strains complied with a percentage of similarity superior to 99% (MS-P-07, MS-P-07-1, MS-P-05, MS-P-03 and MS-P-03-2). During their validation process, no higher levels of ambiguities that generate some uncertainty in the identification process are reported, except for MS-P-03-1, which did not meet the condition of similarity for rpb2, when compared with its closest peers, showed a value different to that established by the protocol. This identification indicates that this strain could be recognized as an ambiguous or new species. Therefore, it deserves to be evaluated by the phylogeny of maximum verisimilitude by means of a phylogenetic tree for rpb2 and tef1.

Consequently 5 of the 6 strains accomplished the condition $\exists!$ (rpb2 99 $\cong$ tef1 97). Therefore, two species were identified, *Trichoderma koningiopsis* and *Trichoderma asperellum,* for the first case the strains MS-P-07-1, MS-P-05 and MS-P-03-2 belongs to *T. koningiopsis,* while MS-P-07 and MS-P-03 belongs to the *T. asperellum*.

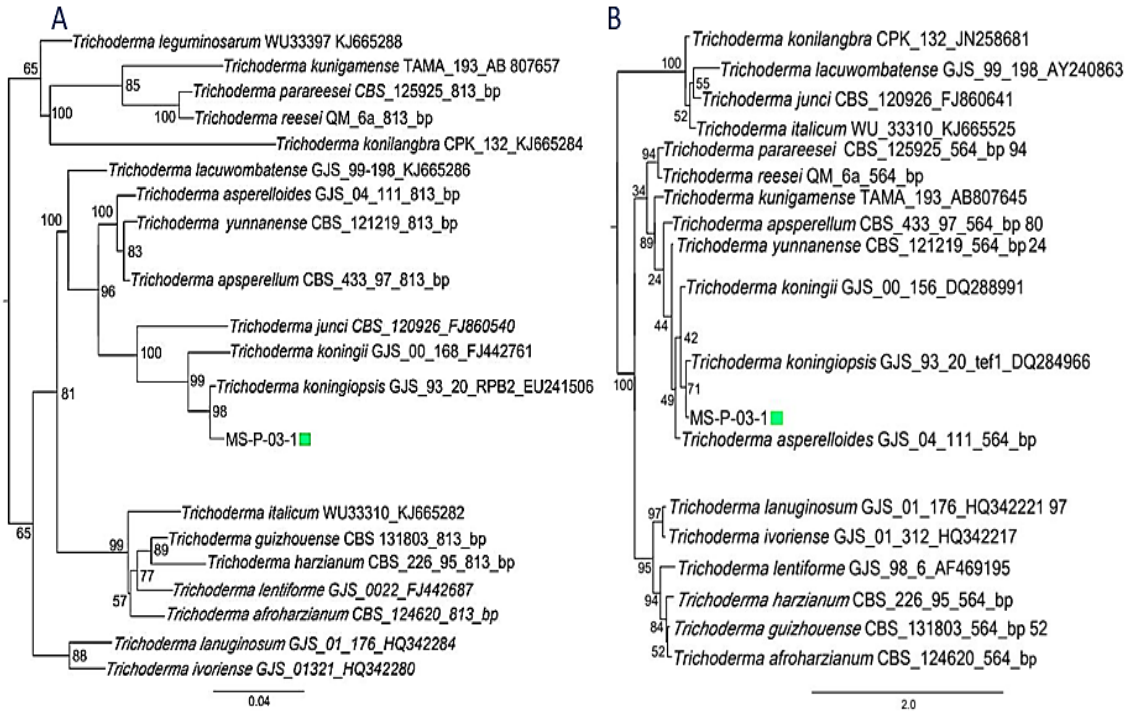

Figure 3: Phylogenetic tree based on Maximum Likelihood analysis of the rpb2 (A) data set and tef1-α (B) data.
Made by: Jimenez, 2023.

The result **(Figure No.3)** showed topologically similar trees with minor differences, which reflect Bootstrap values, for tef1 of 98% and rpb2 of 71%, values higher than 50%. Therefore, it can be established that strain MS-P-03-1 belongs to *Trichoderma koningiopsis* species. However, these results could merit additional studies that include methods with an integrative approach, such as statistical parsimony [SPN], generalized mixed coalescent [GMYC] and phylogenetic-Bayesian phylogeography [BPP] (Cai and Druzhinina 2021), which will allow delimiting the species and reporting strain MS-P-03-1 as a new species for science.

On the other hand, the isolated strains coded representatively as Alternaria negra and Alternaria blanca (**Annex D**), according to the phylogenetic tree obtained, it is reported that the Alternaria blanca strain belongs to the species *Alternaria burns* with a Bootstrap value of 94% and Alternaria negra as *Alternaria alternata* with a value of 91% (**Figure No.4**).

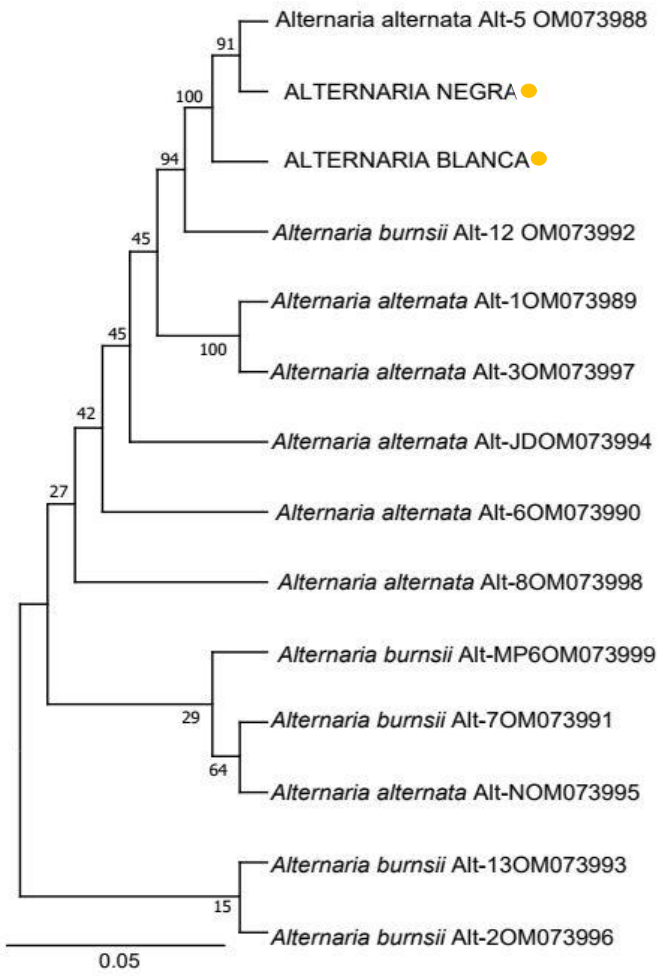

Figure 4: Bayesian phylogenetic tree based on the combined genetic sequences of ITS, tef1, gpd and LSU of two *Alternaria* strains isolated from yellow pitahaya crops.

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This information is corroborated with previous research conducted by Valencia et al. (2016) and by Patel and Zhang (2017); that identified *Alternaria burnsii* and *Alternaria alternata* as highly aggressive species for yellow pitahaya crop, capable of causing significant economic losses for farmers as reported in other cities of Ecuador, in research conducted by Vilaplana and Valencia (2017), Trujillo (2014) and Vilaplana et al.(2018).

2.2. Antibiosis of *Trichoderma* spp. on *Alternaria* spp.

The result revealed that all the extracts tested at the three different concentrations inhibited the growth of the pathogen compared to the control with a significant difference of (P<0.001). In addition, increased efficacy was observed with increasing concentration. Among the three concentrations used, the maximum reduction of mycelial growth of

Alternaria burnsii, was observed when the extract of *Trichoderma* was15% (**Annex E** and **F**), reaching an inhibition percentage of 48% corresponding to T34; followed by T22, T23, T25, T28 and T35 treatments with inhibition percentages of 26.7%, 26.6%, 26.4%, 26.2% and 25.5%, respectively. The treatment with the lowest inhibition capacity was T30, with 9.9%.

Meanwhile, in the case of growth control of *Alternaria alternata,* the treatment with the highest efficiency was T13, with an inhibition percentage of 38.8%, followed by T16, with an inhibition percentage of 34.8% and T10, with a percentage of 33.9%, in all these cases the extract of *Trichoderma* was 15%. The least effective strains to control this species were T3, T9, T12, T2 and T1 with percentages of 0.8%, 1.9%, 3.2%, 6.5% and 8.1%, respectively, as shown in **Figure No.5.**

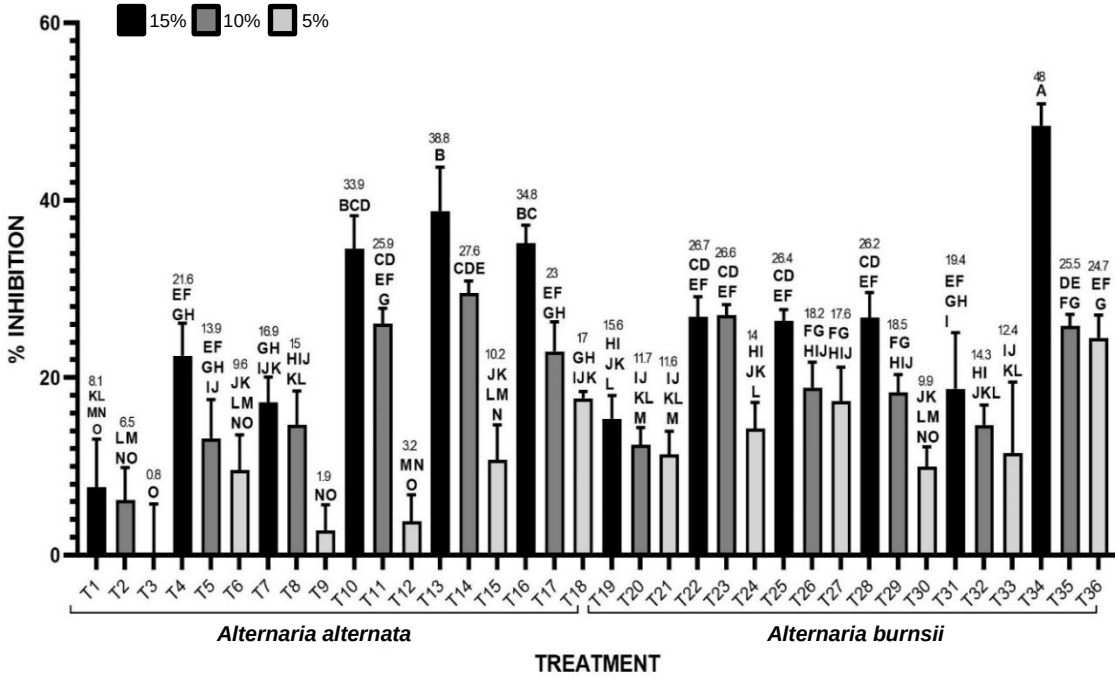

Figure 5: Mean of the percentage of inhibition obtained by Tukey test at 95% confidence of six extracts of *Trichoderma* spp. in three different concentrations (15%, 10%,5%).
Made by: Jimenez, 2023**.**

The extract with the highest percentage of inhibition of mycelial growth of *Alternaria burnsii*, was obtained from strain MS-P-03 identified as *Trichoderma asperellum*; while for *Alternaria alternata,* the highest inhibition was obtained from strain MS-P-03-1 molecularly identified as *Trichoderma koningiopsis*. One of the primary reasons the genus *Trichoderma* is regarded as a potential biocontrol agent is its ability to generate antagonistic chemicals, such as proteins, enzymes, and volatile and non-volatile secondary metabolites that can operate as antibiotics (Hernández-Melchor et al. 2019;

Díaz et al. 2020). However, some authors believe that antibiosis should not be an antagonist´s primary mechanism of action since resistant pathogens are likely to emerge (Martínez 1998; Benítez et al. 2004).

(Dennis and Webster 1971) related the antibiotic activity of *Trichoderma asperellum* and *Trichoderma koningiopsis* to non-volatile compounds such as astrichodermin, glyoxin and viridin trichodermin, suzukacillin, alamethicin, dermadin, trichothecenes and trichorzianin. However, years later, Gupta et al. (2020) observed volatile compounds produced by *Trichoderma* spp. such as: isobutyl alcohol, isopentyl alcohol and 3-methylbutanal exerted a positive effect in inhibiting the growth and reproduction of pathogenic fungi, making them more susceptible to non-volatile compounds.

Results that years later were supported in a study by Negrete (2012) in which it was shown that volatile compounds cause up to 47% of the antifungal activity. However, the same author mentions that the results obtained in his research only provide a general overview. This statement is supported by (Martinez et al. 2013) argue that each of the strains belonging to the same species may present differences in their modes of action and produce different volatile and non-volatile compounds.

2.3. Antagonism tests

The statistical analysis allowed to set the normality of the data (P<0.0029) in the Shapiro Wilks test. The antagonistic activity of *Trichoderma* strains showed a significant difference (P<0.0001). It was observed the mycelial growth between *Trichoderma* spp. and *Alternaria* spp. Mycelial growth of *Trichoderma* was quite more accelerated in comparison with the plant pathogen (**Annex G** and **H**) since *Alternaria* spp. is a slow-growing microorganism (Fernández and Suárez 2009), Hyder et al. (2017) state that the *Trichoderma* genus produces enzymes such as chitinases that degrade the cell wall of the phytopathogenic fungus, hindering its growth and survival, being as a potential option in the field of biotechnology.

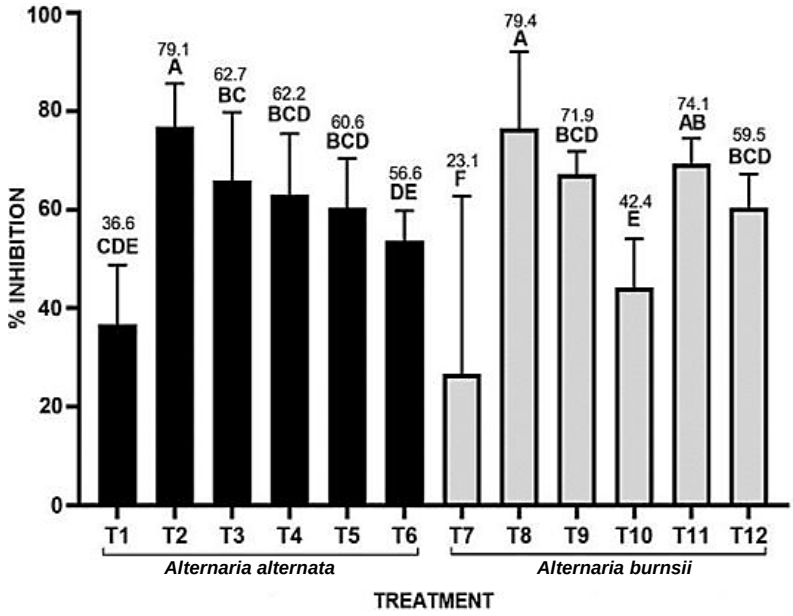

Figure 6: Represents the mean percentage inhibition of *Trichoderma* spp. established by Tukey test at 95% confidence.

Made by: Jimenez, 2023.

The highest percentage of inhibition (**Figure No.6**) of *Alternaria burnsii* was obtained with T8 with 79.4%, followed by T11 with 74.1%; and the treatment with the lowest control of this pathogenic fungus was T7 with 23.1%. In the case of *Alternaria alternata*, the highest percentage of inhibition was obtained with T2 with 79.1%, followed by T3 with 62.7% and the lowest was T1 with 36.6%.

Cotes et al. (2007) that the species of *T. asperellum* and *T. koningiopsis* used as biocontrollers are effective in the control of foliar and soil pathogens due to the antagonistic properties of the genus *Trichoderma* against phytopathogenic fungi.

All the properties of the *Trichoderma* genus such as: antibiosis, mycoparasitism, plant phytohormone promotion and nutrient competition against pathogens (Hernández-Melchor et al. 2019) can be tested by dual confrontation assays.

When comparing the results obtained between antibiosis and dual confrontation crops, it can be determined that the highest percentages are obtained in confrontation crops since the combination of the mentioned mechanisms increases the control of the phytopathogenic fungus and decreases the possibility of resistance development of the pathogen (Vero and Mondino 1999).

Mendoza et al. (2011) mentioned that one of the most relevant mechanisms of *Trichoderma* spp. is mycoparasitism since this genus can develop on pathogenic fungal colonies; however, Cabrera et al.,2020) affirmed that the inhibition efficacy of *Trichoderma* species depends on their capacity to produce volatile and non-volatile antifungal metabolites.

Several studies have shown that *T. koningiopsis* produces three main volatile compounds with antifungal and antimicrobial capacity: azetidine, 2-phenylethanol and ethyl hexadecanoate (Choi et al. 2012; Angel et al. 2016; Deep et al. 2016) On the other hand, *T. asperellum* species has the ability to produce compounds such as 2-methyl-1-butanol, 3-methyl-1-butanol, toluene, ethylbenzene, p-xylene, m-xylene, α-pinene, 3-ethyl-cyclopentanone, phenol and 2-phenylethanol (Ruiz 2011).

Mejía et al. (2008) argue that the effectiveness of plant extracts tends to decrease under *In vivo* conditions, suggesting that field trials represent a challenge for organic agriculture. Therefore, it is convenient to scale up biocontrol trials to *In vivo* conditions in greenhouses and agricultural fields.

On the other hand, the capacity of *T. asperellum* and *T. koningiopsis* to induce the production of plant phytohormones in studies carried out by Cotes et al. (1996), Clavijo and Cotes (1998) and (Castillo et al. 2007) establish that it is one of the most important characteristics since the activity of pathogenesis-related proteins, such as β-1,3-endoglucanases and endochitinases, induces the production of these enzymes in plant tissue, causing hydrolysis in the walls of phytopathogenic fungi and also inhibiting conidial germination and the growth of germ tubes.

In the present investigation was found the maximum inhibition of 79.4%, which is validated by similar investigations with an inhibition percentage of 71.6% and 69.8 % between *Trichoderma* spp. against *Alternaria* spp. in Rios et al. (2016) and inhibition percentages between a range of 25% to 56% in Camacho (2015).

However, thanks to the study carried out by Chávez et al. (2008), it is possible to increase the antagonistic capacity and to obtain better percentages of inhibition. The author based on his research, suggests that for better growth of *Trichoderma* spp. the light conditions must be constant during 8 days and the substrate or medium must be solid or semi-solid enriched with molasses at 10%.

Based on these considerations and the favorable results obtained *In vitro*, it is necessary to scale up these trials and test the antagonistic effectiveness of *T. asperellum* and *T. koningiopsis* in the field.

3. CONCLUSION

In the study, the biocontrol species *Trichoderma* obtained from Pitahaya crops located in Palora city are *Trichoderma koningiopsis* and *Trichoderma asperellum*; and the pathogenic species were *Alternaria alternata* and *Alternaria burnsii.*

In the antibiosis tests, inhibition percentages ranged from 0.8% to 48% as maximum values were obtained corresponding to T34 corresponding to *T. asperellum* (MS-P-03) against *Alternaria burnsii*. On the other hand, during the antagonism tests, inhibition percentages higher than 23.1% were obtained, and the maximum inhibition rate was 79.4%, corresponding to the treatment of *Trichoderma koningiopsis* (MS-P-07-1) against *Alternaria burnsii*. This leads us to conclude that the Antibiosis assay showed a lower percentage of inhibition in relation to the Antagonism assay.

The genus *Trichoderma* spp. has the capacity to control the growth of the phytopathogen *Alternaria spp.* specifically the species identified as *Trichoderma koningiopsis* and *Trichoderma asperellum* in this study, presenting better percentages of inhibition in the tests carried out by dual culture. It is recommended to use the species of *Trichoderma koningiopsis* and *Trichoderma asperellum,* as a method of prevention to control or mitigate the brown disease and perform more trials involving all the mechanisms of action of *Trichoderma* to obtain greater efficacy in the control of the phytopathogen, and therefore less damage to the crop. The perspective of this research is to generate a biofungicide with the strains of *Trichoderma* MS-P-03 and MS-P-07-1 to benefit the yellow pitahaya crops; decrease the use of chemical fungicides and, while reduce the cost of production for farmers; considering that this massification process of *Trichoderma* it can be replicated in rural contexts after the training provided to farmers.

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5. ANNEXES

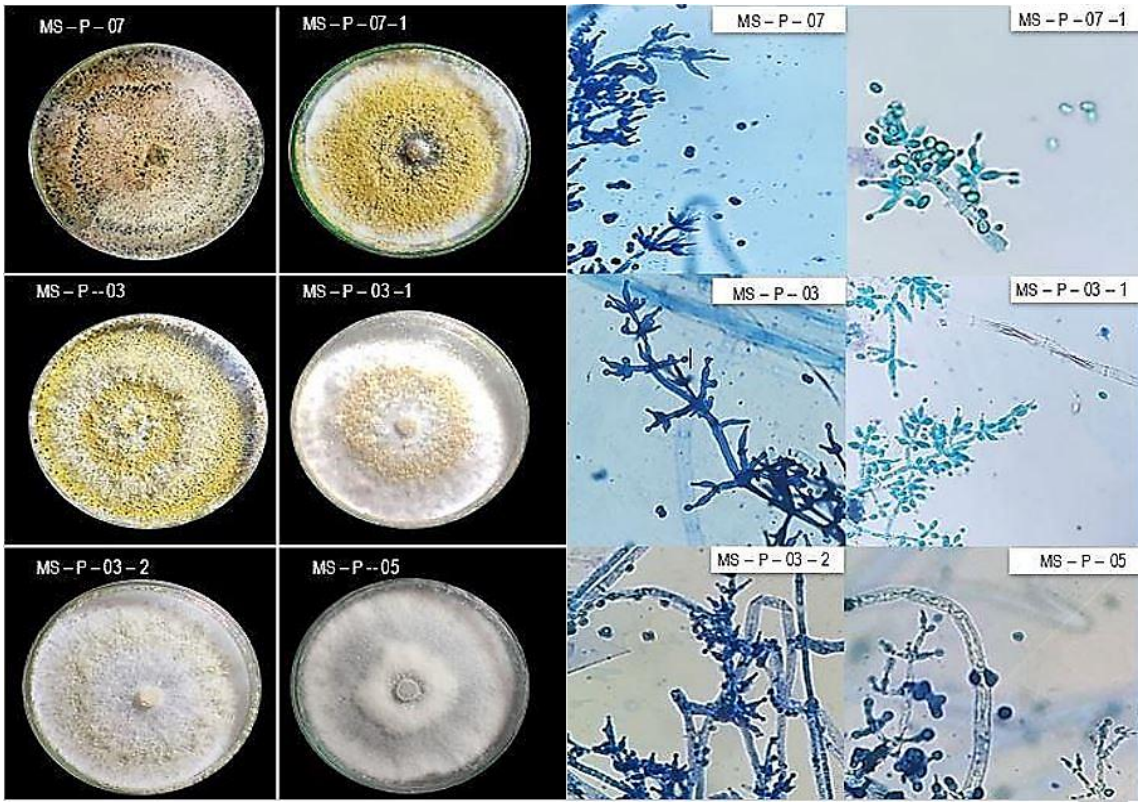
Annex A: Primers for the identification of *Trichoderma* strains

Primers for the identification of *Trichoderma* strains							
		ITS		**rpb2**		**tef1**	
Primers (5' - 3')		**ITS1F** GGAAGTAAAAGTCGTAACAAGG		**fRPB2-5f** GAYGAYMGWGATCAYTTYGG		**EF1** ATGGGTAAGGARGACAAGAC	
		ITS4 TCCTCCGCTTATTGATATGC		**fRPB2-7cr** CCCATRGCTTGTYYRCCCAT		**EF2** GGARGTACCAGTSATCATGTT	
PCR protocol		**T (°C)**	**Time**	**T (°C)**	**Time**	**T (°C)**	**Time**
Pre -denaturation		94	5	94	3	94	5
35 cycles	Denaturation	94	30”	94	1	94	30”
	Alignment	50	45”	57	1	50	30”
	Extension	72	1	72	1	72	1
Final extension		72	7	72	7	72	7

Annex B: Primers for the identification of *Alternaria* strains

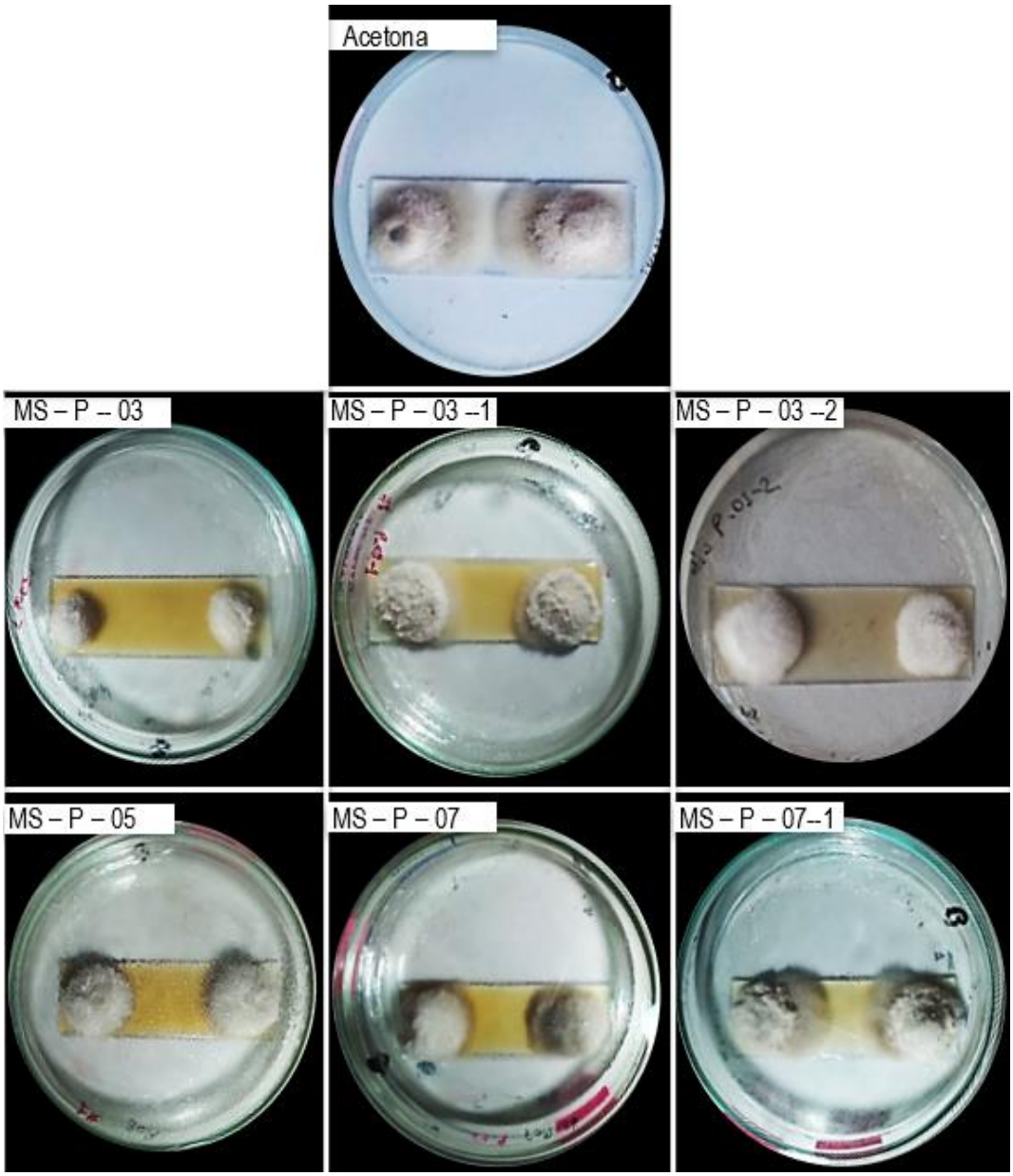
Primers for the identification of *Alternaria* strains									
		ITS		**LSU**		**tef1**		**gdp**	
Primers (5' - 3')		**ITS1F** GGAAGTAAAAGTCGTAACAAGG		**LR0R** ACCCGCTGAACTTAAGC		**EF1-728F** CAT CGA GAA GTT CGA GAA GG		**gpd1** CAACGGCTTCGGTCGCATTG	
		ITS4 TCCTCCGCTTATTGATATGC		**LR5** ATCCTGAGGGAAACTTC		**tef1-EF1-986R** TAC TTG AAG GAA CCC TTA CC		**gpd2** GCCAAGCAGTTGGTTGTGC	
PCR protocol		**T (°C)**	**Time**	**T (°C)**	**Time**	**T (°C)**	**Time**	**T (°C)**	**Time**
Pre -denaturation		94	5	94	3	96	3	95	5
35 cycles	Denaturation	94	30”	94	30”	95	30”	94	30”
	Alignment	50	45”	55	50”	54	45”	55	30”
	Extension	72	1	72	1	72	45”	72	1
Final extension		72	7	72	10	72	7	72	10

Annex C: *Trichoderma* spp. strains at macro (PDA culture medium) and micro (methylene blue staining) levels.

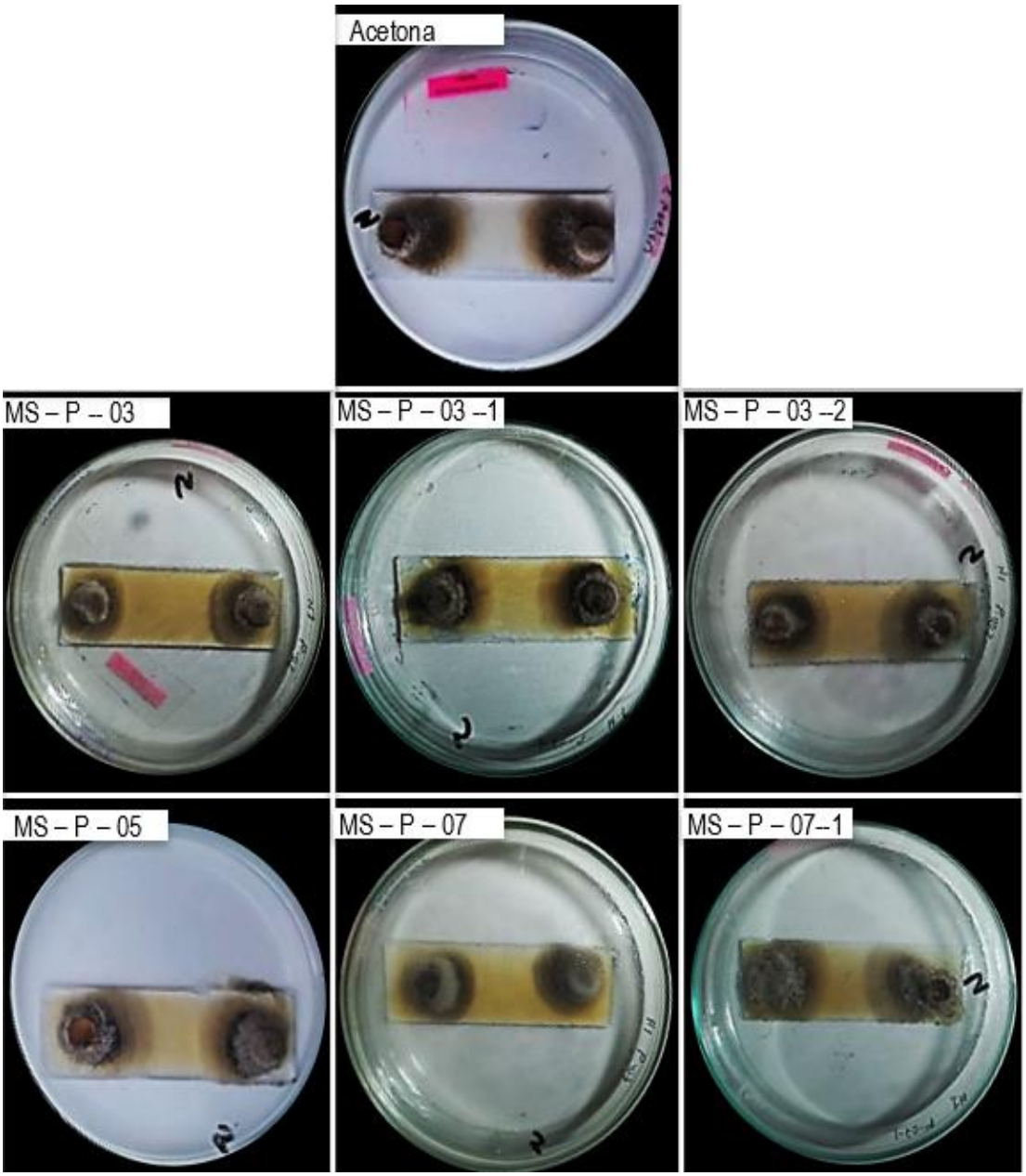


Annex D: *Alternaria* spp. strains at macro (PDA culture medium) and micro (methylene blue staining) levels.

Annex E: Antifungal evaluation by *In vitro* antibiosis assays of *Trichoderma* spp. against Alternaria blanca identified as *Alternaria burnsii.*



Annex F: Antifungal evaluation by *In vitro* antibiosis assays of *Trichoderma* spp against Alternaria negra identified as *Alternaria alternata.*



Annex G: Antifungal evaluation by *In vitro* antagonism assays of *Trichoderma* spp against Alternaria blanca identified as *Alternaria burnsii*

Annex H: Antifungal evaluation by *In vitro* antagonism assays of *Trichoderma* spp against Alternaria negra identified as *Alternaria alternata*.